

# Book of Abstracts



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**European Conference on  
Allostery in Drug Discovery**



**9 | 11 April 2025  
Athens GREECE**

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# Table of Contents

Welcome .....	3
Programme.....	4
<b>Wednesday, 9 April 2025</b> .....	4
<b>Thursday, 10 April 2025</b> .....	5
<b>Friday, 11 April 2025</b> .....	6
Oral Presentation Abstracts .....	8
Poster Abstracts .....	39
Participant List .....	52

# Welcome

Dear Colleagues and Friends,

We are delighted to announce the European Conference on «Allostery in Drug Discovery», which is organized by the “Allostery in Drug Discovery” (ALLODD) Marie Skłodowska-Curie Innovative Training Network Consortium and funded by the European Commission. The conference takes place between 9-11 April 2025 at the Biomedical Research Foundation of the Academy of Athens, in Athens, Greece. More information about the Consortium can be found here: <https://www.allodd-itn.eu/>.

For the past 3 years, the ALLODD Consortium has organized dynamic meetings providing a forum to discuss and extend the impact of computational and experimental methodologies to study protein allostery in the fields of biology, chemistry, pharmacology, physics, and computer science.

This final ALLODD conference is designed to foster the concept of ALLODD via a small format meeting designed to maximize interactions amongst the participants and to provide an opportunity for young scientists to present their work. The conference will be held over three days of scientific talks and includes a poster session and discussions on the topic.

We are excited to host the Conference in the historic city of Athens, Greece and we are looking forward to welcoming you to an inspiring meeting and conference!



Dr. Zoe Cournia  
ALLODD Project Coordinator  
Biomedical Research Foundation,  
Academy of Athens, Greece

# Programme

## Wednesday, 9 April 2025

10:00 – 10:15	<p style="text-align: center;"><b>Opening Remarks</b>  <b>Zoe Cournia</b> (ALLODD Coordinator)</p>
10:15 – 11:15	<p style="text-align: center;"><b>Opening Plenary Lecture:</b>  <b>Jean-Pierre Changeux</b> (Institute Pasteur, France) - The concept of allosteric interaction and its usefulness to understand the higher brain functions and their pathology</p>
11:15 – 15:30	<p style="text-align: center;"><b>Session 1: Towards a global understanding of allostery</b>  <b>Chair: Christoph Rademacher</b></p>
11:15 – 11:45	<p style="text-align: center;"><b>Charalampos G. Kalodimos</b> (St Jude's Hospital, USA) - Leveraging the dynamic conformational ensemble to develop kinase inhibitors</p>
11:45 – 12:15	<p style="text-align: center;"><b>Coffee Break</b></p>
12:15 – 12:45	<p style="text-align: center;"><b>Simone Fulle</b> (Novo Nordisk, Denmark) - Generative Protein Design for Therapeutic Applications</p>
12:45 – 13:15	<p style="text-align: center;"><b>Christoph Rademacher</b> (University of Vienna, Austria) - Allostery of C-type lectins</p>
13:15 – 13:45	<p style="text-align: center;"><b>Antoine Taly</b> (CNRS, France) - The opportunities and limitations of AlphaFold to model allosteric proteins: A case study on P2X receptors</p>
13:45 – 14:00	<p style="text-align: center;"><b>Francho Nerin Fonz</b> (BRFAA, Greece) - Machine Learning for allosteric site prediction</p>
14:00 – 15:00	<p style="text-align: center;"><b>Lunch Break</b></p>
15:00 – 15:30	<p style="text-align: center;"><b>Yiorgo Skiniotis</b> (St. Jude Children's Research Hospital, USA) - Non-Equilibrium Structural Pharmacology of the <math>\mu</math>-Opioid Receptor</p>
15:30 – 18:00	<p style="text-align: center;"><b>Session 2: Identification of allosteric pockets and ligands</b>  <b>Chair: Marco Cecchini</b></p>
15:30 – 15:45	<p style="text-align: center;"><b>Sonja Peter</b> (Nxera, UK) - Comparative Study of Allosteric GPCR Binding Sites and Their Ligandability Potential</p>
15:45 – 16:00	<p style="text-align: center;"><b>David Sotillo Núñez</b> (University of Urbino, Italy) -Automatic detection of cholesterol hot spots in GPCR-membrane systems</p>
16:00 – 17:00	<p style="text-align: center;"><b>Coffee Break</b></p>



17:00 – 17:30	<b>Efstratios Stratikos</b> (University of Athens, Greece) - Targeting the regulatory site of ER aminopeptidase 1 for the development of next-generation immunomodulatory agents
17:30 – 18:00	<b>Demetres D. Leonidas</b> (University of Thessaly, Greece) - Structure driven drug discovery targeting allosteric sites of glycogen phosphorylase
18:00 – 19:00	<b>Poster Session</b>

## Thursday, 10 April 2025

<b>9:30 – 17:00</b>	<b>Session 2: Identification of allosteric pockets and ligands</b> <b>Chair: Ijen Chen</b>
9:30 – 10:00	<b>Reto Walser</b> (Astex, UK) - Fragment screening for the identification of allosteric binding sites
10:00-10:15	<b>Varbina Ivanova</b> (University of Barcelona, Spain) - Multiple-copies Association Studies (MAS) – a computational approach for binding mode elucidation
10:15 – 10:45	<b>Steven Jerome</b> (Schrodinger, Europe) - A Novel Workflow for the In-Silico Identification and Prioritization of Potential Allosteric Binding Sites based on Mixed Solvent Simulations and SiteMap
10:45 – 11:15	<b>Coffee Break</b>
	<b>Chair: Patrick Scheerer</b>
11:15 – 11:45	<b>George Spyroulias</b> (University of Patras, Greece) - NMR evaluation of ligand binding among protein players in the ADP-ribosylation pathway
11:45 – 12:00	<b>Nina-Louisa Efrém</b> (FMP, Germany) - From active site- to allosteric SHP2 inhibitors: Discovery and structure-activity relationship of N-aryl-1H-azaindazoles
12:00 – 12:15	<b>Vincenzo di Lorenzo</b> (RCNS, Hungary) - Advancing Covalent Allosteric Inhibitors: Insights into SHP2 Modulation on the Latch side
12:15 – 12:30	<b>Hryhory Sinenka</b> (University of Strasbourg, France) - Towards the accurate prediction of modulatory ligands activity in synaptic receptors
13:00 – 14:00	<b>Lunch Break</b>



	<b>Chair: György Keserü</b>
14:00 – 14:30	<b>Athanasios Papakyriakou</b> (National Research Center Demokritos, Greece) - Discovery of novel allosteric inhibitor hits for Insulin-Regulated Aminopeptidase provides insights on enzyme mechanism and regulation
14:30 – 15:00	<b>Anastassis Perrakis</b> (National Cancer Institute, Netherlands) - Allosteric Autotaxin inhibitors: a key to clinical success?
15:00 – 15:30	<b>Marco De Vivo</b> (Italian Institute of Technology, Italy) - Protein-protein and protein-nucleic acid interactions through alchemical free energy calculations
15:30 – 16:00	<b>Coffee Break</b>
16:00 – 16:30	<b>Petros Giastas</b> (Agricultural University of Athens, Greece) - Allosteric Activation of ACE2: Structural Insights and Future Perspectives in Drug Discovery
16:30 – 17:00	<b>Xavier Barril</b> (Boehringer-Ingelheim, Germany) - Virtual Screening for Allosteric Drugs: Where are We?"
17:00 – 20:00	<b>Free time</b>
20:00	<b>Conference Dinner</b>

## Friday, 11 April 2025

<b>9:30 – 12:30</b>	<b>Session 3: Understanding allosteric response</b> <b>Chair: Giulia Rossetti</b>
9:30 – 10:00	<b>Judith Günther</b> (Bayer, Germany) - Joys and tribulations pursuing allosteric inhibitors – an industry perspective
10:00 – 10:15	<b>Enrico Guarnera</b> (Merck KGaA, Germany) - Hijacking EGFR Signaling: Computational Insights into Allosteric Antibodies Engineering
10:15 – 10:45	<b>Minos Matsoukas</b> (University of Western Attica, Greece) - Exploring Allosteric Modulation through Virtual Screening: From GPCR Heteromers to a Cancer-linked Target
10:45 – 11:00	<b>Sigrid Pedersen</b> (Charité, Germany) - Elucidating the structure and dynamics of the Melanocortin-4 receptor (MC4R)
11:00 – 11:30	<b>Coffee Break</b>

	<b>Chair: Marc Nazaré</b>
11:30 – 12:00	<b>Adnan Sljoka</b> (RIKEN, Japan) - Probing allosteric activation and biased signaling with rigidity transmission allostery (RTA) theory, NMR, and geometric Monte-Carlo simulations
12:00 – 12:15	<b>Simone Mariani</b> (Forschungszentrum Jülich, Germany) - Predicting Binding Affinity and Kinetics in GPCR Oligomers: Insights from Enhanced Sampling Simulation
12:15 – 12:30	<b>Bohdana Sokolova</b> (Karolinska Institute) - Above-Filter Digestion Proteomics Identifies Drug targets and estimates ligand binding site
<b>12:30</b>	<b>Closing remarks and farewell - End of conference</b>



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## Oral Presentation Abstracts

## **The concept of allosteric interaction and its usefulness to understand the higher brain functions and their pathology**

Jean-Pierre Changeux

Institut Pasteur & Collège de France, Paris.

Classically, the field of drug therapy relied upon “the relationship between isosterism & competitive phenomena”. The new strategy proposed for drug design in neuropharmacology is based upon the concept of allosteric interaction initially proposed to account for the inhibitory feedback mechanism mediated by bacterial regulatory enzymes. In contrast with the classical mechanism of competitive, steric, interaction between ligands for a common site, allosteric interactions take place between topographically distinct sites and are mediated by a discrete & reversible conformational change of the protein.

The concept was soon extended to membrane receptors for neurotransmitters and shown to apply to the signal transduction process which, in the case of the nicotinic receptor for acetylcholine (nAChR), links the ACh binding site to the ion channel. Pharmacological effectors, referred to as allosteric modulators, such as Ca<sup>++</sup> ions or ivermectin, were discovered that enhance the transduction process when they bind to sites distinct from the orthosteric ACh site and the ion channel. The recent X-ray structures, at atomic resolution, of the resting & active conformations of prokaryotic homologs of the nAChR and eukaryotic receptors including the nAChR itself, in combination with atomistic molecular dynamics simulations reveal several distinct quaternary transitions in the transduction process with tertiary changes which profoundly modify the boundaries between subunits. These interfaces host orthosteric and allosteric modulatory sites which structural organization changes in the course of the transition. The model emerging from these studies has led to a new mode of regulation of synaptic efficacy and to the conception and development of several new pharmacological agents.

Such allosteric transitions are hypothesized to mediate the modulation of brain circuits involved in pathologies such as, among others, autosomal dominant frontal lobe nocturnal epilepsy, congenital myasthenia, schizophrenia, the dual action of nicotine both as a drug of abuse and as a cognitive enhancer.

By capitalizing on the constantly developing structural and molecular dynamics information at high resolution, the ultimate aim is the design of orthosteric and allosteric modulators for receptors which are not only selective but also specific to their site on one conformation of the receptor over the others. The strategy has been termed state-based pharmacology which should lead to the rational design of pharmacological agents with a characteristic physiological action such as activators vs inhibitors vs desensitizers. These developments pave the way to a new orientation of neuropharmacology.

## Leveraging the dynamic conformational ensemble to develop kinase inhibitors

Charalampos G. Kalodimos

Department of Structural Biology, St. Jude Children's Research Hospital, Memphis, TN, USA.

Evolutionary pressure has forced kinases to adopt similar active states so that regions required for catalysis are precisely positioned. By contrast, inactive states can be distinct and may in principle vary considerably between kinases. A prime example is imatinib (Gleevec), which selectively binds to an inactive state of Abl. We now have evidence that several kinases populate inactive conformational states that are unique. I will be discussing our efforts to leverage transiently populated conformational states to design selective inhibitor for protein kinases.

## **Generative Protein Design for Therapeutic Applications**

Simone Fulle

Novo Nordisk A/S, Digital Science & Innovation, R&ED, Måløv 2760, Denmark

In recent years, protein design has undergone a transformation, shifting from a physics-based to a machine learning-based discipline, resulting in improved hit rates and the development of tools that facilitate de novo design and optimization tasks. A significant advantage, compared to traditional hit identification methods such as display technologies, is the ability to confine the designs to selected epitopes, paving the way for more knowledge-driven protein binder designs. In the presentation, I will outline our in-house initiatives to establish a scalable GenAI platform for protein design, share benchmarking results, and discuss experimentally verified designs. These applications encompass the design of therapeutic proteins and chemical probes that target specific epitopes of the desired targets.



## Allostery of the C-Type Lectins

Christoph Rademacher<sup>1,2</sup>

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<sup>2</sup>Department of Microbiology, Immunology and Genetics, Max F. Perutz Labs, Biocenter 5, 1030 Vienna, Austria

The C-type lectin protein family is the largest and most complex family of mammalian carbohydrate-binding proteins. They are explored as therapeutic targets in various disease settings such as infectious diseases, immunotherapy, anti-inflammatories, allergies, vaccine development and drug delivery in general. Consequently, there is a broad interest in developing specific ligands that could modulate the function of C-type lectins. However, selective chemical probes are limited. This is due to the shallow, polar, and promiscuous carbohydrate binding sites in many C-type lectins. For several of the members of the C-type lectin family the presence of distant allosteric secondary sites has been implied by previous research. For a few lectins, this has been explored even further, and probes are being developed to expand our insights into the mechanism and function of these sites. Taken together, this approach could offer increased druggability and selectivity while regulating receptor activity.

In this presentation, I will outline our ongoing efforts in leveraging the structural flexibility inherent to C-type lectins to develop small molecule modulators of their receptor functions. To illustrate the practical application of our ligands, I will provide insights from our latest research focused on two C-type lectins: DC-SIGN [1,2] and langerin [3,4]. For DC-SIGN, we have identified an allosteric site and its underlying molecular mechanism of information transfer. In case of langerin, we found a previously unreported and unexpected mode of action for a series of small molecules inhibitors. To expand on these individual insights, a computational approach was developed to predict and characterize allosteric secondary sites in C-type lectins. Here, we identified 20 structurally conserved pockets, at least four of which were predicted to have allosteric potential. These pockets overlapped with known binding sites for ligands and protein-protein interactions, suggesting a conserved function that could be explored for targeting C-type lectins.

- [1] Aretz, J. et al. Identification of Multiple Druggable Secondary Sites by Fragment Screening against DC-SIGN. *Angewandte Chemie International Edition* 56, 7292-7296 (2017).
- [2] Wawrzinek, R. et al. A Remote Secondary Binding Pocket Promotes Heteromultivalent Targeting of DC-SIGN. *Journal of the American Chemical Society* 143, 18977-18988 (2021).
- [3] Hanske, J. et al. Intradomain Allosteric Network Modules Calcium Affinity of the C-type Lectin Receptor Langerin. *Journal of the American Chemical Society* 138, 12176–12186 (2016).
- [4] Aretz, J. et al. Allosteric Inhibition of a Mammalian Lectin. *Journal of the American Chemical Society* 140, 14915–14925 (2018).



## The opportunities and limitations of AlphaFold to model allosteric proteins: A case study on P2X receptors

Antoine Taly<sup>1</sup>, Thomas Grutter<sup>2</sup>

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<sup>2</sup>University of Strasbourg, CNRS, CAMB UMR 7199, F-67000 Strasbourg, France

P2X receptors are trimeric ligand-gated ion channels, activated by ATP. The function of P2X receptors is known from data obtained from biochemical, electrophysiological and molecular engineering experiments as well as structural studies of their 3D structure and gating cycle [1].

The year 2021 was marked by the formidable achievement of predicting the three-dimensional structures of proteins from their amino acid sequences, thanks to AlphaFold 2 (AF2) [2] and RoseTTAFold (RF) [3]. This achievement was even recognized by the 2024 Nobel Prizes in Chemistry. However, predicting the structure of membrane proteins remains challenging for both AF2 and RF due to difficulties in capturing conformational ensembles and interactions with the membrane [4]. Furthermore, although very powerful, AF2 and RF have many limitations, such as the inability to take into account post-translational modifications (PTMs), ions, ligands, DNA and RNA [4].

A new generation of deep-learning tools, AlphaFold 3 [5], RoseTTAFold All-Atom [6], but also boltz [7] and chai1 [8] partly fill the gaps with the possibility to generate models with PTMs, ions, ligands, DNA and RNA. In addition, other tools, like BioEmu [9] allow for the generation of conformational ensembles.

The application of these tools to the study of P2X receptors will be presented showing opportunities but also remaining limitations arguing for the hybridization with more classical modeling approaches.

- [1] Illes, P., Müller, C. E., Jacobson, K. A., Grutter, T., Nicke, A., Fountain, S. J., ... & Di Virgilio, F., **2021**, *British Journal of Pharmacology*, 178(3), 489-514.
- [2] Jumper J, Evans R, Pritzel A, et al. *Nature* 2021 ; 596 : 583–9.
- [3] Baek M, DiMaio F, Anishchenko I, et al. *Science* 2021 ; 373 : 871–6.
- [4] Versini R, Sritharan S, Aykac Fas B, et al. *J Chem Inf Model* 2024 ; 64 : 26–41
- [5] Abramson J, Adler J, Dunger J, et al. *Nature* 2024 ; 630 : 493–500.
- [6] Krishna R, Wang J, Ahern W, et al. *Science* 2024 ; 384 : ead12528.
- [7] Wohlwend, J., Corso, G., Passaro, S., et al. (2024). *bioRxiv*, 2024-11.
- [8] Chai Discovery, Boitreaud, J., Dent, J., et al. (2024). *bioRxiv*, 2024-10.
- [9] Lewis, S., Hempel, T., Jiménez Luna, J., et al. (2024). *bioRxiv*, 2024-12.



## Machine Learning for allosteric site prediction

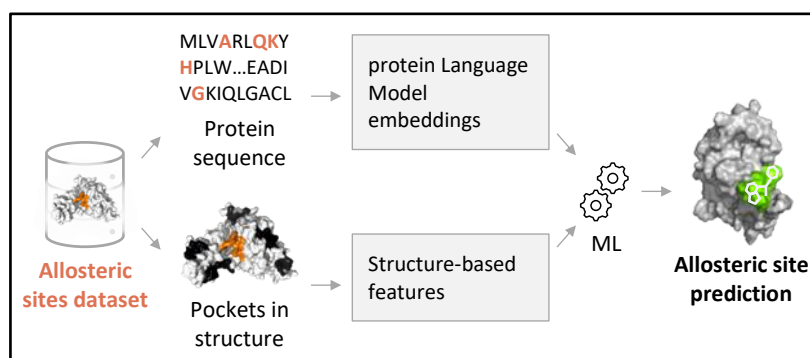
Francho Nerín-Fonz<sup>1,2</sup>, Alexios Chatzigoulas<sup>1</sup>, Zoe Cournia<sup>1,2</sup>

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Allosteric regulation is a fundamental biological mechanism that can control protein signaling via allosteric modulator binding to protein distal functional sites. The advantages of allosteric modulators acting on these distal sites over orthosteric ones have sparked the development of numerous computational approaches, such as the identification of allosteric binding sites, to facilitate allosteric drug discovery<sup>1</sup>. Building on the success of Machine Learning (ML) approaches in solving complex problems in biology and chemistry, several ML tools for the prediction of allosteric sites that use descriptors of the protein structures and ligand binding pockets have been developed<sup>2</sup>. However, these tools are built upon limited and outdated datasets, and, in addition, state-of-the-art methodologies such as protein Language Models (pLMs) have not yet been thoroughly explored for allosteric site prediction.

In this work, we present our efforts to 1) enhance an existing dataset of allosteric sites and 2) investigate the utility of pLMs for allosteric site prediction. First, we curate and expand a widespread structural database of annotated allosteric sites<sup>3</sup>, enriching it with new allosteric sites retrieved from recent literature for kinases, G-protein coupled receptors, and ligand-gated ion channels. This effort increased the number of non-redundant, diverse structures with annotated allosteric sites from 147 in the existing dataset<sup>4</sup> to 289. Then, using these structures, we trained ML models to classify residues in allosteric sites versus non-allosteric residues, and allosteric versus non-allosteric binding pockets, using pLM embeddings as features. However, we obtained suboptimal predictions, which reveal the limitations of relying solely on pLM embeddings for this task. To address this challenge, we combined structure-based features with pLM embeddings to train allosteric pocket-based classifiers. This approach significantly improved prediction accuracy, resulting in a model that performs comparably to the current state-of-the-art tools on an independent test set of newly described allosteric sites, achieving a ~75% F<sub>1</sub> score. Interestingly, we found that pocket physicochemical descriptors were the most important features for prediction, while pLM embeddings were not discriminatory.



- [1] Chatzigoulas, A.; Cournia, Z. Rational Design of Allosteric Modulators: Challenges and Successes. *WIREs Comput. Mol. Sci.* 2021, 11 (6).
- [2] Nerín-Fonz, F.; Cournia, Z. Machine Learning Approaches in Predicting Allosteric Sites. *Curr. Opin. Struct. Biol.* 2024, 85, 102774.
- [3] He, J.; Liu, X. et al. ASD2023: Towards the Integrating Landscapes of Allosteric Knowledgebase. *Nucleic Acids Res.* 2024, 52 (D1), D376–D383.
- [4] Huang, W.; Wang, G.; Shen, Q.; Liu, X.; Lu, S. et al. ASBench: Benchmarking Sets for Allosteric Discovery. *Bioinformatics* 2015, 31 (15), 2598–2600

## Non-Equilibrium Structural Pharmacology of the $\mu$ -Opioid Receptor

Georgios Skiniotis

Center of Excellence for Structural Cell Biology, Department of Structural Biology, St. Jude Children's Research Hospital, Memphis, TN, USA.

Agonists of different efficacy for the same receptor can activate intracellular signaling partners to varying degrees, but a structural characterization of how they achieve this remains elusive. Here, we leveraged time-resolved cryoEM to visualize the GTP-induced activation of  $G_i$  when bound to the  $\mu$ -opioid receptor in the presence of either a partial, a full, or a super agonist. We identify several ensembles of states characterized by distinct transitions of the alpha helical domain (AHD), disengagement of the  $G_{\alpha i}$  C terminus from the receptor, and initial separation of the  $G_{\alpha}$  subunit from  $G\beta\gamma$ . Notably, we observe substantial ligand-dependent differences in relative populations and stabilities of the various ensembles, many of which seem to derive from increased dynamics at the base of TM5 and TM6 in the presence of increasingly potent agonists. The findings also demonstrate key differences in the GTP-induced activation of MOR/ $G_i$  compared to our recent work with  $\beta_2$ AR/ $G_s$ , which likely have kinetic consequences for the activation and signaling of the two G proteins. Together these results allow us to define major contributing factors in the transduction of partial vs full vs super-agonism, and demonstrate how pushing toward dynamic ensembles of complexes in non-equilibrium conditions can help resolve complicated questions of pharmacological mechanism.



## Comparative Study of Allosteric GPCR Binding Sites and Their Ligandability Potential

Sonja Peter, Lydia Siragusa, Morgan Thomas, Tommaso Palomba, Simon Cross, Noel M. O'Boyle, Dávid Bajusz, György G. Ferenczy, György M. Keserű, Giovanni Bottegoni, Brian Bender, Ijen Chen, Chris De Graaf

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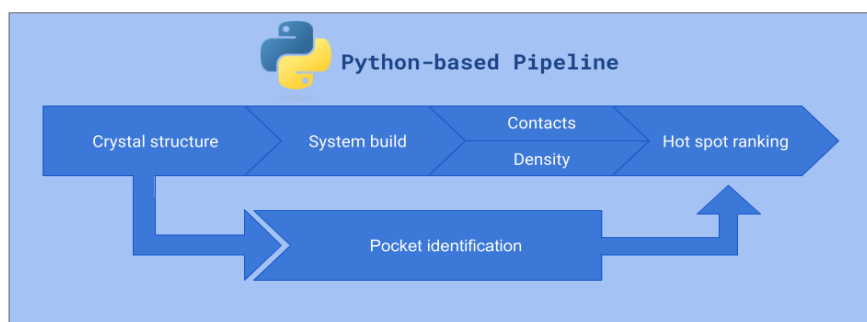
The steadily growing number of experimental G-protein-coupled receptor (GPCR) structures has revealed diverse locations of allosteric modulation, and yet few drugs target them. This gap highlights the need for a deeper understanding of allosteric modulation in GPCR drug discovery. The current work introduces a systematic annotation scheme to structurally classify GPCR binding sites based on receptor class, transmembrane helix contacts, and, for membrane-facing sites, membrane sublocation. This GPCR specific annotation scheme was applied to 107 GPCR structures bound by small molecules contributing to 24 distinct allosteric binding sites for comparative evaluation of three binding site detection methods (BioGPS, SiteMap, and FTMap). BioGPS identified the most in 22 of 24 sites. In addition, our property analysis showed that extrahelical allosteric ligands and binding sites represent a distinct chemical space characterized by shallow pockets with low volume, and the corresponding allosteric ligands showed an enrichment of halogens. Furthermore, we demonstrated that combining receptor and ligand similarity can be a viable method for ligandability assessment. One challenge regarding site prediction is the ligand shaping effect on the observed binding site, especially for extrahelical sites where the ligand-induced effect was most pronounced. To our knowledge, this is the first study presenting a binding site annotation scheme standardized for GPCRs, and it allows a comparison of allosteric binding sites across different receptors in an objective way. The insight from this study provides a framework for future GPCR binding site studies and highlights the potential of targeting allosteric sites for drug development.

## Automatic detection of cholesterol hot spots in GPCR-membrane systems

David Sotillo Núñez, Giovanni Bottegoni

Dipartimento di Scienze Biomolecolari, Università degli Studi di Urbino Carlo Bo, Campus Scientifico Enrico Mattei, Via Ca' Le Suore, 2/4, Urbino 61029, ITALY

Cholesterol interacts with G protein-coupled receptors (GPCRs), acting as an allosteric modulator at the protein–membrane interface [1]. Numerous studies have investigated cholesterol's allosteric role using biophysical, molecular dynamics, or a combination of both [2], focusing on individual GPCRs or small subsets of the receptor superfamily. Our goal is to contribute to this understanding by offering a broader and more systematic perspective. The results presented here are instrumental to enable a large-scale computational analysis, comparing an extensive set of 3D crystallographic structures of GPCRs to examine cholesterol's allosteric modulation in greater detail. Two major challenges hamper the systematic analysis of a large number of structures: (i) the setup and analysis processes are complex and difficult to automate, and (ii) simulating large protein–membrane–solvent systems for a biologically relevant timescale quickly becomes computationally expensive. To address these challenges, we developed a largely automated protocol to build, simulate, and analyse GPCR structures embedded in simple membrane models. This workflow, integrated through Python scripts, leverages the coarse-grained representation provided by the MARTINI force field to enhance efficiency [3]. The generated trajectories are analysed by identifying statistically significant cholesterol-contacting residues (i.e., outliers) and examining the spatial distribution function (SDF) of cholesterol around the receptor, according to the protocol originally reported by Ferraro and colleagues [4]. These results are then mapped to pockets detected by NanoShaper [5] on the original crystal structures, enabling the characterization and ranking of hot spots. Our protocol has been currently tested on a benchmark of 30 rationally selected GPCR-membrane systems. Cannabinoid receptor 1 will be discussed as an in-depth case study (**Figure 1**).



**Figure 1.** Flowchart of the Python integrated pipeline. From a starting crystal structure, a system is built, simulated and analyzed, based on both cholesterol-contacting residues and cholesterol density distribution. By mapping the results to the pockets identified on the original structure the detected hot spots are ranked.

- [1] Ray, Arka Prabha, et al. “Dual Mechanisms of cholesterol-GPCR Interactions That Depend on Membrane Phospholipid Composition.” *Structure*, vol. 31, no. 7, May 2023, pp. 836-847.e6.
- [2] Conflitti, Paolo, et al. “Functional Dynamics of G Protein-coupled Receptors Reveal New Routes for Drug Discovery.” *Nature Reviews Drug Discovery*, Jan. 2025.
- [3] Souza, Paulo C.T.; Alessandri, Riccardo; Barnoud, Jonathan et al. “Martini 3: a general purpose force field for coarse-grained molecular dynamics.” *Nature Methods*, vol.18, no.4, pp 382-388.
- [4] Ferraro, Mariarosaria, et al. “Mapping Cholesterol Interaction Sites on Serotonin Transporter Through Coarse-Grained Molecular Dynamics.” *PLoS ONE*, vol. 11, no. 12, Dec. 2016, p. e0166196.
- [5] Decherchi, Sergio. “NanoShaper”. <https://gitlab.iit.it/SDecherchi/nanoshaper>



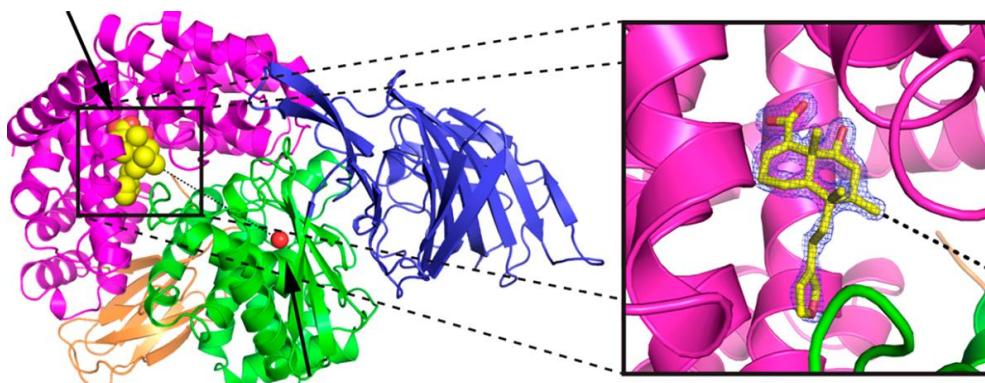
## Targeting the regulatory site of ER aminopeptidase 1 for the development of next-generation immunomodulatory agents

Efstratios Stratikos<sup>1,2</sup>

<sup>1</sup>Department of Chemistry, National and Kapodistrian University of Athens

<sup>2</sup>National Centre for Scientific Research Demokritos

ER aminopeptidase 1 (ERAP1) is an intracellular enzyme that generates antigenic peptides and is a pharmacological target for cancer immunotherapy and the control of autoimmunity. ERAP1 inhibitors described previously target the active site and are limited in selectivity, minimising their clinical potential. To overcome these limitations, we focused on an allosteric regulatory site critical for long substrate recognition which normally accommodates the C-terminus of antigenic peptide precursors<sup>1</sup>. I will describe the discovery and functions of this allosteric site as well as ongoing efforts to exploit it to design highly selective and potent modulators as part of an effort for the preclinical development of small compounds that regulate the antigenicity of cells for therapeutic applications<sup>2,3,4</sup>.



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## Structure driven drug discovery targeting allosteric sites of glycogen phosphorylase

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Glycogen phosphorylase (GP) is a critical enzyme in glycogen metabolism, functioning as the rate-limiting factor in glycogenolysis, the process by which glycogen is broken down into glucose-1-phosphate. This enzyme is particularly significant in the context of type 2 diabetes, where its activity contributes to elevated blood glucose levels. The allosteric regulation of GP, which involves the binding of various metabolites, plays a crucial role in its activity and, consequently, in glucose homeostasis. The development of allosteric inhibitors targeting GP has emerged as a promising strategy for the design of new antidiabetic agents, as these inhibitors can modulate the enzyme's activity without directly competing with the substrate, thereby offering a more refined approach to therapy. The interplay between the enzyme's structure and its regulatory mechanisms is essential for the development of inhibitors that can effectively target the allosteric sites without disrupting the enzyme's overall function. The physiological implications of GP inhibition extend beyond glucose metabolism. Recent studies have indicated that inhibiting GP can have beneficial effects on cell survival in various contexts, including cancer and glioblastoma. These findings suggest that GP inhibitors may have therapeutic potential not only in diabetes management but also in cancer treatment, where metabolic reprogramming is often a hallmark of tumorigenesis. Furthermore, the role of GP in brain metabolism has garnered attention, particularly regarding its involvement in cognitive functions and neuroprotection. The exploration of GP inhibitors in the context of neurodegenerative diseases represents an exciting frontier in drug discovery, where metabolic modulation could offer new avenues for treatment. The targeting of allosteric sites on glycogen phosphorylase represents a promising strategy in drug discovery, particularly for the treatment of type 2 diabetes and potentially other metabolic disorders. The combination of structural biology, computational modelling, and pharmacological evaluation has paved the way for the development of novel GP inhibitors that can effectively modulate the enzyme's activity. As research continues to elucidate the complex regulatory mechanisms governing GP, it is anticipated that new therapeutic agents will emerge, offering improved management of glucose homeostasis and broader implications for metabolic health.

## **Fragment screening for the identification of allosteric binding sites**

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Allosteric sites are of great interest in drug discovery, offering the opportunity to affect the behaviour of proteins while not directly competing with the natural substrate or ligand. We will disclose a number of examples where X-ray crystallographic fragment screening has been used to identify novel ligand binding sites on a number of different protein classes. The talk will discuss computational methodologies used to characterise the binding pockets and the structure guided optimisation work undertaken to develop hits into chemical probes which were used to elucidate the functional relevance of these sites.



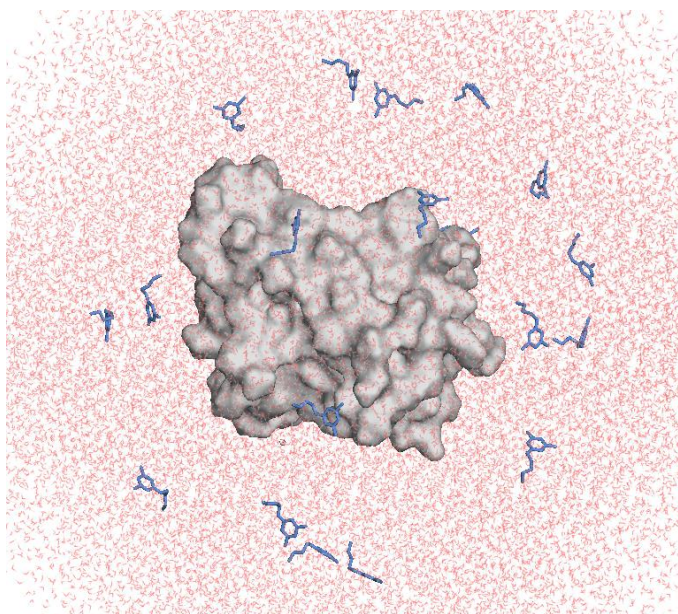
## Multiple-copies Association Studies (MAS) – a computational approach for binding mode elucidation

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In computer-aided drug design, the identification of binding modes is crucial for optimizing drug candidates. When binding site information is available, docking methods and MM/PB(GB)SA scoring together with molecular dynamics (MD) simulations are commonly used to predict accurate binding modes. [1] However, predicting ligands' binding modes without prior knowledge of the binding pocket remains challenging. Techniques like FTMap [2] and mixed-solvent MD approaches [3] can identify potential binding hotspots on the protein, but binding mode elucidation of known binders is more cumbersome. This task becomes especially difficult in the case of allosteric ligands, the binding sites of which are often poorly described or completely unknown.

In this work, we introduce the Multiple-copy Association Studies (MAS) approach – a workflow that combines classical MD simulations with high ligand concentrations (~30 mM) and employs a post-MD clustering algorithm to identify the most probable binding mode. We have developed a novel LJ potentials adjustment approach to mitigate small molecule aggregation typically observed in water simulations with high concentrations of organic molecules, enabling MAS to be conducted at high concentrations of ligand. To optimize and validate the method, we utilized a benchmarking set comprising different protein-ligand systems with crystallographic and binding affinity data. The MAS approach holds promise for uncovering unknown binding modes for known small molecule binders which could enhance not only fragment-based drug discovery and high-throughput screening campaigns but also the design of allosteric drug candidates.



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## **A Novel Workflow for the In-Silico Identification and Prioritization of Potential Allosteric Binding Sites based on Mixed Solvent Simulations and SiteMap**

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Allosteric modulation is a promising strategy for developing drugs against difficult targets where traditional orthosteric site targeting faces challenges with selectivity, resistance, or developability. The increasing availability of high-resolution protein structures and advancements in computational power and in-silico algorithms have expanded the potential of structure-based drug design (SBDD) for allosteric drug discovery. However, there remains a significant need for effective tools to identify and prioritize potential allosteric binding sites, particularly those not accessible in the apo protein structure. To address this, we have developed a novel workflow that leverages mixed solvent molecular dynamics (MxMD) simulations to reveal potential binding sites, coupled with an improved SiteMap for scoring the druggability of these sites. Our combined approach achieved an 86.4% Top-5 found rate of known allosteric binding sites in apo structures from a set of 22 apo/holo PDBs, compared to only 63.6% with SiteMap alone and 54.5% with MxMD alone. We further evaluated our workflow on a curated set of five pharmaceutically relevant targets with multiple known allosteric binding sites, and our method outperformed popular machine learning methods, p2rank and DiffDock, as well as SiteMap, in all systems except one. Finally, we report the successful application of this workflow to an active drug discovery project within our therapeutics group. Our new workflow offers an improved method for characterizing binding sites in allosteric systems. Furthermore, efforts are underway to refine the predicted binding sites to enable virtual screening campaigns. This combined approach demonstrates a significant improvement over existing methods for identifying allosteric binding sites and has the potential to enable effective hit discovery campaigns for novel binding sites.



## NMR evaluation of ligand binding among protein players in the ADP-ribosylation pathway

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The macro domains (MDs) are highly conserved structural modules found across all life forms, including viruses, often as components of larger proteins. These domains adopt an  $\alpha/\beta/\alpha$  sandwich fold and function as "readers" and "erasers" of ADP-ribosylation, a post-translational modification mainly mediated by PARPs, that regulates key biological processes such as DNA repair, immune responses, and intracellular signaling. Human PARPs like PARP9, PARP14, and PARP15—together called "macroPARPs" because of their tandem macro domains—have MDs that seem very important for their in-cell localization and their interactions with other cellular elements. While PARP9 and PARP14 are involved in interferon signaling, the specific function of PARP15 remains unclear. However, not all MDs within macroPARPs have been fully characterized, either structurally or biochemically. Cancer, neurological disorders, and developmental abnormalities are associated with the dysregulation of macroPARPs and other MD-containing proteins. Regarding viruses such as Coronaviruses, Alphaviruses, and Rubella virus, MDs help shape host-pathogen interactions by countering the role of PARPs in immune response. Viral MDs have two functions: their "reader" activity participates in the replication of (+)ssRNA, and their de-MARylation activity prevents PARP-mediated antiviral mechanisms from functioning, which makes the virus more harmful.<sup>1,2</sup>

Our research focuses on the comparison of viral and human macroPARP MDs to describe their structure and biochemical features of members that haven't been studied before. The aim is to figure out small differences that can be exploited in the design of selective binders while dealing with sequence identity problems that come up among MD family members. We therefore use NMR-driven methods, biophysical techniques, and biochemical assays to prove the binding, the selectivity, and the functional properties of a series of compounds (<20) identified through *in silico* screening over millions of commercially available compounds and probe the factors that modulate the binding affinity as done previously with a selective de-MARylation inhibitor (GS-441524) of the SARS-CoV-2 MD.<sup>3</sup>

### Acknowledgements

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## **From active site- to allosteric SHP2 inhibitors: Discovery and structure-activity relationship of N-aryl-1H-azaindazoles**

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The cytosolic protein tyrosine phosphatase SHP2 is a promising anticancer drug target given its central role in receptor tyrosine kinase signaling and the modulation of the tumor microenvironment. In a rescaffolding study, we have discovered a novel series of allosteric SHP2 inhibitors that feature striking similarities to orthosteric SHP2 inhibitors previously developed in our group. N-aryl-1H-azaindazole derivatives with nanomolar potency in biochemical assays and ability to inhibit key downstream signaling events in cancer cell lines were identified in structure-activity relationship investigations, supported by co-crystal structures. This talk will present insights into the molecular recognition phenomena underlying SHP2 modulation by this novel series of allosteric inhibitors, which are currently being evaluated for potential synergism with orthosteric SHP2 inhibitors in cellular assays.



## Advancing Covalent Allosteric Inhibitors: Insights into SHP2 Modulation on the Latch side

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Src homology domain-containing phosphatase 2 (SHP2) is a pivotal allosterically regulated enzyme implicated in receptor tyrosine kinase signaling and oncogenesis, with its activation leading to oncogenic responses via the Ras/Raf/ERK and PI3K/AKT pathways<sup>1</sup>. Additionally, SHP2 contributes to immune evasion in tumor cells through programmed death receptor signaling, altering the tumor microenvironment<sup>2</sup>. Aberrant SHP2 activity contributes to cancer progression<sup>1</sup> and immune evasion<sup>2</sup>, making it a prime target for therapeutic intervention. While historically deemed “undruggable,” the discovery of allosteric inhibitors has enabled significant advancements in SHP2-targeted therapies, with several modulators currently in clinical trials<sup>3</sup>.

We investigated SHP2 allosteric modulation at the “latch” site through a comprehensive platform combining fragment-based screening, computational design, targeted covalent inhibitor synthesis, biochemical assays and mass spectrometry. Screening of lysine and cysteine-targeting fragment libraries initially resulted in 17 and 11 hits ( $IC_{50} < 100 \mu M$ ), respectively. These findings guided the rational design of a set of lead-like inhibitors equipped with diverse electrophilic warheads, such as NHS ester-modified SHP844 derivatives, achieving potent activity with  $IC_{50}$  values at the nanomolar range. Molecular dynamics and docking studies elucidated key interactions, including the significance of hydrogen bonding, aromatic interactions, and stereoelectronic effects. Mass spectrometry and enzymatic digestion experiments provided insights into covalent labeling, highlighting specific amino acids and binding site dynamics.

Our research advances the understanding of SHP2 allosteric modulation and the efficiency of previously developed allosteric inhibitors<sup>4</sup> offering a robust framework for covalent allosteric inhibitor development. The methodologies applied herein provide a foundation for extending these strategies to other allosterically regulated proteins, paving the way for novel therapeutic interventions in SHP2-associated diseases.

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## Towards the accurate prediction of modulatory ligands activity in synaptic receptors

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Nicotinic acetylcholine and Glycine receptors (nAChRs and GlyRs) play a central role in the intercellular communication in the brain and the nervous system and are involved in fundamental processes such as attention, learning, and memory [1]. They are pentameric protein assemblies that convert a chemical signal into an ion flux through the postsynaptic membrane. Their pharmacology is currently under development for the treatment of Alzheimer's, Parkinson's, schizophrenia, depression and pain.

Recently, it was found that pharmacological attributes such as potency, efficacy and selectivity of modulatory ligands can be expressed in terms of their binding affinity for the multiple conformational states of the receptor [2]. In addition, due to recent improvements in the structural determination of transmembrane proteins, several high-resolution structures of nAChRs and GlyRs in different physiological states and in complex with modulatory ligands have become available [3, 4].

The peculiarity of the neurotransmitter-receptor recognition is the presence of essential cation- $\pi$  interactions in the ligand-binding site. These interactions are difficult to describe with classical force fields, which hinders the possibility of accurate affinity predictions via free-energy simulations. A thorough assessment of the quality of the existing force fields when cation- $\pi$  interactions are involved is necessary for any rational drug design for brain disorders.

The present work is moving in two directions.

On the one hand, we found that classical force fields stabilize binding modes for glycine and taurine at GlyR that are different compared to recently deposited cryo-EM structures. The refinement of their binding pose is essential to reveal the fundamental nature of the neurotransmitter-receptor interaction and assess whether classical force fields are adequate for binding free energy calculations at synaptic receptors. For this purpose, *ab initio* QM/MM molecular dynamics simulations were started from two cryo-EM structures of GlyR using the MiMiC QM/MM framework and dispersion-corrected DFT functionals, and the QM/MM results compared with classical MD simulations.

On the other hand, the quality of classical force fields could be assessed by comparing calculated ligand-binding affinities at synaptic receptors with experiments. For this purpose, absolute binding free energy (ABFE) calculations were carried out on the acetylcholine binding protein (AChBP), a model system of the extracellular domain of nAChR featuring the same cation- $\pi$  interactions in the nicotine's binding site. As AChBP is characterized by several X-ray high-resolution structures with ligands as well as high-quality binding affinity data, these calculations allow for a direct comparison with experiments. In this context, the accuracy of different force fields was analyzed.

The results of both investigations will be presented at the Conference.

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## Discovery of novel allosteric inhibitor hits for Insulin-Regulated Aminopeptidase provides insights on enzyme mechanism and regulation

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Insulin-Regulated Aminopeptidase (IRAP) is a transmembrane zinc metalloprotease with various important biological roles, including fibrosis, cognitive functions and immune system regulation. As a result, IRAP is an emerging pharmacological target for several diseases. However, the development of selective inhibitors that specifically regulate its activity remains challenging due to its high sequence and functional homology with many other enzymes that have highly conserved active sites. To circumvent this limitation, we targeted the malate allosteric site, a site that has yielded highly selective inhibitors for the homologous enzyme ERAP1.[1] We performed virtual screening to discover drug-like compounds that bind with high affinity to this allosteric site in IRAP.[2] From molecular docking of a library comprising of 38 million diverse compounds of the ENAMINE database we selected a subset of 240 highly ranked compounds that were evaluated with molecular dynamics simulations and MM/GBSA free energy calculations. Among the compounds exhibiting high stability and the lowest free energy of binding we purchased 17 compounds for further *in vitro* evaluation of their inhibitory activity for IRAP. A fluorogenic assay using a dipeptidic model substrate validated 3 hits, the best of which was highly selective and displayed an uncompetitive mechanism of action consistent with the allosteric nature of the targeted site. Compounds were also evaluated by two orthogonal assays based on MALDI-MS analysis, and following the degradation of a model antigenic peptide precursor and the cyclic peptidic hormone vasopressin. These results are discussed in the context of enzyme inhibition mechanism and regulation.

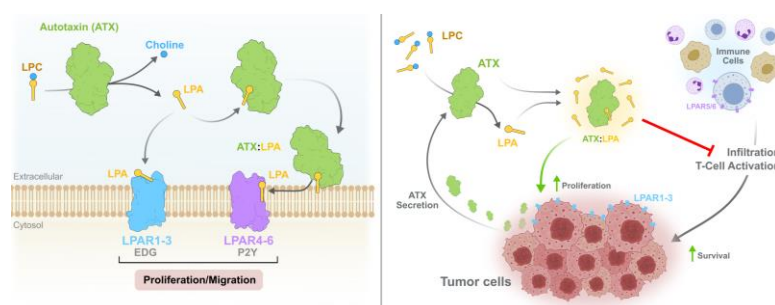
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## Allosteric Autotaxin inhibitors: a key to clinical success?

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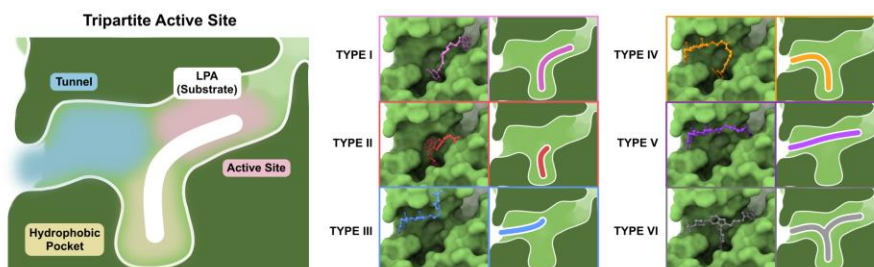
Autotaxin (ATX) is an extracellular lysophospholipase D secreted by diverse cell types to convert lysophosphatidylcholine (LPC) into bioactive lysophosphatidic acid (LPA). LPA is a lipid mediator that acts through two families of G protein-coupled receptors (GPCRs), LPA1-3 (EDG) and LPA4-6 (P2Y), to regulate a great variety of biological functions including cell migration, proliferation, survival, angiogenesis, and inflammation (**Figure 1**). ATX was originally characterized as an inducer of tumour cell motility and metastasis-enhancing enzyme. Since then, numerous preclinical studies implicated ATX-LPA signalling in tumour progression, acting mainly via LPA1, making ATX an attractive drug target.



**Figure 1:** The ATX/LPA signalling axis (A) and its effect on tumor and immune cells (B)

While ATX inhibitors occupying the **orthosteric** LPA binding site have been available early on, the first clinical success was with an inhibitor occupying the ATX **allosteric** site (GLPG-1690, later called **ziritaxestat**). That led to defining four “**types**” of ATX inhibitors according to their binding mode, which our team recently extended to **six types, I-VI** (**Figure 2**). The ATX inhibitors are defined by their binding mode on the ATX “**tripartite site**” that is made up by three components: i) the **active site** that normally binds the LPA substrate glycerol moiety bringing the scissile phosphodiester to the bimetallic active site; ii) the **hydrophobic pocket** that normally accommodates the aliphatic lysolipid chain; and iii) an **open tunnel** that can bind lysolipids and other biological molecules, and acts as an **allosteric** site modulating ATX activity and as a chaperone site for delivering LPA to its cognate GPCRs.

Most notable ATX inhibitors currently on clinical trial are **cambritaxestat** (under evaluation in patients with pancreatic cancer) as well as **BLD-0409** and **BBT-877** (for idiopathic pulmonary fibrosis). I will show the new Type V and VI inhibitors developed by our team; the latter utilize a three-point lock binding mode occupying the active site, the hydrophobic pocket, and the allosteric tunnel, completing the ATX inhibitors combinatorial puzzle. I will also describe the NKI Type IV **Cpd17** inhibitor, which we have shown to be reducing inflammation response in liver cells *in vitro* and in reducing both steatosis and fibrosis in two relevant mouse models and is currently assessed in combination with immunotherapy in mouse models at the NKI.



**Figure 2:** The six types of ATX inhibitors, with distinct structural binding modes.

## **Protein-protein and protein-nucleic acid interactions through alchemical free energy calculations**

Marco De Vivo

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In our lab, we study enzymatic catalysis of pharmaceutically relevant targets to uncover mechanistic insights that are potentially valuable for drug discovery and computer-aided enzyme design. Here, I will present recent examples where we have applied alchemical free energy calculations to quantitatively compute the effect of mutations at the protein-protein interface, which are targets for drug design and allosteric modulation of function. As a test case, we have studied the protein complex involving small Rho-GTPases, which provide a wealth of structural, mutagenesis, and binding affinity data due to their central role in cellular signaling and cancer progression. In addition, I will share recent results of our efforts on free energy calculations applied to protein-nucleic acid interactions, specifically in the case of polymerase enzymes interacting with nucleotides and DNA. Lastly, I will highlight our efforts in guiding RNA-targeted small molecule design using free energy perturbation calculations.



## Allosteric Activation of ACE2: Structural Insights and Future Perspectives in Drug Discovery

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Angiotensin-converting enzyme 2 (ACE2) plays a pivotal role in the renin-angiotensin system by converting angiotensin II (Ang II) into angiotensin 1-7 (Ang 1-7), a peptide with vasoprotective and anti-inflammatory properties. Beyond its physiological significance, ACE2 has attracted considerable attention due to its role as the primary receptor for SARS-CoV-2 entry. However, its catalytic function remains an underexplored therapeutic target, despite its potential for cardiovascular and hypertensive disease modulation [1, 2]. Here, we present a structural and biochemical investigation into the allosteric regulation of ACE2, highlighting the discovery of a small-molecule activator that enhances its enzymatic function.

Our research targets the catalytic domain of ACE2, which we have successfully expressed and purified in a monomeric state, providing a robust platform for structural and functional studies. To elucidate the conformational landscape of ACE2 during substrate processing, we co-crystallized the enzyme with a phosphinic angiotensin II analogue, a non-hydrolyzable transition state mimic. This structural snapshot reveals a striking hinge-bending motion, wherein the initially open, substrate-receptive groove collapses into a concave, semi-enclosed cavity, likely representing a catalytically competent state. Using a targeted screening approach, we identified a small-molecule activator that enhances ACE2 enzymatic activity. Structural analysis revealed that this activator binds within a previously unreported allosteric pocket within the internal cavity of ACE2, distinct from the active site. While this pocket was identified by soaking the activator in the open conformation of ACE2, our structure with the phosphinic angiotensin II analogue confirmed that the activator does not compete with the natural substrate, reinforcing the physiological relevance of this allosteric site. Our initial data regarding the precise activation mechanism suggest that this activator enhances the catalytic turnover without affecting the affinity for the substrate.

Our future efforts will focus on comprehensive kinetic and thermodynamic characterization of this activator, alongside structure-based optimization to enhance its efficacy and potency. These efforts aim to pave the way for novel therapeutics targeting ACE2-related pathologies, such as hypertension and acute respiratory distress syndrome. This talk will highlight how integrating biochemical and medicinal chemistry tools can unlock the allosteric potential of ACE2, contributing to the broader field of allostery in drug discovery.

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## Virtual Screening for Allosteric Drugs: Where Are We?

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Discovering bioactive molecules that target novel allosteric sites remains a significant challenge. Over the years, my research group has developed computational tools to systematically tackle this problem—specifically, MDmix for identifying and characterizing uncharted binding sites [1-5] and docking+DUck for targeting these sites via virtual screening.[6-11] In this talk, I will present recent examples of successfully discovered allosteric modulators across unrelated protein families, demonstrating that efficient computational strategies can make this a feasible goal.[12-14] However, translating allosteric ligands into effective drugs presents far greater challenges. Structural elucidation, biochemical-to-biological translation, and potency optimization are just a few of the many hurdles that appear uniquely complex for allosteric drug discovery. Drawing from our recent experiences, I will highlight key bottlenecks in this process and discuss potential strategies to overcome them.



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## **Joys and tribulations pursuing allosteric inhibitors – an industry perspective**

Judith Günther

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Within pharmaceutical industry pipelines, allosteric modulators continue to represent an opportunity to exploit the advantages of classical SMOLs while offering elegant solutions to target-specific challenges, such as druggability and selectivity. This presentation will show Bayer research examples which illustrate how allosteric modulation can be exploited, despite the challenge to the optimization for other end points. The talk will also address the scope and limitations of current computational methods, with an emphasis on their interdependence on wet lab experiments.



## Hijacking EGFR Signaling: Computational Insights into Allosteric Antibodies Engineering

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In this talk, we explore the critical role of EGFR (epidermal growth factor receptor) regulation and its allosteric modulation in health and disease, focusing on the therapeutical use of monospecific and biparatopic antibodies to modulate EGFR activity. We introduce a novel strategy for evaluating allosteric modulations induced by anti-EGFR allosteric antibodies [1,2], aimed at designing optimal biparatopic formats. By combining AlphaFold with a structure-based statistical mechanical model of allostery (SBSMMA, [3–5]), we assessed inter-domain communication and rationalized experimental results. Our findings unveil the complexity of EGFR allosteric networks that significantly affect signaling pathways and dimerization processes. Importantly, not all antibody combinations prove effective, as dual binding can non-trivially redistribute allosteric signaling. This work paves the way towards the computational design of biparatopic formats with enhanced therapeutic potential, offering promising strategies to overcome drug resistance and advancing personalized medicine.

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## Exploring Allosteric Modulation through Virtual Screening: From GPCR Heteromers to a Cancer-linked Target

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Protein structural information can be used for virtual screening approaches to identify novel small molecules as potential hits which may act as antagonists, agonists, or allosteric modulators. Pharmacophore modeling and docking are well-established methods for screening large databases for drug design and can be a feasible approach in targeting the signaling outcomes of the different types of biological systems. Using the structures or computational models of druggable protein targets, we have such pipelines to perform virtual screening of large compound databases and identify small molecule bioactives applied to two allosterically modulated systems.

The CB<sub>1</sub>R-5HT<sub>2A</sub>R heteromer is implicated in CB<sub>1</sub>R agonist-induced cognitive impairment. Following a helical peptide design approach to disrupt the heteromer, in which the peptide “replaces” one of the TMs of 5HT<sub>2A</sub>R, we sought to identify small molecules that would mimic the activity of the designed peptide, modulating heteromer stability. The identified allosteric modulators, similarly to the active peptide, were shown to prevent heteromerization in fluorescence assays and alter heteromer-dependent cell signaling. This provides a proof-of-principle for developing optimized ligand-based disruptors of the CB<sub>1</sub>R-5HT<sub>2A</sub>R heteromer for therapeutic applications [1].

Using a similar approach, we have targeted the allosteric cavity of Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) paracaspase to identify potential anticancer agents. Out of a few hits, a nanomolar affinity modulator GM-22 has been identified. Enzymatic assays, followed by 3D spheroid cell-based assays under- and over-expressing MALT1, have demonstrated that GM-22 ameliorates cancer cell survival in patient derived cell lines.

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## Elucidating the structure and dynamics of the Melanocortin-4 receptor (MC4R)

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The melanocortin-4 receptor (MC4R) is a G-protein coupled receptor, well-known for the role it plays in energy expenditure and the modulation of satiety. Indeed, MC4R mutations are the most frequent cause of monogenic obesity with about 160 variants identified so far<sup>[1]</sup>. However, only one FDA-approved drug is currently being used for treatment: setmelanotide<sup>[2,3]</sup>. This drug is also known to be non-specific for MC4R and able to activate other members of the melanocortin family<sup>[4]</sup>. Thus, it would be highly valuable to obtain a high-affinity drug specific to MC4R and not to the other four melanocortin receptor subtypes.

Interestingly, a recent paper by the company ConfoTherapeutics published in 2024 showed the structure of MC4R in complex with pN162, a nanobody obtained using their proprietary platform (Confobody), which they claim does showcase these properties<sup>[5]</sup>. ConfoTherapeutics then further improved this nanobody, resulting in an optimized version called pN2121. As part of the Allostery in Drug Discovery (ALLODD) funding program, I was able to obtain a complex of MC4R with this new confobody. We are currently trying to solve the structure of this new complex MC4R-pN2121 using cryo-electron microscopy (cryo-EM)<sup>[3]</sup>.

To better understand how a specific agonist activates MC4R structurally and dynamically, other promising methods can also be employed. For example, the electron paramagnetic resonance (EPR) and double electron-electron resonance (DEER) techniques have been used to study the dynamics and characteristics of changes in other GPCRs caused by different ligands or under different environmental conditions<sup>[6]</sup>. However, this method relies on the insertion of a couple of EPR labels at reactive cysteines at key sites of the MC4R, and thus requires first and foremost a minCys MC4R variant, in which all naturally reactive cysteines are mutated to serines in order for it to be used as a negative control. Together with our partners at the lab of Matthias Elgeti (Leipzig, Germany), we have now identified two naturally reactive cysteines in wild-type MC4R. After testing this minCys variant and confirming its functionality with a cAMP signaling assays, we can focus on producing and testing new MC4R variants with reactive cysteines inserted at key sites. The distances between these EPR probe-labeled cysteines before and after the addition of different MC4R ligands will then provide new information about the structural dynamics as well as the similarities and differences in the activation of MC4R and the subsequent binding to different coupled G-proteins.

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## Probing allosteric activation and biased signaling with rigidity transmission allostery (RTA) theory, NMR, and geometric Monte-Carlo simulations

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Allostery predictions algorithms, which are based on mathematical rigidity theory, provide a mechanical interpretation of allosteric signaling in protein structures. Rigidity Transmission Allostery (RTA) methods are designed to predict if perturbation of rigidity at one site of the protein can propagate across a network and in turn cause a change in rigidity at a second distant site, resulting in allosteric transmission. Here we focus on the application of RTA on allosterically driven functional dynamics in G-Protein Coupled Receptors (GPCRs). We show how ligands trigger allosteric changes that propagate to functionally important regions; the role of beta-gamma subunit in G proteins as critical facilitators of allostery within the ternary complex with adenosine A2AR; the role of differentiated allosteric pathways in GPCR promiscuity and selectivity mechanisms in A2AR in the presence of different G protein subtypes. We combine 19F-NMR and RTA to delineate allostery and distinct key functional activation and precoupled intermediate states in Adenosine A2A GPCR complexed with heterotrimeric G-protein. NMR showed that binding of G-protein stabilizes a precoupled activation intermediate state and two distinct active states which facilitate nucleotide exchange by full or partial agonists. RTA showed beta-gamma subunit in G proteins is critical in facilitating allostery within the ternary complex. We present results on several GPCR structures and recent work which investigates the selectivity and efficacy in A2AR in the presence of different G protein subtypes.

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## Predicting Binding Affinity and Kinetics in GPCR Oligomers: Insights from Enhanced Sampling Simulations

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The investigation of G protein-coupled receptor (GPCR) oligomers has become an increasingly important area of research in recent years [1]. GPCR oligomerization significantly impacts receptor function, including ligand binding, receptor signaling, and downstream physiological effects [2,3]. The relevance of studying GPCR oligomerization has been particularly demonstrated in the Central Nervous System [4], with the dopamine D2 receptor being a notable example. This receptor forms transient homodimers, and increased dimerization has been linked to the development of schizophrenia [5].

Here we examined how the dimerization of the D2 receptor influences the thermodynamic properties of ligand binding using Well-Tempered Metadynamics [6], as done already for monomeric GPCRs [7]. We also employed Infrequent Metadynamics as already done in our lab [8] to analyze the differences in residence time of risperidone between the monomer and the two subunits of the dimer. Our insights could contribute to the development of more effective therapeutic strategies by taking into account the altered ligand binding properties in different receptor states. This work is supported by the Helmholtz Partnering Project between IIT and FZJ, and funded by the project "Allostery in drug discovery" (ALLODD) within the Horizon 2020 - Marie Curie Sklodowska Foundation framework.

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## Above-Filter Digestion Proteomics Identifies Drug targets and estimates ligand binding site

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Identification of drug targets is the main goal of chemical proteomics. While some methods focus primarily on target determination, others additionally give insights into ligand-protein interaction surface. Each of the existing methods has limitations, necessitating the development of complementary approaches.

Here we present such an approach, Above Filter Digestion Proteomics (AFDIP), capable of identifying drug targets, and detecting areas of the protein structure affected by drug-target binding. The method relies on time-dependent trypsin digestion of the extracted proteome above a membrane filter with a 3kDa molecular weight cut-off. Peptides located near the binding site change their relative abundance in digestion between drug-treated and control samples, allowing for binding site (and target) identification.

AFDIP has proven highly efficient in drug target identification across multiple compounds including methotrexate, rapamycin, FK506, staurosporine, and acetyl CoA. The method identified DHFR as methotrexate's main target and multiple FK506-binding proteins for rapamycin and FK506, while distinguishing between different downstream interaction pathways of the two drugs. Notably, AFDIP achieved structural resolution within 10 Å of crystallography-determined binding sites. The method provided deeper proteome coverage and higher sequence coverage compared to similar techniques, while also offering structural insights through analysis of shifting peptides, as demonstrated in the FKBP4-FK506 interaction study.

With its ability to identify binding sites with high precision, even in complex cellular environments, AFDIP is positioned to become an essential proteome-wide tool for target identification and characterization of drug-target interactions.



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## Poster Abstracts





## From active site- to allosteric SHP2 inhibitors: Discovery and structure-activity relationship of N-aryl-1H-azaindazoles

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Src homology domain-containing phosphatase 2 (SHP2) is a cytosolic protein tyrosine phosphatase that has gained much attention as an anticancer drug target thanks to its central role in receptor tyrosine kinase signaling and its potential to modulate the tumor microenvironment. Several allosteric SHP2 inhibitors currently undergo clinical testing, most as part of combination therapies for cancer indications. We have previously developed N-arylated azaindole-based orthosteric inhibitors of SHP2. In an endeavor to identify novel scaffolds for allosteric SHP2 inhibitors, we observed a switch in the mechanism of inhibition of N-arylated azaindole-based inhibitors from active site- to allosteric inhibition upon the exchange of peripheral substituents on the N-aryl azaindole core with privileged allosteric site-targeting substructures. An extensive structure-activity relationship study assisted by co-crystal structures revealed key structural determinants necessary to shift from an orthosteric to an allosteric binding mode. Investigation of a single atom exchange of the azaindole core at various positions led to the identification of N-aryl-1H-azaindazoles as allosteric SHP2 inhibitors with nanomolar potency in biochemical assays and the ability to inhibit key downstream signaling events in cancer cell lines. We are currently investigating whether synergistic inhibition of SHP2 by allosteric and orthosteric inhibitors is a strategy to target clinically relevant activated SHP2 mutants.



## Allosteric Modulation of the C-type Lectin DC-SIGN

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DC-SIGN, a C-type lectin receptor, plays a pivotal role in orchestrating an adequate immune response towards invading pathogens by bridging innate and adaptive immunity. The receptor is expressed on antigen-presenting cells and exhibits functional versatility across multiple biological processes including the mediation of cellular adhesion, antigen presentation, and immune activation. In addition to exploiting the receptor as a potential entry point to the immune system for targeted delivery applications, the critical role of DC-SIGN in mediating pathogen recognition and dissemination, particularly in the context of HIV-1 and SARS-CoV-2, has driven substantial interest in the development of selective small-molecules capable of modifying the receptors function. However, the intrinsic structural constraints of its Ca<sup>2+</sup>-dependent carbohydrate-binding site, characterized by a shallow topology and high polarity, pose significant challenges for the design of selective modulators. Recent studies have provided evidence for the existence of remote secondary binding sites, of which one is an allosteric site, modulating the carbohydrate-binding site<sup>1,2</sup>. Exploiting this allosteric site represents a compelling approach to circumvent the low druggability and intrinsic promiscuity of the carbohydrate-binding site while enabling modulation of receptor function. I will present our latest research elucidating the allosteric network governing long-interaction between the allosteric secondary site and the primary, Ca<sup>2+</sup>-dependent carbohydrate-binding site of DC-SIGN<sup>1</sup>. By leveraging a broad set of biophysical methods, with a particular emphasis on advanced Nuclear Magnetic Resonance spectroscopy, we delineate the molecular mechanisms underlying the allosteric communication. Beyond the application as antivirals, I will showcase how these insights can be utilized for the rational design of heteromultivalent liposomal constructs that exploit the allosteric network to achieve highly selective DC-SIGN targeting in the context of cell-based assays. This strategy holds significant potential for promoting precise immune system modulation and targeted therapeutic delivery.

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## Development of Allosteric Inhibitors for Langerin: Mechanistic Insights and Structural Characterization

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Langerin, a member of the C-type lectin (CTL) superfamily, is a  $\text{Ca}^{2+}$ -dependent carbohydrate-binding protein involved in pathogen recognition, immune signalling, and modulation of immune responses. Given its critical role in antigen uptake and immune regulation, langerin has emerged as a promising target for inhibitor development, both to elucidate its biological functions and to explore potential therapeutic applications. Various classes of langerin inhibitors have been reported, including glycomimetic compounds, antibodies, and allosteric non-carbohydrate inhibitors. Among the latter, allosteric non-carbohydrate inhibitors are of particular interest due to their non-canonical binding site, offering advantages such as higher selectivity, a novel mechanism of inhibition, and calcium-independent regulation. In this study, we present two distinct types of inhibitors: calcium-competitive and calcium-independent. The calcium-competitive inhibitors were rigorously characterized using ITC,  $^{43}\text{Ca}$  NMR, and X-ray crystallography, providing strong evidence for their competitive binding mechanism. On the other hand, the calcium-independent inhibitors, which were grown from the calcium-competitive inhibitors but possess larger molecular structures, were systematically analyzed through structure-activity relationship (SAR) studies and characterized using ITC, molecular dynamics (MD) simulations, and various NMR techniques. These studies provided detailed insights for their binding sites and inhibition mechanism, highlighting their potential for modulating langerin activity and regulating immune response.

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## Unravelling Allosteric Pathways in Haloalkane Dehalogenase LinB using Enhanced Sampling Simulations

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Allostery plays a crucial role in regulating the enzyme function, yet identifying allosteric sites in highly dynamic proteins remains very challenging. We have performed Gaussian accelerated molecular dynamics (GaMD) simulations in haloalkane dehalogenase LinB, and have uncovered a newly discovered side tunnel (ST)[1]. The ST and cryptic pocket at the mouth of ST, shows potential involvement in allosteric regulation[1], [2]. By employing computational allosteric site predictor tools such as PASSer 2.0[3], FTMove[4], we consistently identified the ST's mouth region as a high-ranking allosteric hotspot and the ST cryptic pocket showed comparable importance with active-site pocket.

In an independent study, ST pocket was shown experimentally to bind ligands (1-chlorohexane and 1-bromocyclohexane), suggesting that this mouth pocket possess capacity to modulate haloalkane dehalogenases' activity from a remote site[2]. GaMD further revealed the conformational transitions that govern ST opening particularly the side helix movement, which were less frequently sampled in conventional molecular dynamics. Together, these findings suggest that the ST and its cryptic entrance pocket may enable fine-tuned allosteric control of substrate transport and catalysis in LinB. From a biotechnological perspective, targeting such hidden pockets could broaden strategies for enzyme engineering or inhibitor design.

By emphasizing rare conformational states connected to rare tunnels, our results highlight the utility of advanced sampling approaches in identifying and characterizing allosteric pathways. It overcomes the sampling limitations of standard classical simulations, paving the way for new insights into the regulation of buried active-site enzymes.

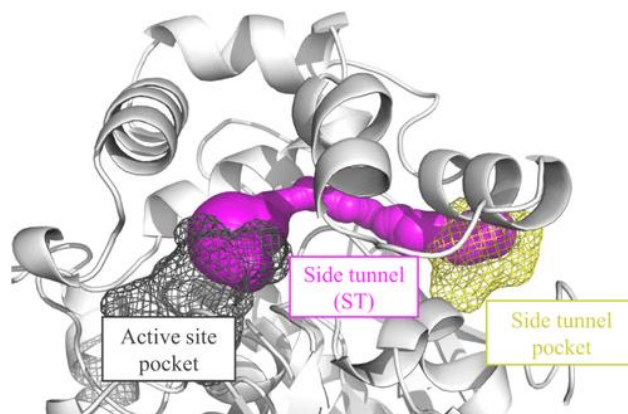


Figure: Cryptic pockets detected in the LinB-Wt structure by FTMove. The active site and ST pockets are shown in dark gray and yellow, respectively. The ST pocket is located at the mouth of the ST, which connects these two pockets.

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## MAVISp: Module for Identification and Validation of Candidate Allosteric Mutations

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The role of genomic variants in diseases, including cancer, continues to expand thanks to the advent of advanced sequencing techniques integrated into clinical practice [1]. The rapid growth in the identification of genomic variants has led to the classification of many variants as Variants of Uncertain Significance (VUS) or those with conflicting evidence, posing challenges in their interpretation and application. Additionally, current methods for predicting pathogenic variants do not necessarily provide information on the mechanisms underlying pathogenicity [2-4].

We introduce MAVISp (Multi-layered Assessment of Variants by Structure for proteins), a modular structural framework for variant effects, to handle high-throughput saturation variant analysis with a standardised workflow, integrating results with various pathogenicity predictors [5]. Currently, MAVISp offers analyses for 600 different proteins, encompassing more than 5 million variants.

Here, we present the LONG\_RANGE module of MAVISp, which operates in two modes: simple and ensemble. The simple mode leverages AlloSigMA2 [6] to estimate long-range effects by quantifying allosteric free energy changes caused by mutations, refined through a filtering workflow. The ensemble mode extends this by integrating molecular dynamics (MD) simulations and the graph-based tool PyInteraph [7] to identify stable long-range interactions and reduce false positives from static structures. Together, these workflows improve variant effect predictions and provide deeper insights into how mutations impact allosteric mechanisms.

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## Computational Pipeline for Designing Protein Binders Targeting MALT1

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MALT1 (Mucosa-associated lymphoid tissue lymphoma translocation protein 1) is a key mediator of NF- $\kappa$ B signaling, playing a crucial role in immune cell survival and proliferation. Dysregulation of MALT1 activity has been implicated in various hematological malignancies and solid tumors, including glioblastoma multiforme (GBM) where it performs a critical role in promoting GBM pathogenesis. Given its central role in both tumor cells and immune regulation, MALT1 protease inhibition has emerged as a promising therapeutic strategy to cancer therapy. [1] While small-molecule inhibitors have been explored, the design of high-affinity protein-based inhibitors remains a challenge. Moreover, given the limitations of irreversible covalent MALT1 inhibitors, the development of selective, reversible allosteric inhibitors represents a promising alternative. [2] Here, we present a computational pipeline that integrates state-of-the-art AI-driven protein design methods to generate and validate novel protein binders targeting MALT1 allosteric sites.

Our approach leverages RFDiffusion, which generates de novo backbone scaffolds designed to bind to the MALT1 allosteric site. We employ ProteinMPNN to assign sequences to the binders followed by structural validation with AlphaFold3 (AF3) to assess whether the designed binders adopt the intended conformation when folded alongside MALT1. Further filtering is applied based on key structural confidence metrics, including backbone RMSD, predicted local distance difference test, and predicted aligned error, ensuring selection of high-confidence binders. Using this pipeline, we generated 1000 binders against the apo state of human MALT1, following the guidelines suggested by Watson et al. [3]. Our results demonstrate that a subset of designed binders meets the predefined criteria, indicating their potential as viable MALT1 allosteric binders with modulatory potential.

This study underscores the feasibility of AI-driven protein binder design in the context of allosteric drug discovery, providing a framework for developing novel protein therapeutics against challenging drug targets like MALT1. Future work will focus on molecular dynamics simulations and *in vitro* validation of the top-ranked binders, for binding specificity and affinity, and further optimizing their therapeutic potential through experimental and computational approaches.

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## Targeting NAD-dependent Histone Deacetylase 6 (SIRT6) with Small Molecule Activators

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Aging is the primary factor that increases susceptibility to morbidity from natural causes. The aging phenotype arises from a diminished capacity for cellular regeneration, largely driven by the depletion of cells responsible for maintaining epigenomic integrity [1]. One protein whose enhanced function has been linked to improved epigenetic and genomic stability is Sirtuin 6 (SIRT6), an NAD-dependent enzyme primarily responsible for the deacetylation of Histone H3 Lysine 9 (H3K9ac), among other substrates [2]. Despite extensive efforts to identify small molecules that enhance the catalytic efficiency ( $K_m$ ) of SIRT6, few candidates have demonstrated significant cellular effects. To address this challenge, we explored the chemical space of the Enamine library to identify potential allosteric binders targeting a pocket near the N-terminal region of SIRT6. Although multiple experimental structures of SIRT6 are available, we utilized an AlphaFold3 model, as existing structures lacked NAD<sup>+</sup> and H3K9ac, both of which influence allosteric site binding, particularly the positioning of the nicotinamide moiety, which differentiates between the active and the inactive state. Given the inherent flexibility of the SIRT6 N-terminal tail, we incorporated the most potent known activator (12q) [3], into the structure prediction process. Molecular dynamics simulations of the complex revealed high flexibility of the SIRT6 N-terminal tail, while the ligand remained stable due to favorable interactions in the cavity, guiding the final model for virtual screening. We used GNINA and virtually screened ~1.3 million compounds after filtering based on Lipinski's Rule of Five, as well as PAINS and Brenk filters. Compounds were ranked by using a combination of scoring functions, and a Tanimoto similarity clustering was applied to obtain diverse chemotypes. After visual inspection, 75 promising candidates were selected for validation in cell line assays.

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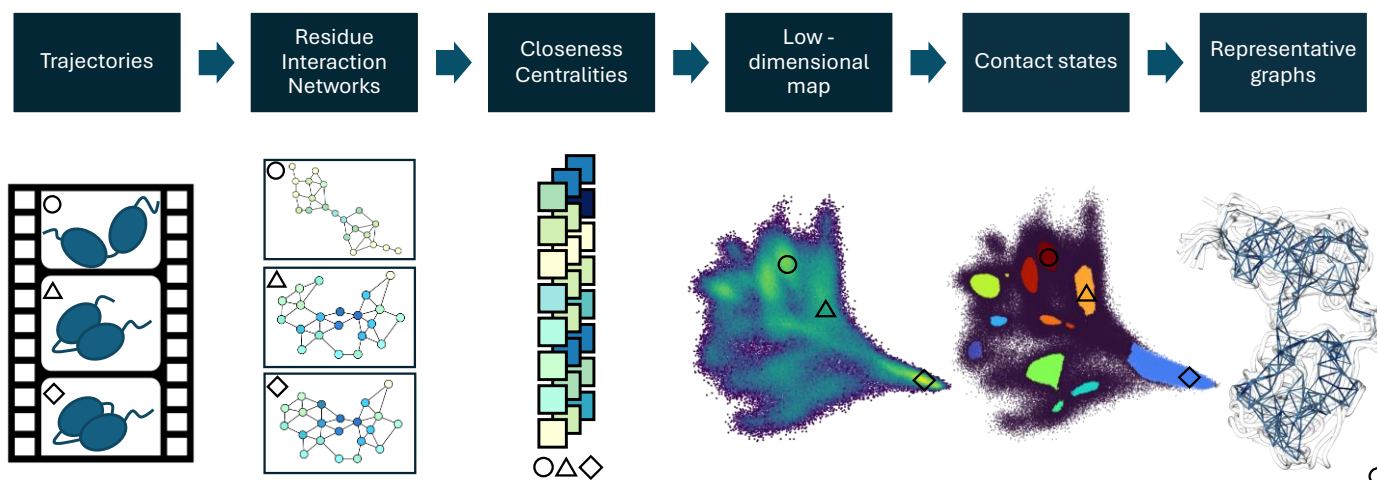


## Clustering and Analyzing Ensembles of Residue Interaction Networks from Molecular Dynamics Simulations

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Network methods and molecular dynamics (MD) simulations have become essential tools for studying protein allostery. While network descriptions capture the intricate interactions within a protein, MD simulations provide high-resolution modeling of how these interactions change over time. Due to methodological or computational constraints, using network-based analysis methods for MD data often requires careful selection of a limited set of simulation frames. This poses a significant challenge especially for large data sets from extensive MD simulations, which can encompass multiple trajectories and high conformational heterogeneity. Here, we propose a graph-based workflow for extracting meaningful insights from large MD data sets and for making an informed selection of representative data for further down-stream processing, such as the construction of allosteric networks. The basis of the workflow is formed by a graph theoretic featurization of protein structures. After converting each frame from the simulation into a residue interaction network, the closeness centralities of each residue in the protein are extracted as features, which were previously shown to be an expressive fingerprint for capturing the contact topology of proteins [1]. This feature set is then used as input for unsupervised machine learning algorithms to perform dimensionality reduction and clustering. Once suitable representations of the resulting clusters are determined, they are characterized in detail based on tools from the upstream workflow. On the example of FAT10 - a two-domain protein with intrinsically disordered regions - we demonstrate how this approach can be used to understand the protein's conformational landscape on different, interconnected levels - from the global to the local - and to characterize its most populated states. The modularity of the workflow allows for adaptability and interpretability, which makes it a suitable method to support network-based analyses of MD simulations for a wide variety of proteins.



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## Predicting protein-membrane interfaces of peripheral membrane proteins using ensemble machine learning

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Abnormal protein-membrane attachment is involved in deregulated cellular pathways and in disease. Therefore, the possibility to modulate protein-membrane interactions represents a new promising therapeutic strategy for peripheral membrane proteins that have been considered so far undruggable [1]. A major obstacle in this drug design strategy is that the membrane binding domains of peripheral membrane proteins are usually unknown. The development of fast and efficient algorithms predicting the protein-membrane interface would shed light into the accessibility of membrane-protein interfaces by drug-like molecules. Herein, we describe an ensemble machine learning methodology and algorithm for predicting membrane-penetrating amino acids. We utilize available experimental data in the literature for training 21 machine learning classifiers and a voting classifier [2]. Evaluation of the ensemble classifier accuracy produced a macro-averaged  $F_1$  score = 0.92 and an MCC = 0.84 for predicting correctly membrane-penetrating amino acids on unknown proteins of a validation set. Predictions were further verified in independent test sets. The Python code is available at <https://github.com/zoecournia/DREAMM>.

The allosteric modulation of peripheral membrane proteins by targeting protein-membrane interactions with drug-like molecules represents a new promising therapeutic strategy. We also present a drug design pipeline for drugging protein-membrane interfaces using the DREAMM (Drugging pRotein mEmbrAne Machine learning Method) web-server [3]. DREAMM works in the back-end with a fast and robust ensemble machine learning algorithm for identifying protein-membrane interfaces of peripheral membrane proteins. Additionally, DREAMM also identifies binding pockets in the vicinity of the predicted membrane-penetrating residues in protein conformational ensembles provided by the user or generated by DREAMM. DREAMM has been made accessible via a web-server at <https://dreamm.ni4os.eu>.

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## Predicting protein-membrane interfaces of peripheral membrane proteins using machine learning

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Abnormal protein-membrane attachment plays a critical role in deregulated cellular pathways and in disease. Therefore, the possibility to modulate protein-membrane interactions represents a new promising therapeutic strategy to target PMPs. However, the membrane-binding interfaces of peripheral membrane proteins (PMPs) are usually unknown due to the limited PMP-membrane structural information and the complexity of PMP-membrane binding modes. To address this challenge, we aim to use machine learning techniques to predict membrane-interacting from non-membrane-interacting amino acids for PMPs with unknown membrane-interacting regions. The first step of this process is to build a robust dataset. Previously, we curated a dataset of experimentally validated membrane-interacting amino acids to train an ensemble machine learning classifier model [1], and utilizing the PePrMInt dataset [2], we trained deep learning classifier models [3].

Building on this work, we have now curated an updated dataset by expanding the experimentally validated membrane-interacting amino acids using the current literature and combining it with the PePrMInt dataset. By extracting a diverse set of physicochemical, structural, and sequence-based features on this dataset, we aim to train a new ensemble machine learning classifier model to improve accuracy and generalization in predicting membrane-binding interfaces of PMPs. This new ensemble machine learning classifier model will be made available through an open and free web-based application [4], paving the way for new therapeutic strategies for drugging protein-membrane interfaces.

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## Structural insights into PI3K $\alpha$ activation by the H1047R mutation at the protein-membrane interface

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PI3K $\alpha$  is the most frequently mutated kinase in human cancers with mutations often occurring at the C-terminus. The C-terminus has a dual function in regulating the kinase, playing an auto-inhibitory role for kinase activity and also mediating protein binding to the cell membrane. When PI3K $\alpha$  attaches to the cell membrane, it phosphorylates its substrate PIP2 and converts it to PIP3, initiating a signaling cascade for cell proliferation.[1] The oncogenic mutation H1047R in the C-terminus of PI3K $\alpha$  leads to overactivation of the kinase.[2,3] However, the precise molecular mechanism by which C-terminal oncogenic mutations cause PI3K $\alpha$  overactivation has remained poorly characterized, and the membrane-bound active conformation of the H1047R mutant is still unresolved.

To understand how C-terminal mutations-particularly H1047R-alter kinase activity, we performed both unbiased and biased molecular dynamics simulations of the wild-type (WT) and H1047R mutant. We report the free energy associated with the C-terminal “closed-to-open” transition and the conformational changes involved, with the H1047R mutant exhibiting the lowest free energy for this transition compared to the WT.[4]

To further investigate membrane interactions and how the H1047R mutation affects them, we performed Molecular Dynamics simulations of the active-like PI3K $\alpha$  WT and H1047R mutant states in complex with HRAS on a model membrane. Our simulations reveal that the H1047R mutation enhances substrate coordination and induces an open C-terminal conformation that interacts with the membrane, stabilizing the protein on the lipid bilayer. Moreover, H1047R strengthens allosteric coupling between the C-terminus and membrane-binding loop 2 in both solution and membrane-bound states.

These findings provide new insights into the molecular mechanisms underlying the activation of oncogenic PI3K $\alpha$  by the H1047R mutation in complex with a model membrane, and offer a mechanistic basis for the enhanced membrane-binding ability of the mutant.

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## Investigating KRAS-4B Conformational Dynamics: Insights from Coarse-Grained Molecular Dynamics Simulations

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KRAS-4B, the primary isoform of KRAS, is essential for regulating cellular proliferation and differentiation through its GTPase activity. The G12D KRAS-4B mutant is implicated in pancreatic, colorectal, and lung malignancies. Structurally, KRAS-4B comprises a catalytic GTP/GDP-binding G-domain and a hypervariable C-terminal region (HVR) that anchors the protein to the membrane via farnesylation. Membrane composition has been shown to significantly influence the KRAS-4B function<sup>1-3</sup>, while molecular dynamics (MD) simulations have identified three key orientations of the monomeric KRAS-4B on the membrane: OS1, an active-like conformation that promotes effector interactions, OS2, an inactive membrane-bound state, and OS0, a metastable intermediate that bridges OS1 and OS2<sup>4,5</sup>. Studying the monomeric KRAS-4B in the membrane would enhance our understanding of how its interactions with the lipid environment may influence its conformational dynamics leading in turn to insights into the mechanisms governing its function.

Herein, we perform atomistic and coarse-grained MD of KRAS-4B on the membrane to achieve two primary objectives: (i) assess the ability of the CGMD Martini 3 force field to reproduce dominant KRAS-4B orientations on the membrane as well as lipid-protein interactions that have been previously observed by atomistic MD simulations and (ii) investigating the impact of different lipid bilayer molecular composition on KRAS-4B orientation and dynamics. To achieve these goals, we conducted both atomistic and CGMD simulations on four lipid bilayers of varying composition. Our findings demonstrate that the CGMD simulations using Martini 3 force field reproduce the OS2 conformation of KRAS-4B that is observed in atomistic MD simulations. However, the CGMD simulations exhibit limited sampling of the OS1 state and the transient metastable state OS0, which appear in atomistic MD simulations in larger populations. This finding suggests that the accelerated dynamics of the Martini 3 force field may lead to a rapid convergence towards stable states, bypassing short-lived intermediate conformations. Moreover, the OS2 state emerged as the dominant conformation across all simulated membrane environments largely being independent of lipid composition. This finding suggests that the conformational preferences of the monomeric KRAS-4B are primarily governed by intrinsic structural properties rather than membrane-driven effects. Further analysis revealed that the OS2 state consistently exhibits higher diffusion across all lipid bilayers, indicating weaker or more transient membrane interactions of KRAS-4B compared to the OS1 and OS2 states. In contrast, OS1, the active-like state, displayed reduced diffusion, likely due to stronger lipid interactions that facilitate effector binding. These results highlight the ability of the Martini 3 force field to capture the dominant OS2 KRAS-4B orientation and highlight that the lipid compositions studied herein do not affect KRAS-4B conformational transitions.

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