CeTPD Journal Club

Targeted protein degradation, medicinal chemistry, chemical structural biology & cell biology

Journal Club

September 2023

Centre for Targeted Protein Degradation
University of Dundee

innovate
collaborate
inspire

Content

Content **0**

Meet this Month's Editors **1**

CeTPD Outreach in September **2**

Targeted Protein Degradation **4**

Chunlong Zhao *et al.*, *Angew. Chem. Int. Ed*., Histone deacetylase 3-Directed PROTACs have anti-inflammatory potential by blocking polarization of M0-like into M1-like Macrophages 4

Anna Pasieka *et al.*, *ChemMedChem*, Click Chemistry and Target Degradation: A Winning Combination for Medicinal Chemists? 4

Chunli Song *et al.*, *J. Am. Chem. Soc*., Selective Protein of Interest Degradation through the Split-and-Mix Liposome Proteolysis Targeting Chimera Approach 5

Weiguo Xiang *et al.*, *J. Med. Chem*., Discovery of ARD-1676 as a Highly Potent and Orally Efficacious AR PROTAC Degrader with a Broad Activity against AR Mutants for the Treatment of AR + Human Prostate Cancer 6

Zhiziang Chen *et al.*, *J. Med. Chem*., Discovery of ERD-3111 as a Potent and Orally Efficacious Estrogen Receptor PROTAC Degrader with Strong Antitumor Activity 6

Markella Konstantinidou *et al.*, *J. Am. Chem. Soc.*, Structure-Based Optimization of Covalent, Small-Molecule Stabilizers of the 14-3-3σ/ERα Protein−Protein Interaction from Nonselective Fragments 7

Abigail Brewer *et al.*, *bioRxiv*, Mapping the substrate landscape of protein phosphatase 2A catalytic subunit PPP2CA 8

Andrew J. Tao *et al.*, *bioRxiv*, A Biotin Targeting Chimera (BioTAC) System to Map Small Molecule Interactomes *in situ* 8

Ananya Basu *et al*., *bioRxiv*, A CRISPR activation screen identifies FBXO22 as an E3 ligase supporting targeted protein degradation 9

Other Paper Highlights **10**

Lan Phuong Vu *et al.*, *J. Med. Chem.*, Expanding the Structural Diversity at the Phenylene Core of Ligands for the von Hippel−Lindau E3 Ubiquitin Ligase: Development of Highly Potent Hypoxia-Inducible Factor-1α Stabilizers 10

Luca S. Dobson *et al.*, *Org. Lett.*, Aryl (β,β',β''-Trifluoro)-tert-butyl: A Candidate Motif for the Discovery of Bioactives 10

Lukas T. Henneberg *et al.*, *Nat*. *Chem. Biol.*, Activity-based profiling of cullin–RING E3 networks by conformation-specific probes 11

Meet this Month's Editors

This month's editors are (from left to right): Hirotake Furihata, Luke Simpson and Qingzhi Zhang

"*The JC delivers the latest research outcomings in the field of Target Protein Degradation to us. Hopefully the treasures within JC will propel us a significant leap forward in the future".*

Hirotake grew up and accomplished PhD in Japan. His doctoral research is about thalidomide-dependent teratogenesis. They identified SALL4 and PLZF as being involved in thalidomide-dependent teratogenicity. They proposed thalidomide hydroxylation enhanced SALL4 degradation through structure-based analysis of CRBN-SALL4 complex.

"*Throughout my PhD, the Ciulli Group monthly Journal Club was a fantastic way to keep up-to-date with relevant TPD-based literature. As an editor of this month's issue, I hope that others find this month's issue a helpful resource."*

Luke completed his MSci in Biomedical Science at the University of Aberdeen and his PhD under the supervision of Professor Gopal Sapkota and Dr Ian Ganley in the MRC PPU at the University of Dundee. Here, Luke's doctoral research centred around exploring technologies for targeted protein modification and involved the combined use of nanobody- and PROTAC-based technologies for targeted protein degradation. Luke joined the Ciulli Group in March 2022 as a Cell Biologist as part of the PROTAC Drug Discovery collaboration with Boehringer Ingelheim.

"*The CeTPD Journal Club covers the latest development in the field of Target Protein Degradation. It is a great resource to navigate beyond my specialty and broaden my mind and knowledge."*

Qingzhi received her BSc and MSc degree from China. She had been teaching Organic Chemistry at Henan Normal University before pursing PhD under the supervision of Prof Derek Woollins at St Andrews. She stayed in St Andrews working with the late Dr Nigel Botting as postdoctoral research associate on food chemistry and ^{13}C-labelling. She then sprinted to industry at EPP for one year and returned to St Andrews working with Prof David O'Hagan on fluorine chemistry.

CeTPD Outreach in September

Contributor: Giorgia

Playful Gardens

Myself, Javi, and Alejandro represented CeTPD and joined lots of other groups and organisations from around Dundee for a family fun day in the University Botanic Gardens this month.

We took along our Ubiquitination dart board, and protein fishing games to play in the sun with members of the local community of all ages! These allowed us to chat to children, as well as their parents and carers about what work is going on in the centre and give an insight into how PROTAC's work and why we are so interested in them.

Massive thank you to Vicky Armstrong for organising and Shabnam Wasim for giving us the opportunity to take part!

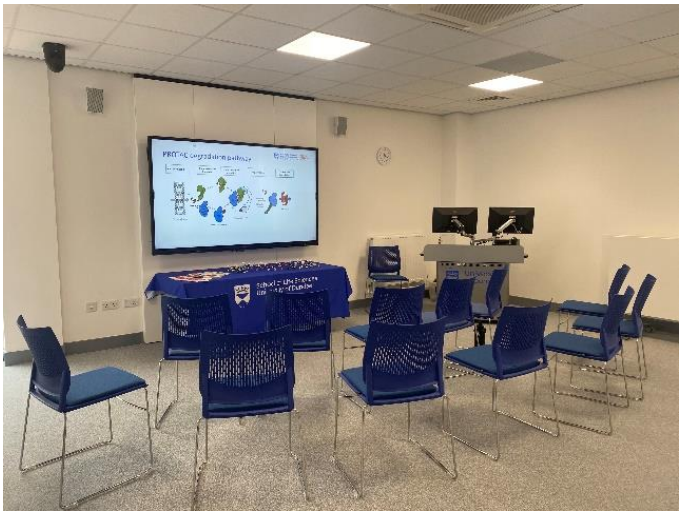
Doors Open Days 2023

In September, we also took part in this year's Doors Open Day, here in Dundee, with members of the local community joining us for tours of our labs and some family fun activities.

This gave us the opportunity to show these enthusiastic people our facilities and highlight the science going on within the Centre, and within the wider School of Life Sciences. Again, we had our tabletop activities, as well as the badge maker, which was a smash hit, with everyone making badges with PROTACs, future career goals (plenty of future scientist badges were made!), and even the occasional meme was turned into a badge.

We had a short video and chats about what a PROTAC is, and the different disciplines we have in the centre, showing our attendees how we work together to try to understand how the body works better, or in drug development programs.

Massive thank you to our tour guides Will and Andre, and Valentina, Denzel, Darren, Sohini and Louise who helped on the stalls/set up making the day run smoothly! Extra thanks go to Amy Cameron from the SLS public engagement team for helping with the planning/resources for the day!



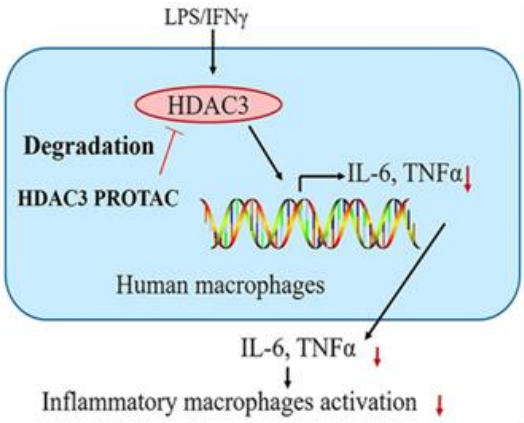
Targeted Protein Degradation

Chemistry | Cell Biology

Contributor: Qingzhi and Simon Krols

Histone deacetylase 3-Directed PROTACs have anti-inflammatory potential by blocking polarization of M0-like into M1-like Macrophages

Chunlong Zhao[§], Shipeng Chen[§], Deng Chen[§]…, Frank J. Dekker*
Angew. Chem. Int. Ed. **2023**, e202310059



Macrophage polarization plays a crucial role in inflammatory processes. Histone deacetylase 3 (HDAC3) has a deacetylase-independent function that can activate pro-inflammatory gene expression in M1-like macrophages but cannot be blocked by traditional small-molecule HDAC3 inhibitor. In this work, the authors designed two series of VHL-based PROTACs to target the deacetylase-independent function of HDAC3. They started from a HDAC3-selective inhibitor BRD3308 comprised of two benzamides, using the acetyl group on the second benzamide as an exit vector to tether VHL-021 *via* a linear aliphatic linkage with various lengths. However, PROTACs obtained exhibited either none or limited HDAC3 degradation in THP-1 cells. Replacement of 2-aminobenzamide with an acylhydrazine moiety led to more potent and selective HDAC3 degraders **P6**-**P8**. Among them, PROTAC **P7** displayed excellent inhibitory potency and highest selectivity to HDAC3. **P7** induced degradation of HDAC3 at low micromolar concentrations in both THP-1 cells and human primary macrophages. Degradation of HDAC3 by **P7** increased the secretion of anti-inflammatory cytokine in THP-1 derived M1-like macrophage and reduced the pro-inflammatory cytokine expression in human primary macrophages. The blockage of macrophage polarization by **P7** demonstrated the potential of PROTACs in inflammation suppression beyond oncology.

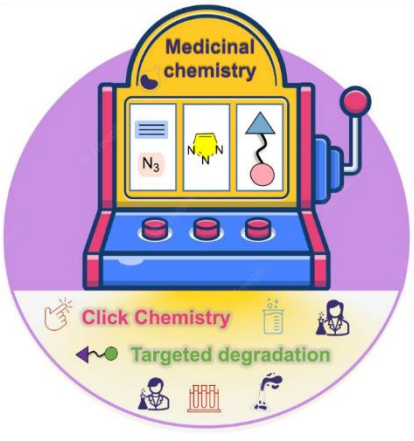
Compared with other complex PROCACs, **P7** is surprisingly simple with an acylhydrazide warhead and a plain linkage. The biology seems highly novel indeed but caveat. While the authors show some HDAC inhibition/degradation selectivity, a proteomic study is needed to prove that compound **P7** is a selective degrader and that the biology observed is indeed due to HDAC3 degradation.

Chemistry

Contributor: Qingzhi

Click Chemistry and Target Degradation: A Winning Combination for Medicinal Chemists?

Anna Pasieka, Eleonoro Diamanti, Elisa Uliassi, and Maria Laura Bolognesi*
ChemMedChem **2023**, e202300422



Click chemistry and target protein degradation are two flourishing trends in medicinal chemistry. Bioorthogonal chemistry is very similar to click chemistry but with a crucial difference that bioorthogonal reaction involves in non-toxic reagents to living organisms and the reaction can be performed *in vivo.* This study presents the application of the Nobel Prize-winning technology of click/bioorthogonal chemistry in the synthesis of protein degraders. It covers a wide range of synthesis of small and non-small molecular degraders using Cu(I)-catalyzed alkyne-azide cycloaddition (CuAAC). In particular, CuAAC has been used in conjunction with DNA-encoded library screening (DECL) for POI and solid-phase synthesis to accelerate the generation of PROTACs. Similarly, sulfur(VI) fluoride exchange (SuFEx) strategy allows high-throughput synthesis and screening of inhibitors for POI and thus speeds up the development of PROTACs. For bioothogonal reactions, they provide an excellent example

named as *in-cell* click-formed PROTAC (CLIPTACs). The reaction is based on the inverse electron demand Diels-Alder (IEDDA) cycloaddition between the small and cell membrane crossable tetrazine-tagged thalidomide and *trans*-cyclooctene-tagged ligand of POI. Also included is the copper free strain-promoted azide-alkyne cycloaddition (SPAAC) of bicyclooctyne-terminated VHL ligands and azide-tagged transcription factor ligand. And more…

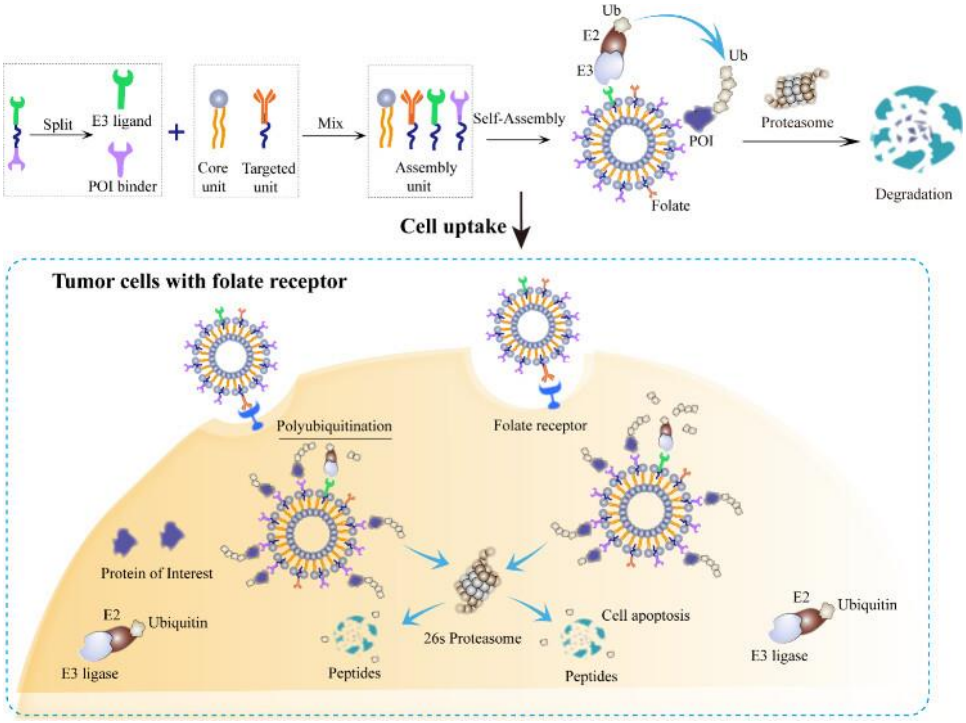
While the 1,2,3-triazole motif could change the physicochemical properties of PROTACs and improve cell permeability, solubility and stability, there is a concern about its metabolism mediated by cytochrome P450. Overall, the simplicity, speed, ease, modular nature and robustness in complementary building block-assembly make click/biorthogonal reaction very powerful in the synthesis and screening of heterobifunctional target degraders. The combination of click/biorthogonal chemistry can be a winning campaign for medicinal chemists.

Cell Biology **Chemistry**

Contributor: Qingzhi

Selective Protein of Interest Degradation through the Split-and-Mix Liposome Proteolysis Targeting Chimera Approach

Chunli Song[§], Zijun Jiao[§], Zhangfeng Hou[§] …, Zigang Li*, Feng Yin*
J. Am. Chem. Soc. **2022**, DOI: 10.1021/jacs.3c05948



The development of conventional bifunctional molecules for targeted degradation remains a time-consuming process that requires tedious optimization. In this study, the authors designed and prepared a novel liposome self-assembly-based split-and-mix PROTAC platform (LipoSM-PROTAC) for facile screening and self-optimized biomolecules recognition. They firstly conjugated the DSPE-PEG2000-NHS with the E3 ligand VHL or the binder for POI Erα. Self-assembly of the E3 ligand-bearing DSPE-PEG2000-VHL and ERα binder-loaded DSPE-PEG2000-Tam with the core unit forms well-structured liposome nanosphere. The ERα and E3 recruiters are randomly exposed on the hydrophilic surface of the nanosphere, bringing target protein ERα at spatial close to E3 ligase for degradation. To make the delivery of LipoSM-PROTAC target selective, they also incorporated folate (FA) into the liposome for FA receptor-positive cells (FR+) recognition. The triple functionalised liposome PROTACs FA-V-Tam-L could be efficiently and selectively taken up into the cells by FA receptor-positive cells (FR+) and degrade the POI with lower concentration (single digital micromolar) than that of previously reported peptide-based nanoplatform (two digital micromolar level).

The excellent biocompatibility and biodegradability, stable sphere structure, multiple ligand-loading capacity and drug efficacy suggest that LipoSM-PROTACs could serve as a universal platform for proximity-induced applications.

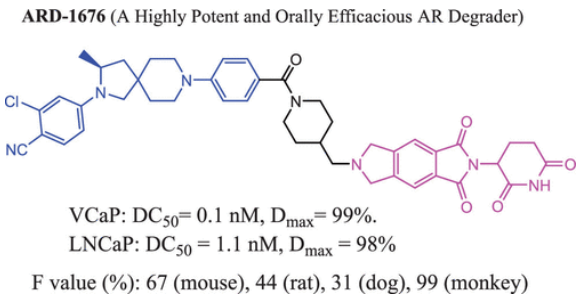
Cell Biology | Chemistry

Contributor: Qingzhi

Discovery of ARD-1676 as a Highly Potent and Orally Efficacious AR PROTAC Degrader with a Broad Activity against AR Mutants for the Treatment of AR + Human Prostate Cancer

Weiguo Xiang[§], Lijie Zhao[§], Xin Han[§], Tianfeng Xu[§], Steven Kregel[§], Mi Wang[§] …, Shaomeng Wang*

J. Med. Chem. **2023**, *66, 13280*



The androgen receptor (AR) and AR signalling play a pivotal role in the initiation and progression of human prostate cancer. PROTAC induced degradation of AR emerged as a promising therapeutic strategy. Cereblon-based AR degraders show better oral bioavailability as exemplified by phase-II clinical trial candidates ARV-110 and ARV-766. Previously Wang's group disclosed several potent cereblon-based AR degraders including ARD-2128, ARD-2585 and ARD-2051 with variable oral bioavailability across different species. In this study they reported another potent AR degrader AR-1676 with excellent oral bioavailability. This work started from design and screening of new AR ligands bearing a spiro ring and novel tricyclic cereblon ligands. The optimized 5,6-spiro ring-containing AR ligand was then tethered with the 5-membered ring fused tricyclic cereblon ligands, leading to the potent degrader ARD-1623. Further optimization by installation of a methyl group as *S*-isomer onto the 5,6-spiro ring resulted in the titled PROTAC with excellent degradation potency. ARD-1676 potently inhibits cell growth in AR+ prostate cancer cell lines and effectively reduces the expression of AR-regulated genes.

This work was well designed with systematic optimisation. I am really impressed by the creation of the chiral centre with a methyl group. While the *S*-methyl derivative increases 57-fold of potency of the lead, the *R*-methyl analogue is 3000 times less potent than the *S*-isomer. The excellent pharmacokinetic profile and the 99% oral bioavailability in monkey makes ARD-2051 a highly promising candidate for clinical trial.

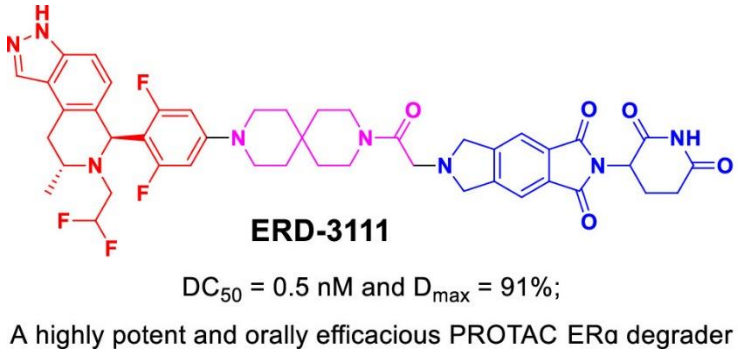
Cell Biology | Chemistry

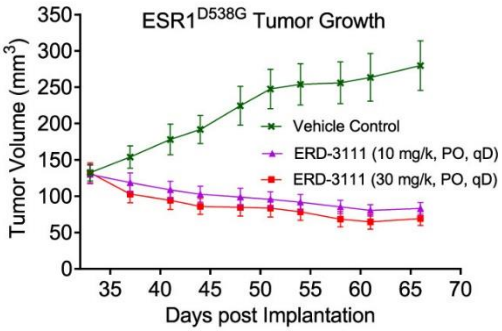
Contributor: Hirotake

Discovery of ERD-3111 as a Potent and Orally Efficacious Estrogen Receptor PROTAC Degrader with Strong Antitumor Activity

Zhixiang Chen[§], Biao Hu[§], Rohan Kalyan Rej[§], Dimin Wu[§], Ranjan Kumar Acharyya[§], Mingliang Wang[§], Tianfeng Xu[§], …, Shaomeng Wang*

J. Med. Chem. ***2023***, *66, 17, 12559–12585*





The estrogen receptor (ER), a nuclear hormone transcription factor takes an essential role in the development and progression of breast cancer. Prohibiting the signal transduction by endocrine therapy is greatly accomplished for both early and advanced stages of breast cancer. The third-generation aromatase inhibitors (letrozole, anastrozole and

exemestane), selective ER modulators and degraders have been mainly developed for endocrine therapy over 30 years, but de novo and acquired resistance to these therapies causes cancer recurrences, metastasis, and mortality. The discovery of ARV-471 improved extraordinary poor physiochemical properties of orally bioavailable ER PROTACs for a promising therapeutic treatment of ER positive breast cancer. Authors developed **ERD-3111** as a potent and orally bioavailable ER PROTAC to show the inhibition in vivo growth of breast cancers with either WT or mutated ER in mice. They implemented spiro-ring containing linkers because of the high conformational rigidity, low polar surface area, and exclusive sp3 atoms. The 6,6-spiro ring-containg linkers showed the best degradation potency and efficiency. Further optimisation of ER ligand core with either a tricyclic indole or a tricycle indazole core led to **ERD-3111** as the best ER-degrader. **ERD-3111** induced degradation of ER protein in MCF-7 and T47D cells. Oral administration of **ERD-3111** reduced WT and mutated ER protein in xenograft tumour tissues by PK/PD studies. **ERD-3111** treatments did not exhibit signs of toxicity in mice.

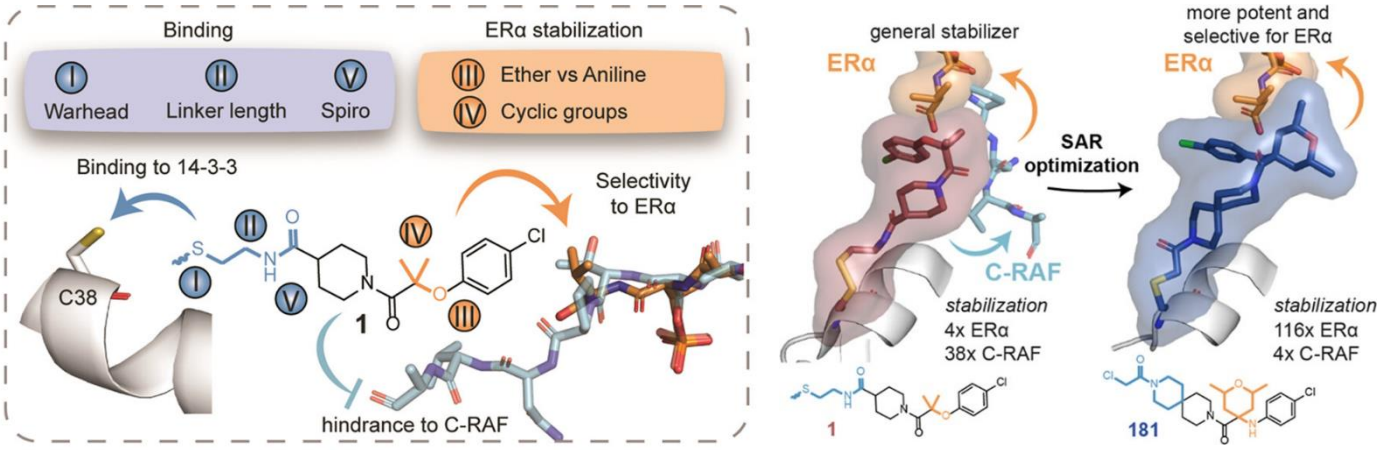
The new type of CRBN ligand by Shaomeng's group was also applied to AR PROTAC and may produce different angular linkers and substrates.

Cell Biology | Chemistry | Structural Biology/Biophysics

Contributor: Hirotake

Structure-Based Optimization of Covalent, Small-Molecule Stabilizers of the 14-3-3σ/ERα Protein–Protein Interaction from Nonselective Fragments

Markella Konstantinidou[§], Emira J. Visser[§], …, Christian. Ottmann*, Luc. Brunsveld*, Michelle R. Arkin*
J. Am. Chem. Soc. ***2023****, 145, 20328–20343*



14-3-3 protein is a highly abundant adaptor and scaffolding protein that binds to hundreds of phosphorylated and intrinsically disordered protein domains. Among 14-3-3 binding partners, there are therapeutic targets such as Estrogen Receptor, RAF kinases in the RAS/MARK signalling and LRRK2, tau and α-synuclein in neurodegeneration pathways. Michelle's group pursued the disulfide-tethering study to develop 14-3-3/partners glues for peptides with diverge shapes and binding modes. They carried out the structure-guided optimization of selective small compound stabilizers for the 14-3-3σ/ERα complex. The developed compound **181** and the natural product Fusicoccin-A (FC-A) had a comparable stabilization and selectivity profile. Their primary design principle focused on increasing stable orthostatic interactions with the phosphopeptide of partners in the 14-3-3σ/ERα. In the crystal structure, 14-3-3 and phosphopeptide took same conformations with their compounds. This strategy showed to apply not only other selective stabilizers of 14-3-3 partners but also the general structure-guided optimization and development for molecular glue to regulate PPI.

Along the new developed PROTACs or molecular glues, the structure-based optimization from fragment hits would enable to produce selective small molecule stabilizers for diverse targets by use of PPI stabilization.

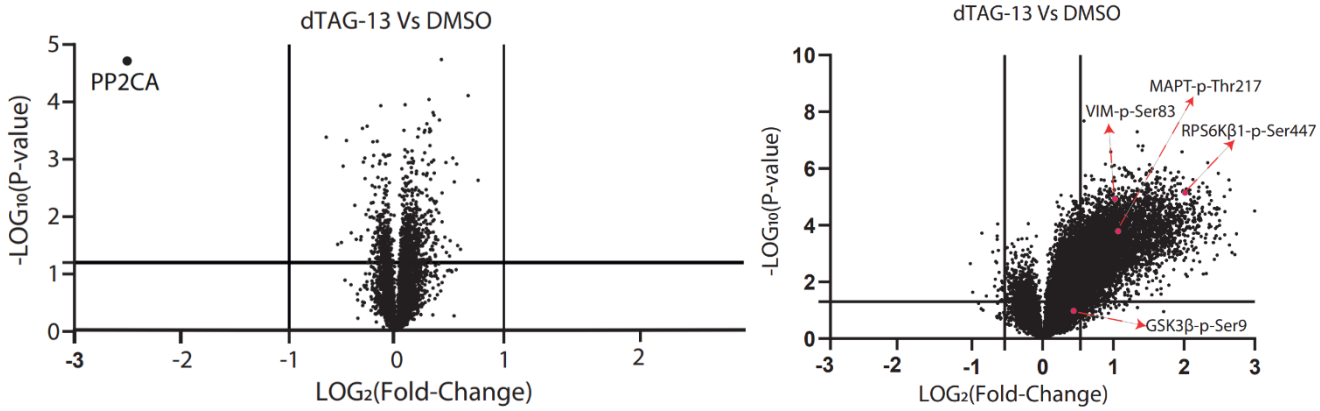
Cell Biology

Contributor: Luke

Mapping the substrate landscape of protein phosphatase 2A catalytic subunit PPP2CA

Abigail Brewer[§], Gajanan Sathe[§], …, Gopal Sapkota*

bioRxiv **2023**, DOI: 10.1101/2023.09.19.558429



Almost all aspects of mammalian cell biology are regulated by reversible protein phosphorylation. Protein phosphorylation is catalysed by kinases and is hydrolysed by phosphatases. Protein phosphatase 2A (PP2A) is an essential Ser/Thr phosphatase that functions as a holoenzyme complex, consisting of a scaffolding subunit, a regulatory subunit, and a catalytic subunit (PPP2CA). Many proteins have been previously reported as substrates of PP2A, however the full range of PP2A substrates has yet to be determined.

In this paper, the authors employ CRISPR/Cas9 technology to generate cells where PPP2CA is knocked in with dTAG ($FKBP12^{F36V}$). Following confirmation that the tagging of PPP2CA with dTAG was homozygous, the authors confirm that dTAG-PPP2CA can be efficiently degraded with dTAG-recruiting PROTACs (e.g. CRBN-recruiting dTAG-13) in a selective manner. Unbiased, TMT-based global phospho-proteomics was then performed following dTAG-13-mediated degradation of dTAG-PPP2CA. The abundance of phospho-peptides corresponding to more than 2,200 proteins were identified to be significantly increased following dTAG-PPP2CA degradation, identifying them as potential PP2A substrates. Some of these PP2A substrates were then validated by immunoblotting using phospho-specific antibodies.

It is exciting to see tag-based protein degradation systems being used to study challenging biology, such as phosphatases, and will be exciting to see applied to additional phosphatases, as the authors suggest, and other difficult-to-study enzymes!

Cell Biology | Chemistry

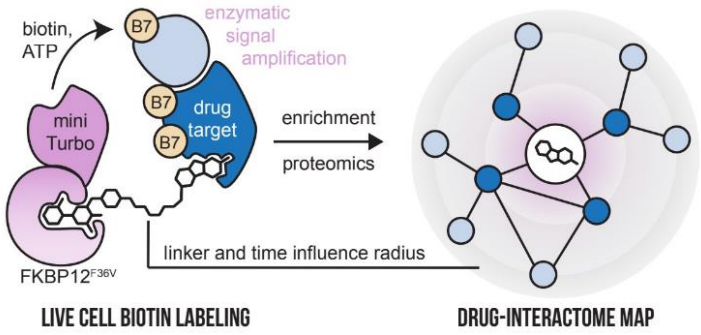
Contributor: Luke

A Biotin Targeting Chimera (BioTAC) System to Map Small Molecule Interactomes *in situ*

Andrew J. Tao[§], Jiewei Jiang[§], …, Justin English*, Fleur Ferguson*

bioRxiv **2023**, DOI: 10.1101/2023.08.21.554211

Robust profiling methods are needed for determining the cellular interactomes of small molecules, to validate their selectivity and identify any potential off-target proteins, or neosubstrates induced by molecular glue effects. In this paper, the authors develop the biotin targeting chimera (BioTAC) system, a ligand-guided miniTurboID method, to evaluate ligand-target interactome changes promoted by inhibitors or molecular glues.



The BioTAC system requires the functionalisation of the ligand of interest with a linker and the dTAG recruiter orthoAP1867, and the expression of miniTurbo (mTurbo) conjugated to FKBP12^{F36V} (dTAG) in cells. Alternative systems involve the substitution of the dTAG for the SNAP- or Halo-tag and their corresponding ligands. Following the treatment of mTurbo-dTAG expressing cells with the dTAG-recruiting ligand and biotin, biotinylated proteins can be isolated from cell lysates by streptavidin bead pulldown and analysed by mass spectrometry. The authors validate the BioTAC system by mapping the interactome of the inhibitor JQ1, including BRD4, and the molecular glue Trametinib.

It will be exciting to see the future applications of the BioTAC system, for example in screening mode as the authors suggest, for unbiased profiling of molecular glue libraries!

Cell Biology | **Chemistry** | **Computational Chemistry** | **Modelling/Simulation**

Contributor: Luke

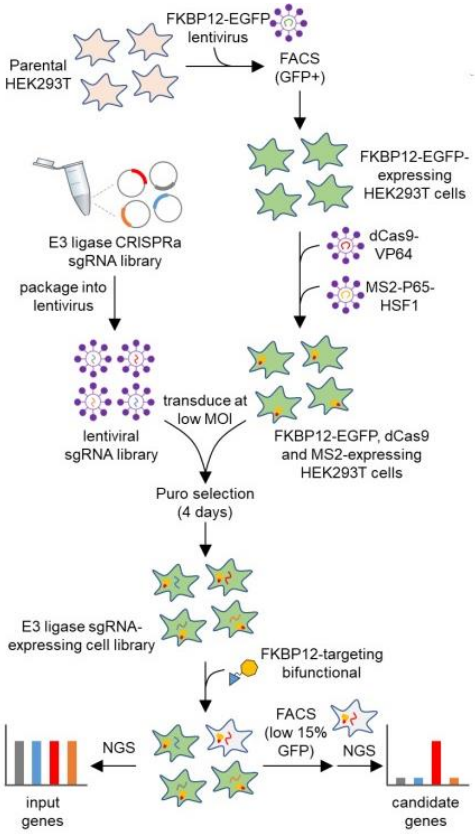
A CRISPR activation screen identifies FBXO22 as an E3 ligase supporting targeted protein degradation

Ananya Basu[§], …, Xiaoyu Zhang*
bioRxiv **2023**, DOI: 10.1101/2023.09.15.557708

Within the TPD field, there are huge ongoing efforts to identify additional E3 ligases that can be hijacked for degrading neosubstrates via small molecule approaches. By expanding upon the E3s that currently can be recruited to mediate TPD, we may be able to increase the number of proteins we can efficiently degrade, specifically those proteins with varying subcellular localisations and expression patterns, depending on that of the E3.

To identify additional E3s suitable for TPD applications, the authors employ an E3-focussed CRISPR-based transcriptional activation screen (workflow diagram attached to the right), minimising limitations due to differential E3 expression across different cell lines. Via this approach, the authors identify that the compound 22-SLF promotes the degradation of FKBP12 when there is transcriptional activation of the FBXO22 gene. The authors then validate that 22-SLF-mediated FKBP12 degradation requires FBXO22 in multiple cell lines by FBXO22 CRISPR-Cas9 knockout (KO) and determine that 22-SLF interacts with FBXO22 at residues C227 and/or C228. To demonstrate the versatility of FBXO22 for TPD, the authors then successfully target BRD4 for degradation through recruiting FBXO22.

It will be exciting to see the applicability and the benefits of employing FBXO22 for targeting additional proteins for degradation, as well as the employment of the E3-focussed CRISPR-based transcriptional activation screen to identify additional E3s for TPD!



Other Paper Highlights

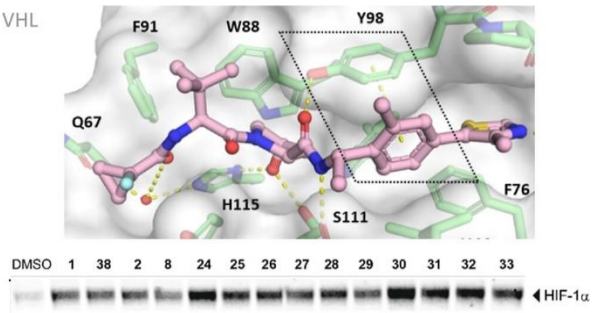
Cell Biology | Chemistry | Structural Biology/Biophysics

Contributor: Maria Rodriguez-Rios

Expanding the Structural Diversity at the Phenylene Core of Ligands for the von Hippel–Lindau E3 Ubiquitin Ligase: Development of Highly Potent Hypoxia-Inducible Factor-1α Stabilizers

Lan Phuong Vu[§], … Alessio Ciulli * and Michael Gutschow*

J. Med. Chem. **2023**, 66, 18, 12776–12811



In this work, Vu et al. explore the chemical space of Von Hippel–Lindau E3 ligase to aid development of highly potent optimised VHL ligands.

The authors developed a comprehensive library of optimised VHL ligands. Design was guided by thorough analysis of co-crystal structures of the "state-of-the-art" VHL inhibitor VH298 bound to VHL in the VCB complex. The structural analysis identified the phenylene core of the VHL ligand as the most appropriate vector for introducing chemical variability to leverage higher affinity in the VHL pocket. Following two SAR campaigns for ligand optimisation, the authors found a highly potent optimised VHL ligand, that introduced two methyl groups in the structure. The optimised ligand (compound **30**) outperforms VH298 both with regard to binding affinity and cellular potency. Compound **30** was evaluated, structurally, biophysically and biologically with a variety of independent experiments and showcased excellent binary binding affinity with VHL with a dissociation constant of 40 nM and inducing HIF-1 alpha stabilisation in a dose dependent manner, upregulating HIF-1α-dependent processes.

These optimised series of VHL ligands will serve as potent probes to chemically induce upregulation of HIF-dependent pathways and information compiled in these studies can help driving further optimisation of VHL-inhibitors. Besides, these ligands can help optimisation of VHL-recruiting PROTACs, currently underrepresented in the clinical stages, with a considerable higher number of orally bioavailable CRBN-based PROTAC degraders currently investigated in clinical trials. The findings of this work could help drive design of new generations of more drug-like VHL-based PROTAC degraders.

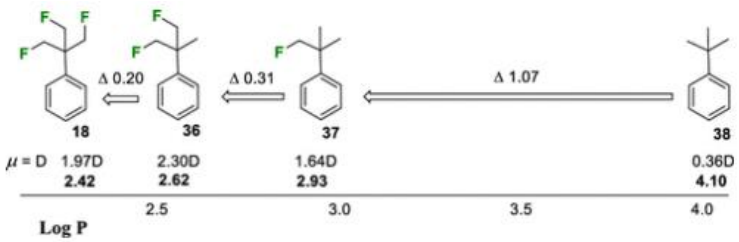
Chemistry | Modelling/Simulation

Contributor: Qingzhi

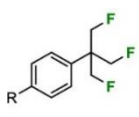
Aryl (β,β',β''-Trifluoro)-tert-butyl: A Candidate Motif for the Discovery of Bioactives

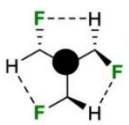
Luca S. Dobson[§], …, David O'Hagan*

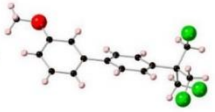
Org. Lett. **2022**, 22, 6802



tert-Butyl group is a common structure in organic chemistry. However, its application in medicinal chemistry is limited due to high lipophilicity and LogP. While it is well known that fluorinated aromatic rings such as Ar-F and Ar-CF_3 tends to have increased lipophilicity, it is less articulated that selective fluorination of aliphatic moiety can reduce their LogP. In this work, the authors demonstrated the trend of LogP decrease by sequential fluoromethylation of *tert*-butylbenzene, ultimately by 1.7 LogP unit in (β,β',β''-trifluoro)-*tert*-butylbenzene (PhTFTB) relative to *tert*-butylbenzene (Ph^{t}Bu). X-ray







crystal of a Suzuki derivative reveals a propeller arrangement of C-F bonds in TFTB moiety, in agreement with the conformational analysis by DFT simulation. The neighbouring C-H and C-F bonds lie approximately antiparallel with electrostatic attraction between the electron-positive hydrogens (polarised by their geminal fluorine) and the electron-negative fluorines on an adjacent fluoromethyl group. The higher water solubility, lower LogP and slower metabolism of PhTETB than that of Ph^{t}Bu suggests that TETB motif may have a potential application in discovery of bioactives, which was exemplified by the synthesis of an aryl-TFTB analogue of the *tert*-butyl containing pesticide pyridaben.

This novel trifluoro-motif would be of general interest to medicinal chemists.

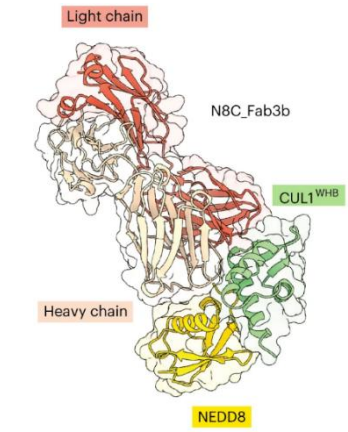
Cell Biology | **Structural Biology/Biophysics**

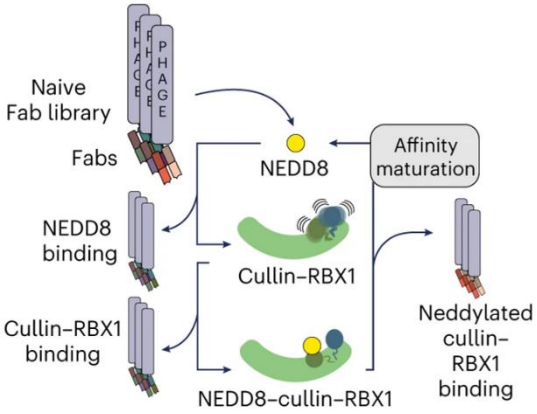
Contributor: Hirotake

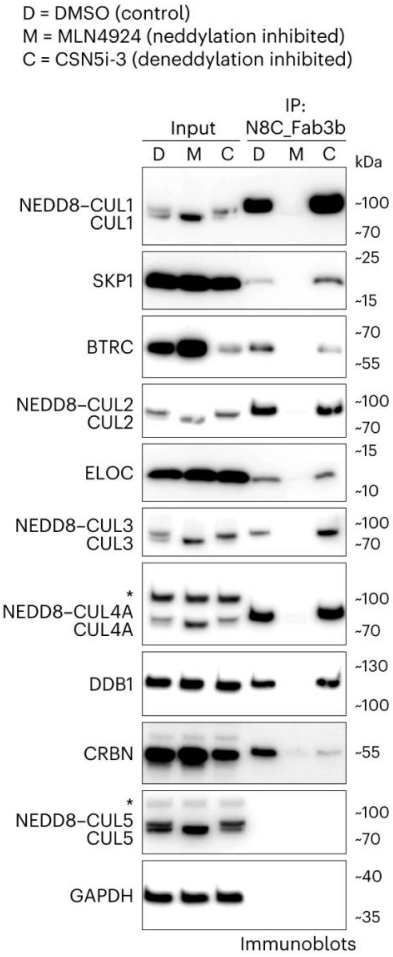
Activity-based profiling of cullin–RING E3 networks by conformation-specific probes

Lukas T. Henneberg[§], Jaspal Singh[§], David M.Duda[§], …, Sachdev Sidhu*, Brenda A. Schulam*

Nat. Chem. Biol. **2023**







The cullin-RING ubiquitin ligases (CRLs) compose of E3 ligases (E3s) and comprises over 300 unique complexes that regulate ubiquitination on widespread biological reactions. A CRL's ubiquitination function is switched on by the ubiquitin-like protein NEDD8 linkage to a specific site conserved across cullin C-terminal WHB domain called Neddylation. However, CRLs lack an active site like probes reacting with the catalytic cysteine such as other E3s. Regulation by Neddylation included cell division, immune signalling, DNA replication and repair, responses to redox stress and hypoxia, tumorigenesis and hijacking by bacterial and viral pathogens, and also targeted protein degradation is significantly dependent on Neddylation. To prove NEDD8-activated CRLs, CRLs required endogeneously tagged in the current method, which limits studies and can be challenging for primary cells. The anti-NEDD8 antibodies would not recognize NEDD8 binding with cullin given that NEED8's surface is buried in the interface within cullin. To generate probes binding neddylated CRLs, the authors selected specific sequences from a library of Fabs on phage.

From eight generated affinity reagents selectively binding neddylated cullins, the authors developed one, **N8C_Fab3b** which recognized NEDD8 and a cullin (CUL1-CUL4) in active arrangement. The binding of **N8C_Fab3b** suggests that the active arrangement is conserved for Neddylated CUL1-CUL4. IPs with **N8C_Fab3b** revealed probability that substrate-binding modules association in the CRL system does not always involve Neddylation and deneddylation. These features highlight lacking universal and uniform mechanism of assembly disassembly, activation, and deactivation of different CRLs. Although **N8C_Fab3b** would potentially interfere with deneddylation and substrate ubiquitylation, using **N8C_Fab3b** IPs can identify pathways involved in CRLs without endogenous tagging. They also found neddylated CRL repertoires vary across macrophage activation states and proposed CRL repertoires might adjust to resolve stress arising from toxic effectors as well as dynamically rearrangement of CRL networks.

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