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Linkers as Game-changers in PROTAC Technology: Emphasizing General Trends in PROTAC Pharmacokinetics for their Rational Design

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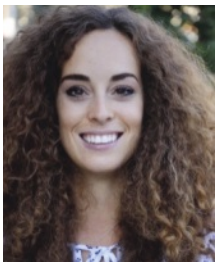
Abstract: Proteolysis Targeting Chimeras (PROTACs) are heterobifunctional molecules that act as degraders. They selectively remove disease-associated proteins by hijacking the Ubiquitin-Proteasome System (UPS). Chemically, they consist of three parts: an E3 ligase ligand, a target of interest (TOI) ligand, and a linker, which connects the two moieties. The rapid expansion of PROTAC technology as an innovative therapeutic modality in cancer fostered the drug discovery effort to optimize their physicochemical properties. Due to their large size, their features are far from the traditional 'drug-like' properties. This short review highlights some of the main structural features in the linker component to optimize the PROTAC Drug Metabolism and Pharmacokinetics (DMPK) profile. In particular, we discuss aspects related to solubility, cell permeability, active transporters efflux, and metabolic stability.

Keywords: Drug metabolism and pharmacokinetics · Linker optimization · Proteolysis Targeting Chimeras (PROTACs) · Targeted protein degradation



Carlotta Cecchini studied Pharmaceutical Sciences in Bologna (Italy), where she was awarded a scholarship for two consecutive years (2015/2016). She joined the group of Prof. Leonardo Scapozza (Biochemistry/Chemistry) at the University of Geneva to perform her research Master thesis. After receiving her Master's degree in 2017, she started her PhD in the same group at the University of Geneva. Concomitantly to the PhD, she obtained her title as Qualified Pharmacist at the University of Ferrara in 2018. Carlotta's research focuses on designing and synthesizing heterobifunctional molecules called Proteolysis Targeting Chimeras (PROTACs), which act as targeted degraders. Through this innovative approach in drug discovery, her work aims at investigating dysregulation of the Ubiquitin-Proteasome System (UPS) in cancer.

From Lipinski's rule of 5 (Ro5) to bRo5 (beyond Ro5)

Modulating the chemical structure of a drug candidate to optimize its absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties represents a challenging task in drug discovery. The available ADMET tools have been mainly used for predicting and measuring small molecules properties, which stick to Lipinski's rule of 5 (Ro5). These guidelines describe the 'drug-like' features – MW ≤500, logP ≤5, H-bond donors (HBDs) ≤5, H-bond acceptors (HBAs) ≤10 – of orally active compounds that have driven the classical drug discovery in the last decades. The rapid expansion of Ro5 chemical space led to the development of new chemical entities, ranging from peptides or peptidomimetics to Proteolysis Targeting Chimeras (PROTACs).[1] PROTAC technology has emerged as a new modality to selectively degrade disease-related proteins by hijacking the ubiquitin-proteasome system (UPS).[2]

PROTACs belong to the bRO5 class of compounds since their physicochemical features are far from the traditional chemical space. This includes high molecular weight (MW >800), high polar surface area (PSA >200 Å), a high number of rotatable bonds, low solubility, and low permeability.[2a] Although new *ad hoc* property-based design strategies are emerging, the development of orally available degraders is still empirical and very laborious.[3]

PROTAC Pharmacokinetics is Affected by its Building Blocks

Each component of a given PROTAC – an E3 ligase binder, a target of interest (TOI) ligand, and a linker – is responsible for affecting its final physicochemical properties. Hence, ligands with good pharmacokinetic (PK) properties should be favored as a starting point to design new degraders. PROTAC warheads usually consist of orally available small-molecule inhibitors, which have been modified with suitable linker attachment points.[2a] Simultaneously, traditional E3 ligase binders used in PROTAC technology, such as thalidomide and its derivatives, are considered acceptable in terms of physicochemical properties and safety.[4] The linker provides the largest flexibility in terms of structural modifications for PROTAC optimization since the structures of the TOI ligand and E3 ligand are restricted by the structure-activity relationship (SAR) and target binding.

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Indeed, other than playing a crucial role in defining the ternary complex geometry, the linker composition and length can affect the final intrinsic solubility, permeability, and metabolic stability of a given PROTAC. Therefore, an attentive focus on structural modifications in the linker is pivotal for PROTAC development.

Linker Impact on PROTAC Solubility

Notably, as the ClogP and the size of compounds increases, their solubility usually decreases.[5]

Low solubility negatively affects compound absorption and bioavailability after oral dosing, and it may result in erratic values from bioassays during the drug development, thus hampering the hit-to-lead process. In general, the minimum solubility required for a compound is strictly dependent on its permeability and dose. High-permeable compounds require lower solubility than low-permeable compounds to achieve maximum oral absorption.[6]

In the context of PROTACs, which have high MW and generally low permeability, an early focus on solubility is pivotal for achieving consistent preclinical efficacy results and oral efficacy in drug discovery.[7] Choosing warheads and E3 ligases that have moderate/high solubility is preferable, but their selection is contingent on the target and the E3 ligase tissue expression.

Chemical modifications in the linker provide the most exploitable route to optimize PROTAC solubility. Increasing ionization at physiological pH, or hydrogen bonding capacity, are among the most common and practical strategies used by medicinal chemists to enhance the aqueous solubility of organic compounds. In a recent work, Wang *et al.* showed that the replacement of traditional linear alkyl and polyethylene glycol (PEG) moieties with saturated nitrogen heterocycles, such as piperidines and/or piperazines, significantly improved the solubility of their compounds, resulting in a more potent degrader such as ARD-69 (Fig. 1a).[8] Likewise, the incorporation of polar piperazine and pyrimidine moieties into the linker led to more soluble and potent compounds (PROTAC 4 and PROTAC 6) (Fig. 1b).[9] It should be noticed that rigidifying the linker with heterocycles may disfavor the ternary complex formation in such a way as negatively affecting the bioactivity of degraders. Therefore, *in silico* and/or experimentally structural studies should always drive the optimal spatial conformation of a degrader prior to the introduction of more rigid linkers.

Linker Impact on PROTAC Cell Permeability

Cell permeability has drawn much attention in PROTAC development.[2a] In particular, increased size of compounds leads to decreased permeability, limiting oral drug absorption.[5]

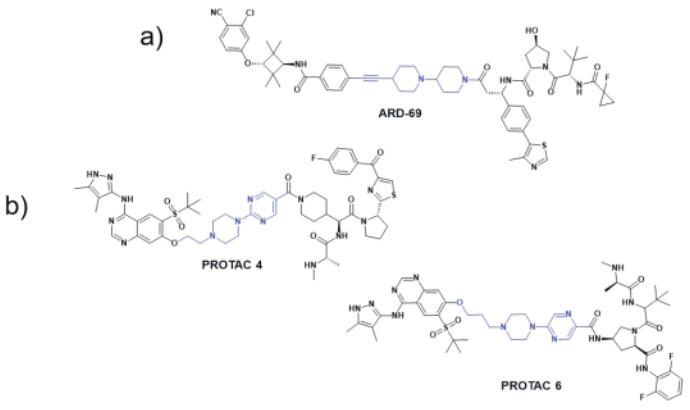


Fig. 1. a) Structure of a potent androgen receptor (AR) degrader for the treatment of prostate cancer (ARD-69); b) Structure of potent PROTACs that selectively degrade Receptor-Interacting Serine/Threonine Protein Kinase 2 (RIPK2) (PROTAC 4 and 6).

The large size of PROTACs makes them very prone to be low permeable compounds. However, despite poor permeability, some degraders still showed potent cellular activity, probably due to their catalytic, event-driven pharmacology.[10]

Besides the MW, the structural modifications to improve compound permeability consist of reducing polarity, modulating lipophilicity (within the range of 3–5 AlogP), and reducing HBDs and HBAs.[10a,11] Therefore, achieving a good balance in terms of compound solubility/permeability is very challenging and still difficult to predict, despite the rapid expansion of *in silico* tools.[1b,3]

In some cases, compound lipophilicity was successfully increased by using short alkyl linkers or by incorporating tertiary amines that minimize the topological polar surface area (TPSA), resulting in improved cell permeability.[10b,12]

Lokey *et al.* provided insight as to how structural changes in the linker affect PROTAC permeability by combining a parallel artificial membrane permeability assay (PAMPA) and a lipophilic permeability efficiency (LPE) metric.[10a] It is noteworthy that these label-free assays consider only passive permeability without the confounding effects of active transport. Their findings showed that solvent-exposed NH groups (*i.e.* amides) of VHL ligand close to the linker attachment points likely contribute to decreasing permeability. Therefore, reducing the presence of solvent-exposed HBDs (by *N*-methylation or removal of amide) or shielding the –NH polarity from solvent (by increasing steric hindrance in proximity) are both powerful strategies to improve compound permeability. This was shown in a recent work, where the *tert*-Leu amide –NH in PROTAC MZ1 could be shielded from the solvent by the *tert*-Leu side chain and by an intramolecular hydrogen bond (IMHB) formed between –NH and the PEG ether (Fig. 2).[10a] Interestingly, the partial shielding provided by the PEG linker generated a PROTAC that was even more permeable than its analog bearing an aliphatic linker.

The same effect is observed in the presence of molecular chameleons, such as macrocycles, where the flexible shielding of amide bonds can enhance compound permeability.[13]

Consistent with this, a PROTAC bearing a relatively long, elongated, and flexible PEG linker showed improved cell permeability by changing its conformation to a less polar one, where IMHBs shielded some of the PROTAC's polarity.[14] These findings prove that PROTAC permeability can be finely modulated in different ways. However, designing degraders that act as molecular chameleons is very challenging as it requires attentive conformational studies.

Recently, Ciulli *et al.* provided a simple strategy to enhance PROTAC permeability and bioactivity through a bioisosteric amide-to-ester substitution in the linker (Fig. 3).[15]

Higher lipophilicity (within the range of 1–4 ALogP) combined with the reduced number of solvent-exposed HBDs were likely responsible for the increased permeability of several linker types.[15] To hamper the susceptibility of esters to hydrolysis, bulky groups were added to the chemical space surrounding the area, thus improving plasma stability.

Overall, these findings suggest that the intrinsic lipophilicity of warhead and E3 ligase binder moieties directly influences the choice of the optimal linker composition.

Linker Impact on PROTAC Efflux

P-glycoprotein (P-gp) is a 170 kDa member of the ATP-binding cassette transporter superfamily (ABCB1) and it has also been referred to as multi-drug resistant protein 1 (MDR1).[16] P-gp is abundant in cell barriers that have a protective function, such as the blood-brain barrier, small and large intestine, liver, kidney, adrenal gland, pregnant uterus.[16]

P-pg-mediated drug efflux represents a major limitation for oral absorption. It has a greater effect when the drug concentration on the luminal surface is low and when the compound has

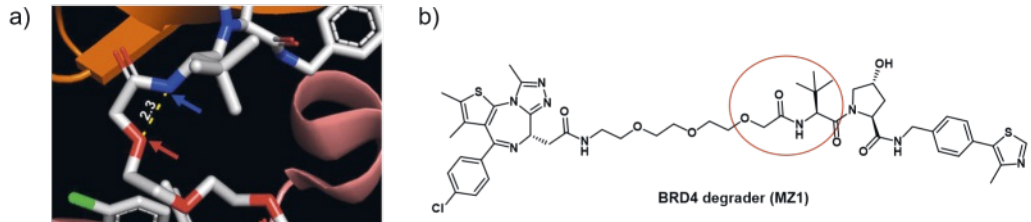


Fig. 2. a) Crystal structure of PROTAC MZ1 (colored by element) in complex with VHL (orange) and Brd4 (pink) (PDB:5T35).[10a] The VHL ligand *tert*-Leu -NH (blue arrow) is shielded by the *tert*-Leu side chain and is within hydrogen-bonding distance of the VHL ligand PEG oxygen (red arrow). b) Chemical structure of PROTAC MZ1. The *tert*-Leu amide is highlighted in the red circle. Figure adapted from ref. [10a].

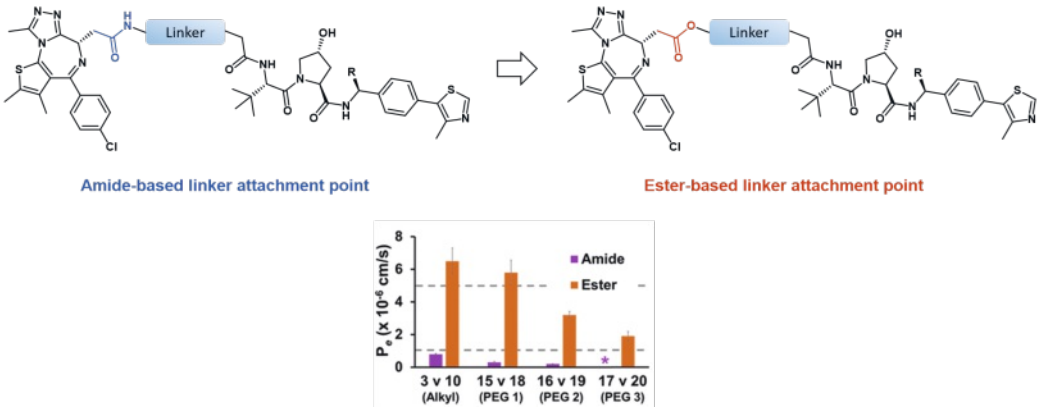


Fig. 3. Amide-to-ester substitution on a set of VHL-based BET degraders to improve cell permeability. The graphic shows the PAMPA permeabilities of model compounds organized by amide (purple) and ester (orange) matched pair. Dashed gray lines represent categorical threshold for poor (P_e <1 × 10^{-6} cm/s), moderate (1 × 10^{-6} cm/s < P_e < 5 × 10^{-6} cm/s), and good (P_e > 5 × 10^{-6} cm/s) membrane permeabilities. Adapted from ref. [15]

low passive diffusion.[6] High P-gp efflux results in poor ADMET processes and reduced exposure of the compound to the therapeutic target. Therefore, in most cases it is generally desirable to circumvent this effect.

Compounds that have N + O ≥8, MW >400, and acid with pKa >4 are more likely to be P-gp substrates.[6] Being large and low permeable molecules, PROTACs are very expected to undergo P-gp efflux.[15,17]

In principle, cancer cells expressing high levels of MDR1 would likely exhibit resistance to degraders. However, even tissues with low or non-detectable MDR1 protein levels might acquire resistance to chronic exposure of PROTACs by upregulation of MDR1.[17] Consequently, PROTACs that displayed high efflux ratios may require concurrent blockade of MDR1 to achieve durable protein degradation and therapeutic response in cancer.

Alternatively, structural modification strategies to evade or reduce the P-gp substrate affinity of compounds might overcome the need for MDR1 blockage. Since P-gp does not have a well-defined drug-binding pocket, the range of its substrate specificity is very broad, including aromatic, aliphatic, or charged compounds.[18] Structure–efflux relationships on a series of lead compounds showed a direct correlation between efflux and the presence of HBAs.[19] In particular, aromatic amides were revealed to be especially susceptible to efflux.[19]

Bulky group introduction, *N*-methylation, removal of hydrogen bonding groups such as amides, are among the structural modifications that were successful in reducing P-gp efflux.[6]

Moreover, the introduction of a strong acid, such as carboxylic acid, may interfere with P-gp binding, resulting in decreased efflux.[6] In principle, applying some of these modifications in the linker might reduce PROTAC affinity for P-gp.

In a recent work, the effect of the linker on Caco-2 permeability and efflux was explored with a set of PROTACs bearing the same warhead and E3 ligand.[10c] Interestingly, the addition of fluorine atoms in the linker motif resulted in low efflux ratios compared to linear linkers, probably due to the increased steric hindrance which lowered the affinity of compounds for P-gp.[10c]

In the attempt of improving PROTAC cell permeability through amide-to-ester substitutions on the linkers, Ciulli *et al.* observed that efflux ratios increased with compound lipophilicity up to an ALogP of around 4.[15] Therefore, if on the one side, highly permeable PROTACs – within the range of 3–5 AlogPs – are very desirable, on the other hand, their higher propensity to actively undergo efflux represents a significant limitation for oral absorption.

The increasing number of experimental data on PROTAC efflux will likely drive the design of PROTACs toward lower P-gp efflux ratios.

Linker Impact on PROTAC Metabolism

Metabolic stability is defined as the susceptibility of a chemical compound to biotransformation and is expressed as *in vitro* half-life ($t_{1/2}$) and intrinsic clearance (Cl_{int}).[20] Based on these values, *in vivo* pharmacokinetic parameters such as bioavailability and *in vivo* half-life can be calculated.[20] Sufficient drug exposure is highly desirable to elicit the biological activity, which, in the case of degraders, includes ternary complex formation and target protein knockdown. Metabolically unstable drugs have relatively high values in Cl_{int} and low values in $t_{1/2}$, which are associated with poor ADME properties. For this reason, strategies to optimize drug metabolic stability are among the most resource-intensive tasks in the pharmaceutical panorama. Identification of soft spot location through metabolite identification experiments (MetID) seems to be the most effective strategy for driving the medicinal chemists' effort towards better metabolic stability.

In a recent work, Goracci *et al.* showed that the linker length and composition have significant impact on PROTAC metabolic stability.[21] Compared to the individual ligands, the introduction of a third element – the linker – was shown to increase the number of soft spots that are responsible for PROTAC liability. PROTACs bearing short linear linkers resulted in better metabolic stability due to fewer soft spots and to the higher steric hindrance

of warhead/E3 binder, which can hamper the interaction with metabolism-devoted enzymes. It was found that the introduction of cyclic groups into the linker, such as piperazine and triazole, resulted in higher metabolic stability. Interestingly, their findings showed that even if the number of soft spots is higher in PEG-like PROTACs due to the multiple *O*-dealkylation reactions, the overall metabolic rate is not necessarily negatively affected. Furthermore, linker attachment points to both warhead and E3 ligand resulted in very labile soft spots, due to *N*-dealkylation and amide hydrolysis reactions.[21]

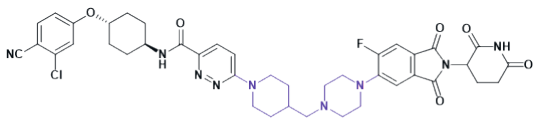
Overall, this study suggests that longer linkers increase the likelihood of degraders to undergo fast metabolism, but a clear trend for improving PROTAC metabolic stability by linker modifications is still missing. Early identification of soft spots may help to accelerate this process towards the generation of metabolically stable compounds.

Synopsis of Linker Structural Modifications

Properties such as solubility and permeability can be fine-tuned by modifying the linker nature – polar/lipophilic – and length, but also the number of HBDs and HBAs.

In principle, short lipophilic linkers containing cyclic ionizable groups would result in more permeable and soluble compounds, which are less prone to metabolic instability and P-gp efflux, but their increased rigidification might change the entire geometry of the ternary complex in such a way as to affect PROTAC efficacy. Therefore, ideal linker modifications would combine both stable ternary complex formations with good DMPK properties (Table 1).

Not surprisingly, the first two PROTACs to entering phase II clinical trials, ARV-110 and ARV-471, are consistent with this trend (Fig. 4).[22] Both compounds bear a short, rigid linker, with incorporated piperidine and piperazine moieties.



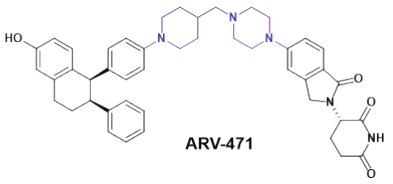


Fig. 4. Structure of ARV-110 and ARV-471. The linker part is shown in violet.

Conclusion

PROTACs' potential as future therapeutics is nowadays evident and supported by a wealth of preclinical efficacy data. Nevertheless, PROTAC intrinsic features – high MW, PSA, number of rotatable bonds, low cell permeability, and poor solubility – make their delivery and bioavailability the most significant hurdles to overcome on the way to the clinic.

So far, optimization of oral degraders have been shown to require a lot of empiricism.

As PROTACs represent independent chemical entities, their DMPK profile cannot be directly predicted from their components. Linker modifications on a set of degraders bearing the same warhead/E3 ligand seem to be the most successful approach for providing a general trend in the DMPK profile and bioactivity of PROTACs. An increasing amount of experimental data on their DMPK properties is fervently expected for guiding PROTAC development into a less time-consuming and more rapid-straightforward process.

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Table 1. Linker structural features affecting PROTAC DMPK profile.

Solubility	Permeability	P-pg efflux	Metabolic stability
• Decrease at high ClogP(>3)	• Decrease at low ClogP (<3)	• Increase with HBAs	• Increase with steric hindrance
• Increase with polar and ionizable groups	• Decrease with polar and ionizable groups	• Decrease with steric hindrance	• Decrease with longer linkers
	• Decrease with HBAs and HBDs	• Decrease with strong acid	• ClogP has a minor effect
	• Increase with IMBDs	• ClogP has a minor effect	

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