Multispecific Drug Design: Advances in Event and Occupancy-Driven Pharmacology

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Abstract

Rational drug design has historically consisted of 1-target-1-drug approaches that – while having yielded hundreds of drugs for clinical use – run into limitations with "undruggable" targets. The invention of proteolysis-targeting chimeras (PROTACs) signified the birth of a pharmacological paradigm shift, whereby drugs are now being rationally designed to partake in more than one binding event. Such drugs, termed multispecific drugs, can harness natural biology to link a target to a biological effector or use cooperative binding to localize therapeutic agents at relevant locations. Due to their differing mechanisms of action, multispecific drugs can circumvent traditional limitations and expand the undruggable therapeutic space. Here, I review an array of notable multispecific technologies and discuss their development, design considerations, limitations, challenges, and clinical scope.

Acknowledgements

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Introduction

Since the advent of aspirin, medicinal chemistry has experienced several transformative discoveries. 1-target-1-drug approaches signified the earliest stages of rational drug design, where a biologic or small molecule drug was engineered to bind a target in a lock-and-key fashion to activate or inactivate it. This model describes an occupancy-driven approach in which drug-target binding needs to be productive (competitive against an endogenous substrate or conformationally modulatory) and the drug needs to reside in its pocket to elicit its effect. Small molecules soon met limitations with this approach due to their inability to bind expansive target surfaces (as in transcription factors), an issue that did not plague the larger protein modalities as significantly. Biologics such as antibodies have since become the most successful drugs in the 21st century. Recently, a paradigm shift has taken place with the rise of multispecific drugs that are able to partake in more than one binding event.[1] These drugs contain two or more binding elements such that they can bring together between targets and effectors to redirect natural biology for therapeutic purposes (molecular matchmakers), or even use cooperative binding to introduce precision (tetherbodies).[1] Only a handful of multispecifics have obtained approval, but they have been rapidly gaining traction and are beginning to dominate drug discovery pipelines (e.g., Amgen).[1]

Druggability or ligandability can be defined as the extent to which a target can be modulated upon binding of a drug-like molecule.[2,3] Estimates using sequence homology with known drug targets suggest that, of the approximate 20,000 genes encoded by the human genome, only the products of 3,000 – 5,000 genes (15 - 25%) are druggable by such molecules.[2,3] Druggability alone does not ensure that a protein can serve as a viable target, however, as its involvement in disease is also paramount to elicit therapeutic responses.[3] Analyses have shown that an overlap between disease-linked and druggable genomes yields a predicted 600 – 1,500 viable drug targets in the human genome (Fig. 1).[3] Numerous geometry and energy-based approaches that calculate free-energy of ligand binding have also been taken to predict druggability.[4–6] However, these analyses tend to rely on Lipinski's rule of 5 to define drug-like binders (hydrogen bond donors < 5, hydrogen acceptors < 10, molecular weight < 500 Da, Log *P* < 5). As emerging successful technologies are shifting away from rule of 5 properties (antibodies, multispecifics), estimates deeming 85% of the human proteome "undruggable" can be somewhat limiting. There are typically only a handful of parameters needed to identify protein pockets for high-affinity and selective binding: a pocket should be significantly hydrophobic, concave, deep,

large, and lacking high proportions of charged residues.[5] The lack of such a pocket is highly correlated with "undruggable" targets, as seen in cancer drivers such as c-Myc and Tau. Due their differing mechanisms of action that circumvent conventional drug needs, multispecific drugs offer opportunities to tackle these "undruggable" targets.

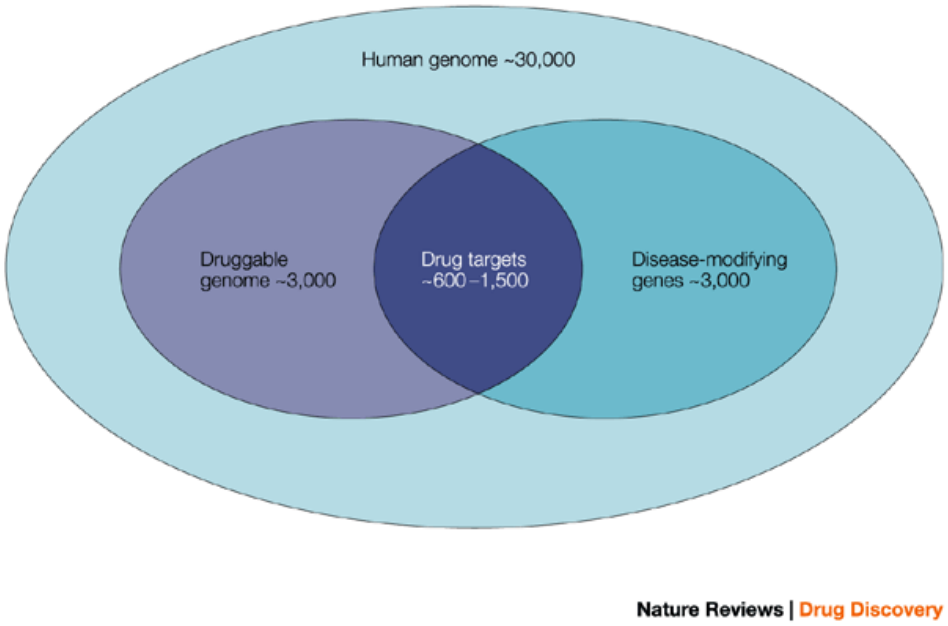


Figure 1 - Work by Hopkins and Groom (2002) displays the overlap of disease-linked and druggable genomes.

Molecular Matchmakers That Induce Targeted Degradation

PROTACs: Leveraging the Cell's Proteasome Pathway to Degrade Targets

A drastic expansion of the druggable space has been brought about with the emergence of the field of targeted protein degradation (TPD), of which proteolysis-targeting chimeras (PROTACs) have been at the forefront since 2001.[7,8] PROTACs are heterobifunctional small-molecules that consist of a POI (protein-of-interest)-binding element tethered to another element that recruits an E3 ubiquitin ligase. Through simultaneous binding of both components, a ternary complex is formed (Fig. 2) to facilitate ubiquitination of the POI by the ligase and subsequent degradation by the ubiquitin-proteasome system (UPS). A key differentiator between PROTACs and conventional small molecules is that, theoretically, PROTAC binding to the POI need not be competitive against an endogenous ligand. If a productive ternary complex is formed, PROTACs

can completely eradicate the presence of a disease-linked protein from the cell and be recycled for repeated targeting of a POI, holding inherent catalytic advantages over conventional inhibitors.[7,8]

By redirecting natural effectors (ubiquitin ligases) to POIs, the focus of drug design shifts from inducing physical change through occupancy to association, largely removing the need to find the ideal binding pocket. Recent clinical proof-of-concepts of PROTACs display high degrees of efficacy in clinical trials while maintaining safe profiles and showcase that the technology can expand the clinically druggable space. On the other hand, these molecules are associated with their own challenges: namely worsened pharmacokinetic properties due to higher MW, a largely limited scope with targets that require up-regulation/activation, a dependency on ideal linkers, and a need for the POI-ligase interface to allow ubiquitin transfer in a manner that will induce degradation.[7,8] With the exception of a few antibody-based PROTACs that engage membranous E3 ligases (AbTACs, PROTABs), they are also unable to induce degradation of extracellular and membrane proteins.[9] The hook effect additionally describes a counterintuitive bell-curve relation between concentration and efficacy of PROTACs, where higher concentrations of PROTAC supplementation can produce smaller biological responses. This occurs because each binding element in the PROTAC molecules starts saturating its respective target due to entropic gain, limiting the possibility for both elements to simultaneously bind and facilitate ternary complex formation.[8]

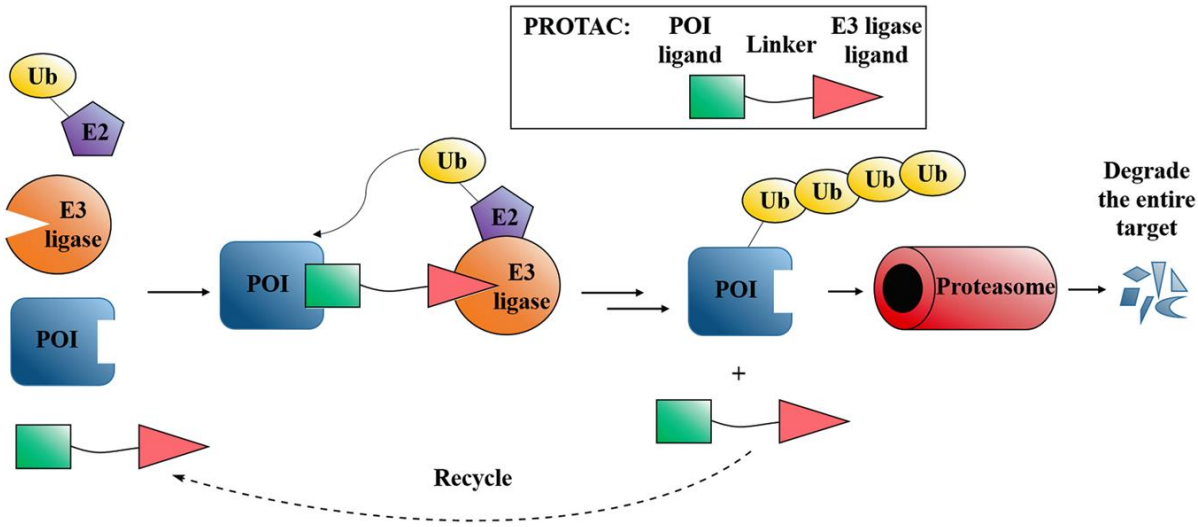


Figure 2 - Mechanism of TPD through the UPS by PROTACs. Ternary complex formation (complex of POI, drug, and E3 ligase) is depicted.[10]

The UPS is a canonical player in inducing PROTAC-mediated degradation using ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-protein-ligases (E3). Over 600 of these ligases exist in the human proteome, each providing novel target opportunities due to differences in protein subset specificities.[11,12] The most recruited ligases are cereblon (CRBN), as the ligase-binding moiety in PROTACs tends to be an immunomodulatory imide drug (IMiD) – an active target of CRBN, and VHL (von Hippel-Lindau tumour suppressor). Heavy reliance on these brings drug resistance into question with cancers, as it has been shown in work done on BET-PROTACs by *Zhang et. al* that treatment of tumour cells with such drugs can fail to induce complete eradication.[8] It was found that following generations of tumor cells contained mutant VHLs and CRBNs, pointing to a resistance mechanism that evolves such ligases instead of the target protein. Ligase expression varies across and within cells as expression profiles are influenced by tissue and tumor type, cellular compartment, and cell state, presenting an opportunity for increased precision.[11,12] Some ligase-POI complexes cannot even exist long enough for ubiquitin transfer to exposed lysine residues, thereby limiting the scope of the degradable proteome. Drug resistance can also possibly be countered by relying on an essential E3 ligase for ubiquitination. These combined reasons point to a need for exploring alternative E3 ligases and several opportunities to confer greater degrees of specificity and modulation with the design of the second binding moiety.

BioPROTACs: Fusion Proteins and pepTACs

BioPROTACs extend the PROTAC methodology to protein modalities consisting of three subgroups. PepTACs are the most similar in binding mode to the traditional small-molecular PROTAC as they consist of POI-binding and E3-ligase binding peptide-based warheads conjugated together with a linker.[8] To allow for cell penetration, such molecules are usually tethered to a cell-penetrating peptide (CPP), but an additional mode of delivery can be transfection of cells with DNA/mRNA encoding the pepTAC.[8] Some researchers have constructed fusion proteins to directly repurpose endogenous E3 ligases for binding of a POI.[8] This has been shown through the replacement of the substrate-binding domain of some E3 ligases for domains specialized to bind the POI – e.g., the MYC-associated factor X (MAX). While these constructs are unlikely to be orally bioavailable, they present advantages in that they can bind to more expansive and blander surfaces due to being larger binding elements. Consequently, bioPROTACs have the potential to even drive forward small-molecule PROTAC discovery: they can help

provide proof-of-concept for degradation in undruggable targets that cannot be modulated with conventional small-molecules, assess the benefits of degradation over occupancy-driven binding, and even probe ideal sites for ternary complex formation.[8]

Targeted RNA Degradation

While messenger RNA (mRNA) molecules encode proteins and account for 3% of all RNA transcripts, the remaining majority (non-coding RNAs; ncRNAs) have key regulatory roles in cellular processes such as mRNA translation, cell development, and apoptosis.[13–15] Improper ncRNA function – especially of microRNAs (miRNAs) – is emerging as significant a contributor of cancers and other diseases (e.g., neurological, cardiovascular).[13–15] Clinically approved nucleotide-based therapeutics such as small interfering RNAs (siRNAs) and antisense oligonucleotides (ASOs) target RNAs for degradation but are faced with poor pharmacokinetics and toxicity profiles.[16–18] Small molecule inhibitors have been developed to circumvent such issues; however, they tend to exhibit limited efficacy, selectivity, and restriction to productive binding sites.[17,18]

Ribonuclease-targeting chimeras (RIBOTACs) are multispecific molecules that apply PROTAC design to induce proximity between an RNA molecule and a ribonuclease for targeted RNA degradation.[16] RIBOTACs consist of an RNA-binding element linked to an RNase recruiting element that activates the nuclease.[1] The initial RIBOTAC proof-of-concept was demonstrated by the Disney Lab, which recruited RNase L to the precursor of oncogenic pre-miR-96 to induce its selective and catalytic degradation through confirmed ternary complex formation.[16] This RIBOTAC showed improved efficacy and selectivity over its inhibitor component (Targaprimir-96) while also inducing apoptosis of MDA-MB-231 cells. Since, RIBOTACs have been developed to degrade pre-miR-210, pre-miR-21, and even SARS-CoV-2 RNA. While RIBOTACs are novel degraders that open the relatively undruggable RNA space to small molecule modalities, they are held back by short supplies of small molecule RNA binders and the cytosolic RNase L localization. Thus, the progression of the technology will be dependent on novel platforms to design selective RNA binders and the identification of other RNases that could degrade RNA targets in other locations such as the nucleus.[16,17]

Moving Beyond the UPS to the Lysosome

Around 40% of the human genome encodes proteins that are either secreted to the extracellular environment or associated with the cell membrane. PROTACs thus leave behind a

vast space of therapeutic opportunity due to the intracellular localization of the UPS and largely limit their scope to proteins containing cytosolic domains. A need for extending TPD to this space consequently arises, pointing to a solution that lies in leveraging the natural processes by which such extracellular proteins are degraded.

The Lysosome in Membranous and Extracellular Degradation

Most membrane and extracellular proteins are degraded via the endosome/lysosome system: a dynamic interplay involving initial endocytosis of membranous or captured extracellular cargo, subsequent fusion with an early endosome, maturation of the endosome and, ultimately, its fusion with the lysosome for degradation (Fig. 3).

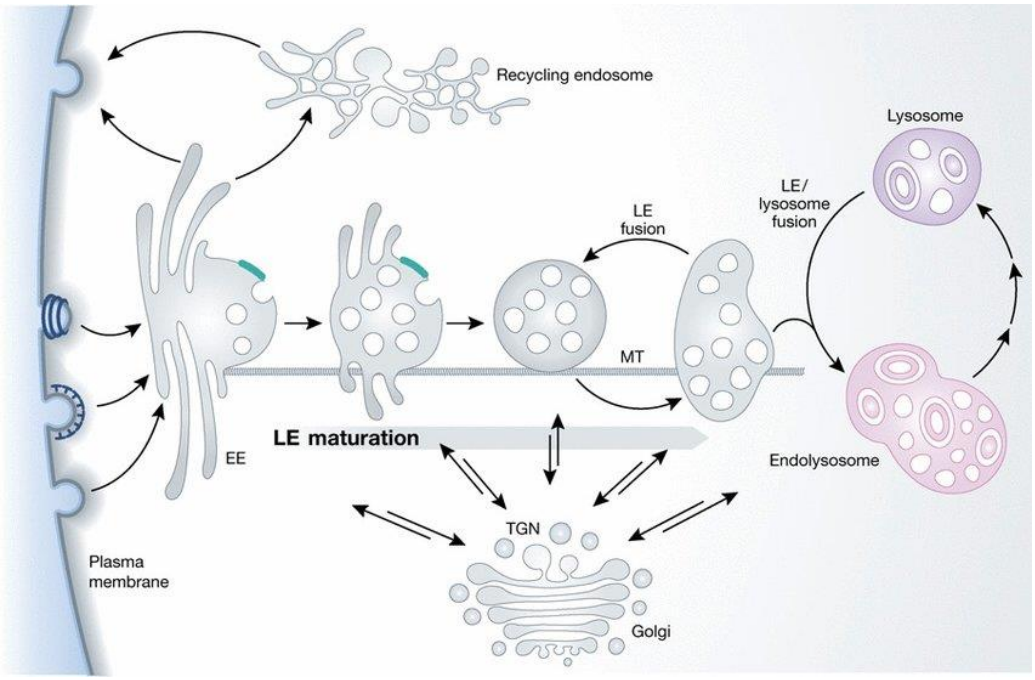


Figure 3 – A depiction of the endosome/lysosome system starting from endosome maturation to lysosomal fusion.[19]

Receptor-mediated endocytosis is the mechanism of key relevance in extracellular protein degradation, whereby proteins are usually tagged with a recognition element such that they become bound by a membranous receptor.[20] These receptors are associated with clathrins present at the cytosolic portion of the membrane which, upon receptor-substrate binding, facilitate the formation of vesicles that collapse and enter the cell.[20] Upon removal of their clathrin coating, the cargo-containing vesicles fuse with early endosomes that then mature into late endosomes through

several mechanisms (e.g., acidification).[20] This is followed by fusion of the endosomes with lysosomes such that the cargo is degraded via hydrolases.[20]

Leveraging the Lysosome for Targeted Protein Degradation: **LYTACs**

A novel technology that can engage the natural lysosome/endosome system for TPD has recently emerged from the Bertozzi group.[21] This is accomplished via bifunctional molecules termed LYTACs (lysosome-targeting chimeras), which consist of a POI binding element (small molecule or peptide/antibody) tethered to a lysosomal trafficking shuttle recognition element (Fig. 5).[22,23] A prominent example of such a shuttle is shown in CI-M6PR (cation-independent mannose-6-phosphate receptor), which recognizes proteins with mannose-6-phosphate-capped N-glycans and transfers them to pre-lysosomal vesicles.[22,23] The CI-M6PR is a prototypical trafficking shuttle primarily involved in directing M6P-capped hydrolytic enzymes from the golgi complex to the lysosome (Fig. 4).[22,23] When it is localized on the membrane, however, it directs similarly capped extracellular and membranous biomolecules to the endosome/lysosome system for degradation. Binding of cargo by CI-M6PR induces clathrin-coated pit formation and early endosome fusion, and the increasing acidity associated with maturation results in weakened binding to cargo and release of it to the lysosome.[22,23]

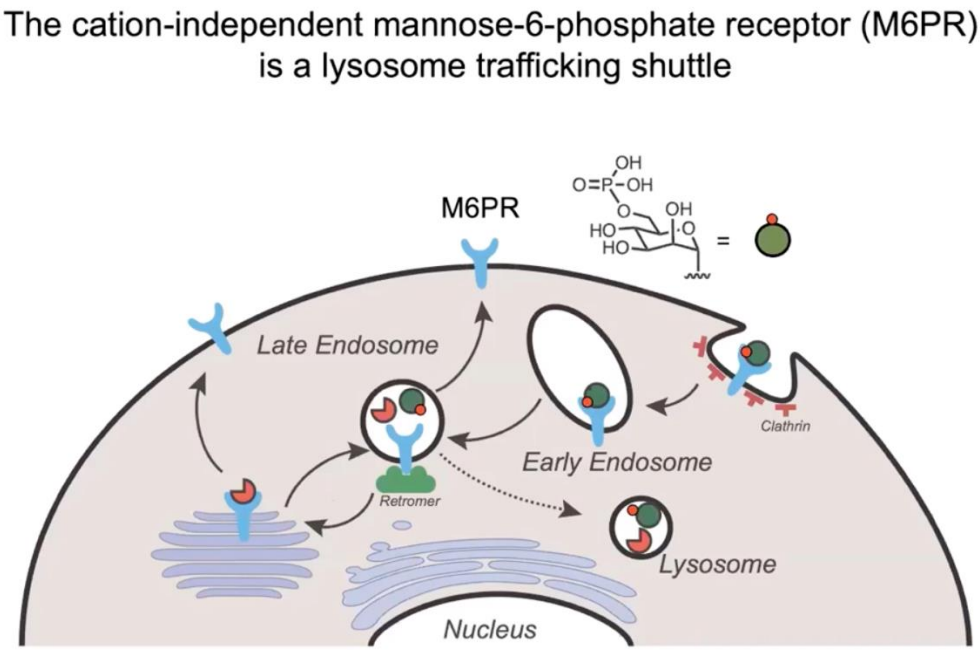


Figure 4 – The dynamic cycling of the M6PR. Figure obtained from Dana-Farber TPD webinar series.

The first proof-of-concept study by the Bertozzi Group centered around a M6PR recognition element made of oligopeptides conjugated to M6P analogues at serine and lysine. As a preliminary test for intentional uptake and degradation by LYTACs, the oligopeptide was biotinylated such that it would be theoretically bind fluorescent NeutrAvidin-647 and initiate its lysosome-mediated degradation. Upon supplementation, 5 - 6-fold increases in cellular fluorescence were observed relative to control, indicating uptake as a function of M6P and M6Pn (mannose-6-phosphonate, a non-hydrolysable analogue) presence. Interestingly, the length of the M6PR-binding glycopolymers only contributed minor effects in uptake while it was the identity of the glycan label (M6Pn vs M6P) that defined major differences in uptake efficiency. M6P-LYTACs showed less efficient degradation than their M6Pn counterparts, which was possibly a consequence of M6P being more susceptible to hydrolysis and metabolism. The use of biotin demonstrated the potential of small-molecule LYTACs but, for therapeutic applications, the glycopolymer was "clicked" onto antibodies. The first of these was used to demonstrate efficacy on an extracellular protein – apolipoprotein E4 (ApoE4) – which is implicated in neurodegenerative diseases such as Alzheimer's. LYTACs were able to induce a 13-fold increase in uptake of ApoE4, and additional inhibition of serine and cysteine proteases led to accumulation of intracellular ApoE4. Overall, these observations signified the efficacy of antibody based LYTACs in degradation of an extracellular target through a confirmed lysosomal mechanism of action.

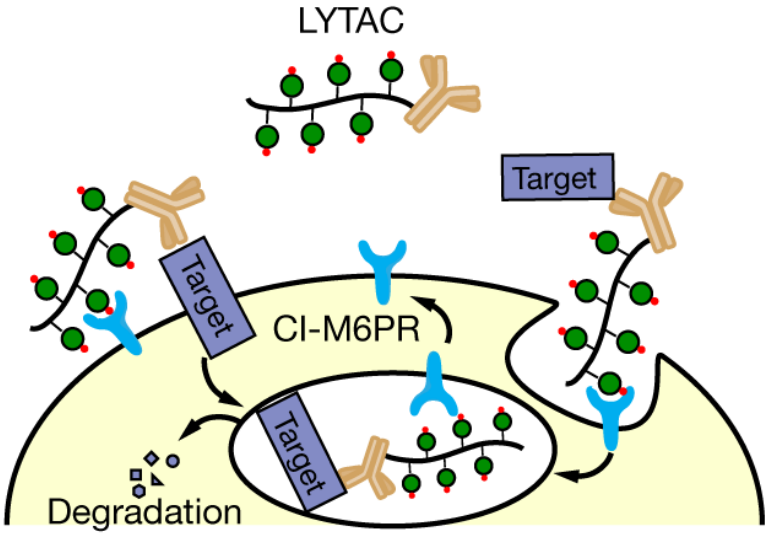


Figure 5 – A simplified cartoon of LYTAC mechanism of action. Figure obtained from Dana-Farber TPD webinar series.

Extracellular targeting in lysosomal treatments has been preestablished in lysosomal treatments, meaning proof-of-concept for degradation of membranous proteins was a significant next step. The Bertozzi Group shifted to targeting the epidermal growth factor receptor (EGFR), a driver of cancer proliferation. Upon 10 nM treatment of cells with cetuximab (monoclonal antibody) conjugated to M6Pn, degradation of greater than 70% was observed at 24 h after treatment and the effect was sustained till 72 h.

The Bertozzi group then extended LYTACs to PD-L1, a therapeutically relevant membrane protein primarily involved in immune checkpoints. PD-L1 undergoes an independent endosomal pathway that is partly regulated by a chaperone CMTM6. When CMTM6 associates with the cytosolic domain of PD-L1, it effectively increases PD-L1 steady state levels observed on the membrane by promoting endosomal recycling as opposed to the competing degradation pathway. Thus, the potential of LYTACs to override a target's endosomal equilibrium for therapeutic benefit could be tested. Treatment with 5 mM M6Pn-Tecentriq resulted in 70% degradation at 48 h, showcasing the ability of LYTACs in targeting proteins engaging their own endosomal recycling pathways.

Second-Generation LYTACs

Several opportune questions in the potential of LYTACs remained. First, could other lysosome trafficking receptors be harnessed by LYTACs? Second, could different receptor profiles be used to introduce tissue specificity? Third, could LYTAC structures be simplified and optimized? To address these questions, the Bertozzi group shifted to the asialoglycoprotein receptor (ASGPR), a liver-specific uptake receptor that is involved in internalizing numerous biomolecules such glycoproteins and aging platelets from circulation.[24] ASGPR recognizes N-acetyl galactosamine (GalNAc) and galactose residues to mediate steady-state protein levels through the endo-lysosomal system. The receptor exists as a highly expressed trimer on hepatocyte surfaces (10^6 trimers/cell) and, in the siRNA therapeutic space, multivalent GalNAc/galactose displays have been made to bind it with high affinity.[1] Thus, this methodology was readily extended to the LYTAC platform with the construction of lysine-conjugated tri-GalNAc LYTACs. The construct was able to induce ASPGR-mediated uptake of soluble targets into hepatocytes at much higher rates than those observed in comparable M6Pn platforms.[24] However, both constructs induce similar degradation of EGFR (membrane target) on hepatocyte cell lines containing both M6PR and ASGPR despite the latter's uptake being more efficient. This suggested that there was

limited reliance on the nature of the trafficking system for observed cargo degradation when the endosome/lysosome pathways are engaged. As expected, Cetuximab-LYTACs to uptake through ASGPR or M6P reduced EGFR signalling more effectively than Cetuximab alone, showing potency benefits associated with TPD.[24]

HEPG2 (hepatocyte cellular carcinoma) xenografts in immunocompromised mice were also tested with HER2 (the driver of carcinoma proliferation) as the target. The mice were supplemented with 3 varying doses of Pertuzimab-M6Pn and Pertuzimab-GalNAc LYTACs. 78% degradation was observed with Ptz-M6Pn dosing, but Ptz-GalNAc only yielded 11% downregulation. This was later attributed to worsened pharmacokinetic properties associated with oversaturation of Ptz with GalNAc, a consequence of unspecific labelling chemistry. 10 dendrimers were being attached to each Ptz and, since each dendrimer contains three GalNAc residues, this was resulting in around 30 GalNAcs being presented on each antibody. Shifting to site-selective labelling introduced the possibility of decreasing to 2 dendrimers/antibody and even moving dendrimer locations around the antibody as desired, significantly improving half-life and allowing for LYTAC structure optimization.[24]

The new site-specific and single-dendrimer GalNAc-LYTACs resulted in 45% degradation of HER2 relative to untreated control, better downregulation than that of oversaturated Ptz (0%) and unlabelled Ptz (10%). Groups other than the Bertozzi group have also implemented GalNAc constructs in a similar triantennary fashion and even in small-molecule modalities. Zheng et. al also recently reported an integrin-facilitated lysosomal degradation (IFLD) strategy, where they developed bifunctional compounds that potently trafficked both extracellular (HaloTag constructs of apoE4) and membrane proteins (PD-L1) through integrin $\alpha_v\beta_3$.[25] These integrin LYTACs were able to effectively degrade POIs through ternary complex formation both *in vivo* and *in vitro*. The scope of LYTACs is being tested on various major disease targets and even undruggable targets on which occupancy-driven pharmacology fails to work. Before LYTACs enter the clinic, several areas of investigation remain: it is unknown if LYTACs can trigger adverse immune responses in patients and the extent to which the antibody portion itself is degraded is unclear. Additionally, desirable properties of membrane targets to pursue and the role of the hook effect must be elucidated. At the same time, LYTACs seem to be more forgiving of orientation and sequence considerations that PROTAC design must incorporate, which is likely a consequence of the M6PR being a transporter and not an effector that modifies the POI. Going forward, LYTAC development

will be reliant on probing the space of usable trafficking shuttles as a source of precision and the degree to which their mechanisms of action are understood will be paramount in efficient design.[26]

MADTACs to Engage the Autophagy Pathway

PROTACs have been shown as adept inducers of cytosolic protein degradation, but there exist several other intracellular disease drivers such as damaged organelles, protein aggregates, intracellular debris, other biomolecules, and invasive microbes. As the UPS is inherently limited to proteins, the autophagy system arises as the pathway suited for targeted degradation of non-proteinaceous cytosolic contents.[27] Macroautophagy is a type of autophagy characterized by sequestration of substrates with cytosolic double-membrane vesicles called autophagosomes.[27] The process initiates with phagophore nucleation, an isolation membrane that is assembled at the phagophore assembly site (PAS) by a protein complex involving LC3-I (microtubules-associated protein light chain 3), the ULK complex, PI3K kinases, Beclin1, and p150 kinase.[26,27] Phagophore elongation follows such that substrates destined for degradation are sequestered and a mature autophagosome forms upon complete membrane enclosure. The autophagosome then fuses with the lysosome to create an autolysosome with hydrolases which, in the presence of the internal acidic environment, degrade the cargo. [27]

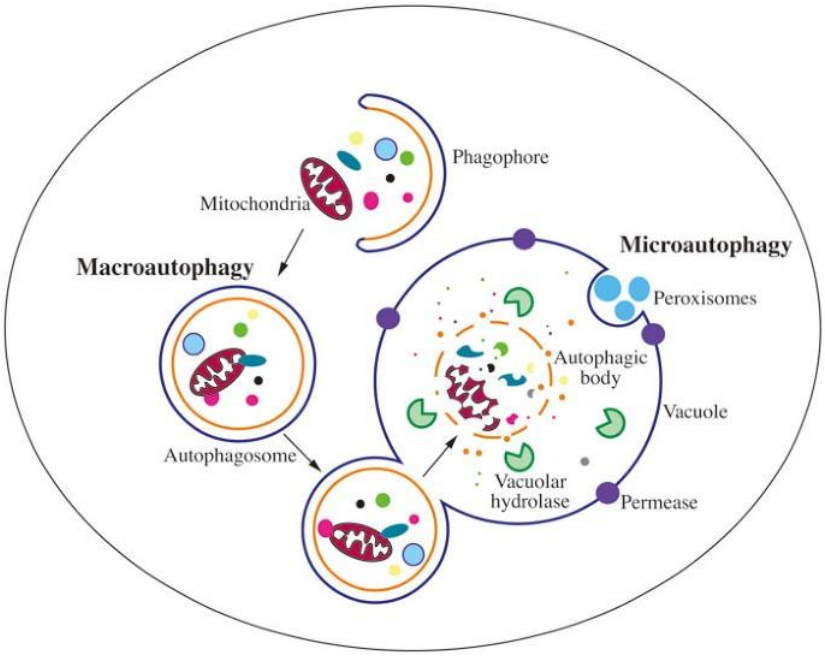


Figure 6 – The processes of macro and microautophagy starting from phagophore formation.[27]

In antibacterial autophagy (xenophagy, the most studied autophagy pathway) of group A streptococci (GAS), S-guanylation (cysteine conjugation to 8-nitro-cGMP) of GAS surface proteins is a key post-translational modification that is associated with K63 polyubiquitination and subsequent lysosomal degradation of the microbe. As of now, all that is understood about the process is that it is Atg5-dependent and results in LC3-positive-puncta accumulation in the cell.[28]

The Arimoto laboratory had been a leader in understanding the role of S-guanylation in xenophagy and, consequently, the group was poised to exploit the natural process for targeted degradation.[28] First, they showed that cGMP's covalent conjugation to HaloTag-GFP can induce the protein's selective autophagy, identifying S-guanylation as a standalone tag that can trigger cargo-selective autophagy.[28] This was done through the observation that the covalently labelled construct localized with the autophagosome players such as LC3-II and K63 polyubiquitin. Mechanism of action was investigated with the knockout of key players in the autophagy cycle, such Agt5 and p61/SqSTM-1.[28] Localization with LC3-II and K63 polyubiquitin was minimized upon knockout, providing validation of the mechanism of action.[28]

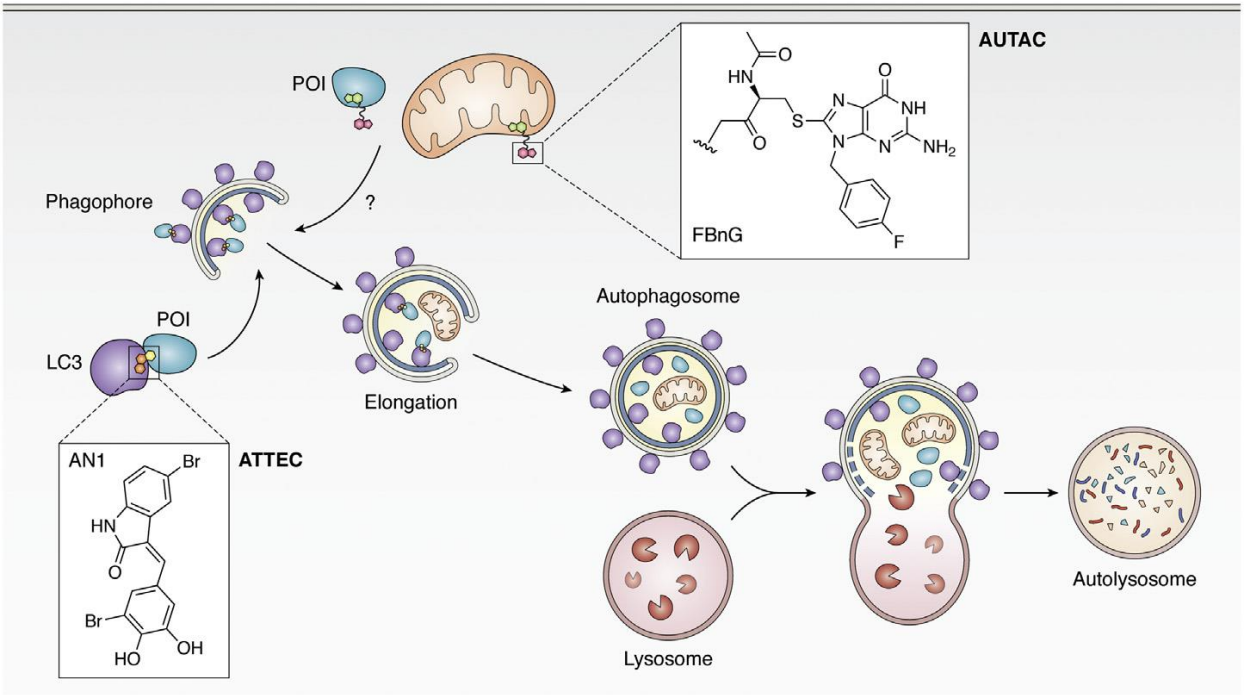


Figure 7 – Autophagy mechanism of action of AUTACs.[26]

To circumvent natural processes in which cGMP is involved, the Arimoto group developed a standalone tag that resembles cGMP: p-fluorobenzylguanine (FBnG).[28] FBnG was shown to be sufficient in inducing selective autophagy and its linkage to known binders of MetAP2, a very

early PROTAC target, generated the first bifunctional autophagy targeting chimera (AUTAC1). AUTAC1 consisted of FBnG linked to fumagillol, a hydrolyzed product of fumagillin that covalently binds MetAP2. The group showed AUTAC1 was able to induce ~80% degradation of MetAP2 in HeLa at 1μM dosage, which likely also reflected the benefits associated with covalent binding. To demonstrate AUTAC potential in non-covalent binding modes, AUTAC2 was created as a degrader of FK506-binding protein (FKBP12). AUTAC2, a chimera of SLF (FKBP12 binder) and FBnG, was able to induce approximately 70-80% degradation in HeLa cells at 10μM dosage.

AUTAC3 was created to target the BET family of proteins, a series of transcriptional regulators that have been a focus of several therapeutic efforts due to their central role in diseases such as melanoma.[28] Outside therapeutic relevance, targeting the BET proteins was of particular interest due to their nuclear localization that presents challenges in drug discovery efforts, meaning the potential of autophagy-mediated targeted degradation on similar proteins could be tested. AUTAC3 was created from covalently linking JQ1, a known binder of the BET family, to FBnG and A459 cells were dosed at varying concentrations. Only slight BRD4 degradation of ~30% was seen as a function of AUTAC3 which, through a cell-cycle synchronization technique, was identified to occur when nuclear contents were exposed to the cytoplasm during the mitotic cell phases. These findings suggest that the AUTAC platform has little application to nuclear proteins due to the autophagy system's cell-cycle dependence and limited nuclear reach.

To demonstrate the application of the AUTAC platform on organelles, the Arimoto group tested AUTAC-induced degradation of mitochondria. This was done by expressing a fusion protein of OMM (outer mitochondrial membrane) protein 25 and EGFP-HT in HeLa cells, and treating them with FBnG-Halotag ligand. Approximately 50% degradation was observed at concentrations > 10 μM after 12 hrs, providing proof-of-concept for autophagy-mediated targeted non-proteinaceous degradation. They also show that AUTACs can induce mitophagy in systems with dysfunctional mitochondrial systems to provide therapeutic effects. As the process of macroautophagy is not fully understood yet, clinical optimization of MADTACs will prove difficult.

Inducers and Removers of Non-Degradative Post-Translational Modifications

Phosphorylation in Cell Homeostasis

Estimates suggest that 90% of the human proteome is subject to phosphorylation at some point, making it one of the most commonly occurring post-translational modifications in the cell.[29] Phosphorylation/dephosphorylation can induce conformational and functional changes in proteins, trigger signalling cascades, and modify interactomes.[29–32] The dynamics of phosphorylation are intricately controlled by kinases (proteins that facilitate phosphorylation) and phosphatases (proteins that facilitate dephosphorylation) to regulate physiological processes such as protein synthesis, cell division, growth, and aging. Dysregulation in phosphorylation pathways is greatly associated with disease, and kinase inhibition has thus served as a hotspot for drug discovery efforts in cancerous, inflammatory and neurodegenerative diseases.[30–32] ~98% of phosphorylation events occur at serine (ter) and threonine (thr) residues with ser/thr-specific protein kinases while, with the aid of Tyr-specific kinases that can be RTKs (receptor tyrosine kinases) and non-receptor tyrosine kinases, 2% of phosphorylation events occur at tyrosine residues.[30–32] Conventional kinase inhibition efforts are met with significant side effect profiles and other issues such as those associated with occupancy-driven pharmacology (non-catalytic mechanisms). Thus, using small molecules to catalyze phosphorylation events through induced proximity becomes an increasingly attractive approach.

Phosphorylation-Inducing Chimeric Small Molecules (**PHICS**)

The Schreiber group rationalized that targeted protein phosphorylation or neo-phosphorylation could be used to alter function, evoke immune responses, and modify interactions of a target with other biomolecules such as DNA/RNA which are negatively charged. In a proof-of-concept concept study, they showed that bifunctional PHICS are capable of redirecting ser/thr AMPK (AMP-activated protein kinase) and PKC (protein kinase C) to induce both native and neophosphorylation of the non-substrate BRD4 (bromodomain-containing protein 4).[33]

The Schreiber group created a binder of AMPK using a known allosteric activator as a reference while using a previously reported synthetic activator of PKC as its binding element.[33] Both molecules were confirmed as activators of their respective kinases and then tethered to (*S*)-JQ1 to generate PHICS with varying linker lengths, of which PHICS1 for AMPK and PHICS2 for

PCK were found to be the most optimal inducers of BRD4 phosphorylation. These compounds displayed characteristics associated with ternary-complex formation (hook effect, linker dependence) and PHICS-dependent neophosphorylation was shown to occur on BRD4 residues that had not been previously reported in the literature. The PHICS were also observed to elicit catalytic mechanism of action (turnover) in ADP-Glo assays. Further examination showed that PHICS1 was unable to phosphorylate BRD4 through an AMPK isoform due to differential binding of the activator element, an effect also seen in PHICS2 when PCK was substituted with α, βI, βII, γ, and δ isoforms. Additional specificity amongst BRD paralogs (2/3/4) was observed with BRD4 showing the greatest extent of phosphorylation. Although these findings collectively showed benefits and properties associated with bifunctional molecules, they were unable to induce BRD4 phosphorylation in cells, which was likely a consequence of BRD4's nuclear localization that contrasts with the cytosolic localization of the kinases.

The Schreiber group then shifted to targeting BTK (Burton's Tyrosine Kinase) with PHICS due to its cytosolic localization, the availability of known BTK ligands and their co-crystal structures with BTK, and the fact that its phosphorylation at S180 is functionally important to prevent its membranous translocation.[33] BTK was not known to be a natural substrate of AMPK, which posed a question of whether PHICS could induce AMPK-mediated phosphorylation of a non-substrate kinase. Ibrutinib, a known binder of BTK, was tethered to the AMPK activator with an aliphatic carbon chain to yield PHICS3. With a confirmed dependence on PHICS3, BTK was phosphorylated at S180 in a dose-dependent manner with fast kinetics. This preliminary study highlights the potential of PHICS in drug design toolkits and subsequent studies are being conducted to induce tyrosine kinase-mediated phosphorylation, phosphorylation at DNA-binding domains of hard-to-drug transcription factors, and neo-phosphorylation to elicit anti-tumor responses.

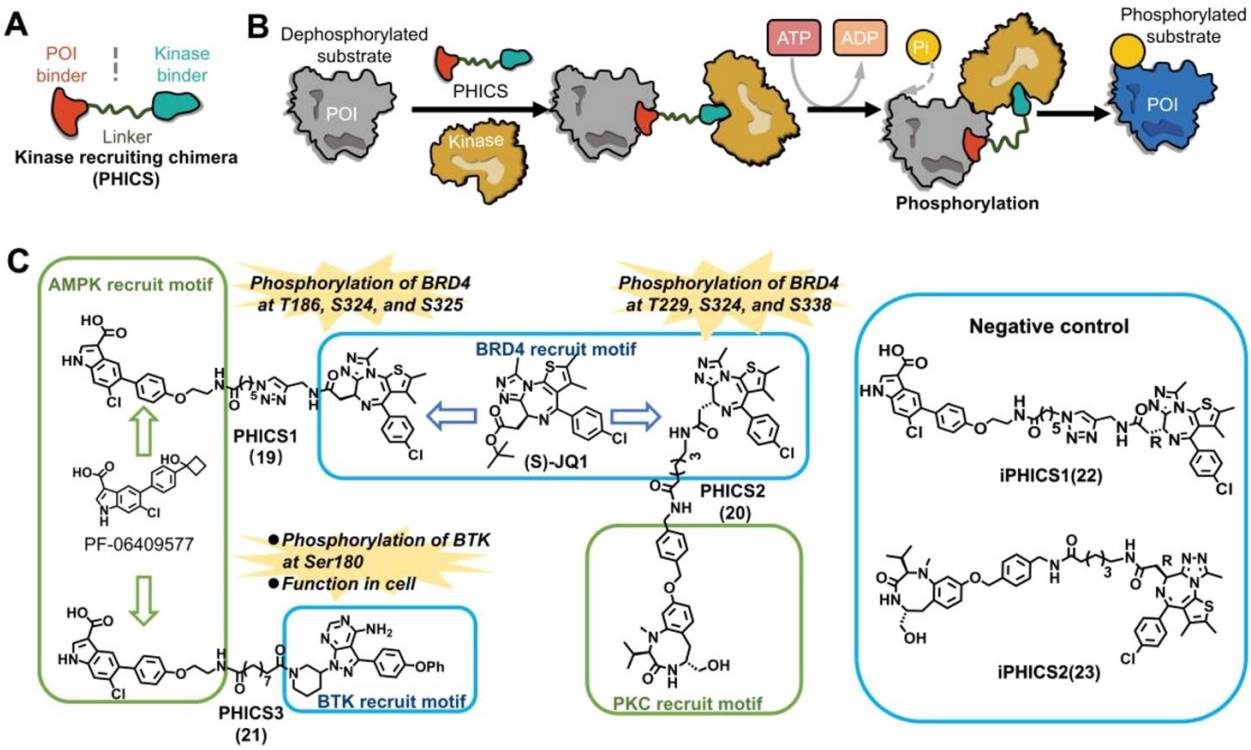


Figure 8 – PHICs design and mechanism of action.[34]

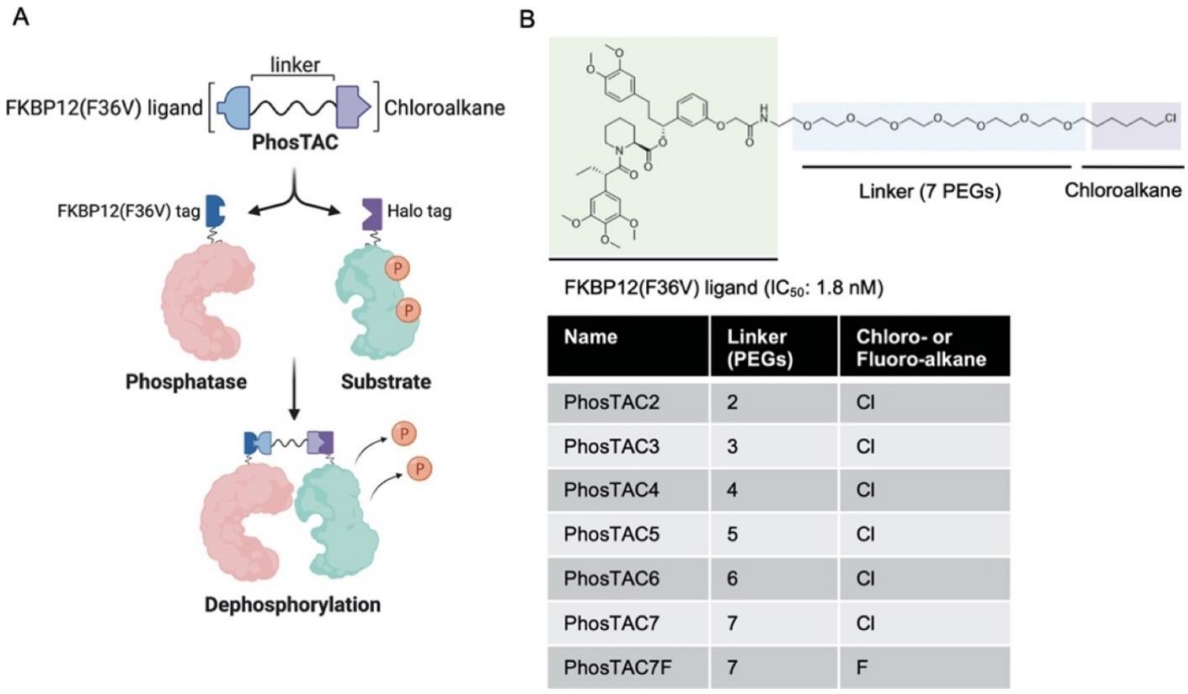
Phosphorylation Targeting Chimeras and Phosphatase Recruiting Chimeras to Dephosphorylate On-Demand.

While targeted protein phosphorylation adds to the arsenal of tools to modulate cell-signalling, targeted dephosphorylation presents a vast space of therapeutic opportunity as well, especially in cases where hyperphosphorylation is associated with pathogenesis.[31,32] In a proof-of-concept study, Yamazoe et al. aimed to inhibit the PI3K (phosphoinositide 3-kinase)/AKT (protein kinase B) pathway by dephosphorylating AKT through induced proximity to PP1 (protein phosphatase 1).[35] In doing so, PHORCs (phosphatase recruiting chimeras) were created with the PROTAC methodology, wherein the E3 ligase binding component is replaced with a phosphatase recruiting element.[35]

PHORC 1 was constructed by linking an AKT binding element to a HaloTag-reactive and chemoselective chloroalkyl group with the aim of forming a ternary complex between AKT and a HaloTag-PPI-FLAG fusion construct. AKT, which has maximal activity that is dependent on simultaneous phosphorylation of Ser473 and Thr308, was significantly dephosphorylated at Thr308 upon 10 µM dosage of the compound in LNCaP, MCF7/neo HER2, and PC-3 cell lines. This experimental validation led the authors to develop PHORC 3, a chimera of the previously

used AKT binding element linked to the PP1-activating synthetic peptide PDP1. Supplementation of purified $pAKT^{Thr308}$ with PHORC 3 at 2.5 μM displayed maximal dephosphorylation of greater than 80% (relative to untreated control), where greater and lower PHORC 3 concentrations resulted in submaximal dephosphorylation in accordance with the hook effect. Dephosphorylation effects were not replicated in LNCap cells at concentrations up to 10 μM, which was hypothesized to be a consequence of poor membrane permeability or proteolytic susceptibility of the peptide-based binding element. Thus, a new PHORC of an allosteric AKT binder (4a) linked to the PP1-binding RVSF was generated. 10 μM of 4a induced significant dephosphorylation of both $pAKT^{Thr308}$ and $pAKT^{Ser473}$ in LNCaP, MCF7/neo-HER2, and PCP-3 cell lines after 8 h. Maximal dephosphorylation was observed at 6-8 h where dephosphorylation efficiency varied amongst cell lines. Yamazoe et al. also shifted to creating EGFR (a receptor-tyrosine kinase) dephosphorylating molecules by linking EGFR inhibitor AZD-9921 to the HaloTag-reactive chloroalkyl group, resulting in PHORC 7. 10 μM dosage of PHORC 7 in HaloTag-PP1-FLAG transfected HCC827 cells for 8 h resulted in substantial decrease of $pEFGR^{Tyr1068}$ levels, which suggests that even tyrosine dephosphorylation with the ser/thr phosphatase PP1 can be incurred through proximity-inducing small molecules.

The Crews laboratory built on the PHORC platform in a separate study by generating bifunctional PhosTACs (phosphorylation targeting chimeras) that instead leverage PP2A (protein phosphatase 2A), the most abundant protein phosphatase in mammals.[36] As with the PHORCs study, they generated HaloTag fusions of target proteins PDCD4 (programmed cell-death 4) and FOXO3a (Forkhead-box O3a transcription factor) which are both involved in tumor suppression. Fusions of FKBP12(F36V) and PP2A were also constructed, as in similar PROTAC proof-of-concepts, since an existing ligand of FKBP12 binds to it with high affinity (IC_{50} = 1.8 nM) and selectivity.[8] The FKBP12 ligand was tethered to the HaloTag-reactive chloroalkane through PEG (polyethylene glycol) to generate PhosTACs of varying linker length, which were then dosed to wild-type HeLa cells at 5 μM. Through Halo-Trap assays conducted after 24 h, PhosTAC7 (linker of 7 PEGs) was observed to induce significant dephosphorylation of HaloTag-PDCD4 through ternary-complex formation. PhosTAC7 was also able to induce HaloTag-FOXO3a dephosphorylation in a similar manner, an effect observed through the altered FOXO3a phosphorylation kinetics and increased transactivation activity in luciferase assays upon treatment.



Name	Linker (PEGs)	Chloro- or Fluoro-alkane
PhosTAC2	2	Cl
PhosTAC3	3	Cl
PhosTAC4	4	Cl
PhosTAC5	5	Cl
PhosTAC6	6	Cl
PhosTAC7	7	Cl
PhosTAC7F	7	F

Figure 9 – The construction of PhosTACs (and similarly, PHORCs) and a depiction of their mechanism of action.[36]

Bispecific Antibodies as Cellular Matchmakers

Most of the technologies discussed have centered around inducing proximity between a molecular target and an effector to incur a pharmacological event such as degradation, stabilization, or phosphorylation. These events take place inside the cell and are thus suited for the more membrane-permeable small-molecule modalities. In the current therapeutic space however, the targets and effectors have even been extended to the cellular scale, where interactions between T-cells and disease-causing cells are facilitated through induced proximity.[37]

Bispecific antibodies (bsAbs) are molecules that can bind two epitopes to engage pharmacological consequences such as bridging two receptors to activate/inactivate them through cross-linkage, bringing together an enzyme and a substrate that can serve as a cofactor mimetic for treatment, and 'piggybacking' where binding one epitope can result in transport of the bsAb to the second epitope (Fig. 10).[37] The design of bsAbs that act in such mechanisms has yielded significant clinical success, but another distinct early example of the capabilities of these molecules has appeared in the bridging of T-cells and cancer cells through induced proximity. These types of bsAbs are termed BiTEs (bispecific T-cell engagers), of which BCEs (bispecific CD3 engagers) have been the most successful examples**.** BsAbs outside BCEs to link together biomolecules (Fig.

10 b-d) have not been explored or emphasized as deeply, which is partially due to conventional antibody-mediated inhibition of such targets being sufficient. BCEs consist of a domain that binds to the CD3 subunit of TCR (T-cell receptor, a complex of proteins found on T-cell surfaces) and a second domain that binds to a cancer-specific surface antigen.[1,37] Upon simultaneous engagement of the two targets, the effective molarity of T-cells surrounding the cancer cells increases, resulting in T-cell-mediated cytotoxic activity of therapeutic benefit**.** Where poor pharmaceutical properties and manufacturability plagued the modality, blinatumomab emerged as the only FDA-approved BCE that is in still in use today. Blinatumomab's success has sparked the development of T-cell redirecting drugs, leading to at least 40 BCEs that have entered the clinic (all of which target CD3). The conceptual similarity of BCEs to their small-molecule counterparts (PROTACs and proximity-inducers) illustrates the benefits associated with such a therapeutic approach: catalytic and immunogenic eradication of disease-causing cells.

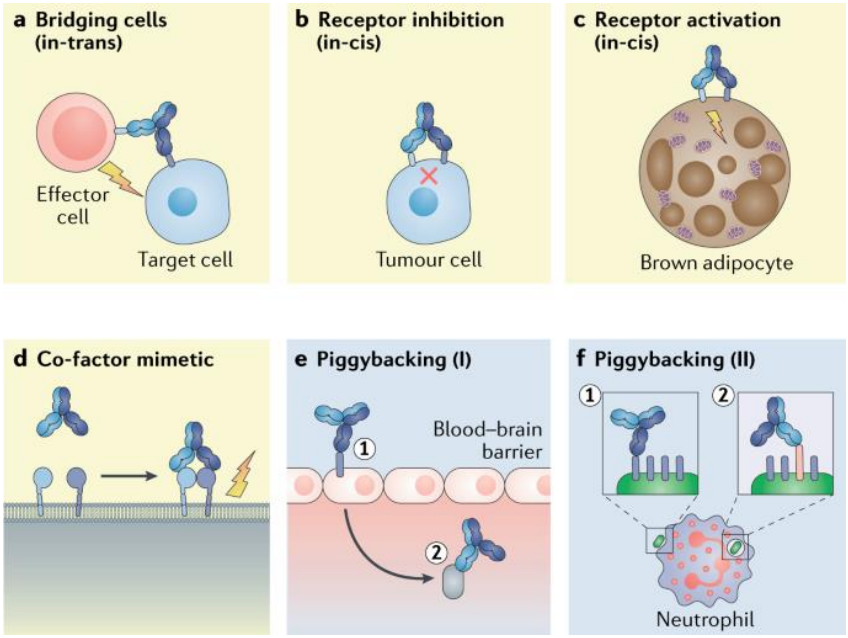


Figure 10 – Various bsAB mechanisms of action.[37]

BCEs (and similar immunotherapeutics such as BiTEs) have several common hurdles that are considered in drug development, the first which of which is a direct consequence of immunogenic mechanisms of action. In cases where T-cells are extensively activated upon treatment, patients can experience cytokine release syndrome (CRS) due to release of inflammatory cytokines by white blood cells. This initiates positive feedback loops to induce further cytokine release, possibly leading to an array of adverse effects such as organ failure upon

dysregulation. Drug-resistance through epitope loss is another key challenge that can invalidate BCE efficacy, as with most cancer-therapeutics, although there have been measures taken to circumvent it. There also exist inherent limits in the scope of amenable epitopes due to their presence on healthy cells as well, whereby targeting them would result in significant side-effect profiles. Avenues to counter this limitation are being explored with specific delivery that leverages matrix metalloproteases unique to the cancers and BCEs that rely on two surface epitopes to engage their mechanism of action.[1]

Tetherbodies as Precision Payload Carriers

A different type of multispecific drug is one that does not act through induced proximity upon engaging a target and an effector, but rather uses one binding element to enrich the other at a relevant location. Such drugs, termed “tetherbodies” by Raymond J. Deshaies, pose benefits associated with precise delivery: decreased toxicity and lower dosage/higher efficacy.[1] The siRNA (small interfering RNA) space has implemented said principles by constructing siRNA-triGalNAc constructs that operate similarly to LYTACs. As triGalNAc-labelled cargo binds in a multivalent fashion to the liver-specific ASGR1 membrane protein such that the cargo is endocytosed, resulting in specific siRNA uptake by liver cells for genetic knockout (Fig. 11b). Antibody-cytokine fusions work similarly but instead cooperatively bind targets to enact their function (Fig. 11c) from the extracellular surface. Concentrating cytokines at cytokine-receptors on cancers has been used to promote immune responses, but it is an approach met with toxic effects. An antibody-cytokine fusion strategy is also being implemented where isolated cytokine binding is weakened, but also cooperatively compensated for by antibody-mediated antigen binding. The fusion will thus only effectively bind at cell surfaces that contain both the antigen for antibody-recognition and the cytokine receptor, therefore introducing precision.

The most successful example of a “tetherbody” is the antibody-drug conjugate (ADC), which comprise a cytotoxic payload tethered to a monoclonal antibody (mAb) through a chemical linker.[38] The antibody component binds to a cancer-specific antigen (CD3 or HER2, for example) that, upon binding, facilitates cellular internalization of the ADC. The cytotoxic payload is released after internalization through different mechanisms depending on the linker’s nature: non-cleavable linkers release the payload after the mAb is degraded in the endosome/lysosome system, and cleavable linkers release the payload upon protease (lysosome/endosome protease, β-

glucuronidase) or glutathione activity. Mylotarg (or gemtuzumab ozogamicin) was the first cancer-targeting ADC to obtain FDA-approval in 2001, which is a status 11 more ADCs have obtained since.

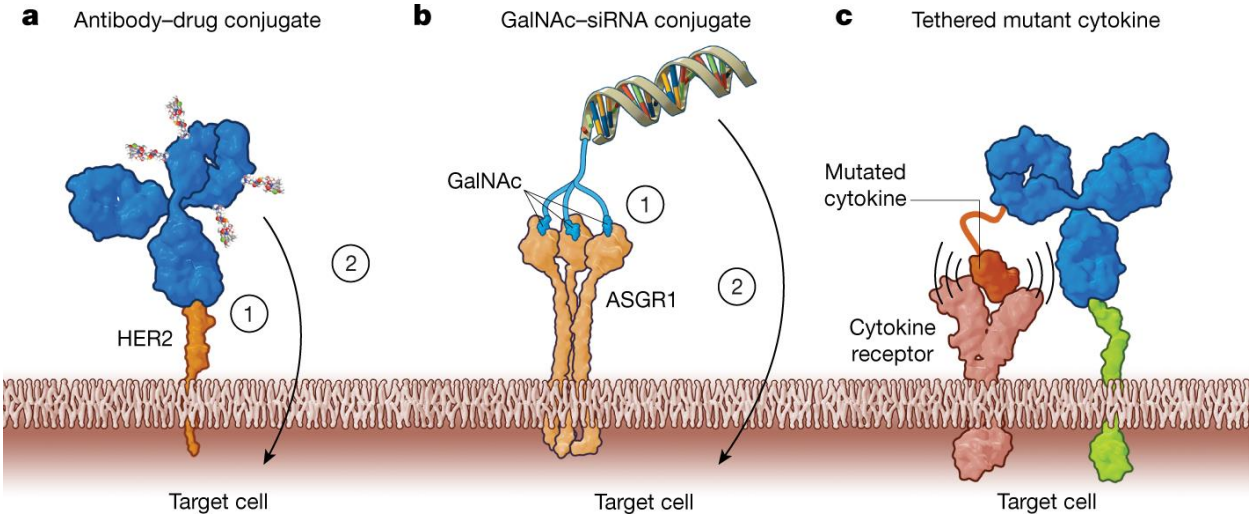


Figure 11 – Examples of tetherbodies that localize drugs at relevant locations.[1]

Bitopic Drugs: Orthosteric + Allosteric Pharmacophore Fusions

Classic examples of multispecific drugs illustrate both the power of inducing proximity between two targets (matchmakers) and the precision that comes with sequential/concurrent binding of two targets (tetherbodies). In the construction of bitopic ligands, however, the principles of multispecific binding have been applied to a single target.[39–43] Such ligands consist of an allosteric binding element linked to an orthosteric binding element to engage two distinct sites on the same protein. Due to their immense physiological importance and engagement in complex allosteric and signalling pathways, G-protein coupled receptors (GPCRs) have historically been the primary subject of bitopic design efforts.[39–43]

G-protein coupled receptors (GPCRs) are integral membrane proteins characterized by a hepta-helical transmembrane structure. Their involvement in signalling pathways spans a vast number of physiological processes such as olfaction, taste, and vision. They bind endogenous extracellular ligands and are coupled to numerous signaling partners such as G proteins and β-arrestins. GPCRs are thus ideal druggable and disease-linked targets in medicinal chemistry efforts, serving as the targets for 30-40% of drugs currently on the market. GPCR drugs are challenged with a lack of subtype specificity and signalling bias (selectively engaging one signalling pathway over another), both of which are associated with a host of negative

pharmaceutical properties. Orthosteric sites are highly conserved across GPCR subtypes while allosteric sites tend to demonstrate greater differentiation and can even be more allosterically responsive. Bitopic drugs aim to leverage this by simultaneously binding both sites to overcome said challenges in a manner reminiscent of antibody-cytokine fusions, all the while possibly introducing greater affinity due to cooperativity.

The muscarinic acetylcholine receptor (mAChr) was the first GPCR target of engineered bitopics, and it has since been the most studied model for bitopic design. Reasons for this include its allosteric depth and the availability of defined extracellular structures near its orthosteric pocket (such as extracellular loops). In a pioneering study, Disingrini et. al developed subtype-specific "hybrid" agonists for M_2 receptors consisting of the orthosteric agonist oxotremorine and various allosteric modulators. The advent of GPCR structural and pharmacological elucidation has facilitated drug design, resulting in the emergence of bitopic ligands with greater affinity and subtype-specificity for various GPCRs (e.g., ß-adrenergic receptors, adenosine receptors, µ-opioid receptors) over their single-pharmacophore counterparts.[39–43]

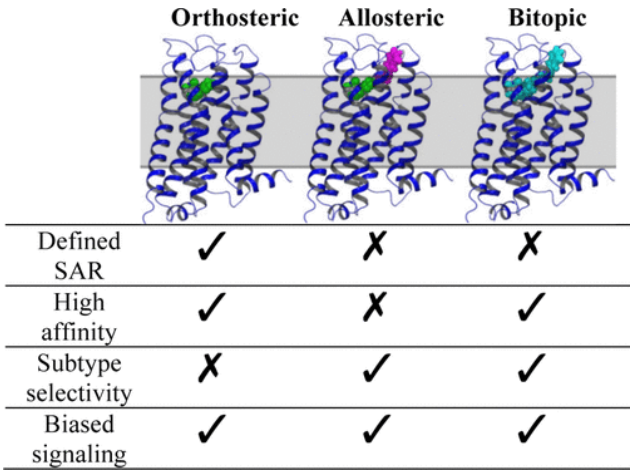


Figure 12 - Contrasts between conventional and bitopic GPCR ligands.[40]

Conclusion and Scope

The paradigm shift from 1-target-1-drug design to multispecific approaches has been tightly associated with the illumination of novel chemical biology. This is historically apparent in the development of molecular matchmakers – one of the most successful and popular types of multispecific drugs – as they harness and redirect natural biology for therapeutic purposes, circumventing the needs of occupancy-based approaches. The invention of rationally engineered PROTACs sparked the next generation of proximity-inducing molecules that engage pathways

other than the ubiquitin-proteasome system, resulting in a toolkit of chimeric small molecules that can be applied to several indications. Some notable technologies that were not discussed here include molecular glues that are able to induce proximity using a single binding element and deubiquitinase-targeting chimeras (DUBTACs) that stabilize targets instead of degrading them.[1,44,45] The concept has been extended beyond molecular targets and effectors to even the cellular scale (BCEs), such that more comprehensive immunogenic responses can be incurred. At the same time, "tetherbodies" such as antibody-conjugates illustrate multispecificity as a tool to localize cytotoxins at therapeutically relevant locations, a strategy that has yielded significant success in the clinic. Perhaps most distinctly, bitopic ligands depict a return to occupancy-driven pharmacology where multispecific approaches are being implemented to create safer, more efficacious, and functionally selective drugs for classical targets such as GPCRs.

Such molecules bring improved efficacy and pharmaceutical properties, but they also face unanimous challenges to overcome before entering clinical settings.[1] First, they have an increased tendency to be of higher molecular of weight and non-compliant of Lipinski's rule of 5 when compared their monospecific counterparts. This can negatively impact pharmacokinetics: multispecific molecules are more prone to aggregation and proteolysis or metabolism. Their manufacturability and mechanisms are also more complex, meaning synthetic/conjugation techniques and thorough elucidation of relevant biology can serve as limiting factors when navigating later stages of drug development. While multispecific drugs are a large source of optimism in the next generation of pharmaceuticals, their success will hinge on the mesh between chemistry, biology, and the computational sciences.

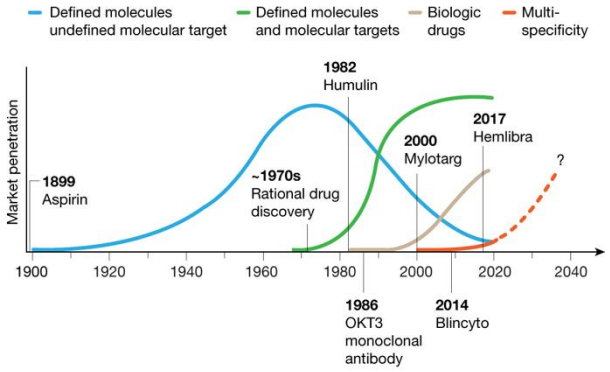


Figure 13 - The major waves of pharmaceutical discovery throughout history.[1]

References:

1. Deshaies, R. J. Multispecific drugs herald a new era of biopharmaceutical innovation. *Nature* vol. 580 329–338 Preprint at https://doi.org/10.1038/s41586-020-2168-1 (2020).
2. Hajduk, P. J., Huth, J. R. & Tse, C. Predicting protein druggability. *Drug Discov Today* **10**, 1675–1682 (2005).
3. Hopkins, A. L. & Groom, C. R. The druggable genome. *Nat Rev Drug Discov* **1**, 727–730 (2002).
4. Perola, E., Herman, L. & Weiss, J. Development of a Rule-Based Method for the Assessment of Protein Druggability. *J Chem Inf Model* **52**, 1027–1038 (2012).
5. Liu, T. & Altman, R. Identifying Druggable Targets by Protein Microenvironments Matching: Application to Transcription Factors. *CPT Pharmacometrics Syst Pharmacol* **3**, 93 (2014).
6. Volkamer, A., Kuhn, D., Grombacher, T., Rippmann, F. & Rarey, M. Combining Global and Local Measures for Structure-Based Druggability Predictions. *J Chem Inf Model* **52**, 360–372 (2012).
7. Li, K. & Crews, C. M. PROTACs: past, present and future. *Chemical Society Reviews* vol. 51 5214–5236 Preprint at https://doi.org/10.1039/d2cs00193d (2022).
8. Békés, M., Langley, D. R. & Crews, C. M. PROTAC targeted protein degraders: the past is prologue. *Nature Reviews Drug Discovery* vol. 21 181–200 Preprint at https://doi.org/10.1038/s41573-021-00371-6 (2022).
9. Cotton, A. D., Nguyen, D. P., Gramespacher, J. A., Seiple, I. B. & Wells, J. A. Development of Antibody-Based PROTACs for the Degradation of the Cell-Surface Immune Checkpoint Protein PD-L1. *J Am Chem Soc* **143**, 593–598 (2021).
10. Sun, X. *et al.* Protacs: Great opportunities for academia and industry. *Signal Transduction and Targeted Therapy* vol. 4 Preprint at https://doi.org/10.1038/s41392-019-0101-6 (2019).
11. Lee, J. *et al.* Discovery of E3 Ligase Ligands for Target Protein Degradation. *Molecules* vol. 27 Preprint at https://doi.org/10.3390/molecules27196515 (2022).
12. Kramer, L. T. & Zhang, X. Expanding the landscape of E3 ligases for targeted protein degradation. *Current Research in Chemical Biology* **2**, 100020 (2022).
13. Esteller, M. Non-coding RNAs in human disease. *Nat Rev Genet* **12**, 861–874 (2011).
14. Cooper, T. A., Wan, L. & Dreyfuss, G. RNA and Disease. *Cell* **136**, 777–793 (2009).
15. Connelly, C. M., Moon, M. H. & Schneekloth, J. S. The Emerging Role of RNA as a Therapeutic Target for Small Molecules. *Cell Chem Biol* **23**, 1077–1090 (2016).
16. Costales, M. G., Suresh, B., Vishnu, K. & Disney, M. D. Targeted Degradation of a Hypoxia-Associated Non-coding RNA Enhances the Selectivity of a Small Molecule Interacting with RNA. *Cell Chem Biol* **26**, 1180-1186.e5 (2019).
17. Childs-Disney, J. L. *et al.* Targeting RNA structures with small molecules. *Nat Rev Drug Discov* **21**, 736–762 (2022).
18. Meyer, S. M. *et al.* Small molecule recognition of disease-relevant RNA structures. *Chem Soc Rev* **49**, 7167–7199 (2020).
19. Huotari, J. & Helenius, A. Endosome maturation. *EMBO Journal* vol. 30 3481–3500 Preprint at https://doi.org/10.1038/emboj.2011.286 (2011).

20. Eldeeb, M. A., Zorca, C. E. & Goiran, T. Extracellular protein degradation via the lysosome. *Communications Chemistry* vol. 3 Preprint at https://doi.org/10.1038/s42004-020-00397-8 (2020).
21. Banik, S. M. *et al.* Lysosome-targeting chimaeras for degradation of extracellular proteins. *Nature* **584**, 291–297 (2020).
22. Ghosh, P., Dahms, N. M. & Kornfeld, S. Mannose 6-phosphate receptors: New twists in the tale. *Nature Reviews Molecular Cell Biology* vol. 4 202–212 Preprint at https://doi.org/10.1038/nrm1050 (2003).
23. le Borgne, R. & Hoflack, B. *Mannose 6-Phosphate Receptors Regulate the Formation of Clathrin-coated Vesicles in the TGN*. *The Journal of Cell Biology* vol. 137 (1997).
24. Ahn, G. *et al.* LYTACs that engage the asialoglycoprotein receptor for targeted protein degradation. *Nat Chem Biol* **17**, 937–946 (2021).
25. Zheng, J. *et al.* Bifunctional Compounds as Molecular Degraders for Integrin-Facilitated Targeted Protein Degradation. *J Am Chem Soc* **144**, 21831–21836 (2022).
26. Alabi, S. B. & Crews, C. M. Major advances in targeted protein degradation: PROTACs, LYTACs, and MADTACs. *Journal of Biological Chemistry* vol. 296 Preprint at https://doi.org/10.1016/j.jbc.2021.100647 (2021).
27. Feng, Y., He, D., Yao, Z. & Klionsky, D. J. The machinery of macroautophagy. *Cell Res* **24**, 24–41 (2014).
28. Takahashi, D. *et al.* AUTACs: Cargo-Specific Degraders Using Selective Autophagy. *Mol Cell* **76**, 797-810.e10 (2019).
29. Ramazi, S. & Zahiri, J. Post-translational modifications in proteins: resources, tools and prediction methods. *Database* **2021**, (2021).
30. Tarrant, M. K. & Cole, P. A. The Chemical Biology of Protein Phosphorylation. *Annu Rev Biochem* **78**, 797–825 (2009).
31. Cohen, P. The role of protein phosphorylation in human health and disease. . *Eur J Biochem* **268**, 5001–5010 (2001).
32. Ardito, F., Giuliani, M., Perrone, D., Troiano, G. & Muzio, L. lo. The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review). *Int J Mol Med* **40**, 271–280 (2017).
33. Siriwardena, S. U. *et al.* Phosphorylation-Inducing Chimeric Small Molecules. *J Am Chem Soc* **142**, 14052–14057 (2020).
34. Hua, L. *et al.* Beyond Proteolysis-Targeting Chimeric Molecules: Designing Heterobifunctional Molecules Based on Functional Effectors. *Journal of Medicinal Chemistry* vol. 65 8091–8112 Preprint at https://doi.org/10.1021/acs.jmedchem.2c00316 (2022).
35. Yamazoe, S. *et al.* Heterobifunctional Molecules Induce Dephosphorylation of Kinases-A Proof of Concept Study. *J Med Chem* **63**, 2807–2813 (2020).
36. Chen, P. H. *et al.* Modulation of Phosphoprotein Activity by Phosphorylation Targeting Chimeras (PhosTACs). *ACS Chem Biol* **16**, 2808–2815 (2021).

37. Labrijn, A. F., Janmaat, M. L., Reichert, J. M. & Parren, P. W. H. I. Bispecific antibodies: a mechanistic review of the pipeline. *Nature Reviews Drug Discovery* vol. 18 585–608 Preprint at https://doi.org/10.1038/s41573-019-0028-1 (2019).
38. Khongorzul, P., Ling, C. J., Khan, F. U., Ihsan, A. U. & Zhang, J. Antibody–Drug Conjugates: A Comprehensive Review. *Molecular Cancer Research* **18**, 3–19 (2020).
39. Kamal, M. & Jockers, R. Bitopic ligands: all-in-one orthosteric and allosteric. *F1000 Biol Rep* **1**, (2009).
40. Lane, J. R., Sexton, P. M. & Christopoulos, A. Bridging the gap: bitopic ligands of G-protein-coupled receptors. *Trends Pharmacol Sci* **34**, 59–66 (2013).
41. Valant, C., Robert Lane, J., Sexton, P. M. & Christopoulos, A. The Best of Both Worlds? Bitopic Orthosteric/Allosteric Ligands of G Protein–Coupled Receptors. *Annu Rev Pharmacol Toxicol* **52**, 153–178 (2012).
42. Faouzi, A. *et al.* Structure-based design of bitopic ligands for the μ-opioid receptor. *Nature* (2022) doi:10.1038/s41586-022-05588-y.
43. Fronik, P., Gaiser, B. I. & Sejer Pedersen, D. Bitopic Ligands and Metastable Binding Sites: Opportunities for G Protein-Coupled Receptor (GPCR) Medicinal Chemistry. *J Med Chem* **60**, 4126–4134 (2017).
44. Toriki, E. S. *et al.* Rational Chemical Design of Molecular Glue Degraders. *bioRxiv* 2022.11.04.512693 (2022) doi:10.1101/2022.11.04.512693.
45. Henning, N. J. *et al.* Deubiquitinase-targeting chimeras for targeted protein stabilization. *Nat Chem Biol* **18**, 412–421 (2022).
46. Zarrin, A. A., Bao, K., Lupardus, P. & Vucic, D. Kinase inhibition in autoimmunity and inflammation. *Nat Rev Drug Discov* **20**, 39–63 (2021).
47. Huehls, A. M., Coupet, T. A. & Sentman, C. L. Bispecific T-cell engagers for cancer immunotherapy. *Immunol Cell Biol* **93**, 290–296 (2015).
48. Nolan, O. & O'Kennedy, R. Bifunctional antibodies: concept, production and applications. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* **1040**, 1–11 (1990).
49. Bhullar, K. S. *et al.* Kinase-targeted cancer therapies: progress, challenges and future directions. *Mol Cancer* **17**, 48 (2018).
50. Borsari, C., Trader, D. J., Tait, A. & Costi, M. P. Designing Chimeric Molecules for Drug Discovery by Leveraging Chemical Biology. *J Med Chem* **63**, 1908–1928 (2020).
51. Li, Z., Zhu, C., Ding, Y., Fei, Y. & Lu, B. ATTEC: a potential new approach to target proteinopathies. *Autophagy* **16**, 185–187 (2020).
52. Ji, C. H. *et al.* The AUTOTAC chemical biology platform for targeted protein degradation via the autophagy-lysosome system. *Nat Commun* **13**, 904 (2022).
53. Disingrini, T. *et al.* Design, Synthesis, and Action of Oxotremorine-Related Hybrid-Type Allosteric Modulators of Muscarinic Acetylcholine Receptors. *J Med Chem* **49**, 366–372 (2006).
54. Kruse, A. C. *et al.* Muscarinic acetylcholine receptors: novel opportunities for drug development. *Nat Rev Drug Discov* **13**, 549–560 (2014).

55. Corson, T. W., Aberle, N. & Crews, C. M. Design and applications of bifunctional small molecules: Why two heads are better than one. *ACS Chemical Biology* vol. 3 677–692 Preprint at https://doi.org/10.1021/cb8001792 (2008).
56. Li, Z. *et al.* First small-molecule PROTACs for G protein-coupled receptors: inducing α1A-adrenergic receptor degradation. *Acta Pharm Sin B* **10**, 1669–1679 (2020).
57. Goebeler, M. E. & Bargou, R. C. T cell-engaging therapies — BiTEs and beyond. *Nature Reviews Clinical Oncology* vol. 17 418–434 Preprint at https://doi.org/10.1038/s41571-020-0347-5 (2020).
58. Smalley, J. P., Cowley, S. M. & Hodgkinson, J. T. Bifunctional HDAC therapeutics: One drug to rule them all? *Molecules* vol. 25 Preprint at https://doi.org/10.3390/molecules25194394 (2020).
59. Gerry, C. J. & Schreiber, S. L. Unifying principles of bifunctional, proximity-inducing small molecules. *Nature Chemical Biology* vol. 16 369–378 Preprint at https://doi.org/10.1038/s41589-020-0469-1 (2020).
60. Modell, A. E., Lai, S., Nguyen, T. M. & Choudhary, A. Bifunctional modalities for repurposing protein function. *Cell Chemical Biology* vol. 28 1081–1089 Preprint at https://doi.org/10.1016/j.chembiol.2021.06.005 (2021).
61. Ponomarenko, E. A. *et al.* The Size of the Human Proteome: The Width and Depth. *Int J Anal Chem* **2016**, 1–6 (2016).
62. Samarasinghe, K. T. G. & Crews, C. M. Targeted protein degradation: A promise for undruggable proteins. *Cell Chem Biol* **28**, 934–951 (2021).
63. Rath, V. L. *et al. Human liver glycogen phosphorylase inhibitors bind at a new allosteric site*. (2000).
64. Doak, B. C., Over, B., Giordanetto, F. & Kihlberg, J. Oral druggable space beyond the rule of 5: Insights from drugs and clinical candidates. *Chemistry and Biology* vol. 21 1115–1142 Preprint at https://doi.org/10.1016/j.chembiol.2014.08.013 (2014).
65. Finan, C. *et al. The druggable genome and support for target identification and validation in drug development*. https://www.science.org.
66. Aebersold, R. *et al.* How many human proteoforms are there? *Nature Chemical Biology* vol. 14 206–214 Preprint at https://doi.org/10.1038/nchembio.2576 (2018).
67. Coleman, N. & Rodon, J. Taking Aim at the Undruggable. *American Society of Clinical Oncology Educational Book* e145–e152 (2021) doi:10.1200/edbk_325885.
68. Li, K. & Crews, C. M. PROTACs: past, present and future. *Chem Soc Rev* **51**, 5214–5236 (2022).
69. Hanan, E. J. *et al.* Monomeric Targeted Protein Degraders. *Journal of Medicinal Chemistry* vol. 63 11330–11361 Preprint at https://doi.org/10.1021/acs.jmedchem.0c00093 (2020).