KINASES, PROTEIN PHOSPHORYLATION AND CANCER JUNIOR GROUP

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OVERVIEW

Rational and precise targeting of oncogene-driven signalling is a crucial and yet today outstanding challenge in cancer research. Understanding the structural and molecular bases of oncogene activation and signalling is key for the design and development of better therapeutics. Our research focuses on the structural and molecular understanding of protein kinase function: how protein kinases are activated and regulated by post-translational modifications and allosteric inputs, and how they assemble into macromolecular protein complexes to transmit signals inside the cell. We put a special emphasis on how these mechanisms are corrupted in cancer due to oncogenic mutations and other oncogenic insults. Crucially, such atomic and molecular information can be translated into the design and development of next generation protein kinase inhibitors for targeted and personalised therapies.

We apply an integrated and multidisciplinary approach by combining: molecular biology for the generation of suitable constructs; protein biochemistry and biophysics for protein purification, quality assessment and functional evaluation; mass spectrometry (MS) for the identification and quantification of post-translational modifications; X-ray crystallography for the 3D-visualisation of proteins; and *Drosophila* as an *in vivo* model for data validation. Furthermore, we use structure-guided drug discovery and MD simulation approaches to exploit structural and functional vulnerabilities for drug design and development.

Gradutate Students
Nicolás Cuesta (until November), Ana Martín-Hurtado, Moustafa Ahmed Shehata (until April)

Visiting Gradutate Student
Yanara Astudillo (*Universidad Tecnológica Equinoccial - Fundación Carolina,* Quito, Ecuador)

Students in Practice
Marina Rodríguez (Bachelor's Degree Final Project, *Univ. Autónoma de Madrid*; Master's Thesis, *Univ. Complutense de Madrid,* Spain), Alejandro Sánchez (March-September) (Master's Thesis, *Univ. de Alcalá de Henares,* Spain)

RESEARCH HIGHLIGHTS

Our main strategic lines are:

1. Structural and molecular determinants that control protein phosphorylation. Auto-phosphorylation controls the transition between discrete functional and conformational states in protein kinases, yet the structural and molecular determinants underlying this fundamental process remain unclear. In our recent work, we show that c-terminal Tyr 530 is a *de facto* c-Src auto-phosphorylation site with slow time-resolution kinetics and strong intermolecular component. By contrast, activation-loop Tyr 419 undergoes fast kinetics and a cis-to-trans phosphorylation-switch that controls c-terminal Tyr 530 auto-phosphorylation, enzyme specificity, and strikingly, c-Src non-catalytic function as a substrate. In line with this, we visualised by X-ray crystallography a snapshot of Tyr 530 intermolecular phosphorylation in which a c-terminal palindromic phospho-motif flanking Tyr 530 on the substrate molecule engages the G-loop of the active kinase for ready entry prior catalysis. Perturbation of the phospho-motif accounts for c-Src dysfunction as indicated by viral and a colorectal cancer (CRC) associated c-terminal deleted variants. We showed that c-terminal residues 531 to 536 are required for c-Src Tyr 530 and global auto-phosphorylation, and this detrimental effect is caused by the substrate molecule inhibiting allosterically the active kinase. Our work reveals a bi-directional crosstalk between the activation and c-terminal segments that controls the allosteric interplay between substrate and enzyme acting kinases during auto-phosphorylation (Cuesta and Contreras *et al.*, under revision. *BioRXiv*. doi: https://doi.org/10.1101/2022.10.16.512342).

2. Structure, function, and pharmacology of protein kinase-gene fusion products. Gene fusion products are known drivers in human cancers and are current drug targets for personalised therapy. A second strategic line in the lab was established and directed to dissect the functional and structural determinants for two RET oncogenic fusion products, namely CCDC6-RET and KIF5B-RET, which are drivers and therapeutic targets in lung (NSCLC) and thyroid cancers. We have successfully purified these challenging proteins using a baculovirus expression system in different isoforms and length-variants. By applying an integrated approach, we demonstrated that full-length constructs behave like active dimers in solution. Auto-phosphorylation and enzymatic assays demonstrated fast kinetics compared to wild-type RET, and further phospho-proteomic characterisation by mass spectrometry highlighted important roles for catalytic activity and substrate specificity through unexpected allosteric inputs by distant elements to the catalytic site (Hurtado *et al.*, submitted).

3. Structure-guided drug discovery for next generation protein kinase inhibitors. A third main research line is focused on the exploitation of structural and functional vulnerabilities in RET for the rational design and development of highly specific inhibitors. Our current paradigm is based on the recently developed second generation RET inhibitors LOXO-292 and BLU-667 that showed excellent results in both preclinical models and early clinical trials, resulting in timely FDA approval for the treatment of RET-rearranged or -mutated cancers. We are applying an integrated approach combining structural data, molecular docking, structure-guided molecular dynamics simulations, and screening with both virtual and chemical libraries together with biophysical and biochemical tools for functional validation. Following this approach, we identified an allosteric interface in RET with good druggability score that can be potentially targeted with allosteric inhibitors. Furthermore, we found a cryptic and druggable pocket within the ATP-binding site that is exploited by LOXO-292 and BLU-667 (Shehata and Contreras *et al., J Adv Res 2022)*. This information will be crucial to designing and developing highly specific third generation RET inhibitors able to overcome refractory RET mutations. Based on these results we are optimising chemical scaffolds of second generation RET inhibitors to maximise contacts and interactions with the cryptic pocket, in collaboration with CNIO's Experimental Therapeutics Programme. ■

PUBLICATIONS

- Shehata MA, Contreras J, Martín-Hurtado A, Froux A, Mohamed HT, El-Sherif AA, Plaza-Menacho I (2022). Structural and dynamic determinants for highly selective RET kinase inhibition reveal cryptic druggability. *J Adv Res.* PMID: 35595215.
- Cuesta N, Contreras J, Sánchez-Walder-mer J, Soriano-Maldonado P, Martín-Hur-tado A, Muñoz IG, Llimargas M, Muñoz J, Plaza-Menacho I (2022). An allosteric switch between the activation loop and a c-terminal palindromic phospho-motif drives c-Src function. *BioRXiv*. doi: https://doi.org/10.1101/2022.10.16.512342.

PATENT

- Plaza Menacho I. Identification of a cryptic and druggable pocket in the active site of RET with therapeutic potential: the post-lysine pocket. PCT application (2022). *PCT/EP2022/077036. WO2023052462A1.*