XXVI National Meeting in Medicinal Chemistry

XII Young Medicinal Chemists' Symposium

ABSTRACT BOOK

Department of Pharmaceutical Sciences (DISFARM) - University of Milan

The Department of Pharmaceutical Sciences (DISFARM) was created in 2012 from the Department of Pharmaceutical Sciences “Pietro Pratesi” and the section of Organic Chemistry "Alessandro Marchesini" of the Department of Molecular Sciences Applied to Biosystems. DISFARM is organized in the three sections of Medicinal Chemistry "Pietro Pratesi", Pharmaceutical Technology and Legislation "Maria Edvige Sangalli", and General and Organic Chemistry "Alessandro Marchesini", combining specific scientific skills, which favor the promotion and coordination of interdisciplinary research lines in the framework of pharmaceutical sciences.
The goals are those of improving knowledge in the field of basic research, a major cultural investment to design a qualified research aimed at the discovery of novel drugs and able to boost an efficient technology transfer.
The research at DISFARM covers a range of activities focused on the design, synthesis, development, and control of novel biologically active molecules, and of new pharmaceutical drug delivery systems, food supplements, cosmetics, biocides and medical devices. DISFARM’s scientific activities are financially supported by Italian and European grants, and are assisted by collaborations with public institutions and national/international companies.
With the purpose of training young researchers, the Department hosts the PhD course in “Pharmaceutical Sciences” as well as the specialization school in “Hospital Pharmacy”, an interuniversity master and three post-graduate programs.

The University of Milan

The mission of the University of Milan, established in 1924, is to contribute to society through the pursuit of teaching/education and research at the highest international levels of excellence. It is the only Italian member among the 23 prestigious Universities of the League of European Research Universities (LERU).

With a teaching staff of about 2,200 tenured professors and with almost 60,000 students, the University of Milan (UNIMI) is the largest university in Lombardy, one of the most dynamic and internationally-oriented EU regions. The University of Milan offers several study programmes covering three macro-disciplinary areas: *i)* Humanities, Social Sciences and Law, *ii)* Medicine and Healthcare, *iii)* Natural Sciences.
The broad range of subjects taught, in the running 79 Undergraduate programmes, 57 Master programmes, 9 Single-cycle programmes, 21 Doctoral Schools, and several Advanced Vocational programmes, attracts students from all of Italy and the whole world.

In addition to its excellent level of education, UNIMI has an established reputation as one of the institutions most strongly committed to basic and applied research in Europe.
Research activities are conducted in 33 Departments and 22 Inter-departmental Research Centres, with more than 24,000 scientific publications in the last three years, 222 patent family and 24 active spin-off. 5 scientists from the University that have been ranked as *highly cited* by the ISI – Institute for Scientific Information (USA).
The University’s researchers occupy leading positions in numerous research programmes conducted both at a national and international level. 4 technological platforms equipped with next-gen instrumentation are available for implementing research activities: COSPECT (spectroscopic, spectrometric and diffractometric

analysis- spectroscopy), INDACO (computing power and data storage), NOLIMITS (imaging - light, confocal and electron microscopy) e OMICs (proteomic and metabolomics).

In order to foster researcher participation to grant funding programmes, the University of Milan developed a Transition grant programme (5000€-80000€ per project) that supports proposers that applied to H2020 (over threshold, not funded) and ERC (evaluated A at step 1, not funded).

The protection and exploitation of the scientific productivity is a strategic focus of the University of Milan. It has thus developed a policy to facilitate and shorten the time and mode of results transfer from research to market. In this view, it has also built relationships with different third parties such as industries, not profit organizations, institutional entities, SMEs. Several patent applications, in co-ownership with partners, havebeen filed to protect such results.

The University of Milan is constantly developing projects in cooperation with some of the most relevant international research groups, often acting as activities coordinator. The interdisciplinary and intersectoral nature of such projects is enabled by the wide spectrum of basic and applied research activities present in University of Milan, spanning from Life, Physical and Social Sciences to Humanities.
EU programmes represent a major source of funding for the University of Milan, which has signed 147 grants under the 7th Framework Programme (2007-2013), for a total value of € 50.344.153, and 120 grants up to April 2019 under the Horizon 2020 Programme, for a total value of more than € 30.000.000. Up to now, UMIL Researchers have been participating in around 40 COST Actions.

UNIMI is actively promoting and supporting its internationality with English being the official language in the 31 PhD programmes, and the only language in one Bachelor programme and 10 Master and Single-cycle programmes. Other courses are partially held in English with 3 Masters of two years and one Single-cycle university programme of 5 years. UNIMI organizes 2 international Master programmes with courses held both in Milan, as well as in other countries, and with the possibility of obtaining a double degrees in two different countries of the consortium and 3 joint international PhD programmes organized in Milano together with other foreign Universities. Exchanges of students, scientists and professors with foreign universities are also encouraged and promoted by UNIMI that not only participates to several European and non-European exchange programmes (e.g. Erasmus+, LLP – Lifelong Learning Programme, Erasmus Mundus, Socrates, European Research Council, Business Exchange and Student Training, Fulbright, Galileo, Vinci and Vigoni programmes), but also have several specific international agreements with universities and research centers from all over the world. Finally grants for excellent researchers and students to specialize abroad are also funded by UNIMI.

Società Chimica Italiana

The Italian Chemical Society, founded in 1909 and established as a Moral Organization with Royal Decree no. 480/1926, is a scientific association with over three thousand five hundred members. The members carry out their activities in universities and research institutions, in schools, in industries, in public and private research and control laboratories, in the liberal profession. They are united not only by their interest in chemical science, but also by their desire to contribute to the cultural and economic growth of the national community, to the improvement of the quality of human life and to the protection of the environment.

The Italian Chemical Society aims to promote the study and progress of chemistry and its applications, and in particular:

- to encourage and increase scientific research in all fields of Chemistry;
- to spread the knowledge of Chemistry and the importance of its applications in the context of the progress and well-being of humanity;
- to promote and encourage the study of chemistry in universities and in all schools of all levels;
- to promote in every field the development of the Sciences.

To achieve these aims, and with the exclusion of profit, the Italian Chemical Society promotes, also through its Peripheral Organs, (Sections, Divisions, Interdivisional Groups), publications, studies, surveys, events.

The Sections pursue the aims of the Society at a regional level. The Divisions bring together Members who follow a common scientific and research policy. The Interdivisional Groups bring together members interested in specific interdisciplinary topics.

The governing bodies of the Society are the President, the Executive Committee and the Central Council. Sections and Divisions are also governed by a President and a Board of Directors.

The Society organises numerous conferences, courses, schools and seminars both at national and international level. To spread the principles of chemical science in the upper secondary school organizes annually the Chemistry Games, a competition that allows young people to test their knowledge in this field and that selects the national team for the International Chemistry Olympics.

Important is the publishing activity with the publication, together with other European Chemical Societies, of scientific journals of high international level. It also publishes the journal La Chimica nella Scuola.

The official organ of the Society is the journal La Chimica e l'Industria.

EFMC - European Federation for Medicinal Chemistry

The European Federation for Medicinal Chemistry (EFMC) is an independent association founded in 1970 that represents 25 scientific organisations from 23 European countries. Its objective is to advance the science of medicinal chemistry & chemical biology by promoting cooperation and encouraging strong links between the national adhering organisations in order to deepen contacts and exchanges between medicinal chemists & chemical biologists in Europe and around the World. EFMC fulfils this objective by organizing symposia and short courses, by sponsoring meetings and medicinal chemistry schools, by publishing on relevant topics and by conferring awards and prizes.

Its most important organisation is the biennial International Symposium on Medicinal Chemistry (EFMC-ISMC). These symposia, with an average attendance of 1,200 delegates, are highly international with a broad range of speakers and attendees from the pharmaceutical industry and academia. Next to the ISMC, EFMC is involved in the organisation of the Frontiers in Medicinal Chemistry (FMC) and the international symposium on Advances in Synthetic and Medicinal Chemistry (ASMC). EFMC also organises intensive short courses on specific topics in medicinal chemistry.

An important part of the EFMC activities is sponsorship of national scientific meetings and Medicinal Chemistry Schools. EFMC also awards travel grants for younger scientists to attend EFMC symposia and schools.

Furthermore, in collaboration with the Royal Society of Chemistry, EFMC is publishing MedChemComm, the official journal of EFMC. MedChemComm is a peer reviewed journal publishing concise articles and reviews covering medicinal chemistry research. The electronic newsletter MedChemWatch as well as the yearbook "Medicinal Chemistry in Europe" help to further develop contacts between medicinal chemists around the world.

EFMC also acknowledges the excellence of medicinal chemists' work, by conferring three major awards: the Nauta Pharmacochemistry Award for Medicinal Chemistry and Chemical Biology, the UCB-Ehrlich Award for Excellence in Medicinal Chemistry and the Prous Institute-Overton and Meyer Award for New Technologies in Drug Discovery, which are given every two years for outstanding achievements in the field of Medicinal Chemistry. From 2010 on, EFMC established two new prizes to acknowledge the scientific accomplishments of young medicinal chemists, both in industry and academia.

EFMC-YSN: THE YOUNG SCIENTISTS NETWORK

Building a strong network at an early stage for young scientists' career is crucial: therefore, EFMC decided to create a new network for young scientists: the EFMC-Young Scientists Network.

The aim of the EFMC-YSN is to inspire, connect and provide opportunities to medicinal chemists and chemical biologists in their Early Career. Its main activities:

- Networking activities
- Training activities
- Support to young scientists
- Events & Meetings

BREAKTHROUGHS IN GPCR STRUCTURAL BIOLOGY AND THEIR IMPACT ON COMPUTER-AIDED DRUG DESIGN

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We discover and characterize new receptor ligands to modulate purinergic signaling using chemical, pharmacological, and structural approaches. Purine receptors encompass 4 G protein–coupled receptors (GPCRs) for adenosine, 8 GPCRs activated by nucleotides (P2YRs), and 7 ATP-gated P2X ion channels. High-resolution X-ray structures of several adenosine receptors (ARs) and P2YRs, determined through our collaboration with Ray Stevens and colleagues (Univ. So. California), facilitate the rational design of ligands, either by modification of known agonists and antagonists or by virtual screening to discover novel chemotypes. The $P2Y_1R$ is a target for neurodegeneration, and we reported the first antagonist of nM affinity, the nucleotide MRS2500. A $P2Y_1R$ antagonist, BPTU, displayed both surmountable and insurmountable antagonism, which was not interpretable structurally prior to the determination of its high-resolution structure in a $P2Y_1R$ complex. It was the first extrahelical, allosteric modulator of a GPCR to be reported, suggesting that GPCRs can be modulated by small molecule binding at many different sites. The $P2Y_1R$ interactions of both allosteric and orthosteric ligands were predicted using docking and molecular dynamics simulation, to recapitulate the known $P2Y_1R$ structure activity relationship (SAR) and to aid in rational ligand design. We have discovered potent and selective ligands of the A_3AR and $P2Y_{14}R$, both inflammation-related GPCRs, with the aid of molecular modeling based on homology to X-ray structures of closely related receptors. $P2Y_{12}R$ structures were applied to discovery of novel heterocyclic antagonists of the $P2Y_{14}R$ for treating inflammation. We have also introduced sterically constrained rings to mimic native ribose in nucleosides and nucleotides, to determine their preferred conformation when bound to P2YRs or ARs. Modeling of adenosine derivatives in complex with the A_3AR suggested that structural plasticity of transmembrane helix 2 (TM2) is present. Several of our ribose-containing A_3AR agonists have advanced in clinic trials for treatment of inflammatory diseases and cancer, and other second generation, conformationally-locked agonists of even greater selectivity are being considered for chronic neuropathic pain treatment. Highly specific (>10,000 fold selective) A_3AR agonists were designed using a structure-based approach and screened using an in vivo phenotypic pain model, which reflected both pharmacokinetic and pharmacodynamic parameters. Thus, purine receptor structures have enabled novel ligand discovery, the elucidation of their biological role and the conceptualization of future therapeutics.

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PL2

INDUCING PROTEIN DEGRADATION WITH SMALL MOLECULES: HOW PROTACS WORK

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Targeted protein degradation with small molecule is a new modality of chemical intervention that could lead to new medicines against targets conventionally thought of as undruggable.

Proteolysis-targeting chimeras (Protacs) are double-headed molecules that recruit a target protein to an E3 ubiquitin ligase, thus co-opting the ligase activity leading to the protein being ubiquitylated and degraded by the proteasome.

My talk will take the audience to a journey of key discoveries from the past 10 years, from my laboratory and others that have collectively contributed to making targeted protein degradation by Protacs a powerful tool to probe biology and a disruptive therapeutic approach.

IMPLEMENTING MOLECULAR INTERACTION KINETIC ANALYSIS FOR DRUG DISCOVERY

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The development and application of biophysical methods for structural and kinetic analysis of biomolecular interactions has transformed pharmaceutical research and the strategies used for development of drugs. Our focus has been to exploit biosensor technology for detailed studies of enzyme-inhibitor interactions and other important recognition processes in the life science area. The work has involved studies of a variety of enzymes, and other proteins involved in important disease processes. Through kinetic analysis of interactions at varying conditions, we have demonstrated the importance of defining the mode-of-action via characterization of the complexities of molecular interactions, including the types of interaction mechanisms or the location of ligand binding sites relative to active sites. This has revealed the possibility to design specific and safe drugs, even when multiple isoforms with similar characteristics exist. The work also suggests that structure kinetic relationship analyses and identification of dominating interactions for optimization of lead compounds should ideally be based on intrinsic rate constants instead of the more easily accessible observed kinetic constants, which also account for binding linked reactions.

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ARTIFICIAL INTELLIGENCE. NOT JUST ANOTHER TOOL IN THE TOOLBOX

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The explosion of interest in Artificial Intelligence for new Medicine R&D driven by 'big data' as well as new technologies from the Tech industry. However, 'AI' technology is not new to our industry which has a long history of investment in cheminformatics and other computer-based methods and which are well established as essential elements of modern drug discovery. This presentation asks whether this new wave of AI computer-based methods do represent a step-change in Medicines R&D or whether they are 'just another tool in the toolbox'.

The talk defines Artificial Intelligence as a system of autonomous software agents that operate independently of human control but which still produce results equivalent to those produced by human experts. This is sometimes described as 'Weak AI', since it doesn't imply that the software system is in some way conscious, an ambition which remains in the realm of science fiction. Building autonomous software to carry out the tasks that meet the goals of a medicinal chemistry project without expert intervention is still very challenging and is much more difficult than building and using the same software as tools for experts, for reasons that will be described in the talk. As well as the software challenges of delivering performance, continuous assessment of quality must be baked in to the software if results are to be trusted and the software must be able to manage the critical decisions that experts make based on experience, without asking their opinion.

The benefits of using computed aided drug discovery tools as autonomous systems *vs* tools are in the extreme scalability, comprehensive coverage of biological and chemical information as well as transparency and objectivity in assessment of the results. Autonomy allows the software systems to build continuously updated repositories of an organisation's tacit knowledge or beliefs which are valid across its areas of interest and survive the loss of individual experts, all of which can operate in the background as a strategic, cross-project process, accessible to project teams. They are likely to operate alongside medicinal chemists and molecular design specialists as a 'trusted colleague', providing a live view of the organisation's knowledge and bringing new insight to the project.

Artificial intelligence as autonomous software systems that build on medicinal chemistry know-how, our rich heritage of computer aided drug design systems and the massive chemical and biological information sources have breakthrough potential for our industry. It is not unreasonable to be cynical about this statement, given the history of overpromise, but there is a good case to be made that real AI, if defined differently from 'just another tool', has the potential to deliver real changes in productivity and output of new medicines.

INTEGRATED STRUCTURAL BIOLOGY: APPROACHING INFECTIOUS AND DEGENERATIVE DISEASES

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Discovering the structural details and dynamic properties of proteins is the challenging but necessary step for the rational design of new drugs. The biophysical and biochemical tools currently available allow us to tailor molecules for maximal target specificity and affinity, thus encoding two key properties for an effective drug. The field of Structural Biology has been developing over the past few decades in an exponential way, thanks to several technological developments that include the availability of synchrotron radiation for crystallographic applications, super-computing for exploring the dynamics properties of protein targets, efficient isolation of recombinant soluble and membrane proteins, and single particle cryo electron microscopy. It can be safely stated that the discovery search for any new drug should rely on an integrated structural biology approach, synergizing with all the powerful development methods of medicinal chemistry and drug design.

X-ray crystallography, coupled to *in silico* screening provides a flexible tool for the discovery of molecules directed *vs*. new protein targets (*e.g.* enzymes from pathogens). This approach may entail a search through virtual compound libraries (but also fragment libraries), followed by crystallographic analyses of the best *in silico* hits and the ensuing *in vitro* tests. I will present examples in the context of a search for antiviral compounds.[1]

Structural biology can be exploited for the design of new vaccines, based on fundamental protein structure principles and simulations that allow identifying epitopes through the analysis of three-dimensional structures of protein antigens. The same approach can also deliver (peptide) epitopes for the use in diagnostic microarrays. Part of the structure-based search for protective epitopes targeting the pathogen *Burkholderia pseudomallei* will be presented.[2]

Single particle cryo-EM provides a new approach that allows reaching residue resolution in complex protein aggregates that cannot be crystallized. The potential of this recently introduced branch of Structural Biology will be presented by exposing the study of ex-vivo amyloid deposits from a patient affected by systemic amyloidosis with severe heart involvement.[3]
At the same time, single particle cryo-EM currently provides the best approach to the study of membrane proteins, well known targets of at least 30% of the active drugs. Specifically, heart voltage-gated ionic channels are a target on reach, for which preliminary results will be presented.

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ANNELATED MEDIUM-SIZED AZAHETEROCYCLES AS ATTRACTIVE SCAFFOLDS FOR CNS TARGETED LEADS

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Medium-sized nitrogen heterocycles (7-to-15-membered) have widespread interest in organic synthesis and medicinal chemistry. Indeed, such heterocyclic rings are found as subunits or core structures in natural and bioactive molecules, including pharmaceutical products, whereas on the other hand they often can serve as key intermediates in the synthesis of bicyclic compounds by selective transformations (e.g., transannular ring-contractions, cycloadditions). These molecular frameworks, particularly annelated 7-to-10-membered aza-heterocycles, have long drawn our attention as potential scaffolds for developing new multitarget-directed ligands (MTDLs) for treating Alzheimer's disease (AD) and other neurodegenerative syndromes.

AD, the most common form of dementia affecting people worldwide, is a progressive neurodegenerative disorder, whose multifactorial pathogenesis is still not completely understood. The main histopathological changes include synaptic dysfunction and neuronal loss resulting from intracellular and extracellular fibrillar aggregates of β-amyloid (Aβ), hyperphosphorylated and β-folded tau proteins, cholinergic impairment, oxidative stress, neuroinflammation, metal dys-homeostasis and mitochondrial damage. Among others, *N*-methyl-D-aspartate receptors (NMDARs) play a major role in learning and memory, and their overactivation causes excessive calcium influx and consequent excitotoxicity, which is associated with CNS diseases, including Parkinson's disease.

Starting from our old[1,2] and recent[3] findings on the suitability of partially hydrogenated benzo-, chromane-4-one- and indole-fused azepine and azocine derivatives targeted at enzymes, receptors and biochemical pathways involved in the pathogenesis of AD, we extended the investigation to novel derivatives of annelated azonines and azecines.

Herein, our recent advances of benzo- and indol-fused 7-to-10-membered nitrogen heterocycles as molecular tools for AD-associated targets (e.g., butyryl- and acetylcholinesterase, monoamine oxidases A and B, Aβ aggregation, ROS insult, NMDAR antagonism), along with the results from investigation on cell and ex vivo/in vivo animal models, will be presented and discussed in an effort of rationalizing structure-activity relationships and progressing drug optimization of the examined CNS-targeted lead compounds.

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HYDROXYAZOLES AS CARBOXYLATE BIOISOSTERES: AN EFFICIENT TOOL FOR DESIGNING ACTIVE COMPOUNDS WITH ADDED INTELLECTUAL PROPERTY VALUE

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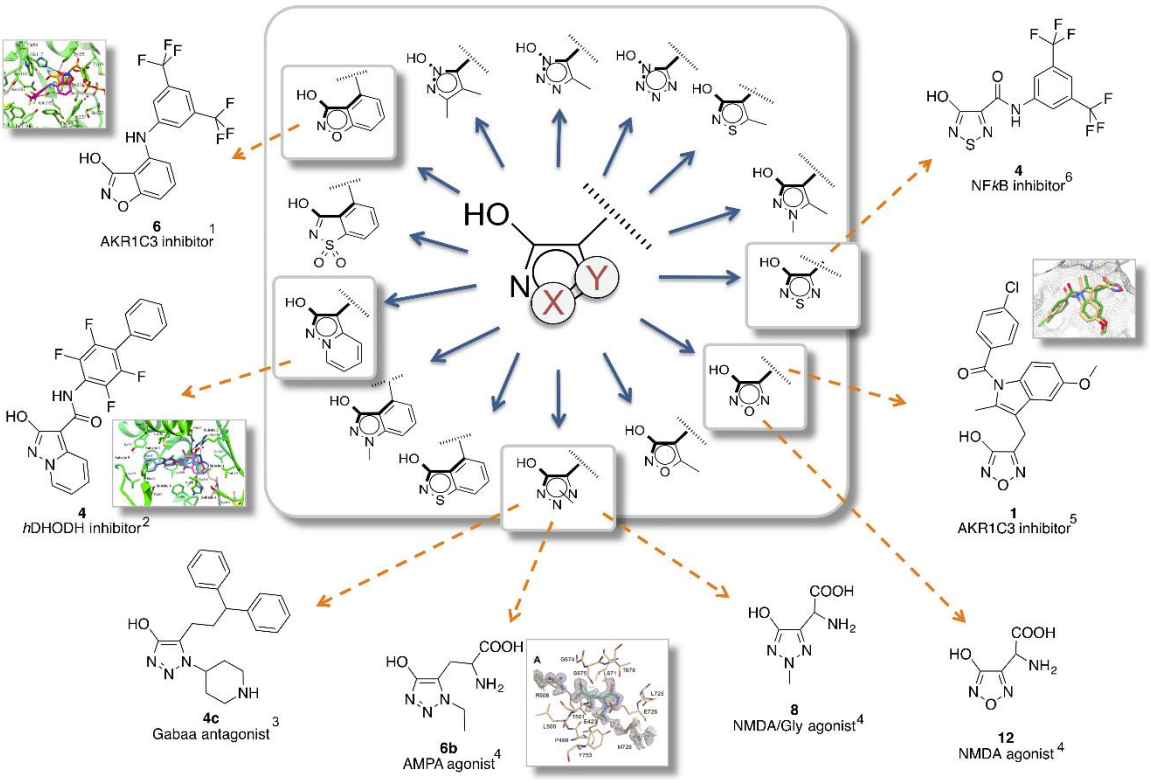
During the design of drug candidates, *bioisosterism* is often the winning approach to improve potency/selectivity, achieve optimal ADME-T profiles and acquire novel intellectual property (IP). In some ways, the eternal confrontation between the concepts of *isostere* (defined by a chemistry-related context) and *bioisostere* (defined by a biological-related context) is well representative of the deep soul of a Medicinal Chemist. The frequent absence of correlation of biological activity between isosteres is often a brutal remind of how the translation of a chemistry-based design into a living organism context could be challenging.
In the last fifty years, acidic ***hydroxyazoles***, because of their isosteric connection to the carboxylic group, represent an efficient tool for designing active compounds with added IP value. Recently, we and other groups, while expanding the chemical space of these hydroxylated heterocycles, systematically explored these systems in the framework of *hit-to-lead* optimization processes.
The presentation, while covering the acidic hydroxyazoles research field, will detail some of their most recent application in *Medicinal Chemistry* (among them *h*DHODH, AKR1C3, GABAa and Glu neurotransmission, NF*k*B, …).



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FROM ZINC TO ZINClick: OUR WAY TO EXPLORE THE CHEMICAL SPACE

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We all know that the chemical space is an almost infinite set of all possible chemical entities, and it is not possible to create all molecules to subsequently search for the interesting ones. There are plentiful chemical databases available online that are useful for assessing (and accessing) different areas of the chemical space.[1] One of the most widespread approaches involves the use of the ZINC database and, through virtual screening, going to identify which molecules within it have a potential activity on a specific target.
Over the years we have successfully pursued this strategy by identifying, for example, new inhibitors of DGKα suitable for clinical trials in X-linked lymphoproliferative disease 1 (XLP1).[2] Pharmacological inhibition of DGKα in XLP1 animal models limits CD8+ T cell expansion and interferon-γ production, suggesting the development of DGKα inhibitors for XLP1 therapy.
Besides the idea to select compounds "easy to buy" we have focused our attention also on the creation of a new library of compounds which represent an unknown portion of chemical space. We have already demonstrated the ability of the 1,2,3-triazole scaffold to give important and pivotal binding interactions with biological targets.[3]
In the last years, we have investigated the click-chemical space covered by molecules containing the triazole ring, we generated a database of 1,2,3-triazoles called ZINClick,[4] starting from literature-reported alkynes and azides synthesizable in no more than three synthetic steps from commercially available products. This combinatorial database contains millions of 1,4-disubstituted-1,2,3-triazoles that are easily synthesizable, new and patentable. The library is regularly updated and it is freely downloadable (http://www.ZINClick.org).[5] The new implementation of ZINClick will be discussed as well as our new strategy about clustering the chemical space covered by 1,4-disubstituted-1,2,3-triazoles around their availability: from direct purchase to different degree of synthetic feasibility of the compounds (Figure 1).

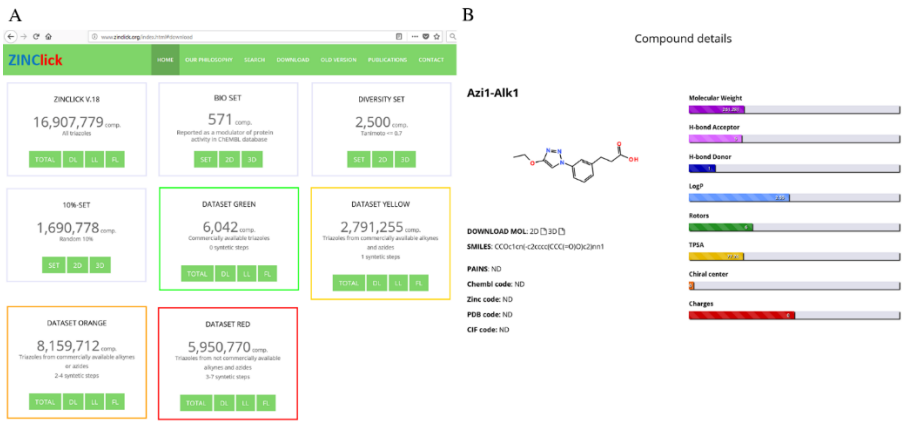

Figure 1. Detailed view of the ZINClick v.18 webpage: (**A**) details of the subsets available to download, and (**B**) details page of a ZINClick triazole.

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HOW WE TURNED A WEAK AND METABOLICALLY UNSTABLE CXCL12-MIMIC PEPTIDE INTO A STABLE, POTENT AND SELECTIVE CXCR4 ANTAGONIST: A SUCCESSFUL PEPTIDE OPTIMIZATION STORY

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Among the different signaling pathways, directly or indirectly involved in cancer immune-resistance and migration, the C-X-C motif chemokine 12 (CXCL12), also known as SDF-1 alpha (stromal-cell-derived factor-1alpha), and its receptor CXCR4 have been extensively investigated for their implication in both tumor growth and metastasis.[1] Aberrant expression of CXCR4 has been found in more than 20 different cancer types, characterized by high aggressiveness and metastatic potential, where CXCL12 is released.[2]
With the aim of discovering novel CXCR4 antagonists suitable for anticancer therapy, we identified a CXCL12-derived small cyclic peptide, that binds the CXCR4 receptor in the μM regimen.[3] Despite its promising *in-vitro* activity, this peptide suffered from two main drawbacks, which hampered any further biological evaluation: a) low metabolic stability in biological fluids; b) unsuitable potency for *in-vitro* to *in-vivo* translation in a preclinical model. In this presentation, the results of a successful lead optimization campaign aimed at enhancing both the metabolic stability and the target affinity will be described. The newly obtained analogs displayed longer *in vitro* half-life, enhanced affinity for CXCR4, [4-6] better selectivity versus other related and unrelated chemokine receptors, and ability to block CXCL12-dependent cell migration and CXCL12-mediated CXCR4 internalization.[5-6]
These encouraging results also set the stage for the following study aimed at converting our potent and selective CXCR4 antagonist in a radiolabeled probe to detect pertinent tumors *in-vivo*. *In vivo* studies demonstrated that the NOTA-functionalized is able to selectively mark CXCR4 overexpressing CHO cells in PET imaging experiments using a proper xenograft mouse tumor models.

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LIGAND-BASED FLUORINE NMR SCREENING: PRINCIPLES AND APPLICATIONS IN DRUG DISCOVERY

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Over the last few years, fluorine NMR spectroscopy has emerged in pharmaceutical industries and academic institutions as a powerful tool for performing binding and functional screening. The binding assay FAXS (Fluorine chemical shift Anisotropy and eXchange for Screening) performed in direct and/or competition mode and the functional assay n-FABS (n-Fluorine Atoms for Biochemical Screening) are robust and have now found widespread applications in the early phase of drug discovery projects. In addition, the versatility of these methodologies allows novel applications with complex chemical and biological systems. The large dynamic range of the binding assay permits the detection of very weak affinity ligands where other biophysical techniques might fail. This is particularly relevant for fragment-based screening, where the fragments, due to their small size, interact only very weakly with the receptor. The principles of the FAXS along with some applications to challenging pharmaceutical targets with low ligandability will be presented.

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DRUGGABLE TARGETS IN NUCLEIC ACIDS

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Nucleic acids are among the lowest represented class of clinically used drug targets: a recent list of "druggable" targets reports only three categories of compounds acting on nucleic acids with diverse molecular mechanism of action, namely, alkylation, complexation and intercalation.[1] As the most important feature in drug development is specificity, it is hardly surprising that nucleic acids, and particularly DNA, are not ideal drug targets: the (almost) universal nature of the genetic code represents a reasonable obstacle in the search of chemicals aimed selectively at the information "storage" in cells.

Despite these fundamental limitations, compounds acting on nucleic acids are used clinically, and still investigated with the aim of biochemical exploitation and pharmacological intervention. When touching the "untouchable" it is proper to consider the context or, in other words, the effect and consequences on the complex system imbalanced by the initial drug-target binding event. In the nucleic acids-druggable world, often the nature and entity of these perturbation events are still not completely known.

While DNA double-helix is essentially invariable, its older sibling and more "social" RNA makes for a more dynamic horizon of druggable targets. RNA is a fundamental player in the communication inside our cells, its structure is flexible and it has enzymatic activities. Unfortunately, a structure-based approach toward RNA targets is still hampered by the size, the complexity and the intrinsic flexibility of this macromolecule.[2] Technological tools directed at studying RNA are still underrepresented in comparison to protein-directed tools. However, considering the "sociological" nature of RNA stem-loop organization,[3] small folded structures inside the complex RNA fold are often sufficiently meaningful targets. The dynamic nature of RNA is worth exploring, as it has been demonstrated that restricting conformational changes in small tridimensional motifs is exploitable in drug development, either by stabilizing an RNA fold crucially implicated in a biochemical event, or in inhibiting a protein recognition event. Recognizing the druggability of unique RNA folding is therefore the main driver for medicinal chemists targeting nucleic acids. Tuning small molecules ability to recognize preferentially the "social" relative to the "storage" is the hard endeavor of our work.

Finally, understanding how drugs impact the biochemical pathways responsible for the desired as well as the undesired effects leads to the acknowledgment that *the more we know, the more we know that we don't know*. Far from being discouraged by the sobering complexity of life within and around us, medicinal chemists can make wise use of the molecules they develop not only to (possibly) cure diseases, but also as powerful tools to understand the complexity of life's molecules and machines, as the lesson learned from aminoglycosides.[4]

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TOWARD THE COMPUTATIONAL DESIGN OF NANORECEPTORS WITH INTELLIGENT RECOGNITION ABILITIES

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We present our recent progress[1,2] in the rational design of functionalized nanoreceptors using molecular dynamics (MD) simulations integrated with experiments. These intelligent nanoreceptors are used to detect the presence of different organic analytes in solution via NMR chemosensing measurements. We show that it is possible to rationally design functionalized coating thiols that self-organize to form transient pockets in monolayer-protected gold nanoparticles (AuNPs). These may find applications for detecting small molecules such as drugs, metabolites, illegal drugs, and small molecular markers for cancer.

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HISTONE DEACETYLASE INHIBITORS AS MULTITARGET LIGANDS: NEW PLAYERS IN ALZHEIMER'S DISEASE DRUG DISCOVERY?

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In the recent years, we have witnessed an explosion in the design of Multitarget Ligands (MTLs) able to interact with a plethora of biochemical targets believed to be involved in Alzheimer's Disease (AD) pathogenesis.[1] These molecules hit multiple targets acting on different and not strictly related biochemical pathways. Among these targets, Histone Deacetylases (HDACs) have recently emerged as an appealing opportunity in drug discovery.[2] The application of HDACs inhibitors (HDACIs) as neuroprotective agents is gaining a lot of considerations due to the role of HDACs in neurodevelopment, memory formation and cognitive processes.[3] Indeed, HDACIs control gene expression by modulating the acetylation status of histone proteins inhibiting the activity of HDACs. Moreover, they modulate the activity of cytoplasmic non-Histone proteins. Given their mechanisms of action and the complex nature of AD, HDACIs, such as Vorinostat, have been proposed for the design of novel MTLs.[4] Indeed, thanks to the peculiar geometry of the HDACs active site, HDACIs could be easily modified equipping them with additional biochemical actions. Consequently, HDACIs could be used to design MTLs with innovative mechanisms of action (Figure 1). The reported examples of HDACI-based MTLs are very promising in terms of anti-AD properties and toxicity.[5,6]

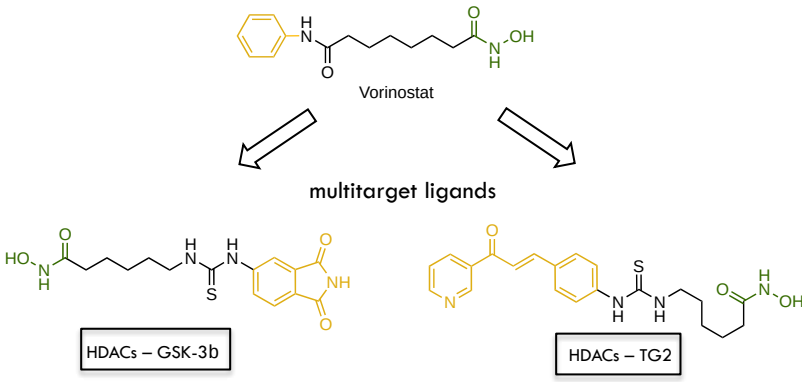

Figure 1

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THE NEW ERA AGAINST NEGLECTED TROPICAL DISEASES: FIGHTING PROTOZOA STRIKING KNOWN AND INNOVATIVE TARGETS

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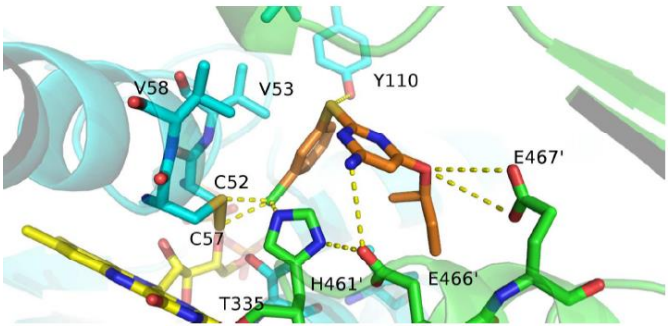
Protozoan infections, including African sleeping sickness, Chagas disease and leshmaniases, are challenging life-threating diseases causing million of deaths annually and are categorized as Neglected Tropical Diseases. The current antiprotozoal therapy is based on a limited number of drugs suffering of poor efficacy, high toxicity. Therefore, there is a strong need to develop new, effective, safe and affordable antiprotozoal drugs. Trypanothione reductase (TR) is an attractive target involved in the redox metabolism of trypanosomatids. Differently from its human homologue GR, TR displays higher affinity towards positively charged substrates. Recently, we discovered RDS 777 as a promising antiprotozoal compound, endowed with micromolar inhibitory activities against a panel of clinically relevant parasites and high affinity towards TR of L. infantum. X-ray structure of LiTR in complex with RDS 777 (see Figure), gave the basis for a rational design of a new class of TR inhibitors. The data coming from the biological assays will be shown and discussed.[1]
A further promising protozoan target is 14α-demethylase (CYP51) enzyme involved in the ergosterol biosynthesis. Depending on parasite species and stages, trypanosomatids show dependence from de novo sterol biosynthesis whose inhibition affect parasitic survival. Ergosterol is also the main sterol in fungi and CYP51 inhibition represents the mechanism of action of currently employed antifungal drugs.
Following a drug-repurposing approach, we discovered a series of in-house azole antifungal compounds as potent antiprotozoal agents targeting CYP51. A few compounds displayed nanomolar activities against T. cruzi in in vitro assays and dramatically reduced parasitemia in T. cruzi mouse model without acute toxicity.[2]



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UNVEALING UNCHARTED POCKETS OF IDO1 FOR NOVEL THERAPEUTIC OPPORTUNITIES

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Indoleamine 2,3-dioxygenase 1 (IDO1) catalyzes the oxidative cleavage of L-Tryptophan (L-Trp) to yield N-formylkynurenine in the first and rate limiting step of the kynurenine pathway.[1] Bioactive metabolites, involved in the regulation of important immunological responses and neurological processes, are then produced by downstream enzymes along the pathway.[2] In this communication, we present results of an integrated approach combining biophysical methods with supervised molecular dynamics (SuMD)[3] and mutagenesis experiments which was aimed at investigating the molecular recognition path of L-Trp to IDO1. Results allow disclosing for the first time high and low affinity dissociation constants of L-Trp to IDO1, and the presence of a metastable interaction site. While casting new lights on structural and dynamics properties of the enzyme, these results support the notion that ligands may exist binding different sites with diverse pharmacological outcomes, as indeed resulting from our screening campaigns. A case study is illustrated for one of such ligand showing therapeutic potential in the treatment of multiple sclerosis.

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ALKYLPHOSPHOLIPID CURES CANINE LEISHMANIASIS. DISCOVERY, PHARMACOLOGICAL CHARACTERIZATION AND MECHANISM OF ACTION THROUGH GENOMIC APPROACH

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Leishmaniasis is a complex of diseases caused by different *Leishmania* species, affecting more than 12 million people worldwide, with 1.6 million new cases each year. Only five drugs are available but they show a sub-optimal profile, safety concerns and/or decreased efficacy. Among these, Miltefosine (MIL) is the only oral drug.[1] Zoonotic infections are critical and Leishmaniasis in particular is particularly severe and affects both animal and humans. Dogs-humans transmission is at risk and over 84millions dogs in Europe, 2.5 are infected, with 700 cases of dog-humans infections estimated in the last year. Cure of the infections is impossible if the dogs reservoir Leishmanisis is not cure, therefore One Health cosndierations should be taken into account when a drug discovery program is approached.[2] During the FP7 project NMTrypI,[3] some leads were identified and among the one was very interesting and could be developed at the final pre-clinical phase.

A novel drug lead (NMT-A2) was identified and pharmacologically characterized. NMT-A2 shows oral availability and low toxicity in the three animal models studied (mice, hamsters and dogs). Compared to MIL, the candidate demonstrates a better pharmacological and therapeutical profile against Visceral Leishmaniasis (VL) in dog trial. NMT-A2 shows impressive decrease of immediate adverse events no hemolytic effects, positive immune stimulation and significant accumulation in target organs, lower toxicity, faster and longer-lasting activity and overall better profile compared with miltefosine.

The drug resistance profile was also studied using a genomic and proteomic approach and revelead that it is

effective against parasites resistant to known anti-leishmania drugs such as antimonials. Structural studies led to the identification of main mutations linked to resistance mechanisms. Mechanism of action investigations suggest that the candidate shows a mutitargeting profile.

Two years dogs follow-up studies, after the end of the treatment, prove that our compound leave the dogs in a healthy status better than for Miltefosine. NMT-A2 improves the oral treatment of canine leishmaniasis and it has a promising profile for human leishmaniasis.

The story of the trial in dogs and follow-up can be found at the below reported YouTube, web address: https://www.youtube.com/watch?v=hkm0ropFR7Y&feature=youtu.be

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INDOLE-BASED PRIVILEGED STRUCTURES VIA MULTICOMPONENT REACTIONS

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Multicomponent Reactions (MCRs) represent nowadays an efficient one-pot synthetic strategy to access complex biologically active scaffolds. Thanks to their convergent nature, atom economy, and efficiency, MCRs are considered straightforward methodologies for both medicinal and organic chemists. Recently, we identified a novel interrupted Ugi reaction starting from an amphoteric sulfonylamino-arylaldehyde, a secondary amine and an isocyanide providing *N*-alkyl- 2,3-diaminoindoles (Figure 1). Surprisingly, the use of pyrrolidine as the secondary amine gave an unexpected organocatalytic triple domino process, switching from *N*-alkyl-2,3-diaminoindoles to 2-iminoisatins (Figure 1). Reaction conditions were hence optimized by performing the reaction on water, while irradiating with an ultrasound bath, in accordance with the green chemistry principles. Additionally, RP HPLC-DAD and UHPLC-HRMS real-time monitoring of the reaction provided experimental data supporting the reaction mechanism.

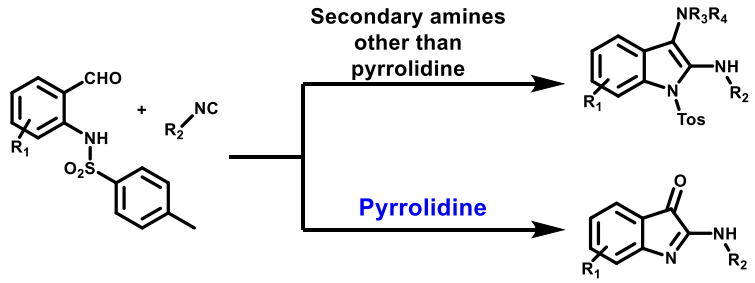

Figure 1. One-pot multicomponent synthesis of *N*-alkyl-2,3-diaminoindoles and 2-iminoisatins.

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BENZOYLPIPERIDINE-BASED COMPOUNDS AS REVERSIBLE, POTENT AND SELECTIVE MAGL INHIBITORS

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Monoacylglycerol lipase (MAGL) is a serine hydrolase with a widespread distribution, since: *1*) it is present in peripheral tissues, cleaving monoacylglycerols into fatty acids and glycerol and, therefore, being involved in the formation of pro-tumorigenic signaling molecules; *2*) MAGL is highly expressed in the central nervous system, in which it is the main responsible of the degradation of 2-arachidonoylglycerol, an endocannabinoid implicated in several physiological processes. Targeting MAGL is considered a valid therapeutic approach to treat many pathological conditions, including several types of cancer.[1] The main drawback of most MAGL inhibitors reported in the literature is their mechanism of action, since they irreversibly inhibit MAGL leading to undesired side effects. A structure-based virtual screening study led to the identification of a reversible MAGL inhibitor bearing a benzoylpiperidine-based scaffold, which was later subjected to a chemical optimization process with the aim of improving both potency and selectivity.[2,3] The best compounds of this class are selective over other hydrolases and cannabinoid receptors, prove to efficiently inhibit MAGL in *in vivo* studies and are also able to reduce proliferation of cancer cells. Recent developments led to the obtainment of nanomolar inhibitors (IC_{50} = 35 - 78 nM, Figure 1), which, to the best of our knowledge, represent the most potent reversible MAGL inhibitors reported so far.

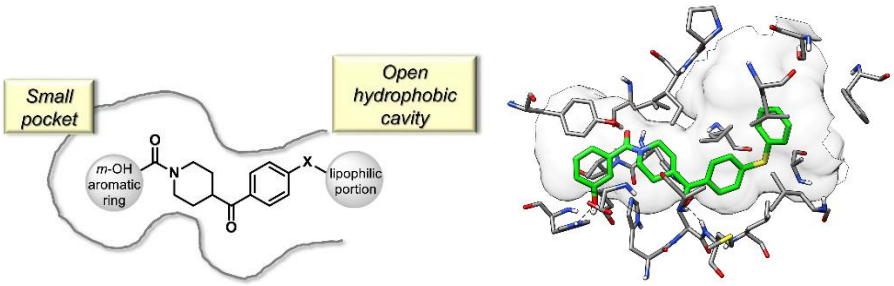

Figure 1. Putative binding disposition of one of the best inhibitors in MAGL active site.

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NANOTECHNOLOGICAL APPROACHES FOR BIOMEDICAL APPLICATIONS

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The family of carbon nanostructures consists of many forms and dominated the last 30 years of studies on nanomaterials, with great potentialities in biomedicine.
Among its components, nanotubes (CNTs) are characterized by a cylindrical structure presenting an inner space and practically consist of rolled up graphene sheets mainly presenting sp^2 carbon, NDs have an sp^3 diamond core and could have a surface with a mixture of sp^2 and sp^3 hybridized atoms, with various functional groups depending on the production methodologies.
One of the most promising applications of these materials is as vector for bioactive molecule and herein we will consider the use of CNTs to intracellular deliver macromolecules into cells and the capability of the tubes to reach mitochondria.
Moreover, the exploitation of the inner cavity of the tubes led to the incapsulation of different materials as radioactive salts, used for both imaging and therapy. Their confinement into the tubes ensured the absence of leakage from the vectors but at the same time reduces the possible chemical approaches to functionalize the nanocapsules, being necessary to avoid any damage on the carbon cage.
Also the preparation and the used of carbon-based nanocapsules, presenting great potentialities in biomedicine, will be presented.

SCOUTING SIGMA RECEPTOR LIGANDS AS NEW TOOLS FOR THE TREATMENT OF NEURODEGENERATIVE DISEASES AND CANCER

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Sigma receptors (σRs) are nowadays recognized as a unique class of membrane receptors divided into two subtypes, σ_1R and σ_2R. σRs regulate a number of physiological functions and their role has been evaluated in many disorders. Deficits in σ_1R are associated with neurodegeneration while their activation may represent a valuable strategy for the treatment of a number of neurodegenerative disorders. Moreover, σ_1R is overexpressed in a variety of cancer cells and selective σ_1R antagonists are reported to modulate cancer cell viability.[1] σ_2R are also highly expressed proliferating tumors. σ_2R agonists are giving promising results in preclinical studies for the treatment of resistant or hardly treatable tumors and σ_2R ligands have been proposed as biomarkers for tumors proliferation.[2] However, the identification of potent and selective ligands and the comprehension of the chemical features behind agonism/antagonism still remain a primary challenge in this field. With this aim, following a ligand-based approach, a library of over 120 ligands have been designed and synthesized over the years, by combining different substituted five-membered heterocyclic rings with appropriate σR pharmacophoric amines. Compounds were tested for σ_1R and σ_2R affinity showing K_i values in the micromolar / sub-nanomolar range, with a selectivity mainly shifted toward the σ_1R. A detailed SAR, supported by molecular modelling, was drawn up. The intrinsic activity was determined *in vivo* for the most promising molecules. According to their profile, σ_1R agonists were tested for neuroprotection, whereas σ_1R antagonists / σ_2R agonists for anticancer activity. Preliminary results in SH-SY5Y neuroblastoma cells showed the ability of some compounds to protect neuronal cells from death induced by four toxicity models.

Cell viability assays were performed on different cancer cell lines to assess the anti-proliferative potential of selected molecules. In particular, dose and time dependent treatments were done on prostate cancer cells, which express higher levels of both σ_1R and σ_2R compared to normal samples. Similarly, we assessed the effect of the compounds on melanoma cells: BS148, a potent and selective σ_2R agonist, showed anti-proliferative activity on immortalized and PDX (metastatic melanoma patient-derived xenografts) cell lines.[3] Confocal microscopy studies with BS148 fluorescent probe revealed the internalization of BS148 within melanoma cells, with a cytoplasmatic localization, mostly in the perinuclear region, according to σ_2R distribution. Finally, to verify whether TMEM97 / σ_2R mediates BS148-antiproliferative activity, we stably overexpressed the TMEM97 gene in HeLa cells: TMEM97-Hela were more sensitive to BS148 anti-proliferative activity compared to control cells, which express endogenous σ_2R levels.

Taken together, these results support the idea that σ_2R is an innovative target in cancer, paving the way for improved tools for cancer diagnosis, monitoring and therapy.

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DISCOVERY OF FIRST-IN-CLASS INHIBITORS OF APOBEC DNA CYTOSINE DEAMINASES

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APOBEC enzymes are a family of single-stranded DNA cytosine-to-uracil deaminases with overlapping protective roles in innate immunity by targeting foreign DNA, thereby promoting its degradation. However, the mutagenic capacities of endogenous APOBEC enzymes are exploited by cancer cells and viruses to enable favorable genomic mutations that contribute to disease progression and the development of drug resistance. Therefore, inhibitors of APOBEC enzymes may ultimately find utilities as combination therapies with existing drugs to slow or prevent the accrual of drug resistance mutations that contribute to failure of the primary therapy. Our research team has utilized high-throughput screening, computational design, and rational design to discover first-in-class inhibitors of multiple APOBEC-family enzymes. This presentation will focus on our efforts to discover novel APOBEC chemical probes that may ultimately find utilities in human medicine.

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5-(1H-IMIDAZOL-2-YL)-4-METHYL-4H-THIENO[3,2-b]PYRROLE DERIVATIVES AS NEW POTENT REVERSIBLE INHIBITORS OF HISTONE LYSINE DEMETHYLASE (KDM1A/LSD1)

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Lysine-specific demethylase 1 (LSD1 or KDM1A) is a FAD-dependent monoaminoxidase enzyme that acts as a transcription corepressor or coactivator by regulating the methylation status of histone H3 lysine K4 and K9, respectively.
Several lines of evidences, which include over-expression in solid and hematological malignancies[1,2] as well as the correlation in certain tumor types between over-expression and poor prognosis,[3] indicate LSD1 as a promising anticancer target.
While the main medicinal chemistry strategy towards LSD1 inhibition is based on the optimization of ligands that irreversibly bind the FAD co-factor within the enzyme catalytic site, we have recently reported a series of thieno-[3,2-b]pyrrole-5-carboxamide derivatives[4] that are novel, potent and reversible LSD1 inhibitors. The SAR exploration around these derivatives led to the discovery of a series of imidazole derivatives endowed with picomolar inhibitory biochemical activity.
Here we report on the identification of these novel LSD1 inhibitors (Figure 1), their preliminary SAR supported by ligand/LSD1-CoRest co-crystal structures, and their biological activity up to evidence of in vivo efficacy in murine leukemia models.

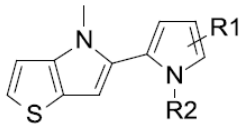

Figure 1

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ACYCLIC BORONIC ACIDS MIMIC BETA-LACTAMASES TETRAHEDRAL INTERMEDIATES LEADING TO BROAD SPECTRUM INHIBITORS

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Worldwide bacterial resistance is mining the efficacy of available antibiotics and the continuous dissemination of pan resistant bacteria represents a real menace to public health.[1] Among the several mechanism of resistance bacteria employ, the production of β-lactamases (BLs) is the prevalent one against β-lactams antibiotics and has rapidly led to BLs with extended spectrum of action, e.g. the carbapenemases BLs able to inactivate last resort carbapenems.[2] β-lactamases include enzymes which utilize serine for β-lactam hydrolysis (SBLs) and class B metalloenzymes (MBLs) which require divalent zinc ions for substrate hydrolysis. While inhibitors are available in therapy for SBLs, for MBLs no inhibitor have been at the present approved.[3] As a consequence, infections caused by bacteria strains coproducing different type of BLs and carbapenemases result not susceptible to available antibiotics. The design of a BLs inhibitors (BLI) active against SBLs and MBLs despite its attractiveness, is challenged by the targets structural and mechanistic peculiarities. A chance of success is however represented by compounds able to mimic those tetrahedral intermediates common to all BLs.

Among recently disclosed novel not β-lactam like BLI, boronic acids (BAs) BAs demonstrated their potentiality in the design of pan-spectrum BLI.[4] Following this direction, we recently disclosed a small library of benzo[b]thiophen-2-ylboronic acids as the first acyclic BAs active against all four BLs classes and with biological activity *vs* clinical strains (**Figure 1**).

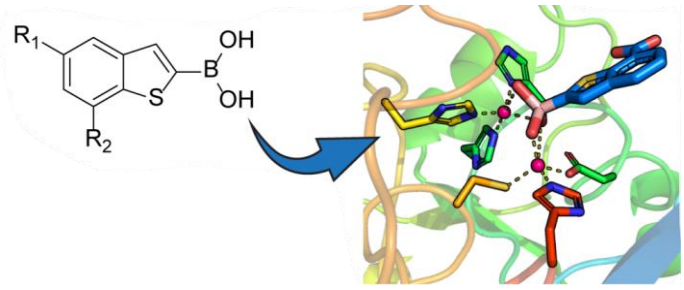

Figure 1. Boronic acids mimic natural NDM-1 tetrahedral intermediate, disrupt the active nucleophile and contribute to Zn coordination.

The ability of these compounds to inhibit structurally and mechanistically different types of BLs has been structurally investigated depicting the role played by the boronic group in driving molecular recognition, especially in the interaction with MBLs.[5] The evidence our derivatives mimic the tetrahedral intermediates is of particular interest and explains their ability to overcome active site architecture peculiarities along BLs classes. They represent a productive route toward potent broad-spectrum inhibitors.

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NEW STILBENE-AMMONIUM BASED NICOTINIC LIGANDS: INSIGHTS INTO ALPHA9-10 AND ALPHA7 NACHR RESPONSES

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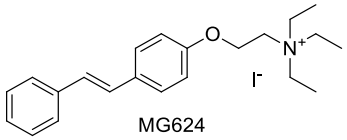
Homomeric α9-α10 and α7 nicotinic acetylcholine receptors (nAChRs) are overexpressed in several extra-neuronal tumours, like non-small-cell lung carcinomas, small cell lung carcinomas, gliomas and glioblastomas. Whereas nicotine induces the hyperproliferation of tumoral cell lines expressing the α9-α10 and α7 subtypes, α9-α10 and α7 specific antagonists like α-bungarotoxin, methyllycaconitine or selective conotoxins revert it.[1] MG624 (Figure 1) is a known nicotinic ligand, able to block agonist-evoked currents at the α9-α10 and α7 subtypes. We recently demonstrated that the elongation of its ethylene linker provides ligands with higher potency in blocking the agonist-evoked currents and with a high anti-adenocarnicoma and anti-glioblastoma activity, which increases with the length of the linker.[2,3]



MG624

Figure 1

On a continuation of the project, aimed at identifying key structural features modulating α9-α10 and α7 affinities and activities, MG624 has been modified at the ammonium head, at the oxyethylene linker and at the stilbene scaffold. In detail, the triethyl ammonium group of MG624 was replaced with other quaternary ammonium heads, to probe the steric requirements of the binding sites. Then, the oxyethylene bridge was incorporated within constrained alicyclic rings, to investigate the role of the linker flexibility. Furthermore, the geometry, the planarity and the electronic properties of the stilbenic core were assessed by replacement with other aromatic systems.

All these analogues were tested in radioligand binding assays at the α7 subtype and in electrophysiological functional assays at the α9-α10 and at the α7 nAChRs. Selectivity against other nicotinic subtypes, such as α4β2 and α3β4, was considered.

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UNEXPECTED ACTIVITY OF BROMIPHEN AGAINST PROKARYOTIC AND EUKARYOTIC INFECTIOUS AGENTS

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Control and prevention of infectious diseases are perpetual and global challenges above all when considering the increasing of antimicrobial resistance. To prevent infection spreading, disinfection and hygiene habits are crucial, especially when the microorganism can persist and survive on textiles, surfaces and medical devices[1]. Such measures become particularly relevant in healthcare-associated environments, to avoid that infections occur in patients under medical care. The WHO estimates that about 15% of all patients suffer from these infections, prolonging their hospital stay and worsening their underlying clinical condition. Nosocomial infections, which are due to different pathogens including bacteria, viruses and fungi, represent an important financial burden for health care systems. To support the extensive infection control programs already applied by the hospitals, there is an increasing demand for novel and improved disinfectants active against a wide range of potential pathogens.

Cationic agents are an important class of antinfectives widely used for environmental infection control. In particular, quaternary ammonium compounds (QACs) are largely recognized as antiseptics and disinfectants. During our recent study[2,3] regarding the stability of domiphen bromide, which belongs to the QACs family, *p*-bromo-domiphen bromide (bromiphen bromide)[4] came out as by product due to the unpredictable reactivity of the parent compound. Surprisingly, the “undesired” bromiphen bromide showed a comparable, if not better, activity on bacteria respect to the domiphen bromide and a better activity on yeasts.

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UNVEILING TARGET ASSOCIATIONS FOR POLYPHARMACOLOGY FROM ANALYSIS OF CRYSTALLOGRAPHIC LIGANDS IN THE PROTEIN DATA BANK

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The design of a single drug that specifically modulates a set of disease-related targets (*i.e.*, polypharmacology) is gaining increasing consideration in current drug discovery.[1]
Indeed, the polypharmacology approach allows to favorably exploit additive and synergistic multi-target activities, circumventing unwanted or unexpected issues potentially associated to single-target or combination therapies (*e.g.*, drug resistance, toxicity and side effects).[2]
Although the selection of appropriate target associations has a key role in the design of polypharmacological modulators, data reported on structural databases have not yet been systematically explored to identify promising target combinations for polypharmacology. Here, a systematic analysis of the Protein Data Bank (PDB), which provides experimentally-validated structural information on targets and ligands, was conducted to identify novel multi-target possibilities for which the *in silico* drug design could benefit of the available crystal structures and activity data. To this aim, a workflow integrating different ligand- and structure-based *in silico* approaches was performed in order to analyze crystal structures of human proteins reported in the PDB. Moreover, analyses of structural, bioactivity and therapeutic data reported in public databases, and comparisons with the obtained in silico results were also performed to identify promising target associations for polypharmacology.
A number of target combinations with potential therapeutic value were identified, the modulation of some of them having also recognized synergistic effects standing on current literature. Moreover, to provide a proof of concept of the validity of the proposed *in silico* approach, one of the identified target associations was experimentally validated, leading to the identification of a CHK1/EGFR dual ligand with room for medicinal chemistry optimization.

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THE UNDERSTANDING OF THE MECHANISM OF ACTION OF PYRIDOBENZOTHIAZOLONES REVEALS MUTATIONS IN THE 3'-UTR: IDENTIFICATION OF A COMPOUND PROTECTING MICE FROM DENGUE-2 LETHAL INFECTION

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Belonging to Flaviviridae, the Flavivirus genus consists of arthropod-borne RNA viruses which include important human pathogens like Dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Yellow fever virus (YFV), Zika virus (ZKV), and tick-borne encephalitis virus (TBEV).[1] Flavivirus infections cause from mild flu-like symptoms to hemorrhagic fevers, hepatitis, and neuropathies depending on the infective agent.[2-4] In particular, an estimated 40% of the world is at risk of dengue fever, with around 390 million infections per year. WHO has included Dengue and Zika among the ten threats to global health in 2019.[5] Although several flavivirus inhibitors mainly targeting viral proteins have been discovered, no drugs are available while few specific vaccines show limitations such as poor efficacy and population coverage. Hence, the identification of anti-flavivirus compounds is a hot-topic in drug discovery.

We identified a series of potent, selective and broad-spectrum anti-flavivirus pyridobenzothiazolones able to inhibit the cellular replication of most relevant pathogens such as DENV, ZIKV, WNV, YFV and JEV. Structurally, the best derivatives are characterized by the presence of an aminoacyl pendant bound to the heterocyclic scaffold thorough an amide link. Although pyridobenzothiazolones inhibit the isolate NS5 RNA-dependent RNA polymerase (RdRp), the compounds do not affect viral RNA synthesis in cell but strongly impair virions infectivity.[6,7]

In this contribution, with the main aim to definitively demonstrate the mechanism of action for this compound class, we designed and synthesized a new series of derivatives mainly characterized by structural modifications of the amide region; meanwhile a size reduction and opening of the central pyridobenzothiazolone core was also realized. The new aminoacyl derivatives showed good antiviral activity and NS5 RdRp inhibition. Interestingly, docking studies on the RdRp thumb region suggested the compounds binding to hot-spots relevant in the NS5 RdRp/NS3 protease interaction and this property reflects on potent protein-protein inhibition in an experimental model. One compound (**HeE1-2d**) was fruitfully employed to identify escape resistant mutants possessing three main alterations in the 3'-UTR genome sequence. Thanks to these relevant findings, **HeE1-2d** was tested in an in-vivo DENV-2 lethal mouse model[8] and we observed a 100% protection with ½ log reduction in viremia. Pyridobenzothiazolones thus represent one of the most potent and well characterized classes of anti-flavivirus agents.

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DESIGN AND RECEPTOR INTERACTIONS OF A1 ADENOSINE RECEPTOR AGONISTS IN THE NATIVE RIBOSE AND CONFORMATIONALLY-CONSTRAINED (N)-METHANOCARBA SERIES

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A_1 adenosine receptor (A_1AR) agonists have a therapeutic interest, given their antiarrhythmic, antilipolytic, antinociceptive, cerebroprotective, cardioprotective, antidepressant, sleep-enhancing and antiseizure properties. It has recently emerged that A_1AR activation in the brain induces hypothermia, independently from peripheral A_3AR activation, with consequent potential clinical applications, such as cerebroprotection in brain ischemia. Most of the available A_1AR agonists have cardiovascular effects that make them poor drug candidates, so there is a need for new A_1AR agonists and partial agonists. In this work, new (N)-methanocarba ([3.1.0]bicyclohexyl) adenosines and corresponding ribosides were synthesized and their binding affinity to ARs determined to identify novel A_1AR agonists for central or peripheral applications. Different N_6, C2 and C5' substitutions were explored, positioning cycloalkyl groups at N_6 position, which typically increase A_1AR selectivity. On the basis of in vitro and in vivo ADME-tox testing, the N_6-dicycloalkyl ribose agonist (compound MRS7469) turned out to be more than 2000-fold selective for A_1AR in two species and drug-like. Selected analogues were tested for the ability to induce hypothermia in mice, and most of them were inactive or weak, despite mA_1AR full agonism and variable mA_3AR efficacy. MRS7469 was able to induce strong hypothermia, which was dependent only on A_1AR activation, without a contribution from A_3AR (determined using A_1AR or A_3AR knockout mice). Molecular modeling enabled us to suggest a putative binding mode of MRS7469 at the hA_1AR orthosteric site, and the stability of the complex was investigated through molecular dynamics simulations. Conserved hA_1AR interactions were preserved according the suggested model.
In conclusion, in this work novel ribose and methanocarba nucleosides able to potently activate A_1AR were identified and characterized in vivo and computationally.[1]

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TRPM8 CHANNEL MODULATORS: IDENTIFICATION OF A SELECTIVE SUPERANTAGONIST BY STRUCTURAL SIMPLIFICATION AND HIT REFINEMENT APPROACHES

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Transient receptor melastatine-8 (TRPM8) is a non selective Ca^{2+} permeable transmembrane cation channel, expressed in different tissues and involved in a wide range of physiological and pathophysiological processes, such as pain and allodynia, cancer, overactive and painful bladder syndromes, dry eye syndrome.[1,2] These findings underscore the high therapeutic potential of this target and the efforts made in pursuing the development of selective TRPM8 modulators. Starting from the evidences that tryptamine represents a privileged scaffold for the design TRPM8 ligands,[3,4] we decided to synthesize a small library of tryptamine-based derivatives (Figure 1) to explore the structural requirements determining TRPM8 modulation. Using a structural simplification approach a set of tryptamine based TRPM8 agonists and antagonists was designed from known naturally derived TRPM8 modulators. The pharmacological properties of the novel derivatives were evaluated by HTS fluorescence-based screening assay and patch-clamp recordings. Molecular modelling studies led to the generation of a new homology model of TRPM8 that was used to rationalize pharmacological results, to highlight SAR clues and to identify the chemical features discriminating TRPM8 agonists from antagonists. The homology model was also used for in silico driven hit refinement approach, leading to a second set of tryptophan-based TRPM8 antagonists. These molecules were characterized in vitro for their potency, efficacy and selectivity, leading to the identification of the most potent and selective TRPM8 antagonist described so far (IC_{50} 0.2 ± 0.2 nM, Figure 1). Moreover, in vivo, the antagonist showed significant target coverage in both an icilin-induced WDS and oxaliplatin-induced cold allodynia mice models. The results obtained confirmed that the tryptophan moiety provides a new pharmacophoric scaffold for the design of highly TRPM8 potent modulators.

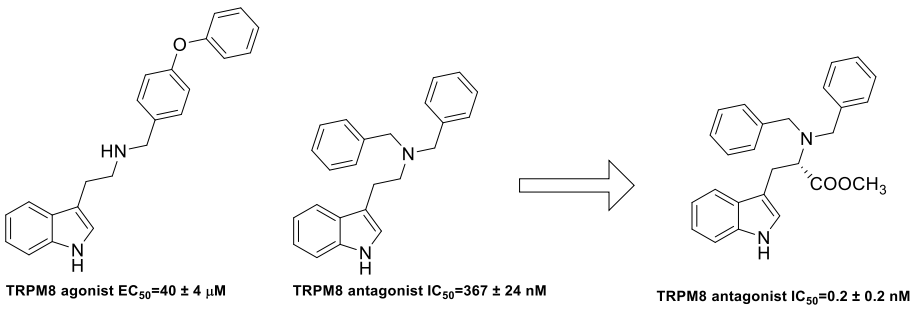

Figure 1. Exemplary structures of the I and II series of TRPM8 modulators.

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IDENTIFICATION OF A NOVEL QUINOLINE-BASED DNA DEMETHYLATING COMPOUND HIGHLY POTENT IN CANCER CELLS

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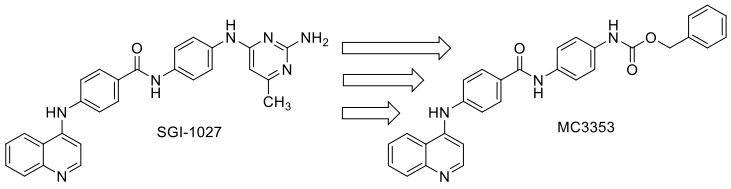
Background: DNA methyltransferases (DNMTs) are epigenetic enzymes involved in embryonic development, cell differentiation, epithelial to mesenchymal transition and control of gene expression, whose overexpression or enhanced catalytic activity has been widely reported in cancer initiation and progression.[1] To date, two DNMT inhibitors (DNMTi), 5-azacytidine (5-AZA) and 5-aza-2'-deoxycytidine (DAC), are approved for treatment of myelodysplastic syndromes and acute myeloid leukemia. Nevertheless, they are chemically instable and quite toxic for healthy cells, thus the discovery of novel DNMTi is urgent.[1,2]



Scheme 1. Design of MC3353

Results: We will present the identification of a new quinoline-based molecule, MC3353, as a non-nucleoside inhibitor and downregulator of DNMT. The design of MC3353 is based on the known non nucleosidic DNMTi SGI-1027 replacing the 4-methyl-2,6-diaminopyrimidine moiety of the template with a benzyl carbamate function. This compound was able, in promoter demethylating assays, to induce enhanced green fluorescence protein (EGFP) gene expression in HCT116 cells and transcription in a cytomegalovirus (CMV) promoter-driven luciferase reporter system in KG-1 cells. Moreover, MC3353 displayed strong antiproliferative activity when tested on HCT116 colon cancer cells after 48 h of treatment at 0.5 µM. At higher doses, this compound provided a cytotoxic effect in double DNMT knockout HCT116 cells. MC3353 was also screened on a different panel of cancer cells (KG-1 and U-937 acute myeloid leukemia, RAJI Burkitt's lymphoma, PC-3 prostate cancer, and MDA-MB- 231 breast cancer), where it arrested cell proliferation and reduced viability after 48 h of treatment with IC50 values ranging from 0.3 to 0.9 µM. Compared to healthy cell models, MC3353 induced apoptosis (e.g., U-937 and KG-1 cells) or necrosis (e.g., RAJI cells) at lower concentrations. Importantly, together with the main DNMT3A enzyme inhibition, MC3353 was also able to downregulate the DNMT3A protein level in selected HCT116 and PC-3 cell lines. Additionally, this compound provided impairment of the epithelial-to-mesenchymal transition (EMT) by inducing E-cadherin while reducing matrix metalloproteinase (MMP2) mRNA and protein levels in PC-3 and HCT116 cells. Last, tested on a panel of primary osteosarcoma cell lines, MC3353 markedly inhibited cell growth with low single-digit micromolar IC50 ranging from 1.1 to 2.4 µM. Interestingly, in Saos-2 osteosarcoma cells, MC3353 induced both expression of genes and mineralized the matrix as evidence of osteosarcoma to osteoblast differentiation.

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PRECLINICAL STUDIES ON THE ANTITUMOR ACTIVITY INDUCED BY NOVEL OXADIAZOLE TOPSENTIN ANALOGS AGAINST PANCREATIC CANCER CELLS

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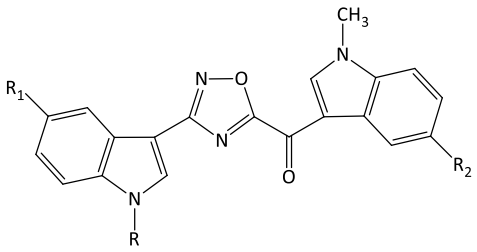
Pancreatic ductal adenocarcinoma (PDAC) accounts for the majority of pancreatic malignancies and is one of the most lethal solid malignancies worldwide, mostly because of its metastatic spread and multifactorial resistance to chemotherapy. In order to improve the outcome of pancreatic cancer, in this study we conveniently synthesized a new series of topsentin analogs (Figure 1), a well-known marine alkaloid endowed antitumor activity.[1]



R = H, CH_3; R_1 = R_2 = H, Br, F, OCH_3

Figure 1. Structure of the new series of topsentin analogs.

Growth inhibition assays showed that these compounds inhibited cell growth of human pancreatic cancer cell lines (SUIT-2, PANC-1 and CAPAN-1) within micromolar concentrations, as confirmed by the range of IC_{50} values, from 0.4 to 7.1 μM. The compounds' ability to interfere with cell cycle was evaluated through cell cycle analysis with cytofluorimetry, and a significant increase of G2/M phase was observed, highlighting an additional induction of apoptotic cell death. Furthermore, scratch wound healing assay was used to evaluate whether oxadiazole analogs could interfere with cell invasiveness; the results obtained from this assay demonstrated that two of these compounds were capable of interfering with cells' ability to migrate and recover from wounds, as assessed by the reduced cell migration of CAPAN-1 cells by 40-50%. Finally, the over-expression of MMP-9 and SNAIL2 genes suggests the induction of feedback mechanisms to counteract the anti-migration and anti-proliferative activity of these compounds.
Together, all this data showed the activity of our new analogs against PDAC cells, revealing their ability to interfere with cell proliferation, induce apoptosis and reduce invasiveness.
In conclusion, the findings of the present study provide evidence of the activity of neo-synthetic compounds against PDAC cells, offering a novel tool for optimizing chemotherapy against this tumor.

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TARGETING TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 (TRPV1) CHANNEL SOFTLY FOR TOPICAL TREATMENT OF INFLAMMATORY SKIN DISORDERS

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TRPV1 is arguably the best-characterized member of the transient receptor potential (TRP) family: it is a calcium permeable nonselective ion channel gated by noxious heat, extracellular protons, and bioactive lipids. TRPV1 is also activated by vanilloid substances, among which capsaicin (Fig. 1), an active component of chili peppers. Capsaicin is used as an analgesic, in topical ointments and dermal patches, to relieve pain.

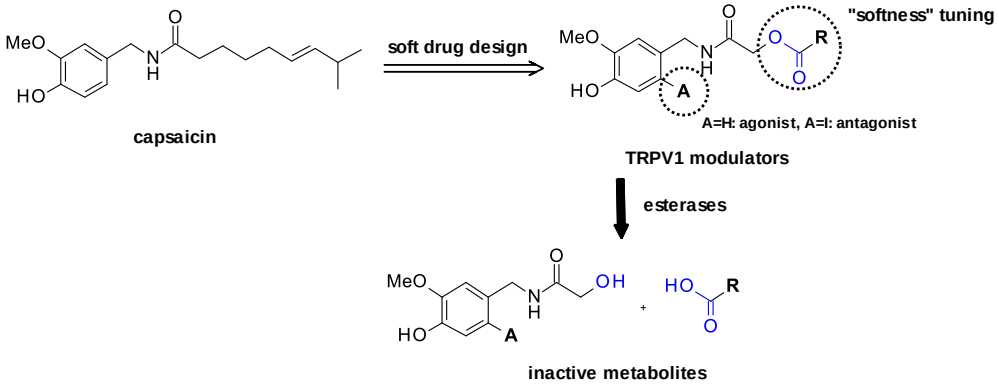

Figure 1

Early studies on TRPV1 modulators were centered on agonists. They lead to the initial opening of TRPV1, followed by desensitization of the channel which is linked to their therapeutic effects. Due to this mechanism, the most notorious clinical limitation of capsaicin and related vanilloids is the TRPV1-coupled acute excitation, which results in a marked burning sensation and reduced patient compliance. Besides, capsaicin is a very lipophilic, non-water soluble compound and can reside in human skin relatively unchanged for a long period in the stratum corneum leading to frequent erythema reactions depending on drug concentration. Recently, it has also been reported that chronic, long-term topical application of capsaicin increases skin carcinogenesis in mice treated with a tumor promoter.

The insertion of an ester group in the lipophilic chain of capsaicinoids (by the Passerini multicomponent reaction) afforded both agonists and antagonists that retained TRPV1 modulating activity being susceptible to hydrolysis in skin, blood and liver while forming inactive metabolites. Thus, “softness” of the capsaicinoids - intended as hydrolytic susceptibility - was proven to be finely tuned by changing the nature of the acyl substructure.[1] The most promising antagonist (A= iodine, R= undecyl linear chain - Fig. 1) showed *in vivo* anti-nociceptive activity on pruritus and hyperalgesia without producing hyperthermia, thus validating it as novel treatment for dermatological conditions that implicate TRPV1 channel dysfunction.[2]

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BIO-INSPIRED G-QUADRUPLEX BINDERS POWERED BY MULTICOMPONENT REACTIONS

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In the search for new drug-like selective G-quadruplex binders, a bio-inspired design focused on the use of nucleobases as synthons in a multicomponent reaction (MCR) proved to be viable and successful. Hence, a new class of multi-functionalized imidazo[2,1-*i*]purine derivatives, easily synthesized with a convergent approach, allowed the identification of the first dual *BCL2*/*c-MYC* gene promoter G-quadruplex ligand. Biophysical studies along with biological investigations have been carried out to assess the potency and to characterize the binding mode of the newly identified lead compound. The absence of toxicity towards normal cells, together with the small molecular weight (≅ 500), the water solubility, the ease of functionalization, and the selectivity profile are promising and desirable features to develop G-quadruplex binders as safe and effective anticancer agents.

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TARGETING CHAGAS DISEASE USING FRAGMENT-BASED LEAD DISCOVERY: VALIDATION AND SCREENING OF FPPS ENZYME

Opassi, G.;[a] Nordström, H.;[a,b] Lundin, A.;[c] Napolitano, V.;[d] Magari, F.;[e] Dzusz, T.;[a] Klebe, G.;[e] Jahnke, W.;[f] Danielson, U. H.[a,b]

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Fragment-based lead discovery (FBDL) is one of the most efficient approaches when comes to the exploration of the available chemical space. The restricted molecular weight of fragment molecules (< 300 Da) allows a superior sampling of the chemical space using libraries containing few but structurally diverse compounds. Principal requirement for screening fragment libraries is the use of sensitive biophysical methods for the identification of hit compounds which interact with the target. The weak affinity that typically fragments have for the target of interest is actually one of the major challenges in this approach. A successful screening therefore implies the use of very sensitive methods, high quality preparations of the target protein and an extensive understanding of its physicochemical properties under various conditions. Here we described a Surface Plasmon Resonance (SPR) biosensor-based driven fragment-based discovery of novel leads targeting farnesyl pyrophosphate synthase (FPPS) from *Trypanosoma Cruzi*, causative agent of the neglected infectious tropical disease known as Chagas Disease. A panel of orthogonal biophysical methods have been initially applied to confirm folding, structural homogeneity and thermal stability of the produced enzyme. A real time luminescence-based enzymatic assay for pyrophosphate detection was developed and used to confirm FPPS activity. A 90 fragments library was screened using SPR against FPPS from both human and *Trypanosoma*, resulting in the identification of few selective hits. The study resulted in the validation of FPPS from *Trypanosoma Cruzi* as a suitable target for FBDL and in the identification of weak but selective hits for the parasitic enzyme.

TARGETING DNA G-QUADRUPLEX STRUCTURES: TOWARD THE DEVELOPMENT OF NEW ANTICANCER AGENTS

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G-quadruplexes (G4s) are DNA secondary structures constituted of two or more stacked guanine tetrads, which can form within G-rich tracts of human genomic DNA. The interest in these non-canonical DNA structures as targets for novel and selective anticancer drugs is exponentially growing, due to their regulatory role in basic nuclear functions. Indeed, G4-promoting sequences have been mapped at key regulatory genomic sites such as oncogene promoters and telomeres, and much effort is currently underway to discover effective and selective ligands.[1] This study is focused on hydrazone-based compounds, designed as analogues of a promising lead[2] which was recently proved to stabilize G4s and simultaneously increase R-loop levels in human cancer cells.[3] Some analogues turn out to be potent G4 ligands with high selectivity over duplex DNA and preference for one G4 topology over others. Interestingly, the only mono-hydrazone of the series proved to be the most selective, indeed it significantly stabilized only the c-myc G4 and has been shown to trap G4 structures in the nucleus of cancer cells (Figure 1). This behavior correlated with efficient cytotoxic activity in human osteosarcoma cells.[4] In 2014, another approach led us to study bis-indolinone derivatives able to selectively stabilize G-quadruplex over duplex DNA.[5] These molecules were formed by an aromatic core linked with two indolinone moieties. Molecular docking calculations showed that only one indolinone moiety is involved in specific interactions with the target. These findings have inspired us the design of new G-quadruplex binders which can be viewed as "hybrid" molecules characterized by an aromatic core linked with only one indolinone nucleus, whereas the other indolinone was substituted by an iminoguanidine or a hydrazinylimidazoline.[2,4] The G4 binding properties of the new compounds have been evaluated by CD-melting experiments employing both human telomeric and oncogene promoter G4s with different folding topologies. On the basis of the results obtained, one derivative has been selected to investigate the effects in human cancer cells. Specifically, nuclear G4 structures have been visualized by immunofluorescence microscopy using the BG4 antibody, which is specific against G4s, and the cytotoxic activity has been determined against U2OS and HeLa cells.

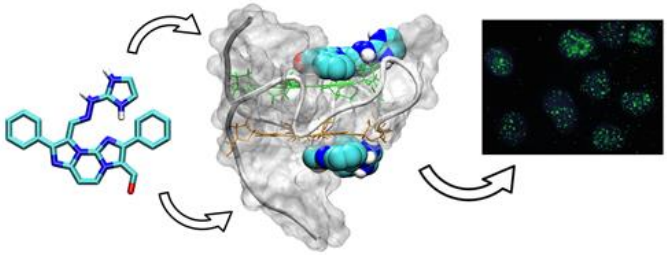

Figure 1

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ANOPHELES GAMBIAE 3-HYDROXYKYNURENINE TRANSAMINASE: LC-MS/MS ACTIVITY AND INHIBITION STUDY OF NEW POTENTIAL INHIBITORS

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In *Anopheles gambiae*, the most efficient vector of malaria parasite *Plasmodium falciparum*, 3-hydroxykynurenine (3-HK) represents a toxic metabolite. In the adult mosquitoes, the excess of this toxic metabolite is removed by a specific 3-HK transaminase (*Ag*-HKT) which converts the 3-HK into the more stable xanthurenic acid. Interfering with 3-HK metabolism in *A. gambiae* is a potential approach for the development of transmission-blocking drugs and insecticides. LC-MS/MS method was optimized for the evaluation of the kinetic parameters of *Ag*-HKT and the kinetic characterization of inhibitors of the enzyme. An enzymatic assay was set up by using the recombinant *Ag*-HKT. The chromatographic separation of analytes was performed in a gradient mode on a Synergi column, using an acidic mobile phase. Mass spectrometric detection was achieved with an ion trap equipped with an ESI source, in positive ionization scan, operating in SRM mode. The LC-MS/MS method was used for the determination of kinetic constants values for *Ag*-HKT (Km and Vmax). Moreover, this selective method was applied for the evaluation of *Ag*-HKT inhibition by using a compound whose synthesis was previously described in the literature (INI) and a new promising inhibitor (3-OH-INI). The results obtained for 3-OH-INI revealed a competitive mechanism of action and a Ki value that suggested a more potent activity in respect to INI [1].
Given the interest to identify novel and more potent *Ag*-HKT inhibitors, we have decided to make further changes to the structure of 3-HK preparing other Cα-desaminated analogues and conformationally restricted derivatives. 25 new compounds were tested in terms of inhibition using the new optimized and validated LC-MS/MS method. The flow chart of the work and the structures are reported in **Figure 1**.

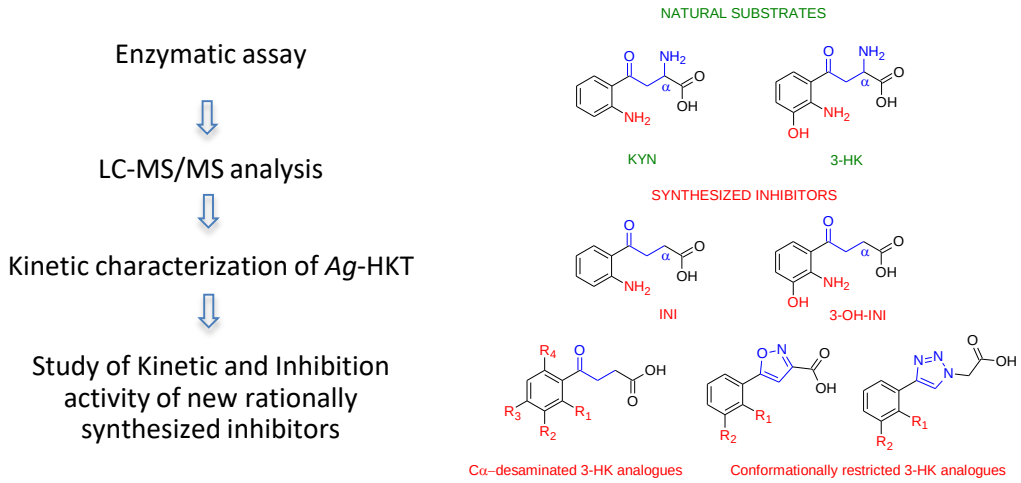

Figure 1

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FIRST ASYMMETRIC SYNTHESIS OF NMS-P528, A NEW HIGHLY PROMISING AGENT FOR ANTIBODY-DRUG CONJUGATES GENERATION

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Traditional cancer chemotherapy widely used in clinic is often accompanied by systemic toxicity to the patient with narrow therapeutic windows. Approaches aimed at selectively delivering the drug to the site of action, sparing healthy tissues, are of great relevance nowadays, taking also advantage from the development of nanotechnologies.
A wide spectrum of low- and high-molecular-weight carriers can be used for targeting anticancer drugs and, amongst others, antibody-drug conjugates (ADCs) are increasingly employed in different oncology settings. ADCs are targeted cancer medicines that deliver a payload of a cytotoxic chemotherapy directly to cancer cells via a linker attached to a monoclonal antibody that binds to a specific target expressed on cancer cells.
At present, there are more than sixty ADCs in clinical evaluation and more than 60% of the antibodies result to be conjugated to auristatin or maytansine, two well-known tubulin binding agents. New toxins with a different mechanism of action in cells, possibly acting also on not proliferating or slowly proliferating cells are therefore strongly needed.
Duocarmycins (antibiotics including yatakemycin, and CC-1065) are DNA minor groove alkylating agents and several semisynthetic derivatives of CC-1065 evaluated in early clinical trials (1992-94) resulted highly toxic. Their use as warheads for antibody conjugation (as MDX-1203 or more recently as SYD985 in clinical evaluation) besides their high cytotoxic potency show tendency to induce antibody aggregation once conjugated. In this context, we approached a new proprietary class of alkylating agents, the thienoindole derivatives, with the aim to obtain new and highly potent toxins with the proper physico-chemical profile suitable for the generation of non-aggregating ADCs.
Here we describe the design, the synthesis and related proof of concept studies for this novel chemical series, characterized by both potent antitumor activity and physicochemical properties highly compatible with deployment as antibody payloads. Optimisation of this class of agents and in vitro profiling led to the selection of a potent cytotoxic compound (NMS-P528). Extensive process research has been completed for the production of NMS-P528, resulting in establishment of an efficient stereoselective method for preparation of GMP material on 100 g scale.
In summary, we have identified a novel class of toxins, the thienoindoles, characterized by both potent antitumor activity and physicochemical properties highly suited to deployment as antibody payloads.

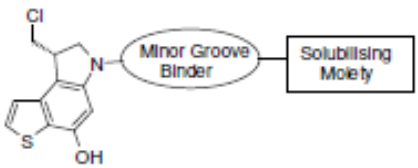

Figure 1. NMS-P528

CHEMICAL CHARACTERIZATION AND EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS FROM FIBRE-TYPE *CANNABIS SATIVA* L. (HEMP)

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Volatile terpenes represent the largest group of *Cannabis sativa* L. components and they are responsible for its aromatic properties. Even if many studies on *C. sativa* have been focused on cannabinoids, which are terpenophenolics, little research has been carried out on its volatile terpenic compounds. In the light of all the above, the present work was aimed at the chemical characterization of seventeen essential oils from different fibre-type varieties of *C. sativa* (industrial hemp or hemp) by means of GC-MS and GC-FID techniques. In total, 71 compounds were identified and the GC analysis revealed that α- and β-pinene, β-myrcene and β-caryophyllene are the major components in all the essential oils analyzed. In addition, a GC-MS method was developed here for the first time and applied to quantify cannabinoids, and in particular cannabidiol (CBD), in hemp essential oils.

The antibacterial activity of hemp essential oils against some pathogenic and spoilage microorganisms isolated from food and food processing environment was also determined. The inhibitory effects of the essential oils were evaluated by both the agar well diffusion assay and the minimum inhibitory concentration (MIC) evaluation. By using the agar diffusion method and considering the zone of inhibition, it was possible to preliminarily verify the inhibitory activity on most of the examined strains. The results showed a good antibacterial activity of six hemp essential oil against the Gram+ bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and against *Bacillus* spp. spoilage bacteria.

The antimicrobial activity of pure CBD and of the major terpenes detected in hemp essential oils was also evaluated in the present study in comparison with conventional antibiotics. In general, all the pure compounds tested in this work exhibited an antibacterial activity towards the strains considered. In particular, a good activity was observed for CBD and for the monoterpenes (α-pinene, β-pinene and β-myrcene) especially toward *Listeria* and *Enterococcus* strains. For what concern *Staphylococcus* and *Bacillus* strains, all the tested compounds exhibited MIC values higher than those of common antibiotics, with the exception of α-pinene and β-pinene, which showed lower MIC values than amoxicillin against *Bacillus cereus*.

By focusing on the chemical composition-bioactivity relationships, it is difficult to identify the compounds responsible for the antimicrobial activity of the essential oils tested. The observed antibacterial activity of hemp essential oil may probably arise from a synergism established among the different compounds of this rich phytocomplex. Further studies on synergistic activity of pure compounds are necessary in this ambit.

In conclusion, the results obtained in this study demonstrate that hemp essential oil can inhibit or reduce bacterial proliferation, thus proving to be a valid support to reduce microorganism contamination, especially in the food processing field.

INTEGRINS & P53: DUAL-TARGETING PEPTIDOMIMETICS TO CHALLENGE GLIOBLASTOMA MULTIFORME

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α5β1 integrin and p53 are convergent pathways in the control of glioma apoptosis.[1,2] In particular, the inhibition of the α5β1 integrin signalling pathway can represent a new opportunity to overcome the p53-dependent resistance to temozolomide (TMZ) in Glioblastoma Multiforme (GBM) cells. In fact, selective ligands of the α5β1 integrin reactivate the p53wt function inducing apoptosis in TMZ-treated cells, whereas p53-MDM2/4 interaction inhibitors dramatically reduce the expression of α5 integrin in p53wt GBM. These outcomes prompted us to seek a molecule able to simultaneously modulate both target families.
Indeed, in a recent virtual screening campaign against our in-house database, we found that Arg-Gly-Asp (RGD)-mimetic molecules can act as dual MDM2/4 inhibitors. Thus, our presentation will focus on an optimization campaign of RGD-mimic molecules based on the general structure depicted in Figure 1. The study culminated in the discovery of one compound acting as a potent MDM2/4 and α5β1/αvβ3 blocker.[3] Additionally, NMR and modeling studies defined the molecular basis of interaction with its targets.

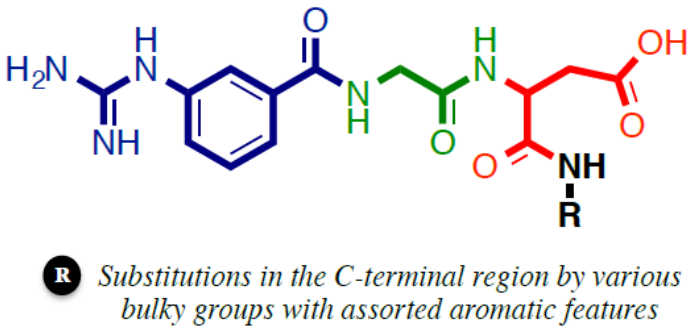

Figure 1. General structure of novel peptidomimetics potentially acting on both RGD-integrins and p53 axes.

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DELVING INTO SPIRULINA PLATENSIS PEPTIDOME: DISCOVERY OF A NOVEL DECAMERIC PEPTIDE WITH POTENT IN VIVO ANTIHYPERTENSIVE ACTIVITY

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Arthrospira Platensis, better known as "Spirulina" is the top-sell product in the nutraceutical market. This blue-green microalga possesses exceptional nutritional properties, in particular a high protein content.[1] While numerous scientific evidences point out the healthy properties of peptides deriving from the protein fraction of this microalga little is known about the bioavailability of the resulting peptides after ingestion, and hence, their bioactivity is far from be unveiled. To highlight these questions, in this work the protein fraction of Spirulina was subjected to in vitro simulated gastro-intestinal digestion, and the resulting digest was characterized by a multi-step peptidomic workflow based on the combination of untargeted and targeted mass spectrometry methods. A novel decameric peptide was identified which exerted direct endothelium-dependent vasodilation of ex vivo vessels, an effect occurring via a PI3K/AKT pathway converging on NO release. In vivo, administration of SP6 evoked a significant hemodynamic effect, reducing blood pressure, an action absent in eNOS (endothelial nitric oxide synthase)-deficient mice. Finally, in an experimental model of arterial hypertension, SP6 exerted an antihypertensive effect, improving endothelial vasorelaxation associated with enhanced serum nitrite levels. Based on our results, this novel decameric peptide may extend the possible fields of therapeutical application for spirulina-derived peptides

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DIRECT PREPARATION OF MELATONIN DERIVATIVES VIA C-H ACTIVATION/FUNCTIONALIZATION PROTOCOL, A USEFUL TOOL IN SYNTHETIC MEDICINAL CHEMISTRY

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Selective carbon−hydrogen bond activation and functionalization is highly attractive to provide more complex molecules by building C−C and C−heteroatom bonds with high efficiency, low cost, and minimal environmental impact.[1] Of particular interest is the borylation of arenes and heteroarenes, which represents one of the most elegant ways to obtain (hetero)-arylboronate esters, versatile synthons in the construction of carbon-carbon bonds or C-heteroatom bonds.[2] Melatonin (MLT) **1** is a widely studied natural neurohormone with a wide range potential therapeutic activities both as modulator of its receptors (MT_1 and MT_2) and as antioxidant.[3] Tipically, the synthesis of MLT derivatives involves either a multistep sequence for the introduction of the ethylene amine side chain in position 3- of the indole ring[4] or the construction of the pyrrole ring using Fischer or Larock annulation reaction. We sought that a direct and selective late-stage functionalization of MLT could be more practical and efficient way to rapidly generate an array of derivatives both in C-2 and C-7 positions.

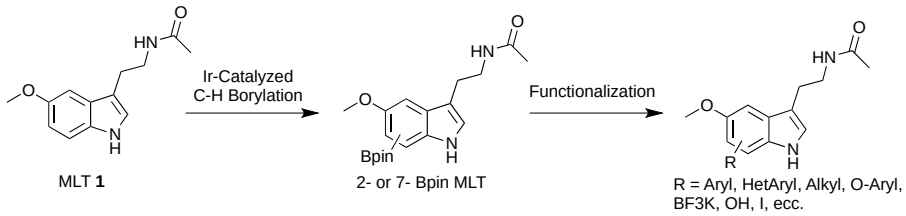

Figure 1. Late-stage functionalization of MLT.

Inspired by previous work by Movassaghi and Hartwig,[5] we used an Iridium complex $[Ir(cod)OMe]_2$ with a bipyridine-based ligand (dtbpy) and B_2pin_2 to obtain 2-Bpin MLT and 7-Bpin MLT; conversion and selectivity of the reaction can be partially modulated by changing the reaction conditions. More importantly, a wide range of reactions could be applied to the borylated MLT such as Suzuki-Miyaura, Chan-Lam coupling, Rh-catalyzed additions to aldehydes and enones, oxidation, halogenation and boron interconversion so that several different MLT-derivatives were synthesized (Figure 1). In addition, other direct late stage functionalization reactions have been developed on MLT such as C-H silylation and thioether formation. Finally, N-methyl-substituted naphtyl derivative showed the presence of two interesting atropisomers that have been isolated after derivatization.

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SUBSTITUTED ACETAMIDE AS A CYSTEINE TRAP FOR THE DEVELOPMENT OF NOVEL IRREVERSIBLE TYROSINE KINASE INHIBITORS WITH ANTIPROLIFERATIVE ACTIVITY

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In the treatment of several human cancers, including Squamous Non-Small-Cell Lung Cancer (SQCLC), irreversible Tyrosine Kinase Inhibitors (TKIs) have produced extensive clinical benefits. They feature an acrylamide moiety to target specific cysteine residues that undergo alkylation by virtue of a hetero-Michael addition. With the aim of decreasing the innate, promiscuous reactivity of the acrylamide, yet maintaining the ability to bind the target cysteine, we designed a panel of substituted acetamide warhead-groups to functionalize the appropriate TKI, in the hope of obtaining irreversible inhibitors with improved selectivity. We synthesized a 2-chloro-*N*-(4-(phenylamino)quinazolin-6-yl)acetamide UPR1303 as a prototype of EGFR inhibitor able to react with Cys797 by nucleophilic substitution. We modulated the reactivity of the acetamide fragment by replacing the chlorine atom with (hetero)-aromatic thiols or carboxylate esters to act as leaving groups. Optimization of the leaving group structure, supported by modulation of reactivity and QM/MM simulation of the Cys797-alkylation mechanism, led to the 2-((1H-imidazol-2-yl)thio)acetamide UPR1381, which showed long-lasting inhibition of wild-type EGFR autophosphorylation in A549 cells and resulted able to bind recombinant EGFR L858R/T790M in a time-dependent manner, while showing negligible reactivity with cysteine in solution. UPR1381 inhibited both EGFR autophosphorylation and proliferation in gefitinib-resistant H1975 lung cancer cells (expressing EGFR L858R/T790M mutant) at low micromolar concentration.[1] From these premises, we approached the development of irreversible FGFR inhibitors, where targeting cysteine residues represents a more challenging task, owing to lower accessibility. Relying on the validated compound FIIN2, we synthesized and investigated a set of novel inhibitors targeting FGFR, among which the chloroacetamide UPR1376 resulted able to irreversibly inhibit FGFR1 phosphorylation in FGFR1 H1581 over-expressing H1581 cells, with a higher potency than the reference reversible inhibitor BGJ398, while sparing FGFR1 low-expressing cells.
Our findings suggest that the insertion of a suitably substituted acetamide warhead on a scaffold with high affinity for its target is a valuable strategy that might supersedes the use of acrylamide moieties in the construction of novel irreversible TKI inhibitors.

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TARGETING THE OPIOID RECEPTORS: BIPHALIN ANALOGUES DESIGN

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Biphalin is an opioid octapeptide with a dimeric structure based on two identical portions derived from enkephalins joined tail to tail by a hydrazine brigde. During several years of research, we have modified the biphalin's structure by applying a hierarchical peptidomimetic design approach (Figure 1). In this presentation, I will report the most interesting modifications obtained so far, such as: 1) substitution of the α-amino acids residues with the relative hβ3 amino acids, 2) the substitution of the hydrazine bridge and the introduction of a fluorine atom on phenylalanine, 3) the cyclization by disulphide bridge, 4) cyclization by Clips technology and 5) cyclization by Ring Closing Metathesis.[1-3]

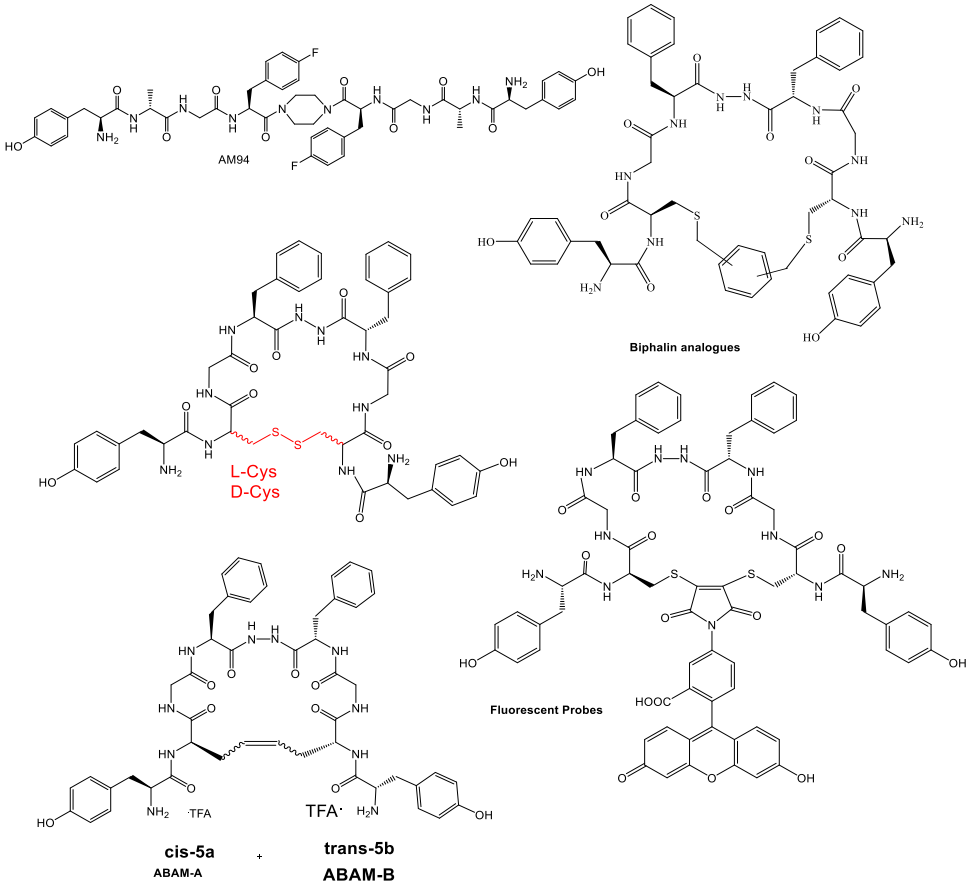

Figure 1. Biphalin analogues.

Several cyclic analogues biphalin have been prepared and tested for binding assays, GTP stimulation, and in vivo by several routes of administration.[1-3]

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A FLUORESCENT MITOCHONDRIA-TARGETED NO PHOTODONOR ACTIVABLE WITH HIGHLY BIOCOMPATIBLE GREEN LIGHT

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Nitric oxide (NO) is an endogenous messenger involved not only in maintaining the physiological homeostasis but also in an extensive number of different diseases, among them cancer. It has been shown that low NO concentrations (pM-nM) induce cancer growth whereas high concentrations (μM) are toxic and reduce cancer progression.[1] The photogeneration of NO achieved using compounds able to release NO under the action of the visible light, namely NO photodonors (NOPDs), has received a great attention as potential new anticancer therapy. NOPDs allow the action of NO to be confined to the irradiated area with high spatial precision, and its dosage to be controlled with accuracy by tuning the duration and intensity of the irradiation. Moreover, the visualization of NOPDs in cellular environment through fluorescence techniques represents an indispensable requisite in view of image-guided phototherapy.[2] Another relevant issue in anticancer therapy is the targeting of mitochondria. In fact, high levels of NO in mitochondria induce toxicity, principally via inhibition of mitochondrial respiration. In addition NO stimulates the production of an excess of reactive nitrogen (RNS) and oxygen (ROS) species that lead to mitochondrial apoptosis.[3] Rhodamine B is a chromofluorogenic unit well-known to act as a mitochondrial probe. We recently reported a molecular hybrid in which this mitochondrial targeting ligand was linked to a nitroaniline derivative as NOPD activatable by blue light.[3] We report here a novel fluorescent NOPD activatable by the more biocompatible green light, consisting of a nitrosoaniline derivative appendage covalently linked to the Rhodamine B. In this case this chromofluorogenic unit acts also as a light harvesting antennae, allowing a clean NO release from nitrosoamine appendage, through an intramolecular photinduced electron transfer, and stimulating intense orange emission useful to localize the hybrid in the cells (Figure 1). Preliminary biological assays are also presented.

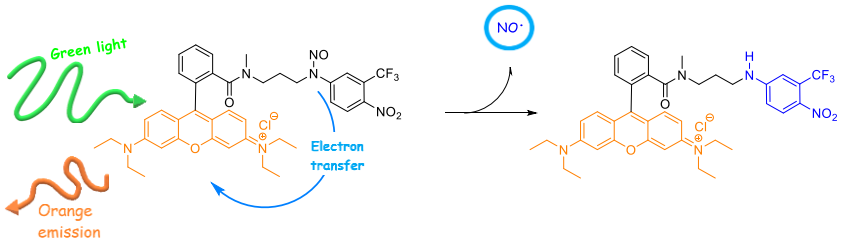

Figure 1

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PLASMODIUM FALCIPARUM DIHYDROOROTATE DEHYDROGENASE INHIBITORS WITH HYDROXYPYRAZOLE CORE: NEW SCAFFOLDS AGAINST MALARIA DISEASE

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Pyrimidines are essential for the cell surviving and the proliferation in both human and parasitic organisms. In human cells, the pyrimidine building blocks are synthesized through both *de novo* biosynthesis and salvage pathways, while many parasitic organisms, such as *Plasmodium* species causing malaria, cannot access to the salvage pathway. For this reason, blocking the *de novo* biosynthesis is an effective therapeutic opportunity, and inhibitors of *dihydroorotate dehydrogenase* (DHODH), one of the six enzymes involved in pyrimidine biosynthesis, are expected to have many antiparasitic activities.[1] Recently, a *Pf*DHODH inhibitor demonstrated clinical efficacy in patients with uncomplicated *P. falciparum* malaria infection in a phase 2a study.[2]

Assaying some hydroxylated heterocycles present in our laboratory,[3] we identified pyrazole **1** as a *Pf*DHODH inhibitor in the µM range, selective versus the human isoform. In order to ameliorate its potency, we performed an extensive study of its SAR modulating four different positions of its structure. The best derivative **2**, able to inhibit PfDHODH with IC50 at 2.8 µM, was co-crystallized with the protein obtaining its experimental binding mode. Compound **2** shows also activity against *P. falciparum*-infected erythrocytes, and good selectivity versus the human DHODH and mammalian cells.[4] In this occasion, the design, synthesis and biological chacterization of these molecules, that represent new chemical entities in the field of *Pf*DHODH inhibition, are discussed.

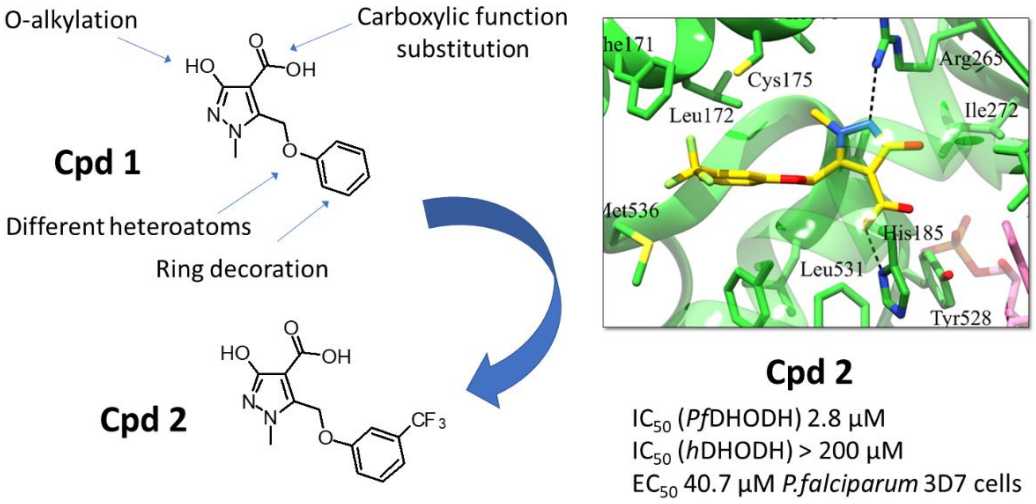

Figure 1. Structure, binding mode and biological activity of discussed compounds.

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DESIGN, SYNTHESIS AND PHARMACOLOGICAL CHARACTERIZATION OF SELECTIVE BLOCKERS OF 2-ARACHIDONOYLGLYCEROL DEGRADATION

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The endocannabinoid system (ES) is composed of endogenous lipid-signalling molecules defined as endocannabinoids (ECs) and their cellular targets, the G-protein coupled receptors CB1R and CB2R, along with the transporters and enzymes responsible for ECs biosynthesis and metabolism. N-Arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) are two members of the ECs signaling molecules, with the Fatty Acid Amide Hydrolase (FAAH) principally involved in AEA catabolism and the Monoacylglycerol Lipase (MGL) playing a pivotal role in the regulation of 2-AG levels.[1] Accordingly, inactivation of MGL elevates the endogenous concentrations of its substrate, thus potentiating the beneficial effects on the modulation of pain, inflammation and neurodegenerative disorders, whilst diminishing the side effects related to direct CBRs stimulation. MGL therefore, represents a potentially valuable therapeutic target and the selective inhibition of this enzyme can contribute to the development of agents useful for the treatment of chronic pain, Fragile-X syndrome and Multiple Sclerosis (MS).[2] To this end, we exploited the β-lactam scaffold for the first time to deliver potent and selective β-lactam-based MGL inhibitors, achieving compounds with pronounced in vivo activity and favorable drug-like properties.[3] The compounds inhibit MGL through an irreversible and stereoselective mechanism, which we elucidated using computational, mass spectrometry and racemate resolution analyses. The lead compound in this β-lactam series, NSD1819, displays substantial metabolic stability, high permeability through artificial membranes, and low toxicity in vitro. Oral administration of NSD1819 produces marked analgesic effects in rodent models, which is suggestive of a potential application of this new class of MGL inhibitors in the treatment of chronic pain. In mice experimental autoimmune encephalitis models, significant decreases in clinical severity during remission phases were observed with NSD1819 treatment, which undoubtedly reverts the clinical progression of MS in a CB1R/CB2R-dependent manner.

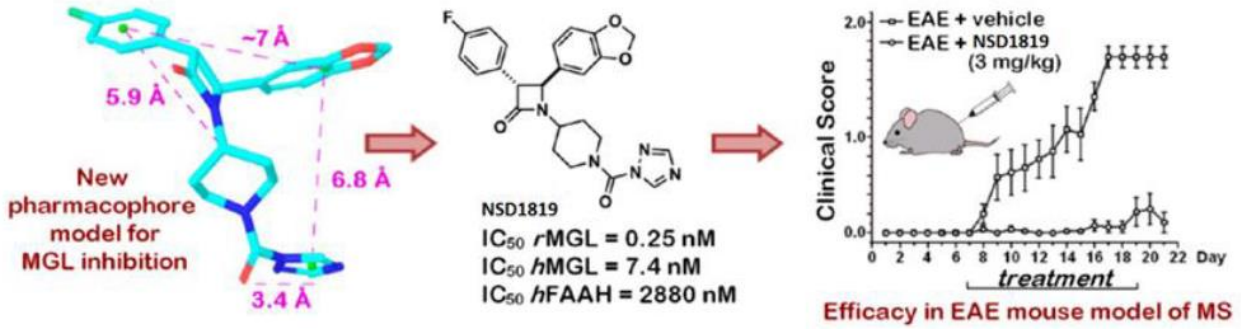

Figure 1. Structure of the β-lactam based MGL inhibitor and its activity on animal models of MS.

*This abstract is based on a recently published and multidisciplinary paper (Reference no 3) where all the other co-authors are listed with their affiliations. Further enquiries can be submitted to the corresponding author of the paper, Prof. Giuseppe Campiani, e-mail: campiani@unisi.it

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STRUCTURAL INSIGHTS FOR THE DEVELOPMENT OF NOVEL ANTITUBERCULARS TARGETING THE IRON UPTAKE PATHWAY

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Tuberculosis nowadays ranks among the leading causes of death worldwide, and the growing emergence of resistant strains of *Mycobacterium tuberculosis* (Mtb) poses a serious threat to the public's health; therefore, the discovery of new antitubercular agents is assuming critical importance.[1]

In this context, the inhibition of the mycobacterium-specific salicylate synthase MbtI, which is involved in the iron uptake pathway, has been recently validated as a pharmacological target for the development of novel antitubercular agents.[2]

A structure-based virtual screening allowed us to identify a competitive furan-based inhibitor of MbtI (VS1, IC_{50} = 21.1 µM). With the aim of increasing its inhibitory effect, we explored the chemical space around this hit through a thorough structure-activity relationship study, leading to the identification of more potent MbtI inhibitors. These compounds exhibited a promising antimycobacterial action, related to the reduced production of siderophores.[3,4]

The novel lead compounds were submitted to co-crystallization experiments to empirically define the binding mode of this class of compounds within the active site of MbtI. The structural data allowed us to evidence the interactions of the inhibitors at a molecular level, revealing previously undetermined roles of key residues. These results may lead to the identification of new strategies for rational modifications intended to improve the inhibitory properties of the molecules.

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PREDICTING RESISTANCE OF CLINICAL ABL MUTATIONS USING COMPUTATIONAL METHODS

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The therapeutic effect of targeted kinase inhibitors can be significantly reduced by intrinsic or acquired resistance mutations that modulate the affinity of the drug for the kinase. The emergent use of deep sequencing in a clinical setting is now able to reveal genetic alterations in tumor cells in a convenient timeframe but this information may be useless in case of rare mutations. In these cases, the lack of prior knowledge prevents the adoption of a fully personalized treatment.

In this case, a purely physics-based approach, which does not require any previous knowledge of the clinical outcome of the observed mutation may be of great help. Here we explore the possibility of adopting a computational tool called alchemical free energy perturbation (FEP+) to support clinical decisions, using a retrospective set of data from Sloan-Kettering Institute.

FEP+ is a computational technique that is now broadly applied to calculate the difference in binding affinity between ligands. The FEP+ approach can also be used in the context of drug resistance emerging from point mutations where protein sidechains interact differently with inhibitors due to steric clash or reduced interactions. In this talk I will present the Schrödinger FEP+ methodology and highlight the relevant components (OPLS3e forcefield, replica-exchange solute-tempering, Desmond-GPU MD engine, alchemical water, Gran-Canonical Monte Carlo). Using the example of Abl kinase, I will discuss how FEP+ allows classifying a panel of 144 clinically-identified point mutations as resistant or susceptible with 88% accuracy.[1]

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YOUR MANUSCRIPT THROUGH THE EDITOR'S EYES

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Have you ever wondered what happens to your manuscript once you submit? Or how editors conduct peer review? Or are you curious about what exactly editors do? Dr. Joseph Unsay, Associate Editor at *Chemistry - A European Journal*, *ChemBioChem* and *ChemistrySelect* (Wiley-VCH and ChemPubSoc Europe Journals) talks from his experience as a scientific editor and opens up the "black box" of science publishing. Along the way, he'll talk about a few tips including questions authors can ask themselves to improve their next submission. This is a great opportunity to learn about editorial work, which is an excellent career option for those with graduate degrees in the sciences.

DEVELOPMENT OF A SCREENING PLATFORM FOR THE IDENTIFICATION OF SMALL-MOLECULE LIGANDS FOR THE READER PROTEIN PHF20

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Epigenetic proteins are categorized in writers, erasers and readers.[1] Despite their role in the development of several pathologies, methyl reader proteins have been less pursued as therapeutic targets and few chemical probes are available for this class of proteins. In addition, the lack of a validated screening procedure for these domains complicates the discovery of new compounds. With the aim to obtain a robust methodology for the drug discovery process, this project is focused on the development of a versatile screening platform for the identification of small-molecule modulators for the reader protein PHF20, a multidomain protein.[2,3] Among all, the second Tudor domain of PHF20 is capable of binding methylated residues (one of the most common is H4K20me2) on histone tails. On the basis of the compound UNC1215, (first identified as a chemical probe for the reader protein L3MBTL3 but which is able also to interact with PHF20),[4] a small library of analogues was synthesized at EMCL (Epigenetic Medicinal Chemistry Laboratory) with the aim to identify new ligands of PHF20. The first part of the project concerned the expression and purification of the protein PHF20. In particular, four different constructs of PHF20 were expressed and purified (Tudor2 wt, Tudor2 mut, Tudor1-2 with and without GST tag). Subsequently, the proteins were used for the application of different biophysical and biochemical techniques (nanoDSF, MST, SPR, AlphaLISA) in order to evaluate the effect of the compounds. The nanoDSF was used as a primary screening method then the use of SPR and MST allowed the determination of the K_D. In addition, it was developed and optimized an AlphaLISA assay, which was used for the determination of IC_{50}. The combined use of these techniques allowed the discovery of "true hits", overcoming the limitations of each method that often lead to the identification of false positives during drug discovery programs.

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ENRICHING THE TOOL-BOX OF NORA INHIBITORS BY COMPUTER-AIDED SCAFFOLD HOPPING APPROACH

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Antimicrobial resistance (AMR) is a health issue of global concern, inserted by WHO among the worst ten treats in 2019.[1] It is believed that this phenomenon will carry us in a "post-antibiotic era", a time in which we were unable to easily manage common microbial infections; indeed recent reports estimate that by 2050, ten million people every year will die for AMR. Bacterial efflux pumps (EPs) and their overexpression represent the early step mechanism lying beneath the resistance development to antibiotics.[2] EPs by extruding antibiotics from bacterial cell allow the microbe to survive in presence of sub-optimal antibacterial drug concentration and mount with specific high level target-based resistance. Therefore the use of EP inhibitors (EPIs) is widely recognized as a powerful tool to counteract AMR.[2]

Since many years, we contributed to the AMR research field though the design and synthesis of EPs inhibitors against different bacteria. NorA is the most studied EP of *Staphylococcus aureus*, one of the ESKAPE species causing common and difficult-to-treat nosocomial infections, and represents the target of several medicinal chemistry works. We have identified and developed the 2-phenyl-4-hydroxyquinoline (2PQs) derivatives that resulted the most promising NorA inhibitors so far reported.[3] Structure-activity relationship profile indicate the *p*-propiloxy group at the C-2-phenyl ring and the alkylamino chain at the 4-hydroxy position as essential chemical moieties for a potent NorA inhibition.[3] Recently, we also built two pharmacophore models using potent NorA inhibitors belonging to four different chemical families, including 2PQs.[4] In the search of new potent and safe NorA EPIs, herein, we applied a scaffold hopping of 2PQs. First, we built a library of small molecules based on scaffolds commonly found in FDA approved drugs and then we screened the library against the two pharmacophore models, retaining only molecules that showed fitness ≥1.5. Among the selected hits, we decided to synthesize some of the new derivatives considering also synthetic accessibility. The new compounds will be tested for their NorA EPI activity against a resistant *S. aureus* (SA1199B - *norA*+/A116E GrlA mutation) strain in two different assays: i) EtBr efflux inhibition to select compounds showing >70% inhibition, ii) checkerboard assays at scalar dilution to evaluate synergism with ciprofloxacin. Experiments are still ongoing thus results and further development will be detailed within this scientific contribution.

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NOVEL STRATEGIES TO IRON-STARVE *STAPHYLOCOCCUS AUREUS*: STRUCTURAL AND DYNAMIC CHARACTERIZATION OF THE HEMOPHORE-HB INTERACTION

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Antimicrobial resistance represents one of the main current risks for human health. The emergence of bacteria resistant to last resort antibiotics is responsible for more than 700,000 annual deaths and to respond to this emerging threat, new targets, new treatments and alternative therapies are urgently needed. Among the so-called superbugs, *Methicillin-Resistant Staphylococcus Aureus* (MRSA) has been prioritized as the most threatening multidrug resistant Gram+.[1] MRSA expresses Iron-regulated Surface Determinants (Isd) proteins to bind human hemoglobin (Hb), extract the heme and degrade it to seize iron, which is essential for bacterial growth and virulence. Among Isd proteins, IsdB and IsdH are responsible for Hb capturing and heme mining. To date, however, the mechanism of heme extraction has not been completely understood and, despite the crucial role of this pathway in bacterial infection and virulence, no inhibitors of the IsdB:Hb or IsdH:Hb interaction have been developed.

To rationalize the IsdB:Hb recognition and the heme extraction process and prove the druggability of the Hb-hemophore complex we coupled *in silico* with biochemical analyses. We investigated the mechanism of Hb binding and heme extraction by IsdB by multi-wavelength stopped-flow UV-visible spectroscopy, both with wild type IsdB and the Y440A mutant, unable to complete the heme extraction.[2] Equilibrium dissociation constants of wild type and mutated IsdB for Hb were assessed by a newly developed colorimetric 96-well plate assay. Extensive Molecular Dynamics (MD) simulations (about 7 µs) were run to explore the IsdB:Hb interaction and to identify the critical residues involved in the complex formation and in the first steps of heme extraction. In particular, electrostatic interactions formed at the IsdB:Hb interface have a key role in stabilizing the transition state, accomplished through a disruption of the F helix of Hb, and in extracting the heme. Concurrently, the most representatives Hb conformations were extracted from MD trajectories and used to perform Structure-Based Virtual Screenings (SBVS) of commercial compound libraries, to identify molecules able to interfere with the Hb:IsdB complex formation and, thus, affect the bacteria iron supply. The ligands selected by SBVS were then submitted to docking analyses. The most promising candidates have been purchased and *in vitro* experiments to verify their activity are currently ongoing (Figure 1).

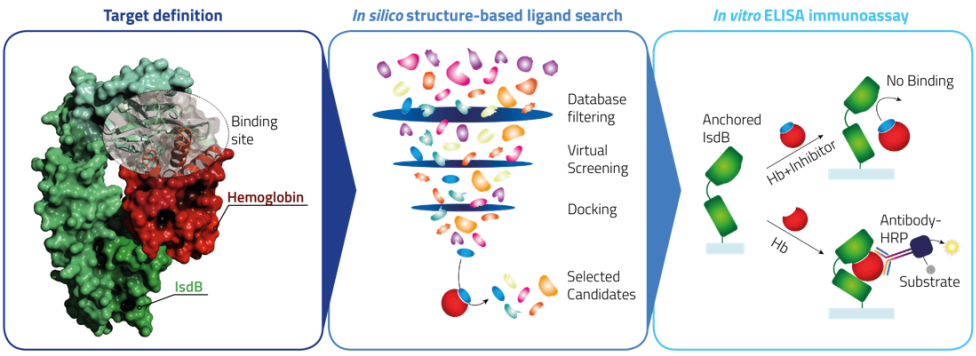

Figure 1. Compound selection pipeline.

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A NEW CXCL12/HMGB1 HETEROCOMPLEX INHIBITOR

Sgrignani, J.;[a] Fassi, E. M. A.;[a] D'Agostino, G.;[a] Cecchinato, V.;[a] Garofalo, M.;[a] Grazioso, G.;[b] Uguccioni, M.;[a] Cavalli, A.[a]

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HMGB1 (high mobility group box 1) is small protein with multiples roles in inflammation and cancer.[1-3] HMGB1 is produced in a reduced form (i.e., without disulfide bond between cysteines 23 and 45 of Box-A) and released in the extracellular environment during inflammation or tissue damage. Moreover, HMGB1 can also translocate into the nucleus where promotes gene expression.

From the structural point of view, HMGB1 is formed by two domains "Box-A" and "Box-B" connected by a small loop and by a disordered acidic C-terminal tail. Experimental investigations demonstrated that the reduced form of HMGB1 promotes the recruitment of inflammatory cells by the formation of a heterocomplex with a chemokine (CXCL12).[1] In particular, the more accredited mechanistic hypothesis foresees that one HMGB1 molecule could bind two CXCL12 and then activate two dimerized CXCR4 receptors.[2,4]

Applying several computational tools, we identified a peptide able to bind HMGB1 and reduce the cellular migration. The affinity between the peptide and the target protein was then measured by microscale thermophoresis (MST). Finally, computational and experimental alanine scanning procedures were used to derive structure-activity relationships (SARs).

Considering the importance of the CXCR4 axis in inflammation and in the cancer onset and progression our work represents an exciting step towards the development of innovative drugs.

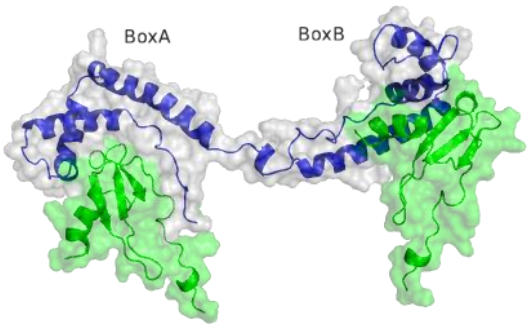

Figure 1. Computationally derived structure of the CXCL12/HMGB1 heterocomplex.

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RESCUING DEFECTIVE CFTR APPLYING A DRUG REPOSITIONING STRATEGY

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Cystic Fibrosis (CF) is the most common lethal monogenic disorder in Caucasians. It is due to different mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), the most common one is the deletion of phenylalanine 508 (F508del). This mutation causes an inappropriate folding and structural instability of CFTR, that for this reason, remains trapped in the endoplasmic reticulum and is rapidly degraded. The deficit of CFTR at the plasma membrane has a major impact on the respiratory system, determining an impairment of innate defence against bacteria.[1,2]

Current therapies are mostly aimed at treating CF symptomatically and although they have significantly pushed forward the mean survival age of patients, the burden of CF care continues to be very high and life quality and expectancy for most CF patients are still limited.

An ambitious therapeutic alternative is to address CF systemically, by means of small molecules able to restore the trafficking (correctors) or the gating (potentiators) capacity of mutated CFTR. Some interesting compounds are emerging in academic laboratories and pharmaceutical companies, however, up to now, marketed drugs are only Lumacaftor, Tezacaftor (both correctors) and Ivacaftor (potentiator), whose mechanism of action remains to be fully characterized.

To this lack of knowledge surely contributes the fact that only in 2018 the X-rays of a human wild type experimental structure in its active conformation were released,[3] making thus difficult until now to gain useful insights into the binding mode of small ligands to the mutated protein.

It derives that the main needs in the field of CF therapy are the full knowledge of the mechanism of action of the approved drugs and, above all, the availability of novel candidates, whose discovery, in turn, depends on appropriate computational and experimental models to be combined to generate new pipelines of analysis.

Relevant to this point, are here presented: i) an integrated computational and surface plasmon resonance (SPR) approach targeted to better clarify the molecular mechanism of action of Lumacaftor and analogues; ii) a preliminary computational drug repositioning strategy performed using a specific pipeline implemented in an OpenStack Hybrid Cloud Infrastructure, applied on the AIFA database and a library of binding pockets identified using a human F508Δ-CFTR computational model.[4] Computational and experimental results so far obtained, suggest a binding pocket for Lumacaftor between NBD1 and ICL4, for Tezacaftor at NBD2 and highlight some AIFA drugs as potentially promising in correcting CF.

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DEVELOPING GLYCOPROTEINS AS POTENTIAL VACCINES AGAINST TUBERCULOSIS

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Tuberculosis (TB) is the first cause of death from infectious diseases worldwide. Only a single anti-TB vaccine is currently available for clinical use, but its efficacy is debatable. Conjugation of antigenic oligosaccharides, such as lipoarabinomannane (LAM), with antigenic proteins from *Mycobacterium tuberculosis* (MTB), has been recently proposed as a new strategy for developing efficient vaccines.[1] Glycosylation with α(1-6) polymannan has been also investigated in order to improve the biological activity of antigenic proteins.[2] This evidence was the rationale leading to the design, synthesis, and analytical characterization of the *neo*-glycoconjugates herein reported as potential vaccines against TB.

A number of semi-synthetic glycoconjugates were prepared, starting from mannose, di- and tri α(1-6) mannan analogues. Glycans were activated with a thiocyanomethyl group at the anomeric position to address protein glycosylation by a selective reaction with lysines of recombinant Ag85B (rAg85B). Since the immunogenicity of rAg85B was decreased upon glycosylation,[3] the mutants K30 and K282 were designed by replacing lysines involved in the main T-epitope sequences with an arginine residue (R) to prevent their glycosylation.

The effect of K30R, K282R and K30R+K282R mutations on the T-cell activity of rAg85B were assessed by an immunological assay. The same test was carried out on the glycosylation products of the mutants. After glycosylation, the K30 mutants completely retained the original T-cell activity, thus resulting in antigenic carriers which might be suitable for the development of glycoconjugate-based vaccines against TB.[4] Moreover, the epitope of rAg85B involved in the interaction with antibodies from different sources was identified by proteolytic affinity-MS. The affinity of rAg85B, mutants and rAg85B-glycoconjugates for the monoclonal antibody anti-Ag85 was compared by SPR analyses to support the mutagenesis approach.[5]

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CAN WE IMPROVE DRUG DESIGN BY ILLUMINATING DRUGGABLE TARGETS WITH BDDCS?

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The Biopharmaceutics Drug Disposition Classification System (BDDCS) allows medicinal chemists to predict pharmacokinetics (PK) properties[1] and to forecast drug disposition issues[2] for new chemical entities (NCEs) based on aqueous solubility and extent of metabolism. During the process of drug development, NCE "discovery" (identification) and NCE (lead) "optimization" remain sequential, not fully integrated steps. This limiting factor has severe repercussions on both cost and time, and could be improved by implementing a parallel identification-optimization process. One way to address this issue is to introduce BDDCS relevance in early drug discovery by cross-referencing it with information on drug targets. In this study, we mapped BDDCS categories for over 1,000 approved drugs to the mode-of-action that triggers their therapeutic[3] and off-target effects. We used the online drug compendium DrugCentral[4] to extract data for the major drug target families, which have been extensively explored and are largely considered "druggable".[5] In this manner, BDDCS classes were annotated for each target family. This allows the recognition of protein families that have higher preference for a particular BDDCS class, information that can be used to better understand how drug targets influence PK properties of NCEs. Such a "pharma-shortcut" could focus virtual screening studies on smaller sets of chemicals with PK properties specific for that target family.

The concept is illustrated in **Figure 1**, which shows clear evidence that "druggable" target families show higher preference for a specific BDDCS class: G-protein coupled receptors (GPCRs) and ion channels have preference for BDDCS class 1 (high solubility, high metabolism) drugs, whereas nuclear receptors and kinases have preference for BDDCS class 2 (low solubility, high metabolism) drugs. BDDCS and target druggability integration in more detail for specific protein families by discussing drug ATC (Anatomic, Therapeutic and Chemical) classification codes as well as protein subcellular compartment location. Although it is known that PK properties influence drug-target interactions (pharmacodynamics), we show that drug targets themselves have specific preference for drugs with certain PK profiles. We will also discuss a newly developed computational model to forecast the BDDCS class for novel compounds, as well as the web-app that we are building to access this type of information. Finally, we introduce a revised version of BDDCS enriched with transporter (extent of transport, EoT) effects as a third major BDDCS parameter. In this novel schema, high and low EoT characterizes each BDDCS class respectively. These concepts may lead to the development of an integrated "discovery" and "optimization" system that supports parallel property optimization, which may significantly influence the drug design process.

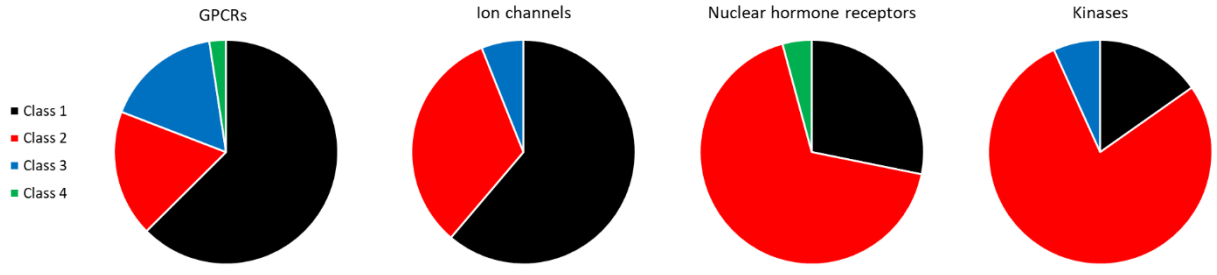

Figure 1. BDDCS drugs distributions for the major target families.

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KINASE INHIBITORS AS POTENTIAL NEUROPROTECTIVE AGENTS: PLAYING WITH THE TRIAZOLOTRIAZINE SCAFFOLD ON GSK-3β AND CK-1δ

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The glycogen synthase kinase 3β (GSK-3β) and casein kinase 1δ (CK-1δ) are serine/threonine protein kinases phosphorylating more than hundred different substrates. In recent years, many researchers have proved GSK-3β and CK-1δ implications in the neuroinflammatory process. Furthermore, some of their substrates are present in the protein aggregates or inclusions bodies typical of neurodegenerative diseases, comprising tau, α-synuclein, TDP-43 and parkin.[1] In order to investigate GSK-3β and/or CK-1δ inhibitors as potential therapeutic agents for neuroinflammation-related disorders, we synthesized [1,2,4]triazolo[1,5-*a*][1,3,5]triazine derivatives, which could be opportunely decorated at three different positions. First derivatives were built taking inspiration by the well-known kinase inhibitor Roscovitine (Figure 1). Obtained derivatives showed inhibitory activity for one or both kinases in the micromolar range. In order to obtain stronger inhibitors a recognized strategy is to design a covalent inhibitor, thus a derivative bearing the cyanoacrylamide group able to give a thia-Michael reaction with cysteine 199 (Cys199), was designed.[2] Compound underwent molecular docking studies on both GSK-3β and CK-1δ demonstrating that the reactive moiety was oriented toward the nucleophilic Cys199 in GSK-3β and interacted through a hydrogen bonding with key residues in CK-1δ. In fact, IC_{50} values decreased to the sub-micromolar range. In order to prove the covalent interaction with GSK-3β, a co-crystallization of ligand and protein was performed. X-ray crystallographic studies confirmed the presence of electron density between the α-carbon atom of the cyanoacrylamide group and the sulfur atom of Cys199. This is the first time that a covalent interaction was proved for GSK-3β. Preliminary studies on in vitro models of Parkinson's disease revealed that selected compound is not cytotoxic and shows neuroprotective activity. These results encourage further investigations to validate GSK-3β/CK-1δ inhibition as a possible new strategy to treat neuro-inflammatory/degenerative diseases.[1]

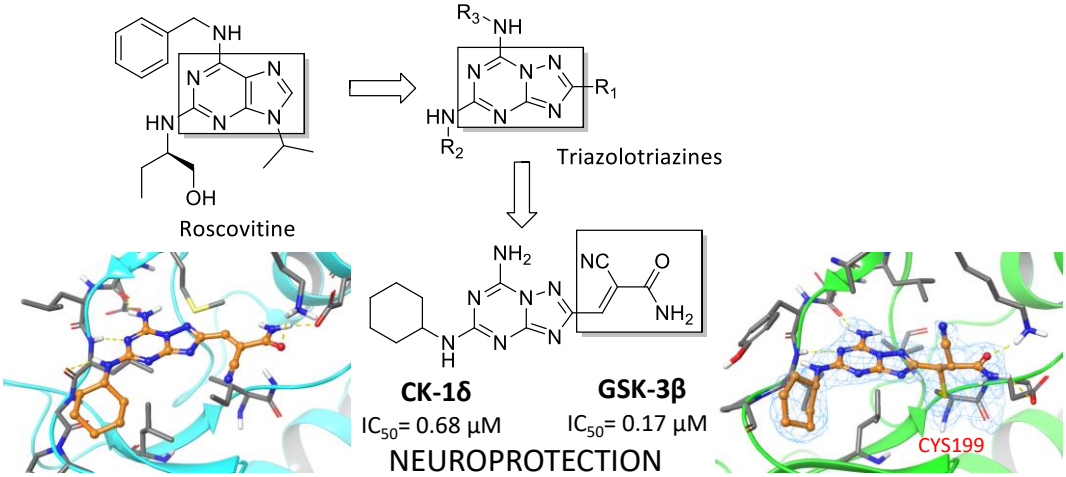

Figure 1. Synthetized triazolo-triazine derivatives led to a dual GSK-3β/CK-1δ inhibitor, which mediated neuroprotection *in vitro*.

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FLOW NANOPRECIPITATION OF SIZE-CONTROLLED D-LEUCINE NANOPARTICLES AS EXCIPIENT FOR SPRAY-DRYING FORMULATIONS

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During the last decades, nanoparticles (NPs) have attracted a great deal of attention for biomedical applications.[1] The ability to design and produce NPs through economical and scalable processes that allow the fine tuning of size, size distribution and morphology, represents a big challenge especially for drug delivery where reproducibility is a crucial issue. In this regard, seminal reports have shown the benefits of flow chemistry over conventional batch methods for the preparation of NPs for nanomedicine and theranostic applications.[2] In this communication we report the development of a cheap and scalable flow-assisted flash nanoprecipitation (FNP) of D-leucine NPs as glidant in dry powder formulations for pulmonary drug delivery. D-leucine has indeed demonstrated excellent properties as excipient in dry powder inhaler (DPI) devices[3] that, interestingly, well combine with its antimicrobial effect mediated by the inhibition of bacterial biofilm growth.[4] Starting from a preliminary batch screening, the process was successfully translated, optimized and scaled-up under mesofluidic conditions. The suspensions obtained were immediately spray-dried to recover the powders that were characterized. The aerodynamic evaluation of a formulation obtained by physical mixing of D-leucine powders with micronized budesonide, selected as poorly water-soluble and poorly flowable model drug, will be herein described and analyzed.

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IDENTIFICATION OF CHROMENO[3,2-*c*]PYRIDINE AS SUITABLE SCAFFOLD OF NOVEL MULTITARGET-DIRECTED LIGANDS FOR THE TREATMENT OF ALZHEIMER'S DISEASE

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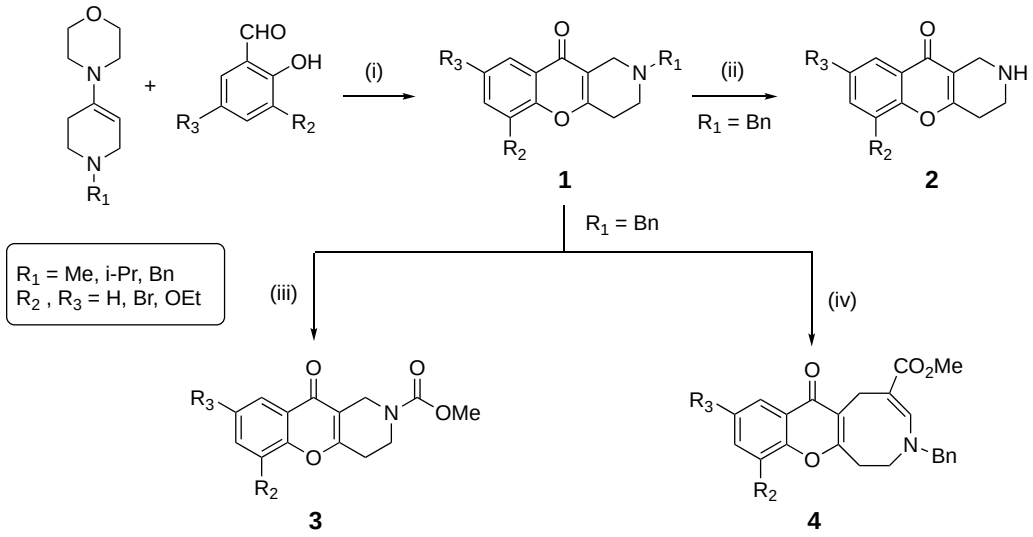
Despite huge efforts to develop effective disease-modifying drugs for Alzheimer's disease (AD), the currently available treatments (acetylcholinesterase, AChE, inhibitors and the NMDA receptor antagonist memantine) can provide at best symptomatic relief in earlier stages of disease. The research recently shifted to the more promising Multi-Target-Directed Ligands (MTDLs). Our efforts have long been devoted to discovering dual coumarin-based inhibitors of monoamine oxidase B (MAO B) and AChE, whereas more recently we usefully decorated some other heterocyclic scaffolds to obtain novel MTDL leads for AD.[1-3]

Herein, the synthesis (Scheme 1) and biological activities toward some AD-related target of 1,2,3,4-tetrahydro-10*H*-chromeno[3,2-*c*]pyridine-10-one (THCP) derivatives is reported.



Scheme 1. Reagents and conditions: (i) 1) n-hexane, rT, 24h; 2) CrO_3 2Py, dichloromethane; (ii) H_2, Pd/C, EtOH, 40 °C; (iii) $ClCO_2Me$, toluene; (iv) methyl propiolate, CF_3CH_2OH, -16 °C.

As major findings of this study: i) compound **1**, bearing benzyl and ethoxy as R_1 and R_2 groups, respectively, inhibited in vitro human MAO B (IC_{50} of 0.9 µM) and displayed neuroprotective effects in neuroblastoma SH-SY5Y cell line, significantly recovering cell viability when impaired by $A\beta_{1-42}$ and pro-oxidation insults; ii) the carbamate derivative **3** proved to be a moderate dual MAO/AChE inhibitor; iii) the chromeno[2,3-*d*]azocines **4**, irrespective of their substituents R_2 and R_3, lost activity toward the assayed enzymes and pathogenic pathways. Overall, this study highlighted THCP as useful and versatile scaffold for developing new small molecules targeting enzymes and biochemical pathways involved in the pathogenesis of AD.

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QUINOLINE-BASED EFFLUX INHIBITORS AS ANTIMICROBIAL RESISTANCE BREAKERS (ARBs)

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Antimicrobial resistance (AMR) represents a hot topic in drug discovery. Besides the identification of new antibiotics, the use of antimicrobial resistance breaker (ARB) molecules to block resistance mechanisms is a powerful alternative.[1] Microbial efflux pumps exert an early step in AMR development by allowing microorganisms to grow at sub-inhibitorial drug concentrations. Thus, efflux pump inhibitors (EPIs) offer a great opportunity to fight AMR.[2]

Starting from some 2-phenylquinolines, previously reported as potent EPIs of the *Staphylococcus aureus* and *Mycobacterium avium*,[3,4] a large series of quinoline/quinolone analogues has been designed and synthesized to improve EPI activity and obtain an acceptable safety profile towards human cells.

Regarding the search for *S. aureus* EPIs, the introduction of methoxy group/s on the benzene ring of the quinoline core afforded potent derivatives endowed with high EPI activity, as shown by the strong synergism of these compounds with the antibacterial ciprofloxacin against resistant *S. aureus* strains. The best compounds were further evaluated to confirm their mechanism of action, human cell toxicity and pharmacokinetic properties. Of all, two C-6 substituted derivatives appeared the best analogues thus resulting the new lead compounds. On this basis, additional C-6 functionalized quinoline analogues were designed and synthesized in order to expand SAR and further improve the pharmacokinetic profile.

In parallel, based on the most active class of *S. aureus* EPIs, *in silico* pharmacophore models were built and used to perform a drug repurposing approach that led to the identification of nicardipine drug as a potent *S. aureus* EPI.

Regarding the search for *M. avium* EPIs, the quinoline scaffold was combined with the isoflavone nucleus of biochanin A, a natural EPI of nontuberculous mycobacteria, to afford the 3-phenylquinolone chemotype. Initially, various substituents on the quinolone *N*-1 position afforded a first set of derivatives still suffering from human cell toxicity but with an improved *M. avium* EPI activity with respect to the starting quinoline analogues. The second round of chemical optimization of this 3-phenylquinolone class was focused on the exploration of different substituents on the C-6 and C-7 positions. Some compounds exhibited very promising results and, in particular, two of them displayed a strong EPI activity by synergizing at very low concentrations with ciprofloxacin and clarithromycin against various *M. avium* strains. Interestingly, both of them exhibited a lower toxicity towards human cells, thus resulting the first compounds in literature acting as *M. avium* EPIs at concentration non-toxic for human cells. This finding enabled us to perform *ex-vivo* experiments on *M. avium*-infected human macrophages to confirm the EPI effect upon a surrogate of *M. avium in vivo* infection.

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DEVELOPMENT OF NEW ANTIPROTOZOAL AGENTS: DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION

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Trypanosomatid diseases caused by protozoa belonging to the genera *Leishmania* and *Trypanosomoma* represent an increasing global health concern affecting more than 20 million people worldwide. Currently employed drugs are unsatisfactory in terms of efficacy and safety, requiring the development of new therapeutic options.

To this purpose, a rational approach should rely on metabolic pathways essential for protozoa but absent or sufficiently different in human hosts.[1,2] In the search of innovative antiprotozoal agents, we focused on trypanothione reductase (TR) and CYP51, two among the most promising parasitic targets involved in critical processes whose disruption deeply affect protozoal survival.[1,2,3]

TR is the central enzyme of the unique thiol-based metabolism of trypanosomatids from which key biological processes, including prevention of oxidative damage and DNA replication, depend. The pivotal role played in trypanosomatids together with its absence in human host and structural peculiarities render TR an innovative and extremely prominent target for the development of novel antitrypanosomatid drugs.[1,2,4]

Via a structure-based screening, we identified our in-house diaryl sulfide derivative **RDS 777** as a promising antiprotozoal agent endowed with high affinity toward *L. infantum* TR (*Li*TR). Moreover, the solved X-ray structure of this compound within the enzyme highlighted a series of peculiar interactions with TR catalytic residues.[4] Starting from these data, seventeen sulfide and sulfone analogues of **RDS 777** previously described as antiviral agents were re-synthesized, with the aim of exploiting the influence of both complementary and alternative chemical modifications on protozoal inhibition. Alternative synthetic pathways were applied in order to optimize yields, reaction times and safety. Most active compounds displayed antileishmanial inhibitory potencies within the micromolar range and peculiar binding modes in docking studies.[5]

Moreover, basing on the solved X-ray structure and in order to increase the enzymatic affinity, nineteen new derivatives were designed and synthesized by means of batch and microwave-assisted reactions. Promising inhibitory potencies and alternative mechanism of inhibition were highlighted in enzymatic assays.

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EXPLORING THE 1-(4-FLUOROBENZYL)PIPERAZINE SCAFFOLD FOR THE DEVELOPMENT OF NEW POTENT TYROSINASE INHIBITORS

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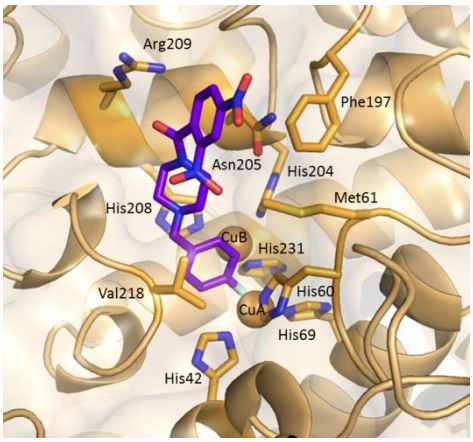
Tyrosinase (Ty, EC 1.14.18.1) is a copper containing enzyme widely distributed among the different species. Its active site is well conserved and it is characterized by the presence of two copper ions each coordinated by three histidine residues. Ty catalyzes the hydroxylation of tyrosine in L-dopa and its subsequent oxidation in dopaquinone which is converted through other interrelated enzymatic reactions in melanin pigments. Although melanin plays a crucial role in the protection of the skin against UV radiation damaging, its abnormal production leads to several hyper-pigmentary disorders, neurodegeneration associated with Parkinson's disease and melanoma. Consequently, the inhibition of Ty represents an attractive strategy for the therapy of hyperpigmentation-related pathologies. Several Ty inhibitors (TyIs) have been reported but their use is limited by their low stability and toxicity.[1,2] Recently, through a combination of crystallographic and docking based studies, we have designed a new series of TyIs bearing the 1-(4-fluorobenzyl)piperazine fragment which proved to be more active than the reference compound kojic acid, a secondary metabolite commonly used for the treatment of hyperpigmentation disorders.[3] Among the designed compounds, the derivative 4-(4-fluorobenzyl) piperazin- 1-yl](2-methylphenyl)methanone showed an inhibitory activity in the very low micromolar range and therefore it was used as starting point for the design of a new series of TyIs containing the 1-(4-fluorobenzyl) piperazine scaffold by combining *in silico* and structural approaches. The resulting compounds were synthesized and their Ty inhibitory activity was evaluated. Among them, the most promising inhibitor was the [4-(4-fluorobenzyl)piperazin-1-yl](2,4-dinitrophenyl)methanone (IC_{50} = 0.96 µM) which showed low toxicity in B16F10 cells and the ability to reduce melanin content in α-MSH-stimulated B16F10 cells. Crystallographic studies further clarified the binding mode of this inhibitor into the catalytic pocket of Ty enzyme (Figure).



Figure

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DESIGN, SYNTHESIS AND INTERACTION STUDIES OF NEW COMPOUNDS TARGETING THE RNA-BINDING PROTEIN HUR

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The essential role of RNA-binding proteins (RBPs) in the regulation of post-transcriptional processes has been well established, as their complexes with RNA are involved in numerous dysfunctions (*i.e.*, cancer, inflammation and neurodegeneration). In recent years, the interesting question has been posed of whether they could be exploited as therapeutic targets with clinical relevance.[1]
Challenged by this concept, the research efforts of our team in this study field have been aimed at identifying compounds able to modulate protein–RNA interactions, with a special focus on the ELAV/Hu (embryonic lethal abnormal vision) protein family, in particular HuR. In fact, it has been demonstrated that HuR is highly abundant in many cancers and it is upregulated and dysregulated in cancer cells; it may either represent a marker for malignancy or have an oncogenic role in numerous tumor systems. For this reason, HuR is considered a promising candidate target for governing gene regulatory mechanisms by developing compounds able to modulate its complexes with RNA.[2,3]
Here, we report on the synthesis and binding mode elucidation of *ad hoc* designed HuR ligands.[4]
Specifically, we applied a structure-based virtual screening approach to produce diverse scaffolds, easy to obtain through multi-component reactions or equally efficient processes. The most interesting structures were synthesized and their binding to HuR evaluated according to a STD (saturation transfer difference)-NMR and *in silico* combined strategy. The information so gathered constitute the foundation to further develop these structures aimed at obtaining compounds able to act on the stability of HuR–RNA complexes to modulate gene expression.

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A NOVEL NO-PHOTORESPONSIVE POLYMERIC PLATFORM AS AN ENHANCER OF DOXORUBICIN DELIVERY

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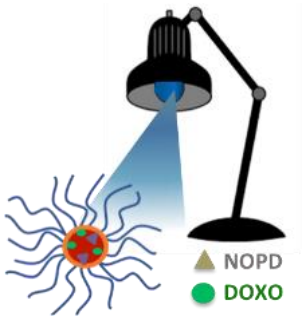
Nitric oxide (NO) is an endogenous messenger involved in cancer biology, low levels (pM-nM) of NO promote cancer growth, while high levels (µM) reduce cancer progression. The photogeneration of NO achieved using NO photodonors (NOPDs), namely, compounds able to release NO under the action of the visible light, has received a great attention as potential new anticancer therapy. Although the NOPDs previously studied,[1-2] showed to release enough NO to perform an anticancer activity, with the purpose of further increasing its performance, a novel NO-photoresponsive polymeric platform as an enhancer of doxorubicin delivery was developed. An amphiphilic block-copolymers has been designed and synthesized as the drug carrier, *via* friendly Ring-Opening Polymerization (ROP), and thoroughly characterized. As monomers lactide and an in-house prepared cyclic carbonate bearing a boc-protected amine, named tert-butyl (2-oxo-1,3-dioxan-5-yl) carbamate (referred as CT) were used, while as macro-initiator methoxy-polyethylene glycol with molecular weight 5000 Da (mPEG5000). The amine groups present in this polymeric system were deprotected and activated on-demand and enabled the introduction of a variety of drugs.[3] Namely, two grafted-polymers were produced via *N,N'*-disuccinimidyl carbonate (DSC) chemistry, coupling doxorubicin and NOPD separately, and then formulated in nanoparticles by means of solvent displacement technique. Comparing free DOXO, DOXO-nanoparticles and the mixed micelles (formed by the co-precipitation of NOPD-polymer and DOXO-polymer) both in dark (normal lab conditions) and light (under blue light irradiation) conditions, a remarkable enhancement in killing activity against lung, intestine, and skin cancer cell lines was observed when the mixed micelles were tested. The intra-cell co-localization study of each nanoparticle formulation was also performed by exploiting the intrinsic fluorescence in the red range of cyanine-5 previously coupled with the same polymeric platform and then co-self-assembled in the same nanoparticles. The cell-uptake assay has highlighted that all formulations were, to some degree, trafficking to the lysosomes during the endocytosis with an absence of cyanine-5 signals in nuclei. In addition, all nanoparticle formulations were spectroscopically and photochemically characterized, by confirming that the enhanced anticancer activity was a consequence of the synergic effect between the DOXO-polymer and NOPD-polymer.



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IDENTIFICATION OF RAD51-BRCA2 DISRUPTORS TO INHIBIT HOMOLOGOUS RECOMBINATION AND SYNERGIZE WITH OLAPARIB AS NEW ANTICANCER DRUG DISCOVERY CONCEPT

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Homologous recombination (HR) is an error-free DNA repair pathway triggered in the presence of DNA double-strand breaks (DSBs). BRCA2 protein plays a key role in HR by recruiting RAD51, the ATP-dependent recombinase that directly initiates DSBs repair. Because cancer cells rely on efficient HR activity, the inhibition of BRCA2-RAD51 protein-protein interaction (PPI) represents an attractive strategy for the development of novel therapeutics. In this context, we recently proposed a new anticancer drug discovery concept, which combines RAD51-BRCA2 disruptors with the PARP inhibitor Olaparib.[1] Indeed, according to our working hypothesis, administering RAD51-BRCA2 disruptors to BRCA2-functional cancer cells would chemically mimic the enhanced sensitivity to Olaparib observed in BRCA2-defective oncology patients, leading to a synthetic lethal effect.[2] Targeting PPI is an attractive strategy for designing innovative drugs. However it was proven to be challenging due to the typical protein-protein large and flat interfaces[3]. In-depth studies of the two protein structures allowed identifying two PPI critical "hotspots" on RAD51 surface, zone I and II, as suitable targets for the design of small molecule PPI inhibitors. Following a structure-based approach focused on zone I, a virtual screening campaign allowed us to identify a triazole-based hit compound **1** that showed to inhibit BRCA2-RAD51 PPI in biochemical assay.[2] To discover more effective compounds and depict general structure-activity relationship (SAR) studies, the chemical space around **1** was explored by optimizing a general synthetic strategy and building a library of new triazole analogues (Figure 1).[1,2] As proof of principle, compound **2** proved to inhibit HR and increase the response to Olaparib in pancreatic cancer cells expressing a functional BRCA2, supporting the idea that small organic molecules can mimic genetic mutations. To promote sustainable chemistry, we privileged protocols that exploit microwave-assisted synthesis.

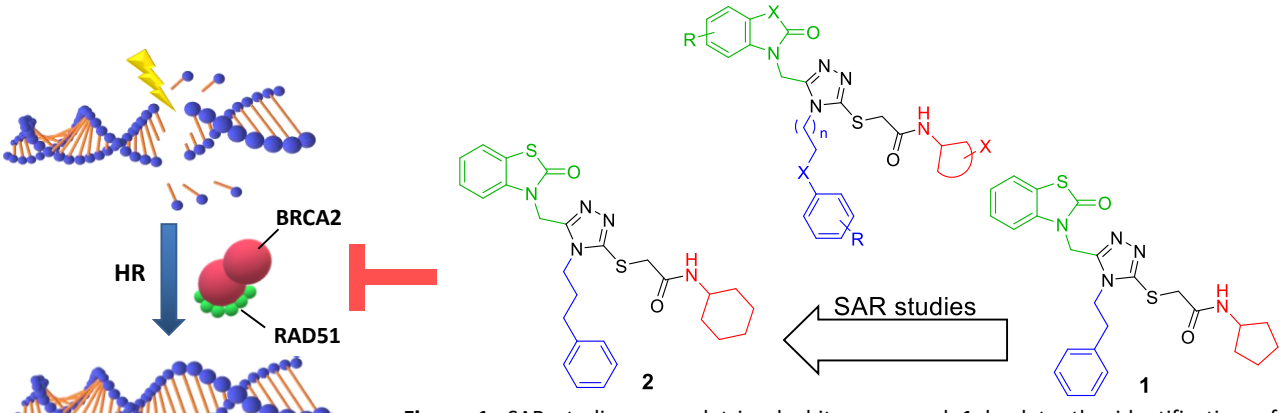

Figure 1. SAR studies around triazole hit compound **1** lead to the identification of compound **2** as HR inhibitor.

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MULTISCALE APPROACH TO PREDICT THE BINDING MODE OF METALLO BETA-LACTAMASE INHIBITORS

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The growing resistance against antibiotics has become a major threat in the global public health.[1] One of the causes of this resistance is the activity of the β-lactamases which catalyse the opening of the β-lactam ring leading to the inefficacy of the drug. The metallo β-lactamases (MBL) represent a challenging target for which no inhibitor has been approved so far for clinical use.[2] Many promising compounds have been proposed and inhibitors based on the coordination of the metal ions showed promising results.[3,4] However, from a computational point of view, the presence of the metal ions can affect the performance of the methods.[5] In this work, we propose a multiscale approach to predict the binding mode of a novel zinc-coordinated inhibitor of the IMP-1 enzyme, belonging to the MBL-B1 subclass, which presents two catalytic zinc ions within the binding site. First, we docked the inhibitor into the binding site of two different crystal structures of IMP-1. Then we evaluated different protocols for molecular dynamics simulations and we identified the best approach to stabilize the system and to better describe the coordination of the zinc ions. Finally, the complexes were refined through high-level QM/MM optimization. As a result, we predicted the binding mode of a new thiol-based inhibitor which is able to firmly coordinate both the zinc ions preventing the entrance of the substrates inside the catalytic site. We also identified a reliable pipeline that can be applied for the drug-design of β-lactamase inhibitors.

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LEAD OPTIMIZATION OF [1,2]OXAZOLE[5,4-*e*]ISOINDOLES AS TUBULIN POLYMERIZATION INHIBITORS

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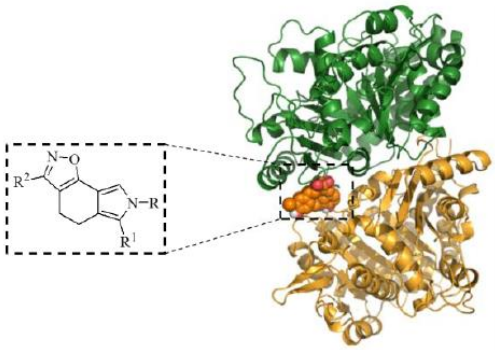
In recent decades, both natural and synthetic products have appreciably contributed to the development of a large number of anticancer drugs. Among these, tubulin-binding molecules represent an important class of antineoplastic agents. The therapeutic potential of antimicrotubule agents has been extensively exploited in clinical practice.[1,2] Recently, combretastatin A-4 (CA-4), a natural product, has been considered a promising lead compound for the design and synthesis of novel microtubule targeting agents.[3,4] CA-4 analogues containing the [1,2]oxazole ring as a linker of the two heterocyclic moieties displayed higher antitubulin activity than CA-4.[5] The [1,2]oxazolo[5,4-*e*]isoindole system, previously investigated by us, gave excellent results in preclinical studies reducing *in vitro* cell growth, impairing cell cycle progression and inducing apoptosis, as a consequence of the inhibition of tubulin polymerization, in experimental models of diffuse malignant peritoneal mesothelioma (DMPM).[6] Moreover, selected derivatives showed significant *in vivo* antitumor activity at well-tolerated doses in a DMPM xenograft model.

To obtain compounds with better drug-like properties and to gain structure-activity relationship (SAR) information regarding the position and the type of substituents, structural changes in the tricyclic core were further investigated. In particular, introduction of aminoalkyl chains or amides from primary and secondary amines in specific positions of the tricyclic scaffold were explored. Some of the synthetized compounds, screened at the National Cancer Institute in Frederick MD, confirmed strong antiproliferative effects in the micromolar-submicromolar range against the full panel of 60 tumor cell lines. Additionally, all new [1,2]oxazoles were also tested against different lymphoma cell lines at the Institute of Oncology Research (IOR). The effects of compounds on tubulin polymerization *in vitro* and on the binding of colchicine to tubulin were examined. Results will be discussed.



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THE MECHANISM OF N-PALMITOYLETHANOLAMINE HYDROLYSIS CATALYSED BY N-ACYLETHANOLAMINE ELUCIDATED BY QM/MM SIMULATIONS

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N-acylethanolamines (NAEs) are a large family of endogenous lipid transmitters characterised by an acyl chain, which differs among the NAEs for length and degree of unsaturation, linked to ethanolamine through an amidic bond. NAEs exert several biological effects, and among them N-palmitoylethanolamine (PEA) takes a major role in analgesia and in anti-inflammatory and neuroprotective processes mediated by the activation of PPARα.[1] The activity of PEA is terminated by its degradation to palmitic acid and ethanolamine catalysed by N-acylethanolamine acid amidase (NAAA), a member of the N-terminal nucleophile (Ntn) hydrolases superfamily mainly expressed in B lymphocytes and macrophages,[2,3] where it concentrates in lysosomes and shows an optimal activity at acidic pH.[4] NAAA is produced as proenzyme autocatalytically activated through the proteolysis that releases the N terminal cysteine C126, which is the catalytic residue.[3] As revealed by recent X-ray experiments,[5] the catalytic cysteine is placed at the entrance of a narrowed and hydrophobic channel that accommodates the benzothiazole-piperazine scaffold of the non-covalent inhibitor ARN19702,[6] and the lipophilic chain of the covalent inhibitor ARN726.[7] On the solvent-exposed access of the channel, the N-terminal amino group is caged between the side chain of D145, R300, and N287, which forms the oxyanion site with the backbone of E195. Even though NAAA represents a promising target for the identification of novel analgesic and anti-inflammatory agents, the mechanism through which it degrades its substrates is still not clear. Moreover, the catalytic mechanism of cysteine Ntn hydrolases has been poorly investigated.
In this work, the reaction mechanism of the PEA hydrolysis catalysed by NAAA has been studied by applying free-energy studies in the QM/MM framework. These computational results offer an insight on cysteine Ntn hydrolases and provide important information for the design of novel NAAA inhibitors.

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NEW ENANTIOPURE HYDROXYETHYL-PIPERAZINES AS CARBONIC ANHYDRASE INHIBITORS

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The hydration of CO_2 into bicarbonate and protons and the optimal equilibrium between these chemical species is essential for the vitality of organisms in all life kingdoms.[1] This reaction is catalyzed by the metalloenzyme Carbonic Anhydrase (CA), one of the most efficient enzyme known in nature, evolved in seven genetically different families (α-θ). A large number of isoforms are described among the different organisms, their presence being crucial for pH regulation, secretion of electrolytes and for other essential physiological or pathological processes.[2]For these reasons, CAs are important targets for drugs that can be used for different pathologies, providing that it could be possible to exploit the existent differences between families or isoforms to achieve a selective activity. This may not be an easy task, since the catalytic sites are well conserved, at least among the sixteen human α isoforms (I-XVI); however, variability can be found in hydrophilic and lipophilic accessory sites close to the Zn-binding domain. Aiming to further investigate the structure activity relationships (SAR) of a previously synthesized series of CA inhibitors **I-A** and **I-B**, bearing an enantiopure benzyl-piperazine scaffold,[3] two series of new chiral hydroxyethyl-piperazine **II-A** and **II-B**, carrying a 4-sulfamoylbenzoyl moiety on one nitrogen (**Figure 1**) have been designed and prepared from L- or D-Aspartic Acid.[4] In this communication the synthesis and inhibitory activity of the new compounds, assessed against four physiological relevant human CA isoforms (I, II, IV, IX), will be reported and compared to the already characterized benzyl-piperazine analogues **I-A** and **I-B**.

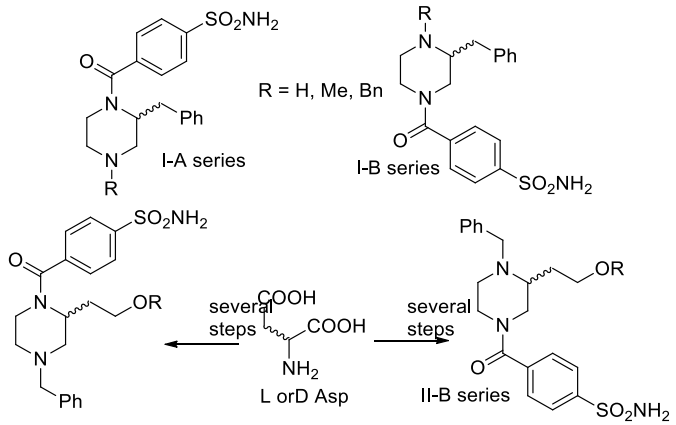

Figure 2

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MEMANTINE-FERULIC ACID HYBRIDS AS MOLECULAR PROBES FOR THE STUDY OF ALZHEIMER'S DISEASE

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Loss of synaptic function is closely associated to cognitive decline in Alzheimer's disease (AD), albeit the mechanism of synaptic damage remains incompletely understood. N-methyl-D-aspartate (NMDA) receptors (NMDAR) play a crucial role for synaptic plasticity in the healthy brain. The localization of NMDAR seems to affect their functional properties, with synaptic NMDAR contributing to cell survival and plasticity, while extrasynaptic NMDAR preferentially activating apoptotic signaling cascades.[1]

Memantine is an uncompetitive NMDAR antagonist approved by FDA to treat moderate-severe AD patients. It acts as an open-channel blocker with a relatively rapid off-rate from the channel. Due to this peculiar kinetics, memantine mainly enters the channel in conditions of excessive and prolonged glutamate exposure, preferentially acting on extrasynaptic/tonically-activated NMDAR over synaptic/phasically-activated NMDAR.[2]

Based on these premises, we envisioned memantine as a guiding motif which could specifically convey bioactive functions at glutamatergic extrasynaptic sites, where NMDAR have been proposed to trigger neurotoxic events mediated by amyloid β peptide (Aβ) and oxidative stress. To this aim, we designed and synthesized a set of hybrid compounds between memantine and ferulic acid (FA), which is known to protect the brain from Aβ neurotoxicity and neuronal death caused by ROS.[3]

Electrophysiological studies have shown that the new molecules behave, like memantine, as open-channel blockers of NMDAR, albeit with lower efficacy with respect to prototype. Furthermore, the most promising compound exerts antioxidant properties both directly and indirectly through the activation of Nrf-2 pathway in SH-SY5Y cells, and the ability to modulate Aβ production, as revealed by the observed increase of the non-amyloidogenic sAPPα in H4-sw cells.

Acknowledgments. This work was supported by the University of Bologna (grants from the RFO).

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DISCOVERY OF A NOVEL CLASS OF POTENT AND NON CYTOTOXIC *TRYPANOSOMA BRUCEI* GROWTH INHIBITORS

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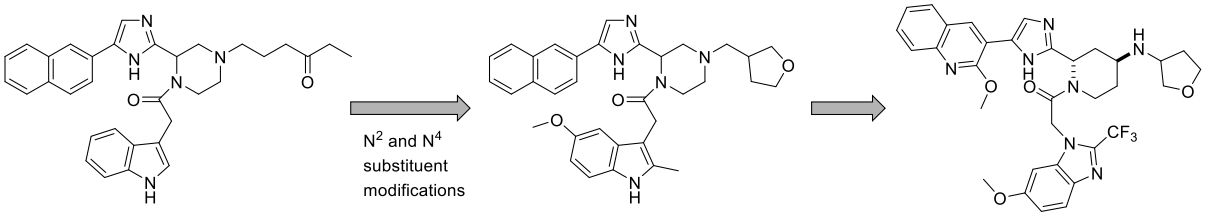
Human African Trypanosomiasis (HAT), also known as sleeping sickness, is a tropical disease caused by parasites *Trypanosoma brucei gambiense* (west Africa) and *rhodesiense* (east Africa). The disease affects less than 15000 people across sub-Saharan Africa (with 1446 cases recorded in 2017), where it is predominantly transmitted to humans by tsetse flies. HAT disease progression involves an initial blood stage and a second more severe stage once the parasite crosses the blood-brain barrier into the CNS. The CNS stage is always fatal if left untreated and the current front-line treatments for chronic *T. b. gambiense* (nifurtimox-eflornithine combination therapy), and *T. b. rhodesiense* HAT (melarsoprol, an organo arsenic compound), even though represent a substantial improvement over previous therapies, suffer from disadvantages such as inadequate efficacy, toxicity, high cost, emerging resistance and complex treatment regimens. HAT's designation as a neglected disease, with 70 million people at risk of infection, underlines that there remains a strong humanitarian need for new anti-parasitic agents to treat *T. brucei* infections.



1
T. brucei EC_{50} 489 nM
HeLa CC_{50} >25000 nM

2
T. brucei EC_{50} 5 nM
HeLa CC_{50} >25000 nM
Hepatocyte $t_{1/2}$ (m,h) 0.4 h, 2.4 h

3
T. brucei EC_{50} 7 nM
HeLa CC_{50} 7486 nM
Hepatocyte $t_{1/2}$ (m,h) > 4 h, 3.1 h

In this presentation, we will report a novel class of *T. brucei* growth inhibitors that show excellent potency *in vitro* with more than 1000 fold selectivity index and characterized by a fast-killing mode of action and high permeability in a blood-brain barrier model. The chemical class was identified by screening of subset of CNCCS compound collection in a whole parasite growth assay that measures the killing of the parasite. The hit compound **1** showed sub-micromolar activity in the *T. brucei* growth assay and no cytotoxicity in HeLa cells. The optimization of the series was initially focused on the exploration of the moieties at N^2 and N^4 of the piperazine ring and led to compounds (e.g. compound **2**) that inhibited *T. brucei* growth at low nanomolar concentrations (EC_{50} 5 nM), maintained no activity against proliferation of HeLa cells, but showed low *in vitro* stability (Hepatocyte stability $t_{1/2}$ 0.4 h (mouse), 2.4 h (human)). Further SAR in this series improved the ADME profile of the series (e.g. Compound **3** Hepatocyte stability $t_{1/2}$ >4 h (mouse), 3.1 h (human)), whereas maintained nanomolar activity in the parasite growth assay and good selectivity over HeLa cells. Biological *in vitro* and *in vivo* profile resulted suitable for obtaining proof-of-concept in an *in vivo* efficacy model. The SAR leading to this compound with biological and pharmacokinetic profile of the series will be described.

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BUFFALO RICOTTA PEPTIDE REDUCES OXIDATIVE STRESS IN INTESTINAL EPITHELIAL CELLS AND ANGIOTENSIN II-INDUCED VASOCONSTRICTION ON MICE MESENTERIC ARTERIES BY INDUCTION OF NRF2 TRANSLOCATION

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Buffalo dairy products are an important source of bioactive peptides. These are inactive, since encrypted in their parent sequences, but turn active when released by fermentation or ripening during food processing, or by digestive enzymes during gastrointestinal transit.[1] Once released, the bioactive peptides are able to exert either local or systemic pharmacological effects, involving specific biochemical pathways and leading to the identification of undisclosed drug-target interactions. This is why food-derived bioactive peptides, particularly from dairy products, are attractive tools for drug discovery campaigns.[2]

In the present study, to evaluate the biological activities of encrypted peptide sequences from buffalo ricotta cheese, a simulated gastrointestinal (GI) digestion of the raw material was performed.[3] Chemical and pharmacological characterization of the digest leads to the identification of a novel peptide endowed with antioxidant and anti-hypertensive action. The GI digest was fractionated by Semiprep-HPLC and fractions were tested against reactive oxygen species (ROS) release in H_2O_2-treated intestinal epithelial cell line. UHPLC-PDA-MS/MS analysis revealed the presence of an abundant β-lactoglobulin peptide (*SFNPTQL*, **BPR2**) in the most active fraction. The peptide was synthesized *via* Fmoc chemistry solid phase peptide synthesis and pharmacologically characterized. Pharmacological assays revealed the antioxidant activity of **BPR2**, involving ROS reduction, Nuclear factor erythroid 2-related factor 2 (Nrf2) activation and cytoprotective enzymes expression. Bioavailability studies through Caco-2 cell monolayer[3] revealed equal bi-directional transport and linear permeability of **BPR2**, consistent with a passive diffusion mechanism. In addition to its local effects, administration of **BPR2** on mice mesenteric arteries counteracts the Angiotensin II-induced vasoconstriction by Nrf2 nuclear translocation, reduction of active form of Ras-related C3 botulinum toxin substrate 1 (Rac1) and NADPH oxidase activity. These data suggest a specific further highlight the role of buffalo ricotta cheese-derived peptides against oxidative stress related diseases and suggest their health promoting potential. Further studies are ongoing to identify the specific **BPR2** structure-activity relationship and to identify its biological targets.

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HALOGENATED PHENOTHIAZINES WITH ANTI-TB ACTIVITY THROUGH THE NDH-II INHIBITION

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Antimicrobial resistance is one of the most public health threat owing to the ability of the microorganisms to become rapidly resistant to commercially available antibiotics. To overcome the resistance issue, the research for new drugs with mechanism of action different from those of the approved drugs is essential.

Over the years, the enzymes involved in the generation of energy in form of ATP gained attention for their fundamental importance both in *M.Tuberculosis* growth and in latency state.[1] In fact, in 2012 FDA approved bedaquiline, a completely new anti-tubercular drug that inhibits F_1-F_0 ATP synthase.[2] Another most investigated respiratory chain enzyme is the type II of the NADH dehydrogenase (NDH-II), of which mammalian cells are lacking. Various NDH-II inhibitors have been reported in literature that confirm its validity to obtain an anti-tubercular effect. Among them, some phenothiazine derivatives, including therapeutic thioridazine and chlorpromazine.[3]

Thus, by a repurposing approach, a set of in-house phenothiazines, designed as anti-HIV compounds, were assayed against *M.Tuberculosis* H37Rv. Based on promising results, additional derivatives were designed. In particular, various side chains were studied as N-10 substituents, coupled with halogen atoms placed in different positions of the phenothiazine core to exploit the ability of halogen bonding to improve the target recognition, an approach that have recently attracted the attention of medicinal chemists to design innovative compounds.[4]

All the new derivatives were assayed for their activity against *M.Tuberculosis* H37Rv strain in parallel with the cytotoxicity. The best compounds were further investigated for their ability to inhibit NDH-II enzyme, for their bactericidal effect, and in combination with known anti-tubercular drugs to evaluate the synergistic effect. Finally, the most promising compounds are under evaluation against a panel of dopamine/serotonin receptors in order to study the safety profile.

From the whole biological characterization, interesting derivatives with clear SAR insights emerged, that will be the object of the presentation.

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SMALL MOLECULE LIGAND-BASED FLUORESCENT PROBES FOR THE IMAGING OF THE NOREPINEPHRINE TRANSPORTER

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Fluorescent ligands provide a class of valuable pharmacological tools[1, 2] for the identification of transporters trafficking pathways in living cells and for the study of their recycling and degradation mechanisms. Novel tropane-based fluorescent ligands that bind with high affinity to dopamine transporter (DAT) and serotonin transporter (SERT) have been successfully used for transporter visualization, colocalization and trafficking studies.[3-5] Building on this strategy, we have focused on the design and synthesis of novel molecular probes with high affinity and selectivity for the norepinephrine transporter (NET) and on the evaluation of their binding affinities and NET selectivities over DAT and SERT. The general structure of the compounds is based on a ligand linked by a linker chain of variable length and atomic composition to a fluorescent dye. The parent ligands, Nisoxetine and Talopram, were selected taking into account affinity, selectivity and synthetic feasibility. Using previously described structure activity relationships (SAR) we chose two different positions on each of these parent molecules for "fluorescent tagging". The design, synthesis and binding affinities of this first set of novel fluorescent tools will be reported with a plan for further characterization.

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INHIBITION OF FTSZ TO BLOCK BACTERIAL REPLICATION: DEVELOPMENT OF POTENT ANTIMICROBIALS

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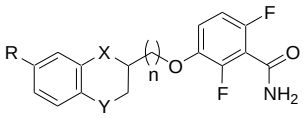
The need for developing efficient antibiotics is urgent and the lack of them represents a severe problem for public health. Focusing on innovative mechanisms of action is one potential approach. Among the promising targets, the bacterial divisome, a complex of copious division proteins, is crucial for bacteria viability. Divisome proteins are widely conserved among several bacteria and are absent in eukaryotic cells, thus enabling high selectivity and a low cytotoxicity of the novel antimicrobics.
FtsZ is one of these proteins; it is a β-tubulin functional homologue, responsible for auto-polymerization at the cell mid-point with the consecutive formation of the Z-ring, cell constriction, mesosome formation and thus the separation in the two daughter cells.[1]
In the recent years, starting from the synthetic molecules risen in literature, we prepared 2,6-difluorobenzamide derivatives, structurally similar to the most interesting FtsZ inhibitors lead compounds.[2]
Our class of compounds (Figure 1) were designed by introducing a differently substituted 1,4-benzodioxane, 1,4-benzoxathiane or 1,4-benzodithiane, in addition to the 2,6-difluorobenzamide. Methylenoxy- or ethylenoxy- bridges linked the two moieties together.[3-5]



R= H, lipophilic substituent
X= O, S
Y= O, S
n= 1,2

Figure 1

In this work, we report the most recent results. It was proven that FtsZ is the real target of these molecules by performing peculiar biochemical assays. Moreover, the compounds were tested for MICs against several Gram-positive and Gram-negative strains and over mutated *E. coli,* showing very promising results. We further evaluated cytotoxicity over human MRC-5 cells for the best derivatives.
Finally, we performed molecular modelling studies, aimed at defining and validating a docking protocol for FtsZ to decipher the binding mode of our class of compounds. *In silico* studies were also performed to detect potential pockets in the enzyme and to study the physico-chemical and drug like properties of the chemical compounds.

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CANNABIS LIGHT PREPARATIONS IN ITALY

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The increase of *Cannabis sativa* crops found in Italy in the last few years is due to the rising demand of Cannabis products. In 2016 the law 242 defined legal in the crop a total THC content not exceeding 0.2 % and in any case, not exceeding 0.6 % only for farmers.
A further note was published from the Ministry of the Interior (20/07/2018 number of protocol 2018/43586) in which it was remarked that the sale or the presence in the markets of products (inflorescences, concentrates, essences and resins) or plants with concentrations higher than 0.5% fall in the definition of illicit drugs or psychotropic substances subjected to the supervision and control of the Ministry of Health and thus their detention and marketing constitutes a crime. In this ambiguous legislative panorama arises the need to control the so-called Cannabis light preparations to avoid to deal with illegal products. This is the reason for the recent sentence of Cassation (31 May 2019) which wants to put an end to a market border-line with legality and potentially harmful to health. Almost 1000 inflorescences of Cannabis light, obtained from Italian growers, were analyzed in our laboratory by a reliable LC-UV system, in order to determine the presence of 11 cannabinoids and the amount of THC, THCA, CBD, CBDA and CBN.[1,2,3]
18% of the crops are to be considered legal for the market (THC tot < 0.2%), a substantial amount of Cannabis light preparations (24%) would be considered illegal (THC > 0.5%), but the most of the inflorescences (58%) have a THC tot content comprised between 0.2% and 0.5%, and it is not clear whether these products could be sold or not (Fig.1). All the analyzed samples belong to chemotypes III and our results showed a linear correlation between CBD and THC levels in each Cannabis sample. It is likely that our results can represent a view of Cannabis Light products on the Italian market.

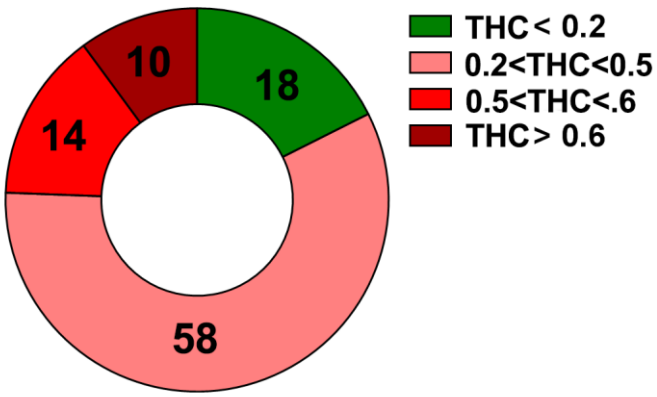

Fig. 1 - Cannabis light samples represented in a part of whole graph depending on THC tot (%) concentration (922 samples).

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CANNABIS OLIVE OIL: COMPARISON AMONG DIFFERENT PREPARATION METHODS

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The therapeutic properties of *Cannabis sativa* are widely recognized nowadays. Due to an increasing interest and the lack of authorized medicinal products, Italian pharmacists are involved in compounding Cannabis magistral preparations, mostly based on the olive oil extraction of cannabinoids from inflorescences. The main extracted components are Delta9-tetrahydrocannabinol (THC) and cannabidiol (CBD), and their corresponding acid, THCA and CBDA. A compendial standardized procedure is not yet available, so methods proposed in scientific literature are followed to prepare Cannabis olive oils.[1-4] The methods most frequently used are four, all based on maceration of vegetable materials in olive oil at high temperature. Two methods[1-2] don't involve a preliminary decarboxylation, step required to convert THCA and CBDA, molecules pharmacologically less active, into THC and CBD, the active neutral compounds. Decarboxylation can be obtained heating the plant materials at a temperature above 100°C before maceration in olive oil[3] or by sonication.[4]

Aim of this work is to evaluate the Efficiency of Extraction (E. E.) of THC and CBD total contents in Cannabis olive oils compounded in the Italian pharmacies, using four Cannabis varieties (Table 1) and following four different preparation conditions.[1-4] Over 3000 samples were analyzed in 2017 and 2018. The E. E. was calculated considering the standardised cannabinoid total content declared in the data sheet of each variety versus the same data obtained in analyzed samples. Cannabis olive oils are prepared with the recommended ratio cannabis/solvent 1g/10 mL (Table 1). Due to the very low content (less than 0.2% w/w), CBD in Bedrocan and THC in Bedrolite was not considered.

The E. E. of total THC and CBD in all Cannabis varieties and for any preparation method resulted quite similar, slightly higher for CBD (almost always over 80%) than for THC (less than 75%). In case of varieties with similar CBD and THC content, Bediol and FM2, homogeneous E.E. values were observed. High variability was observed for the methods without decarboxylation.[1,2]

In conclusion, independently by the used method, cannabinoid extraction is never complete, but probably it can be further improved using a standardized preparation procedure.

Table 1 - Extraction efficiency (%) of CBD and THC measured in cannabis oil samples obtained using different cannabis varieties and preparation methods.

% w/w	**Bedrocan (THC 2.2 w/w)**	**Bediol (CBD 0.8 w/w, THC 0.7 w/w)**		**Bedrolite (CBD 0.9 w/w)**	**FM2 (CBD 1.2 w/w, THC 0.8 w/w)**	
	THC+THC-A	CBD+CBD-A	THC+THC-A	CBD+CBD-A	CBD+CBD-A	THC+THC-A
Romano/ Hazekamp (1)	71.5±23.5	83.9±53.1	72.8±34.9	82.7±60.1	84,1±60,0	85,0±55,1
Cannazza (2)	74.5±32.0	83.9±32.7	63.1±22.0	80.8±46.0	83,2±41,1	71,6±29,6
SIFAP-SIFO (3)	71.6±19.0	79.0±27.9	62.8±16.4	73.2±32.8	74,8±21,5	69,9±20,5
Calvi (4)	59.7±13.7	74.1±20.0	62.6±8.8	-	-	-

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EXPLORING THE ROLE OF THE HETEROCYCLIC MOIETY OF 5-(4-NITROPHENYL)-2-FUROIC ACID, A PROMISING INHIBITOR OF MBTI FROM *MYCOBACTERIUM TUBERCULOSIS*

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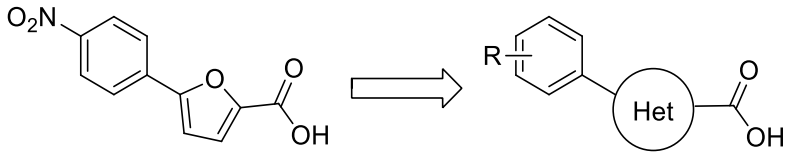
Tuberculosis nowadays ranks as the first leading cause of death from an infectious disease worldwide,[1] especially because of the appearance of multi-drug resistant forms. Efficient iron acquisition is crucial for the pathogenesis of *Mycobacterium tuberculosis* (Mtb), since it serves as a cofactor in many essential biological processes. Given the high toxicity of free iron, Mtb synthesizes siderophores to assimilate it. The bifunctional salicylate synthase MbtI catalyzes the first step of mycobactin biosynthesis through the conversion of the primary metabolite chorismate to salicylic acid *via* isochorismate.[2,3] By means of a receptor-based virtual screening approach, we identified MM40 a new furan derivative targeting this salicylate synthase (MbtI). Herein, we report on a structure activity relationship study on MM40 to investigate the role of the heterocyclic moiety for the interaction with the enzyme.



MM40

IC_{50} = 7.6 μM

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5,6-DIMETHYL-1,3-DIHYDROBENZO[*c*][1,2,5]THIADIAZOLE-2,2-DIOXIDE AS NEW STAT3 INHIBITOR

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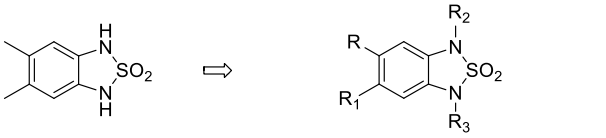
As part of our ongoing investigations into the development of novel STAT3 inhibitors,[1] through a computational repositioning approach, we identified the 5,6-dimethyl-1,3-dihydrobenzo[*c*][1,2,5] thiadiazole-2,2- dioxide **I** (NCI database, Figure 1)[2] as a new candidate targeting the STAT3 SH2 domain. Since it exhibited an interesting inhibitory activity (inh. % = 81.6 at 30 µM), we investigated the molecular basis of its binding mode by synthesizing a set of derivatives showing different substitution patterns (Figure 1). The SAR study on these analogues highlighted several essential features to ensure inhibitory activity against STAT3. Furthermore, spectroscopic and spectrophotometric studies suggested a possible mechanism of interaction for product **I**. The results of our investigation will be presented.



I

R = H, CH_3, CF_3, NO_2, Cl, $NHCOCH_3$, Ph
R_1 = H, CH_3, Br
R_2, R_3 = H, CH_3, CH_2Ph, $CONH(CH_2)_2Ph$

Figure 1. Product **I** and its derivatives (general structure).

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MASS SPECTROMETRY IDENTIFICATION OF PROTHROMBIN AS A NOVEL BINDING PARTNER OF THE HUMAN RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE)

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The human Receptor for Advanced Glycation End products (RAGE) is a multi-ligand pattern recognition receptor proposed to be involved in propagating inflammatory responses. It was named after its ability to bind Advanced Glycation End products (AGEs), a heterogeneous group of damaging molecules derived from non-enzymatic reactions between sugar and protein. Other ligands identified so far are S100 proteins, HMGB1, Mac-1, amyloid-beta peptide and extracellular matrix proteins.[1] RAGE is a type I transmembrane glycoprotein with an ectodomain, a single transmembrane domain and a carboxyl-terminal tail responsible for intracellular signaling that culminates in the activation of NF-κB. RAGE ectodomain contains 3 IgG like domains: V, C1 and C2. V and C1 constitute an integrated domain to which most ligands bind.[2] Soluble RAGE (sRAGE) is a scavenger receptor that just contains the ectodomain. Although several studies evidenced an important role of RAGE in mediating inflammation, the molecular mechanisms are still poorly understood. In particular, RAGE damaging or protective roles remain to be elucidated.

To identify RAGE ligands in human plasma, we set up an *in vitro* pull-down assay based on VC1 as a bait and on the subsequent identification of the proteins by mass spectrometry.[3] Out of 46 interacting partners, proteins of the blood coagulation and the complement processes were the most representative. Prothrombin (PT) was the most abundant interactor identified and it shares with other 4 interactors the γ-carboxyglutamic acid (Gla) domain, a high-affinity calcium binding domain. The recalcification of plasma gradually reduced the affinity of PT to VC1. The Gla-domain mediated the binding of PT to VC1 as confirmed by the lack of interaction with VC1 of a Gla-domain less PT (des-Gla-PT), obtained by *in vitro* limiting proteolysis with α-chymotrypsin. The affinity of VC1 for purified PT was measured in solution by Microscale thermophoresis (MST). In the presence of increasing concentrations of $CaCl_2$, MST analysis revealed a reduced affinity of VC1 for PT. Moreover, we examined the effect of RAGE on blood coagulation using a method based on the aPTT-reagent that activates the intrinsic pathway. The presence of different concentrations of VC1 delayed plasma clotting time with respect to the control by a value that was dose-dependent. This is the first evidence that RAGE could affect blood coagulation via targeting the Gla domain of coagulation factors.

In healthy adults, RAGE expression is very low except in Type I alveolar cells of the lung. Besides being a major mediator of pulmonary inflammation, our results suggest that RAGE could prevent blood coagulation upon loss of the alveolar-capillary membrane integrity. This study could set the basis to understand the molecular mechanisms of RAGE-PT interaction in modulating inflammation/coagulation processes, in particular related to pulmonary diseases.

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ADVANCED QUANTITATIVE PROTEOMICS TO EVALUATE THE SIGNALING PATHWAYS INDUCED BY BIOMOLECULES IN HUMAN DERMAL FIBROBLASTS

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Background

4-hydroxynonenal (HNE) is an α,β-unsaturated carbonyl compound, a subgroup of reactive carbonyl species involved in many molecular pathways including inflammation, skin aging and actin elastosis. These effects are mediated by a direct reaction with extracellular matrix proteins (i.e. covalent adducts with collagen and elastin and protein cross-links) as well as by an intracellular activity.[1] Hyaluronic acid (HA), a linear polysaccharide of the extracellular matrix, is responsible of the hydration and elasticity of tissues. Low molecular weight fragments of HA are produced, for example, in the wound healing process inducing pro-inflammatory effects and stimulating macrophages to release cytokines. The extracellular signals reach the intracellular space thanks to HA receptors, such as CD44 on fibroblasts.[2] HA low-molecular-weight form also demonstrated to penetrate across the *corneum stratum in vitro*.[3] At last, the epidermal activity of retinoic acid (RA) (i.e. stimulation of procollagen synthesis, fibroblasts proliferation) could be considered as positive control being already well confirmed.[4,5]

Aims and Methods

Basing on *in vitro* proteomic approach, we tested the toxicity of HNE and the intra-/extra-cellular effects of HA low molecular weight and RA on human dermal fibroblasts. In details, we identified and quantified the up- and down-regulated cellular proteins by label-free quantitative proteomics thanks to nLC-MS/MS (Orbitrap Fusion Tribrid Mass Spectrometer). The analysis of data was performed by MaxQuant and Perseus software for the quantitative analysis, Cytoscape and other suitable software for the network analysis.

Results and Conclusions

Among the results, HNE up-regulated enzymes as the farnesyltransferase, prenylcysteine oxidase 1 (PCYOX1) and protein genes (i.e. AKR1C1/2, SLC25A2); it supported the release of hydrogen peroxide that leads to an oxidative/electrophilic stress response by the Nrf-2 pathway (i.e. thioredoxin reductase, superoxide dismutase aldo-keto reductase); it affected the matrix components up-regulating fibrinogen and inducing proteins related to the collagenase gene family and elastin synthesis with the deposition of the elastotic material. The incubation of different concentrations of hyaluronic acid promoted several structural constituents and regulators of the cytoskeleton (i.e S100A6 protein, thymosin beta-10, calponin) improving the cell shape and also cell proliferation (i.e. chondroitin sulfate proteoglycan 4) sustaining the fibroblasts role. About the retinoic acid instead, we confirmed most of the cellular pathways so far reported for all-trans RA, including, among others, up-regulation of cytokeratins and down-regulation of metalloproteinases.[6] To summary, counting on label-free quantitative proteomics and high-performance analytic instruments, we identified a huge number of proteins as well as the intracellular pathways into human dermal fibroblasts reaching a more complete comprehension of the molecular actions of HNE and of two very common cosmetic ingredients, HA and RA. This method can easily be also applied to understand the activity, the mechanism of action and the toxicity of many other active principles.

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VACCINIUM MACROCARPON URINE METABOLITES IDENTIFICATION AND QUANTIFICATION AND EVALUATION OF THEIR EFFECT ON CANDIDA ALBICANS ADHESION

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The resistance to antimicrobial therapies leads to persistent infections, therefore alternative approaches to conventional therapies such as the use of natural products can be a promising way. *Vaccinium macrocarpon* (cranberry) has been widely used for decades in the prevention of urinary tract infections (UTIs).[1] Proanthocyanidins (PACs), especially the A-type, are the main responsible for the *in vitro* activity.[2] Nevertheless, there are controversial results on their presence in human urine after cranberry oral intake.[3,4] The reason of such dissimilar results can be the use of different dosages as well as non-standardized cranberry products. The aim of the work was i) to identify and quantify cranberry components and metabolites in human urine after the oral intake of a highly-standardized cranberry extract (Anthocran™, Indena S.p.A.), ii) to evaluate the urine effect on *C. albicans* adhesion and biofilm formation and iii) to test the *in vitro* activity of a mixture of metabolites identified in the active fractions. Ten young healthy female volunteers took 2 capsules Anthocran™/day for 7 days. Urine samples were collected before supplementation and at the following time-points after the last dose: 1, 2, 4, 6, 10, 12, 24 hours. An HPLC-MS/MS method was set-up using a LTQ-XL-Orbitrap working in data dependent scan mode to perform the analyses. A targeted and an untargeted approach was used to identify 33 known metabolites and compounds hereto unreported in the literature. The identification was confirmed by the use of pure standards, if commercially available, and their quantification was performed by using ethylgallate as internal standard. 5-(3',4'-dihydroxyphenyl)-gamma-valerolactone, one of the main PAC metabolites, was synthesized to have a reference standard of this class of compounds. Collected urine were then tested on the reference strain *C. albicans* SC5314: urine fractions collected after 1 and 12 hours were found to significantly reduce the adhesion compared to the control. The *ex vivo* effect of cranberry metabolites was then confirmed by evaluating the significant inhibitory effect of a reconstituted mixture of metabolites on *C. albicans* adhesion and biofilm formation. In conclusion, the data reported in the present work demonstrate that i) PACs are metabolized after cranberry oral intake, ii) urines collected following one week of cranberry treatment are able to significantly reduce *C. albicans* adhesion and biofilm formation, iii) the activity can be due to a synergistic effect of identified cranberry metabolites including PACs metabolites.

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COMPUTATIONALLY DRIVEN STRUCTURE OPTIMIZATION, SYNTHESIS, AND BIOLOGICAL EVALUATION OF NOVEL PCSK9 INHIBITORS

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The high degree of organization into secondary structures (helices, turns and β-sheets) of proteins engenders the modulation of an array of biological functions. Several of these functions are affected by the reciprocal interactions of proteins, known as "protein-protein interactions" (PPIs). Recently, the disruption of PPIs is considered an attractive way to manage the activation/deactivation mechanism of proteins involved in numerous pathological conditions.

In this respect, the discovery and development of innovative drug candidates targeting cardiovascular diseases may start from the identification of novel compounds able to inhibit the PPI between PCSK9 and LDLR. In fact, the suppression or impairment of this PPI increases the liver intracellular cholesterol pool and decreases the plasma LDL-C level and, consequently, the atherosclerosis progression.

To interfere with the PCSK9/LDLR interaction, we have identified the **peptides** responsible for the hypocholesterolemic effects of lupin proteins, by performing *docking, MD simulations* and *MM-GBSA calculations* on a freshly developed PCSK9 model.[1] Then, the small-peptides showing the highest theoretical activity were synthesized and biologically tested. Interestingly, one of these peptides (**P5**) was recognized as a potent PCSK9-LDLR interactions inhibitor, constituting a promising candidate for designing new potent inhibitors of the PCSK9/LDLR PPI. Another one (**T9**) displayed an encouraging activity on the Gain-Of-Function (GOF) variant of PCSK9, the $PCSK9^{D374}$, for which no inhibitors have been described in literature.[2] Then, aiming to improve the potency of this peptide, the T9 residues mainly responsible for the interaction with $PCSK9^{D374Y}$ (hot spots) were computationally predicted. Subsequently, the “non-hot” residues were properly substituted by new amino acids capable to increase the structural complementarity between T9 and $PCSK9^{D374Y}$. The outcomes of this study were confirmed by *in vitro* biochemical assays and cellular investigations.[3]

In a parallel approach, new **peptidomimetics** have been designed by means of combined computational methods. In fact, starting from a poli-imidazole showing IC_{50} value in the micro molar range and mimicking a poli-Ala peptide,[4] new imidazole analogs were designed, synthesized, and biologically characterized. Finally, one of them displayed a PCSK9 inhibitory activity 10-fold lower than the template compound and, remarkably, at the concentration of 1 μM, it successfully prevented the LDLR degradation mediated by PCSK9 on HepG2 cells. As well as increasing the LDL uptake at the same concentration, this compound represents currently one of the most potent small molecules targeting the PCSK9/LDLR PPI.

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GLYCOSIDASE-CATALYZED SYNTHESIS OF GLYCOSYLATED NUTRACEUTICAL INGREDIENTS

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Hydroxyphenyl propenoic acids (hydroxycinnamic acids) and their alcohol derivatives are common components of the human diet which often occur in plants in the form of various glycosides. As the diets rich in polyphenols have repeatedly been related to low incidence of cardiovascular, neurodegenerative, and oncological diseases, various food supplements containing these compounds are becoming increasingly popular among the general population.

In quest of a biocatalytic route to structurally complex phenolic glycosides, we built a sustainable and convenient, one-pot two-enzyme method for the glucosylation of arylalkyl alcohols based on the synthetic exploitation of a fungal rutinosidase from *A. niger* and rhamnosidase from *A. terreus*. Both these enzymes were available to us as heterologous proteins produced by a recombinant strain of *P. pastoris.*

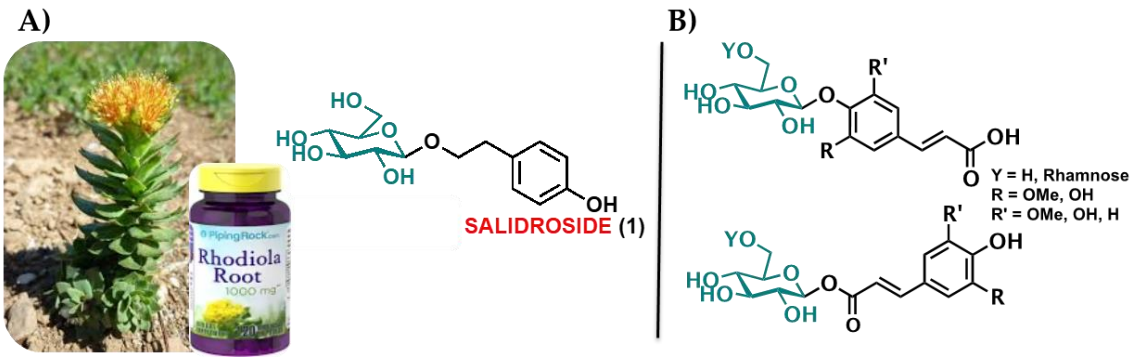

Figure 1. Examples of the synthetized glycosyl phenols.

As an example, the α-glucoside salidroside (**1**, **Figure 1A**), a compound endowed with various pharmacological effects and commercialized in *Rhodiola rosea* nutraceutical formulations, was obtained in high isolated yield and purity from tyrosol thanks to our one-pot enzymatic process. Furthermore, during the course of our investigation, we found that the rutinosidase from *A. niger* not only efficiently converted hydroxylated aromatic acids (*e.g. c*oumaric and ferulic acids) into the respective phenolic rutinosides, but surprisingly could also catalyze the formation of the respective glycosyl esters (**Figure 1B**).

Here the results of our systematic study about the glycosidase-based biocatalytic preparation of glycosylated nutraceutical ingredients, which lead us to the discovery of a unique enzymatic entry to naturally occurring glycosyl esters, are reported.

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STRUCTURAL OPTIMIZATION, IN VITRO CHARACTERIZATION AND IN SILICO PHARMACOPHORE MODELING OF A NEW ANTIPLASMODIAL CHEMOTYPE

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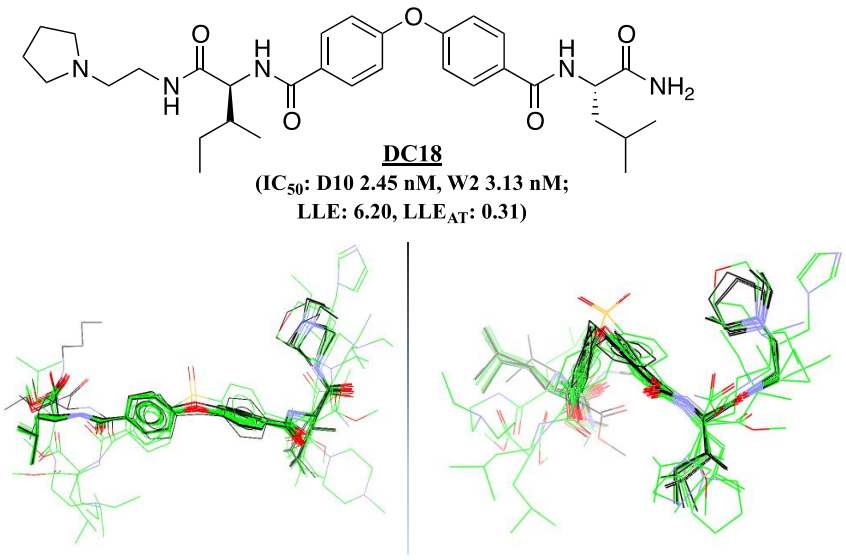
Malaria is the most common parasitic disease worldwide, and the third deadliest infection after HIV and tuberculosis. Due to the risk of diffusion of artemisinin resistance in Africa, new drugs for ensuring efficacious antimalarial treatment are urgently needed. In one of our previous communications, we reported the discovery of a new 4,4'-oxybisbenzoic acid-based chemotype with promising antimalarial activity against both CQ-sensitive and CQ-resistant *P. falciparum* (Pf) strains.[1] Here, we present our exploration of the structural requirements for the inhibition of Pf growth by this new chemotype through a detailed SAR investigation focused, also, on the improvement of its drug-likeness parameters. Our investigation led to the identification of the potent antiplasmodial compound, DC18.

An *in silico* analysis was run to build a pharmacophore model for this new, still target-less, antiplasmodial chemotype.



DC18

(IC_{50}: D10 2.45 nM, W2 3.13 nM; LLE: 6.20, LLE_{AT}: 0.31)

Figure 1. Structure of DC18 (up) and its ligand overlay-based pharmacophore modeling (down).

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TARGET-ORIENTED DEVELOPMENT OF NOVEL ANTIPROTOZOAL AGENTS: CELASTROL CARBOXAMIDES AS INHIBITORS OF *LEISHMANIA* Hsp90

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The *Leishmania* isoform of the 90kDa Heat Shock Protein (*Ls*Hsp90), a chaperone known to assist the folding of more than 200 client proteins, was reported to be generally involved in parasite differentiation from promastigote to amastigote possessing a pivotal role during heat-induced cellular stress. Moreover, it was demonstrated that an impair of the native functions of *Ls*Hsp90 through the action of active-site inhibitors can exert a detrimental effect on the natural parasite life-cycle ultimately leading to its death.[1,2]

Celastrol (**Figure 1**) is a natural triterpene exhibiting a plethora of *in vitro* and *in vivo* activities. Among them, this pentacyclic compound is reported to possess a promising antiproliferative activity thanks to its ability of interacting with the chaperone cycle of the human isoform of Hsp90 (*h*Hsp90).[3] Moreover, celastrol derivatives (*e.g.* the methyl ester pristimerin, **Figure 1**) have also exhibited an interesting antiprotozoal activity.[4,5]

With the aim of building a target-oriented approach to treat *Leishmania* infections based on the inhibition of *Ls*Hsp90, we prepared two basic carboxamides celastrol derivatives (**SS-1** and **SS-2**, **Figure 1**) to enhance its leishmanicidal activity and selectivity of action by deducting its unspecific cytotoxicity (measured as IC_{50} on HMEC-1 cell lines). Accordingly, celastrol and the two basic derivatives **SS-1** and **SS-2** (**Figure 1**) were *in vitro* tested for their leishmanicidal activity against promastigotes of *Leishmania tropica* and *L. infantum* and, in parallel, their mechanism of action was investigated as well *via ad hoc in vitro* experiments using a recombinant Hsp90 from *L. braziliensis* (LbHsp90).[6]

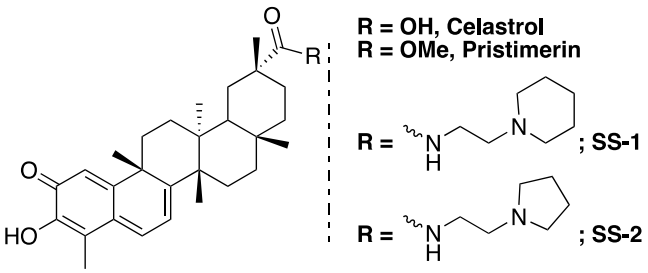

Figure 1. Structures of celastrol, pristimerin and the basic derivatives SS-1 and SS-2.

In virtue of their pH sensitive basic heads, both **SS-1** and **SS-2** were found to be more potent (IC_{50} in the nanomolar range) and selective leishmanicidal agents than celastrol itself. Furthermore, we were able to demonstrate that **SS-1** and **SS-2** successfully (*in vitro*) inhibited the native kinase activity of *Lb*Hsp90 highlighting the key role of the inhibition of this chaperone in their mechanism of action.

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THERANOSTIC NANOCAGES FOR IMAGING AND PHOTOTHERMAL THERAPY OF PROSTATE CANCER CELLS BY ACTIVE TARGETING OF NEUROPEPTIDE-Y RECEPTOR

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Gold nanocages (AuNCs) have been shown to be a useful tool for imaging and hyperthermia therapy of cancer, thanks to their unique optical properties, low toxicity and facile surface functionalization. Herein, we use AuNCs for selective targeting of prostate cancer cells (PC3) via specific interaction between neuropeptide Y (NPY) receptor and three different NPY analogs conjugated to AuNCs (Figure 1). Localized surface plasmon band of the nanoconjugates was set around 800 nm, which is particularly promising for in vivo applications. Long-term stability of nanoconjugates in different media was confirmed by UV-vis and DLS studies. Active NPY receptor targeting was observed by confocal microscopy showing time-dependent AuNCs cellular uptake. Activation of ERK1/2 pathway was evaluated by Western blot to confirm the receptor-mediated specific interaction with PC3. Cellular uptake kinetics were compared as a function of peptide structure. Cytotoxicity of nanoconjugates was evaluated by MTS and Annexin V assays, confirming their safety within the concentration range explored. Hyperthermia studies were carried out irradiating the cells, previously incubated with AuNCs, with a pulsed laser at 808 nm wavelength, showing a heating enhancement from 6 to 35 °C above the culture temperature dependent on the irradiation power (between 1.6 and 12.7 W/cm2). Only cells treated with AuNCs underwent morphological alterations in the cytoskeleton structure upon laser irradiation, leading to membrane blebbing and loss of microvilli associated to cell migration. This effect is particularly promising in view of possible inhibition of proliferation and invasion of cancer cells. In summary, our Au-peptide NCs proved to be an efficient theranostic nanosystem for targeted detection and activatable killing of prostate cancer cells.

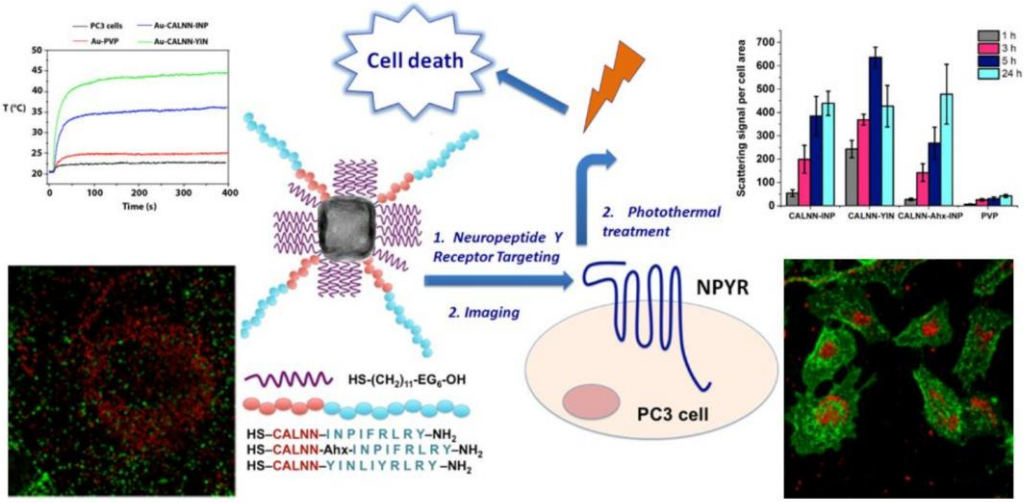

Figure 1. Schematic representation of NPYR targeting on prostate cancer cells by peptide-functionalized gold nanocages and subsequent hyperthermia by NIR irradiation.

NEW HYDROPHILIC RIMINOPHENAZINES AS POTENT ANTIPROTOZOAL AGENTS

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Malaria and leishmaniasis are life-threatening human parasitosis caused by protozoa-infected insect vectors. In most of affected countries, the expansive and hazardous therapies available to fight protozoan infections are generally harmed by the spread of drug resistance phenomena upon prolonged treatments. This scenario highlights the need of novel antiprotozoal agents hopefully able to act trough new mechanism(s) of action.

Interestingly, the fat-soluble antimycobacterial drug Clofazimine (**Figure 1**) was reported to exhibit a moderate antiprotozoal action and some interesting antileishmanial *in vitro* and *in vivo* effects were reported in few preliminary, yet promising, studies.[1,4]

Intrigued by these results, we have previously prepared a series of basic Clofazimine analogues which demonstrated the beneficial effects of the introduction of a basic head on the antiprotozoal activity.[5] Here, to further investigate the role of balancing between the lipo- and hydrophilicity on the antiparasitic activity of these riminophenazines, we report the synthesis and the *in vitro* evaluation as leishmanicidal (*L. tropica* and *L. infantum* promastigotes) and antiplasmodial (chloroquine sensitive and resistant *P. falciparum* strains) agents of a family of hydrophilic C-2 aminopyridinyl substituted riminophenazines, bearing in C-3 differently decorated basic side chains (**Figure 1**).

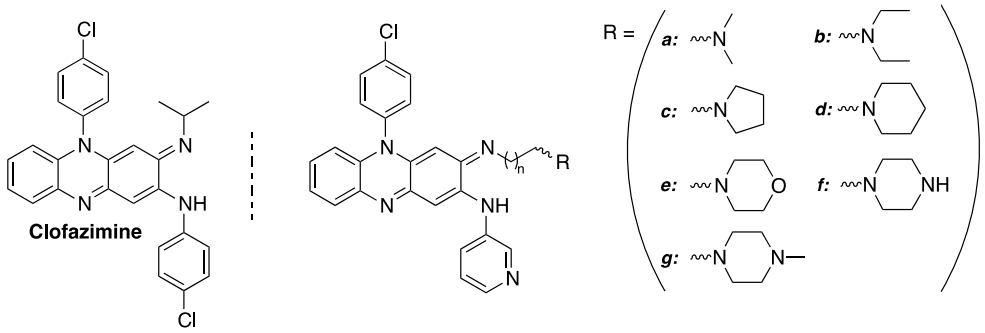

Figure 1: Structures of Clofazimine and C-2 aminopyridine substituted riminophenazine analogues.

Results showed that most of the new compounds potently inhibited the growth of protozoa with IC_{50} in the high nanomolar range and underlined the key role of the hydrophilic C-2 aminopyridinyl moiety to improve the leishmanicidal activity. In addition, the length and the nature of the basic side chain differently influenced the antiprotozoal activity and the selectivity index versus mammalian cells, providing useful information for further structural optimizations.

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SYNTHESIS AND APPLICATION OF ISOTOPE-LABELED CARNOSINE IN LCMS/MS

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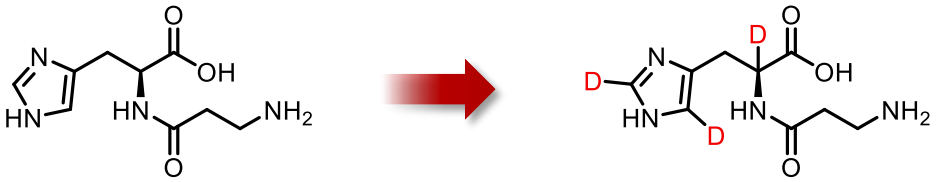
Carnosine is an endogenous dipeptide, composed of β-alanine and L-histidine, and is highly concentrated in skeletal muscle and other excitable tissues. Its physiological roles, based on its biochemical properties, include pH-buffering, metal-ion chelation and antioxidant capacity as well as the ability to protect against the formation of advanced glycation and lipoxidation end-products.[1] For these reasons, besides its nutritional ergogenic application in the sport community,[2] carnosine supplementation offers a therapeutic potential for the treatment of numerous diseases in which ischemic or oxidative stress is involved.[1] Quantitation of carnosine in biological matrices appears to be crucial for these applications, and LC-MS procedures with isotope-labeled internal standards are the state-of-the-art approach for this analytical need.[3] The use of these standards allows to account for variations during the complex sample preparation process, different matrix effects between patient samples, and variations in instrument performance.



Carnosine **Carnosine-d$_3$**

Figure 1

In this work, we present a fast and highly efficient synthetic route to obtain a deuterated carnosine analogue (Figure 1) starting from the trideuterated L-histidine (α-d$_1$, imidazole-2,5-d$_2$). Moreover, the use of Carnosine-d$_3$ in the validation of a multiple reaction monitoring (MRM) LC-MS/MS method for the analytical quantitation of carnosine in a biological matrix will be reported.

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DESIGN, SYNTHESIS AND PRELIMINARY BIOLOGICAL EVALUATION OF 3-CYCLOPROPYL-4-PHENOXY-1*H*-PYRAZOLE DERIVATIVES AS SMALL MOLECULAR LIGANDS OF RAGE

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Receptor for advanced glycation end products (RAGE) is a multiligand receptor belonging to the immunoglobulin superfamily and plays a crucial role in the development of many human diseases such as neurodegenerative diseases, diabetes, cardiovascular diseases and cancer.[1] RAGE is involved in a number of cell processes such as neuroinflammation, apoptosis, proliferation and autophagy, and therefore it is of considerable interest as a promising drug target for innovative therapeutic approaches. It consists of an extracellular region, a short hydrophobic transmembrane spanning region, and a highly charged amino acid cytoplasmatic tail. The extracellular region contains a signal peptide, followed by one *N*-terminal V-type immunoglobulin domain and two *C*-type (C1 and C2) immunoglobulin domains.[2] RAGE is able to interact with a large number of pro-inflammatory and regulatory molecules, such as advanced glycation end-products (AGEs), quinolinic acid, beta amyloid (A*β*), high mobility group box 1 (HMGB1), S100/calgranulin family proteins.[3,4] However, due to the structural heterogeneity of these endogenous ligands, little is known about the key pharmacophore elements for ligand-RAGE interaction and the specific mode of binding.

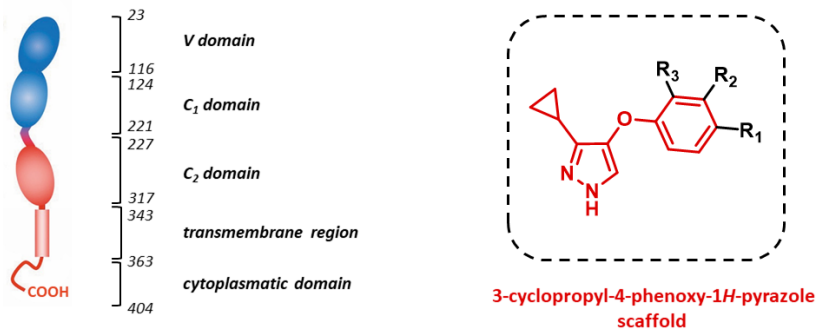

Figure 1. Schematic depiction of RAGE structure and general structure of the new pyrazole-based RAGE ligands.

On these grounds, we aimed at designing new small molecules able to bind the VC1 extracellular domains of RAGE, in order to clarify the structural features that account for RAGE affinity and activation, and to identify new drug-like compounds. Following a process of structural simplification of known pyrazole-5-carboxamide RAGE ligands,[1] we planned a set of novel derivatives characterized by a variously functionalized 3-cyclopropyl-4-phenoxy-1*H*-pyrazole scaffold (Figure 1). The design and synthesis of the new putative RAGE ligands will be presented and discussed, together with the results of their in vitro screening by means of a surface plasmon resonance (SPR)-based assay to estimate their binding ability to the RAGE extracellular domain.

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MULTI-TARGET ANTITUBERCULAR DRUGS: IDENTIFICATION OF NOVEL DUAL MBTI AND MPTPB INHIBITORS

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Tuberculosis, the infectious disease caused by *Mycobacterium tuberculosis*, has high levels of mortality worldwide and has already gained resistance to first- and second-line drugs.[1] In this context, the study of new chemical entities with promising activities assumes paramount importance for the improvement of the current therapeutic strategies in the treatment of the patients affected with this disease. In particular, the development of multi-target agents has significantly grown in recent years, due to the reduction of financial costs and to the more pronounced therapeutic activity that they can exert, becoming one of the main research topics in the pharmaceutical industry.

In this poster, we report the identification of a new class of compounds that can simultaneously interact with two molecular targets of *Mycobacterium tuberculosis*, namely MbtI and MptpB, already validated for the development of novel antitubercular agents.[2,3]

Among a series of competitive furan-based inhibitors of MbtI previously discovered by our research group,[4] we disclosed promising ligands of MptpB, showing IC_{50} values in the range of about 30 μM. These preliminary results may be useful for the rational design of new derivatives, with the aim of increasing their inhibitory potency.

Acknowledgments. We acknowledge the University of Milan (Piano di Sostegno della Ricerca 2018 – Linea 2-PSR2018_FMENE_01) for financial support.

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HPLC ANALYSIS AND DEPIGMENTING EFFECTS OF UMBELLIPRENIN-CONTAINING EXTRACTS OF *ANETHUM GRAVEOLENS*, *PIMPINELLA ANISUM*, AND *FERULAGO CAMPESTRIS*

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During the last two decades extensive studies have been carried on the phytochemical and pharmacological properties of a rare class of secondary metabolites of plant, fungal, and bacterial origin, the oxyprenylated phenylpropanoids. In this context umbelliprenin (7-farnesyloxycoumarin) has been revealed as one of the most promising compound (Figure 1).[1,2]

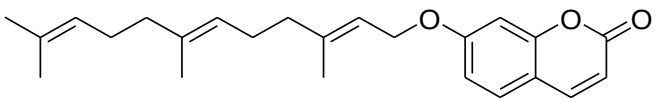

Figure 1. Structure of umbelliprenin.

From a pharmacological point of view, umbelliprenin exhibits anti-inflammatory, immunomodulatory, cancer chemopreventive (papilloma, colonrectal, melanoma, and breast carcinomas), pro-apoptotic, and anti-melanogenic effects. In particular for this latter biological activity, we have recently shown how umbelliprenin has a great potential as a skin-whitening agent. Moreover the recorded effects to this concern were comparable or even better to those exhibited by known and widely used depigmenting substances like arbutin, kojic acid, some flavonoids, and others.[1] In this poster communication, adopting a reverse pharmacognosy approach (e.g. from molecules to plants), we wish to describe a simple and reliable HPLC analytical procedure to quantify umbelliprenin in extracts obtained from seeds of selected Apiaceae plant species, namely *Anethum graveolens* L. (dill), *Pimpinella anisum* L. (anise), and *Ferulago campestris* (Besser) Grecescu (field ferula), and to assess the skin whitening properties of these latter using cultured murine Melan A cells as the pharmacological model. EtOH was shown to be the best solvent, providing yields in umbelliprenin ranging from 1.7 % to 14.4 % (measured respect to the total amount of extract obtained). The extracts with the highest content of this farnesyloxycoumarin were then assayed as modulators of melanogenesis in cultured murine Melan A cells, employing the umbelliprenin obtained by chemical synthesis, as the reference drug. A parallelism between the content of the coumarin and the recorded depigmenting effect (60 % for the EtOH extract of *F. campestris* as the best value) was revealed for extracts of all plants applied at a dose of 100 μg/mL. The obtained results indicate that the HPLC process set up can be easily adopted to reveal the content of umbelliprenin in plant extracts and in addiction the title species can be effectively considered as therapeutic remedies for the cure of hyperpigmentation syndromes and as ingredients for cosmetic preparations to lighten the skin.

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BIOASSAY-GUIDED ISOLATION OF DRYPETES KLAINEI STEM BARK EXTRACT WITH TISSUE REPAIR PROPERTIES AND IDENTIFICATION OF THE BIOACTIVE COMPOUND

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Drypetes klainei Pierre ex Pax is used in Cameroon by Baka Pygmies in the wound healing process and for the treatment of burns.[1] The stem bark is directly applied on the skin injury as fine powder or orally assumed as water macerated. The validation of the traditional use has been described in a previous work were the extracts abilities to accelerate wound healing were studied on murine and human fibroblasts in terms of cell viability and migration (scratch wound-healing assay.[2]

Starting from these published results, we carried out a bio-guided fractionation of the most active DME extract, with the aim of identifying and isolating the bioactive compound/s responsible for the wound repair activity. The DME extract was analyzed by RP-HPLC and purified by flash chromatography giving rise to three fractions.

The fraction Fr2, that exhibited the higher activity on wound closure, was re-fractioned into sub-fractions, which were subjected to the bioassays. Among the five sub-fractions obtained (Fr2subA, B, C, D and E), the Fr2subE resulted the most active in terms of effects on cell viability and growth as well as on migration by scratch wound assays.

MS analyses and NMR spectroscopy were used for identifying the isolated compound. The results obtained allowed to unambiguously identify the bioactive compound as Nigracin, a known phenolic glycoside firstly isolated and characterized from bark and leaves of *Populus Nigra* in 1967. However, this is the first time that Nigracin is identified in the *Drypetes* genus and that a wound healing activity is demonstrated for this molecule. Specifically, we established that Nigracin significantly stimulates fibroblast growth and improves cell motility and wound closure of fibroblast monolayer in a dose-dependent manner, without any toxicity at the concentrations tested, and is still active at very low doses. This makes the molecule particularly attractive as a possible candidate for developing new therapeutic options for wound care.

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USE OF PolyHIPEs-BASED STATIONARY PHASES AS INNOVATIVE SUPPORTS FOR BIOCATALYSIS

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Polymerized high internal phase emulsions (PolyHIPEs) are highly porous solid materials obtained by the polymerization of the external phase of HIPEs, w/o emulsions with an internal phase volume grater than 74% of the total volume. In here we developed polyacrylate-based emulsion templated stationary phases in form of epoxy monolithic columns. Monoliths were obtained by an in-situ polymerization procedure occurred in 24 h at room temperature in empty glass Omnifit® columns. The starting emulsions were obtained by mixing the external phase composed by the lipophilic monomers butyl acrylate and glycidyl methacrylate as structural and functional monomer respectively, the polymeric surfactant synperonic PE/L 121 and the trifunctional crosslinker trimethylolpropane triacrylate with the internal phase (degassed deionized water) in a volume ratio of 20:80. The radical polymerization technique was achieved by the redox initiator potassium persulfate/tetramethyletylendiamine. The synthesis of polyHIPEs and the selection of the most appropriate preparation conditions were performed by a design of experiment (DoE) approach. Therefore, the monolithic composition was selected, as reported in a previous work,[1] and applied for the covalent immobilization of ATA-117, an (R)-selective ω-transaminase, leading to the obtainment of an immobilized enzyme reactor (IMER). The ATA-117-based IMER, after the evaluation of immobilization yield and reactor efficiency, was applied for the in-flow biocatalytical synthesis of chiral amines, such as (R)-1-(4-methoxyphenyl)propan-2-amine and (R)-1-methyl-3-phenylpropylamine, intermediates for the synthesis of formoterol and dilevalol respectively. For the simultaneous monitoring of the reaction rate, the IMER was on-line coupled with an achiral RP stationary phase by a switching six port valve leading to a bidimensional chromatographic system. The enantiomeric excess (ee) of the obtained chiral amines was also evaluated by an off-line assay after an in-batch derivatization procedure with Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide reagent. In conclusion, polyHIPE-based monolithic columns resulted as valuable innovative stationary phases for the immobilization of enzymes, for biocatalysis applications and for the in-flow synthesis of intermediates of pharmaceutical interest. Moreover, the possibility to obtain monoliths in any dimensions and shapes, highlights the potential of polyHIPEs-based IMERs in developing a scaled up biocatalysis process.

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NOVEL BIOLOGICALLY ACTIVE NUTRACEUTICAL PRINCIPLES FROM SPINACH, GOJI, AND QUINOA

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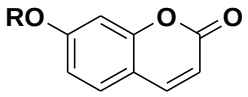
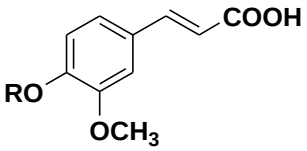
Spinach leaves, goji berries, and quinoa seeds are commonly consumed ingredients of salads and soaps all over the world. These plant food are known to contain several phytochemicals having well established beneficial effects for human welfare. In particular spinach (*Spinacia oleracea* L., Amaranthaceae) contains bioactive components like flavonoid glycosides, leuteolin, glycolipids and coumaric acid derivatives able to act as effective radicals and reactive oxygen species scavengers, to modulate the expression of genes encoding proteins playing a key role in modulating metabolism, the cell proliferation, the inflammation, and overall anti-oxidant defense of human body, and finally to slow compulsive food intake by promoting the secretion of satiety hormones.[1] Goji (*Lycium barbarum* L., Solanaceae) berries have been used for centuries in traditional medicine practices in China and also in form of food products supplements like juices, jams, bakery products, energy bars, and capsules. They contain mainly polysaccharides, polyphenols, and carotenoids able to exert beneficial effects for the prevention of chronic diseases (cancer, atherosclerosis, obesity and diabetes), and to promote weight loss and longevity.[2] Quinoa (*Chenopodium quinoa* Willd, Amaranthaceae) seeds are also classified as "pseudocereals" as they are featured by the same nutritional value as cereals (high content of essential aminoacids and polysaccharides), but without gluten. They also contain polyphenols, tocopherols and carotenoids able to lower the risk of oxidative stress related diseases like cancer, cardiovascular diseases, stroke, diabetes and obesity, as well as to exert effective anti-oxidant and anti-inflammatory effects.[3] In this poster communication we wish to describe the extraction and UHPLC analysis and photodiode array (PDA) detection of selected umbelliferone and ferulic acid oxyprenylated derivatives (Figure 1), namely 7-isopentenyloxycoumarin **1**, auraptene **2**, umbelliprenin **3,** boropinic acid **4** and 4'-geranyloxyferulic acid (GOFA) **5** from the title plant species. The samples of these plants were extracted with different solvent mixtures (e.g. EtOH, H_2O/EtOH 3:7, and H_2O/EtOH 7:3) and the extractions were accomplished using a microwave apparatus. Subsequent UHPLC analysis and photodiode array detection were employed for the quantification of the title secondary metabolites. EtOH was found to be the best solvent in terms of extractive yields and the above-mentioned phytochemicals were recorded in the concentration range 2.01 – 49.22 μg/g dry extract. The findings depicted herein revealed that spinach, goji, and quinoa are good sources of oxyprenylated umbelliferone and ferulic acid derivatives.



1 R = 3,3-dimethylallyl
2 R = geranyl
3 R = farnesyl



4 R = 3,3-dimethylallyl
5 R = geranyl

Figure 1. Structures of natural oxyprenylated compounds under investigation

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BIOEQUIVALENCE DETERMINATION OF PHARMACEUTICAL FORMULATIONS BY PARALLEL ARTIFICIAL MEMBRANE PERMEABILITY ASSAY

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The demonstration of bioequivalence is fundamental for the approval of generic medicinal formulations. The concept of bioequivalence is based on the assumption that two medicinal products, displaying equivalent bioavailability, will have the same therapeutic effects. This ensures the same *in vivo* performance in terms of efficacy and safety.[1] The determination of dissolution and adsorption profile of a drug product is crucial in order to assess its bioavailability.[2] In this context the application of an optimized pharmacokinetic model is essential to improve drug efficacy and to save money and time during the drug development process and ADME (absorption, distribution, metabolism and excretion) assessment. For this porpouse we developed an *in vitro* model for the determination of the biovailability of levonorgestrel released by a generic and branded named tablet. A dissolution test[3] was performed in order to simulate the intestinal fluid and to compare the release and the solubility of levonorgestrel from the two oral formulations over time. In the mean time the optimization of PAMPA (Parallel Artificial Membrane Permeability Assay) test[4] allowed to analyse the obtained solutions and to determine the passive oral absorption of levonorgestrel released by both formulations.
The developed model resulted useful for predicting differences in the dissolution and adsorption profile of levonorgestrel contained in the two different oral formulations.

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PHARMACOKINETIC STUDIES OF RESVERATROL AND DERIVATIVES IN HUMANS

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3,4,5-Trihydroxystilbene-3-beta-monoglucoside or resveratrol 3-beta-mono-D-glucoside (piceid) is a stilbenoid glucoside and is a major trans 3,5,4' trihydroxystilbene (trans resveratrol) derivative in grape juice.[1] Both piceid and resveratrol are known for their antioxidant and anti-inflammatory properties. Indeed a recent study revealed the antioxidant effect of piceid to be higher than that of resveratrol in mice.[2]
Piceid is more resistant to enzymatic oxidation, it enters in the cells with an active transport mechanism involving glucose transporters.[3] Moreover, its higher intestinal absorption due to the higher water solubility makes piceid more bioavailable than resveratrol. For all these reason several nutritional supplements based on piceid or resveratrol have been launched on the marked in the last few years. As consequence their pharmacokinetic characterization becomes necessary. For this porpoise we developed a very fast liquid chromatographic analysis for the detection of piceid and resveratrol in human plasma. The plasma samples were processed by liquid-liquid extraction (using methanol). Then the gradient elution allowed the separation of piceid and resveratrol in less than 2.5 min. The limit of detection (LoD) and the limit of quantification (LoQ) values were found to be 0.01 µM and 0.03 µM respectively. Moreover an accuracy value of 95% was determined. All these parameters makes the developed method suitable for the fast detection of piceid and resveratrol in human plasma and for its pharmacokinetic characterization.

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HYDROPHOBIC AMINO ACIDS IN ONIONS AS POTENTIAL FINGERPRINTS OF GEOGRAPHICAL ORIGIN

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In the present study, we have compared the amino acid profile in a specific onion variety, namely, the "Rossa da inverno sel. Rojo Duro", cultivated in two different Italian sites: Cannara (Umbria region) and Imola (Emilia Romagna region). Onions were cultivated in a highly comparable way, especially for that it concerns mineral fertilization, seeding, and harvesting stages, and good weed control. Moreover, in both sites, onion plants were irrigated by the water sprinkler method and exposed to similar conditions (temperature, weather, etc.). An additional group of Cannara onions, which were grown through microirrigation, was also included in the study. The extracted proteinogenic free amino acids were analyzed *via* an ion-pairing reversed-phase HPLC (IP-RP-HPLC) method; while, an efficient detection was assured by the use of an evaporative light scattering detector (ELSD). The chromatographic analysis revealed that only leucine (Leu), phenylalanine (Phe), and tryptophan (Trp), were present in all the investigated samples and the corresponding peaks were completely unaffected by the matrix interferences. Statistical methods such as beeswarm/box plots with ANOVA analyses, indicated that the content of Leu and Phe were noticeably affected by the geographical origin of the onions (***$p << 0.001$ for Phe), but not by the irrigation technique. Prior to the quantitative assay of Leu, Phe, and Trp in the onion samples, the applied HPLC-ELSD method was validated in terms of specificity, linearity, limit of detection (LOD) and of quantification (LOQ), accuracy (≥ 90% for inter-day Recovery percentage), and precision (≤ 10.51 for the inter-day RSD percentage). Based on the evidences collected in the study, it is possible to speculate that the quantity of specific amino acids in onions could be regarded as potential fingerprints of their geographical origin.

EVALUATION OF ANTI-INFLAMMATORY AND ANTIOXIDANT PROPERTIES OF BIOACTIVE COMPOUNDS IN A NOVEL FUNCTIONAL FOOD: DEHYDRATED POTATOES

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Several studies showed that an uncontrolled reactive oxygen species (ROS) production can cause cellular damage and death also leading to many diseases such as atherosclerosis, arthritis, diabetes, cancer, and inflammatory bowel disease (IBD). In order to maintain the redox homeostasis, ROS are neutralized by the endogenous enzymatic and non-enzymatic antioxidant systems. An imbalance in these mechanisms lead to excessive levels of ROS and subsequently the oxidative stress.[1] Therefore, dietary antioxidants can supplement the antioxidant system and help to reduce the degenerative oxidative damage. Vegetable matrices represent important sources of several classes of antioxidant compounds such as polyphenols and bioactive peptides, which are often used as ingredients for developing functional foods and nutraceutical products.[2]

Among them, the potatoes (*Solanum tuberosum*) are of particular interest because, in terms of production, potato is the second largest protein-supplying crop per hectare grown, and in terms of consumption since it has been a staple food in many traditional diets of the Western world. To date, more than half of all potatoes consumed are chips, fried and roast potatoes or processed potato products, especially among older children and young adults. Therefore, potatoes represent a contradictory food because they contain macronutrients such as proteins and fibers able to exert beneficial effects on human health. On the other hand, the frequent consumption of fried potatoes appears to be associated with developing obesity, diabetes, and cardiovascular disease due to their large starch content and high glycemic index.[3] In addition, potatoes provide other important micronutrients such as vitamins and organic acids, which are all associated with a decreased risk of morbidity and mortality.[4]

For these reasons, in order to maintain the beneficial properties of potatoes and decrease the higher intakes of trans fatty acids, oxidized lipids, acrolein, acrylamide, furan, and glycidamide of fried potatoes, in this work we have investigated the antioxidant properties of dehydrated potatoes.

In detail, an on-line HPLC-2,2'-diphenyl-1-picrylhydrazyl (DPPH•) assay for the screening of antioxidant polyphenol was developed. To identify bioactive peptides, a simulated gastrointestinal digestion was performed. Peptide extract was tested on intestinal epithelial cells (IEC-6) under inflammatory condition. Our results demonstrate that the peptide fraction was able to significantly inhibit pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cycloxygenase-2 (COX-2) expression and oxidative stress markers such as nitrotyrosine formation and ROS release. The results demonstrate a potential use of dehydrated potatoes in the prevention the inflammatory and oxidative stress state at the intestinal level.

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PRUNUS AVIUM L. FRUIT: ANALYTICAL CHARACTERIZATION AND *IN VIVO* ANTI-OXIDANT ACTIVITY EVALUATION

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Prunus avium L. is a tree belonging to the *Rosaceae* family. It fruit is a well-known edible product, rich in nutrients and antioxidant compounds.[1,2] This fruit is considered to exert beneficial effects on human health: indeed, its consumption has been associated with the prevention of cell oxidative injury and inflammation, thanks to its free radical scavenging and anti-inflammatory activities[2]. These beneficial effects are closely related to the rich polyphenolic composition of sweet cherry fruit, which encompasses numerous chemical classes of compounds.[1-3] Therefore, sweet cherry fruit represents a source of bioactive natural compounds to be exploited in the nutraceutical field.[1-3]

In the light of all the above, the development of efficient analytical methods, together with the establishment of suitable extraction procedures, are crucial features in order to highlight the great nutraceutical potential of this product. In this view, the aim of the present study was the development of a new analytical method for the comprehensive multi-component analysis of polyphenols in sweet cherry. In particular, a RP-HPLC-UV/DAD and HPLC-ESI-MS^2 method was successfully optimized for the qualitative and quantitative analysis of sweet cherry polyphenolic compounds. Sample preparation was based on two sequential dynamic maceration steps. The optimized analytical method was finally applied to different sweet cherry samples. Four anthocyanins were identified in sweet cherry extracts, with cyanidin-3-O-rutinoside being the most abundant one. Caffeoyil-quinic and cumaroyil-quinic acids were also identified as the most representative phenolic acids.

Given the high biological value of the polyphenolic fractions of sweet cherry fruit, the extracts were submitted to in vivo antioxidant assays using the *Caenorhabditis elegans* model to test their capacity to increase the worm resistance to oxidative and thermal stress.

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OPTIMIZATION OF COMPREHENSIVE HYDROPHILIC INTERACTION CHROMATOGRAPHY × REVERSED-PHASE ULTRA-HIGH-PRESSURE LIQUID CHROMATOGRAPHY PLATFORM FOR THE ANALYSIS OF *PUNICA GRANATUM* FRUIT JUICE

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Punica granatum L., better known as pomegranate, is an ancient fruit widely consumed as fresh fruit and juice. Moreover, it is increasingly used in cosmetic and pharmaceutical fields for its antioxidant, anti-inflammatory, anti-microbial and anti-proliferative properties related to the presence of several bioactive phytochemicals.[1] In order to characterize in detail the bioactive compounds present in the pomegranate juice, we developed an online comprehensive Hydrophilic High performance Liquid Chromatography × Reversed Phase-Ultra High performance Liquid chromatography (HILIC-HPLC × RP-UHPLC) platform hyphenated to Ion Trap-Time of Flight (IT-TOF) mass spectrometry.[2] A narrowbore hydrophilic interaction chromatography column (150 × 2.0 mm, 3.0 µm, cross-linked diol) was employed in the first dimension, while a reversed-phase column based on monodisperse sub-2 µm fully porous particles (50 × 3.0 mm, 1.9 µm d.p.) with high surface area (410 m^2/g) was employed in the second dimension. The modulation interface consists of two C18 trapping columns (C18 10 × 3.0 mm × 1.9 µm, 80 Å), instead of sampling loops. The combination of a trapping column modulation interface with the high retentive fully porous monodisperse reversed-phase column in the second dimension resulted in higher peak capacity values, increased sensitivity, sharper and more symmetrical peaks. Hyphenation with an ion trap time-of-flight mass spectrometer led to the tentative identification of 73 analytes, showing how this platform could be a powerful analytical tool for the accurate profiling of complex polyphenolic samples.

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DEVELOPMENT OF ONLINE COMPREHENSIVE REVERSED-PHASE × REVERSED-PHASE ULTRA-HIGH-PRESSURE LIQUID CHROMATOGRAPHY APPROACH FOR THE ANALYSIS OF ONCONUTRACEUTICAL SMOOTHIES

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Many studies have demonstrated that a healthy lifestyle and diet can help in prevention of chronic pathology such as cardiovascular and neurodegenerative diseases, diabetes and cancer. Several scientific evidences described the potential health effects that dietary polyphenols are able to exercise on biological systems like anti-proliferative, anti-cancer, anti-inflammatory, cardioprotective and antioxidant activities. Nowadays, high attention has been paid to natural components in fruits and vegetables with potential anticarcinogenic, antimutagenic, antioxidant and anti-inflammatory activities.[1] In particular, the consumption of dietary supplements and nutraceuticals in cancer therapy has as its main purpose the chemoprevention, the reduction of the drug resistance, the identification of synergistic effects whit the anti-cancer treatment and the decrease of drug concentrations.[2]

In this work we have realized a superfood consisting of three different natural matrices, such as annurca (*M. pumila Miller cv Annurca*) apple, red grape (*Vitis vinifera L. cv. Aglianico N*) and orange (*Citrus sinensis*) fruits, able to reduce the side effects associated with current cancer treatments that induce unintended side effects compromising also health and well-being of patients.[3-5]

Mono dimensional LC-MS method is overwhelmed by the complexity of this samples, and, as result, important information can be lost. For these reasons in this work the functional smoothie was characterize by developing and optimizing a comprehensive two dimensional liquid chromatography approach, based on the online coupling of two Reversed Phase (RP × RP) with two different pH conditions. The two dimensions were coupled by a multiport switching valve equipped with two C18 trapping columns, which trap and concentrate the analytes during the transfer. The system was hyphenated to High Resolution LTQ-Orbitrap XL mass spectrometry.

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NATURAL AND SEMISYNTHETIC OXYPRENYLATED PHENYLPROPANOIDS AND MELANOGENESIS

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Melanin is the main pigment in humans and other mammals found in skin, eyes, nasal cavity, inner ear, and hair. It is responsible for skin color and represents the most effective defense for these tissues and organs against overexposure to UV-B radiations.[1] It has been very recently shown how naturally occurring oxyprenylated coumarins, like 7-isopentenyloxycoumarin and umbelliprenin, are effective modulators of melanogenesis.[2] In this poster communication we wish to generalize the potentialities as skin tanning or whitening agents of a wider panel of natural and semisynthetic aromatic compounds, including coumarins, cinnamic and benzoic acids, and benzaldehyde derivatives (Figure 1). A total number of 41 compounds have been tested assaying their capacity to inhibit or stimulate melanin biosynthesis in cultured murine Melan A cells. The wider number of chemicals herein under investigation allowed to depict a detailed structure-activity relationship, as the following: a) benzoic acid derivatives are slightly pigmenting agent, for which the effect is more pronounced in compounds with longer *O*-side chains; b) independently from the type of substitution, cinnamic acids are able to increase melanin biosynthesis, while benzaldehydes are able to decrease it; c) coumarins with a C5 or shorter skeletons as substituents in position 7 are tanning agents, while coumarins with farnesyloxy groups are whitening ones; d) double oxyprenylation in position 6 and 7 and C5 or C10 skeletons have slight depigmenting capacities, while C15 skeletons tend to marginally increase the tanning effect; e) the presence of electron withdrawing groups (acetyl, COOH, and -Cl) and C10 or C15 oxyprenylated chains respectively in positions 3 and 7 of the coumarin nucleus lead to a whitening effect, and finally f) oxyprenylated anthraquinones have only a weak depigmenting capacity.

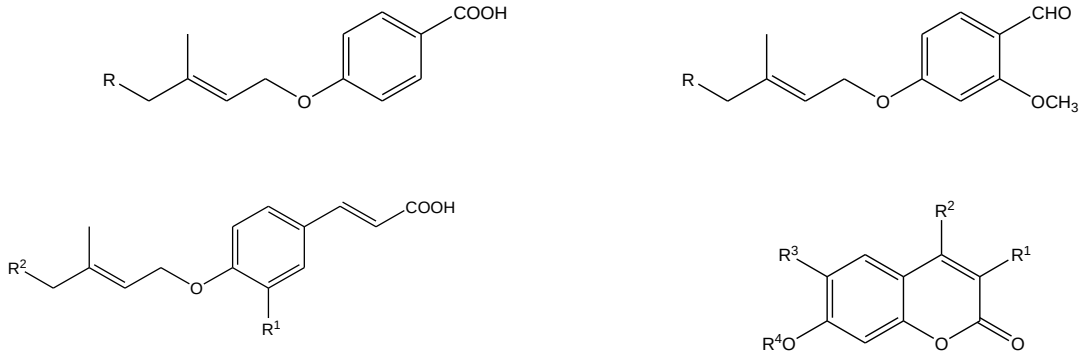

Figure 1. Natural and semisynthetic oxyprenylated compounds under investigation.

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DEVELOPMENT OF AN IN VITRO AGGREGATION ASSAY BASED ON TAU R3 DOMAIN

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Deposition of tau aggregates in neurons is an important neuropathological hallmark in multiple neurodegenerative disorders including Alzheimer's disease (AD).

Due to the pathological relevance of this phenomenon, great efforts have been made to understand tau aggregation and find new chemical entities able to interfere with it, as therapeutic strategy for tau-associated disorders. Notwithstanding the considerable interest, early screening of small molecule inhibitors and definition of clear structure-activity relationships have been slowed down because of issues related to the use of the full-length tau protein, the relatively high variability of the aggregation process, and the high costs of long tau fragments such as K18 and K19.[1] In this light, the development of a reliable *in vitro* screening assay for monitoring tau misfolding and aggregation is beneficial to drug discovery, enabling the preliminary prioritization of potential tau inhibitors. In human brain tau protein exists in six different isoforms which share a common third repeat domain (R3, residues 306–336). Importance of the R3 domain in inducing tau aggregation has been highlighted by mutagenesis studies.[2] Based on these considerations and on the recent evidence that 306–336 peptide is able to induce aggregation of tau's microtubule-binding region in cells,[3] we undertook a study aimed at defining the experimental conditions for a reproducible aggregation assay involving tau 306–336 peptide (R3 domain). Requirements for acceptable conditions were set as follow: (i) presence of a suitable lag phase prior aggregation; this should ensure monitoring of conformational changes and interference with the conformational shift by inhibitors (ii) no use of aggregation inducers, which are often used in *in vitro* assay to promote aggregation of full tau or long tau fragments; (iii) suitable analysis time and format for screening campaigns. To reach this goal we started from our previous findings[4] on the aggregation behavior of the tau derived hexapeptide AcPHF6 (Ac-VQIVYK-NH_2), which was identified as essential element for aggregation.[2]

Formation of tau fibrils were monitored in real time by using thioflavin T (ThT) as fluorescence probe and scanning fluorescence intensity each 10 min for 16 h. Pretreatment and solubilization conditions, peptide concentration, assay temperature and shaking conditions were optimized. In the final conditions reproducible sigmoidal aggregation profiles with about a 3.5h-lag phase and 1.5-2h-exponential phase were achieved.

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GLYCATED HUMAN SERUM ALBUMIN INTERACTION WITH RAGE ECTODOMAIN

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The receptor for advanced glycated end-products (RAGE) is a pattern recognition receptor which is thought to be involved in the pathological setting of several chronic diseases including diabetes complications. Although RAGE was first described as receptor for advanced glycated end-products (AGEs), an emerging view is that RAGE is a multiligand receptor, able to recognize both AGEs and not glycated substrates such as amyloid peptides. Activation of RAGE induces intracellular signaling that results in the production of proinflammatory cytokines and an increase of the oxidative stress. Hence, because of its ability to recognize binders with different features and considering clinical implications related to its activation, many efforts have been devoted to define structural requirements for RAGE binding. AGE-albumins are likely the most studied RAGE ligands. On the other hand, albumin is the most abundant protein in plasma and accounts for over 80% of total glycated serum proteins.[1] In this scenario, this study aimed to investigate the interaction between VC1, i.e. RAGE ectodomain, and glycated human serum albumin (HSAgly) by combining surface plasmon resonance (SPR) technology and affinity-mass spectrometry to provide affinity parameters along with structural features for VC1 recognition. The selected HSAgly was characterized in terms of glycation sites by two-dimensional liquid chromatography electrospray tandem mass spectrometry (2D-LC-ESI-MSMS) while the extent of glycation was quantified by top down LC-MS. The ability of HSAgly to interact with VC1 was confirmed by SPR studies using a HSAgly sensing surface and affinity proteomics employing a purposely developed VC1 column. The equilibrium dissociation constant (K_D) highlighted a moderate affinity between the two interactants while displacement studies using chondroitin sulphate, a known RAGE binder, validated the VC1-HSAgly interaction assessed by the SPR-based assay. Furthermore, the combination of "epitope extraction method"[2] with LC-MSMS analysis allowed further insights into the region(s) of HSAgly involved in the binding. Outcomes of these analyses highlighted a recognition pattern involving glycated Lys525 (subdomain IIIB) and subdomain IA. Computational analysis[3] helped elucidating residues belonging from the two regions involved in the interactions with RAGE, highlighting a key role for glycated Lys525. Nonetheless, ionic interactions involving residues surrounding Lys525, namely Asp512 and Glu520, and Arg78 residue of RAGE are mandatory for the stabilization of the complex. Finally, clusters of ionic interactions involving charged residues in the IA subdomain of HSA may further stabilize RAGE-HSAgly.

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TSPO DENSITY ASSESSMENT IN HUMAN GLIOMA CELL LINES USING THE FLUORESCENT LIGAND F-PIGA

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The Translocator Protein 18 kDa (TSPO) is a protein located on the outer mitochondrial membrane and it is implicated in a number of cellular functions, mainly steroidogenesis and immunomodulation.[1] Clinical investigation highlighted that TSPO is upregulated in inflammatory diseases, such as cancer and brain injuries.[2] For these reasons, several studies have attempted to validate TSPO as a biomarker of brain injury using both radiolabeled and fluorescent TSPO ligands. Fluorescently labeled ligands represent a safer, faster, and less-expensive alternative to radioligands in probing the ligand receptor complex.
In this context, we synthesized and biologically evaluated a TSPO fluorescent probe (F-PIGA **II**), designed on the basis of *N*,*N*-dialkyl-2-phenylindol-3-ylglyoxylamides **I**, bearing the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) fluorescent moiety linked to the *N*-hexyl chain (Figure 1).[3] According to our pharmacophore/topological model (Figure 1),[3] the fluorescent group should interact with one of the lipophilic pockets termed L3 and L4 in the receptor binding cleft, thus maintaining the high affinity of the parent ligand **I**.[4] Indeed, F-PIGA **II** displayed nanomolar TSPO affinity, high specificity and attractive spectroscopic properties. All these features make this compound suitable for application in a fluorescence-based assay useful to evaluate the TSPO density *in vitro*.[4]

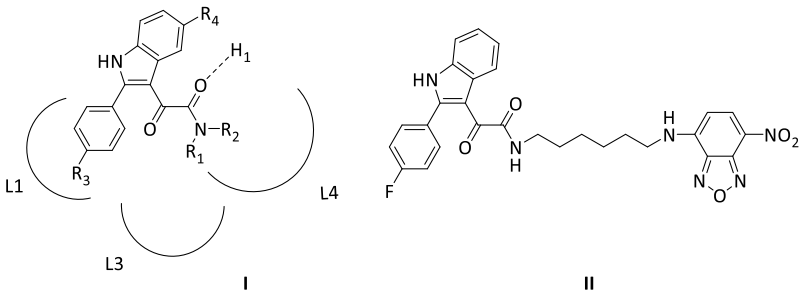

Figure 1. 2-Phenylindolglyoxylamide derivatives I in the pharmacophore/topological model and fluorescent probe F-PIGA II.

A number of human cell lines were selected for their different content of TSPO and F-PIGA **II** was evaluated to test its feasibility to measure TSPO *in vitro* density, comparing the data with those obtained quantifying the TSPO levels by the classical radioactive method. Results evidenced that low micromolar concentration of F-PIGA **II** gave a reproducible and TSPO specific intracellular fluorescent labeling. Moreover, the data obtained by fluorescence assays were completely in accordance with radioligand binding assays, suggesting that F-PIGA **II** may represent a useful and safe probe to evaluate the TSPO density in *in vitro* studies on the localization, expression level, structure and physiological/pathological roles of this protein.

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DESIGN, SYNTHESIS AND IN VITRO EVALUATION OF ANTIOXIDANT AND UV-FILTERING PROPERTIES OF 2-ARYL-BENZOTHIAZOLE

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Free radicals can be defined as molecular species associated with various neurodegenerative diseases, including atherosclerosis, Parkinson's, Alzheimer's and others.[1] Scientific evidences correlate the damage induced by solar radiation to the genesis of free radicals: for this purpose, several substances with both photo-protective and antioxidant properties have been designed and developed. This alert notice explains our aim to develop a new series of dualistic molecules, able to combine antioxidant and UV filtering activities. The new compounds here reported derive from the isosteric replacement of 2-Phenyl-1H-benzimidazol-5-sulfonic acid (PBSA), a commercial UV-filter known for its photo-protective properties towards UVB rays,[2] with a benzothiazole nucleus to improve the spectrum activity (UVA-UVB range). Afterwards in position 2 several polyphenolic moieties were introduced to provide their antioxidant capacity and the benzothiazolic functional group in position 5 was also replaced. First, the antioxidant activity was evaluated *in vitro* on the 21 compounds obtained, with the use of both DPPH assay (evaluating the antioxidant capacity against one of the most stable nitrogen radicals) and FRAP test (measuring the ability to reduce the ferric ion). The Photo-protective capacity was carried using *in vitro* diffuse spectrophotometric transmittance technique to determine the sun protection factor (SPF), the UVA / UVB ratio, the UVA Protection Factor (UVAPF), and the critical wavelength (λc) of the new compounds in solution. From this work, four compounds have emerged with marked antioxidant activity showing IC_{50} values for the DPPH assay between 6.42-11.29 µg/ml and FRAP values between 6268.43-7402.73 µmolTE/g. A promising UV filter profile was supported by the remarkable values obtained: SPF 2.82-7.42; UVA/UVB 0.40-0.81; UVAPF 2.23-7.34 e λc 353-381. Finally, these results confirm the dualistic properties of the designed molecules.

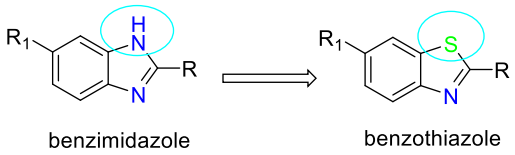

Figure 1. Isosteric replacement of benzimidazole nucleus of PBSA with variously substituted benzothiazole. R= polyphenolic ring, R_1 = H, COOH, SO_2NH_2

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SAIDs-H_2S RELEASING HYBRIDS: SYNTHESIS AND BIOLOGICAL EVALUATION IN INFLAMMATORY DISEASES

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Hydrogen sulfide is an endogenous gasotransmitter that plays significant physio-pathological roles in several biological systems.[1] It is endogenously synthesized mainly by two pyridoxal-5'-phosphate-dependent enzymes: cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE).[1] Secondary pathways involve the enzymes cysteine aminotransferase (CAT)/3-mercaptopyruvate sulfurtransferase (3MST) and the more recently identified enzymes D-amino-oxidase (DAO)/3MST, that produce H_2S from the amino acid D-cysteine.[2]

Our interest has been mainly focused on the involvement of H_2S in inflammatory diseases and, in particular, those affecting skin and lung. In the skin, H_2S participates in important functions and in vitro and in vivo studies have shown the anti-inflammatory effects of H_2S as well as its participation in the resolution of inflammation and repair processes.[3]

Moreover, H_2S has shown an anti-inflammatory activity in many models of respiratory diseases, including asthma and COPD.[4,5]

On this basis we have designed an extensive research project focused on the synthesis of molecular hybrids obtained by the condensation of several NSAIDs with different moieties able to release H_2S (4-hydroxy-thiobenzamide, TBZ; 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione, ADT-OH; 4-hydroxy-phenyl-isothiocyanate, HPI; ethyl 4-hydroxybenzodithioate, HBTA) (Fig. 1).

The starting corticosteroids were converted to the corresponding 21-succinates that were coupled with the H_2S-releasing moieties leading to the formation of the desired hybrids.

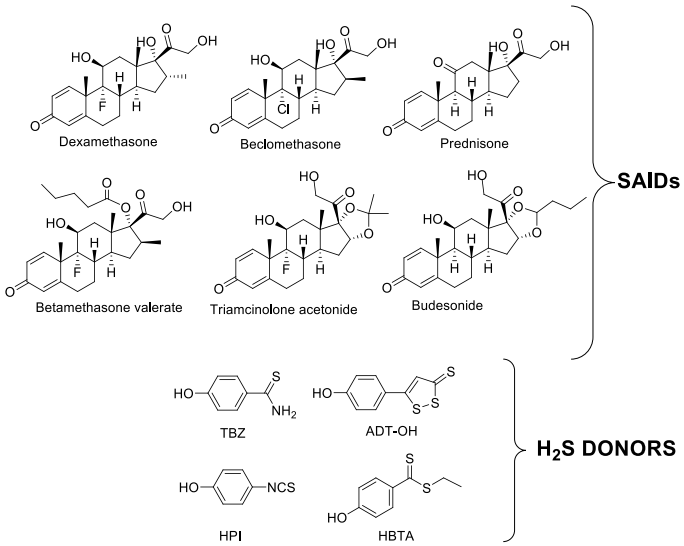

Figure 1

All the molecules were characterized for their ability to release H_2S both by an amperometric approach, allowing a real-time qualitative and quantitative evaluation of the H_2S-release, and by using a fluorescent probe, allowing the determination of the intracellular release of H_2S. Finally, selected compounds were evaluated in vivo in murine models of psoriasis and chronic allergic lung inflammation.

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OVERCOMING EXTENDED SPECTRUM BETA-LACTAMASE RESISTANCE IN *ACINETOBACTER BAUMANII* WITH BORONIC ACID TRANSISTION STATE INHIBITORS (BATSIs)

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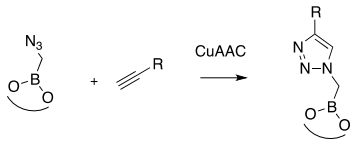
The World Health Organization (WHO) has recently identified antimicrobial resistance as one of the most important problems facing human health. *Acinetobacter baumannii* is a gram-negative bacterium associated with hospital-acquired infections, designated as a "red alert" human pathogen. Bacterial production of β-lactamase enzymes represents the most clinically concerning mechanism of resistance to β-lactam antibiotics.[1] A significant portion of resistance in *A. baumannii* results from a unique family of class C β-lactamase enzymes, the Acinetobacter-derived cephalosporinases (ADCs). Among these enzymes, ADC-7 demonstrated to exhibit extended-spectrum activity against numerous β-lactams. BATSIs are a new class of non β-lactam-β-lactamase inhibitors, which have recently reached the market: for instance, Vaborbactam, an α-acylamidoboronic acid, is now used for the treatment of complicated urinary tract infections and pyelonephritis as Vabomere®. In these compounds the boronic moiety mimics the highly reactive β-lactam ring of antibiotics and the carbon α to the boron is substituted with different groups able to confer high affinity and selectivity towards β-lactamases.[2] Whereas boron moiety acts as the "warhead" blocking the catalytic site, the α-amido group enhances molecular recognition by mimicking natural substrates.[2]



Scheme 1. Copper-catalyzed Azide-Alkyne Cycloaddition.

Herein, we reported the synthesis of a library of α-acylamidoboronic acid bioisosters, bearing a 1,4-disubstituted 1,2,3-triazole instead of the amide, through Copper-catalyzed Azide-Alkyne Cycloaddition (CuAAC) between 1-azidoalkylboronates and terminal acetylenes (Scheme 1). In fact, amide and triazole share several chemical properties such as planarity, size, dipole moment and hydrogen-bond capabilities. However, they also have important differences: triazole restricts conformational flexibility and improve hydrolysis and oxidation stability.[2] A small library of 24 BATSIs was obtained, displaying Ki *vs* ADC-7 in the low micromolar-nanomolar range; these data are comparable to those obtained for a number of α-acylamidoboronic acids and confirm the bioisosterism. Among all the compounds synthesised, ME096 (Figure 1) demonstrated the highest affinity towards ADC-7, displaying Ki= 0.09 μM. In addition, when associated to the β-lactam antibiotic ceftazidime ME096 restored antibiotic activity (MIC decreased from 16 to 2 μg/ml). Co-crystallization of ME096 and ADC-7 was performed and X-ray analysis further confirmed our hypothesis. Beyond the canonical interactions with the core boronic acid group of the BATSIs, the triazole displays a hydrogen bond with the side chain amide nitrogen of Asn152 as already observed for α-acylamido and α-sulfonamido-boronic acids.

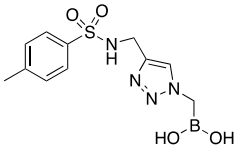


Figure 1. ME096.

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IDENTIFICATION OF NOVEL ANTI-FIBROTIC AGENTS

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Fibrosis is a common pathological process associated to many chronic diseases, especially of the lung, liver, heart, and kidney, characterized by excessive deposition of extracellular matrix (ECM) that affects both tissue architecture and function.[1,2] The evolution of the fibrotic process leads to end-stage organ failure, a common cause of morbidity and mortality. So far, only few therapeutic agents have been approved by regulatory agencies for the treatment of fibrosis-related pathologies, and organ transplantation is often the final option. Therefore, there is an urgent need to identify new effective anti-fibrotic drugs.[3]

The cytokine Transforming Growth Factor-β (TGF-β) is a master regulator of both physiological and pathological fibrogenesis, and induces the trans-differentiation of epithelial cells to myofibroblasts, which are able to produce and secrete ECM components.[4] On the other hand, Bone Morphogenetic Proteins (BMPs), which are members of the TGF-β family, trigger an intracellular signaling pathway that can counteract the effect of TGF-β. The activation of the BMP pathway by BMP-7 has been recently reported[5] as a strategy to antagonize fibroproliferative events. BMP-7-stimulated signaling opposes pro-fibrotic mechanisms, and its antagonism to profibrogenic TGF-β signaling has been demonstrated in several experimental models of organ fibrosis.[6]

The modulation of the BMP signaling pathway may therefore represent a new anti-fibrotic strategy, and the development of small molecule activators of this pathway could be envisaged as a promising therapeutic approach.

Here, we report the identification of small molecule activators of the BMP pathway starting from the high-throughput screening of ca. 11,000 compounds on ATDC5 cells stably expressing a specific luciferase reporter gene (BRE-Luc).[7] The anti-fibrotic activity of the initial hits was confirmed in an *in vitro* model of TGF-β1-induced 2D ECM deposition (Picrosirius Red Assay)[8,9] performed on human fetal lung (MRC-5) and normal rat kidney (NRK-49F) fibroblasts. About 150 analogues of the most interesting hit were synthesized and tested to obtain structure-activity relationship (SAR) information and increase the hit's anti-fibrotic activity (Figure 1). The most active compounds were tested in dose-response experiments. A few compounds with activity comparable to that of the anti-fibrotic agent IN-11309 were obtained. Additional functional studies are underway to characterize the biological profile of the most interesting compounds.

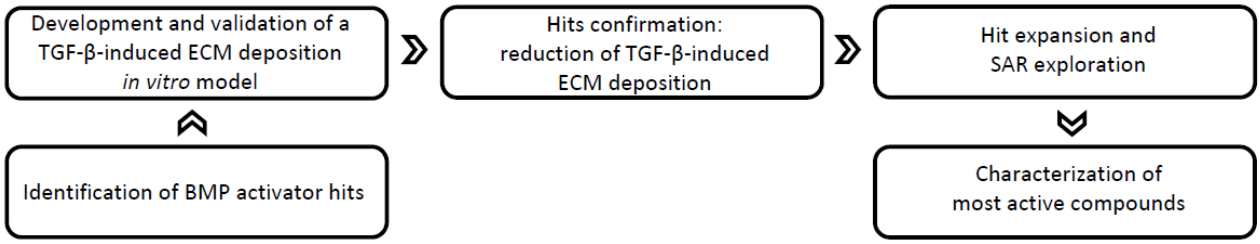

Figure 1. Screening strategy for the identification of new anti-fibrotic compounds.

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DISCOVERY OF NEW SECOND SITE CORRECTORS OF CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR)

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Cystic fibrosis (CF) is a genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene.[1] Of the about 2000 known CF mutations, deletion of phenylalanine at position 508 (F508del) in the CFTR protein is the most common one.[2] F508del alters CFTR biosynthesis, resulting in a misfolded, rapidly degraded protein that is poorly trafficked out of the endoplasmic reticulum to the cell surface. At present two orally-bioavailable small molecule targeting mutant CFTR are commercially available for CF patients: the corrector VX-809 and the potentiator VX-770 combined in a drug named Orkambi.[3] Correctors specifically address the folding and trafficking defects of F508del-CFTR protein.[4] There is a general belief that treatment with a single corrector is not enough to achieve a clinically-relevant rescue of F508del defect and that a combination of correctors having complementary mechanisms is desired.[5]

We started our study aiming at finding new chemotypes to maximize the rescue of mutant CFTR for the correction of the basic defect and with a particular ability to synergize with first generation correctors. Thus nearly 300 compounds synthetized by us were tested at the Telethon Institute of Genetics and Medicine (TIGEM) from which one lead compound was initially identified (PP7) and further improved (PP8). PP8 showed an interesting ability to functionally rescue F508del-CFTR, particularly in combination with VX-809. Importantly, it was also active in primary bronchial epithelial cells producing a marked synergic effect on transepithelial chloride secretion. Supported by our experience in the chemistry of nitrogen heterocycles, we synthetized new molecules and on the basis of structure-activity relationship (SAR), the pharmacological insights inspired the synthesis of new compounds. All compounds were tested as correctors using the halide-sensitive yellow fluorescent protein (HS-YFP) assay on CFBE41o- cells co-expressing F508del-CFTR. Activity of F508del-CFTR was measured as quenching rate (QR) of HS-YFP due to CFTR-dependent iodide influx and the most promising further tested on primary airway epithelial cells (bronchial and/or nasal) to confirm activity. Biochemical assay by western blot analysis on CFBE41o- cells expressing F508del-CFTR, treated with and without correctors showed an increase of the C band indicating an effect on F508del-CFTR maturation. Several new analogues of the starting lead compound demonstrated promising activity. Among the best derivatives PP28 stood out for its increased potency and efficacy compared to PP8 also when tested in primary bronchial epithelial cells. PP28 showed higher activity than VX-809 and a remarkable synergistic effect when used in combination with it corresponding to approximately 60% recovery of the normal CFTR functionality. The results obtained so far indicate PP28 as highly effective F508del corrector, capable to synergize with class 1 correctors, with a new complementary mechanism of action. For the relevance of the results obtained, a patent application with Telethon Foundation (IT 102018000010466) for this class compounds as correctors of the basic defect in CF is ongoing.

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3,4-DISUBSTITUTTED GIBBILIMBOL ANALOGUES: EFFECT OF THE SUBSTITUENTS ON BIOLOGICAL ACTIVITY AND SELECTIVITY AGAINST *TRYPANOSOMA CRUZI*

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Chagas' disease is a tropical neglected disease caused by Trypanosoma cruzi and currently has only two options of drugs available for treatment, both relatively toxic and lacking efficacy in the chronic phase. The LINS03 series was designed based on the simple structure of natural 4-alkylphenols, gibbilimbols A and B. The analogues proposed have different substitution patterns on the aromatic ring and functional groups in the alkyl chain.[1] An exploratory data analysis showed that the most active analogues, carboxylic acid derivatives (EC_{50} from 20.1 to > 100 µM) and amines (EC_{50} from 1.33 to 13.3 µM), are separated from the rest by being more hydrosoluble suggesting that compounds with a higher hydrosolubility have a better activity.[2] This led to the design and synthesis of new analogues of these promising derivatives (Figure 1) bearing an additional substituent in the *meta* position of the aromatic ring (3-OMe or 3-OH), as a way to enhance hydrosolubility.

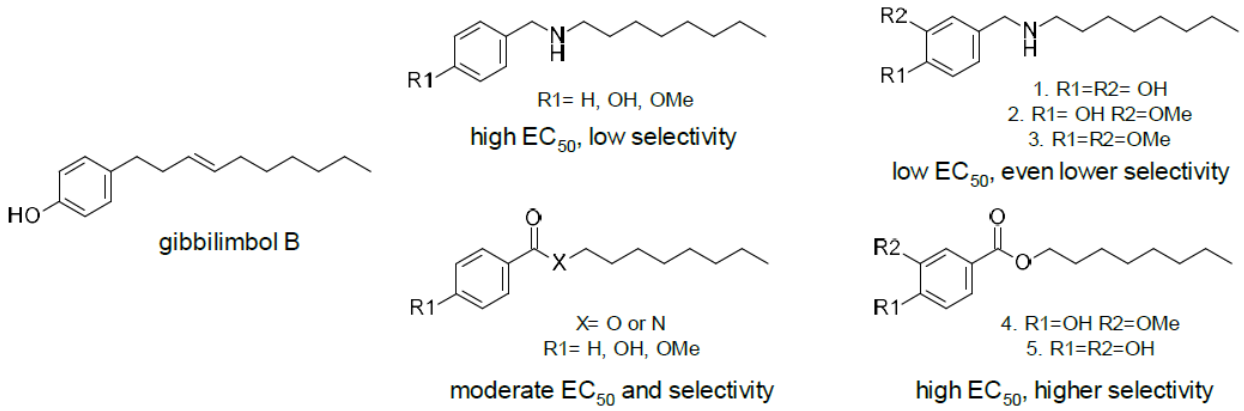

Figure 1. Design and structure of LINS03 analogues.

For the amines 1-3, the addition of a *meta* substituent abolish the activity against the amastigote form of T. cruzi (EC_{50} > 100 µM) and leads to more cytotoxic analogues. Whereas for the disubstitutted esters 4 and 5 this addition contributes to a higher EC_{50} (6.8 and 13.9 µM) and selectivity (18.2 and 7.7) against the amastigotes, respectivel.[3] These results showed opposite relations between the basic (amines) and neutral analogues, indicating that there is an ideal range of hydrosolubility for these compounds to and have an effect. Excessively hydrosoluble molecules may have trouble penetrating the infected cells, where the amastigotes are replicating, and therefore have a lower activity.

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DISCOVERY AND DEVELOPMENT OF A NOVEL NANOMOLAR F508-del CFTR CORRECTOR FOR THE TREATMENT OF CYSTIC FIBROSIS

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Cystic Fibrosis (CF) is a lethal genetic disease caused by mutations in the CF Transmembrane conductance Regulator (CFTR) chloride channel, resulting in reduced anion conductance on epithelial cells of multiple organs. Nearly 2000 mutations of the CFTR gene have been identified;[1] the most frequent is the deletion of phenylalanine at position 508 (F508-del). This mutation causes a severe defect in protein folding and stability, and affects the gating behavior. An effective treatment for F508-del CF patients requires at least a *corrector*, to increase CFTR levels at the cell surface, and a *potentiator,* to increase the opening frequency of the mutant CFTR channel.[2] At the moment, only two correctors for the treatment of CF patients bearing the F508del-CFTR mutation have been approved, i.e. *lumacaftor* (*VX-809*) and *tezacaftor* (VX-661), in combination with a potentiator, *ivacaftor* (*VX-770*). However, these drugs are unable to effectively rescue the folding defects of F508-del CFTR and, thus to substantially ameliorate CF clinical phenotype. Therefore, there is a clear need to continue developing new CF therapies.[3]

Using a high-throughput functional phenotypic assay, based on the Halide-Sensitive Yellow Fluorescent Protein (HS-YFP),[4] a collection of about 15,000 maximally diverse commercial small-molecules was screened in two different cell types (FRT and CFBE41o-) stably expressing F508del-CFTR. This activity led to the identification of some primary hits, belonging to different chemical classes. One of these chemo-types was investigated extensively. Rounds of chemical modifications of the hit and functional evaluation in different secondary assays provided the information to build the Structure-Activity Relationships (SARs) within this novel chemical class. Hit-to-Lead and Lead-Optimization campaigns led to compounds with high potency and efficacy in rescuing the activity of F508-del CFTR in bronchial epithelial cells from CF patients homozygous for the F508del mutation, as measured by electrophysiological assays. The best correctors displayed a very good efficacy and potency in the low nanomolar range. Among them, few analogs showed drug-like properties suitable for further development upon evaluation in *in vitro* DMPK assays. This work allowed the discovery of a novel, potent CFTR corrector[5] that is currently under preclinical development investigation.

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DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL RHODESAIN INHIBITORS FOR THE TREATMENT OF HUMAN AFRICAN TRYPANOSOMIASIS

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Human African Trypanosomiasis (HAT), commonly known as sleeping sickness, is an endemic parasitic disease caused by two subspecies of *Trypanosoma*, *T. brucei gambiense* and *T. brucei rhodesiense,* that affects 36 countries of sub-Saharan Africa, where it continues to be an important cause of morbidity and mortality, mainly in the rural areas, in spite of the significant decrease in the number of new reported cases over the last years.

Rhodesain, a cysteine protease of *T. brucei rhodesiense,* represents one of the most valuable targets for the development of new antitrypanosomal agents. The importance of the rhodesain is due to its several functions: it is required to cross the BBB, inducing the neurological stage of HAT; it is involved in the turnover of the VSGs of trypanosome, which forms a densely packed coat surrounding the parasite, whose periodic antigenic variation allows the parasite to evade the host immune system. In addition rhodesain takes also part to the degradation of parasite proteins and intracellularly transported host proteins within the lysosomes.[1]

Recently, we synthesized a panel of dipeptidyl derivatives as rhodesain inhibitors, bearing a reactive warhead able to inactivate the enzyme. In particular, compound **1** showed a k_{2nd} value of 67,000 x 10^3 M^{-1} min^{-1} and a high binding affinity expressed by a K_i value of 38 pM. The strong potency towards rhodesain was coupled with a good antitrypanosomal activity (EC_{50} value against *T. b. brucei* of 2.97 μM).[2] Based on these results, we developed derivatives **2a-h**, **3** and **4** (Fig. 1) with the aim to increase the target selectivity and the potency against the parasite; structural variations at P1', P2 and P3 sites were carried out whereas the reactive vinyl ketone warhead was kept unchanged, due to its high reactivity.

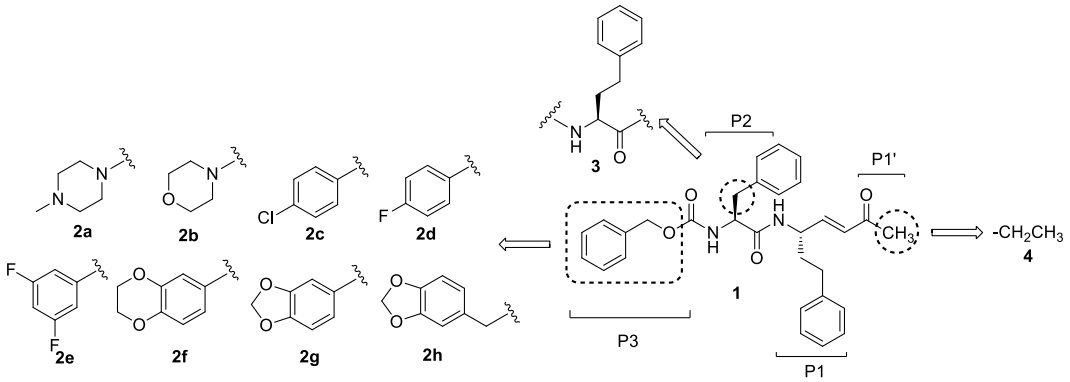

Figure 1, Development of novel rhodesain inhibitors.

The results of the biological investigation against rhodesain and *T. b. brucei* as well as molecular modelling studies will be reported and discussed.

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SYNTHESIS AND EVALUATION OF SUGAR-BASED ALIPHATIC AND AROMATIC FATTY ACID ESTERS AS ANTIMICROBIAL AND ANTIBIOFILM AGENTS

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Food pathogens are responsible of significant economic losses in food industry each year and cause various human diseases.[1] Indeed, a recent study by the Centers for Disease Control and Prevention estimated new cases of food-related illness in the United States at around 50 million, resulting approximately in 3,000 deaths and 130,000 hospitalizations every year.[2]

Some of the common disinfection practices in food industry, such as chemical-based and irradiation, are very effective. However, they are frequently expensive, dangerous and risky for the human health and the environment. In addition, these approaches may have no effect against bacteria organized in biofilm, a well-known tridimensional structure that confers to bacteria more resistance to environmental stresses as well as to antimicrobial treatments. Other practices, such as the use of common food preservatives (e.g. maleic acid or potassium sorbate), are now proving to be unsafe and toxic. Recently, the use of natural products in combating foodborne bacterial and fungal pathogens has become a trend. However, the antimicrobial activities of most natural products are too low to enable their practical use. In short, there is still a great need for effective antimicrobial food additives that are generally regarded as safe (GRAS) by Food and Drug Administration.

In this contest and as a part of our research interests,[3-5] we report enzymatic and chemical synthetic procedures applied to obtain sugar-fatty acid esters, and based on the esterification of three different sugars (monosaccharides glucose and mannose, and disaccharide lactose) with aliphatic (C_8-C_{16} saturated fatty acids) or aromatic (phenylacetic, biphenylacetic, triphenylacetic and *p*-phenylbenzoic) acids. These compounds have been characterized and tested in order to determine their minimum inhibitory concentration (MIC) values against different Gram-positive and Gram-negative bacteria and fungi. Secondarily, the most promising compounds of the series were also tested to evaluate both their antibiofilm activity at different time of development (24 and 48 h, 5 days) of representative food-borne pathogens and biocompatibility profile. The obtained data reveals an elevated percentage of biofilm formation inhibition up to 5 days (> 90% in some cases) and at MICs values no toxicity on Caco-2 cell line.

Overall, the analyzed sugar-based surfactants could be considered possible biocompatible and safe preservatives for food and other industrial applications.

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CARVACROL PRODRUGS AS ANTIBACTERIAL AND ANTIFUNGAL AGENTS

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The ability of bacteria to form biofilms contributes significantly to the pathogenesis of many microbial infections. Among natural products produced by essential oils, carvacrol (CAR) has recently attracted much attention due to its ability to inhibit the growth of preformed biofilms and interfere with biofilm formation. From a chemical point of view, it is a phenolic monoterpenoid endowed with antimicrobial, fungicidal, anticarcinogenic, and antitumor activities. CAR exerts its antimicrobial activity mainly against Gram positive bacteria, causing destabilization of bacterial membrane, decreasing the membrane potential, resulting in dissipation of pH gradients and perturbation of lipid fractions of bacterial cytoplasmatic membranes. The presence of the hydroxyl group and a delocalized electron system are the structural requirements for the antibacterial activity of CAR.[1-3]

In drug discovery, the poor water solubility and dissolution in the gastrointestinal fluids are limiting factors to the *in vivo* bioavailability of compounds after oral administration. In this context, CAR has limited water solubility (0.11 mg/mL). Moreover, its low distribution to target sites and poor bioavailability are additional factors complicating the use in a clinical setting.

In this study, the synthesis and antimicrobial evaluation of 23 CAR derivatives (**WSCP1-23**) against a panel of selected gram-positive and gram-negative bacteria are reported. Using the prodrug approach, CAR hydrophilic (**WSCP1-17**) and lipophilic (**WSCP18-23**) prodrugs were prepared. Notably, CAR water solubility was increased by using polar neutral groups (such as natural amino acids) with the aim of improving oral drug delivery. On the other hand, CAR lipophilic prodrugs, obtained by prenylation of CAR hydroxyl group, were designed to promote membrane permeation and oral absorption.

Our results revealed that **WSCP1-3**, showing the highest water solubility (> 1700-fold compared to that of CAR), possessed good antibacterial activity against gram-negative bacteria with MIC values comparable to those of CAR and antifungal properties against different species of *Candida*. **WSCP18-19** were the most promising prodrugs, showing good antibacterial profiles against gram-positive bacteria by interfering with the biofilm formation of *Staphylococcus aureus* and *Staphylococcus epidermidis*. Moreover, **WSCP18-19** resulted more stable in simulated fluids and human plasma than **WSCP1-3**. Toxicity studies performed on human erythrocytes and HaCaT cells revealed that all **WSCPs** were not toxic at the tested concentrations.

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TARGETING BACTERIAL SOS-RESPONSE TO SAVE THE ANTIMICROBIALS ARSENAL

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Drug Resistant Bacteria represent a global emergency, limiting the effective treatment of bacterial infections. The development of novel strategies fighting bacterial infections is strongly desirable.[1] The SOS pathway has been recently validated as a key target for combating the evolution of antibiotic resistance.[2] In bacteria, the SOS response is orchestrated by two proteins: RecA, the sensor protein, and LexA, the regulator one. As a consequence of damage to the DNA, RecA monomers assemble into large nucleoprotein filaments on single stranded damaged DNA and promote self-cleavage of the repressor LexA, binding a palindromic sequence of 16 base pairs (lexA binding Box), thus inducing the expression of more than 40 genes involved in DNA repair and mutagenesis.[3] Despite the high potentially of inhibiting LexA thus modulating antibiotic resistance evolution, very little as been done so far in this direction.[4]

Starting from the available LexA structural information,[5] a structure based virtual screening of a library of available chemicals was performed with the aim to identify LexA inhibitors able to block the proteolytic activity of LexA C-terminal domain.[6,7] Moreover, a full gene-to-crystal structure pipeline of a sequence coding for recombinant LexA C-terminal domain have been optimized to characterize and screen the most promising hits.

As a parallel, highly promising strategy, a nanobody libraries is currently under development and screening in our laboratory. Such an approach has the potential to open up novel opportunities for efficaciously targeting the SOS response, reversing drug resistance and rehabilitating abandoned antibiotics.

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CYCLOHEPTATHIOPHENE-3-CARBOXAMIDE PROTEIN-PROTEIN INTERACTION INHIBITORS POSSESS POTENT ANTI-INFLUENZA ACTIVITY AND A HIGH BARRIER TO DRUG RESISTANCE

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In the search for next-generation of anti-influenza virus (flu) agents, the viral RNA-dependent RNA polymerase (RdRP) has been validated as a valid drug target. The RdRP is a heterotrimeric complex composed of PB1, PB2, and PA subunits, which extensively interact with each other in a tightly associated and coupled fashion. Their correct assembly is essential for the RdRp functions of RNA transcription and replication.
After almost 20 years from the approval of flu neuraminidase inhibitors oseltamivir and zanamivir, a potent inhibitor of the PA endonuclease function, baloxavir marboxil, has been approved in Japan and the United States. Moreover, the PB1 polymerase inhibitor favipiravir was approved in Japan in 2014, and the PB2 cap-binding inhibitor pimodivir advanced in phase 3 clinical trials. The approval of these drugs confirmed the high profile of the RdRp as target. Nevertheless, they suffer from some limitations, of which the most important is the rapid development of drug-resistant viruses.
Interfering with the correct assembly of the three subunits using a protein-protein interaction inhibition approach is an alternative way to inhibit flu polymerase, which could led to the development of compounds with a high barrier to drug resistance.
Our group has identified many of the PA-PB1 complex formation inhibitors reported to date,[1] some of which based on the cycloheptathiophene-3-carboxamide (cHTC) scaffold. In particular, starting from a cHTC-based hit compound emerged by an initial SBVS approach,[2] its optimization permitted to obtain compounds endowed with an increased ability to interfere with polymerase PA-PB1 interaction but, above all, to acquire anti-flu activity at no-toxic concentration.[3-5]
In this study, the further elaboration of the cHTC scaffold produced among the most potent anti-flu compounds within the PA-PB1 interaction inhibitors. The new compounds possess potent and broad-spectrum anti-influenza activity interfering with the fluA polymerase in a cellular context, without showing cytotoxicity. Most important, no viral variants with reduced susceptibility to the compounds emerged after serial passages of fluA under drug selective pressure.

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DEVELOPMENT OF NEW ANTIFOLATES AS HOST-BASED THERAPEUTICS TO CONTROL INFLUENZA VIRUS

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Influenza and other acute respiratory viral diseases are of major global public health importance, causing 3–5 million severe cases of disease and 1 million deaths a year. The emergence in the last decade of high morbidity viruses have considerably increased the research efforts for preventing and fighting respiratory virus diseases. Besides approaching with the direct-acting antivirals (DAAs) and vaccines, therapeutics targeting host cellular machinery (host targeted antivirals, HTAs), which is essential for viral infection, are considered useful in developing broad-spectrum agents to overcome viral mutations and drug resistance.

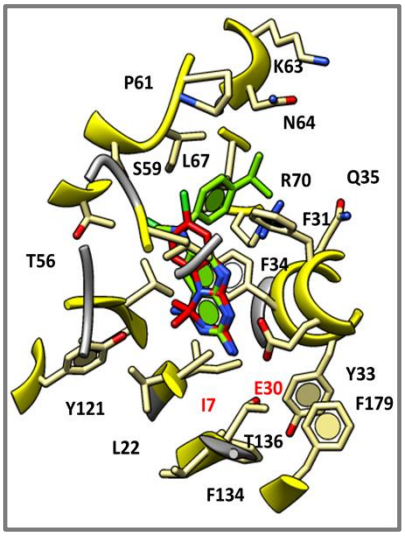
The present work represents a follow-up of previous studies on two series of 2,4-diamino dihydrotriazine-containing derivatives, which were shown dual antiviral agents against influenza and respiratory syncytial virus (RSV) by interaction with the host (human) dihydrofolate reductase (DHFR) enzyme.[1,2]

Docking studies reported key contacts with the host enzyme through H-bonds with the carbonyl group of E30 and with I7.

Herein, the novel conceived 2-aminotriazino[1,2-a]benzimidazoles were synthesised and screened against the hDHFR to confirm the mechanism of action as potential host targeting antivirals, and also against a panel of both human and protozoan folate-dependent enzymes, with the aim of estimating the specificity profile. Then the new compounds were tested in vitro for cytotoxicity and for antiviral activity against influenza virus, RSV and other virus strains.

Docking studies performed for the novel triazino-benzimidazoles within the X-ray data of the hDHFR allowed to reveal the most important features underlying protein/ligand binding, gaining a better comprehension of their mechanism of action as host factor-directed compounds. The new molecules displayed the H-bond interaction with the carbonyl group of E30 through its NH_2 group on triazine ring and π-π stacking interactions with residues Y33 and F34 thanks to the tricyclic moiety. However, they lacked the H-bond interaction with the previously mentioned I7 residue, which, together with E30, is considered a key point for an effective hDHFR inhibition.

Thus the 2-amino dihydrotriazino substructure is proven again to be a valuable molecular framework for developing promising HTAs against influenza virus and RSV.



Docking pose of 2-amino-7,8-dichloro-4,4-dimethyl-3,4-dihydrotriazino[1,2-a]benzimidazole (C atom; red) within the X-ray crystallographic structure of the hDHFR in complex with the inhibitor I (C atom; green;

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STRUCTURE-ACTIVITY RELATIONSHIP STUDY ON A NOVEL POTENT DECAMERIC PEPTIDE OF SPIRULINA PLATENSIS

Sala, M.;[a] Scala, M. C.;[a] Sommella, E.;[b] Carrizzo, A.;[c] Novellino, E.;[b] Vecchione, C.;[c,d] Campiglia, P.[a,e]

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Arthrospira platensis, known as spirulina, a filamentous fresh-water planktonic cyanobacterium, has an enhanced nutritional profile with high bioavailability of essential amino acids, biliproteins and other pigments, B and E vitamins, mineral substances and trace elements, glycolipids, sulpholipids, and essential polyunsaturated fatty acid. They provide therapeutic properties in the treatment and prevention of a variety of disorders.[1] Interestingly, Spirulina has been reported to exert biological activities and have beneficial properties in the management of cardiovascular diseases.[2]

In a recent work, through a peptidomic analysis of a spirulina formulation subjected to gastrointestinal digestion, we identified a decameric peptide, GIVAGDVTPI, named **SP6** (spirulina peptide 6). In vivo administration of **SP6** reduced blood pressure, improved endothelial vasorelaxation, and exerted an antihypertensive action in experimental models of hypertension, working through a NO-dependent mechanism.[3]

Considering the above highlighted, the aim of this study was to elucidate structure-activity relationships of **SP6**, in particular, in order to identify the primary sequence of the peptide, we synthesized a library of overlapping peptides, with specific length and specific offset covering the entire **SP6** sequence (Figure 1). The synthetized peptides were evaluated for their ability to modulate vascular function.

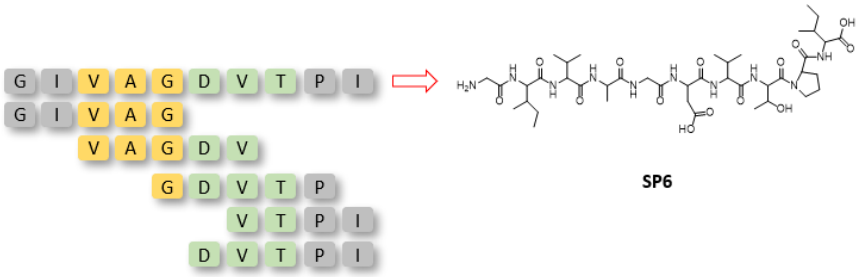

Figure 1. SP6 overlapping peptide library.

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STRUCTURE-ACTIVITY RELATIONSHIP STUDIES AND BINDING ASSAY OF C-LOBE LACTOFERRIN DERIVED PEPTIDES ACTIVE TOWARDS INFLUENZA

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Influenza is a highly contagious, acute respiratory illness, which represents one of the main plagues worldwide.[1] Even though some antiviral drugs are available, the alarming increase of virus strains resistant to them, highlights the need to find new antiviral compounds.[2]
Previously, Superti et al. have deeper investigated the mechanism of the anti-influenza virus effect of bovine Lactoferrin (bLf) and the role of its tryptic fragments (the N and C-lobes) in the antiviral activity.[3] In particular they have evaluated the influence of bLf on hemagglutinin-mediated functions. Recently, through a truncation library, we identified the tetrapeptides, SKHS (**1**) and SLDC (**2**), derived from Blf C-lobe fragment 418-429, which were able to bind HA and inhibit cell infection in a concentration range of femto- to picomolar.[4]
Starting from these results, in this work we initiated a systematic SAR study on the peptides mentioned above, through an Alanine scanning approach, a classical chemical technique to check the relevance of side chains of each aminoacidic residue in the interaction with the target molecule. We carried out binding affinity measurements by microscale thermophoresis (MST) and hemagglutination inhibition assay on peptide synthesized. Results obtained led to the identification of an interesting peptide endowed of broad anti-influenza activity and able to inhibit viral infection in a greater extent of reference peptide.

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IMMOBILIZATION OF γ-GLUTAMYL TRANSPEPTIDASE FOR THE SYNTHESIS OF *KOKUMI* PEPTIDES

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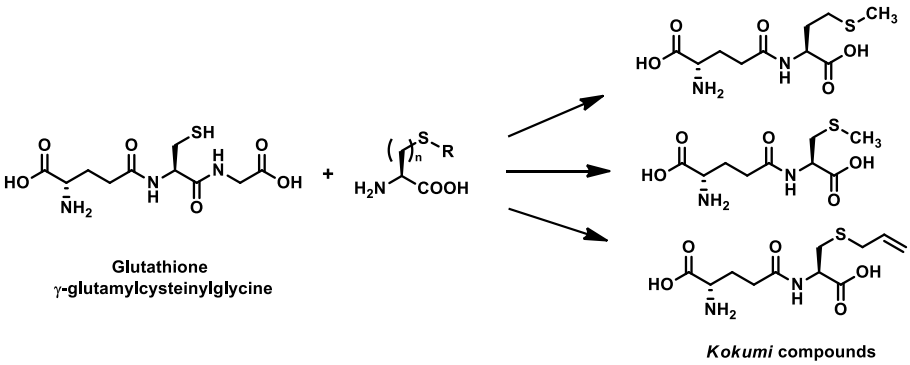
γ-Glutamyl transpeptidase from equine kidney (ekGGT, E.C. 2.3.2.2)[1] is an intrinsic membrane enzyme able to catalyze the transfer of the γ-glutamyl moiety of glutathione to amino acids or modified amino acids,[2] thus producing γ-glutamyl derivatives[3,4] which are known as *kokumi* compounds (Scheme 1). *Kokumi* is a Japanese word that refers to taste perception defined as having mouthfulness, thickness and a long-lasting savory sensation. Although being nearly tasteless in themselves, *kokumi* compounds are able to elicit strong taste sensations, especially when associated with protein-rich food, thus acting as true flavor enhancers.



Scheme 1. ekGGT-catalyzed synthesis of *kokumi* compounds.

Despite their simple chemical structure, the synthesis of γ-glutamyl derivatives through the classical peptide chemistry is wearisome. Biocatalysis can provide, instead, a more straightforward synthetic route.
An immobilization study of ekGGT was carried out to develop a robust biocatalyst for the synthesis of *kokumi* compounds. A tailor-made immobilization protocol was set up by assaying several types of binding chemistry, chemical activation of the support, and immobilization conditions. Octyl-glyoxyl heterofunctional agarose[5] resulted in a high immobilization yield and activity recovery (93% and 88%, respectively). The immobilized ekGGT retained 95% activity under reaction conditions (pH 9, 50 mM Tris HCl)[1] for 6 days. The residual activity after 7 reaction cycles (21 days) was 85% and 31% upon storage at 4 °C for 6 months. The immobilized ekGGT was also characterized by Raman spectroscopy and is currently being used for the preparative synthesis of γ-glutamylmethionine.

This project (TailGluTran, ID 2016-0741) was funded by Cariplo Foundation (Italy).

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AN EFFICIENT APPROACH TO AROMATIC AMINOMETHYLATION USING DICHLOROMETHANE AS METHYLENE SOURCE

Vestuto, V.;[a] Ostacolo, C.;[b] Di Sarno, V.;[a] Musella, S.;[c] Ciaglia, T.;[a] Pepe, G.;[a] Merciai, F.;[a] Campiglia, P.;[a] Gomez Monterrey, I. M.;[b] Bertamino, A.[a]

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The indole nucleus is present in a wide range of bioactive natural products and it is considered as privileged structure in the fields of pharmaceutical and material chemistry.[1] Research of new synthetic metal- or organo-catalyzed methodologies for the rapid construction of functionalizated indoles has seen relevant progress in recent years.[2] Indole amino methylation, one of the most important methods for the direct formation of C–C and C–N bonds,[3] continues to be a challenge for chemists, especially indole amino methylation at N-1 position. Mannich and Mannich-type Friedel–Crafts reactions using imines, N, O acetals or N, N aminals in the presence of a Lewis acid, constitute the most commonly used chemical approaches for the construction of amino methylated indoles.[4]
Our procedure involved the reaction of free indole with amine in basic medium using dichloromethane (DCM) as C_1 donor source, and ultrasound waves as catalysts (Fig.1). This innovative amino methylation protocol results in good to excellent yields of multifunctional indole derivatives. The method is also applicable to other aza heterocyclic compounds and, interestingly, affords direct access to amino methyl-substituted aryl alcohols.

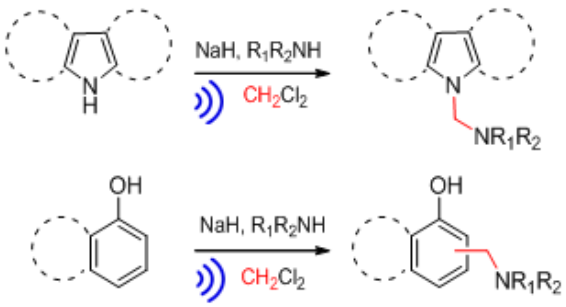

Figure 1. Synthetic route of N- and C-aminomethylation on different heterocyclic scaffolds.

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NOVEL BIORTHOGONAL APPROACHES TO FUNCTIONALIZE THE GOLD NANOPARTICLE SURFACE VIA HETEROBIFUNCTIONAL LINKERS

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The development of tailored methods to engineer the surface of gold nanoparticles (AuNPs) is a key issue for their use in biotechnological applications. In this respect, stable conjugation of a controlled number of biologically active molecules and their proper orientation on the AuNP surface, is essential to obtain selective interactions with the biological interface.

The aim of this project is the development of advanced conjugation strategies to expand the range of biomolecules that can be effectively coupled to PEGylated AuNPs.[1,2] Therefore, we selected two different reaction classes known for their efficacy and versatility in bioconjugation protocols:

- strain-promoted azide–alkyne cycloadditions (SPAAC);[3]
- inverse electron demand Diels–Alder reactions (DAinv) with 1,2,4,5-tetrazines.[4]

Despite their successful application in solution, their exploitation on nanoparticles has hardly been explored. In this contribution we will show the preparation of variety of heterobifunctional linkers and report their use for the functionalization of nanoparticles in aqueous medium under very mild reaction conditions. We have also developed a series of fluorescently labeled molecules, which are useful to investigate the reactivity of the linkers. Under comparable reaction conditions, the DAinv strategy resulted in systematically better conjugation yields compared to the SPAAC strategy.

Finally, we have applied the DAinv reaction to conjugate a 21-amino acid long glycopeptide to the AuNPs, demonstrating the usefulness of the developed strategy.

We envision this approach could be extended to others peptides and proteins, to improve the performance of biofunctionalized AuNPs in diagnostics, imaging, and drug delivery.

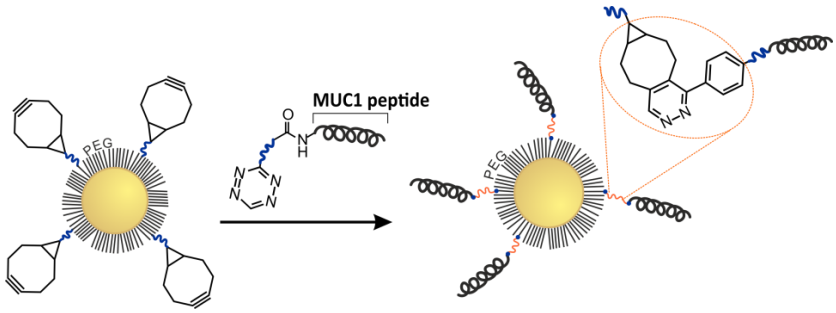

Figure 1. A schematic representation of the conjugation of an antigenic peptide derived from MUC1 on AuNPs via heterobifunctional linkers.

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ROLE OF CHIRALITY IN THE BINDING TO σ RECEPTORS AND DOPAMINE TRANSPORTERS AND EFFECT ON BINGE EATING EPISODE OF A POTENT σ_1 ANTAGONIST ANALOGUE OF SPIPETHIANE

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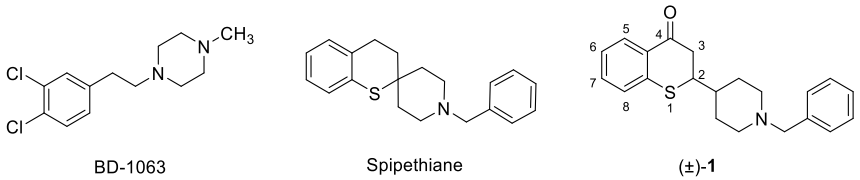
Sigma (σ) receptors are transmembrane proteins implicated in several cellular functions and are among the most poorly understood proteins in cell biology. At present, they are considered to be a unique receptor family comprising two pharmacologically distinct subtypes, namely σ_1 and σ_2 receptors.[1] Recently, the crystal structure of the human σ_1 receptor in complex with the potent σ_1 ligands has been resolved.[2] Because of its wide expression throughout the human body and its involvement in many physiological functions, the σ_1 receptor is considered a highly attractive target for the treatment of several diseases.[1] While a collection of evidence has demonstrated the role of σ_1 receptors in drug abuse,[3,4] just one study reports their involvement in binge eating disorder (BED). Such a study showed that the σ_1 antagonist BD-1063 (Figure 1) (σ_1 pK_i = 8.05, σ_1/σ_2 selectivity ratio = 71) reduced binge-like eating in a dose-dependent manner and blocked the increased eating rate in palatable rats.[5] From a SAR study about a series of spipethiane analogs showing high affinity and selectivity for the σ_1 receptor over the σ_2 receptor, compound (±)-**1** (Figure 1) emerged as a potent σ_1 receptor antagonist (σ_1 pK_i = 10.28) and, to our knowledge, is the most selective σ_1 ligand reported to date (σ_1/σ_2 selectivity ratio = 29510).[6]



BD-1063 Spipethiane (±)-**1**

Figure 1. Chemical structures of BD-1063, spipethiane and (±)-**1**.

Since compound (±)-**1** shows both a markedly higher σ_1 affinity and σ_1/σ_2 selectivity with respect to BD-1063, to better clarify the role played by the σ_1 receptor system in compulsive-like eating disorders, it was evaluated in a female rat model of BED. Moreover, the presence of a centre of chirality in (±)-**1** prompted us to preliminarily separate the two enantiomers (+)-**1** and (-)-**1** and evaluate their affinities for the σ_1 and σ_2 receptors. The affinities of (±)-**1** and its enantiomers were also evaluated at dopamine transporters (DAT), considering that DAT have been demonstrated to be involved in binge eating disorders and that several σ_1 ligands also bind DAT.[7,8] Analogously to (±)-**1**, both the enantiomers showed very high affinity for the σ_1 receptor and unprecedented selectivity over both the σ_2 receptor and DAT. The lack of enantioselectivity between (+)-**1** and (-)-**1** indicated that the stereocenter in 2-position of the benzothiochromane nucleus doesn't play a crucial role in the interaction with any of the studied targets. Docking studies confirmed that the configuration of the enantiomers has only marginal effects on the molecular interactions with the σ_1 receptor. In *in vivo* studies in a female rat model of BED, (±)-**1** dose-dependently decreased the binge eating episode elicited by a history of intermittent food restriction and stress, confirming and strengthening the important role played by the σ_1 receptor in bingeing-related eating disorders.

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SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL ADENOSINE RECEPTOR LIGANDS

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Adenosine is a regulatory nucleoside that can be generated in response to cellular stress and tissue damage as well as during episodes of tissue hypoxia or inflammation. It acts on specific G-protein coupled receptors that have been classified into four subtypes (A_1, A_{2A}, A_{2B} and A_3) on the basis of their structures and signal transduction systems.[1] Adenosine receptors (ARs) are found in almost all kinds of tissue: central nervous system (CNS), peripheral neurons, cardiovascular system, respiratory tract and immune system.
It has been demonstrated that A_1 and A_3ARs play a crucial role in the modulation of the pain stimulus;[2] therefore, they represent interesting targets for the development of new analgesic drugs.
Substitutions at both 5' and N^6 positions of the adenosine scaffold furnished derivatives endowed with different affinity profiles towards AR subtypes.
Our previous work showed that the replacement of the 5'-hydroxy- group by a chlorine atom in N^6-substituted adenosine derivatives gave potent and highly selective A_1AR agonists. 5'-Chloro-5'-deoxy-N^6-(±)-(*endo*-norborn-2-yl)-adenosine (5'Cl5'd-(±)-ENBA) displayed high A_1AR affinity and selectivity.[3] It was shown to reduce both mechanical allodynia and thermal hyperalgesia in a mice model of neuropathic pain without affecting motor and cardiovascular functions.[4] Moreover, it reduced dyskinesia evoked by L-DOPA in a mice model of Parkinson's disease.[5]
In this work, novel N^6/5'-disubstituted adenosine derivatives were synthesized and their pharmacological profile was assayed. The results of this study will be discussed.

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FORMYL PEPTIDE RECEPTOR 2 AGONISTS FOR THE TREATMENT OF NEURODEGENERATIVE DISEASES

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Formyl peptide receptor 2 (FPR2) is a G protein-coupled receptor, belonging to the N-formyl peptide receptors family, which plays critical roles in neuroinflammatory responses. Lipoxin A4, member of the specialized pro-resolving mediators (SPMs), interacts with FPR2 to contribute to the resolution of inflammatory processes by switching of macrophages from a pro-inflammatory (M1) to an anti-inflammatory and reparative (M2) phenotype.[1] Dysregulation of these events produces continuous release of pro-inflammatory mediators, which causes chronic inflammation. This is a central pathological process in neurodegenerative disease, such as Alzheimer's Disease, Parkinson's Disease, and Multiple Sclerosis.[1] Therefore, mimicking the effects of LXA4 by means of drug-like molecules could have the potential to treat neurodegenerative diseases. We have recently identified the FPR2 agonist MR39, which is able to reduce the production of pro-inflammatory mediators (NO, IL-β e TNF-α), showed neuroprotective properties in an *in vitro* model of neuroinflammation, promising *in vitro* pharmacokinetic properties (resistance to oxidative metabolism in rat microsomes, passive diffusion and permeation rate in a hCMEC/D3 cell monolayer).[2] Starting from our lead compound, we have designed new potential FPR2 agonists by modifying the "right hand" part of the molecule which is crucial for the interaction with both FPR2 and enzymes responsible for degradation. Here we report the optimization of our FPR2 agonists in terms of potency and in vitro pharmacokinetic properties and their effect on viability/metabolic activity, necrotic death, and production of pro-inflammatory mediators in microglial cells under normal conditions and after stimulation with different inflammatory stimuli.

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STUDY ON THE RESIDENCE TIME OF SEROTONIN 7 (5-HT$_7$) RECEPTOR LIGANDS

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The properties of a pharmacological tool are defined by the affinity for the target receptor (K_i) and by the agonist (EC_{50}) or antagonist (IC_{50}) potency. In recent years, there is interest also to investigate how a given ligand kinetically interacts with the target receptor by measuring ligand residence time, i.e. how long a ligand is bound to its target.[1] Drug-target residence time may be a useful additional parameter as it is thought to represent a surrogate marker of drug clinical efficacy: the longer the drug occupies the receptor, the more profound the drug may exert its effect.[1,2]

Over the years, our research groups have been involved in the identification and characterization of pharmacological tools to study the serotonin 7 (5-HT$_7$) receptor subtype.[3] 5-HT$_7$ receptor is a G protein-coupled receptor involved in a variety of central nervous system processes including the regulation of circadian rhythms, mood, cognition, pain processing, and mechanisms of addiction. 5-HT$_7$ receptor has been recently shown to modulate neuronal morphology, excitability, and plasticity, thus contributing to shape brain networks during development and to remodel neuronal wiring in the mature brain. Therefore, the activation of 5-HT$_7$ receptor has been proposed as a therapeutic approach for neurodevelopmental and neuropsychiatric disorders associated with abnormal neuronal connectivity.[3]

In order to identify novel pharmacological tools to study 5-HT$_7$ receptor characterized by different residence times, we have selected from our chemical library a set of 5-HT$_7$ receptor ligands structurally related to the 5-HT$_7$ receptor agonist BA-10 (Figure 1) characterized by different lipophilic properties (1.78 < ClogP < 4.13) and determined their residence times.

We have found that it is not the overall lipophilicity of the molecule that determines ligand residence time, but rather the lipophilicity at a specific position of the scaffold.

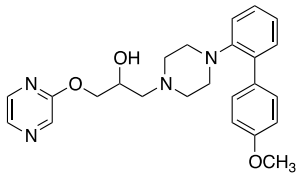

Figure 1. Structural formula of the 5-HT$_7$ receptor agonist BA-10.

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SYNTHESIS AND BIOLOGICAL EVALUATION OF SELECTIVE PDE4D INHIBITORS

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Type 4 phosphodiesterase (PDE4), an enzyme that hydrolyzes cAMP to 5'-AMP, is considered an important pharmaceutical target because of its crucial involvement in the signaling of the central nervous system. Indeed, some PDE4 inhibitors (PDE4Is) have been shown to improve memory and cognitive functions under both physiological and pathological conditions. Selective inhibition of the PDE4D isoform has been shown to represent a possible strategy for achieving memory improvements along with reduced side effects (mostly emesis and sedation).[1]

In an effort to develop new PDE4D selective inhibitors and with the aim to elucidate the molecular bases of selective inhibition of different PDE isoforms, we provided a structural and functional characterization of a large library of compounds that we synthesized in recent years (named **GEBR derivatives**). These compounds are characterized by a catecholic moiety, which is typical of Rolipram-related PDE4Is, linked with a terminal amino function (basic end) by a different alkyl chain (linker)(**Figure 1**).[1,2]

In detail, we identified three major ligand conformational classes, which we classified as protruding, twisted, and extended.[3] Our results clearly suggested that the subset of protruding ligands is the most promising candidate for further library development. Based on the acquired crystallographic information, we therefore set out to design and synthesize new derivatives in order to improve enzyme interaction and ligand potency. In particular:

a) compounds **1** (**Figure 1**), which feature greater steric hindrance, obtained by replacing the hydrogen atom of the iminoether linker with a methyl group;

b) compounds **2** (**Figure 1**), which are characterized by a reduced structure flexibility.

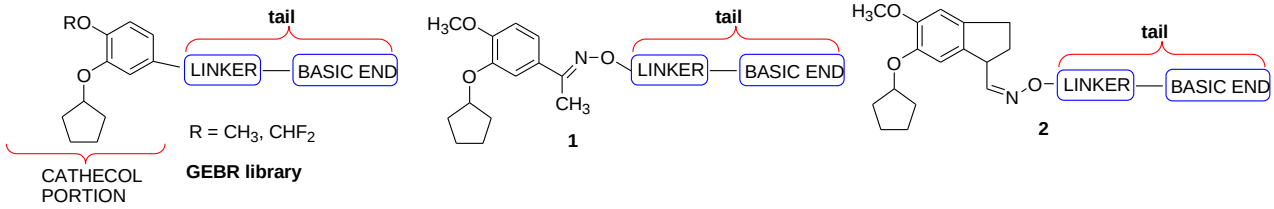

Figure 1. Molecular formula of **GEBR library** and new synthesized compounds **1** and **2**.

Synthesis, enzyme inhibition and crystallographic data will be reported in the poster session.

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SYNTHESIS OF KETOXIMIC DERIVATIVES AND THEIR OPTIMIZATION AS POTENT REVERSIBLE MAGL INHIBITORS

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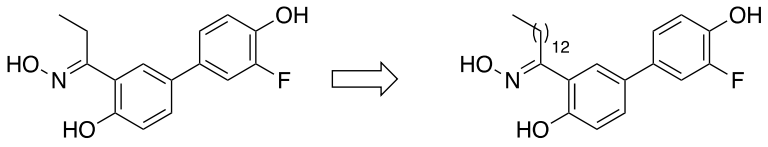
Monoacylglycerol lipase (MAGL) is a serine hydrolase, which represents the major contributor to the hydrolysis of brain 2-arachidonoylglycerol, an endocannabinoid neurotransmitter involved in many physiological and pathological processes, such as inflammation, pain, neuroprotection and cancer. MAGL is overexpressed in aggressive cancer cells, where it generates free fatty acids and glycerol, and promotes cancer aggressiveness, invasiveness and proliferation.[1] Thus, MAGL inhibition can be a good strategy in order to develop new potential therapeutic agents, such as: anti-inflammatory, anti-nociceptive and anti-cancer agents. Several MAGL inhibitors have been reported in the literature; however, many of them interact with the enzyme by means of an irreversible mechanism, provoking a chronic MAGL inactivation which determines undesired side effects. At present, few reversible and selective MAGL inhibitors have been developed. Hence, with the purpose to identify new reversible MAGL inhibitors, an in-house library of published compounds was screened, and compound **1** (Figure 1) showed a promising MAGL inhibition activity [2] (IC_{50} = 11 μM). This salicylketoxime was optimized, leading to the identification of new potent and reversible MAGL inhibitors. The most active of these ketoximes is compound **2** (IC_{50} = 0.68 μM), which is characterized by a good selectivity towards other targets of the endocannabinoid system and showed a relevant antiproliferative activity in cancer cells.[3] The newly developed salicylketoximes were further modified in order to improve MAGL inhibition activity together with a modulation of the lipophilicity of the molecules. These new compounds also demonstrated to efficiently inhibit MAGL enzyme.



1 **2**

Figure 1. Chemical structures of ketoximes **1** and **2**.

Acknowledgments. We are grateful to the University of Pisa (Progetti di Ricerca di Ateneo, prog. n. PRA-2018-18) for funding.

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DESIGN, SYNTHESIS AND ANTIPROLIFERATIVE ACTIVITY OF NEW BENZYLIDENE DERIVATIVES OF PYRAZOLE- AND IMIDAZO[1,2-*b*]PYRAZOLE-CARBOHYDRAZIDES

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The approval of T cell checkpoint inhibitory antibodies as ipilimumab, pembrolizumab and nivolumab has started the new era of anticancer immunotherapy. However, there are still many alternative pathways that are open to intervention by traditional small molecules drugs. Moreover, small-molecules drugs offer several advantages over recombinant protein approaches, such as access to specific intracellular targets, more options for drug formulation and pharmacokinetic challenges, lower cost in production and delivering. In addition, re-examination of classical antitumor agents (e.g. doxorubicin or cyclophosphamide) revealed that they promote an immunogenic cells death; other (such as taxanes) block tumor growth and also affect, as off-target, immune cell function in the tumor microenvironment. Small molecules may therefore have a cooperative role in cancer immunotherapy. In the last ten years, we identified several pyrazole and imidazopyrazole derivatives as inhibitors of the neutrophil-mediated inflammatory response.[1] More recently, our pyrazolyl-urea **GeGe-3** was found to control the activation of MAPK signaling, to block angiogenesis and tumor growth both in vitro and in vivo tests.[2,3] Having in mind these considerations, we try to investigate on the possible antiproliferative activity for a new series (compounds **1a-j** and **2a-j**, Figure 1) in which our previous pyrazole and imidazopyrazole scaffolds are linked to a differently decorated phenyl ring through an acyl-hydrazone linker.

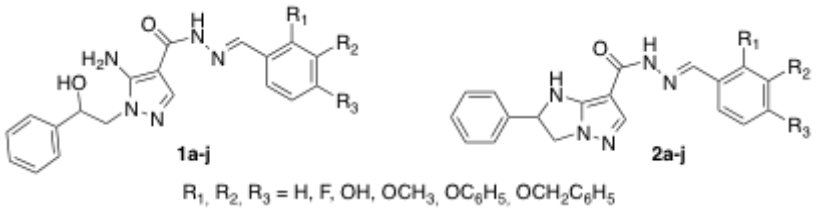

Figure 1. General structure of title compounds

The library was submitted to the National Cancer Institute (USA) to obtain preliminary information about antiproliferative activity. They were firstly tested at a single high dose (10^{-5} M) in the full NCI 60 cell panel. The best compounds **2g** (R_1 = H, R_2 = OCH_3, R_3 = OC_6H_5) and **2i** (R_1 = H, R_2 = OCH_3, R_3 = $OCH_2C_6H_5$) were progressed to the full 5-dose assay on the same NCI 60 cell panel. The promising results obtained, prompted us to look for further confirmation. Compounds **2g** and **2i** were then tested for the inhibiton of cell proliferation, the induction of apoptosis, and the effect on the cell cycle. Both compounds, in particular **2i,** showed a good growth inhibition, with IC_{50}s mainly ranging in the low micromolar range of concentrations, associated with the ability to induce apoptosis above all at the higher concentration used (IC_{75}). Furthermore, both compounds were able to induce alterations of the cell cycle phases with the time- and concentration-dependent appearance of polyploidy cells.
Further investigations are in progress to identify the molecular target.
Synthetic procedures and detailed biological results will be presented during the session poster.

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TOWARD ENRICHED *VHTS* FOR CDK2 INHIBITORS: MOLECULAR DYNAMICS, PHARMACOPHORE MODELLING, AND DOCKING

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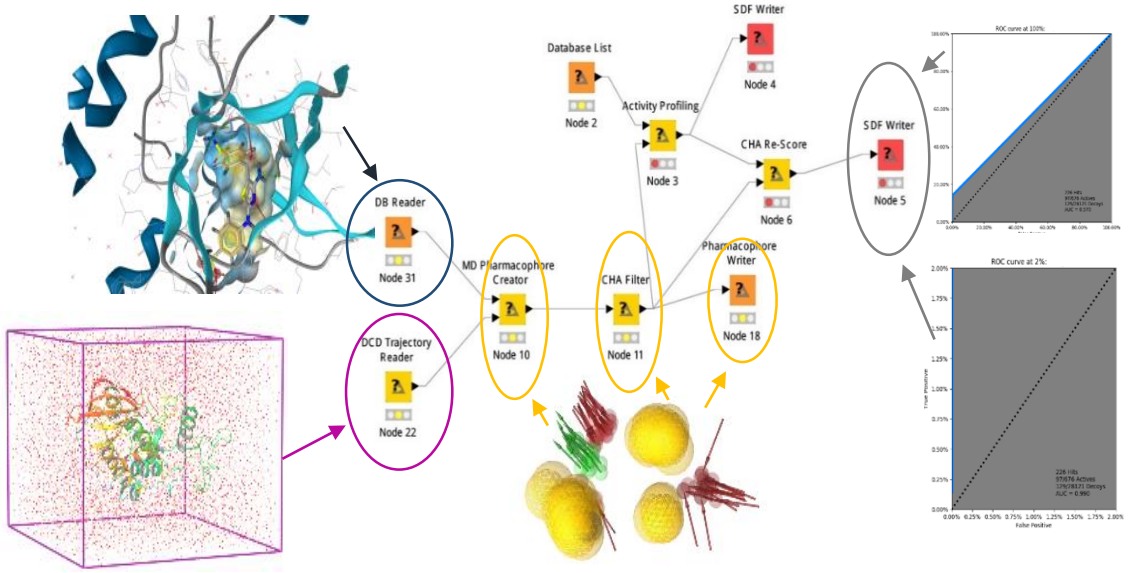
Cyclin-Dependent Kinases-2 (CDK2) are members of the serine/threonine protein kinases family. They play an important role in the regulation events of the eukaryotic cell division cycle, especially during the G1 to S phase transition. Experimental evidence indicates that excessive expression of CDK2s should cause abnormal cell cycle regulation. Therefore, since a long time, CDK2s have been considered potential therapeutic targets for cancer therapy. In this work, we collected one-hundred and forty-nine complexes of inhibitors bound in the CDK2-ATP pocket submitting to short MD simulations (10ns) and free energy calculation by means of MM-GBSA. The calculate ΔG values have been compared with experimental data (K_i, K_d, and IC_{50}). Information collected on short MD simulations of protein-ligand complexes has been used to perform molecular modeling approaches that incorporates flexibility into structure-based pharmacophore modeling (Common Hits Approach, CHA,[1] and Molecular dYnamics SHAred PharmacophorE, MYSHAPE[2] approach) and constraints docking, to enrich the hits list of virtual screening. Short simulations proved to be exhaustive to examine the crucial ligand-protein interactions within the complexes.



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ANTIOXIDANT-CONJUGATED 1,2,4-TRIAZOLO[4,3-*a*]PYRAZIN-3-ONES: HIGHLY POTENT AND SELECTIVE A_{2A} ADENOSINE RECEPTOR ANTAGONISTS EFFECTIVE IN NEUROPATHIC PAIN

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The A_{2A} adenosine receptor (AR) is expressed on neurons and in glial cells where it regulates several functions related to excitotoxicity. Activation of A_{2A} AR in microglia shows facilitating action on the release of pro-inflammatory cytokines and ROS which are associated with neuronal damage occurring in neurodegenerative diseases and in neuropathic pain.[1] Several studies highlighted that A_{2A} AR blockade confers neuroprotection in diseases in which inflammatory and oxidative processes play a significant role.[2] Neuropathic pain results of several factors leading to impairment in nerve function and oxidative stress might contribute to its development.[3] The role of the A_{2A} AR in pain still needs to be clarified. Selective A_{2A} AR antagonists proved to be effective anti-nociceptive agents in several animal models and they have been indicated as possible agents for the management of neuropathic pain.[3]
As a part of our recent research on AR ligands,[4] we designed new 8-amino-6-aryl-2-phenyl-1,2,4-triazolo[4,3-a]pyrazin-3-ones (Figure 1) to obtain human A_{2A} adenosine receptor (hA_{2A} AR) antagonists endowed with antioxidant moieties, since we envisaged they would possess increased protective properties in neuropathic pain. The lipoyl and 4-hydroxy-3,5-di-terbutylbenzoyl residues were chosen as antioxidant portions and were appended by different linkers on the 6-phenyl ring.

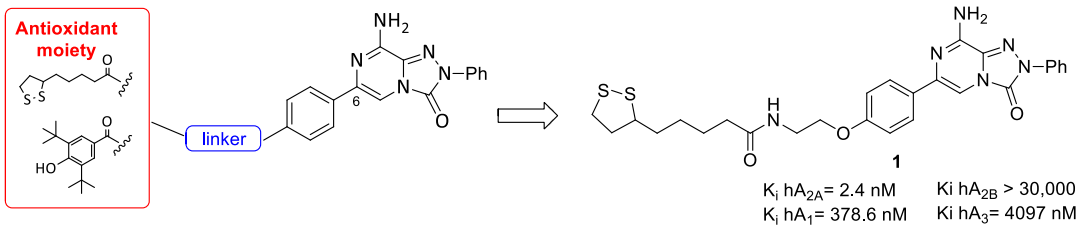

Figure 1

Several new triazolopyrazines were potent and selective hA_{2A} AR antagonists (K_i = 0.6-54.5 nM). Molecular docking studies were performed to rationalize the affinity data and to represent the hypothetical binding mode of these antagonists at the hA_{2A} AR. Some selected triazolopyrazines reduced oxaliplatin–induced toxicity in microglia cells. In this model, the lipoyl-derivative **1** was the most active compound, being effective in reducing the oxygen free radical level. Moreover, it was able to revert oxaliplatin-induced neuropathy in mouse. Efficacy of **1** can be due to a synergistic action of the two components: the hA_{2A} AR antagonist and the antioxidant lipoic acid function. This dual activity makes **1** a promising neuroprotective agent in oxidative stress-related diseases.

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INDAZOLONES: AN EXAMPLE OF SCAFFOLD HOPPING FOR THE SYNTHESIS OF A POTENT AND SELECTIVE PARP-1 INHIBITOR

Posteri, H.; Borghi, D.; Casale, E.; Cirla, A.; Donati, D.; Felder E. R.; Galvani, A.; Isacchi, A.; Montagnoli, A.; Poma, E.; Rainoldi, S.; Papeo, G.

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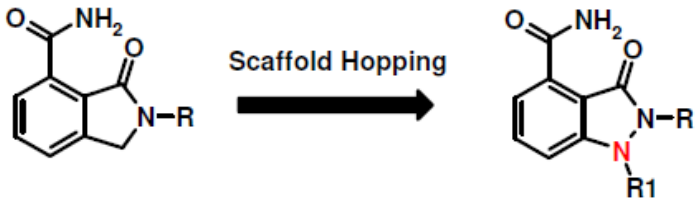
PARP-1 (Poly(ADP-ribose) polymerase-1) is a nuclear enzyme involved in the detection and repair of DNAdamage. It is the most abundant protein of a family of some 17 proteinsn named ADP-Ribosyl-Transferases Diphtheria Toxin-like (ARTDs),[1,2] that share the PARP signature motif, a highly conserved sequence within the catalytic domain. Among those isoforms, only PARP-1 and PARP-2 contain a DNA-binding domain that facilitates the localization to the site of DNA damage. PARP-1 and PARP-2 catalyze the transfer of ADPribose units from nicotinamide adenine dinucleotide (NAD^+) to nuclear acceptor proteins, leading to the formation of ADP-ribose polymers.[3] This is a key process for the repair of DNA damage caused by DNA-damaging chemotherapeutic agents and radiations. Selective inhibition of these targets in specific DNA-repair deficient settings may provide an innovative therapy for the treatment of tumors both as single agent and in combination, as confirmed by the PARP-1 inhibitors actually on the market (e.g. Lynparza™, Rubraca™, Zejula™, Talzenna™).
Aiming at discovering new proprietary PARP-1 privileged scaffolds, we explored indazolones, a chemical class obtained *via* a "Scaffold Hopping" approach from isoindolinones. In this poster, we reported SAR studies and the synthetic pathway toward the preparation of different compounds, together with their corresponding pharmacological data.



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DEVELOPMENT AND VALIDATION OF A PIPELINE PILOT VIRTUAL SCREENING WORKFLOW: TARGETING THE HECT E3 LIGASE

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Virtual Screening (VS) approaches consist of an ensemble of computational tools aimed at the identification of promising compounds within large libraries database of chemical structures. The top-ranked compounds (virtual hits) are selected to be biochemically evaluated for their ability to interact with target of interest. Active compounds in biochemical assays represent validated hits, which will be further optimized in classical medicinal chemistry cycles. Fundamental aspects to consider in performing VS studies are (i) selection of libraries to be screened and (ii) selection of algorithms and tools. A good balance between size of screening set and the accuracy of methods used is paramount in order to identify, at reduced computational resources, high quality virtual hits with chances to be active in biochemical assays, and to ultimately ensure the success of the VS campaign.

In this context we have developed a two-steps Pipeline Pilot protocol, called “Pipeline Pilot virtual screening workflow” (PPvsw). In the first step virtual libraries are submitted to Bemis-Murcko fragmentation and structure cluster analysis in order to identify a reduced pool of representative scaffolds (centroids) able to describe well the chemical space of such libraries. Each of the representative scaffold identified in the first step is than submitted to the next step of the PPvsw. Such step is based on (i) docking studies by means of three different software (Vina, Glide and Plants); (ii) binding mode cluster analysis, regardless of the software docking scoring function; (iii) identification of 5 most populated binding modes; (iv) selection of the top-ranked binding mode applying a scoring function based on short MD-simulations, PBSA and MM-GBSA methodologies; (v) visual inspection. Being independent from any docking scoring function, PPvsw has the advantage of circumventing the well-known issue related to the ranking power of docking methods, thus allowing the accurate exploration of the chemical space defined by the screening set. Moreover, the use of centroids (PPvsw step 1) as representative of structurally-related families of compounds provides a solid base for classical hit expansion activities.

Using our internal computational infrastructure PPvsw was validated on an internal project targeting the HECT E3 ligase super-family and allowed the identification of a hit compound with micromolar IC_{50} on an enzymatic assay. PPvsw also provided a putative binding mode of the hit, useful to guide medicinal chemistry follow-up for potency and selectivity optimization.

Acknowledgments: This work was supported by Associazione Italiana Ricerca sul Cancro (AIRC IG 21328) to C. M. and by a generous donation to IFOM from Ravelli Family.

SMART: A WEB-BASED PLATFORM FOR COMPOUND MANAGEMENT, IN-SILICO EXPERIMENTS, AND SCREENING DATA HANDLING

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Nowadays, in both small research groups and pharmaceutical companies, an efficient management of large chemical libraries, sample tracking, and handling of associated data are of paramount importance toward successful drug discovery projects. This requires the use of a user-friendly integrated informatics infrastructure, supporting the access to various resources at different stages of a project. However, the diversity of infrastructures in existing drug discovery groups makes impossible to find a common standard solution. Even more, the development of such integrated platform must accomplish several essential requirements: (i) flexibility in compound management, (ii) integration of diverse laboratory equipment, (iii) accurate sample tracking from chemical library to screening assay, (iv) high throughput/content screening data handling. All these attributes heavily contribute at different stages of the decision-making processes to successfully reach the final goal.

In this context, we developed ***S****ample* ***M****anagement and* ***A****ssay* ***R****egistration* ***T****ool* (SMART), an informatics web-based platform, which integrates chemistry, modeling, chemoinformatics, and biology domains. SMART follows a standard three-tier architecture, having Oracle as data-tier, Biovia's Pipeline-Pilot as logic-tier, and Pipeline Pilot's Web-Port as client-tier. SMART integrates: (i) compounds management and substances library registration procedures into an ORACLE database; (ii) in-silico experiments (chemoinformatics and modeling tools); (iii) wet biology experiments (cherry picking, sample dilutions, assay plate generation and data acquisition); (iv) screening data handling (registration, analysis, and representation). SMART interfaces to different IT resources (e.g. Oracle DB, Citrix Platform, Biovia's ELN, Perkin Elmer's Columbus server) and integrates different laboratory instruments (e.g. Tecan's Evo150, Hamilton's MICROLAB STAR Line, Perkin Elmer's Operetta CLS). Our solution, using as middle-tier the Biovia's Pipeline Pilot platform, proves to be a successful approach that can be easily adapted and extended to other drug-discovery realities.

Acknowledgments: This work was supported by a generous donation to IFOM from Ravelli Family.

DESIGN AND PRELIMINARY BIOLOGICAL EVALUATION OF (N-HYDROXY-CARBONIMIDOYL)PYRIDINE-4-CARBOXYLIC ACID DERIVATIVES AS NEW KDM4C INHIBITORS

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The Jumonji KDM4 family members are site-specific N-methyl lysine demethylases which function enzymatically by way of an iron and 2-oxoglutarate (2-OG)-dependent oxygenase mechanism. The KDM4 family members catalyze the demethylation of histone-3-lysine-9 trimethyl and dimethyl (H3K9me3/H3K9me2), which is a process linked to transcriptional activation.[1-3] Additionally, KDM4A-C catalyze the demethylation of histone-3-lysine-36 trimethyl and dimethyl (H3K36me3/H3K36me2), which is associated with gene repression, aberrant initiation and the prevention of transcriptional elongation.[2,3] KDM4 family members have been implicated in events that may contribute to cancer development, such as cell-cycle progression[4,5] and hypoxia.[6] As a result, KDM4 family members have generated significant interest as therapeutic targets in oncology.

Here we report on the identification of (N-hydroxy- carbonimidoyl)pyridine-4-carboxylic acid derivatives (Figure 1) as a new class of KDM4C inhibitors through a Medicinal Chemistry/SBDD approach, their preliminary SAR and target modulation in cell.

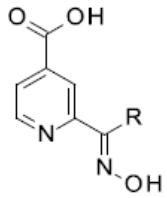

Figure 1

Acknowledgments. This research was supported by a generous donation to IFOM from Ravelli Family.

Authors information. The work by D. F., C. M., L. S., M. P., P. T., P. V., and M. V. was performed in part at the European Institute of Oncology, Drug Discovery Program.

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IDENTIFICATION OF A POTENT P-GLYCOPROTEIN LIGAND ABLE TO OVERCOME MULTIDRUG RESISTANCE (MDR) IN CANCER STEM CELLS

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P-Glycoprotein (P-gp) is a transmembrane protein belonging to the ATP-binding cassette (ABC) transporters superfamily, a class of proteins able to transport a variety of molecules using the energy produced by ATP hydrolysis. P-gp plays a pivotal defense role recognizing and binding endogenous or exogenous toxic substances and mediating their efflux.[1] Moreover, P-gp is a major cause of cancer chemotherapy failure because it is overexpressed in tumor cells where it causes the efflux of several chemotherapeutic agents and thus the phenomenon of multidrug resistance (MDR). It has been noticed that a relevant population of cancer cells, i.e. Cancer Stem Cells, shows an overexpression of P-gp which promotes tumor by stem cells renewal, survival, differentiation and chemoresistance.[2]

Due to the interest in overcoming MDR in cancer therapy, we developed a series of P-gp ligands, starting from the previously studied P-gp inhibitor **MC70** (EC_{50} = 0.69 μM), with the aim of producing novel P-gp inhibitors able to restore chemotherapeutic drugs sensitivity of tumor cells.[3,4] We focused our attention on the phenolic group of the lead compound, introducing alkyl and oxyalkyl chains, moieties containing different substituted furazan (1,2,5-oxadiazole) rings and several functional groups possibly acting as phenol bioisosteres. Among all the compounds, **5b**, characterized from an *N*-(benzenesulfonyl)benzenesulfonamide moiety, emerged for its selective activity toward P-gp (EC_{50} = 15 nM). Notably, **5b** restored doxorubicin toxicity in resistant cancer cells when co-administered at nontoxic concentration. Finally, we carried out a molecular dynamics study in order to rationalize the binding mode between the most active compounds of the series and P-gp.

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IDENTIFICATION OF POTENTIAL mTOR INHIBITORS THROUGH STRUCTURE-BASED VIRTUAL SCREENING APPROACHES

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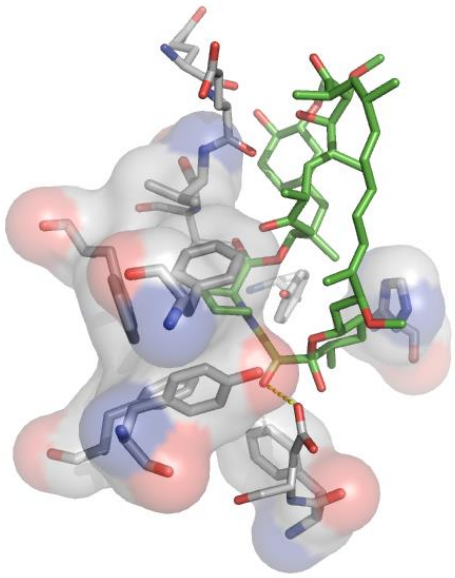
The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine protein kinase with multiple cellular functions influencing physiological and pathological neuronal processes. mTOR has been associated with synaptic plasticity, memory function and neuronal repair mechanisms and .recent evidence suggests that an altered mTOR pathway is implicated in several neurological disorders.[1] The aim of our work was to identify new molecules with potential neurological effects acting as mTOR inhibitors. mTOR assembles into two complexes with distinct inputs and downstream effects: mTOR complex 1 (mTORC1) regulates cell growth by promoting translation, ribosome biogenesis and autophagy and is sensitive to rapamycin; while mTOR complex 2 (mTORC2) promotes cell cycle entry, cell survival, actin cytoskeleton polarization, anabolic output and is insensitive to rapamycin.



To identify new chemical entities able to inhibit mTOR with a rapamycin-like mechanism (binding to FKBP12), we performed computational techniques.

Our starting point was the atomic structure of rapamycin human immunophilin FKBP12 complex (1FKB, Figure 1),[2] which was employed to create a simplified pharmacophore model by means of LigandScout software.

Subsequently, through a web tool SwissSimilarity was selected a library of compounds presenting a chemical structure similar to rapamycin. A similar approach was used in PubChem and SciFinder platforms thus creating different databases that were combined. As result we obtained a wider collection of compounds that were screened by pharmacophore model.

The selected compounds were further analyzed and refined using drug-like filters. The most promising hits with different scaffolds were picked out for docking studies and the selected ligands were purchased to perform specific biological tests.

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HETEROCYCLIC HYDRAZONES AS MULTIFUNCTIONAL ANTIOXIDANT, PHOTOPROTECTIVE AND ANTIPROLIFERATIVE POTENTIAL DRUGS

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In recent years, a new concept of drug design has emerged that has revolutionized pharmacology: the multi-target approach. A multi-target active ingredient can be designed to act simultaneously on multiple targets. There are numerous advantages that it can offer: not only from a pharmacological point of view, characterized by a better efficacy and minimal adverse effects,[1] but there is also a reduction in costs and development times before reaching the market.[2]
In this context, our attention has been focused on the research of compounds with multifunctional activities, that are equipped with both antioxidant and UV-filtering capabilities, to counteract the oxidative damage caused by free radicals and, at the same time, protect during exposure to UV radiation. Heterocycles such as indole, benzimidazole and benzofuran are widespread naturally occurring compounds as component of biologically active products. Due to their broad range of biological activities, they are privileged structures in medicinal chemistry. In this context we designed new series of heterocyclic hydrazones (Figure 1), in which the strong antioxidant properties were associated with photoprotection and antiproliferative activity allowing both to neutralize the oxidative damage promoted by free radicals, generated by UVA radiation, and to protect against UVB, responsible for direct damage to DNA and the onset of cancer in the skin.

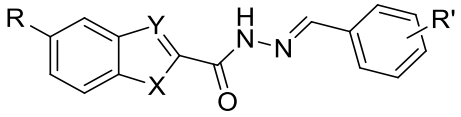

Figure 1. General structure of the heteroarylhydrazones studied

Here we report synthetic pathways, antioxidant, photoprotective and antiproliferative results and SAR studies on the new compounds.

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UNVEILING THE BIOCHEMISTRY OF THE EPIGENETIC REGULATOR SMYD3 TO EASE DRUG DISCOVERY IN CANCER DISEASE

Fabini, E.;[a,d] Talibov, V. O.;[b] Manoni, E.;[d] Mihalic, F.;[b] Naldi, M.;[a,c] Bertucci, C.;[a] U. Danielson, U. H.;[b,e] Bartolini, M.;[a] Del Rio, A.[d]

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SET and MYND domain-containing protein 3 (SMYD3) is a lysine methyltransferase that plays a central role in a wide variety of cancer diseases, exerting its pro-oncogenic activity by methylation of key substrates, both nuclear and cytoplasmic.[1] However, the precise pathophysiology of SMYD3 in the insurgence and progression of cancer is not yet fully understood.[2]

In this context, availability of specific chemical probes is of key importance for unveiling the biochemistry of SMYD3 and ease the development of new drugs for cancer treatment. Hence, to address these issues, we developed a combination of biophysic and biochemical assays to investigate SMYD3 biology and identify new chemical tools. Specifically, a novel surface plasmon resonance (SPR)-based assay was used to characterize interactions between peptide ligands and immobilized SMYD3 while a tailored liquid chromatography-mass spectrometry (LC-MS)-assay was employed to specifically monitor SMYD3 methyltransferase activity. SPR binding-assay results showed a complex biochemistry for the enzyme, which is characterized by broad substrate specificity at the histone site. Interestingly, the enzymatic assay revealed only mitogen activated kinase kinase kinase 2 (MAP3K2)-mimicking peptide was efficiently methylated. Combination of binding analyses and activity assays revealed a random bi-bi mechanistic model for SMYD3 methylase activity. To identify new chemical tools and dissect SMYD3 cellular roles in carcinogenesis, the developed assays were employed to characterize a small library of compounds from which a new chiral binder emerged. Racemate and enantiomers, named EH05, (S)-EH05 and (R)-EH05, were characterized in terms of binding affinity and inhibitory capacity. Interestingly, the X-ray analysis of SMYD3 in complex with (S)-EH05 revealed a peculiar binding mode in which (S)-EH05 binds at SMYD3 C-terminal domain and occupies a surface involved in the interaction with the molecular chaperone HSP90. This suggests (S)-EH05 could possibly interfere with SMYD3 localization within the cell. Set of assays developed in this study constitutes a multi-methodological platform to identify and characterize new inhibitors of SMYD3 enzyme; the identified tool compound with a novel binding mode can contribute to further unveil SMYD3 biology and provide insight into alternative inhibition strategies.

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SUBSTRATE PREFERENCE OF THE LYSINE METHYLTRANSFERASES SMYD3 REVEALED THROUGH A COMBINATION OF BIOPHYSICS AND BIOCHEMICAL APPROACHES

Fabini, E.;[a,b] Talibov, V. O.;[c] Manoni, E.;[b] Mihalic, F.;[c] Naldi, M.;[a,d] Bertucci, C.;[a] U. Danielson, U. H.;[c,e] Bartolini, M.;[a] Del Rio, A.[b]

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Cancer is arguably one of the most intricate diseases the healthcare community is dealing with and chemical entities struggle to reach the market because of poor efficacy in *in vivo* tests.[1] Translatability in drug discovery heavily depends on the collection of reliable data during *in vitro* analyses so that lead compounds can have a higher chance to advance pre-clinical phases towards clinical trials. In this context, having access to robust methods for target characterization is a critical step.[2] Epigenetic enzymes, which are responsible for the spatial-temporal regulation of gene expression, have become key targets for the oncotherapy.[3] Among epigenetic enzymes SET and MYND domain-containing protein 3 (SMYD3) is a lysine methyltransferase whose aberrant activity has been linked to several type of cancer thus emerging as attractive target for oncotherapy[3]. However, its role in the cancer disease is not fully understood.[3]

In the present work we aimed at investigating features of interest underlying the complex *in vitro* biology of SMYD3. The interactions of SMYD3 with peptides mimicking its putative substrates were studied via a direct kinetic binding assay using surface plasmon resonance (SPR)-based technology. Amino acid sequences were selected to comprise reported binding motif of known SMYD3 interactants, i.e. mitogen-activated protein kinase kinase kinase (MAP3K2), vesicular endothelial growth factor-1 (VEGFR) and histones H3 and H4, and to varying in length and methylation of target lysine in order to establish minimum requirements for binding. Interactions with co-factor S-adenosyl-methionine (SAM) and reaction by-product S-adenosyl-cystein (SAH) was also probed and revealed striking differences, with SAM forming a very stable and slowly dissociating complex with the enzyme. The methylation activity of the enzyme on the same peptide selected for the binding assay was investigated through a liquid chromatography (LC)-mass spectrometry (MS)-based assay. Interestingly, despite multiple substrates could be specifically recognized by SMYD3 in the kinetic interaction analysis, methylation marks were detected only on the 26 a.a. MAP3K2 peptide and at limited extent on 26 a.a. VEGFR-1 peptide. Results showed that SMYD3 has low affinity for peptide substrates and can recognize multiple binding motifs, although only some can yield catalytically-productive binding. The use of two orthogonal techniques increases reliability of the study as well as allowed to highlight complementary aspects of the enzyme behavior towards substrates. Combination of data acquired in the kinetic interaction analysis with data obtained in the biochemical assay a suggested a random bi-bi mechanism for SMYD3 catalytic cycle. Overall, results obtained in this study can be seen as a general methods to investigate epigenetic enzymes and can constitute a starting point for the development of novel chemical tools to probe inhibition of SMYD3.

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DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF DUAL INHIBITORS OF HISTONE DEACETYLASES (HDAC) AND METHYLTRANSFERASE (EZH2)

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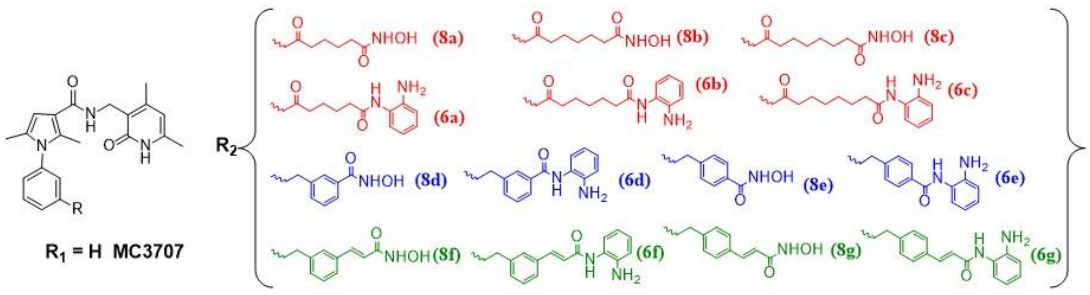
The development of polypharmacology therapies, able to modulate simultaneously multiple targets involved in the onset of the pathology, is considered an attractive approach to treat multifactorial diseases such as cancer.[1] On the basis of these evidences we have designed and synthesized potential dual inhibitors of HDACs (classes I, II) and EZH2, important epigenetic targets overexpressed in cancer and contributing to its initiation and progression. Classes I and II HDACs are Zn^{2+}-dependent histone deacetylases, while EZH2, the catalytic subunit of PRC2 complex, is a SAM-dependent histone methyltransferase. Specifically, it catalyzes the H3K27 mono-, di- and tri- methylation. For the development of the before mentioned dual inhibitors we combined the well-known HDACi moieties shown in approved drugs[2] to the already optimized EZH2i scaffold MC3707. As suggested by previous investigation, the most favourable substitution on phenyl ring bound to the N1-pyrrole was the *meta* one. Thus, we designed to link the HDAC targeting element in that position. According to the HDACi pharmacophoric model, we have chosen different type of spacer: the aliphatic one (vorinostat), the benzoic one (entinostat) and the cinnamic one (panobinostat and belinostat). As zinc binding group we used in turn a hydroxamic acid or an *orto*-amino anilide. In biochemical screening all the compounds showed a comparable inhibition potency against EZH2 (2 µM > IC_{50} < 7 µM), while the inhibition activity on HDAC isoforms 1-6 and 8 proved an interesting isoform selectivity. In particular, the hydroxamic acids **8b** and **8c** showed a strong selectivity and single digit nanomolar potency for HDAC6 (IC_{50} = 3.7 nM and IC_{50} = 5.4 nM, respectively) while still able to inhibit EZH2 (IC_{50} = 4.57 µM and IC_{50} = 7.37 µM, respectively). Among the *orto*-amino anilides, the most potent compound **6e** displayed an IC_{50} of 83 nM against HDAC1, whereas compound **6a** provided the best inhibition value towards HDAC3 (IC_{50} = 0.925 µM). Moreover, compound **8c**, once tested in U937 AML cells, showed a dose-dependent α-tubulin hyperacetylation, a decrease of H3K27 tri-methylation and impaired dose- and time-dependent cell vitality.



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DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL SIRT3 ACTIVATORS

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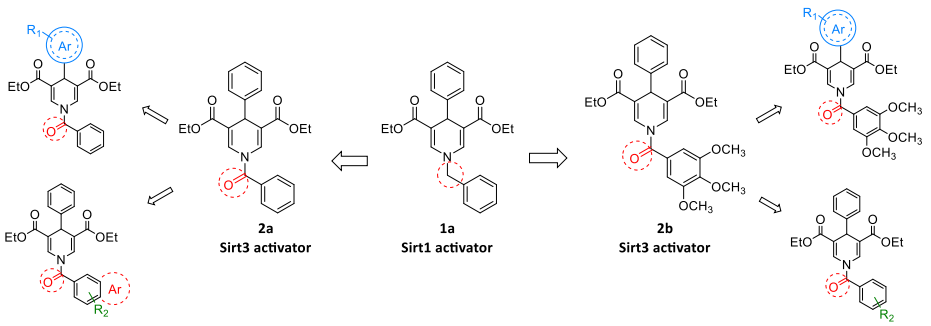
Sirtuins are NAD^+-dependent protein deacetylases involved in metabolic regulation and aging. Among the mitochondrial sirtuins, SIRT3 is the only one exhibiting robust deacetylase activity. It regulates a wide range of targets, from signaling to metabolic proteins. SIRT3 is implicated in responses to exercise or nutritional regime, in aging-related disorders (e.g. type II diabetes, cardiovascular diseases) and in caloric restriction effects. Based on these evidences, the development of SIRT3 activators is considered a promising strategy to prevent/ameliorate many diseases and health deficiencies related to aging.[1] In a previous study, among a panel of 1,4-dihydropyridine(DHP)-based compounds, we identified selective SIRT1 activators like compound **1a** (**Fig.1**), as well as non-specific SIRT1-3 activators.[2] We hypothesized that by chemical manipulation on this scaffold we could also obtain SIRT3-specific activators. Thus, we synthesized a novel set of compounds based on **1a**. Once tested in biochemical assays, the novel derivatives bearing an acyl function at the N1-position of the DHP scaffold (**2a** and **2b** in **Fig.1**) displayed selective activation of SIRT3 over SIRT1, 2, and 5, being respectively 3.5 and 5 folds more potent than **1a**. The moderate potencies of **2a** and **2b**, prompted us to seek for more potent derivatives. We generated new series of compounds by varying their "top" or "bottom" with other substituents at N1 or C4 position of the DHP scaffold, respectively. According to the enzymatic assay results, in addition to compounds **2a** and **2b**, **3a** (3,4-dimethoxy-benzoyl derivative), **3b** (3,5-dimethoxy-benzoyl derivative) and **3c** (3-methoxy-benzoyl derivative) showed the strongest SIRT3 selectivity at 10 and 100 µM. Once tested in MDA-MB-231 breast cancer cells, **2a** and the methoxy compounds **2b**, **3a**, **3b** and **3c** activated GDH, a SIRT3 substrate.



Additionally, we also analyzed the deacetylation of MnSOD, another established SIRT3 substrate, in MDA-MB-231 cells. SIRT3 knock-out, as expected, caused an increase in MnSOD-Lys68 acetylation, whereas treatment with **2a** at 50 µM resulted in an increase in MnSOD-Lys68 deacetylation. Thus, we concluded that our DHP-based compounds act as SIRT3 activators in cellular system.
Additionally, in HBV-infected cells **2a** led to histone H3 and/or H4 hypoacetylation and reduction of the transcription from viral cccDNA template, accompanied by reduction in HBV replication. Overall, these results indicate that the novel and selective SIRT3 activator **2a** modulates the acetylation status of SIRT3 substrates in MDA-MB-231 cancer cells and of cccDNA-bound H3/H4 histones. Our findings provide a novel tool to study SIRT3 functions in cancer and in HBV infection, possibly leading to novel and alternative therapeutic strategies. Moreover, further changes of the DHP-based scaffold could provide in the future the identification of novel selective activators of the other Sirtuins.

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NOVEL CURCUMIN ANALOGUES AS INHIBITORS OF AMYLOID β-INDUCED NEUROINFLAMMATION IN ALZHEIMER'S DISEASE

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Alzheimer's Disease (AD) is a multifactorial neurodegenerative disorder, mainly characterized by an abnormal deposition of protein aggregates and by the presence of activated microglia and astrocytes surrounding Aβ plaques in the brain. Many unanswered questions still remain regarding the inflammatory component of AD, including the dual nature of microglia activation (i.e., beneficial *vs* detrimental) and the role exerted in this context by different Aβ oligomeric populations. The identification of novel pharmacological strategies able to control microglia activation is currently a research area of great interest in the treatment of inflammatory conditions in AD. Based on these considerations, in this work we optimized conditions for the preparation, analytical separation and isolation of two different populations of Aβ42 oligomers namely monomers-dimers (LMW) and aggregates larger than dodecamers (HMW),[1] to test their ability to stimulate microglia inflammatory response in the absence or presence of curcumin and curcumin analogues. Curcumin is a well known natural compound that participates in a wide range of AD-related pathways, including neuroinflammation. We recently showed that curcumin and some analogues attenuate lipopolysaccharide-induced microglia activation in *in vitro* and *in vivo* models;[2,3] moreover, curcumin and an analogue characterized by a prenyloxy moiety on one aryl ring (CUR6) exhibited an inhibitory activity on Aβ oligomerization and fibril deposition.[4] Thus, evaluation of the effect on Aβ-induced microglia activation was here carried out on curcumin, CUR 6 and a brominated curcumin analogue (CUR 16). The last derivative is likely to be more chemically stable and to be endowed with an improved BBE permeability than curcumin. To this end, microglial cells were stimulated for 6, 24 and 48 h with increasing concentrations (5-20 μM) of Aβ1-42 oligomers (LMW, HMW, or Aβ1-42 *in toto*). Supernatants and cell lysates were collected and subjected to ELISA to measure the concentration of the pro-inflammatory cytokines TNF-α and IL-1β. The release of TNF-α and IL-1β and the intracellular concentration of IL-1β were increased, in a similar manner, in response to stimulation with HMW oligomers and Aβ1-42 *in toto*, starting from 24 h. In contrast, LMW Aβ1-42 oligomers failed to induce an inflammatory response in microglial cells. Next, the anti-inflammatory effect of curcumin, CUR6 and CUR16 was examined. Microglia were exposed for 1 h to concentrations of the three compounds not affecting cell viability (1-10 μM) and then stimulated with 10 μM HMW Aβ1-42 oligomers for 24 h, so to induce an inflammatory response. The release of TNF-α and IL-1β and the intracellular concentration of IL-1β induced by Aβ1-42 oligomers were significantly suppressed by curcumin, CUR6 and CUR16.

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DISCOVERY OF THE FIRST IN CLASS GSK-3β/HDAC DUAL INHIBITOR AS DISEASE MODYFING AGENT TO COMBAT ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is the most common cause of dementia and a cure for this pathological condition is not available so far. The complexity of AD suggests that it could be better contrasted by a chemical entity able to simultaneously modulate multiple targets involved in the onset of the disease.[1] Several evidences point out the role of epigenetics in AD[2] and the connections between epigenetic and "classical" AD targets. Due to the reported connection among histone deacetylases (HDACs) and glycogen synthase kinase 3β (GSK-3β),[3-6] we designed and developed the first-in-class hit compound able to exert promising anti-AD effects by modulating epigenetic and tau-related targets, such as HDAC1, HDAC6, and GSK-3β in the low micromolar range of concentration.[7] Compound **11** increases histone acetylation and causes a reduction of tau phosphorylation. It is nontoxic and protective against H_2O_2 and 6-OHDA stimuli in SH-SY5Y and in CGN cell lines, respectively. Moreover, it promotes neurogenesis and displays immunomodulatory effects. Compound **11** shows no lethality in a wt-zebrafish model (<100 μM) and high water solubility.

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NEW NON-COVALENT INHIBITORS OF NLRP3 INFLAMMASOME ATPASE ACTIVITY

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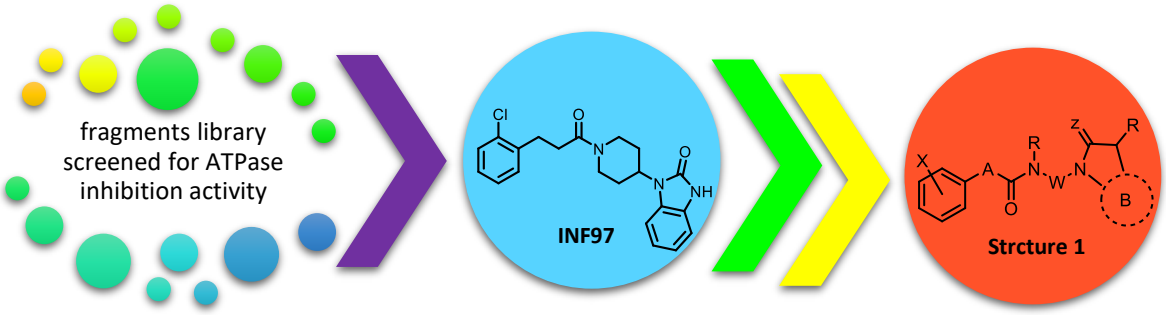
NLRP3 inflammasome is a multiprotein complex playing a key role in the intracellular activation of the innate immune system through activating cleavage of pro-inflammatory interleukins (IL)-1β, IL-18 and triggering of pyroptotic cell death. It plays a protective response involving immune cells and molecular mediators.[1] However, chronic inflammation leads to cell damage, cell death, and progressive loss of tissue functions. Immune system-mediated chronic inflammation is involved in the onset and/or progression of different pathologies such as autoinflammatory diseases (e.g. cryopirinopathies), autoimmune diseases (e.g. Inflammatory Bowel Disease, rheumatoid arthritis), neurodegenerative diseases (e.g. Alzheimer's disease, Parkinson's disease, Multiple sclerosis).[2]

All the different NLRP proteins possess a NACHT domain mediating ATP-binding and hydrolysis. Only NLRP3 and few others inflammasome possess ATP-binding potential and intrinsic ATPase activity. Mutations in these ATP-binding regions abolished their ATP-binding and ATPase activities and thereby resulted in impaired IL-1β maturation.

From a screening of different molecular fragments on recombinant human (rh)NLRP3 protein, a benzo[d]imidazol-1-one sub-moiety was identified as a weak inhibitor of ATPase activity (Scheme 1). Interestingly, this moiety was recently confirmed to compete against ATP for the binding of the NLRP3 protein.[3] In this project, the fragment has been used as a starting point for further chemical modulation. The fragment was functionalized using other structural motifs present in derivative **INF39**,[4] previously identified as able to bind to NLRP3 and to hamper ATPase activity. Through this study, a series of compounds able to inhibit NLRP3 ATPase was obtained, among them a new non-covalent hit compound (**INF97**), has been identified and characterized. Modulation of selected molecular moieties (*Structure 1*: A; B; X; W; Z; R1, R2) was performed using classical medicinal chemistry techniques such as bioisosteric replacement and refining of conformational flexibility. **INF97** elicits an interesting concentration-dependent ATPase inhibition (IC_{50}: 17.2 µM, 15.4 – 19.2 C.L. 95%) and inhibits LPS/ATP triggered pyroptosis.



Scheme 1.

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INCAPSULATED NO-GEMCITABINE: GASEOUS SOLUTION FOR MDR PANCREATIC CANCER

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Since the late 1990's, gemcitabine (GEM) has been available as the standard of care regimen for advanced pancreatic ductal adenocarcinoma (PDAC). However, the resistance to chemotherapy treatment is the main issue against a successful anti-cancer therapy.[1] Nitric oxide (NO) is a small free radical molecule involved in many physiologic and pathologic pathways in human cells. It plays also a key role in tumor biology and at micromolar concentrations it displays antitumor effects.[2,3] In our previous study we have successfully used a multitarget approach, combining antitumor drug doxorubicin with NO donor moieties, overcoming MDR in the colon and breast cancer cells.[4,5]

In this study, we synthesized a small library of seven NO-releasing gemcitabine conjugates (NO-GEMs) in order to improve the effectiveness of GEM in PDAC cell lines (**Figure 1**). Among the new NO-GEM multitarget compound GCB2 was identified as the most effective compound in inhibiting cancer cell growth. To improve its uptake and minimize side effects we encapsulated it in liposomes (LIPO-GCB2), increasing its antiproliferative and apoptotic effect, especially in GEM-resistant Panc1 cells, as compared to standard GEM. We observed that GCB2 and LIPO-GCB2 were able to nitrate tyrosine residues of the multidrug resistant protein MRP5, which is involved in the extrusion of GEM, favoring the intracellular accumulation of GEM and triggering apoptotic cell death in GEM-resistant PDAC cells. Our results support the development of a new antitumor strategy against GEM-resistant PDAC cells based on the usage of NO-releasing GEM encapsulated in liposomes.

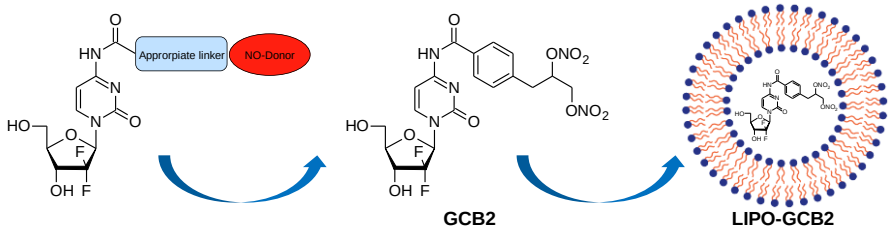

Figure 1. NO-GEM, GCB2 and LIPO-GCB2.

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DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF 6-SUBSTITUTED THIENO[3,2-*d*] PYRIMIDINE ANALOGUES AS DUAL EPIDERMAL GROWTH FACTOR RECEPTOR KINASE AND MICROTUBULE INHIBITORS

Oliva, P.;[a] Romagnoli, R.;[a] Kimatrai Salvador, M.;[b] Lopez-Cara, L. C.;[b] Brancale, A.;[c] Hamel, E.;[d] Viola, G.[e]

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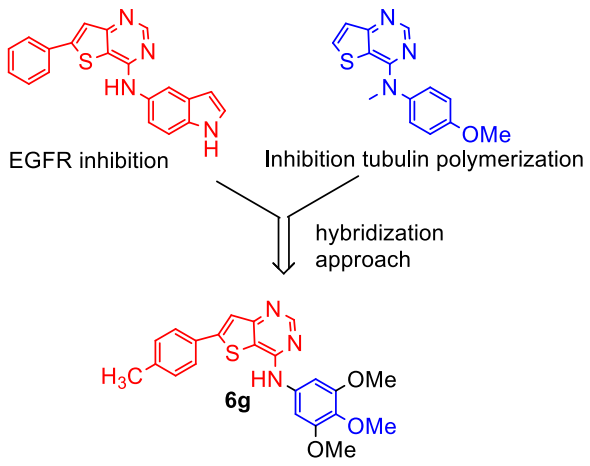
The clinical evidence for the success of tyrosine kinase inhibitors in combination with microtubule targeting agents prompted us to design and to develop single agents which possess both epidermal growth factor receptor (EGFR) kinase and tubulin polymerization inhibitory properties.[1,2] A series of 6-aryl/heteroaryl-4-(3',4',5'-trimethoxyanilino) thieno[3,2-d] pyrimidine derivatives were discovered as novel dual tubulin polymerization and EGFR kinase inhibitors. The 4-(3',4',5'-trimethoxyanilino)-6-(*p*-tolyl)thieno [3,2-*d*]pyrimidine derivative **6g** was the most potent compound of the series as an antiproliferative agent, with IC_{50} values in the single- or double-digit nanomolar range. Compound **6g** bound to tubulin in the colchicine site and inhibited tubulin assembly with an IC_{50} of 0.71 μM, and **6g** inhibited EGFR activity with an IC_{50} of 30 nM. Our data suggested that the excellent *in vitro* and *in vivo* profile of **6g** may be derived from its dual inhibition of tubulin polymerization and EGFR kinase.



IC_{50}: 1-19 nM as antiproliferative agent
IC_{50}: 0.7 μM as inhibitor of tubulin polymerization
IC_{50}: 30 nM EGFR kinase inhibition activity

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RATIONAL DESIGN, SYNTHESIS AND CHARACTERIZATION OF NEW SPIROTHIAZAHETEROCYCLES WITH POTENTIAL ACTIVITY ON CENTRAL NERVOUS SYSTEM

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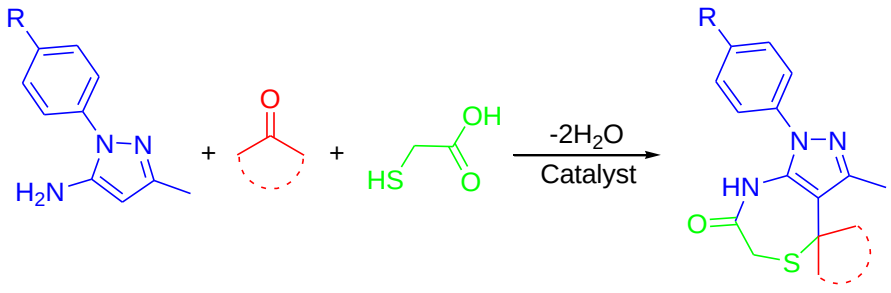
Drugs designed to treat disorders in central nervous system (CNS) have been questioned because patients consuming this type of products often suffer from adverse reactions or side effects, which is why in recent decades, attempts have been made to create molecules sorting such undesirable effects.[1-3] In this work, an articulation of organic chemistry, computer-aided drug design tools and fundamentals of physiology of CNS (GABAergic function, specifically) was employed to perform the modeling, synthesis and characterization of compounds with potential activity on $GABA_A$ receptors.

As part of the contribution to the health sciences and taking advantage of the existing computational tools, we designed and worked on the synthesis of hybrid molecules with pyrazole and thiazepinone privileged scaffolds and a spirofused carbocyclic rings. *Thiazepinone* moiety could be considered a structural analogue of the recognized *diazepine* core already used in several drugs, but this last pharmacophore unit has exhibited undesirable effects, leading to the development of novel drugs with better selectivity.

This work started with the generation of a virtual library of compounds, all with the spiropyrazolo[3,4-*e*][1,4]thiazepinone nucleus, which were prefiltered by determining their ADMET properties, followed by a study of molecular docking using a refined human $GABA_A$ receptor. Virtual screening of the aforementioned compounds was carried out using *clonazepam* as reference drug and the selected compounds were synthesized and fully characterized by IR, NMR, melting point and HRMS.



Scheme 1. Synthesis of the selected spiropyrazolo [3,4-*e*][1,4]thiazepinone molecular hybrids.

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PIPERAZINE AND 4-AMINOPIPERIDINE DERIVATIVES AS POTENT HUMAN CARBONIC ANHYDRASE INHIBITORS

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Carbonic Anhydrase (CA) is a metallo-enzyme widely expressed in nature. Seven genetically-different families have been found; in humans, sixteen different isoforms belonging to the α family have been characterized, differing for cellular and tissue distribution, and catalytic activity. The reversible hydration of CO_2 is the most important reaction catalyzed by these enzymes, affecting several important physiological processes such as pH and electrolyte balance; an abnormal activity often leads to pathological effects.[1]
CA inhibitors have been used in the clinic for more than 50 years; their main therapeutic uses are as diuretics, antiglaucoma and antiepileptic agents, although their use may be limited by side effects due to poor isoform selectivity.[2]

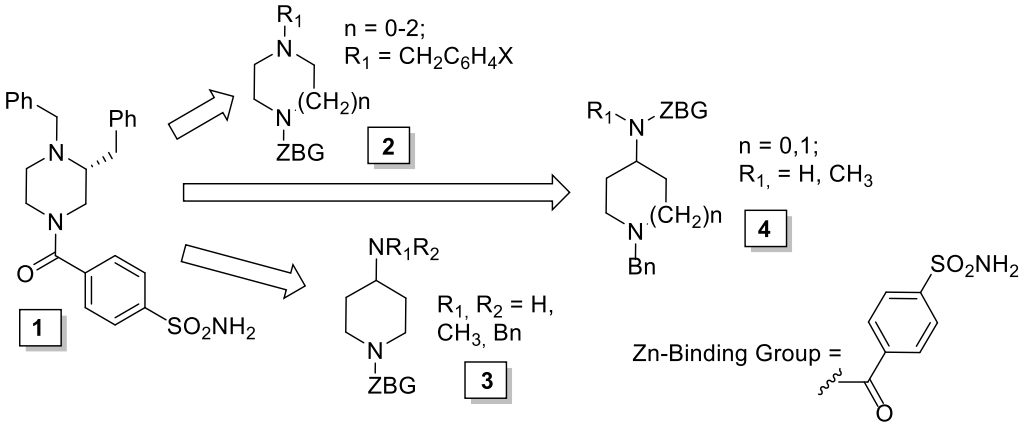

Figure 1. Structure of the lead and the designed compounds

We have recently reported a series of 2- or 3-benzylpiperazines (exemplified by **1**, Figure 1) endowed with selectivity for human CA IV.[3] In order to derive further structure-activity relationships useful for designing isoform selective compounds, the structure of the lead compound **1** was simplified into a series of piperazines (**2**) and 4-aminopiperidines (**3, 4**) carrying a 4-sulfamoylbenzamide moiety as Zn-binding group. The new compounds have been tested on human hCA I, II, IV and IX isoforms using a stopped flow CO2 hydrase assay. The structural modifications changed the selectivity profile of the analogues from hCA IV to hCA I and II, and improved potency. In fact, several new compounds showed subnanomolar activity on hCA II. X-ray crystallography of ligand-hCA II complexes was used to compare the binding modes of the new piperazines and the previously synthesized 2-benzyl-piperazine analogues.

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DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF STEREO- AND REGIOISOMERS OF AMINO ARYL ESTERS AS MULTIDRUG RESISTANCE (MDR) REVERSERS

Braconi, L.;[a] Contino, M.;[b] Riganti, C.;[c] Bartolucci, G.;[a] Manetti, D.;[a] Romanelli, M.N.;[a] Trezza, A.;[d] Athanasios, A.;[d] Spiga, O.;[d] Perrone, M. G.;[b] Giampietro, R.;[b] Gazzano, E.;[c] Colabufo, N. A.;[b] Dei, S.;[a] Teodori, E.[a]

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The anticancer chemotherapy is often impaired by the resistance that cancer cells develop to cytotoxic drugs after an exposure period. This resistance is often due to an enhanced energy dependent drug efflux caused by the overexpression of transmembrane proteins belonging to the ATP-Binding Cassette (ABC) transporter family. These proteins perform an ATP-dependent active outward transport of a variety of structurally and mechanistically unrelated chemotherapeutic drugs resulting in the so-called multidrug resistance (MDR).[1] A variety of compounds able to modulate the efflux activity of these proteins, named chemosensitizers, have been identified and studied in order to reverse MDR.[2]

As a continuation of our research on MDR modulators with the aim of developing potent and selective P-gp ligands able to counteract MDR,[3,4] in the present study we explored the consequence of introduction of a methyl group on the position 2 of a three methylenes chain giving origin to a stereogenic center. The two possible enantiomers, (*R*) and (*S*), could show different biological activity. For this purpose, we synthesized a new series of compounds with a 2-(methyl)propyl chain combined with a 3-, 5- or 7-methylenes long chain. All the possible stereo- and regioisomers were obtained (Chart 1).

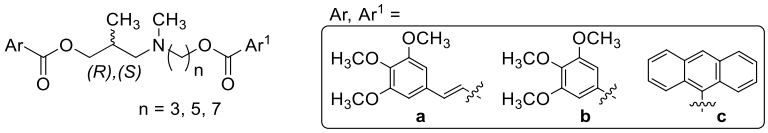

Chart 1. General structure of designed compounds.

The P-gp modulating ability of these compounds was evaluated by the pirarubicin uptake assay on K562/DOX doxorubicin resistant cells that overexpress only the membrane glycoprotein P-gp. Docking simulations were employed to elucidate the binding mode of these compounds in the P-gp pocket site. Their P-gp interaction profile and selectivity towards other ABC transporters, such as MPR1 and BCRP, was evaluated on MDCK transfected cells overexpressing the three transporters. Moreover, these compounds have been tested for their ability to induce collateral sensitivity (CS) against P-gp or MRP1. The stability of this series of molecules towards spontaneous or enzymatic hydrolysis was also investigated.

The promising preliminary results of the new molecules will be reported and discussed.

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REPOSITIONING OF DANTROLENE FOR ALZHEIMER'S DISEASE: NEW AND OLD MULTITARGET ACTIVITIES TOWARDS AD-RELATED TARGETS

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Dantrolene is a life-saving drug used for the treatment of malignant hyperthermia, a pharmacogenetic disease triggered by many anesthetics used in clinical surgery. It acts by inhibiting ryanodine receptors (RyRs), which are responsible for calcium mobilization in striatal muscles and brain. This feature has suggested a possible use of dantrolene in AD, as demonstrated by several reports in animal models of the disease.[1] Recently, we presented previously undisclosed activities of dantrolene (Figure 1), namely the inhibition of monoamine oxidase B (MAO B), acetylcholinesterase (AChE), and beta amyloid (Aβ) aggregation, and the activation of the carrier of L-acylcarnitine (CACT), as concomitant biological features responsible for neuroprotection.[2] To overcome the low aqueous solubility of dantrolene, either as free base or sodium salt, we also dealt with the synthesis of selected structural congeners, with the aim of confirming their multitarget profile while improving the solubility. The newly synthesized compounds retained good AChE/MAO B inhibitory activity, with moderate to good activation of CACT. Results from ongoing studies will be presented and discussed.

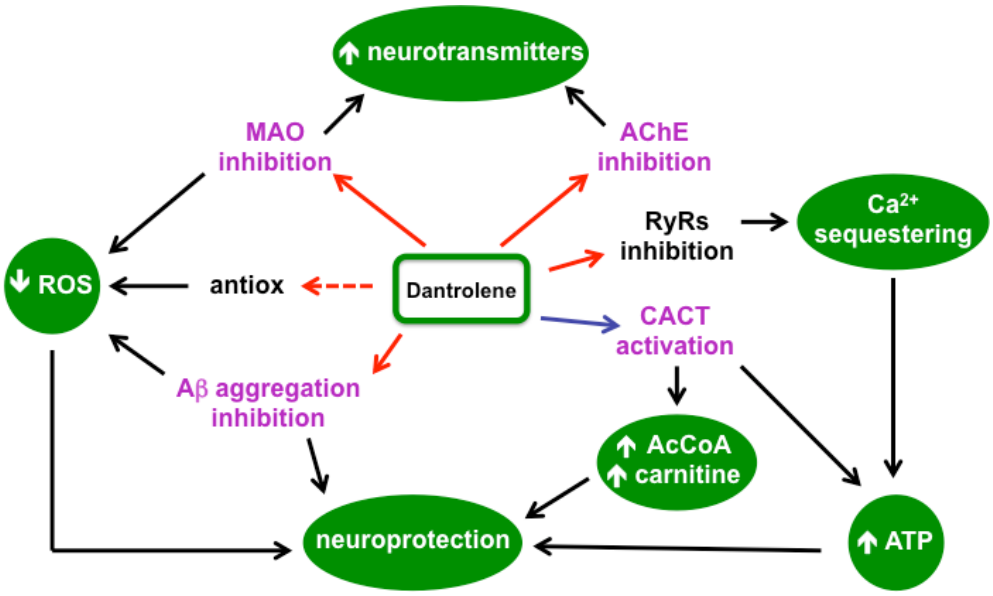

Figure 1. Multitarget AD-related activities of dantrolene.

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DESIGN, SYNTHESIS AND IN VITRO EVALUATION OF BIVALENT CHEMICAL PROBES TARGETING THE TANDEM BD1/BD2 IN BROMODOMAIN AND EXTRA TERMINAL DOMAIN (BET) PROTEINS

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The Bromo and Extra-Terminal Domain (BET) family of proteins is characterized by the presence of two tandem bromodomains: BD1 and BD2. Through their bromodomains, members of this family (BRD2, BRD3, BRD4 and BRDT) specifically recognize the acetyl-lysine residues of histones and they are involved in numerous disease states.[1] Actually, the role in chromatin remodeling and transcriptional regulation correlate these proteins to several disease states such cancer, inflammation, and viral infection, making them an excellent therapeutic target. Several reports suggested that the two bromodomains have different functions, but their high homology limited an efficient drug design process for the development of selective inhibitors.

As a result, most of the known inhibitors bind indistinctly on both domains and the need of a simultaneous or a selective inhibition has not been elucidated.[2,3] With the aim to finally clarify this point, we hypothesized to merge two known inhibitors: the RVX-208, a selective inhibitor of BD2 domain and a triazolobenzotriazepine-based compound, an inhibitor of BD1 domain.

Here we describe the design, synthesis and preliminar biochemical evaluation of a new class of bivalent chemical probes able to bind BD1 and BD2 bromodomains simultaneously (Figure 1).

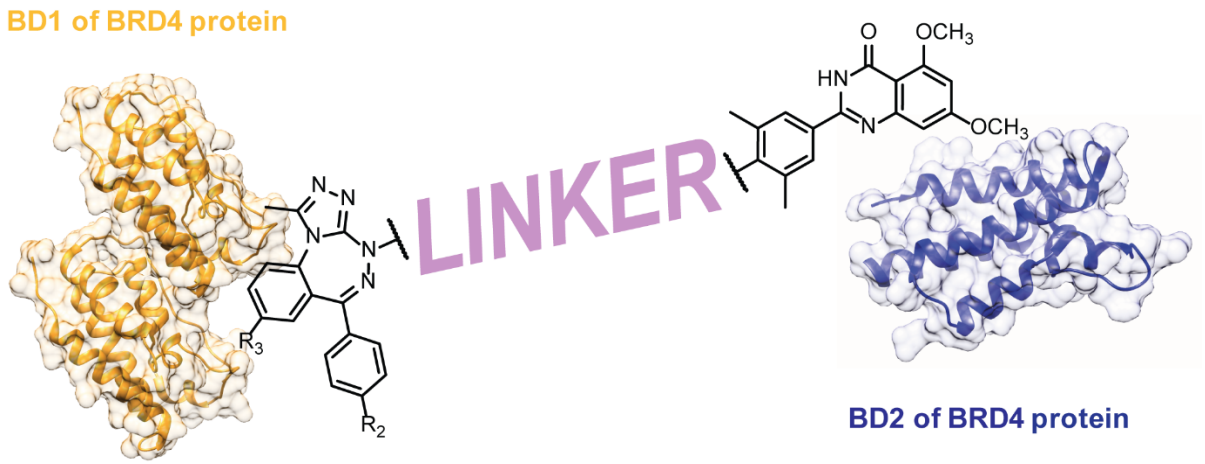

Figure 1. Bivalent chemical probe.

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IDENTIFICATION OF NOVEL SMALL-MOLECULE LIGANDS OF METHYL-LYSINE BINDING PROTEIN PHF20

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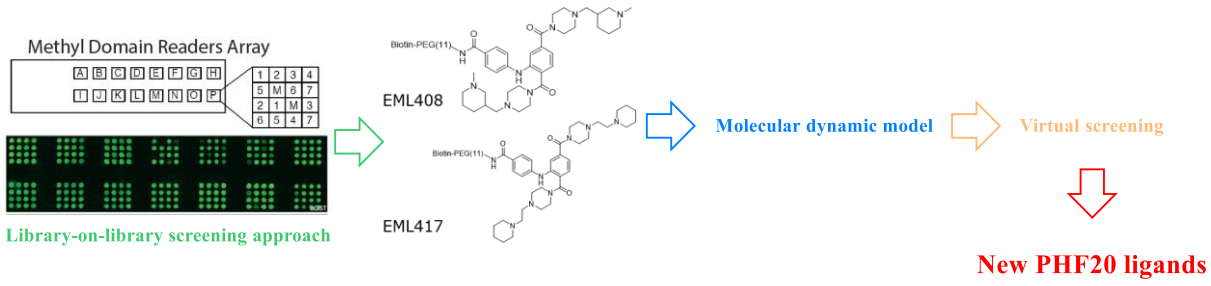
Methylation of histone tails represents a crucial post-translational modification involved in gene regulation. Recognition of these methyl marks has been attributed to the "Royal Family" of proteins, including the Tudor domain subfamily. If the ability of these enzymes in binding lysine-methylated protein substrate has been well documented,[1,2] much remains to be elucidated with regard to precise mechanisms by which such interactions influence the processes of transcription, translation and RNA splicing. Among the "readers", the Plant Homeodomain Finger protein 20 (PHF20) is a transcription factor, which was originally identified in glioma patients.[3] While little is known about its cognate cellular role, PHF20 is prevalent in hepatocellular tumors of stage I[4] and is also abundantly expressed in both advanced small-cell lung cancer and adenocarcinoma, indicating that PHF20 might be tumor-associated antigen and could play a role in cancer progression.

Starting from a 'library-on-library' screening approach, compounds that selectively bound the Tudor domains of PHF20 were identified (EML408 and EML417, Figure 1). A molecular dynamic model was also used to understand the right length to allow optimal interactions of the new ligands with the two cages of PHF20 dimer.

Prompted by our interest in the discovery of small molecule modulators of epigenetic targets, after structural optimization as well as virtual screening studies, here we report the identification of a series of inhibitors of PHF20, that might represent new opportunities to investigate the role of this protein in chromatin biology and drug discovery.



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FINE-TUNING OF STORE-OPERATED CALCIUM ENTRY BY PYRTRIAZOLES AS POTENTIAL TREATMENT FOR CALCIUM-RELATED RARE DISEASES

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The term Store-Operated Calcium Entry (SOCE) is referred to the ability of cells to sense a decrease in endoplasmic reticulum luminal calcium and to induce calcium entry across the plasma membrane. The main components of SOCE machinery are Orai, a family of calcium channels on the plasma membrane, and STIM, the proteins that act as calcium sensors on endoplasmic reticulum. Calcium represents a ubiquitous intracellular second messenger and the fine-tuning of Ca^{2+}-signals to generate highly specific response is heavily involved in numerous physiological processes.[1] On the other hand, calcium dys-regulation is the underlying cause of a vast array of pathological conditions, such as asthma, allergic disorders, cancer, acute pancreatitis and rare genetic diseases.[2]

Starting from already reported compounds known as Pyrs,[1] a novel class of SOCE modulators has been synthesized using click chemistry approach. Among this family of compounds, named Pyrtriazoles, one potent inhibitor **1S** and two positive modulators, that are the first non-borinate SOCE enhancers, have been discovered. Compound **1S**, however, is affected by a poor hydrolytic stability due to the ethyl ester portion that was broadly modified within our SAR study. This effort led to the identification of two promising compounds, **31** and **39**, that display inhibitory activity at the low micromolar level, no cytotoxicity up to 100 micromolar, improved plasma stability compared to **1S** and excellent selectivity profiles (Figure 1).[3] Compound **31** has proven its efficacy in *ex vivo* studies on muscle biopsies of patients affected by tubular aggregate myopathy (TAM), a rare genetic diseases caused by SOCE loss-of-function mutations. Compound **39** is well tolerated as a single intraperitoneal administration in mice and has shown *in vivo* efficacy in a mouse model of acute pancreatitis. Both pathological conditions are currently without pharmacological treatments.

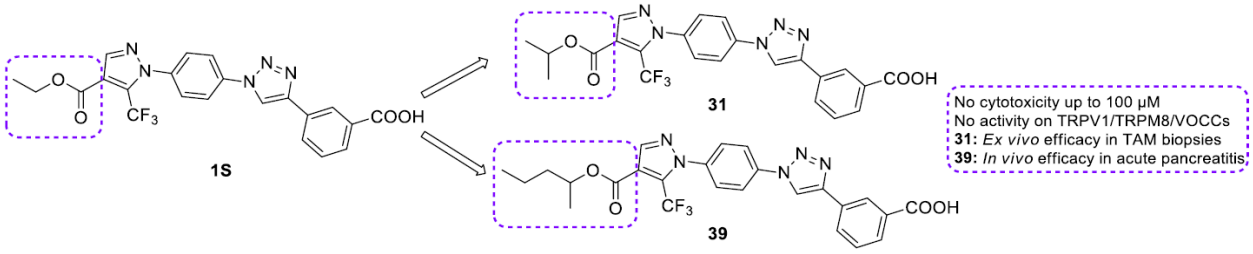

Figure 1

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DESIGN AND SYNTHESIS OF POTENTIAL ALLOSTERIC MODULATORS OF AROMATASE FOR TREATMENT OF BREAST CANCER

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Breast cancer is the most common cancer among women and approximately 75% of patients are hormone receptor (ER/PR) positive, so that after surgery they can be treated with hormonal therapy alone, in the absence of more toxic chemotherapy, with a relatively favorable prognosis. Different approaches can be used for targeted therapy of breast cancer: interference with binding of estrogens to their receptor *via* selective estrogen receptor modulators (SERMs), such as tamoxifen, and selective estrogen receptor degraders (SERDs), such as fulvestrant, or inhibition of the synthesis of estrogens *via* inhibition of CYP450 aromatase, key enzyme of their biosynthetic process.

Recently, some metabolites of tamoxifen were found to act as aromatase inhibitors (AIs).[1] In particular, endoxifen showed non-competitive inhibition and this opened the way for the hypothesis that an allosteric mechanism might contribute to the pharmacologic regulation of aromatase and could thus be exploited to modulate its activity for therapeutic benefit. In addition, a non-competitive or mixed inhibition mechanism was postulated for the marketed AI letrozole.[2]

Using computational methods, three potential allosteric pockets were then identified,[3] two of them being of particular interest: site 1, lying along one of the possible substrate access channels to the catalytic site and site 2, (heme-proximal cavity) corresponding to the CPR binding site, thought to be involved in the electron transfer from CPR to the heme group.

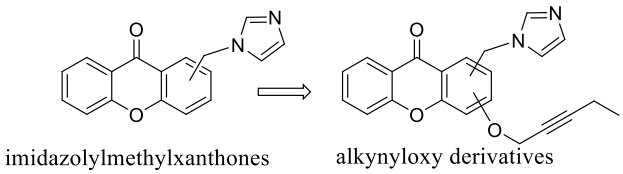

Figure 1. Design strategy.

In view of these findings, and starting from a series of imidazolylmethylxanthones previously reported as heme-coordinating non-steroidal AIs,[4] some new derivatives were designed (Figure 1) by inserting a long alkynyloxy side chain, in an attempt to reach one of the pinpointed binding pockets obtaining non-competitive or mixed inhibition of the enzyme and, on the other hand, to deeper investigate the possibility for allosteric modulation of aromatase.

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NEW INHIBITORS OF THE INDUCIBLE NITRIC OXIDE SYNTHASE AS ANTICANCER AGENTS

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High levels of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are highly involved in the malignancy of different cancers, as well as in chemoresistance, due to the activation of several signaling mediators. In this contest, the dysregulated production of the free radical Nitric Oxide (NO) by inducible Nitric Oxide Synthase (iNOS) plays a recognized role, and NO inhibition can be considered an emerging therapeutic possibility to treat these diseases.[1]

From the development of the acetamidine 1400W by GlaxoSmithKline, recently we have identified a new small potent acetamidine proline-derived (CM544), able to selectively inhibit iNOS in rat glioma cells without interacting with the constitutive NOS isoforms. Results of our work showed that iNOS inhibition by CM544 reduced glioma cells proliferation by enhancing PARP-1 cleavage and compromising the adaptive responses of glioma cells, which are involved in chemoresistance. In fact, the co-administration of temozolomide and CM544 potentiated effects on cell viability.[2]

In order to identify more potent and selective iNOS inhibitors, in this work a new set of acetamidines was synthesized, and the biological evaluation against NOS was performed by the fluorimetric RP-HPLC detection of L-Cit produced by the catalytic activity of NOS.[3] New compounds are structurally related to the leading scaffold of 1400W, and were obtained by substitution of the *m*-methylamino group with other *p*-substituents; moreover, in some compounds, the methylene bridge between acetamidino group and aromatic moiety was deleted or substituted with thiazole (**Figure 1**).

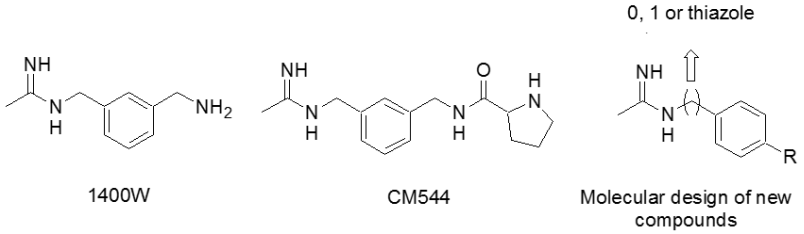

Figure 1

All the assayed molecules are able to inhibit iNOS; in particular the benzamido-amidine FAB1020 emerged as the most interesting compound of the series, due to biological results and physicochemical properties. This compound reduced rat glioma cells viability after 24h treatment, with dose-response effects on metabolic activity. Interestingly, this compound did not impair non tumoral cell viability. The evaluation of antiproliferative and cytotoxic effects on other tumor cell lines is ongoing.

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SAR OF THE LINS01 SERIES THROUGH DOCKING ANALYSIS AND BINDING ASSAYS AT HISTAMINE H_3 AND H_4 RECEPTORS

Corrêa, M. F.;[a] Kiss, D.;[b] Silva, A. B.;[c] Simões, S. C.;[c] Fernandes, A. B.;[a] Varela, M.T.;[a] Keserü, G.;[b] Fernandes, J. P. S.[a]

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Histaminergic receptors are important therapeutic targets for drug design. The H_3R is expressed in the central nervous system as an autoreceptor and heteroreceptor, controlling the synthesis and releasing of several neurotransmitters beyond histamine. Accordingly, it present therapeutical potential to the treatment of neurological and psychiatric disorders.[1] The H_4R is mainly expressed on immune cells and also on neuronal terminals, thus involved in inflammatory and immunological processes. Therefore, antagonists of such receptor may be useful to the treatment of immune-inflammatory disorders.[2] Our group have been exploiting the LINS01 compounds as H_3R/H_4R antagonists, which have already shown promising *in vivo* activity on memory and asthma models.[3] However, some key questions about the chemical motifs of the LINS01 compounds had raised, especially regarding the enantioselectivity, lack of aromaticity and of the carbonyl group, and also the role of the substituents on binding affinities. Thus, a docking study of the LINS01 molecules binding modes on H_3R and H_4R was carried out. Homology models of H_3R and H_4R were built and used as target using the Glide software, including molecular dynamics simulations. The compounds ABT-239 (for H_3R) and JNJ-7777120 (for H_4R) were used as references. Regarding H_3R binding modes, the LINS01 compounds showed to interact with Glu206 related to the antagonist binding mode and in accordance to the experimental data. Moreover, the results indicated that bulky hydrophobic substituents in the dihydrobenzofuran decrease the H_4R affinity, while for the H_3R they are well acomodated. Furthermore, the inclusion a phenyl group in this part of the molecule (as present in ABT-239) would improve the affinity to H_3R. Therefore, LINS01016 was synthesized and evaluated, showing better affinity than the previous compounds (H_3R: IC50 = 116 nM). Moreover, the chirality seems not important to the binding modes of LINS01 compounds. Regarding H_4R, the results suggested that the carbonyl group would increase the affinity, and thus the benzofurancarboxamide LINS01018 was prepared and tested, also showing improved affinity (H_4R: IC50 = 352 nM) (Figure 1). In summary, this work is a nice example of the synergy between CADD and experimental results to achieve molecules with improved activity.

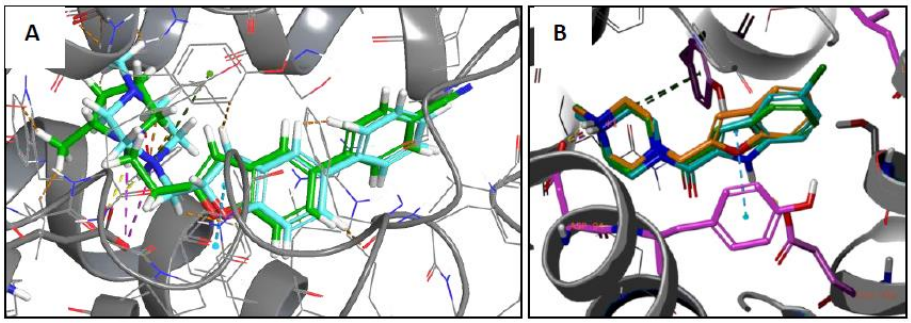

Figure 1. (A) Binding modes of ABT-239 (green) and LINS01016 (cyan) at H_3R; **(B)** binding modes of JNJ-7777120 (cyan), LINS01017 (orange) and LINS01018 (green) at H_4R.

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PROTONATABLE RILUZOLE ANALOGUES AS USE-DEPENDENT INHIBITORS OF VOLTAGE-GATED SODIUM CHANNELS: DESIGN, SYNTHESIS, AND BIOLOGICAL EVALUATION

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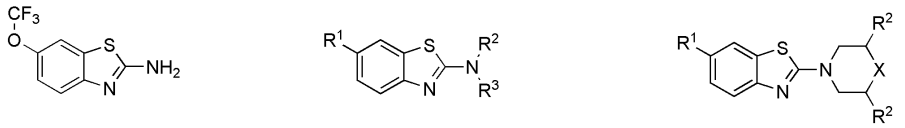
Background. Riluzole (Fig. 1)[1] is a clinically relevant drug indicated for amyotrophic lateral sclerosis. Riluzole is also a potent inhibitor of skeletal muscle voltage-gated sodium channels hNav1.4 and showed potent activity in an animal model of myotonia congenita, a rare disease characterized by skeletal muscle overexcitability and stiffness.[2] The sodium channel blocker mexiletine is the first-line drug for myotonia, but some patients show sub-optimal response or side effects.

Design. Starting from the hypothesis that the introduction of a protonatable nitrogen atom would confer use-dependent blocking activity (that is, higher activity on tissues with higher rate of stimulation), we performed chemical maneuvers on riluzole with the aim of exalting potency and use-dependency of block of sodium channels in order to enlarge the selectivity window of the so-obtained riluzole analogues with respect to the parent lead compound.

Methods. Two series of riluzole analogues (**A** and **B**, Fig. 1) were designed and prepared (when not commercially available) in order to explore the stereoelectronic features possibly involved in the sodium channel blocking activity. Sixteen riluzole analogues were tested on sodium currents recorded with patch-clamp in HEK293 cells transfected with hNav1.4. Riluzole and some analogues were tested in vitro on rat muscle fibre excitability using two-intracellular microelectrode current-clamp technique.

Results. As expected, the introduction of protonatable nitrogen atoms conferred significant use-dependent behaviour. The introduced amine groups should be secondary since tertiarization lowered both potency and use-dependency. The removal of the 6-CF_3O group or its replacement with ±M effect exerting substituents were detrimental.

Conclusions. Riluzole appears like a well-optimized compound since its analogues did not show increased potency on sodium currents. However, the possibility to increase use-dependency by introducing a protonatable nitrogen atom opens the way to obtain safer drugs.



riluzole

A

R^1 = H, Cl, OH, MeO, *i*Pr, CF_3, CF_3O, NO_2
R^2 = H, Me, NH_2, CH_2CH_2NHMe
R^3 = H, Me

B

X = CH_2, O, NH, NMe
R^1 = *i*Pr, CF_3, CF_3O, PhO
R^2 = H, Me

Figure 1. Structures of riluzole and analogues (series **A** and **B**).

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CHARACTERIZATION OF NEW TRPM8 MODULATORS IN PAIN PERCEPTION

Di Sarno, V.;[a] De Caro, C.;[b] Cristiano, C.;[b] Avagliano, C.;[b] Bertamino, A.;[a] Ostacolo, C.;[b] Campiglia, P.;[a] Gomez Monterrey, I. M.;[b] La Rana, G.;[b] Gualillo, O.;[c] Calignano, A.;[b] Russo, R.[b]

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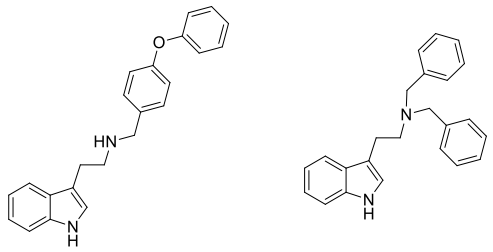
Transient receptor potential melastatin type-8 (TRPM8) is a transmembrane non-selective Ca^{2+} permeable cation channel, considered as the major sensor for peripheral innocuous cool, and its modulation contributes to a wide range of (patho)physiologic processes.[1] One of the most investigated effects produced by TRPM8 modulation is the analgesia against chronic and neuropathic pain.[2]
Recently, we have identified two tryptamine-based derivatives acting as selective activator (**1**) and inhibitor (**2**) of TPRM8 channel (Figure 1).[3] In this communication we present the pharmacological characterization of these compounds.



1
IC_{50}= 367± 24 nM

2
EC_{50}= 40± 4 µM

Figure 1. Selective activator (**1**) and inhibitor (**2**) of TPRM8 channel.

We tested both compounds in acute and chronic animal pain models, after systemic and local administration. The wet-dog shaking test and change in body temperature have highlighted the antagonist and agonist activities of **2** and **1** on TRPM8 channel, respectively. The antagonist **2** also produced an analgesic effect in formalin-induced orofacial pain and in chronic constriction injury-induced neuropathic pain, demonstrating the involvement of TRPM-8 channel in pain. On the other hand, local administration of agonist **1** at low concentration produced hypersensibility in CCI mice model of cold allodynia and anti-allodynia effect at media and high dosis, suggesting a dowregulation of other Ca^{2+} channels.

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STEREOSELECTIVE INHIBITION OF HEME OXYGENASE-1 BY PHENYLETHANOLIC-IMIDAZOLE DERIVATIVES

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Heme oxygenases (HOs) are a family of enzymes involved in the catabolism of heme. The development of selective heme oxygenase-1 (HO-1) inhibitors is emerging as a novel strategy in the treatment of specific cancer (*e.g*. chronic myeloid leukaemia, prostate and breast cancer).[1] The lack of potent HO-1 inhibitors and the poor selectivity towards HO-2 currently represent a limiting step in pursuing such strategy. Recently in our laboratory, we identified two among the most potent and selective HO-1 inhibitors known so far (Fig. 1).[2]

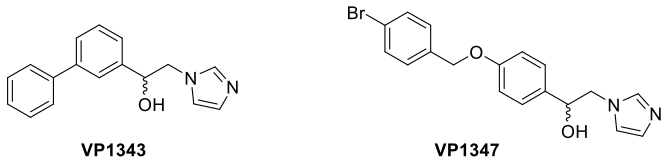

Figure 1. Chemical structure of HO-1 inhibitors VP1343 and VP1347

A stereoselective inhibition of HO-1 emerged from SAR studies around azalanstat, the first identified HO-1 inhibitor. Since our compounds present a stereocenter (at the C_1 ethanolic position), herein we investigate the biological effect of the single enantiomers of VP1343 and VP1347.
The "racemic approach" was used to isolate the enantiomers, being it the most convenient way for a rapid access to small amounts of highly pure optical isomers.[3] Accordingly, the CHIRALCEL OD-H polysaccharide-based chiral stationary phase, containing cellulose tris(3,5-dimethylphenylcarbamate) was identified as the optimal choice in combination with a mobile phase consisting of *n*-hexane/ethanol - 85/15 (v/v). Off-line electronic circular dichroism (ECD) studies on the isolated enantiomers, combined with time-dependent density functional theory (TD-DFT) calculations allowed to characterize the stereochemistry of the enantiomers and determine a (R) < (S) enantiomer elution order (EEO). Prior to biological evaluation, the two isolated enantiomers were submitted to a reversed-phase HPLC purification step via preparative-scale chromatography, and then their chemical integrity was checked through high-resolution mass spectrometry (HRMS) analysis.
Biological evaluation showed a stronger inhibitory potency for the (R) enantiomers. Biological data were rationalized by docking studies.

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SYNTHESIS AND PHARMACOLOGICAL CHARACTERIZATION OF CONFORMATIONALLY RESTRICTED RETIGABINE ANALOGUES AS NOVEL NEURONAL KV7 CHANNELS ACTIVATORS

Ciaglia, T.;[a] Ostacolo, C.;[b] Miceli, F.;[b] Nappi, P.;[d] Iraci, N.;[a] Soldovieri, M.;[e] Di Sarno, V.;[a] Ambrosino, P.;[f] Vestuto, V.;[a] Lauritano, A.;[d] Musella, S.;[c] Pepe, G.;[a] Basilicata, M.;[a] Manfra, M.;[g] Novellino, E.;[a] Bertamino, A.;[a] Gomez Monterrey, I. M.;[b] Campiglia, P.;[a] Taglialatela, M.[a]

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The Kv7 K^+ channels play a fundamental role in controlling neuronal excitability, representing an attractive pharmacological target for the treatment of different neurological disorders, particularly epilepsy.[1,2] Retigabine, the only antiepileptic drug approved for human use, acts as Kv7.2/7.3 agonist. However, it has been withdrawn from the market due to the formation of unsafe oxidized metabolites.[3] In order to improve both chemical and metabolic stability, we designed and synthesized three series of conformationally restricted analogues of retigabine (Figure 1). The pharmacological effects of these series were investigated by electrophysiological and patch-clamp experiments. The indole-based derivatives **23a** (EC_{50} = 0.08 ± 0.04 μM) and **24a** (EC_{50} = 0.63 ± 0.07 μM) acted as potent Kv7.2 agonists with improved potency and efficacy than retigabine (EC_{50} = 0.93 ± 0.43 μM).

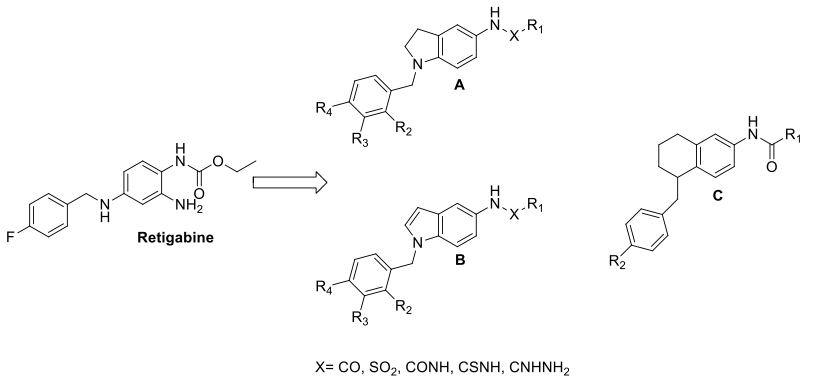

Figure 1. Design and synthesis of conformationally restricted analogues of retigabine.

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IDENTIFICATION OF AN INDOL-BASED MULTI-TARGET KINASE INHIBITOR THROUGH PHENOTYPE SCREENING AND TARGET FISHING USING INVERSE VIRTUAL SCREENING APPROACH

Musella, S.;[c] Ostacolo, C.;[b] Di Sarno, V.;[a] Ciaglia, T.;[a] Lauro, G.;[a] Pepe, G.;[a] Vestuto, V.;[a] Autore, G.;[a] Bifulco, G.;[a] Marzocco, S.;[a] Campiglia, P.;[a] Gomez Monterrey, I. M.;[b] Bertamino, A.[a]

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The development of multi-target-based drug approach, which could be considered complement of the well know target-based and phenotype-based drug discovery approaches, is still a challenge for the scientific community.[1,2] We applied this approach to the identification of the potential target(s) of the most potent compound of a series of 1,3,5-substituted indoles designed as anti-proliferative agents, in particular 3-(((2-([1,1'-biphenyl]-4-yl)ethyl)(methyl)amino)methyl)-N-(4-fluorophenyl)-1H-indole-5-carboxamide (**27**).
Target fishing of our hit compound using a combination of inverse virtual screening (IVS) approach and ligand-based shape similarity study, identified the top-ranked targets of **27** as belonging to kinome (Fig.1). In vitro binding assays of **27** against a panel of 40 kinases confirmed and identified this hit as multi-target kinase inhibitor. Compound **27** was further characterized for its antiproliferative activity by in cell studies, showing a mechanism of action involving modification of the cell cycle, increase in ROS release and caspase 3-expression and decrease in ERK expression.

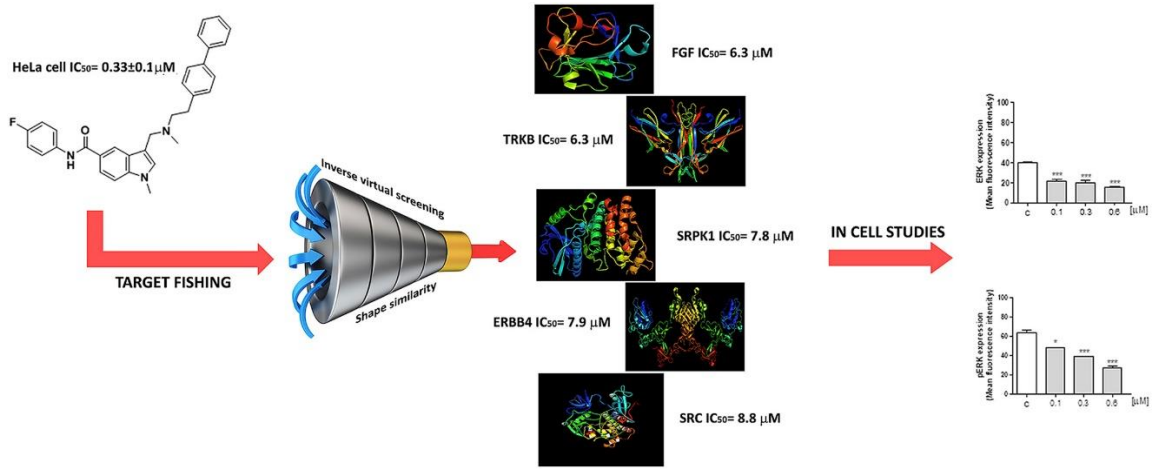

Figure 1. Synthesis, target fishing and in cell studies of 1,3,5-substituted indole derivates.

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NOVEL ADAMANTYL RETINOID-RELATED MOLECULES WITH A BROAD-SPECTRUM PRECLINICAL ANTITUMOR ACTIVITY

Catalano, M.;[a] Cincinelli, R.;[a] Musso, L.;[a] Guglielmi, M. B.;[b] Esposito, A.;[b] La Porta, I.;[b] Signorino, G.;[b] Cardile, F.;[b] Colelli, F.;[b] Pisano, C.;[b] Dallavalle, S.[a]

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In recent years the so-called atypical retinoids (AR) (or retinoid-related molecules (RRMs) have been developed as a promising class of antitumor compounds. These agents are attracting growing attention because of their unique mechanism of action, which appears different from that of classical cyto-differentiating retinoids such as 13-cis-RA and ATRA. Indeed, RRMs induce apoptosis in ATRA-resistant cells. One of the most investigated compound of this class is E-3-(3'-adamantan-1-yl-4'-hydroxybiphenyl-4-yl)acrylic acid (Adarotene, Figure 1).

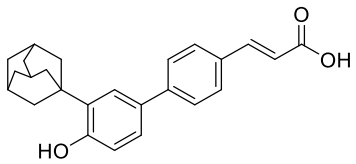

Figure 1

Extensive SAR studies have been performed to identify the putative key portions of the molecule. In the present work we report the results of our efforts to more thoroughly explore the chemical space around the carbon 3 of Adarotene.
The effects of the synthesized compounds on cell growth were determined on a panel of human tumor cells and a panel of hematological cancer cell lines. The results showed that the structural requirements for optimal activity are rather strict. Essentially, the data indicated that both the size and electron density of the substituent impacted antiproliferative activities, which were retained if the group had some electrodonating capacity (H-bond-accepting) and restricted volumes. The most promising compounds showed a broad spectrum of antitumor activity against several tumor histotypes and increased cytotoxic activity against Adarotene-resistant cell line, compared to the parent molecule.
The antitumor activity of a selected compound was evaluated on a lung cancer model (NCI-H460) xenografted s.c. and in orthotopic models of hepatocellular carcinoma (Hep G2) and human mesothelioma (MM487). Particularly significant was the in vivo activity of the compound as a single agent or in combination with Cisplatin, against mesothelioma. Moreover, the administration did not induce negative effect on mice body weight, thus suggesting a high tolerability.

SYNTHESIS AND IN VITRO PHARMACOLOGICAL EVALUATION OF 2-HYDROXYPROPYL-4-ARYLPIPERAZINE DERIVATIVES AS SEROTONINERGIC LIGANDS

Fiorino, F.;[a] Magli, E.;[a] Sparaco, R.;[a] Struga, M.;[b] Bielenica, A.;[b], Lesniak, A.;[b] Pawłowska, A.;[b] Bujalska-Zadrożny, M.;[b] Perissutti, E.;[a] Santagada, V.;[a] Caliendo, G.[a]

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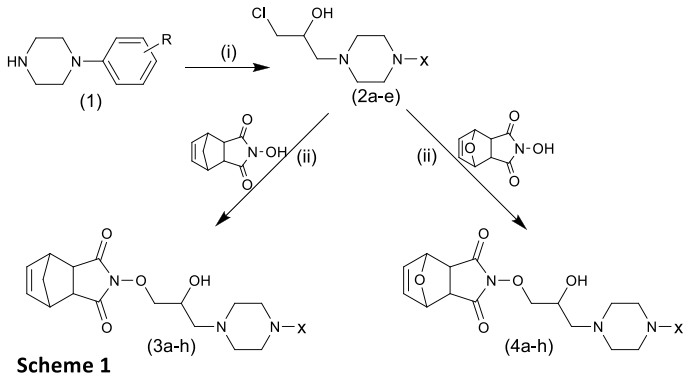
Pharmacological regulation of the 5-HT system have a great therapeutic potential, and therefore it is the subject of intense studies.[1] One of the most interesting 5-HT receptors, involved in mediating 5-HT biological effects, is the 5-HT_{1A} receptor that is involved in a wide range of psychiatric disorders, and even in the proliferation of human tumor cells (PC3). 5-HT_{2A} receptor stimulates the secretion of various hormones and mediate several processes such as intestinal motility and secretions, whereas, the 5-HT_{2C} receptor is probably the one with the most widespread distribution in the CNS and is able to mediate the action of 5-HT in virtually all brain areas. In this study we report the synthesis of two series of compounds, embodying norbornene and exo-N-hydroxy-7-oxabicyclo[2.2.1]hept-5-ene-2,3-dicarboximide nuclei, that have been evaluated for their binding to the 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors. The designed molecules have been prepared following the procedure depicted in scheme 1. The combination of structural elements (heterocyclic nucleus, hydroxyalkyl chain, and 4-substituted piperazine) known to be critical for affinity to 5-HT_{1A} receptors and the proper selection of substituent led to compounds with high specificity and affinity toward serotoninergic receptors. Moreover, the compounds displaying better affinity and selectivity binding profiles towards 5-HT_{1A} and 5-HT_{2A} receptors were selected in order to be tested by *in vitro* and *in vivo* assays to determine their functional activity. Compounds **4e** and **4g** showed the most interesting affinity/selectivity profile towards 5-HT_{1A} receptors. Besides the remarkable 5-HT_{2A} receptor affinity and selectivity of compound **3d** supporting a 3,4-dichlorophenyl group (K_i = 32.7 nM), another interesting K_i value was that of compound **3g**, supporting a naphthalen-1-yl moiety (K_i = 80.0 nM). Finally, all the synthesized derivatives, showed no significant affinity profile towards 5-HT_{2C} receptors; these data are very interesting considering the high degree of homology existing between the considered receptors. The selected derivatives have been tested by *in vitro* assay to determine their activity profile towards serotonin evoked contractions on the rat ileum evidencing an antagonistic profile. The derivative **4e**, supporting a piridyl group, was showwed an agonist profile (pEC_{50} = 6.4 ± 0.4; E_{max} (%) = 128 ± 5.4) whereas for the compound **4g**, supporting a naphthalen-1-yl moiety, was observed an antagonist activity (pEC_{50} = 6.4 ± 0.4). These results further support the choice of the norbornene nucleus for the preparation of serotoninergic ligands endowed with 5-HT_{2A} affinity and activity, while the exo-N-hydroxy-7-oxabicyclo[2.2.1]hept-5-ene-2,3-dicarboximide scaffold resulted as a useful scaffold for the preparation of derivatives characterized by a selective 5-HT_{1A} affinity/activity profile.



Scheme 1

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USING NETWORK-BASED APPROACHES ON A SET OF ANTI-PROLIFERATIVE COMPOUNDS: FROM CHEMICAL SPACE TO HETEROGENEOUS NETWORKS

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Nowadays, the systems pharmacology paradigm, according to which many drugs act interacting with multiple biomolecules (in contrast to the "one disease, one target, one drug" outdated assumption), is well accepted. In this context, inferring drug-target interactions assumes a fundamental role, both for the identification of targets and mechanism of action (MoA) of drug candidates, and for drug repurposing. Recently, computational modelling and particularly network-based approaches have been shown to be useful strategies to characterize chemical space, as well as to tackle the major challenges of systems pharmacology.[1]
The aim of the project is to investigate potential targets of a set of 228 compounds using network-based approaches. Within our data set, 143 compounds were previously tested for antiproliferative activity on K562 cell line, and the remaining on HL60 cell line as human leukaemia models, so the IC_{50} values are available.
We first designed coordinate-free similarity-based networks (Figure 1), in which nodes represent compounds and edges represent pairwise molecular similarity relationships to provide a readily interpretable global representation of the compounds data set.[2] To assess chemical structure similarity, we generated a matrix using Tanimoto coefficient (Tc) as similarity measure of fingerprint descriptors.
Molecular or biological properties could be displayed in the network through, e.g., nodes colour or size, and edges were weighted in order to show the variation of similarity values above the pre-defined Tc threshold value.
Secondly, we applied a validated (random walk-based) prediction algorithm for identifying novel drug-target association from the heterogeneous network constructed by merging drug-drug, target-target and drug-target networks integrating chemical and biological data extracted from public databases.[3]
In perspective, the network analysis might give us the possibility to gain a system-wide understanding of our compound's MoAs, to support experimental studies and to assist the decision making process on the prosecution of the ligand discovery project.

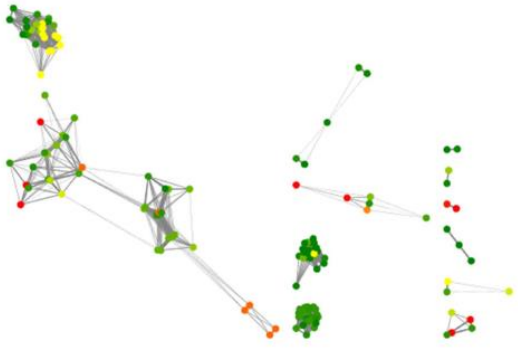

Figure 1. Chemical space network of 143 K562-tested compounds. Tc threshold value was set to 0.7, nodes were colour-coded on the basis of IC_{50} values from high (red) to low (green). The figure was obtained with Cytoscape.[4]

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AKR1C3 INHIBITORS DESIGNED BY A BIOISOSTERIC APPROACH TO HIT PROSTATE CANCER

Boschi, D.;[a] Pippione, A. C.;[a] Sainas, S.;[a] Bonanni, D.;[a] Oliaro-Bosso, S.;[a] Daga, M.;[a] Boscaro, V.;[a] Gallicchio, M.;[a] Marini, E.;[a] Chiorino, G.;[b] Friemann, R.;[c] Lolli, M. L.[a]

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The aldo-keto reductase 1C3 (AKR1C3) isoform plays a vital role in the biosynthesis of androgens and is considered an attractive target in prostate cancer (PCa). No AKR1C3-targeted agent has to date been approved for clinical use. Flufenamic acid and indomethacine are non-steroidal anti-inflammatory drugs known to inhibit AKR1C3 in a non-selective manner as COX off-target effects are also observed. We employed a scaffold hopping approach to design a new class of potent and selective AKR1C3 inhibitors based on an N-substituted hydroxylated triazole pharmacophore. Applying a conformational restriction strategy, we designed a second series focused around an acidic hydroxybenzoisoxazole moiety, which was rationalised to mimic the benzoic acid role in the flufenamic scaffold. Finally, we designed indomethacin analogues by proposing a bioisosteric connection between the indomethacin carboxylic acid function and either hydroxyfurazan or hydroxy-1,2,5-N1-methyltriazole rings. Through iterative rounds of drug design, synthesis and biological evaluation, several compounds were discovered to target AKR1C3 in a selective manner. The most promising compounds of the series (1 - 2) were found to be highly selective for AKR1C3 over the 1C2 isoform with minimal COX1 and COX2 off-target effects. In cell-based assays, the compounds of all the series reduced the cell proliferation, prostate specific antigen (PSA), testosterone production in AKR1C3-expressing 22RV1 prostate cancer cells and showed synergistic effect when assayed in combination with abiraterone and enzalutamide. Structure determination of AKR1C3 co-crystallised with one representative compound from each of the series clearly identified both compounds in the androstenedione binding site, hence supporting the biochemical data. The evaluation of AR protein level after cellular treatment by most active compounds revealed a significant down-regulation of the AR protein level while the microarray analysis didn't highlight a down-regulation at the AR messenger RNA level suggesting a possible interference of the inhibitors with the degradation process of the AR protein. Therefore, these AKR1C3 inhibitors can be used to provide further insight into the role of AKR1C3 in prostate cancer.

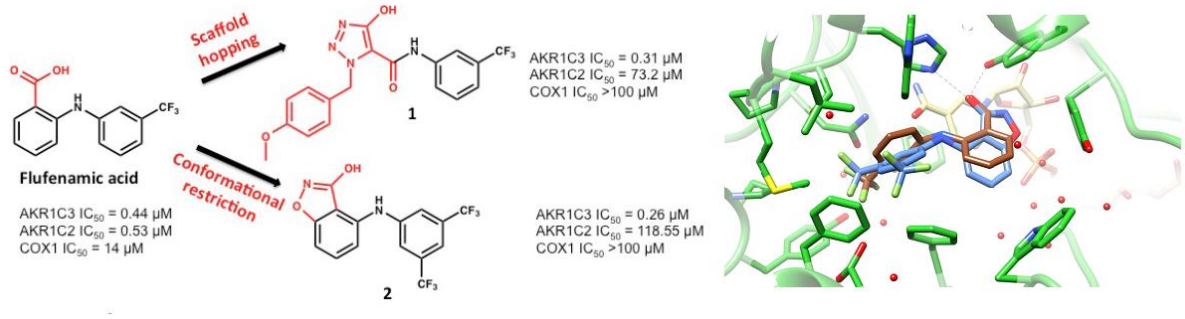

Figure 1. Structure, binding mode and biological activity of two of discussed compounds.

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FROM *HIBISCUS SABDARIFFA* LINN A NEW OPPORTUNITY FOR FIGHTING MULTIPLE MYELOMA

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Despite recent advances in its therapy, Multiple Myeloma (MM) still causes many deaths every year and its incidence is increasing worldwide. Thus, current therapies sometimes fail and are associated with severe adverse effects, among all neurotoxicity.[1] As a part of our ongoing efforts to discover new potential therapies against MM, we prepared *Hibiscus sabdariffa* extracts obtained by a microwave-assisted solvent extraction and investigate its activity by in vitro assays on RPMI-8226 cell line.[2] From the results of cell viability assay (MTT) emerged that the crude extract has an interesting cytotoxic activity (IC_{50} = 21.3 mg/mL). Building on this promising result, we performed a bio-guided fractionation and identified the HsFrC as the most active fraction, with an IC_{50} value of 3.5mg/mL. The subsequent fractionation of HsFrC allowed the isolation of Hibiscus acid dimethylester (HibA-ester) and 5-hydroxy-2*H*-pyran-6-carbaldehyde (Hib-carbaldehyde) as active secondary metabolites (Figure 1). Of note, Hib-carbaldehyde has never been isolated before.
Of both compounds the *in vitro* profile has been drawn, assessing cell viability (MTT and Tripan blue test), cell migration (Boyden chamber assay), proteasome inhibition (proteasome activity test) and neurotoxicity (DRG neurotoxicity assay). We demonstrated that they are able to significantly reduce cell migration and to inhibit both proteasome activity and autophagy process. HibA-ester shows the highest activity (IC_{50} = 2,3 mM).

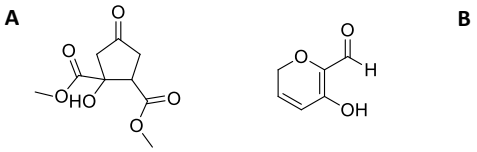

Figure 1. Structure of Hib-ester (**A**) and Hib-carbaldehyde (**B**).

Herein, we present the results of the study, mainly focusing on compounds extraction, identification and biological characterization.

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NOVEL *trans*-Pt (II) COMPLEXES: BIOLOGICAL INTERACTIONS AND CELL EFFECTS

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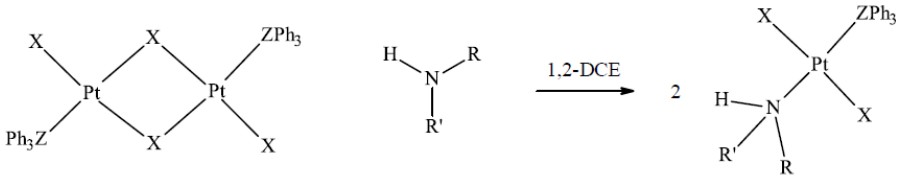
Cisplatin is a widely used antitumor agent approved for the treatment of numerous solid cancers.[1] Nevertheless, the clinical success of cisplatin is limited by significant side effects, mainly neurotoxicity and nephrotoxicity, and intrinsic or acquired resistance. The resistance appears as a multifactorial event and indeed resistant cells show a myriad of phenotypic changes that involve drug uptake, DNA damage repair and apoptosis.[2]

Thousands of platinum compounds have been synthesized and screened *in vitro* and *in vivo* for the anticancer activity and many tens have entered the clinic in the past years. Nevertheless, little success has been achieved in finding novel platinum analogues able to overcome cisplatin resistance.

The *trans* isomer of cisplatin was much less active, due to the fact that *trans* chloro species are kinetically more reactive than the corresponding *cis* isomers, and the high cytotoxic 1,2-intrastrand DNA lesion, induced by cisplatin, is stereochemically hindered.[3] Nevertheless, substitution of amine ligands in transplatinum with more bulky ligands led to compounds with significant antiproliferative effect and often active towards cisplatin resistant tumor cells.

In a previous paper we prepared and studied some *trans*-dichloro(triphenylphosphine)platinum(II) complexes characterized by N-(butyl),N-(arylmethyl)amino ligands showing an interesting antiproliferative activity on both cisplatin- sensitive and -resistant cell lines.[4]

In this context, complexes *trans*-[$PtX_2(ZPh_3)(NHRR')$] (X = Cl, Br; Z= P, As; R,R' = Bu, $PhCH_2$- or R = R'= $HOCH_2CH_2$-) were synthesized by ring opening of the corresponding dinuclear precursors *trans*-[$PtX(\mu\text{-}X)(ZPh_3)]_2$ by RR'NH amines. The complexes are well soluble and stable in DMSO solutions.



The antiproliferative effect was assayed on a panel of human tumor cell lines and in particular on the A2780wt and A-2780cis cell line pairs, cisplatin-sensitive and -resistant, respectively. The interaction with macromolecules and intracellular organelles was investigated by spectroscopic and electrophoretic methods. In detail, the ability to bind to DNA and to be accumulated in cells was determined and discussed with a special focus on the comparison between resistant and sensitive cells.

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FUSED 1,2,4-TRIAZOLO DERIVATIVES AS CASEIN KINASE 1δ INHIBITORS: ROLE OF THE SUBSTITUENTS ON THE CARRIED PHENYL RING

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Casein kinase 1δ (CK-1δ) is a monomeric serine/threonine kinase which was found to be involved in different pathological conditions including neurodegenerative diseases, sleeping disorders, parasites infections and cancer.[1] CK-1δ has a role on both Wnt and Hedgehog pathways, thus seems that its inhibition could be efficacious in counteracting neuro-inflammatory states.[2] In addition, among its substrates can be found tau, α-synuclein and TDP-43 proteins, all involved in protein aggregation processes observed in neurodegenerative diseases.[2] Consequently, we are interested in developing potent CK-1δ inhibitors as potential neuroprotective agents. During an initial screening on 2,5,8-trisubstituted[1,2,4]triazolo[1,5-*c*]pyrimidines derivatives (Figure 1), the presence of a phenyl group at the 2 position led to a slight kinase inhibition. Thus, substituents with different steric and electronic properties were introduced at the 3 or 4 position of the phenyl ring. The best result was obtained with a hydroxy group at the 3 position. Docking studies using the crystallographic structure of CK-1δ suggested that a hydrogen bonding is formed between the hydroxyl group and a Lysine in the ATP binding pocket. Observing the binding pose, it was possible to observe that in the opposite site of the hydroxyl group there is an Aspartic residue which could be also involved in hydrogen bonding. For this reason, 3,4- and 3,5- dihydroxyphenyl derivatives were synthesized. The specular substitution on the phenyl ring (at the 3 and 5 positions) increased the inhibitory activity on the kinase, proving the starting hypothesis. Thus, the same strategy was applied to 2,5,7-trisubstituted[1,2,4]triazolo[1,5-*c*]pyrimidines and [1,2,4]triazolo[1,5-*a*][1,3,5]triazine and the 3,5-dihydroxyphenyl derivatives displayed the best results, reaching an IC_{50} value in the sub-nanomolar range (0.18 μM). These compounds represented a good starting point for structural optimization on the other positions of the considered heterocyclic scaffolds.

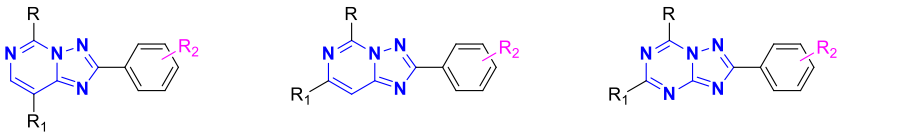

Figure 1. Fused 1,2,4-triazolo scaffolds used to develop CK-1δ inhibitors.

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SMALL MOLECULE INHIBITORS OF KDM5 HISTONE DEMETHYLASES INCREASE THE RADIOSENSITIVITY OF BREAST CANCER CELLS OVEREXPRESSING JARID1B

La Regina, G.;[a] Naccarato, V.;[a] Masci, D.;[a] Puxeddu, M.;[a] Nalli, M.;[a] Coluccia, A.;[a] Negri, R.;[b] Silvestri, R.[a]

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KDM5 enzymes are H3K4 specific histone demethylases involved in transcriptional regulation and DNA repair.[1] These proteins are overexpressed in different kinds of cancer, including breast, prostate and bladder carcinomas, with positive effects on cancer proliferation and chemoresistance.[2,3] For these reasons, these enzymes are potential therapeutic targets.

In the present study, we analyzed the effects of three different inhibitors of KDM5 enzymes in MCF-7 breast cancer cells over-expressing one of them, namely KDM5B/JARID1B.[4] In particular, compounds RS3195, RS3152, RS3183, R5033 and RS4995 were assayed in terms of H3K4 demethylation (western blot), radio-sensitivity (cytoxicity and clonogenic assays) and damage accumulation (COMET assay and kinetics of H2AX phosphorylation) (Figure 1). We showed that three compounds can selectively inhibit KDM5 enzymes and are capable of increasing sensitivity of breast cancer cells to ionizing radiation and radiation-induced damage. These findings confirmed the involvement of H3K4 specific demethylases in the response to DNA damage, showed a requirement of the catalytic function and suggested new strategies for the therapeutic use of their inhibitors.

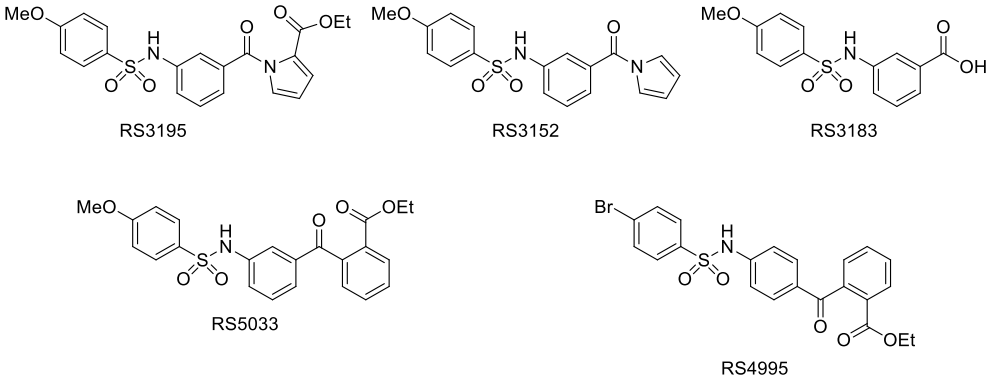

Figure 1. Chemical structures of compounds RS3195, RS3152, RS3183, RS5033 and RS4995.

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DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF PDZ1 TARGETING NHERF1 INHIBITORS AS ANTICANCER AGENTS

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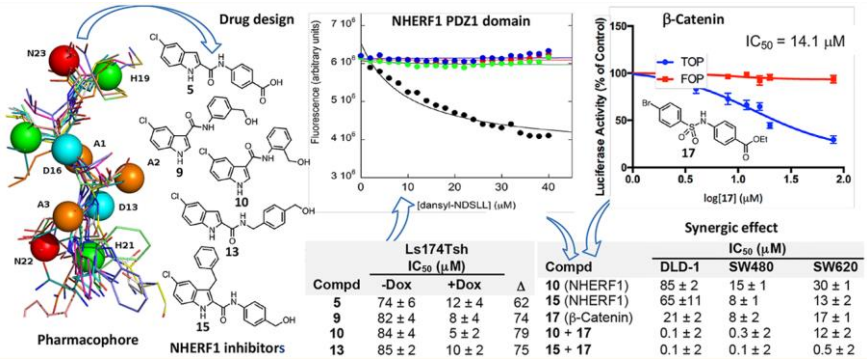
NHERF1 (Na^+/H^+ exchanger 3 regulating factor 1) is an integral membrane adaptor protein carrying two NH_2-terminal PDZ (postsynaptic density 95/discs large/zona occludens 1) tandem domains.[1] PDZ1 (11-97 amino acids) and PDZ2 (150-237 amino acids) show 74% identity to each other and bind to specific carboxyl-terminal motifs on target proteins, such as β-catenin and PTEN, that may have a pivotal role in tumorigenesis. Oncogenic activity of NHERF1 is strictly dictated by changes on its subcellular localization.[1,2]

A pharmacophore model was used to filter out an in-house training set of about 6000 compounds, leading to identify a potent inhibitor of NHERF1.[3] We herein reported the design and synthesis of new NHERF1 inhibitors (Figure 1).[4] The new compounds were synthesized by treating the appropriate indole with thionyl chloride and the proper amino derivative in the presence of pyridine in dichloromethane at room temperature for 12 h; alternatively, the coupling reaction was carried out using (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate and triethylamine in *N,N*-dimethylformamide at room temperature for 12 h. Compounds **5**, **9**, **10** and **13** exhibited a remarkable cytotoxicity in Ls174Tshβ-Cat cells. The binding to NHERF1 PDZ was confirmed by means of a dansylated peptide corresponding to the *C*-terminal sequence of β2-AR. When used in combination with antagonists of β-catenin, the new derivatives increased the apoptotic death of colorectal cancer cells refractory to currently available Wnt/β-catenin-targeted agents.



Compd	Ls174Tsh IC50 (μM) -Dox	+Dox	Δ
5	74 ± 6	12 ± 4	62
9	82 ± 4	8 ± 4	74
10	84 ± 4	5 ± 2	79
13	85 ± 2	10 ± 2	75

Compd	IC50 (μM) DLD-1	SW480	SW620
10 (NHERF1)	85 ± 2	15 ± 1	30 ± 1
15 (NHERF1)	65 ±11	8 ± 1	13 ± 2
17 (β-Catenin)	21 ± 2	8 ± 2	17 ± 1
10 + 17	0.1 ± 2	0.3 ± 2	12 ± 2
15 + 17	0.1 ± 2	0.1 ± 2	0.5 ± 2

Figure 1. Design, chemical structures and biological activity of new NHERF1 inhibitors.

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IDENTIFICATION OF POTENT 1,3-DIOXOLANE-BASED 5-HT$_{1A}$ RECEPTOR AGONISTS FOR THE TREATMENTS OF CNS DISORDERS AND PAIN

Linciano, P.;[a] Sorbi, C.;[a] Franchini, S.;[a] Tait, A.;[a] Bardoni, L.;[b] Ronsisvalle, S.;[c] Denora, N.;[d] Lesniak, A.;[e] Bujalska-Zadrożny, M.;[e] Pawłowska, A.;[e] Cichero, E.;[f] Fossa, P.;[f] Brasili, L.[a]

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5-HT$_{1A}$ receptors (5-HT$_{1A}$R) are still recognized as a valuable target for drug discovery. High affinity 5-HT$_{1A}$R ligands have been considered to play an important role in the treatment of anxiety, depression, neurodegeneration, Parkinson's disease and neuropathic pain.[1] The 1,3-dioxolane scaffold has proved to be a versatile and useful scaffold for different classes of ligands. In a lead optimization program, two series of 1,3-dioxolane-based phenoxyethylamines were prepared and tested for affinity, potency and efficacy at 5-HT$_{1A}$R. In the first series, we explored the expansion, opening and simplification of the 1,3-dioxolane ring as a way to increase the intrinsic activity and selectivity for 5-HT$_{1A}$R. Within this series, the 1,3-dioxane derivative (**1**) emerged as a potent, selective and full 5-HT$_{1A}$R agonist (pK_i = 8.8; pD_2 = 9.22; $\%E_{max}$ = 92). *In vitro* assays showed the ability of **1** to penetrate the BBB. This was confirmed by pharmacokinetic studies in rats that revealed a high bio-distribution in the brain compartment. Behavioral studies in rats treated orally with **1** demonstrated an anxiolytic and anti-depressant effect.[2]

In the second series, retaining unaltered the 1,3-dioxolane- scaffold, a wide variety of heteroaryls was introduced in *ortho* position on the phenoxy ring. The 4-pyridyl derivative (**2**) resulted a potent and selective 5-HT$_{1A}$R partial agonist and was chosen for further studies. Compound **2** was able to protect SH-SY5Y human neuroblastoma cells from oligomycin A and H_2O_2 induced cytotoxicity, revealing neuroprotective potential.[3]

In addition, the antinociceptive activity of **2** was evaluated *in vitro* and *in vivo. In vitro,* electrophysiological recordings from dorsal horn neurons in mouse spinal cord slices showed that **2** produces an outward hyperpolarizing current. *In vivo*, formalin test was performed on mice and **2** was able to decrease the late response to the noxious stimulus. In both experiments, the answer exerted by **2** was reverted by WAY-100635.

Since **2** presents one chiral center, the two enantiomers were synthesized following enantioselective synthetic procedures. Binding affinity results showed a slight preference for the (S) enantiomer. Docking calculation were performed to elucidate the binding mode of the two enantiomers, supporting the experimental results. Interestingly, in addition to its 5-HT$_{1A}$R agonism, the (S)-**2** exerted a full D_2 antagonism, with a eudismic ratio S/R of 100. Compound **2** may represent a potential multitarget lead compound for the treatment of schizophrenia, since the simultaneous stimulation of 5-HT$_{1A}$R can ameliorate extrapyramidal motor disorder induced by pure D_2 antagonists. Considering those potential therapeutic applications, further *in vivo* investigation are ongoing.

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DESIGN AND SYNTHESIS OF NOVEL RC-106 DERIVATIVES FOR DISCOVERING NEW AGENTS AGAINST MULTIPLE MYELOMA

Listro, R.;[a] Stotani, S.;[a] Rossino, G.;[a] Malacrida, A.;[b] Miloso, M.;[b] Cortesi, M.;[c] Tesei, A.;[c] Rossi, D.;[a] Collina, S.[a]

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Multiple myeloma (MM) is a cancer that affects the plasma cells, key components of the immune system, and it is related to the proliferation of neoplastic plasma cells in the bone marrow. In recent years, the novel effective chemotherapeutic drugs have been discovered. The drugs commonly used for treating MM are proteasome inhibitors, like bortezomib, or immunomodulators such as lenalidomide and thalidomide. Nowadays, bortezomib is the first- and second-line treatment in MM. However, multiple cycles of chemotherapeutic treatments cause drug resistance, leading to refractory MM and it is associated with the so-called chemotherapy induced peripheral neuropathy.[1] Consequently, there is still an urgent need of new treatment for this pathology. Prompted by the experience of team members in this field, we directed our current efforts to the identification of novel agents against MM.

Our starting point is **RC-106** (Figure 1),[2] a pan-SR modulator with good *in vitro* antiproliferative activity toward a panel of different cancer cell lines, recently developed by our group. **RC-106** is effective against pancreatic and glyoblastoma cancer cell lines, and have a modest activity against MM.

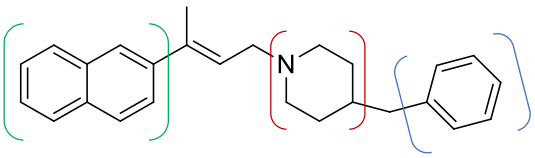

Figure 1. [Structure of **RC-106**]

With the aim to discover new agents effective in MM treatment, we designed a 65 member compound library with three point of diversity: the aromatic nucleus, the aminic- and the benzylic- moieties, as shown in figure1. The library has been prepared following a combinatorial chemistry approach. As a result, 40 compounds have been obtained in sufficient amount and suitable purity for biological assays. The MMT test on MM cell lines (RPMI 8226) evidenced that only 4 molecules have a good value of IC_{50} (in the range is 26-29 μM). An in depth in vitro profile of the most active compounds is ongoing.

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INCREASED STABILITY OF THERAPEUTICALLY-RELEVANT PEPTIDES IN BIOLOGICAL MATRICES: A MICROSAMPLING INVESTIGATION

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The growing market of biotherapeutic peptides and the increasing interest in the monitoring of peptide compounds as diagnostic biomarkers of pathophysiological states has highlighted the need for effective bioanalytical methods useful for drug discovery and development, to obtain effective diagnostic tools and to tailor personalised therapies on a precision-medicine perspective. Clinical needs and regulatory requirements are encouraging bioanalytical laboratories to develop methodologies that are time- and cost-effective, while still producing assays that are sensitive enough to cope with biological matrices. On the other side, the emerging phenomenon of sport doping by means of performance-enhancing peptides poses the need for their rapid and accurate identification and quantitation in biological samples. Among the main challenges facing the analysis of this broad range of compounds, sample stability represents one of the main concerns. In fact, proteolytic degradation as well as bacterial activity can alter the quali-quantitative composition of these specimens after biological fluid sampling, even when stored under controlled temperatures, thus potentially leading to underestimation of peptide levels.[1] From promising preliminary data, it emerged how miniaturised biosampling strategies in dried form (e.g. dried matrix spot, DMS and volumetric absorptive microsampling, VAMS) are able to improve the stability of different classes of small molecules with respect to classic cryopreservation protocols of fluid samples, even when such microsamples are stored at room temperature.[2] However, similar stability data related to large molecules are still sparse in the scientific literature on the subject.

In this research work we aimed to carry out an in-depth and systematic assessment of mid-term stability of a selected set of peptide compounds in urine sampled by DMS and VAMS, by comparing their degradation rate with that of fluid samples stored at different temperatures (-20, -80°C). The test compounds included therapeutic and/or diagnostic peptide compounds (ACTH, hCG, oxytocin, desmopressin, triptorelin) as well as drug candidates under clinical evaluation (growth hormone releasing factors, hexarelin, alexamorelin, CJC-1293, AOD9604, TB-500), some of which are also included in the list of prohibited substances in sport compiled by the World Anti-Doping Agency (WADA). An original LC-MS/MS method was developed and fully validated, while all the main parameters involved in DMS and VAMS sampling and pretreatment were studied and optimised. This allowed to reach optimal extraction performances and the implementation of a workflow featuring high reproducibility and throughput. The mid-term stability study revealed that DMS and VAMS provided satisfactory results, with all studied compounds recovered in the 80-90% range after 3 months. Moreover, dried matrix stability performance was significantly better than the corresponding one obtained from fluid samples, even when storage was at -80°C. Dried micromatrices proved to be attractive and advantageous alternatives to fluid one for the sampling, storage and testing of low-stability peptide compounds.

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TACKLING CANCER: EXPLORING THE ARYLISATIN SCAFFOLD TO GET THE IDEAL MMPI SELECTIVITY PROFILE

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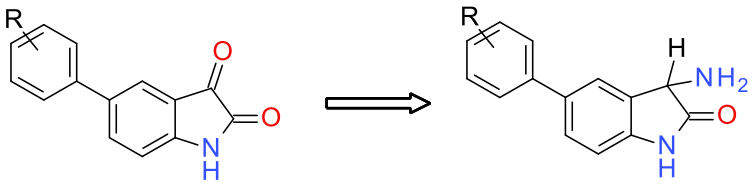
The matrix metalloproteinases (MMPs) are cation-depended enzymes involved in the catabolic degradation of matrix proteins, and playing an important function in the oncogenesis.[1] Based on the MMP-2, MMP-13 and MMP-8 role in cancer pathology,[2] it clearly emerges the advantages that a modulator with the correct selectivity pattern versus these three proteases could slow and/or stop the metastasis progression. Despite the initial efforts in finding MMP inhibitors (MMPIs) for cancer, most of them resulted in Phase III failure due to their lack of specificity.[3] A possible strategy to overcome the selectivity issue is to design compounds not binding the catalytic zinc ion and that exploit all possible interactions in the S1' site to gain potency and selectivity.

To this aim, in a previous study, a structure-based approach has been applied to prioritize metalloprotease oriented fragments. Top ranking hits were tested on a biological assays bringing to the identification of isatin-based compounds.[4]

In the present work, an expanded SAR study has been carried out on the isatin scaffold. Taking into account the arylisatin synthetic pathway, isatin isosteric replacements, chemical feasibility, and following a drug-like guided peripheral decoration, a virtual tangible library of 1000 analogues was generated, enumerated and submitted to the docking evaluation in the MMP S_1' site, to identify the most promising compounds for synthesis. A total of 46 compounds out of that set were selected for synthesis and following enzyme inhibition assays. From this work, it emerges a better understanding of the MMP modulatory properties of the arylisatin scaffold; in particular, we build a SAR model which can be used for further research activity around the 5-phenyl-isatine core. Moreover, we identified a new chemical scaffold that presented an interesting activity profile with the advantage, in addition to provide novel chemistry, to move away from the promiscuous isatin function.[5] A detailed structure-based computational study completes the analysis of the new ligand binding to MMPs.



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MECHANISTIC INSIDE MULTI-ACTION PLATINUM(IV) ANTICANCER AGENTS: FROM MAGIC BULLETS TO CLUSTER BOMBS

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Although all the Pt drugs in the clinic are Pt(II) complexes, Pt(IV) complexes have attracted a lot of attention recently since their chemical properties allow great flexibility in design of novel drugs. Pt(IV) complexes are octahedral complexes that are kinetically more inert than their Pt(II) precursors and may be administered orally. Pt(IV) complexes are believed to act as prodrugs that are activated inside the cancer cells by reductive elimination resulting in the concurrent release the two *axial* ligands, and the Pt(II) complex.[1] Recently it became popular to prepare "dual action" Pt(IV) prodrugs that are Pt(IV) derivatives of cisplatin or oxaliplatin with bioactive axial ligands.[2-4] The main advantage of the “dual action” drugs is the simultaneous release of two anti-proliferative agents that have different mechanisms of action and attack different cellular targets thereby increasing the chances of overcoming resistance to a single drug.

We tried to extend this underlying concept and we prepared also “triple” and “quadruple action” Pt(IV) based prodrugs and studied their properties. Most of the “multi-action” compounds were very potent against a variety of cancer cells, and some were highly effective against KRAS mutated cancer cells. Mechanistic studies revealed that bioactive axial ligands can be effectively used to tune the molecular mechanism of action and the anticancer potential of these Pt(IV) based prodrugs.

Overall, these results open new options for using Pt(IV)-based “multi-action” prodrug as anticancer agents.

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DESIGN AND SYNTHESIS OF NEW QUINAZOLIN-4(3H)-ONE HYBRIDS AS DUAL INHIBITORS OF TUBULIN AND DIHYDROFOLATE REDUCTASE

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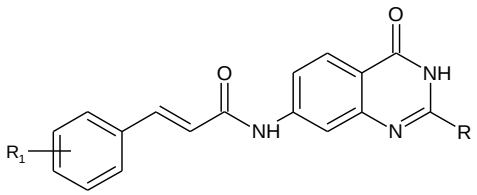
New strategies are needed for fighting anti-cancer with the goal to improve efficacy of anti-cancer therapy and to limit the onset of drug resistance. Indeed, cancer cells are able to set cellular mechanisms for survival and multiple pathways support their survival. The inhibition of one pathway may then result in the activation of an alternative pathway. One strategy useful for combatting this phenomenon is represented by multi-target drugs.
Herein we will present our work aim at identifying new anticancer compounds, which combine dihydrofolate reductase (DHFR) properties with tubulin inhibition. DHFR is a key enzyme involved in the synthesis of raw material for cell proliferation and the inhibition of tubulin protein family is able to suppress mitotic spindle dynamics and causes mitotic arrest and cell death.[1,2]
To identify compounds able to simultaneously recognize the above-mentioned targets and using a molecular modelling approach, we designed a small library of potential DHFR/tubulin inhibitors. The quinazolin-4(3H)-one moiety is a common scaffold of DHFR and tubulin inhibitors and therefore it was select as starting pharmacophore.[2,3] Specifically, we designed compounds **1a-n**, and performed docking studies on both tubulin and DHFR, in comparison with colchicine (a tubulin inhibitor reference compound) and LIH, a lipophilic antifolate. The docking results showed that seven compounds (**1h-n**), characterized by a 2-styryl group in the quinazolinone moiety, exhibited a good interaction with both tubulin and DHFR active sites with score values in the range of -10.7 – -10.2 and -11.0 – -9.9 kcal/mol, respectively. Among all, compound **1k** emerged as the most interesting one, showing a potential good affinity for both enzymes. Building on these in silico results, we synthetized the whole compound series. Future preclinical studies are ongoing to confirm the DHFR and tubulin inhibition properties and therefor their efficacy on cancer cells cytotoxic activity.



1a-n

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NOVEL DIARYL UREA DERIVATIVES AS CANNABINOID TYPE 1 RECEPTOR ALLOSTERIC MODULATORS

Meini, S.;[a] Bertini, S.;[a] Pertwee, R. G.;[b] Stevenson, L. A.;[b] Gado, F.;[a] Digiacomo, M.;[a] Macchia, M.;[a] Manera, C.[a]

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Cannabinoid receptor type 1 (CB1R) is an important target to address several pathological conditions like nausea, pain, multiple sclerosis, obesity, nicotine- and alcohol-addiction. Compounds **1-4**[1] and **5-8**[2] were recently reported in literature as analogs of the diaryl ureic CB1R-allosteric modulator PSNCBAM-1,[3] (Fig. 1). These derivatives showed good results in increasing the binding affinity and in decreasing the receptor functionality of the orthosteric reference compound CP 55,940. In order to identify more potent and selective CB1R-allosteric modulators and to expand the knowledge on the structure-activity relationships for these type of molecules, we designed and synthesized new derivatives combining the structural modifications described in different recently published works,[1,2] and performed the biological assessment of three series of compounds: biphenyl derivatives (A), compounds carrying an amine spatial linker with bipyridinyl structure (B) or without the pyrrolidine ring (C) (Fig. 1).

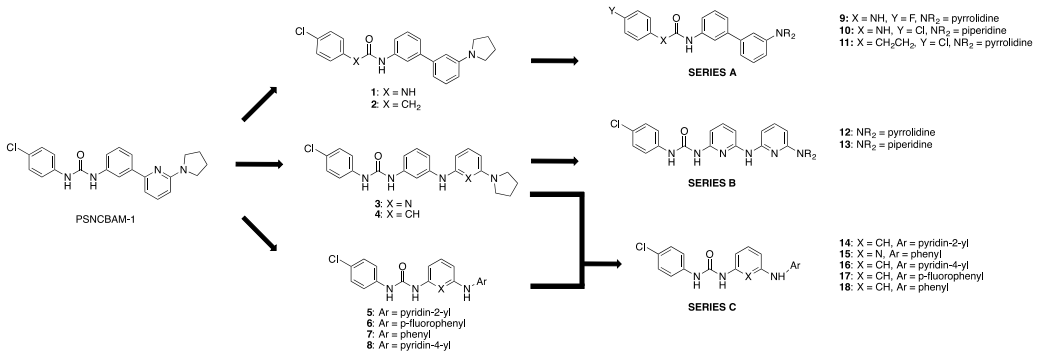

Figure 1. Design of novel structural modifications of PSNCBAM-1 based on the derivatives **1-4**[1] and **5-8**,[2] recently reported in literature.

The compounds were subjected to radioligand binding assays (1 nM - 10 µM), using hCB1-CHO cell membrane preparations. Then, through [^{35}S]GTPγS functional assays, we made a selection of compounds based on their inability to significantly alter the cellular response in absence of the orthosteric agonists. These derivatives were furtherly tested in presence of CP 55,940. Compounds **10** and **17** gave the best biological results, behaving as positive allosteric modulators (PAMs) in binding assays and as negative allosteric modulators (NAMs) in functional assays, in line with the parental compound PSNCBAM-1.[3] This outcome seems to suggest that the combination of the biphenyl system and the piperidine ring and, the association of the *p*-fluorophenyl moiety and the amine spatial linker are significant structural features, which could be taken in account for further developments.

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FLAVONOID DERIVATIVES AS PROMISING THERAPEUTIC ALTERNATIVES IN LEISHMANIASIS AND CHAGAS DISEASE

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Neglected tropical diseases (NTDs) seriously impact on health outcomes in every region of the world.[1] Populations living in poverty with sub-standard sanitation and close contact with infectious vectors are the most affected.[2] According to World Health Organization reports, leishmaniasis, and Chagas disease are the most common NTDs, especially in the Americas.[3] Recently, flavonoids have shown promising activity against leishmaniasis and Chagas disease.[4,5] The objective of the present work was to design and synthesize some flavonoid-based derivatives and evaluate their efficacy against leishmaniasis and Chagas disease. Flavonoid derivatives were synthesized according to standard chemical procedures. Antileishmanial and antichagasic activities were carried out in *Trypanosoma cruzi* and *Leishmania infantum* according to reported methods with slights modifications. The cytotoxicity of the flavonoids was evaluated in human leukemic monocyte cell line (THP-1 cells). As a result, a total of 14 flavonoid compounds (X0-X13) were obtained with good to excellent yields (46.71-98.19%). Compounds X0, X1, and X6 showed good antileishmanial activity (EC_{50} < 10 µg/mL), whilst X11-X12 exhibited potent antichagasic activity (EC_{50} < 40 µg/mL). These bioactive compounds also showed good selectivity against THP-1 cells (Selectivity index > 5). These flavonoids could serve as baseline for the discovery of new antileishmanial and antichagasic agents.

Key words: Neglected tropical diseases; Flavonoids; Leishmaniasis, Chagas disease; Selectivity.

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A NEW CLASS OF POTENT AND SELECTIVE HDAC6 INHIBITORS: SAR ANALYSIS AND DOCKING RESULTS

Vergani, B.; Rocchio, I.; Marchini, M.; Pavich, G.; Sandrone, G.; Caprini, G.; Steinkuhler, C.; Fossati, G.; Stevenazzi, A.

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Histone deacetylase 6 (HDAC6) is the only isoform among the 11 human known HDACs featuring two catalytic domains and a ubiquitin binding Zinc-finger domain. It is usually localized in the cytoplasm and it acts mainly on non-histonic substrates, like tubuline, Hsp90, cortactine. Through acetylation of these important proteins it contributes to the regulation of many different physiologic functionalities, like microtubules dynamics and immune system homeostasis. HDAC6 is an interesting therapeutic target, as it has been demonstrated to be involved in many different pathologies. Some HDAC6 selective inhibitors are under investigation against auto-immune diseases, such as lupus,[1] myasthenia gravis, rheumatoid arthritis[2] and GVHD,[3] oncological disorders, such as melanoma[4] and multiple myeloma,[5] and some CNS syndromes, like Huntington's and Alzheimer's diseases.[6] Moreover, their activity as immune check point modulators is also under exploration.[7] We have recently discovered a new class of potent and selective benzohydroxamate-based HDAC6 inhibitors with a pentaheterocyclic scaffold (see Fig.1).[8] We present here the SAR analysis and docking results on this class of compounds, showing the importance of a methylene or sulfur bridge between the central pentaheterocycle and the benzohydroxamic moiety, the right distances between the cap-term and the central core and, especially, the influence of the benzohydroxamic ring fluorine substitution on the selectivity toward HDAC6 compare to the other isoforms.

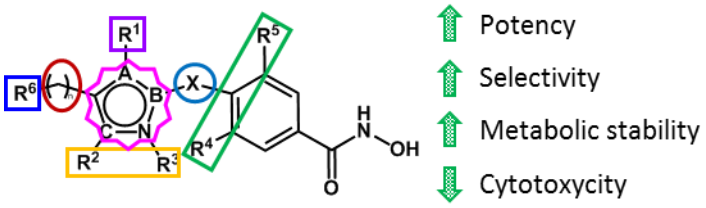

Figure 1. General structure of new potent and selective benzohydroxamate-based HDAC6 inhibitors with a pentaheterocyclic scaffold.

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NEW MULTI-TARGET MODULATOR OF ENDOCANNABINOID SYSTEM FOR AN INNOVATIVE THERAPEUTIC APPROACH IN MULTIPLE SCLEROSIS

Gado, F.;[a] Bertini, S.;[a] Digiacomo, M.;[a] Meini, S.;[a] Reynoso-Moreno, I.;[b] Micheli, L.;[c] Nieri, P.;[a] Carpi, S.;[a] Polini, B.;[a] Cervetto, C.;[d] Tuccinardi, T;[a] Gertsch, J.;[b] Marcoli, M.;[d,e] Ghelardini, C.;[c] Di Cesare Mannelli, L.;[c] Chicca, A.;[b] Macchia, M.;[a] Manera, C.[a,e]

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In a research program aimed at obtaining CBRs ligands, a series of 2-oxo-1,2-dihydropyridine-3-carboxamide derivatives variously substituted on central nucleus were synthesized.[1,2] From these studies we selected the novel multi-target modulator **FM6b** that exerts "pro-cannabinoid" activities by acting with different mechanisms of action (orthosteric agonist activity for both cannabinoid receptors and inhibitory activity on degrading enzymes and AEA transporter) and that is characterized by good drug-likeness.

FM6b was investigated *in vitro* to verify its potential to modulate the production of pro- and anti-inflammatory cytokines in activated mouse BV2 microglial cells and to evaluate its potential neuroprotective effects by limiting neuronal glutamate release, using isolated nerve terminals (synaptosomes) in superfusion system (Figure 1).

Subsequently, the same compound was tested *in vivo* to evaluate its efficacy on the experimental autoimmune encephalomyelitis model of multiple sclerosis in mice and to assess its antinociceptive effect in an animal model of neuropathic pain (Figure 1).

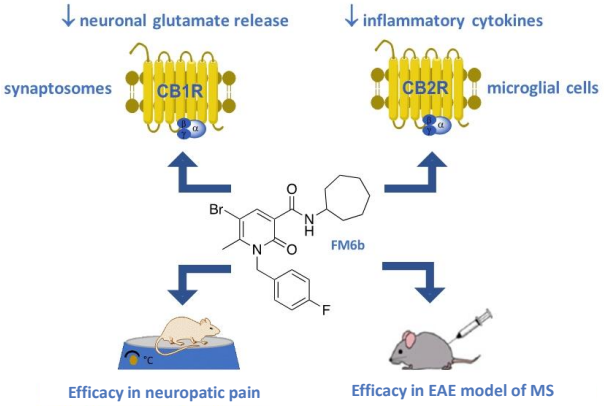

Figure 1. *In vivo* and *in vitro* activity of **FM6b**.

The obtained results suggest that multi-target modulation of endocannabinoid system might represent an innovative therapeutic approach to control the disease progression and at the same time the symptoms of MS.

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