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Perspective

Inhibitors to degraders: Changing paradigm in drug discovery

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SUMMARY

The chemical understanding of biological processes provides not only a deeper insight but also a solution to abnormal biological functioning. Protein degradation, a natural biological process for debris removal in the cell, has been studied for years. The recent finding that natural degradation pathways can be utilized for therapeutic purposes is a paradigm shift in the drug discovery approach. Methods such as Proteolysis Targeting Chimera (PROTAC), lysosomal targeting chimera, hydrophobic tagging, AUtophagy TArgeting Chimera, AUTOphagy TArgeting Chimera and several other variants of these methods have made a considerable impact on the way of drug design. Few selected examples testify that a huge wave of change is on the way. The drug design based on the targeted protein degradation is a powerful tool in our arsenal. More molecules will be invented that will uncover the hidden secrets of biological functioning and provide enduring solutions to several unmet medical needs.

INTRODUCTION

Living systems are always challenged by a variety of life-threatening diseases and the solutions to some exist, but far more awaits discovery. Our understanding of biological processes, coupled with chemical creativity will be cornerstones of future development in drug discovery. The interaction of biomacromolecules with small molecules and restricting its function is the underlying principle of drug development. The design of these bioactive molecules could be based upon natural products, on traditional knowledge systems or are totally synthetic non-natural chemical products. The use of small molecules to trigger, suppress, alter, or control biological systems is a strategy of drug design and has been in use to correct pathological conditions.

Utilizing the cellular machinery for correcting the aberrant cellular behavior, by using small chemical molecules is a powerful approach but not fully tapped by scientists. The reason lies in our incomplete knowledge of complex cellular processes and inadequate utilization of biochemical approaches.

The enormous progress in drug discovery resulted from the symbiosis of chemistry and biology. The response of biological systems upon interference using synthetic molecules will provide new insights into the living system. Engineered designer small molecules could act as chemical tools to either disturb, control, or streamline biological processes.

Protein homeostasis is the strategy by which cells regulate the intracellular concentration of proteins and eliminate damaged and unfolded proteins. In eukaryotes, the dysfunctional proteins are cleared by two pathways; proteasomal and lysosomal pathway. The proteasomal clearance removes short lived proteins and involves ubiquitination followed by its degradation and the pathway is known as ubiquitin-proteasome system (UPS).[1] Ubiquitin-mediated proteolysis is a major cellular pathway for intracellular protein degradation, besides lysosome-mediated protein degradation and maintains cellular homeostasis by eliminating misfolded and damaged proteins. The UPS participates in the degradation of more than 80% of cellular proteins.[2] Ubiquitin is a 76-residue protein that is attached to target proteins via a lysine iso-peptide bond. Ubiquitin has seven lysine (K) residues: K6, K11, K27, K29, K33, K48, and K63. K48 and K63 linkages are most abundantly found in mammalian system. The K48 of ubiquitin is conjugated in proteasomal degradation targeted proteins and directs tagged protein to 26S proteasome for degradation, while K63 linked protein is directed to the lysosomal pathway. The targeted protein degradation (TPD) cellular pathway, is exploited as a therapeutic strategy, for the first time in 1999.[3] TPD ensures the near-complete removal of pathogenic proteins within minutes to hours of action[4] in contrast to conventional inhibitors that only sterically hinders with the activity of target protein.

The ubiquitination of the target protein is performed by the sequential action of three enzymes namely E1-E2-E3 in a catalytic cascade mode acting sequentially.[5] This process starts with the C-terminal activation of ubiquitin by adenosine triphosphate (ATP), followed by a reaction with E1 (ubiquitin-activating enzyme), leading to an E1-ubiquitin complex. The activated ubiquitin is then transferred to the ubiquitin-conjugating enzyme E2, generating an E2-ubiquitin complex. Finally, E3 (ubiquitin ligase) recognizes the target protein and catalyzes the transfer of ubiquitin from E2 to the target protein at ε-amino group of lysine residues. The development of an engineered small molecular system that targets desired protein and directs it to degradation by proteasome is an effective and enduring therapeutic strategy. Hijacking

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the UPS, using small molecules, to direct the undesirable protein, or mutated protein from the cytosol/nucleus for degradation is like imposing a cellular correction strategy.

This approach of using small molecules as messengers for self-correction of cellular mistakes is unlikely to invite any biological resistance. The initial idea for the development of degrader systems, was proposed by Nabet et al.,[6] who synthesized dTAG (degradation tag) molecules of varying selectivity and potency, that induced the degradation of $FKBP^{F36V}$ (destabilizing domain) fused endogenous protein, by linking it with the E3 ligase complex, cereblon (CRBN). The dTAG system hijacks the cellular proteasomal system to degrade cellular proteins fused with $FKBP^{F36V}$ tag. This approach was successfully utilized to chemically downregulate divergent proteins such as HDAC1, MYC, EZH2, and PLK1. Both the heterobifunctional degraders, dTAG7 and dTAG13 were potent enough to degrade the target proteins at sub micromolar concentrations.[6]

Further, the proof-of-principle of targeted degradation of protein of interest (POI) was developed in 2001.[7] The authors developed a molecule with one end binding to POI and the other end acting as a ligand to the E3 ligase. This resulted in the ubiquitination of POI, followed by proteasomal degradation and the molecule is regenerated in the end.

RATIONALE

Disease phenotypes are majorly associated with an underlying aberrant functioning of pathological proteins. The traditional drugs act by binding to the active site of the protein and impeding its activity, leading to its functional silencing. Targeting of pathological proteins by traditional inhibitors comes with the disadvantage of off-target effects. Also, some proteins such as membrane proteins and transcription factors lack a defined catalytic pocket, making them undruggable by traditional therapeutics. Moreover, enzymatic inhibitors pose the risk of mutation driven drug resistance in the target protein. Recent advances in chemical approaches have led to the discovery of chimeras that are capable of TPD. These chimeras can eliminate "undruggable targets," protein aggregates and even cellular organelles, that was previously unachievable by traditional inhibitors. These "degraders" bring about chemical knockdown of pathological proteins, have a long-lasting effect, and poses minimum risk of mutation. These unique features have extended the applicability of these degraders in both research and clinical settings.

Molecular glues: Chemical molecule that mediates protein-protein interaction

Molecular glues (MGs) are small molecules that possess intrinsic property to induce interaction between two proteins. They promote the dimerization or colocalization between two unrelated proteins via formation of a ternary complex. These induced interactions can be between a target protein and a ubiquitin ligase, resulting in its proteasomal degradation or between two unrelated proteins, resulting in perturbation of signaling cascades.

Thalidomide is one such example which was serendipitously discovered as a molecular glue degrader. Thalidomide and its derivatives were found to have immunosuppressive, immune-modulatory, and anti-cancer activities.[8] These pharmacological activities of thalidomide were discovered, years after its ban in clinical settings due to its serious side effects, such as birth defects (deformed limbs). Thalidomide and its derivatives bind to E3 ligase and the target protein leading to its E3 mediated ubiquitination and targets it for degradation. Thalidomide and lenalidomide act as the MG that facilitates protein-protein interaction (PPI) between E3 ligase and the transcription factors, IKZF1 and IKZF3,[9] while pomalidomide links E3 to $IKZF_1$ and $CK1_\alpha$. The glueing of these transcription factors with E3 ligase, induces their proteasomal degradation and this explains the anti-multiple myeloma activity of these drugs.[10]

Another example of MG is, cyclosporin A (CsA) and FK506 (Figure 1) which are powerful immunosuppressants, that functions by inhibiting T cell activation.[11] Though both are structurally different, but their mechanisms of action are similar. CsA made organ transplants possible, though it was discovered before the term MG was even introduced. CsA inhibits "peptidyl-prolyl" isomerase activity of cyclophilin protein. The 1:1 complex of CsA-cyclophilin then binds to calcineurin and inhibits its phosphatase action. The calcineurin mediated de-phosphorylation of the nuclear factor of activated cells (NFAT) is crucial for its nuclear translocation, where it induces transcription of downstream cytokine genes such as IL-2, IL-4, TNF-α, and IFN-γ. Thus, CsA blocks the nuclear translocation of NFAT and NFAT mediated transcription of cytokine genes that are crucial for T cell activation.[12] These are examples of non-degrader MGs that does not induces the UPS mediated degradation of the target protein, however, they inhibit crucial cellular signaling pathways.

Lu and coworkers discovered four MGs, based on a small-molecule microarray-based screening that specifically tethers mutant huntingtin protein with the autophagosome protein, LC3 thereby promoting its autophagic clearance.[13] These include the compounds AN1, 10O5, AN2, and 8F20 (Figure 1C). Another MG, BI-3802 induces the homopolymerization of the B cell lymphoma-6 (Bcl-6) oncogenic transcription factor.[14] These filaments are then ubiquitinylated by the E3 ligase SIAH1 resulting in its proteasome-mediated degradation.

Since MGs are chemical species of low molecular weight, they are more drug alike, with good bioavailability and pharmacodynamic parameters. Also, MG can also be used as warheads for designing of Proteolysis Targeting Chimera (PROTAC) molecules.

Limitations of molecular glues

Since many MGs such as immunomodulatory drugs target crucial transcription factors, they can induce teratogenicity in the system. The mode of action of many MGs is poorly understood. Also, MG binds to a variety of targets which are often unpredictable and could exhibit distinct biological activities.[15] For instance, CsA promoted the gefitinib (EGFR inhibitor)-induced apoptosis of non-small cell lung cancer, via inhibition of the STAT3 pathway.[16] CsA was also found to suppress growth of prostate cancer by inhibiting the CaMKKβ/AMPK/mTORC1 signaling arm.[17]

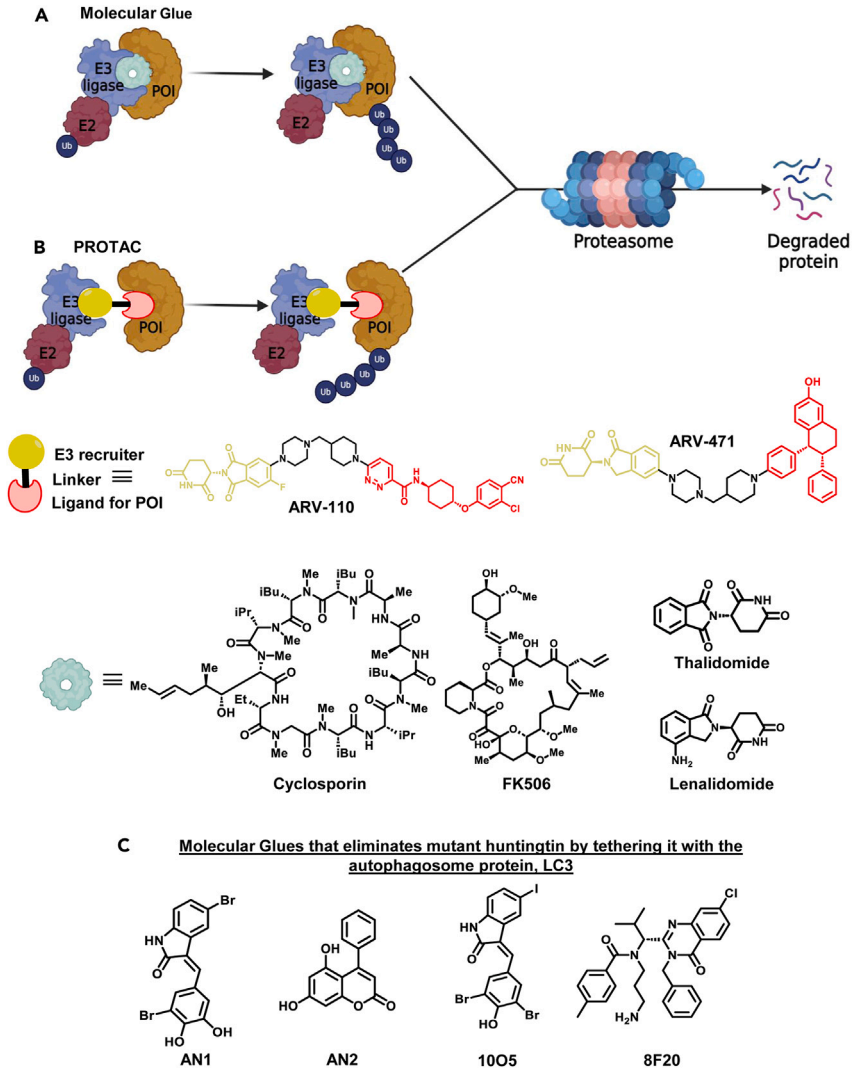

Figure 1. Mode of action of molecular glues and PROTACs

(A) Schematic diagram showing the mechanism of action of molecular glues. Cyclosporin, FK506, Thalidomide, and Lenalidomide act as molecular glues that brings protein of interest (POI) in proximity with E3 ligase, mediating its polyubiquitination and ultimately its proteasomal degradation.

(B) PROTACs are heterobifunctional molecules composed of an E3 recruiter and ligand for the target protein, connected by a linker. PROTACs also promote the close association between E3 ligase and POI, thereby mediating polyubiquitination of target protein. The polyubiquitinated target protein is then degraded by the cellular proteasome machinery. Structures of the PROTACs, ARV-110 and ARV-471, that targets the oncogenic proteins; androgen, and estrogen receptors are also shown, respectively.

(C) Chemical structures of molecular glues that tethers mutant hungtintin with the autophagosome protein LC3, mediating its autophagic clearance.

Overcoming the limitations of molecular glues

It is believed that more than about 3,00,000 protein-protein interactions (PPIs) takes place in human cells.[18] MGs are molecules that bind with POI and E3 ligase, thereby facilitating PPIs between two proteins. The MG degraders have been discovered mostly by fortuity, not by rational design approaches.[19] Therefore, *in silico* modeling and virtual screening approaches could facilitate their discovery process. Additionally, the high-throughput proteomics methods will speed up the discovery of MG degraders. Moreover, the molecular mechanisms, degradation rate, pharmacokinetic properties of each lead MG degrader should also be determined.

Advancements in PROTAC strategies

PROTACs are heterobifunctional molecules that are composed of two moieties connected by a linker. One moiety binds to the POI and the other binds and recruits E3 ubiquitin ligase. The E3 ligase initiates the polyubiquitinylation of the target protein, directing it for degradation by UPS, thereby enabling the knockdown of the target protein.[5] The formation of ternary complex (POI-PROTAC-E3) is crucial for the action of

PROTAC molecule. This brings the POI and E3 ligase in close proximity, to facilitate the ubiquitin transfer reaction, and achieving this is a challenging task. After triggering the polyubiquitinylation of the target protein, PROTAC molecule is recycled back for binding to another POI molecule. Unlike traditional inhibitors, PROTACs are catalytic in nature and can promote target protein degradation, even at low concentrations[20] (Figure 1B). Thus, PROTACs have a utility at low concentration, in the range of nanomoles to picomoles and exhibits higher target selectivity.[21] PROTACs were mostly designed to recruit the human E3 ligases such as Von Hippel-Lindau (VHL) tumor suppressor and CRBN.

Over the years, several PROTAC technologies such as selective androgen receptor degrader (SARD),[22] hydrophobic tagging (HyT), and selective estrogen receptor degrader (SERD)[23] were developed, that entered preclinical and clinical trials. However, several cellular E3 ligases are not exploited for PROTACs and absence of a versatile pan ubiquitinating E3 ligase is one of the limiting factors of the PROTAC strategy.

The conventional drug designs that are based upon small molecule binding to a protein target has serious disadvantage, as only 25% of the human cellular proteome can be targeted.[24] The remaining 75% of proteins including membrane proteins and transcription factors lacks a binding/catalytic site, hence the occupational model fails to work. Moreover, conventional inhibitors bind to allosteric or catalytic sites to mediate its inhibitory action, whereas PROTAC requires only an accessible binding surface on the target protein.[25] This feature has extended the targetability of PROTACS to undruggable proteins such as scaffolding proteins, transcription factors, that lacks a well-defined catalytic site. The utilization of PROTAC to such proteins expands the scope of protein-degradation-based drug discovery.

Within the limited time, two potential cancer targeting PROTAC compounds, ARV-110 and ARV-471 have entered Phase II clinical trials (Table 1).[5] ARV-110 targets the androgen receptor which is a driver of prostate cancer, while ARV-471 targets the estrogen receptor which drives breast cancer. However, the PROTAC strategy could only target cytosolic protein, thereby sparing a large number of transmembrane and extracellular proteins.

Subsequently, the targetability of PROTAC was further extended to non-cytosolic proteins, by using a specific DNA aptamer as a warhead. Aptamers are short DNA or RNA oligonucleotides, which folds into a three-dimensional structure and binds to the target protein with high specificity. Tan and co-workers,[26] designed a nucleolin targeting PROTAC (ZL216), consisting of a 26 base ssDNA aptamer namely AS1411 conjugated with the VHL-E3 ligase recruiter AHPC.[26] This Aptamer-based PROTAC induced the degradation of cell surface localized nucleolin and effectively killed breast cancer cells both *in vitro* and *in vivo*.

Limitations of PROTAC

Though PROTAC opened numerous opportunities in drug discovery and attempted to revolutionize the area of chemical biology, it also faces several challenges. The PROTACs so far reported are designed to target very few ubiquitin ligases (VHL, CRBN, MDM2, and cIAPs), though approximately more than 600 E3 ligases are known. One of the remarkable highlighted features of PROTACs is its ability to act on "undruggable" biological targets; however, only limited cases have been reported. More examples of PROTACs in this category awaits discovery. An accurate method for evaluation of the pharmacokinetics (PK) and pharmacodynamics (PD) properties of PROTACs is still lacking.[20] Most of the designed PROTACs target cytosolic domain, thereby limiting its scope. The evaluation of protein degradation and avoiding off-target effects require more studies.

Also, the relatively high molecular weight of these molecules (700–1500 g/mol) results in low solubility, reduced oral bioavailability, poor cell permeability, and low blood brain barrier penetration capability.[27,28]

Overcoming the limitations of PROTAC

As of now, only very few design attempts are made on PROTACs. The use of high throughput screening and utilization of combinatorial chemistry approaches will speed up the discovery process. Very limited examples of peptide based PROTACs are reported, and more attempts in this direction are needed. The live cell platform for monitoring the real time degradation kinetics will provide much needed insight into the future PROTAC designs.[29]

PROTAC molecules can be developed into nanoformulations in order to enhance the cellular permeability and its retention in the biological system. These nanoformulations include lipid, polymeric or metallic based nanoparticles which offers advantages of biocompatibility, biodegradability, and increased stability.[30]

Nanoparticles carrying PROTAC molecules can also be surface conjugated with antibody against membrane proteins expressed specifically on cancer cells.[30] This antibody conjugated nanoparticles, can be utilized to specifically target solid tumors, and reduce the associated toxicity on normal cells.

HyT: Targeting cellular proteins by making them appear hydrophobic

Another extension of TPD is HyT which harnesses the fact that exposure of hydrophobic residues on the protein surface marks it as an unfolded protein,[31,32] ultimately eliminating the protein through UPS or autophagy. HyT consists of a hydrophobic tag (such as adamantane) linked with the target binding ligand. The binding of HyT to a POI, increases the hydrophobicity of the target protein that results in the recruitment of heat shock proteins, ultimately targeting it for proteasomal degradation.[27] Recently, Xu and co-workers[33] reported a bridged cyclic hydrocarbon, norbornene as a tag for TPD. The binding of a hydrophobic norbornene unit to the target protein, mimics misfolded protein, thereby degrading the POI by chaperone-mediated UPS, bypassing the E3 ligase-mediated polyubiquitination (Figure 2). The authors used ligands for anaplastic lymphoma kinase (ALK) fusion protein, which is oncogenic in nature. For targeting of ALK, alectinib was linked to a hydrophobic tag (Norbornene) by various linkers (Hyt-7 to Hyt-10). Hyt-9 was found to be the best candidate amongst all and degraded ALK with

Table 1. Summary table of TPD technologies

TPD Platform	Representative cellular target	Degradation Machinery	Preclinical/Clinical Trial Status		
			Name of molecule	Target protein	Clinical Indication
PROTAC	Intracellular cytosolic proteins	Ubiquitin-Proteasome system	Phase II clinical trial		
			ARV-110	Androgen receptor	Prostate cancer
			ARV-471	Estrogen receptor α	Breast cancer
			Phase I clinical trial		
			AC682	Estrogen receptor	Breast cancer
			ARV-766 & CC-94676	Androgen receptor	Prostate cancer
			DT2216	BCL-X_L	Liquid and solid tumors
			KT-474	Interleukin-1 receptor associated protein 4 (IRAK4)	Autoimmune diseases
			KT-333	STAT3	Liquid and solid tumors
			KT-413	IRAK 4	MYD88-mutant diffuse large B-cell lymphoma
			NX-2127 & NX-5948	Bruton tyrosine kinase (BTK)	B cell malignancies
			FHD-609	Bromodomain containing 9 (BRD9)	Synovial sarcoma
			HSK29116 & BGB-16673	BTK	B cell malignancies
			GT-00029	Androgen receptor	Androgenic alopecia and acne
LYTAC	Extracellular and membrane proteins	Lysosomal degradation system	Cetuximab (Ctx)-M6Pn	EGFR	Breast and liver cancer
			Apt-LYTAC	Platelet-derived growth factor (PDGF)	Cancer
Hydrophobic tagging (HyT)	Cytosolic proteins	Chaperone-mediated ubiquitin proteasomal system	HyT-9	anaplastic lymphoma kinase (ALK)	Tumors
			MS1943	enhancer of zeste homolog 2	Triple-negative breast cancer cells
			TX1-85-1	oncoprotein Her3	Lung and ovarian cancer cell lines
AUTAC	Cellular proteins and damaged organelles	Ubiquitin-mediated selective autophagy	AUTAC1	Methionine aminopeptidase 2 (MetAP2)	–
			AUTAC2	FK506-binding protein (FKBP12)	–
			AUTAC3	transcriptional regulator Brd4	–
			AUTAC4	translocator protein of outer mitochondrial membrane	–
AUTOTAC	Degradation resistant protein aggregates	Selective macroautophagy (ubiquitin independent)	PHTPP-1304	Estrogen receptor β	Renal carcinoma and breast cancer cells
			Vinclozolin M2-2204	Androgen receptor	Prostate cancer cells
			Fumagillin-105	MetAP2	Glioblastoma cells
			PBA-1105 & PBA-1106	Mutant tau protein (tauP301L)	Proteinopathies
			Anle138b-F105	Mutant tau protein (tauP301L)	Proteinopathies

Comprehensive table indicating each TPD platform, their representative cellular targets, degradation machinery utilized by them and their detailed preclinical/clinical trial status. The preclinical/clinical trial status of each TPD platform is subcategorized into: name of the molecule, their target protein, and the clinical indication for which the molecule is designed.

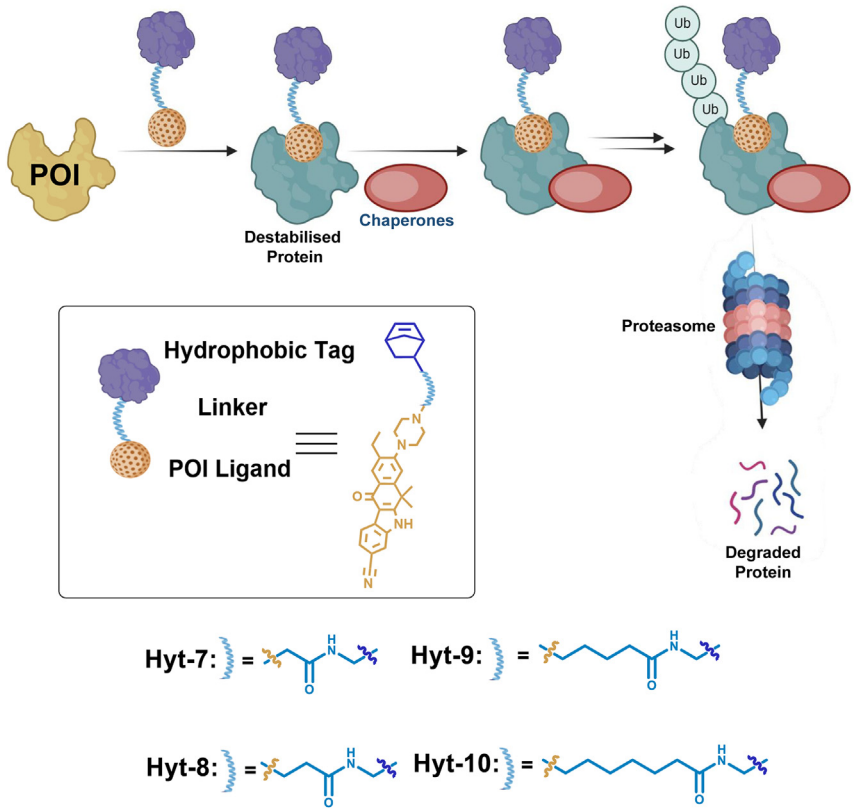

Figure 2. Schematic diagram of targeted protein degradation with hydrophobic tagging strategy

Hydrophobic tag (HyT) degrader consists of a hydrophobic tag (Norbornene here) linked with a target specific ligand. When bound to the target protein, it increases its hydrophobicity that mimics a misfolded protein thereby recruiting cellular chaperones and the target protein is cleared through chaperone mediated ubiquitin proteasomal system. The chemical structure of four HyT molecules, targeting the oncoprotein ALK and utilizing Norbornene as the hydrophobic tag is also shown. The four HyT molecules differ only in the length of linker.

a half-maximal degradation concentration (DC_{50}) of 134 nM. 100% inhibition of ALK phosphorylation was observed after 2 h of treatment with Hyt-9.

HyT technology has also been employed for silencing of oncoproteins such as the pseudokinase Her3, transcriptional co-activator SRC-1, androgen receptor.[25] These molecules were also found to have growth attenuating effect upon a variety of cancers.

Limitations of HyT

The off-targeting effect of HyT has not been explored as it might possibly perturb the cellular unfolded protein response (UPR) pathway.[34] Also, the hydrophobic moiety of HyT might tend to bind non-specifically to hydrophobic stretches of other cellular proteins.

Possible solutions

The propensity of HyT in inducing UPR should be investigated in a cell line model and on a variety of cell lines. This can be monitored by detecting phosphorylated forms of UPR markers namely, IRE1α, PERK, and eIFα in cells treated with HyT by western blotting.[35] The cleavage pattern of ATF6α can also be assessed in cells treated with HyT, as it undergoes cleavage into a 50kDa form, in an ER stress responsive manner. The accumulation of unfolded cellular proteins structures, upon exposure to HyT, can also be visualized by transmission electron microscopy (TEM).

LYTACs: Expanding the scope of protein degradation

Extracellular proteins that include various cytokines, immune complexes, and growth factors play a crucial role during disease progression. However, their extracellular localization makes them inaccessible to UPS and hence untargetable by PROTACs.[36] To overcome this challenge, lysosomal targeting chimera (LYTAC) was discovered, which targets non-cytosolic proteins thereby expanding the scope of protein degradation. Lysosomes are a site of degradation for both intracellular and extracellular proteins through autophagy and endocytosis.[37] Lysosomal degradation is an evolutionarily conserved pathway for clearing non-functional proteins and proteins of extracellular origin.

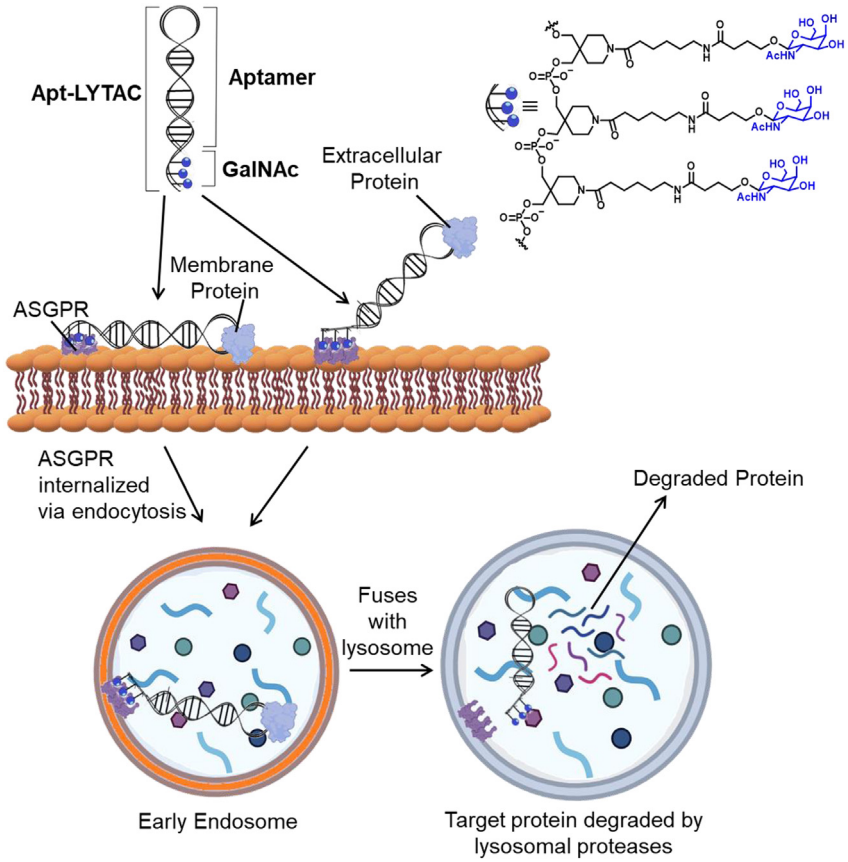

Figure 3. Schematic diagram of the mode of action of aptamer based LYTAC (Apt-LYTAC) for degradation of extracellular/membrane proteins

Apt-LYTAC utilizes RNA based aptamers that are specific to the target protein, conjugated to trivalent N-acetyl galactosamine (GalNAc), which binds to asialoglycoprotein receptors present on cell membrane. LYTACs induces the bridging of extracellular target proteins and asialoglycoprotein receptors (ASGPR), facilitating receptor mediated internalization of the protein. The internalized target protein is finally degraded by lysosomal proteases. The receptor and LYTAC molecule remain intact and are recycled back to the cell membrane.

The first generation LYTAC utilized the ubiquitously expressed cation independent mannose-6-phosphate receptor (CI-M6PR) that transport proteins bearing N-glycans capped with mannose-6-phosphate residues to the lysosomal compartment.[38] The LYTAC consisted of a target protein specific antibody or inhibitor molecule conjugated with mannose-6-phosphonate oligopeptide, which serves as agonists of CI-M6PR. This directs the POI to the recycling receptor, CI-M6PR facilitating its internalization and further lysosomal degradation.[39]

Another study exploited the rapidly recycled hepatocytic receptor, asialoglycoprotein receptor (ASGPR), for facilitating the depletion of extracellular and membrane proteins. The natural ligand for ASGPR, triantennary N-acetylgalactosamine (tri-GalNAc) was conjugated with antibodies and antibody fragments for generating diverse LYTAC molecules.[40]

ASGPR was further exploited by Zhu and coworkers[41] who designed an aptamer-based LYTAC (Apt-LYTAC) system, wherein a target protein specific RNA-aptamer was linked to tri-GalNAc. This Apt-LYTAC bound the ASPGR at a nanomolar concentration, and internalizing the receptor ligand complex via clathrin-mediated endocytosis (Figure 3).

The aptamer in LYTAC binds to platelet-derived growth factor (PDGF)-BB, which is involved in cancer metastasis, proliferation, and invasion. The evaluation of the Apt-LYTAC in HepG2 cells showed efficient degradation of extracellular PDGF. The authors also extended their system to a *trans*-membrane receptor, protein tyrosine kinase-7 (PTK7) which plays a role in tumor initiation and invasion.[42] The chimera harbors an aptamer specific for PTK7 and the tri-GalNAC tag to facilitate lysosomal-mediated degradation of PTK7. The smaller sizes of aptamers compared to antibodies encourages their internalization, and are also easy to synthesize.

This research quickly evolved from curiosity-driven investigation to the discovery of life-saving drugs. One of the most powerful features of this strategy is that the approach can be tailor-made for the discovery of drugs for many diseases.

Limitations of LYTACs

Despite of its substantial applications in depleting extracellular and membrane proteins by targeting them to lysosomal degradation pathway, LYTACs have some shortcomings. The relatively large molecular size and less *in vivo* stability, reduces its effectiveness.[43] The antibodies and glycopeptides moieties in LYTACs, can also induce a strong immune response in the system.[43,44]

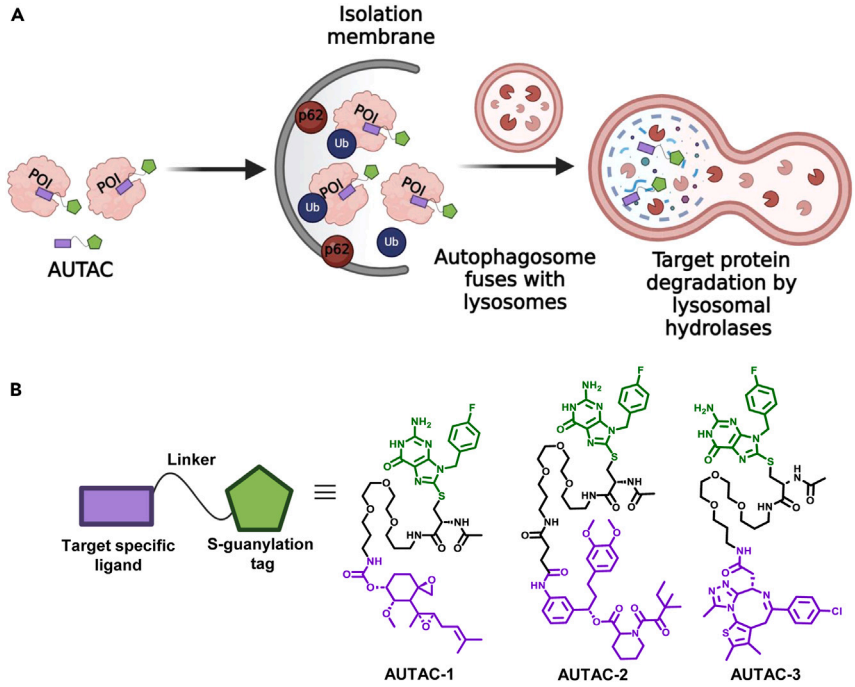

Figure 4. Mechanism of action of ubiquitin-mediated autophagy targeting molecular degrader: AUTAC

(A) Schematic diagram showing the mechanism of action of AUTAC, which is composed of a modified guanine tag, linked to a target specific warhead tag. AUTAC binds to the target protein and triggers its K63-linked polyubiquitination, which is then cleared through lysosome-mediated degradation.

(B) Structures of AUTACs designed to chemically downregulate a variety of target proteins (MetAP2, FKBP12, and BET). The modified guanine tag moiety in each AUTAC is highlighted in green and the target specific warheads are highlighted in violet.

Overcoming the limitations of LYTACs

The immune responses against LYTAC moieties can be reduced by modifying the mode of delivery, thereby minimizing its direct encounter with the immune system. Additionally, the antibody and glycopeptide moieties can be selectively masked and made available only at the time of its biological action. Moreover, the mannose-6-phosphonate oligopeptide can be replaced with small molecules that bind potently to CI-M6PR, which could reduce the effective size of LYTAC molecule, thereby minimizing the immune activation.

AUTAC and AUTOTAC for targeted autophagy-based protein disposal

The eradication of large protein aggregates, damaged and dysfunctional organelles, that are resistant to degradation poses a problem for proteasomal degradation; hence macroautophagy-based degradation pathway is a desirable option. Macroautophagy is the process by which cytoplasmic contents such as aggregated proteins, lipid droplets, and even damaged organelles are selectively sequestered into autophagosomes and fused with lysosomes for degradation.[45] This cellular homeostasis pathway is utilized by both AUtophagy TArgeting Chimera (AUTAC) and AUTOphagy TArgeting Chimera (AUTOTAC). AUTAC marks a protein for ubiquitin mediated selective autophagy and hence are ideal for clearance of proteasomal degradation-resistant protein aggregates and even damaged organelles. AUTOTAC on the other hand targets a protein for degradation through ubiquitin independent selective autophagy.

AUTAC utilizes modified guanine as a degradation tag, a warhead to target POI and a flexible linker. AUTAC is composed of an S-guanylation tag and target specific binder, that facilitates accelerated K63 polyubiquitination (Figure 4A). These Ub chains are recognized by the ubiquitin-associated (UBA) domain of adaptor protein p62, followed by autophagic degradation of the target by lysosomal hydrolases. Mitochondria targeting AUTAC (mito-AUTAC), also promoted mitophagy of dysfunctional fragmented mitochondria.[46]

The proof-of-concept was demonstrated using AUTAC-1, which could bind to the endogenous protein methionine aminopeptidase 2 (MetAP2) and an S-guanylation tag for tagging MetAP2 for autophagic degradation. Fumagillol was used as a warhead, as it is known to bind covalently to MetAP2.[46] Experiments revealed that AUTAC could chemically silence MetAP2 via lysosomal degradation pathway through autophagy.

In another design, the epigenetic anti-cancer agent JQ-1 acid was used as the warhead to design AUTAC-3. JQ-1 is a known binder of bromodomain and extra terminal domain (BET) family consisting of Brd2, Brd3, and Brd4. Brd4 is a transcriptional regulator that plays a critical role in maintenance of melanoma and its nuclear localization makes it a challenging target. Increasing concentrations of AUTAC-3 reduced the level of Brd4, expanding the horizons of AUTACs in targeting nuclear proteins as well. The chemical structures of designed AUTAC 1, 2, and 3 is represented in Figure 4B.

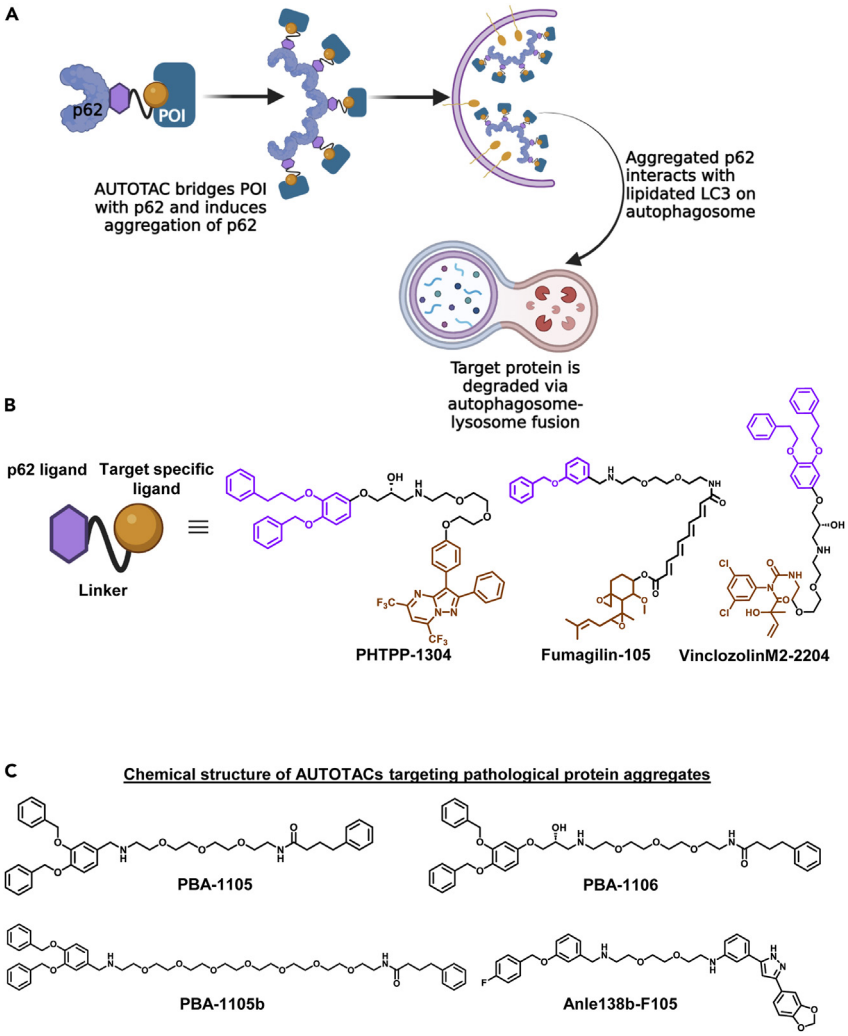

Figure 5. Mechanism of action of macroautophagy targeting AUTOTACs

(A) AUTOTACs are bifunctional molecules composed of autophagy targeting ligands and target binding ligands, that bridges together the autophagic receptor, p62 and POI. This bridging induces a conformational change in p62, which induces its polymerization along with the target protein and activates macroautophagy at the site, resulting in autophagy mediated degradation of the target protein. AUTOTACs are effective in elimination of oncoproteins and aggregated proteins.

(B) Chemical structures of various synthetic AUTOTAC molecules designed to degrade the oncoproteins, ERβ (PHTPP-1304), MetAP2 (Fumagillin-105), and AR (VinclozolinM2-2204).

(C) Chemical structure of various AUTOTACs that are designed to eliminate pathological protein aggregates. PBA-1105, PBA-1106 targets mutant tau protein, PBA-1105b targets mutant desmin protein and Anle 138b-F105 targets mutant huntingtin protein, resulting in their clearance via macroautophagy.

Thus, the AUTAC-based system with an S-guanyl entity utilizes ubiquitination-dependent lysosomal degradation.[46]

In order to develop a degradation platform, that is independent of ubiquitin and proteasomal machinery, AUTOTAC, namely PHTPP-1304, vinclozolin M2-2204, fumagillin-105 were developed by Kwon et al.[47] These bifunctional molecules bind with POI and ZZ domain of p62/sequestosome 1, resulting in a conformational change and subsequent oligomerization of p62, forming target-p62 complex. This also exposes the LC3 interacting region (LIR) domain on p62, that further interacts with lipidated LC3 present on autophagic membranes, ultimately leading to sequestration and autophagic degradation of the target protein, in a multi-step manner[47] (Figure 5A). The pull-down assay using biotinylated p62 ligands (YT-8-8, YOK-2204, YOK-1304, YTK-105) confirmed the mechanism of degradation. The synthesized AUTOTAC, PHTPP-1304 composed of p62 binding moieties linked to ERβ synthetic ligand (PHTPP), by a repeating PEG moiety, caused degradation of ERβ at DC_{50} of ~2 nM in HEK293T and <100 nM in MCF-7 breast cancer cell line. Similarly, androgen receptor (AR) and MetAP2 targeting AUTOTACs vinclozolin M2-2204 and fumagillin-105 efficiently degraded the target protein in nanomolar concentrations (Figure 5B).

Interestingly, AUTOTACs were also designed for the *in vivo* and *in vitro* degradation of misfolded tau protein aggregates, that are responsible for the pathology of Alzheimer's disease and escape the proteasomal clearance, due to their large size. The authors used 4-phenylbutyric acid (PBA) and Anle138b as target binding ligands (TBL) that recognizes hydrophobic regions present on the misfolded

and aggregated proteins. These TBLs were linked with the p62 ligands, YTK-1105 and YTK-105 and the resulting AUTOTACs (PBA-1105 and Anle138b-F105) efficiently induced autophagic degradation of pathological tau aggregates. The chemical structures of few protein aggregate targeting AUTOTACs are presented in Figure 5C. The evidences obtained so far, validates the robust efficacy of AUTOTACs in targeted proteolysis of both intracellular proteins and protein aggregates.

Limitations of AUTACs and AUTOTACs

Despite of the versatility of AUTAC in clearing oncogenic proteins and damaged organelles, they do carry some limitations. The molecular mechanism operating behind the S-guanylation mediated K63-ubiquitination of target proteins is unclear and hence poses a risk of off-targeting.[27]

Moreover, the global effect of AUTOTACs on cellular machinery is not explored yet as AUTOTACs molecules may influence the cellular autophagy pathways.[48] Thus, further investigation is required to probe the potential off-target effects of AUTOTACs.

Overcoming the limitations of AUTACs and AUTOTACs

For elucidating the mechanism for S-guanylation mediated K63 polyubiquitination, pull-down assay followed by mass spectrometry, using this moiety could be utilized for finding interacting protein partners from cell lysates. The probable protein binders/interactors of S-guanylation tag can provide a glimpse about the protein mediators that recognize the tag and recruit the ubiquitination machinery at the site.

The global effect of AUTOTACs on cellular proteome can be checked by carrying out comparative proteomics under untreated and AUTOTAC treated conditions. The changes in protein abundance or their post-translational modification status in response to AUTOTAC treatment, can reveal the cellular pathways perturbed by these molecules.

Future perspectives

PROTACs have been exploited for wide ranging diseases such as cancers (Table 1), neurodegenerative diseases, fatty liver disease, and cardiovascular diseases. Recently they have been implemented for management of bacterial and viral diseases as well; however, PROTACs have not been utilized for tackling of infectious diseases. Parasitic diseases such as Malaria (caused by *Plasmodium falciparum*), leishmaniasis and trypanosomiases have limited therapeutic options. Reports of artemisinin resistance in some regions of Southeast Asia are an alarming situation, and novel approaches to target the intraerythrocytic development of the parasite are in great demand. To our knowledge, there is no report of PROTACs molecules designed to target parasitic proteins.

The *P. falciparum* genome encodes for nine ubiquitins or Ub-like proteins, eight E1 or E1-like activating enzymes, fourteen E2 or E2-like conjugating enzymes and fifty four E3 or E3-like ligases.[49] Inhibitors of ubiquitylation pathway were effective in inhibiting the growth and development of *P. falciparum* (in submicromolar range), suggestive of a functional UPS during the intraerythrocytic development stages.[50] Another study reported the expression of UBA1 (E1), UBC (E2), HRD1 (E3), and confirmed their *in vitro* ubiquitylation activity.[51]

Also, various studies have reported the rapid growth inhibitory action of proteasome inhibitors against asexual stages of *Pf* and the stage specific ubiquitinome of parasite is also reported.[50] All these body of evidences suggest that the proteasomal machinery of *Pf* can be harnessed for targeted degradation of parasitic undruggable targets. Interestingly, *Pf* encoded E1 and E2 enzymes are conserved across eukaryotes, whereas *Pf* E3 ubiquitin ligase has high level of divergence,[52] which can be exploited to identify parasite specific E3 recruiters. This can be carried out by *in silico* screening of chemical libraries against the specific *Pf* E3 ligase. The ligands with favorable docking scores can then be taken forward for confirmation of their interaction with cellular E3 ligase by pull-down assays and cellular thermal shift assays. The potent E3 recruiter molecules can thus be linked with a variety of ligands specific to crucial parasitic protein, to yield novel TPD based antimalarials.

Moreover, parasite encoded host cell invasion ligands, transcription factors, virulence factors, scaffold proteins mediate their function through PPIs and lacks a functional active site. Since PROTAC transiently binds to accessible binding surface on the target protein, these pathogen encoded proteins can serve as worthwhile candidates for TPD. Also, parasites such as *Toxoplasma gondii* and *Leishmania* spp. infects differentiated human cells, adding an advantage for employing human E3 ligases to degrade parasite crucial proteins and exported virulence factors.

Moreover, increasing body of evidence suggests the presence of ubiquitin-proteasomal pathway proteins in mature erythrocytes.[53,54] Another study reported the presence of functional 20S proteasome in mature RBCs.[55] The erythrocytic proteasomal machinery can be utilized to degrade mutated forms of hemoglobin such as HbS, HbC, HbE, by designing degrader molecules that specifically targets abnormal hemoglobin variants. These degraders can be used as adjunctive therapy, (along with drugs to induce HbF and blood transfusions) to ameliorate the complications associated with hemoglobinopathies.

CONCLUSION

The investigations so far unequivocally showed that protein degradation strategy is a powerful yet very promising druggable strategy for perpetual removal of pathological proteins. Conversion of protein degradative pathway, a natural process, to small molecule-directed destruction of the protein of our choice, has unlimited possibilities in both academia and industry. Already several designs such as PROTAC, AbTAC, AUTAC, AUTOTAC, and LYTAC have emerged as a result of the last two decades of research. The utility of the discovery is evident from

numerous drugs entering the drug discovery pipeline and clinical trials. The coming decade will revolutionize drug discovery at an unprecedented rate, presumably by introducing drugs to most of the terminal diseases. TPD is an example of how academic curiosity leads to a solution to the long-standing sufferings of humanity.

The emergence of drug resistance is an ongoing issue and needs urgent intervention. In response to this, and to propel the future drug exploration, novel strategy that involves leveraging cellular machinery to rectify irregular biological functions via small molecules, is need of the hour. Although hurdles do exist due to incomplete comprehension of cellular processes and lack of appropriate chemical biology tools. Contemporary advancements are centered around the application of TPD as a therapeutic avenue to chemically downregulate pathological proteins. Here, small molecules are tailored to guide specific proteins toward degradation pathways, thus offering a versatile means to rectify cellular errors while circumventing biological resistance. Approaches such as PROTAC, HyT, LYTAC, AUTAC, and AUTOTAC, as discussed, are being investigated for a variety of ailments such as cancer, neuropathies, broadening the horizons of drug discovery beyond conventional practices. This field exhibits substantial potential to transform drug exploration and tackle the prevailing issue of drug-resistance and concerning health issues.

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AUTHOR CONTRIBUTIONS

V.H.: conception of idea, manuscript writing, proofreading of the manuscript, S.D.: manuscript writing, construction and compilation of figures, A.M.: manuscript preparation, compilation of table and figures S.S.: conception of idea, proofreading of the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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