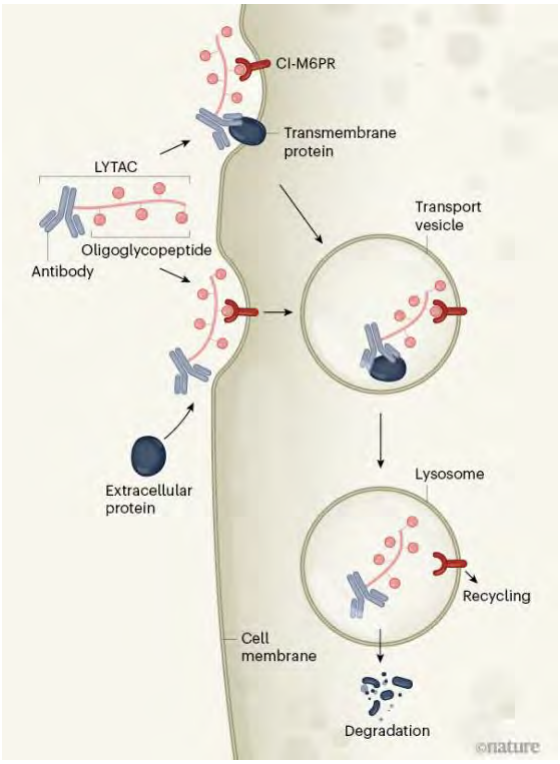
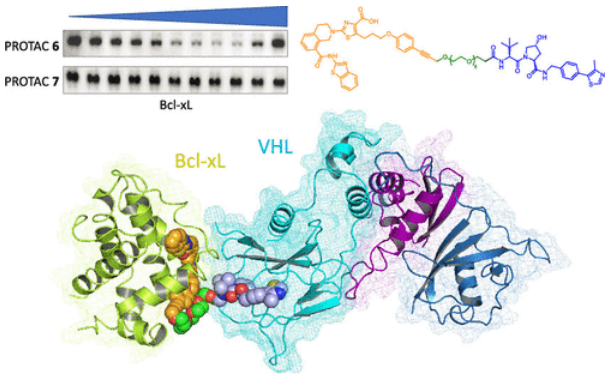
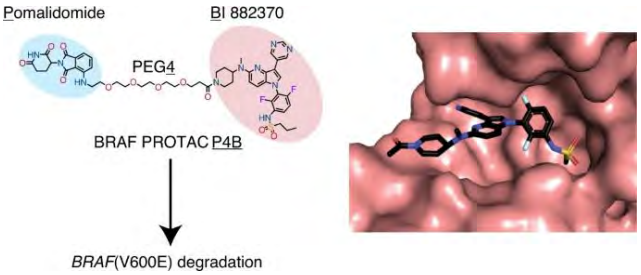
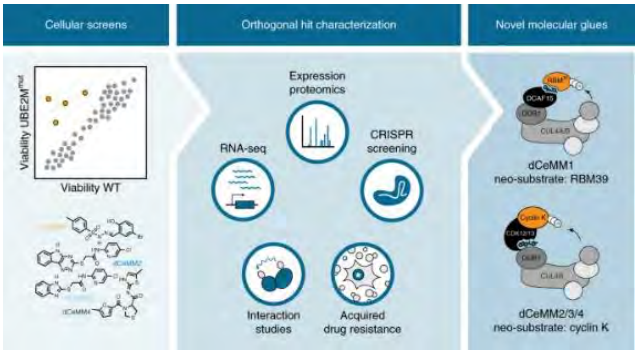
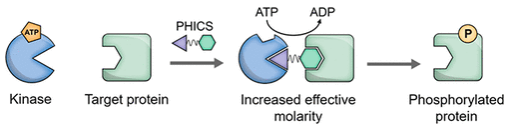
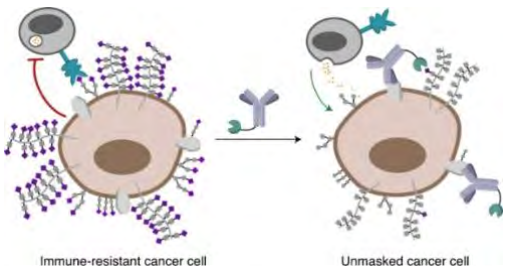


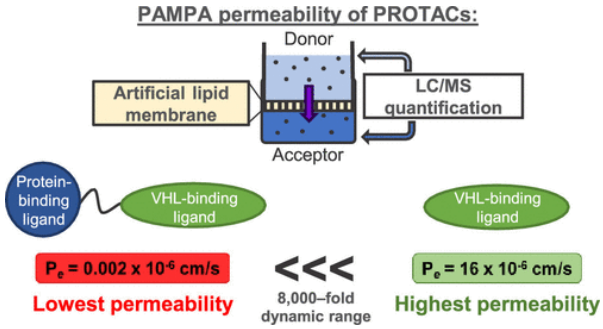












Ciulli Group Journal Club

Targeted protein degradation and

Other literature highlights

August 2020 Edition

Ciulli Journal Club Contents

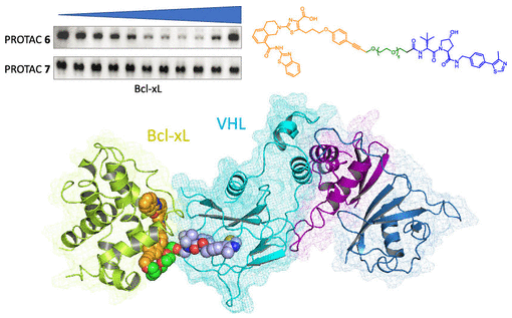
Targeted Protein Degradation

Contributor: Angus

Structural Insights into PROTAC-Mediated Degradation of Bcl-xL

Chun-wa Chung[§]*, ….., Andrew B. Benowitz

ACS Chem. Biol. **2020**, *13*, 103.



Bcl-2 family proteins regulate the intrinsic apoptotic pathway. Pro-survival Bcl-2 family members antagonise the activity of pro-apoptotic Bcl-2 proteins to prevent permeabilization of the mitochondrial outer membrane and subsequent release of apoptogenic factors from the intermembrane space. In cancer, pro-survival Bcl-2 proteins such as Bcl-2 and Bcl-X_L are often upregulated, preventing cancer cell death. Structure-based drug design has played an essential role in the development of protein-protein interaction (PPI) inhibitors of pro-survival Bcl-2 family members, with the Bcl-2-selective inhibitor Venclexta recently receiving FDA approval for treatment of chronic lymphocytic leukaemia and small lymphocytic leukaemia. Bcl-X_L is overexpressed in many solid tumours and haematological malignancies and is correlated with poor prognosis and resistance to chemotherapy. Although PPI inhibitors of Bcl-X_L have been developed, they suffer from on-target platelet toxicity due to platelet dependence on Bcl-X_L for survival. Recently, a VHL-based Bcl-X_L-degrading PROTAC (DT2216) was demonstrated to circumvent platelet toxicity due to low expression of VHL in platelets, thereby maintaining Bcl-X_L levels in platelets and degrading it on other cell types. In addition, while the parent Bcl-X_L compound also bound to Bcl-2, PROTAC-induced degradation was Bcl-X_L-specific, highlighting the importance of productive ternary complex formation between the target and the E3 that leads to target ubiquitination. In this paper, the authors design a novel PROTAC by linking the Bcl-X_L-specific potent inhibitor A-1155463 via a flexible PEG linker to the VHL-binding moiety VH032 (compound 6). The PROTAC displayed negative cooperativity as measured by SPR but displayed a respectable DC_{50} value of 4.8 nM and a D_{max} value of 76% in THP-1 cells. The authors solved the structure of the ternary complex of VBC:PROTAC:Bcl-X_L to 1.9 Å. Interestingly, in spite of the negative cooperativity value, the ternary complex involves hydrophilic and hydrophobic neo-PPIs between VHL and Bcl-X_L, as well as neo-noncognate-protein–ligand interactions between the VHL and A-1155463 and between Bcl-X_L and VH032. The entropic cost of the interaction that may explain the observed negative cooperativity likely comes from the compact folded conformation of the linker. The authors suggest shortening and optimisation of the linker to reduce entropic cost and increase cooperativity that could be experimentally explored in future work.

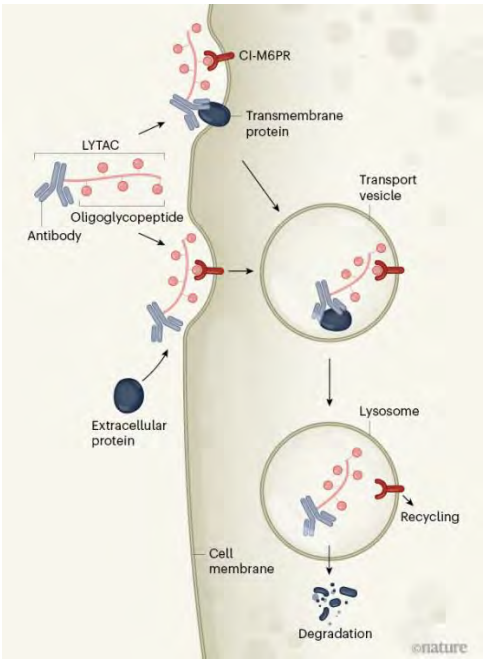
This is an interesting addition to the small but growing number of PROTAC ternary complex crystal structures. The paper highlights the exceptional importance of structural information in PROTAC design, given the difficulty in predicting neo-protein–protein interfaces, neo-noncognate-protein–ligand interactions and linker interactions which all contribute to the stability of the ternary complex. Only with structural information in hand can these factors can be optimised rationally to develop extremely potent and specific degrader moelcules.

Contributor: Sarath

Lysosome-targeting chimaeras for degradation of extracellular proteins

Steven M. Banik[§], ….., Carolyn R. Bertozzi*

Nature **2020**, *584*, 291.



Application of current protein-degradation platforms such as proteolysis-targeting chimaeras (PROTACs), molecular glues and others (for example, dTAGs, Trim-Away, chaperone-mediated autophagy targeting and SNIPERs) are limited to intracellular proteins or proteins with cytosolic domain. Extracellular and membrane-associated proteins—the products of 40% of all protein-encoding genes—are key agents in cancer, ageing-related diseases and autoimmune disorders, and so a general strategy to selectively degrade these proteins has the potential to improve human health. Here the authors establish the targeted degradation of extracellular and membrane-associated proteins using conjugates that bind both a cell-surface lysosome-shuttling receptor and the extracellular domain of a target protein. Lysosome-targeting chimaeras (LYTACs) consist of a small molecule or antibody fused to chemically synthesized glycopeptide ligands that are agonists of the cation-independent mannose-6-phosphate receptor (CI-M6PR). The article demonstrates the scope of this platform through the degradation of therapeutically relevant proteins, including apolipoprotein E4, epidermal growth factor receptor, CD71 and programmed death-ligand 1.

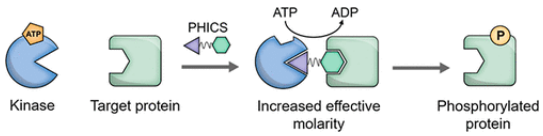
The figure (above, right) is from a News and Views piece on the article by our own Claire and Alessio (Nature 584, 193-194 (2020)), highlighting the proposed mechanism of action of LYTACs. LYTACs promise to serve as a new tool for targeted protein degradation strategies by targeting extracellular and membrane proteins for lysosomal degradation. Therapeutic application of the platform would benefit from further insights in to the mode of activity and kinetics of the ternary complexes formed by LYTACs, which could guide the design of optimal linkers. Exploring alternate lysosome-shuttling receptors for LYTACs could help overcome any potential resistance issues.

Contributor: Sarath

Phosphorylation-Inducing Chimeric Small Molecules

Sachini U. Siriwardena[§], Dhanushka N. P. Munkanatta Godage[§], Veronika M. Shoba[§], Sophia Lai[§], ….., Amit Choudhary*

J. Am. Chem. Soc. **2020**, *142*, 14052.



In this article, the authors attempt to mimic the PROTAC approach by utilising a bifunctional small molecule to bring a protein kinase in close proximity to a protein of interest to mediate native or neo-phosphorylation. Herein, they describe phosphorylation-inducing chimeric small molecules (PHICS), which enable two exemplary kinases—AMPK and PKC—to phosphorylate target proteins that are not otherwise substrates for these kinases. PHICS like PROTACs exhibit several features of a bifunctional molecule, including the hook-effect, turnover, isoform specificity, dose and temporal control of phosphorylation, and activity dependent on proximity. Using PHICS, they were able to induce in vitro phosphorylation of BRD4 by AMPK or PKC. The authors attributed the inability of PHICS to phosphorylate BRD4 in cells to different cellular localisation of the kinase and BRD4. A cytoplasmic target protein Bruton's tyrosine kinase was used to ascertain cellular activity of the PHIC. They envision that PHICS-mediated native or neo-phosphorylations will find utility in basic research and medicine.

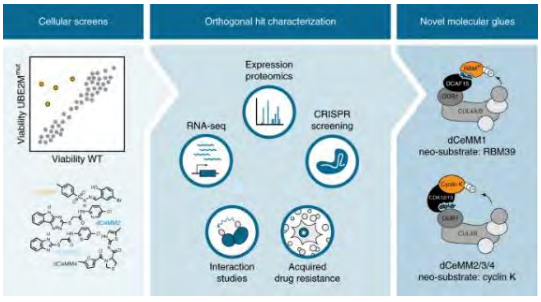
An important design feature of the PHICS in the article involves utilising allosteric small-molecule kinase activators as kinase recruiters. Allosteric kinase binders ensure the active site of kinase is accessible to the target protein. Phosphorylation is a key post-translational regulator of many cellular processes including cell cycle, growth, apoptosis and signal transduction pathways. The ability to phosphorylate specific protein offers a potential avenue to alter protein's localisation, activity and lifetime. A logical extension would be to apply this approach to modulate activity and specificity of other enzymes.

Contributor: Sarath

Rational discovery of molecular glue degraders via scalable chemical profiling

*Cristina Mayor-Ruiz[§], ….., Georg E. Winter**

Nat. Chem. Biol. **2020**. DOI: 10.1038/s41589-020-0594-x



Both molecular glues and PROTACs rely on bringing E3 ligases in close proximity to the target protein to mediate ubiquitination and subsequent proteasomal degradation. The modular approach of the bifunctional PROTACs involving linking E3 binders with ligands engaging target proteins, has enabled a rapid expansion of this platform. Discovery of molecular glue on the other hand has been serendipitous. This article describes a scalable strategy towards glue degrader discovery that is based on chemical screening in hyponeddylated cells coupled to a multi-omics target deconvolution campaign. A comparative viability and profiling of cytotoxic or cytostatic compounds in WT v/s isogenic hyponeddylated

cells facilitates identification of potential lead glue scaffolds. CRISPR-Cas9 screen using a custom-designed single-guide RNA library covering all known CRLs and associated regulators along with quantitative profiling allows for determination of complementary CRL-degraded protein pair. As a proof of concept, the article describes identification of molecular glue candidates mediating degradation of RBM39 and CyclinK via gluing to DCAF15 and DDB1, respectively.

The article attempts to provide a framework towards a rational search for molecular glue degraders. Although the RBM39 degrading molecular degrader identified through this study is structurally similar to previously published sulfonamide molecules, the CyclinK degraders identified through the study are structurally diverse. CyclinK degraders from this study (like CDK inhibitor CR8 https://doi.org/10.1038/s41586-020-2374-x) function by gluing the CDK12/13-CyclinK heterodimer to Cul4 adaptor protein DDB1 directly without a substrate receptor.

Contributor: Angus

In vivo target protein degradation induced by PROTACs based on E3 ligase DCAF15

Liang Li[§], Dazhao Mi[§], Haixiang Pei[§], ….., Mingyao Liu*, Yihua Chen*

Sig. Transduct. and Target. Ther. **2020**, *5*, 129.

To date, only a handful of the >600 E3 ligases present in humans have been hijacked by PROTACs and the call has been put out to expand the targeted protein degradation toolbox. The CRL4 receptor protein DDB1-CUL4-associated factor (DCAF)15 has recently been identified as the E3 ligase hijacked by the sulfonamide molecular glues to degrade RBM39 and is a promising candidate for PROTAC development. In this study, the authors design several PROTACs by tethering the sulfonamide compound E7820 to the well characterised BET inhibitor JQ1 and test them in the SU-DHL-4 lymphoma cell line for degradation of the BET protein BRD4. The most potent compound DP1 (DC_{50} = 11 ± 1 μM, D_{max} = 98%) displayed dose-dependent degradation of BRD4, BRD2 and BRD3, and was also active in several cell lines derived from hematopoietic and lymphoid lineages. The cellular and in vivo effects of DP1 were also evaluated and in a xenograft tumor model, DP1 administration attenuated tumor growth and reduced levels of BRD4 and c-MYC.

DCAF proteins are a promising family of E3 ligases receptors for PROTAC development, as most contain a WD40 domain which is a demonstrated ligandable fold. Electrophilic PROTACs have been developed for DCAF16 and the molecular glue sulfonamide compounds hijack DCAF15 to degrade RBM39, a neosubstrate. Attempts have been made previously to harness the sulfonamide compounds as handles for PROTACs, but this is the first example of successful hijacking of DCAF15 with a PROTAC. The authors hit a barrier in potency at the micromolar level, a feature which they attribute to a flat binding pocket of DCAF15 and weak affinity of the binary interaction between sulfonamide compounds and DCAF15. Follow up biophysical and structural studies on ternary complex formation would help determine whether this is the case. It's unclear whether the affinity of sulfonamides for DCAF15 will lead to a potency barrier for all sulfonamide-based DCAF15 PROTACs, but one could imagine that if cooperativity in formation of the ternary complex were high

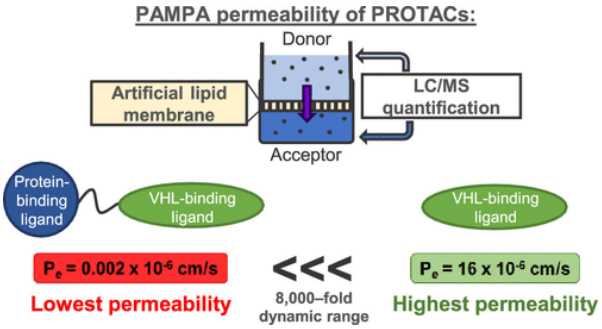
enough it would overcome the problem with affinity (as in the original molecular glue compounds themselves). Moreover, these sulfonamide-based PROTACs could likely be improved by optimising the binding affinity of sulfonamide compounds for DCAF15 alone, or by discovering new ligands with higher affinity.

Contributor: Angus

Understanding and Improving the Membrane Permeability of VH032-Based PROTACs

Victoria G. Klein[§], ….., Alessio Ciulli, R. Scott Lokey*

ACS Med. Chem. Lett. **2020**. DOI: 10.1021/acsmedchemlett.0c00265



PROTACs break the "rule of 5" guidelines that have been widely used to assess "drug-likeness" and predict oral bioavailability. The relationship between chemical structure and permeability of PROTACs has proven difficult to assess and predict.

Here, the authors outline a simple but powerful label-free method for determining structure-permeability relationships of PROTACs and their components using parallel artificial membrane permeability assays (PAMPA) and lipophilic permeability efficiency (LPE) (a measure of the efficiency at which a compound achieves membrane permeability at a given lipophilicity). The study tests this method on several series of PROTACs previously published by our group, that hijack the $CRL2^{VHL}$ E3 ligase and reveals factors that influence the membrane permeability of these molecules. Interestingly, and in line with recent reviews on the subject, the molecular weight (a "rule of 5" parameter) of compounds tested did not strongly correlate with membrane permeability when considered alone. Measuring LPE of these compounds revealed the effect of the chemical environment of hydrogen bond donors (HBD) on permeability. Shielding of HBDs through intermolecular hydrogen bonds (IMHBs) correlated with improved membrane permeability, as did replacing HBDs with hydrogen bond acceptors (HBAs) (particularly in cases where the HBD was unshielded). The authors highlight that efforts to improve permeability should always be performed while monitoring the effects of any changes on ternary complex formation, because ternary complex stability has been shown here and previously to sometimes override permeability properties.

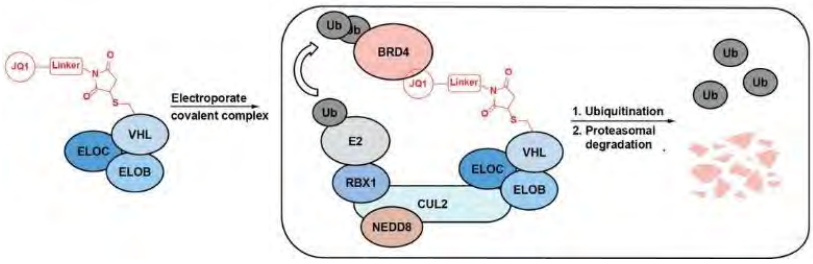
Finding the right balance between affinity and physicochemical properties is key to progressing molecules to the clinic. While great strides have been made in measuring and optimising the affinity of PROTACs, optimisation and understanding of their physicochemical properties has lagged behind. This paper provides a simple, inexpensive and high-throughput method to systematically bridge this gap in knowledge. Flexible PROTACs that can adopt conformations that form IMHBs, such as the ones tested here, will benefit particularly from characterisation by this method.

Contributor: Angus

A Strategy to Assess the Cellular Activity of E3 Ligases against Neo-Substrates using Electrophilic Probes

Benika J. Pinch[§], ….., William C. Forrester*, Dustin Dovala*, Lynn M. McGregor*, Claudio R Thoma*

bioRxiv, **2020***.* DOI: 10.1101/2020.08.13.249482



Assessing "hijackability" of E3 ligases for targeted protein degradation requires either genetic modification or the availability of a ligand that binds to the E3 which can be developed into a PROTAC. In this pre-print article, the authors describe a method for assessing the potential of E3 ligases to degrade neosubstrates that bypasses these informative but time-consuming methods. Covalent Functionalization Followed by E3 Electroporation (COFFEE) involves in vitro chemical modification of the E3 of interest and subsequent introduction of the modified E3 into cells through electroporation. The method requires a surface-exposed cysteine residue on the E3 that is reacted with a maleimide group connected to a warhead (in this case either the BET inhibitor JQ1 or the multi-kinase inhibitor dasatinib). The method is first validated using the well-characterised E3 ligase VHL. VHL, co-expressed with adaptor proteins elongin B^{C89S} (mutated to avoid functionalisation of C89) and elongin C (VBC), was chemically modified on Cys77 with a maleimide linked to either JQ1 or dasatinib and the reaction was monitored by intact mass spectrometry (unfortunately the supplementary data containing this validation are not yet available for this publication). Electroporation of the JQ1- or dasatinib-functionalised VBC into cells lacking VHL resulted in concentration-dependent degradation of the BET protein BRD4 or ABL1 kinase, respectively. The authors then tested the method on SPSB2, another SOCS box E3 ligase that has not previously been hijacked for targeted protein degradation. SPSB2 was co-expressed with adaptor proteins elongin B^{C89S} and elongin C and functionalised with the maleimide-linker-dasatinib probe on Cys53. Introduction of the modified protein into HEK293A cells by electroporation led to degradation of ABL1 kinase. Finally, VBC was functionalised with the dasatinib probe on residue C89 of elongin B to assess the potential for hijacking the adaptor rather than the receptor subunit. Degradation of dasatinib targets by this functionalised protein is assessed by quantitative proteomics, with modest decreases observed in levels of two dasatinib targets (EPHB2 and EPHB4).

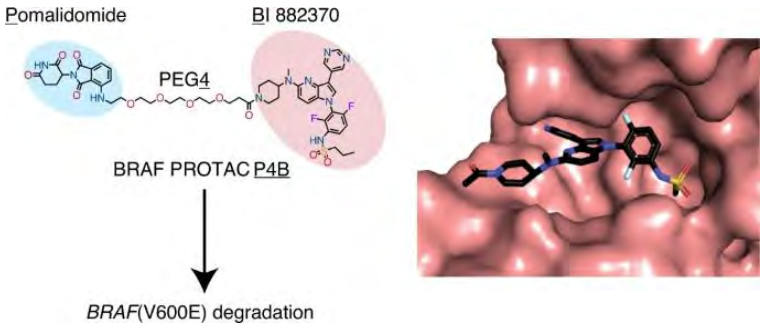
This method provides an interesting alternative to genetic modification for validation of the hijackability of E3 ligases in the field of targeted protein degradation. The method will be simpler than genetic modification in some cases, but it comes with some caveats that may limit wider application; the ligase in question requires one exposed surface cysteine residues or mutation to introduce or remove surface cysteine residues; it requires recombinant expression of the ligase (a not insignificant challenge in many cases); and it requires formation of a productive ternary complex between functionalised ligase and neosubstrate. The latter challenge will likely require synthesis and testing of a large number of probes and potentially different functionalisation points on the ligase to unequivocally prove a ligase hijackable or otherwise. Still, this method should prove useful in ideal cases with a library of functionalised probes and could inform design of covalent PROTACs by identifying which cysteine residues to target with electrophilic ligase ligands.

Contributor: Sarath

Functional characterization of a PROTAC directed against BRAF mutant V600E

Ganna Posternak[§], Xiaojing Tang[§], Pierre Maisonneuve[§], Ting Jin[§], Hugo Lavoie[§], ….., Frank Sicheri*

Nat. Chem. Biol. **2020**. DOI: 10.1038/s41589-020-0609-7



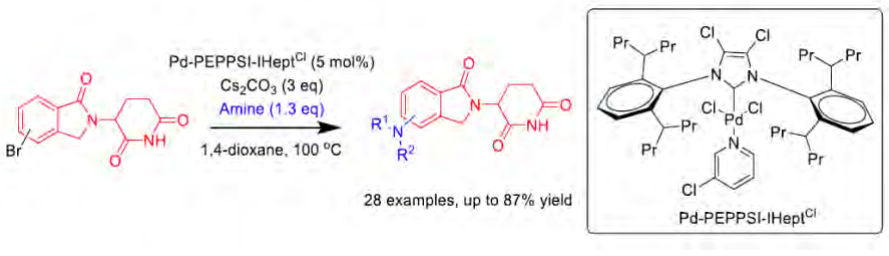
The RAF family kinases function in the RAS–ERK pathway to transmit signals from activated RAS to the downstream kinases MEK and ERK. This pathway regulates cell proliferation, differentiation and survival, enabling mutations in RAS and RAF to act as potent drivers of human cancers. Drugs targeting the prevalent oncogenic mutant BRAF(V600E) have shown great efficacy in the clinic, but long-term effectiveness is limited by resistance mechanisms that often exploit the dimerization-dependent process by which RAF kinases are activated. Here, the authors investigated a proteolysis-targeting chimera (PROTAC) approach to downregulating BRAF signaling. PROTAC P4B displayed superior specificity (unlike nonspecific off-target interaction of parent inhibitor BI 882370 to RAF family member KSR1 or SRMS) and downregulation of signaling (as measured by levels of phosphorylated ERK and MEK) relative to non-PROTAC controls in BRAF(V600E) cell lines. At the 24-h time point, P4B displayed a Dmax of 82% for BRAF(V600E) with a DC50 of 15 nM in A375 melanoma cells that are homozygous for the BRAF(V600E) mutation. In addition, P4B displayed utility in cell lines harbouring alternative BRAF mutations (V600D and G466V) that impart resistance to conventional BRAF inhibitors. This work provides a proof of concept for a substitute to conventional chemical inhibition to therapeutically constrain oncogenic BRAF.

BRAF targeting PROTAC P4B displayed better efficacy in comparison to parent inhibitors but was still ineffective against some alternate BRAF mutations (e.g. G596R and G469A) and RAS driver mutations. Although the parent inhibitor shows similar affinity for WT and V600E BRAF, the PROTACs reported lower degradation efficacy towards WT BRAF. The PROTAC approach seems complicated for this system as the pathway is prone to mutations in BRAF and activation of upstream pathway components conferring alternate resistance mechanisms.

A Buchwald-Hartwig Protocol to Enable Rapid Linker Exploration of Cereblon E3-Ligase PROTACs

Thomas G. Hayhow[§]*, Rachel E. A. Borrows, ….., James S. Scott, and David W. Watson

Chemistry A European Journal **2020***.* DOI: 10.1002/chem.202003137



This paper describes a convergent approach to the synthesis of lenalinomide based PROTACs which utilize the Cereblon E3 ligase. The low electrophilicity of the lenalinomide core prevents the

synthesis of PROTACs based on this motif using a standard nucleophilic aromatic substitution approach. A 5-step synthetic sequence is commonly used which prevents the rapid exploration of PROTACs based on the 5-substituted lenalinomide core. To circumvent this, the authors describe the optimisation of a Buchwald-Hartwig cross-coupling, a ubiquitous catalytic cross-coupling in medicinal chemistry as the final step in their synthetic strategy.

The study began with the optimisation of cross-coupling conditions on 3-(5-Bromo-1-oxoisoindolin-2-yl)piperidine-2,6-dione i.e. 5-bromoisoindolinone analogue of lenalidomide, and Boc-piperazine. Using high-throughput experimentation the authors identified Pd-PEPPSI-IPent containing an N-heterocyclic carbene ligand as the most successful catalyst, which displayed optimal conversion as well as product to internal standard ratio (P/IS). Further investigations revealed that use of NaO*t*-Bu resulted in complete consumption of starting material (3 h), albeit with reduced P/IS. The authors believe that this is likely due to an increase in side reactions. The catalyst loading could be reduced (10, 5 and 2 mol%) without a significant negative effect on reaction progression and a variety of solvents were tolerated.

Scaling up of the conditions led to some additional modifications. Reactions needed to be performed at higher temperatures (90 °C) and 1,4-dioxane was selected for ease of handling during aqueous extraction. Incorporating (*N*-Boc-4-amino)piperidine as the nucleophilic amine to expand the scope led to the identification of Pd-PEPPSI-IHept^{Cl} as the optimal catalyst based on isolated yields. The final optimised conditions were the aforementioned catalyst, 3 equivalents of dried Cs_2CO_3 at 0.1 M in dioxane (degassed) combined with 3 equivalents of amine. Notably, these conditions performed very well (88% isolated yield) on 19 g scale.

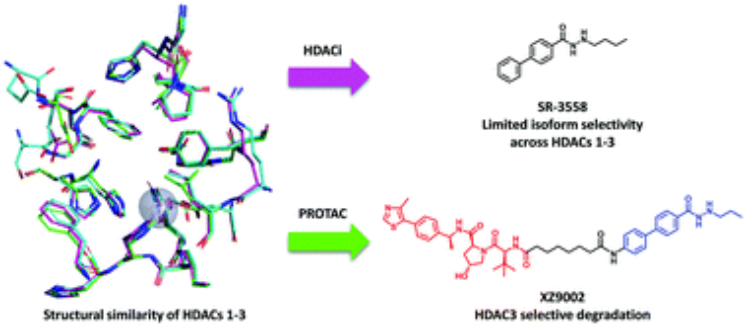
A diverse set of nucleophiles could be used which illustrated the scope of the conditions. A variety of amine nucleophiles underwent efficient cross-coupling. In particular, anilines, di-basic substrates, and base stable (acetal, *tert*-butyl esters) functional groups underwent smooth cross-coupling. Use of sterically hindered amines presented a challenge which was partially overcome by increasing the number of equivalents and careful choice of a less sterically hindered catalyst (Pd-PEPPSI-IPent^{Cl}). Importantly, the authors highlighted some examples of those nucleophiles which were not successfully coupled under the conditions. Gem-dimethyl piperazine, piperidine-4-carboxylic acid, piperidinone, pyrazole and imidazole showed no evidence of the desired products.

The reaction conditions were tested using regioisomers of the 5-bromoisoindolinone scaffold, which provided the corresponding products in moderate yields and illustrated the utility of the conditions in exploring different exit vectors. As a final hurdle the reaction was tested to couple the 5-bromoisoindolinone CRBN binder with piperazine-based linker pre-conjugated at the other end to the BET bromodomain ligand (+)-JQ1. The final PROTAC was achieved in 27% yield. Despite the low yield this represents the first reported introduction of a lenalinomide-based motif as the final step in the preparation of a CRBN-based PROTAC. This paper highlights the difficulty in carrying out a common transformation in medicinal chemistry on complex molecules. This will no doubt prove to be a useful tool for the synthesis of lenalinomide based PROTACs to drive discovery in a highly challenging area.

Discovery of histone deacetylase 3 (HDAC3)-specific PROTACs

Yufeng Xiao[§], Jia Wang[§], ….., Xuan Zhang* and Daiqing Liao*

Chem. Comm. ***2020***. DOI: 10.1039/d0cc03243c



HDACs are an important class of drug targets in epigenetic cancer therapy with four HDAC inhibitors (HDACi) currently approved by the FDA. A majority of HDACi are pan-HDACi and so far have not been successful as single agents and suffer from dose limiting toxicity. Increasing HDAC isozyme selectivity could be advantageous and may result in greater success in a clinical setting. In this arena, PROTACs could be particularly useful due to the possibility of increasing isozyme selectivity, made possible through stable ternary complexation. The authors have described the discovery of XZ9002, which degrades HDAC3. This is an important target involved in oncogene expression and may be a critical factor in breast cancer metastasis. So far HDAC3 has proved to be difficult to degrade compared to other HDACs.

The authors used molecular docking to identify a suitable solvent exposed exit vector on SR-3558, a potent and selective inhibitor of class I HDACs that preferentially binds HDAC3, which was previously disclosed by the authors. PROTACs were designed and synthesised based on this motif using CRBN and VHL E3 ligases. Evaluation of the degradation properties of all PROTACs was undertaken with a triple-negative MDA-MB-468 breast cancer cell line and ER+ breast cancer cell line T47D cells. VHL-based PROTACs proved to be the most successful and could induce HDAC 3 degradation at 100 nM.

XZ9002 was identified as the most potent and selective HDAC3 degrader and displayed a DC_{50} of 42 nM at 14 h. The mechanism of action was confirmed by pre-incubating MDA-MB-468 cells with excess VHL ligand (VH032) or proteasome inhibitor (MG132) which blocked HDAC3 degradation. Further, when using a negative control (XZ9002-NC) which lacks VHL affinity, no HDAC3 degradation was observed. A washout assay also indicated that protein levels took greater than 12 hours to return to appreciable levels. Histone acetylation was dose-dependently increased by XZ9002, albeit with moderate effect compared to CRBN-based PROTACs. The authors contend this may be due the fact that CRBN-based PROTACs maintained their broad spectrum *in vitro* HDAC binary affinity compared to VHL PROTACs, which showed a decrease (10 to 20-fold) in HDAC binding/inhibition. XZ9002 was significantly more potent than XZ9002-NC at supressing clonogenic growth of T47D, HCC1143, and BT549 breast cancer cells, while only moderately more potent in MDA-MB-468 cells. The authors suggest that this may indicate that HDAC3 degradation rather than inhibition may be more effective as an anticancer therapy. However, comparison of the antiproliferative effects of XZ9002 with SR-3558 or indeed an FDA approved pan HDAC inhibitor was not reported. Since XZ9002-NC is likely to display lower cell permeability than an inhibitor, it is perhaps not surprising that its inhibitory properties alone could not compensate for the lack of degradation activity in an antiproliferative assay. Therefore this comparison should be treated with caution.

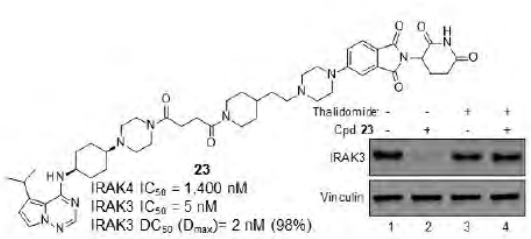
Further work to understand the structural and biophysical basis for degradation of HDAC3 by XZ9002 will be required to drive improvements in its degradation properties, which need to be assessed by in-depth functional analysis in cells. This work demonstrates that such studies towards this end may be achievable in the near future.

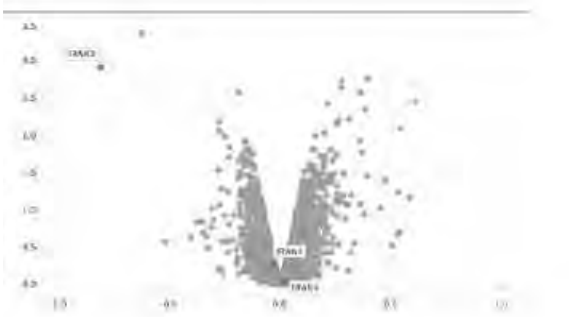
Discovery of PROTAC molecules that selectively degrade the IRAK3 pseudokinase

Sebastien L. Degorce*, Omid Tavana*, ….., Bin Yang, and Scott Edmondson

J Med. Chem. ***2020***. DOI: 10.1021/acs.jmedchem.0c01125

IRAK3 is a member of the IRAK family of proteins which consists of IRAK1, IRAK2 and IRAK4. IRAK3 plays an important role in the innate immune response and may represent a promising target in immuno-oncology. IRAK3 is a pseudokinase bearing an ATP-binding site whose function is not well understood and signals through a non-catalytic mechanism making it an ideal candidate for PROTAC mediated degradation.





To identify a suitable IRAK3 binder the authors performed a search of a vast inventory of kinase data with a focus on selectivity. In particular, the authors desired selectivity for IRAK3 against IRAK4. An IRAK3 selective compound which was serendipitously discovered during an IRAK4 inhibitor campaign, led to the identification of a *cis*-isomer bearing an advanced pyrrolopyrimidine core as a suitable starting point. Analysis of X-ray crystallographic data of *trans* analogues bound to IRAK4 and docking studies using an IRAK3 homology model with *cis*-analogues revealed a solvent exposed exit vector. Subsequently, the authors were able to identify a superior series based upon pyrrolotriazines, with improved selectivity (>100 vs rest of the kinome) and one less HBD. This provided a binder which displayed excellent intrinsic permeability and low efflux (P_{app} = 29 x 10^{-6} cm/s, ER = 0.5, Caco-2 monolayer permeability method). With a potent, selective, cell permeable binder displaying desirable physicochemical properties in hand, the search for a degrader could begin.

Their investigations began by initially utilizing PEG based linkers of different lengths connecting to either CRBN or VHL E3 ligase binders. Despite a small loss (4- to 10-fold) in binary affinity to IRAK3, selectivity against IRAK4 was retained. CRBN-based PROTACs with longer PEG linkers proved to be competent degraders in THP1 cells. The use of all carbon-based linkers did not initially lead to degradation being observed. However, more fruitful efforts explored a previously described linker scaffold bearing a piperazine-piperidine moiety.

The authors identified PROTAC **23** as a potent degrader (DC_{50} = 2 nM, D_{max} = 98%, 16 h). Despite inhibiting four other kinases (CDK11, CDK8, TRKC and GSG2, IC_{50}s ranging from 16 to 110 nM), proteomic profiling in THP1 cells revealed

that only IRAK3 and DHCR24 levels were reduced to a significant extent ($p < 0.05$). Time-course experiments indicated degradation of IRAK3 was maintained for up to 72 h and washout experiments (PBS at 16 h) showed that protein levels recovered after 24 h. Degradation was not observed when pre-treating with either an excess of IRAK3 binder, excess thalidomide, proteasome inhibitor (MG132) or neddylation inhibitor (MLN2924). A PROTAC negative control which lacked binding to CRBN also failed to produce degradation of IRAK3, further supporting the notion that degradation of IRAK3 by **23** is mediated by the ubiquitin proteasome system. IRAK3 degradation was also observed in human primary macrophages which was sustained for up to 32 h of treatment. When the macrophages were pre-treated with lipopolysaccharides (LPS), IRAK3 degradation was maintained throughout the duration of stimulation.

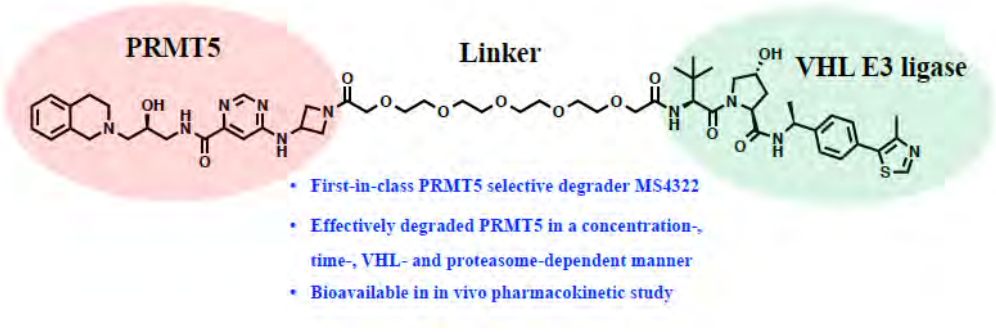
This work reports the first example of IRAK3 degradation in the literature. Furthermore, it provides an account of how careful selection of a target protein binder can provide a robust foundation upon which one can launch a PROTAC campaign. No doubt this will prove to be useful reading to scientists in both academia and industry.

Discovery of First-in-class Protein Arginine Methyltransferase 5 (PRMT5) Degraders

Yudao Shen[§], Guozhen Gao[§], …., Jing Liu*, Mark T. Bedford*, Jian Jin*

J. Med. Chem. **2020**. DOI: 10.1021/acs.jmedchem.0c01111

PRMT5 catalyses the methylation of histone and non-histone proteins and is an important regulator of many biological processes. Knockdown of PRMT5 has demonstrated that it plays a role in cancer cell proliferation and tumour growth. In particular, in patient derived xenograft (PDX) glioblastoma (GBM) tumours and AR-positive LNCaP prostate cancer cell xenograft models, PRTM5 knockdown supressed tumour growth. While many potent inhibitors are described for this target protein, PRMT5 may possess activity which is independent of its catalytic function. Therefore this target represents an ideal arena within which to pursue a PROTAC approach.



With EPZ015666 (a potent and selective PRMT5 inhibitor) in hand, five PROTACs were initially designed and synthesized with PEG linkers of different lengths appended from a suitably placed (solvent exposed) aziridine ring. Degradation assays were performed in MCF-7 cells (ER+ breast cancer cells) and evaluation of three different concentrations indicated that a longer PEG based linker was sufficient to provide degradation at 1 µM (6 day treatment). This degrader was then modified with (*S*,*R*,*S*)-AHPC-Me, a different VHL ligand bearing a methyl group at the benzylic position, presumably to improve the degradation properties of their lead by increasing its binary affinity for VHL.

The authors used a biochemical assay with radiolabelled SAM (^{3}H-SAM) cofactor and H4 (1 – 15) Biotin substrate to determine the potency of their lead PROTAC (**15**) against PRMT5, which was comparable (IC_{50} = 18 nM) to EPZ015666 (IC_{50} = 30 nM). Not surprisingly, a negative control (**17**) which lacks binding for VHL also retained affinity (IC_{50} = 12 nM) for PRMT5. Compound **21** was designed to diminish the PRMT5 affinity and displayed significantly reduced binding to PRMT5 (58 ± 1% inhibition at 5 µM). PROTAC **15** (DC_{50} = 1 µM, D_{max} = 74%) proved to be the most promising degrader, albeit significant degradation was not observed for 2 days (time-course study, 5 µM for 0 – 8 days) with maximum degradation observed between 6 and 8 days. The degradation properties of **15** were demonstrated to be proteasome dependent by co-treatment of MCF-7 cells with excess of EPZ015666, VH298, MLN4924 or MG-132. Further, use of negative controls lacking VHL (**17**) or PRMT5 binding affinity (**21**), or EPZ01566 itself did not lead to degradation of PRMT5 in MCF-7 cells. **15** also showed comparable activity to EPZ01566 at inhibiting global arginine symmetric dimethylation. Washout experiments after treatment with 5 µM for 6 days of **15** indicated that proteins levels started to recover over a 48 h period.

Cell proliferation assays (MCF-7) indicated that inhibition by **15** was comparable to EPZ015666 and that **21** showed no antiproliferative effect. Importantly, control compound **17**, lacking the degradation properties of **15**, whilst maintaining comparable binary affinity for PRMT5, displayed much weaker antiproliferative activity than **15**. Thus demonstrating that the antiproliferative effect of **15** is primarily due to degradation of PRMT5. Global proteomics analysis (MS-based label-free quantitative) of MCF-7 cells treated with **15** or DMSO control indicated that PRMT5, WDR77 (PRMT5 binding partner) and AGRN were the only proteins with significantly reduced levels. Further investigations also confirmed that **15** degraded PRMT5 levels in different cancer cell lines (HeLa, A549, A172 and Jurkat) when dosed with 5 µM for 6 days. PROTAC **15** also inhibited proliferation of these cancer cell lines in a concentration dependent manner.

Pharmacokinetic analysis using intraperitoneal (IP) dosing of Swiss Albino mice at 150 mg/Kg showed that **15** achieved good plasma levels (14 µM, 2 hours post dosing). Of note, plasma protein binding of **15** was not reported and plasma concentrations were not corrected for this in the PK analysis. Since plasma protein binding of PROTAC molecules is typically very high (<1% free fraction), the corresponding fraction unbound in plasma (f_u,plasma) should be carefully determined together with the concentration of fraction unbound in plasma (Cu,p). This would allow the suitability of **15** as a chemical tool for investigating PRMT5 degradation *in vivo* to be thoroughly understood. However, due to the event driven pharmacology of a PROTAC, a low value of f_u,plasma may not present the same challenges as an inhibitor in achieving suitable exposure levels for demonstrable pharmacodynamic effect. Further, since PRMT5 knockdown in PDX GBM tumours supressed tumour growth (*vide supra*), it will be important to measure levels of unbound concentrations of **15** in brain tissue of treated mice. Optimization of **15** to improve its degradation properties should also be undertaken in order to fulfil the full potential of PRMT5 as a target in protein degradation. This paper provides a starting point and hopefully will inspire further work to come.

Other Highlighted Papers

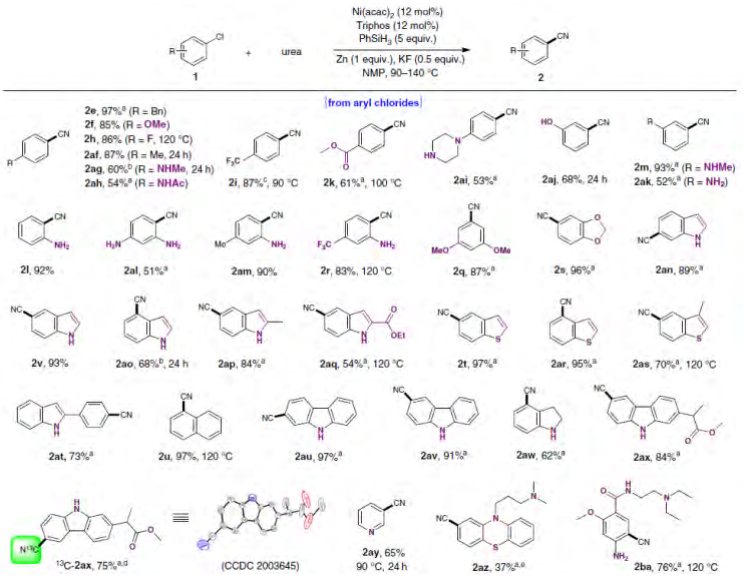
Contributor: Tasuku

Reductive cyanation of organic chlorides using CO_2 and NH_3 via Triphos–Ni(I) species

Yunan Dong[§], ….., Yuehui Li*

Nat. Commun. **2020***, 11,* 4096. DOI: 10.1038/s41467-020-17939-2

Cyano group is one of most frequently found moieties on bioactive compounds, but traditional cyanation reactions require highly toxic cyanide salts that need special care for handling, storing, and disposing. In this paper, Dong et al. reported novel reductive cyanation to arylchlorides by using CO_2/NH_3 as a cyano source. A broad range of substrates were well-tolerated to afford corresponding products in moderate to high yields. In addition, they found that urea can be used as a cyano source instead of CO_2/NH_3. In this reaction condition, alkyl chlorides also provided corresponding cyanated products in low to moderate yield.



Their proposed reaction mechanism showed that no cyanide species (CN^-) are produced in the reaction, therefore this new method has less concern for toxic cyanide species and should be easier and safer than traditional cyanation reactions.

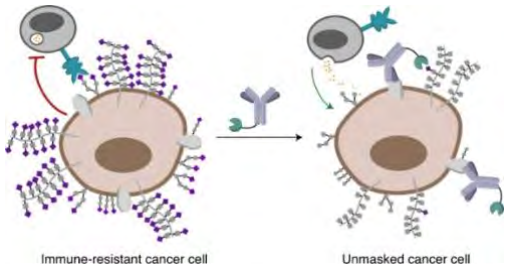
Contributor: Will

Targeted glycan degradation potentiates the anticancer immune response in vivo

Melissa A. Gray[§], ….., Carolyn R. Bertozzi*

Nat. Chem. Biol. **2020***, 83,* DOI: 10.1038/s41589-020-0622-x

In this article the Bertozzi lab further develop sialoglycan targeted destruction as an alternative concept to protein checkpoint targeted immuno13-oncology. The authors identified a sialidase from *Salmonella typhimurium* (ST) that had low intrinsic sialoglycan binding affinity to maximise the window between on-target activity in the tumour microenvironment and off-target tissue activity. Hydrazino-iso-pictet-spengler and click reactions were used to construct a stable linker conjugating the sialidase enzyme with Trastuzumab, a HER2 receptor monoclonal antibody (marketed as Herceptin). I.P. dosing at 10 mg/kg was able to slow tumour growth and extended survival in a HER2+ syngeneic breast cancer model, with subsequent studies using carefully



designed/selected controls showing these effects were driven by sialoglycan reduction in the tumour microenvironment and not driven via antibody-dependent cytotoxicity. Whilst tumours showed highest levels of sialoglycan degradation, at the top dose this was also observed in tissues throughout the mice.

This manuscript represents a significant stride in demonstrating therapeutic potential of a creative and exciting concept for addressing immune checkpoint blockade. Whilst both off-target selectivity and on-target efficacy likely require further progress, it is early days for a fundamentally novel modality and it will be fascinating to see to what can be achieved when applied to a wider array of antibodies/disease models.

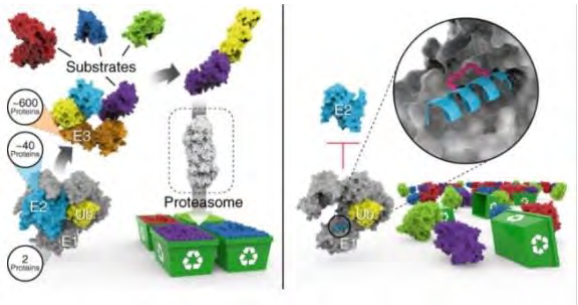
Contributor: Siying

Targeting a helix-in-groove interaction between E1 and E2 blocks ubiquitin transfer

Ann M. Cathcart[§], ….., Loren D. Walensky*

Nat. Chem. Biol. **2020***,* DOI: 10.1038/s41589-020-0625-7

Inhibition of the ubiquitin-proteasome system (UPS) has been identified as a viable strategy for cancer treatments. In this paper, the authors detailed a new strategy to disrupt E1-E2 ubiquitin transfer by targeting the helix-in-groove interactions between the E2 N-terminal alpha-1 helix ($E2^{h1}$) and a pocket within the E1 ubiquitin-fold domain ($E1^{UFD}$).



Staple scanning of the $E2^{h1}$ binding interface residues from four different E2 enzymes leads to the discovery of SAH-UBE2A, a stapled peptide mimetic that directly binds to the E1 enzyme in mammalian cells (UBE1) and specifically blocks E1 thioester transfer with an IC_{50} value of 2.3 M. The structure-activity relationship established by alanine scanning, charge-reversal mutagenesis, and triple-alanine mutagenesis was found to be consistent with the binding mode observed in the $E1^{UFD}$-$E2^{h1}$ X-ray crystal structure and docking studies. The effect on the conformation of E1 enzyme upon SAH-UBE2A binding was studied by hydrogen-deuterium exchange mass spectrometry. It was found that SAH-UBE2A binding induces a change in the conformation of $E1^{UFD}$ that is consistent with an inhibitory mechanism. The generalised inhibitory capacity of SAH-UBE2A is studied in more detail. It was found that SAH-UBE2A blocks ubiquitin transfer from E1 to 20 different E2 enzymes. Furthermore, experiments in HeLa cell lysates showed that SAH-UBE2A can compete with the full-length E2, engage with the endogenous E1 and inhibit polyubiquitination of proteins in the cell lysate.
