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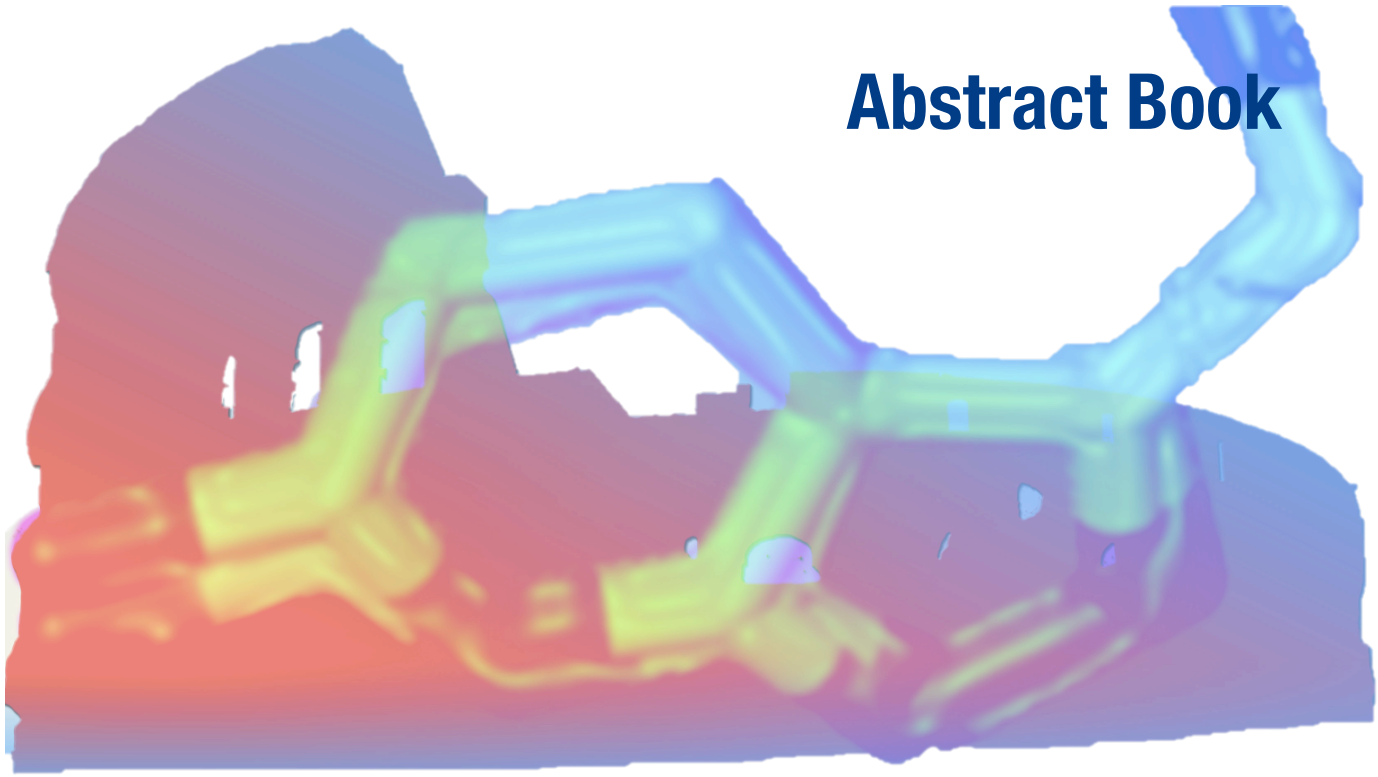

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THE WINDING ROAD OF MEDICINAL CHEMISTRY IN DRUG DISCOVERY: A PERSONAL EXPERIENCE SPANNING THE PAST FOUR DECADES

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In the past three decades continuous paradigmatic shifts have affected the process of drug discovery and development, and the role of the medicinal chemist within this process has undergone major changes because of introduction of new approaches and technologies, such as for example structure-based drug design and combinatorial chemistry.(1) Medicinal chemistry, while maintaining the fundamental character of a chemistry-based discipline centered on design and synthesis of small bioactive molecules, has broadened the focus on multidisciplinary research fields encompassing natural product research synthetic organic chemistry , bioanalytical and computational chemistry in close combination with chemical biology, molecular and structural biology and bioinformatics. The emerging chameleonic role of medicinal chemist in the discovery of innovative drugs is the subject of a debate that, for instance, has recently led the Division of Medicinal Chemistry of the ACS to discuss about the proposal to change its current name in a more inclusive one, that is "Division of Drug Discovery".

This lecture will discuss the changing role of the medicinal chemist in the light of a personal scientific journey along four decades in the field of drug discovery, from the initial synthesis of new heterocyclic compounds "with potential biological activity", through QSAR and early molecular modeling approaches, (2) to the *in silico* design, synthesis and bio-pharmacological evaluation of new molecular entities addressing single or multiple targets in cancer and neurological diseases. (3-5) Particular attention will be paid also to the development of novel methods for data modeling and lead finding and optimization, including multi-objectives optimization. (6)

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TURN-KEY VIRTUAL SCREENING: ARE WE ALREADY THERE?

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Receptor-based virtual screening (VS) is a computer-based technique for identifying promising compounds to bind to a target molecule of known structure. Given the rapidly increasing number of protein and nucleic acid structures this methodology continues to grow as an effective tool that now permeates all aspects of drug discovery. While a plethora of different methods have been devised to qualitatively describe the ligand-target interactions a current challenge is now to quantify them in a short amount of time and major advances are still needed to make VS a turn-key method for lead discovery.(1)

In this presentation, a perspective of our successes in using VS for pharmaceutical lead discovery will be given. These successful lead discovery stories have included a diverse range of targets, including enzyme active sites (APS Reductase,(2) ALR2,(3) and BACE-1)(4) and nucleic acids (G-quadruplex).(5) In all these studies VS methods have been tuned to address the challenges posed by the different targets requiring significant expertise and manual intervention to yield a successful result. All the different approaches will be described from a hands-on point of view along with the results achieved.

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DISCOVERY OF A NEW CLASS OF POTENT INHIBITORS OF ACID CERAMIDASE: SYNTHESIS AND STRUCTURE-ACTIVITY RELATIONSHIP STUDIES

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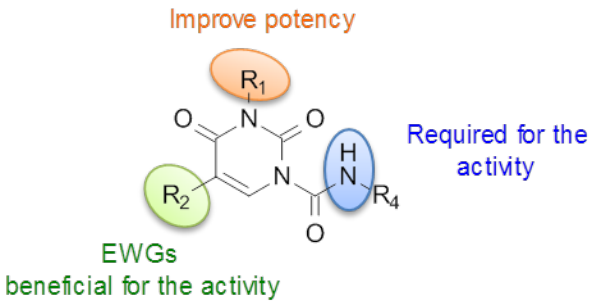
Acid ceramidase (AC) is a ubiquitous cysteine amidase that is located within the lysosome and is responsible for the degradation of the lipid messenger, ceramide.(1) By regulating ceramide concentration within cells, AC is involved in several disorders associated with deregulation of sphingolipid metabolism. In particular, AC is emerging as an important enzyme in cancer progression and the response to tumor therapy, making its inhibition a promising strategy for cancer treatment. Acid ceramidase inhibitors previously reported in the literature have limited potency (medium-high micromolar range) and lack drug-like properties.(2)

Screening a commercial chemical library, we identified the anti-cancer agent carmofur (5-fluoro-*N*-hexyl-2,4-dioxo-pyrimidine-1-carboxamide) as the first nanomolar AC inhibitor (rat AC, IC_{50} = 29 nM), and showed that this compound strongly enhances the anti-proliferative effects of two mechanistically distinct anti-tumoral agents, 5-fluorouracil and taxol.(3) These findings suggested that carmofur might be a good starting point for the discovery of new cancer sensitizing drugs. To explore this possibility, we synthesized and tested a series of uracil derivatives with the aim of studying structure-activity relationships (SARs) for this class of compounds and discovering more potent AC inhibitors.

The SAR study allowed a first elucidation of the structural features of uracil derivatives that are critical for AC inhibition, as well as the identification of the first single-digit nanomolar inhibitors of AC.(4)



The results confirmed that substituted 2,4-dioxo-pyrimidine-1-carboxamides represent a novel class of potent drug-like AC inhibitors. Selected derivatives in this series may provide useful probes to further characterize the functional roles of AC, and assess the therapeutic potential of AC inhibitors, alone or in combination with established treatments, in cancer therapy.

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ARMED ANTIBODIES AND TARGETED CYTOTOXICS: FROM THE BENCH TO THE CLINIC

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Antibodies can be used to deliver bioactive molecules (drugs, cytokines, photosensitizers, radionuclides, etc.) to the tumor environment, thus sparing normal tissues.
The targeting of modified sub-endothelial extracellular matrix components using armed antibodies is particularly attractive, because of:
(i) the abundance and stability of some of these antigens (e.g., splice isoforms of fibronectin and tenascin-C); (ii) the dependence of cancer on new blood vessels; (iii) the accessibility of these structures from the blood-stream;
(iv) the fact that some of these ECM antigens are very abundant in many different cancer types, while being virtually undetectable in most normal adult tissues.[1-3] Similar approaches can be used for other serious non-oncological conditions, which are characterized by the over-exuberant proliferation of new blood vessels.

While cytokines can be conveniently delivered at site of disease by the construction of fusion proteins with antibody vehicles, the targeted delivery of cytotoxic drugs requires more sophisticated strategies. We have recently explored the development of linkerless strategies for the coupling of potent cytotoxic drugs to tumor-targeting antibodies. [4, 5]

Advanced preclinical and clinical data, obtained in collaboration with Philogen (www.philogen.com) will be presented in this lecture.

In addition, we have recently started to explore whether small organic ligands, specific to tumor-associated antigens, can be used for pharmacodelivery applications *in vivo*. Selective ligands can be conveniently isolated from large DNA-encoded chemical libraries.[6]

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CROSS TALK BETWEEN SMALL MOLECULES AND BIOLOGICAL SYSTEMS: POTENTIAL FOR SYSTEMS MEDICINE IN INDUSTRY

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Small molecule drug discovery continues to advance as a science as new tools in synthesis and drug design become available. Case studies will be used to highlight current state of the art. Nevertheless, whereas progress in pharmacokinetics has led to strong reliability of the translation from animal models to human, safety and toxicology cannot be modeled sufficiently yet. By far the most significant reason for failure in drug development is a lack of efficacy in clinical studies.

Systems biology can help to overcome these problems by helping to identify fruitful indications and selecting promising targets with larger potential. Recent investigations for the identification of targets involved in insulin secretion using models for the mathematical representation of processes in the pancreatic beta-cell controlling insulin secretion will be presented. These models e.g. show that GPR119 is a good target to overcome insulin deficiency as it can regulate insulin release in a glucose concentration dependent manner. Additionally, the use of physiological models will be highlighted to determine, whether compounds need to be given in a specific rhythm to deliver best efficacy.

NEXT GENERATION PROTEINKINASE INHIBITORS: WHEN SELECTIVITY COUNTS

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Proteinkinases and their inhibitors have been a major success story both scientifically and commercially. With 518 genes encoding a proteinkinase, this class of enzymes represent 22% of the druggable genome. More than 250 are considered to be disease related, making them an attractive target for new drug („drug targets of the 21st century“)[1]. More than 500.000 papers and 20.000 patents have been published, 20-30% of all early drug discovery programs in the pharmaceutical industry are targeting a proteinkinase. As of today, 23 inhibitors are registered as drugs[2].

The first representative Imatinib (Gleevec) was launched in 2001, 2009 the global market for of this novel class of drugs was already 15 billion USD, estimated to climb to 20 billion in 2014, making proteinkinase inhibitors a major commercial success story. In 2020, the annual sales volume of small molecule protein kinase inhibitors is expected to be more than 25 billion U.S. dollars. The great success of Proeteinkinase Inhibitors in the field of anti-cancer drugs stimulated the study of similar compounds in other therapeutic areas such as inflammation, autoimmune- and neurodegenerative diseases[3].

Are proteinkinases major drug targets of the 21st century, probably yes, but for sure difficult ones! The protein structure of kinases is highly conserved, this is especially true for the ATP-cleft which is the predominant binding site for inhibitors. Including splice variants, 518 genes result in ~2.500 kinase proteins, numerous mutations in cancer cells increase the number of target proteins as well. Selectivity is therefore a major challenge in the design of inhibitors. Currently registered anti-cancer proteinkinase inhibitors are more or less unselective („multiselective“). As in cancer cells, more than one kinase is misregulated, this can even seen as an advantage.

In non-lethal chronic diseases which need a life long treatment, selectivity becomes a major issue. Lack of selectivity is clearly linked to side effect as some kinases are known anti-targets. However not all „untouchables“ are known, making highly selective molecules a must in indications as rheumatoid arthritis, chronic obstructive pulmonary diseases, inflammatory bowel disease and psoriasis or CNS diseaes as alzheimer, parkinsons and neuropathic pain. Candidates are currently in clinical development, an overview will be presented.

Even in cancer, selectivity becomes an essential issue. Second generation Tyrosine kinase inhibitors circumvent acquired resistance to the first-generation inhibitors and are tested in patients with refractory disease[4]. In addition, kinase mutations in the primary tumor become an important or even essential target. The “ideal drug” would be an inhibitor of such a mutated proteinkinase without hitting any of the wild-type kinases. The issue of selectivity reaches an even higher level. In any case, the challenge for the medicinal chemist remains the same, hitting on target kinase beside several thousand highly homolog off-targets.

Our group is working on extermely selective inhibitors for inflammatory diseases and EGFR-mutants. Structure elucidation of ATP complexes bound to protein kinases, have revealed that there are regions within or close to the binding cleft that ATP does not fully occupy. These regions, unoccupied by ATP (hydrophobic region I and II) show structural diversity between members of the kinase family. Another way to induce selectivity makes use of a peptide flip at the hinge region, induced by a carbonyl-interaction of the inhibitor with two backbone NH-groups. We tried to combine both approaches by using carbonyl-groups for targeting the hinge region and aryl-residues to interact with the HR I and/or II. In addition, we minimized the structures by using only templates, interacting directly with the hinge region and the hydrophobic regions (“linear binders”). A third structural requirement was reducing conformational flexibility of the inhibitors. Rigid structures should allow less induced fit to “off-target” kinases.[5]

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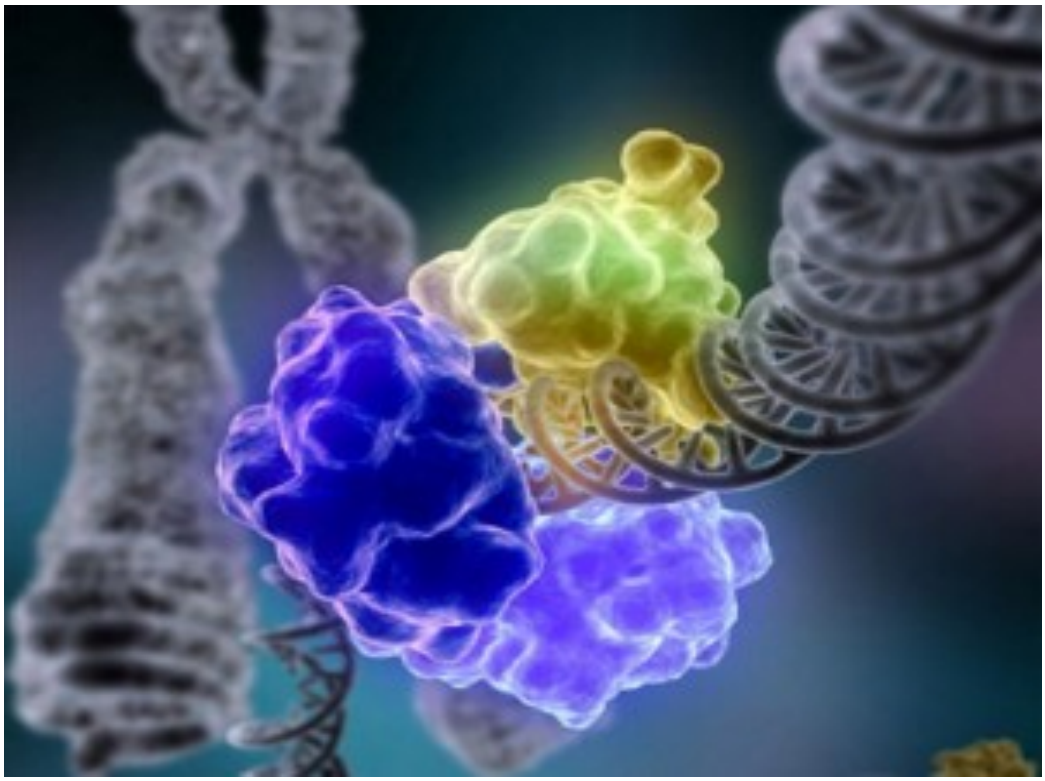

NEW ANTITUMOUR AGENTS TARGETING THE UBIQUITIN-PROTEASOME SYSTEM

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The ubiquitin-proteasome system plays a fundamental role in a number of cellular processes, notably the selective (poly)ubiquitination of lysine residues of proteins prior to proteasomal degradation. Protein ubiquitination is orchestrated by a series of enzymes (E1, E2 and E3), a number of which have being studied as potential anticancer drug targets at the E2 and E3 level. The approval of the proteasome inhibitor bortezomib for the treatment of multiple myeloma and mantle cell lymphoma has helped to drive interest in this class of agents as novel cancer therapeutics.

For example, the E3 ubiquitin ligase BCA2 (breast cancer associated protein 2) provides a potentially useful target for invasive breast cancer therapy.(1) BCA2 is over-expressed in clinical breast cancer samples, and via interaction with binding partner Rad7 provides a mechanism for recycling of growth stimulatory tyrosine kinase receptors such as EGF-R. The double Zn^{2+}-binding motif of the BCA2 RING-domain, essential for E3 ubiquitination activity, is a target for small molecule zinc-ejector molecules. Disulfiram is an aldehyde dehydrogenase-inhibitory drug registered for the treatment of alcoholism, which has additional anticancer activity through inhibition of BCA2 catalytic activity. The presentation will overview the design and synthesis of novel BCA2 inhibitors based on disulfiram,(2) and their anticancer activity in BCA2-expressing breast cancer cells, including induction of EGF-R degradation.

The ubiquitin conjugating enzymes or E2s mediate protein ubiquitination by selective interactions with ubiquitin activating (E1) and ubiquitin ligase (E3) enzymes. We have focused our studies on Rad6B, an E2 ubiquitin conjugating enzyme that is over-expressed in human breast cancer, and associated with loss of epithelial polarity, aneuploidy, resistance to chemotherapeutics drugs, and β -catenin stabilization.(3)

We have used the Rad6B crystal structure to guide the design and synthesis of new diamino-triazines that were found to directly interfere with the Rad6B active site, inhibiting histone H2A ubiquitination and additionally proliferation, colony formation and migration in MDA-MB-231 breast cancer cells.(4) Novel triazine-based compounds were available in three chemical steps, featuring cyclisation between appropriately functionalized biguanides and carboxylic esters as the key synthetic transformation. We have made further design modifications to candidate Rad6B inhibitors based on molecular modeling considerations, to provide further triazine-based compounds for synthesis and evaluation towards the discovery of further selectively active E2-inhibitory anticancer compounds, and to study structure activity relationships.

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CARBONIC ANHYDRASE INHIBITORS AS ANTICANCER AND ANTIMETASTATIC AGENTS

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Carbonic anhydrase IX (CA IX) is a membrane-bound, hypoxia-inducible enzyme that is highly expressed in many types of solid tumors, but shows very restricted expression in normal tissues [1]. CAIX plays an important functional role in processes critical for tumor cell growth and metastasis, including pH regulation, survival, adhesion and migration.[1, 2] The tumor-specific expression of CAIX and its association with cancer progression and poor treatment outcome has led to interest in targeting this enzyme for cancer therapy. The development of pharmacologic inhibitors that selectively target tumor-associated, extracellular CAs without "off-target" inhibition of cytosolic CAs is critical for their use as cancer therapeutics.[1, 2] We have recently described a series of novel ureido-substituted benzenesulfonamides and glycosyl coumarins that selectively and potently inhibit CA IX activity in vitro, and reduce breast tumor growth and metastasis in vivo.[3, 6] Ee further characterized several of these inhibitors in cell-based assays and investigate their efficacy when used in combination with conventional chemotherapy or radiation in vivo. Incubation of selected compounds with 67NR cells constitutively expressing human CA IX significantly suppressed the drop in extracellular pH in a cell-based CA IX activity assay. Inhibition of invasion and/or induction of cell death were observed when highly metastatic MDA-MB-231 LM2-4 breast cancer cells were cultured in hypoxia. Similarly, treatment of A549 non-small cell lung cancer cells cultured as monolayers in hypoxia with CAIX inhibitors resulted in a significant increase in cell death. Preclinical evaluation of selected ureidosulfonamide inhibitors demonstrated a correlation between the amount of intratumoral CAIX expressed in vivo and the degree of inhibition of tumor growth. Treatment of animals harboring highly CAIX positive MDA-MB-231 LM2-4 orthotopic breast tumors resulted in significant inhibition of tumor growth and increased survival times. Furthermore, treatment of mice harboring human orthotopic breast tumors with an ureido-sulfonamide in combination with paclitaxel or doxorubicin resulted in significantly reduced tumor growth compared to either treatment administered alone. Bioluminescence imaging of lungs resected from treated mice revealed that lung metastases were virtually absent from animals treated with the combination therapy. Collectively, these studies provide strong proof of principle data for the therapeutic use of CAIX inhibitors for inhibiting tumor growth and metastasis formation, especially when used in combination with conventional chemo- and radiotherapy.

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DISCOVERY OF HISTONE LYSINE DEMETHYLASE INHIBITORS

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Recent studies have revealed that chromatin remodeling, caused by DNA methylation and histone modifications such as acetylation, methylation, and phosphorylation, plays a pivotal role in the regulation of epigenetic gene expression. Among the posttranscriptional histone modifications, lysine methylation is one of the most widely studied modifications, and methylation at various sites has been shown to lead to transcriptional activation or silencing.

In contrast to other histone modifications, such as acetylation and phosphorylation, histone lysine methylation had been regarded as irreversible because of the high thermodynamic stability of the N-C bond. Indeed, while a number of histone lysine methyltransferases (HKMTs) had been identified by 2003, histone lysine demethylases (KDMs) had not been identified. However, two classes of KDMs have been identified since 2004. One class includes lysine-specific demethylase 1 (LSD1, also known as KDM1A) and LSD2 (also known as KDM1B), which are flavin-dependent amine oxidase domain-containing enzymes. The other class comprises the recently discovered Jumonji domain-containing protein (JMJD) histone demethylases, which are Fe(II) and α-ketoglutarate-dependent enzymes. The identification of these KDMs established that histone methylation is reversibly regulated by HKMTs and KDMs.

As there is increasing evidence that KDMs are associated with various disease states, they have emerged as attractive targets for the development of new therapeutic drugs.[(1)] To date, several classes of KDM inhibitors have been identified, including LSD1 inhibitors and JMJD inhibitors, and the feasibility of using KDM inhibitors as therapeutic agents has been suggested. In this meeting, the design, synthesis, and evaluation of our most recent KDM inhibitors[(2-4)] and their possibility as anticancer agents will be presented.

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CHEMICAL INSTRUMENTS TO PLAY IN THE EPIGENETIC ORCHESTRA: SOUND, SILENCE, AND THE NOTES IN BETWEEN

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Epigenetics describes inherited and acquired modifications of DNA, histones and proteins, which play critical roles in the regulation of gene expression, in chromosome stability, genomic imprinting, and stem cell fate. Alterations in epigenetic signaling are involved in major diseases including cancer, metabolic and neurodegenerative diseases. A dissection of the molecular mechanisms of epigenetic signaling will be facilitated by the availability of small molecule chemical probes that are selective for specific components of the epigenetic machinery.

Together with knockouts and RNAi approaches, chemical probes are invaluable tools to dissect these biological networks. Yet, chemical probes for epi-targets are quite often difficult to identify because of the lack of gold standard screening techniques for these proteins.

Here we will describe our approach combining synthetic medicinal chemistry skills, biomolecular and biophysical techniques (Surface Plasmon Resonance, SPR; Differential Scanning Fluorimetry, DSF; Microscale Thermophoresis, MST; AlphaScreen technology; Isothermal Titration Calorimetry, ITC) and strategic collaborations to generate chemical probes and interrogate epigenetic proteins and related biological pathways.

SYNTHESIS AND STRUCTURE ACTIVITY RELATIONSHIP STUDIES OF MODIFIED BISEBROMOAMIDE ANALOGUES

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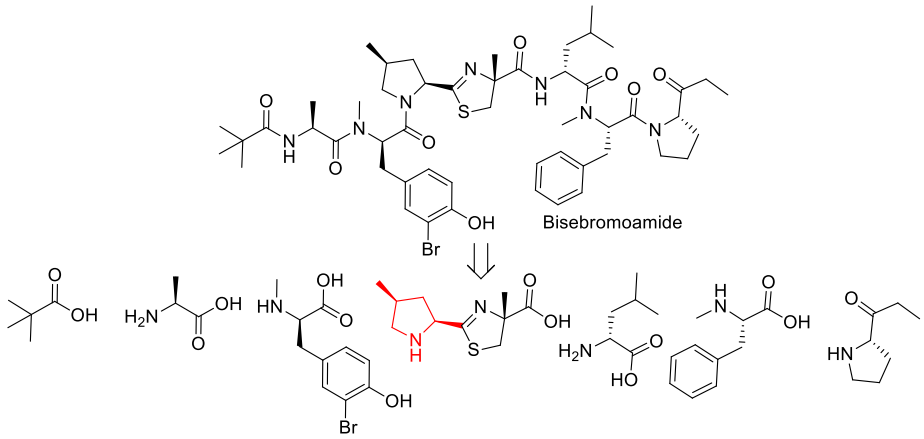
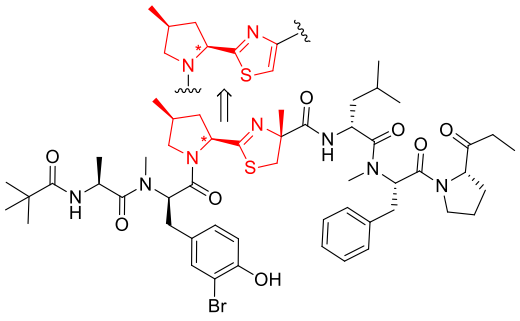
The peptide bisebromoamide was isolated from a marine cyanobacterium *Lyngbya* sp. by the Suenaga group in 2009 and exhibits antiproliferative activity at nanomolar levels.[1] Current SAR data indicates that there is some flexibility in the structure with respect to stereochemistry,[2] but the range of modifications that have been biologically tested is limited. Progress towards the total synthesis of bisebromoamide analogues, via a solid phase peptide synthesis approach which will enable facile modification of the final structure, will be reported.



Bisebromoamide contains a number of non-commercial amino acids and an oxopropyl pyrrolidine moiety which had not been found in a natural product previously.(1) Several new synthetic routes towards the non-commercial amino acid fragments have been developed, including two ring-closure-based approaches to the substituted proline derivative 4-MePro.

While the presence of six amide bonds makes SPPS an appealing approach to synthesising bisebromoamide, the 4-MePro moiety is attached to a thiazoline and it is well documented that the α-position of an amino acid will racemise, under both acidic and basic conditions, when attached to a thiazoline or oxazoline.(3) Previous reports indicated that the methyl group of the thiazoline was not essential for biological activity[4]_ENREF_4_ENREF_4 and so to increase stability it is to be replaced with a thiazole.



The promising anticancer activity of bisebromoamide lends itself to SAR studies; modifications including an alanine scan, truncations and incorporation of modified proline derivatives are envisaged. The biological activity of some of these analogues will also be reported.

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IDENTIFICATION OF SELECTIVE NON-NUCLEOSIDE INHIBITORS OF HUMAN DNA METHYLTRANSFERASES HIGHLY ACTIVE IN CANCER INCLUDING CANCER STEM CELLS

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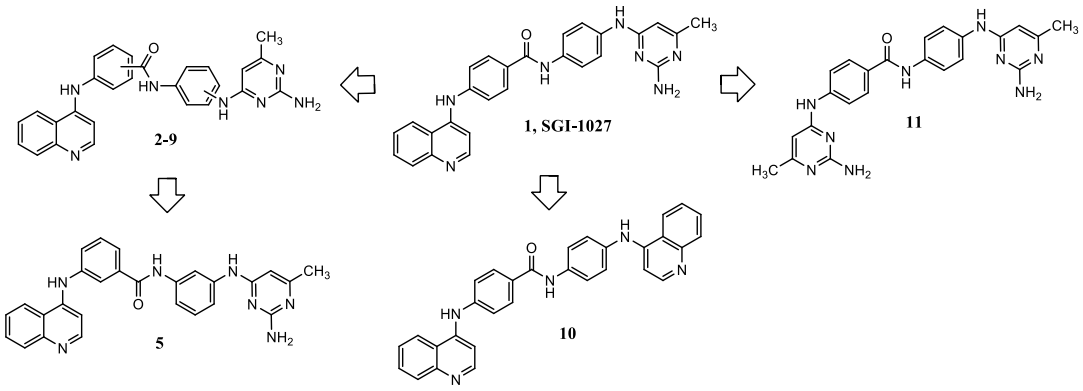
DNA methylation occurs by transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to the C5-position of cytosine predominantly in the CpG dinucleotides (CpG islands) and is catalyzed by three DNA methyltransferases (DNMTs), DNMT1, DNMT3A and DNMT3B.(1) DNA methylation is essential for embryonic development or differentiation and is involved in various pathologies included cancer.(2) Disruption of DNMT1 can stop tumor growth and reverse the nondifferentiation state,(3) and the use of specific inhibitors of DNMTs (DNMTi) can reactivate silenced tumor suppressor genes (TSG) and induce the reprogramming of cancer cells, leading to their proliferation arrest and then to death.(4) Two DNMTi (azacitidine and decitabine, nucleoside analogues) have been approved by FDA for clinical use against hematological malignancies and some compounds have been reported as non-nucleoside DNMTi, among them, SGI1027 (**1**) was described able to reactivate TSG in cancer cells.(5) Hence we designed and synthesized different SGI1027 regioisomer analogs (**2-11**) for evaluating them both in vitro (DNMTs) and in cancer cells (Raji, PC-3, U-937, MDA-MB-231) including medulloblastoma stem cells (MbSCs). Compound **5** showed increased inhibitory potency against DNMTs and more specificity for DNMTs when tested on other AdoMet-dependent enzymes (PRMT1, a protein arginine methyltransferase and G9a-like protein (GLP), a histone H3 lysine 9 methyltransferase), in comparison with **1**. Moreover, both docking studies and competition experiments performed with **5** and DNMT1 by varying concentration of either DNA or AdoMet confirmed that the mechanism of inhibition of **5** was competitive with the DNA substrate and not with the AdoMet cofactor as for **1**. In addition, dose-dependent antiproliferative, apoptotic and cytodifferentiating effects were shown by compound **5** and its analogs both in cancer and cancer stem cells.



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PYRAZOLO[3,4-*h*]QUINOLINES AS POTENT PHOTOSENSITIZER AGENTS

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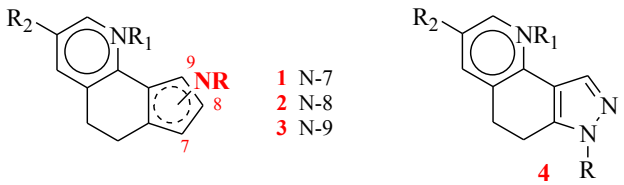
The photodynamic therapy (PDT) is an interesting therapeutic option for the treatment of various tumors, including carcinomas of the esophagus and lung. It requires systemic administration of a photosensitizing agent (PS), followed by irradiation of the tumor with light of proper wavelength in accordance to the absorption spectrum of the PS. PDT is a rapidly expanding field, and is becoming widely recognized as a valuable treatment option, expecially for localized tumors. Up to date it is used for the treatment of skin diseases like psoriasis, vitiligo, cutaneous T-cell lymphoma (CTCL), and it has also been applied to T-cell mediated autoimmune diseases (progressive systemic sclerosis, lupus erythematosus, pemphigo vulgaris and AIDS).[1] The NCI has approved the use of a linear furocumarin, 8-methoxypsoralen (8-MOP), as photosensitizer for the treatment of T-cell lymphoma and it is still in clinical trials also for Hodgkin and non-Hodgkin lymphomas. In the past years, our research group, studied different classes of compounds with antitumor properties. Among these pyrrolo[2,3-*h*]quinolin-2-one **1**, pyrrolo[3,4-*h*]quinolin-2-one **2** and pyrrolo[3,2-*h*]quinolin-2-one **3** showed very promising photosensitizing properties in some cases with higher cytotoxicity than 8-MOP (GI_{50} 0.4-16.4 μM, 1.1-15.0 μM and 0.2-7.4 μM respectively).[2] Moreover they usually localize in mitochondria producing reactive oxygen species (ROS) responsible of the cellular death. Additionally, the class of pyrrolo[3,2-*h*]quinolin-2-one **3** demonstrated a great potential in the modulation of long term side effects, as they do not induce DNA damage at variance of 8-MOP. This result is of extraordinary importance to cover this products with an international patent.[2]



In this light, we planned the synthesis of the *pyrazolo[3,4-h]quinolin-2-one* ring system **4** in which a pyrazole is fused to the quinolinone moiety, with the aim of evaluating the influence on the antiproliferative activity of the substitution of pyrrole ring with pyrazole. Cytotoxicity was determined against 6 human tumor cell lines: K-562, Jurkat, HL-60, A-431, A-549, LoVo and MCF-7. In contrast with the previous series, some of the new compounds showed antiproliferative activity in the low micromolar range already in the dark. Phototoxicity studies indicated a UVA-dose dependent growth inhibitory effect, some derivatives reaching GI_{50} values at nanomolar concentrations (0.04-14.50 μM). Studies on the mechanism of action demonstrated that pyrazoloquinolines photoinduced extensive lipid peroxidation as demonstrated by the TBARs test and by the increase in cell survival when irradiation was carried out in the presence of vitamin E. Moreover, no DNA photodamage was shown *in vitro*. Furthermore pyrazoloquinolines photoinduced cell death by different mechanism: in some cases, necrosis prevails whereas other derivatives induce mostly apoptosis. Mitochondria and later lysosomes were involved in pyrazoloquinoline cell death.

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SYNTHESIS AND ANTITUMOR ACTIVITY IN PERITONEAL MESOTHELIOMA EXPERIMENTAL MODELS OF 1*H*-PYRROLO[2,3-*b*]PYRIDINE DERIVATIVES

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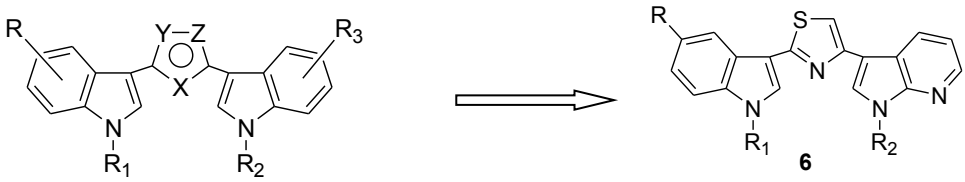
Diffuse malignant peritoneal mesothelioma (DMPM) is a rare and rapidly fatal disease, that develops from mesothelial cells that line the peritoneal cavity and accounts for approximately 10-15% of all malignant mesotheliomas.[1,2] Considering the poor response to conventional therapies, a considerable effort has been made to identify and develop new molecules based on natural compound scaffolds as possible novel cancer therapeutic agents. In particular, bis-indole alkaloids represent one of the most important class of pharmaceutically interesting compounds due to their potent biological activities such as antiinflammatory, antimicrobial, antiviral and antitumor.[3-6] In particular, Nortopsentins A–C having a characteristic 2,4-bis(3'-indolyl)imidazole skeleton, isolated from *Spongosorites ruetzleri*, exhibited *in vitro* cytotoxicity against P388 cells (IC_{50} values: 4.5–20.7 μm). [7-9] Due to the interesting biological activities Nortopsentin is used as lead compound to obtain more active derivatives. In particular, we reported the synthesis and antitumor activity of a different series of bis-indolyl-5-membered heterocycles **1–5**, in which the imidazole moiety of nortopsentin was replaced by thiophene, pyrazole, isoxazole, furan and pyrrole rings. Some of these compounds showed antiproliferative activity against a wide range of human tumor cell lines with GI_{50} values from micromolar to sub-micromolar concentrations.[10-13]



Nortopsentin X=N; Y=CH; Z=NH; **1**: X=S; Y=Z=CH; **2**: X=CH; Y=N; Z=NH; **3** X=CH; Y=N; Z=O; **4**: X=O; Y=Z=CH; **5**: X=NH; Y= Z=CH.

In our attempts to search for new antitumor compounds, we synthesized 3[2-(*1H*-indol-3-yl)-1,3-thiazol-4-yl]-1*H*-pyrrolo[2,3-*b*]pyridines of type **6**, in which the spacer is constituted by the thiazole ring and one of the indole units is replaced by a 7-azaindole moiety. All compounds were selected by the NCI for evaluation against the full panel of human cancer cell lines and some compounds showed GI_{50} values from micromolar to sub-micromolar range. The biological activity was also investigated in STO and MesoII cells, derived from human DMPM. The most active compounds, that act as cyclin-dependent kinase 1 inhibitors, consistently reduced DMPM cell proliferation and induced a caspase-dependent apoptotic response, with a concomitant reduction of the expression of the active Thr34-phosphorylated form of the anti-apoptotic protein survivin. Moreover, the combined treatment of DMPM cells with the most active derivative and paclitaxel produced a synergistic cytotoxic effect, which was parallel by an enhanced apoptotic response. In the mouse model, i.p. administration of active derivatives was effective, resulting in a significant tumor volume inhibition of DMPM xenografts (range, 58%-75%) at well-tolerated doses, and two complete responses were observed in each treatment group.

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IDENTIFICATION OF LIGANDS FOR THE POLYMORPHIC G-QUADRUPLEX DNA TARGETS BY *IN SILICO* APPROACHES

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G-quadruplex (G4) structures are non-canonical nucleic acid conformations occurring in guanine-rich sequences connected *via* Hoogsteen's type hydrogen bonds[(1)] among four guanines and stabilized by monovalent cations (Figure 1).

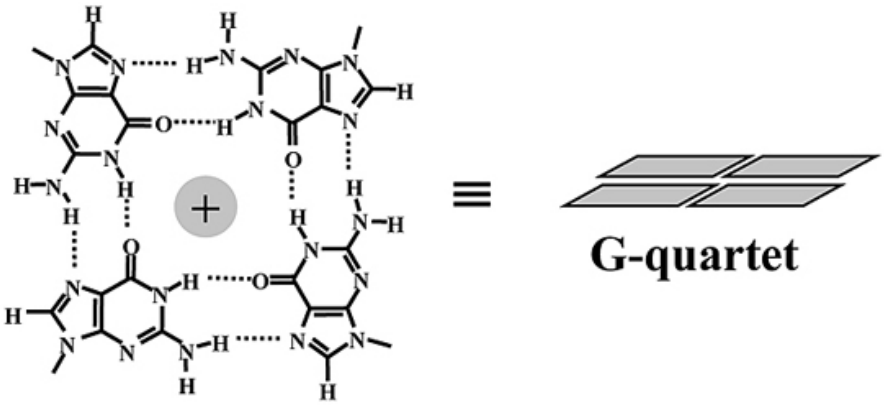

Figure 1: Hoogsteen's type hydrogen bonds in G4 core forming typical G-quartets.

G4 common locations in the genome are at key positions, such as at telomeric ends, ribosomal DNA, RNA, or gene promoter regions. Typically, neoplastic pathologies are related to these G4 regions, but recently also viral infections have been found involved, and likely other diseases will be too. The Protein Data Bank (PDB) includes structural models of G4 sequences with and without stabilizing agents determined by several methods. These models are ideal starting points for rational drug discovery campaigns.

With this respect, our *in silico* work started with the characterization of PDB models of human telomeric sequence *d*[$AG_3(T_2AG_3)_3$] (h-TELO) with conformational studies, docking simulations[(2)] and, more recently, virtual screening experiments[(3)] carried out by means of combined ligand/structure based approaches. Most of the above mentioned works were carried out considering, as much as possible, the flexibility[(4)] of the h-TELO structure, able to assume different G4 folds and therefore to recognize ligands with different binding modes and affinities.

In this communication a selection of the *in silico* experiences carried out in our laboratory are presented highlighting especially successful identification of new G4 binders.

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STRUCTURE-BASED DISCOVERY OF THE FIRST ALLOSTERIC INHIBITORS OF CYCLIN-DEPENDENT KINASE 2

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The classic approach of targeting the ATP site of protein kinases has recently come up against selectivity issues, which can be considerably reduced by following an allosteric modulation approach. Allosteric targeting of protein kinases via displacement of the structural αC helix with type III allosteric inhibitors is currently gaining a foothold in drug discovery. Recently, the first crystal structure of CDK2 with an open allosteric pocket adjacent to αC helix has been described,(1) prospecting new opportunities to design more selective inhibitors. However, the structure has not yet been exploited with structure-based design, and type III allosteric inhibitors of CDKs have not yet been reported.

In this work we report the results of a virtual screening campaign (>600.000 compounds, Asinex collection) that allowed us discover the first-in-class type III allosteric ligands of CDK2. Using a combination of high-throughput docking and post-docking analyses made with our in-house tool BEAR (2), seven allosteric ligands (hit rate of 20%) with micromolar affinity for CDK2 were identified, some of them inhibiting the growth of breast cancer cell lines in the micromolar range. Competition experiments in the presence of a potent ATP-competitive type I inhibitor confirmed the truly allosteric nature of these ligands, in agreement with their design. Of these, compound **2** bound CDK2 with an apparent dissociation constant K_d of 3 μM and inhibited the proliferation of MDA-MB231 and ZR-75-1 breast cancer cells with IC_{50} values of approximately 20 μM, while compound **4** had a K_d of 71 μM and IC_{50} values around 4 μM. Hit expansion through analogue search of the most potent inhibitor **4** revealed an additional ligand **4g** with similar *in vitro* potency on breast cancer cells. *In vivo* experiments on compound **4** were also performed.

In conclusion, this study enabled the structure-based identification of the first type III allosteric ligands of a member of the CDK family of kinase targets. In particular, compounds **2** and **4** can be considered promising enough to enter into a drug optimization phase aimed at improving their potency and cellular permeability.

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DESIGN AND SYNTHESIS OF AZOLYLMETHYL-PYRROLOQUINOLINES AS POTENT AND SELECTIVE NON STEROIDAL CYP19 INHIBITORS

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Nowadays, aromatase CYP19 competitive inhibitors (AI) are the first choice as adjuvant therapeutics for postmenopausal breast cancer patients. However, aimed to overcome the little specificity and the resistance drawback of the clinically used inhibitors such as Letrozole, the search for potent and selective AIs still remains an engaging subject. Our design strategy consisted in combining the angular tricyclic pyrroloquinoline core from our antimitotic phenyl-pyrroloquinolinone derivatives (PPyQs) with a determinant structural element derived from non-steroidal CYP19 inhibitors of the third generation. In particular, the 2-PPyQs endowed with a certain aromatase inhibitory activity[(1)] and antimitotics 7-PPyQs were chemically modified by introducing an imidazolylmethyl or triazolylmethyl group at the selected position (Figure). By this way, the proposed compounds are endowed with an aza-ring for a strong interaction with the heme iron atom of CYP19[(2)] and the bulky tricycle structure having a geometry similar to that of the natural substrate (Figure). Their inhibitory activity was evaluated both by an enzymatic HTS kit and the tritiated water release assay in H295R cells. The results showed that, among the synthesized compounds, imidazol derivatives (**11**, **13**, **14** and **21**) and the triazolylmethyl derivative **22** exhibited an inhibitory potency against aromatase comparable to Letrozole chosen as reference compound. The molecular modelling study along with the predict pharmacokinetics profiles of compounds demonstrated that the pyrroloquinoline scaffold represents a starting point for the development of new pyrroloquinoline-based aromatase inhibitors. Experiments aimed to evaluate the affinity of the new inhibitors against CYP11B1 and CYP17 revealed interesting results and structure activity relationships.

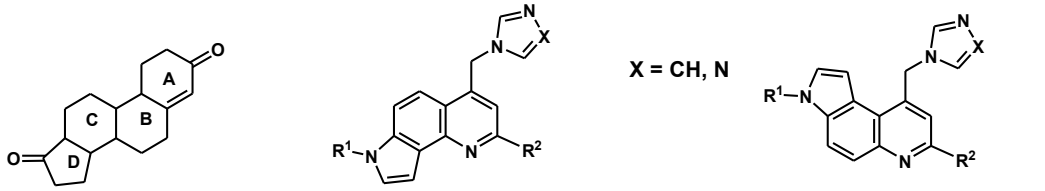

Figure. Structural similarities between the aromatase substrate androstenedione and the proposed CYP19 inhibitors 4-azolylmethyl-PQs.

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N,N-DIALKYL-5-ISOTHIOCYANATO-2-PHENYLINDOL-3-YLGLYOXYLAMIDE: AN IRREVERSIBLE LIGANG TO STUDY THE TRANSLOCATOR PROTEIN

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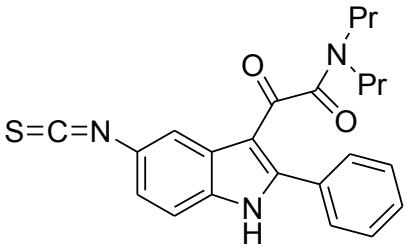
Irreversible ligands are maybe useful tools to study receptor functions in various physiological processes.

In the last decade, our research group has been involved in the medicinal chemistry of the 18 kDa translocator protein (TSPO), a mitochondrial protein implicated in a variety of biological processes (steroidogenesis, cell growth and differentiation, apoptosis induction, etc.), and whose basal density is altered in several diseases, including a variety of tumours, neuropathologies and neuroinflammations, anxiety and mood disorders.[(1,2)] We have previously described a series of *N*,*N*-dialkyl-(2-phenylindol-3-yl)glyoxylamides as potent and selective TSPO ligands.[(3,4)] Starting from these derivatives, we designed novel TSPO irreversible ligands bearing an electrophilic isothiocyanato group, without or together with a NBD-fluorescent probe, showing high affinities for the target protein.

Biological characterization of the fluorescent irreversible TSPO probe, carried out by using fluorescent spectroscopy in human glioma cells, revealed its ability to specifically and irreversibly label TSPO.[(5)] In the present study, we investigated the functional effect induced by the irreversible interaction of derivative **I** with TSPO. Our results showed that nanomolar dose of **I** influenced the functionality of mitochondrial permeability transition (MPT)-pore complex, of which TSPO is an essential component. Furthermore, in a tumor cell model, compound **I** resulted effective to activate mitochondrial apoptosis pathways.



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ANTIPROLIFERATIVE ACTIVITY AND INTRACELLULAR TARGETS OF NOVEL COPPER(II) COMPLEXES

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Copper is the third most abundant transition metal in biological systems. Its main role is the catalysis of crucial oxido-reduction processes and it is an essential cofactor for several proteins involved in a variety of biological processes necessary for life. (1) Indeed, the ability of copper to cycle between its oxidized and reduced forms, makes it a suitable cofactor for redox active metalloenzymes.(2) Nevertheless, when copper is present in excessive concentration, the same redox properties can lead to cellular oxidative damage.(3)

As a consequence of their altered metabolism, cancer cells show an enhanced copper uptake with respect to normal ones. This characteristic prompted us to develop copper complexes as potential antitumor agents.

Different mechanisms of action can be responsible for copper complexes cytotoxicity. Firstly, the intracellular copper uptake can cause an oxidative stress through the production of reactive oxygen species and depletion of reduced glutathione. The strong oxidative damage can lead to cell death either by necrosis or by the activation of the apoptotic process. Moreover, it was demonstrated that some copper compounds can induce a distinct pathway of programmed cell death, named paraptosis, characterized by a massive cytoplasmatic vacuolization, mainly derived by the endoplasmic reticulum, and by the absence of caspases activation.(2) Finally, some copper complexes showed also the ability to inhibit proteasome.(4)

In the present study we report the synthesis, the antiproliferative activity and the investigation on the possible intracellular targets accountable for the cellular effect of some Cu(II) dithiocarbamates designed as novel anticancer agents.

In particular, the antiproliferative effect was assayed on three human tumor cell lines: HeLa (cervix adenocarcinoma), H460 (large cell lung cancer) and A549 (non-small cell lung cancer), and the intracellular complex uptake was determined by ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy).

The cell death pathway was studied by flow cytometry using FITC-labeled annexin V and propidium iodide staining and using an anti-active caspase-3 antibody.

The investigation on the intracellular targets accountable for the cytotoxicity highlighted the ability to interfere with the relaxation activity of the nuclear enzyme topoisomerase II and most interestingly, the capacity at very low concentration, to promote the phenomenon of mitochondrial permeability transition (MPT) in the presence of Ca^{2+} on isolated rat liver mitochondria. The induction of MPT is the result of an oxidative stress, related to a significant oxidative effect on mitochondrial critical thiols. The release of pro-apoptotic factors, such as cytochrome c and AIF (Apoptosis Inducing Factor), consequence of the MPT, allowed us to hypothesize that the active Cu(II) complexes trigger the apoptotic pathway by damaging mitochondrial functions, and this mechanism can account for the antiproliferative effect.

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SYNTHESIS AND BIOLOGICAL EVALUATION OF TRANSPLATINUM COMPLEX DERIVATIVES

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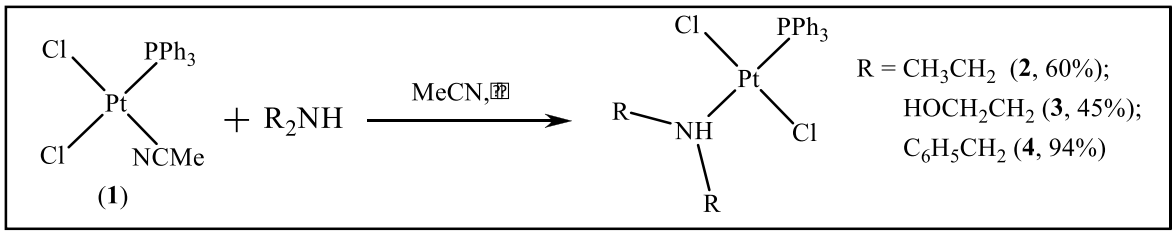
Cisplatin [*cis*-diamminedichloroplatinum(II)] is an effective anticancer agent that shows clinical efficacy against some solid cancer types including testicular, ovarian, head, neck and non-small cell lung cancers.(1) However, the therapeutic efficacy of cisplatin is often limited by intrinsic and acquired resistance and by significant adverse side-effects, such as neuro- and/or renal-toxicity and bone marrow-suppression.(2) The main goal of scientific research focusing on platinum complexes is to identify compounds that show superior efficacy, reduced toxicity, lack of cross-resistance or improved pharmacological characteristics with respect to the parent compound. In this contest, some platinum(II) derivatives carrying one or more phosphine ligands being *trans* to a secondary amine, afforded quite active complexes, even towards cisplatin resistant cells.(3,4) For these derivatives a different mode of action was suggested and in particular, it was proposed that the hydrophobic phosphine ligand could help the complex go through the cell membrane, then enhancing the amount of platinum derivative available for the interactions with intracellular targets.

In the frame of an ongoing project devoted to the synthesis of new platinum(II) derivatives, we developed convenient synthetic procedures for the preparation of complexes $[PtCl_2(PPh_3)(R_2NH)]$,(5,6) starting from easily available *cis*-$[PtCl_2(NCCH_3)(PPh_3)]$ (**1**).(5) In the present study we report the synthesis and the biological evaluation of the *trans*-$[PtCl_2(PPh_3)(R_2NH)]$ (R = CH_3CH_2 (**2**),(6) $HOCH_2CH_2$ (**3**) and $PhCH_2$ (**4**)(Scheme 1).



Scheme. 1. Synthesis of *trans*-$[PtCl_2(PPh_3)(R_2NH)]$.

In detail, the antiproliferative activity was assayed on three human tumor cell lines, HeLa (cervix adenocarcinoma), H460 (large cell lung cancer) and A549 (non small cell lung cancer) and the mechanism of cell death was investigated by flow cytometry using FITC-labeled annexin V and propidium iodide staining. The significant cytotoxic activity of **3**, along with a different behavior in cell death with respect to cisplatin, prompted us to investigate its intracellular targets. Experiments performed on whole cells and on isolated rat liver mitochondria highlighted the ability to affect mitochondrial functions through two different dose-dependent mechanisms.

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SYNTHESIS AND ANTIDERMATOPHYTIC ACTIVITY OF SOME ALLOMALTOL DERIVATIVES WITH CYTOTOXICITY

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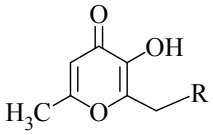
Dermatophytosis, superficial mycoses and candidosis are important causes of morbidity worldwide(1). Especially in patients with impaired immunity, the incidence and severity of these fungal diseases has increased recently. Besides this, a small number of available antifungals often show only fungustatic activity; therefore antifungal resistance occurs frequently(2). Nowadays, the search for new drugs, which are more effective and less toxic than those already in uses, is a current subject. Previously, some novel Mannich bases of 3-hydroxy-6-chloromethyl/hydroxymethyl/methyl-2-substituted 4*H*-pyran-4-one derivatives were synthesized and examined for their antimicrobial, antiviral and anticonvulsant activities by our research group(3-7). In our current studies, chlorokojic acid derivatives were found to have significant antimicrobial and antiviral effects(6-9). Hence, in this study, we will describe the synthesis of ten Mannich bases of allomaltol and present the results of a preliminary evaluation of their cytotoxic and antidermatophytic activities.



R: *acetyl piperazine, piperazine ethylcarboxylate, piperazine tertierybutylmethylcarboxylate, benzyl carboxylate, 4-chlorobenzhydryl piperazine, trans-1-cinnammylpiperazin, 2-chlorophenyl piperazine, 3-chlorophenyl piperazine, 4-chlorophenyl piperazine,4-acetylphenyl piperazine*

In vitro antidermatophytic activity of the derivatives against *Microsporum gypseum*, *Trichophyton mentagrophytes var. erinacei* and *Epidermophyton floccosum* will be screened as broth microdilution method. Terbinafine, itraconazole, ketokonazole, flukonazole, and griseofulvin will be used as the control agents(10). Cytotoxicity will be evaluated by the maximum non-toxic concentrations (MNTCs) of each samples, which will be determined by the method described previously by Özçelik *et al.*(11) based on cellular morphologic alteration.

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NEUTROPHIL CHEMOTAXIS INHIBITORS: NEW TOOLS AGAINST CANCER METASTASIS?

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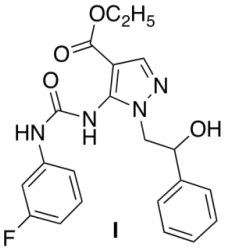
Over 90 % of cancer deaths are due to metastasis development and, at the moment, there is no anti-metastatic drug on the market. The essential prerequisite for invasion and metastasis formation is the tumor cell migration (chemotaxis), a process regulated by different signals that are common of cells responsible for immune processes and inflammation.(1) In this context, it has been observed that tumor cells use the same molecular tools (adhesion molecules, cytokines, chemokines, chemokine receptors) and pathways used by granulocytes in order to spread to distant anatomical sites.(2) Direct associations between tumor infiltration by neutrophils and poor clinical outcome of patients have been described for several types of cancer.(3) A number of studies found that neutrophils modulate the tumor microenvironment to promote tumor progression and cancer cell migration.(4) Conversely, inhibition of neutrophil recruitment by CXCR2 (IL-8 receptor) antagonists resulted in a slower growing ability of tumors *in vivo*.(5) In the last years, our search on neutrophil chemotaxis gave several potent inhibitors able to block granulocyte migration by interfering with intracellular downstream pathways involving different PKC isoforms and p38MAPK.(6) The p38MAPK pathway is known to regulate cancer development by modulating not only angiogenesis, but also cell motility and invasion. Among solid tumors, the high-risk neuroblastoma (NB) (stage-IV) is a pediatric malignant tumor resulting in metastatic dissemination. Moreover, the amplification of MYCN proto-oncogene has been associated with adverse prognosis of NB. Our co-workers recently demonstrated that migration of etoposide-treated NB cells is dependent on p38MAPK and also suggested that the inhibition of this pathway could be a new strategy in limiting the invasiveness of stage-IV NB.(7)

To develop new therapeutic tools for metastasis prevention, we have selected three compounds, among our potent chemotaxis inhibitors (**I**, **II** and **III**),(8,9,10) and their effects on two human NB cell lines, ACN (without MYCN amplification) and SK-N-BE-(2C) (with MYCN amplification) have been tested by the invasion assay. The present study shows that our inhibitors can reduce NB cell migration with a marked effect on ACN cell line. Taking into account the well-assessed potency of these compounds in reducing also neutrophils recruitment, this study strongly supports the hypothesis that our molecules could be very interesting as new multi-targeting preventatives of metastasis.

Detailed results will be reported in poster session.



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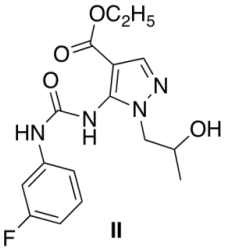

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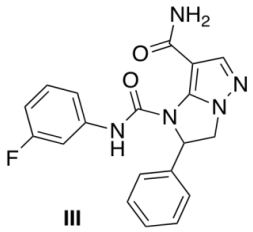

III

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BENZOTHIENO[2,3-c]QUINOLIN-6(5H)-ONE DERIVATIVES AS A NOVEL CLASS OF SELECTIVE TANKYRASE INHIBITORS

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Tankyrases, TNKS-1 and TNKS-2 belong to the PARP superfamily as PARP-5a and PARP-5b. Due to their ability in the transfer of polyADPribose chains (PAR) to targeted proteins they are also referred as ADP-ribosyltransferases ARTD-5 and ARTD-6, respectively.(1) By having an active role in telomere maintenance and Wnt pathway regulation TNKSs take part in the arena of complex processes that orchestrate tissues differentiation and renewal. Aberration of these processes may result in pathological setting as those of many cancers. Therefore, inhibition of TNKSs activity has been recently proposed as new promising strategy in the treatment of cancers.(2)

We have been engaged for several years in the search for novel and potent PARP-1 and -2 inhibitors, and in the unravelling of their mechanism of actions.[3] However, the high sequence homology within the catalytic domain of PARP enzymes hampers the search and development of isoforms-selective compounds.(4) Recently, as an extension of the work in this area, we have been embarked in a project aimed at the search of novel selective TNKS-1 and -2 inhibitors. Thus, a virtual screening procedure was applied to the search of novel TNKSs inhibitors resulting in the selection of 34 virtual hit compounds. Among them a total of six compounds were found able to disrupt, at the concentration of 10 μM, the Wnt signaling pathway in a Wnt gene reporter assay. Two methoxy[*l*]benzothieno[2,3-*c*]quinolin-6(5*H*)-one based hit compounds were discovered also to be potent tankyrases inhibitors at 1μM concentration endowed also with a moderate profile of selectivity (5 fold degree) against the main members of the family PARP-1 and -2.

Starting from these results, an hit to lead optimization process has been carried out in order to infer the structural basis responsible of the inhibitory potency and TNKSs selectivity. A new series of derivatives was designed, synthesized and evaluated for their TNKSs inhibitory activity. With the aim to further investigate the role of each ring of the benzothienoquinolinone nucleus in driving TNKSs selectivity and activity, a 'deconvolution like' approach has been also followed. Wnt pathway disruptor properties were also assessed for the most potent compounds. Finally, different computational approaches spanning from the calculation of physico-chemical properties to deeper docking calculation were employed to gain insights into the activities displayed by the new synthesized compounds.

These findings could be instrumental for further scaffold selection, on the way to more potent and selective PARP or TNKSs inhibitors.

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CARBAZOLE DERIVATIVES AS POTENTIAL NOVEL AGONISTS OF GPER

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Estrogens regulate many aspects of human physiology and influence diverse pathological processes, including the development of hormone-dependent tumors.(1) Although the biological responses to estrogens are mainly mediated by the classical Estrogen Receptors (ERs), the G protein-coupled receptor GPR30/GPER has been recently shown to mediate estrogen signaling in a variety of normal and cancer cells. In particular, GPER mediates relevant physiological responses in the reproductive, nervous, endocrine, immune and cardiovascular systems as well as is a key mediator in the development and progression of several types of tumors.(2-3)

The possibility to differentiate the pharmacology of GPER over that ERs by targeting each receptor subtype has represented a central point in dissecting estrogen signaling. The recent identification of compounds able to bind to and activate or inhibit GPER in a selective manner has greatly advanced our understanding of the role of GPER in numerous biological systems as well as in cancer. (4-7)

In this context, we have *in silico* designed, synthesized and functionally characterized novel carbazole derivatives, one of which presented the ability to selectively activate the GPER-mediated signaling in ER-negative breast cancer cells.

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NEW TITANOCENE DERIVATIVES: SYNTHESIS AND CYTOTOXIC ACTIVITY

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The remarkable antitumor activity shown by *cis*-platinum and other platinum complexes[1-2] has meant that new metal-based anticancer drugs are become a noteworthy subject of research.
Among all synthesized compounds, a great deal of research has been focused on titanium-based complexes, whose cytotoxic activity against solid tumors is well known. In particular, titanocene dichloride, Cp_2TiCl_2 (TDC, Fig.1) shows medium antiproliferative activity *in vitro,* but promising results *in vivo,* [3,4] reaching Phase II clinical trials.

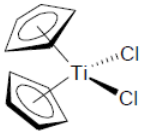

Figure 1: Titanocene dichloride (TDC).

The interesting results obtained with titanocene dichloride have encouraged research to develop new complexes of this metal which might have greater hydrolytic stability and a higher cytotoxic activity.[5]
In this regard, in the present study, novel titanocene-complexes **I** (Fig.2) have been synthesized and evaluated for their growth regulatory effects in MCF7 and SkBr3 breast cancer cells.

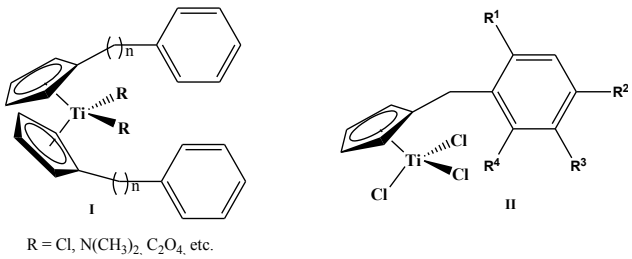

Figure 2: Titanocene-complexes **I** and **II**.

Several of the compounds used have demonstrated a stronger anti-proliferative activity on both MCF7 and SkBr3 breast cancer cells than the titanocene derivatives **II** (Fig. 2) recently synthesized and evaluated on the same model systems.[6]
Therefore, the capability of the novel titanocene complexes to elicit strong repressive effects on cancer cell growth might be relevant towards innovative and promising anti-cancer drug strategies.

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NMR STUDIES OF GRK2 INHIBITORS

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Emerging evidence suggests that GRK2, the most widely studied member of the G protein-coupled receptor kinases (GRKs), is up regulated in pathological situations such as heart failure, hypertrophy and hypertension, and its inhibition offers a potential therapeutic solution to these diseases.(1) Short peptides derived from HJ loop of GRK2 showed to be both potent and selective inhibitors of GRK2.(2) Analysis of the 3D structure of this loop within the X-ray structure of GRK2(3) suggested that cyclization could be a suitable way to stabilize the active conformation of these peptides. Cyclic peptides demonstrated to increase the inhibitory potency of the linear parents. We studied, by solution NMR, the conformational preferences of the most interesting derivatives and their potential interaction with GRK2. In particular, we distinguished between ATP competitive and non-competitive inhibitors using ligand-based NMR techniques.

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DESIGN, SYNTHESIS AND BIOSTRUCTURAL CHARACTERIZATION OF CARBONIC ANHYDRASE INHIBITORS

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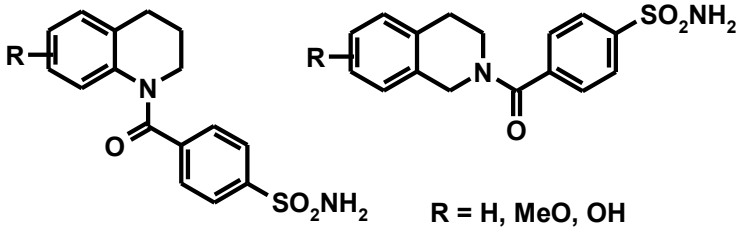
Carbonic anhydrases (CAs, EC 4.2.1.1) play a key role in many physiological processes. However, they are involved in some pathological pathways, thus resulting interesting targets for the treatment of glaucoma, cancer, obesity, and epilepsy. The diffuse localization in many tissues of the 16 human CA (hCA) isoforms limits the potential clinical applications of some well-known carbonic anhydrase inhibitors (CAIs) such as acetazolamide, zonisamide, and topiramate. So the development of newer CAIs possessing high potency and selectivity against specific isoforms might be an attractive strategy to obtain safer compounds acting against this "old target". The CA inhibition is mainly mediated by interaction with the coordinating zinc ion in to the catalytic binding site. The CAIs generally display R-SO_2NH_2 as a key structural requirement. In fact, the deprotonated nitrogen atom of sulfonamide group substitutes the zinc-bound water molecule thus inhibiting enzyme activity. Moreover, several additional interactions of the most CAIs with the hydrophilic and/or hydrophobic region of the active site can be mediated by R-portion. These interactions are generally considered responsible to control both activity and selectivity toward specific isoforms.

Searching for new potent and selective CAIs, we focused our efforts on the introduction of sulfonamide moiety at *N*-position of a large series of 1,2,3,4-tetrahydroisoquinolines.[(1-5)] The evaluation of CA inhibitory effects confirmed that these new heterocyclic-based sulfonamides were active CAIs at nanomolar concentration. The crystal structures of some of these derivatives in complex with the isoform hCA II confirmed the interactions within the catalytic site.



Based on these results herein we report the synthesis of a small series of quinoline and isoquinoline derivatives that have been designed as analog compounds of previously investigated sulfonamide inhibitors. Through a facile synthetic pathway we prepared the designed compounds that were tested to evaluate their enzyme inhibitory effects toward some selected isoforms (hCA VII, hCA IX, and hCA XIV). We also performed computational studies to explore the chemical features that control the enzyme recognition process as well as selectivity. Moreover, binding interaction analysis has been also carried out through the co-crystallization of selected inhibitors with hCA II isoform thus describing the main protein-inhibitor interactions within CA catalytic pocket.

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NOVEL 1,2,4-TRIAZOLE DERIVATIVES AS ATP COMPETITIVE TYROSINE KINASE RET INHIBITORS. SYNTHESIS AND FUNCTIONAL EVALUATION

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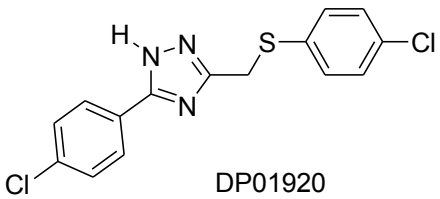
Thyroid carcinoma (TC) is a malignant endocrine tumour arising from either follicular or parafollicular epithelial thyroid cells. Its treatment of choice is represented by medical surgery, radioiodine and hormone suppressive therapy. However, disease can persist or recur, with local and distant metastases which are often fatal. Recent advances in the knowledge of TC development identified receptor tyrosine kinase RET as a viable and promising target. Actually, gain of function mutations and over-expression of RET are causally linked to tumour growth and aggressiveness. Accordingly, a number of ATP competitive RET inhibitors are current involved in clinical trials, showing a promising degree of efficacy.[(1,2)]

Exploiting a receptor-based virtual screening campaign, a novel 1,2,4-triazole hit has been disclosed, DP01920, which proved to inhibit effectively both wild-type and V804L mutant RET. Moreover, tested on a panel of both receptor and cytoplasmic kinases, DP01920 showed good inhibitory properties against VEGFR-1, VEGFR-2 ;VEGFR-3 and PDGFRβ, thus standing out as a novel and promising multi-effective kinase inhibitor. Moving from this exciting result, a small library of 1,2,4-triazole derivatives, of general formula **1**, has been developed, to analyze thoroughly structure-activity relationships of this class of compounds and get to even more effective analogues.

Here we present the synthesis and the functional evaluation of the novel compounds.



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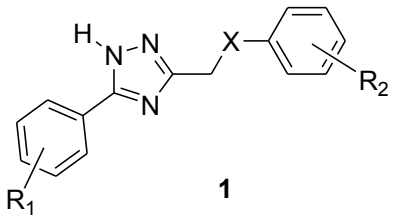

1

X = S, O, CH_2
R_1 = R_2 = H, Cl, CF_3, CH_3, NO_2

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NOVEL 2-ARYL SUBSTITUTED PYRIDOTHIOPYRANO-FUSED PYRIMIDINES: SYNTHESIS AND VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 2 (VEGFR-2) INHIBITION

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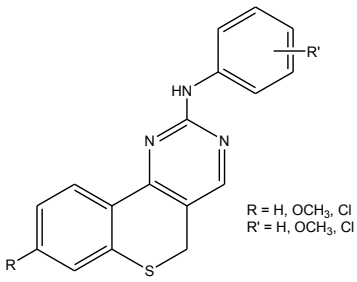
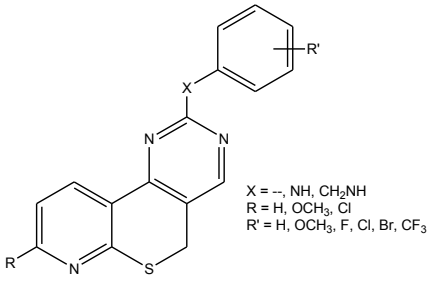
Angiogenesis is a process implicated in tumour growth and metastatic dissemination. Hence, the molecular basis of tumour angiogenesis has been extensively studied and the Vascular Endothelial Growth Factor (VEGF) pathway has emerged as one of the most important positive modulators of this process. The VEGF ligands belong to a family of highly homologous growth factors, that signal through cell surface receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2. These receptors have the peculiarity to be highly conserved throughout the whole class, to be largely expressed in endothelial cells and primarily involved in angiogenesis.[(1,2)] From the understanding of angiogenesis mechanisms in tumorigenesis, the therapeutic strategies blocking VEGF/VEGFR-2 signalling systems became a promising and well-validated approach for suppression of pathological neovascularisation and, consequently, for the treatment of both solid tumours and haematological malignancies, leading to the rational design and development of agents targeting this pathway. The new agents range from anti-VEGF monoclonal antibodies, such as bevacizumab, to small-molecule ATP-competitive VEGFR inhibitors, including compounds from distinct heterocyclic classes.[(3)] In this regard, moving from the structures of some reported anilino substituted pyrimidines, we recently designed the series of benzothiopyrano-fused derivatives **I**, bearing the aniline substituted pyrimidine core. These compounds showed to be appreciable VEGFR-2 competitive inhibitors, possessing interesting antiangiogenic effects.[(4)] On the basis of these results, we describe the preparation of the novel pyridothiopyrano-fused pyrimidines of general formula **II**, characterized by a substituted aryl moiety in the 2-position.



I



II

The capacity of targeting VEGFR-2 was assayed *in vitro* using as reference compound the specific inhibitor SU5416. The antiproliferative activity was evaluated in HUVEC and human tumor cell lines (HeLa, A-431 and MSTO-211H) and the antiangiogenic activity was confirmed in an *ex vivo* rat aortic ring assay.

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NEW THIADIAZOLINE AND THIADIAZOLE ANALOGUES OF K858: ANTIPROLIFERATIVE ACTIVITY IN A MODEL OF PROSTATE CARCINOMA

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Despite the presence of many effective anti-neoplastic agents, their severe side effects together with the appearance of mutant tumors limit the use of these drugs and increase the need for new anticancer drugs. Kinesin Eg5, a microtubule-based motor protein important for many key cellular functions such as mitotic spindle assembly and chromosome segregation, represents an attractive chemotherapeutic target: it is over-expressed in many proliferative tissues while it is almost absent in non-proliferative tissues.(1) Starting from thiadiazoline K858, a promising Eg5 inhibitor (IC_{50} = 1.3 μM against HCT116 cell line),(2) we designed a large number of analogues provided with this heterocyclic nucleus. The chemical exploration of this scaffold has been performed by the introduction of aliphatic, cycloaliphatic, and (hetero)aryl substituents at C5 of the thiadiazoline ring (R and R_1) and by the modification of the lateral amidic chains (R_2 = Me, Et, haloalkyl). Spirothiadiazoline isomers (R_3 = alkyl) were separated by chiral HPLC and their structure characterization was performed. The two-steps synthesis has been carried out by reacting different carbonyl compounds with thiosemicarbazide in ethanol under acid catalysis and by the consequent cyclization of the thiosemicarbazone intermediates into 1,3,4-thiadiazolines with different anhydrides. Moreover, oxidation of the thiadiazoline nucleus gave the corresponding K858-thiadiazole. In conclusion, compounds reported in Figure 1 could possess the pharmacophoric requirements to display a potential antitumoral activity.

Investigation of the pharmacological effects of newly synthesized derivatives, at two discrete concentrations (1 and 10 μM) and three times of exposure (24, 48, and 72 h), gave important information regarding the structure-activity relationships for this scaffold in a cancer model never studied so far (PC3, a human prostate cancer cell line) and in normal cell lines with respect to K858 and other tubulin-binding drugs.

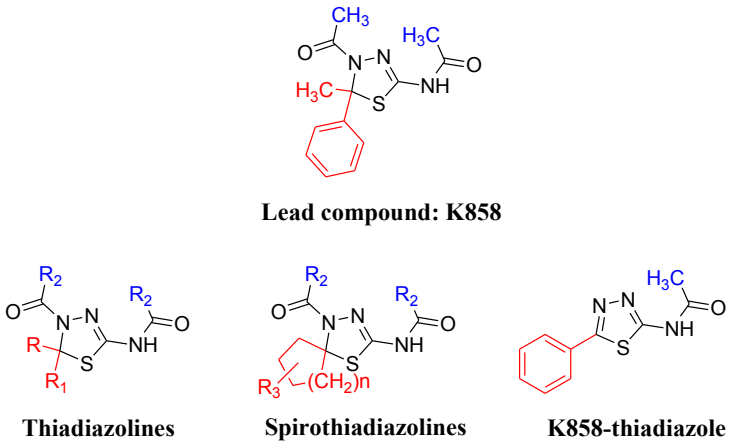

Figure 1: structures of lead compound K858 and its newly synthesized analogues.

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BINDING OF N6-ISOPENTENYLADENOSINE TO FARNESYL DIPHOSPHATE SYNTHASE: AN NMR INVESTIGATION

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The enzyme farnesyl diphosphate synthase (FPPS) a key enzyme in the mevalonate, isoprenoid biosynthesis pathway, has been identified as an interesting target for anti-tumor and anti-infective drug leads. FPPS inhibitors, represented by bisphosphonates drugs are nowadays used in the treatment of malignant bone disease but their employment in different tumor or infective diseases is limited by their adverse pharmacokinetic properties. Therefore there is an increasing interest in the development of new antitumor or ant infective molecules acting as FPPS inhibitors and endowed with improved pharmacokinetic properties.
N6-Isopentenyladenosine (IPA) is a modified nucleoside exhibiting anti-tumor effects on human and murine cells. Mounting biochemical evidence show that this molecule is able to modulate pathways controlled by FPPS. However the mechanism by which IPA may control cancer cell growth and its potential biological target remain unknown.
Here we present experimental data evidencing that FPPS is target of IPA activity. NMR competition binding experiments based on saturation transfer difference (STD) and transfer NOE (TrNOE) measurements show that IPA binds FPPS in the active site normally occupied by biphosphonate, while it does not interact with the recently identified allosteric site.
NMR data validate an "inverse virtual screening" procedure where a set of protein targets are screened in the search of IPA target.

CHEMISTRY AND BIOLOGICAL ACTIVITY OF PLATINUM AMIDINE COMPLEXES

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Pt amidine complexes represent a new class of potential antitumor drugs that contain the imino moiety HN=C(sp2) bonded to the Pt center, similarly to the iminoether derivatives, which were recently shown to be the first Pt(II) compounds with a trans configuration endowed with anticancer activity.(1,2) Furthermore, the chemical and biological properties of Pt amidine complexes, and more generally of Pt imino derivatives of the type depicted in Figure 1, can be tailored given that some features can be rationally modified, such as: 1) The cis or trans geometry of the complex; it is generally observed that the geometry of the starting nitrile complex is maintained in the final amidine products. 2) Either the Z or E configuration can be favored depending on the nature of the nitrile ligand, the entering HNR1R2 amine reactant, and the reaction conditions. Notably, Z conformers of amidines are often more biologically active than the corresponding E isomers, in contrast to what has been observed for the iminoether derivatives.(3) 3) The lipophilicity of the final complex can be modified by changing R and X as a tool to improve the biological activity and to overcome tumor resistance.(4,5) 4) The charge of the complex can be modified by substitution of ancillary anionic ligands with entering nucleophile reagents. 5) The nature of the ancillary ligands with different trans-influence can be varied, thus modifying the ligands substitution kinetics. 6) The introduction of heteroatoms and/or suitable functional groups can allow additional interactions with DNA(6,7) and can modify the biodistribution of the Pt drug.(8) Herein we summarize published results concerning Pt imino complexes endowed with improved cytotoxic activity over that of classical platinum(II) chemotherapeutics.(9, 10)

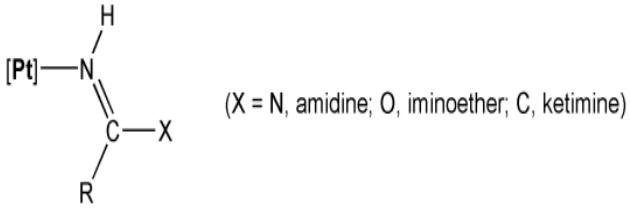

Figure 1. General structure of platinum imino derivatives.

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IDENTIFICATION OF A NOVEL STAT3 INHIBITOR STRUCTURALLY RELATED TO THE OXADIAZOLE DERIVATIVE MD77

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Signal Transducer and Activator of Transcription 3 (STAT3) is a latent cytoplasmic protein over-expressed in various cancer cell lines.[1,2] STAT3 participates in oncogenesis by stimulating cell proliferation and preventing apoptosis; it has been proven as a suitable and selective[3,4] target for anticancer therapy. With the aim to discover new STAT3 inhibitors,[5-7] we focused the efforts on a compound previously synthesized by our research group, **MD77** [4-(4-chloro-phenyl)-1,2,5-oxadiazol-3-N-(4-trifluoromethyl-phenyl)-amide],[8] which had been shown to be able to bind the STAT3 SH2 domain. To improve **MD77** activity and selectivity SAR studies were performed, which allowed us to identify compound **1** as novel STAT3 direct inhibitor, provided with a better profile with respect to the lead.

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The synthesis, crystallographic studies and biological activity of compound **1** will be presented.

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UROTENSIN II RECEPTOR REGULATES CELL MOTILITY/INVASION AND DETERMINES PROGNOSIS OF BLADDER CANCER

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Non Muscle Invasive Bladder Transitional Cancer (NMIBC)/superficial and Muscle Invasive Bladder Transitional Cancer (MIBC)/invasive have different genetic profile and clinical course. However, among NMIBC the prognosis is not completely predictable, since 20% of the cases experience a relapse, even in the form of MIBC and, therefore, the search for new molecular prognostic markers is urgently needed. In this light, we have recently reported that expression of Urotensin II (U-II) Receptor (UTR) is correlated to the prognosis of prostate adenocarcinoma.

Here, we have investigated UTR expression in 4 bladder cancer cell lines and we have found that it was expressed in all bladder cancer cell lines at different extent. Moreover, we have evaluated the biological effects of human agonist (U-II) and antagonist (urantide) on proliferation of human bladder cancer cell lines. UT-II did not induce significant effects on cell growth while the UTR antagonist urantide inhibited cell proliferation.

To investigate upon the role of UTR in bladder cancer we also studied the biological functions of UTR on in vitro bladder cancer cells using either the UT-II antagonist urantide or knocking down UTR expression through the use of a specific shRNA in RT112 and T24 bladder cancer cells. We found that the downregulation of either the function or expression of UTR significantly blocked the motility and invasion of cancer cells. Interestingly, the addition of urantide to transfected cells potentiated the effects of shRNA-induced UTR downregulation in RT112.

UTR expression clearly discriminated between NMIBC and MIBC. In addition, we have evaluated in a series of NMIBC a positive and significant correlation between low UTR expression and shorter disease free survival.

The evaluation of UTR expression can discriminate between NMIBC at high and low risk of relapse. Moreover, our data suggest that UTR is involved in the regulation of motility and invasion of bladder cancer cells.

BICYLIC ACETALS AS POTENTIAL INHIBITORS OF GOLGI α-MANNOSIDASE II

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Cancer causes 5.9 % of deaths in high-income countries and 2.4 % of deaths in the world.(1) Selectins, carbohydrate recognizing proteins, play a crucial role in binding metastasizing tumors.(2) Ligands for selectins are often modified glycosylation patterns on the outer surface of the cancer cells.(3) Studies have shown an overexpression of different sugar-hydrolyzing enzymes in these cells, making such enzymes promising targets for new anti-cancer drugs.(4) Especially inhibition of the Golgi α-mannosidase II (GM II) has shown tumor repression.(5)

GM II, a glycosyl hydrolase, is a 125 kDa type II transmembrane protein(6) that plays an essential role in the *N*-glycosylation pathway of asparagine side chains. The high specific cleavage of two mannose units (α-(1,3) and α-(1,6)) of the intermediate $GlcNAcMan_5(GlcNAc)_2$ takes place in the active site of the enzyme, two aspartate residues and a zinc cation involved. The GM II is a retaining glycosidase and cleaves the sugars in a two-step-S_N2-mechanism that preserve the configuration of the anomeric C-atom.(7)

Already available inhibitors which are mostly derivatives of swainsonine have different side effects due to low selectivity.(8) The future goal is the synthesis of selective, covalent-reversible inhibitors with a long resting time in the catalytic site of the enzyme. QM-calculations and docking-simulations have shown that bicyclic acetals are promising candidates in terms of both, high affinity to the target enzyme and reaction kinetics. Based on *L*-gulose, we synthesize 1-2 and 1-6 bridged species as the reactive subunit of more complex inhibitors. The analogous *D*-glucose derivatives were synthesized in order to validate the theoretical construct and examine β-Glucosidase inhibitor quality. We use known ways(9-12) for the synthesis of potential inhibitors. New strategies like cyclo-addition and especially olefin ring-closing metathesis (RCM) are alternative promising ways to the desired acetals. IC_{50} and Km values were determined via enzyme absorption assays.

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BONE-SEEKING MMP INHIBITORS FOR THE TREATMENT OF MULTIPLE MYELOMA

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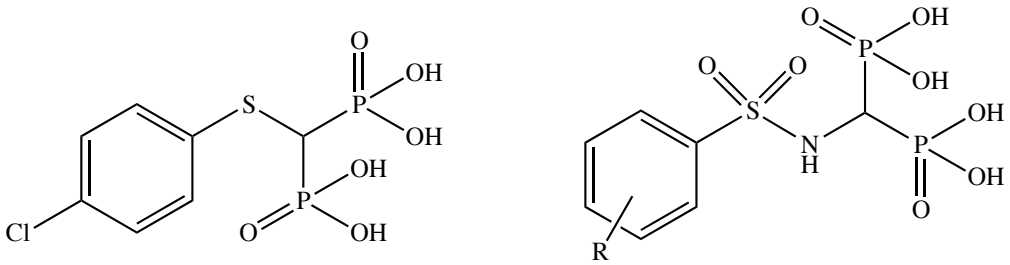
Multiple myeloma (MM) is a type of cancer that originates in the plasma cells in bone marrow. Plasma cells are important in fight infection by producing proteins called antibodies. With multiple myeloma, plasma cells grow out of control in the bone marrow and form tumors in the areas of solid bone. The growth of these bone tumors weakens the solid bones, causing skeletal related events (SRE) and impairing patient's quality of life.

Recently, we have identified a strong correlation between tumor derived MMP-2 and MMP-9 and the progression of many cancers in the bone microenvironment, including MM.(1) The design of selective inhibitors of individual MMPs for the treatment of multiple myeloma is important to overcome the undesiderable effects of broad-spectrum matrix metalloproteinase (MMP) inhibitors as showed by the failure of previous clinical trials. Therefore we have developed novel bone seeking MMP inhibitors (BMMPIs) that are highly selective to MMP-2 and structurally based on the bisphosphonate clinically used, Tiludronate (Fig. 1).(2)



Tiludronate BMMPIs

Fig. 1. BMMPIs structure

In the current study the biological effect of our best BMMPIs has been evaluated in more depth. In vitro, BMMPIs showed greater enzyme inhibition, and specificity for MMP-2 than previously synthesized bisphosphonates (e.g. Zoledronate, Etidronate and Tiludronate) with IC_{50} values in the nanomolar range. Further treatment of the 5TGM1 mouse MM cell line with low micromolar concentrations of the BMMPI inhibitors significantly reduced cell viability through inhibition of cell growth ($p<0.05$). *In vivo,* 5TGMI tumor-bearing mice receiving BMMP inhibitors three times a week, showed significant increase in overall survival compared to vehicle and bisphosphonate treated mice over 80 days ($p<0.05$). This was associated with a reduction in tumor burden and protection from tumor-associated bone loss compared to vehicle treated mice.

X-rays and histomorphometry showed a significant reduction in the number of osteolytic lesions of treated mice. These data suggest that given the roles for MMPs in tumor progression in bone, our novel BMMPIs may be effective in the treatment of MM. We predict that MMP-2 specific BMMPIs may be more effective than current clinically used bisphosphonates for the treatment of MM, and may eliminate undesirable side effects of broad-spectrum MMP inhibitors due to their high specificity and bone seeking nature. We are currently continuing to characterize these BMMPIs both as single agents, and in combination with existing MM treatments (eg. Bortezomib) for the treatment and eradication of MM.

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SELECTIVE G-QUADRUPLEX STABILIZERS: SALPHEN-LIKE COMPLEXES WITH ANTIPROLIFERATIVE ACTIVITY

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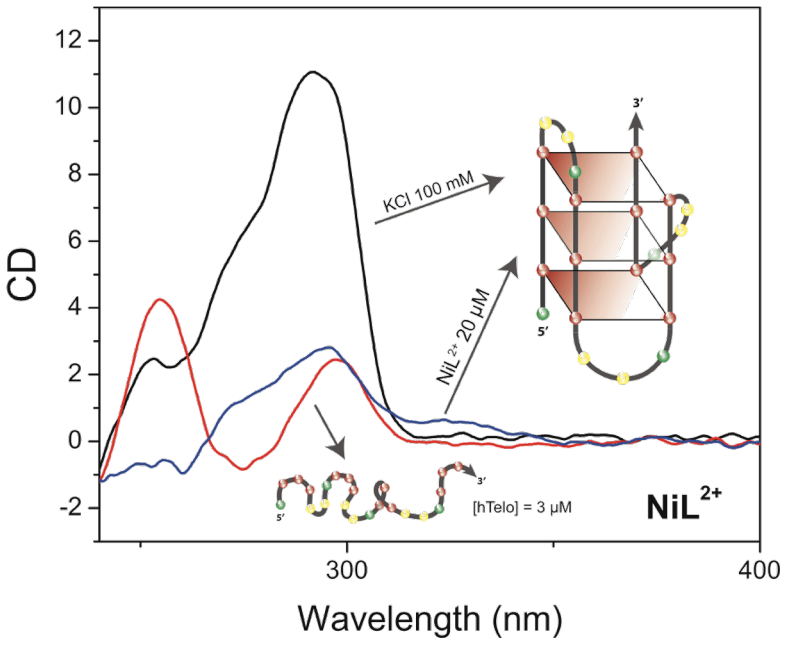
The main goal of anticancer research is to develop therapeutic agents with improved biological activity against cancer cells. Chasing this purpose, recent years have seen an increased interest in the study of small molecules able to bind the deoxyribonucleic acid (DNA) when it assumes secondary structures known as G-quadruplexes (G4) in preference over its B form.[1] G4-DNAs are found in chromosomal DNA mainly in telomeric sequences, but also in some sequences that seem to play important roles in regulating the expression of genes (among them some oncogenes such as *c-myc*).[1,2]

Schiff base complexes derived from *N*,*N*′-bridged tetradentate ligands involving N_2O_2 donor atoms present very favourable features to act as G4 binders. Thus, a series of square-planar and square pyramidal metal complexes, ML^{2+} (M = Ni, Cu, and Zn), have been synthesized and characterized. Their affinity for wild-type *hTelo* and *c-myc* G-quadruplexes DNA and for ct-DNA was investigated by UV absorption spectroscopy, circular dichroism and viscosimetry. The experimental data together with computational approaches collectively suggest that the complexes bind effectively to G-quadruplexes by direct end-stacking with high selectivity with respect to B-DNA. The best G4-DNA stabilizer was found to be NiL^{2+} with a binding constant of about 6.0×10^6 M^{-1}. More interestingly NiL^{2+} is able to induce conformational changes favoring *hTelo* quadruplex DNA formation without the presence of KCl.



The compounds showed concentration- and time-dependent cytotoxity towards HeLa and MCF-7 tumor cell lines. Furthermore, the complexes achieved significant effects on cell cycle distribution with G2/M arrest in HeLa cells and G0/G1 arrest in MCF-7 cells. The distinct cell cycle arrest phase observed in cells treated with the ML^{2+} complexes might be due to the different consequences of DNA binding properties in different cancer cells.

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FROM HISTONE METHYLTRANSFERASE TO HISTONE DEMETHYLASE INHIBITORS: NOVEL QUINAZOLINES ANALOGUES

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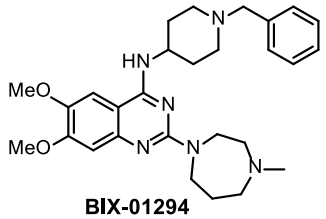
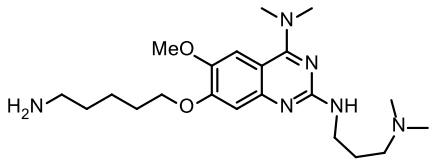
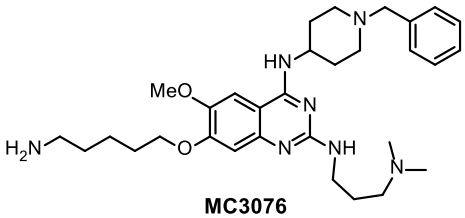
DNA is wound around clusters of eight histone proteins, and together, histones and DNA within the nucleosomes make up the chromatin. Histones not only keep DNA organized, but they are also known to regulate gene expression. Specifically, modifications of histone proteins, such as acetylation, methylation, phosphorylation, ubiquitylation and sumoylation, as well as ribosylation interfering with RNA polymerase activity and DNA transcription, represent one of the main mechanisms of the epigenetic modulation of gene expression. Aberrant expression of histone-modifying enzymes has been linked to various human diseases, such as neurological disorders and cancer. While studies on histone acetylation are known from years, studies on histone methylation and relative modulators are still at the beginning.(1)



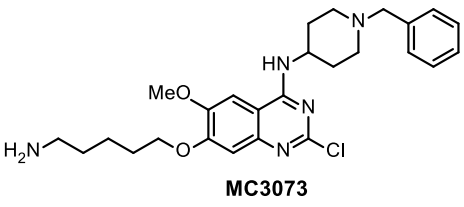
BIX-01294



MC3074



MC3076



MC3073

BIX-01294, a diazepin-quinazoline-amine derivative inhibits G9a and G9a-like protein (GLP) lysine methyltransferases and reduces methylation levels of H3K9 at several G9a target genes.(2) BIX represented our lead compound to design novel potential inhibitors of histone methyltransferases and/or demethylases acting on H3K9, (G9a/GLP and KIAA1718, respectively). Indeed, G9a and KIAA catalyze opposite reactions on H3K9 (the former adding and the latter removing the Me2 histone mark), and may recognize Lys in either unmethylated or methylated state, it being the substrate or reaction product, alternatively. Thus, we replaced the *C*7-methoxy group of BIX with a Lys-mimic side chain (5-aminopentyloxy chain), obtaining increased G9a/GLP inhibition (MC3076) respect to BIX.(3) Since MC3076 as well as BIX were found able to inhibit also the demethylase KIAA1718, according to crystallographic studies performed on MC3076 complexed with either GLP or KIAA and supported by docking simulations we prepared a new series of quinazoline analogs in order to improve their potency and selectivity against KIAA demethylase.

We started with the simplification of the MC3076 structure by insertion of small substituents at the *C*2 and/or *C*4 quinazoline ring positions to obtain new quinazolines (MC3073, MC3074, etc.) highly potent against KIAA1718 and devoid of any GLP inhibiting activity.[4]

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IDENTIFICATION OF A NEW POTENTIAL LIGAND OF THE ESTROGEN RECEPTOR BETA, INHIBITING HUMAN OVARIAN CANCER CELL GROWTH

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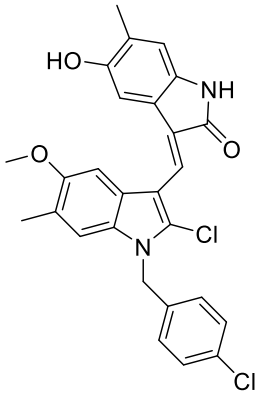
Estrogens have important functions in regulation of cellular processes like growth and differentiation in human ovary and endometrium. Their effects are mediated by two estrogen receptors (ER), ERα and ERβ, which belong to the nuclear receptor superfamily acting as ligand-activated transcription factors. In particular ERβ appears to exert tumor-suppressing actions in ovarian cancer development, since the loss of ERβ expression was shown to have great impact on proliferation, apoptosis and motility of ovarian cancer cells.

In this communication we wish to describe the activity profile of compound NSC 734203 which was found to complex ERβ receptor.



NSC 734203

Compound NSC 734203 emerged as a new lead from a small library of Knoevenagel adducts, that we synthesized by reacting oxindoles with indole aldehydes properly substituted,[(1)] owing to its significant growth inhibition of IGROV1 ovarian cancer cells.

In order to achieve a possible explanation of its antiproliferative effect, some in depth studies have been performed. Based on flow cytometric analysis, the compound inhibited the proliferation of these cells and caused their accumulation in the G0/G1 phase of the cell cycle, moreover, the proliferative response after stimulation with 17-β-estradiol was inhibited. Mass spectrometry analysis showed that NSC 734203 is able to reach the nucleus already 6 hours after administration and the chromatin binding assay, for the estrogen receptor (ER)/DNA complex, demonstrated that it forms a complex with ERβ colocalized on chromatin. The amount of c-myc and cyclin D1 mRNA was decreased significantly in the treated cells, while the p21WAF1 gene expression was not influenced. These data suggest that NSC 734203, complexed with ERβ receptor, could interfere with the transduction mediated by ERβ, supporting its activities as oncosuppressor.

Finally, the synthesis of a new series of analogues of the lead compound NSC 734203 will also be discussed.

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PROPERLY SUBSTITUTED QUINAZOLINE ANALOGUES OF BIX-01294 LOSE THE ABILITY TO INHIBIT H3K9 METHYLTRANSFERASES AND GAIN SELECTIVITY IN DNMT3A INHIBITION

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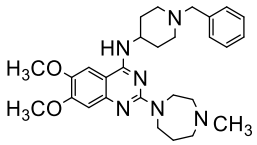
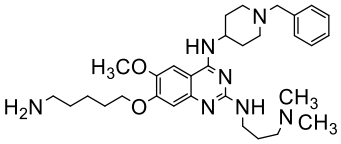
Methylation of DNA occurs at the C5 position of the cytosine ring in a CpG dinucleotide context, through the action of three active enzymes (DNA methyltransferases, DNMTs): DNMT1, DNMT3A, and DNMT3B. Aberrant DNA methylation patterns are associated to initiation and progression of various cancers, as well as to other pathologies such as psychiatric disorders and immune system diseases.[(1,2)]

Chemical manipulation applied on the scaffold of BIX-01294[(3)] (**1**), a specific G9a/G9a-like protein (GLP) histone H3K9 methyltransferase inhibitor, led to E67[(4)] (**2**), bearing a 5-amminopentyloxy substituent at the C7 position of the quinazoline ring and endowed with enhanced G9a/GLP inhibitory activity *in vitro* and reduced toxicity *in vivo*. Chemical changes performed by other authors on **1** led to UNC0638[5] (**3**), a potent and selective G9a/GLP inhibitor which was found totally inactive towards other HMTs, but displayed a slight inhibition of DNMT1. Thus, the quinazoline moiety, used to develop HMT and HDM inhibitors, could be also suitably used to design, with appropriate substitutions, compounds able to inhibit DNMTs. In addition, we thought that if such quinazoline-based compounds lack the C6 methoxy group, crucial for the binding with G9a/GLP, they should be devoid of anti-H3K9 methyltransferase activity. From these observations, we prepared new 6,7-desmethoxy quinazoline derivatives, keeping fixed the 1-benzyl-4-piperidinylamino moiety (typical of **1** and **2**) at the C4 position of the quinazoline ring, and changing the substituent at C2, spanning from differently sized cyclic amines (containing or not heteroatoms and/or lipophilic groups) in addition to hydrazino substituents and open-chain amines. Most of the tested compounds were unable to significantly inhibit DNMT1 at 100 μM but selectively inhibited DNMT3A. The most potent quinazoline DNMT3A inhibitors (**4**, **5** and **6**) were tested against the H3K9 methyltransferase GLP in comparison with **1**, used as reference drug. As expected, **4** was totally inactive against GLP, and **5** and **6** showed 3.3 and 10% of GLP inhibition at 8 μM, thus they showed selective inhibition for DNMT3A.



1
(BIX-01294)



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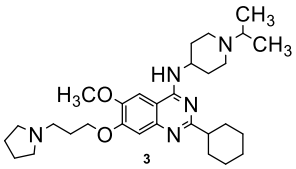
(E67)



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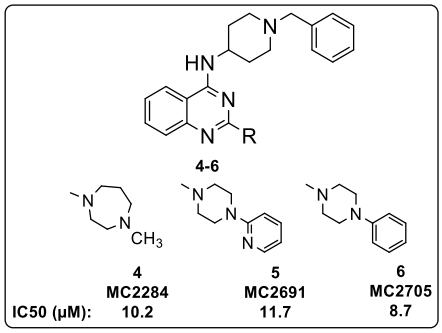

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FROM CARBAZOLES TO PSEUDOPEPTIDES, DESIGN AND SYNTHESIS OF NEW SIRTUIN INHIBITORS

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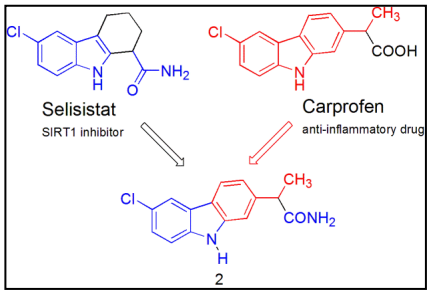
The class III histone deacetylases, also called sirtuins (SIRT1-7) have gained growing attention for their involvement in many biological processes such as cellular metabolism regulation, neuroprotection, apoptosis, inflammation, telomere maintenance etc.(1) SIRT1/2 have been found to be involved in tumorigenesis through their inhibitory effect on p53 associated with tumor suppression. Lately the hypothesis that targeting SIRT1/2 may represent an intriguing anticancer therapeutic option has made its way.(2) Inspired by Selisistat a tetrahydro-1H-carbazole a potent SIRT1 inhibitor and nonsteroidal anti-inflammatory drug (NSAID) Carprofen we prepared a small library of carprofen-related derivatives as SIRTs inhibitors.



Furthermore we focused our efforts on the preparation and biological evaluation of a novel fragment based library of pseudopeptidic inhibitors generated considering our previous binding hypothesis(3) in which a potential sirtuin inhibitor should create an H-bond network (Figure1).

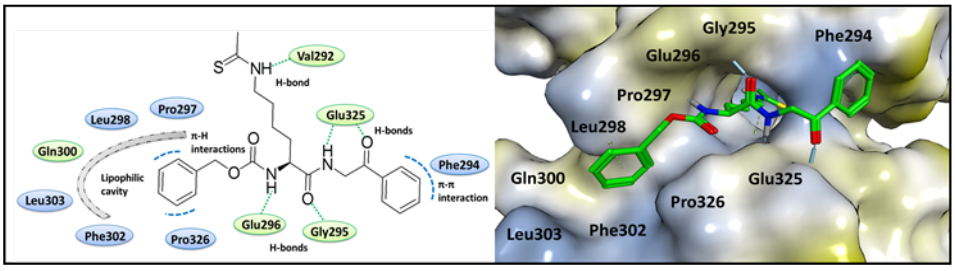


Figure 1. A scheme of the interaction pattern reported for a pseudopeptidic inhibitor in SIRT3.

A small array of Carprofen derivatives were synthesized and tested for their ability to inhibit human Sirtuins 1/2. The primary amide (**2**) demonstrated to inhibit the two enzymatic isoforms also in functional assays by increasing of p53 and α-tubulin acetylation levels. Furthermore the new library of thioacetylated pseudopeptidic compounds was synthesized and screened against human SIRT1-3. The selected compounds were subjected to IC_{50} profile and cellular studies. All of them showed increase in acetylation of Lys382 of p53 after DNA damage. Furthermore, two of the compounds were able to inhibit both A549 lung carcinoma and MCF-7 breast carcinoma cell growth in micromolar concentration with the ability to arrest cancer cell cycle in the G1 phase.

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DESIGN, SYNTHESIS, AND PHARMACOLOGICAL EVALUATION OF NEW ANTITUMORAL DERIVATIVE – LQFM030

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In this work we investigated the cytotoxic effect of the compound LQFM030, which was design from molecular simplification from Nutlins[(1)] lead compounds, on leukemic cells. In this study, the *in vitro* mechanisms of cell death and the *in vivo* antitumoral efficacy, using the Ehrlich ascites tumor, were investigated. In parallel, the *in vivo* oral toxicity of LQFM030 also was investigated. The mechanisms involved in K-562 cell death and cell cycle analysis were investigated by microscopy, colorimetric assays and flow cytometry. The results revealed that LQFM030, inhibited the growth of human leukemia cells, in a concentration-dependent manner, induced apoptosis mediated by mitochondrial-dependent mechanisms and arrested the cells at the G2/M phase, along with modulation on the expression of Bax, Citocromo-c and Bcl-2. *In vivo*, the LQFM030 increased the survival of tumor-bearing animals, when compared to non-treated animals. In the *in vivo* oral toxicity investigation, the LQMF030 was classified as class 5, with low toxicity, in the *Globally Harmonized System of Chemical Classification.*

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SUBSTITUTED PYRAZOLO[1,2-a]BENZO[1,2,4]TRIAZINE-3-ONES: DESIGN, SYNTHESIS AND ANTIPROLIFERATIVE ACTIVITY

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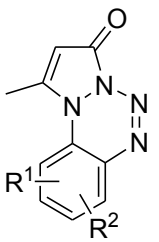
Recently a new series of pyrazolo[1,2-a]benzo[1,2,3,4]tetrazin-3-one derivatives **1** have been explored for their anticancer potential. Because of their peculiar chemical behavior in soft acid environment towards charged or neutral nucleophiles, a probable alkylating mechanism of action is proposed. This latter has been also confirmed by the prediction of the chemiometric virtual lock and key protocol (VLAK). Additionally, the effect on apoptosis induction, cell cycle and proliferation have been approached with the aim to better clarify possible common pathways(1). Several of the new synthesized derivatives screened against more than 50 types of human tumor cell lines, showed promising antiproliferative activity reaching in some cases micromolar values. Here, with the aim of tuning the biological profile, in terms of activity and selectivity, we planned to switch our interest to a deaza analog of the tricyclic 1,2-dihydropyrazolo[1,2-a]benzo[1,2,4]triazine-3-one **2** either to verify the electronic effect resulting from a deaza isosterism and to exploit the advantages of introducing a new point of chemical diversity (in R^3) useful for combinatorial developments. Thus, starting from commercial nitroaniline, aldehydes and a high yield prepared furanone precursor a small library of selected compounds was built for a preliminary biological screening.



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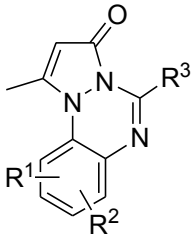

2

Variously fused pyrazolo[x,y-z]triazines are endowed with antiproliferative activity as novel potent inhibitors of kinase CK2(2) and of CYP1A1, including enzymes involved in the metabolism of chemical carcinogens.(3) Moreover, hydrazide derivatives have exhibited remarkable inhibitory activity against SiHa and LS180 human tumor cell lines, with no toxicity towards the HSF control cell line.(4,5)

In the present study, we designed and synthesized a set of new derivatives of type **2** containing selected functional groups. The preliminary NCI one dose screening evidenced moderate antiproliferative activity ($IC_{50} \approx 10$ μM) against UO31 (of the renal cancer panel). Further *in silico* optimization studies are in progress.

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ANTIPROLIFERATIVE OXIME DERIVATIVES THAT INHIBIT GLUCOSE TRANSPORTER 1 (GLUT1) IN CANCER CELLS

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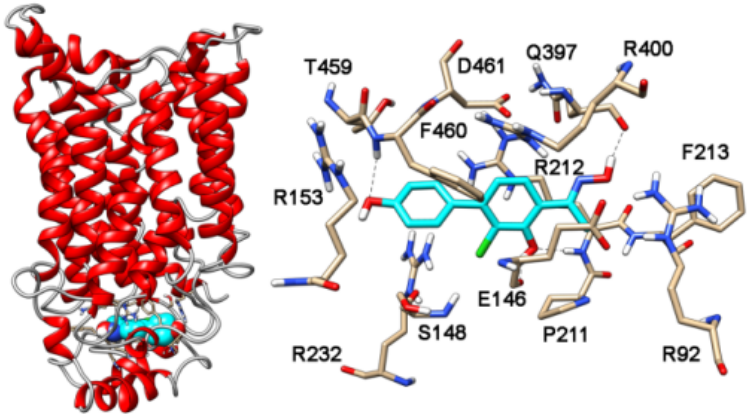
The Warburg effect, consisting in alterations of the glucose metabolism in cancer cells, where glucose mostly undergoes glycolysis with production of lactate, is currently being considered as one of the most intriguing hallmarks of cancer.[1] Therefore, the discovery of new agents able to block the glycolytic processes in tumor cells holds promise for developing relatively nontoxic anticancer treatments.[2]

In terms of energy (ATP) production, glycolysis is dramatically less efficient than oxidative phosphorylation (OXPHOS). In fact, most normal cells rely on OXPHOS for glucose degradation, since they are generally well-oxygenated. On the contrary, invasive tumor tissues are often exposed to more-or-less transient hypoxia, which cannot guarantee the proper functioning of OXPHOS. Under these hypoxic conditions glycolysis leading to lactate production is mainly preferred, since it does not depend on oxygen availability. However, due to the lower efficiency of the glycolytic process, cancer cells commonly show a remarkably high glucose uptake, which is supported by the overexpression of the glucose transporters (GLUTs). GLUT1 is one of the most commonly transporters that are overexpressed by cancer cells and, therefore, represent a potential target for selectively hitting them,[3] although only a very limited number of GLUT1-inhibitors have been reported so far.[4]



On the basis of an analysis of the pharmacophoric features displayed by some previously reported GLUT1-inhibitors, we have identified a series of oxime derivatives[5] as potentially active on this transporter. A preliminary screening of these compounds in H1299 lung cancer cells demonstrated that some of them are able to effectively counteract glucose uptake and cell growth, displaying IC_{50} values in the low micromolar range. We have then developed a new computational model of GLUT1, which provided us with valuable clues about the possible binding site and the most important interactions occurring with some representative oxime derivatives and GLUT1. These indications may prove to be very valuable for the future development of novel potent and selective GLUT1-inhibitors.

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INHIBITORS OF JMJC-DOMAIN CONTAINING HISTONE DEMETHYLASES

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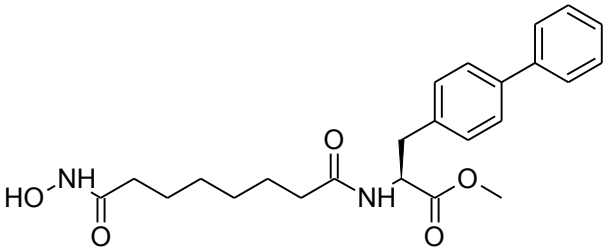
Different epigenetic mechanisms can regulate gene expression in cells, acting both on DNA as well as on the proteins around which it is wrapped, the histones. One of these mechanisms is the reversible methylation of histone lysine residues, mediated by distinct histone methyltransferases and histone demethylases, and implicated also in the manifestation of numerous diseases, such as cancers.(1-3)

The Jumonji-type demethylases (JmjDs) present an iron and α-ketoglutarate dependent mechanism, and they are able to remove up to three methyl groups from methylated lysine residues.(2, 3)

Up to date different JmjD inhibitors have been reported, but, generally, they are not selective. A strategy to inhibit these demethylases is to chelate the iron atom present in the active site. A good chelating moiety is the hydroxamic acid, and some known histone deacetylases (HDAC) inhibitors, bearing this functional group, inhibit also the JmjC demethylases.(3-5)

In a first screening we found that the HDAC inhibitor SW55 was also able to inhibit Jumonji demethylase 2A with an IC_{50} value of 25.4 µM.(6) This compound was taken as starting point to develop a series of analogs with modifications on the alkyl chain, the biphenyl and the methyl ester moiety, in order to improve its potency and selectivity. All compounds were tested on JmjD2A as well as on human HDAC1 and 6.



SW55
JmjD2A IC_{50} = 25.4 µM
HDAC IC_{50} = 0.29 µM

References:

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SYNTHESIS OF A NEW SERIES OF PYRROLE DERIVATIVES WITH POTENTIAL ANTILEUKEMIC ACTIVITY

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High-throughput screening (HTS) is a method for scientific experimentation especially used in drug discovery and relevant to the fields of biology and chemistry. Through this process one can rapidly identify active compounds, antibodies or genes which modulate a particular biomolecular pathway. The results of these experiments provide starting points for drug design and for understanding the interaction or role of a particular biochemical process in biology.

Through High Throughput Screening different pyrrolic and indolic structures have been identified as potential hits with anti-tumor activity. On the basis of the obtained results was carried out a study on the reactivity of these cores to evaluate the positions more responsive by derivatization with several aromatic and aliphatic residues.

There were designed and synthesized several series of molecules, with different substituents, in order to assess the cytotoxic activity and subsequently tested on cell lines L1210(murine leukemia), CEM(T lymphocytes), HeLa(human cervical carcinoma). Molecules with pyrrole nucleus have proved among the most active showing un'IC50 in micromolar order towards the cell line L1210. Then there were selected the two most active molecules made and tested on cell lines K562(human myeloid leukemia) and MCF7(human breast adenocarcinoma) and MiaPaCa2 (pancreatic cancer). Encouraging results were obtained for the compound 386 which showed a significant cytotoxic activity towards the leukemia cell line K562 and for which it has also been highlighted a cell selectivity, while activity towards the control cell line HEK293(embryonic kidney cells) it wasn't observed. The selectivity of these molecules to cancer cells offers great potential for future targeted antileukemic therapy.

ONE-POT SYNTHESIS OF NOVEL 2-ARYLPYRROLO[2,3,4-KL]ACRIDIN-1(2*H*)-ONES

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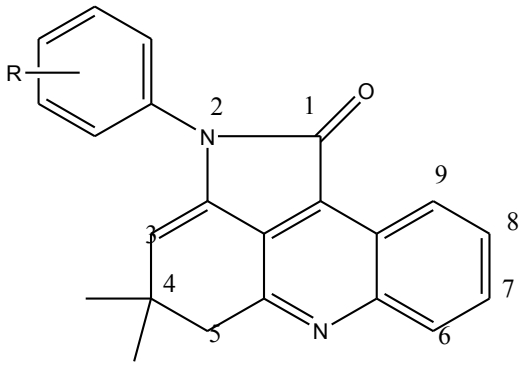
Pyrroloacridines and pyrroloacridones are of particular interest because they have a variety of interesting biological activities. Significantly, members of this family are active in assays for antihelmintic, 1 antitumor,[1,2] antifungal,[3] and DNA binding.[4–6] These abilities are specifically important in inhibiting the growth of cancerous cells, making these compounds ideal for developing novel anticancer drugs.

Plakinidines and alpkinidine are pyrroloacridines that have been obtained from marine sources.[1,7–10] Only a few reports are available for the synthesis of pyrroloacridines and therefore the synthetic versatility of these compounds needs to be explored.

As a result of their significant potential as therapeutics, a considerable synthetic attention has been directed at the development of efficient methods toward the construction of pyrroloacridine moiety. So, in this research we wish to introduce a new method for the synthesis of 4,5-dihydro-4,4-dimethyl-2-arylpyrrolo[2,3,4-kl]acridin-1(2H)-one as a new class of pyrroloacridin.



R=H,Cl,I,CH_3,NO_2,OCH_3,

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N-(2-HYDROXYARYL)AMIDES ENDOWED WITH ANTIPROLIFERATIVE ACTIVITY

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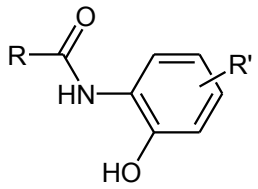
In the discovery of new anticancer agents amide derivatives, both synthetic and naturally derived, have proved to be cancer cell growth inhibitors. Thus diterpenoids amide derivatives exhibited broad spectrum in vitro anticancer activity and IC_{50} value better than cisplatin against HL-60 human promyelocytic leukemia cell line[(1)], and 5-sulphosalicylic acid amides were found endowed with antitumor activity against human breast adenocarcinoma cell line[(2)]. Anthranilic acid diamides have been described as potential anticancer agents[(3)]. Moreover, an increasing number of fluorinated molecules have become a focus in the development of new therapeutics as anti-cancer agents. In this context our research group has described the interesting anticancer activity of a new class of 2-arylamino-6-trifluoromethylnicotinamides [(4)].
In this communication we describe the molecule design, convenient synthesis, and antiproliferative activity of novel series of N-(2-hydroxyaryl)amides bearing a 2-(trifluoromethy)pyridyn-4-ylamino group. The designed amide derivatives were evaluated for their anticancer activity toward human tumoral cell lines by the National Cancer Institute (NCI) and showed antiproliferative activity against human tumoral cell lines, having GI_{50} values in the low micromolar to submicromolar concentration range.



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HIGHLY POTENT CANCER AGENTS BY MODULATING THE C-2 GROUP OF THE ARYLTHIOINDOLE CLASS OF TUBULIN POLYMERIZATION INHIBITORS

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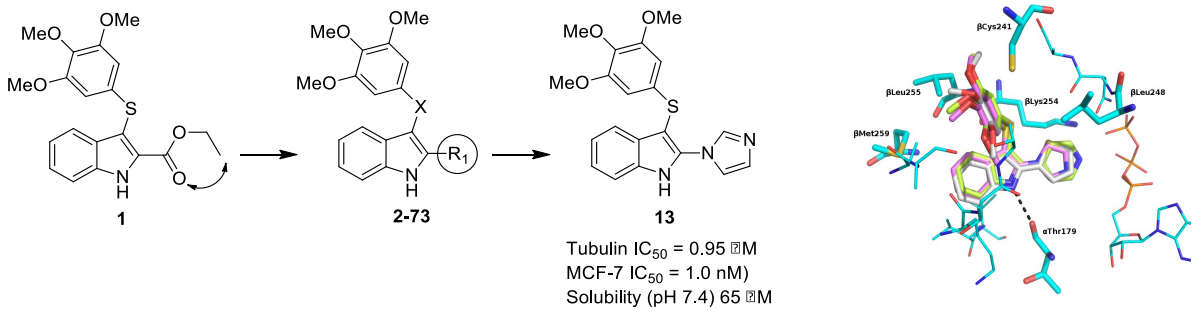
Microtubules (MTs) are highly dynamic cylindrical structures, composed of α,β-tubulin heterodimers. MTs are required for many essential functions, including the maintenance of cell shape, cell motility, intracellular transport and cell division. Our previous study showed that arylthioindoles (ATIs) bearing an alkoxycarbonyl group at position 2 of the indole, were potent tubulin assembly inhibitors. In this study we included the ester function in a heterocyclic nucleus. New arylthioindole derivatives (**2-73**) were potent tubulin assembly inhibitors that bound to the colchicine site. Furthermore, they showed metabolic stability superior to the previously synthesized ATIs (e.g., **1**).([1-3]) Compound **13** was exceptionally potent as an inhibitor of cell growth and it was uniformly active in the whole panel of cancer cells and superior to colchicine and combretastatin A-4. It showed water solubility and high metabolic stability in human liver microsomes.



R_1 = azolyl, azinyl, phenyl or substituted phenyl, naphthyl, biphenyl, benzofused heterocyclyl, alkyl, cycloalkyl; X = S, CO, CHOH, CH_2.

Figure 1. General Structure of ATIs and binding mode into the colchicine site of tubulin.

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A NEW, SIMPLE AND HIGH YIELDING SYNTHESIS OF 2,9-DIHYDRO-1*H*-PYRIDO[3,4-*b*]INDOL-1-ONES

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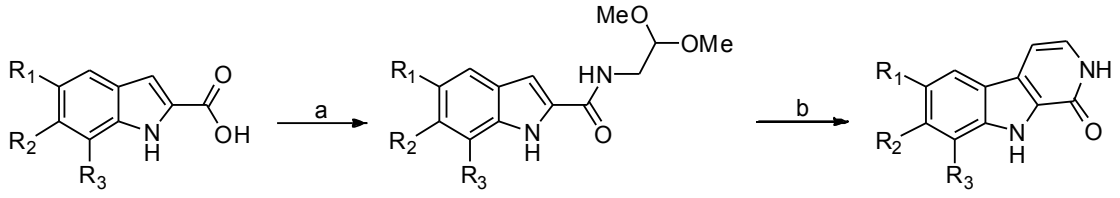
β-Carboline is a key pharmacophore present in a large number of natural tricyclic alkaloids, which can be found in numerous plants and animals, exhibiting potent biological activities. As a key member of the β-carboline family, its structural variant tricyclic β-carbolinone (2,9-dihydro-1*H*-pyrido[3,4-*b*]indol-1-one) has served as an important intermediate for the preparation of complex alkaloids[(1-3)] and has been found to possess potent bioactivities. The natural and synthetic β-carbolinones are reported to have pharmacological effects in several aspects, such as the anticancer activity against colon and lung cancers, central nervous system activity in mammals, and also as the biological control agent for receptor research on bio-enzyme inhibitors, such as the inhibition of human leukocyte elastase.[(4-6)]

We developed a new, simple and high yielding (88-98%) method to prepare 2,9-dihydro-1*H*-pyrido[3,4-*b*]indol-1-ones by treating the appropriate *N*-(2,2-dimethoxyethyl)-1*H*-indole-2-carboxamide with polyphosphoric acid (PPA) at 110 °C for 30 min. The method was poorly affected by the presence of electron-donating and -withdrawing substituents on the indole nucleus. The reaction of the right 1*H*-indole-2-carboxylic acid with aminoacetaldehyde dimethyl acetal in the presence of Et_3N and BOP reagent in DMF at 25 °C for 2 h furnished the requested *N*-(2,2-dimethoxyethyl)-1*H*-indole-2-carboxamides.

Scheme 1. Synthesis of 2,9-dihydro-1*H*-pyrido[3,4-*b*]indol-1-ones[a]



R_1 = H, Me, Alogen, OMe, NO_2; R_2 = H, OMe; R_3 = H, NO_2

[a]Reagents and reaction conditions. (a) Aminoacetaldehyde dimethyl acetal, Et_3N, BOP reagent, 25°C, 2 h, 90-98%. (b) PPA, 110 °C, 30 min, 88-98%.

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BIOLOGICALLY ACTIVE HETEROAROMATIC DERIVATIVES WITH AMINO SIDE CHAINS AS POTENTIAL ANTICANCER AGENTS

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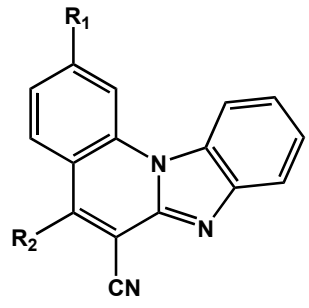
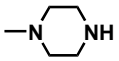
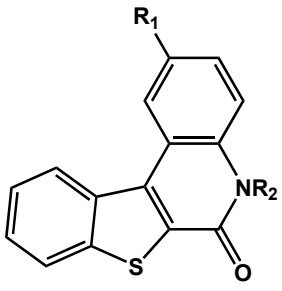
Over the past few years substituted heterocyclic derivatives have been one of the most extensively studied classes of organic compounds due to their well known biological activities. The development of effective antineoplastic drugs has become one of the most intensively studied aspects of contemporary medicinal chemistry and therefore has been tremendous growth in the number and types of new anticancer agents. One of the most used classes of chemotherapeutic agents in cancer therapy comprise molecules that interact with DNA, such as groove binders, DNA alkylating agents or intercalators.

In this report we are presenting the synthesis and antitumor activity of some polyfunctional heterocyclic compounds regarding cyclic benzimidazole, quinolone and isoindoline derivatives bearing different amino side chains.[1-3] For the synthesis of novel compounds were used classical methods of organic synthesis, photochemical cyclizations and microwave assisted reactions. Structures of prepared compounds were confirmed by means of ^{1}H, ^{13}C NMR, IR and UV spectroscopy. Antiproliferative activity *in vitro* was tested on several human tumor cell lines. Some of tested compounds showed a differential and significant antiproliferative effect at micromole concentrations. To shed more light on the mechanisms of biological action, additional experiments of interaction with ct-DNA of some active compounds were performed by using UV/Vis, fluorescence and CD spectroscopy and thermal melting experiments as well as topoisomerases I and II inhibition, cell cycle perturbances, subcellular localization and the influence on Enhanced Green Fluorescence protein.

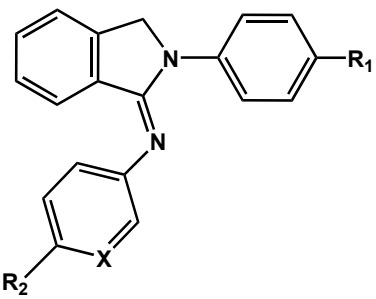


$R_1 = R_2 =$ H, $NH(CH_2)_3N(CH_3)_2$, $NHCH_2CH(CH_3)_2$





$R_1 = NHCOCH_3$, $N(CH_3)_2$, $N(CH_3)_2$ x HCl, $N^+(CH_3)_3I^-$
R_2 = H, CH_3, $(CH_2)_3N(CH_3)_2$, $(CH_2)_3N(CH_3)_2$ x HCl



$R_1 = R_2 = NH_2$, NH_2 x HCl
X = CH, N

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siRNA-BASED THERAPEUTICS: DELIVERY AND TARGETING TO PEL TUMOR BY USING CATIONIC LIPOSOMES

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Aims of this research was to develop a "nanomedicine" approach based on siRNA delivery for the treatment of primary effusion lymphoma (PEL). The therapeutic use of antitumoral siRNA requires the development of specifically designed functional vectors, allowing improvement of siRNA stability after systemic administration, and enabling targeted delivery directly into the neoplastic cells. In this context, liposomes, and particularly cationic liposomes, appears particularly suitable to generate complexes with highly degradable siRNAs, as well as to specifically deliver siRNAs directly into the cytoplasm of the target tumor cells, where RNA interference processes take place. Generally, the electrostatic interaction between the positively charged lipids and the negatively charged nucleic acids leads to the formation of stable lipoplexes, protecting the cargo against nuclease attack and improving the cellular uptake and activity.(1)

In this context, we are investigating innovative target strategies to improve the treatment of human herpesvirus 8 (HHV8)-associated primary effusion lymphoma (PEL). Primary effusion lymphoma (PEL) is an aggressive B-cell non-Hodgkin's lymphoma, affecting the serous cavities (such as the pleural, pericardial and abdominal cavities) and preferentially arising in immunocompromised or elderly patients, typically affected by several comorbidities and organ function impairments. PEL therapy has been revealed to be unsuccessful in the vast majority of patients, who are invariably characterized by a poor prognoses. Recently, small interfering RNAs (siRNAs), able to knock-down viral oncogenic proteins, were shown to induce efficient PEL cell apoptosis in vitro and PEL regressions in mice treated with intracavitary injection of lentiviral vectors expressing siRNA precursors.(2)

Moving from our promising preliminary results in the field of nanotechnologies,(3-4) we are developing different lipid-based nanocarriers (cationic and stealth-cationic liposomes), to deliver specific siRNAs to knock-down novel molecular targets (HHV8-encoded microRNAs, viral oncogenic proteins, or host transcription factors) with relevant functions in PEL pathogenesis.*(5)* We are presently testing the delivery efficiency of these nanocarriers and the antineoplastic activity *in vitro* and *in vivo* using different PEL-derived cell lines and a previously established PEL mouse model.(6)

We performed several preliminary technological experiments aimed at optimizing the operative condition to obtain the efficient liposomes/siRNAs complexes. Chemic-physical properties of both liposomes and lipoplexes were evaluated by exploiting microscopic, spectroscopic and gel electrophoresis techniques.

In vitro experiments demonstrated a high transfection efficiency of some of our carriers, which stably protected and efficaciously delivered siRNAs into PEL cells. Preliminary experiments using a mixture of siRNAs targeting a specific cellular gene showed a remarkable dose-dependent apoptosis, measured by annexin-V staining, in lipoplexes-transfected PEL cells. Moreover, the *in vivo* delivery of these therapeutic siRNAs significantly increased the survival time of treated mice compared with control treatment (log-rank test, lipoplexes vs empty liposomes, p=0.002), indicating that our lipoplexes exerted a significant antineoplastic activity. The empty carriers were not toxic in control mice and did not delay PEL development respect untraeted mice.

Our data indicate that our lipoplexes may therefore be considered as the basis for the development of useful short interfering RNA delivery vectors to treat PEL tumor. Moreover, we identified a target gene whose suppression exerts a relevant tumoricidal activity on PEL cells *in vitro* and *in vivo*, opening new perspectives for PEL treatment.

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NOVEL TYROSINE KINASES INHIBITORS BEARING THE PYRAZOLO[3,4-*D*]PYRIMIDINE HETEROCYCLIC CORE. SYNTHESIS AND FUNCTIONAL EVALUATION

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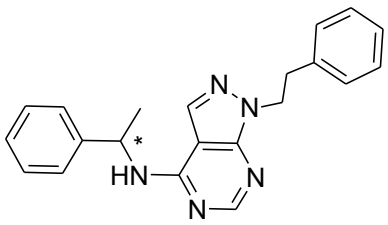
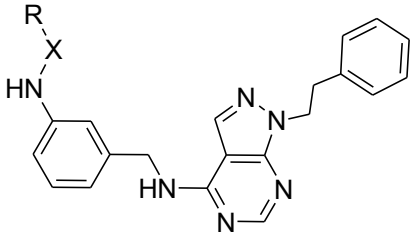
Thyroid carcinoma (TC) is the most common endocrine tumour, whose treatment of choice relies in its complete surgical resection, possibly associated with external radiation therapy. However, disease can persist or recur, with local and distant metastases, often associated to a marked resistance to conventional cytotoxic chemotherapy. Therefore, novel therapeutic strategies to treat TCs are urgently needed. Recent advances in the knowledge of pathogenic mechanisms leading to TCs clearly demonstrated that oncogenic tyrosine kinases, mainly VEGFR2 and RET, sustain their development and/or progression, thus identifying these proteins as new and promising targets.(1)

Recently, we disclosed a novel pyrazolo[3,4-*d*]pyrimidine derivative, namely CLM3, which combines an excellent anti-angiogenic efficacy with an inhibitory activity against receptor tyrosine kinase RET. Moreover, when administered to CD nu/nu mice xenotransplanted with the DePTC cell line, CLM3 proved to inhibit tumour growth and weight, without showing any appreciable toxicity.(2,3)

Taken together, these results affirmed CLM3 as a sound and viable lead candidate, deserving of further development. Accordingly, we embarked in rational optimization of CLM3, to obtain novel and more effective derivatives of general formula **1**. Here we present the synthesis and the functional evaluation of the novel compounds, whose efficacy has been tested on both endothelial and thyroid cancer cell lines.



CLM3



1

X = CO, CONH, SO_2
R = C_6H_5, subst-C_6H_4

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4,11-BIS[(ω-GUANIDINOALKYL)AMINO]ANTHRA[2,3-*b*]THIOPHENE-5,10-DIONES AS POTENT G-QUADRUPLEX INTERACTIVE AGENTS FOR CANCER THERAPY

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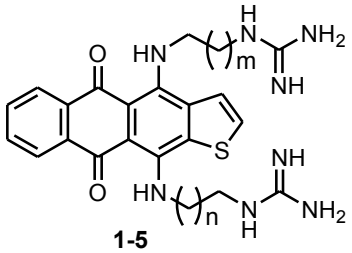
To explore the anticancer potential of anthraquinone derivatives, we have developed the synthesis of DNA-binders based on anthra[2,3-*b*]thiophene-5,10-dione scaffold.(1) The selected designed agents demonstrated high inhibitory potency against topoisomerase I and promising cytotoxicity for mammalian tumor cells including multidrug resistant sublines. We discovered that the introduction of the guanidine groups into the side chains of anthra[2,3-*b*]thiophene-5,10-diones increased affinity for human telomeric G-quadruplex. Thus, we identified bis(guanidine) derivative **1**, as a G-quadruplex ligand with a potent inhibitory activity against telomerase (1). In this work, a series of analogs of **1** with two alkyl side chains of different lengths has synthesized. We investigated the capacity of anthra[2,3-*b*]thiophene-5,10-diones to interact with DNA and RNA G-quadruplexes, to penetrate the cell membranes and modulate gene expression.

The synthesis of the new series of DNA-ligands **2-5** was accomplished by an early developed methodology,(1) which was based on a nucleophilic substitution of the alkoxy groups of 4,11-dibutoxyanthra[2,3-*b*]thiophene-5,10-dione with diaminoalkanes and subsiquent guanidation of the terminal amino groups.



1-5

1 m=n=1;
2 m=1, n=2;
3 m=2, n=1;
4 m=n=2;
5 m=n=3.

Flow cytometry showed that the designed anthra[2,3-*b*]thiophene-5,10-diones **1-5** enter much more efficiently in malignant T24 bladder cells than the nonmalignant kidney 293 and NIH 3T3 embryonic cells. The mechanism by which the designed molecules are taken up in T24 malignant cells depends on the size of the side chains attached to the anthrathiophene chromophore. The uptake of compound **1** with two ethyl side chains occurs by endocytosis, while the uptake of **4** and **5**, with respectively two propyl and butyl side chains, takes place by passive diffusion. Confocal microscopy and biophysical studies showed that, the anthra[2,3-*b*]thiophene-5,10-diones **1-5** localize in the cytoplasm and nucleus and tightly bind to naturally occurring DNA (K_D: 0.15-0.37 μM) and RNA (K_D: 0.06-0.38 μM) G-quadruplexes.

All the designed molecules stabilized G-quadruplex structures and showed a good quadruplex/duplex specificity. Dual-luciferase assays showed that the anthra[2,3-*b*]thiophene-5,10-diones **1-5** decrease the activity of the *HRAS* promoter, while immunoblotting data revealed that they strongly reduce the level of protein $p21^{HRAS}$ in malignant T24 bladder cells. The obtained results suggest that the designed G-quadruplex ligands **1-5** may be useful for down-regulating *HRAS* expression in bladder cancer cells. Anthra[2,3-*b*]thiophene-5,10-diones **1-5** also showed to inhibit the proliferation of T24 cells, but not of nonmalignat 293 or NIH 3T3 cells, through the accumulation at G_2/M. Taken together, our data suggest that the G-quadruplex ligand **1** are promising chemotype for developing novel therapeutic drugs for bladder cancer.

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INTRAMOLECULAR HYDROGEN BOND AS A TOOL TO ENHANCE POTENCY AND SPECIFICITY OF TRIMETHOXY BENZAMIDES P-GP MODULATORS

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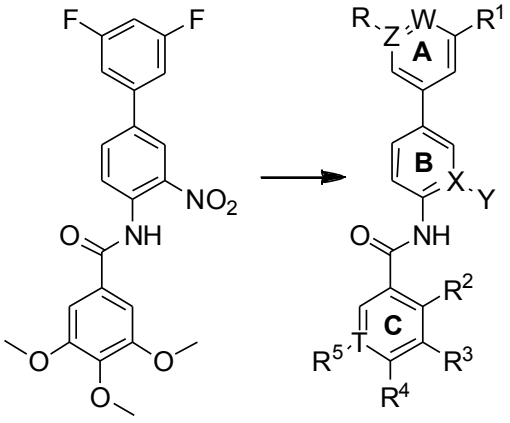
Multidrug resistance (MDR) could be considered one of the main reasons of chemotherapy failure. It was demonstrated that MDR is unrelated to the pharmacological activity of the drugs and that could be triggered by diverse supposed mechanisms such as drug metabolism acceleration, apoptotic pathway modulation, cellular damage repair and drug efflux pump overexpression. (1)

P-gp, the first protein transporter to be discovered nearly 35 years ago, is a single polypeptide arranged in two six transmembrane helices and one nucleotide binding domain (NDB) in the cytoplasmatic side of the cell.(2)

The common molecular requirement pointed out by numerous SAR and QSAR studies was the high lipophilicity of the best P-gp modulators, consistent with the proposed hypothesis that compounds could join the binding site through an entry pathway through the membrane bilayer.[3] Very recently we published[4] some galloyl-based modulators, targeting P-gp and MRP1 and also in our case a trend between lipophilicity and the strength of P-gp modulation was observed.

Our previous best modulator (compound **1**, that showed an IC_{50} 2.6 µM on P-gp inhibition and 2.5 µM on MRP1, Chart 1) has two not negligible problems: it brings a toxicoforic nitro group and it is poorly soluble.



1 General Structure

So we planned the synthesis and the biological evaluation of a new series of trimethoxy benzamides (general structure in chart 1) in order to gain ameliorated physicochemical properties besides high potency. Very potent P-gp modulators were achieved, for example, by combining a 2-fluorophenyl with a pyridyl as B and A ring, respectively. The trimethoxy substituents were systematically moved around the C ring, by the synthesis of a number of regioisomers. The 2,4,5-trimethoxybenzamide derivatives, in particular, exhibited the highest activity reaching the submicromolar range at P-gp. The most striking difference among regioisomers may be seen in the possibility of the , 2,4,5-trimethoxy derivatives to establish an intramolecular hydrogen bond[5] between the 2-methoxy group and the NH of the benzamide that may induce a planar conformation to the molecules. As a consequence, 2,4,5-trimethoxy regioisomers will be more soluble in the lipidic membrane bilayer where they could probably better interact with the pump. NMR studies (NOESY and variable temperature), conformational analysis, ΔlogP measurements and permeability experiments coherently corroborate this hypothesis.

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SMALL MOLECULES TARGETING HPV E6 AND E7 ONCOPROTEINS EXPRESSION

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Infection with high-risk human papillomavirus (HPV) is responsible for the induction of cervical cancer (CC), one of the most common malignancies for incidence and mortality in female population worldwide.(1) The HPV E6 and E7 proteins play a key role in the transformation of primary human keratinocytes into malignant cells by abrogating the function of tumor suppressor proteins p53 and pRb. The continuous expression of these oncoproteins proved to be necessary for the maintenance of the transformed phenotype, representing an ideal molecular target for the development of innovative anti-CC therapies.(2) While macromolecule-based approaches have proved to be effective in silencing this expression,(3,4) the development of small molecules remains highly desirable.

The availability of a large series of quinolone-based compounds endowed with antiviral activity due to the inhibition of viral transactivation, prompted us to test their ability to reduce the E6 and E7 transcription using a cell based high-throughput assay previously developed by us.(5) Some of the tested compounds were active in inhibiting HPV-16 long control region activity with IC_{50} values in the low micromolar range at no toxic concentrations. The ability to downregulate E6 and E7 mRNA transcripts was confirmed in a RT-PCR assay using CaSki cells, that are HPV-16 positive cervical carcinoma-derived cells.

Starting from the most promising derivative, an enlarged series of analogues were designed, synthesized and biologically tested leading to identify more potent compounds and delineate a preliminary structure-activity relationship.

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NEW MULTIDRUG RESISTANCE (MDR) REVERTING AGENTS ENDOWED WITH GOOD POTENCY AND EFFICACY

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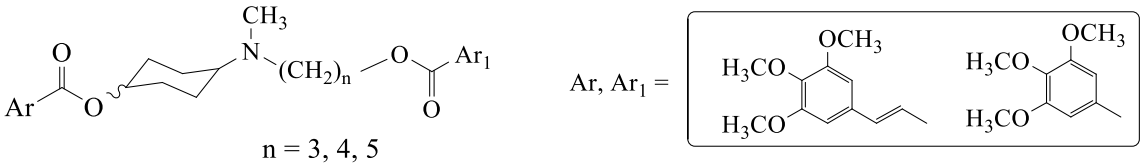
The development of resistance in cancer cells and microorganisms is the main obstacle to achieving success with chemotherapy. Frequently, following drug treatment, cells become resistant to a variety of chemotherapeutic drugs that are even structurally and mechanistically unrelated, thus resulting the so-called multidrug resistance (MDR).(1) In humans, MDR is associated with the over-expression of transporter proteins such as ABCB1 (Pgp) and ABCC1 (MRP1) acting as extrusion pumps that perform an ATP-dependent active outward transport of chemotherapeutic drugs.(2) Inhibition of the functions of Pgp and related proteins, is considered a suitable approach to circumvent MDR. This is the main reason prompting the design and synthesis of Pgp modulators to co-administrate with cytotoxic substrates of Pgp.(3)

In a continuing search for potent P-gp-dependent MDR reversers started a few years ago, we synthesized and studied two new families of MDR modulators, *N,N*-bis(alkanol)amine aryl esters and *N,N*-bis(cyclohexanol)amine aryl esters, endowed with fairly good potency.(4,5) Now we report a new series of derivatives that are hybrid compounds since they are characterized by the presence of two linkers with different flexibility: a methylene chain of different length and a cyclohexylic scaffold.

Figure 1. General structure of *N*-alkanol-*N*-cyclohexanolamine aryl esters.



The new compounds show good potency and efficacy in the preliminary pharmacological tests. Therefore, a deeper pharmacological characterization is in progress. The pharmacological profile of the new molecules will be reported and discussed.

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ST7612AA1, A DRUG CANDIDATE BELONGING TO A NEW CLASS OF HDAC INHIBITORS

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Within the huge field of the so-called "lysine-deacetylase", there are the well-known histone deacetylases (HDACs), a class of enzymes acting removing acetyl groups from ε-amino-lysine residues on many different substrates (histone, non-histone nuclear and cytoplasmatic proteins). The acetylation / deacetylation ratio of these substrates works as a regulatory signaling network in cells.

Over the last years, great efforts have been spent on the HDAC inhibitors in the oncology field: so far two drugs (SAHA and Romidepsin) have emerged as promising anticancer drugs and approved by the US Food and Drug Administration (FDA).

ST7612AA1 is a compound selected from a sigma-tau research program aimed at the investigation of thiol-ω(lactam-amide) analogues of SAHA, as a new class of histone deacetylase inhibitors (HDACi), for the treatment of cancer.

These new compounds are highly competitive respect to known and marketed HDACi, displaying sub-micromolar to low nanomolar inhibitor activity on HDACs, being especially powerful on HDAC6 isoform. They exhibit higher anti-proliferative activity than SAHA on different human cell lines and, in vivo, they were orally administered, showing a higher potency than SAHA, with a negligible toxicity. Besides, they showed a favorable druggability profiles versus Romidepsin.

The overall profile of this new class of HDAC inhibitors, including synthesis and a comprehensive pharmacological characterization, will be presented.

These encouraging results prompted us to select a drug candidate (ST7612AA1) which is currently in a pre-clinical evaluation phase, as anticancer agent, and under investigation as a treatment for other diseases.

DEVELOPMENT OF NOVEL PSEUDOPEPTIDES TARGETING THE 20S PROTEASOME AS ANTICANCER AGENTS

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Proteasome is a multicatalytic threonine protease complex responsible for the turnover of cellular proteins including those involved in signal transduction, cell cycle control and apoptosis. Defects in the proteasome activity can lead to anarchic cell proliferation and tumors development. For these reasons, proteasome is a target of great interest in drug discovery for anticancer therapy.(1) Among the three active sites of proteasome, the greatest interest has been focused on the inhibition of *β*5 subunit (chymotrypsin-like, ChT-L).

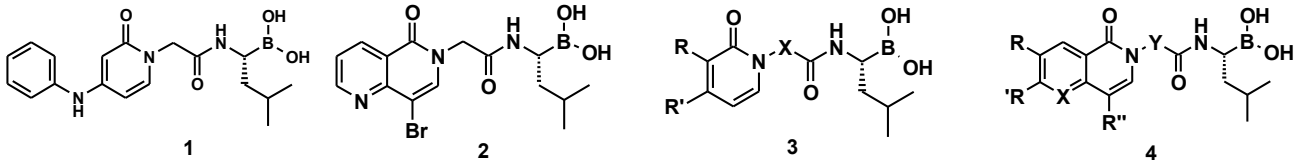
Starting from the structure of bortezomib as lead compound, we recently synthesized a series of conformationally constrained analogs that showed a promising inhibitory profile by blocking primarily the ChT-L activity of the proteasome with K_i values in submicromolar/micromolar range. The obtained results were rationalized by means of docking experiments that also provided essential insights for the optimization of the inhibitors.(2)

On these bases, starting from the most active peptidomimetic boronates **1** and **2** (K_i = 0.098 and 0.17 μM), we designed a novel series of boronic acids **3-4** (Fig. 1). In the optimization of compound **1**, the 1*H*-pyridin-2-one ring has been kept unchanged in view of its ability to enclose the amide moiety of the pyrazinamide of bortezomib. Also the anilino moiety at position 4 of the pyridone nucleus has been maintained, since docking studies pointed out that this substituent projected towards S3 binding pocket and that NH form a *hydrogen* bond with the side chain of D114 of β6 subunit.(2) The anilino moiety has been also shifted in position 3 to verify if its ability to interact with β6-D114 is retained. Additional structural modifications were the introduction of *β*-alanine and phenylalanine residues at P2 position to optimize the interactions with S2 pocket.

Regarding compound **2**, docking studies clearly indicated that this compound adopted a folded conformation that flip the constrained scaffold to the S2 pocket instead of the originally assumed S3 pocket. Therefore, the replacement of the Gly residue at P2 with *β*-alanine or rigid synthons should impede the folding and optimize the interactions with the target. Furthermore, we decided to investigate the real contribution of N1 (corresponding to N4 of the pyrazine ring of bortezomib) to the inhibitory activity by replacing the naphthyridinone scaffold with the isosteric isoquinolin-1(*2H*)-one.

The results of the biological evaluation of the new compounds will be reported and discussed.

Figure 1



R, R' = secondary or tertiary amine
X= CH_2CH_2, $CHCH_2Ph$

R = CH_3O, Br, H; **R'**= CH_3O, H
R''= Br, H; **X** = N, CH
Y = CH_2, $(CH_2)_2$, $CH_2CH{=}CH$

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TNF
Hyperglycemia
High Cholesterol
Diabetes
Metabolic Syndrome
PKR
Obesity
Lifestyle
Inflammation
Visceral Fat
Adiponectin

DESIGN OF AN ORAL C5AR NONCOMPETITIVE ALLOSTERIC INHIBITOR FOR PAIN RELIEF BASED ON A CONSERVED SITE IN CHEMOATTRACTANT RECEPTORS

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Inflammatory and neuropathic pain are the most prevalent types of pathological pain and represent important health problems. Whereas inflammatory pain is one of the classical symptoms of the inflammatory process, neuropathic pain arises from any of multiple nerve lesions or diseases with symptoms including hyperalgesia or allodynia.[(1,2)] Some of the most powerful painkillers, including opioids and non-steroidal anti-inflammatory drugs are generally only partially effective and prolonged exposure can cause unwanted effects.[(3,4)] As a result, there is continuous effort to identify novel therapeutics for pain control with alternative biological mechanisms.

Inflammatory mediators, including cytokines/chemokines, play a critical role in the pathogenesis of inflammatory and neuropathic pain.[(5-7)] Emerging evidences suggest that C5a, the anaphylatoxin produced by the complement activation, has potent nociceptive activity in several models of inflammatory and neuropathic pain by interacting with its selective receptor C5aR.[(8)]

In the present study, we report the successful de novo design of a novel non-peptidic C5a allosteric small molecular weight inhibitor driven by the hypothesis that a minor pocket, previously characterized as the binding site of reparixin and other related CXCR1/2 inhibitors, could be structurally and functionally conserved across the GPCR family. The lead compound, DF2593A, is a potent and orally active C5a noncompetitive allosteric inhibitor with significant antinociceptive effects in a wide range of inflammatory and neuropathic pain models.

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BILE ACID DERIVATIVES: AN EMERGING CLASS OF COMPOUNDS IN INFLAMMATION AND METABOLIC DISORDERS

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During the past decade, it has become clear that inflammation is no longer confined to immune system and infectious diseases, but it is an important component of the initiation, propagation, and development of chronic and metabolic disorders such as obesity, type 2 diabetes, atherosclerosis, and cancer.(1) In this framework, recent evidences have demonstrated the involvement of bile acid receptors in modulating the inflammatory response,(2) opening new avenues to the prevention and treatment of these diseases.

Although the molecular mechanisms are still not completely understood, the bile acid nuclear receptor FXR and membrane G protein-coupled receptor TGR5 are thus receiving a great deal of attention on part of both academia and pharmaceutical companies, with potent and selective ligands being developed as new, efficacious pharmacological tools and clinical candidates.(3)

This communication will highlight our current efforts in defining the complex role and biological relevance of bile acid signalling pathways in inflammatory-related pathophysiological conditions, as well as the reasons for the increasing interest of biliary compounds in drug discovery. In particular, the potential associated with tuned structural modifications of the bile acid scaffold will be exemplified reporting the design, synthesis and profiling of potent and selective FXR/TGR5 agonists that, in view of their pharmacological and PK/ADMET properties, represent promising lead compounds on the way to novel therapeutic agents for chronic liver and metabolic diseases.

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METABOLOMICS INVESTIGATION OF URINARY TRACT INFECTION

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Urinary tract infection (UTI) is a common bacterial infection leading to substantial morbidity, mortality and health care expenditure across all ages. In fact, the term UTI encompasses a variety of clinical syndromes with one common denominator, namely a positive urine culture (i.e., bacteriuria $\geq 10^3$ CFU/ml). The diagnostic and clinical management of UTI are rather straightforward but a reliable clinical scoring system of the disease severity and outcome is still missing. Metabolomics may provide an analytical foundation for such a system. To this end, the aim of this work is to provide a comprehensive overview of the UTI-induced changes in the urinary metabolic pattern. In this context, a molecule-based approach has not only an added value in the development of a clinical scoring system of the disease severity but also provides new insight for the explanation of an infection, the degree of morbidity and the process of recovery. .

A cross-platform approach employing such analytical techniques as nuclear magnetic resonance, liquid and gas chromatography-mass spectrometry has offered a comprehensive overview of the metabolic changes associated with UTI. Firstly, we have detected 'classical' compounds as expression of bacteria contamination such as acetate and trimethylamine oxide. Secondly, we have proposed some physiological markers. For instance, we have indicated well-established molecules for uremia alongside diagnostic agents for the measurement of the renal plasma flow (para-aminohippuric acid) and the ascending course of the infection (hydroxyhippuric acid). Moreover we could also identify and structural characterized unique O-glycopeptides, which are at our knowledge, the first demonstration of glycosilation of human fribrinogen alpha 1-chain. Although the clinical significance of these findings should be validated, these molecules fully represent the multivariate frame of this common as well as complex disease as UTI and they might describe an ongoing process (e.g recovery/infectious process) or the kidney functionality. The implementation of such discoveries in a clinical set up might support the quick identification of the uropathogens as well as a more accurate disease classification and a better understanding of the morbidity severity.

SYNTHESIS AND BIOLOGICAL EVALUATION OF NEW SUBSTITUTED 4-AMIDOCARBAZOLES AS HUMAN mPGES-1 INHIBITORS

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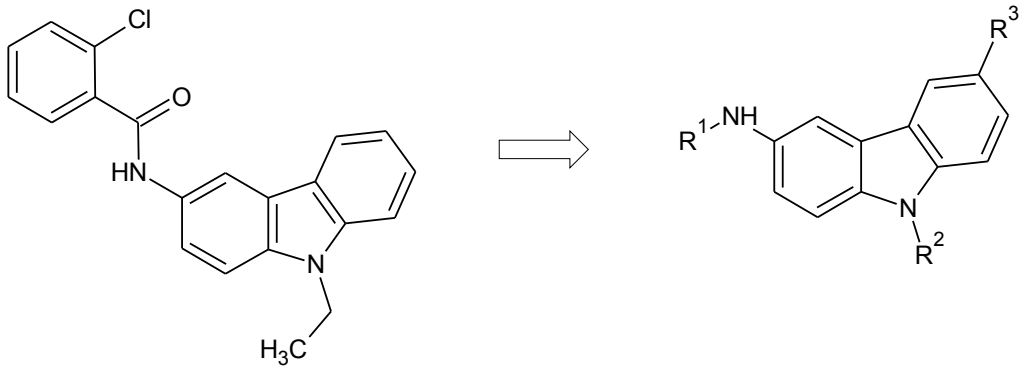
Prostaglandin E_2 (PGE_2) is an important lipid mediator in acute and chronic inflammation, pain and fever and is found in the synovial fluid of patients with osteoarthritis (OA) and rheumatoid arthritis (RA). [(1)] Microsomal prostaglandin E synthase-1 (mPGES-1) constitutes an inducible glutathione-dependent integral membrane protein that is responsible for the conversion of cyclooxygenase-derived prostaglandin PGH_2 into PGE_2.[(2)] mPGES-1 is the major source of inducible PGE_2 and is up-regulated under inflammatory conditions. Studies with mPGES-1 knockout mice have highlighted that mPGES-1 is a promising target for the suppression of increased PGE_2 levels without the crucial side effects of NSAIDs (gastrointestinal toxicity) and COX-2 inhibitors (thrombogenesis). [(3)] Thus, selective mPGES-1 inhibitors are expected to be useful in the treatment of PGE_2-related disorders without the critical drawbacks of existing drugs.

With the aim to find novel mPGES-1 inhibitors, we carried out a high-throughput screening (HTS, enzyme assay) on a commercially available collection of 5000 compounds. Compound **I** emerged as a moderately potent human mPGES-1 inhibitor (79% inhibition at 1 μM).



I **II**

A structure-activity relationship (SAR) study on the scaffold of **I** was conducted focusing the interest on the effect of moieties R^1, R^2 and R^3 in **II**.

In this presentation we will illustrate the results of enzymatic and cellular (A549 human cell line) PGE_2-production inhibition assays of compounds derived from this SAR investigation.

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NOVEL GLUCOSE-CONJUGATED HIGHLY POTENT DUAL THROMBIN AND FACTOR Xa INHIBITORS AS POTENTIAL ANTITHROMBOTICS

Giorgia Zaetta,[1]* Modesto de Candia,[1] Francesco Campagna,[1] Benny D. Belviso,[2] Rocco Caliandro,[2] Francesca Incampo,[3] Cosimo Carrieri,[3] Mario Colucci,[3] Cosimo D. Altomare[1]

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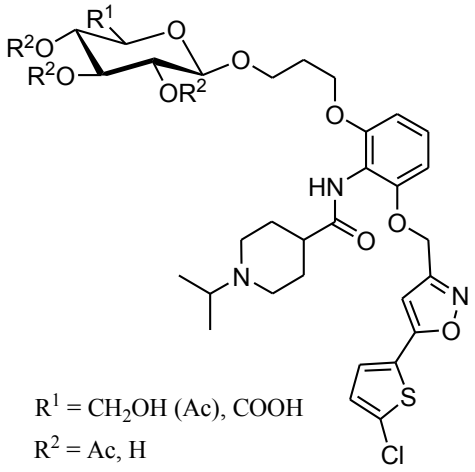
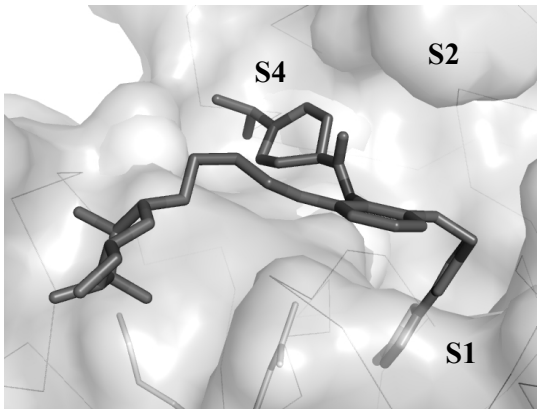
Novel *O*-glucosides of the recently reported potent factor Xa (fXa) inhibitors,[(1)] which bear 5-chlorothien-2-yl moiety and 1-isopropylpiperidine as the fragments binding the S1 and S4 enzyme pockets, respectively, were synthesized. In particular, β-D-glucose was conjugated through an ether-linked C3-alkyl spacer to the central phenyl ring of the most potent inhibitor, providing a β-D-glucosyl derivative which showed picomolar inhibition potency against human fXa (*K*i = 60 pM), nanomolar potency against thrombin (fIIa, *K*i = 60 nM) and high selectivity over a panel of other serine proteases, including trypsin and leukocyte elastase, as well as in vitro sub-micromolar anticoagulant activity in the prothrombin time (PT) clotting assay and a statistically significant 1.6-fold prolongation of the basal PT in an ex vivo assay in mice.

The crystal structures of human thrombin in complex with two highly potent glucose-based compounds were solved, which provided us with useful information on the binding modes of these inhibitors. While as expected from previous studies[(2)] the chlorothiophene group binds in the S1 pocket and the *N*1-isopropylpiperidine group in the S4 region, the sugar moiety binds in a protein region hitherto unexploited by small-molecule direct fIIa inhibitors, which is located near the S4 subsite, where the glucose O2 form strong H-bonds with two basic residues, i.e., Arg221A and Lys224.



$R^1 = CH_2OH$ (Ac), COOH
R^2 = Ac, H



X-Ray crystal structure of human thrombin (active site region) in complex with the most potent *O*-glucoside inhibitor ($R^1 = CH_2OH$, R^2 = H).

The potential of the newly synthesized glucose-based dual fXa and fIIa inhibitors as antithrombotic agents has been further supported by our recent results, which showed favorable effects on the thrombin generation, as determined by calibrated automated thrombography (CAT), and profibrinolytic activity, as assessed by plasma turbidimetric assay.

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SYNTHETIC APPROACH TO NOVEL HINGE-BINDING MOTIFS FOR JAK3-INHIBITORS VIA NUCLEOPHILIC AROMATIC SUBSTITUTION

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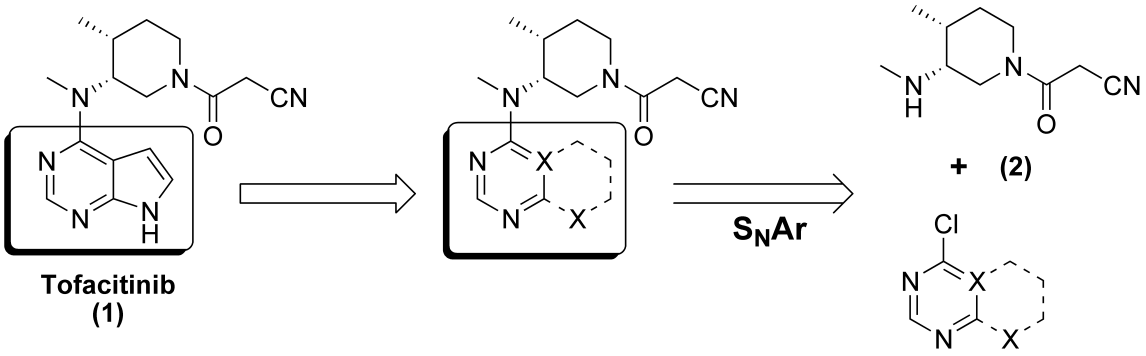
Janus kinases play a key role in many signalling pathways of cytokines, interleukins and interferons. The JAK kinase family consists of four members, JAK1-3 and TYK2. Belonging to the non-receptor tyrosine kinases they mediate extracellular signals via the phosphorylation of STAT-proteins (signal transducer and activator of transcription). Whereas the other members of the JAK family are expressed ubiquitously, JAK3 is predominantly found in cells of the haematopoietic system, having a key function in the maturation of immune cells. Patients with a malfunction of JAK3 are lacking T-lymphocytes and natural killer cells resulting in severe combined immunodeficiency (SCID). The restriction of its function to the immune system makes JAK3 a promising target for the treatment of autoimmune diseases, inflammation and allograft rejection.[1] The recently FDA-approved Tofacitinib **(1)** is the first small-molecule JAK3-inhibitor for the treatment of rheumatoid arthritis.[2]



In the design of ATP-competitive kinase inhibitors the hinge-binding motif plays a crucial role. The hinge region connects the N- and C-terminal lobes of the kinase and forms the back side of the catalytic cleft of the enzyme. ATP binds to the residues of the hinge region by forming two hydrogen bonds between the adenine heterocycle and the protein backbone. The hinge-binding motif of a kinase inhibitor is mimicking the adenine-moiety of ATP. It usually consists of a nitrogen containing heterocycle forming between one and three hydrogen bonds to the hinge region.

During the development of Tofacitinib, the side chain attached to the heterocycle was optimized extensively, but the hinge-binding pyrrolopyrimidine was left untouched.[3] In our attempt to obtain novel JAK3-inhibitors we used highly potent Tofacitinib as template and varied the hinge-binding motif. We developed a high yielding synthesis for the aminopiperidine side chain **(2)** of Tofacitinib, which can be coupled with electron deficient chlorinated heterocycles via nucleophilic aromatic substitution (S_NAr). These heterocycles are common building blocks and readily accessible. Therefore our approach is an efficient way for modifying the hinge-binding motif while reducing synthetic effort to prepare new JAK3-inhibitors.

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NEW CHALCONE DERIVATIVES BEARING A METHYLSULFONYL MOIETY AS POTENTIAL COX INHIBITORS

Mehlika Dilek Altıntop,[1]* Ahmet Özdemir,[1] Zafer Asım Kaplancıklı,[1] Gülhan Turan-Zitouni,[1] Gülşen Akalın-Çiftçi[2]

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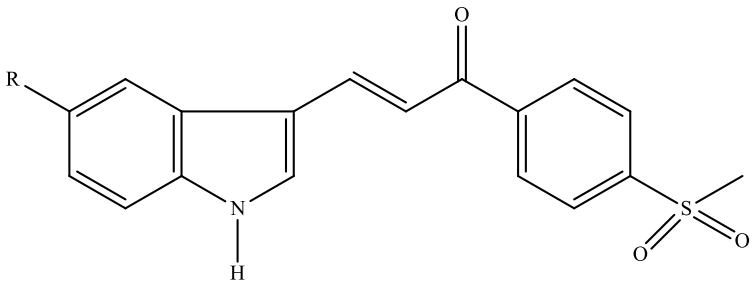
Chalcones are considered as precursors of open chain flavonoids and isoflavonoids present in edible plants. In the last few decades, chalcones and their analogues have attracted a great deal of interest due to their synthetic and biological importance in medicinal chemistry.[(1-2)]

3-(5-Substituted-1*H*-indol-3-yl)-1-(4-(methylsulfonyl)phenyl)prop-2-en-1-ones were synthesized *via* the base-catalyzed Claisen-Schmidt condensation of 4'-(methylsulfonyl)acetophenone with 5-substituted-1*H*-indole-3-carboxaldehydes. The obtained compounds were evaluated for their COX inhibitory activity. The compounds displayed different levels of COX inhibitory activity.



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ANTIARRHYTHMIC AGENTS ENDOWED WITH ANTIOXIDANT PROPERTIES

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Due to the presumed role of oxidative stress in a range of human diseases such as cardiovascular disorders (e. g. atherosclerosis and ischemia-reperfusion injury),(1) a number of efforts have been recently focused on the development of new effective antioxidant drugs. Mexiletine, a voltage-gated Na^+ channel blocker with antiarrhythmic effect, can also act as antioxidant by inhibiting hydroxyl radical-mediated lipid peroxidation in brain membranes.(2) Moreover, a pyrroline derivative of mexiletine has been demonstrated to be capable of providing marked protection against ischemia-reperfusion myocardial injury.(3) In the last ten years we have prepared several mexiletine analogues (Fig.1), most of which act more potently than mexiletine in blocking skeletal muscle voltage-gated sodium channels.(4) These compounds performed as more potent antiarrhythmic agents than the parent compound. Thus, under the hypothesis that a synergism between antiarrhythmic activity and antioxidant properties may contribute to cytoprotection, we linked mexiletine and its analogues to a pyrroline ring, a well known ROS scavenging moiety, in order to obtain potential dual-acting drugs. The resultant pyrroline derivatives evaluated for their ability to block native skeletal muscle sodium channels (Nav1.4) by voltage-clamp recordings, showed improved activity with respect to mexiletine. Importantly, these compounds are among the most potent use-dependent Mex analogues described so far, thus representing interesting pharmacological tools.(5) Herein, the synthesis and the pharmacological characterization of the mexiletine analogues and their pyrroline derivatives as antiarrhythmic and antioxidant agents will be presented.

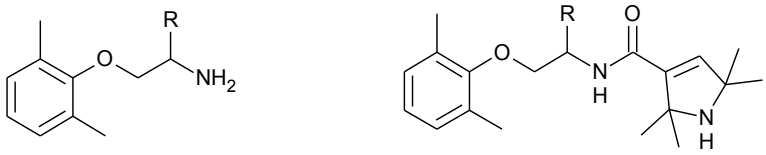

Fig 1: R = Me, *i*Pr, *t*Bu, Ph

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NOVEL SEMISINTHETYC DOXORUBICINS WITH REDUCED CARDIOTOXICITY

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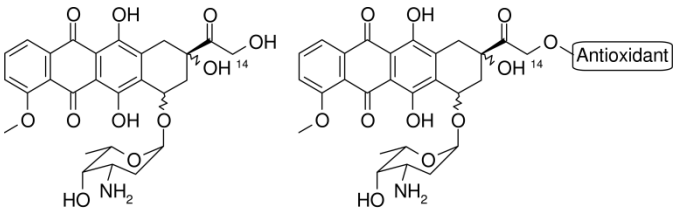
Doxorubicin (DOXO) (**1**, Figure 1) is a potent broad-spectrum antineoplastic antibiotic belonging to the anthracycline family. It is widely used, as single agent or in combination with other anticancer drugs, in treating a variety of cancers including solid tumors, soft-tissue sarcomas, lymphomas, and leukemias.(1) DOXO displays a number of clinical toxicities, of which cardiomyopathy is the most important. Two kinds of cardiomyopathies can occur: an acute form and a chronic, cumulative dose-related form. The former kind is rarely a serious problem, while the latter can lead to congestive heart failure that is unresponsive to digitalis.(2) The mortality rate in patients with congestive heart failure is close to 50%. The classic molecular mechanisms underlying both the anticancer and the toxic effects of DOXO operate at two distinct levels: by modifying DNA, and by inducing oxidative stress. The heart is very sensitive to oxidative stress, because of its strongly oxidative metabolism and poor antioxidant defenses. DOXO can generate free radicals in a number of different ways: it is reduced by various biological systems to a semiquinone free radical giving rise to a number of ROS, including peroxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH\cdot$). It is known that the heart is very rich in mitochondria, which contain a phospholipid called cardiolipin, for which DOXO displays great affinity; (3) in consequence, DOXO accumulates in the mitochondria, with production of high levels of ROS. The final result is the cardiomyocyte damage, mainly deriving from the impairment of mitochondrial functioning.(4,5)



1 **2**

The use of the variety of natural and synthetic antioxidants to prevent DOXO induced cardiotoxicity has been considered,(1,6) and combinations of DOXO with agent(s) capable of blocking its ROS-mediated cardiotoxicity effect have been investigated. As a development of our studies on semi-synthetic doxorubicin, we here report preliminary results obtained with DOXO hybrids of general structure **2**, in which the antibiotic is linked with selected antioxidant moieties through appropriate spacers.

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PHENYLDIAZENYL ANALOGUES OF FIBRATES AS PPAR AGONISTS

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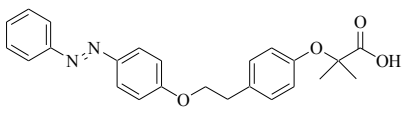
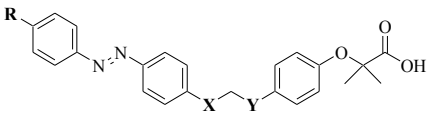
Peroxisome Proliferator-Activated Receptors (PPARs) are nuclear hormone receptors expressed in metabolically active tissues. It has been recognized that metabolic syndrome lead an increase of atherosclerosis, myocardial and cerebral stroke. Insulin resistance and lipid homeostasis are factors involved in metabolic syndrome. Thiazolidinediones, used in therapy as antidiabetic agents, and fibrates, used as antihyperlipidemic drugs, are PPARγ and α agonists, respectively. Unfortunately, fibrates are poor activators of PPARα, their subtype selectivity is not high and their use is associated with an increase of myopathy and hepatotoxicity. Thiazolidinediones have also frequent side effects such as weight gain, edema, and heart failure.[1,2] For these reasons, the potential beneficial effects of activating both PPARα and PPARγ receptors have stimulated large interest in development of PPARα/γ dual agonists with the benefits of fibrates on plasma lipids and thiazolidinediones on insulin sensitivity.[3,4]

The typical chemical structures of synthetic PPAR agonists are referable to a pharmacophore that include a carboxylic acid head, an aromatic ring, and a lipophilic cyclic tail, connected by linkers.

Recently we reported a series of fibrate analogues based on a combination of the classical clofibric acid moiety and lipophilic groups derived from natural products such as chalcone and stilbene. We kept unaltered the clofibrate scaffold and systematically varied the cyclic tail and the length of linker between the aromatic centre and the lipophilic tail. Interesting results were found for the phenyldiazenyl analogue of clofibric acid **I**; it was identified as a dual PPARα/γ agonists (αEC_{50}=0.6 μM and γEC_{50}=1.4 μM).[5]



I



II

R=H, Br, Cl, CF_3, CN, NO_2; **X**= CH_2, O; **Y**= CH_2, O

On the basis of these results, this molecule was chosen as lead compound and it was crystallized with the PPARα and PPARγ to better understand the interaction with the receptors. In the poster session we describe the crystal structures of the complexes of PPARα-LBD and PPARγ-LBD with lead compound.

Moreover, in order to gain more insight on the structure-activity relationships, we also report the synthesis and biological evaluation of the new phenyldiazenyl derivatives **II** with different substituent in *para* to the phenyldiazenyl moiety and with the oxygen of the linker in *para* to the 2-methyl-2-phenoxypropanoic group.

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DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF NEW ANALOGS OF THE DUAL PPARα/γ AGONIST LT175

Antonio Laghezza,[1*] Giorgio Pochetti,[2] Luca Piemontese,[1] Sabina Sblano,[1] Antonio Carrieri,[1] Giuseppe Fracchiolla,[1] Giuseppe Carbonara,[1] Roberta Montanari,[2] Antonio Lavecchia,[3] Paolo Tortorella,[1] Fulvio Loiodice[1]

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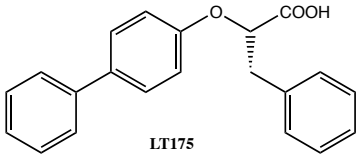
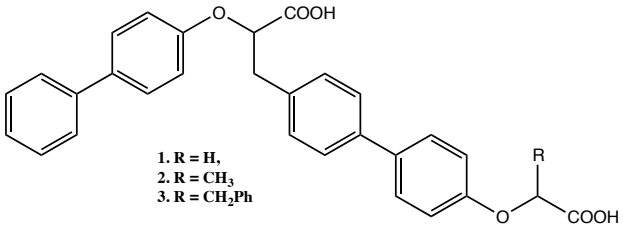
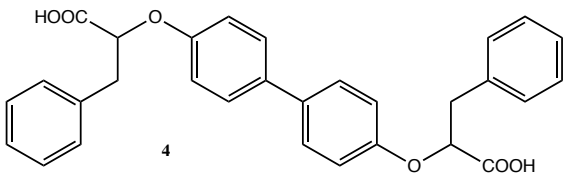
Peroxisome Proliferator-Activated Receptors (PPARs) are members of the nuclear receptor superfamily playing a crucial role in the regulation of metabolic homeostasis. As such, the three PPAR isoforms designated α, γ and δ, certainly represent attractive targets for molecules that could be used in diabetes and dyslipidemia.(1) Following the recent indications available in the literature, research is currently addressed to the preparation and characterization of new molecules able to simultaneously activate more PPAR subtypes (PPAR dual agonists and/or pan-agonists), and/or to selectively modulate them (SPPARMs). Recently, we reported the synthesis and biological activity of some chiral carboxylic acid derivatives showing an interesting dual activity towards PPARα and PPARγ with the stereochemistry playing a crucial role in the receptor activation.(2,3) In particular, one of these newly identified PPAR ligands (**LT-175**, see Figure) has been shown to occupy a branch of the PPARγ ligand-binding domain (LBD), named "diphenyl pocket", exhibiting the typical profile of partial agonists.(2) The corresponding R enantiomer, instead, is less active fitting another part of the receptor binding pocket. With the aim to evaluate the effects resulting from both interaction modes, we synthesized and tested the dimeric analogs **1-4** characterized by the presence of two carboxylic functions which represent critical contact points for the formation of hydrogen bond networks with the receptor. Preliminary results on PPARα and PPARγ show very interesting activity profiles of these ligands with marked differences depending on the configuration of the two stereogenic centers. The critical influence of stereochemistry on the activity prompted us to accomplish a structural simplification which led to the monocarboxylic derivative **5** containing only one chiral centre. The S enantiomer of this compound (**LJ-570**) turned out to be the most potent agonist on both receptors. The X-ray structure of the PPARγ/**LJ-570** complex was resolved to investigate the interaction mode and gain structural biology insight into the role played by the carboxylic group and the diphenyl systems present in this ligand.



LT175



1. R = H,
2. R = CH_3
3. R = CH_2Ph



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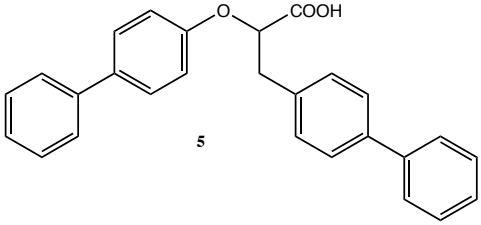

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SYNTHESIS AND BIOLOGICAL EVALUATION OF NEW SUBSTITUTED 4-AMIDOCARBAZOLES AS HUMAN mPGES-1 INHIBITORS

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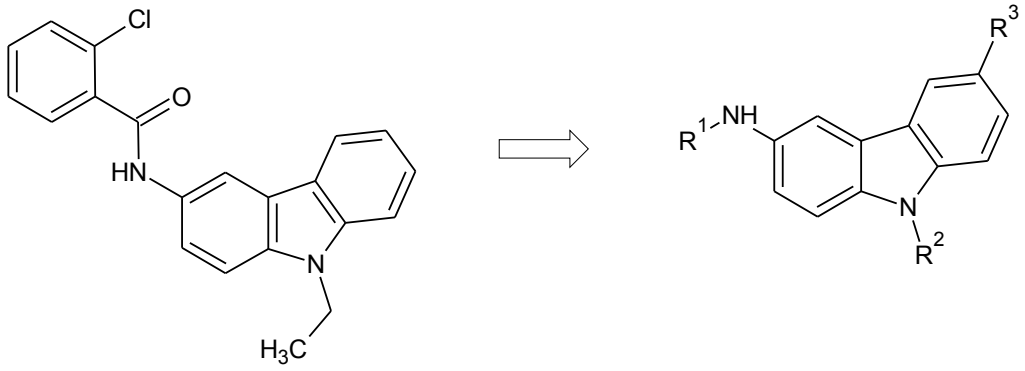
Prostaglandin E_2 (PGE_2) is an important lipid mediator in acute and chronic inflammation, pain and fever and is found in the synovial fluid of patients with osteoarthritis (OA) and rheumatoid arthritis (RA). [(1)] Microsomal prostaglandin E synthase-1 (mPGES-1) constitutes an inducible glutathione-dependent integral membrane protein that is responsible for the conversion of cyclooxygenase-derived prostaglandin PGH_2 into PGE_2.[(2)] mPGES-1 is the major source of inducible PGE_2 and is up-regulated under inflammatory conditions. Studies with mPGES-1 knockout mice have highlighted that mPGES-1 is a promising target for the suppression of increased PGE_2 levels without the crucial side effects of NSAIDs (gastrointestinal toxicity) and COX-2 inhibitors (thrombogenesis). [(3)] Thus, selective mPGES-1 inhibitors are expected to be useful in the treatment of PGE_2-related disorders without the critical drawbacks of existing drugs.

With the aim to find novel mPGES-1 inhibitors, we carried out a high-throughput screening (HTS, enzyme assay) on a commercially available collection of 5000 compounds. Compound **I** emerged as a moderately potent human mPGES-1 inhibitor (79% inhibition at 1 μM).



I **II**

A structure-activity relationship (SAR) study on the scaffold of **I** was conducted focusing the interest on the effect of moieties R^1, R^2 and R^3 in **II**.

In this presentation we will illustrate the results of enzymatic and cellular (A549 human cell line) PGE_2-production inhibition assays of compounds derived from this SAR investigation.

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DISCOVERY OF NOVEL QUINAZOLINONE BASED mTOR INHIBITORS ENDOWED WITH ANTIFIBROTIC PROPERTIES

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In tissues after a prolonged injury or a chronic inflammation the persistence of repair processes leads to the aberrant extracellular matrix (ECM) deposition (*i.e.* tissue fibrosis).(1) Fibrosis ultimately compromise the function(s) of the organ resulting in diseases (*i.e.* idiopathic pulmonary fibrosis, liver cirrhosis, or renal fibrosis, etc) responsible of several thousands of deaths per year. The massive ECM deposition is mediated by excessive/persistent release of pro-inflammatory cytokines and growth factors that stimulates myofibroblasts expansion, de-regulates metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) expression. To date the pharmacological care in patients with tissue fibrosis relies on corticosteroids and immunosuppressant drugs and it is rather unsatisfactory since often the ultimate resource is an organ transplant. Modern pharmacological strategies aim to take inhibiting specific components of the fibrogenic pathways by mean of monoclonal antibodies or small molecules.(2)

Along this line, mTOR, a serine/threonine kinase belonging to the family of phosphoinositide 3-kinase related kinases, has emerged as a potential target for anti-fibrotic therapies.(3)

Herein we report the discovery of novel quinazolinone based mTOR inhibitors that showed interesting antifibrotic properties while not showing cytotoxic activities (Figure 1).

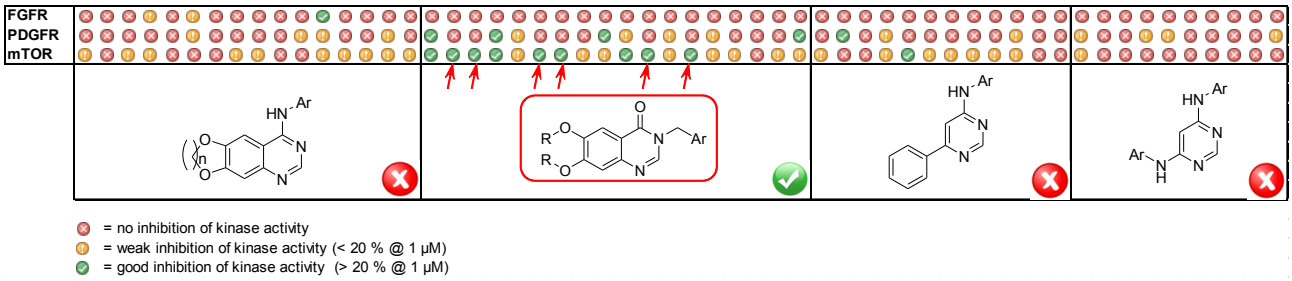

Figure 1. Preliminary assays that lead to the identification of novel mTOR inhibitors

The title compounds have been identified starting from the screening of an in-house library of properly designed potential kinase inhibitors. Those compounds that showed to be selective mTOR inhibitors have been then submitted to cytotoxic experiments. The molecules unable to impair cell viability have been then submitted to specific assays in order to investigate the antifibrotic properties. In particular, the selected molecules (at 1 μM) reduced the fibrosis-related mRNA transcript levels (*i.e.* collagen type I and fibronectin) in primary human hepatic stellate cells and intestinal myofibroblasts, two different cellular populations involved in the fibrogenic process of liver and gut, respectively.(4)

Finally, molecular modelling studies (based on an homology model of mTOR kinase) have been used to propose a plausible binding mode for quinazolinone compounds.

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DESIGN, SYNTHESIS, BIOLOGICAL TESTING AND SAR OF 5 PYRIDINYL-2-THIOIMIDAZOLE DERIVATIVES AS APPROACH FOR JNK3 SELECTIVE INHIBITORS OVER P38A

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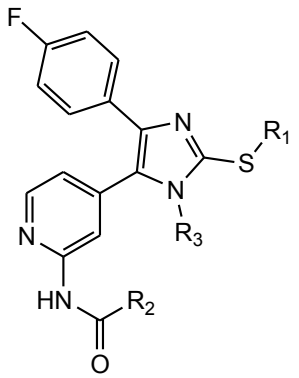
Many inflammatory conditions are driven by both JNK3 and p38α MAPK.(1) To discriminate between the effects of each kinase, selective inhibitors are required. For p38α this is the case, (2) but to date only few selective JNK3 inhibitors with good ADME (Absorption, Distribution, Metabolism, Excretion) properties are available.

JNKs are c-jun NH_2-terminal serine/threonine mitogen activated kinases. Cytokines and environmental influences are the main activators.(3,4) JNK3 is believed to play a central role in the pathology of neurologic diseases such as cerebrovascular accidents, Parkinson's and Alzheimer's disease.(5) Therefore it has become a valid and attractive drug target.



Pyridinyl-thioimidazole derivatives are well known p38α inhibitors.[6] Due to the sequential and steric similarity of p38α and JNK3, we assumed to gain active and selective JNK3-inhibitors by substituting moieties R_1, R_2, and R_3. The amino acid residues Asn152 and Gln155 are located at the margin of the hydrophobic region II in JNK3, whereas Asp112 and Asn152 are presented in p38α.(3) In order to achieve repulsion between Asp112 and p38α, we introduced anionic substituents at the aminopyridine scaffold. Furthermore, we tried to hit Asp112(3) by introducing carboxylic acid moieties at the imidazole nitrogen. In order to target the conserved but steric diverse Arg107 and Asn194[3], we synthesised carboxylic acid substituents linked by a sulfide to the tetra substituted imidazole resulting in an IC_{50} value of 50 nM for JNK3 with about 10 fold selectivity against p38α.

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SIDE CHAIN MODIFIED HDCA DERIVATIVES: SYNTHESIS, CMC DETERMINATION AND MOLECULAR MODELLING STUDIES

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The elucidation of the interconnection between the biological pathways under the bile acid (BA) control and manifestations of chronic hepatic and metabolic diseases have extended the scope of biliary derivatives as promising chemical tools and lead compounds for preclinical and clinical investigations.[(1,2)] Accordingly, the design and synthesis of novel BA-based receptor modulators have required a deep understanding of both structure-activity and structure-property relationship issues.

In this connection, we have been engaged in the preparation and the critical micellization concentration (CMC) evaluation of a series of hyodeoxycholic acid (HDCA) derivatives characterized by a diverse side chain length and by the presence of a methyl group at the alpha position to the terminal carboxylic moiety. The collected data depict a clearer scenario on the structure-CMC relationships of this class of compounds, unveiling some peculiar properties shared by the molecular shape of BAs. In particular, we have demonstrated the strong effect on the CMC value of the substituent optical configuration at the alpha position to the carboxylic group with the (*R*)-epimer being more prone to form micelles with respect to the corresponding (*S*)-one. As an additional observation, the side chain elongation was found to reduce the different micellization ability between the diverse couple of epimers, as quantitatively confirmed by root-mean-square deviation (RMSD) analysis. The results presented can be of great utility in combination with model of receptor activity, to guide the development of novel BA modulators with improved pharmacokinetic profile and drug-like properties.

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DESIG, SYNTHESIS AND IN COMBO PHARMACOLOGICAL STUDIES OF AZA AND THIAZAHETEROCYCLES WITH ANTIDIABETIC ACTIVITY

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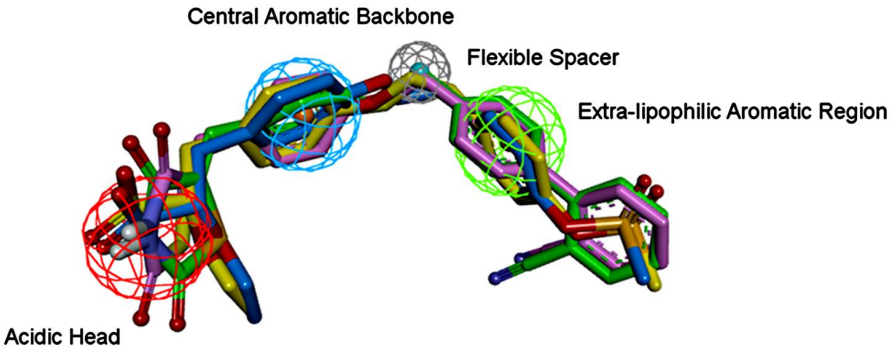
Aza and thiazaheterocycles such as benzimidazole, (benzo)thiazole and thiazolidine-2,4-dione, are privileged structures which shows several pharmacological activities.(1) In this research, we used these scaffolds in order to get new antidiabetic compounds.

A series of thiazolidine-2,4-dione (TZD), barbituric acid, benzothiazole and benzimidazole derivatives were prepared using a short synthetic route, and all compounds were characterized by elemental analysis, mass spectrometry, and NMR spectroscopy. Their in vitro relative expression of peroxisome proliferator-activated receptor (PPAR) alpha and peroxisome proliferator-activated receptor gamma was evaluated only for TZD bioisosteres . Some compounds showed an increase in the mRNA expression of both peroxisome proliferator-activated receptor isoforms, as well as the GLUT-4 levels and FATP-1.

The antidiabetic activity of compound the most active compounds was determined at 50 mg/Kg single oral dose using a non-insulindependent diabetes mellitus rat model. The results indicated a significant decrease in plasma glucose levels. Additionally, we performed a molecular docking of the most active compounds into the ligand binding pocket of PPARα and PPARγ. In these binding models, thiazolidene-2,4-dione may bind into the active site of both isoforms showing important short contacts with PPAR gamma residues: Tyr 473, His 449, Ser 289, His 323; and PPAR alpha residues: Tyr 464, His 440, Ser 280 and Tyr 314. With these results, we propose that the features could be taking into consideration for designing novel dual PPAR α/γ modulators should consist of four parts(2):

(a) An acidic head group, such as thiazolidine-2,-4-dione, carboxylic acid, or related bioisoteres;
(b) A central aromatic backbone;
(c) An extra-lipophilic aromatic region;
(d) A flexible spacer that connects regions (b) and (c), and allow the structure to adopt specific conformation.



The remaining compounds were tested as inhibitors of the enzymes: Protein tyrosine Phosphatase 1B and 11β-Hydroxysteroid dehydrogenase. Inhibition of both enzymes has been considered as attractive therapeutic targets for diabetes, obesity and metabolic syndrome.[3] The most active inhibitors showed significant lowering of plasma glucose concentration in the *in vivo* antidiabetic assay.

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SYNTHESIS AND COX INHIBITORY ACTIVITY OF NEW CHALCONE DERIVATIVES

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Cyclooxygenase (COX) enzymes have attracted a great deal of interest as important targets for drug discovery due to their essential role in prostaglandin biosynthesis. Inhibition of COX enzymes is a promising approach for pharmacologic intervention in inflammation. Among therapeutic agents in clinical use today, nonsteroidal anti-inflammatory drugs (NSAIDs) exert their therapeutic action by inhibiting COX enzymes.

Chalcones have gained great importance in medicinal chemistry and considerable research on them in relation to inflammation has been accomplished.

In the present work, new chalcone derivatives were obtained *via* the reaction of 1-methylindole-3-carboxaldehyde with appropriate acetophenones. The synthesized compounds were investigated for their potential COX inhibitory activity.

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IMPROVING THE SOLUBILITY AND THE BIOLOGICAL PROFILE OF A NOVEL CLASS OF DERIVATIVES AS SELECTIVE COX-2 INHIBITING NITRIC OXIDE DONORS

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The family of cyclooxygenase-2 (COX-2) selective inhibitors (COXIBs) is a class of compounds developed to overcome gastrointestinal toxicity caused by use of traditional non steroidal anti-inflammatory drugs (t-NSAIDs).(1) Moreover, it is well known that the cardiovascular side effects of COXIBs are due to the selective COX-2 inhibition;(2) therefore, in the last years research attention focused on developing pharmacodynamic hybrids that conjugate the COX-2 selective skeleton with different Nitric oxide (NO)-releasing chains, to make use of NO-mediated vasorelaxing effects.(3) In the past years we synthesized a first class of COXIBs characterized by a 1,5-diphenyl-pyrrole scaffold and a NO releasing moiety;(4-5) these derivatives showed good biological profiles, despite their low water solubility. On these grounds and on the basis of literature data, the study progressed by means of modifying the central core in order to improve the solubility of this class of derivatives. Following the previous adopted protocol, selected compounds have been tested: i) *in vitro*: to assess their cyclooxygenase-1 (COX-1) and COX-2 inhibition activities; ii) *ex vivo*: to investigate their NO-releasing properties; iii) *in vivo*: to evaluate their efficacy in analgesia, by means of the abdominal writhing test.

Furthermore, since COX-inhibitors play important opposite roles in carcinogenesis and they are also involved in reducing the Histone Deacetylase Inhibitors (HDACi) side effects of upregulating COX-1 and COX-2,(6) in our future studies, we aim to investigate antitumor activity of these compounds, also in association with HDACi.

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BOUND BY BLUE: A NEW, HIGH-AFFINITY AND FAST HEPARIN SENSOR IN COMPETITIVE MEDIA

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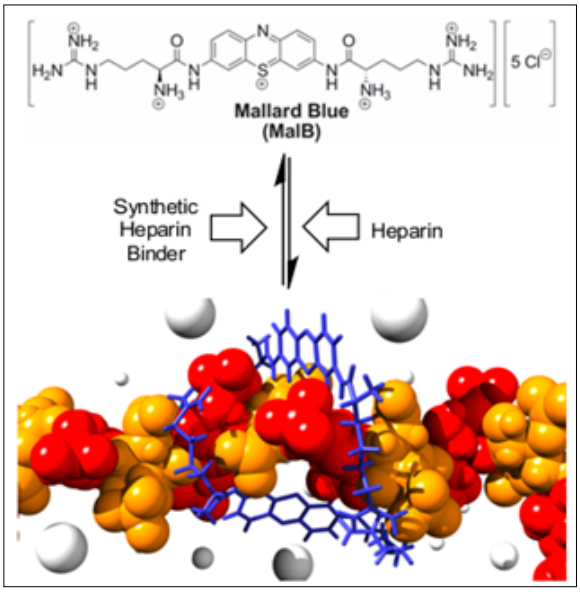
Heparin, the highest negatively charged, naturally occurring biopolymer, is widely used as anticoagulant agent, especially during surgery. As a result of its clinical importance, there has been a surge of interest in developing heparin sensors able to operate under biologically relevant conditions and in highly competitive media.[1,3] In the present contribution we report the design, synthesis and full investigation of an arginine-functionalized thionine – termed Mallard Blue (MalB)– as novel heparin binding dye. This molecule works efficiently as heparin sensor under harsh conditions, including water with high levels of competitive electrolytes, buffered aqueous solution, and human serum. Molecular dynamics simulations provide detailed insight into heparin/Mallard Blue binding mode and interactions. Importantly, we clearly demonstrate that MalB outperforms standard dyes currently used as heparin sensors, such as azure A.[4,6]

Furthermore, we describe the use of MalB in a novel spectroscopic assay to probe the heparin binding ability of synthetic systems – i.e. different generations of polyamidoamine (PAMAM) dendrimers – both in buffer and human serum. This, with the final goal of potential applications for post-surgical heparin removal.[7] Currently, the only licensed heparin reversal agent is protamine sulfate; protamine causes adverse reactions in up to 10% of patients and 2.6% of cardiac surgeries experience serious complications.[8,9] There is therefore interest in developing a replacement heparin reversal agent. Interestingly, the assay identifies G2-PAMAM – the often neglected and yet the less toxic member of the PAMAM family – as the preferred dendrimer in terms dose/effect terms. *In silico* experiments support these data and a molecular rationale for these findings is proposed. Remarkably, the new assay also works using heparin delivered in 100% human serum – indeed, G2-PAMAM remains an effective heparin binder under these fundamental physiological conditions.



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SYNTHESIS AND MOLECULAR DYNAMIC STUDIES ON 2-(BENZISOTHIAZOLYL)-*N*-(4-OXO-2-ARYL-THIAZOLIDINYL)-PROPANAMIDES AS POTENT INHIBITORS OF METALLOPROTEINASES

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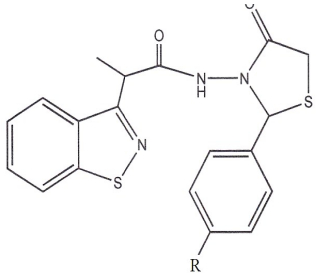
The matrix metalloproteinases (MMPs) are a large family of zinc dependant proteases with a central role in degradation and remodelling of the extracellular matrix (ECM). These enzymes are implicated in many pathological conditions, such as inflammation, cancer, Alzheimer's disease and osteoarthritis (OA). The use of MMPs inhibitors, potent and selective, could represent an excellent strategy for the treatment of these pathologies.(1) In our previous study, 4-thiazolidinones have shown to possess anti-inflammatory and anti-degenerative properties.(2) In particular 2-benzisothiazolylimino-5-benzylidene-4-thiazolidinones inhibited MMP-3 and -13, the major MMPs involved in cartilage degradation in OA disease.(3) Among these 5-(4-methoxybenzylidene)-2-(benzisothiazol-2-ylimino)-thiazolidin-4-one proved to be the most potent and selective MMP-13 inhibitor (IC_{50} = 0.036 μM). Docking studies indicate a non-chelating zinc interaction mode.(4)

On the basis of this considerations, we synthesized 2-(benzo[*d*]isothiazol-3-yl)-*N*-(4-oxo-2-arylthiazolidin-3-yl)propanamides (figure1), a novel class of potential antidegenerative multi–targeted drugs combining two pharmacophoric heterocycles and the propanoic chain of the well known antinflammatory profene drugs.



Comp	R
1a	H
1b	-Cl
1c	-OCH_3
1d	-COOH
1e	-NH_2

Figure 1. 2-(benzo[*d*]isothiazol-3-yl)-*N*-(4-oxo-2-arylthiazolidin-3-yl)propanamides

This study reports molecular dynamic interaction and inhibition activity (IC_{50}) of 2-(benzo[*d*]isothiazol-3-yl)-N-(4-oxo-2-arylthiazolidin-3-yl) propanamides **1a–e**, on MMP-9 and MMP-13, particularly involved in tissue degeneration.(5) The results show how the inclusion of hydrophilic, lipophilic, electron-withdrawing and donors groups, influences the interaction and inhibition towards MMP-9 and -13.

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SOLID PHASE SYNTHESIS OF RAGE LIGANDS

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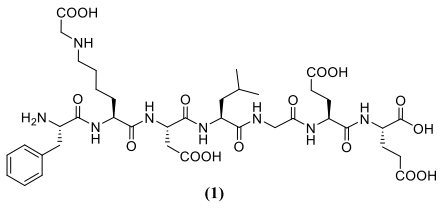
RAGE (AGEs receptor) activity has been associated with pathological conditions characterized by persistent inflammation, such as diabetes mellitus cardiovascular complications, atherosclerosis, Alzheimer's and autoimmune diseases.(1)

Among AGEs, the major RAGE ligands are the protein adducts carboxy-methyl-lysine (CML) and carboxy-ethyl-lysine (CEL) that are known to bind the receptor only if they are incorporated into a peptide or a protein.

Numerous studies related to AGEs or RAGE are reported, but there is a substantial lack of a solid phase synthesis protocol for the obtainment of CML and CEL peptides. In fact, most of the synthetic procedures for CML or CEL peptides use liquid-phase synthesis or the synthesis of the CML/CEL-amino acid in liquid phase followed by its inclusion by solid phase synthesis in the peptide sequence.(2-4)

We report here a solid phase protocol for the direct carboxymethylation or carboxyethylation of peptides on solid phase, obtaining a streamlined process that gives these compounds in high yields.



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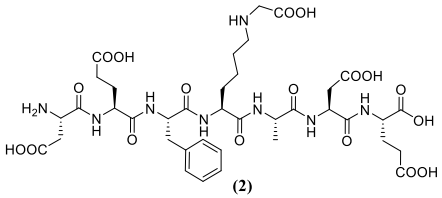

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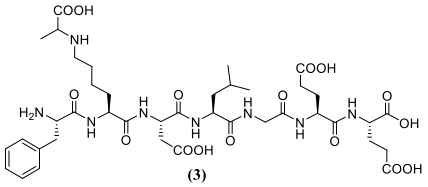

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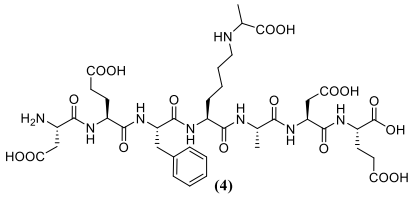

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DESIGN AND SYNTHESIS OF DIBENZOOXEPINONES AS p38α MAP KINASE INHIBITORS: EXTENDING INTERACTIONS WITH HYDROPHILIC AMIDE AND ESTER SUBSTITUENTS TOWARDS THE HYDROPHOBIC REGION II

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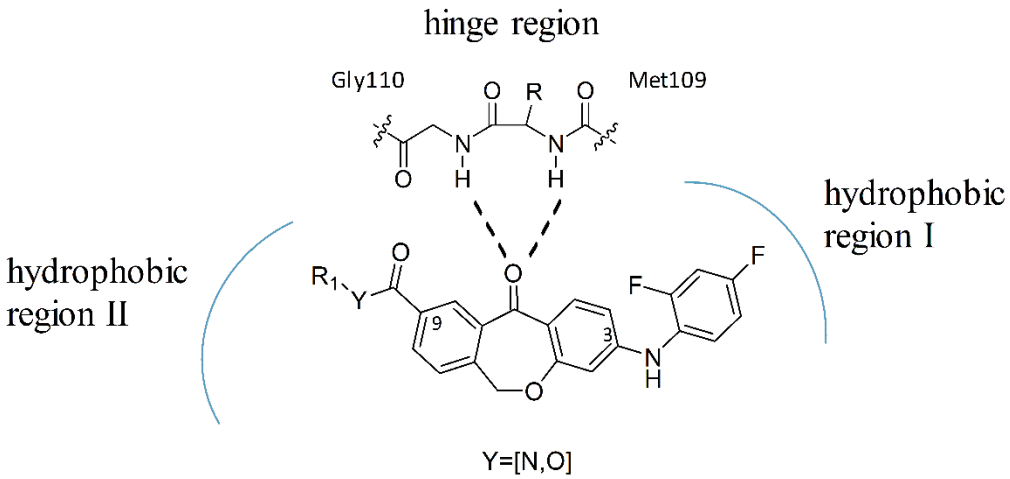
p38α kinase is a mitogen activated protein (MAP) kinase playing a central role in several severe inflammatory diseases, like psoriasis and inflammatory bowel disease.[1] Modulating angiogenesis and apoptosis[2] it's also involved in cancer. Regulating the signaling pathways of a variety of inflammatory cytokines, such as TNFα[3] and Il-1β[4], it seems promising to inhibit this cascade in order to suppress inflammation. A highly potent class of molecules inhibiting signal transduction via p38α MAP kinase are substituted dibenzosuberones and dibenzooxepinones formerly developed in our group.[5,6]



We previously observed that hydrophilic moieties like (poly-)alcohols in position 9 of this scaffold templates can form additional, highly favorable interactions with the hydrophobic region II leading to highly potent inhibitors with outstanding whole blood activity.[5] Apart from enthalpic effects, an increase in entropy by displacing water, which is ubiquitous in this region, seems to be a key driving force for increasing potency. We formerly prepared dibenzooxepinones with these hydrophilic moieties connected to the scaffold by ether linkers which proved to be highly potent. Since these structures are difficult to access, the moieties were replaced by ester and metabolically more stable amide substituents to increase the affinity and selectivity to the enzyme. Therefore a new synthetic strategy had to be established. According to expectations, the modification of the dibenzooxepinone scaffold led to a range of highly potent inhibitors.

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DIBENZOSUBERONES AS p38 MITOGEN-ACTIVATED PROTEIN KINASE INHIBITORS WITH LOW ATP COMPETITIVENESS AND OUTSTANDING WHOLE BLOOD ACTIVITY

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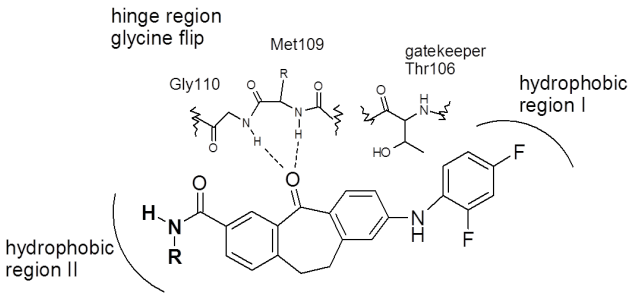
The p38α mitogen-activated protein (MAP) kinase plays a central role in signaling pathways regulating the biosynthesis of pro-inflammatory mediators such as TNF-α and IL-1β. These cytokines are known to be involved in the development of many inflammatory diseases. Therefore p38 is a valuable target for novel anti-inflammatory drugs.

Various approaches can be used to design selective inhibitors of p38 MAP kinase: 1. Exploiting the glycine flip;(1) 2. Occupation of the hydrophobic region I which lies adjacent to the ATP binding site; 3. Design of rigid inhibitors.(2)

Combination of these three selectivity features led to Skepinone-L, an ATP-competitive p38 MAP kinase inhibitor with outstanding potency and selectivity.(2) The example of Skepinone-L showed that introduction of hydrophilic residues directing towards the hydrophobic region II led to improved potency compared to compounds without this feature. Therefore we introduced promising hydrophilic moieties. In comparison to Skepinone-L the novel class of dibenzosuberones features a carboxylic acid in position 7 of the dibenzosuberone scaffold. Thereby the introduction of amides and esters could be achieved. The amide dibenzosuberone compounds show nanomolar IC_{50} values with respect to p38α MAP kinase, down to an IC_{50} of 2 nM, and low nanomolar IC_{50} values with respect to TNFα-release in human whole blood, down to an IC_{50} of 17 nM.(3) This approach led to an improvement of activity compared to the outstanding IC_{50} values of Skepinone-L.



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Analytical Tool in Drug Discovery and Development

MS BINDING ASSAYS IN MEDICINAL CHEMISTRY – FROM BINDING STUDIES TO DRUG SCREENING

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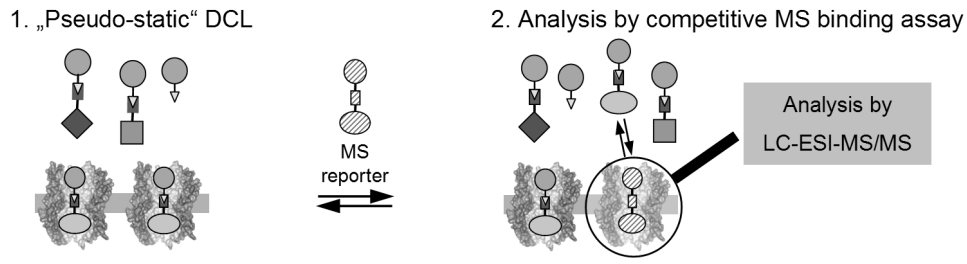
MS binding assays are a novel technique for the characterization of ligand-target interactions.[1] They are closely related to radioligand binding assays, but avoid all drawbacks that result from radioactivity associated with radiometric assays as no labelling of the ligands for quantitation by MS is required. They can be used in saturation experiments, competition experiments or kinetic studies. Like radioligand binding assays they deliver high quality data for the ligand target interaction. This will be demonstrated for the GABA transporter mGAT1 (SLC6a1), a target representing the most important subtype of the γ-aminobutyric acid transporters. In addition, competitive MS binding assays can also be employed for drug screening. Even libraries generated by Dynamic Combinatorial Chemistry (DCC) may be screened provided appropriate measures are taken to render them pseudostatic. This approach, that will be exemplified for mGAT1 as well, is highly rewarding as it combines the efficiency of MS binding assays to determine ligand binding with the ease of library generation by DCC.



For the screening, one dimensional hydrazone libraries were generated by reacting sets of aldehydes with a hydrazine derivative delineated from binding motifs known from common GAT1 inhibitors. Libraries to be “pseudo-static” has been ascertained by employing the hydrazine derivative in large excess. Moreover, as libraries are generated with the protein target already present and under conditions compatible for screening, they can be directly subjected to hit detection by MS binding assays.[2] In the present study in a first screening campaign two hit compounds were identified, which were further optimized by a second screening process of focussed pseudostatic compound libraries leading to even more potent mGAT1 inhibitors which served as templates for the synthesis of stable carba analogues.[3] Enabling screening of libraries independent whether generated by conventional chemistry or DCC and being applicable to all kind of targets including membrane bound targets such as GPCRs, ion channels or transporters, the described strategy displays high potential in the drug discovery process.

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APPLICATIONS OF NONCOVALENT ESI-MS IN DRUG DISCOVERY

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One of the first steps of the drug discovery process is the study of new chemical entity interactions with a biomolecule of therapeutic interest. Generally biomolecules are peptides, proteins, deoxyribonucleic acids (DNA), and ribonucleic acids (RNA). The use of electrospray ionization mass spectrometry (ESI-MS) in the study of non covalent target-ligand interactions is considered a well established approach.[(1,2)] This method directly detects non covalent ligand-target complexes in the gas phase, and allows inference of affinity (and specificity) of the ligand-target interaction in solution. Although non covalent complexes have been investigated by other techniques, the ESI-MS method has some key advantages. The MS methods are rapid and automatable, and the high sensitivity of the mass detector requires minimal amount of macromolecular target. Another key advantage of mass spectrometry is specificity; in fact, the identity of different complexes can be directly deduced by the mass of each molecule that acts as intrinsic label. Consequently, labeled targets or ligands are not required. Moreover, ESI-MS is also able to identify, within a mixture, components that selectively bind the active site of the biopolymer and could be profitably exploited to screen libraries of known compounds.

The presentation gives an overview of a binding assay based on ESI-MS and its application to the study of the interactions between small molecules and biopolymers (protein, DNA) will be exemplified. The use of the ESI-MS method for discovery of novel protein-protein interaction inhibitors will be also presented.

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ANALYTICAL APPROACHES FOR THE MONITORING OF HEMODIALYSIS PATIENTS UNDERGOING OXYCODONE THERAPY

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The administration of drugs to patients suffering from renal impairment can often be problematic, since the drug elimination process can be impaired, with potentially severe results for the patient's health and for the therapy success. Subjects with total renal failure undergoing hemodialysis are obviously more problematic, since the practice itself can have a different impact on different drugs: some of them can be eliminated almost completely through the dialysis membrane, others can be totally retained in the blood.

In order to personalize the therapy and adjust the dose, it is necessary to carry out preliminary studies for the determination of drug plasma levels before entering ("arterial blood") and after exiting ("venous blood") the dialysis machine, and also before and after the hemodialysis practice. This peculiar "therapeutic drug monitoring" [(1)] allows the adjustment of the administered dose according to the possible different elimination rate, thus avoiding both over- and under- doses.

The determination of the drug "dializability" is particularly important for compounds having a narrow therapeutic window, such as opioids and related substances used for pain management in oncology and in other contexts. Of course, all active metabolites an their dializability should also be taken into account, to avoid any underestimation of the total pharmacological effect.

Aim of this research is thus the development of analytical approaches that can be used for the study of the dializability of opioid drugs used for pain management in patients undergoing hemodialysis, focusing in particular on oxycodone, one of the most widely used drug for this purpose, together with the two active metabolites noroxycodone and oxymorphone.

Two methods have been developed and compared, one based on UHPLC with DAD detection and one based on LC coupled to tandem mass spectrometry (MS/MS). The pre-treatment of plasma samples includes a solid phase extraction (SPE) procedure on a reversed phase sorbent. Preliminary results are promising in terms of extraction yields and sensitivity for the three analytes. The methods are currently undergoing validation and the study will continue with the analysis of plasma samples from several patients, drawn before and after subjecting them to hemodialysis.

This research was supported by grants from Regione Emilia-Romagna (POR-FESR funds), Italy and Mundipharma Pharmaceuticals Srl.

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POLYMERIC NANOPARTICLES FOR BRAIN DELIVERY

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Nanoparticles (NPs) are rapidly revolutionizing many areas of medicine and technology and they are recognized as promising and powerful tools to fight against the human brain diseases such as multiple sclerosis or Alzheimer's disease. The development of new delivery systems to increase drug bioavailability and reduce adverse effects has been claimed as a good option. In this study, the design, production and characterization of novel fluorescent polymeric NPs to target brain tissues is proposed and their in vivo distribution is followed. As far as polymeric nanoparticles are concerned, two innovative fluorescent nanospheres were designed: ethylcyanoacrylate-based nanospheres coated with polysorbate 80 (ECA-TW80) and human serum albumin-based (HA) nanospheres. NPs designs took advantage of physiological mechanisms to cross the blood brain barrier (BBB) already existing for endogenous molecules: receptor-mediated transcytosis in the case of NPs coated with Tween 80 and adsorptive transcytosis in the case of plasma proteins.(1) Ethylcyanoacrylate-made nanospheres were prepared by emulsion polymerization method,(2) while human serum albumin-made nanospheres by coacervation method and chemical cross-linking with glutaraldehyde.(3) Nanospheres were characterized in terms of dimensional analysis, polydispersity and Zeta potential, morphology, encapsulation efficacy and loading capacity, stability of the fluorescent probe. Ethylcyanoacrylate- and albumin-made nanospheres were produced with good yields (65% and 85%, respectively). Both nanospheres were suitable for the intraperitoneal administration (mean diameter ≤300 nm; Polydispersity: 0.2), had a sphere-like shape. In particular, HA-based NPs are made fluorescent by incapsulating fluorescein sodium salt; encapsulation efficacy (≈98%) and loading capacity (≈60%) are good. Intracerebrally injected ethylcyanoacrylate- and albumin-made nanospheres into the nucleus basalis magnocellularis of anesthetized rats did not induce any glial reaction and inflammatory response. Differently from albumin-made fluorescent nanospheres that remained in loco 24 hours and one week after the intracerebral administration, ethylcyanoacrylate-made fluorescent nanospheres mobilized from the injection site and distributed unilaterally in the injected hemisphere. Preliminary experiments demonstrate that, one week after injection, ethylcyanoacrylate-made fluorescent nanospheres were detected in the brain parenchyma within blood vessels, microglial and neuronal cells indicating their passage through the cell membranes in addition to endothelial cells. Intraperitoneally administered ethylcyanoacrylate- (400 and 200 mg/kg) and albumin-made (200 and 100 mg/kg) fluorescent nanospheres to C57BL/6 mice were detected in the brain parenchyma one hour after administration, indicating their cross through the BBB. A subchronic intraperitoneal administration for two weeks of C57BL/6 mice with ethylcyanoacrylate- (200 mg/kg/die) and albumin-made (100 mg/kg/die) did not result in any side effects, impairments in locomotor activity and cognitive deficits in the "step down" inhibitory avoidance test and object recognition test (Discrimination Score>0.05), as compared to vehicle (PBS) treated mice. In conclusion, the selected strategies are effective to overcome the blood brain barrier and to distribute within the brain parenchyma, thus they may provide innovative drug delivery tools for Alzheimer's disease treatment.

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LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY IN THE SYNTHESIS AND RATIONAL DESIGN OF A NEO-GLYCO-VACCINE AGAINST TUBERCULOSIS

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Tuberculosis (TB) is still one of the deadliest human diseases together with malaria and HIV that are named the big three, mostly in developing countries. The current strategy for TB control is based on reducing the spread of infection through effective treatment of individuals with active TB and vaccination of children. However, the only vaccine against TB provides efficient protection only in newborns, not in adults and for these reasons the search of new effective vaccines is an important challenge.

Mycobacterium tuberculosis (MTB) presents a cell wall constituted by lipoarabinomannans, which carbohydrate moieties evokes a strong antibody response. Moreover, a number of highly expressed and immunogenic proteins have been discovered in MTB which induce cellular response.

In this work we describe the rational synthesis of a new glyco-vaccine against *M. Tuberculosis* obtained by conjugating highly immunogenic *Mycobacterium* proteins, namely TB10.4 and Ag85B presenting strong T-cell epitopes, with glyco-components based on arabinose and mannose residues evoking antibody response. Different synthetic strategies were exploited for protein glycosylation using carbohydrates with modelled chemical activation [IME (2-iminomethoxymethyl) or homobifunctional (adipate 4-nitrophenyl diester)] to evaluate the influence of the coupling reagent in the reaction of glycosylation of the target proteins.

The glycosylation degree was monitored by direct infusion of intact proteins in a linear ion trap mass spectrometer (ESI-LIT-MS), while glycosylation sites and relative abundances were characterized by liquid chromatography mass spectrometry peptide mapping after an appropriate proteolytic cleavage of the synthesised glycoproteins. The detailed characterization allowed the study of the effect of the different chemical activations on the reactivity, the selectivity and the efficiency of the glycosylation process.

The selected glycan activation was then used to couple TB10.4 and Ag85B with Ara-Man disaccharides under optimized experimental conditions. The synthesized glyco-vaccines were characterized in terms of purity, glycosylation yields and sites, epitope preservation, conformational changes induced by carbohydrate chains using ESI-MS and nanoESI-MS-based analytical approaches. The immunogenicity of the prepared glycoproteins was also determined.

DEVELOPMENT OF A NEW MICRO-PATTERNED BIO-SENSING SURFACE TO SCREEN FOR ACETYLCHOLINESTERASE PAS BINDERS

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In the life sciences current search for speed and automation, sensing surfaces which operate with immobilised biomolecules have found a broad range of applications. In particular, miniaturization and automation of in vitro screening systems may be advantageous in the drug discovery process, in which a large number of new chemical entities needs to be screened for affinity towards specific target proteins.

Specifically, in drug discovery programs, acetylcholinesterase (AChE) inhibitors acting at the catalytic binding site (CAS) of the enzyme are of great interest for treatment of cholinergic deficiencies in the central nervous system (e.g. Alzheimer's disease). Other then CAS binders, more recently, AChE's peripheral binding site (PAS) binders have attracted the attention of several research groups.(1) The rational basis of this interest is the experimental evidence that the interaction of soluble amyloid-beta (Aβ) peptide with AChE's PAS may promote the deposition of the neurotoxic Aβ oligomers/fibrils and accelerate the onset and progression of the AD pathology.(2)

The available *in vitro* screening assay for the selection of PAS binders implies the use of a large amount of the target enzyme (a micro-molar concentration is needed).(3) Therefore, in the attempt of keeping the screening costs within an academic laboratory budget, the assay is usually performed with *electric eel* AChE instead of the human isoform, making the selection of a proper drug candidate more difficult.

On the light of these premises, and in view of the advantages in terms of miniaturization and increased stability and efficiency of the immobilized enzyme, in this talk the initial development of a new AChE-based fluorescence sensing surface for the identification of PAS binders through propidium displacement studies will be presented.

To achieve this goal, different micro-patterned silicon wafers (diameter 4 in) with a reflective layer (either platinum or silicon) and SiO_2 pillars, lines, and holes were fabricated and tested. Indeed, reflective surfaces may have the advantage of higher output signals when fluorescence detection is used. Therefore, an initial selection was performed to obtain a suitable multi-layered material of optimal pattern thickness, required to maximize fluorescence signal and maintain chemical stability. Then, the selective immobilization of recombinant human AChE on the SiO_2 architectures with optimal geometry and chemistry was achieved. Measurements with CLSM, AFM and scanning Auger microscopy–scanning electron microscopy (SAM-SEM) supported the conclusion that AChE was mostly confined to the top of SiO_2 structures. This confinement might be because of chemical contrast which resulted from either the structuring of the wafer or the cleaning procedures used before derivatisation.

In the optimal design, the AChE-based bio-sensing surface showed an efficient fluorescence emission after labelling with propidium, a selective fluorescent probe of AChE's PAS,[4] thereby having potential application as a selective tool for the verification of the mode of action of PAS ligands.

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POLY IMPLANT PROTHÈSE (PIP) FAULTY MAMMARY PROSTHESES: ANALYTICAL INVESTIGATIONS

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In 2010, the French health authority was the first to ban the use of implants from Poly Implant Prothèse (PIP). It has been estimated that a huge number of these adulterated implants have been implanted into hundreds of thousands of unknowing women around the world (around 500.000), from Europe to South America. They were fraudulently manufactured with substandard, non-medical grade silicone, and recent clinical studies consistently confirmed their significant higher rupture rate, migration to axillary lymph nodes and incidence of silicone locoregional spread compared to implants from other manufacturers. [1,2] To date, fifteen thousands French women have been explanted.

Aim of our recent ongoing studies[3,4] was to understand on analytical grounds the reasons of the exceptional rupture rate of these implants and whether there are potential relevant toxicological consequences from the exposure of human body to the silicones present in their composition unapproved for medical applications.

The study was conducted on filler silicone and elastomeric shells from (i) non implanted intact PIP breast prostheses, (ii) PIP implants from n=3 patients explanted for therapeutical reasons (capsular contraction), (iii) late periprosthetic fluids (LPF) from n=4 patients with ruptured PIP implants. Further informations were obtained by comparison of the results with those from a virgin Mc Ghan 410 MX prosthesis and from a sample of technical-grade non-cohesive silicone.

The specimens were analysed using rheological techniques, attenuated total reflectance infrared spectroscopy (ATR-FT-IR), nuclear magnetic resonance (^{1}H NMR), gas chromatography coupled to mass spectrometry (GC–MS), high performance liquid chromatography (HPLC-UV-DAD) and flow injection electrospray mass spectrometry (FI-ESI-MS). Filler silicones, elastomeric shells and LPF were also submitted to phase contrast microscopy to investigate their morphological characteristics.

Our results indicate that the higher rupture rated of PIP implants are due to the combination of different factors involving both the elastomeric shell and filler silicone (and LPF):

(i) the presence of extraneous non silicone residuals (2-hydroxy-isobutyrophenone, HIBP; 2,2-diethoxyacetophenone, DEAP; biphenyl-4-carboxaldehyde, BP-4-CA), known as UV sensitive radical initiators may elicit the inflammatory reactions accelerating capsular formation and contracture; (ii) the consequent mechanical stress enhances silicone bleeding and rupture of the weakened elastomeric shell; (iii) the non cohesive (as demonstrated by comparison of its properties with those of an approved implant [3]) filler silicone is incorporated by emulsification into the surrounding LPF.

Finally, the exposure of this silicone/LPF microemulsion to the draining breast lymphatic system, leads to its irreversible active migration and accumulation to axillary, neck and infra-thoracic mediastinal lymph nodes, thus to evolve to the severe siliconomas formation and inflammatory reactions often diagnosed in the patients carrying this faulty, adulterated product. The presence of HIBP, DEAP and BP-4-CA demonstrated that the PIP implants envelopes were manufactured using undeclared, and of unknown efficacy, procedures.

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^{1}H NMR AND HPLC-MS MEASUREMENTS OF URINARY METABOLIC CHANGES IN CURCUMIN-TREATED RATS: A PRELIMINARY METABOLOMIC STUDY

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There is a growing interest towards so-called "nutraceuticals", namely food or plant-derived mixtures that possess health promoting effects. Curcuma extracts, derived from the rhizome of *Curcuma longa*, are used as spices in foods and are largely used as traditional medicines in several asiatic countries. Many studies dealing with Curcuma were published showing several pharmacologic effects including anti-inflammatory, antimicrobial, antioxidant and chemopreventive. Despite being one of the most widely studied plant extracts, its mode of action *in vivo* remains still unclear.

In the present paper, the effects of the supplementation of curcuma extract on the metabolic status of healthy rats was investigated. A metabolomic strategy based on ^{1}H-NMR and HPLC–MS data in conjunction with statistical analysis was applied to rat urine.

24-Hour urine samples of twelve rats, randomly divided into a control- and a curcumin-treated group (80 mg/kg daily corresponding to 56 mg/kg of curcumin), were collected on day 1, 5, 9, 14, 19, and 25 during the animal experiment. Curcumin extract was administered each day by oral gavage. ^{1}H-NMR and HPLC-MS measurements were performed on all urine samples and data were used for statistical analysis. Spectral profiles of individual metabolites as a function of diet were obtained by independent deconvolution of NMR and HPLC-MS data, using Parallel Factor Analysis (PARAFAC) and Batch Statistical Process Control (BSPC) and Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA).

Changes in metabolic profiles of the treated group compared to control were observed both in the HPLC-MS and 1H-NMR datasets. These changes include N-acetyl-L-cysteine, allantoin, and fatty acids. Identification of other metabolites related to these changes is in progress.

The different levels of these metabolites may provide a preliminary indication of the biochemical pathways modified by curcumin supplementation. Our findings indicate a new approach for the study of the health related effects of plant extracts in an *in vivo* healthy subject model.

STUDY OF MENADIONE AS A NEW USEFUL DERIVATIZATION REAGENT FOR LC ANALYSIS OF ALIPHATIC THIOLS IN ALIMENTARY SUPPLEMENTS AND PHARMACEUTICALS

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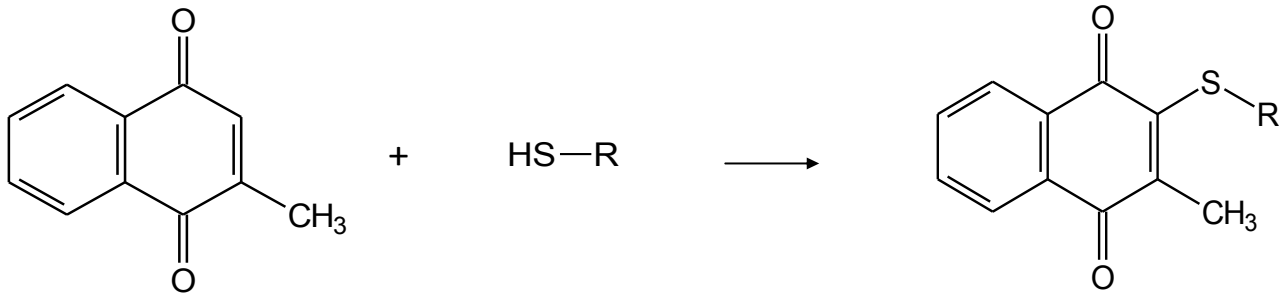
Thiol compounds, such as N-acetylcysteine (NAC), L-cysteine, homocysteine and glutathione, play an important role in metabolic processes. Dietary sulphur-containing amino acids or protein supplementation may be indicated for vegan athletes, children or patients with AIDS, because of increased risk of sulphur-containing amino acid deficiency in these groups.(1)

The analysis of aliphatic thiols often presents difficulties largely due to the absence of a strong chromophore and fluorophore group and their tendency to oxidize to disulphides. Chemical derivatization combined with HPLC (high performance liquid chromatography) represents a powerful and versatile tool able to improve the detectability and the chromatographic behavior of a variety of compounds.(2)

On the basis of the satisfactory results recently obtained proposing 1,4-naphthoquinone, as a derivatization pre-column reagent for thiol function,(3) the aim of this work is to present a new HPLC method to evaluate the potential usefulness of its 2-methyl analogue, menadione (**I**), as a new reagent for the selective and sensitive detection of aliphatic thiols. Menadione is a synthetic chemical compound sometimes used as a nutritional supplement because of its vitamin K activity.



I II

The derivatization reaction was carried out at room temperature for 5 min at pH 8.5 under magnetic stirring in a micro-reaction vessel. The reaction between NAC and MD was first carried out on preparative scale and analytical data (^{1}H NMR, IR and UV) for the isolated derivative were found to be in agreement with the reported structure II. In these conditions the reaction showed to be quantitative and reproducible in comparison with an authentic specimen of NAC synthesized adduct.

LC (Liquid chromatography) separations of derivatized thiols were performed at room temperature on a Phenomenex Synergi 4 μm MAX-RP stainless steel column combined with a guard column packed with the

same stationary phase using a mobile phase consisting of methanol/TEA phosphate (pH 3). UV-diode array detection was used setting the wavelength at $\lambda = 260$ nm.

The developed and validated method was applied successful to thiol analysis in complex matrices. The proposed method can be useful for quality control of nutraceuticals and pharmaceuticals in any analytical laboratory, not requiring a sophisticated instrumentation.

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AN ORIGINAL METHOD FOR THE ANALYSIS OF γ-HYDROXYBUTYRIC ACID (GHB) IN BIOLOGICAL MATRICES

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Gamma-hydroxybutyric acid (GHB, Figure 1) is a short-chain hydroxylated fatty acid that occurs naturally in all cells of the human body and binds to specific receptors, whose precise function remains unclear. It is a powerful central nervous system depressant and is currently used in medicine for the treatment of narcolepsy and of alcohol dependence.(1) In recent years, it has gained popularity among illegal *club* drugs, mainly due to its euphoric and aphrodisiac effects. GHB has also been postulated to have anabolic effects due to its inducing deep sleep and thus growth hormone synthesis, and has been used by body-builders for muscle building and fat reducing. Moreover, GHB is odourless and colourless and may be combined with alcohol and given to unsuspecting victims prior to sexual assault: this use has resulted in GHB being known as a *date rape* drug.(2) Much of the GHB found on the streets or over the Internet is produced in illegal labs. Its production usually involves the use of lye or drain cleaners mixed with gamma-butyrolactone (or GBL), a chemical cousin of GHB and an industrial solvent.

In forensic toxicology, testing for GHB in biological matrices is challenging because of its polar properties, its equilibrium state favouring GBL at low pH, and the complete conversion of GBL to GHB at high pH values.

Aim of this study is the development of a reliable analytical method for the analysis of GHB in different biological matrices (e.g. plasma and urine) of healthy volunteers and both alcohol-dependent patients and users/abusers of the drug.

A new HPLC method coupled with electrochemical detection has been developed to quantify GHB in specific matrices. The analysis was performed on a reversed-phase C18 column, with a mobile phase consisting of a mixture of phosphate buffer and acetonitrile. Electrochemical detection was carried out setting the detector at a suitable oxidation potential value. A careful and rapid microextraction by packed sorbent (MEPS) procedure was chosen for sample purification obtaining good extraction yield values. Promising results were obtained in terms of linearity and sensitivity and assays are in progress in order to completely validate the method and to apply it to real samples.

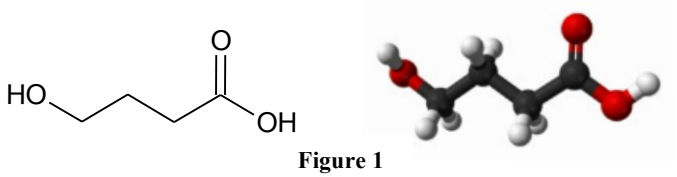

Figure 1

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THE COMPUTATIONAL ANALYSIS OF BIOMOLECULAR INTERACTIONS AND ITS POTENTIAL IMPACT ON DRUG DISCOVERY

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The synergy between experimental and computational biology has greatly benefited both fields, providing invaluable information in many different areas of the life sciences. Available method for predicting the three-dimensional structure of a protein, which is in turn the main determinant of its biological function and therefore essential to interfere with it, cannot only rely on our understanding of the basic laws of physics, because of the enormous complexity of a protein structure. We therefore need to rely on empirical methods for the prediction of a protein structure and its interactions with both macromolecules and smaller compounds.

These methods, even if approximate, are essential for understanding the details of the molecular function and give valuable insights for the development of effective rational strategies for experiments such as studies of disease related mutations, site directed mutagenesis, or structure based drug design.

I will describe some of the methods that we developed to this end and show some examples of their effectiveness in providing relevant information about systems of biomedical interest.

For example, we developed a system to automatically compare and analyze structurally similar regions of proteins of known structure interacting with a common partner that permits to identify mutually exclusive interactions and that often correctly recognize at least one residue (five on average) belonging to the interaction interface.(1) We have also provided the community with several tools for the prediction and analysis of antibody structures,[2] for the design of peptides able to interact with specified regions of proteins (in preparation), for exploring protein sequence, structure and function relationships,[3, 4] as well as a database of therapeutic targets in pathogens and associated tools.(5)

All these tools and others are publicly available from out web page www.biocomputing.it and have been applied to a number of relevant biomedical problems such as the role of synaptic ADAM10 in Alzheimer's disease, (6) the effect of Auranofin and Arthemeter for Plasmodium falciparum and Schistosoma mansonii inhibition, (7, 8) chronic lymphocytic leukemia (9,10) and others.

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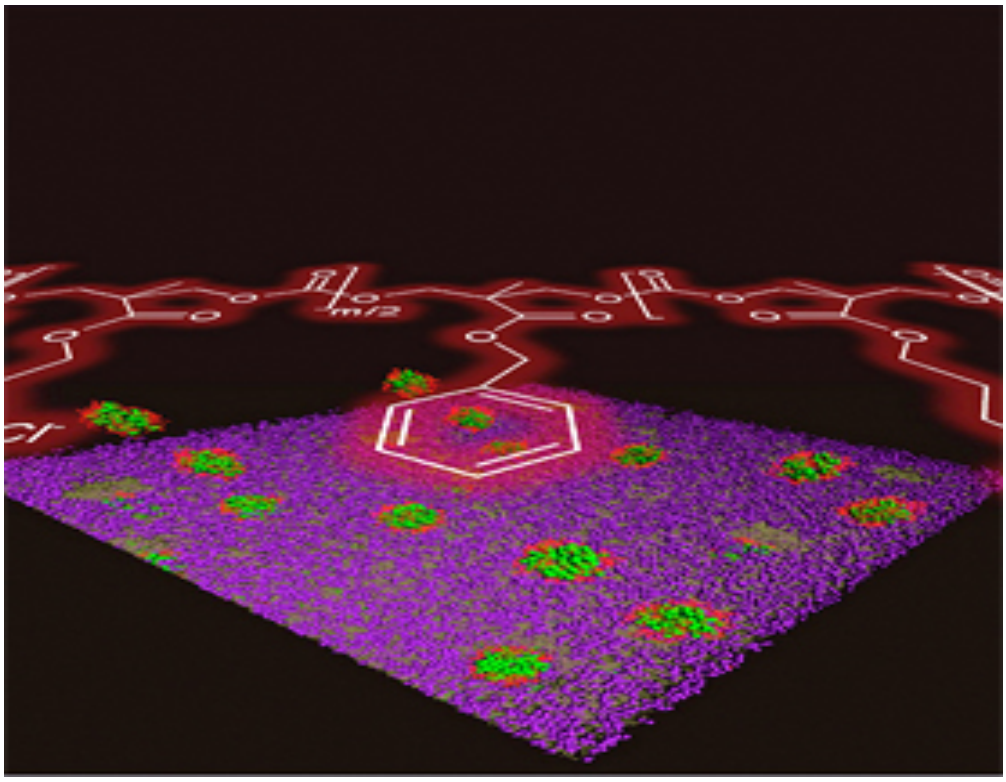

NEW STRATEGIES AND NEW TARGETS TO COMBAT DRUG RESISTANCE IN HIV CHEMOTHERAPY: CHEMISTRY MEETS VIROLOGY

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Since the first diagnosis of AIDS in 1981 great efforts were made to treat HIV infections. Among the strategies, the introduction of HAART in the mid '90s was an important improvement in combating HIV. HAART drugs primarily target the viral enzymes. However, the occurrence of drug resistance and potential side-effects in long-term HAART require the search for new targets and subsequent development of novel drugs.

One still up-to-date option is the use of nucleoside analogues as potent RNA/DNA-polymerases. Several examples are known and are used very successfully in the clinic, e.g. d4T. In addition to nucleoside analogues bearing a glycon-moiety, carbocyclic nucleosides showed also interesting antiviral properties. We reported on the carbocyclic 2′-deoxythymidine (*carba*-dT **1**), which proved highly active against several viruses, e.g. HIV-1. Primer extension assays using RNA-templates and reverse transcriptase revealed a new mechanism of the inhibition in which DNA synthesis was blocked by a so-called delayed chain termination. This makes D-*carba*-dT a very promising lead for the development of an antiviral that will be effective against nucleoside-reverse transcriptase-inhibitor (NRTI)-resistant viruses.

Secondly, cellular cofactors as the eukaryotic initiation factor 5A (eIF-5A) play an important role in the HIV replication cycle. eIF-5A is involved in the transport of the unspliced spliced viral mRNAs from the nucleus to the cytoplasm. A unique post-translational modification of a specific lysine residue to the unusual amino acid hypusine by two human enzymes (deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH)) is mandatory for activation of eIF-5A (see Scheme). CNI-1493 efficiently inhibits DHS and thereby suppressing HIV replication. The synthesis, the evaluation of DHS inhibition and the *in vitro*-inhibitory potency on HIV-1 replication of several CNI-1493 derivatives will be discussed. Finally, structure-based drug design approaches were applied for the development of novel, active-site DHS inhibitors showing marked inhibitory activity against DHS.

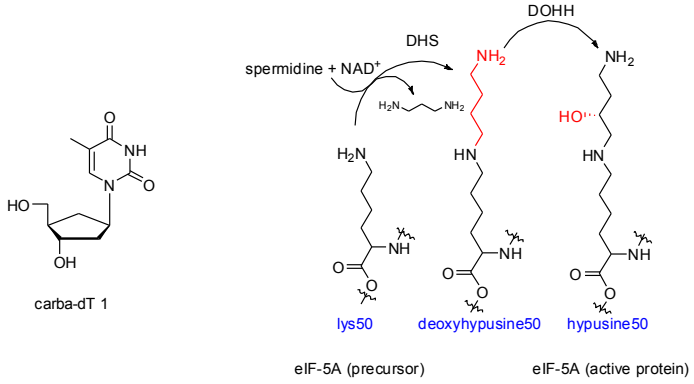

Fig. 1. Chemical structure of *carba*-dT and the post-translational modification of eIF-5A

TUBERCULOSIS DRUG DISCOVERY IN GSK

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In recent years, *Mycobacterium tuberculosis* has been steadily re-emerging as a major worldwide health concern. With both the number and prevalence of multi-drug resistant (MDR) strains increasing, there is a clear need for the development of new drugs to combat this deadly disease. The Tuberculosis group of GSK exists as part of the Diseases of the Developing World (DDW) unit in Spain, which relies increasingly on external partnerships to foster innovation in drug discovery.

This talk will summarize GSK´s approach in this challenging disease area. Internal phenotypic and target-based screening approaches will be discussed, including key learnings around the inclusion of multiple assay platforms for prioritizing new hits. Additionally, our extensive work with collaborators, both through European/international consortia and through the Tres Cantos Open Lab will be highlighted for their potential to open new modes of research.

DESIGN, SYNTHESIS AND EVALUATION OF NOVEL ANTI-CHIKV COMPOUNDS

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Chikungunya virus (CHIKV) is an *Arbovirus* that belongs to the *Alphavirus* genus of the *Togaviridae* family. It is transmitted to humans by mosquito *Aedes Aegypti* and is associated with an acute pathology characterised by fever, rash and arthralgia.(1) In particular, the latter is often severe and may persist for several months or become chronic in the 10% of infected individuals. CHIKV infection was first described in Tanzania in 1955 and since 2005 it re-emerged with a previously unknown virulence in Africa, Indian Ocean, India and South-East Asia, reaching even Europe and the US. The virus ability to adapt to a new vector, the mosquito *Aedes Albopictus*, has almost certainly contribute to the worldwide spread of the infection.(2,3) Clinically approved compounds such as chloroquine, alpha-interferon and ribavirin, even if showing some antiviral effect *in vitro*, demonstrated poor *in vivo* activity against CHIKV infection and, to date no specific treatment is available, nor a vaccine is approved for human use: the therapy is still limited to supportive treatment of the symptoms.

CHIKV is an enveloped virus with an 11.8 kb single-stranded positive-sense RNA genome. It contains two open reading frames and encodes four non-structural proteins (nsP1, nsP2, nsP3, nsP4), three structural proteins (capsid, E1, E2) and two small polypeptides (E3, 6K). All the non-structural proteins are essential for virus replication and can be considered suitable targets for the development of an antiviral therapy.

In this presentation we will give an overview of the work we are currently carrying out in this research field, which has lead to the discovery of a novel series of anti-CHIKV compounds.(4)

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DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF NEW MACROLIDE CLASS ACTIVE AGAINST RESISTANT RESPIRATORY PATHOGENES

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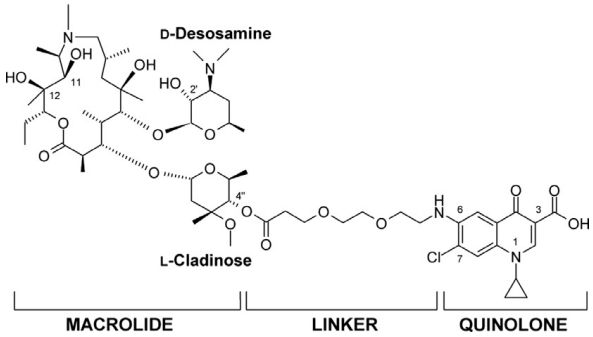
Macrolide antibiotics are globally among the most widely prescribed broad-spectrum antibacterials. The compounds containing a 14- or 15-membered lactone ring in their structure linked to L-cladinose and D-desosamine sugar molecules, particularly clarithromycin and azithromycin, have had the greatest impact on the market, especially for respiratory infections.(1) They characterise distinct pharmacokinetic properties, macrolides accumulate to a high degree in cells and tissues, and their concentrations in tissues often exceed by 10–100-fold those found in plasma.(2) However, as with all antibacterial agents continuous use of macrolides has greatly increased the number of infections caused by macrolide-resistant bacteria.(3) Macrolide compounds modified at the 4”-position present a considerable opportunity for the development of novel antibiotics to effectively address the growing problem of macrolide resistance.

Recently, we reported the design and synthesis of a new class of compounds, consisting of a macrolide scaffold and a 4-quinolone-3-carboxylic acid moiety attached at position C(4”) of the cladinose through various linker fragments.(4-13) The obtained compounds, have demonstrated antimicrobial activity superior to that of the known macrolides/ketolides and fluoroquinolones as also interesting PK properties allowing favourable dosing regimen



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TARGETING PROTEIN-PROTEIN INTERACTIONS AS A PROMISING ANTI-HIV STRATEGY

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Protein-protein interactions (PPIs) are attractive targets for therapeutic intervention because of their crucial roles in several biological processes. Targeting these interactions with small molecule inhibitors is a research area of considerable interest in medicinal chemistry. Recently, a lot of information on PPIs in the HIV (human immunodeficiency virus) life cycle has been made available through chemical biology experiments providing many opportunities in the fight against AIDS (acquired immunodeficiency syndrome).[1] Herein, we report the successful application of computational strategies for the identification of novel HIV inhibitors targeting PPIs. In detail, a structure-based virtual screening approach was applied with the aim of identifying novel HIV-1 entry inhibitors targeting the interaction between CD4 and HIV-1 gp120.[2] As a result, four novel classes of inhibitor emerged that have significant anti-HIV-1 activities in cells. Remarkably, biological investigations supported the hypothesis that these compounds interfere with the binding of gp120 and CD4. Similarly, we successfully combined different computational techniques in order to identify small molecule inhibitors of HIV-1 Integrase (IN) dimerization.[3-4] Two hit compounds were identified which represent the first small molecule IN inhibitors operating through this mechanism of action.

On the other hand, computational methodologies were applied to study the effect of compounds targeting an IN allosteric binding site (sucrose's binding site).[5] A natural compound emerged as a potential IN-stabilizing agent.

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PYRROLYL AND QUINOLINONYL DERIVATIVES AS NOVEL ANTI-HIV AGENTS TARGETED TO THE RIBONUCLEASE H FUNCTION OF THE HIV-1 REVERSE TRANSCRIPTASE ENZYME

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The HIV-1 RT has two associated activities: i) the DNA polymerase activity (both RNA and DNA dependent) and ii) the RNase H activity that selectively degrades the RNA strand of the hybrid RNA/DNA which is formed during the synthesis of the minus (-) strand DNA that uses (+)RNA as a template. The HIV-1 RT-associated RNase H function is one of the several steps of the HIV-1 life cycle that are potentially vulnerable to a specific inhibition. Indeed, several studies have demonstrated that the abolition of the HIV-1 RNase H function stops the virus replication. Therefore, it is a validated and attractive target for the development of new anti-retroviral agents. Despite this, it has been little explored and it needs to be further developed through the support of new HIV/AIDS drug discovery programs, in order to identify more efficient anti-HIV drugs that could be used for therapy.[(1-5)]

The RT inhibitors currently approved for the treatment of HIV infection inhibit the RT polymerase activity, while none of them block the RT RNase H activity. Until now, only a few compounds have been described to inhibit the HIV-1 RNase H function. However, with very few exceptions, they are not truly selective for the HIV-1 RT-associated RNase H activity since most of them inhibit also the HIV-1 RT-associated RDDP activity or the RNase H from other organisms.

Since several years we have been engaged in the designed and synthesis of RNase H inhibitors.[6] Recently, we have discovered new classes of RNase H selective inhibitors characterized by a pyrrolyl or quinolinonyl backbone. The preliminary data will be shown and discussed.

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MMPL3 INHIBITORS ENABLING NEW POSSIBILITIES FOR TB TREATMENT

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M. tuberculosis, the causative agent of tuberculosis, infects one third of the world's population and it is the second leading cause of mortality worldwide.(1) New shorter and simpler drug regimens with bactericidal mechanism that differ from those of current drugs are needed.

In this context we identified a new chemical class of 1,5-diphenyl-pyrroles endowed with potent antimycobacterial activity through a screening of a library of azole compounds. Among them, BM212 proved to be active against multidrug-resistant clinical isolates, *M. tuberculosis* residing within macrophages, and against *M. avium*.(2) The identification of BM212 as a hit within this compound class provided the stimulus to a synthetic hit structure optimisation effort. Initial SAR data produced by these libraries have been used to drive exploratory medicinal chemistry efforts, producing compounds with an improved lead-like profile. Thus, some of the newly synthesised compounds showed very good biological profile with a MIC (against MTB) ranging from 0.016 to 1 μg/ml and PI (Protection Index= CC_{50} /MIC ratio) ranging from 104.48 to 1180.62. BM212 congeners proved also to inhibit intracellular mycobacteria, and a good MIC in the LORA assay. The best derivative was progressed to *in vivo* efficacy studies and it exhibited potent anti-TB activity.(3,4)

Here we describe the hit-to-lead chemistry process for identifying new analogs with improved potency, reduced off-target activities, and physiochemical/metabolic properties suggestive of reasonable *in vivo* pharmacokinetics. Moreover we describe the target identification process applied for defining the molecular target for BM212, by generating spontaneous *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* H37Rv mutants resistant to BM212. By screening of genomic libraries and by whole genome sequencing we found that all the characterized mutants show mutations in the mmpL3 gene.(5)

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A CHEMICAL PROTEOMIC APPROACH IN ANTIMALARIAL DRUGS DISCOVERY

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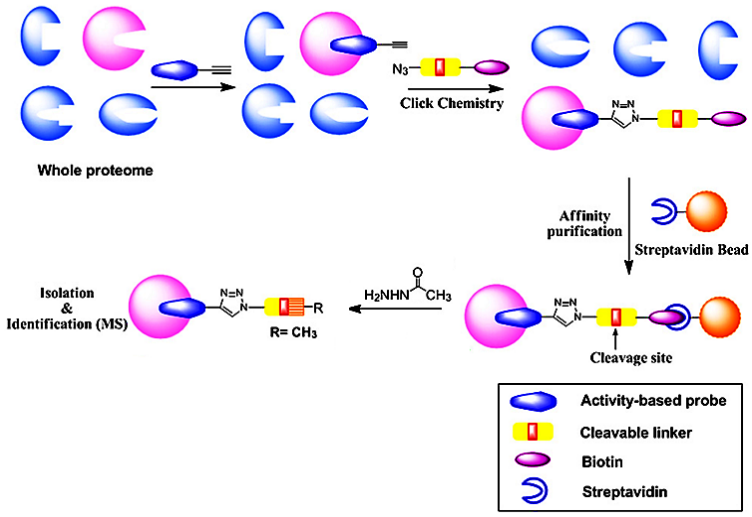
We have previously identified new compounds with high in vitro efficacies against the intraerythrocytic stages of *Plasmodium falciparum* (*P.f.*) (IC_{50} <10 nM), but the molecular target is unknown and preliminary results indicate that indeed multiple targets may exists.[(1-4)]

Proteomics offers a unique tool for target identification and several proteomic approaches are available. One of the most interesting is the so called "chemical proteomics", which couples affinity purification methods with mass spectrometry and therefore permits to increase selectivity and sensitivity.

Several limitations can affect the results of chemical proteomics approaches. For example, it is necessary to use tag compounds that present high activity and affinity for the target protein. Moreover, considering that LC-MS analysis is a very sensitive methodology used for identification of the target protein, non-specific protein binding could cause high background noise, therefore greatly complicating the proteomic analysis. Chemical proteomics applied to antimalarial drug discovery is a difficult task and several attempts have been made. The main problem derives from the *Plasmodium* which is a very small organism living inside the red blood cells and therefore only a very limited amount of protein is available and a noteworthy interference is expected from the host proteins.

We present here a chemical proteomics study using a new selective approach (Figure1) that overcomes non-specific protein binding using a cleavable linker that allows the selective release of the tagged protein from the affinity beads. Furthermore, the cleavable linker containing the affinity tag (Biotin) is introduced through a click reaction after incubation with the lysate, reducing therefore the perturbation in the structure of the starting inhibitor. Preliminary results indicate that one or two specific proteins are involved in the mechanism of action of this new class of *P.f.* inhibitors.



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MYCOBACTERIUM TUBERCULOSIS PKNB INHIBITORS BY IN-SILICO APPROACH

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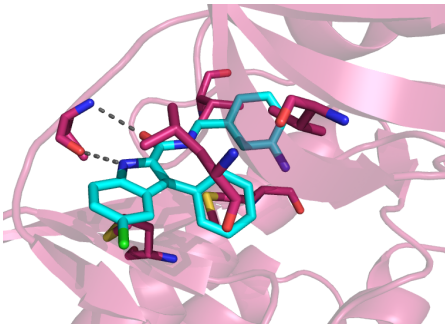
Mycobacterium tuberculosis (MTb) remains one of the world's most devastating pathogens, with more than 9 million people suffering from an active tuberculosis infection and 1.4 million resulting deaths in 2011.(1) The emergence of multi-drug resistant strains has highlighted the need for new agents to treat tuberculosis.

The *MTb* serine/threonine protein kinases (STPKs) are attractive targets because of their importance in *MTb* survival(2). Among them, PknB is an essential transmembrane enzyme that is upregulated upon infection within macrophages.(3-4) The early success stories from the development of eukaryotic kinase inhibitors suggested that similar drugs could be developed to treat bacterial infections. The crystal structure of the kinase domain of PknB in complex with either ATP analogue(4) or inhibitor(5) showed a striking conservation of both protein fold and catalytic mechanism between eukaryotic and prokaryotic STPKs.

Starting from available X-ray structures we screened an in-house compounds' small library (about 5000 molecules) carrying out docking experiments, which resulted in the selection of a molecular scaffold. The hit to lead process has proceeded by mixing ligand-based with structure-based *in silico* methods. We followed a re-branching approach to create a new *in silico* library by the introduction of focused substituents at the selected scaffold. The whole library was first scored by docking analysis and then evaluated by a MMGBSA calculation. The most promising derivatives were synthesized and tested. Thanks to the computer aided drug design methods, we were able to identify a new series of *MTb* PknB inhibitors.



Autodock proposed binding mode at the ATP pocket

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1,3-BENZOTHIAZOLE DERIVATIVES: DESIGN, SYNTHESIS AND EVALUATION OF THEIR ANTIMICROBIAL ACTIVITY

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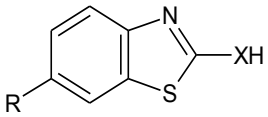
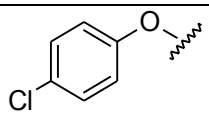
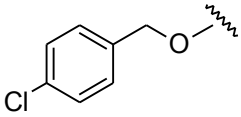
The global diffusion of new antimicrobial infections as well as the continuously increasing resistance of pathogens against many of the commonly used antibiotics imposes a considerable effort to develop alternative therapies to the use of classical drugs. Many heterocyclic nuclei have been reviewed as antimicrobial agents.(1) Our attention was focused to the benzothiazole nucleus. In the past, our research group was interested in a series of 2-mercapto-1,3-benzothiazole derivatives showing antibacterial activity against Gram positive and negative (series **1**).(2) Looking for new lead compounds as new potent antimicrobial agents, actually at the isosteric relationship between SH and NH_2 groups, we have recently synthesized a series of 2-amino-1,3-benzothiazoles (series **2**) and tested *in vitro* the antimicrobial activity against bacteria strains and *Candida* species.(3) We observed that the isosteric substitution of SH with NH_2 brought to the loss of activity against both Gram positive and negative bacteria, while, quite surprising, all the compounds exerted antifungal activity. Actually two of the newly synthesized compounds were very attractive, showing MIC values similar to those of Fluconazole, used as reference drug (results shown in the table). In addition, these two compounds were screened for their cytotoxicity and they did not show any toxic effect for human THP-1 cells: these compounds could be considered promising scaffolds for the development of novel agents against *Candida* spp.



series **1:** X = S
series **2:** X = NH

R	X	*Microorganism (MIC, µg/mL)*			
		C.a. 10231	*C.p.* 22019	*C.t.* 750	*C. k.* 6258
	NH	8	4	8	32
	NH	4	8	4	64
Fluconazole		2	2	4	32

Abbreviations: *C.a.*: *C. albicans; C.p.: C. parapsilosis; C.t.: C. tropicalis; C.k.: C. krusei.*

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TARGETING CDKs TO INHIBIT THE HIV-1 TAT-MEDIATED TRANSCRIPTION

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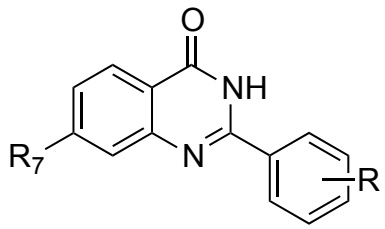
The activity of the CDKs is critical for HIV-1 Tat-mediated transcription and represents a promising target for antiviral therapy.[1] Taking advantage of the crystallographic data of CDK9 in complex with flavopiridol,[2] we have recently performed computational studies that, along with preliminary synthetic efforts, allowed us to identify and characterize a new class of nontoxic anti-CDK9 agents based on the 2-phenylquinazolinone scaffold. CDK2, which plays a supportive role within the transcriptional machinery and shares structural features with CDK9, was also inhibited by these derivatives at comparable concentrations.

Inhibition of CDKs translated into the ability to interfere selectively with Tat-mediated transactivation of the viral promoter and in the inhibition of HIV-1 reactivation from latently infected cells, with the most potent derivative showing an IC_{50} = 4.0 μM.[3]



Since the identified 2-phenylquinazolinones are still fragments, they are largely optimizable paving the way to derivatives with improved potency. To this end, the available CDK9 and CDK2 experimental structures are under investigation in order to identify the best templates to use in structure-based design efforts. Using these templates, we will design novel derivatives as CDK9 and/or CDK2 inhibitors in an attempt to study the optimal balance between the inhibition of the two enzymes, and also obtain more potent anti HIV-1 activities.

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IMIDAZOLE DERIVATIVES WITH IN VITRO ANTICHAGAS ACTIVITY: SYNTHESIS, BIOLOGICAL EVALUATION AND DOCKING STUDIES

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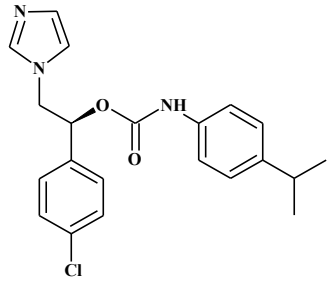
Chagas disease (CD), caused by the parasite *Trypanosoma cruzi*, affects approximately 10 million people worldwide. Currently the treatment of CD is limited to nifurtimox and benznidazole, effective only in the acute phase of the disease. Therefore the development of new drugs has become an urgent need. Similar to fungi and yeasts, *T. cruzi* is strictly dependent on endogenously produced sterols, essential cellular components, that modulate membrane fluidity/permeability and also play multiple regulatory functions related to cell division, growth and developmental processes. Sterol 14α-demethylase (CYP51) is an essential enzyme in the sterol biosynthesis and its inhibition causes the block of sterol production and the accumulation of toxic methylated sterol precursors followed by pathogen growth arrest and death.[(1, 2)]

Our approach focused on selecting a series of compounds, available from our laboratory library, chosen for their structural analogy with *T.cruzi* CYP51 (CYP51*Tc)* inhibitors. The racemic selected compounds have been *in vitro* tested against different parasites: *T. cruzi, T. brucei rhodesiense, L. donovani* and *P. falciparum*. Some compounds possess a high activity towards *T. cruzi* with IC_{50} values in the low nM range and are characterized by low cytotoxicity and low interference with human liver microsomes, as well. Furthermore, all molecules proved to be highly selective against *T. cruzi*, as evidenced by low IC_{50} values compared to the other studied parasites. The high activity of the racemic azole derivatives prompted us to define the differences in the anti-trypanosomal activity of the single enantiomers. The results have showed that the (*S*) enantiomers were more active than the corresponding (*R*) ones; in particular the compound ***(S)*-1** was 1000 times more active than the reference drug benznidazole.



***(S)*-1**

Based on the structural similarity between the studied compounds and the current CYP51 inhibitors, a docking study was performed. The results are consistent with the hypothesis of CYP51*Tc* inhibition:

moreover they agree with the higher activity of *(S)* enantiomers over *(R)*-enantiomers and the high selectivity *vs T. cruzi*.

Recently, we focused our research on design, synthesis, biological evaluation and docking studies of novel imidazoles compounds modifying the side chains of (1H-imidazol-1-yl)-ethyl derivatives.

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NOVEL PEPTIDOMIMETIC INHIBITORS, CONTAINING A 3-BROMO ISOXAZOLINE MOIETY, FOR THE TREATMENT OF NEGLECTED TROPICAL DISEASES

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Neglected tropical diseases (NTDs) affect populations living in the third world countries which are characterized by poverty, disadvantage and often by civil wars. Over 1 billion people, one sixth of the world's population, suffer from one or more neglected diseases.(1) At present, there is an obvious and urgent need to develop new drugs to treat Human African Trypanosomiasis (HAT) and malaria and, in particular, to find new targets for drug design due to the lack of an efficacious and non toxic chemotherapy for their treatment.

In this context, it has been demonstrated that rhodesain is a key enzyme of *T. brucei rhodesiense*,(2) belonging to the cathepsin L subfamily of the papain-like (clan CA, family C1) cysteine proteases. Rhodesainis required by *T. brucei* to cross the blood-brain barrier, leading to the second stage of the sleeping sickness, degrade host immunoglobulins and to perform the turnover of variant surface glycoproteins of its coat. On the other hand falcipain-2 (FP-2), a papain-family (clan CA, family C1) cysteine protease isolated from *P. falciparum*, is among the prime targets for the development of novel antimalarial agents.(3) FP-2 is involved in the hemoglobin degradation, essential for the parasite growth; it is also responsible for the erythrocyte rupture by cleaving ankyrin and band protein 4.1, cytoskeletal elements vital to the stability of the red cell membrane.

We recently developed novel cysteine protease inhibitors (e.g. **1**, Figure 1), characterized by the presence of a 1,4-benzodiazepine (BDZ) scaffold, which represents the constrained form of the D-Ser-Gly fragment, different aryl carbamates linked to the hydroxyl group, and by the 3-bromo isoxazoline nucleus as an innovative electrophilic moiety capable to react with the active site Cys of rhodesain and falcipain-2. All the synthesized inhibitors were proven to possess K_i values in the micromolar/submicromolar range towards both enzymes.

Subsequently, we have developed a second series of inhibitors (i.e. **2**, Figure 1) in which spacers of different length have been introduced between the BDZ scaffold and the warhead, to investigate which is the optimal distance for the interaction with the target enzymes. Molecular simplification have been also carried out on the BDZ core, by converting the conformationally constrained P3 serine into a glycine residue, in order to evaluate if the carbamoyl portion, appended to the serine hydroxyl group, established key interaction with the binding sites of rhodesain and falcipain-2. The results of this investigation will be reported and discussed.

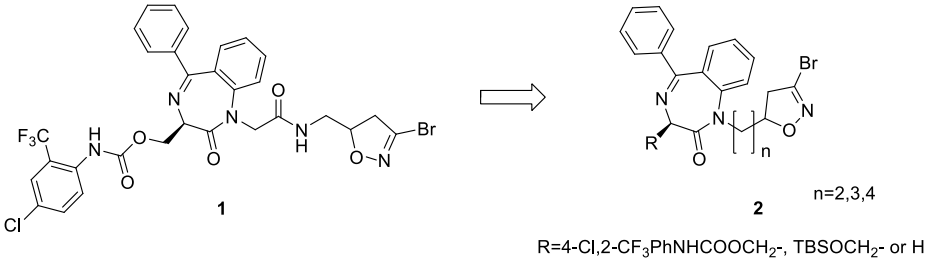

Figure 1.

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SYNTHESIS AND BIOLOGICAL EVALUATION OF NEW INHIBITORS OF ENZYMES INVOLVED IN c-di-GMP METABOLISM

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Recently, much attention has been focused on the need for new antimicrobial agents with new targets or mechanisms of action against multidrug-resistant bacteria. Heavy antibiotic use and person to person spread of bacteria have greatly increased antibiotic resistance due to genetic mutation and this problem is continually increasing in severity. Biofilm-forming bacteria, resistant to antibiotics, cause over 65% of hospital infections. Biofilms are structural communities of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inhert or living surface. In the biofilm, bacteria are 1000-times more resistant to conventional antibiotic treatment. Despite the central role that bacterial biofilm plays in infection, there is currently no anti-biofilm drugs in clinical use. Therefore, the development of original compounds that specifically target the formation of biofilm is of great need in view of a rational use of antibiotics.

Cyclic di-GMP (c-di-GMP) is a second messenger unique of bacteria that plays a central role in biofilm formation and also in the expression of virulence traits. Synthesis of c-di-GMP occurs via diguanylate cyclase enzymes (DCGs), while degradation of c-di-GMP occurs via phosphodiesterase enzymes (PDE). Small molecules interfering with c-di-GMP metabolism could potentially inhibit biofilm formation and virulence in a variety of bacteria. Inhibition of bacteria virulence rather than growth is an alternative strategy that allows to combat bacterial infections without exerting strong selective pressure for the bacteria to evolve resistance mechanisms. The exact details of c-di-GMP signaling is currently being studied by several laboratories and it is expected that analogs of c-di-GMP or other small molecules able to inhibit the enzymes involved in the c-di-GMP metabolism will become useful as either antivirulence or antibiofilm drugs. To date, only few molecular scaffolds have been identified and new small molecules that are able to prevent or destroy biofilm formation are needed.

Based on these findings new c-di-GMP analogs has been synthesized and their ability to inhibit both PDE or DCG enzymes has been evaluated using an innovative approach to follow the enzymatic c-di-GMP formation and degradation in real-time.(1) The enzymes assayed are RocR and the cytoplasmatic portion of PA1120 from *P. aeruginosa* (PDE and DCG, respectively) and PleD from *C. crescentus*, as a reference of DCGs. The newly synthesized compounds showing the highest activity *in vitro* are currently being analyzed for their ability to inhibit c-di-GMP signaling, biofilm formation and/or virulence factors production *in vivo*, using the human pathogen *P. aeruginosa* as model bacterium. Results of these studies will be discussed.

References

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DEVELOPMENT OF COVALENT INHIBITORS OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE, A PROMISING TARGET FOR THE TREATMENT OF PROTOZOAL INFECTIONS

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The incidence of protozoal infections such as malaria, leishmaniasis, and trypanosomiasis has been steadily increasing. The current chemotherapeutic regimens suffer from the lack of safe and effective drugs and the emergence of drug resistance. Thus, there is an urgent need for novel, potent antiparasitic agents.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a key enzyme of the glycolytic pathway, which catalyzes the conversion of glyceraldehyde 3-phosphate (G3P) into 1,3-biphosphoglycerate (1,3-BPG), coupled to the NAD^+/NADH reduction, has been highlighted as a potential antiparasitic target because protozoa rely solely on glycolysis for energy production.

We focused our efforts on the development of GAPDH inhibitors using the covalent inhibitor approach, based on the fact that GAPDH is characterized by a catalytic Cys residue. In recent years, the development of covalent irreversible inhibitors has been re-emphasized, based on the consideration that selective covalent binding of a drug candidate to the desired target can be beneficial owing to the increased efficiency associated with the non-equilibrium binding mechanism. We previously identified the 3-Br-isoxazoline as an efficient warhead for the covalent irreversible inhibition of enzymes containing a Cys residue within the active site.(1)

Thus, we designed and synthesized a set of potential inhibitors characterized by the presence of a 3-Br-isoxazoline nucleus as a warhead, and by different substituents at the C-5 position of the heterocycle.

To test the alkylating properties of these compounds, we cloned and expressed GAPDH from *Plasmodium falciparum* and set up an inhibition assay, complemented with mass spectrometry experiments to evaluate the selectivity towards the catalytic cysteine.(2) Our data show that the 3-Br-isoxazoline compounds produce a time- and concentration-dependent inhibition. Depending on the nature of the substituent R, the inhibition rates can vary up to 10-fold, indicating a specific recognition at the active site.

These data suggest that it is possible to tune the inhibitory activity by coupling the reactive warhead to a suitable recognition moiety. Future efforts will be devoted to further improving the affinity and specificity of this new class of inhibitors towards GAPDH through a target-based drug design approach. To this aim, efforts are ongoing to obtain a crystal structure of the enzyme covalently bound to one of our inhibitors.

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SYNTHESIS AND ANTIFUNGAL ACTIVITY OF A NEW SERIES OF 2-(1H-IMIDAZOL-1-YL)-1-PHENYLETHANOL DERIVATIVES

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The increasing incidence of serious fungal infections is a well-recognized problem. *Candida* species are the main agents responsible for nosocomial fungal infections; especially *Candida albicans*, which is commensal in healthy individuals, is the most common pathogen isolated in invasive candidiasis. Furthermore, an increasing rates of invasive candidiasis caused by non-*albicans Candida* species have been reported worldwide; these species, typically less sensitive to the principal antifungal drugs, include *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, and *Candida krusei*. The azole derivatives including triazoles (*e.g.* fluconazole) and imidazoles (*e.g.* econazole, miconazole, clotrimazole, and ketoconazole) are commonly used as first line drugs to treat *Candida* infections.

The rationale design of new antifungal azoles is based on the homology modeling and pharmacophore modeling techniques, reported in literature.[(1,2)] According to these models, the main azole drugs possess at least three principal pharmacophoric groups: (A) iron coordinating group, consisting of imidazole or triazole ring, able to interact with the heme iron, (B) a first hydrophobic moiety (typically aromatic) near the iron coordinating group and (C) a second aromatic region. Certain active compounds present an additional hydrophobic area referred as region (D).

In a previous paper,[(3)] we have reported new 2-(1H-imidazol-1-yl)-1-phenylethanol derivatives with antifungal activity and low cytotoxicity.

Here, we described new compounds prepared and tested *vs Candida albicans* and non-albicans *Candida* species. Some compounds showed an activity *vs Candida albicans* in the range 0.20-0.67 μg/ml expressed as geometric mean of minimal inhibitory concentration (GM MIC) compared to 1.27 of the reference drug fluconazole; moreover the activity *vs* non-*albicans Candida* was in the range 0.51-2.06 μg/ml compared to 2.91 of fluconazole. The most active compounds have been synthesized as pure enantiomers and tested *vs* the same fungal strains. The results showed that the (*S*) enantiomers were more active than the corresponding (*R*) ones; in particular the most active compound was 6 times more active than the reference drug. We also tested our compounds *vs C. albicans* and *C. glabrata* resistant strains; the results showed that all the resistant strains are more sensitive to our products respect to fluconazole.

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NEW INDOLYLARYLSULFONES AS POTENT AND BROAD SPECTRUM HIV-1 NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

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Acquired immune deficiency syndrome (AIDS) pandemic remain among the leading causes of death worldwide. HIV-1 non-nucleoside reverse transcriptase inhibitors (NNRTIs) are key drugs of highly active antiretroviral therapy (HAART) in the clinical management of AIDS/HIV-1 infection.

Our recent studies showed that indolylarylsulfones (IASs) bearing a cyclic moiety at the 2-carboxamide nitrogen linked through a short spacer group were endowed with potent antiretroviral activity[1]. Thus, we have expanded the SAR studies by the introduction of a number of (hetero)aryl or heterocyclyl moieties at the 2-carboxamide nitrogen through a methylene/ethylene bridge (Chart 1 and Figure 1)[2].

Chart 1.

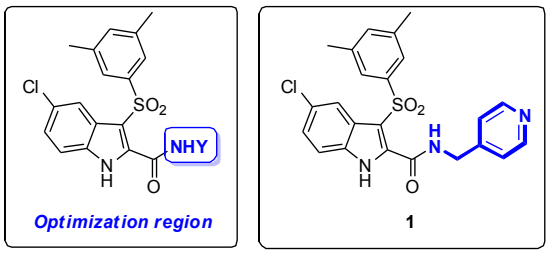
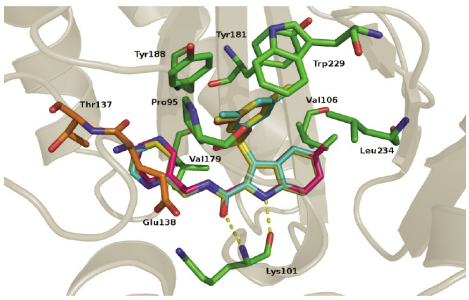


Figure 1.



Several new derivatives were highly active against HIV-1 replication in MT-4 cells with inhibitory concentrations in the low subnanomolar range. The most active compounds were highly effective against HIV-1 WT and mutant HIV-1 strains carrying resistance mutations to the commonly used NNRTI drugs nevirapine and efavirenz.

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POLYPHENOLS: NATURE HERITAGE TO DISCOVER NEW ANTI-HIV-1 AGENTS?

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The urgent need for new anti-HIV drugs is a global concern. Side effects and the emergence of drug resistance have limited the therapeutic usefulness of anti-HIV drugs, and new targets for anti-retrovirals are explored. Our attention has recently focused on an emerging and promising target, HIV-1 Nucleocapsid protein (NC), a nucleic acid chaperone. Compounds able to impair NC activities would lead to inhibition of viral replication since the protein is critically implied in several steps of the HIV-1 life cycle.(1) Its strict conservation goes with the fact that all the known mutations in the protein sequence are lethal for the virus and raises the possibility that HIV-1 will be unable to generate NC mutants resistant to drugs.(2) For all these reasons the NC protein is an attractive candidate for drug development.

NC acts as a nucleic acids chaperone that destabilizes stable nucleic acid structures and then promotes the formation of the annealed nucleic acid helices, substrate for Reverse Transcriptase after strand transfers. We focused our attention on the minus strand transfer, the first of the two obligatory strand transfer. This event involves the annealing of the Trans Activation Responsive (TAR) region of the viral RNA to the complementary sequence (cTAR) at the 3'-end of the DNA template. Although thermodynamically favored, the reaction does not occur extensively in the absence of NC, being cTAR and TAR sequences highly structured and stable. Compounds able to inhibit NC activity were discovered by high throughput screening and did not show common structural features.(3) The screening of libraries of different molecules is therefore valid to identify potential anti-NC agents as lead compounds suitable for the development of more powerful derivatives.

To this aim, we developed and optimized a simple, fast and reliable assay to test the inhibitory activity of a large number of molecules on the NC-mediated nucleic acid helix-destabilizing activity. We screened a library of natural and synthetic polyphenols and identified highly active hits. The hits did not stabilize the TAR or cTAR structure and did not compete with NC for the binding to nucleic acids. Instead, they interacted directly with NC, resulting to be inhibitors of the NC protein with a mechanism of action distinct from that of zinc ejectors developed in past years. The effective impairment of the NC-mediated melting of TAR and cTAR led to a corresponding reduction of the TAR-cTAR hybrid formation analyzed in vitro on the NC-assisted annealing of TAR with cTAR. Compounds active in vitro showed also activity in HIV-1 infected cells. This approach would ultimately bring new insights into the development of compounds acting through NC targeting. As it has always been the case, "mother nature" offers great promises, and natural bioactive compounds and their derivatives could be excellent sources for the development of new anti-HIV therapeutics.

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SOME MONO AND DISUBSTITUTED BENZIMIDAZOLES AND THEIR ANTIMICROBIAL ACTIVITIES

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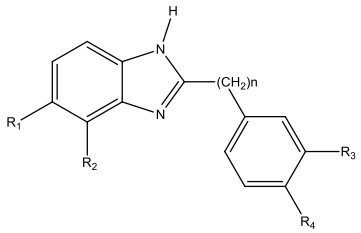
In the recent years, the human population have been affected with life-threatening infectious diseases caused by multidrug-resistance pathogen bacteria and fungi. It has been an increased use of antimicrobial agents and this resulted the development of resistance to antimicrobial drugs.(1) Currently use of standart antimicrobial therapies (β-lactam, macrolides, quinolones, and vancomycin for bacteria and azoles, polyenes, allylamines and echinocandis for fungi) are limited because of diminishing efficacy rate.(2)

One way to fight with this challenge is build up novel agents which have different mechanism of actions and contribute to the development of resistance to antimicrobial therapy.

One of the new structures is benzimidazole which is known to exhibit a wide variety of pharmacological properties including antimicrobial and antitumor activity as well as inhibition of nucleic acid synthesis.(3)

In the present study, chemical structure of synthesized 19 compounds having 2,4- or 2,5-disubstituted and 2-substituted benzimidazole were confirmed by using IR, NMR, LC/MS/MS. In order to evaluate *in vitro* antimicrobial activities and structure-activity relationships of this compounds against two Gram positive *S. aureus* (ATCC 25923), *S. pyogenes* (ATCC 6303) and three Gram negative bacteria *E. coli* (ATCC 35218), *P. aeruginosa* (ATCC 27853), *A. baumanii* (RSHM 2026) and two pathogen fungi *C. albicans* (ATCC 10231) and *C. glabrata* (RSHM 4019) were used.



R_1= -H, -CH_3, -OCH_3 **R_2**= -H, -CH_3 **n**= 0, 1, 2 **R_3**= -H, -OCH_3 **R_4**= -H, -OH, -OCH_3

All of these compounds inhibited the growth of Gram positive and Gram negative bacteria at MIC (Minimum inhibitory concentration) values between 15.625 and 250 µg/mL, which was one order magnitude less than that of ampisilin which was used as a control (7.812 µg/mL), and MIC values between 15.625 and 125 µg/mL for the fungi which was inhibited equal to the growth of the all screened fungi in which fluconazole used as a control (15.625 µg/mL). Considering all results obtained from antifungal tests, it can be concluded that entire compounds tested more active towards fungus than bacteria used. The experiments regarding the structure-activity relationship are still contuining and particularly cell viability will be determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

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CINNAMOYL DERIVATIVES AS INHIBITORS OF HISTONE ACETYLTRANSFERASE ENZYMES

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Epigenetic modifications have been defined as the structural alteration of chromosomal regions to register, signal or perpetuate altered activity states. The actions of HATs in gene transcription can be divided into gene specific effects and global effects. Histone acetylation targeted to promoters mediates activation or repression of specific genes, whereas histone acetylation over large regions of chromatin, including coding regions and nonpromotor regions, affects global gene expression levels. It has been shown that global histone modification levels are predictive of cancer recurrence. This indicates that histone acetylation is a versatile regulatory event that plays a key role in multiple cellular processes.[1]

Our interest on HAT enzymes started a few years ago when we designed and synthesized polyphenols derivatives related to curcumin, a natural compound that was proven to inhibit HATs. In that study we described the good activity of these polyphenols against HATs; in particular we identified the bisbenzylidene cyclohexanone derivative RC 56 as selective p300 inhibitor able to penetrate the cell membrane.[2]

Here we present the ongoing research in this field, based on virtual library design and molecular docking studies aimed at supporting the design of RC 56 analogues. The synthesis of the newly designed molecules has been planned by the means of a literature research on available building blocks and was performed by microwaves irradiation. The newly synthesized derivatives have been tested for their inhibition on p300 and PCAF HAT enzymes. The biological assays allowed us to identify some inhibitors selective for p300 and/or PCAF.

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AZOLE BASED COMPOUNDS TARGETED TO LANOSTEROL 14α-DEMETHYLASE OF *T. CRUZI*

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Chronic infection with *Trypanosoma cruzi* (Tc) is a major cause of morbidity and mortality in Latin America with as many as 200000 new cases of infection occurring per year. Drugs to treat its associated illness, Chagas disease, are toxic and frequently unsuccessful. A series of compounds that were determined to be highly potent inhibitors of the *T. cruzi* lanosterol 14α-demethylase (L14DM) enzyme are in preclinical development for Chagas disease. Moreover, the only drugs accepted for clinical use are the two nitroheterocyclic compounds, benznidazole and nifurtimox, which are inadequate and no vaccines are available up to day.(1) A new approach for therapy is the block of the sterol biosynthesis pathway. Within this pathway, the sterol 14α-demethylase is one of the most important enzyme, which is a member of the cytochrome P450 superfamily (CYP51).(2) During the last two decades, our group was engaged in the research of novel anti-mycotic agents targeted to lanosterol demethylase of fungi. Since *T. cruzi* L14DM is considered as a fungal-like enzyme,(3) we decided to screen our library of antifungal agents on a panel of parasites and identified RDS 416 as a hit active against Tc at 14 ng/ml concentration (EC_{50}). We tested RDS 416 for its binding to TC L14DM in vitro and found that it binds this target, consistently with the EC_{50} data on the parasite. The essential features leading to the inhibition of the L14DM were found by the means of molecular modelling studies that have highlighted the binding mode of RDS 416.

According to the found binding mode, a series of derivatives of this hit was synthesized and tested against Tc. As a parallel approach we decided to try to validate the binding mode of the RDS 416, by means of the design of further azole derivatives characterized by the lack of one or more essential features.

The results of the biological assays will be shown.

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NOVEL 2-SUBSTITUTED 2'/3'-*C*-METHYL-ADENOSINE DERIVATIVES: SYNTHESIS AND BIOLOGICAL EVALUATION AGAINST TRYPANOSOMA BRUCEI

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Human African trypanosomiasis (HAT), which is also known as sleeping sickness, is a devastating parasitic disease that affects more than 300000 people of sub-Saharan Africa each year. The causative agent of this affliction is the protozoan *Trypanosoma brucei*, which is introduced in the mammalian host by the tsetse fly. *T. brucei* attacks the central nervous system leading to dementia, epileptic attacks, coma, and, if left untreated, death. Current treatment for this disease, including suramin, pentamidine, melarsoprol, and difluoromethylornithine (DFMO), is often antiquated, highly toxic and frequently ineffective. Therefore, new highly effective and not toxic drugs are needed.

Like most obligate intracellular parasites, *T. brucei* has lost the capacity to synthesize purines *de novo* and depends on the salvage pathway of nucleosides from the body fluids of the host. Bloodstream *T. brucei* can take up different types of purines and interconverts them into essential cellular nucleotides.

Cordycepin (3'-deoxyadenosine) is an adenosine derivatives able to cure mice inoculated with the human pathogenic *T. brucei* even after parasites have penetrated into the brain, but requires co-administration with the adenosine deaminase (ADA) inhibitor coformycin to prevent deamination. However, the toxicity of coformycin has stimulated the search of adenosine analogues active against the parasite, but resistant to ADA.

In our previous work, we found that the introduction of a methyl group in position 2'- or 3'- of the sugar moiety of adenosine (2'-*C*-methyladenosine and 3'-*C*-methyladenosine, respectively) confers a certain grade of resistance to ADA.(1) In fact, 3'-MeAdo is resistant to ADA, while 2'-MeAdo is deaminated by ADA even though the rate of deamination was 1/25 that observed with adenosine. Moreover, some 2, N^6-disubstituted adenosine analogs have been reported to show antitrypanosomal activity.(2)

Based on these findings a new series of 2-substituted-2'-*C*-methyl-, and 3'-*C*-methyl-adenosine derivatives were synthesized and tested for their antiprotozoal activity.

The results of this study will be discussed.

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IN VITRO ANTI -*Trypanosoma cruzi* EFFECT OF THE FLAVONE HORTENSIN ISOLATED FROM THE BRAZILIAN ORCHID *Miltonia flavescens* L

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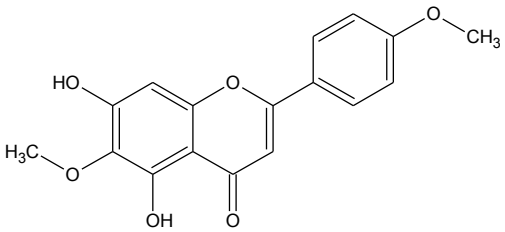
Trypanosoma cruzi is the agent of Chagas' disease which is an endemic disease that constitutes a serious public health problem in many tropical and subtropical areas.(1) Currently, there are only two drugs available for the treatment, benzonidazole and nifurtimox, but they cause serious toxic side effects. Thus plants and natural compounds provide a potential alternative source for the treatment of American trypanosomiasis.(2) In this context, the South Brazilian orchid *Miltonia flavescens* was studied aiming the isolation of bioactive metabolites. As a result, the flavone hortensin (6,8-dihydroxy-7,4'-dimethoxy-flavone) was isolated from the flowers of the plant and assayed to evaluate its growth inhibition effect on epimastigote and trypomastigote forms of *T. cruzi*.



Antiproliferative assays against epimastigote forms were performed with 10^6 epimastigotes/ml exposed to different concentrations of hortensin and kept at 28°C for 96 h. For the viability assay, 10^7 trypomastigotes/ml were incubated at 37°C for 24 h with different concentrations of hortensin. Additionally, to evaluate the safety of this compound, mammalian cells ($LLCMK_2$) were treated with increased concentrations, and cell viability was assessed using MTT assay. Our data showed that hortensin presented activity against epimastigote and trypomastigote forms, with an IC_{50} of 35±7.07 µM and 38.2±2.40 µM, respectively. Moreover, cytotoxicity effects against $LLCMK_2$ cell line were observed just with concentrations up to 80±14.14 µM. This study shows the potential anti-*Trypanosoma cruzi* effect of hortensin. Tests with rats are being performed now. Acknowledgements: This study was supported through grants from CNPq, Fundação Araucária, FINEP, and CAPES.

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D,L-PEPTIDES AS STRONG INHIBITORS OF HIV-1 GP120

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The viral envelope glycans, in particular the glycoprotein gp120,(1) due to their crucial role in viral transmission, are a relatively new and important target for anti-HIV drug development. For this reason, many attempts are devoted to find out new compounds able to block the HIV-1 gp120 inhibiting the viral infection and transmission.

Starting from the experimental results obtained with D,L-peptides as carbohydrate-binding proteins,(2,3) we aim to identify cyclic and linear peptides with regular enantiomeric sequence able to bind to the HIV-1 gp120 glycan portion. Peptides with such a particular feature are an important class of sequence-specific peptidomimetics known to produce different biological activities. In particular, they have the strong propensity to self-organize in flat, ring-shaped systems and constitute a class of synthetically accessible biomaterials having unique structural and functional properties,(4) e.g. as artificial carbohydrate receptors.

In this contest we have identified new D,L-peptides with the potential capability of inhibiting HIV. Preliminary studies revealed that the octapeptide For-(D-Phe-L-Lys)$_4$-OH may be particularly interesting for its strong HIV-1 antiviral activity. Therefore, our attention focuses on a class of linear and cyclic D,L-peptides, ranging from six to ten residues starting from lysine-phenylalanine dimer units in order to incorporate regular enantiomeric sequences with different hydrophobicity.

Here, molecular modelling, design and preparation of this class of D,L-peptides are reported. The linear and cyclic peptides were synthesized by solid-phase peptide synthesis and characterized by ESI-MS and NMR spectroscopy.

The interaction between some D,L-linear oligopeptides and mannose derivatives was investigated using ESI-MS spectrometry and NMR and CD spectroscopies. Preliminary results seems to be coherent with the designed flexibility of the outer surface and the internal diameter of these peptides which enable these ring-shaped structures to fit in the glycans of the gp120 and make a stable noncovalent complex as suggested by theoretical models (one of the possible structure is reported in Fig. 1).

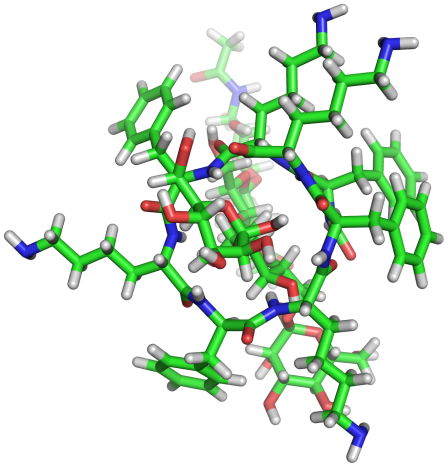

Fig. 1 Structural model of the association complex between the octapeptide For-(D-Phe-L-Lys)$_4$-OH in right-handed β-like conformation and a pentasaccharide chain extracted from the PDB 2BF1 crystal structure of the glycoprotein gp120.

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ANTIBACTERIAL ACTIVITY OF NEW PYRROLE DERIVATIVES

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There is a growing concern that we are moving toward a second pre-antibiotic era since the current therapeutic armamentarium is not enough to prevent and manage a number of bacterial infections.(1) The widespread of so-called "superbugs" has worsened dramatically in the last decade with the risk of severe social, and economic implications.(2,3) Consequently, the need of novel and highly effective antibacterial agents is imperative.

In this study a series of pyrrole derivatives were tested against three Gram-positive and three Gram-negative pathogens. Tested isolated included: 10 *Staphylococcus aureus* (7 MSSA and 3 MRSA), 10 *Enterococcus faecalis* (8 susceptible and 2 resistant to vancomycin), 10 *Enterococcus faecium* (6 susceptible and 4 resistant to vancomycin), 10 *Escherichia coli* (6 of which ESBL-producing), 10 *Klebsiella pneumoniae* (3 of which carbapenemase-producing), and 10 *Proteus vulgaris* (3 of which ESBL-producing). The most active compounds exhibited MIC_{90} values in the range of 8-32 μg/mL, and did not show significant cytotoxic activity at 500 μg/ml against HeLa cells using Alamar blue assay (cell viability). Here we present the synthesis, and the biological results of the newly synthesized compounds. Further studies are in progress to elucidate their mechanism of action.

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STRUCTURAL INVESTIGATIONS OF THE 2-PHENYL-4 HYDROXYQUINOLINE CLASS OF *S. AUREUS* NORA EPIS

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The worldwide increase of bacteria resistant to almost every common antibacterials is now considered an alarming health emergency.(1) Among the strategies by which resistance can be acquired, overexpression of the efflux pumps leads to a sub-lethal antibacterial concentration at the target site that in turn may predispose to the development of high-level target-based resistance. The blockage of the activity of such pumps through the use of efflux pump inhibitors (EPIs) might thus be a powerful approach for improving the efficacy and/or extending the clinical utility of existing antibiotics, giving new life to old drugs with secure economic, social and health benefit. Of particular concern is the overwhelming rise of methicillin-resistant *S. aureus* (MRSA), which is highly virulent and contagious. MRSA accounted for 44% of healthcare-associated infections and 22% of attributable extra deaths in 2008 in EU. The increased expression of one or more MDR efflux pump genes was identified in 151 out of 309 *S. aureus* clinical strains (49%). Among those overexpressing a single gene, *norA* was most common (43%), followed by its strict homologue *norB* (23.2%) and *mepA* (9.9%).(2) In *S. aureus*, the NorA protein is able to extrude from the bacterial cell several antibacterials, including hydrophilic fluoroquinolones such as ciprofloxacin, and dyes. Taking into account the strong inhibitory activity on NorA efflux pump displayed from our previously reported 2-pheny-4-hydroxylquinoline derivatives,(3,4) in this work we have explored the effects of the introduction of a single methoxy group, a substituent frequently recurrent in both natural and synthetic NorA EPIs, at different positions of the quinoline core, maintaining at C-4 position the *O*-alkylamino chains that previously showed the best EPI activities (Fig. 1). The main aim was to obtain new and potent small molecules capable of restoring CPX activity on *S. aureus* resistant strains through the inhibition of NorA efflux pump and to get data for the refinement of our pharmacophore model,(4) that highlights the structural requirements necessary for the inhibition activity of NorA EPIs.

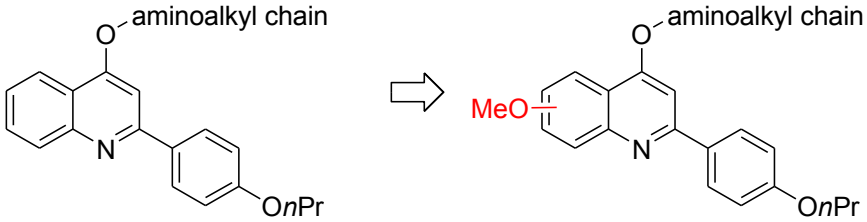

Figure 1

Activity data of the synthesized compounds, regarding the ethidium bromide (EtBr) efflux inhibition and the synergistic activity with ciprofloxacin against several *S. aureus* strains having different levels of NorA pump expression will be reported.

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DESIGN AND SYNTHESIS OF NEW INTEGRASE INHIBITORS

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The human immunodeficiency virus type 1 (HIV-1) integrase (IN) catalyzes integration of the reverse-transcribed viral DNA into the host cell genome. This is an essential step in the viral replication cycle. IN has no mammalian homologues and inhibiting IN is a major target for specific anti-retroviral drugs.

Structurally, integrase comprises three domains: an N-terminal zinc-binding domain (residues 1–55), a catalytic core domain (CCD; residues 50–212), and a C-terminal DNA binding domain (residues 220–270). Recently it is shown that the IN N-terminal domain plays a key role in the dimerization process and, at least indirectly, in the binding with viral DNA. (1)

In this communication, we report the obtained results with a small library of mimotopic peptide inhibitors derived from IN N-terminal domain.

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CLOFAZIMINE ANALOGS WITH ANTILEISHMANIAL AND ANTIMALARIAL ACTIVITIES

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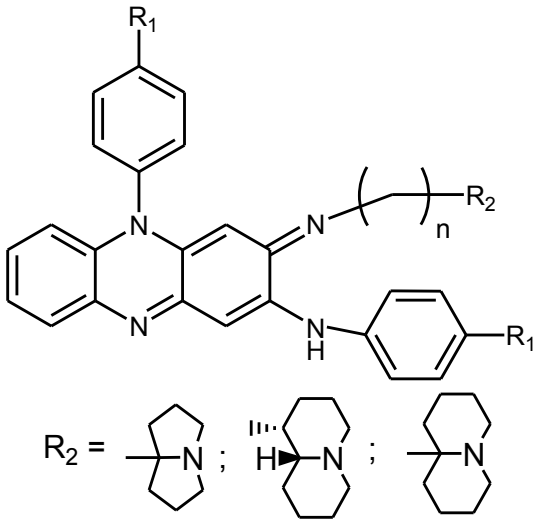
Clofazimine is a fat-soluble riminophenazine dye used in combination with rifampicin and dapsone as multidrug therapy (MDT) for the treatment of leprosy. This agent is known to have antileishmanial effects both in vitro and in vivo.(1)

Tetramethylpiperidine –substituted phenazines, structurally related to clofazimine, have been described to be endowed with activity against multidrug resistant strains of *Plasmodium falciparum*.(2)

In the search for more effective alternatives to the presently used antileishmanial drugs and with the aim to study more thorougly the antimalarial potentialities of this kind of structures, we synthesized a set of novel iminophenazines bearing a bicyclic basic head linked through an alkylic chain to the imino nitrogen in position 3 on the phenazine nucleus.

The new compounds inhibited the growth of different strains of *Leishmania* promastigotes as well as chloroquine sensitive (CQ-S) and chloroquine resistant (CQ-R) strains of *P. falciparum* with IC_{50} in submicromolar range whereas clofazimine was 10-20 –fold less active.



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CYCLOHEPTATHIOPHENE-3-CARBOXAMIDE DERIVATIVES AS INFLUENZA A VIRUS POLYMERASE INHIBITORS

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The currently available anti-influenza (Flu) drugs, i.e. M2 blockers and neuraminidase inhibitors are still inadequate to treat Flu virus,(1) an important pathogen responsible for both yearly seasonal epidemics and more extensive global pandemics. Clearly, next-generation antivirals are needed to efficiently combat flu, preferably with an innovative mechanism of action. The viral RNA-dependent RNA polymerase (RdRP), a heterotrimer formed by the PB1, PB2, and PA subunits, provides an attractive target,(2) being essential for viral replication and involved in virus pathogenicity.(3) Moreover, it is highly conserved among flu A, B, and C while no homologue has been found in mammalian cells. With the aim to disrupt the RdRP correct assembly through protein-protein interaction inhibitors, an *in silico* screening of small molecule libraries using the crystal structure of a truncated form of PA bound to a PB1-derived peptide,(4) has been recently performed. Some interesting compounds showing the ability to specifically interfere with the PA-PB1 interaction, which translated in the capacity to block virus growth in cell cultures at non-cytotoxic concentrations, have been identified.(5,6) In this work, one of these hits, a cycloheptathiophene-3-carboxamide derivative, has been structurally investigated in order to increase the anti-PA/PB1 activity and achieve potent anti-flu activity, also encompassing clinical isolates and drug-resistant strains.

The design and synthesis of a large series of derivatives that led to identify improved anti-flu compounds along with preliminary SAR information, will be presented.

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SYNTHESIS AND ANTIMICROBIAL EVALUATION OF 6-(4-METHYLPIPERAZINE)-3(2*H*)-PYRIDAZINONE-2-ACETYL-2-SUBSTITUTED BENZAL)HYDRAZONE DERIVATIVES

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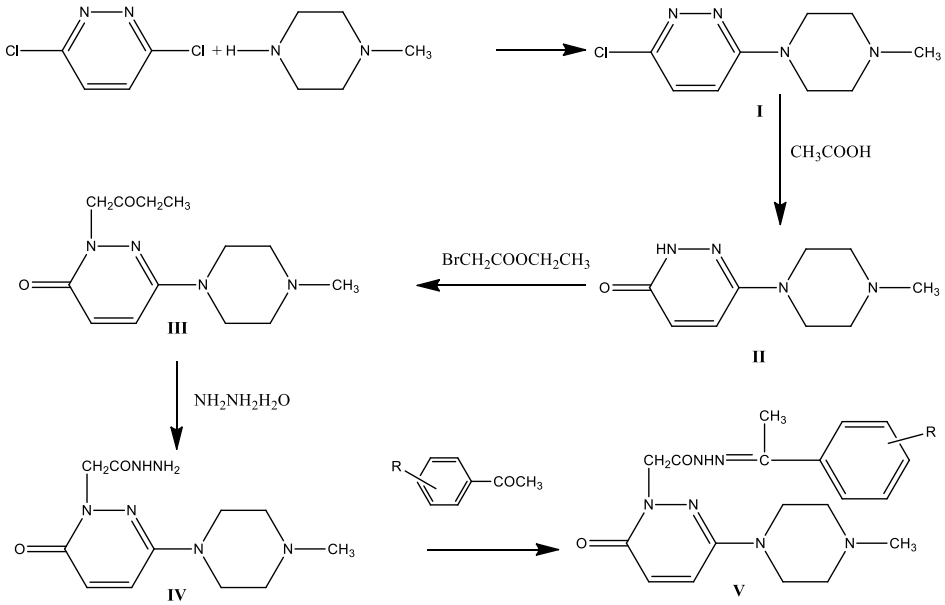
The pyridazinone nucleus has been incorporated into a wide variety of therapeutically interesting molecules to transform them into better drugs[1]. Some of the present day drugs such as emorfazone (analgesic), pimobendan (positive inotropic, vasodilator), levosimendan (calcium sensitizer), imazodan (cardiotonic), zardaverin (cardiotonic) medazonamide (antitussif) are the best examples for potent molecules possessing pyridazinone nucleus. Due to favorable presence a pyridazinone moiety in known active structures, pyridazinone derivatives provoked a special interest in the search for new antibacterial agent[2-3]. Also, it is well known that the hydrazone group plays an important for the antimicrobial activity a number of hydrazone derivatives have been claimed to possess interesting antibacterial and antifungal activities.Considering above, we report synthesis of fifteen of 6-(4-methylpiperazine)-3(2H)-pyridazinone-2-acetyl-3-(Substitue/nonsubstitue) benzalhydrazon **V** derivatives by the condensation reaction 3(2H)-pyridazinone-2-acetohydrazides with substituted benzaldehyde derivatives. The structures of these new pyridazinone derivatives were confirmed by their IR, ^{1}H-NMR spectra and elementary analysis. Antimicrobial activities of the synthesized compounds were also investigated.



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SYNTHESIS AND BIOLOGICAL EVALUATION OF (HETERO)ARYL-METHYLOXYPHENYLDERIVATIVES AS POTENT P-GLYCOPROTEIN MODULATING AGENTS

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P-gp is the main component of the blood-brain barrier (BBB), and limits or prevents the input of several chemotherapeutic agents, small peptides, antibiotics, HIVprotease inhibitors and antidepressant drugs in the CNS.(1) The high and homogeneous distribution of P-gp in the CNS shows that this kind of efflux pump may be essential both for brain detoxification andfor protection against xenobiotics.

The P-gp pumps out of the brain capillary endothelial cells several drugs thus limiting their accumulation within the endothelial cells and consequently reducing their efficacy. While on the one hand this effect results in a protection of the brain from toxic substances, on the other hand it may represent the main limiting factor involved in the reduced effectiveness of some therapies for the treatment of neurodegenerative diseases (i.e. Parkinson's (PD) and Alzheimer's diseases (AD)(2)as well as different types of cerebral tumors. Recently, we developed a new class of small molecules having arylmethyloxyphenyl structure that displayed moderate P-gp modulating activity.(3)Among these previously studied derivatives, compound **1a** (EC_{50}= 17.2 μM) (Figure 1), has shown the best results in modulating P-gp activity. Previous studies evidenced that the P-gp inhibition activity was influenced by the presence of a methoxy group on the C-ring. In the present work, new compounds have been synthesized in order to investigate the following: (i) the influence of the extent of methoxylation on the A-ring, C-ring or both for P-gp modulating activity, (ii) the effect of the position of the methoxy groups on the P-gp activity (iii) the effect of the halide on the C-ring, and (iv) the effect of the replacement of C-ring with heteroaromatic cycles such as thiophene and pyrimidine. The P-gp inhibition activity for each compound was studied by three combined biological assays: (i) inhibition of [3H]- vinblastine transport, (ii) ATP cell depletion, and (iii) apparent permeability (*P*app) in a Caco-2 cells monolayer. By these assays, it was possible to determine the structural requirements that favor P-gp inhibition and the mechanism involved in P-gp modulation. Moreover, the best P-gp modulators have been tested for both their MRP1 activity and cytotoxicity.

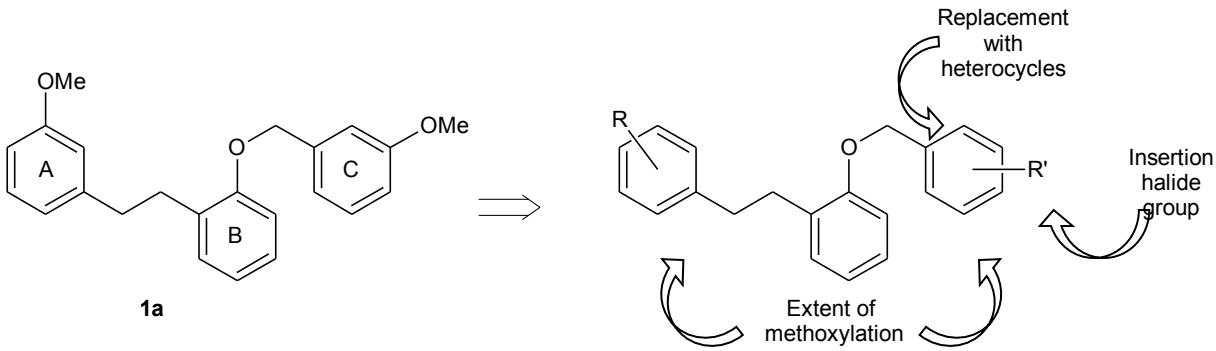

Figure1. Lead optimization of arylmethyloxyphenyl derivative**1a**

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APPLICATION OF METABOLOMICS IN THE INVESTIGATION OF THE FATE AND ROLE OF FOOD BIOACTIVE COMPOUNDS

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Mass spectrometry-based metabolomics provides an invaluable tool to identify, measure and interpret the complex time related concentration, activity and flux of exogenous and endogenous metabolites in cells, tissues, biofluids and organs.

One example of such application can be provided by the study of the metabolism and distribution of cyanidin 3-glucoside in mammals combining the results of both targeted and untargeted UPLC-MS experiments. In rats, cyanidin 3-glucoside is rapidly taken up from blood into tissues (t ½ = 0.36 min), where they accumulate up to their bioactivity threshold.[1] Methylation appears faster than other metabolic pathways. Both cyanidin 3-glucoside and its methylated form, peonidin 3-glucoside, can be detected in plasma, kidneys, and liver. Traces of other minor metabolites of cyanidin 3-glucoside (delphinidin 3-glucoside and its methylated forms petunidin and malvidin 3-glucoside) were also observed. The capacity of cyanidin 3-glucoside and their metabolites to affect mammalian metabolism was demonstrated in an investigation of the transient metabolomic changes in the brain and the plasma of adult rats after intravenously administration of cyanidin 3-glucoside. It was shown that cyanidin 3-glucoside alters certain important cellular metabolites, such as bile acids, glutathione, oxidized glutathione, and some lipids in the blood, kidneys, and liver of rats.[2] Moreover, these results demonstrating the ultra-fast distribution and metabolism of IV-administered anthocyanins in rats suggest that in mammalian plasma the sink could largely exceed the capacity of the source. This raised the question if the fast-changing concentrations of anthocyanins measured in plasma can be at all wise indicators to estimate their putative bioefficacy.[3]

In some case, the presence in food of a natural phyto-complex of oligomeric structures can be addressed with a combined approach requiring MS-based targeted metabolomics and NMR. We have indeed still a very partial understanding of the presence of some important bioactives in food, as in the case of berry ellagitannins.[4-6] While the metabolic fate of the ellagitannins in the gut is expected to follow a common mechanism, the precise characterisation of their native structure is important for example in explaining their anti-inflammatory activity at gastric level, which has been only recently investigated.[7]

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USING NATURAL COMPOUNDS, NUTRIGENOMICS AND METABOLOMICS IN FUTURE TARGETED THERAPEUTIC MOLECULE DEVELOPMENT

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There are many possible causes of disease which are linked to both genetic and environmental factors. Using data from the emerging field of nutrigenomics and metabolomics new therapeutic strategies are now feasible that cover many important factors involved in disease initiation and progression. In particular the role of metabolic dysfunction and increased oxidative stress and inflammation is becoming more apparent.

The positive actions of nutrients and minerals are in part related to the classical and well known actions related to energy metabolism but what is also important is that the right balance of that make us healthier do so in part because they induce the expression of health-promoting genes and reduce the expression of disease-promoting genes.

Many recent studies have shown that certain biochemical and genetic alterations occur in the cells of the organism and form part of the natural history underlying disease progression. This also presents large opportunities for prevention. The identification and evaluation of appropriate genetic, metabolic and biochemical markers correlating with early steps of disease onset and progression, may thus be a key preventing this disease.

The data being accumulated on natural compounds such as resveratrol, carnitine, curcumin, green tea and other compounds such as Coenzyme Q10 and lipoic acid show great potential as disease modifying agents.

The natural molecule carnitine and its derivatives are appropriate to illustrate this point. Carnitine is a natural product synthesized in mammals from the essential amino acids lysine and methionine or obtained from dietary sources. It is essential in fatty acid metabolism. Recent important metabolomic and nutrigenomic data strongly stress the importance of the carnitine system and oxidative metabolism in many physiopathological states. Carnitine is a well recognised scavenger and antioxidant function to control ROS, showing specific preventive action against lipid peroxidation and membrane damage, which are molecular phenomenon substantiating the chain of events that culminate in the cellular damage. In addition, acetylated carnitine (acetyl-L-carnitine, ALC) appears to protect stressed cells exposed to neurotoxins.

New therapeutic rationale using carnitine derivatives are emerging and we will discuss a few therapeutic examples which target on the following systems of the body:

- CNS and neurodegenerative disease
- Reproductive system and fertility
- Cardiovascular system and heart failure in aging

These points will be discussed further using experimental data from our and other groups.

NEW PREBIOTIC COMPOUNDS FROM AGRO-FOOD WASTES AND BY-PRODUCTS: NEW PERSPECTIVES FOR NUTRACEUTICALS AND FUNCTIONAL INGREDIENTS DEVELOPMENT

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Human health is a complex system, ruled by different parameters, where food is principal actor in the scene. Beside this concept, the idea "*we are what we eat*" is a classic – but renewed - parameter useful to improve the so call *well-being* state in humans.

Moreover, the crucial role of the whole gut microbiota in humans is largely recognised by scientists today worldwide. It is possible to trigger a *healthy state* simply by balancing microbiota, leading a health status in the body? This is the challenge in this field today, particularly studying all the interactions among foods, microbiota and molecular markers able to describe their action in humans. Moreover, the balance between benign "probiotic" bacteria and pathogens in the gut is a key topic in food safety. As showed and confirmed by a lot of recent papers, the role of food - particularly prebiotic foods - is the preferred performing solution to simply trigger the development of probiotic bacteria in the gut. The concept of "tailored foods", beside the possibility of use of specific food supplements or nutraceuticals, is considered another cool strategy to improve *well-being* and health in humans.

Prebiotic compounds (molecules of different molecular weight, principally isolated from plants and principally belonging the class of carbohydrates) are largely studied today, beside the characterization of new probiotic microorganism with peculiar characteristic. The study of the cryo-resistance of probiotic bacteria, as well as increasing their shelf life, is another technological challenge for pharmaceutical industry.

Some "classic" prebiotic substances like inulin, fructooligosaccharides (FOS) and galactooligosaccharides (GOS) are largely exploited by food industry, mainly in food supplements, nutraceuticals as well as in dietetic products and infant formulas. The prebiotic activity of these substances is well characterized; despite this fact, some negative traits (particularly correlated to flatulence, gut disorders in children treated with large quantities of prebiotics and negative aromas, affecting the product palatability and acceptability) are highlighted in literature.

The core of our studies on this topic during the last years was focused on the identification of new safe and performing prebiotic substances in food, both from plant and animal source, as well as their chemical characterization, showing the bioactive prebiotic properties towards some probiotic strains.

The aim of this oral communication will be a schematic report on the isolation, chemical characterization and in vitro bioactivity determination of some prebiotic carbohydrates isolated from wastes (whey from bovine milk and pellicles from roasted hazelnuts).

The sialic acid-rich olygosaccharides (concentrated by ultrafiltration), isolated from bovine whey and chemically characterized by mean of MALDI-FTICR, nano LC-Chip-Q-ToF and Gas Chromatography, are more similar to those present in human milk (the "key" reference for the prebiotic activity), when compared to "classic" prebiotic fibers. Moreover, the protein-free fibre and oligosaccharides isolated from the pellicles released from hazelnut (a waste obtained from hazelnut roasting process) showed an interesting cryo-protectant capacity during lyophilisation of probiotic strains, as well as a significant prebiotic activity towards *L. plantarum* P17630 and *L. crispatus* P17631.

Finally, in this communication, we will show that unusual by-products and wastes from agro-food industry can be considered an interesting and powerful source of prebiotic substances useful to food and nutraceutical design.

LISTENING TO NATURE'S HINTS: EXPLOITING A NATURAL PRODUCT SCAFFOLD TO SIMPLIFY A COMPLEX CHEMICAL STRUCTURE

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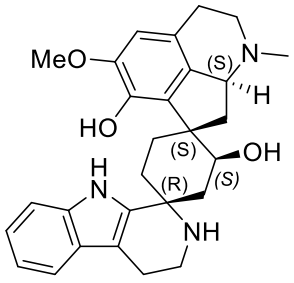
Natural products are important sources of bioactive compounds. Their complex chemical structure is at the same time their main perk and the major problem involved in their use as therapeutic agents. Total synthesis is, in many cases, long and low yielding while relaying completely on extraction from natural sources could lead to environmental issues. An alternative to total synthesis may be the exploitation of a three-dimensional scaffold that guide the interaction of different functional group with their biological target. Prof. Quinn group at the Eskitis Institute recently identified 1-azaspiroundecane as a highly conserved privileged scaffold embedded in many natural products extracted from different sources. This structure is present in Phoebegrandine D, an alkaloid found in *Phoebe grandis* leaves, whose extract owns a good antimalarial activity (IC_{50} < 8 μg/ml).(1) We present here the synthesis of 8-amino-1-azaspiroundecane scaffold realized in six high yielding steps. The scaffold was used as a starting point for a series of compounds mimicking the aromatic systems present in Phoebegrandine D. This strategy led to a compound more active than the plant extract (IC_{50}: 4.7 μg/ml), and could be used in the future to simplify other bioactive natural products, circumventing total synthesis.



Phoebegrandine D

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ANTIMICROBIAL ACTIVITY OF *PHYLLANTHUS MUELLERIANUS* (KUNTZE) EXCELL STEM BARK: FROM CRUDE EXTRAC TO BIOACTIVE COMPOUND

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The plant kingdom is an endless source of chemically diverse bioactive compounds which are used in traditional folk medicine for the treatment of a wide array of diseases. Since many plant extracts have been shown to exert biological activity in vitro and in vivo, researches on traditional medicine focused on the characterization of active compounds of these plants is justified. In addition, in recent years there have been a renaissance of interest in natural or herbal remedies worldwide, partly because of the realization that modern medicine is unable to provide a "cure-all" solution against human diseases.

In this work, we present the studies providing an evidence for traditional uses of *Phyllanthus muellerianus* and suggesting the future research opportunities for this plant.

Particularly, *Phyllanthus muellerianus* stem bark is used in the pygmies traditional medicine for the treatment of tetanus and wound infections.(1) A previous investigation on the stem bark extracts of *P. muellerianus*(2) demonstrated an interesting activity of the defatted methanol extract against *Clostridium sporogenes* (MIC= 100 µg/ml) and *Streptococcus pyogenes* (MIC= 300 µg/ml), responsible for gas gangrene and suppurative and non suppurative diseases, respectively, which supported the traditional use of the extract by local populations in Cameroon. A further advancement on this research is represented by a bioguided fractionation of the defatted methanol extract. After acquisition of the HPLC fingerprinting, the defatted methanol extract was purified by reverse phase flash chromatography in order to isolate and identify the active component/s. This purification step afforded six fractions and their biological properties were compared to that of the starting crude extract. Fractions 4 and 5 exhibited the most significant antimicrobial activity against C. sporogenes and S. pyogens with MIC values of 25 µg/ml and 50 µg/ml (F4), and 37.2 µg/ml and 56 µg/ml (F5), respectively. Although F4 and F5 showed a comparable activity, the most abundant F4 was further purified by SPE affording a bright yellow solid whose HPLC profile highlighted the presence of only one peak with MIC and MBC values in the same order of magnitude of the control. A full chemical characterization of the bioactive compound is under investigation and results will be presented.

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ANALYTICAL CHARACTERIZATION AND BIOLOGICAL EVALUATION OF THE MAIN CONSTITUENTS OF *CROCUS SATIVUS* L. (SAFFRON) FROM DIFFERENT GEOGRAPHICAL ORIGINS

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The chemical composition of *Crocus sativus* L. stigmas is the most important indicator of its quality and of its commercial value in accordance with ISO 3632-1 and 3632-2 procedures. Among the most relevant compounds, safranal, crocin and picrocrocin (Figure 1) are definitely the analytes with the highest chemical/biological interest. These elements give saffron its organoleptic properties (color, odor and taste) and characterize markedly its economic value. In order to counter the growing number of adulteration and due to the high cost of this spice, we compared, in terms of qualitative/quantitative recovery, microwave- and ultrasound-assisted extractions with the classical solvent extraction under magnetic stirring. Recovery of the bioactive compounds was affected by more than one parameter, such as extraction technique, solvent, temperature, length of the process, and particle size. Then, we optimized the microwave-assisted extraction maximizing recovery and minimizing the use of solvents and extraction time. For this purpose, an experimental design has been performed to allow us to identify, in a limited number of experiments, type and volume of extraction solvent, time, and the instrumental parameters of microwave irradiation (power, pressure, temperature). The developed method was then validated by HPLC in terms of recovery, intra-day and inter-day precision, limit of detection (LOD) and limit of quantification (LOQ), and linearity.

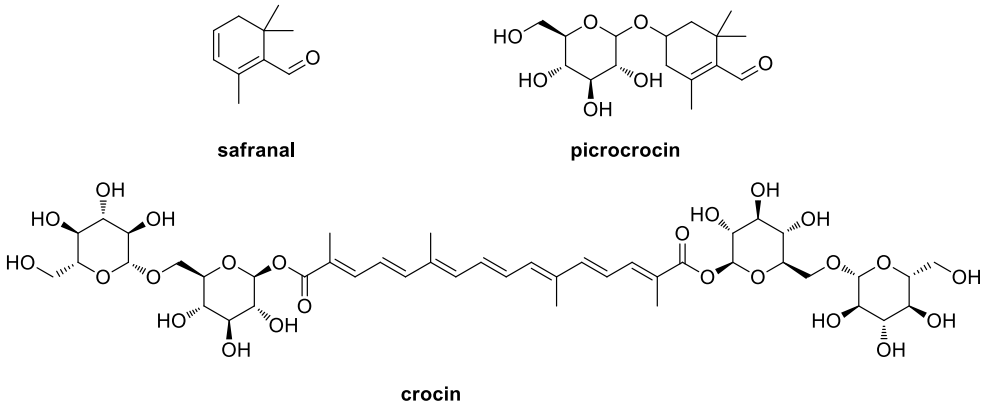

Figure 1: Structures of the main bioactive components of *Crocus sativus* L. (saffron spice).

The method of extraction and subsequent chromatographic analysis was then applied to 106 samples of saffron from Greece, Turkey, Spain and Italy (Sardinia and Lazio). Moreover, a discriminant analysis with near-infrared (NIR) spectroscopy has been carried out on the ground stigmas without any sample manipulation to determine the chemical composition and geographical origin. The spectra were acquired in reflectance mode and have been used for the construction of chemometric models of classification. Our aim was to correlate HPLC and NIR techniques to correctly classify the geographical origin and absence of adulteration of each collected sample of saffron.

To enhance the value of the product, we also assessed the biological activity of its major components as regards inhibitory activity against human monoamine oxidase (MAO-A and MAO-B), carbonic anhydrase isoforms (CA1 and CA2), and *Helicobacter pylori.*

THEORETICAL DETERMINATION OF THE pK_as OF BETALAMIC ACID RELATED TO THE FREE-RADICAL SCAVENGER CAPACITY: COMPARISON BETWEEN SEMI-EMPIRICAL AND *AB INITIO* METHODS

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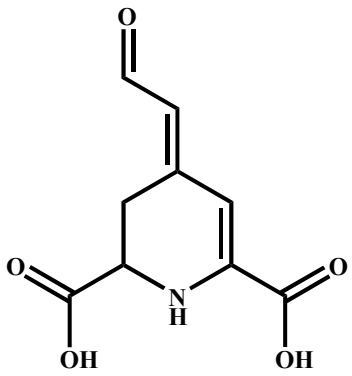
Health benefits of dietary phytochemicals have been suggested in recent years. Among thousands of different compounds, Betalains, which occur in a number of vegetables of the *Cariophyllalae* order with cactus pear fruits and red beet as the principal dietary sources, have been considered because of reducing power and potential to affect redox-modulated cellular processes[1-3]. The antioxidant power of Betalains is strictly due to the dissociation rate of the acidic moieties present in all the molecules of this family of phytochemicals. Experimentally, only the pK_as of Betanin have been determined, and recently, it was evidenced as the acid dissociation, at different environmental pHs, affects on its electron donating capacity, and further on its free-radical scavenging power[4]. The same correlation was studied on another betalain family compound, Betalamic Acid, but no pK_as values were experimentally measured [5].



With the aim to justify its behaviour as free-radical scavenger, we calculated *in silico* the pK_as of Betalamic Acid by means different approaches. Starting from the known experimental pK_as of a number of acid compounds, both phytochemicals and small organic, we compared two semi-empirical approaches and DFT calculation to give a realistic prediction of the pK_as of Betalamic Acid. Obtained results by means these computational approaches are concordant with the experimental results of Gandia-Herrero[5] who showed that the free-radical scavenging capacity drastically decrease at pH>5 in stable solution of the free radical $ABTS^{\circ+}$. In fact as showed by us *in silico*, at the experimental pH>5, in solution, the dianionic species is predominant exploiting the high electron donating capability (HOMO energy) to decrease the concentration of the colorant. Therefore the computational calculated pK_as values of Betalamic Acid resulted very reliable

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RADICAL SCAVENGING EFFECT OF PHENOLICS FROM THEOBROMA CACAO ON RAT H9C2 CARDOMIOBLASTS EXPOSED TO OXIDATIVE STRESS

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Cacao and its derivatives display antioxidant and antiradical properties, which can have a role in the maintenance of human health. On the basis of many studies of the in vivo bioavailability of polyphenols contained in the cacao, it can be inferred that some of them (phenolic acids, epicatechin and catechin, clovamide, dimeric procyanidins, other minor flavonoids) may be considered bioavailable at significative degree. Cardiovascular diseases are the first cause of morbidity and mortality in western countries. Phenolic compounds contained in cocoa have been reported to be beneficial in pathologies linked to hypertension, dyslipidemies and other inflammatory diseases related to cardiovascular diseases.[(1,2)] The radical scavenging properties of clovamide, a minor cacao component,[(3)] potential anti-platelet aggregant,[(4)] could help in reducing the consequences of cardiac ischemic damage, which is followed by the oxidative stress in the reperfusion phase, at the end responsible for the loss of myocytes.[(5)]

Herein we have studied the radical scavenging properties of clovamide, epicatechin and rosmarinic acid (this last one as reference compound) in a model of rat cardiomyoblasts (H9c2 cell line), evaluating their inhibition on ROS (reactive oxygen species) release induced by hydrogen peroxide. Also polyphenols extracted from cacao (fractioned by Solid Phase Extraction in low, medium and high molecular weight, then characterized by HPLC-DAD) were studied in this cell model. The anti-apoptotic activity of these compounds in cells treated with H_2O_2 was evaluated as well. At micro-nanomolar concentrations, clovamide, epicatechin and rosmarinic acid dramatically inhibited ROS release and protect H9c2 cells from H_2O_2–induced apoptosis, evaluated both in a TUNEL assay and cytofluorimetrically.

These data are a further support of the potential bioactive beneficial role of the cocoa based products in the context of cardiovascular pathologies, particularly in the protection towards ischemic injury.

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BOTANICAL FOOD SUPPLEMENTS: REGULATORY FRAMEWORK

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Currently, in the European Union (EU) two parallel regulatory frameworks allow the use of botanicals in both food supplements(1) and medicinal products.(2)

Food supplements EU legislation will be reviewed in this paper, followed by a discussion of progression towards harmonisation in the use of botanicals as ingredients, ending with a description of the Italian system of post-marketing surveillance.

Food supplements are regulated under the framework of foodstuffs, which means that they have to comply with all provisions of food laws, starting from the Regulation (EC) 178/2002 ("General Food Law").(3) In addition, food supplements are subject to the specific requirements of core Directive 2002/46/EC,(1) according to which, they are defined as "concentrated sources of *nutrients* or *other substances with a nutritional or physiological effect*", alone or in combination. Such products are marketed as capsules, pastilles, etc. (in order to be taken in measured small unit quantities), with the scope of supporting or optimizing the normal physiological functions within the "homeostasis" (4).

Among the mentioned "other substances" there is an increased interest in the use of botanical ingredients (commonly referred to as "natural") for their health-enhancing properties, but, due to the fact that the food supplements Directive does not contain specific provisions for their use, different national rules for marketing botanical food supplements are in place.

In recent years, the publication of Regulation on nutrition and health claims (NHCR)(5) has stressed the importance of effectively communicating to the consumers about the health effects that could be ascribed to foodstuffs in order to help them to better understand the product's aim and make the proper choice. NHCR application on botanicals is a critical point about validation criteria of health claims since traditional use as evidence of efficacy is not accepted in food supplements, despite of its applicability in traditional herbal medicinal products.

Italy, like France and Belgium, has regulated the use of botanicals in food supplements with national rules based on positive lists of plants that are allowed, establishing, in some cases, maximum levels of certain herbal constituents and specific additional label warnings for the correct use of products. Recently, in an initial effort to harmonize this sector, the Competent Authorities of Italy, France and Belgium, started the so called "BELFRIT project", aimed to obtaining a common list of eligible plants for the use in food supplements by comparing the three lists of allowed plants.(6) Furthermore, since at European level an open debate on validation parameters for botanical health claims has kept them "on hold", a key objective of BELFRIT project is to promote the extension of traditional use for botanical physiological effects also to food supplements field.

An important Italian initiative for health protection is the "natural products surveillance system"("fitovigilanza"),[7] based on the collection and evaluation of suspected adverse reactions arising from food supplements intake. This system provides relevant data for a proper risk management of botanicals in such products, also taking into account that the national market is the largest in EU.

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BIOPARTITIONING MICELLAR CHROMATOGRAPHY AS *IN VITRO* TECHNIQUE IN PREDICTION OF ORAL HUMAN ABSORPTION OF PLANT EXTRACTS COMPONENTS

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Raw materials quality assessment is a very important factor enabling products creation up to the international standards. In this investigation qualitative composition of bioactive plant extracts has been checked. Tested extracts have been obtained by the use of supercritical carbon dioxide extraction and can be utilized as raw materials in a commercial production of wide range of many different goods. Plant extracts of raspberry (*Rubus idaeus*), strawberry (*Fragaria ananassa*), blackcurrant (*Ribes nigrum*), aronia (*Aronia Medik.*), Japanese rose (*Rosa rugosa Thunb*.) seeds and palmetto palm (*Sabal minor*) fruit have been examined. In these extracts saturated, mono- and polyunsaturated fatty acids as well as polyphenols have been identified. Appropriate content of these components tends to be a determinant of human organism operation.

From a pharmaceutical and medicinal point of view, one of the most important biological activity descriptor of oral absorption has been examined. For *in vitro* determination of human oral absorption, the usefulness of Biopartitioning Micellar Chromatography (BMC) has been confirmed.

Chromatographic parameters have been collated with steric, electronic and physicochemical ones using QRAR (*Quantitative Retention – Activity Relationships*) and QSAR (*Quantitative Structure – Activity Relationships*) models. Moreover, retention BMC data have been compared with lipophilicity parameter, $\log P_{o/w}$ (n-octanol – water partition coefficient). Moreover, for prediction of human oral absorption of the compounds tested, the Abraham model has been utilized. On the basis of Abraham descriptors as well as of chromatographic, lipophilicity, steric and different physicochemical parameters, new models of human oral absorption of the investigated compounds have been constructed.

NUTRACEUTICALS & GUT: PHYTOSTEROLS & ULCERATIVE COLITIS

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Background and Aim: Ulcerative colitis(UC) is a worldwide chronic, idiopathic, inflammatory bowel disease[1] of the rectum and colon, presenting with acute phases and remission periods. (1)

The current status of drug therapy for UC consists, mainly, of aminosalicylates, corticosteroids, immunosuppressive drugs, antibiotics and biological drugs. These drugs, however, are associated with unpredictable and serious side effects, particularly in the long term. (1)

Recently, considerable attention has been devoted on identifying naturally occurring chemopreventive natural substances, particularly those present in dietary and medicinal plants. (2)

Phytosterols, such as β-sitosterol, campesterol, stigmasterol, Δ5-Avenasterol, are plant-derived sterols, structurally similar and functionally analogous to cholesterol in vertebrate animals, mainly found in nuts, fruits, and seeds.(3) These molecules have been found to exert several biological activities, including anti-inflammatory, antibacterial, antifungal, antiulcerative, and antitumor activities, in addition to cholesterol-lowering activities. (4) In this study the preventive, therapeutic and reparation effects of a phytosterols mixture, mainly constituted by β-sitosterol, campesterol, stigmasteroland brassicasterol has been evalueted in a model of acute DSS induced colitis in male Balb/c mice (8 weeks old, 25-30 gm b.w.).

Methods: The animals were divided into 2 groups: in the first group they were fed a normal diet over 14 days and administered DSS (5%v/w) over 10 days, followed by control diet for 14 days. In the second group, animals were fed phytosterols (400 mg/kg/day) enriched diet over 14 days, followed by phytosterols + DSS (5%v/w) over 10 days, followed by phytosterols over 14 days.

In both groups, after DSS assumption, the clinical (Disease Activity Index)(DAI), intestinal inflammation parameters (intestinal histology, micro Positron Emission Tomography (micro PET) with 18F-FDG) and ileal, colonic and gallbladder motility were evaluated.

Results: Phytosterols did not either prevent colitis, but slightly reduced severity of colitis, induced a significant remission of colitis with respect to control diet animals, as indicated both by histology, DAI and uptake of 18F FDG by micro PET (Fig. 1).

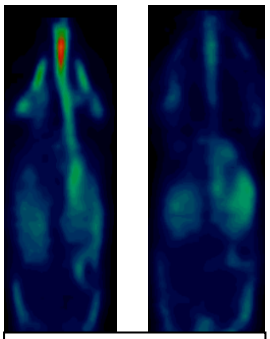

Fig. 1: 18F-FDG uptake in control (Left) and phytosterols fed (Right) animals during remission phase

In addition, phytosterols administration to healthy animals slightly alters ileum, colon and gallbladder smooth muscle response to Carbachol and Atropine. In DSS induced colitis animals, the smooth muscle response to Carbachol and Atropine is severely altered both in animals fed phytosterols and normal diet. The administration of phytosterols restores the response of these tissues to Carbachol and Atropine to the values previous to DSS administration.

Conclusion: Phytosterols exhert anti-inflammatory properties at the level of the reparation of tissue damage: this support their clinical use both in acute phases, to improve reparation and in the remission phases of UC.

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BIOLOGICAL EVALUATION OF SOME CHALCONE DERIVATIVES

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Chalcones and their analogues are versatile and convenient starting materials or intermediates for the synthesis of naturally occurring flavonoids and various nitrogen-containing heterocyclic compounds. Many chalcones have also been reported to exhibit a wide spectrum of biological effects.(1)

Some chalcone derivatives were synthesized *via* the base-catalyzed Claisen-Schmidt condensation of 5-substituted-1*H*-indole-3-carboxaldehydes with appropriate acetophenones. The obtained compounds were evaluated for their COX inhibitory activity.

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TARGETING NF-kappaB: EXTRACTION, CHARACTERIZATION AND ANTINFLAMMATORY ACTIVITY IN CYSTIC FIBROSIS CELLS OF DIETARY POLYPHENOLS FROM OLIVE MILL WASTE WATER (OMWW)

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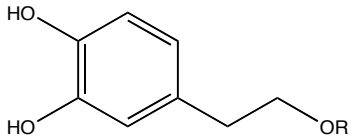
Waste water from the work up of vegetables are a rich source of active molecules. A dietary polyphenols rich extracts, > 98% of glycosylated polyphenols, derived from olive mill waste water (OMWW) and obtained by a selective extraction Molecular Imprinting approach, have been investigated in order to discover compounds able to reduce IL-8 expression in human bronchial epithelial cells (IB3-1), derived from a CF patient with a DF508/W1282X mutant genotype and stimulated with TNF-alpha. A persistent recruitment of neutrophils in the bronchi of cystic fibrosis (CF) patients contributes to aggravate the airway tissue damage, suggesting the importance of modulating the expression of chemokines, including IL-8. The identification of innovative drugs, to reduce the excessive lung inflammation in Cystic Fibrosis (CF) patients, is considered a therapeutic target to prevent the progressive lung tissue deterioration. The final aim of the present study is to determine the activity of components, present in the new OMWW-extract, on the expression of IL-8 gene, the major chemokine released from CF cells under the control of NF-kB transcription factor (TF). The "core" functional structure, more represented in the components of the extract, both in free form and as contained within the molecular structure of other polyphenols, is hydroxytyrosol: glycosides containing this structure represent 80% of the components of the mixture for an overall content >35%. After a preliminary screening in inhibiting TF/DNA complexes, among the different molecules present in the extract, three compounds were particularly intriguing to us: apigenin, oleuropein and cyanidin chloride. NF-kappaB is known to be a very interesting target molecule for the design of anti-tumor, pro-apoptotic and anti-inflammatory drugs; these natural derivatives have proved to be excellent lead compounds for the inhibition of NF-kB-p50 biological activity. Finally, we demonstrated that apigenin and cyanidin chloride are able to modulate the expression of IL-8 gene regulated by NF-kB TF in CF IB3-1 cells, while oleuropein showed no effect in controlling the IL-8 expression.



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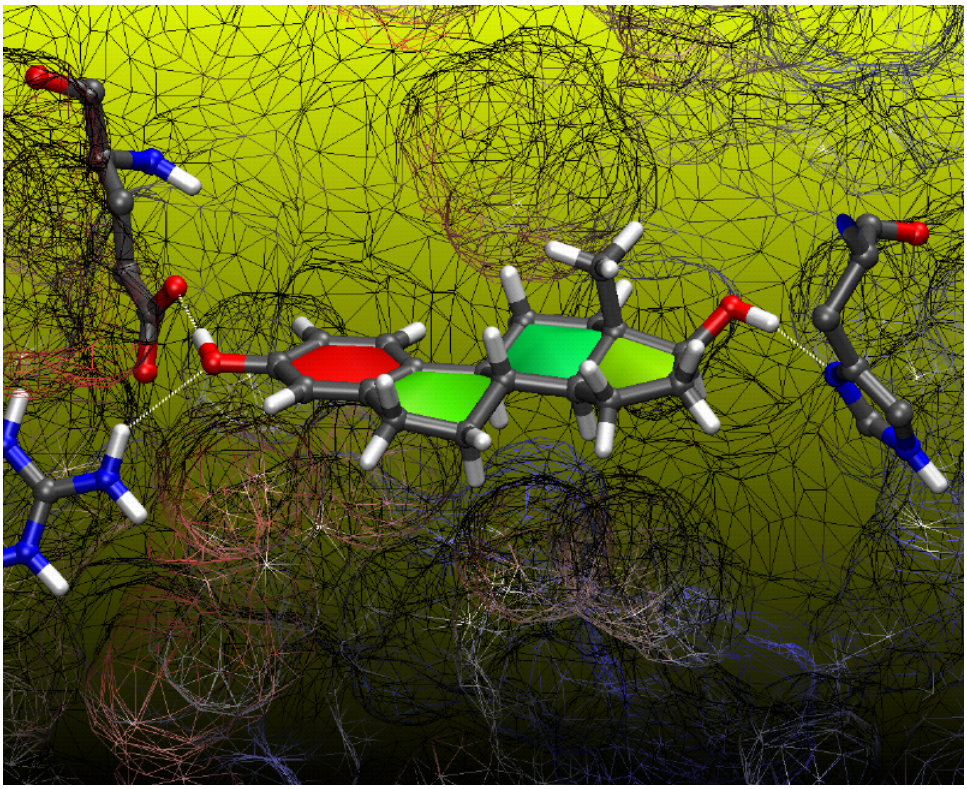

PROTEIN-LIGAND BINDING INVESTIGATED VIA MOLECULAR DYNAMICS SIMULATIONS: THE CASE STUDY OF A TRANSITION-STATE ANALOG BINDING TO THE PNP ENZYME

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The purine nucleoside phosphorylase (PNP) enzyme has been proposed as a target for disorders caused by T-cell malfunctioning. Transition-state analog PNP inhibitors have already been synthesized and tested. The inhibition has also been experimentally determined and biochemically characterized. It shows several peculiarities such as a slow onset followed by a tight binding and "one-third-the-sites" inhibition. Based on these experimental observations, we have chosen PNP as a suitable, and definitely non-trivial, test bed to study protein-ligand binding from thermodynamic and kinetic standpoints. In this work, we perform 10 unbiased Molecular Dynamics (MD) simulation runs (at least 250 ns each, overall over 7 μs of MD), made affordable by the GPU architecture. We compare a few MD engines, such as the NAMD and ACEMD packages, in terms of accuracy and performance. To increase the chances of seeing a binding event, we start with an artificially higher ligand concentration (a few mM) relative to the experimental conditions. We then use the statistics coming from the trajectories that led to binding as a pool of samples in the configuration space from which we identify the most relevant features. In these simulations, we are able to see the binding (up to a RMSD of 0.6 Å from the crystal structure) and three different routes to it. By means of a purposely-developed clustering algorithm, we define a set of meso-states and identify several possible intermediates to the binding. From the trajectories, we also derive some insights concerning transitions between these states, and we are now performing more targeted simulations in order to build a Markovian model to get quantitative estimation of the corresponding kinetics rates. In this lecture, learned lessons, results, consequences and possible outlooks of extensive molecular dynamics simulations in drug design will be discussed.

INVESTIGATING THE BINDING MECHANISM OF THE GLYCOSYLATED AND NON-GLYCOSYLATED CSF114 PEPTIDE AT MEMBRANE INTERFACE

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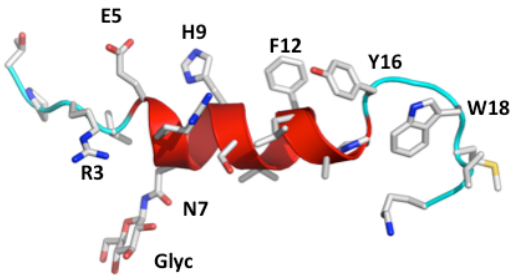

Glucosylations play a fundamental role in antigen-antibody recognition. The majority of these interactions occurs on the surface of biological compartments where the sugar moieties are crucial for the binding to the antibody counterpart. Prompted by the immunogenic response of the glycosylated fragment of mielin oligodentrocyte glycoprotein (MOG), we have very recently designed and synthesized the glycosylated peptide CSF114(Glc).[(1)] This peptide turned out to be a potent antigenic probe that accurately measures IgM auto-Abs in the sera of patients affected by multiple sclerosis.

Unfortunately, which are the key interactions during the peptide-antibody binding mechanism are still unclear. This information is extremely important since disclosing molecular details on the positioning of this peptide in the binding to the antibody, can provide precious tools to improve the diagnostic and prognostic accuracy of CSF114 biomarker.

As the recognition between the antigen and antibody generally occurs at extracellular level close to the outer layer of the cellular membrane, we have decided to investigate the interaction mechanism of the peptide CSF and its glycosilated form with a membrane model through a very extensive computational study. In particular, we have run 10 independent molecular dynamics (MD) simulations of both glycosylated and non-glycosylated peptides forms in explicit DMPC bilayer environment. A total amount of 1 microsecond MD simulations on each system has been carried out and the use of a funnel-restraint potential[(2)] has allowed to reduce the conformational space to explore, enhancing at the same time the sampling of the peptide/membrane bound states. These simulations have identified the main interacting sites between the peptides and the membrane highlighting the key roles played by some residues and the sugar during the peptide/membrane binding event.

The results coming from the MD simulations have been confirmed and complemented through a series of experiments. In particular, the theoretical model together with the data collected from NMR and Electron Paramagnetic Resonance (EPR) experiments in presence of membrane models, have shown the main interacting sites between the glyco-peptides and the membrane, highlighting the key role played by specific residues and the sugar moiety during the glycopeptide/membrane and the glycopeptide-antibody binding event.

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IN EVERY DARK CLOUD THERE IS A SILVER LINING: THE ROLE OF S45F BETA CATENIN MUTANT IN DESMOID TUMORS

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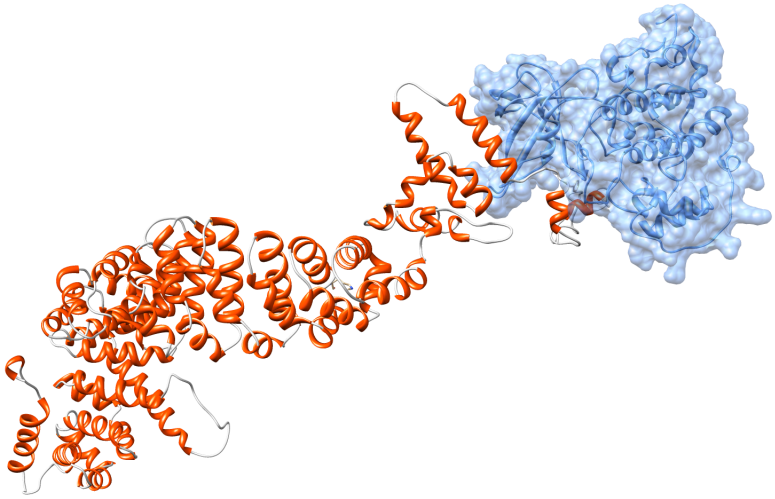
Desmoid tumors (DTs) are rare mesenchymal lesions with fibroblastic proliferation characterized by a variable clinical course. In a clinical study involving 179 patients a high frequency of mutations (85%) in the gene encoding for β-catenin protein was found [(1)]. In particular three different mutants were identified: T41A, S45F and S45P. All this residues are phosphorylation/regulation sites for this protein.

Specifically, phosphorylation of Serine 45 increases the affinity of β-catenin for GSK3, thus promoting its ubiquitination and degradation. Actually, only S45F mutant seems to play a fundamental role in tendency for local recurrence after complete surgical resection. Exploiting molecular simulation technique [(2)], we then studied the interactions of the wild-type (WT) and T41A, S45P, and S45F β-catenin mutants with GSK3. Of note, a proprietary 3D homology model of the full β-catenin structure was employed in this study. The detailed analysis of the binding free energy and its per residues deconvolution for all these supramolecular complexes shows that replacing Serine with Phenylalanine results in a stronger interaction of β-catenin with GSK3. In detail, the side chain of F45 reaches deeply into the specific phospho-serine pocket, thus preventing the disruption of the complex and, contextually, hampering the kinase activity of GSK3 [(3)] on T41, S33 and S37. The results of this *in silico* investigation support the clinical evidence for the high aggressiveness of the S45F mutant and its importance as a potential prognostic marker.



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UNDERSTANDING BINDING AND SPECIFICITY DETERMINANTS IN α-2 ADRENOCEPTOR SUBTYPES

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α-2 Adrenoceptors (ARs) are G Protein-Coupled Receptors (GPCR) that bind the endogenous hormone adrenaline and neurotransmitter noradrenaline. α2-ARs are potential drug targets with many clinical applications, e.g. in the treatment of elevated blood pressure and intraocular pressure, alleviating withdrawal symptoms from opiates and alcohol, and could be used as an anesthetic adjuvant in surgery.

In human, the α2-ARs are divided into three subtypes A, B and C that share about 66% of identical amino acids in the transmembrane region. Subtype-specific ligands have been long thought to be useful for therapeutic applications or as chemical probes to better understand the function of these receptors. To date there is not a general explanation about subtype specificity, although for a few specific cases a reasonable explanation exists.[1, 2, 3]

Recently, we have tested more than 17.000 compounds in a miniaturized robotic microplate assay, finding 93 nM active ligands tested to the three α-2 AR subtypes.[4] This dataset represents an ideal set to study subtype specificity.

Here, we build homology models of all three subtypes since there are no available crystal structure for the α-2 ARs. A close homologue the dopamine receptor (pdb code: 3PBL)[5] has been recently crystallized.

All of the 93 active compounds have been flexibly docked in the α-2 ARs homology models. In this communication we will discuss some key features of binding and specificity determinates in α-2 ARs subtypes.

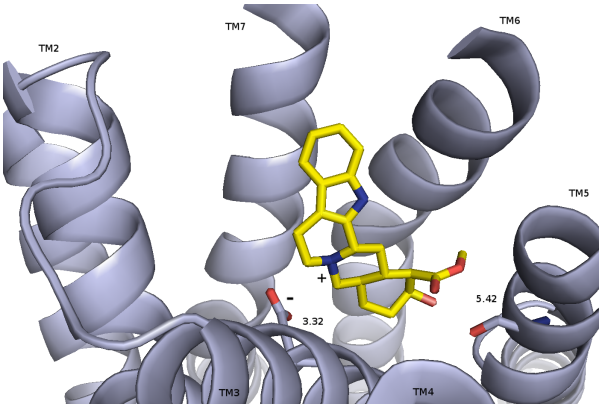

Figure: Docking pose of yohimbine in α-2_A AR subtype, two important interactions are shown: ASP 3.32 and SER 5.42

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TARGETING THE HUMAN TELOMERIC G-QUADRUPLEX THROUGH VIRTUAL SCREENING CALCULATIONS

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Targeting of DNA secondary structures, such as G-quadruplexes (G4), is now considered an appealing opportunity for drug intervention in anticancer therapy.(1) However, the majority of G4 ligands developed so far are polyaromatic compounds endowed with poor drug-like properties and quadruplex *vs* duplex selectivity.(2) Thus, the identification of brand new chemotypes able to bind and stabilize G4 is still of great demand. In this scenario, our research group has already identified a small set of drug-like binders targeting the simple G4 $[d(TGGGGT)]_4$ and displaying promising in vitro antitumor activity.(3,4) Here, we have selected the more biologically relevant 24-nt telomere G4 forming sequence ($d[TTGGG(TTAGGG)_3A]$ – Tel24)(5) as target of our receptor-based virtual screening campaign. We have, thus, succeeded in identifying three effective drug-like telomeric G4 new ligands. The best one among these compounds has displayed impressive G4 binding and stabilizing properties. Moreover, its ability to induce selective DNA damage at telomeric level and induction of apoptosis and senescence in several tumour cells lines has been experimentally proven.

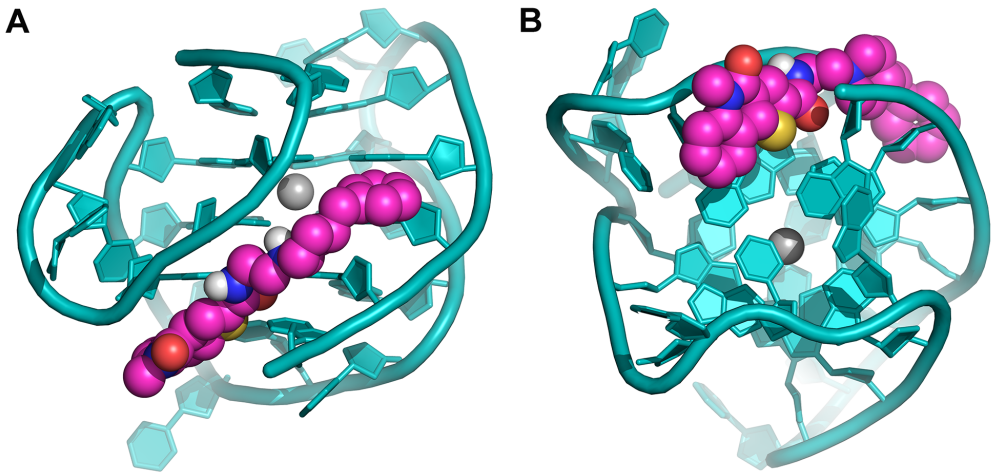

Figure 1. Side (A) and bottom (B) view of the binding mode of the best VS-derived ligand to Tel24. The ligand is depicted as magenta spheres. The DNA is shown in cyan cartoons, and the metal ions are represented as gray spheres.

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ZinClick: A DATABASE OF 16 MILLION OF NEW AND PATENTABLE 1,4 DISUBSTITUTED TRIAZOLES EASILY SYNTHETIZABLE

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Since Professor Barry K. Sharpless first introduced the concept of the "click reactions" in 2001 as powerful tools in drug discovery, the 1,4-disubstituted-1,2,3-triazoles have gained an important status in medicinal chemistry thanks to the discovery of the perfect click 1,3-dipolar cycloaddition reaction between azides and alkynes catalysed by copper salts. These triazoles have been suggested to be *aggressive pharmacophores* able to actively participate in the drug-receptor interactions, maintaining, at the same time, an excellent chemical and metabolic profile. Surprisingly, despite these interesting features, only 14% of the published works on click chemistry were related to drug discovery and no virtual libraries of 1,4-disubstituted-1,2,3-triazoles which attempted to systematically investigate the click-chemical space have been generated. In this presentation it will be shown the preparation of ZinClick, a database of triazoles, generated using *existing* alkynes and azides, synthesizable in no more than three synthetic steps from commercially available products. This resulted in a combinatorial database of over 16 million of 1,4-disubstituted-1,2,3-triazoles (Molecular Weight < 600), each of which is **easily synthesizable**, but in the same time **new** and **patentable**!

Exploration of the structural diversity of the ZinClick database, its comparison with other available database will be discussed as well as its application for the design of novel bioactive molecules containing the triazole nucleus.

Figure 1. Schematic representation of click chemistry galaxy in the chemistry universe.

COMPUTATIONAL STUDIES OF COLCHICINE SITE COMPOUNDS

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The microtubules (MT) play a crucial role in many functions of the cell cycle. It is not wrong to affirm, as the most important is the formation of the mitotic spindle during mitosis. The MT are formed by the aggregation of heterodimers of α and β tubulin in a process called dynamic instability.

As consequence of this role played by MT an than by tubulin, all compounds able to induce an interference in the dynamic instability bring to mitotic spindle disaggregation with loss of chromosomes and arrest of cell division in G2-M phase. Because of cancer cells high rate of proliferation, tubulin arose as an important target for anti cancer drugs development.

Among the classes of anti tubulin compounds we identify ArylThioIndoles (ATIs) family. ATIs are able to induce the arrest of tubulin polymerization into MT by binding at the interface between α and β tubulin sub-units. In the development of the ATIs family the computer aided drug design (CADD) approach proved to be very useful. Thanks to CADD we identify the ATIs binding mode; we solved some metabolism problems rising from *in vivo* oxidation/hydrolysis; we identified the pharmacophore interactions at the Colchicine binding site and we evaluated their stability by molecular dynamics. The CADD approach led as to get to new compounds featured by better biological activity and ADME profile. The worthy correlation between *in-silico* models with the biological assays results, confirmed the goodness of the followed approach. Thanks to carried out molecular modelling studies, we was also able to identify a new pharmacophoric portion of the colchicine binding site: a flexible loop (T7) close to binding site. It is known that the T7 loop movement allows the inhibitors binding but less clear is its role in the bound compounds stability. We observed by molecular dynamics simulations that the ATIs most active compounds never exceeded the bound distance from T7 loop residues. This led as to affirm that stable interactions with T7 are linked with better anti-tubulin activity.

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NEW BENZOSUBERONE p38α MAP KINASE INHIBITORS: EXTENDING INTERACTIONS TO THE DEEP POCKET / FROM TYP I TO TYP II INHIBITORS

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The p38 mitogen-activated protein kinase (p38 MAPK) is a key player in the pathogenesis of many inflammatory and autoimmune diseases of RA, COPD, IBD, Psoriasis etc. and their pro-inflammatory cytokines like TNF-α and IL-1β.

p38 inhibitors supress the synthesis of these cytokines. In general there are three different types of p38 MAPK inhibitors: typ I, typ II and typ III inhibitors. Typ I inhibitors are ATP competitive. Typ II inhibitors use an extra hydrophobic pocket ("deep pocket"), which is only available when the activation loop changes its conformation. Typ III inhibitors bind in an allosteric region of the enzyme.[1,2]

Recently we described Benzosuberone[3] compounds as selective Typ I inhibitors. The main goal of this project is to synthesize and evaluate new Benzosuberone analogues designed to reach the "deep pocket" of p38 MAPK to combine both extreme selection of this class of compounds with the slow off-kinetik of typ II inhibitors. The employed strategy to design new compounds as Typ II inhibitors was based on the insertion of hydrophobic aromatic side chains or hydrophilic groups on Benzosuberone scaffolds, aiming to identify which position and substituent is the most effective on reaching the "deep pocket", finally contributing to the increase of affinity.[4]

The designed Benzosuberone compounds were synthesized and evaluated by an enzymatic assay to determine their ability to inhibit the p38 alpha MAPK through the quantification of substrate phosphorylation.[5] The insertion of different hydrophobic and hydrophilic groups resulted in novel Benzosuberone derivatives designed as p38α MAPK inhibitors with IC_{50} down to 85 nM.

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IDENTIFICATION OF A NEW CLASS OF POTENT BACE-1 INHIBITORS THROUGH SCAFFOLD HOPPING

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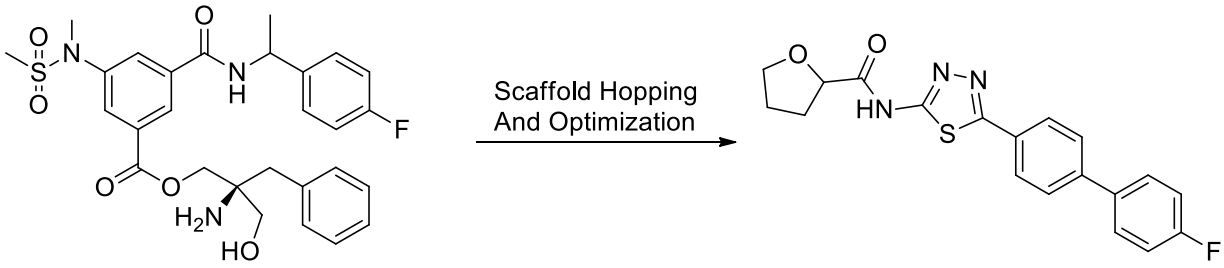
Due to the constant growth in the number of patients affected with Alzheimer disease (AD), the need of effective drugs able to stop the neuronal degeneration is increasing. Considering the therapeutic necessity of new molecules, we focused our attention in designing new BACE-1 inhibitors with good pharmacokinetic and pharmacodynamics properties, able to cross the blood-brain barrier. Using a scaffold hopping approach, we moved from a known BACE-1 ligand with good activity but poor values of MW and tPSA to a new aminothiazole scaffold with improved properties. Further optimization, aimed to improve pharmacokinetic properties as well as metabolic stability, led to the synthesis of an aminothiadiazole scaffold library of compounds that have been tested to assess their ability to inhibit BACE-1 activity.



I03
MW: 557.20
CLogP: 2.96
tPSA: 139.03

13
MW: 369.09
CLogP: 4.05
tPSA: 63.05

THE STRUCTURE OF THE LAMININ β_1 NONAPEPTIDE PROBED THROUGH LONG-TIMESCALE TEMPERATURE REPLICA-EXCHANGE MOLECULAR DYNAMICS SIMULATIONS IN EXPLICIT SOLVENT

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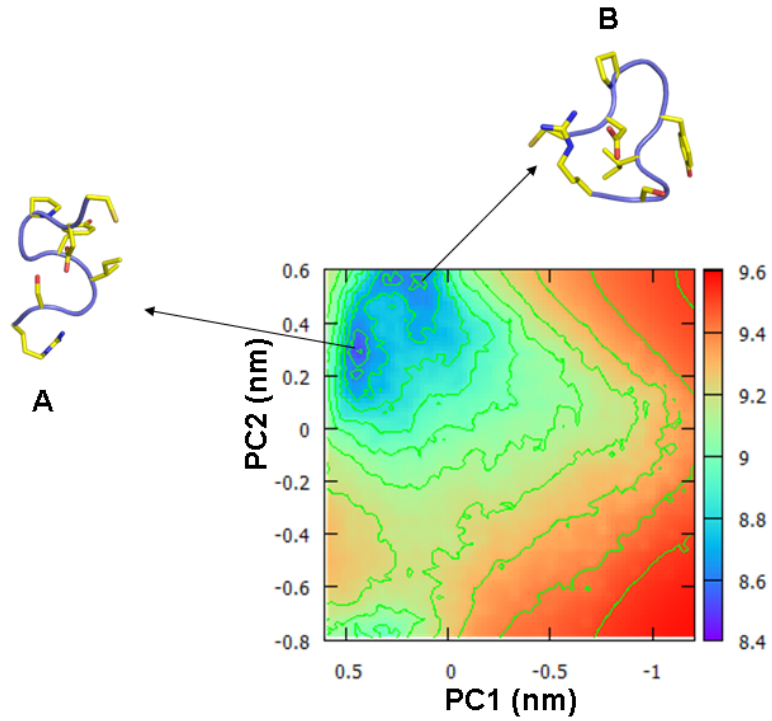
Laminin-1 is a member of a heterotrimeric glycoproteins family belonging to basement membrane. These proteins interact with cell surface receptors involved in adhesion, proliferation, differentiation and angiogenesis processes. The laminin-1 β_1 nonapeptide CDPGYIGSR, known also as peptide 11, from domain III of the β_1 chain of laminin-1, has been identified as the putative primary binding site for the 67 kDa Laminin Receptor (LR).(1) Overexpression of LR showed strong correlations with poor clinical prognosis in several solid tumors. Because of its critical role in cancer progression, the potential laminin-1 bioactive conformation has been the focus of a number of structural and biological studies. Here, the conformational dynamics of peptide 11 was probed by temperature replica-exchange molecular dynamics (T-REMD) simulations in explicit solvent.(2-3) T-REMD simulations were completed starting from an initial mutated structure of the murine epidermal growth factor peptide (mEGF-(33–42 residues)). Each replica was run for 100 ns. The structural characters were studied based on parameters such as distributions of backbone dihedral angles, free energy surface, stability of folded structure, and favourite conformations. The results showed that the peptide 11 in water adopted two different conformational states: the first state (**A**) was a bend ensemble with an open β-turn$_{2-5}$ and nine hydrogen bonds, the second state (**B**) was a bend ensemble with a open β-turn$_{2-5}$ and five hydrogen bonds. These findings allowed us to define a pharmacophore model useful for the design of small molecules able to destabilize LR/laminin-1 interaction.



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DISCOVERY OF NOVEL PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ (PPARγ) SCAFFOLDS WITH PARTIAL AGONIST BINDING PROPERTIES BY INTEGRATED *IN SILICO*/*IN VITRO* WORK FLOW

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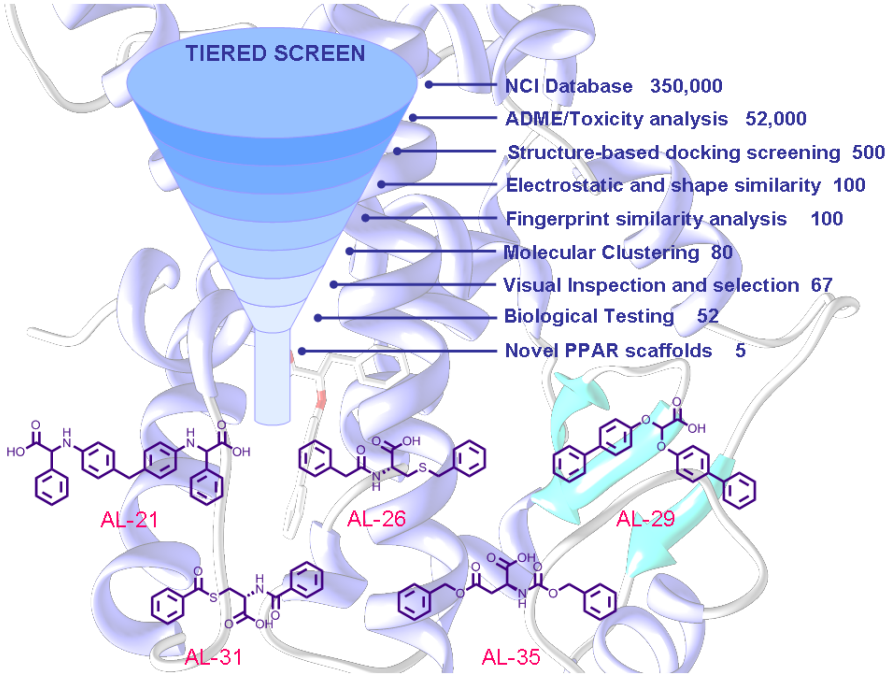
Full agonists to the peroxisome proliferator-activated receptor γ (PPARγ), such as rosiglitazone, have been associated with a series of undesired side effects, such as weight gain, fluid retention, cardiac hypertrophy, and hepatotoxicity. Nevertheless, PPARγ is involved in the expression of genes that control glucose and lipid metabolism and is an important target for drugs against type 2 diabetes, dyslipidemia, atherosclerosis, and cardiovascular disease.(1) In an effort to identify novel PPARγ ligands with an improved pharmacological profile, emphasis has shifted to selective ligands with partial agonist binding properties. Built on structure- and ligand-based computational techniques (2), a consensus protocol was developed for use in the virtual screening of chemical databases, focused toward retrieval of novel bioactive chemical scaffolds for PPARs. Consequent from application, five novel PPAR scaffolds displaying distinct chemotypes have been identified, namely (**AL-21**), S-benzyl-N-(phenylacetyl)cysteine (AL-26), bis([1,1'-biphenyl]-4-yloxy)acetic acid (**AL-29**), N,S-dibenzoylcysteine (**AL-31**), O-benzyl-N-((benzyloxy)carbonyl)-4-oxohomoserine (**AL-35**) with good ADME properties. In vitro transactivation assays demonstrated partial agonism of PPARγ by all five compounds. Additionally, differential PPAR isotype specificity was demonstrated through assay against PPARα and PPARδ subtypes. This work showcases the ability of target specific "tiered screen" protocols to successfully identify novel scaffolds of individual receptor subtypes with greater efficacy than isolated screening methods.



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DISCOVERY OF NEW SMALL MOLECULES TARGETING THE VITRONECTIN BINDING SITE OF THE UROKINASE RECEPTOR THAT BLOCK CANCER CELL INVASION

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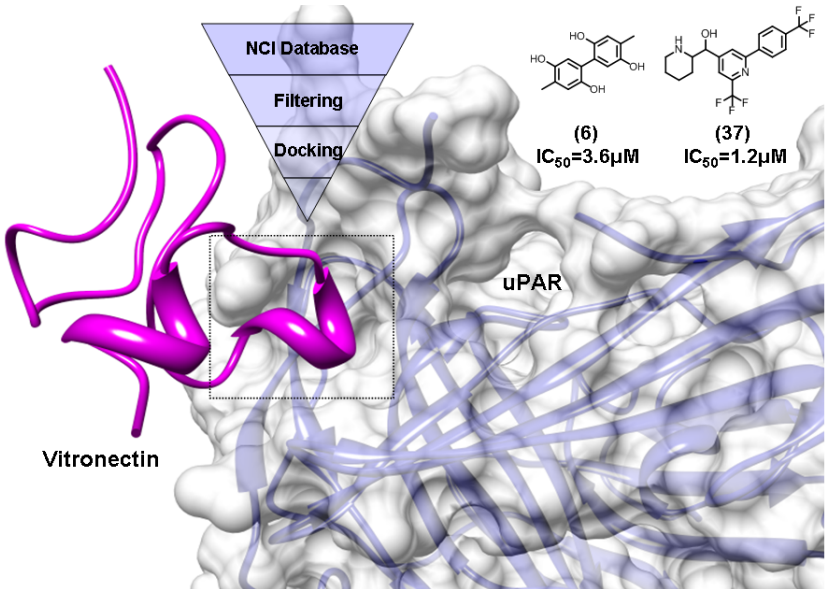
Besides focusing urokinase (uPA) proteolytic activity on the cell membrane, the urokinase receptor (uPAR) is able to bind vitronectin (VN), via a direct binding site. Furthermore, uPAR interacts with other cell surface receptors, such as integrins, receptor tyrosin kinases and chemotaxis receptors, triggering cell-signalling pathways that promote tumor progression.(1) The ability of uPAR to coordinate binding and degradation of extracellular matrix and cell signalling makes it an attractive therapeutic target in cancer. We used structure-based virtual screening (SB-VS) (2) to search for small molecules targeting the uPAR binding site for VN. 41 compounds were identified and tested on uPAR-negative HEK-293 epithelial cells transfected with uPAR (uPAR-293 cells), using the parental cell line transfected with the empty vector (V-293 cells), as a control. Compounds, **6** and **37** selectively inhibited uPAR-293 cell adhesion to VN and the resulting changes in cell morphology and signal transduction, without exerting any effect on V-293 cells. **6** and **37** inhibited uPAR-293 cell binding to VN with IC_{50} values of 3.6 and 1.2 μM, respectively. Compounds **6** and **37** targeted S88 and R91, key residues for uPAR binding to VN but also for uPAR interaction with the f-MLF family of chemotaxis receptors (fMLF-Rs). As a consequence, **6** and **37** impaired uPAR-293 cell migration toward FCS, uPA and f-MLF, likely by inhibiting the interaction between uPAR and FPR1, the high affinity fMLF-R. Both compounds blocked in vitro extracellular-matrix invasion of several cancer cell types and could represent new promising leads for pharmaceuticals in cancer.(3)



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SYNTHESIS AND BIOLOGICAL TESTING OF JNK3-INHIBITORS: A CASE STUDY TO EXAMINE HALOGEN BONDING IN PROTEIN-LIGAND INTERACTIONS

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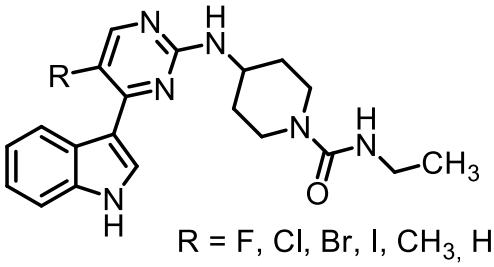
Halogen bonding is still an underestimated "nonclassical" interaction, first found during the 1970's in material science.(1) Even though a lot of these noncovalent interactions could be found by retrospective search of crystallographic databases, there are only few small molecule ligands that were designed to use this interaction for enzyme binding.(2) For drug design, the interaction with a nucleophile (O, N, S) of an aminoacid sidechain of the protein can be seen as an interesting tool to increase selectivity and/or activity.

R-X ------ D-R'

In analogy to hydrogen bonds, halogen bonding can be simplified as above, where X is Chlorine, Bromine or Iodine and D is an electron donor. An anisotropic arrangement of the electrostatic potential of the halogen is necessary to form halogen bondings. This anisotropic arrangement, called "sigma hole", forms a round positive spot in elongation to the covalent carbon-halogen bond. This sigma hole, that is necessary for halogen bonding, is more pronounced from Chlorine to Iodine. However it does not exist when X is Fluorine.(3) In order to examine the influence of halogen bonding in protein-ligand interactions, we synthesized a series of different substituted small-molecule JNK3-Inhibitors and tested their inhibitory effect in a JNK3 enzyme assay.



For R = Cl there is already an X-Ray structure published, where we found that the Chlorine atom is directly pointing towards the Met149-Gatekeeper of JNK3 and angle and distance should be in a manner that halogen bonding is possible.(3, 4)
We have synthesized the three halogen derivatives that are able to form halogen bonding and tested them in a biological JNK3 assay. In order to compare their IC_{50}-values with compounds that are not able to do halogen bonding, we synthesized the other three derivatives mentioned above.

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AUTOMATIC GENERATION OF 3-D QSAR MODELS: A QUASI-SISTEMATIC APPROACH

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Quantitative structure–activity relationship (QSAR), is a ligand-based (LB) method to develop mathematical/statistical models which attempts to find a statistically significant correlation between structure and function using chemometric techniques. 3-D QSAR is a broad term encompassing all those QSAR methods which correlate macroscopic target properties with computed atom-based descriptors derived from the spatial (three-dimensional) representation of the molecular structures.

In its simplest form the development of a 3-D QSAR model comprises several steps: training set selection (molecules active against a given target), conformation generation and superimposition (alignment rules), molecular interaction field calculation (MIF), correlation of bioactivity and MIF, graphical analysis.

When no information are available on the target structure and hence the binding site of the training set molecules, the alignment rules definition is the most critical step, especially if the training set is composed of flexible molecules.

In continuing our search in the 3-D QSAR field in this report we focus on the developing an automatic procedure to build LB 3-D QSAR models whose alignment rules are defined through pruning hundreds of models with different alignment approaches. The 3-D QSAR engine of the process rely on our recent 3-D QSAutogrid/R procedure[1] which with its implemented multi-probe approach allowed the definition of quantitative pharmacophore models.[2]

In this report we focus on a sort of systematic alignment search by using several alignment programs to derive different alignment rules on pre-existent training sets. The final 3-D QSAR model is then selected on the basis of the statistical coefficients as follows: r^2, SDEC, q^2 and SDEP, and also on the lack of chance correlation as measured by a scrambling procedure.

Details and results will be presented at the poster session.

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RE-VALUATION OF THE INVERSE DOCKING TECHNIQUE TO COMPUTE DRUGS IN MULTI-TARGET STUDIES

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The molecule-receptor interaction studies are limited by the possibility to analyse only one target without having the possibility to consider interactions with systemic protein systems. This is restrictive limitation taking in consideration that these molecules could represent potential hits for the treatment of several diseases. To fully develop the potentiality of the Inverse Docking (ID)[(1,2)] technique an exhaustive analysis of the interactions between a number of ligands and a protein database, such us the Protein Data Bank (PDB), was conducted using different softwares like MOE[(5)], AutoDock Vina[(3)], Knime[(4)] and Amber[(6)] with NAMD[(7)], in a concerted manner.

We were capable to automate several different processes with a good yield with respect computer time consuming and to ameliorate time operator, at least in small-scale. The docking studies have been conducted on a multi-conformers system, as suggested by Chen et al..[1] Multi-conformers have been obtained through an automatized conformational search conducted by Knime, implemented with MOE nodes. Moreover, docking analysis were conducted with scripts that culminate in an automated Autodock Vina starting, thus obtaining accurate docking scores and RMSD values. The best ligand-protein interaction will be submitted in a brief semi-automatized dynamic simulation.

The protein systems involved in the present study are metalloproteinases (MMPs), namely MMP-13 and MMP-3, and recently opioid co-crystallised receptors (μ, δ, κ).

To evaluate the goodness of the depicted workflow, we selected co-crystallised molecules like a blank control and a series of compounds developed and published by us and by other research groups. The protein systems involved in the present study are metalloproteinases (MMPs), namely MMP-13 and MMP-3, and new opioid co-crystallised receptors (μ, δ, κ).

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NITROGEN-CONTAINING BISPHOSPHONATES AS FPPS INHIBITORS: A COMPUTATIONAL STUDY

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Nitrogen containing bisphosphonates (N-BPs) are important drugs, inducing the FPPS inhibition, that are widely used in the treatment of a variety of bone diseases such as osteoporosis, Paget's disease, hypercalcemia and tumor-induced osteolysis.(1) Although advanced studies explored the use of N-BPs or related FPPS inhibitors as a promising approach to the above mentioned pathologies,(2) the exact mechanism of inhibition is not fully understood yet. In particular, comprehensive structure-activity relationships of N-BPs as inhibitors of the human or mammalian enzyme, have not been determined so far.(3)

The aim of this study is, therefore, the investigation of the binding of these compounds at the FPPS active site and the correlation between the calculated binding energy and the experimental pIC50 of known N-BPs.

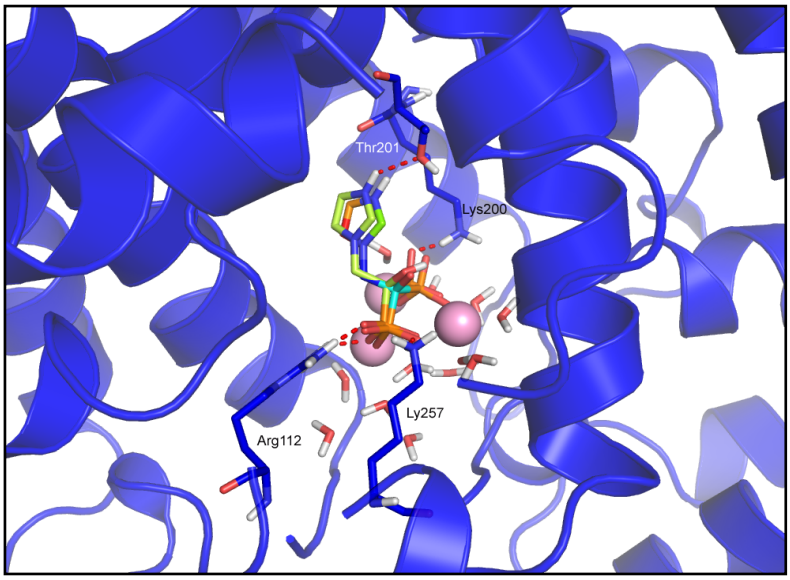

Figure 1. Superposition between Prime pose for zolendronic acid and ligand in crystallographic complex (PDB: 1ZW5).

At this purpose, several docking and post-docking strategies were employed in the prediction of the binding mode and energy for 49 N-BPs collected from the CHEMBL database.

A significant correlation between calculated and experimental binding affinities was only found dividing the whole group of N-BPs inhibitors in two subsets on the basis of the structure's size, suggesting that the target enzyme may be able to discriminate the ligand size and demonstrating that FPPS inhibition is mainly controlled by steric factors.

The good performances obtained with Prime/MM-GBSA in the prediction of binding-free energies and the high quality of poses (Figure 1), suggest that it could be used in the lead discovery and optimization of newly designed N-BPs inhibitors.

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INSIGHTS INTO TLR2 ANTAGONISM BY SMALL MOLECULES THROUGH MOLECULAR MODELING

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Recently, Toll-like receptors (TLRs) have been reported to contribute to several chronic inflammatory and autoimmune diseases and have thus been suggested as potential drug-targets for the treatment of these conditions(1). Through a recent structure- and ligand-based virtual screening campaign we identified several TLR2 antagonists representing novel chemical classes.

Here, we present an extensive study aiming to identify plausible binding modes for the novel small molecule TLR2 antagonists. The compounds were first classified into main structural categories and analyzed for potential activity cliffs necessary for their activity. Then, docking studies were performed for each structural category separately. This led to the detection of a binding site in the front region of the TLR2 lipopeptide binding cavity for the newly identified benzotropolones(2) as well as for most of the other identified antagonists. In contrast, compounds containing a phenyl-urea scaffold were found to bind to an alternative region in the back part of the lipopeptide binding cavity.

In a last step, the gained knowledge was integrated into a 3D pharmacophore collection that contains the currently available information on TLR2 antagonism through small molecules. The models were optimized and validated and can now be used for further virtual screening for TLR2 antagonists.

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THE ROLE OF A CONSERVED WATER MOLECULE IN BINDING OF NITROSOPYRIMIDINES AND RELATED ANALOGUES TO CDK2

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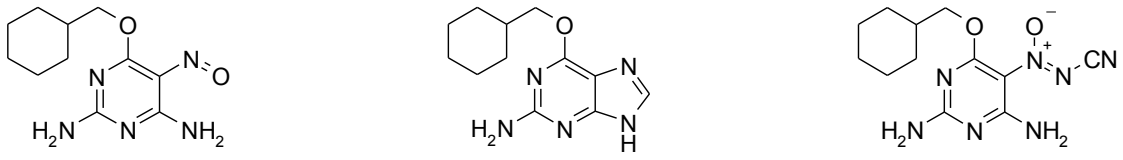
The division of eukaryotic cells occurs in four phases (G1, S, G2, M), and cell-cycle progression is regulated by members of a family of cyclin-dependent serine/threonine kinases (CDKs). Inhibition of CDKs by small molecules continues to attract considerable attention as a strategy to exploit in developing anticancer drugs. A variety of these inhibitors have been described, some of which are currently under clinical evaluation.(1) In particular, the chemotype exemplified by NU6027 (6-(cyclohexylmethoxy)-5-nitrosopyrimidine-2,4-diamine, Figure 1) has been developed as a potent and selective CDK2 inhibitor. In this structure, the intramolecular hydrogen bond between the adjacent 5-nitroso and 4-amino groups has long been considered as the main responsible of its pseudo-purine geometry, reminiscent of the purine scaffold of NU2058 (6-(cyclohexylmethoxy)-9*H*-purine-2-amine, Figure 1), another selective CDK2 inhibitor.(2) Indeed, X-ray analysis of the CDK2/NU2058 and CDK2/NU6027 crystal complexes shows that the two products establish the same key interactions with the backbone of amino acid residues within the ATP-binding site of CDK2, namely a triplet of H-bonds (2-NH2 to Leu83, N3 to Leu83, N9-H to Glu81, and 2-NH2 to Leu83, N1 to Leu83, 4-NH2 to Glu81, respectively).(2) Structure-activity relationships (SAR) have been studied in detail for these two leads, confirming the crucial role of the nitroso group at the 5-position of the pyrimidine; only nitro, formyl, and acetyl moieties are tolerated as possible alternatives. We recently showed that potent and quite selective CDK2 inhibitors can be obtained by replacing the NO group in NU6027 with a cyano-*NNO*-azoxy moiety (NC-N(O)=N, Figure 1).(3)



NU6027
IC_{50} = 1.3 μM

NU2058
IC_{50} = 12 μM

cyano-*NNO*-azoxy analogue of NU6027 IC_{50} = 0.9 μM

Figure 1. Structures of NU6027, NU2058 and our novel cyano-*NNO*-azoxy derivative.

As part of our work we carried out a molecular modelling study to investigate the binding mode of our newly synthesised analogues. Our results suggest that a conserved crystal water molecule plays a crucial role in stabilizing the pseudo-purine geometry of NU6027 and related analogues, and may be even more important than the aforementioned intramolecular hydrogen bond in determining the high affinity of these ligands for CDK2.

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KINETICS IN DRUG DISCOVERY. A CASE FOR G PROTEIN-COUPLED RECEPTORS

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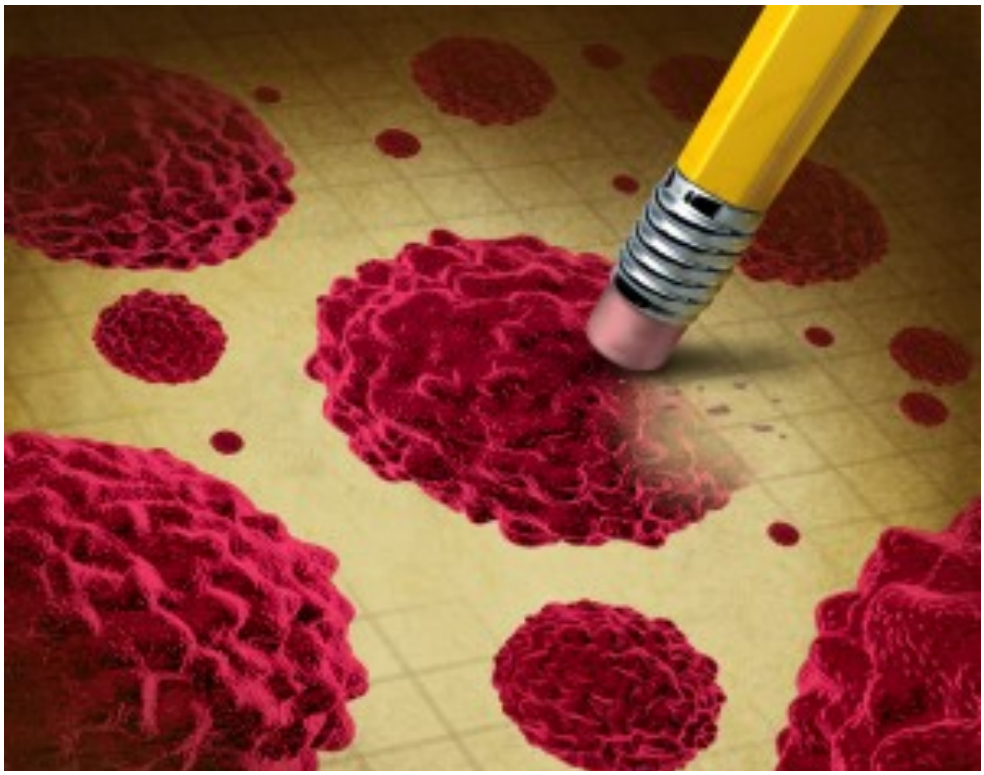
In early drug discovery one strives to optimize the properties of drug candidates for a given therapeutic target, usually focusing on standard pharmacological parameters of affinity, potency and intrinsic activity. There is mounting evidence, however, that the often ignored *kinetic* aspects of the interaction between a drug and its target in the body are highly relevant for *in vivo* efficacy and clinical success. This ignorance may be one of the reasons for the high attrition rates in drug discovery, as it has been analyzed that quite a few recently marketed drugs had indeed improved kinetic profiles.

G protein-coupled receptors (GPCRs) are attractive drug targets, and a few examples of FDA- or EMA-approved drugs acting on GPCRs indicate that their beneficial effects in patients may result from prolonged receptor occupancy, i.e. a long residence time. Such retrospective observations, however, provide neither a theoretical nor a technological framework for the 'titration' of this novel design criterion into new chemical entities. To make this happen it seems imperative to understand the molecular mechanisms of kinetic action better, to come up with suitable 'off-the-shelf' assays, and to make the translational effort from early *in vitro* screening to *in vivo* profiling of new lead candidates.

In this presentation we will provide an overview of our own recent efforts in this respect, while addressing the GPCR protein superfamily. We will first briefly introduce some concepts of receptor kinetics, then move towards the development of higher-throughput assays, and finally study the possible link between residence time and other pharmacological parameters such as affinity and intrinsic efficacy. Ultimately our efforts may lead to a better understanding of structure – kinetics relationships (SKR) to be used in the design of kinetically appealing molecules.



INHIBITION OF METALLOPROTEASES WITH DIMERIC LIGANDS – A CRYSTALLOGRAPHIC STUDY

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Many biological systems use dimerization as a regulatory mechanism in signal transduction. The precise mode of dimerization is critical to determine agonostic or antagonistic signalling. Dimerization is also important in non-signalling systems since the average free energy of interaction between a ligand moiety and a receptor moiety in a polyvalent interaction can be greater than, equal to, or less than the free energy in the analogous monovalent interaction. The improvement, if any, will depend on the degree of cooperativity.

A good theoretical understanding has been developed to analyse the results of binding tests, but there is no effective guide to help in the design of suitable ligands that can be used in a pharmacological context. We have chosen matrix metalloproteinases (MMPs) as a model system to study and control protein homodimerization using synthetic twin-inhibitors. The chemical approach contributes to the discovery process. Some monomeric ligands that are excessively hydrophobic have a tendency to associate through their exposed hydrophobic surfaces and spontaneously dimerize causing dimerization of their protein targets. However, to add precision to the system and reproduce the correct signal, the linker between the two monomers must be carefully crafted.

A rational discovery pathway that starts with monomeric ligands, involves a spacer design step and finally leads to a bi-functional or self-dimerizing ligand has been put in place. Achieving the exact design is not essential since ligands that induce incorrect dimerization, are valuable as antagonists and those that have sufficient flexibility to act both as agonists or antagonists can be effective as modulators. However, it is important to characterize their effect precisely.

Crystallographic data is an essential asset to guide spacer design and to understand its function. As analogues are analyzed small positional variations can be understood and correlated with the activity of the polyvalent ligand, which depending on its structure can change from agonistic to antagonistic through subtle variations in the homodimer assembly.

Since dimerization is difficult to handle in a crystallization setting, mainly because nucleation can get out of control, we have had to modify the crystallization techniques to cope with these ligands [(1)]

With this obstacle overcome, the second step has been to analyse how linker length, its structure, its flexibility and point of attachment can alter its properties. This is the main focus of the results that will be presented here.

Finally we have looked at the effectiveness of our best ligands in a cellular setting with good results in cellular proliferation tests in Matrigel.

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BATTLING DRUG RESISTANCE IN TARGETED CANCER THERAPIES

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The discovery of mutations in protein kinases has marked a dramatic change in the treatment of non-small cell lung cancer. Patients with mutant lung carcinoma receiving tyrosine kinase inhibitors have a median overall survival of more than 2 years, contrasting with the survival of unselected patients receiving chemotherapy. Acquired resistance to these targeted drugs is in 50% of the cases mediated by a secondary point mutation at the gatekeeper position in the catalytic domain of the kinase. The particular size and physicochemical properties of the amino acid found at this position are critical determinants for kinase inhibitor affinity and selectivity. Gatekeeper mutations affect the thermodynamic and kinetic binding characteristics of classic kinase inhibitors. The impact of current and next generation kinase inhibitors to overcome drug resistance in mutant kinases will be discussed.

POLYSACCHARIDE NANOHYDROGELS AS DRUG DELIVERY PLATFORMS

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In recent years, significant efforts have been devoted to the development of amphiphilic polymers based on hydrophilic polysaccharides and hydrophobic moieties because of their ability to form self-assembling nanoparticles in aqueous media.(1) The intermolecular interactions between the hydrophilic and hydrophobic segments respectively allow to create a hydrophilic shell toward the solvent and a hydrophobic core with the minimal interaction with the aqueous environment. Self-assembled nanohydrogels (NHs) based on hydrophobically modified polysaccharides have been intensively studied due to their significant potential applications as drug delivery systems. Polysaccharides are actually suitable for drug and protein delivery applications because of their several advantages over synthetic polymers because of their natural abundance, generally low cost, ease of manipulation, facile derivatization and, in most cases, biocompatibility.(2) Among natural polysaccharides, gellan (Ge) and hyaluronic acid (HA) are promising candidate for biomedical applications due to their peculiar physico-chemical and bioactive properties.(3,4) The purpose of this research line is the development of NHs based on these two polysaccharides to carry hydrophobic as well as hydrophilic drugs, exploiting the internalization of these carriers into the cells. The first step of the research was the reduction of the molecular weight of the starting polymers by means of ultrasound treatment, in order to obtain a chain length suitable for the formation of NHs. Bi-dimensional NMR experiments showed that the primary structure of these polysaccharides was not affected by sonication. Polymer chains were then hydrophobized by chemical conjugation with a derivative of prednisolone (for the Ge) or with a derivative of cholesterol (for HA chains). The derivatization degree of the products was in the range 6-20 % mol/mol. NHs were thus obtained by nanoprecipitation in water or by ultrasound treatments. Ge-Pred and HA-Chol NHs were almost spherical in shape, their size are in the range 80-300 nm and their ζ-potential was negative (< - 20 mV); moreover NHs are highly stable in storage conditions at least up to 1 month. NHs can be easily sterilized and can be freeze-dried without damage of the dosage form. The NHs can be easily loaded with drugs. The HA-NHs were also exploited to carry protein and enzymes. The cytocompatibility of NHs, their activity and the availability of the drug was also assessed.(5,6)

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ON THE ROUTE TO FLOW GLUCURONIDATION: DESIGN OF EXPERIMENTS AND KOENIGS-KNORR REACTION OPTIMIZATION

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In humans, the glucuronidation reaction is the most common phase II metabolic pathway, being the primary responsible of xenobiotic elimination including drugs, food additives, pollutants and several endogenous compounds such as hormonal steroids and bile acids.[1] Moreover, although a potential biological effect of glucuronyl metabolites is generally excluded, they can directly or indirectly contribute to the pharmaco-toxicological activity of the parent compounds. Therefore, the monitoring of glucuronide levels in biological fluids has a great relevance in pharmacokinetic and toxicological studies, in medical diagnosis of metabolic disorders as well as in forensic investigations.[2] Currently, the available methods for the preparation of glucuronides suffer from several problems including the presence of protection-deprotection extra steps, low yields, laborious protocols and purifications.[3]

Based on these premises and following our interest in the development of flow synthesis of medicinally relevant products,[4] in this communication, we describe a new synthetic protocol for the generation of glucuronidated derivatives under flow conditions. In particular, we report our initial efforts in the optimization of a convenient flow set-up for the Koenigs-Knorr reaction of ursodeoxycholate, chosen as the model reaction (Figure 1). The fine-tuning of the reaction conditions by Design of Experiments (DoE), the obtained results and the related statistical analysis will be presented and discussed.

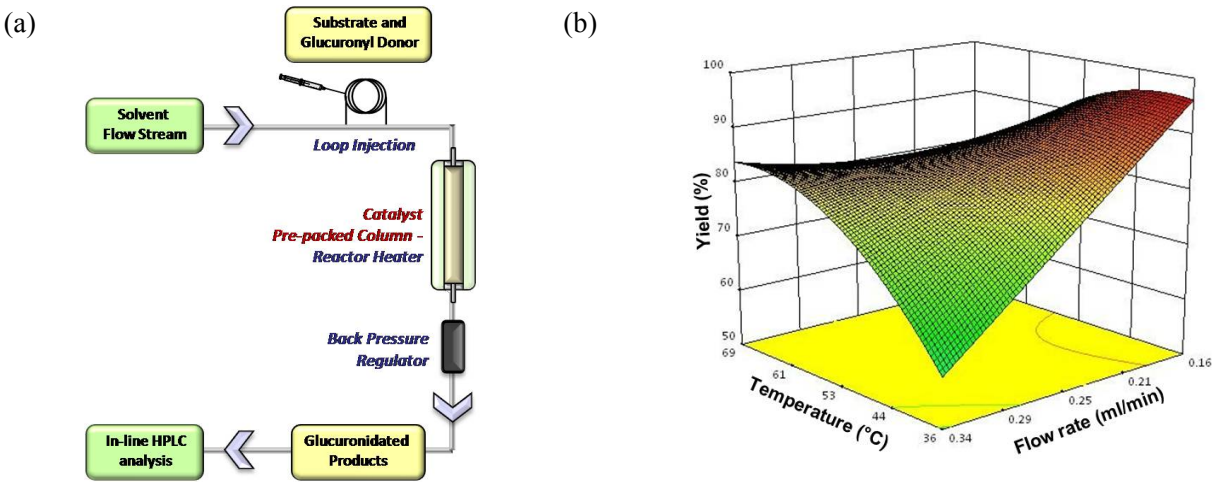

Figure 1. Flow set-up (a) and DoE response surface modelling (b) for glucuronidation reaction optimization.

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SMART NANOVESICLES FOR THERANOSTICS: PREPARATION AND CHARACTERIZATION

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In recent years, enormous efforts have been made to translate nanotechnology innovations into medical practice. The main focuses are diagnosis and therapy, that can be achieved at the same time in the theranostic approach.

The development of effective theranostic nanocarriers must allow for imaging sensitivity, accuracy of targeting, and controlled drug release. Currently, the technologies available for imaging of theranostic nanoparticles include optical imaging, magnetic resonance imaging (MRI), nuclear imaging, computed tomography (CT), and ultrasound (US). All approaches and type of theranostic nanoparticles show, at the same time, advantages and disadvantages. In our study lipid coated nanobubbles for ultrasound imaging and magnetic nanoparticles (MNPs) loaded niosomes for MRI applications have been evaluated.

Ultrasonography is a widely used imaging technology, non invasive and cost-effective, which is playing a vital role in clinical imaging and diagnosis. Commercial ultrasound contrast agents (UCAs), consisting of encapsulated gas microbubbles, enable only a qualitative visualization of the microvascularization for a short period of time since they are rather unstable. In a strategy to develop more stable UCAs, nanobubbles (Fig. 1) have been prepared with phospholipid coating for their use as contrast agents in ultrasound imaging.

The superior magnetic properties of iron oxide MNPs along with their non-toxicity, biodegradability and as well as low costs, made them a material of choice in many bio-applications, such as contrast probes in MRI. In order to confer colloidal suspendability to the particles, additives, typically hydrophilic polymers, are added during the particle formation process, which passivate the nanocrystal surface and protect against particle aggregation. To offer the opportunity for targeted, non invasive diagnosis by MRI, iron oxide MNPs have been encapsulated into surfactant vesicles to develop niosomal contrast agents, *i.e.*, magnetic niosomes (MNs - Fig. 2).

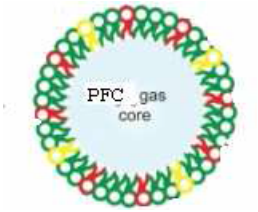

Fig 1: Lipid coated nanobubble entrapping perfluorocarbon (PFC)

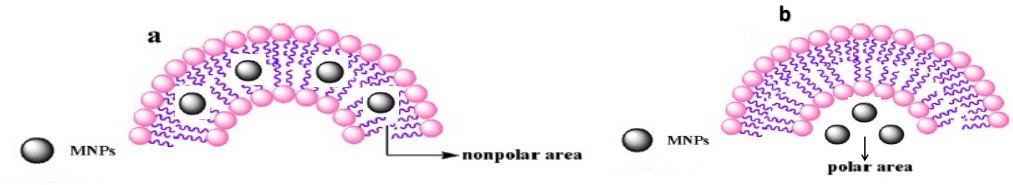

Fig 2: Niosomes loaded with lipophilic (a) or hydrophilic (b) MNPs

A HYPERSENSITIVE DECTION OF A PROTEIN KINASE USING RADIO PHOSPHORYLATION OF A FUSED ENZYME SUBSTRATE IN A LAB-ON-A-CHIP

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Microarrays have attracted worldwide interest and can be used as components of unique approaches to gene expression and protein sequence analysis, in clinical diagnostics, in high-throughput screening (HTS), and in drug discovery and screening.[(1, 2)]

A protein-fused substrate for calcium/calmodulin-dependent protein kinase (CaMKII) was constructed by genetically fusing a gold binding polypeptide (GBP) to the putative spermidine synthase (PSPD) of *Selenomonas ruminantium*. DNA encoding the CaMKII substrate Autocamtide-2, the amino acid sequence of which is KKALRRQETVDAL, was cloned and fused to the C-terminal 32 kDa of PSPD. In the present study, the GBP-PSPD-fusion Autocamtide-2 substrate (GBP-SP-AC2) was used to detect the signal intensities reflecting CaMKII action, on gold-coated glass slides. The construction of a lab-on-a-chip (LOC) employing radioisotopes (RIs) requires the appropriate design, fabrication, and testing of plastic microfluidic devices allowing on-chip substrate preparation and employment of protein microarrays. We investigated the feasibility of RI detection techniques used to measure the phosphorylation of a GBP-fused substrate, in turn facilitating the highly sensitive detection of CaMKII, employing an LOC. The LOC that we developed can be used to analyze CaMKII in complex solutions of biological samples. Our LOC device, which was designed for use in applications employing radioisotopes, is architecturally simple, affords rapid testing, is cost-effective, and generates minimal amounts of radioactive waste.[(3)]

This strategy can be used in the general development of LOCs for high-throughput screening in biological and medical research. This LOC device embracing the use of RI will make significant contributions to the fields of biomedicine and drug discovery.

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EXPERIMENTAL AND THEORETICAL STUDY OF POLYMORPHIC AND SOLVATE AMPICILLIN FORMS

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In literature, many active pharmaceutical ingredients (APIs) are known to crystallize in different crystalline packings (polymorphs) or with solvent molecules as an integral part of their structures (pseudopolymorphs). In a pharmaceutical dosage form, the active ingredient solid-state phase identity or conversion could dramatically alter the final pharmaceutical properties. In particular, the solid state administrated drug can influence important properties like bioavailability. In this study four α-aminobenzylpenicillin forms were crystallized[(1)] and the molecular vibrations of the various ampicillin forms were investigated by ATR/FT-IR,[(2,3)] micro-Raman and SERS (surface enhanced Raman spectroscopy) [(4)] spectroscopies (firstly reported). The HSRM (hot stage Raman microscopy) was also able to follow the transition from the trihydrate ampicillin to the amorphous monohydrate. The same technique allowed of controlling the solid-solid convertion from trihydrate to anhydrous forms. DSC, TGA, XRPD data were also afforded. For the first time, the Raman spectra of the four ampicillin forms are reported. Finally, for assisting experimental assignment bands quantum mechanical calculations were also performed and the density functional theory (DFT) predictions were used. [(5.6)]

Refereces

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ODD MAN OUT: MOLECULAR DYNAMIC SUDIES ON DENDRIMERS AND THEIR INTERACTIONS WITH ALBUMIN AND OLIGODEOXYNUCLEOTIDES

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Dendrimers are a very promising tool for the delivery of bio-active molecules. They allow a careful tailoring of their physiochemical characteristics, giving the possibility to alter the ADME profile of a candidate molecule. Specifically, cationic dendrimers can easily form complexes with nucleic acids, and are widely studied as carriers for the transfection of cells with DNA-RNA in vitro [(1)].

In this work, we focused our efforts on unveiling the tangled relationship between different dendrimers and nucleic acids (NAs) from the host-carrier point of view. Also, we specifically devised how dendrimers can actively play in the formation and stabilization of more efficient gene delivery complexes.

As an example of this peculiar interaction we will discuss the key role played by a fifth generation TEA-core PAMAM dendrimer in the formation of nanocarrier/cargo ensembles with "sticky" small interfering RNA (ssRNA). Specifically, using a combined computational/experimental we discovered that both the nature and length of the ssRNA overhangs concur in modulating the strength of their binding with the dendrimer and, hence, the formation of the relevant complexes.[(3)] However, by applying more sophisticated simulation techniques we were also able to explore some aspects connected to the ssRNA release.

Another major obstacle in gene delivery is the interaction of NAs with albumin, the main protein in bloodstream. Being negatively charged, albumin can compete with NAs for their positively charged nanocarriers, thereby decreasing their efficacy. We hence studied this "ménage a trois" particularly considering a new series of carbosilane dendrimers, and describing both their interaction with albumin and with two oligodeoxynucleotides. As an innovative concept, we applied steered molecular dynamics (SMD) experiments to mimic the formation of the complex between dendrimer and oligodeoxynucleotides.

Last but not least, another key issue in the behavior of dendrimers as nanocarriers is their ability to cross the cell membrane to ultimately release their cargo inside the cell. Thus, we are currently stydying the crossing of a bilayer membrane by dendrimers using a combined umbrella sampling/SMD approach.

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THREE COMPONENT SYNTHESIS OF *C*-OXIMINOAMIDINES: VERSATILE BUILDING BLOCKS USEFUL IN DRUG DISCOVERY

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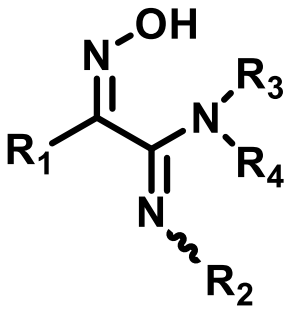
Multicomponent reactions (MCRs) are reactions where three or more substrates combine in one step to give a product that contains essential parts of all of them. They are a powerful tool for the rapid and innovative construction of molecules either, not easily accessible via the classical two-component chemistry, or never reported in literature. Over the last decades, one of the aim of our research group has been the discovery of *novel isocyanide-mediated multicomponent reactions* with the goal of unveiling innovative synthetic routes for unknown molecular scaffolds or for identifying shorter synthetic strategies for the construction of medicinally important drugs. In this poster communication, we present a straightforward one pot-three component synthesis of *elusive* C-oximinoamidines and their subsequent chemical manipulations.



NEW OPPORTUNITIES FOR MEDICINAL CHEMISTS: THE REACH CHALLENGE

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Despite QSAR has played a central role[1] in many front-line research projects (e.g., experimental design, ADME modeling, lead finding and optimization), its actual application has sometimes brought to misleading results. In recent years, such pitfalls have been the object of criticism of a number of milestone papers indicating to QSAR practitioners how to properly apply QSAR avoiding the risk of non-sense conclusions.[2]

In this scenario, the REACH (Registration, Evaluation, Authorization and restriction of Chemical substances) legislation has indeed strengthen (Q)SAR potential and renewed its intimate purpose that is the prediction of endpoints necessary for the registration and, more importantly, marketing of chemicals in the light of the REACH slogan "no data no market".

In this respect, it is worthy saying that the right application of QSAR within REACH is supervised by the Organization for Economic Co-operation and Development (OECD) as well as of European Chemicals Agency (ECHA).

In our investigation, we have applied QSAR for the prediction of the Bioconcentration Factor (BCF), a relevant ecotoxicological endpoint quantifying the ratio of the concentration of a substance in an organism with respect to that in water. Normally, the BCF assessment takes place according to the experimental test OECD 305, which requires for each compound more than one hundred of fishes, months for test execution and tens of thousands Euro cost. However, the replacement of the experimental test by alternative methods, like QSAR, can have the effect of reducing economical and time costs as well as the sacrifice of animals. To this end, we herein present new QSAR models for the prediction[3, 4] of BCF derived from a pool of easily interpretable biokinetics descriptors and trained, as well validated, on a large dataset of more than 800 chemicals. The descriptive and predictive power of our models is by far better than existing ones. [5]

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4-QUINOLONES MODIFICATION: INFLUENCE OF THE RATIO OF REAGENTS AND SUBSTITUENT POSITION ON THE PRODUCT STRUCTURE IN THE REACTION WITH ETHANOLAMINE

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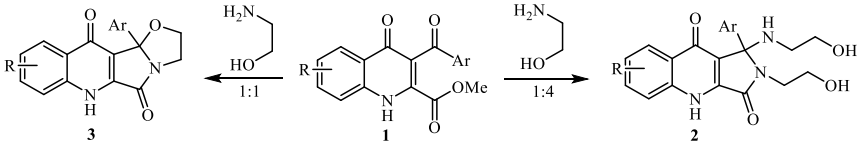
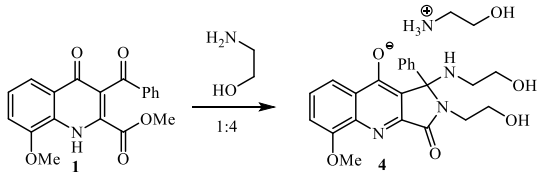
4-Quinolone drugs have been used in clinical practice for 25 years as effective antibacterial agents with broad spectrum of activity.(1) Moreover, compounds containing 4-quinolone moiety display antitumor (2), antiviral(3), antidiabetic(4), antituberculosis(5) and other types of activity. This prompts scientists to more exhaustively explore the 4-quinolone scaffold decoration and investigate the biological effects of the products. The decarbonylation reaction of 1-aryl-3-aroyl-4,5-dioxo-4,5-dihydro-1*H*-pyrrole-2-carboxilates (6) leads to 4-quinolones **1** that contain two functional groups (alkoxycarbonyl and aroyl) readily accessible for further modifications. The reaction of these groups with ethanolamine in different conditions, leads to new heterocyclic systems containing 4-quinolone moiety **2**, **3**. The reaction of methyl 1,4-dihydro-2-quinolinecarboxylates **1** with ethanolamine (1:4 ratio) in 1,4-dioxane affords 2-(2-hydroxyethyl)-1-[(2-hydroxyethyl)amino)]-1-aryl-1*H*-pyrrole[3,4-*b*]quinoline -3,9(2*H*,4*H*)-diones **2**. The same reaction in 1:1 ratio in propanole-2 yields 11b-aryl-2,3,6,11b-tetrahydrooxazolo[2',3':2,1]pyrrole[4,3-*b*]quinoline-5,11-diones **3**. (7)



3 **1** **2**

1: R = 6-Me, 6-OMe, 6-Br, 6-F, 8-Me, 8-Et; **2**: 7-Me, 7-MeO, 7-Br, 7-F, 7-Me; **3**: 9-Me, 9-OMe, 9-Br, 9-F, 7-Et.

Unexpected results were obtained when 8-methoxysubstituted derivative was used as the initial compound. An unusual product was isolated after reflux of **1** (R^2 = OMe) with ethanolamine in ratio 1:4 using propanole-2 as a solvent. Instead of expected annulated 4-quinolone, analogous to **2**, a derivative of 4-quinolone tautomeric form – the 4-hydroxyquinoline **4** – was formed. Attempts to get the similar salt for 6-methoxy substituted derivative **1** were unsuccessful.



The ability of synthesized quinolones to affect the level of glucose in blood is being studied.

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DESIGN, SYNTHESIS AND FREE RADICAL SCAVENGING ACTIVITY OF A NOVEL CLASS OF DUALISTIC FILTERS FOR SKIN PHOTOPROTECTION

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Sun exposure is the main cause of photocarcinogenesis, photoageing, and photosensitivity, because both UVA and UVB can induce the formation of reactive oxygen species leading to gene mutations and immunity suppression; thus, photoprotection is an important issue.

It is possible to substantially reduce the incidence of skin cancer in humans by photoprotective strategies; one of these is the application of cosmetic sunscreen preparations which, when applied on the skin, attenuate the transmission of the solar radiation.

In the present study our interest has been addressed toward a new generation of filters, the aim is to develop dualistic molecules able to filter UV rays and at the same time working as antioxidants to protect by free radicals damages.

Methods: To reach the goal we designed novel molecular scaffold introducing radical scavenging moieties on known UV filters and then testing them for antioxidant and sunscreen activities.

The compounds were screened by different antiox methods: DPPH; FRAP; PCL; ORAC and in the UV-VIS range. The molecules scoring an ABS value similar or better than the commercial UV filters were included in standard cosmetic formulations, these latter were tested to evaluate the antioxidant potency for their SPF in vitro.

Results: Some of the designed molecules displayed very interesting antiox activity combined with an improved broad spectrum (UV-A and B) filtering capabilities, good solubility in water as compared to the reference known filters. The prepared finished cosmetic formulations displayed a very good profile of oxidative and UV protection.

Conclusion: The results here obtained showed that it is possible, not only in line of principle, to obtain effective dualistic molecules with broad spectrum of activity, thus reducing the number of ingredients required for a solar product and also. We believe that this approach deserve certainly applications in the field. (Patent appl. Filed)

Acknowledgments
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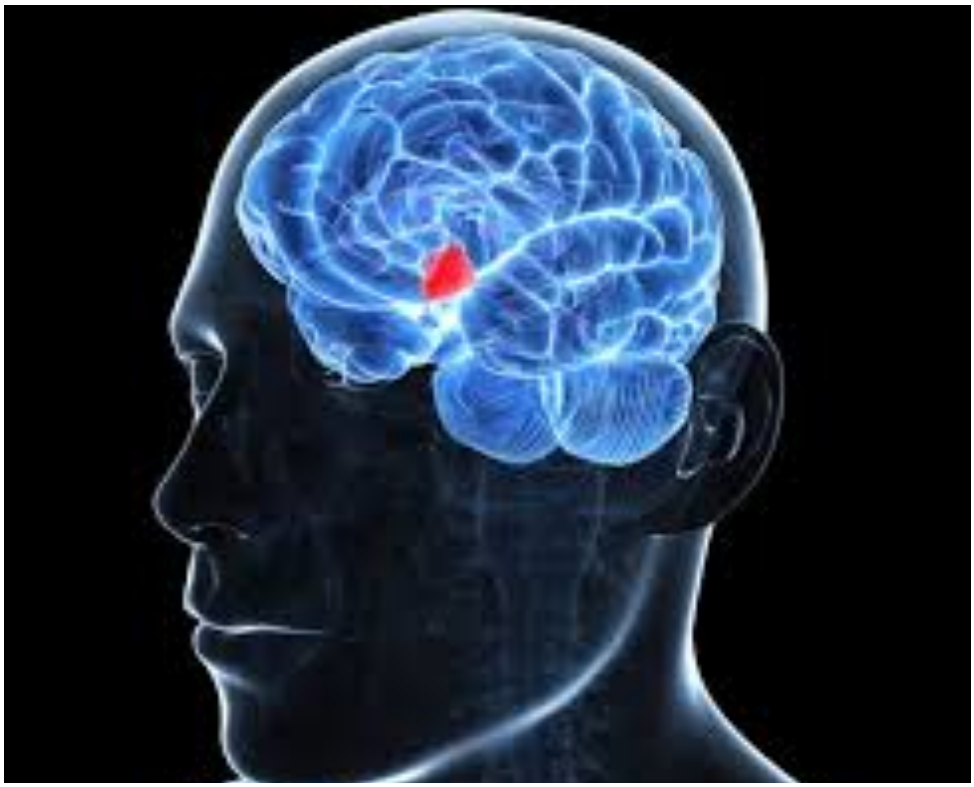

SUBTYPE-SELECTIVE METABOTROPIC GLUTAMATE RECEPTOR LIGANDS IN THE TREATMENT OF CNS DISORDERS

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Metabotropic glutamate (mGlu) receptors form a family of eight subtypes, of which mGlu1 and mGlu5 receptors are coupled to Gq/G11, and all other subtypes are coupled to Gi/Go. mGlu1 receptors show a prominent expression in cerebellar Purkinje cells and play a key role in mechanisms of activity-dependent synaptic plasticity underlying cerebellar motor learning. Positive allosteric modulators (PAMs) of mGlu1 receptors improve motor symptoms in a mouse model of spinocerebellar ataxia type 1 (SCA1) and are promising candidates for the treatment of this disorders in humans. mGlu1 receptors also regulate the activity of the talamocortical network underlying absence seizures, and mGlu1 receptor PAMs are effective in rat models of spontaneous absence epilepsy. mGlu5 receptor NAMs are in the final stages of clinical development for the treatment of Fragile X syndrome and L-DOPA-induced dyskinesias in patients with Parkinson's disease. In the latter disorder, mGlu5 receptor NAMs may also reduce the progressive degeneration of nigral neurons. mGlu5 receptor PAMs are under development for the treatment of psychotic disorders because mGlu5 receptors positively modulate the activity of NMDA receptors in the prefrontal cortex. Orthosteric agonists of mGlu2/3 receptors and mGlu2 receptor PAMs are under clinical development for the treatment of schizophrenia with prevalence of negative symptoms, whereas mGlu3 receptor PAMs should be developed for the treatment of chronic neurodegenerative disorders such as amyotrophic lateral sclerosis and Alzheimer's disease. mGlu3 receptor NAMs inhibit mechanisms of chemoresistance in glioma stem cells, and hold promise as add-on drugs in the treatment of malignant gliomas. Finally, mGlu4 receptor PAMs are under development for the treatment of Parkinson's disease because mGlu4 receptors negatively modulate GABA release in the first synapsis of the indirect pathway of the basal ganglia motor circuit.

POSITRON EMISSION TOMOGRAPHY AS IMAGING BIOMARKER IN CNS DISEASES

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Positron Emission Tomography (PET), presently always coupled to Computed Tomography (CT) in hybrid scanners, is a mature clinical imaging modality, mostly applied to oncology in combination with the administration of the radiotracer 2-[^{18}F]fluoro-2-deoxyglucose (FDG). Indeed, FDG has been and is still a pillar of PET imaging, which exploits its ability to assess and measure regional glucose metabolism differences, in both physiological and pathological conditions, including cancer.

Although more than 90% of PET exams in the clinical settings concerns tumor diagnosis, staging and therapy control, earliest studies and greatest expectations from PET were on brain imaging. In this organ, PET can expect the best results from featured high-sensitivity, absolute concentration quantitation, non-invasivity, and take additional advantage from the fortunate imaging location of the brain, far from confounding effects produced by large vessels, moving organs and anatomical variety. High-resolution MRI, in the newest generation of PET/MRI hybrid scanners, is also adding further value to research on the Central Nervous System, by merging the brain functional assessment by MRI to PET molecular imaging information. Understanding the biochemical and biological characteristics of the disease and monitoring its progression/remittance represent an area of paramount importance not only for patients, who may benefit from better diagnosis and prognosis, but also in evaluating new therapies. A positive impact can also be expected in reducing the heathcare costs by earlier disease detection and relocation of investments from treatment to prevention. All of the above, along with the development of the concept of personalised medicine from one side and the stagnation in the development of new medicines, despite the relevant progression of knowledge in molecular biology and the increasing number of druggable targets, call for innovation in the way we explore and characterise the disease. Major challenges are the identification of effective biomarkers and surrogate clinical end-points that could be used to stratify patients and rationalize clinical trial results. PET imaging has been postulated since long to be an optimal candidate to this purpose. However, these expectations would have remained unmet without a continuos development of new radiotracers,[1] mostly labelled with short-lived carbon-11 (20.4 min half-life) and fluorine-18 (109.7 min half life), able to highlight bioditribution and pharmacokinetics of the radiolabelled drug, as well as to show target engagement (proof-of-target) and proof-of-mechanism (e.g. receptor occupancy) in CNS drug development, or demonstration of efficacy in controlled clinical trials using a reference radiotracer.

In the last decade a step further has been made by PET since it was considered a valuable resource and a support to regulatory filings. Many PET Centres have aligned to higher (common) quality standards, thus attaining increased data robustness and access to larger populations, so to overcome the major hurdles of biomarker validation and qualification.

Beside FDG (glucose metabolism) and cerebral blood flow indicators, many tracers have been prepared having CNS as a target;[2] still only a few has had a real clinical impact. Improved relationships with Regulatory Agencies and the direct participation of industrial partners is helping in discriminating speculative efforts from clinically-oriented developments. Earlier/improved diagnosis in neuropsychiatric diseases on one side and support to drug development in neurodegenerative disorders, neuroinflammation and depression on the other are now progressing on a renewed track. Finally, extended knowledge in multimodal imaging is paving the way to the exploration of brain involvement in very modern "diseases" such as addiction (e.g. compulsive game or internet, food), obesity, searching for functional "cold" indicators beyond typical symptom analysis and evaluation.[3]

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THE DESIGN AND SYNTHESIS OF SELECTIVE α7 AGONISTS FOR THE TREATMENT OF NEUROLOGICAL DISEASE

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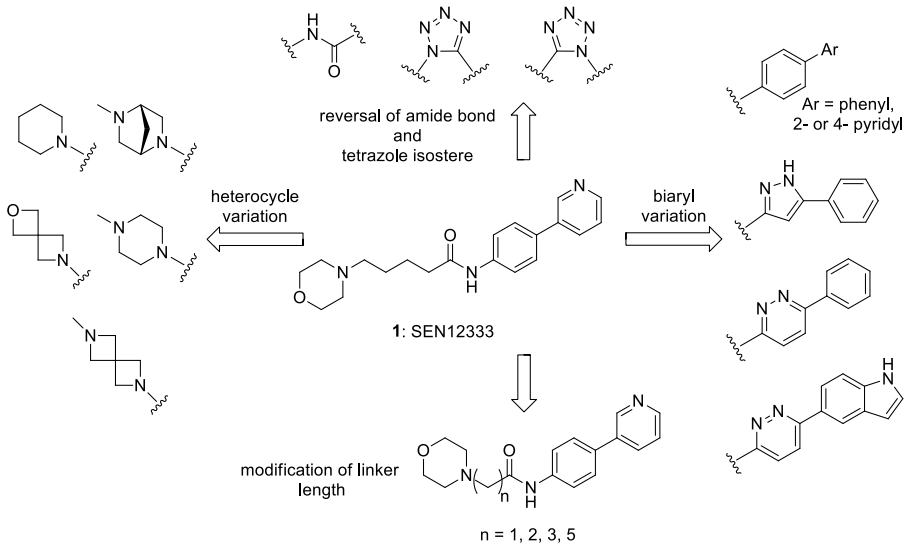
Nicotinic acetylcholine receptors (nAChRs) are ion channels gated by acetylcholine (ACh), one of the major excitatory transmitters in the nervous system. The α7 nAChR subtype is expressed in brain regions associated with cognitive function, and reduced density of α7 nAChRs is evident in both schizophrenia and Alzheimer's disease (AD). Prototypical α7 nAChR agonists have been shown to enhance a variety of cognitive behaviors in animal models and to normalize sensory gating deficits, which are believed to contribute to the cognitive fragmentation in schizophrenia.(1) A neuroprotective effect is also elicited upon treatment of α7 nAChR agonists on cultured neuronal cells exposed to β-amyloid or deprived of NGF.(1) Based on these findings α7 nAChR agonists are predicted to be effective treatment agents for the improvement of cognition in both schizophrenia and AD.

Few selective α7 nAChR agonists have been reported, and none show sufficient brain penetrability or optimal pharmacokinetics for clinical utility.(2) SEN12333 (**1**) was recently identified as an orally bioavailable, brain-permeable, small molecule agonist of α7 nAChR, albeit of low potency. With the objective of improving potency and maintaining selectivity at the α7 nAChR, systematic modification of the biaryl and heterocyclic rings, the amide bond, and the alkyl linker of SEN12333 have been explored.(3) Details of the synthesis, receptor binding, and functional activity of a library of more than 50 compounds will be presented, and will highlight the structural features essential for α7 nAChR selectivity, affinity, and efficacy.



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NEW 2-HETEROCYCLYL-IMIDAZO[2,1-i]PURIN-5-ONE DERIVATIVES AND WATER-SOLUBLE PYRAZOLO[4,3-e][1,2,4]TRIAZOLO[1,5-c] PYRIMIDINES AS POTENT AND SELECTIVE HUMAN A_3 ADENOSINE RECEPTOR ANTAGONISTS

Delia Preti,*[1] Giulia Saponaro,[1] Mojgan Aghazadeh Tabrizi,[1] Stefania Baraldi,[1] Romeo Romagnoli,[1] Allan R. Moorman,[2] Ettore Novellino, [3] Pier Andrea Borea,[4] Pier Giovanni Baraldi[1]

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The development of human (h) A_3 adenosine receptor (AR) antagonists has permitted the identification of lead compounds showing promising potential in preclinical studies for the treatment of several diseases such as stroke, glaucoma, asthma or COPD.(1) Therefore, the search for new selective A_3 AR ligands, still remains an attractive goal. Herein we report our recent results in this field.

Project A: a series of 4-allyl/benzyl-7,8-dihydro-8-methyl/ethyl-2-[(substituted)isoxazol/pyrazol-3/5-yl]-1*H*-imidazo[2,1-*i*]purin-5(4*H*)-ones has been synthesized and evaluated in radioligand binding assays to determine their affinities at the human A_1, A_{2A}, and A_3 adenosine receptors.(2) Efficacy at the hA_{2B} AR and antagonism of selected ligands at the hA_3 AR were also assessed through cAMP experiments. All of the synthesized molecules exhibited high affinity at the hA_3 AR (K_i values ranging from 1.46 to 44.8 nM), as well as remarkable selectivity versus A_1, A_{2A} and A_{2B} AR subtypes. Compound (*R*)-4-allyl-8-ethyl-7,8-dihydro-2-(3-methoxy-1-methyl-1H-pyrazol-5-yl)-1H-imidazo[2,1-*i*]purin-5(4H)-one (*R*-**33**) was found to be the most potent and selective ligand of the series (K_i hA_3 = 1.46 nM, K_i hA_{2A}/ K_i hA_3 > 3425; IC_{50} hA_{2B}/ K_i hA_3 > 3425; K_i hA_1/ K_i hA_3 = 1729).

Project B: A relevant problem of the pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine nucleus, an attractive scaffold for the preparation of adenosine receptor antagonists, is the low water-solubility. We originally functionalized the C^5 position with a salifiable 4-pyridyl-carbamoyl moiety which conferred good water solubility at low pH (<4.0), but poor solubility at physiologic pH, indicative of the dissociation of the pyridinium species. Here we replaced the pyridin-4-yl moiety with a 1-(substituted)piperidin-4-yl-ring so to exploit the higher basicity of this nucleus and the possibility to generate stable, water-soluble salts.(3) The hydrochloride salt of 1-(cyclohexylmethyl)piperidin-4-yl derivative (**10**, K_i hA_3= 9.7 nM, IC_{50} hA_3 = 30 nM, K_i hA_1/ hA_3 = 351, K_i hA_{2A}/ hA_3 > 515, IC_{50} hA_{2B} > 5 µM), showed a solubility of 8 mg/mL at physiological pH and gave a stable aqueous system suitable for intravenous infusion. Molecular modeling studies were helpful in rationalizing the available structure-activity relationships and the selectivity profile of the new ligands.

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EVERYTHING YOU ALWAYS WANTED TO KNOW ABOUT SIGMA RECEPTOR* (*BUT WERE AFRAID TO ASK)

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Originally considered an enigmatic protein, the σ_1 receptor has recently been identified as a unique ligand-regulated protein. Since its discovery, many studies have shown the potential of σ_1 receptor ligands for the treatment of various diseases of the central nervous system (CNS). One distinguishing feature of the σ_1 receptor is its promiscuity in binding a wide range of different pharmacological agents although, how binding of these various compounds translates into function(s) through the σ_1 receptor is currently not clear.[(1)] Moreover, almost no information are available to date not only on the interaction of the receptor with its ligands but also on the differences in the interactions of agonists and antagonists with the σ_1 receptor protein.. Furthermore, since σ_1 is a membrane bound protein, its expression, purification, crystallization, and structure determination is a difficult process. Thus, no evidence about the three-dimensional (3D) structure of the cloned σ_1 receptor has been released so far. For this reason, our group published for the first time a 3D model of the σ_1 receptor protein as obtained from a complex multistep computational recipe based on homology modeling techniques and molecular dynamics simulations (**Figure1 A,B**).[(2)] Then, after extensive model validation by means of computer-aided design, synthesis and testing for activity of novel ligands with high affinity and selectivity for the σ_1 receptor protein,[(3-6)] we recently embarked in a great, jointed effort toward unprecedented understanding of the σ_1 structure and binding site. Accordingly, parallel *in silico* and experimental site directed mutagenesis experiments were carried out, which yielded a preliminary molecular-based rationale for the agonistic and antagonistic effects of the ligands (**Figure 1C**).[(7)] Furthermore, the determination of short and long-term effects of promising σ_1 ligands on intracellular dynamics and post-translational modifications gave deeper insights into the role of σ_1 agonists and antagonists in the molecular mechanism of neuronal plasticity and survival under stressful conditions.[(5)] Altogether, these results afforded an intense improvement in the understanding of the functions and roles of the σ_1 receptor. This, in turn, will foster the development of news drugs aimed at treating frequent and important human diseases such as Alzheimer's disease, anxiety, depression and pain.

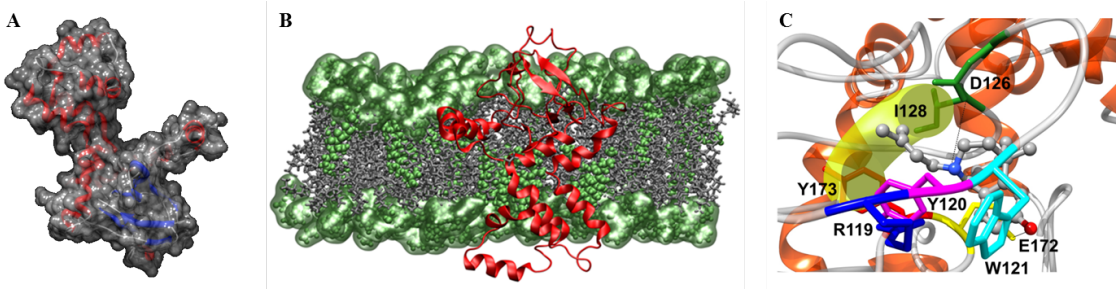

Figure 1. View of the σ_1 receptor MD-optimized homology model represented by its van der Waals surface (**A**) and inserted in the phospholipidic membrane (**B**); (**C**) Equilibrated MD snapshot of the wt σ_1 receptor in complex with its radioligand +-pentazocine (PTZ). The image is a zoomed view of the receptor binding site.

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DESIGN, SYNTHESIS AND BINDING AFFINITY OF NEW LIGANDS FOR THE CENTRAL NICOTINIC RECEPTORS

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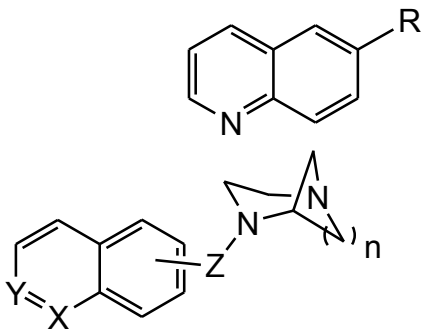
Nicotinic acetylcholine receptors (nAChRs) appear to be involved in various neuropsychiatric disorders, including Alzheimer's and Parkinson's diseases, schizophrenia, addiction and in important functional mechanisms, such as pain control and cognition.[1,2] While their importance as therapeutic target is well known, the main problem to face in the development of modulators is subtype selectivity, needed in order to decrease unwanted side effects.

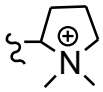
Some years ago we synthesized some quinoline derivatives (compounds **I** and **II**) displaying some selectivity towards α7 nicotinic receptors with respect to the α4β2 subtype.[3] As a continuation of that work, in order to see if selectivity could be improved, quinoline or other similar heterocycles were joined to diazabicyclic moieties frequently found in α7-selective ligands, leading to compounds with general formula **A** and **B**. The norbornane derivatives **C** have been designed on the basis of the result from a 3D search in the Cambridge Structural Database (CSD), using a query containing the structural features of the nicotinic pharmacophore. A similar approach, applied some years ago, led to the synthesis of compounds **I** and **II**.[4]



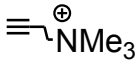
A

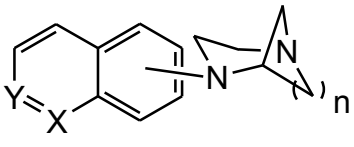
X= N or C
Y= C or N
Z= CO, NHCO, COO, CONH, OCO
n= 1,2

I R =



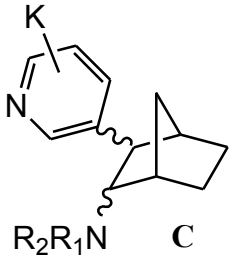
II R=





B

X= N or C;
Y= C or N:
n= 1,2



C

K= nothing, 5-Br, 6-Cl, 5-OCH_3
R_1= H, CH_3
R_2= H, CH_3

The affinity of the compounds was assessed by means of binding studies on α4β2 and α7 nAChRs subtypes. Some derivatives of **B** series display selectivity for the α4β2 subtype, while others for α7 one. The **C** series display good affinity on α4β2 and α7 subtypes with Ki in the nanomolar range. The synthesis and pharmacological evaluation of these compounds will be reported in this communication.

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SYNTHESIS AND BIOEVALUATION OF A NOVEL ESTERASE-SENSITIVE CYCLIC PRODRUG OF S-ALLYL-GLUTATHIONE USING AN (ACYLOXY)ALKOXY LINKER

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Neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD), are a group of pathologies characterized by a progressive and specific loss of certain brain cell populations. Oxidative stress, excitotoxicity, mitochondrial dysfunction, and apoptosis play interrelated roles in these disorders. It is well documented that free radical oxidative damage – particularly on neuronal lipids, proteins, DNA, and RNA – is extensive in PD and AD brains. Moreover, reduced levels of glutathione (GSH) have been found in the brains of PD and AD patients. In particular, the strongest alteration in the antioxidant defense is a decrease in GSH concentration, especially in the *SNpc* of PD patients. In fact, it is hypothesized that the magnitude of its depletion is the earliest indicator of nigrostriatal degeneration.[1]

Unfortunately, the use of GSH as a therapeutic agent is limited by its biochemical and pharmacokinetic properties. GSH has a short life in human plasma and hardly crosses cell membranes, so the administration of high doses is necessary to reach a therapeutic value. As a consequence, the reduced GSH levels observed in these pathologies have stimulated a number of researchers to find new potential approaches for maintaining or restoring GSH levels. The unfavourable biopharmaceutical properties of GSH can be transiently modified using prodrug strategies. Several linear GSH prodrugs are reported in literature but, to date, no example of cyclic GSH prodrugs has been reported, yet.[2]

Here, we describe the synthesis of a novel esterase-sensitive cyclic prodrug of linear allyl-GSH (**CP11**) using an (acyloxy)alkoxy linker. **CP11** was designed to be susceptible to esterase metabolism, leading to a cascade of chemical reactions and resulting in the generation of the linear peptide. We also investigated the anti-inflammatory ability of **CP11** in U937 cells, an immortalized human monocyte cell line that represents a valid model for studying the inflammatory response.

Results showed that LPS stimulation in U937 monocyte cells induces a modification in the redox state of these cells by causing accumulation of ROS responsible for activation of the MAPK pathway. Moreover, the new molecule **CP11,** structurally related to GSH, acts as an anti-inflammatory modulator in LPS-induced inflammation in U937 cells, with a mechanism that prevents the raise of intracellular levels of ROS through a mechanism involving the MAPK pathway. Further results will be reported in the poster session.

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INTERATION OF N-ALKYL-CARBAZOLES WITH Aβ(25-35) AMYLOID PEPTIDE

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Deposition of senile plaques composed of fibrillar aggregates of Aβ-amyloid peptide is a characteristic hallmark of Alzheimer's disease. Amyloid plaques are primarily composed of β-amyloid peptides Aβ(1-40) and Aβ(1-42). Aβ (25-35) represents the biologically active region of Aβ, as it includes the shortest fragment capable to form large β-sheet aggregates.(1)

In the present contribution we present the synthesis of a set of N-alkyl-carbazole compounds. They are analyzed for the ability to interact with the Aβ(25-35) peptide using a combined approach based on circular dichroism, nuclear magnetic resonance, thioflavin fluorescence spectroscopy and in cell ELISA immunoassay. The aim of our research is to develop a better understanding of the critical structural requirements stabilizing the interaction of N-alkyl-carbazole compounds with Aβ(25-35) peptide and the subsequent Aβ(25-35) conformational preferences.

Depending on conditions, amyloid peptides undergo a conformational transition from random coil or α-helical monomers to the highly toxic β-sheet oligomers, which form the mature fibrils.

A widely employed approach in the research of anti-Alzheimer agents involves the identification of substances able to prevent amyloid aggregation, or to disaggregate the amyloid fibrils through a direct interaction with either soluble or aggregated peptide.(2) A selective mode of interaction of these compounds with soluble oligomers or amyloid aggregates has still not been clearly established. The development of small molecules able to interact with amyloid peptides is considered strategic in view of identifying the structural parameters responsible for Aβ stabilization and/or aggregation.(3)

Our data show that N-alkyl-carbazoles analyzed in interaction with Aβ(25-35), are able to preserve the soluble form of the peptide and may be identified as new lead compounds in the search of innovative anti-Alzheimer therapeutics.

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SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF SOME NEW 8-SUBSTITUTED-7-TRIFLUOROMETHYL-4,5-DIHYDRO-4-OXO-1,2,4-TRIAZOLO[1,5-a]QUINOXALINE-2-CARBOXYLATES AS AMPA/KAINATE RECEPTOR ANTAGONISTS

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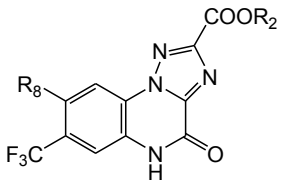
Glutamic acid (Glu), the major excitatory neurotransmitter in the mammalian central nervous system, plays pivotal roles in regulating many physiological processes through activation of metabotropic (mGluRs) and ionotropic receptors (iGluRs), the latter classified as AMPA, Kainate (KA) and NMDA. The NMDA receptor is modulated by several agents, in particular by the amino acid glycine which, binding at the allosteric strychnine-insensitive glycine site (Gly/NMDA), exerts a primary role in receptor activation. Due to the iGluR-mediated excitotoxicity that can lead to neuronal cell death, the AMPA, KA and NMDA receptors have received considerable attention for their involvement in many neurodegenerative disorders such as epilepsy, cerebral ischemia, Alzheimer's and Parkinson's diseases. Moreover, all the three iGluRs have well defined roles in nociception.(1) There is increasing evidence that KA receptor in particular can be considered as an emerging target for the development of new treatments for different type of pain. In fact, KA receptor antagonists demonstrated beneficial effects as analgesics in migraine.(2)



R_2 = Et, H
R_8 = heteroaryl, NHCOR, $NHSO_2R$

TQX Series

As a part of a project aimed at finding novel AMPA and KA receptor antagonists, we synthesized a new set of 7-trifluoromethyl 4,5-dihydro-4-oxo-1,2,4-triazolo[1,5-a]quinoxaline-2-carboxylates (TQX) bearing different substituents at position-8. Previously reported TQX derivatives were AMPA or mixed AMPA and Gly/NMDA receptor antagonists.(3-5) Most of the herein reported compounds bind both AMPA and KA receptors with comparable affinity falling in the low micromolar range, while the 8-(2-carboxybenzoylamino)-4,5-dihydro-4-oxo-7-trifluoromethyl-1,2,4-triazolo[1,5-a]quinoxalin-2-carboxylic acid is 25 fold more active at the KA site with a K_i value of 0.13 μM. Some of the newly synthesized compounds were evaluated in the paw-pressure test, a rat model of mononeuropathy.

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RATIONAL DESIGN AND SYNTHESIS OF NOVEL FATTY ACID AMIDE HYDROLASE (FAAH) INHIBITORS

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Fatty acid amide hydrolase (FAAH)(1) is a membrane-bound serine hydrolase that catalyses the hydrolytic cleavage of endogenous biologically active fatty acid ethanolamides,(2) such as anandamide (AEA), an agonist of cannabinoid receptors, and palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), which are agonists of type-α peroxisome proliferator-activated receptors (PPAR-α). These natural FAAH substrates were demonstrated to play important roles both in the central nervous system (CNS) and in peripheral tissues, where they are involved in several physiological processes. The inhibition of FAAH represents a promising approach for the treatment of several disorders (*e.g.,* pain, inflammation).(3) At present, a wide number of selective and potent FAAH inhibitors, belonging to different chemical classes, have been disclosed (*e.g.* carbamates, ureas, and α-keto heterocycles).(4) *O*-aryl carbamates are one of the most representative families, in terms of drug discovery efforts and drug-likeness.(5,6)

In this context, we designed an efficient and reliable synthetic strategy that allowed us to discover a novel class of carbamate-based FAAH inhibitors characterized by favourable physico-chemical and in vitro ADME properties.

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STRUCTURAL INVESTIGATIONS ON 2-ARYLPYRAZOLO[4,3-*d*]PYRIMIDIN-7-AMINO DERIVATIVES AS NEW HUMAN ADENOSINE RECEPTOR ANTAGONISTS

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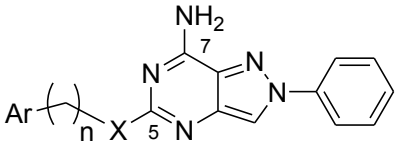
Adenosine exerts many physiopathological effects through activation of G protein-coupled receptors, currently classified as A_1, A_{2A}, A_{2B} and A_3 subtypes. There is growing evidence that ligands for adenosine receptors (ARs) could be promising therapeutic agents in a wide range of diseases. In particular, A_{2A} antagonists are effective in the treatment of central nervous system disorders such as cerebral ischemia, aging-associated neurodegeneration and Parkinson's disease (PD).(1) Balanced A_1/A_{2A} receptor antagonists have also demonstrated efficacy in PD since they counteract both movement (A_{2A}) and cognitive (A_1) disorders associated to the pathology.(2)

In a part of our recent research, a set of pyrazolo[4,3-*d*]pyrimidin-7-amino derivatives (Series **A**) were identified as potent human (h) AR antagonists, whose affinity and selectivity depended on the nature of substituents at the 5 and 7 positions.(3) To shift affinity toward the A_{2A} receptor, arylalkyl(amino) moieties with different length were introduced at the 5 position of the bicyclic scaffold, and the free amino group was maintained at the 7-position.



Series A

X= CH_2, NH, –N⟨⟩N–

n= 0-3

Preliminary binding results show that arylalkyl substituents at the 5-position afford A_{2A} or A_1/A_{2A} receptor antagonists. Molecular docking studies have been carried out to rationalize affinity and selectivity profiles of the new antagonists and to identify their hypothetical binding mode to the AR binding site.

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DESIGN, SYNTHESIS, AND EVALUATION OF (ISO)NIPECOTAMIDE AND PIPERAZINYLUREA DERIVATIVES AS TRPV1 MODULATORS

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The transient receptor potential vanilloid-1 (TRPV1) is a nonselective cation channel, with a preference for calcium, present on polymodal nociceptors. TRPV1, identified as the receptor for the vanilloid compound, capsaicin, is activated by endogenous stimuli such as protons, heat, and ligands including anandamide, and arachidonic acid metabolites. The vanilloid receptor is expressed predominantly on unmyelinated pain-sensing nerve fibers (C-fibers) and small Aδ fibers in the dorsal root, trigeminal, and nodose ganglia. The activation of the vanilloid receptor by agonists triggers cation influx resulting in excitation of primary sensory neurons, and ultimately the central perception of pain. The initial excitation is followed by a refractory state of desensitization, where the C-fiber sensory neurons becomes unresponsive to TRPV1 agonists and other inflammatory mediators. This desensitization represents a basis for therapeutic use of vanilloid receptor agonists in the management of acute and chronic nociceptive pain.(1) On the other hand, the identification of capsazepine, an antagonist of capsaicin binding, was of great importance because it provided a proof of principle for the discovery of novel analgesics based on the blocking of activation of TRPV1 by endogenous stimuli.(2) Given the analgesic effects observed for both TRPV1 agonists and antagonists, in the last years there is a growing interest in developing TRPV1 ligands as potential analgesic drugs. Within the context of our research program aimed to search for new anti-inflammatory/analgesic agents,(3) we designed new (iso)nipecotamide/piperazinylurea based derivatives in order to be evaluated as TRPV1 modulators (Figure 1).

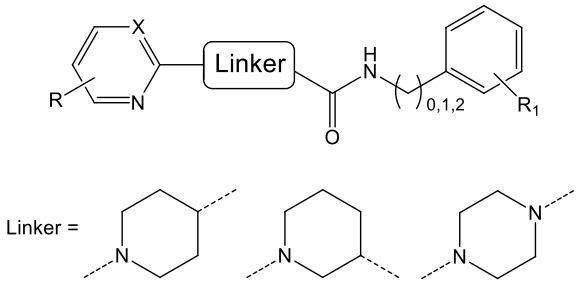

Figure 1

The synthesis of a series of these compounds, results of the in vitro evaluation, and initial SAR study are discussed.

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DESIGN, SYNTHESIS AND EVALUATION OF 3,4-DIHYDROXYBENZOIC ACID DERIVATIVES AS ANTIOXIDANTS, BIO-METAL CHELATING AGENTS AND ACETYLCHOLINESTERASE INHIBITORS

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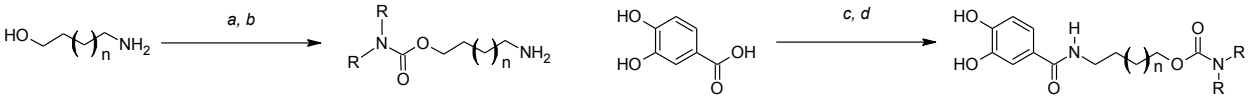
The relationship between the development of neurodegenerative diseases, e.g. Alzheimer's (AD) or Parkinson's (PD) and metals is very complex, but it has become clear that alterations in the homeostasis of metal ions such as iron, copper and zinc can drive the progression of neurological diseases.(1) Moreover, it is well known that iron and copper play a key catalysts role for the production of reactive oxygen species (ROS) and they are considered a major cause of the oxidative stress.(2) Thus, the modulation of biometals in the brain has been proposed as a potential therapeutic strategy for the treatment of neurodegenerative diseases, especially for AD.(3,4)

We designed derivatives **8-11** (Scheme 1) joining antioxidant and chelating properties of 3,4-dihydroxybenzoic acid with anticholinesterase activity due to the presence of a carbamic moiety typical of many acetylcholinesterase inhibitors.



1 n = 1
2 n = 2
3 n = 3

4 n = 1 , R = Et
5 n = 2 , R = Me
6 n = 2 , R = Et
7 n = 3 , R = Et

8 n = 1 , R = Et
9 n = 2 , R = Me
10 n = 2 , R = Et
11 n = 3 , R = Et

Scheme 1. Reagents and conditions: (*a*): 6M HCl, benzene, Dean-Stark distillation; (*b*): R_2NCOCl, CH_3CN, reflux, 12 h; (*c*): N,N'-bis(4-methylphenyl)carbodiimide, CH_3CN, 40 °C, 12 h; (*d*): **4-7**, CH_3CN, 40 °C, 24-72 h.

The synthesized carbamates **8-11**, tested by DPPH methods, showed good antioxidant and radical scavenging properties, as indicated by EC_{50} values lower than trolox. These compounds were also able to chelate the bio-metal cations Cu (II), Fe (II) and especially Fe (III). Interestingly, **8-11** also resulted non-competitive or mixed inhibitors of acetylcholinesterase, with K_i values in the range 1.5 - 18.9 μM, and are poorly active towards butyrylcholinesterase. A molecular docking study indicated that these compounds could interact with AChE in a common binding mode involving the PAS, the access gorge and the active catalytic site.

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DRUG DESIGN AND SYNTHESIS OF GSK-3β INHIBITORS

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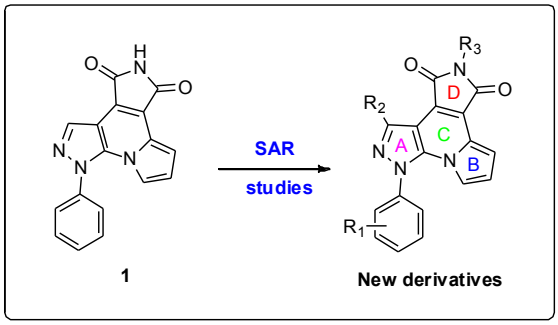
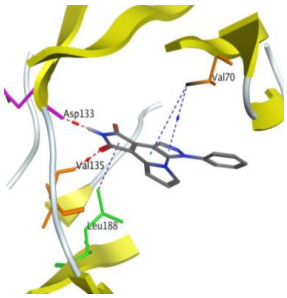
Alzheimer's disease (AD) is a leading cause of death worldwide. The development of AD is accompanied by neuronal loss, presence of intracellular neurofibrillary tangles and the formation of extracellular senile plaques.

Recent evidence suggests that glycogen synthase kinase 3 (GSK3) proteins play key roles in many fundamental processes during neurodevelopment[1]. Over the past decades have been reported a number of diverse GSK-3β inhibitors. We observed that compound **1** synthesized previously by our group[2] shared some chemical feauters common with known GSK-3β inhibitors. We selected compound **1** as an useful hit compound for the development of new GSK-3β inhibitors (Chart 1). Thus, we have carried out SAR studies and synthesized a number of new derivatives that showed potent GSK-3β inhibitory activity in the low micromolar range of concentration.

Chart 1. New GSK-3β inhibitors and their binding mode





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REVISITING A RECEPTOR-BASED PHARMACOPHORE HYPOTHESIS FOR HUMAN A_{2A} ADENOSINE RECEPTOR ANTAGONISTS

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Adenosine is a neuromodulator whose biological functions are accomplished through the activation of specific proteins belonging to the G protein-coupled receptors (GPCRs) superfamily. To date, four distinct Adenosine Receptors (ARs) subtypes, termed A_1, A_{2A}, A_{2B} and A_3, have been identified.(1) Owing to the wide range of effects exerted in numerous organ systems, the activation or blockade of ARs finds potential therapeutic applications in the treatment of several pathologies, such as cardiac and cerebral ischemia, asthma, Parkinson's disease, cancer, and kidney diseases.(2) In view of their potential application for pharmaceutical purposes, several groups have focused their attention on the synthesis of both ARs agonists and antagonists, especially aimed by the pharmacological and biophysical characterization of the receptors.(3)

The application of both structure- and ligand-based design approaches represents to date one of the most challenging strategy in the discovery of new drug candidates. In the present paper, we investigated how the application of docking-driven conformational analysis can improve the predictive ability of 3D-QSAR statistical models. With the use of the crystallographic structure in complex with the high affinity antagonist ZM 241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol) we revisited a general pharmacophore hypothesis for the human A_{2A} adenosine receptor of a set of 751 known antagonists, by applying an integrated ligand- and structure-based approach. Our novel pharmacophore hypothesis has been validated using an external test set of 29 new synthesized human adenosine receptors antagonists.

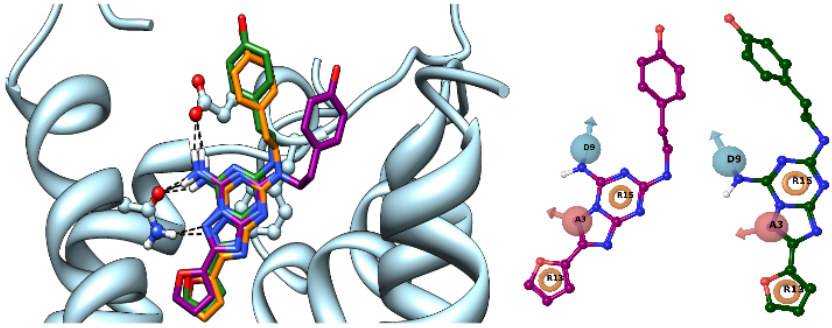

Figure 1. On the left: superposition of ZM 241385 *best pose* (dark magenta) and *selected pose* (green) conformations to the crystal pose of ZM 241385 (orange) in the binding pocket of the hA_{2A} AR; on the right: pharmacophore hypothesis for *best pose* (dark magenta) and *selected pose* (green) protocol.

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[1,2,4]TRIAZOLO[1,5-a][1,3,5]TRIAZINE DERIVATIVES AS ANTAGONISTS FOR THE ADENOSINE RECEPTORS: A PRELIMINARY STUDY ON AFFINITY AT THE A_3 SUBTYPE

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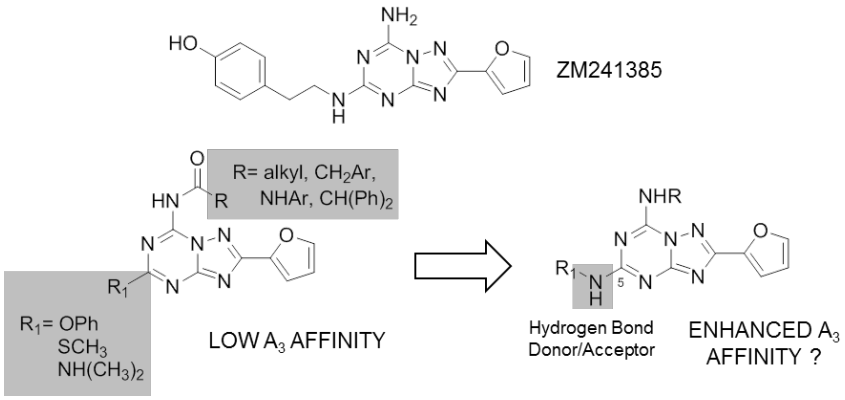
In the last 20 years intense medicinal chemical efforts led to the synthesis of a variety of adenosine receptor (AR) agonists and antagonists for the pharmacological characterization of this family of G protein-coupled receptors.(1) Several classes of heterocyclic derivatives have been reported as AR antagonists with high levels of both affinity and selectivity.

Recently, the synthesis of more simplified heterocyclic derivatives has been strongly investigated in order to obtain derivatives with a better pharmacokinetic profile. In particular bicyclic systems such as adenine, triazolo-pyrazine, triazolo-pyrimidines and triazolo-triazine could be considered some of the most promising targets.(2) One of the most appealing bicyclic core is the triazolo-triazine nucleus, which led in the past to the discovery of potent and selective A_{2A} AR antagonists, such as ZM241385.(3) In the past, our group performed a study on this nucleus trying to optimize substitution at the C5 and N^7 positions with the aim of improving affinity and selectivity versus the $hA_{2B}AR$ and hA_3AR subtypes. In particular, inclusion at the N^7 position of arylcarbamoyl (for A_3) or arylacetyl (for A_{2B}) moieties, which gave good results in the pyrazolotriazolopyrimidine family, has been investigated.(4) Unfortunately, none of these substitutions led to the desired selectivity. Regarding the A_3 subtype, lack of affinity was probably observed because only lipophilic moieties were present at the 5 position (phenoxy, dimethylamino, thiomethyl) avoiding the formation of hydrogen bonds with residues inside the binding pocket of the A_3 adenosine receptor.

Our aim, in this work, was to synthesise new [1,2,4]triazolo[1,5-a][1,3,5]triazines introducing substituents at the 5 position able to form hydrogen bonds, such as monosubstituted amines, in order to verify if it is capable of enhance affinity and selectivity towards the A_3 AR. The analogues were docked in a homology model of the hA_3 AR, and the per residue electrostatic and hydrophobic contributions to the binding were assessed and stabilizing factors were proposed in order to support the experimental binding data.



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SYNTHESIS AND BIOLOGICAL EVALUATION OF IMIDAZOLYL-INDOLES AND HOMOTRYPTAMINES AS NEW POTENTIAL SSRIs

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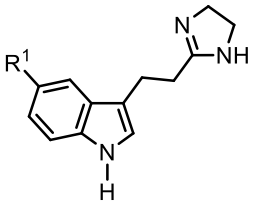
Herein we report the synthesis and evaluation of imidazolylindoles **1a** and **1b**, and homotryptamines **2a** and **2b** as potential selective serotonin uptake inhibitors (SSRIs). The in vitro studies showed that while compounds **1a**, **1b** and **2b** were able to inhibit serotonin uptake by SERT in an only micromolar range (IC_{50} 0.9-4.9 μM), a high inhibition was observed for **2a** (IC_{50} 43.5 nM), similar to that induced by the reference compound fluoxetin (IC_{50} 41.7 nM).

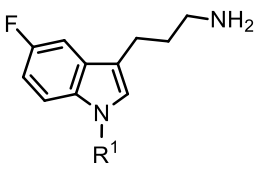
Inhibition of serotonin transporter (SERT) prevents the reuptake of serotonin (5-HT) into presynaptic terminals, regulating the 5-HT levels and potentiating the synaptic function of 5-HT.(1) It forms the full or partial basis for the mechanism of action of many antidepressants (SSRIs).(2) Although many clinically useful SSRIs have been discovered, the development of compounds with higher affinity and target selectivity is still an area of interest.(3) On this basis, we decided to synthetize 5-HT analogues**1a**, **1b**, **2a**, and **2b** modulated by (1) the nature of the electron-withdrawing group on the aromatic ring, (2) substitution on the indolic nitrogen atom, (3) insertion of an additional methylene group into the side chain, and (4) replacing the basic amino group of the side chain by an imidazoline ring, in order to be tested as SSRIs

Compounds **1a**, **1b**, **2a** and **2b** were evaluated for their ability to inhibit the uptake of 5-HT by SERT in frontal cortex synaptosome of male rats. [^{3}H]-5HT were used as specific radiolabelled ligand for SERT. The results of the reuptake inhibition assays, determined and expressed here as SERT IC_{50} values, show that all the synthesized compounds were able to inhibit 5-HT uptake by SERT in a concentration dependent manner with IC_{50} values ranging from 43.5 nM for compound **2a** to 4.85 μM for compound **1a**.



1a: R^1 = H

1b: R^1 = F



2a: R^1 = H

2b: R^1 = 

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NEW FLUORINE-CONTAINING 1,3,5-TRIARYL-2-PYRAZOLINES AS POTENTIAL ANTIDEPRESSANT AGENTS

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1,3,5-Triaryl-2-pyrazoline derivatives have attracted a great deal of interest owing to their synthetic and biological importance.(1) In the present work, we described the synthesis of fluorine-containing 2-pyrazolines, which were tested for their antidepressant-like activity using modified forced swimming and tail suspension tests.(2) Rota-Rod test was carried out to determine probable neurological deficits due to the test compounds, which may interfere with the test results. The compounds displayed different levels of antidepressant-like activity.

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DISCOVERY OF A NOVEL SMALL MOLECULE INHIBITOR TARGETING THE FRATAXIN/UBIQUITIN INTERACTION VIA STRUCTURE-BASED VIRTUAL SCREENING AND BIOASSAYS

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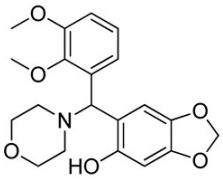
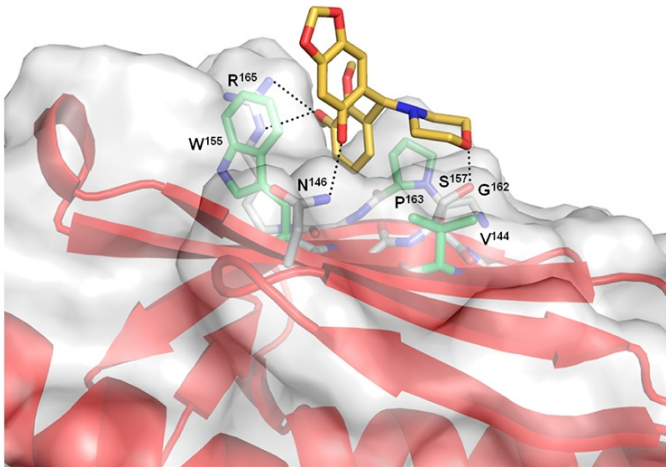
Friedreich's ataxia (FRDA) is an autosomal recessive neuro- and cardiodegenerative disorder for which there are no proven effective treatments. FRDA is caused by decreased expression and/or function of the mitochondrial protein frataxin.(1) Here, we report findings that frataxin is degraded via the ubiquitin-proteasomal pathway and that it is ubiquitinated at residue K^{147} in Calu-6 cells. A theoretical model of the frataxin-K^{147}/Ub complex, constructed by combining bioinformatics interface predictions with information-driven docking, revealed a hitherto unnoticed, potential ubiquitin-binding domain in frataxin. Through structure-based virtual screening (2) and cell-based assays, we discovered a novel small molecule (compound (+)-**11**) able to prevent frataxin ubiquitination and degradation. (+)-**11** was synthesized and tested for specific binding to frataxin by an UF-LC/MS based ligand-binding assay.(3)



(+)-**11**



Follow-up scaffold-based searches resulted in the identification of a lead series with micromolar activity in disrupting the frataxin/Ub interaction. This study also suggests that frataxin could be a potential target for FRDA drug development.

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NEW HETEROCYCLIC DERIVATIVES OF BENZYLPIPERIDIN-4-YL METHYL AND BENZYL(METHYL)AMINOBUTYL SEQUENCES AS SIGMA LIGANDS

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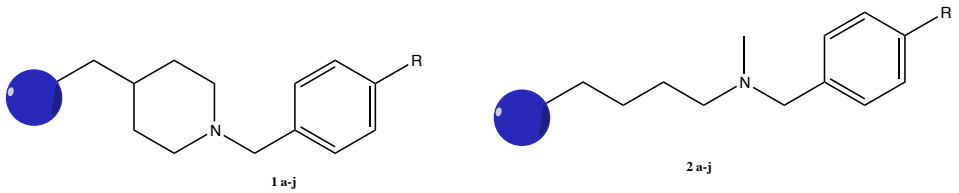
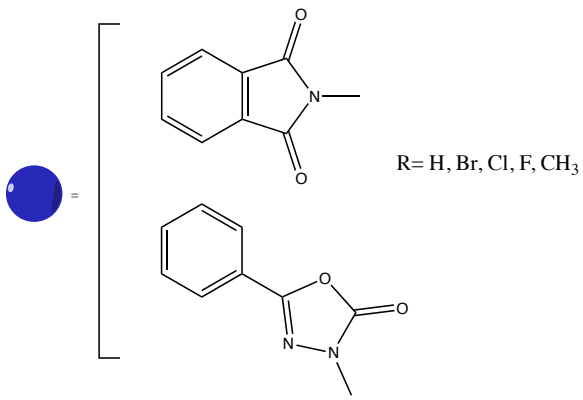
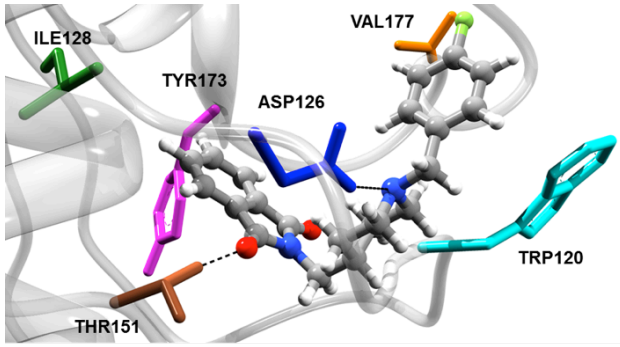
Sigma (σ) receptors are involved in several functions such as modulation and biosynthesis of several neurotransmitters, motor control, cell growth and proliferation. Several classes of structurally unrelated compounds interact with σ receptors, but only few σ ligands are gifted with affinity and selectivity against a specific receptor subtypes.

The interest in σ ligands stems from the possibility to develop clinical agents for the treatment of several CNS diseases, for neuroprotection, tumor treatment and diagnosis. Therefore σ_1 receptor ligands could be involved in treatment for schizophrenia, depression, lack of memorization skill, difficulty of learning and increase of analgesic action.

On the basis of some benzo[d]oxazol-2(3*H*)-one derivatives previously developed by us and gifted with excellent σ_1 affinity and selectivity[1,2], we have synthesized a new series of compounds **1** and **2**, changing the benzoxazolone moiety with similar groups to evaluate their effects toward the sigma affinity and selectivity.







(Fig. 1). Docking of the derivatives **1d** (*p*-F) into the σ_1-putative site.

With the aim to discover some new sigma 2 ligands, we try to modulate the selectivity and the affinity of the compounds towards the two receptors subtypes changing the middle piperidinemethyl spacer (**1a-j**) with a less constrained butylaminomethyl chain (**2 a-j**).

A preliminarily binding study (Discovery studio 2.5) of the *p*-fluoro derivatives of both series confirm the interactions between the structure of our new derivatives and the features of the σ_1 3D-receptor model (Fig.1). Furthermore an initial binding test was performed for the compound **1d** (*p*-F) which gave Ki values of 67 nM and 286 nM for σ_1 and σ_2 respectively. The evaluation on the complete series is still in progress.

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SYNTHESIS AND BIOLOGICAL EVALUATION OF TWO NEW CYCLIC BIPHALIN ANALOGUES

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Cyclization of peptides is a useful approach for developing diagnostic and therapeutic peptidic and peptidomimetic agents. As a continuation of our previous work on design of cyclic analogues of biphalin,(1) we present here two new 22-membered cyclic analogues of biphalin.
Biphalin is an opioid octapeptide with a dimeric structure based on two identical portions derived from enkephalins joined tail-to-tail by a hydrazine bridge (Figure 1).(2-4)
The cyclic analogues have been obtained by replacing the native D-Ala residues in position 2 and 2' with two residues of D-Penicillamine or L-Penicillamine (compounds **1** and **2** respectively) and by closing a disulfide bridge between these two residues (Figure 2).

H-Tyr-D-Ala-Gly-Phe-NH-NH-Phe-Gly-D-Ala-Tyr-H

Figure 1. Structure of Biphalin.

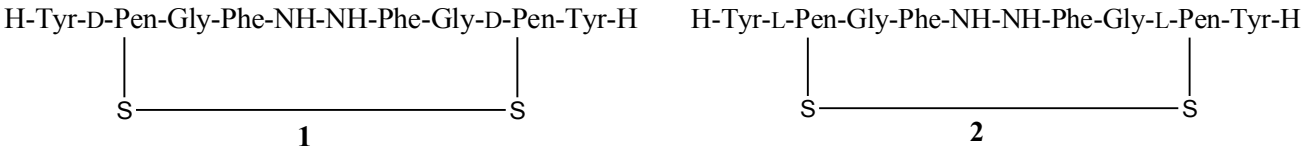

Figure 2. Structures of cyclic biphalin analogues **1** and **2**.

Following *in vitro* binding assays, compound **1**, which presents D-Pen$^{2,2'}$, showed a high affinity toward μ and δ opioid receptors ($K_{i\mu}$ = 7.5 ± 0.4 nM; $K_{i\delta}$ = 20 ± 4.7 nM), with a $K_{i\mu}$ slightly lower than selective μ agonist DAMGO. On the contrary, L-Pen-containing analogue **2** showed no significant affinity.

In vitro bioactivity MVD and GPI bioassays confirmed the remarkable opioid affinity of compound **1** (IC_{50}^{GPI} = 20.80 ± 6.6 nM; IC_{50}^{MVD} = 7.18 ± 1.2 nM), indicating that a reduced degree of freedom induced by cyclization can positively influence the binding and consequently the ability of eliciting an antinociceptive response.
These data are in full accordance with our previous published work [1] on the synthesis of cyclic biphalin analogues containing D-$Cys^{2,2'}$ and L-$Cys^{2,2'}$ residues.
In vivo Hot Plate, Tail flick tests and NMR conformational analysis of compounds **1**, **2** and biphalin in DPC micelles were also performed.

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FROM PAROXETINE TO NEW CHROMANE OXIME ETHER DERIVATIVES: A NOVEL SCAFFOLD FOR SERT INHIBITORS

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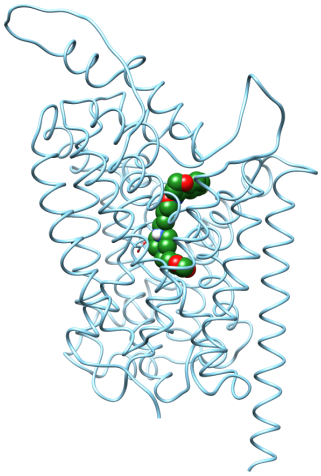
In recent years many research groups interested in the study of new antidepressant drugs dedicated their efforts to improve a dual action on serotonin transporter (SERT) and α2-adrenergic receptors (AR), synthesizing compounds characterized by longer scaffolds with respect to the classical paroxetine.[1,2] The combination of serotonin reuptake inhibition with α2-AR antagonism, either in a single molecule or as combined therapy, seemed to be helpful in the treatment of resistant depression. [1]

This strategy led to the discovery of R226161, possessing low nanomolar activity on SERT. [2] Starting from this evidence, novel scaffolds with longer spacers were designed and some hypotheses able to rationalize their activities through key interactions with SERT binding site were carried out. [3-5]

In this context, new chromane oxime ether derivatives were synthesized in our laboratory, preserving the piperonilic moiety of paroxetine and its distance from the protonated amine. The chromane oxime ether mimics the dihydrobenzopyrano[4,3]-isoxazole of R226161, bounded to a piperonilmethylpiperazine group through a 2C- or 4C- linker. These new compounds showed a high nanomolar affinity profile for SERT, and seemed to interact with the outward-facing SERT model binding site. In order to clarify the structure-activity relationships of this series and to optimize the substitutions on the new scaffold, a docking study on the SERT model was performed and a small library of chroman oxime ether derivatives was synthesized.



Their dockings confirm the key interaction with Asp98,[4] and suggest the insertion of the chromane tail between TM1 and TM3, towards the extracellular loop 4, not directly involved in small inhibitors binding but implicated in serotonin translocation.[5]

The results of the preliminary binding assays at SERT of these new compounds will be discussed.

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SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF NEW *N*-PHENYLPIPERAZINE DERIVATIVES DESIGNED AS HOMOLOGUES OF THE ANTIPSYCHOTIC LEAD COMPOUND LASSBIO-579

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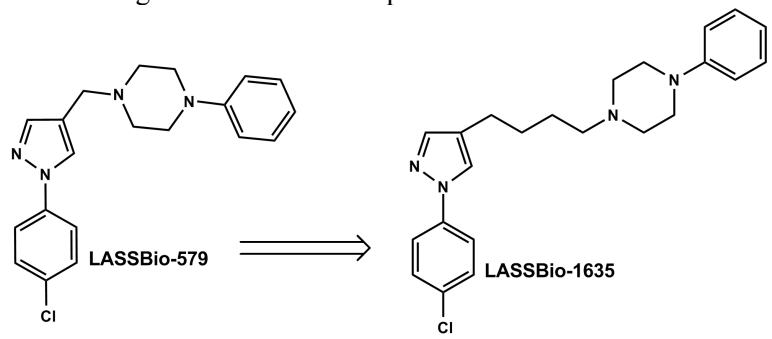
Schizophrenia is a severe neuropsychiatric disorder characterized by positive or psychotic symptoms, negative symptoms and cognitive dysfunction [(1)]. Besides the large number of antipsychotic drugs, there is a high level of treatment discontinuation, supporting the development of more effective and safer antipsychotics [(2)]. Thus, new derivatives were obtained through the extension of the methylene linker between the 4-chloro-*N*-phenylpyrazolyl and *N*-phenylpiperazine subunits of compound LASSBio-579 [(3)], considering that homologation could be a suitable strategy since the presence of two planar aromatic or heterocyclic ring systems separated by an aliphatic or alicyclic chain containing basic protonable nitrogen has been reported to favor binding to the 5-HT2A receptor [(4)].



The binding profiles of these compounds to dopaminergic, serotonergic and alpha-adrenergic receptors relevant to the treatment of schizophrenia as well as their effectiveness in pharmacological models of schizophrenia symptoms in mice were assessed. The results indicate that a significant increase in affinity for the 5-HT2A receptor has been achieved and the compound LASSBio-1635 is a better hit than LASSBio-579 and it is active in two mice models of schizophrenia positive symptoms at doses without effect on spontaneous locomotion.[(5)]

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LIGAND- BASED STRATEGIES TO MODIFY NP_S SURFACE FOR BLOOD-BRAIN BARRIER CROSSING

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The main limitation in the treatment of neurological diseases consists of the presence of the blood–brain barrier (BBB), which precludes the entry of therapeutic molecules from blood to brain.

Specifically engineered nanoparticles (NPs) have gained interest as drug carriers able to ensure an effective brain targeting, overcoming the BBB and carrying drugs to the central nervous system (CNS).

Our research group focused on biocompatible and biodegradable poly(d,l-lactide-co-glycolide) (PLGA) nanocarriers, engineered with different specific ligands able to promote the brain targeting taking advantage of the BBB crossing pathways, such as endocytosis or transcytosis[1-2].

In particular, we explored different ligand to favor the BBB crossing and the cellular interaction; i) a g7 glycopeptide (H_2N – Gly- L -Phe- D -Thr-Gly- L -Phe- L -Leu – L -Ser – O-β - D -glucose-$CONH_2$) ii) a sequence 12–32 (g21) of leptin iii) both glycopeptide (g7) for BBB crossing and sialic acid (SA) residue for interaction with brain receptors.

The brain localization of engineered nanoparticles NPs was evaluated in rats after intravenous administration, by confocal microscopy, fluorescence microscopy and electron microscopy. Studies to evaluate the biodistribution of modified NPs in comparison to the unmodified NPs were also carried out.

Results

i) g7-NPs were able to cross the BBB[3]: in particular, the biodistribution of these NPs showed a localization into the CNS in a quantity about two orders of magnitude greater than that found with the other known NP drug carriers. Not only, the results obtained by quantitative brain biodistribution of Rhodamine-123 loaded g7-NPs (15% of the injected dose) are comparable with the results obtained by antinociceptive assays with loperamide loaded into g7-NPs (at least 13% of the injected dose inferred by ICV studies).

ii) After intravenous administration in rats, the g21-NPs were able to cross the BBB and to enter the brain parenchyma. The biodistribution studies of both unmodified and modified NPs pointed out an uptake at liver and spleen level, whereas only the g21-NPs showed brain localization. The food-intake experiments pointed out that the intravenous administration of g21 conjugated to the NP surface did not produce any anorectic effect in the rats.

iii) the double-covered NPs (with SA and glycopeptide) crossed the BBB owing to the presence of glycopeptide on the NPs' surface, followed by endocytosis as the BBB crossing mechanism. Then, as a consequence of the presence of SA moiety on the NPs' surface, the double-covered NPs could interact with brain SA-specific receptors, thus explaining both the prolonged activity of loperamide delivered by NPs and the prolonged NP brain residence time. Biodistribution studies showed high NP localization (6% of the injected dose into the CNS) over a prolonged time (24 h) along with the qualitative evaluation of the NPs' visualization within the brain, kidney, liver, spleen and lung tissue parenchyma.

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DESIGN, SYNTHESIS, AND PHARMACOLOGICAL PROPERTIES OF NEW CB_2 CANNABINOID RECEPTOR LIGANDS

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Recent developments indicate that CB_2 receptor ligands have the potential to become therapeutically important. To explore this potential, it is necessary to develop compounds with high affinity for the CB_2 receptor. Very recently, we have identified the oxazinoquinoline carboxamides as a novel class of CB_2 receptor full agonist.[(1,2)] Here, we describe the medicinal chemistry of two series of heteroaryl-4-oxopyridine/ 7-oxo-pyrimidine[(3)] and 7-oxo-pyrazolo[1,5-*a*]pyrimidine-6-carboxamides derivatives. [(4)] Some of the reported compounds showed high affinity and potency at the CB_2 receptor while showing only modest affinity for the centrally expressed CB_1 cannabinoid receptor. In 3,5-cyclic adenosine monophosphate (cAMP) assays, the novel series show the dose-dependent effect in the modulation of forskolin-induced cAMP production revealing different behavior as full agonist, partial agonist and inverse agonist.

In particular, among the series of heteroaryl-4-oxopyridine/7-oxo-pyrimidines the functionality of ligands is controlled by the nature of the heteroaryl function condensed with the pyridine ring; while the **7**-oxo-pyrazolo[1,5-*a*]pyrimidine-6-carboxamides in cAMP assays show stimulatory effects on forskolin-induced cAMP production acting as inverse agonists.

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4-(ALKYL)AMINOMETHYL-SUBSTITUTED COUMARINS AS POTENT AND SELECTIVE ACHE AND MAO-B DUAL INHIBITORS WITH A THERAPEUTIC POTENTIAL IN NEURODEGENERATIVE DISORDERS

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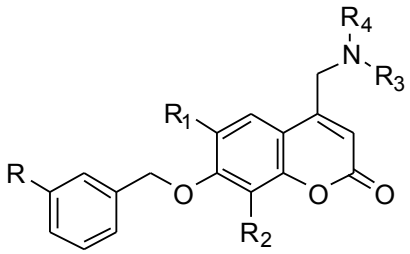
The failure of the ongoing therapeutic protocols to treat neurodegenerative diseases (NDs), has been linked to the multifactorial nature of NDs connoted by a complex network of, often unrelated, cellular events. Although the etiopahtogenesis of many NDs is still obscure, growing evidence suggested that mitochondrial dysfunction, metal dyshomeostasis, oxidative stress, protein misfolding and dysregulated signaling pathways play a pivotal role in ND. The lack of disease-modifying therapies in NDs claimed for a new medicinal chemistry approach rooted on the rational design of molecular entities able to modulate multiple and aberrant biochemical mechanisms. Among the altered biochemical mechanisms, a key role of two enzymes (AChE and MAO) in the onset and progression of neural disorders has been supported by several experimental proofs. AChE is responsible for the catalytic degradation of acetylcholine and MAO is involved in the catabolism of several endogenous and exogenous amines including many neurotransmitters. The possibility of blocking simultaneously the activity of these two enzymes might restore a proper balance of neurotransmitter levels and, in addition, exert an additional beneficial effect by MAO inhibition that reduces the production of hydrogen peroxide thus avoiding the formation of reactive oxygen species.[1]

Among naturally occurring heterocycles, coumarins have been largely explored as MAO inhibitors.[2] and reported also as dual binding sites AChE inhibitors.[3] As a further extension of previous investigations,[4] herein we report the design of coumarin-based dual inhibitors of AChE and MAO-B that bear small/medium sized amino groups at position 4 and a proper substituent at position 7. This design was grounded on previous 3D-QSAR studies that indicated positions 4 and 7 as preferred spatial regions, to assure a strong and selective binding at MAO-B. Moreover, structural modifications at position 4 aimed at an adequate modulation of the pharmacokinetic molecular properties, in particular, brain targeting and lipophilic balance for an in vivo activity at the CNS. The careful exploration of position 4 with basic, linear, unhindered, hydrogen bonding donor amino groups afforded promising dual inhibitors with IC_{50}s in the low nanomolar range for MAO-B and low micromolar range for AChE.



R= F, Cl; R_1 = H, Cl, Me; R_2 = H, Me; $R_{3,4}$ = H, alkyl, cycloalkyl

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2-HYDROXY-1,2,4-BENZOTHIADIAZIN-1,1-DIOXIDE DERIVATIVES AS NEW ANTAGONISTS OF IONOTROPIC GLUTAMATE RECEPTORS

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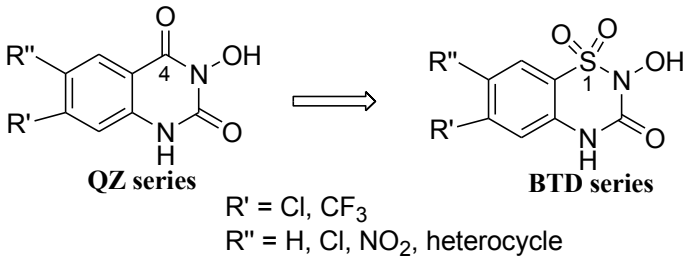
The excitatory neurotransmitter Glutamate (Glu) plays pivotal roles in regulating normal neuronal signaling processes in the central nervous system (CNS). Glu exerts its effects by activating metabotropic and ionotropic receptors (iGluRs) these latter being classified as NMDA, AMPA and Kainate (KA) receptors. The NMDA receptor complex possesses different binding sites including, as an unique feature, the glutamate co-agonist glycine binding site (gly/NMDA). Overstimulation of iGluRs, induced by excess of Glu, causes an uncontrolled Ca^{2+} overload potentially leading to cell damage and death. Several neurological disorders, such as Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, epilepsy, chronic pain and neuropathology ensuing from cerebral ischemia or cardiac arrest, are, at least in part, linked to over-activation of iGluRs. Therefore, iGluRs represent potential targets for therapeutic intervention in such neurological diseases.[1,2,3]



As a part of a research program devoted to the development of new iGluR antagonists, we have studied the 3-hydroxyquinazoline-2,4-dione system (**QZ series**) which was disclosed as a useful scaffold to obtain selective iGluR antagonists.[4,5,6] Thus, to identify novel potent and selective iGluR antagonists, we planned the synthesis of new derivatives (**BTD series**) resulting from replacement of the 4-oxo function of **QZ series** with the 1-sulfonyl moiety (**BTD series**). All the synthesized 2-hydroxy-1,2,4-benzothiadiazin-1,1-dioxide derivatives (**BTD series**) bear, on the benzofused moiety, substituents that in the **QZ series** turned out to be profitable for improving affinity and/or selectivity toward AMPA and KA receptors, or the gly/NMDA site.[4,5,6] Preliminary binding results showed that the **BTD** derivatives possess increased AMPA receptor affinity and selectivity with respect to the previously reported **QZ** derivatives, thus indicating that the **BTD** scaffold, suitably decorated, may afford novel iGluR antagonists.

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DESIGN, SYNTHESIS AND EFFICACY OF NOVEL G PROTEIN-COUPLED RECEPTOR KINASE 2 INHIBITORS

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G protein-coupled receptor kinase 2 (GRK2) is a relevant signaling node of the cellular transduction network, playing major roles in the physiology of various organs/tissues including the heart and blood vessels. Emerging evidence suggests that GRK2 is up regulated in pathological situations such as heart failure, hypertrophy and hypertension, and its inhibition offers a potential therapeutic solution to these diseases. As reported in literature, two short peptides KRX107 (GLLRrHS) and KRX124 (GLLRrHSI) derived from HJ loop of GRK2/3 inhibit GRK2 activity [(1)] and emerge as a valuable starting point for the development of a novel class of GRK2 inhibitors.

We explored the GRK2 inhibitory activity of a library of cyclic peptides derived from the HJ loop of G protein-coupled receptor kinases 2 (GRK2). The design of these cyclic compounds was based on the conformation of the HJ loop within the X-ray structure of GRK2.[(2)] Starting from these data in this communication we report the preliminary results obtained with a small library of short analogues of KRX107 and KRX124.

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NOVEL 1,3-DIPROPYL-8-(3-BENZIMIDAZOL-2-YL-METHOXY-1-METHYLPYRAZOL-5-YL)XANTHINES AS POTENT AND SELECTIVE A_{2B} ADENOSINE RECEPTOR ANTAGONISTS

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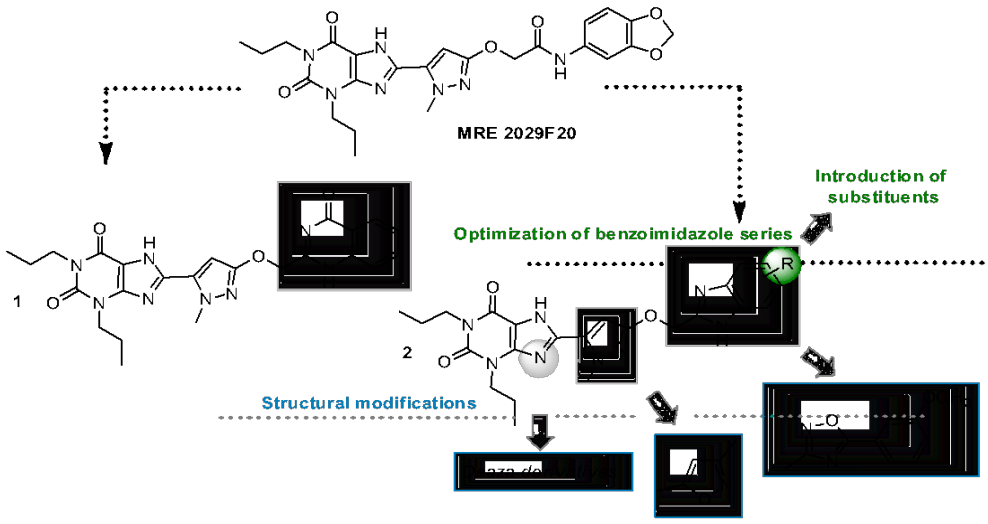
Molecular modeling studies, including the comparative molecular field analysis (CoMFA) method, on 52 antagonists of the A_{2B} adenosine receptor with known biological activity were performed to identify the three-dimensional features responsible for A_{2B} adenosine receptor antagonist activity. On the basis of these and previous results on the potent antagonist effect of 8-pyrazolyl-xanthines at human A_{2B}AR,[(1-3)] a new series of compounds was synthesized and evaluated in binding studies against the human A_1, A_{2A}, A_3 and A_{2B}ARs.[(4)]

The 3D QSAR model led to the design of two new derivatives, including a bioisosteric replacement of the anilide moiety of **MRE2029F20**[(1)] with quinazoline or benzimidazole rings (compounds **1** and **2** respectively).



In this study, a new series of 1,3-dipropyl-xanthines that carry a *N*-methylpyrazolo or isoxazolo group at position 8 was synthesized and biologically evaluated. The selection of the benzimidazol-2-yl-methoxy group at the 3-position of the pyrazole/isoxazole was based on the greater potency of the benzimidazole derivative **2** over that of quinazoline **1**, and various structural modifications were realized using the novel A_{2B} antagonist **2** as the template.

The amide bond was also replaced with the 5-phenyl-1,2,4-oxadiazole nucleus on the basis of other adenosine pharmacophores, such as those reported previously in literature. In this context, considering the good antagonistic potencies of deazaxanthines at human adenosine receptors, a comparison of the affinity and selectivity profiles of 9-deazaxanthines with the corresponding xanthines was afforded by the preparation of four 9-deaza direct analogs. The new compounds were tested in competitive binding assays toward four hARs expressed in CHO cells. cAMP assays were performed to determine their functionality.

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