33rd Camerino-Cyprus Symposium

Receptor Chemistry: Reality and Vision

Camerino May 15-19, 2016

Palazzo Ducale

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33rd CAMERINO-CYPRUS SYMPOSIUM

Receptor Chemistry: Reality and Vision

Organized by University of Camerino

School of Pharmacy

under the sponsorship of

Medicinal Chemistry Division of the Italian Chemical Society

European Federation for Medicinal Chemistry

Camerino, Italy May 15-19, 2016

UNIVERSITÀ DEGLI STUDI DI CAMERINO

Palazzo Ducale

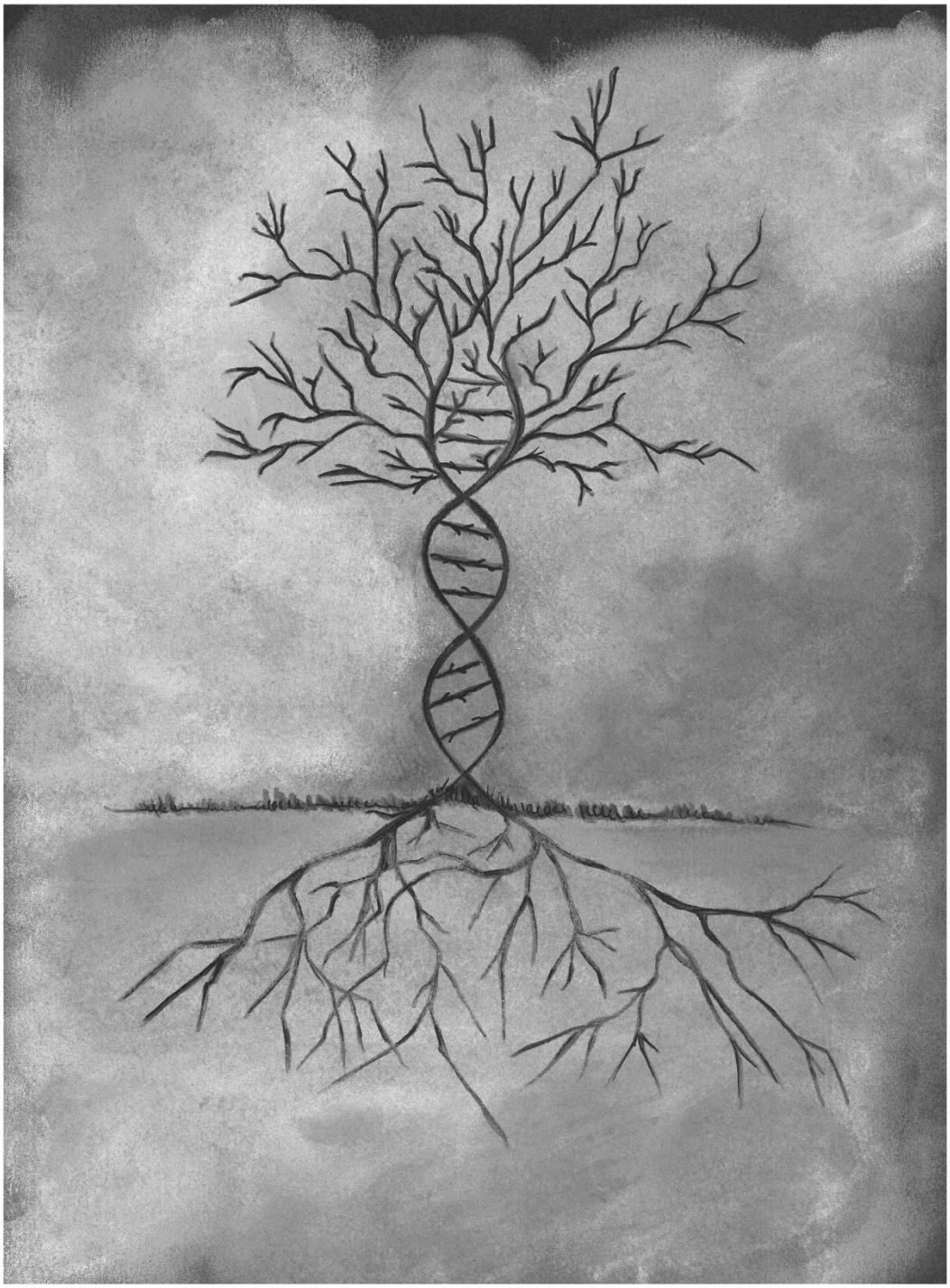

Illustration by Francesca Ghergo

Chemistry and life

Happy molecules orchestrate me
after a coffee or the smile of a friend,
melting from snowflake confetti
into precious raising agents, the basis of all life.

The receptors feel important,
sending positive signals,
opening and closing my pores
like semaphores in every active instant.

Chemistry and life are fused in my body
working together like two good friends
right up to the end, and when I am dead
together they will sever my roots.

Chimica e vita

Molecole felici mi compongono
dopo un caffè o il sorriso di un'amica,
coriandoli di neve che si fondono
preziosi lieviti, la base di ogni vita.

Si sentono importanti i Recettori
che mandano segnali positivi,
si aprono e si chiudono i miei pori
come semafori in ogni istante attivi.

Chimica e vita son fuse nel mio corpo
collaborando come due buoni amici
fino alla fine, e quando sarò morto
recideranno insieme le radici.

Piero Angeli

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PROGRAMME

SUNDAY, MAY 15

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Opening

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Seven Transmembrane Receptors

20:00

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Aula Arangio Ruiz - Palazzo Ducale

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MONDAY, MAY 16

Aula Arangio Ruiz, Palazzo Ducale

Modeling GPCRs in the Structural Biology Era

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Aula Arangio Ruiz, Palazzo Ducale

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Aula Arangio Ruiz, Palazzo Ducale

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12:30
Social Tour to Ascoli Piceno and Social Dinner

Opening Lecture

SEVEN TRANSMEMBRANE RECEPTORS

Robert J. Lefkowitz, MD

Howard Hughes Medical Institute
Duke University Medical Center

Seven transmembrane receptors (7TMRs), also known as G protein coupled receptors (GPCRs) represent by far the largest, most versatile, and most ubiquitous of the several families of plasma membrane receptors. They regulate virtually all known physiological processes in humans. As recently as 40 years ago, the very existence of cellular receptors for drugs and hormones was highly controversial, and there was essentially no direct means of studying these putative molecules. Today, the family of GPCRs is known to number approximately 1,000, and crystal structures have recently been solved for more than two dozen members of the family and even of a receptor-G protein complex. In my lecture, I will briefly review how the field has evolved over the past 50 years, hanging some of the story on my own research throughout this period. Then I will discuss recent developments in the field, which are changing in fundamental ways our concepts of how the receptors function and are regulated. These include the duality of signaling through G-proteins and β-arrestins; the development of "biased ligands"; and the possibility of leveraging this new mechanistic and molecular information to develop new classes of therapeutic agents. Finally, I will discuss recent biophysical and structural studies of receptor-β-arrestin interactions.

Lectures

Session 1

Modeling GPCRs in the Structural Biology Era

MODELING G PROTEIN-COUPLED RECEPTORS IN THE STRUCTURAL BIOLOGY ERA

Stefano Costanzi[a] and Stefano Moro[b]

(Session Chair)

(a) Department of Chemistry and Center for Behavioral Neuroscience, American University, Washington, DC 20016, USA

(b) Molecular Modeling Section (MMS), Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova 35131, ITALY

G protein-coupled receptors (GPCRs) are membrane-bound proteins involved in a wealth of physiological and pathological functions and targeted by many prescription and leisure drugs [1]. Detailed and accurate knowledge of the three-dimensional structure of GPCRs is highly sought after information, as it provides the grounds for rational drug development programs. However, GPCR structures have being historically difficult to solve. The first crystal structure of a GPCR, namely, bovine rhodopsin, was solved only in 2000 [2]. After this initial milestone, a second GPCR, namely the beta2 adrenergic receptor, did not concede to crystallography studies until 2007 [3,4]. However, since then, a number of breakthroughs ushered a new era characterized by the solution of novel GPCR structures at a significantly faster pace.

At the time of this writing, a total of 147 structures for 33 different receptors have been solved (http://www.costanziresearch.com/p/table.html). As this section aims to highlight, the availability of experimental GPCR structures has opened the floodgates for molecular modeling studies meant to design and discover new GPCR ligands and to understand the molecular functioning of the receptors. Notably, the experimental structures, besides directly shedding light onto the topology of the solved receptors, are also invaluable tools for the construction of homology models for those GPCRs for which experimental structures are not yet available.

References:

(1) Pierce, K.; Premont, R.; Lefkowitz, R. *Nat. Rev. Mol. Cell. Biol.* **2002**, 3, 639-650.
(2) Palczewski, K.; Kumasaka, T.; Hori, T.; Behnke, C.A.; Motoshima, H.; Fox, B.A.; Le Trong, I.; Teller, D.C.; Okada, T.; Stenkamp, R.E.; Yamamoto, M.; Miyano M. *Science*. **2000**, 289, 739-745.
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(4) Rosenbaum, D.M.; Cherezov ,V.; Hanson, M.A.; Rasmussen, S.G.; Thian, F.S.; Kobilka, T.S.; Choi, H.J.; Yao, X.J.; Weis, W.I.; Stevens, R.C.; Kobilka, B.K. *Science*. **2007**, 318, 1266-1273.

MODELING AND STRUCTURE BASED DEORPHANIZATION OF GPCRs

Ruben Abagyan, Irina Kufareva, Andrey Ilatovsky, Kirti Kandhwal, Polo Lam

University of California, San Diego, USA; Molsoft LLC

Here we address three challenges related to G-protein coupled receptors, (i) building a 3D model of a complex between a GPCR with its large ligands; (ii) identifying chemical and peptide modulators for an uncharacterized GPCR, and (iii) identify GPCR targets affected by an arbitrary chemical compound. The models are based on the growing body of crystallographic, mutation and sequence data, as well as the data on the screening data of various chemicals. We have done the following: (i) derived and named a set of pockets in all proteins, the Pocketome, with crystallographic structure together with their conformational variation and co-crystallized ligands and quantitative description of the contact residues; (ii) converted these collections into predictive models to predict targets and poly-pharmacology for any new compound; (iii) used the GPCR derived pockets together with alignments of new GPCRs without known three dimensional structures to organize GPCRs by pocket similarity using the weighted pocket sequence distance; (iv) identified and experimentally confirmed modulators of an orphan GPCR by repurposing from close pockets (a collaboration with Tony Ngo, Nicola Smith and Bob Graham, Victor Chang Institute, Sydney, Australia); (v) predicted and experimentally confirmed several known drugs as Smoothened receptor modulators and studied their potential as multi-pathway therapies for the Hedgehog-pathway-dysregulated cancers; and (vi) based on the target screen against the Pocketome-derived models identified and confirmed experimentally two new targets for Praziquantel (a collaboration with Joyce Lee, Bryan Roth, Charles Cunningham and Pauline Cupit). We also applied restrained internal coordinate simulations of a system including a GPCR and one or several ligands to predict their low energy states and the mechanism of action that lead to the following results: (i) designed a crystallizeable construct for a chemokine receptor with a chemokine and explained specificity of chemokines (collaboration with the Handel lab); (ii) built a model for the Succinate receptor and explained the mechanism of action of two new positive allosteric modulators (collaboration with Dio Siegel lab).

(1) Kufareva I, Ilatovsky A, Abagyan R, C. *Nucl Ac.Res.*. **2012**, PMID 22080553, *Database Issue*.

(2) Kufareva I, Handel TM, Abagyan R., *Methods Mol Biol PMID: 26260608*, **2015**

(3) Ngo, T, Kufareva I, Coleman JL, Graham RM, Abagyan R, Smith NJ, C. *Br J. Pharm.* **2016**, *Feb 2*.

STRUCTURAL INSIGHTS INTO TRANSMEMBRANE RECEPTOR SIGNALING

Andrew C. Kruse

Department of Biological Chemistry and Molecular Pharmacology

Harvard Medical School

Transmembrane receptors are critical mediators of virtually every aspect of human biology, but the molecular details underlying the function of these important proteins have remained poorly understood. However, recent advances in membrane protein biochemistry and crystallography have now allowed many transmembrane receptors to be understood an an unprecedented level of detail. In particular, G protein-coupled receptors (GPCRs) are increasingly amenable to structural characterization. The muscarinic acetylcholine receptors represent a prototypical example, and structural studies of these receptors have led to new insights into ligand selectivity, receptor activation, and and GPCR allostery. In addition, techniques developed in GPCR structural biology are now finding application in studies of other important transmembrane receptors. By adapting and extending these methods, we have recently solved the structure of the sigma-1 receptor, an enigmatic non-GPCR receptor with superficial pharmacological similarity to opioid receptors. The structure reveals an unusual fold, with a single transmembrane domain anchoring a membrane-embedded ligand-binding domain. Comparison of structures with multiple bound ligands offers insight into plasticity of ligand recognition and explains prior mutagenesis data. In addition, the structure offers a molecular explanation for receptor destabilization due to disease-associated mutations in humans.

REVELATIONS FROM GPCR PROTEIN-LIGAND STRUCTURES, WITH KEY ROLES FOR WATER AND THE PREDICTION OF KINETICS

Jonathan S Mason and Andrea Bortolato

Heptares Therapeutics Ltd, BioPark, Broadwater Road, Welwyn Garden City, AL7 3AX United Kingdom

An ever increasing number of X-ray structures for G Protein-Couple Receptors (GPCRs) continue to yield interesting and often unexpected revelations on GPCR ligand binding. Structures are now available for all the major Classes (A, B, C, F), crystallized in complex with predominantly antagonist ligands, but also with agonists (full, partial, biased) and allosteric modulators. The unexpected diversity of ligand-binding pocket position, size and shape is now apparent.

The ability to calculate full water networks and estimate the relative free energy of each water molecule is a recent major breakthrough in computational drug design. Water is now considered to be a key component for design along with the ligand and the protein. Initial studies focused on potency, selectivity and druggability and clearly water molecules often play an important role and need to be considered in all structure-based drug design (SBDD), including during docking and scoring. Water-mediated ligand selectivity enables designs where a smaller vs larger molecule can be used, with potentially better drug-like properties. Waters have been shown in many G protein-coupled receptor (GPCR) X-ray structures to play important roles and at times to mediate all polar interactions.

The residence time of a ligand-protein complex is a critical aspect in determining biological effects *in vivo*. We have investigated off-rate kinetics prediction, with encouraging results, using initially a simple model looking just at trapped water energetics, that can be useful for some series but now using molecular dynamics (MD)-based methods. This is needed generally for predicting ligand kinetics as the unbinding event is complex with multiple possible paths; the MD based approach we have developed, based on adiabatic-bias MD and metadynamics[1], aims to decode the role of water dynamics in ligand-protein unbinding. The protocol can provide actionable working hypotheses that are applicable to the rational optimisation of ligand binding kinetics in a drug discovery program, and results of these methods for predicting off-rate kinetics will be presented, together with examples of the critical role of waters in design, for a variety of GPCR targets including the multiple ligand structures from the StaR® (Stabilised Receptor) technology.

(1) Bortolato, A; Deflorian, F; Weiss, D.R; Mason, J.S. *J. Chem. Inf. Model*. **2015**, *55 (9),*1857–1866.

STRUCTURAL BASIS FOR G-PROTEIN SELECTIVITY USING MULTISCALE DYNAMICS AND FRET SENSORS

Nagarajan Vaidehi[1], Ansley Semack[2], Manbir Sandhu[2] and Sivaraj Sivaramakrishnan[2]

[1]*Division of Molecular Immunology, Beckman Research Institute of the City of Hope, Duarte CA- 91006*
[2]*Department of Genetics, Cell Biology & Devleopment, University of Minnesota, Twin Cities, Minneapolis, MN-55455.*

Upon binding to agonists, G protein-coupled receptors (GPCRs) couple selectively to specific G-proteins (such as G_s, G_i and G_q) or β-arrestins to activate specific signaling pathways. Some GPCR agonists exhibit differential responses to signaling pathways, a phenomenon called "functional selectivity".[1,2] The agonists that cause selective signaling with more focused cellular changes are called "functionally selective agonists". Although the discovery of β-arrestin signaling pathway selective agonists has gained traction with growing literature, agonist selectivity for G-proteins (Gs, Gi, Gq, $G_{12/13}$) is relatively obscure. Structure based design of G-protein selective ligands remains a challenge due to the paucity of information on the structural elements of the conformational ensemble of ligand-GPCR-G-protein complexes that lead to G-protein selectivity. There are huge challenges in using crystallography or NMR to determine structural ensembles of multiple ligand-GPCR-effector complexes especially for large number of complexes. We have combined internal coordinate molecular dynamics method[3] at multi-resolution levels with FRET-based measurements[4] of the GPCR-G protein interface in live cells, to build a rational scalable approach to identify structural hotspots that drive effector selection. Using these techniques we have (a) shown that the C-terminus peptide of the Gα-protein is a critical element in G-protein selectivity for various class A GPCRs, (b) identified hotspot residues in the C-terminus peptide of the Gα subunit that contribute significantly to selectivity of the agonist-GPCR pair and (c) switched β2-adrenergic receptor (which is largely a Gαs mediated receptor) to bind and signal to mutated C-terminus peptide of the Gαq protein, and V1AR, vasopressin receptor which is Gq mediated, to signal through mutated Gαs C-terminus peptide. I will discuss the structural basis and provide insight into the Gq versus Gs binding to class A GPCRs. I will also demonstrate the power of using the combination of these scalable and complimentary techniques to discern the G-protein selectivity in several class A GPCRs.

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2) Shukla A.K., Singh G., and Ghosh E., 2014, Trends. Biochem. Sci., 39, 594-602.
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MODELLING THE CANNABINOID 2 RECEPTOR AND STRUCTURE BASED STUDIES OF AGONIST/ANTAGONIST MOLECULAR SWITCHES

Claudio Cavasotto[1], Mariel Spinosa[1], M. Gabriela Aucar[2], Philippe Diaz[3]

[1]IBioBA-CONICET-Partner Institute of the Max Planck Society, Buenos Aires, Argentina; [2]Department of Physics, Universidad Nacional del Nordeste, Corrientes, Argentina; [3]Department of Biomedical and Pharmaceutical Sciences, The University of Montana, Missoula, MA, USA.

There is a growing interest in using agonists of the cannabinoid receptor 2 (CB2) for the treatment of neuropathic pain. Although the structure-based discovery and design of small-molecule modulators is hampered by the lack of CB2 experimental crystal structures, the sustained development of antagonist, inverse-agonist, and agonist-bound G Protein-Coupled Receptors (GPCRs) structures clears the way for reliable homology models (1). Recently, structure-based efforts led to the discovery of non-selective and selective tricyclic carbazole CB2 agonists with activity in the nanomolar range (2). These promising results prompted us to build improved CB2 models, using different modelling strategies, and with ligand-steered optimized binding sites. Our models were validated using published mutagenesis data and small-scale high-throughput docking using the GPCR Ligand Library (GLL) and the GPCR Decoy Database (GDD) (3). The results of our molecular modelling support the successful rationalization of the structure-activity relationship data for agonist and antagonist ligands, especially of a remarkably efficient switch from agonistic to antagonistic behaviour occurred when introducing a methoxy moiety into the series of these novel compounds.

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POLYPHARMACOLOGY OF CONFORMATIONALLY RIGID NUCLEOSIDES: PURINE RECEPTORS AND BEYOND

Kenneth A. Jacobson, Dilip K. Tosh, Antonella Ciancetta

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We substituted a bicyclic ring system ([3.1.0]bicyclohexane) in place of ribose to increase potency/selectivity of nucleosides and nucleotides as ligands at adenosine receptors and P2Y/P2X receptors, respectively (1). This bridged ring system (methanocarba) freezes the conformation of the normally twistable ribose ring into one that is preferred at a given receptor. Two specific methanocarba isomers, North and South, approximate the conformational clusters of ribonucleosides and deoxyribonucleosides found in nature. These ligands contain both flat (nucleobase) and 3 dimensional (ribose-like) moieties that contribute to their versatility. This approach led to the discovery of efficacious agents designed for treating chronic neuropathic pain (A_3AR agonists), thrombosis ($P2Y_1R$ antagonists) and heart failure (P2X4R positive modulators). The recognition of such rigid agonist and antagonist ligands at their purine receptor sites has been modeled by molecular docking or determined by X-ray crystallography. Moreover, we followed the lead of previously undetected off-target interactions of the same series of rigidified adenosines to enhance activity at these unrelated receptors and transporters, and simultaneously to lower affinity at adenosine receptors. Thus, we have repurposed nucleoside ligands to satisfy the pharmacophoric requirements of diverse GPCRs or other targets (2). By comparing docking of congeneric members of this nucleoside series, based on screening data, we can predict the mode of binding to certain off-target GPCRs. Thus, rigid nucleosides can serve as a scaffold for polypharmacology. This polypharmacology can be directed, to either eliminate or enhance off-target activity, through structure-based manipulation of functional groups on the scaffold. The examples of novel allosteric enhancers of tropane binding at the dopamine transporter (DAT) (3) and antagonists of $5HT_2$ serotonin receptors will be presented. The diversity of targets accommodated by this approach suggests that nucleosides are indeed a privileged structure for the design of novel receptor ligands and potentially as therapeutic agents for a wide variety of conditions.

(1) Tosh, D.K.; Finley, A.; Paoletta, S.; Moss, S.M.; Gao, Z.G.; Gizewski, E.; Auchampach, J.; Salvemini, D.; Jacobson, K.A. *J. Med. Chem.* **2014**, *57*, 9901-9914.

(2) Paoletta, S.; Tosh, D.K.; Salvemini, D.; Jacobson, K.A. *PLoS One* **2014**, *9*, e97858.

(3) Janowsky, A. ; Eshelman, A.J.; Tosh, D.K.; Jacobson, K.A. *J. Pharmacol. Exp. Ther.* **2016**, doi:10.1124/jpet.115.229666.

MOLECULAR MODELLING STUDIES ON ADENOSINE RECEPTORS AND THEIR AGONISTS

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The natural occurring nucleoside adenosine (Ado) exerts a number of physiological functions through the interaction with four G protein-coupled receptors called Adenosine Receptors (ARs), which include the A_1, A_{2A}, A_{2B}, and A_3 receptor subtypes (1). ARs represent key targets for the development of pharmacological tools for the treatment of several conditions like CNS disorders, cancer, cardiovascular and inflammation related diseases. The availability of crystal structures of the A_{2A}AR in complex with agonists, antagonists, and inverse agonists provides a set of data useful to interpret the conformational changes of the receptor from inactive to active state, the key residues for the interaction with ligands, and the structural features that make a ligand be able to activate or not the receptor (2). Since the existence of the ARs has been demonstrated, potent agonists have been synthesized by modification and/or substitution of the natural nucleoside Ado. Molecular modelling studies helped to analyse their binding modes at the different receptor subtypes (3,4). In the last years, non-nucleoside structures have been identified as AR agonists. In our recent studies, we analysed non-nucleoside agonists of the ARs to simulate and compare their possible binding modes at these receptor proteins (5,6). Simulations performed at the A_{2A}AR were made by using different arrangements of the binding cavity and various docking tools. Mutagenesis results, reported in literature, worked as reference data for the assessment of the different ligand arrangements observed in this study. The results suggest different possible binding modes, two of which appear compatible with an agonist activity and in agreement with the mutagenesis data. The elucidation of the structural requirements of the non-nucleoside agonists to make them able to activate the ARs could provide a platform to design and develop further compounds with analogue pharmacological profile but belonging to different structural classes.

(1) Chen, J.-F.; Eltzschig, H. K.; Fredholm, B. B. *Nat. Rev. Drug Discov.* **2013,** *12*, 265-286.
(2) Jacobson, K. A.; Costanzi, S. *Mol. Pharmacol.* **2012,** *82*, 361-71.
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(5) Dal Ben, D.; Buccioni, M.; Lambertucci, C.; Thomas, A.; Volpini, R. *In Silico Pharmacol.* **2013,** *1*, 24.
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MUTANT MUSCARINIC RECEPTORS AS NOVEL CHEMOGENETIC TOOLS

Jürgen Wess

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DREADDs (designer receptors exclusively activated by designer drug) represent powerful novel chemogenetic tools to study the physiological relevance of signaling pathways activated by different functional classes of G protein-coupled receptors (GPCRs). Structurally, DREADDs are mutant muscarinic receptors that can be activated by clozapine-N-oxide (CNO), an otherwise pharmacologically inert agent, with high potency and efficacy. Importantly, these new designer receptors cannot be activated by acetylcholine, the endogenous muscarinic receptor agonist. At present, muscarinic receptor-based DREADDs that selectively activate $G_{q/11}$, $G_{i/o}$, or G_s are available. Moreover, we generated an M_3 muscarinic receptor-based DREADD that is unable to active $G_{q/11}$ but retains the ability to recruit beta-arrestins and initiate beta-arrestin-dependent signaling in response to CNO treatment. More recently, we also developed a $G_{q/11}$-biased, M_3 muscarinic receptor-derived DREADD that lacks the ability to interact with beta-arrestins.

During the past few years, we expressed DREADDs with different coupling properties in a cell type-specific fashion in mice, with primary focus on metabolically relevant cell types including hepatocytes, pancreatic beta-cells, and certain neuronal subpopulations of the hypothalamus. CNO treatment of the DREADD-expressing animals leads to the selective stimulation of distinct GPCR signaling pathways only in the DREADD-expressing cells. This approach makes it possible to assess the in vivo consequences of activating distinct GPCR signaling pathways in specific cell types. Clearly, such studies are difficult to perform with native GPCRs which are typically expressed in multiple tissues and cell types.

These studies clearly demonstrated that DREADDs represent highly useful tools to delineate GPCR-dependent signaling pathways that can be targeted for the treatment of various pathophysiological conditions including type 2 diabetes and obesity.

This work was supported by the Intramural Research Program, NIDDK, NIH, US Department of Health and Human Services.

HONORARY DEGREE IN PHARMACEUTICAL CHEMISTRY AND TECHNOLOGY

Camerino 16 May 2016

Encomium

Mario Giannella

Knowing that I would be delivering this encomium, the local newspaper asked me to provide the title for an article dedicated to Nobel Laureate Robert Lefkowitz's visit to Camerino. My suggestion was "A remarkable protagonist of a fascinating story". That story, of course, concerns receptors. The story begins at the end of the 19th Century with a brilliant insight from the distinguished English physiologist John Newport Langley and continues with other equally important scholars. These include Paul Ehrlich, the 1908 Nobel Laureate for Medicine and the father of Chemotherapy, and Otto Loewi, also the recipient of the Nobel Prize for Medicine in 1936. In 1921, Loewi demonstrated for the first time that the transmission of neural impulses depended on a chemical substance (in this case, acetylcholine) being released into the synapse.

But despite these successes, the pharmacology of receptors, or receptorology, continued to be met with a cold skepticism. Even the physical existence of receptors was so controversial that, as late as 1973, the esteemed pharmacologist Raymond Ahlquist published an article outlining an abstract physiological concept, whose only virtue was to explain the responses of tissues to interactions with chemical substances.

It was in this climate that, at the start of the 1970s, Robert Lefkowitz and his collaborators embarked on a voyage of exploration into territory so unknown and beset with uncertainties that we can rightly call it 'odyssean'. At the end, however, they reached their Ithaca. Thanks to their research, receptors were transformed from hypotheses concerning their existence into real chemico-physical entities. On 10 October 2012, the Royal Swedish Academy of Sciences awarded the Nobel Prize in Chemistry to Robert Lefkowitz and Brian Kobilka (a young medical fellow working with Lefkowitz) for "studies of G-protein-coupled receptors (GPCRs)". GPCRs are one of the largest receptor families, with more than 1000 members regulating virtually all physiological processes in mammals. The Nobel committee's decision was welcomed not only by chemists but also by researchers working in scientific disciplines such as biochemistry, toxicology and, above all, pharmacology. Indeed, over the years, researchers in each of those sectors have made important contributions to the story of receptors and will likely continue to do so.

One of the initial keys to the success of Lefkowitz's studies is that they combined, with great intelligence and felicitous inspiration, the development of molecular biology with that of several new technologies, such as the solubilization of receptor proteins, their purification, and their reconstitution. β_2 Adrenoreceptors are of particular interest to him for various reasons, most notably for their role in cardiovascular function. Hence the introduction of beta blockers into clinical practice, thanks to the work of James Black (the 1988 Nobel laureate for medicine). Their specificity allows them to be used as a starting material for constructing radioligands or affinity chromatography matrices. Finally, β_2 adrenoreceptors activate adenylate cyclase, an enzyme studied by the physiologist Earl Sutherland (recipient of the 1971 Nobel Prize for Medicine).

In light of the above, determining the β_2 adrenoreceptor's nature and mechanism of action (technically known as 'transduction') was a particularly intriguing project. The first success came with the purification of the receptor, using an affinity chromatography procedure that was repeated more than 100,000 times with a final yield of 25-50 micrograms. The functional identity of this approximately 60,000-dalton protein was subsequently validated by its reconstitution in phosopholipid vesicles, where it was demonstrated to behave identically to the protein obtained by purification. Finally there was no longer a reason for the existence of skeptisism concerning the existence of receptors!

During their research, Lefkowitz and his collaborators, including Kobilka, cloned the gene for the β_2 adrenoreceptor, definitively clarifying the complex sequence of its mechanism of action: interaction with the ligand, activation of the G-protein coupled to the receptor, activation of adenylate cyclase, and formation of cyclic adenosine monophosphate (cAMP) as the second messenger. The other important result, confirmed by determining in quick succession the structures of the other eight adrenoreceptors as well as many others (including muscarinic receptors and the dopamine D1 receptor), is that the GPCRs all present a similar serpentine structure determined by an amino acid sequence that crosses the cell membrane seven times. Indeed, '7TM receptors' is a more accurate term than GPCRs because, as would later be demonstrated, these receptors can function independently of G-protein activation. The great surprise emerging from this research was that this receptor family presents an extraordinary structural similarity to the visual pigment rhodopsin. Rhodopsin was already the subject of intense investigation by biochemists thanks to its centrality in visual processes and its notable abundance in the bovine retina, which facilitated its isolation and study. As with β-adrenoreceptors, rhodopsin's function depended on G-protein activation, in this case by photons. Thus we see how, as the number of receptors identified grew (Lefkowitz says that he never imagined that the 7TM family would grow to be so large and

diverse), there was also a growth in the number and type of transmitters identified. These included not only small molecules like biogenic amines, but also carboxylic acids and amino acids, neuropeptides, chemokines, lipids, nucleotides, peptide hormones and pheromones, taste and smell mediators, photons, Ca^{2+} ions and protons, and gaseous substances. Who would have imagined, for example, that nitric oxide, found in car exhaust fumes and legally restricted due to its toxicity, could number among the endogenous mediators? This discovery earned Furchgott, Ignarro and Murad the 1998 Nobel Prize for Medicine.

It was a short step from discovering the structure of the receptors to discovering their mechanism of action: the first model of a ligand-receptor-G-protein ternary complex was proposed to understand how these receptors interact with the guanine nucleotides (GTP or GDP), the study of which led to the 1994 Nobel Prize for Medicine being awarded to Alfred Gilman and Martin Rodbell. Activating the G-protein leads to the formation of a second messenger. By stimulating adenylate cyclase, the second messenger will be cAMP, which was one of Lefkowitz's initial research interests. By stimulating phosphodiesterases, the second messengers will be inositol trisphosphate (IP3) and diacylglycerol (DAG) with the subsequent release of calcium ions (Ca^{2+}). Each of these second messengers, coupled to a cascade, can in turn be substrates for other enzymatic systems, greatly amplifying the initial signal.

Using mutagenesis protocols to create chimeric receptors was an inspired move and decisive for understanding the relationship between the structure and function of the receptors. The first example involved assembling, in a single structure, different filaments of the β_2 adrenoreceptor and the α_2 adrenoreceptor. As previously noted, stimulating the β_2 receptor activates adenylate cyclase with the subsequent formation of cAMP. In contrast, stimulating the α_2 receptor inhibits adenylate cyclase. It was thus possible to use the interaction of suitable ligands to identify distinct regions of the hybrid, that is, specific amino acid sequences, as being responsible for G-protein coupling or for ligand binding.

This study produced an additional result, which Lefkowitz describes as a fortunate accident, although we agree with Louis Pasteur that "in the field of observation, fortune favours the prepared mind". Specifically, this study led to the discovery of the intrinsic activity of receptors, that is, their capacity to produce a response even in the absence of an agonist. This activity can be explained by the fact that the receptor can assume a variety of conformations. The active conformation induced by the agonist can exist, albeit in a reduced magnitude, in its absence.

The next step was discovering the mechanism by which the signalling is stopped. This involves a sequential process whereby the receptor is phosphorylated and then reacts with a protein called arrestin. The receptor is then internalized into the cell, where it is either degraded or recycled by dephosphorylation and returned to the cell membrane.

At this point, the receptor can no longer be considered a simple switch, which turns the neural impulse on or off. Instead, it assumes the character of a biological microprocessor, which receives a variety of extracellular signals and transfers them to a wide variety of cytosolic receivers. It's as if the endogenous ligands, receptors, G-proteins, effectors, kinases, second messagers, and arrestin were in perfect harmony, like a single orchestral body, transforming the initial signal into an extraordinary concert to bring about the final pharmacological effect. For researchers working in drug discovery, Lefkowitz's studies revealed a great multitude of potential drug targets at which they can aim new biologically active molecules that are increasingly specific (and therefore less toxic) and increasingly effective. Indeed, structure-based drug design has been made possible by understanding the chemical nature and three-dimensional structure of those sites where binding, through a clearly defined mechanism, brings about specific functional effects.

Not by chance, wishing to repropose for drug-receptor interaction the suggestive "lock and key" image proposed by Fisher in 1894, 30-50% of all prescription drugs behave as the most ideal keys for unlocking access to treatment of pathologies such as migraines, ulcers, hypertension, coronary heart disease and neurodegenerative diseases, asthma, allergies, and some types of cancer. Citing an Italian study, even the path of love is presided over by a whole series of receptors: from adrenaline during the moment of "love at first sight" to dopamine during the phase of passion, from oxytocin during the tender bonding to endorphins in the final tranquility.

Have we reached the end of the story of receptors? On the contrary: many outstanding questions still remain. The dimerization of receptors, the deorphanization of the dozens of receptors discovered in the human genome sequence, the discovery of new mechanisms of transmission, and polymorphisms are still waiting to be fully clarified. Thus we can conclude with Lefkowitz that "However exciting and surprising the past has been, the future will be even more so!"

Session 2

Understanding GPCR-Ligand Interactions

UNDERSTANDING GPCR-LIGAND INTERACTIONS

Rob Leurs

(Session Chair)

Vrije Universiteit Amsterdam, the Netherlands

As one for the preferred drug target classes, insights in G protein coupled receptor structure and function has been crucial for new developments in drug discovery, since the initial discovery of GPCRs and their signalling pathways. With recent developments in the area of GPCR structural biology and the application of new computational and medicinal chemistry approaches, like fragment-based drug discovery, the discovery and rational design of ligands with desired properties (e.g. kinetics of ligand binding) have entered a new era.

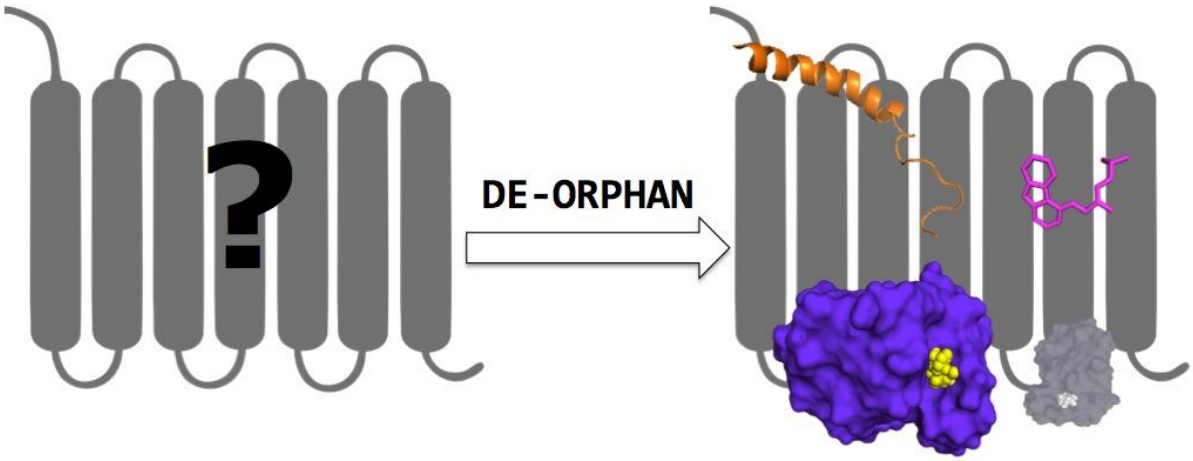
IDENTIFICATION OF ENDOGENOUS AND SURROGATE LIGANDS FOR ORPHAN G PROTEIN-COUPLED RECEPTORS

David E. Gloriam, Hans Bräuner-Osborne

Department of Drug Design and Pharmacology

University of Copenhagen

We recently reported putative endogenous ligands, l-α-amino acids, L-tryptophan and l-phenylalanine, for the orphan receptor GPR139[1]. Herein, we will report a novel peptide that can activate this receptor with higher potency, and could represent the endogenous ligand. We have also identified the first potent and selective small-molecule allosteric antagonists for the orphan receptor GPRC6A[2], and agonists for GPR32 and GPR132[3], by computational methods. Funded by the ERC and the Lundbeck Foundation, two new 5- and 7-year programmes aim to identify endogenous peptide ligands for orphan receptors using bioinformatics, and pharmacological tool compounds by focused screening libraries, and G protein inhibitors by structure-based drug design. Tailored tools for GPCR analysis and ligand design are made available through the GPCR database, GPCRdb[4] (gpcrdb.org).



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STRUCTURAL BIOLOGY OF THE EDG RECEPTORS

Michael A. Hanson

GPCR Consortium

The Edg receptor family are lipid binding GPCR that have been widely studied using biochemical methods and a number of pharmaceuticals have been developed targeting its prototypical member, S1P1. The structure of the S1P1 receptor has been determined and utilized to understand the binding mode of pharmaceutical agents for the treatment of relapsing multiple sclerosis and other autoimmune disorders. Recently, structural analysis of the LPA1 receptor has revealed alternate binding modes for lipid ligands that may be more permissive for binding a wide array of endogenous lipid ligands. Utilizing this structure we discover overlap between the LPA and cannabinoid signaling pathways. Recent activity within the GPCR Consortium promises to provide even more surprises from this diverse class of receptors

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GPCR LIGAND BINDING KINETICS: FROM X-RAY STRUCTURE TO UNDERSTANDING OF ANTAGONIST DISSOCIATION KINETICS

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This presentation will show how the recent breakthroughs in GPCR structural biology can be complemented by computational and experimental medicinal chemistry studies for a more accurate description and prediction of molecular and structural determinants of ligand binding kinetics in different key binding regions of the histamine receptors. Biocomputational approaches including structure-based virtual screening, protein-ligand interaction fingerprint scoring and customized molecular dynamics simulation techniques are used to discover and guide the design and synthesis of new GPCR ligands and elucidate the molecular mechanism of histamine receptor ligand binding kinetics.

FRAGMENT LINKING FOR GPCRs: A CASE STUDY ON DOPAMINE D3 RECEPTORS

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Fragment based drug discovery (FBDD) employs growing and linking strategies for optimization. Structural information on G-protein coupled receptors (GPCRs) made FBDD available on this class of targets, however, most reported programs applied a growing strategy for compounds with optimized properties. We developed a sequential docking methodology employing Glide to support the identification and linking of fragment hits. Predicting the binding mode of multiple fragments bound to a single target we assessed the sampling and scoring accuracy for the first and second site binders in self- and cross-docking situations. The promising results obtained prompted us to test our docking approach prospectively on GPCRs. The sequential docking methodology was applied to computationally predict starting points for fragment linking using the human dopamine D3 receptor crystal structure and a human dopamine D2 receptor homology model. Two focused fragment libraries were docked in the primary and secondary binding sites, and best fragment combinations were enumerated. Similar top scoring fragments were found for the primary site, while secondary site fragments were predicted to convey selectivity. Three linked compounds were synthesized that had 9-, 39-, and 55-fold selectivity in favor of D3 receptor. The structural assessment of the subtype selectivity of the compounds allowed us to identify further compounds with high affinity and improved selectivity.

REVISITING MECHANISM OF ACTION AND CLINICAL PROFILE OF CHOLINE ALPHOSCERATE. AN EXPERIENCE IN THE FIELD OF COGNITIVE DYSFUNCTIONS

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Treatment of adult-onset dementia disorders including Alzheimer's Disease (AD) represents is a challenge for pharmacotherapy. The first therapies introduced in clinical practice for symptomatologic treatment of AD were cholinergic precursors, but trials with choline or the choline-containing phospholipid phosphatidylcholine (lecithin) failed to show relevant effects. Five (acetyl)-cholinesterase inhibitors (ChE-I) [rivastigmine (Exelon), galantamine (Razadyne, Reminyl), tacrine (Cognex), donepezil (Aricept)] and subsequently the NMDA receptor antagonist memantine (Namenda) were approved by US Food and Drug Administration (FDA) and European Agency for the Evaluation of Medicinal Products (EMEA) for the treatment of cognitive manifestations of AD. These compounds slow modestly the progression of cognitive symptoms and reduce problematic behaviors in some people, but problems of lack of efficacy in some individuals and a gradual loss of activity are commonly reported.

It is since 1993 that a group of neuromorphology research started its activity in Camerino for investigating neuroanatomical correlates of hypertensive brain damage primarily using spontaneously hypertensive rats (SHR). These investigations did bring to the suggestion that SHR may represent a model of cerebrovascular brain injury and to some extent of vascular dementia. Research in this field was subsequently extended to the investigation of cholinergic and monoaminergic systems on the above animal model and to the analysis of the influence of drugs (primarily ChE-I and choline-containing phospholipids) on neuroanatomical, neurochemical and behavioural correlates of brain injury in SHR. A main contribution of this research was the identification that the association between a cholinesterase inhibitor and the cholinergic precursor choline alphoscerate is more affective than single compounds in increasing braiun acetylcholine and in countering brain damage occurring in this model. Choline alphoscerate (alpha-gliceryl-phosphoryl-choline, GPC) is among cholinergic precursors the most effective in enhancing acetylcholine biosynthesis and release in animal models.

The association with choline alphoscerate plus donepezil was also investigated in clinical settings, by the ASCOMALVA (Effect of association between a ChE-I and choline alphoscerate on cognitive deficits in AD associated with cerebrovascular injury) trial. It is a double-blind trial investigating if the ChE-I donepezil and choline alphoscerate in combination are more effective that donepezil alone. The trial has recruited AD patients suffering from ischemic brain damage documented by neuroimaging and has completed 2 years of observation in 113 patients of the 210 planned. Patients were randomly allotted to an active treatment group (donepezil + choline alphoscerate) or to a reference group (donepezil + placebo). Cognitive functions were assessed by the Mini-Mental State Evaluation and Alzheimer's Disease Assessment Scale Cognitive subscale. Daily activity was evaluated by the basic and instrumental activities of daily living tests. Behavioral symptoms were assessed by the Neuropsychiatric Inventory. Over the 24/36-month observation period, patients of the reference group showed a moderate time-dependent worsening in all the parameters investigated. Treatment with donepezil plus choline alphoscerate significantly slowed changes of the different items analyzed suggesting that combination of choline alphoscerate with a ChE-I may prolong/increase the effectiveness of cholinergic therapies in AD with concomitant ischemic cerebrovascular injury.

Session 3

Advanced Clinical Studies with Drugs for the Treatment of Alzheimer's and Parkinson's Disease

(Session in memory of Dr. Wolfgang Froestl)

ADVANCED CLINICAL STUDIES WITH DRUGS FOR THE TREATMENT OF ALZHEIMER'S AND PARKINSON'S DISEASE

David A. Lowe

(Session Chair)

AC Immune SA; EPFL Innovation Park; Building B; CH-1015 Lausanne; Switzerland

Neurodegenerative conditions such as Alzheimer's and Parkinson's Disease represent a major challenge to society, with major initiatives across the pharma and biotech industry, not-for profits, academia and government in the search for efficacious therapeutics and diagnostics. In this session '"Advanced Clinical Studies with Drugs for the Treatment of Alzheimer's and Parkinson's Disease" we will hear from three US-based biotech companies about their progress with molecules acting at three interesting molecular targets: Adenosine A2A, Sigma-1 and RAGE. The session was organized by Dr Wofgang Froestl of AC Immune, who had a long-standing association with the Camerino-Cyprus Symposia, but who sadly passed away in September 2015. Dr Froestl had a successful career at Ciba-Geigy and then Novartis, where he made major internally-recognized contributions to the GABAb field. This was followed by several highly productive years at AC Immune in which he made major contributions to building the Company's technical platforms in antibodies, vaccines and small molecular weight therapeutics, as well as PET imaging agents. The organizing committee and AC Immune have dedicated this session to the memory of Wolfgang and his many contributions to the neuroscience field.

TOZADENANT, A POTENT AND SELECTIVE ADENOSINE A_{2A} RECEPTOR ANTAGONIST

Stephen Bandak[1], Robert A Hauser[2], C Warren Olanow[3], Karl D Kieburtz[4], Emmanuelle Pourcher[5], Any Docu-Axelerad[6], Mark Lew[7], Olexandr Kozyolkin[8], Ann Neale[1], Chris Resburg[1], Uwe Meya[1], Christopher Kenney[1]

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Tozadenant is a new chemical entity that potently and selectively inhibits the adenosine A2a receptor (Ki = 5 nM) with >100-fold lower affinity for the other adenosine receptor subtypes and >1000-fold lower affinity for 132 other pharmaceutically important targets tested. Its efficacy and safety were evaluated in a Phase2b randomized, double-blind, placebo-controlled dose-finding study in Parkinson's patients taking levodopa and experiencing end-of-dose wearing off. Eligible patients were aged 30–80 years, had been on a stable regimen of PD drugs for at least 4 weeks and were randomized to placebo or four doses of tozadenant (60, 120, 180 and 240 mg BID) in a 1:1:1:1:1 ratio. In 420 subjects (mean age 63.3 years, mean duration of PD 8.7 years) mean OFF-time at baseline was about six hours per day. The primary endpoint was the reduction in the mean total hours of awake time per day spent in the OFF-state over the 12-week treatment period. Secondary endpoints included the number of total hours spent in the ON state while awake; the number of hours spent in the ON state without/with troublesome dyskinesia, a variety of scores on the Unified PD Rating Scale (UPDRS), and both clinical and patient impressions.

Compared with placebo, mean daily off-time was significantly reduced in the tozadenant 120 mg twice-daily group (−1·1 h, −1·8 to −0·4; p=0·0039), and the tozadenant 180 mg twice-daily group (−1·2 h, −1·9 to −0·4; p=0·0039). Improvements in secondary endpoints were consistent with those in the primary. Tozadenant was generally well tolerated.

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ANAVEX-2-73, A SIGMA-1 RECEPTOR AGONIST: AN OVERVIEW OF THE CLINICAL DEVELOPMENT PROGRAM

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AZELIRAGON; A RAGE ANTAGONIST IN PHASE 3 CLINICAL TRIALS FOR THE TREATMENT OF ALZHEIMER'S DISEASE

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The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin supergene family, a cell surface receptor that is overexpressed in brain tissues of patients with Alzheimer's disease (AD). RAGE is an important cellular cofactor that binds with ligands that are implicated in multiple etiologies of AD, including amyloid beta (Aβ) transport into the brain, the phosphorylation of tau, chronic inflammation, vascular dysfunction, metabolic dysregulation and neurotoxicity. These effects are attenuated following antagonism of the RAGE receptor.

The application of TTP Translational Technology® to the discovery of RAGE antagonists provided multiple chemical series of RAGE antagonists. Azeliragon (TTP488) is a potent and selective member of one of those chemical classes which binds to RAGE. Azeliragon is an orally administered, small molecule drug candidate that has the potential to be among the first FDA approved disease-modifying AD therapeutics due to its novel mechanism of action of inhibiting RAGE.

Azeliragon is efficacious when given in vivo a) in inhibiting plaque deposition and its accompanying inflammation in a mouse model of systemic amyloidosis, and b) in attenuating the Aβ plaque deposition and accompanying cognitive defects in a transgenic mouse model of AD.

Azeliragon has been studied in multiple phase 1 and phase 2 human clinical trials. In an analysis of data collected in our Phase 2b clinical trial, azeliragon slowed the progression of cognitive decline in mild and mild-to-moderate AD patients. Azeliragon has the potential to offer a novel modality in AD therapeutics and represents a new approach for the treatment of AD.

Azeliragon is currently in a Phase 3 clinical trial, the STEADFAST study under an FDA-agreed SPA.

Session 4

Dopamine D2-Like Receptors: from Structure and Function to Potential Therapeutics

DOPAMINE D2-LIKE RECEPTORS: FROM STRUCTURE AND FUNCTION TO POTENTIAL THERAPEUTICS

Stefania Butini,[a] Alessandro Bonifazi,[b] Aloke Dutta,[c] Amy Hauck Newman[b]

(Session Chair)

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The neurotransmitter dopamine (DA) exerts it effects via DA receptors with varied signaling transduction mechanisms and expression patterns in the brain. DA receptors belong to the G protein-coupled receptor (GPCR) superfamily and are divided into two subfamilies. The D1-like DA receptors (D1 and D5) couple to stimulatory G_s proteins and enhance adenylyl cyclase (AC) activity and increase cytosolic cyclic adenosine monophosphate (cAMP) levels. D2-like DA receptors (D2, D3 and D4) couple to inhibitory $G_{i/o}$ proteins that suppress AC activity and decrease cAMP. Within the D2-like receptor subfamily, the D2 and D3 receptors are the most homologous pair, sharing extensive sequence identity in the transmembrane domain (~78%) and the putative ligand binding sites. Herein, we focus on the dopamine D2-like receptors as targets for medication development and efforts to parse out structural determinants of receptor subtype affinity, selectivity and efficacy. Stefania Butini will introduce the topic of D2-like receptors focusing on efforts toward multi-targeted ligands as potential new treatments for schizophrenia. Alessandro Bonifazi will describe the elucidation of structure-activity relationships in a series of D2-like receptor ligands, based on sumanirole, and the discovery of novel G-protein-biased agonists. Aloke Dutta will describe novel dopamine D3 receptor preferring agonists and their potential for the treatment of Parkinson's Disease. Finally, Amy Newman will highlight the development of a highly selective D3 receptor antagonist that attenuates self-administration and reinstatement to drug seeking of the prescription opioid, oxycodone, in rats, and tetrahydrocannabinol (THC), in squirrel monkeys. A discussion of the challenges to translate preclinical findings to the clinic in the D2 receptor class of agents will follow.

D_2-LIKE RECEPTORS AS THE BASE OF MULTI-TARGET DIRECTED LIGANDS FOR THE TREATMENT OF SCHIZOPHRENIA

Stefania Butini

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Dopamine and its five receptor subtypes are intimately involved in numerous neurological disorders and in drug addiction and dependence. In particular, the D_2-like receptors play a pivotal role in the regulation of a variety of behaviors, and have been established as valuable pharmacological targets for the treatment of neurological and psychiatric disorders. Most neurological diseases have a multifactorial nature and the number of molecular mechanisms ascertained as underpinning these diseases is continuously evolving. Neurological diseases such as schizophrenia, depression, and others share a complex origin related to a complicated and intertwined dysregulation of multiple neurotransmitter systems. In this view, these systems cannot be considered separately; hence the treatment of these diseases requires the development of drugs displaying a multireceptor affinity profile including D_2-like receptors and a series of other GPCRs, mostly serotoninergic. In this frame, as a result of our longstanding medicinal chemistry efforts in the field, we gathered considerable experience in the development of multitarget directed ligands as D_2-like receptor antagonists featuring different extents of D_2-like affinity.[1-6]

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NOVEL BITOPIC LIGANDS BASED ON THE SUMANIROLE PHARMACOPHORE REVEAL DOPAMINE D2 RECEPTOR (D2R) BIASED AGONISM

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A remarkable diversity of neuropsychiatric disorders have been associated with dysfunction of dopamine (DA)-containing neurons, including schizophrenia, bipolar disorder (BD), Parkinson's disease (PD), and restless legs syndrome (RLS). In such disorders, DA transmission in discrete pathways may undergo hypoactivation or hyperactivation. Specifically those involving the D_2R subtype provide the rationale for pharmacotherapeutic treatment approaches to activate or block D_2R. Sumanirole, originally developed for the treatment of PD and RLS, is a full agonist showing good D_2R affinity (Ki = 46 nM) and modest D_2R selectivity (D_3R/D_2R = 12). Although it has not been approved for clinical use, it is a valuable preclinical tool and provides a template for lead optimization. G protein-coupled receptors (GPCRs) can adopt several biologically active states differentially stabilized by ligands binding to distinct sites (e.g., orthosteric ligands and allosteric modulators). Although classical approaches to GPCR drug design have targeted the orthosteric binding site (OBS), a secondary binding pocket (SBP) may be an alternative target as many receptors have been reported to possess such sites. The molecular bivalent design has been used successfully over the last decade to synthesize highly selective D_3R antagonists as it combines a primary pharmacophore (PP), for binding in the OBS, and a secondary pharmacophore (SP), able to recognize the SBP by linkers of appropriate length. In the current study, a novel series of D_2R ligands has been designed and synthesized linking sumanirole or N-propyl substituted sumanirole pharmacophores with several secondary molecular fragments, inspired by known GPCR ligands, in both positions *N*-1 and/or *N*-5. The resulting hybrid or 'bitopic' ligands were designed to explore the molecular requirements of the SBP and determine effects on D_2R affinity, selectivity and potential biased agonist properties. To be able to simultaneously reach both OBS and SBP, and to determine the distance between the two sites, the two pharmacophores have been linked with methylene and polyethylene glycol chains of varying lengths. All the newly synthesized compounds were tested in radioligand binding assays using an agonist radioligand [^{3}H]7-OHDPAT to label hD_2R, hD_3R or hD_4R stably expressed in HEK293 cells. It has been recently observed how discrimination amongst D_2R G-proteins and/or β-arrestin activation pathways may be directly involved with the therapeutic actions of antipsychotic drugs. For this reason, on the basis of their binding affinities, the new compounds were also evaluated for their agonist and/or antagonist activities in five different BRET-based constructs: i) Gi protein engagement, ii) Gi protein activation, iii) Go protein activation, iv) adenylyl cyclase inhibition, and v) β−arrestin2 recruitment. The identification of highly potent D_2R biased agonists with different structural templates herein opens the door to understanding the molecular determinants for D_2R functional selectivity and provides clues to improve dopamine D_2R or D_3R selectivity.

This work is supported by the NIDA-Intramural Research Program, National Institutes of Health

IMPLICATION OF MULTIFUNCTIONAL D3 PREFERRING AGONISTS AS THERAPEUTIC AGENTS IN PARKINSON'S DISEASE

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Interest in the search of novel dopamine receptor agonists and antagonists has been very high for a considerable period of time, as these receptors have been implicated as the principal targets for drug development for the treatment of various neurological disorders such as Schizophrenia, Parkinson's disease, drug abuse, etc. Targeting the dopamine D3 receptor, a subfamily of the dopamine D2-class of receptors, for various CNS disorders is drawing much attention because of its unique location in the brain. It has also been shown that selective D3 receptor agonists can provide neuroprotection in Parkinson's disease by inducing brain derived neurotrophic factors (BDNF). In our overall goal to develop molecules either with preferential affinity at D3 receptors or non-selective D2/D3 agonists, we undertook a hybrid drug development approach by combining known dopamine agonists moiety with a substituted piperazine fragment. Our drug development effort resulted in production of number of lead molecules, which showed variable selectivities for D3 over D2 receptor subtypes. In this regard, one of our compounds, D-440, exhibited one of the highest selective agonist potencies for the D3 receptor known to date (EC50 , D2 = 114 nM, D3 = 0.26 nM, D2/D3 = 438). In *in vivo* studies, our lead molecules D-264, D-512 and others were found to be efficacious in PD animal models and were also found to be neuroprotective due to their multifunctional properties. Our hybrid molecular template allows us to introduce a unique set of relevant multifunctional properties without compromising agonist potency. Our overall goal is to develop suitable multifunctional dopamine agonists to address both symptomatic relief (e.g. motor dysfunction) and further development of disease modifying neuroprotective effects to slow or stop the progression of PD. In order to understand the interaction of our hybrid compound with the dopamine receptors at a molecular level, we carried out several studies with mutant dopamine subtype dopamine receptors. Thus, the interaction of our selected compounds with D3 mutant receptors e.g. S192A, T369V, D110N etc. led to greater insight into their molecular interaction. In our presentation, details information of structural and functional characterization along with highlights of in vivo animal data will be provided.

This work is supported by National Institute of Neurological Disorders and Stroke/National Institutes of Health (NS047198, AKD).

VK4-116 - A NOVEL AND HIGHLY SELECTIVE DOPAMINE D_3 RECEPTOR ANTAGONIST THAT ATTENUATES OXYCODONE AND THC SELF-ADMINISTRATION

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The dopamine D_3 receptor (D_3R) is a target for the development of medications to treat substance use disorders. D_3R-selective compounds with high affinity and varying efficacies have been discovered, providing critical research tools for cell-based studies that have been translated to *in vivo* models of drug abuse. D_3R antagonists and partial agonists have shown especially promising results in rodent models of relapse-like behavior, including stress-, drug- and cue-induced reinstatement of drug seeking. However, to date, advancement to human studies has been limited. The high resolution D_3R crystal structure has provided a new opportunity for structure-based drug design in combination with small molecule structure activity relationships (SAR). Using this hybrid approach, a highly potent and selective dopamine D_3R antagonist, VK4-116, has been discovered. VK4-116 demonstrates high D_3R binding affinity, with a K_i=6 nM and ~2700-fold selectivity over D_2 and D_4 receptors. Given that VK4-116 showed excellent metabolic stability in both rats and monkey liver microsomes, we evaluated its *in vivo* efficacy in these species. In rats trained to self-administer the prescription opiate oxycodone, under an FR1 schedule, VK4-116 attenuated self-administration at 15 and 25 mg/kg, inhibited oxycodone seeking during extinction testing and blocked oxycodone-induced reinstatement to drug seeking. VK4-116 also significantly attenuated naloxone-precipitated conditioned place aversion in chronic oxycodone treated rats. These data suggest that D_3R antagonists may be suitable alternatives or adjunctive to opiate-based medications currently used clinically, in treating opiate addiction. As VK4-116 proved successful in this model, we then explored its potential for treatment of cannabis dependence. Due to the lack of a reliable THC self-administration model in rodents, we tested VK4-116 in squirrel monkeys self-administering THC under an FR10 schedule. VK4-116 dose-dependently blocked THC self-administration (4 μg/kg/inf) in the 1-10 mg/kg range without affecting food self-administration. In addition, VK4-116, in the same dose range, blocked THC-induced reinstatement of drug seeking in these animals suggesting that D_3R antagonists may be useful in treating marijuana abuse. This is the first demonstration that D_3R is a medication target for marijuana self-administration and cannabis-seeking behavior and that the highly D_3R-selective VK4-116 is a new lead molecule for development.

This work is supported by the NIDA-Intramural Research Program, National Institutes of Health

$G_{\alpha s}$ / $G_{\alpha olf}$ FUNCTIONAL SELECTIVITY IN DOPAMINE D1 RECEPTOR

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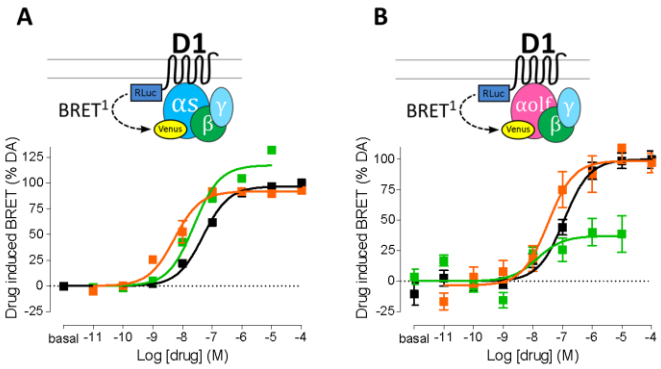
(short communication)

The dopamine D1 receptor (D1R) plays a pivotal role in locomotion, reward, cognition, and memory and learning and therefore is an important drug target in various neuropsychiatric disorders[1]. Despite the distinctive expression pattern of D1R in the brain, little attention has been given to its overlap with Gαs (Gs) and Gαolf (Golf) – two D1R-coupling Gα subunits highly homologous in amino acid sequence yet very discrete in their expression patterns[2]. Typical drug screening and characterization methods focused on downstream effectors cannot readily discriminate Gs and Golf coupling. Here newly developed drug screening methods that can differentiate coupling differences between Gs and Golf have allowed us to uncover a class of functionally selective[3] D1R ligands at Gs / Golf pathways, at which they behave as full agonists for Gs but partial agonists for Golf signaling (seen below in green curves). This finding was confirmed by studying multiple cellular, electrophysiological, and behavioral readouts. The combination of partial efficacy at Golf and full efficacy at Gs may constitute a new therapeutic approach that can dissociate effects on locomotion and cognition. This novel set of assays can be used to identify additional compounds with selective Gs and Golf coupling for potential therapeutics in neuropsychiatric illnesses linked to the D1R and other Gs/olf-coupled receptors.



A. Dose-response curves of drug induced BRET between D1R-Rluc and Gs-Venus (black = DA, orange = SKF81297, green = compound X respectively). B. BRET between D1R-Rluc and Golf-Venus (same color scheme).

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This work is supported by Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health.

NEXT GENERATION SCREENING & PROFILING PLATFORMS FOR DRUG DISCOVERY

Benedetta Montagnini

DiscoverX Corp. Ltd., Birmingham, UK.

G-protein coupled receptors (GPCRs) represent a large class of important therapeutic targets. To simplify and speed exploration of GPCR biology and drug discovery, DiscoverX offers an industry-leading portfolio of over 600 naturally coupled GPCR cell lines designed to detect GPCR signaling through second messenger activation, arrestin recruitment, and receptor internalization. Our broad collection of human and ortholog GPCR assays in a wide variety of functional readouts gives you complete flexibility to choose the ideal screening platform that meets your specific project needs. Regardless of the technology platform, DiscoverX provides the most robust, reliable, high-throughput-friendly, chemiluminescent GPCR assays available.

In addition, DiscoverX offers the most comprehensive menu of *in vitro* biochemical and cell-based assays for evaluating kinase activity. Our *in vitro* biochemical kinase panel includes an industry-leading set of > 460 assays, allowing rapid and efficient determination of kinome-wide potency and selectivity of compounds. By integrating EFC technology, kinase knowledge, and expertise in cell-based assays, we also offer a growing menu of homogenous, functional assays to detect activated kinases in whole cells. Used alone or in combination, these powerful assay platforms can be used at all stages of drug discovery and are ideal for identifying highly potent and selective kinase inhibitors.

Last but not least, the BioMAP Platform consists of primary human cell-based assay systems, a database of reference compound profiles, and computational data mining and analysis tools. In each BioMAP System, various primary human cell types are stimulated such that multiple, disease relevant signaling pathways are active, capturing pathway and interactions that manifest in the diseased tissue. By measuring biomarkers (proteins, small molecule mediators, etc.) in multiple systems and comparing resulting activity profiles to reference compound profiles in the database, BioMAP provides insight into mechanisms of action, compound efficacy and safety-related effects and enable correlation of compound activity with clinical outcomes.

Session 5

Beyond Receptor Chemistry: Transporters

BEYOND RECEPTOR CHEMISTRY: TRANSPORTERS

Richard A. Glennon

(Session Chair)

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Transporters are proteins responsible for regulating the flow of substrates across biological membranes. Historically, this symposium has tended to focus on receptors and little attention, if any, has been devoted to transporters. The purpose of this session is to remedy this situation. Greater than 800 genes encode for transporters and about half have been found to encode for solute carrier (SLC) transporters; the remaining (non-SLC) transporters include voltage-gated ion channels, ligand-gated ion channels, and others. The SLC transporters consists of >50 families (SLC1-SLC53) with some being very substrate selective, whereas others are non-selective. These transporters play a very broad physiological role and also offer great potential for drug development. Perhaps the best known SLC transporters are members of the SLC6 family that include SLC6A2, SLC6A3, and SLC6A4 transporters, more commonly known as the norepinephrine (NET) dopamine (DA), and serotonin (SERT) transporters, respectively. They have been widely investigated because of their involvement in disorders such as, for example, depression, anxiety, obsessive-compulsive disorder, post-traumatic stress disorder, and substance abuse. Yet, these three transporters represent <1% of identified SLC transporters. Many of the other transporters have already been linked to common, and not so common, disorders. In terms of drug development, what little has been achieved is “likely only the tip of the iceberg” (1). Here, Dr. Sitte will discuss SERT and DAT as targets for clinically relevant drugs and amphetamine-like agents, whereas Dr. Loland will desctibe regulation of conformational changes in a neurotransmitter:sodium symporter. Dr. Dukat’s presentation involves the organic cation transporter as a potential target for novel neuropsychiatric agents, and Dr. Glennon describes a group of abused substances, synthetic cathinones, that act at DAT, SERT, and NET.

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TRANSPORTERS AS CLINICAL TARGETS OF DRUGS: AMPHETAMINES, NEW PSYCHOACTIVE SUBSTANCES AND THE MONOAMINE TRANSPORTER CYCLE

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Serotonin and dopamine transporters are the clinically relevant target of antidepressant drugs; they inhibit the reuptake of these monoamines by competitively blocking the transporters' action. Thereby, these compounds enhance the extracellular concentration of serotonin and dopamine, which is relevant for clinical success. These transporters also provide a route for non-exocytotic neurotransmitter release (efflux) triggered by amphetamines.

Recent advancement in the understanding of the structural and molecular mechanisms of amphetamine-induced efflux via serotonin transporters will be discussed. Furthermore, the mechanisms underlying the activity of the recently introduced “bath salts”, a class of amphetamine-like compounds, will be highlighted.

DIRECT ASSESSMENT OF CONFORMATIONAL CHANGES UNCOVERS FUNCTIONAL REGULATION BY K^+ IN A NEUROTRANSMITTER:SODIUM SYMPORTER

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Neurotransmitter:sodium symporters (NSSs) are responsible for Na^+-dependent reuptake of neurotransmitters and represent key targets for antidepressants and psychostimulants. LeuT, a prokaryotic NSS protein, constitutes a primary structural model for these transporters. Here, we show that K^+ competitively inhibits Na^+-dependent binding of substrate to LeuT, promotes an outward-closed/inward-facing conformation of the transporter and inceases uptake. To assess K^+-induced conformational dynamics we measured fluorescence resonance energy transfer (FRET) between fluorescein site-specifically attached to inserted cysteines and Ni^{2+} bound to engineered di-histidine motifs (transition metal ion FRET). The measurements supported K^+-induced closure of the transporter to the outside, which was counteracted by Na^+ and substrate. Mutational locking of LeuT in the outward-open conformation abolished the K^+-effect. The K^+-effect depended on an intact Na1-site and mutating the Na2-site potentiated K^+ binding by facilitating transition to the inward-facing state. The data reveal an unrecognized ability of K^+ to regulate the LeuT transport cycle.

BEYOND MONOAMINE TRANSPORTERS: ORGANIC CATION TRANSPORTERS (OCTs)

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Organic Cation Transporters (OCTs; SLC22 gene family) are alternative and/or complementary regulators of synaptic concentrations of biogenic amines and might offer an underexplored mechanism to treat neuropsychiatric disorders. We have previously reported that 2-amino-6-chloro-3,4- dihydroquinazoline (A6CDQ), a 5-HT_3 receptor competitive antagonist (K_i = 80 nM, IC_{50} = 0.26 μM) produces antidepressant-like action in the mouse tail suspension test (TST) ED_{50} = 0.23 mg/kg (1). Its 7-chloro positional isomer A7CDQ produces equipotent antidepressant–like action as A6CDQ but binds with 25 times lower affinity (K_i = 1,975 nM) at 5-HT_3 receptors, suggesting involvement of an additional mechanism in their antidepressant-like action.

Because the most widely prescribed antidepressant, fluoxetine, a serotonin transporter (SERT) inhibitor, although lacking affinity at 5-HT_3 receptors behaves as a functional antagonist, we examined A6CDQ and A7CDQ for SERT activity. Both A6CDQ and A7CDQ bind at hSERT with insignificant affinity (K_i = 5,852 and >10,000 nM, respectively). In electrophysiological studies using hSERT expressed in *Xenopus laevis* oocytes A6CDQ mimics the action of 5-HT (K_m = 2.8; 0.94 μM, respectively) whereas its positional isomer A7CDQ although 600 times less potent mimics the action of fluoxetine (K_m = 43.6; 0.076 μM, respectively).

OCTs, and in particular OCT3, is widely expressed in CNS regions implicated in mood and antidepressant action. Thus we examined both analogs at hOCT3s expressed in HEK293 cells and found that both A6CDQ and A7CDQ inhibit hOCT3-mediated uptake (IC_{50} = 3.9; 5.9, μM, respectively). To study plausible binding modes for dihydroqinazolines we generated the first 3-D graphics models of hOCT3 using the inorganic phosphate transporter (PDB:4J05) as a template.

It would apper that combination of both serotonergic and OCT components might underlie the antidepressant-like actions of these dihydroqinazolines.

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SYNTHETIC CATHINONES: A NEW CLASS OF ABUSED SUBSTANCES AND THEIR ACTIONS AT MONOAMINE TRANSPORTERS

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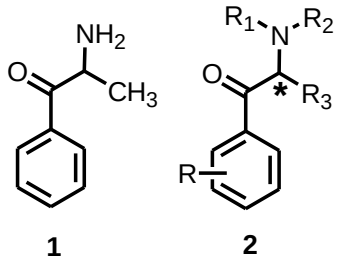
Synthetic cathinones are a popular and rapidly emerging class of illicit drugs that only gained widespread attention for their abuse potential in the last few years (1). Cathinone (Figure 1), or β-ketoamphetamine, is a natural product first identified in 1975. Following a brief period of scientific interest on cathinone in the 1980s, it was not until the identification of methcathinone (i.e., β-keto-methamphetamine) in the 1990s that interest briefly increased. Then, around 2010, a number of synthetic cathinones made their appearance and, today, >100 have been identified on the worldwide clandestine market. One of the first of the new generation of agents was termed "bath salts" and, although perhaps of varying composition, introduced two "new" analogs: mephedrone (4-methyl-methcathinone) and MDPV (methylenedioxypyrovalerone). It was quickly established that mephedrone acted at the DAT (dopamine transporter) as a releasing agent (i.e., as a substrate). In this respect, it behaved similar to methamphetamine and methcathinone. MDPV, on the other hand, was found to act as a cocaine-like reuptake inhibitor at DAT with a potency of about 35 to 50 times that of cocaine.



1 **2**

Figure 1. Cathinone (**1**) and the general structure of synthetic cathinone analogs (**2**).

Our studies have focused on identifying what structural features account for the actions of synthetic cathinones as releasing agents versus reuptake inhibitors at DAT, and how they influence selectivity for DAT versus serotonin and norepinephrine transporters (SERT and NET, respectively). With a primary focus on DAT, it has been found that terminal amine substituents (R_1, R_2; Figure 1), stereochemistry, the nature of the α-side chain (R_3), and the nature and location of aryl substituents (R) are responsible for the action (i.e., as substrates or reuptake inhibitors) and the selectivity of these agents. For example, a tertiary amine and/or an extended side chain favors action as a reuptake inhibitor at DAT, whereas aryl substituents play a role in selectivity for DAT versus SERT. SAR, QSAR, as well as homology modeling and docking studies have provided new hypotheses that are currently being experimentally evaluated in our laboratories.

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REMEMBERING MARIA PIGINI AND HER TWO DECADES OF IMIDAZOLINES

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In the hands of Maria "Nelly" Pigini, imidazolines have been remarkable molecules. The most relevant research achievements in the last two decades will be presented.

PAIN RELIEVER EFFECT OF 2-SUBSTITUTED IMIDAZOLINE DERIVATIVES IN A RAT MODEL OF PERIPHERAL NEUROPATHY

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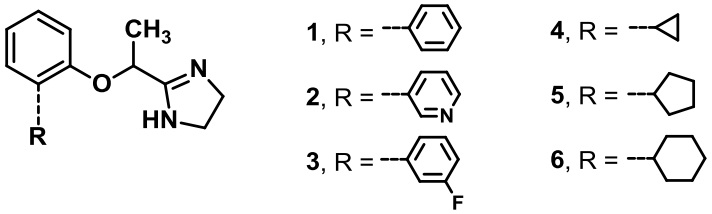
Dedicated to Maria "Nelly" Pigini

Neuropathic pain affects millions of people worldwide causing substantial disability and greatly impairing quality of life. Commonly used analgesics or anti-hyperalgesic compounds are generally characterized by limited therapeutic outcomes. The 5-hydroxytryptamine receptor subtype 5-HT_{1A} is involved in central nociceptive mechanisms with a pivotal role in the inhibitory descending pain pathway. Since 5-HT_{1A} agonists may modulate the nervous signaling altered by neuropathies, the present research aimed to study novel 5-HT_{1A} ligands in a rat model of neuropathic pain induced by the chronic constriction injury (CCI) of the sciatic nerve.

(*S*)-(-)-**1** emerged as a potent and long-lasting antinociceptive agent in previous algesiometric paradigms.[1] In addition, (*S*)-(-)-**1** proved to be able to interact with 5-HT_{1A} receptor. Fourteenth days after injury, the acute administration of low doses of (*S*)-(-)-**1** (0.36-1.08 mg/kg, *per os* - p.o.) was able to significantly increased the pain threshold to noxious stimuli longer than 1 h. (*S*)-(-)-**1** efficacy was confirmed reducing spontaneous pain. The clinically used compound gabapentin (100 mg/kg intraperitoneally – i.p.) induced a pain reliever effect slightly higher than (*S*)-(-)-**1** administered at 100 fold lower dose (1.08 mg/kg). Furthermore, the selected compounds **2-6**, analogues of (*S*)-(-)-**1** and endowed with significant 5-HT_{1A} receptor affinity, were tested (1 mg/kg p.o.) in the same model.



All compounds were effective 30 min after administration. In particular **6** fully reverted the CCI-induced hypersensitivity. The co-administration of **6** with the selective 5-HT_{1A} receptor antagonist WAY 100635 (1 mg/kg i.p. 15 min before **6**) partially reverted the pain reliever effect of the new synthesized compound suggesting the relevance of the serotoninergic system modulation in its pharmacodynamic mechanism.

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DOCKINGS AND MOLECULAR DYNAMICS OF 2-SUBSTITUTED IMIDAZOLINES AS HIGH AFFINITY BINDERS TO THE SEROTONIN 5-HT$_{1A}$ RECEPTOR

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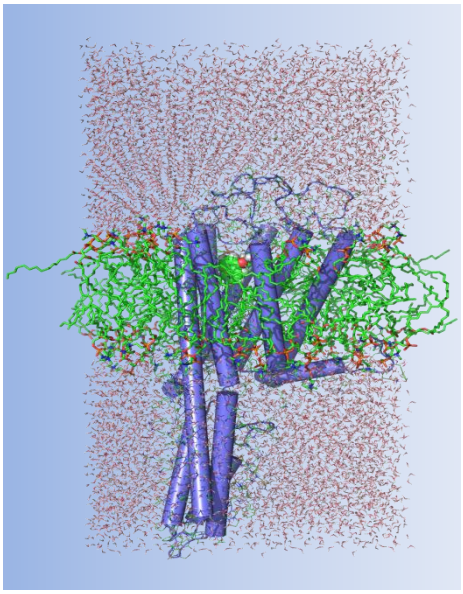
Homology modeling based on the crystallographic coordinates of the human 5-HT1B receptor [1] was applied in the rationale of the serotoninergic profile of several imidazolines endowed with a common scaffold made by a biatomic bridge linking an aromatic area to the position 2 of the imidazoline ring. This study highlights the role of some essential molecular determinants in the 5-HT1A-R recognition and activation like the charged nucleus, a polar function and a methyl group in the bridge, as well as the steric hindrance of substituents linked to the aromatic moiety of the binders.



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Session 6

The Future

THE FUTURE

David J. Triggle

(Session Chair)

State University of New York, Buffalo, NY, USA

This session dealing with the future of receptor chemistry will adopt a deliberately broad perspective with topics ranging from the process of scientific publication, the changing role of medicinal chemistry in drug discovery, to the role of natural products in medicine, and whether the behavior o zombies has anything to teach us.tDocument settings:

Chris. R. Triggle. Weill Cornell Medical College, Qatar. Publish and perish. Where is scientific publication going?

Joel Barrish. Bristol-Myers-Squibb USA. Drug Discovery Innovation. The evolving role of medicinal chemistry.

Shyam S. Chatterjee. Willmar-Schwabe Gmbh, Germany. What can we get from natural products and alternative medicine?

David J. Triggle. State University of New York, USA. Zombies and their pharmacology. What do they teach us?

PUBLISH AND PERISH: WHERE IS SCIENTIFIC PUBLICATION GOING?

Chris R. Triggle

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After almost 50 years in academia with my first research paper published in 1969 it is valuable to reflect on how new technologies have enhanced scientific research, but have all of these "advances" really been positive? This question is of particular relevance for the career advancement of scientists and the role of publications in the assessment of their achievements and future potential. The goal of this talk is to highlight and discuss some of the concerns that have been raised within the scientific community. *"Publish or Perish"* was first stated in an academic sense in the 1940s (1) and although the importance of publications for career advancement has always been a factor it does seem to be particularly critical in today's seemingly highly competitive research environment. The most frequently stated "measure" is the *Impact Factor* (*IF*) of the journal; however, linking the *IF* of a journal to the impact of an individual paper published in that journal is problematic (2,3). The arrival and sheer number of open access journals has complicated the interpretation of *IF*. Therefore, would citations and/or downloads be a better indicator of impact? *"Soon more journals than authors?"* is the title of an editorial by Pontus B Persson in *Acta Physiologica* in 2016 (4). Although the answer to the question raised by Dr. Persson is (currently) *"no"* nonetheless it is true that we are truly facing an information overload. A quote from 2010 reveals the magnitude of the problem: *"There are now 25 400 journals in science, technology, and medicine, and their number is increasing by 3.5% a year; in 2009, they published 1.5 million articles. PubMed now cites more than 20 million papers"* (5). ResearchGate was founded in 2008 and, according to its website, you can find *"over 80 million publications, 7 million researchers and 1 million answers to research questions".* In the field of pharmacology there at least 300 journals that can be linked to the discipline. There is also a near monopoly of the industry by essentially four publishing houses that are responsible for approximately 50% publications; however, Beale also lists >200 so called predatory publishers (6). Indeed, if you are willing to pay then your manuscript will be published - perhaps without peer review; whether it will be read, let alone cited, is the question. Furthermore, publishing in such journals may be detrimental to one's career (7). Jennings stated in a 2006 *Nature* web peer review debate: *"Whether there is any such thing as a paper so bad that it cannot be published in any peer reviewed journal is debatable"* (8). So, what advice do we give to our trainees as how best to compete in the current environment and avoid the *"Publish & Perish"* trap?

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DRUG DISCOVERY INNOVATION: THE EVOLVING ROLE OF MEDICINAL CHEMISTRY

Joel C. Barrish

Achillion Pharmaceuticals

300 George Street, New Haven, Connecticut 06511, USA

The Pharmaceutical Industry is facing significant challenges: a regulatory environment that has become more restrictive and where the safety bar is higher; substantial cost increases at the same time that R&D success rates have decreased and identifying highly validated targets has become even more difficult; and a system of payers worldwide trying to reduce costs and demanding more accountability. Medicinal chemists within the Industry have been particularly affected by changes made in response to these challenges.

Despite the headwinds, there is reason for optimism - not only because drug therapies will continue to be important for an aging population, but especially given considerable scientific advancements within Drug Discovery. Our future success will depend on our ability to continue to innovate. For the medicinal chemist, the ability to rapidly evolve and adapt to new approaches and strategies will be key.

This presentation will be a personal view of the key scientific innovations where Chemistry has the opportunity for greatest impact and will also highlight the attributes and capabilities needed by the future medicinal chemist.

WHAT CAN WE GET FROM NATURAL PRODUCTS AND ALTERNATIVE MEDICINE?

Shyam Sunder Chatterjee

Retired head pharmacology research laboratories, Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany

Ever since the early decades of the past century, several researchers and practitioners of traditionally known systems of medicine, have suggested that natural products derived substances could be promising drug leads for prevention and cure of diverse chronic diseases. Since then, preclinical and clinical observations made with them and with numerous molecules isolated and derived from them have led not only to the discovery of several currently available drugs, but also to the identification of numerous novel therapeutic leads and animal models potentially useful for drug discovery and development purposes. However, several other easily reproducible but unexpected ones still remain unexplored, or are often neglected by modern drug designers. Analogous is the situation also for approximately 200,000 bioactive phytochemicals listed in current dictionaries of natural products. Although their diverse combinations and doses are regularly consumed with everyday meals, or with numerous currently available and popular phyto-pharmaceuticals, nutraceuticals, and other plant derived products, our current understanding on adverse or beneficial health effects of numerous of them and their combinations still remain at the best speculative only.

Efforts to exploit them for drug discovery purposes or for better understanding their roles in regulating human health have often led to paradoxical observations, several similar ones of which are now also consistently being reported for numerous drugs and other natural products currently widely used for health care purposes. Some such drug discovery relevant observations made reproducibly during effort to resolve these paradoxes using numerous phytochemicals commonly consumed with food and other plant derived products will be summarized during this presentation. Potential uses of such observation for better understanding the role of natural products in modulating the functions biological processes and mechanisms involved in health and diseases, or for obtaining urgently needed novel therapeutic leads against malnutrition and other lifestyle associated physical and mental health problems will also be pointed out.

ZOMBIES AND THEIR PHARMACOLOGY. WHAT CAN HOST-PARASITE RELATIONSHIPS TELL US ABOUT NEW PHARMACOLOGICAL APPROACHES?

David J. Triggle

Program in Scientific Ethics, State University of New York, Buffalo, NY, USA

Since at least the time of Paul Ehrlich there has been a consistent effort to define "magic bullet" molecules capable of highly specific and targeted therapeutic actions. There has been considerable success in a search extending over one hundred years to define biological space in a universe of chemical space [1]. It is, however, quite clear that we need a broader systems-biology approach whereby we examine drug targets within the context of the organism [2]. This holistic approach has led to important advances in human health through examination of, for example, the role of the human microbiome in health and disease [3]. And just as the microbiome and more recently the mycobiome [4] are seen to be critical so too are the host relationships with higher organisms. Such host-parasite interactions have been shown to have profound influence on host behavior and survival frequently resulting in behavioral changes in the host critical to the survival and more efficient reproduction of the parasite [5]. The study of such interactions offers potential for new pharmacological approaches to human disease.

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Posters

NOVEL TETRAHYDRODIBENZOOXAAZECINES AS A PROBE TO DISCRIMINATE DOPAMINE D1/D5 RECEPTORS

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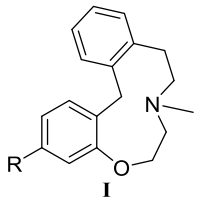
Dopamine is a neurotransmitter that regulates several physiological functions by acting through two different receptor families named D_1-like (D_1 and D_5) and D_2-like (D_2, D_3 and D_4). Dysfunction of the dopaminergic signal transduction is strongly related to neuropsychiatric diseases, such as schizophrenia, Parkinson's disease, and addiction, indicating dopamine receptors as attractive therapeutic targets. Several different compounds have been developed over the last decades as ligands more or less selective toward the dopamine receptor subtypes, but the discrimination between D_1 and D_5 receptors is still an almost unexplored challenge.[1] It has already been reported that activation of dopamine D_5 receptors in the VTA may contribute to the addictive properties of cocaine.[2] This observation indicates dopamine D_5 antagonists as interesting potential tools for the treatment of cocaine addiction.

Based on the literature, a rich panel of data concerning the topographic requirements of dopamine receptors is available. However, as above mentioned, the knowledge of the structural features of dopamine D_5 selective ligands emerged during the last few years. Lehmann et al. reported the discovery of new high affinity D_1-like receptors antagonists (**I**) with slight preference for D_5 receptor subtype and indicated them as "a step toward dopamine D_5 selectivity"[3]. Back in 2006 Nichols' group described[4] a new highly selective dopamine D_1-like receptor full agonist (**II**) endowed with a D_1-like high affinity in binding tests (8 nM) on porcine striatal preparations and a slight preference for D_5 receptors in competition binding assays on cloned human receptor.



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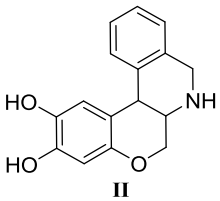

II

Thus, we designed the synthesis of novel tetrahydrodibenzooxaazecines potentially able to bind the D1-like receptors and discriminate between D_1 and D_5 subtypes.

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NEW AMINOPYRIDINE-3,5-DICARBONITRILES AS ADENOSINE A_{2B} RECEPTOR NON-NUCLEOSIDE AGONISTS

Marco Betti[a], Vittoria Colotta[a], Flavia Varano[a], Matteo Falsini[a], Katia Varani[b], Fabrizio Vincenzi[b], Diego Dal Ben[c], Anna Maria Pugliese[d], Daniela Catarzi[a]

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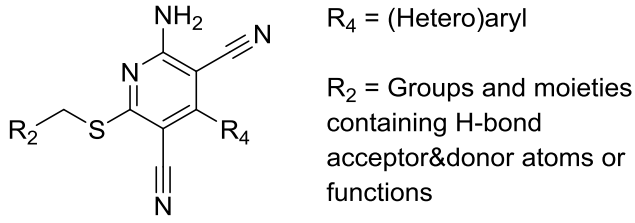
The G protein-coupled adenosine receptors (ARs) have been important targets for drug development since their discovery in 1965. In fact, ligands at ARs are becoming promising therapeutics for many diseases because of the wide range of effects mediated by the endogenous ligand adenosine, fully considered as a homeostatic regulator (1). Though several compounds are in clinical trials for a range of indications, further investigations are needed to clarify the pathophysiological functions of ARs (2). This is especially true for the A_{2B} subtype, which is the less known among all the ARs due to the lack of potent and selective agonists. However, pharmacological studies on this subtype highlighted both its central and peripheral anti-inflammatory role and also envelopment in some heart diseases correlated with metabolic dysfunctions in which A_{2B}AR seems to be involved in synergy with A_1AR. On the basis of molecular modelling studies, some new non-nucleoside aminopyridine-3,5-dicarbonitrile derivatives have been designed to targeting the A_{2B}AR. Most of the synthesized compounds show very good activity at human A_{2B}AR and a partial agonist profile. One selected compound among this set is being used as pharmacological tool for clarifying the role of A_{2B}AR in the CNS, specifically in an oligodendrocytes precursor cell differentiation model (3).



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DUAL MODULATORS OF DOPAMINE D3 RECEPTOR AND FATTY ACID AMIDE HYDROLASE: MODELING AND STRUCTURE ACTIVITY RELATIONSHIP STUDIES

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Multi-target directed ligands are compounds endowed with the ability to concurrently modulate multiple targets, hence holding great potential toward exerting disease-modifying effects on complex conditions. (1) Recently, we have demonstrated that it is possible to rewire computer-assisted drug design methods originally conceived to work according to the classic "one molecule, one target" paradigm to rationally design multi-target directed ligands. (2) Indeed, we have reported the first series of derivatives displaying potent and balanced activities toward both D3 dopamine receptor and fatty acid amide hydrolase (FAAH) enzyme. (3,4) These two targets contribute through different pathways to initiate and maintain nicotine addiction and engaging them simultaneously could represent a viable strategy toward obtaining effective medications. Here, some relevant aspects related to the application of computational methods toward tuning selectivity and efficacy are discussed.

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NOP RECEPTOR ANTAGONISM DECREASES ALCOHOL DRINKING IN C57BL/6J MICE

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The Nociceptin/Orphanin FQ (N/OFQ) peptide and its cognate receptor (NOP) are found throughout the brain, spinal cord and dorsal root ganglia, supporting a role of the N/OFQ-NOP system in the modulation of central functions, including learning and memory, reward, mood, feeding, stress and sensory nociceptive processing. Activation of NOP receptors with exogenously administered N/OFQ or synthetic agonists blunts the reinforcing and motivating effects of many abused drugs including alcohol. The role of the endogenous N/OFQ-NOP system in response to drug-mediated behaviors is still not well clarified. In the present study we show the effects of NOP receptor agonists and antagonists in binge-like alcohol consumption, as measured by the "drinking in the dark" (DID) model in C57BL/6J mice. We found that the potent agonist AT-202 (0.0, 0.3, 1.0, 3.0 mg/kg) failed to reduce binge alcohol drinking. The same response was obtained following acute and chronic administration of a second potent agonist, MT-7716 (0.0, 0.1, 0.3, 1.0 mg/kg). AT-202 also failed to affect DID behavior when administered in mice with a previous history of alcohol intoxication. Conversely, treatment with two chemically distinct NOP receptor antagonist, namely SB612111 (0, 3, 10, 30 mg/kg) and LY2817412 (0, 3, 10, 30 mg/kg) decreased binge drinking both in post-dependent and non-dependent mice. SB612111 also reduced alcohol preference in a two-bottle choice DID model while leaving sucrose intake and locomotor behavior unaltered. Altogether, these experiments provide new evidence for a role of the N/OFQ system in excessive drinking in mice and suggest that NOP antagonists may represent a new approach with clinical potential in humans.

BIOLOGICAL PROFILE AND BIOAVAILABILITY OF IMIDAZOLINE COMPOUNDS AFFECT THEIR MODULATORY ACTIVITY ON MORPHINE TOLERANCE

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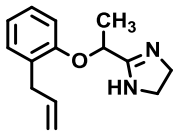
Dedicated to Maria "Nelly" Pigini

Therapeutic use of opioids represents the standard of care in the treatment of severe chronic pain and cancer-related pain (1). Various α_2-adrenoceptor agonists, devoid of α_2 subtype selectivity (i.e. clonidine), have been clinically used in pain management but, due to its α_{2A} subtype activation, they might be responsible for sedation and hypotension side effects. Moreover, to overcome the side effects of opiate drugs, the synergism with compounds interacting with imidazoline I_2 receptors has been reported (2). The aim of the study was to compare the effects of the imidazoline compounds **1**, **2**, and **3** (Figure) on morphine tolerance in an animal model of inflammatory pain in rats. **1**, **2**, and **3** have been selected in that, although bearing a common scaffold, preferentially bind to α_2-adrenoceptors, imidazoline I_2 receptors, or both systems, respectively.



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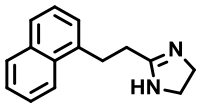

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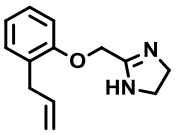

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These compounds have been tested *in vivo* in association with morphine, by measuring the paw withdrawal threshold to mechanical pressure. The sub-chronic 4 days treatment with **1-3**, administered twice a day 15 min before each morphine administration significantly restored at day 4 the morphine analgesic response. The maximal activity of **2** was at t=45 min; instead that of **1** and **3** was at t=90 min. Since the different temporal profile on the tolerance reduction displayed by **1-3** might be associated not only to their different target profile, but also to their bioavailability, we developed an HPLC-mass spectrometry method for the determination of the ligand levels in the rat plasma. The mean serum concentration of **1** was determined to be maximum at 60 minutes (14.71 ng/ml); meanwhile that of **2** was maximal at 30 minutes (58.00 ng/ml). Therefore, this study highlights that both peculiar biological profile and bioavailability of such ligands complement each other to modulate the reduction of morphine tolerance.

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USES AND APPLICATIONS OF GOLD IN NANOMEDICINE

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Gold was used for medical purposes already in ancient China and India (5000 B.C.) and in ancient Egypt (3000 B.C.).[1,2] The medieval alchemists used gold in elixirs for health and life extension in Europe.[3] In the 17th century, gold entered the official drug pharmacopoeias.[2,3] Gold (powder) was mixed into drinks to treat sore limbs, syphilis, and blood circulation, skin ulcers, burns, etc.[1,2] Nowadays, the gold is mostly used in the treatment of rheumatoid arthritis.[4] Radioactive gold-198 is used in the treatment of malignancies and persistent knee effusions.[2] Gold nanoparticles can become useful for applications to "lab on a chip" devices, electronic, photonic and sensing applications based on plasmonics, cancer treatment, etc.[5] Gold has been abundantly used as a contrast agent in biological electron microscopy or computed tomography.[6] In cancer research, gold can be used to target tumors and provide detection, and also as a drug carrier, as gold nanoparticles can be used as vectors for targeting cancer tissue/cells so that the biodistribution of drugs is optimised.[5] Gold nanoparticles are photothermal agents for in-vivo applications. They showed to be effective for the inhibition of pathogenic bacteria cell growth, antibiotic-resistant bacteria[7] and demonstrated effective photothermal destruction of cancer cells and tissue.[5] Gold nanopartices can act as antennas, avoiding damage to healthy tissues. Gold can also destroy the beta-amyloid fibrils whose toxicity has been hypothesized to initiate the pathogenesis of Alzheimer's disease.[8] Gold also possesses well known anti-inflammatory properties and is still used to treat rheumatoid arthritis.[4,10]

Acknowledgements: Fundação para a Ciência e Tecnologia (FCT) for financial support, namely Investigador FCT program (IF/01381/2013/CP1160/CT0007), with financing from the European Social Fund and the Human Potential Operational Program.

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SYNTHESIS AND BIOLOGICAL EVALUATION OF NEW LIGANDS DESIGNED TO SELECTIVELY INTERACT WITH KAINATE RECEPTORS

Cullia G., Mastronardi F., Pinto A., Tamborini L., Marconi L., De Micheli C., Conti P.

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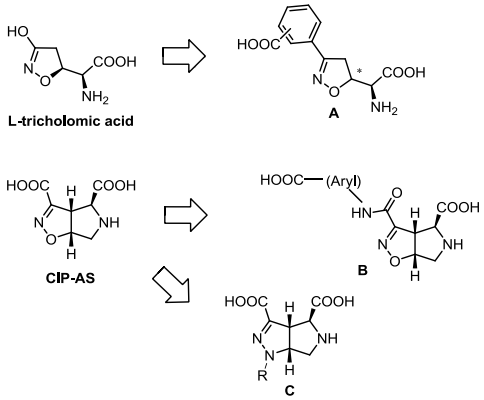
Kainate receptors (KARs) are a family of ionotropic glutamate receptors (iGluRs). KARs are know to play a role in pain, epilepsy, neurodegenerative and psychiatric disorders but the lack of highly selective ligands for KAR subtypes (GluK1-5) prevented so far an exhaustive pharmacological characterization.[1]

Starting for the natural compound L-tricholomic acid, which is a non-selective AMPA/KA agonist, we designed a series of higher homologues of general structure **A**, in which the distal acidic group is linked to the isoxazoline ring through an aromatic spacer. Homologation is aimed at switching the profile from agonist to antagonist, by preventing the ligand binding domain closure, whereas an increased selectivity may arise from additional interactions played by the aromatic ring.

Similarly, starting from CIP-AS, a non selective AMPA/KA agonist previously developed by our group, we designed a series of higher homologues of general structure **B**. Moreover, we designed new analogs of general structure **C**, in which the isoxazoline ring was replaced by a N-substituted-pyrazoline, in order to explore the role played by an additional substituent (e.g. a methyl group) in this position, considering that, due to the presence of less hindered amino acids, KARs are known to accommodate larger substituents than AMPARs.



Preliminar biological data will be presented and discussed.

References:

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ANTIDEPRESSANT-LIKE ACTIVITY OF NOVEL IMIDAZOLINE LIGANDS DIRECTED TO THE SEROTONIN 5-HT$_{1A}$ RECEPTOR

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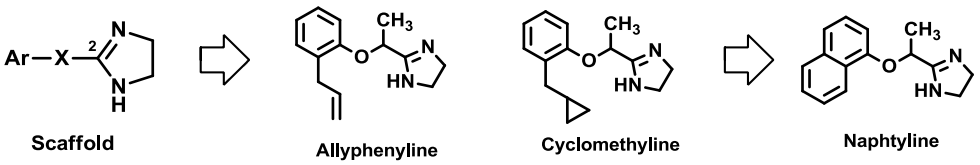
Dedicated to Maria "Nelly" Pigini

For several years our studies have been focused on the design and preparation of biologically active ligands directed to different receptor systems and sharing a common scaffold characterized by an aromatic moiety (Ar) linked by a biatomic bridge (X) to the position 2 of the imidazoline nucleus (Figure). Among these compounds, allyphenyline and its analogue cyclomethyline proved to be able to exert antidepressant activity, also induced by serotonin 5-HT$_{1A}$ receptor (5-HT$_{1A}$-R) activation (1). Therefore, for the first time it has been suggested that such a scaffold might be suitable in the building of ligands addressed to 5-HT$_{1A}$-R. With the aim to individuate novel ligands targeting 5-HT$_{1A}$-R and to identify the structural features favouring the 5-HT$_{1A}$-R interaction, we examined the in vitro 5-HT$_{1A}$ profile of several imidazolines characterized by the common scaffold reported in figure.



Confirming the bioversatility of the 2-substituted imidazoline nucleus, structure-activity relationships, supported by modelling studies, suggested that a polar function and a methyl group in the bridge, as well as an ortho substituent of suitable steric hindrance in the aromatic area of the above scaffold favoured 5-HT$_{1A}$-R recognition and activation. Since 5-HT$_{1A}$-R has been considered as an attractive target for anxiolytic and antidepressive strategies due to a well-known serotonin response, the antidepressant-like effect of the most interesting ligand naphtyline and its enatiomers in the mouse forced swimming test was investigated. Interestingly, the eutomer (*S*)-(+)-naphtyline displayed antidepressant-like effect at very low dose (0.01 mg/kg p.o.), showing a higher efficacy and potency in comparison to the tricyclic antidepressant amitriptyline (15 mg/kg p.o.). The antidepressant-like effect was significantly reduced by the pre-treatment with the 5-HT$_{1A}$-R antagonist WAY100635, demonstrating the involvement of 5-HT$_{1A}$-R in the (*S*)-(+)-naphtyline activity.

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PYRAZOLE-BASED ACID CERAMIDASE INHIBITORS: DESIGN, SYNTHESIS AND STRUCTURE-ACTIVITY RELATIONSHIPS

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Acid ceramidase (AC) is a lysosomal cysteine amidase responsible for the cleavage of ceramide into sphingosine, which is then phosphorylated to sphingosine 1-phosphate[1]. AC regulates the intracellular levels of ceramide and sphingosine, and AC inhibition may be useful in the treatment of disorders, such as cancer, in which ceramide-mediated signaling may be dysfunctional[2]. Despite their potential experimental and therapeutic value, the number of available small-molecule inhibitors of AC activity remains limited. Previous work in our laboratory has identified carmofur (5-fluoro-*N*-hexyl-2,4-dioxo-pyrimidine-1-carboxamide), an antineoplastic drug used in the clinic, as the first nanomolar inhibitor of AC[3]. On this basis, we conducted an *in silico* screening using carmofur as template and we identified 1H-pyrazol-5-ol as promising starting point for the development of a novel class of AC small-molecule inhibitors. In the present work, we describe the synthesis of a series of *N*-acyl pyrazole derivatives bearing different substituents on the heterocyclic ring and we report an in-depth NMR study carried out to elucidate the structures of the isolated pyrazole regioisomers. An extensive structure-activity relationship (SAR) analysis allowed the identification of *N*-hexyl-3-methyl-5-(trifluoromethyl)pyrazole-1-carboxamide which inhibits AC with nanomolar potency causing ceramide accumulation and sphingosine depletion in intact G361 proliferative melanoma cells. By expanding the current armamentarium of AC inhibitors, these results should facilitate future efforts to unravel the biology of AC and the therapeutic potential of its inhibition.

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ACTIVATION OF PPARγ ATTENUATES THE EXPRESSION OF NICOTINE WITHDRAWAL SYMPTOMS BY IMPLICATION OF THE AMYGDALA.

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Nicotine, a potent psychostimulant with high rewarding properties, is the main responsible of the addictive properties of tobacco smoking. Nicotine discontinuation leads to negative abstinence symptoms characterized by somatic (physical) and affective (anxiety) components. These aversive withdrawal symptoms increase the risk of relapse to smoking, thus therapies that prevent the insurgence of these negative state may represent valid candidates for smoking cessation. We have recently found that pioglitazone, an agonist of peroxisome proliferator-activated nuclear receptor (PPARγ, decreases alcohol consumption and attenuates the expression of alcohol withdrawal signs.

Here we studied the effect of pioglitazone on nicotine withdrawal in Wistar rats following 7 days of nicotine treatment through implanted transdermal nicotine patches (5.2 mg/rat/day). The effect of pioglitazone (15.0, 30.0 mg/kg, p.o.) on somatic and affective withdrawal signs, were assessed 16 hours and 6 days after nicotine exposure. In addition, we analyzed the effect of pioglitazone on spontaneous nicotine withdrawal in conditional neuronal PPARγ knock-out (KO) mice and their wild type counterparts (WT). In mice nicotine dependence was induced by injecting nicotine 2 mg/kg (s.c), 4 times per day for 8 consecutive days. The effect of pioglitazone and the effect of the PPARγ antagonist GW9662 (5mg/i.p) was evaluated at 20 hours and 6 days from the last nicotine injection. To identify the brain areas involved in these effects, we performed a Real-time qPCR analysis of PPARγ gene expression in the amygdala, hippocampus and hypothalamus of nicotine-treated mice at 20 hours and 6 days of withdrawal.

The results showed that pioglitazone reduced the total abstinence score for somatic symptoms and nicotine-induced anxiety in rats and in WT mice but not in PPARγ KO mice. These effects were blocked by GW9662 which further confirm the involvement of PPARγ. Gene expression results showed that 6 days of nicotine withdrawal induced a significant increase of PPARγ mRNA levels in the amygdala at both 20 hours and 6 days into nicotine withdrawal.

The results of this study indicate that the amelioration of nicotine withdrawal symptoms by activation of PPARγ and the amygdale appears to be a key area in mediating this effect.

NEW 5-HETEROARYL-PYRAZOLO[4,3-*d*]PYRIMIDIN-7-AMINES AS DUAL HUMAN ADENOSINE A_1 AND A_{2A} RECEPTOR ANTAGONISTS

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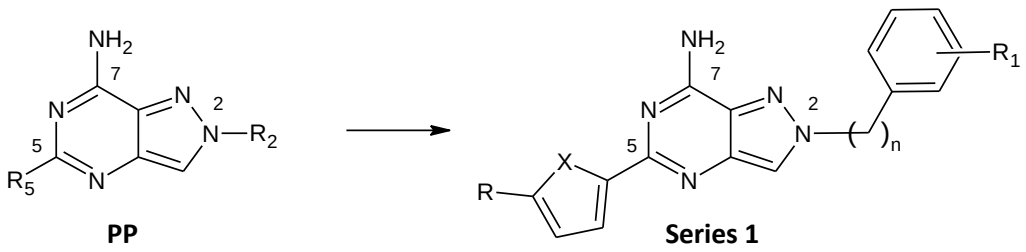
The neuromodulator adenosine exerts its biological effects by activation of GPCR, classified into A_1, A_{2A}, A_{2B} and A_3 subtypes. Adenosine receptor (AR) antagonists emerged as useful therapeutic agents in a wide range of pathologies. In particular, A_{2A} AR and dual A_1/A_{2A} AR antagonists have attracted attention for their efficacy in the treatment of Parkinson's disease (PD) (1,2) because they improve both motor and cognitive impairments associated to the ill. Accordingly, we developed a set of pyrazolo[4,3-*d*]pyrimidin-7-amines **PP** which showed nanomolar affinity at human (h) A_1 and A_{2A} ARs and different degrees of selectivity, depending on the nature of R_5 and R_2 substituents (3,4). Since R_5= 2-furyl enhanced hA_{2A} AR affinity, new pyrazolo[4,3-*d*]pyrimidines 5-(2-furyl)- and 5-(heteroaryl)-substituted, bearing aryl/benzyl groups at position 2 (**Series 1**), were synthesized and in vitro evaluated for their affinities at ARs.



Several of the new derivatives **1** showed nanomolar affinity for the hA_{2A} AR (K_i= 3.6-57 nM) and different degrees of selectivity vs the hA_1 AR (3-22-fold). Molecular docking investigations at the hA_{2A} AR crystal structure and at a homology model of the hA_1 AR allowed us to represent the hypothetical binding mode of these compounds and to rationalize the SARs.

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REGULATION OF INTRA-AMYGDALA ENDOCANNABINOID SIGNALLING MODULATES ALCOHOL DRINKING AND ANXIETY IN msP RATS

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Endocannabinoid system has been implicated in addictive behavior and in the mechanism of action of several drugs of abuse including alcohol (1; 2). The discovery of anandamide, a natural lipid ligand for CB1 receptors and of the mechanisms for its biosynthesis and inactivation, has inspired pharmacological strategies to augment endogenous cannabinoid activity in specific brain areas (3). Fatty acid amide hydrolase (FAAH) is a key membrane protein for metabolism of endocannabinoids, including anandamide and blockade of FAAH increases the level of anandamide in the brain. URB-597 is a selective inhibitor of FAAH considered as a potential therapeutic agent in anxiety, depression and pain (4). To determine if FAAH regulates ethanol consumption, we investigated whether activation of the endogenous cannabinoid tone by URB-597 given into the central (CeA) and basolateral (BLA) amygdala modifies alcohol self-administration in genetically selected Marchigian Sardinian alcohol-preferring (msP) rats, an animal model in which genetic selection for high alcohol preference has co-segregated with anxiety and enhanced sensitivity to stress. Under our experimental condition, administration of URB-597 (0.01, 0.3 and 1.0 μg/rat) reduced alcohol self-administration in msP in the CeA. We hypothesize that exogenous administration of URB-597, enhancing the endocannabinoid signaling might attenuate hyper-anxiety in msP rats, thus removing one of the triggers for the excessive drinking that characterize this rat line. To confirm this hypothesis we evaluated the anxiety-like response to restraint stress after URB-597 microinjection into the CeA in msP rats. Intra-CeA injections of URB-597 (1.0 μg/rat) significantly reduced anxiety-like behavior in restraint rats in the elevated plus maze paradigm. The major finding of the present study is the demonstration that the increase of endocannabinoid tone associated with selective inhibition of FAAH in the CeA leads to a reduction of ethanol consumption and attenuates stress-induced anxiety in the rat.

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DEVELOPMENT OF A LC-MS-MS METHOD FOR THE SIMULTANEOUS DETERMINATION OF STEROIDAL HORMONES IN EQUINE SERUM.

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Monitoring the hormones in equine matrix is crucial to understand the health status of horses, and also to monitor the abuse of these substances before a race or a horse transaction. Few analytical procedures exist which usually are "self made methods", generally based on immunoassay analysis, often impossible to replicate in other laboratories or too complex for the staff. On that, it is necessary to develop new sensitive analytical methods, that can be diffused to external vets and give correct and comparable results. Furthermore, the few analytical procedures involving an HPLC-MS-MS system reported in literature allow for simultaneous quantification of no more than three or four hormones (1, 2). The proposed method makes ~~it~~ possible to detect and quantify seventeen hormones and metabolites in a single assay, in just eleven minutes. Quantifiable hormones with the proposed method are: Pregnenolon, 17-OH-Pregnenolon, Progesteron, 17-OH-Progesteron, Androsteron, Androstenedion, DHEA, DHEAS, Testosteron, Cortisol, Corticosteron, Aldosteron, 11-Deossicortisol, 11-Deossicorticosteron, Diidrotestosteron, Estron, Estradiol. Three deuterated hormones (Cortisol-D4, Aldosteron-D7, Testosteron-D3) have been used as internal standards in order to set a more accurate and precise procedure. The method was developed using the Agilent UHPLC chromatographic system (1290) with a Zorbax RRHD C18 – 1,8 μm column and a 6420Agilent Mass Spectrometer. The procedure is fast and intuitive; the sample preparation is very easy, with the serum deproteinized in vials using a deproteinizing solution, centifuged and injected into the system. The mobile phases are made of water and acetonitrile, both containing formic acid. Overall, the method is very simple and robust and it brings about a remarkable saving of time and money with respect to previously reported methods; in fact, using the UHPLC–Tandem Mass spectrometer enables simultaneous quantification of seventeen Steroidal Hormones.

(1) Methodological approach to the intracrine study and estimation of DHEA and DHEA-S using liquid chromatography– tandem mass spectrometry (LC–MS/MS) Yasuhiro Shibata, Seiji Arai, Seijiro Honma
J Steroid Biochem Mol Biol. 2015 Jan;145:193-9. doi: 10.1016/j.jsbmb.2014.04.002. Epub 2014 May 2.

(2) Simultaneous measurement of testosterone, androstenedione and dehydroepiandrosterone (DHEA) in serum and plasma using Isotope-Dilution 2-Dimension Ultra High Performance Liquid-Chromatography Tandem Mass Spectrometry (ID-LC–MS/MS) Rahel M. Büttler, Frans Martens, Mark M. Kushnir, Mariette T. Ackermans, Marinus A. Blankenstein, Annemieke C. Heijboer
Clin Chim Acta. 2015 Jan 1;438:157-9. doi: 10.1016/j.cca.2014.08.023. Epub 2014 Aug 27.

NOVEL IMIDAZOLINE MOLECULES INSPIRED BY THE PHARMACOLOGICAL PROFILE OF AGMATINE AS POTENTIAL TOOLS IN MANAGING OPIOID ADDICTION

Maria Elena Giusepponi, Carlo Cifani, Maria Vittoria Micioni Di Bonaventura, Fabio Del Bello, Mario Giannella, Valerio Mammoli, Alessandro Piergentili, Wilma Quaglia

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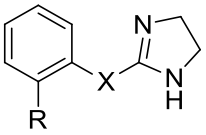
Dedicated to Maria "Nelly" Pigini

Tolerance and dependence associated with chronic opioid exposure result from molecular, cellular, and neural network adaptations. Such adaptations concern opioid and non-opioid systems, including α_2-adrenoceptors (α_2-ARs) and I_1- and I_2-Imidazoline binding sites (IBS) (1). Agmatine, one of the hypothesized endogenous ligands of IBS, targets these systems and, acting as a multitarget agent, also exerts its modulatory action at many other targets (2). Therefore, anxiolitic, antidepressant, antinociceptive, anticonvulsive, antiproliferative and neuroprotective effects characterize its pharmacological profile. Recently, a significant number of studies suggests that agmatine and, in general, molecules displaying high affinity for α_2–ARs and IBS are able to regulate opioid-induced analgesia (3) and to attenuate the development of tolerance and dependence (4). Attracted by the complex pharmacological profile of agmatine and considering the nature of its targets, we synthesized and studied two series of imidazoline molecules, rationally designed to produce simultaneous I_1-/I_2-IBS (**1**-**4**) and I_1-/I_2-IBS/α_2-ARs (**5**-**9**) interactions.

1, R = -CH_3, X = -CH=CH-
2, R = -Cl, X = -CH=CH-
3, R = -$CH_2CH{=}CH_2$ X = -CH=CH-
4, R = -C_6H_5, X = -CH=CH-



5, R = -CH_3, X = -NCH_2-
6, R = -Cl, X = -NCH_2-
7, R = -$CH_2CH_2CH_3$ X = -NCH_2-
8, R = -$CH_2CH{=}CH_2$ X = -NCH_2-
9, R = -C_6H_5, X = -NCH_2-

Our aim was to obtain useful compounds for exploring the biological effects modulated by these target interaction combinations and to discover novel potential therapeutic tools. Therefore, compounds **1** and **5**, showing the highest affinities for I_1-/I_2-IBS and I_1-/I_2-IBS/α_2-ARs, respectively, have been selected for their *in vivo* evaluation on opiate withdrawal syndrome. Interestingly, both of them significantly affected expression and acquisition of morphine dependence, and, therefore, might be considered promising tools potentially useful in managing opioid addiction.

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EFFECT OF THE SELECTIVE CRF-1 RECEPTOR ANTAGONIST R121919 IN AN ANIMAL MODEL OF BINGE EATING

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We recently developed a binge-eating model in which female rats with a history of intermittent food restriction show binge-like palatable food consumption after 15 min exposure to the sight of the food. This "frustration stress" manipulation also activates the HPA stress axis. Here, we determined the role of the stress neurohormones corticosterone and CRF in stress-induced binge eating in our model. We also assessed the role of CRF receptors in the BNST, a brain region implicated in stress responses and stress-induced drug seeking, in this binge-eating model.

We use 4 groups that were first exposed or not exposed to repeated intermittent cycles of regular chow food restriction/re-feeding during which they were also given intermittent access to high caloric palatable food. On test day, we either exposed or id not expose the rats to the sight of the palatable food per 15 min (frustration stress) before assessing food consumption for 2 h.

We found that systemic injections of the CRF 1 receptor antagonist R121919 (10-20 mg/kg) and BNST (25-50 ng/side) or ventricular (1000 ng) injections of the non-selective CRF receptor antagonist D-Phe-$CRF_{(12-41)}$ decreased stress-induced binge eating. This manipulation also increased CRF1 receptor mRNA and Fos (a neuronal activity marker) expression in BNST.

To assess whether corticosterone is involved in the binge eating behavior, rats were treated with metyrapone, a corticosterone synthesis inhibitor at the doses of 50 and 100 mg/kg. It failed to prevent binge eating. Lastly, corticosterone injection (2.5 and 10 mg/kg) did not induce binge eating in restricted and non-stresses rats, in comparison to the control group (non-restricted and non-stressed).

Results demonstrate a critical role of CRF receptors in BNST in stress-induced binge eating in our rat model. CRF1 receptor antagonists may represent a novel pharmacological treatment of stress-induced binge eating.

SEARCHING FOR DOPAMINE D_2 RECEPTOR ALLOSTERIC MODULATORS USING STRUCTURE-BASED VIRTUAL SCREENING

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Allosteric modulation of dopamine D_2 receptor has been suggested as a method to treat schizophrenia and Parkinson's disease (PD). Homocysteine and its analogues, which are negative allosteric modulators of D_2 receptor, have been proposed as a novel treatment for schizophrenia. The tetrahydroisoquinoline derivative SB269,652 is an allosteric antagonist at dopamine D_2 and D_3 receptors; however, its antipsychotic potential has been not evaluated. A peptidomimetic PAOPA which is a dopamine D_2 receptor positive allosteric modulator has been shown to have a neuroprotective effect in MPTP-induced animal model of PD. It has been also proven to be effective in attenuating behavioural abnormalities in rodent models of schizophrenia.

Structure-based virtual screening is nowadays a standard tool in drug discovery used to identify new compounds targeting a protein of interest. We constructed a homology model of dopamine D_2 receptor in active conformation in complex with dopamine and a negative allosteric modulator SCH202676 based on β_2 adrenergic receptor and M_2 muscarinic receptor templates (the latter one in complex with an agonist and allosteric modulator). We used Glide form Schrödinger suite of software to refine docking poses of both orthosteric and allosteric ligand. The docking pose of SCH202676 was additionally corrected by induced-fit docking and molecular dynamics. We used the obtained complex to screen Enamine database using virtual screening workflow of Schrödinger suite of software. The best 100 hits were subjected to visual inspection and 17 most promising compounds were subjected to experimental validation. As a result we identified a number of negative and positive allosteric modulators. In addition, we identified D_2 receptor allosteric modulators among compounds that were initially designed as D_2 receptor orthosteric ligands. Importantly, this was the first successful identification of family A GPCRs allosteric modulators using structure-based virtual screening targeting allosteric site.

3,4-DIHYDROQUINAZOLIN-4-ONES AS NEW LIGANDS OF MGLUR7 RECEPTOR

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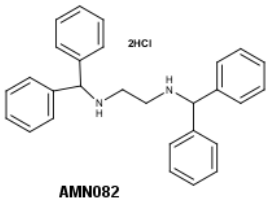
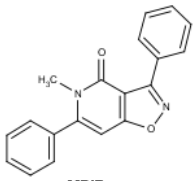
The metabotropic glutamate receptor 7 (mGluR7) is a member of group III family of mGluRs that bind to protein G and inhibit the adenylate cyclase [1]. Anatomic evidence demonstrates that mGluR7 has the highest CNS density of all group III mGluR subtypes due to widely distribution and presence at broad range of synapses [2]. Many studies have shown that mGlu7 receptor could play an important role in treatment of anxiety, post-traumatic stress disorder, depression autism, drug abuse, anxiety, and schizophrenia [1-4]. It was reported that in terms of discovery of new selective ligands the mGluR7 receptor is the most challenging of the all mGluR subtypes [4]. AMN082 is the first discovered mGluR7 agonist ligand [5]. The mGluR7 antagonists activity of MDIP and MMPIP was reported by Suzuki *et al.* in 2007 [1] while negative allosteric modulation for ADX71743 was described by Kalinichev *et al.* [6].

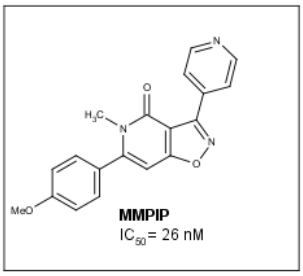
Among the known hit compounds ADX71743 and MMPIP has been selected as a model molecules towards new ligands development as well as standards for *in vitro* study. Variety of chemotypes were synthesized and examined followed by the primary *in vitro* evaluations which lead to the selection of new quinazolinone derivatives as a potent mGluR7 negative allosteric modulators.



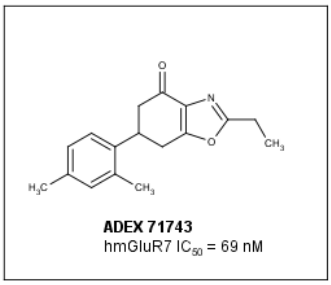
AMN082



MDIP
IC_{50}= 20 nM
poor metabolic stability



MMPIP
IC_{50}= 26 nM



ADEX 71743
hmGluR7 IC_{50} = 69 nM

This study is partially supported by project PBS1/B7/8/2012 financed by *The National Centre for Research and Development (NCBR).*

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ALLOSTERIC PROBE DEPENDENCE ON HUMAN MU OPIOID RECEPTOR

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Allostery is one of the most intriguing features of macromolecules. It is one of the possible answers for the question: how all the complex functions required in sophisticated organisms like primates are performer by relatively low number of proteins encoded in the human genome. Allosteric modulators can alter the response of a protein to its native ligand – e.g. they can modulate response of a receptor to its agonist or rate of a reaction catalyzed by an enzyme. Moreover, they present some unique properties, e.g. signaling bias or probe dependence. The latter is one of the most interesting properties of allosteric modulators. It makes the modulator affect the action of different ligands in a different way. Therefore, it allows for unprecedented selectivity, with different both intensity and quality of response to different ligands. This enables possibility of design of compounds of a very complex pharmacological action.

One of the compounds exerting pronounced probe dependence is BMS986122. The compound is a recently discovered positive allosteric modulator of human mu opioid receptor (MOR). It elicits different influence on number of MOR agonists, depending not on their structure but on their nature – full agonists are affected in a different way than partial agonists. The exact mechanism of this divergence is unknown.

In the presented study, we employed all-atom molecular dynamics (MD) simulations to investigate influence of BMS986122 on binding and action of (R)-methadone and buprenorphine at MOR. Principal component analysis (PCA) was used to sift all relevant information. This approach has proven to be effective in our previous studies [1,2]. The analysis of trajectories indicate that the effect of BMS986122 is mediated mainly by 6^{th} and 7^{th} transmembrane helices of MOR and that the Tyr 7.53 residue is important in signaling and modulation.

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CHEMICAL COMPOSITION, ANTIMICROBIAL AND ANTIPROLIFERATIVE ACTIVITIES OF THE ESSENTIAL OIL FROM CAMEROONIAN *ERIGERON FLORIBUNDUS*

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Erigeron floribundus (Asteraceae) is a herbaceous plant, 1.5 m in height, with pubescent, lanceolate leaves and flowers in yellowish panicles. In Cameroon it is widely used in the folk medicine to treat angina, female infertility, AIDS, dental pain, headache and various diseases of microbial and non-microbial origin (1). Despite the interest in its bioactivity, the plant has received little phytochemical investigation. For this reason, we became interested in the evaluation of *in vitro* antimicrobial and antiproliferative activities of the essential oil obtained from the aerial parts of *E. floribundus*, using agar disc diffusion and microdilution, and MTT methods, respectively. Since essential oils represent an interesting alternative approach against the occurrence of drug resistance in many infectious bacterial pathogens, we completed the work by investigating the inhibitory effects of *E. floribundus* essential oil on nicotinate mononucleotide adenylyltransferase (NadD). NadD occupies a central position in bacterial NAD^+ biosynthesis and is essential for cellular NAD^+ synthesis, as demonstrated by gene deletion, targeted protein degradation, and knocking down experiments (2). Based on this finding, NadD has been recently recognized as a promising new target for developing novel antibiotics (3). Another attractive aspect of targeting NadD is that it is highly conserved in the overwhelming majority of bacterial genomes including most pathogens. Therefore, natural products based on NadD inhibition have the potential of possessing wide-spectrum antibacterial activity. The results of this study will be discussed.

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EVALUATION OF THE *IN VITRO* TRYPANOCIDAL ACTIVITY OF *VERNONIA AMYGDALINA* LEAF EXTRACTS AND ISOLATED COMPOUNDS

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Human African trypanosomiasis (HAT), also known as African sleeping sickness, is a neglected disease caused by two subspecies of the protozoan parasite *Trypanosoma brucei*, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. The parasites are introduced into the mammalian host by the bite of an infected tsetse fly and HAT progresses in two stages: the hemo-lymphatic or early stage and the meningoencephalitic or late stage on which the parasites enter the central nervous system (CNS) (1). HAT threatens primarily rural populations and is fatal unless treated. To date, there are five drugs approved, although none of them are satisfactory, due to treatment failures and toxicity, and the parenteral administration that is inappropriate in settings with poor medical infrastructure. Therefore, there is an urgent need to improve HAT treatment by enhancing the oral administration and the discovery and development of cost-effective new drugs. Drug discovery efforts are nowadays directed towards natural products and medicinal plants represent a validated source for discovery of new lead compounds and standardized herbal medicines against trypanosomiasis (2).

Vernonia amygdalina def., generally called “bitter leaf”, is a small shrub that can grow as tall as 5 m in height, and is member of the Asteraceae (or Compositae) family. The species is indigenous to tropical Africa and is found wild or cultivated all over sub-Saharan Africa. Due to its abundant availability in sub-Saharan countries, *V. amygdalina* has become commonly used in the traditional medicine by local ethnic groups. The leaves of *V. amygdalina* are used in phyto-medicine to treat fever, hiccups, kidney disease and stomach discomfort, as well as anthelmintic and for treatment and prevention of malaria (3). On the basis of the above-illustrated therapeutic properties, the *V. amygdalina* leaf extracts and isolated compounds have been selected as valid candidates for investigation as potential inhibitors of *Trypanosoma brucei*. The results of this study will be discussed.

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VERSATILE SYNTHESIS OF 2,5-DISUBSTITUTED TRIAZOLOTRIAZINE DERIVATIVES AS A_{2A} ADENOSINE RECEPTOR ANTAGONISTS

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Numerous research studies have investigated and confirmed the therapeutic potential of A_{2A} adenosine receptor (A_{2A} AR) antagonists in neurodegenerative disorders such as Parkinson's disease (PD), Huntington's disease, and Alzheimer's disease (1).

We previously developed 8-ethoxy-9-ethyladenine (Fig. 1), which exhibited excellent results in *in vivo* models of PD despite its moderate affinity for the A_{2A} AR (2). Another A_{2A} AR antagonist employed for preclinical characterization is ZM241385 (Fig. 1), endowed with a triazolotriazine nucleus and an excellent affinity profile for the A_{2A} receptor subtype (3).

Hence, we designed and synthesized a new triazolotriazine analogue in which the furan ring of ZM241385 was replaced with the more metabolically stable ethoxy substituent inherent to the potent 8-ethoxy-9-ethyladenine. Furthermore, in order to mimic the structure of this last compound, the corresponding 5-unsubstituted derivative was synthesized (Fig. 1). A novel synthetic approach was developed for the designed triazolotriazine compounds, which permits feasible modification of positions 2 and 5 of the triazolotriazine nucleus.

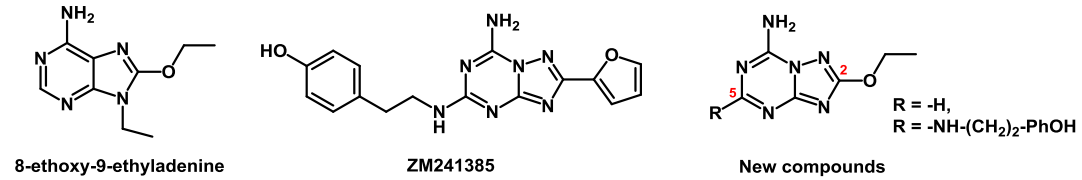

Figure 1: Structures of reference A_{2A}AR antagonists and the general structure of the new compounds.

The new compounds (Fig. 1) were tested in binding studies at human A_1, A_{2A}, and A_3 ARs cloned and transfected in CHO cells. Biological data showed that they behave as A_{2A} AR antagonists with nanomolar or micromolar affinity and could represent a starting point for the design of new therapeutic tools for the treatment of neurodegenerative disorders.

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DOPAMINE D_2, D_3, AND D_4 RECEPTOR AFFINITIES OF 77-LH-28-1 AND ITS ANALOGUES

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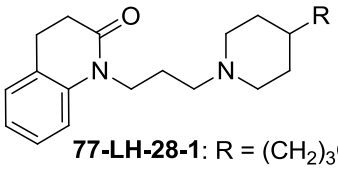
In functional assays, 77-LH-28-1 selectively activates M_1 muscarinic acetylcholine receptor (mAChR) with respect to M_2-M_5 muscarinic subtypes (1). Initially classified as an allosteric agonist, 77-LH-28-1 is actually considered a bitopic agonist (2). This ligand shares a binding domain, which includes both the orthosteric binding site and a putative allosteric region, to selectively engage and activate the M_1 mAChR. 77-LH-28-1 displayed antipsychotic and cognition-enhancing efficacy in pre-clinical models of schizophrenia and Alzheimer's disease (1). Unfortunately, its efficacy was confounded by nonselective effects on other receptors (3). Among these receptors, 77-LH-28-1 has been reported to bind the short isoform of the dopamine D_2 receptor ($D_{2S}R$) (4). In order to know more about its pharmacological dopaminergic properties, 77-LH-28-1 was evaluated for its affinity at dopamine D_2-like receptors ($D_{2L}R$, D_3R and D_4R subtypes) by radioligand binding assays. 77-LH-28-1 showed high affinity and selectivity for D_4R with respect to $D_{2L}R$ and D_3R. To better understand the structural features required for the selective interaction with D_4R, the aliphatic butyl chain of 77-LH-28-1 has been modified and the novel compounds **1**-**6** were prepared. These compounds and the already published compounds **7** and **8** (5) were evaluated in binding studies at dopamine $D_{2L}R$, D_3R and D_4R.



77-LH-28-1: R = $(CH_2)_3CH_3$
1: R = CH_3
2: R = $(CH_2)_2CH_3$
3: R = $(CH_2)_4CH_3$
4: R = C_6H_5
5: R = $CH_2C_6H_5$
6: R = $(CH_2)_2C_6H_5$
7: R = $(CH_2)_2CH_2OH$
8: R = $(CH_2)_4CH_2OH$

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NOVEL POTENT 5-HT$_{1A}$ AGONISTS SELECTIVE OVER α_1-ADRENOCEPTOR SUBTYPES

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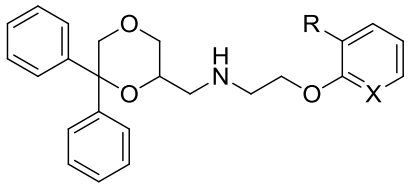
The analogues **2-5** of the potent 5-HT$_{1A}$ receptor agonist and α_{1d}-adrenoceptor (α_{1d}-AR) antagonist **1** (1) were rationally designed and prepared to preliminarily evaluate whether electronic and/or lipophilic properties of substituents in the *ortho* position of its phenoxy moiety could exert any favorable effects on the affinity/activity at 5-HT$_{1A}$ receptor and selectivity over α_1-AR subtypes. The methoxymethylenoxy derivative **6**, an intermediate in the synthesis of **5**, and the 2-pyridyl derivative **7** were also included in this study. The biological profiles of the novel compounds were assessed using radioligand competition binding assays. Moreover, lead **1** and the most interesting compounds **5** and **6** were also evaluated for their affinity for dopamine D$_2$-like receptors. To rationalize the experimental observations and derive information about receptor-ligand interactions of the reported 1,4-dioxane ligands, a retrospective computational study, involving already published and the novel derivatives **2-7**, and docking studies, using two 5-HT$_{1A}$ and one α_{1d} receptor models generated by homology techniques, were performed.



1: X = CH; R = OCH_3
2: X = CH; R = NO_2
3: X = CH; R = Cl
4: X = CH; R = CH_3
5: X = CH; R = OH
6: X = CH; R = OCH_2OCH_3
7: X = N; R = H

The results highlighted that proper substituents in position 2 of the phenoxy moiety of **1** allow to selectively address the ligands toward 5-HT$_{1A}$ receptor with respect to α_1-ARs and D$_2$-like receptors. Indeed, 5-HT$_{1A}$ receptor also accommodates substituents bulkier than methoxy group whereas both α_1-ARs and D$_2$-like receptors have more stringent steric requirements being intolerant to the increase of steric bulk itself. Among the novel compounds, **6** showed the best 5-HT$_{1A}$ selectivity profile and the highest potency at 5-HT$_{1A}$ receptor, behaving as a partial agonist. Finally, **6**, tested in the light/dark exploration test in mice, significantly reduced anxiety-linked behaviors.

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NOVEL 5'-*C*-ETHYL-TETRAZOLYL-N^6-SUBSTITUTED-ADENOSINE DERIVATIVES: SYNTHESIS AND BIOLOGICAL EVALUATION

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Adenosine is an endogenous purine ribonucleoside implicated in the control of the function of many tissues and organs. It exerts a protective action throughout all organs of the body and plays an important role not only in the pathophysiological processes, but also in the modulation of normal processes. Adenosine exerts its effects by interacting with specific G-protein coupled receptors, classified in 4 subtypes: A_1, A_{2A}, A_{2B} and A_3 (1). A_1 adenosine receptor (A_1AR) is widely distributed throughout the brain but also in the heart, aorta, liver kidney, bladder and eye. A_1AR agonists have shown neuro- and cardioprotective effects, but their clinical use is hampered by severe cardiovascular side effects. A_3AR are expressed in multiple organs and at low levels in the CNS. A_3AR agonists are in clinical trials for the treatment of cancer and inflammatory diseases, and recent preclinical studies reported their analgesic effects in chronic neuropathic pain (2).

Our previous work showed that potent dual A_1AR agonists and A_3AR antagonists have been obtained combining a 5'-*C*-ethyl-tetrazolyl moiety and an appropriate N6-substitution in adenosine derivatives (3). A dual A_1 agonist and A_3 antagonist might be useful in the treatment of glaucoma and other diseases, and might have advantages respect to the combination of two drugs.

In order to study the influence of N6-substitution on affinity and selectivity at ARs, a novel series of 5'-*C*-2-ethyl-tetrazolyl-N^6-substituted adenosine derivatives were synthesized and assayed at all human adenosine receptor subtypes. The results of this study will be discussed.

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GENETIC DELETION OF THE NOCICEPTIN/ORPHANIN FQ RECEPTOR IN THE RAT ATTENUATES THE MOTIVATION FOR DRUGS OF ABUSE

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The nociceptin (NOP) receptor is a G-protein-coupled receptor whose natural ligand is the nociceptin/orphanin FQ (N/OFQ) peptide. Evidence from pharmacological studies suggests that the N/OFQ system is implicated in the regulation of several addiction-related phenomena, such as drug intake, withdrawal and relapse.

Here, to further explore the role of NOP system in addiction, we used NOP (-/-) rats to study the motivation for cocaine, heroin and alcohol self-administration in the absence of N/OFQ function. Saccharin (0.2% w/v) self-administration in the NOP (-/-) and Wt counterpart was also investigated. Results showed that, compared to Wt, NOP (-/-) rats showed reduced propensity to self-administer cocaine (0.25 mg/inf) both under a Fixed Ratio 1 and a Progressive Ratio schedule of reinforcement. This effect was not dependent on the cocaine concentration since similar results were obtained also at lower (0.125 mg/inf) and higher (0.5 mg/inf) concentrations. Consistently, cocaine (10 mg/kg, i.p.) was able to induce place preference in Wt but not in NOP (-/-) rats.

To evaluate if lack of motivation for cocaine could generalize to other drugs of abuse, we tested NOP (-/-) rats for heroin (20 μg /inf) nicotine (30 μg /inf) and ethanol 10% v/v self-administration. With the exception of nicotine for which no difference between Wt and NOP (-/-) was determined we found that NOP (-/-) rats showed significantly lower self-administration levels. Saccharin self-administration was also not affected by NOP receptor deletion, excluding the possibility of nonspecific behavioural effects linked, for example, to learning deficits or to generalized disruption of reward mechanisms in NOP (-/-) rats.

In conclusion, our results demonstrate that genetic deletion of NOP receptors confers resilience to drug abuse except nicotine and support a role for NOP receptor antagonism as a potential treatment option for drug dependence.

DESIGN AND SYNTHESIS OF NOVEL BROMINE-CONTAINING PAROXETINE DERIVATIVES TOWARD SEROTONIN TRANSPORTER STRUCTURE-FUNCTION ANALYSES

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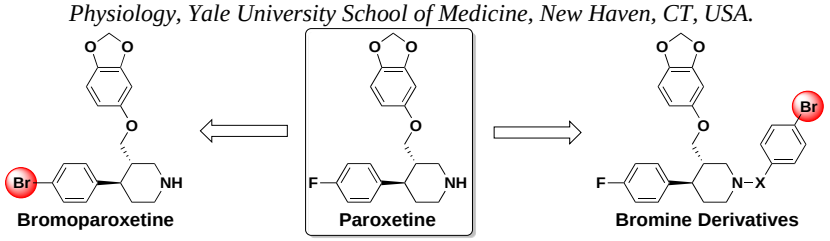
Molecular Targets and Medications Discovery Branch, NIDA-IRP, NIH, Baltimore, MD, USA. Dept. of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT, USA.



The serotonin transporter (SERT) recycles extracellular serotonin into the presynaptic neuron, essential for healthy brain function.[1] Disruption of this tightly regulated mechanism can cause diseases, such as obsessive-compulsive disorder, autism, epilepsy and depression.[2] As such, the SERT is a primary target for the treatment of these disorders; however, despite its success as a drug target, the relationship between the protein's structure on an atomic level and its function is still poorly understood.

Over 80% of the three-dimensional macromolecular structure data in the Protein Data Bank were obtained by X-ray crystallography.[3] Regardless, membrane proteins like SERT are challenging crystallographic targets in part because they often form crystals that diffract to relatively low resolution (e.g., ~3.5-4 Å); therefore, it is difficult to accurately place the functional groups that are critical to determine protein-ligand interactions. This problem can be surmounted if the drug is modified so that one of its atoms is replaced with a chemically similar but more electron dense atom, as would occur with halide replacement (e.g., bromine for fluorine). Because the "heavier" bromine absorbs X-rays much more strongly than the "lighter" fluorine, the X-ray scattering yields an anomalous signal that can then be used as a "beacon" to accurately orient the molecule.[3]

As such, the selective serotonin reuptake inhibitor, paroxetine (Paxil®), which binds to SERT with picomolar affinity, was chemically modified to bear the heavy atom bromine. This was achieved 1) by appending a bromophenyl using varying carbon linker chains to the paroxetine piperidine nitrogen or 2) by replacing the paroxetine fluorine with a bromine via a multi-step, target-driven synthesis. Further studies will include analyzing the binding of these and additional paroxetine analogs at several SERT mutants.

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1,2,4-OXADIAZOLE DERIVATIVES AS NEW POSITIVE ALLOSTERIC MODULATORS OF MGLU4 RECEPTOR

Anna Stankiewicz,† *Ryszard Bugno*,† *Grzegorz Burnat*,‡ *Piotr Brański*,‡ *Joanna M. Wierońska*,‡ *Andrzej J. Bojarski*,† *Andrzej Pilc* ‡

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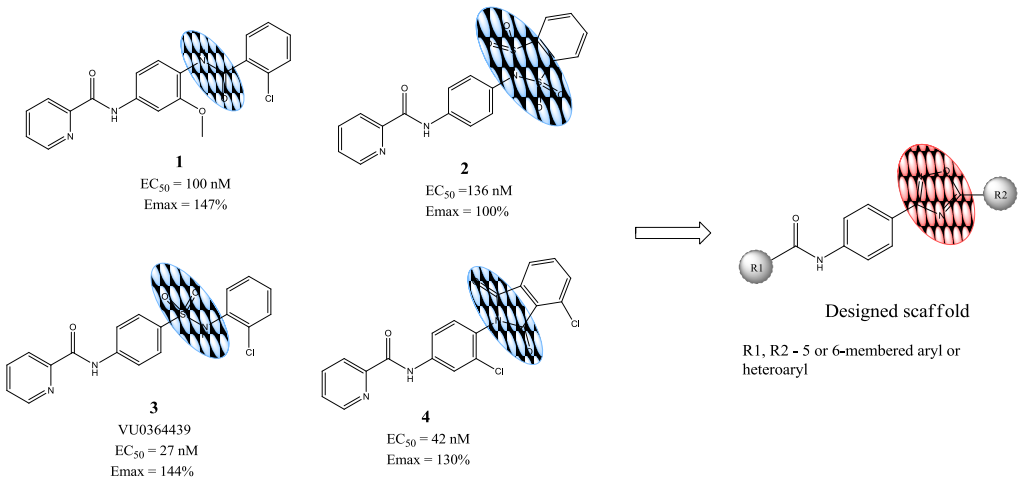
Metabotropic glutamate receptors (mGluRs) are members of the group C family of GPCRs and play important roles in a broad range of central nervous system functions having therapeutic potential in a variety of neurological and psychiatric disorders [1]. Due to the lack of receptor subtype selectivity and physiochemical properties of mGluR orthosteric ligands (poor bioavailability and low potential of blood-brain barrier penetration) a significant effort has been made to identify compounds that can act as allosteric modulators which potentiate the response of endogenous agonists [2]. Number of reviews are available summarizing recent progress in developing new allosteric ligands of mGluRs [3]. Among all, the group III subtypes: mGluR4, mGluR7 and mGluR8 still remains the least explored but with mighty potential for future development of clinical drugs [4].

In present work a development of a new series of potential mGluR4 PAM's is described. Ligands were designed based on structure of known mGluR4 modulators by bioisosteric substitution of mutual parts of molecules with 1,2,4-oxadiazole ring. The results of performed *in vitro* experiments combined with structure-activity relationship determination for new series of derivatives based on developed scaffold will be disclosed.



This study was partially supported by project UDA-POIG.01.03.010-12-100/08-00 co-financed by European Union from the European Fund of Regional Development (EFRD).

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DEVELOPMENT OF NEW DIAGNOSTIC TOOLS TO TARGET Κ-OPIOID RECEPTORS

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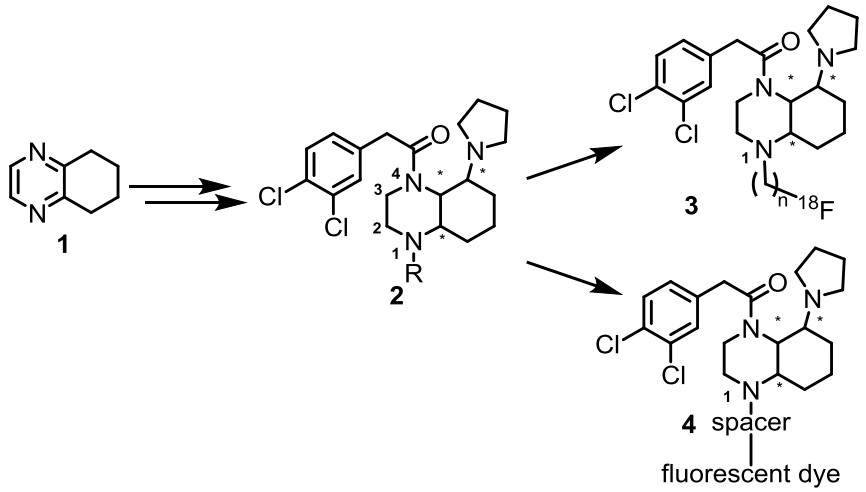
It has been demonstrated that κ-opioid receptors, G-protein coupled receptors belonging to the opioid family, play a key role in pain transmission and also in some neurological diseases, such as major depression, anxiety and addictive disorders. Therefore, the development of new κ selective diagnostic tools is required to better understand the biological pathways related with these pathologies.

The aim of this project is the development of novel imaging tools based on quinoxaline-derived κ-agonists.



Our group has previously demonstrated the high κ affinity of quinoxalines of type **2**. In this project a new synthetic route starting from 5,6,7,8-tetrahydroquinoxaline (**1)** was developed to achieve stereoisomerically defined quinoxalines **2**.

Several substituents in position 1 will be introduced into the building block **2** in order to modulate both the κ receptor affinity and the pharmacokinetic properties. These structural modifications will provide insight into structure activity relationships and will improve our understanding of the dynamics of ligand-active site interactions.

The most promising ligand will be used as starting point for the development of a PET tracer (**3**) by introducing a radioactive ^{18}F atom in different positions of the side chain at position 1.

A fluorescent dye will be attached to position 1 in order to obtain compound **4**, which would allow the expansion to fluorescence-based imaging techniques. Moreover, **4** can be used as alternative to a radioligand in receptor binding studies.

3-FURAN-2-YL-N-P-TOLYL-ACRYLAMIDE, A POSITIVE ALLOSTERIC MODULATOR OF α7 NICOTINIC ACETYLCHOLINE RECEPTORS, ENHANCES MEMORY AND STIMULATES ERK1/2 PHOSPHORYLATION IN MICE

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The α7 nicotinic acetylcholine receptor (nAChR) is considered a potential target for novel psychopharmacological treatments for neurodegenerative disorders such as Alzheimer's and Parkinson's diseases as well as schizophrenia.

Our findings, focused on PAM-2, a positive allosteric modulator of α7 nAChRs (1), indicate that this PAM: (a) improves memory acquisition/consolidation processes after acute treatment as well as memory consolidation after chronic treatment by using the passive avoidance (PA) test in male mice (2). This activity was inhibited by methyllycaconitine (MLA) (an α7-selective antagonist), confirming the role of α7 nAChRs in the promnesic activity of PAM-2 (2); (b) recovers the memory impairment in animals treated with the muscarinic antagonist scopolamine (2). (c) Furthermore, a synergistic (acute) effect between inactive doses of PAM-2 and DMXBA, a selective α7-agonist, was observed (2). Regarding the neuronal mechanisms involved in the promnesic activity elicited by PAM-2, we found that (d) PAM-2 did not affect the α7 nAChR expression but increased the extracellular signal-regulated protein kinase 1/2 (ERK1/2) phosphorylation in the hippocampus and prefrontal cortex in mice (2). Based on our data we demonstrated that PAM-2 may constitute a promising therapeutic agent for the treatment of memory impairment in neurological conditions such as Alzheimer's disease and schizophrenia where the cholinergic tone is altered.

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PROBING THE 2-POSITION OF 9-ETHYLADENINE FOR POTENT AND SELECTIVE A_{2A} ADENOSINE RECEPTOR ANTAGONISTS

Ajiroghene Thomas,[a] Michela Buccioni,[a] Diego Dal Ben,[a] Catia Lambertucci,[a] Gabriella Marucci,[a] Michael Alliance Ngouadjeu,[a] Claudia Santinelli,[a] Andrea Spinaci,[a] Sonja Kachler,[b] Karl-Norbert Klotz,[b] Rosaria Volpini[a]

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The A_{2A} adenosine receptor (A_{2A} AR) represents a key target for the development of potential drugs in treating disorders of the central nervous system (1). Previous studies have demonstrated that the insertion of substituents at various positions on adenine leads to A_{2A} AR antagonists with affinity in the micromolar to nanomolar range (2).

In this work, a series of 9-ethyladenine derivatives bearing phenylalkylamino, phenylakyloxy or phenylakylthio groups of different lengths at the 2-position were synthesized and tested against the human adenosine receptors (Fig. 1). The derivatives showed sub-micromolar affinity for these membrane proteins. The further introduction of a bromine atom at the 8-position has the effect of improving the affinity and selectivity for all ARs and led to compounds that are able to bind to the A_{2A} AR subtype at low nanomolar levels.

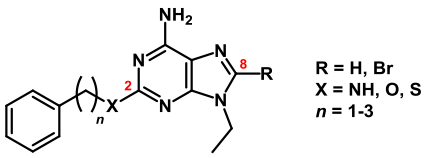

Figure 1: The general structure of the new A_{2A} AR antagonists.

Functional studies confirmed that the new adenine derivatives behave as A_{2A} AR antagonists with half-maximal inhibitory concentration values in the nanomolar range. Molecular modelling studies provide a description of the possible binding mode of these compounds at the A_{2A} AR and an interpretation of the affinity data at this AR subtype (3).

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CONSTRAINED RIBOSE ATP DERIVATIVES AS NEW POTENT ANTAGONISTS FOR THE PURINERGIC P2X3 RECEPTORS

Ajiroghene Thomas,[a] Michela Buccioni,[a] Diego Dal Ben,[a] Catia Lambertucci,[a] Gabriella Marucci,[a] Michael Alliance Ngouadjeu,[a] Andrea Nistri,[b] Claudia Santinelli,[a] Andrea Spinaci,[a] Rosaria Volpini[a]

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Specific targeting of the ATP-gated channels containing P2X3 subunits is proving a promising strategy for the treatment of chronic pain syndromes, chronic cough, overactive bladder disorders, interstitial cystitis, and migraine (2). 2',3'-*O*-(2,4,6-trinitrophenyl)adenosine-5'-triphosphate (TNP-ATP) behaves as a highly potent competitive antagonist of P2X3 receptors with an IC_{50} of 1 nM.

Molecular docking analysis of the agonist ATP and the antagonist TNP-ATP at the P2X3 receptor exposed the role of the 2',3'-*O*- substitution in receptor inhibition mechanism (3). Based on these results, we designed and synthesized TNP-ATP analogues and their monophosphate derivatives as new antagonists of the P2X3 receptor, in which the 2,4,6-trinitrophenyl residue in the 2',3'-*O* position of the sugar moiety was replaced with simpler cyclohexylidene or benzylidene groups (Fig. 1).

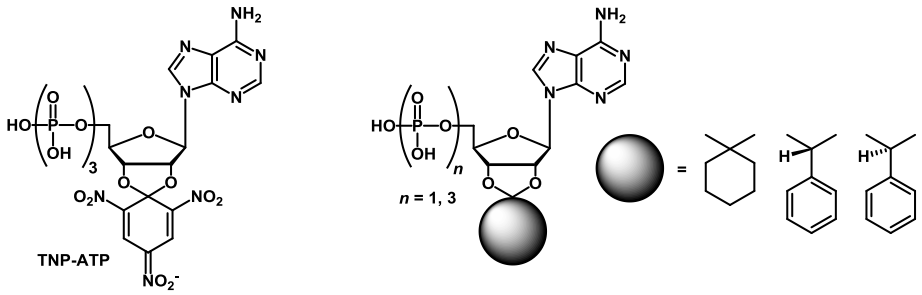

Figure 1: Structures of the reference TNP-ATP and the new P2X3 antagonists.

The newly synthesized nucleotides were evaluated with patch clamp technique on murine P2X3 receptors expressed on trigeminal sensory neurons. The results of the biological evaluation show that the 2',3'-*O*-substituted ATP analogues developed in this study are competitive antagonists of the purinergic P2X3 receptor with potency at nanomolar level. Furthermore, these new P2X3 antagonists are selective against ionotrophic $GABA_A$ and 5-HT_3 receptors, also expressed on the trigeminal sensory neurons.

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BIOACTIVE HETEROCYCLES IN THE LEAVES ESSENTIAL OIL OF *COTULA CINEREA* GROWING IN ALGERIA DESERT

Tigrine Nacéra

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Medicinal plants, including herbs and spices, have many phytochemicals, resulting from secondary metabolism, which are potential sources of natural antioxidants and anti-inflammatory agents, such as diterpenes and flavonoids. These plants have been widely used in traditional practices as treatment for many diseases and to extend the shelf life of foods. Nowadays, a large number of herbal plants have been screened for their potential and exploited for commercial applications, but scientific researches of the efficacy of desertic plants is often lacking in most cases. Consequently, and as part of a chemical investigation program on Algerian native herbs, we have been interested in exploring *C. cinerea*, the medicinal plant growing in Ahaggar which is located in the Hoggar Mountains in the extreme south of Algeria. It has been reported that it is traditionally used for bronchitis, coughs, digestive problems like nausea, vomiting and stomach pain also against sunstroke and rheumatism. Up to now, no chemotaxonomic study of the essential oil has been formally investigated. Our objective was the determination of the chemical composition of the volatile fraction of the leaves. The extraction of the volatile fraction of dried leaves was carried out by Hydro-distillation using Clevenger. The extract was analyzed by gas chromatography with flame ionization detector (GC / FID) and gas chromatography coupled to mass spectrometry (GC-MS). The chemical compositions of qualitative and semi-quantitative have been reported. The identification of compounds revealed the presence of heterocyclic compounds with biomedical power such as 2-Benzylthiophene; Coumarine and Coumarin, 7-methoxy-.

CONFORMATIONALLY CONSTRAINED BENZO[*b*]THIOPHENE DERIVATIVES AS MELATONIN AGONISTS AND ANTAGONISTS

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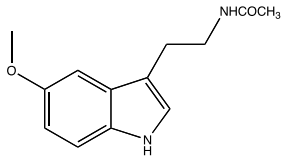
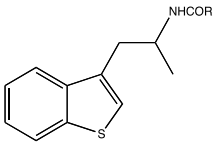
The pineal hormone melatonin (*N*-acetyl-5-methoxytryptamine) is an important neurohormone in the regulation of seasonal and circadian rhythms.[1] Melatonin has been shown to be effective in treating insomnia and jet-lag in humans. The actions of melatonin are believed to be mediated through a family of specific, high-affinity, G-protein coupled cell-membrane receptors. Two receptor subtypes have been cloned in mammals (MT_1, MT_2) which, when expressed in host cells, show the general characteristics of native melatonin receptors. A third receptor subtype, Mel_{1C}, has been cloned from chicken, *Xenopus* and zebrafish, but has not been detected in mammals.

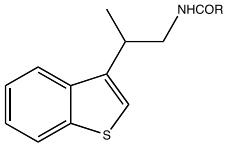
A substantial number of both indole and non-indole derivatives have been evaluated.[2] The 5-methoxy group has been shown to be important for binding to the receptor, but it is not essential for agonist activity. The active conformation of the 3-ethanamine side-chain has been studied in conformationally restricted indole and non-indole analogues. We now report on conformationally constrained benzo[*b*]thiophene derivatives, where the 5-membered pyrrole ring of melatonin has been replaced by thiophene and melatonin's C3-side chain is α- and β-methylated (compounds **1** and **2**, respectively) and describe the effect that these substitutions have on the binding and biology of these molecules.



Melatonin



1: R=Me, Et, n-Pr, *n*-Bu, *c*-Pr, *c*-Bu



2: R=Me, Et, n-Pr, *n*-Bu, *c*-Pr, *c*-Bu

Fig. 1 Structures of melatonin and compounds **1** and **2**

The biological activity of the new analogues was determined in a specific model of melatonin action, the pigment aggregation response of *Xenopus laevis* melanophores, which we have described previously.[3] Both benzothiophene series showed an interesting melatoninergic activity, the **1** (R=*c*-Pr and *c*-Bu) derivatives being more potent antagonists than the control compound, luzindole.

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NEW HYDRAZONES OF 5-NITRO-2-FURALDEHYDE WITH ADAMANTANEALKANOHYDRAZIDES: SYNTHESIS, CONFORMATIONAL ANALYSIS AND *IN VITRO* TRYPANOCIDAL ACTIVITY

Andrew Tsotinis[a], Angeliki-Sofia Foscolos[a], Ioannis Papanastasiou[a], George B. Foscolos[a], Tahsin Kellici[b], Thomas Mavromoustakos[b], Martin C. Taylor[c] and John M. Kelly[c]

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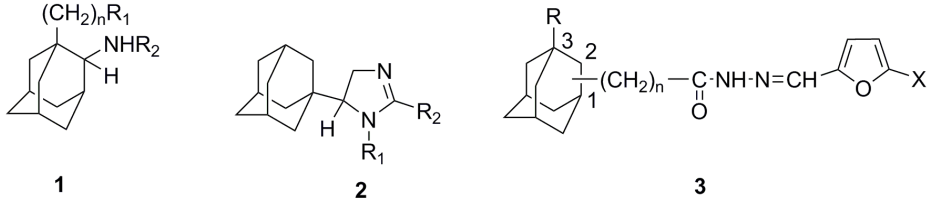
Various anti-influenza A virus aminoadamantanes, e.g. rimantadine, show some trypanocidal activity against the bloodstream forms of *T. brucei*.[1] Furthermore, *N*,1-disubstituted 2-adamantanamines **1**, with hydrophobic substituents R_1 and R_2 (**Fig. 1**) show increased activity.[2] We have previously reported on the synthesis of 5-(1-adamantyl)-2-imidazolines **2**, which were found to be potent against the bloodstream form of *T. brucei*.[3] The present work refers to the design and synthesis of hydrazones of 5-nitro-2-furaldehyde with adamantanealkanohydrazides (**3**). Analogues bearing a nitro-group on the furan ring were active in the nM scale against both *Trypanosoma cruzi* and *Trypanosoma brucei*. In contrast, non-furan ring substituted derivatives were practically inactive against both parasites. NMR data and molecular mechanic studies show that some of these carbohydrazones exist as mixtures of conformers. Conformational analysis shows that hydrazones of 1-adamantane carbohydrazides exist as one conformer, whereas hydrazones of 2-adamantane carbohydrazides, 1-adamantane acetohydrazides and 1-adamantane propionhydazides exist as a mixture of two conformers.



1 2 3

Fig. 1 1-Alkyl-2-adamantaneamines **1**, 5-(1-adamantyl)-2-imidadazolines **2** and adamantane carbohydrazones **3**.

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EPIGENETIC REGULATION OF ADENOSINE A_{2A} AND DOPAMINE D2 RECEPTOR GENE TRANSCRIPTION IN FRUSTATION STRESS-INDUCED BINGE-LIKE PALATABLE FOOD CONSUMPTION

Turchetti Lucia[a], Micioni Di Bonaventura Maria Vittoria[a], Giusepponi Maria Elena[a], Pucci Mariangela[b], Lambertucci Catia[c], Volpini Rosaria[c], D'Addario Claudio[b], Cifani Carlo[a,d]

[a] *School of Pharmacy, Pharmacology Unit, University of Camerino, Camerino, Italy*

[b] *Faculty of Bioscience and Technology for Food Agriculture and Environment, Teramo, University of Teramo, Italy*

[c] *School of Pharmacy, Medicinal Chemistry Unit, University of Camerino, Camerino, Italy*

[d] *NIDA/NIH, Baltimore (MD), USA*

Satisfactory treatments for eating disorders, such as binge eating disorder and bulimia nervosa, are not available at present. Using a well-characterized animal model of binge eating, we investigated the epigenetic regulation of the adenosine A_{2A} Receptor (A_{2A}AR) and dopamine D2 (D2R) gene.

The animal model included four groups (rats fed normally, and then stressed or not, rats exposed to cycles of restriction/refeeding, and then stressed or not).

Gene expression analysis carried out on the amygdala complex of restricted and stressed rats revealed a significant increase of A_{2A}AR and D2R mRNA when compared to non-stressed and non-restricted rats. Administration of the A_{2A}AR agonist (VT 7) induced in restricted and stressed rats a significant increase of A_{2A}AR and D2R mRNA levels when compared to vehicle group, whereas a significant decrease in rats pre-treated with the A_{2A}AR antagonist (ANR 94) was observed.

Pyrosequencing analysis revealed a significant reduction of the % of DNA methylation at A_{2A}AR promoter region in restricted and stressed compared to the non-stressed and non-restricted animals. We did not find any difference in D2R DNA methylation among different groups. Significant changes in the DNA methylation status of A_{2A}AR promoter were found in restricted and stressed rats after administration of VT 7 or ANR 94. We observed a decrease of DNA methylation in VT 7 treated rats and a hypermethylation in ANR 94 rats with respect to the vehicle group. The increase in A_{2A}AR mRNA observed in restricted and stressed rats could be due to a compensatory mechanism to counteract the effect of binge eating, suggesting that the A_{2A}AR activation, inducing receptor gene up-regulation, could be relevant to reduce food consumption. We here demonstrated for the first time the epigenetic regulation of A_{2A}AR in an animal model of binge eating.

TOWARDS THE DEVELOPMENT OF CHEMICAL PROBES FOR *STEMISTRY* APPLICATIONS

Uliassi, E.;(a) Baldassarro, V.A.;(a,b) Massenzio, F.;(a) Petralla, S.;(a) Monti, B.;(a) Calzà, L.;(a,b) Bolognesi, M.L.(a)

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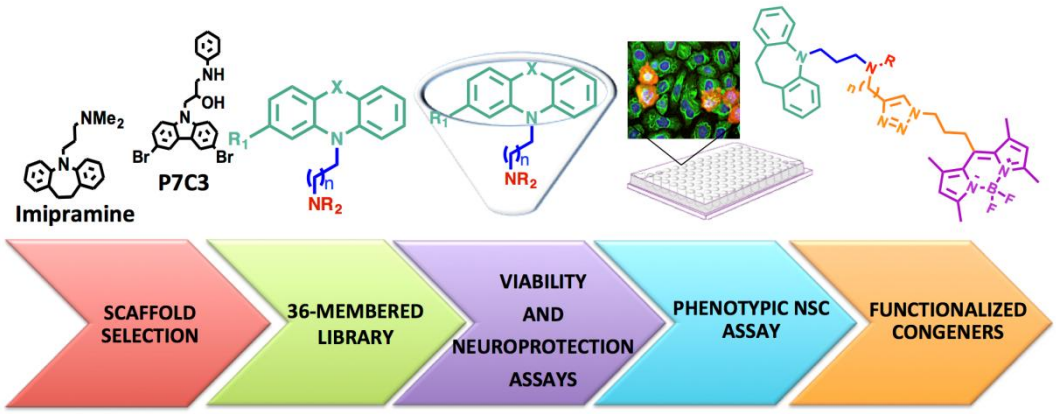
The modulation of endogenous neural stem cells (NSCs) by the use of small molecules (recently defined as *stemistry*)[1] is nowadays one of the most emerging neuroregenerative approach. To date, several mechanisms have been shown to be involved in NSCs fate, however, no validated target for drug discovery purposes has been identified yet. Thus, with the aim to develop new chemical probes able to modulate NSCs, we exploited two strategies (see Figure below): a knowledge-based phenotypic approach (i) and a functionalized congener approach (ii). Toward strategy (i), a focused library of 36 chemical probes has been developed, building on the strong link between drugs (e.g. tricyclic antidepressants)[2] and drug candidates (i.e. P7C3)[3] and the modulation of NSCs. Then, an experimental pipeline, evaluating hepato- and neuro- toxicity, neuroprotection and proliferation in cell lines and primary neurons has been used for prioritizing compounds with better chances to be further investigated in the NSC phenotypic assay. In parallel, the application of the functionalized congener approach (ii) has allowed to design and synthesize fluorescent, multi-target and polyamine congeners of the selected chemical scaffolds. Collectively, these chemical probes can contribute to elucidate NSCs biology.



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DECIPHERING THE SPECIFICS OF RECEPTOR-PEPTIDE INTERACTIONS: THE NEUROPEPTIDE Y2 RECEPTOR-PYY SIGNALLING PEPTIDE SYSTEM

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The neuropeptide Y receptor type 2 (Y_2) belongs to the rhodopsin-like family of G-Protein Coupled Receptors (GPCRs). It is involved in several physiological processes, such as the regulation of appetite. It is activated by three peptides, sorted by decreasing order of affinity: NPY (neuropeptide Y), PYY (peptide YY) and PP (pancreatic polypeptide). These peptides are composed of 36 residues and show great homology in their C-terminal part.

We used our model of the complex between Y_2 and NPY (1) as a starting point for a deeper study of the interactions between the receptor and the preferred natural agonist, PYY. We refined the model combining experimental data and Molecular Dynamics (MD) simulations. The results show the robustness of the binding mode for the C-terminal coil fraction of PYY within the transmembrane section of the receptor. In addition, they shed light on the interactions of the alpha-helical part of the peptide with the extracellular fraction of the receptor. The computational characterization of the mutations, both for the receptor and the ligand, are studied with our Free Energy Perturbation protocol (2). With this strategy, we assist the experimental design and evaluation of the effect of point mutations on receptor-ligand affinities.

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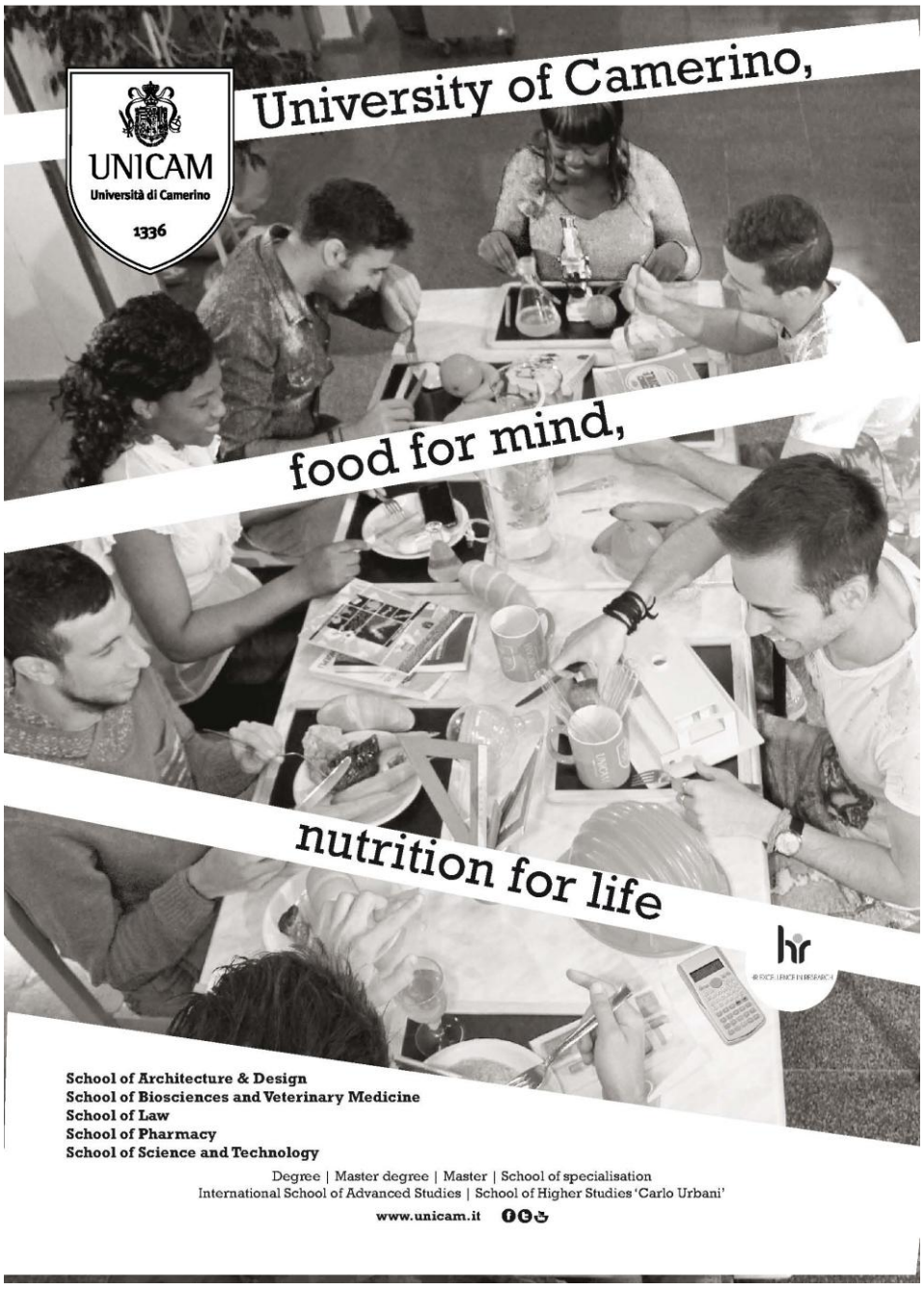
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THE CAMERINO SYMPOSIUM SERIES (1978–2016): A PRIVILEGED OBSERVATORY OF RECEPTOROLOGY DEVELOPMENT

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Integration to the Authorized reproduction of the article published by *In Silico Phamacology* (2013) Dec 20;1:21. doi: 10.1186/2193-9616-1-21. eCollection 2013. Review.

"G protein-coupled receptors are integral membrane glycoproteins, containing a seven-transmembrane helical protein-fold, that mediate a variety of signaling processes, such as neurotransmission, hormonal response, olfaction, and light transduction." It's been a long journey to arrive at this definition. To us, the path really began on Monday September 11, 1978, at Camerino, when the old room of the Chemistry Institute filled with researchers interested in studying receptor chemistry, which was still a very young subject. Despite the publication of several physiological and biochemical works, the physical existence of receptors remained controversial. This skepticism was expressed by Raymond Ahlquist, a respected pharmacologist. Even though he had differentiated the adrenoreceptors as α and β in 1945, Ahlquist still wrote in 1973 "*This would be true if I were so presumptuous as to believe that α and β receptors really did exist. There are those that think so and even propose to describe their intimate structure. To me they are an abstract concept conceived to explain observed responses of tissues produced by chemicals of various structure*" (Ahlquist 1973).

Sitting in the front row at the Chemistry Institute that day were Bernard Belleau, Philip S. Portoghese, Peter G. Waser, and Pietro Pratesi, the leader of one of the few Italian teams devoted to studying receptors, particularly the correlation between the chemical-physical properties of sympathomimetic amines and their biological activity (Pratesi 1958). These four researchers had been invited to Camerino as Speakers at the International Symposium on 'Recent Advances in Receptor Chemistry' by our research team, whose reputation was based on just one paper published (after careful revision!) in the *Journal of Medicinal Chemistry* (Gualtieri et al. 1974).

During the four-day meeting, receptor theory, neurotransmitter membrane receptors, quantitative structure-activity relationships, and computer procedures for rationalizing drug-receptor interactions were the subjects of lively discussion and debate, particularly energized by E. J. Ariëns who, in 1965, had established the prestigious monograph series *Molecular Pharmacology*, published by

Academic Press (Ariens 1965). In his opening lecture, Ariëns hypothesized the existence of sites of action and sites of binding and therewith the existence of silent receptors in membrane proteins with receptor functions. At that time, agonists and competitive antagonists, each of them binding to their own specific receptor sites (two-state model), were thought to be linked by an allosteric interaction. The exceptional scientific atmosphere of the meeting fostered the beginning of a collaboration between Nigel Birdsall, one of the young English talents more involved in receptor study, and Rudolf Hammer, an authoritative researcher at the German pharmaceutical company Boehringer Ingelheim. Birdsall and Hammer eventually worked together to produce an extensive study of Pirenzepine, the first selective muscarinic antagonist, which led to our knowledge of muscarinic receptor heterogeneity. Nevertheless, the content of receptorology then was still so vague that Belleau, in the Preface of the Proceedings published at the end of the meeting, stated "*The hypothetical borders delineating the field of receptorology are so vague and fuzzy that it is hardly possible to provide a clear definition of that science"* (Gualtieri et al. 1979). As an example, transduction mechanisms anticipated that a receptor, interacting with its hormone, could link and activate the enzyme adenylyl cyclase (mobile receptor hypothesis) so forming the second messenger cAMP (Cuatrecasas et al. 1975).

The discovery of a protein acting as transducer between membrane receptor and adenylyl cyclase significantly increased our knowledge of the molecular events that convey signaling from the outside to the inside of the cell. Alfred Gilman, after purifying this protein, called it Gs-protein (Gilman 1987). At the beginning of the 1980s, a number of observations lead to the introduction of the 'ternary complex model' to describe the receptor interaction between G-proteins and endogenous ligands (De Lean et al. 1980) and the quantitation of high (G-protein coupled) and low (not coupled) affinity states of the receptor (Kent et al. 1980). Several novel technologies were developed, including radioligand binding and affinity labeling techniques, detergent solubilization, affinity chromatography purification, and lipid reconstitution. These enabled the fruitful and effective isolation and characterization of receptor processes. For example, the new binding affinity techniques were applied to new large natural and synthetic compounds. This led to the discovery of receptor subtypes in what was previously thought to be a homogeneous system.

Interdisciplinary collaboration between medicinal chemists, pharmacologists, biochemists, and molecular biologists was essential to achieving these advances. This was recognized by the first Camerino Symposium and by every subsequent edition. Such interdisciplinary collaboration led to the isolation and purification of the β_2-adrenoreceptor and its characterization as a glycosylated and phosphorylated polypeptide chain of MW ~ 60–65000 Da (Benovic et al. 1984). The next step was

the reconstitution in phospholipid vesicles of this protein and the verification of its functionality maintenance (Cerione et al. 1983). These advances were reflected in the 1983 meeting, 'Highlights in Receptor Chemistry', whose main topics included Langer's work in describing presynaptic receptors in 1978 (Langer 1978) (subdivided at Camerino for the first time into auto- and hetero-receptors), the description of the dopaminergic receptor's topography with the 'receptor mapping' technique, and a first application of computational procedures in classifying drug and receptor congeners. In the opening article of the Proceedings, David Triggle wrote "*From cloudy and uncertain beginnings we now with confidence can discuss receptor structures, coupling, diseases, defects and can use this knowledge to design new pharmacologic and therapeutic tools*" (Melchiorre and Giannella 1984).

The advent of recombinant DNA technology in the 1980s provided new knowledge of the amino acid sequence of receptors. At the same time, their molecular mechanism of activation was explored using site-directed mutagenesis, chemical synthesis, and molecular modeling in a combined approach. Together with computer graphics, valuable information was obtained concerning a receptor's three-dimensional structure and the specific amino acids involved in a given interaction. The β_2-adrenoreceptor was the first to be cloned and its architecture acknowledged as a homologue of the visual pigmet rhodopsine (Dixon et al. 1986), whose entire amino acid sequence had been determined in 1982 (Ovchinnikov 1982). Hypotheses on the functioning mechanism of the receptor revealed a linkage between the receptor sequence and G-protein transduction. For this reason, researchers began to think that most GPCRs might share a similar arrangement (Dohlman et al. 1987). Robert Lefkowitz, who won the Nobel Prize in Chemistry with Brian Kobilka in 2012 for their pioneering work in studying seven transmembrane receptors (7TMRs, ironically called "The magnificent seven" by Lefkovitz) wrote "*I never imagined that the superfamily of 7TM receptors would grow so large and diverse*" (Lefkowitz 2004). Indeed, ions, organic odorants, amines, peptides, proteins, lipids, nucleotides, and even photons were identified as possible agents able to mediate their message through the 7TMRs. In 1987, it was even discovered that some gases could perform a similar role, with nitric oxide (NO) being the first such finding (Palmer et al. 1987). John Vane, Nobel Prize winner in Physiology and Medicine in 1982, took part in the 1987 Camerino Symposium. In his opening lecture 'Adventures in Bioassay', he wrote of the "*pharmacology and physiology surprise… that one of the most fascinating mediators is a simple one-to-one combination of the main elements of the atmosphere"* (Melchiorre and Giannella 1988).

John Vane
1982 Nobel prize in Physiology and Medicine
Camerino Sept. 6; 1987

At the end of the 1980s, Fulvio Gualtieri's lecture on 1,3-oxathiolane isosteric analogs of muscarinic 1,3-dioxolane ligands (Gualtieri et al. 1988) suggested to Bernard Belleau the synthesis of Lamivudine, a powerful inhibitor of reverse transcriptase (introduced in the following symposium). In this molecule, the 1,3-oxathiolane scaffold, bound to a pyrimidine ring, simulates the ribose nucleus (Soudeyns et al. 1991). This edition of the Symposium saw the beginning of an Italo-Dutch collaboration through a scientific twinning between the Camerino group and a group at Vrije Universiteit (Amsterdam) led by Prof. Henk Timmerman. In the 2007 edition, 'An Overview of Receptor Chemistry', this collaboration was extended to the Cyprus Conference held in Limassol and directed by Prof. Alexandros Makriyannis, director of the Center for Drug Discovery at Northeastern University (Boston). SAR studies yielded more and more selective compounds, allowing the differentiation of many receptor subtypes. As per Fisher's metaphor (Fischer 1894), these were the keys that unlocked the labyrinth. At the same time, the mechanisms preceding and following ligand-receptor interaction were also studied.

The first mutagenesis studies involved the design of structures resulting from the combination of the sequences of multiple receptors (chimeric receptors) (Ostrowski et al. 1992; Strader et al. 1994) or structures with one or more mutated amino acids in specific regions of the receptor polypeptide (site-directed mutagenesis). One of the first chimeras was created by stitching together different sections of α_{2a}- and β_2-adrenergic receptors (Kobilka et al. 1988). It showed that residues in the membrane span produce the ligand-binding specificity, whereas the sequences in the amino and carboxyl terminal portion of the third intracellular loop produce the specificity binding to Gs or Gi. Equally important are the results that Susanna Cotecchia obtained by modifying four amino acids of the third cytoplasmic loop of the α_{1B}-adrenergic receptor (Cotecchia et al. 1992). She presented these results at the 1999 symposium (Cotecchia et al. 2000). These approaches elucidated the role of specific regions of the sequence of the polypeptide chain or of single amino acids, some of which gave rise to constitutively activated receptors. The probable elimination of intermolecular interactions, which are essential in keeping the receptor in an inactive conformation, gives rise to signals that are similar to those of the agonists. As a consequence, it was possible to assume that naturally occurring mutations caused various diseases, including some proliferative disorders

(Spiegel 1998). These observations also led to the discovery of inverse agonism, which is an opposing phenomenon of the constitutive activity, presumably induced by binding and stabilizing the receptor in the inactive state (Lefkowitz 1993). For this reason, inverse agonists are also useful and effective therapeutic tools.

Sir James Black
1988 Nobel prize in Physiology and Medicine
Camerino Sept. 8; 1991

Another important and still unsolved challenge for researchers is the receptor characterization of unknown ligands or functions named 'orphan receptors', obtained with the cloning techniques, whose deorphanization can lead to the discovery of novel physiological responses. The first example of deorphanization was the 5-HT_{1A} receptor encoded by the clone 'G21', isolated from a size-selected human genomic DNA library (Fargin et al. 1988). To date, in spite of the many studies by groups all over the world, only 4% of the proposed pharmacologically relevant 7TMRs are known. Some of the strategies devised to identify the natural ligands of orphan GPCRs were one topic of discussion at the 2007 symposium sessions.

At the end of the 1990s, researchers had defined the universal mechanism that regulates receptor function, which is a sequence of stimulus-dependent receptor phosphorylation by the kinase enzymes (GRKs) followed by arrestin binding (Pitcher et al. 1998; Kohout and Lefkowitz 2003). Thus, Triggle remarked in his opening lecture of the 1999 Symposium "*By the beginning of the 20th Century the foundation had been laid for a definition of receptors that embodied the concepts of specificity, including stereoselectivity, dose–response relationships and transduction-concepts still in use today*" (Triggle 2000). At the beginning of the third millennium, it is possible to synthesize receptors, define their character and properties, and produce genetically modified animals that display our own human receptors. The time is now ripe for advancing our knowledge of those complex mechanisms, which have so fascinated researchers through the years that, in his 'Historical Review' in 2004, Lefkowitz dedicated to them "*entirely his research career*" (Lefkowitz 2004). Receptors can have many faces and acts, as monomeric proteins, as dimers (especially heterodimers), or as oligomers (multimeric quaternary structures). For example, Roberto Maggio's lecture at the Third Millennium Symposium demonstrated that, when co-expressed in the same cells, the M_2 and M_3 muscarinic receptor subtypes can cross-interact with each other forming a

chimeric muscarinic M_2-trunc/M_3-tail receptor with new pharmacological properties (Chiacchio et al. 2000). Consequently we could improve or change our strategies for drug design and development and drug-receptor interaction. The advent of genomics provided new genetically defined targets, which could be associated with disease states, providing new research tools with which to define and validate targets such as knockout mice, siRNA, and so on. The 2003 Symposium, 'Ongoing Progress in Receptor Chemistry', highlighted new tools for medicinal chemists. These included combinatorial chemistry, extremely useful in both generating 'hits' and exploiting molecular space around a 'lead' structure, template-guided synthesis or 'click chemistry'. Moreover, in the 2003 symposium, computational techniques for the study of GPCRs and the rational identification of their ligands are introduced, such as bi-dimensional (2D) and three-dimensional quantitative structure-activity relationships (QSAR), pharmacophore searches, and virtual screening (Triggle 2004).

There was increasing therapeutic interest in molecules which could bind one or more allosteric sites and positively or negatively modulate (PAMs or NAMs) the endogenous ligand response, or which themselves had an agonist or antagonist activity (ago- or antago-allosteric modulators) (Keov et al. 2011). This approach can improve the ligand's subtype selectivity, due to the higher diversity of the allosteric domain relative to the orthosteric one (Christopoulos 2002). Moreover, the allosteric modulators impose a 'ceiling' on the magnitude of their effect (May et al. 2007). These studies led researchers to coin the term 'cooperativity' (positive or negative) to indicate the action of molecules which, by interacting with orthosteric or allosteric sites of one of the two receptors that are part of the homo- or heterodimer, alter the same sites' binding propensity of the other protomer (Milligan and Smith 2007). The introduction of allosteric modulators to the system demands further revision and expansion of the ternary complex model, explaining the drug behavior, which was presented by Nobel prize recipient Whyte Black in his opening lecture "The pharmacology of receptors at the physiological level" to the 1991 symposium. Specifically, the model evolved to the 16-point quaternary complex model. This model takes into account the concomitant binding of orthosteric and allosteric ligands and G protein on the receptor, which can exist in active and inactive conformational states (Christopoulos and Kenakin 2002; Bridges and Lindsley 2008). The selectivity can be engendered by combining both ortho- and allosteric pharmacophores within the same molecule to yield a novel class of 'bitopic' or 'dualsteric' GPCR ligands. This multitarget approach, which somewhat overthrows the one-molecule-one-target paradigm, has been widely applied in the treatment of neurodegenerative and tumor diseases, where a variety of pathological disorders is indicated. Due to the novelty and potential of this therapeutic strategy, an entire session was devoted to the topic at the 2010 Symposium 'Trekking Through Receptor Chemistry'. At the

2010 Symposium, the utility of functional simple domain antibodies (nanobodies) as novel GPCR modulators was illustrated.

Throughout the years, the symposia saw an increasing emphasis on computational techniques. In particular, as we discussed, talks on computer-aided drug discovery became a central part of the Camerino meetings during the early 2000s and underwent a rapid expansion hand in hand with the flourishing of GPCR structural studies. The solution of the crystal structure of rhodopsin in 2000 provided for the first time a reference three-dimensional model (template) for the whole A-family of GPCRs (Palczewski et al. 2000). For seven years, rhodopsin remained the only available receptor solved crystallographically. However, starting in 2007, the field of GPCR structural studies experienced a dramatic expansion. There are currently more than twenty GPCRs for which medium to high resolution crystal structures have been solved, in most cases in complex with multiple small molecule ligands (agonists or antagonists) (Congreve et al. 2011). Recently solved GPCR structures include the M_2 (Haga et al. 2012) and M_3 muscarinic receptors (Kruse et al. 2012), which have been one of the main foci of our research and the symposium for many years.

During a candidate's long journey from hit to lead (described as "crossing the Valley of Death" because of the enormous selection that tested compounds undergo), the candidate too becomes a part of the Camerino symposia's contents. During the 2013 "Receptor Chemistry Skyline" symposium, the opening lecture was delivered by Francesco Bellini, a native of the Italian province Marche, who moved early in his career to Canada, where he has become a successful pharmaceutical manager. His report, "Innovation: transforming an idea into a commercial product", described in detail how an idea (conceived during a Camerino meeting) was translated into a wide-ranging synthesis project, which led to the discovery and clinical use of 3TC (Mateo et al 2012), one of the best AIDS therapy drugs to date. During the same symposium, Roderich Hubbard chaired a session to examine and debate the biophysical techniques used at all stages of the drug discovery process. This topic is of great current interest because of how these studies can elucidate the physicochemical properties (from lipophilicity to binding kinetics and thermodynamics) of successful drugs as well as the success of fragment- and structure-guided discovery and nonconventional targets, such as protein-protein interactions (Tarcsay and Keseru 2013). For example, a drug's binding affinity is modulated by the enthalpic and entropic contributions to the Gibbs energy of binding. Knowing the enthalpic/entropic balance of a compound could thus provide useful information regarding the forces that drive binding affinity. Isothermal titration calorimetry (ITC) can be used to obtain data concerning enthalpy, entropy, and binding affinity.

Thermodynamic optimization plots (TOPs), a novel method developed by Freire (Freire 2009), offer a way to easily organize this data to accelerate drug candidate optimization.

Pain management continues to be a key goal for medicinal chemists, with potential new targets studied in the search for potent and selective drugs. Rottapharm CR4056, for example, exerts its analgesic effect by interacting with the I_2-imidazolinic system (Meregalli 2012). Other potential analgesic agents include the inhibitors of peripherally-restricted fatty acid amide hydrolase (FAAH) (Clapper et al 2010), which deactivates the endocannabinoid anandamide, and of N-acylethanolamine acid amidase (NAAA), which suppresses macrophage activation (Ponzano et al 2013).

Robert J. Lefkowitz
2012 Nobel prize in Chemistry
Camerino May 15; 2016

In conclusion, the receptors are finally a reality. As a closing comment, we offer an evocative line from David Triggle (Triggle 2000): "*Langley and Ehrlich might today be strangers in a strange land were they to return, but they would surely recognize the magnificent fruits of their toil in the vineyards*".

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ACKNOWLEDGEMENTS

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European Federation of Medicinal Chemistry

Comune di Camerino

Comune di Ascoli Piceno
MEDAGLIA D'ORO AL VALOR MILITARE PER ATTIVITÀ PARTIGIANA

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