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 current 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 posttranslational modifications and allosteric inputs, and how they assemble into macromolecular protein complexes to transmit signals inside the cell. We place 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 more potent and specific protein kinase inhibitors, leading eventually to more effective drugs for the treatment of cancer patients.

We apply an integrated and multidisciplinary approach 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-visualization 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 the design, development, and optimization of protein kinase inhibitors as therapeutic agents in cancer.

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RESEARCH HIGHLIGHTS

In 2021, we made significant progress in all our laboratory research projects, which are materializing successfully and, as a result, several papers were submitted for publication and are under review.

1. c-Src codifies a non-receptor tyrosine kinase that is activated by a plethora of signalling receptors that are fundamental in the aetiology of cancer. Despite being the object of intense study over the last 40 years, the precise mechanism by which auto-phosphorylation regulates c-Src intrinsic activity and conformational state independent of external inputs, and how this process is corrupted in cancer, remains elusive. In our work we show that c-terminal Tyr 530 is a *de facto* c-Src auto-phosphorylation site with slow time-resolution kinetics and a strong intermolecular component. By contrast, activation-loop Tyr 419 undergoes very fast kinetics and a cis-to-trans phosphorylation switch that controls c-terminal phosphorylation, substrate specificity and substrate-like properties. In line with these findings, a *Drosophila* mutant at the equivalent residue in the activation loop shows tissue-specific functionality and milder but transforming phenotypes compared with wild-type or constitutive active variants. Furthermore, we provide evidence that the intrinsically disordered N-terminal region of c-Src does not promote direct dimerization in the "apo" or the ATP-complexed states, and that c-Src Tyr 530 auto-phosphorylation is associated with a lowered catalytic status. A crystal structure of the c-Src-Ponatinib complex in a DFG-out state reveals unusual active-like features and provides a clear snapshot of c-terminal Tyr 530 intermolecular phosphorylation between enzyme and substrate acting kinases. Altogether these data indicate that c-Src must adopt an alternative conformation to the inactive-closed state independent of c-terminal Src kinase phosphorylation, and that a sequential and coordinated cis-to-trans phosphorylation switch between the activation and c-terminal segments simultaneously controls c-Src catalytic and non-catalytic functions (Cuesta and Contreras *et al.*, submitted for publication).

2. Gene fusion products are known drivers in human cancers and are current drug targets for personalised therapy. A second research line in the lab was established and directed at dissecting the functional and structural determinants for 2 RET oncogenic fusion products, CCDC6-RET and KIF5B-RET. 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 MS highlighted important roles for catalytic activity and substrate specificity through unexpected allosteric inputs by distant elements to the catalytic site (Hurtado *et al.*, in preparation).

3. A third research line focuses 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 uncovered a cryptic-pocket within the ATP-binding site that is exploited by highly specific second-generation type I RET inhibitors. This information will be crucial to designing and developing highly specific third generation RET inhibitors that are clinically successful and able to overcome refractory RET mutations (Shehata *et al.*, in press).

4. We initiated a new research line focused on the structural and functional characterization of human FAK. We want to explore how auto-phosphorylation drives the functional and conformational landscape of FAK, in a full-length setting, and how phosphorylation interferes with the assembly and interaction with substrates and signalling partners such as RET (both wild-type and oncogenic variants) and c-Src. Using a phospho-proteomic approach we already identified unexpected phospho-sites and revealed a previously unknown switch for FAK catalytic activation by N-terminal elements, which could be therapeutically exploited to design and develop next generation FAK inhibitors.

PATENT

- Plaza Menacho I (2021). *EP21382869.2.*