CeTPD Journal Club

Targeted protein degradation, medicinal chemistry & chemical structural biology literature highlights

May 2022

Centre for Targeted Protein Degradation
University of Dundee

innovate
collaborate
inspire

Journal Club

Content

Meet this Month's Editors

This month's editors are (from left to right): Tom, Selma, Claudia

"It has been great to observe the growth of our Journal Club over the last couple of years and see it become such a useful resource for the TPD community. My shift as editor always serves as a reminder of how fast the field is advancing and certainly helps me keep track of the latest developments!"

Tom is a 2nd year PhD student under the supervision of Alessio, on the MRC Doctoral Training Programme in Quantitative and Interdisciplinary Approaches to Biomedical Science (QIBIOMED). The project is part-funded by AstraZeneca, and co-supervised by Dr John Liddle and Dr Johan Johansson at AZ in Gothenburg. Tom's research focuses on the development of novel methods for the discovery of molecular glues, which can be used for targeted protein degradation.

"Being an editor in the Journal Club fully immerses me into all the disciplines within targeted protein degradation and makes me appreciate everything it teaches and how much we have still to learn."

Selma completed her undergraduate studies in Biomedical Sciences at the University of Dundee before setting off for a BBSRC-funded EASTBIO-DTP at the University of St Andrews. She completed her PhD in Cell Biology under the supervision of Dr Judith Sleeman before returning to Dundee to join the Ciulli lab in March 2021 as a cell biologist within the Almirall collaboration.

"*The Journal Club is a very useful resource to keep up with latest developments in the ever-expanding TPD field and beyond. I particularly enjoy the diversity of the "other paper highlights" section reflecting the diverse research interests of our interdisciplinary group."*

Claudia completed her undergraduate studies in Chemistry at the Justus-Liebig-University Giessen and moved to RWTH Aachen University for her doctorate in transition-metal catalysis in the lab of Prof. Franziska Schoenebeck. In 2021 she joined the Ciulli group as Medicinal Chemist/Chemical Biologist. Claudia is a Marie Skłodowska-Curie Postdoctoral Fellow focussing on rational design and development of novel functional linker motifs.

Feature of the Month

Contributors: Vesna and Valentina

ACBI team visits Boehringer Ingelheim Colleagues in Vienna

Last month the Dundee ACBI team flew to Vienna to meet their Boehringer Ingelheim (BI) colleagues in person for the first time since the start of the pandemic. The two-day meeting was designed to be an engaging forum for scientific exchange and socialising. The mornings were reserved for detailed presentations on subjects that we would not typically have time for in project meetings, while the afternoon featured social activities, including a vineyard hike and a walking tour of Vienna. This trip was particularly important as the Dundee team has doubled in size over the last twelve months, so many of the newer team members have not had the opportunity to meet the BI team outside the confines of a Teams window. It was truly a pleasure to be able to visit Vienna and strengthen our relationship with our BI colleagues. We're now eagerly awaiting our next in-person meeting and welcoming the BI team to Scotland!

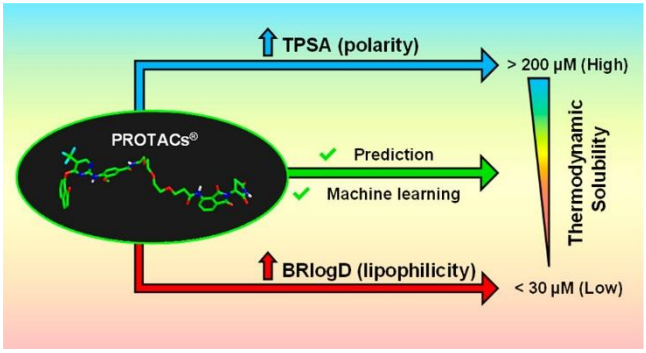
Chemistry | Computational Chemistry

Contributor: Claudia

Designing Soluble PROTACs: Strategies and Preliminary Guidelines

Diego García Jiménez[§], …, Giulia Caron*

J. Med. Chem. **2022**, DOI: 10.1021/acs.jmedchem.2c00201



As "beyond Ro5 compounds", optimising the pharmacokinetic (PK) properties of PROTACs poses challenges, such as oral bioavailability, and optimization of ADME properties is therefore essential for the development of PROTAC degraders as therapeutics. However, to date there are few property-based design strategies available for PROTAC degraders. In the present study, the authors addressed PROTAC solubility by experimentally determining the solubility of 21 commercially available PROTACs covering VHL- and CRBN-recruiting degraders for different proteins of interest (POI), evaluating the performance of common solubility prediction tools and providing a classification system to distinguish soluble from poorly soluble degraders. Generally, only moderate correlation was observed between experimental solubilities and *in silico* predictions, with R^2 < 60% in all cases. Evaluating different potential contributors determining PROTAC solubility, their thermodynamic solubility has been shown to be efficiently classified using two chromatographic descriptors (BRlogD and log k_w^{IAM}) and one computational descriptor (TPSA). Attempting to predict PROTAC solubilities modularly from contributions of both E3 ligase and POI ligands and the linker proved to be challenging and matched the experimental trend in only one out of three examples.

This study provides a valuable classification method to evaluate PROTAC solubility and raises awareness for only moderate reliability of current solubility prediction tools. Though solubility is certainly an important factor to account for, property based PROTAC design might be more balanced and effective by monitoring/optimizing the interplay of both solubility and permeability of degraders simultaneously.

Cell Biology | Chemistry

Contributor: Claudia

Smart PROTACs Enable Controllable Protein Degradation for Precision Cancer Therapy

Lixia Chen[§], Xinqiang Wan, Xiangxiang Shan, Wenzhang Zha, Rengen Fan*

Mol. Diagn. Ther. **2022**, *26*, 283

'Smart' PROTACs aim to release the active degrader upon exposure to an internal or external stimulus. This technique can aid inreduction of off-target effects of PROTAC treatments in cancer therapy, for example those arising from nonspecific accumulation in non-cancerous tissue or from E3-ligase off-target activity. In this review, the authors present currently available stimuli-response mechanisms applied in PROTAC design discussing both internal stimuli – such as reductive environment, hypoxia or enzyme activity as release mechanisms of caged PROTACs – and light as external stimulus for both photocaged and photoswitchable PROTACs. The authors discuss the arising challenges – such as the heterogeneity of tumours and ubiquity of biomarkers used to target cancer cells – as well as opportunities for applying alternative stimuli-response mechanisms, especially using other external stimuli for conditional PROTAC activation.

A good read for a quick overview of current methods for conditional regulation and release of PROTAC degraders!

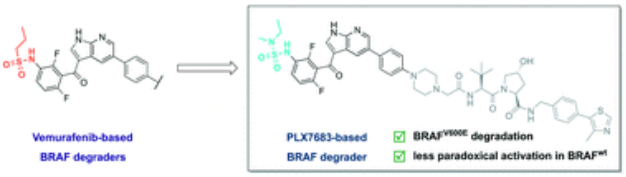
Cell Biology | Chemistry

Contributor: Claudia

Encoding BRAF inhibitor functions in protein degraders

Daniel S. J. Miller[§], …, Christian Steinebach*

RSC Med. Chem. **2022**, DOI: 10.1039/d2md00064d



The BRAF^{V600E} point mutation is one of the most prevalent oncogenic mutations in the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) axis enhancing RAF homo- and heterodimerization leading to uncontrolled kinase activity. Targeting BRAF^{V600E} with kinase inhibitors has proven challenging due to the rapid onset of drug resistance and limited efficacy, as inhibition of kinase function does not prevent RAF dimerization thus promoting paradoxical ERK activation. BRAF^{V600E}-targeting degraders may be able to overcome these limitations, as both the catalytic and non-catalytic functions of BRAF can be silenced. In this study, the authors developed VHL-recruiting BRAF^{V600E}-targeting PROTAC degraders utilising PLX-type BRAF^{V600E} inhibitors as BRAF^{V600E} ligands. While first generation PROTACs did not induce significant BRAF degradation, switching to much shorter cyclohexyl-based linkers and introducing a paradox-breaking BRAF^{V600E}-ligand led to the potent degrader **25**, which selectively degrades BRAF^{V600E} (DC_{50} = 18 nM) without touching wild-type RAF or pERK protein levels.

Dissociating the BRAF^{V600E} depletion from paradoxical activation of the ERK signalling pathway may lead to safer BRAF^{V600E}-targeting PROTAC degraders bearing therapeutic potential. This study highlights the importance of tracing the function and potential down-stream effects of the POI ligand in the context of heterobifunctional PROTAC degraders.

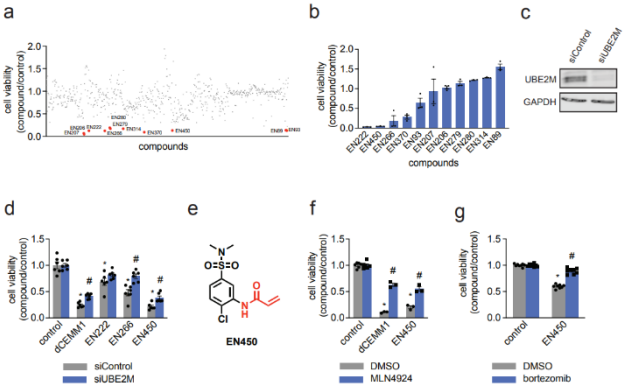
Cell Biology | Chemistry | Computational Chemistry | Structural Biology/Biophysics

Contributor: Tom

Chemoproteomics-Enabled Discovery of a Covalent Molecular Glue Degrader Targeting NF-kB

Elizabeth A. King[§], Yoojin Cho[§], Daniel K. Nomura*

BioRxiv **2022**, DOI: 10.1101/2022.05.18.492542v1



The discovery of molecular glue degraders by design presents significant challenges largely due to the inherently weak nature of the interactions they stabilise, and their potentially extremely weak or non-detectable binary affinities to either component of the ternary complex they induce. Several recent examples have demonstrated the utility of phenotypic screening and counter-screening in the discovery of novel molecular glue degraders. However, there remains a disconnect to determining the mechanism of degradation (deconvoluting the target and the component of the ubiquitin-proteasome system recruited in the ternary complex).

Combining quantitative proteomic and chemoproteomic based screening of a covalent ligand library for anti-proliferative effects in leukemia cells, covalent ligand EN450 was identified. EN450 interacts with the allosteric C111 of E2 ubiquitin ligase UBE2D and was shown to degrade the oncogenic transcription factor NFKB1. The mechanism was shown to be neddylation-dependent, indicating that UBE2D still needs to be recruited to the wider Cullin-E3 ligase complex, however it is not known whether recruitment of the E2 bypasses the necessity for a substrate adapter protein to induce degradation of NFKB1. It is suggested that NFKB1 degradation may occur *via* initial monoubiquitination as a

result of EN450-mediated ternary complex formation, sensitising NFKB1 to further polyubiquitination, or alternatively through a combination of specific E3 ligases.

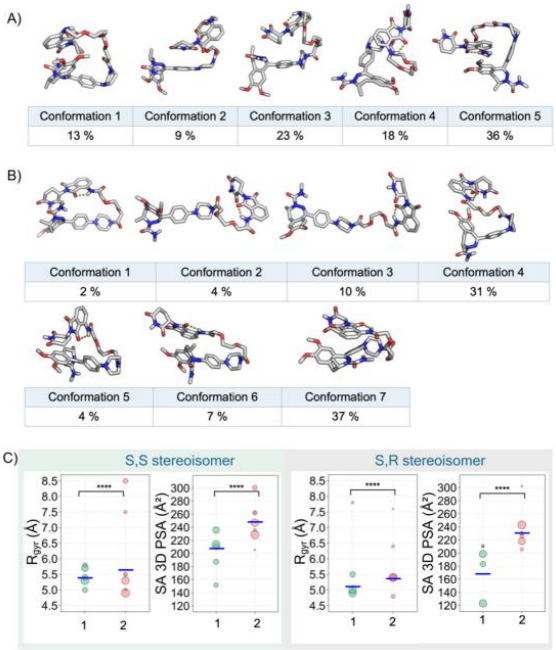
This study highlights important points in relation to the discovery of molecular glue degraders. In particular, the disconnect between phenotypic screening and mechanistic elucidation discussed herein highlights the difficulty in discovering molecular glues by design. Secondly, this presents another example where the need to recruit a specific substrate recognition domain or adaptor protein of an E3-ligase is bypassed. It is noteworthy that there remain very few examples of molecular glue degraders having been discovered in a target-based fashion. Such studies would be hugely significant and dramatically increase the therapeutic viability of molecular glue degraders.

Computational Chemistry | **Structural Biology/Biophysics**

Contributor: Tom

Linker-dependent folding rationalizes PROTAC cell permeability

Vasanthanathan Poongavanam[§], Yoseph Atilaw[§], …, Jan Kihlberg*
ChemRxiv **2022**, DOI: 10.26434/chemrxiv-2022-2gg11



Rationalising cell-permeability for PROTACs has become an important area of focus for the field. Traditional cell-permeability assays, such as Caco-2, are difficult to interpret in the context of degraders since potent degradation can still be observed with poorly permeable PROTACs. The authors highlight limitations in the ability to modify the POI or E3 ligase ligand components of PROTACs and propose that linkerology offers the most interesting opportunity for optimisation of potency and selectivity as well as PK and PD properties and note that despite general agreement within the field on these points, linker-property relationships remain understudied.

Taking 3 CRBN-based BRD4 degraders (all beyond Ro5 compounds) with identical POI and E3 ligases ligands but varying linkers (PEG3, PEG2, alkyl) as model systems, the authors used solution-NMR and unrestrained molecular dynamics to rationalise the influence of the linker on PK and PD properties. In a surrogate analysis of cell permeability (ratio between CRBN binary potency in a cell-based and biochemical assay), these compounds exhibit varying ratios indicating differences in permeability, which were confirmed via PAMPA. However, no correlation could be found between the rank order of permeabilities and calculated PK/PD properties. Using NMR analysis of molecular flexibility in solution (NAMFIS, more here), the authors were able to study potential conformations in chloroform, which has a similar dielectric constant to that of a lipid bilayer (ε = 4.8 and 3.0 respectively). Compounds 1 & 2 (PEG linkers) showed long and medium range NOEs between the E3 ligase ligand and the BRD4 ligand or linker, indicating folded conformations in solution. These NOEs are not observed for compound 3 (alkyl linker), suggesting a more elongated conformation. The polarity and size of the permeating conformations are key properties in the determination of membrane permeability. Folded conformations which minimise the radius of gyration (R_{gyr}) and solvent accessible 3D polar surface area (SA 3D PSA) appear to be important factors in determining permeability for these CRBN PROTACs. MD simulations were used to provide further quantitative analysis of R_{gyr} and SA 3D PSA and the overall conclusions from this analysis were in strong agreement with the NAMFIS data.

The influence of linkers on PROTAC 3D-structure widely acknowledged but remains understudied. We often observe degradation potency drop-offs or PROTAC rank order changes from *in vitro* to cellular assays and attempt to rationalise these with hypotheses around permeability. However, we very rarely take the time to experimentally determine what is driving these changes, and often disregard series which may be possible to optimise if the solution conformation is better understood. This study provides an insight into experiments that can provide structural information to aid design of cell-permeable PROTACs.

Cell Biology

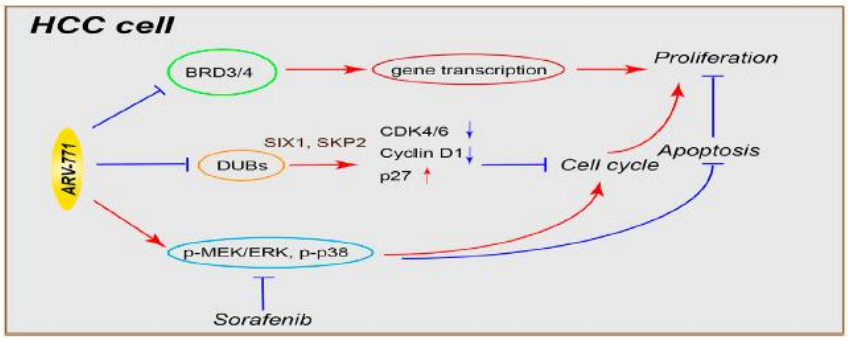
Contributor: Tom

ARV-771 Acts as an Inducer of Cell Cycle Arrest and Apoptosis to Suppress Hepatocellular Carcinoma Progression

Yuanfei Deng[§]*, Cuifu Yu[§], Lushi Chen[§], …, Gengxi Cai*, Fang Liu*

Front. Pharmacol. **2022**, *13*, Article 858901

ARV-771 is a potent BET PROTAC based on VHL which has previously shown anti-proliferation activity superior to that of the parent inhibitor, JQ1, in a range of cancer settings including castration-resistant prostate cancer and acute myeloid leukemia. Herein, the authors assess the ability of ARV-771 to induce cell cycle arrest and apoptosis in hepatocellular carcinoma (HCC).



ARV-771 was shown to inhibit growth of HCC cells via the induction of cell cycle blockade, apoptosis and downregulation of several deubiquitinating enzymes (DUBs) in cells and in vivo. Moreover, ARV-771 aggravates the anti-proliferative activity of the Raf inhibitor sorafenib via a synergistic effect. The authors were also able to demonstrate that ARV-771 can induce apoptosis by reducing expression of Bcl-2 and Bcl-XL. Unlike pioneering studies surrounding the effects of ARV-771 in downregulating BET bromodomains, the authors showed that ARV-771 may affect cell cycle processes via modulating levels of CDK4/6, Cyclin D1 and p27 in HCC, thus proposing multiple pathways to cell cycle arrest, apoptosis, and proliferation suppression. Biomass spectrometry analysis showed limited off-target effects of ARV-771 contributing to cell cycle arrest and apoptosis in HCC cells. These results shed light on novel anti-cancer activities of ARV-771 and highlight the potential of targeted protein degradation to provide a new, promising therapeutic strategy against HCC.

Medicinal Chemistry | Cell Biology | Structural Biology/Biophysics

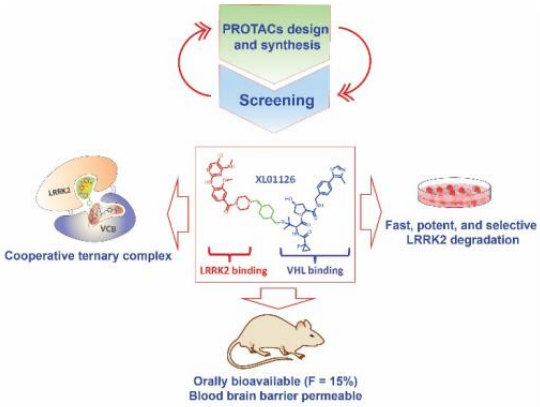
Contributor: Tom

Discovery of XL01126: A Potent, Fast, Cooperative, Selective, Oral bioavailable and Blood Brain Barrier Penetrant Degrader of Leucine Rich Repeat Kinase 2 (LRRK2)

Xingui Liu[§], Alexia F. Kalogeropulou[§], …, Dario R. Alessi*, Alessio Ciulli*

ChemRxiv **2022**, DOI: 10.26434/chemrxiv-2022-4gzm0

LRRK2 is well validated as a promising target for Parkinson's disease (PD). LRRK2 is a large (286 kDa), multi-domain protein that possesses a kinase domain and a GTPase domain as well as several other domains and motifs involved in protein-protein interactions. Several inhibitors have been developed targeting these various domains, however some have been shown to induce unintended effects (interference with vesicle trafficking, on-target side-effects observed on lungs and kidneys). This has given rise to a need for alternative LRRK2 targeting strategies. Of these alternative approaches, PROTACs offer the opportunity to selectively target LRRK2 and chemically remove it at a post-translational level.



Through iterative rounds of medicinal chemistry optimisation, the authors were able to design and characterise XL01126, a VHL-based, fast, potent, co-operative and selective LRRK2 degrader that, significantly, is orally bioavailable and able to penetrate the blood brain barrier. XL01126 qualifies as a chemical probe, offering an extremely useful tool to the community to further validate LRRK2-removal as a potential

strategy against PD. This study adds to the growing number of cases in which covering a broad range of chemical space early on in medicinal chemistry campaigns (including applying this strategy to the linker and not only to the POI and E3 ligase ligands) can quickly yield optimised degraders which can be built upon through iterative rounds of design and testing.

As opposed to numerous 'validation of degradation as a viable mechanism studies', the authors initially sampled a range of structurally diverse linkers. It is noteworthy that moving away from PEG/alkyl chains to motifs with varying degrees of 3D structure and therefore varying degrees of linker vector restraints (phenyl, alkyne, isopropyl and cyclohexyl) allowed for the discovery of XL01126, which has a *trans*-cyclohexyl linker moiety and is more potent and more cell permeable than XL01134, which is identical other than its *cis*-cyclohexyl linker moiety. This highlights the important role three-dimensional structure can play in permeability and potency with respect to PROTACs, and how such subtle changes in linker stereochemistry can play a key role in determining PK/PD properties. In the case of XL01126, had only PEG and alkyl linkers been sampled, the authors would have missed out on the PK/PD improvements gained through linker diversification. We look forward to seeing how the structures of these PROTACs and their linkers progress and how this helps to progress LRRK2 biology and validation.

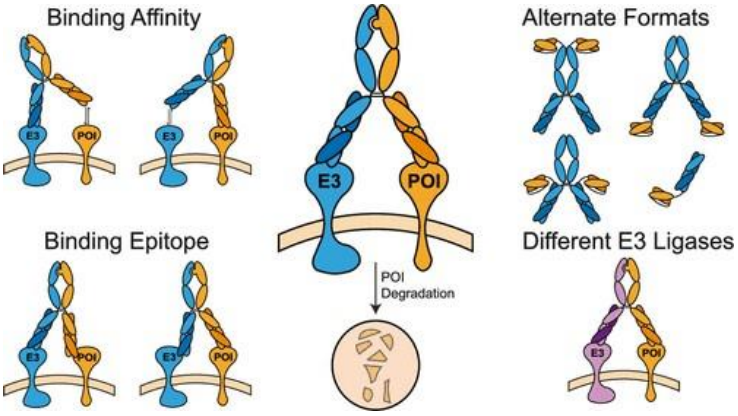
Cell Biology | **Computational Chemistry** | **Structural Biology/Biophysics**

Contributor: Selma

Roadmap for Optimizing and Broadening Antibody-Based PROTACs for degradation of Cell Surface Proteins

Josef A. Gramespacher[§], Adam D. Cotton[§], Paul W. W. Burroughs, Ian B. Seiple, James A. Wells*

ACS Chem. Biol. **2022**, *17*, 1259



Antibody-based PROTACs (AbTACs) can be used to target cell surface proteins for degradation. As most degrader technologies use intracellular mechanisms of action, this can be limiting to targeting membrane proteins. AbTACs use IgG bispecific antibody scaffolds to bring a cell surface E3 ligase, such as RNF43, into close proximity with the target cell surface protein for lysosomal mediated degradation. The study uses immune-checkpoint protein programmed death-ligand 1 (PD-L1) and Epidermal Growth Factor Receptor (EGFR) as target examples, with cell surface E3 ligases RNF43 and ZNRF3. Although the authors do not go into much detail on the importance of targeting these proteins, they provide an exploration into the optimisation of their AbTACs to degrade them efficiently. This included testing the importance of E3 ligase abundancy by showing a ~20% increase of PD-L1 degradation with RNF43 overexpression, and a ~40% increase with ZNRF3 overexpression. It was also demonstrated that although different binding epitopes presented varying levels of degradation, this was not directly correlated with their binding affinities, which also presents their catalytic properties. Lastly, as RNF43 and ZNRF3 have natural processes within the Wnt signalling pathway, the study finishes by confirming that there are no repercussions on using these ligases in the production of AbTACs by showing no unwanted Wnt signalling potentiation using a HEK293-derived luciferase Wnt reporter cell line with AbTAC-mediated PD-L1 degradation. However, it was noted that PD-L1 is not endogenously expressed in these cells and therefore had to be transiently transfected for expression. It would have been useful to observe an alternative method to Wnt signalling tracking within cells that endogenously express AbTAC targeted proteins.

This study presents an interesting approach to targeting cell surface proteins for degradation using AbTACs. It provides an alternative mode to LyTACs (complex glycans conjugated to antibodies) that target membrane proteins through internalisation and trafficking to the lysosome. Moving forward, it was observed the importance of cell surface E3 ligase abundancy through overexpression, it would be interesting to see how this is affected in a system where overexpression is not an option. Particularly in a disease setting, would the level of degradation from endogenous levels of cell surface E3 ligase expression provide sufficient levels of degradation for phenotypic amelioration?

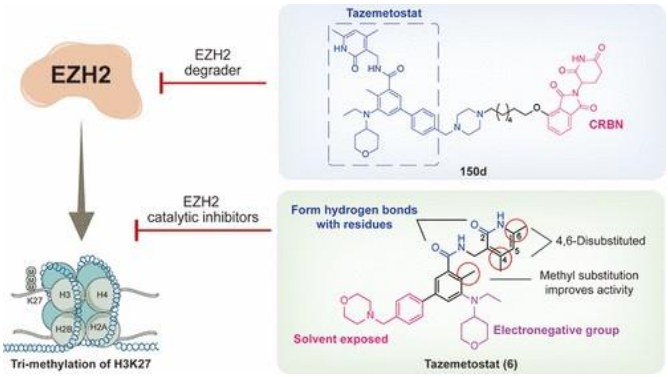
Cell Biology	Chemistry	Computational Chemistry	Structural Biology/Biophysics

Contributor: Selma

Targeting Enhancer of Zeste Homolog 2 for the Treatment of Hematological Malignancies and Solid Tumours: Candidate Structure-Activity Relationships Insights and Evolution Prospects

Juan Xia,[§] …, Xiadong Ren*, Chang Liu*, Chengyuan Liang*

J. Med. Chem. **2022**, *65*, 7016



This review explores the importance of targeting the activity of enhancer of zeste homolog 2 (EZH2) due to its high expression in various solid tumours and hematological malignancies. EZH2 forms the core catalytic subunit of Polycomb repressive complex 2 (PRC2), and is one of the most common epigenetic regulatory factors in human tumours, implicated in tumour proliferation, invasion and metastasis. The authors analyse the benefits and limitations EZH2 inhibitors used in clinical trials, highlighting the success of Tazemetostat, the first EZH2 inhibitor used in treatment of metastatic or advanced epithelioid sarcoma and relapsed or refractory follicular lymphoma. Limitations are discussed with reference to the fact that the carcinogenic function of EZH2 is not completely attributed to its enzymatic activity, and how high doses are needed for efficacy. The authors also highlight the development of acquired drug resistance and insensitivity to solid tumours. The review progresses into the use of targeted protein degradation as a therapeutic strategy against EZH2. Here, it describes two different modalities: hydrophobic tagging (HyT) and PROTACs. HyT makes use of large hydrophobic groups on target protein ligands that increase the hydrophobicity of the protein's surface, thereby inducing instability, misfolding and subsequent turnover of the target protein. Henceforth, the article then finishes its discussion with the use of Tazemetostat and GSK-126 as ligands for EZH2 in PROTAC design using VHL and CRBN as E3 ligases.

With the development of degraders targeting EZH2 still in the Preclinical/Biological testing stages, it will be interesting to see if their progression into clinical stages will overcome some of the limitations highlighted in inhibitors.

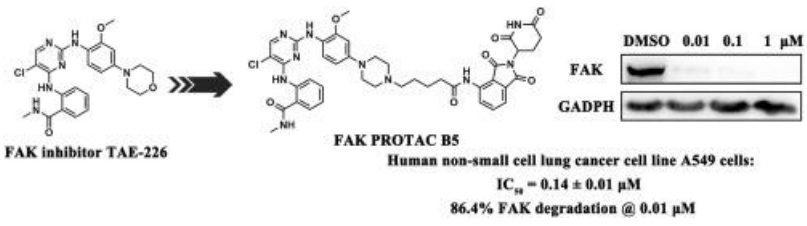
Cell Biology	Chemistry	Computational Chemistry	Structural Biology/Biophysics

Contributor: Selma

Identification of novel and potent PROTACs targeting FAK for non-small cell lung cancer: Design, synthesis, and biological study

Yin Sun[§], Ruifeng Wang, …, Dongmei Zhao*

Eur. J. Med. Chem. **2022**, *237*, 114373



Focal adhesion kinase (FAK, also known as protein tyrosine kinase 2 (PTK2)) is a cytoplasmic tyrosine kinase that is implicated in cancerous cells in both kinase-dependent and –independent ways. As well as it's enzymatic role, FAK also functions as a scaffolding protein, where its properties are closely related to survival and development of different cancers. Thus, despite the promising FAK inhibitors in clinical trials (such as VS-6063/defactinib), targeted protein degradation has been an attractive prospect to tackle both enzymatic inhibition and attenuation of its scaffolding function. This study uses a potent ATP-competitive kinase inhibitor, TAE-226, to develop CRBN-based PROTACs that target FAK. Homogeneous time-resolved fluorescence (HTRF) and Western Blotting (WB) were used to determine enzymatic inhibition and degradation in human non-small cell lung cancer cell line, A549. Whilst the study does not present any ubiquitination assays, it does verify the mechanism of their most

promising compound, **B5,** and proteasome-dependence is demonstrated through competition studies using TAE-226 and Pomalidomide, and using the proteasomal inhibitor MG-132. Lastly, the study finishes by demonstrating that migration of A549 cells with **B5** treatment is suppressed during wound healing assays, and that it also inhibits invasion using a transwell assay.

This study highlights the promising properties of their compound, **B5**, and its potential in tackling tumour progression. Moving forward, with FAK having a role in various cancers, not just lung, it would be interesting to see how **B5** would behave in other cancer-derived cell lines.

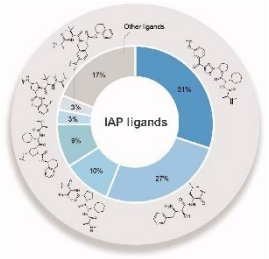
Cell Biology	Chemistry	Structural Biology/Biophysics

Contributor: Selma

Recent advances in IAP-based PROTACs (SNIPERs) as potential therapeutic agents

Chao Wang[§]*, Yujing Zhang*, …, Qian Li*, Dongming Xing*

J. Enzyme Inhib. Med. Chem. **2022**, *37*, 1437



This review reflects on the development of PROTACs using inhibitors of apoptosis (IAP) as the E3 ligand. IAPs are a class of negative regulators of apoptosis and have shown overexpression in cancer cells linked to poor prognoses, thus making them important targets for cancer therapy. IAP-based PROTACs are also referred to as specific and nongenetic IAP-dependent protein erasers (SNIPERs). This review explores the different areas that SNIPERs have been investigated and used in, starting with a discussion on the ligands used for PROTAC design. It then focuses on targets involved in three main areas of disease: fourteen different target groups within cancer, three in immune diseases and two in neurodegenerative diseases. Lastly, it finishes with a discussion of SNIPERs used to target Retinoic Acid Receptors (RAR), due to their involvement in controlling embryonic development and cell differentiation.

This review provides an explorative discussion into the applications of SNIPERs within different disease settings. As IAPs have been implicated in cancer, it provides an interesting modality to PROTAC design. It will be interesting to see how these molecules behave when advanced into a clinical setting.

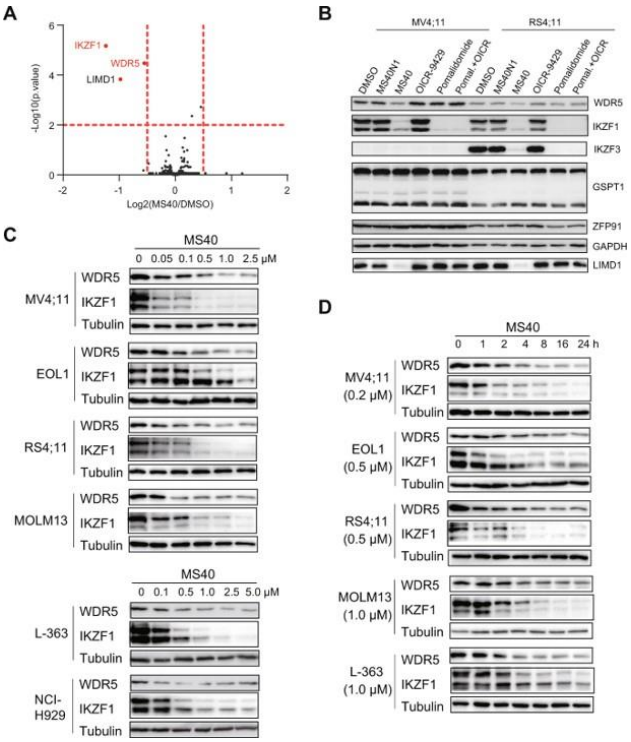
Cell Biology	Chemistry	Computational Chemistry	Structural Biology/Biophysics

Contributor: Selma

Discovery of a dual WDR5 and Ikaros PROTAC degrader as an anti-cancer therapeutic

Dongxu Li[§], …, Jian Jin*, Gang Greg Wang*

Oncogene **2022**, DOI: 10.1038/s41388-022-02340-8



WD repeat domain 5 (WDR5) forms part of the Mixed Lineage Leukemia (MLL/KMT2A) family of histone methyltransferase complexes, that mediate gene expression mainly through methylation of histone H3 lysine 4 (H3K4). Overexpression has been demonstrated in cancers such as leukemia, pancreatic cancer and neuroblastoma, and its scaffolding functions are thought to promote tumorigenesis. Thus, WDR5 provides an attractive onco-target for TPD, especially because protein-protein interaction (PPI) inhibition using small molecule inhibitors exhibits poor antitumour activity. This study reported the development of MS40, a CRBN-based PROTAC using pomalidomide and WDR5 inhibitor, OICR-9429. Interestingly, the study found that MS40 not only induced degradation of WDR5, but also simultaneously promotes degradation of validated drug targets of certain cancers, Ikaros (IKZF1) and Aiolos (IKZF3). The study used immunoblotting in a range of cancer-derived cell lines (primarily MV4;11 and RS4;11, but also included results from five lines of MLL-r AML and eight lines of breast cancer) to monitor degradation, confirming the mechanism of action using proteasome inhibitor carfilzomib, neddylation inhibitor MLN4924, competition studies using pomalidomide and CRBN knockout studies. Moreover, mass spectrometry was used to make a quantitative proteomic analysis of the selectivity of MS40, using MV;411 cells. Increased antitumour effects of MS40 compared to WDR5 PPI inhibitors were demonstrated through increased chromatin displacement of the MLL complex (confirmed using ChIP-seq), altered WDR5-mediated gene expression (observed through RNA sequencing, Global Run-on sequencing and RT-qPCR) and suppressed cancer cell growth *in vitro* using human leukemia cell lines and *in vivo* using a patient-derived xenograft mouse model carrying MLL-AF9 + AML.

This study presented a well-rounded analysis into the applications of their designed PROTAC, MS40, within a few cancer-derived cell lines. After comprehensively confirming the PROTAC's mechanism of action, it effectively analysed its potential in attenuation of cancer cell growth using both *in vitro* and *in vivo* approaches. The authors mention MS40 as a promising anti-cancer strategy, it will be interesting to see how that develops, moving forward.

Cell Biology | Chemistry | Structural Biology/Biophysics

Contributor: Claudia

Ispinesib as an Effective Warhead for the Design of Autophagosome-Tethering Chimeras: Discovery of Potent Degraders of Nicotinamide Phosphoribosyltransferase (NAMPT)

Guoqiang Dong[§], …, Chunquan Sheng*

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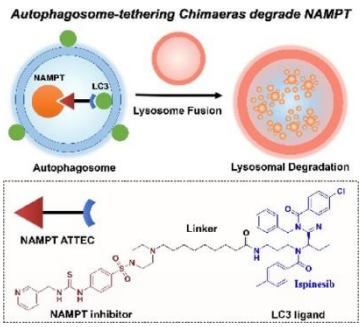

Autophagosome-tethering compounds (ATTECs) hijack the autophagosome for targeted protein degradation using the cellular autophagy-lysosomal pathway (ALP). This offers an alternative degradation pathway to hijacking the ubiquitin-proteasome system (UPS) with PROTACs and allows the degradation of a broader range of biomolecules. ATTECs recruit the microtubule-associated protein 1A/1B-light chain 3 (LC3) attached to the phagophore membrane, thereby delivering, *via* a second ligand bound target, biomolecules to the autophagosome. In this study, the authors identified ispinesib as effective LC3-binding warhead and demonstrated ATTEC-induced autophagosomal degradation of nicotinamide phosphoribosyl- transferase (NAMPT). Initially, an ispinesib-based FP probe was designed validating the suitability of the chosen linker attachment site and colocalization with red lysosomal probe confirmed engulfment of the FP probe into the autophagasome. Exploiting this linker vector on ispinesib, NAMPT-targeting ATTECs were developed based on a NAMPT inhibitor as POI ligand and various alkyl- and PEG-linkers. Degradation assays in human ovarian A2780 cells overexpressing NAMPT showed various degree of NAMPT degradation depending on the linker length, identifying an 8-atom alkyl linker as optimal. This ATTEC **A3** induced up to 91% reduced NAMPT protein levels at 3 µM *via* a lysosomal-mediated autophagy mechanism and showed significantly improved antiproliferative effects compared to its constituting inhibitor.

The herein explored LC3 ligand ispinesib, featuring good binary binding affinity to LC3 and being able to induce target degradation when incorporated in an ATTAC degrader as exemplified with NAMPT as a challenging target, is a promising addition to the ATTEC toolbox. However, ATTEC **A3** showed binding not only to NAMPT, but also to KSP3, the original target of Ispinesib, which might contribute to **A3**'s toxicity and might potentially induce off-target activity. As such, further LC3-ligand optimization starting from ispinesib would be desirable to develop more selective and safer LC3-ligands for ATTEC degraders.

Other Paper Highlights

Chemical biology | Medicinal Chemistry

Contributor: Xingui

Improved Electrophile Design for Exquisite Covalent Molecule Selectivity

José L. Montaño§, …, Balyn W. Zaro*

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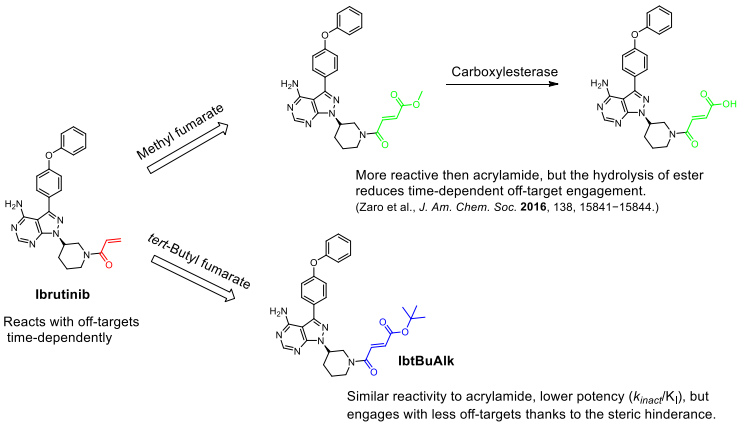


Although acrylamide is one of the most popular warheads for covalent inhibitors, the major disadvantage is promiscuous both time-dependent and time-independent off-target engagements. Using ibrutinib as a model covalent inhibitor, the authors have previously shown that replacing the acrylamide with methyl fumarate resulted in a more reactive covalent inhibitor. However, the slow hydrolysis of the ester by carboxylesterase deactivates the warhead and therefore reduces the time-dependent off-target engagement. In this current work, the authors developed IbtBuAlk where the acrylamide of ibrutinib is displaced with tert-Butyl fumarate. IbtBuAlk has similar reactivity as ibrutinib and is 40-fold less potent than ibrutinib in engaging with Bruton Tyrosine Kinase (BTK). Nevertheless, the selectivity profile of IbtBuAlk has significantly improved over ibrutinib as shown by the gel-based and MS-based ABPP experiments, with much less off-targets engaged by IbtBuAlk in both time-dependent and time-independent manner.

This work offers a new way to tune the selectivity of covalent inhibitors and the authors also thoroughly characterised the potency and selectivity of their covalent inhibitors with a suite of assays. For reactivity and potency (k_{inact}/K_I), the authors used a plate-based cysteine reactivity assay and BTK inactivation kinetic assay, separately. To characterize the selectivity of covalent inhibitors on a proteomic level, gel-based ABPP, ABPP-SILAC, and competitive gel-based ABPP and ABPP-SILAC were employed. These techniques are becoming standard methods to evaluate covalent inhibitors.

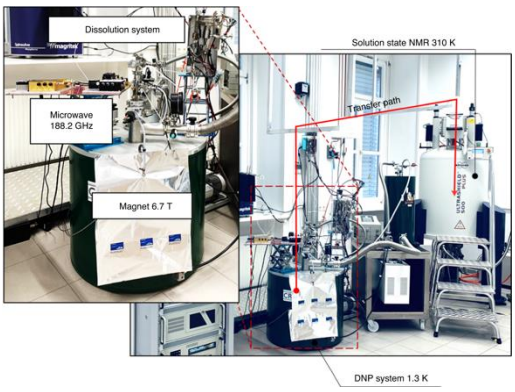
Chemistry | Structural Biology/Biophysics

Contributor: Kevin

Hyperpolarized water as universal sensitivity booster in biomolecular NMR

Christian Hilty*, Dennis Kurzbach*, Lucio Frydman*

Nature Protocols **2022**, DOI: 10.1038/s41596-022-00693-8



Dynamic nuclear polarization (DNP) recently gave rise to an increase insensitivity of solid-state NMR by several orders of magnitude. This is achieved via using the large magnetic momentum of unpaired electrons to build up spin polarization in cryogenic solids that is subsequentially transferred to the nuclear spins of the analyte. Using this effect for solution state NMR has proven difficult due to the requirements for very low temperatures (< 4 K) to preserve hyperpolarization of the solvent. Here a detailed protocol is presented in which ice doped with a persistent radical is hyperpolarized at low temperature and then rapidly molten and mixed with the analyte. A limited number of scans can then be collected before the rapid decay of hyperpolarization. Sensitivity gains of more than 100 times can be achieved this way, enabling trace-analysis in complex mixtures such as cell lysates or rapid structural characterization with high time resolution.

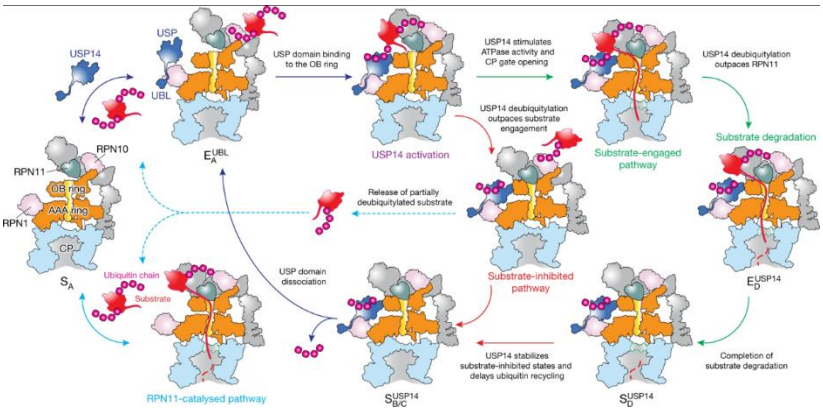
As the article discusses in detail, many challenges remain and both experimental limitation and the need for sophisticated equipment means that solution DNP is far from a routine technique. There might be doubts if all these challenges can be overcome, but the possibilities offered by ultra-sensitive NMR to completely transform chemistry and biophysics certainly make this an exciting development.

Cell Biology	Chemistry	Computational Chemistry	Structural Biology/Biophysics

Contributor: Mark Nakasone

USP14-regulated allostery of the human proteasome by time-resolved cryo-EM

Shuwen Zhang[§], Shitao Zou[§], …, Youdong Mao*
Nature **2022**, DOI*:* 10.1038/s41586-022-04671-8



Zhang and co-workers applied single particle cryo-electron microscopy (cryo-EM) to determine how the deubiquitinase (DUB), USP14, anchors to Rpn1 through its ubiquitin-like (UbL) domain and engages a model polyubiquitinated substrate on the proteasome through its catalytic ubiquitin-specific protease (USP) domain. In this study, $Sic1^{PY}$ was modified with K63 linked polyubiquitin, combined with 26S proteasome, and thirteen structures were determined across a reaction time course of up to 10 minutes. USP14 itself is proteasome-associated and not required for substrate degradation, however the presence of USP14 on the proteasome has consequences for substrate recognition and degradation kinetics. Zhang and co-workers determined USP14 has a tight affinity for proteasome (k_D ~95 nm), and that addition of substrate improves the binding to k_D~44 nM. This basis is revealed in their structures showing extensive contacts with USP14 and subunits of the 19S proteasome: the UBL domain and Rpn1, with the USP domain contacting the AAA-ATPase Rpt1 and the polyubiquitin signal from the substrate. The mechanism of USP14 on proteasome is elucidated through their structures, which capture several distinct conformations of proteasome degrading a model substrate.

Although several previous studies have reported density for USP14 UBL or USP domain(s) on proteasome, this represents one of the most comprehensive structural reports on the function of USP14 at the proteasome. With proteasome DUBs and Ub-binding domains exhibiting preferences for specific Ub-Ub linkages, it is interesting to ponder how such diverse substrates will impact degradation kinetics on the proteasome. Last, their application of the new AlphaCryo4D deep learning framework (https://github.com/alphacryo4d/alphacryo4d/), in combination with time-resolved cryo-EM is a promising approach for determining structural information from related systems.

Cell Biology | Chemistry | Computational Chemistry | Structural Biology/Biophysics

Contributor: Mark Nakasone

Liquid phase separation of NEMO induced by polyubiquitin chains activates NF-κB

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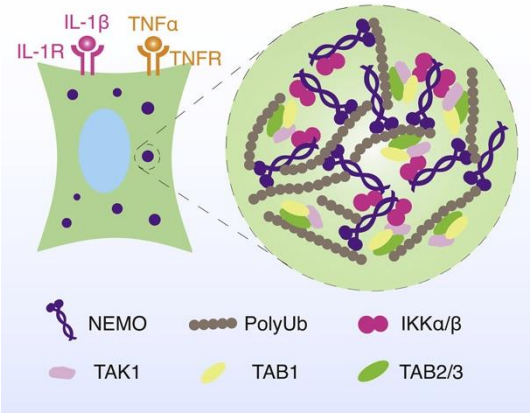

The γ-subunit of the IκB complex (IKK), NF-κB essential modulator (NEMO) employs ubiquitin-binding domains (UBDs) with preferences for M1- and K63-linked polyubiquitin modifications on the cytosolic side of interleukin-1β (IL-1β) and tumour necrosis factor α (TNFα) receptors. As a dimer, NEMO has two UBDs per molecule: NEMO ubiquitin-binding (NUB) and zinc-finger (ZF), providing a total of four Ub binding sites. Du and co-workers demonstrated that only polyubiquitin with K63 or M1 linkages induced liquid-liquid phase separation (LLPS) with NEMO. In addition, longer polyubiquitin resulted in larger condensates and mixed-linkage polyubiquitin containing M1/K63 or K48/K63 linkages also underwent LLPS with NEMO. In the cell E3 ubiquitin-ligases TRAF2/5, TRAF6, RNF31, and cIAP1/2 function to assemble the polyubiquitin for NEMO recruitment.

The authors generated a CRISPR-Cas9 knock-in, mCherry-NEMO and observe association with GFP-TRAF6 following IL-1β or TNFα stimulation. Light microscopy clearly showed both NEMO and TRAF6 in condensates, while a subsequent density gradient ultracentrifugation revealed active IKK (p-IKKα/β) and TAK1 (pThr187-TAK1) in NEMO condensates. Ubiquitination was found to be essential for NEMO condensates, as introduction of deubiquitinases (DUBs) or mutation of NEMO UBDs greatly reduced condensate formation. Du and co-workers then examine NEMO mutations in the UBDs from patients with X-linked ectodermal dysplasia with immunodeficiency (EDA-ID) and also found reduced condensate formation.

This study adds to the growing list of unique biochemical reactions that occur within the "microreactors" associated with LLPS and ubiquitin modification is consistently pivotal.

Cell Biology | Chemistry | Computational Chemistry | Structural Biology/Biophysics

Contributor: Sohini

Validation of an Allosteric Binding Site of Src Kinase Identified by Unbiased Ligand Binding Simulations

Victoria R. Mingione§, …, Markus A. Seeliger*

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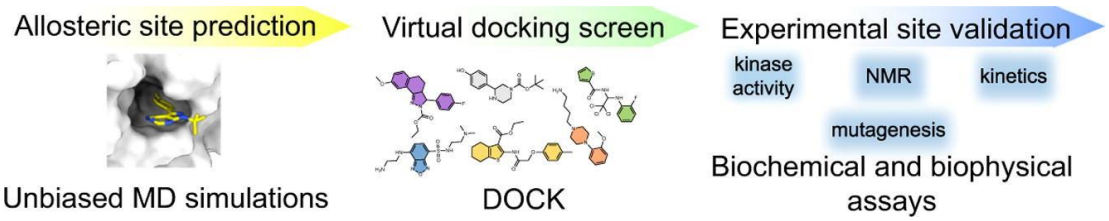

This article presents a rigorous workflow leading to discovery of a novel allosteric ligand binding site on Src kinase, an important drug target. The authors have used unbiased MD simulation to uncover 'cryptic' allosteric sites on the Src kinase which they term as G-loop site. This G-loop site is otherwise unobserved in the static snapshot from the crystal structure of Src kinase. Using virtual screening, the authors have identified several hits that can bind to the G-loop site. The results from computational experiments were validated using biochemical and biophysical assays that included NanoBRET target engagement, NMR and site-directed mutagenesis. Starting from a virtual library of 230,000 compounds, the authors prioritized 49 compounds for experimental testing of which 4 compounds inhibited the kinase

activity and one among these, compound **1C**, was taken forward for thorough analysis. **1C** is specific to Src kinase over its closely related homologues. This work is a perfect example of how integration of computational and experimental workflow can lead to synergistic scientific output.

Developing a high-affinity allosteric inhibitor is more challenging than its orthosteric counterpart but allosteric modulators have several pharmacological advantages, for example lower chances of side-effects. Such allosteric binders could be of particular interest to the TPD community to design allosteric binder based PROTACs, where a high binding affinity is not an absolute requirement.

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