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Proteolysis-Targeting Chimeras (PROTACs)

1.1 An Overview of PROTAC Technology and Drug Metabolism and Pharmacokinetic (DMPK) Research Strategies

Chengyuan Li, Yu Wang, Jing Jin

1.1.1 Introduction

In recent decades, significant advances have been achieved in the development of new therapeutic approaches. Proteolysis-targeting chimera (PROTAC) offers a new approach to some disease treatments, which has increasingly attracted the attention of drug developers in the last five years. Additionally, PROTAC, as a new therapeutic technology, has facilitated substantial investment worldwide. In this chapter, we summarized the PROTAC's structure and mechanism of action, its global landscape, the drug metabolism and PK (DMPK) challenges in PROTAC research, and corresponding potential solutions.

1.1.2 Structure and Mechanism of Action of PROTACs

PROTAC utilizes the body's natural protein degradation mechanism, known as the ubiquitin–proteasome system [1]. PROTAC is a bifunctional molecule that consists of three key structural parts: a ligand that binds the target protein, a ligand that binds the ubiquitin–protein ligase (E3), and a linker that connects the two ligands (Figure 1.1).

Because of its unique structure, PROTAC brings the E3 ligase and the target protein to proximity, which induces the E3 ligase to label the target protein with ubiquitin. This causes the target protein to degrade [2]. The PROTAC molecules that detach after the degradation of the target protein can be recycled (Figure 1.2). In contrast to the occupancy-driven pharmacology of most small-molecule drugs, PROTAC can access proteins that were previously inaccessible without occupying an active pocket or relying on target occupancy to disrupt the function of the target protein. This is known as event-driven pharmacology.

Therefore, PROTAC offers many advantages in drug discovery:

- It has the potential to target many undruggable protein targets that lack active sites.
- While retaining the advantage of directly targeting intracellular proteins, it has the potential to overcome some of the disadvantages of small molecules such as drug resistance.
- To improve patient compliance, it can be administered orally.

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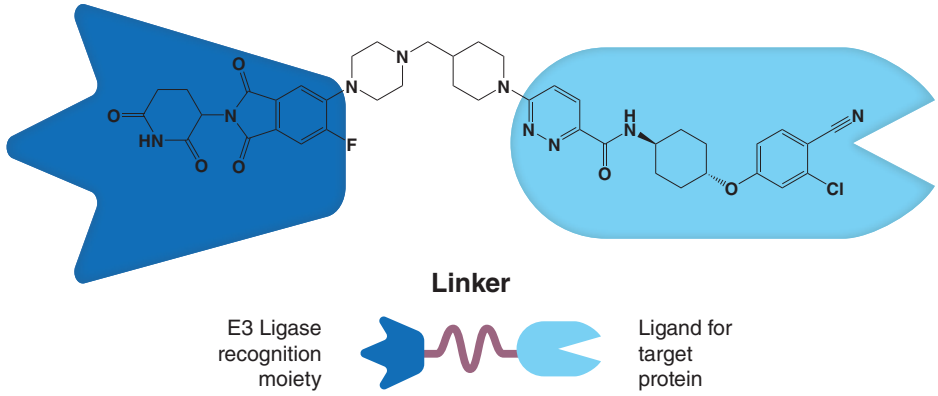


Figure 1.1 Schematic of typical PROTAC structure.

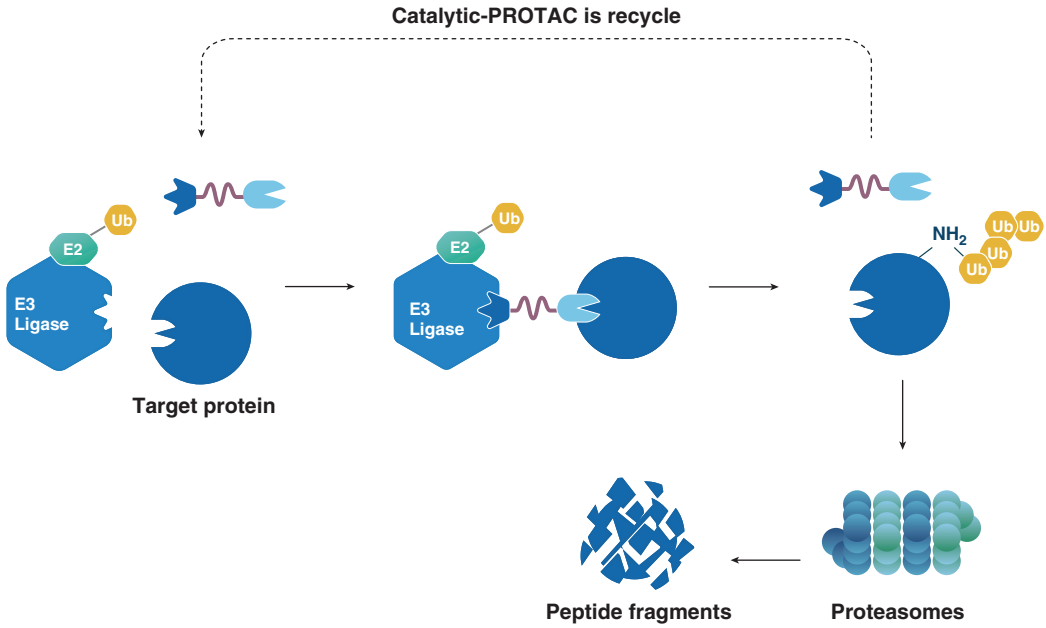


Figure 1.2 Mechanism of action of PROTAC.

1.1.3 PROTAC Landscape Analysis

By using the event-driven pharmacology, PROTAC offers an attractive therapeutic concept to control target protein levels. Although this is a relatively new modality, it has made rapid progress in the drug discovery pipeline.

1.1.3.1 PROTAC Research and Development Progress: International Pharmaceutical Companies

PROTAC has boomed in the last three years with Arvinas' two candidate molecules, ARV-110 and ARV-471, being the first to demonstrate positive clinical data. In addition, ARV-766, the company's

Table 1.1 PROTAC research and development progress of some international pharmaceutical companies.

Company	PROTAC	Indications	Target	Study phase
Arvinas, Inc.	ARV-471	Breast cancer (ER+/HER2-breast cancer)	Estrogen receptor (ER)	Clinical phase III
	ARV-393	B-Cell Malignancies	B-cell lymphoma 6 protein (BCL6)	Clinical phase I
	ARV-102	Parkinson's Disease Progressive Supranuclear Palsy	Leucine-rich repeat kinase 2 (LRRK2)	Clinical phase I
Nurix Therapeutics, Inc.	NX-2127	B cell malignancies	BTK + IMiD activity	Clinical phase I
	NX-1607	Immuno-oncology	CBL-B	Clinical phase I
	NX-5948	B cell malignancies and autoimmune diseases	BTK	Clinical phase I
Kymera Therapeutics, Inc.	KT-474	Allergic dermatitis, hidradenitis suppurativa, rheumatoid arthritis	Interleukin-1 receptor-associated kinase 4 (IRAK4)	Clinical phase II
	KT-621	Immunology	STAT6	Clinical phase I
	KT-333	Solid tumors	STAT3	Clinical phase I
	KT-253	Solid tumors	MDM2	Clinical phase I
C4 Therapeutics, Inc.	CFT7455	Relapsed/refractory non-Hodgkin's lymphoma or multiple myeloma	IKZF1/3	Clinical phase I/II
	CFT8919	Non-small cell lung cancer (NSCLC) with drug-resistant EGFR mutation	EGFR L858R	Clinical phase I
	CFT1946	V600 mutant cancers	BRAF V600	Clinical phase I/II
Bristol-Myers Squibb	AR-LDD	Prostate cancer	AR	Clinical phase I
Dialectic Therapeutics, Inc.	DT-2216	Tumor	BCL-XL	Clinical phase I
Accutar Biotechnology Inc.	AC0699	Breast cancer	ER	Clinical phase I
	AC0682	Breast cancer	ER	Clinical phase I
	Ac176	Prostate cancer	AR	Clinical phase I
	AC0676	Autoimmunity	BTK WT & C481S	Clinical phase I

Note: **The information was obtained from the official websites of the companies** mentioned in the table **and the deadline for information collection is** 15 **December 2024.**
(Only research pipelines with compounds are listed.) Vividion Therapeutics, ERASCA, and other PROTAC companies do not disclose pipeline information.

third PROTAC molecule, is set to enter the clinical phase. Other international companies, such as Kymera Therapeutics, C4 Therapeutics, and Nurix Therapeutics, that focus on developing protein degraders have a molecule in clinical phase I as well. In addition, other pharmaceutical companies such as Bristol-Myers Squibb, Dialectic Therapeutics, and Accutar Biotechnology also own a PROTAC molecule in clinical phase I (Table 1.1).

Table 1.2 PROTAC R&D progress of Chinese pharmaceutical companies.

Company	PROTAC	Indications	Target	Study phase
Lynk Pharmaceuticals Co., Ltd.	LNK01002	Hematological tumor	—	Clinical phase I
Kintor Pharmaceutical Limited	GT20029	Androgenetic alopecia, acne	AR	Clinical phase II
Haisco Pharmaceutical Group Co., Ltd.	HSK29116	B-cell malignancies	BTK	Clinical phase I
BeiGene Ltd.	BGB-16673	B-cell malignancies	BTK	Clinical phase I
Cullgen Inc.	CG001419	Solid tumor	TRK	Clinical phase I/II
Hinova Pharmaceuticals, Inc.	HP518	mCRPC for standard treatment failure	AR	Clinical phase I/II
	HP568	Breast cancer	ER	Preclinical
	HC-X029	Last-line treatment for mCRPC that has failed standard treatment	AR-sv	Preclinical
	HC-X035	KRAS mutated cancers	Protein tyrosine phosphatase (SHP2) containing two SH2 (Src homology 2) domains	Preclinical

Note: **The information was obtained from the official websites of the above companies and the deadline for information collection is 15 December 2024.**
Hangzhou Polymed Biopharma, Fendi Technology, Five Elements Biotechnology, Seed Therapeutics, and other PROTAC companies have not disclosed their pipeline information.

1.1.3.2 PROTAC Research and Development Progress: Chinese Pharmaceutical Companies

In China, PROTAC molecules are also developed by many companies, such as Lynk Pharmaceuticals, Kintor Pharmaceuticals, Haisco, Meizer Pharma, Cullgen, Hinova Pharmaceuticals, BeiGene, and Seed Therapeutics, a subsidiary of BeyondSpring, and all these companies have laid out in the PROTAC field. Among them, Lynk Pharmaceuticals, Kintor Pharmaceuticals, BeiGene, and Haisco own a PROTAC molecule in clinical phase I (Table 1.2).

1.1.4 Why Is DMPK Research Critical to PROTAC Drug Development?

Despite the popularity of PROTAC technology because of its unique mechanism of action, the development of PROTAC molecules still faces multiple challenges (Figure 1.3).

- Oral administration is the ideal drug delivery route for the treatment of most diseases, but because of its unique structure, PROTAC has a high molecular weight and poor solubility, which makes it difficult to meet the classical Lipinski's Rule of Five.
- It has poor *in vivo* and *in vitro* permeability, leading to poor absorption and low bioavailability.
- Several studies of PROTAC have shown that at appropriate or low concentrations, a normal ternary complex is formed. However, at high concentrations, a target protein-PROTAC or

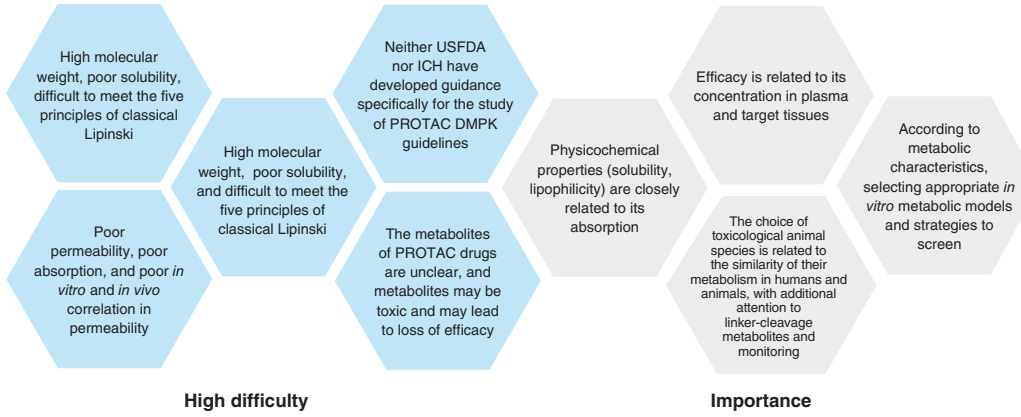
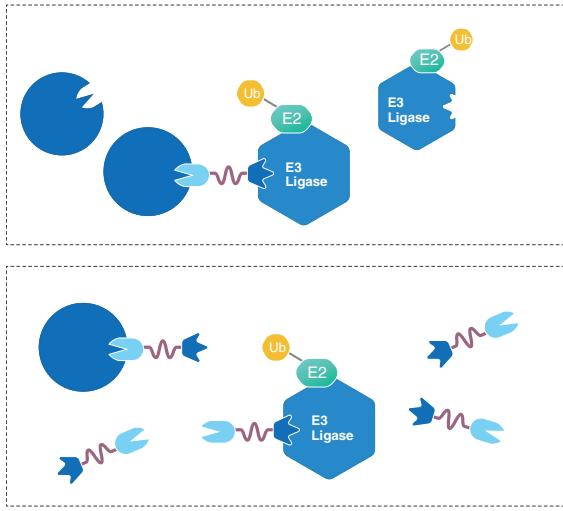
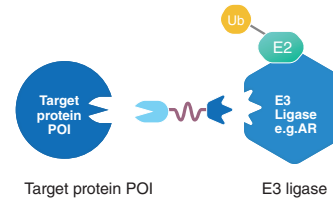


Figure 1.3 Difficulties and importance of PROTAC research.



At low or normal drug concentration

Normal POI-PROTAC-E3 ternary complexes are formed.



At high drug concentration

Competitive formation of POI-PROTAC or E3-PROTAC binary complexes rather than degradable POI-PROTAC-E3 ternary complexes.

Figure 1.4 Schematic representation of the hook effect.

E3 ligase-PROTAC binary complex is formed competitively, leading to reduced efficacy or toxic reactions, also known as the "hook effect" (Figure 1.4). Therefore, it is essential to fully understand the PKPD relationship of PROTAC, to timely modify the dosages.

- These molecules show high plasma protein binding in various species, and their plasma protein binding ratio may not be accurately measured when using the routine plasma protein binding evaluation method. Furthermore, the metabolism of PROTAC compounds in plasma might be affected by the high plasma protein binding.
- Metabolites of PROTACs may result in toxic reactions. Therefore, *in vivo* metabolite monitoring is a crucial part of its preclinical screening, with particular attention to linker-cleavage metabolites.
- There are no guidelines specifically for PROTAC drugs, hence, the guideline for small-molecule drugs is generally used as a reference.

1.1.5 The Strategy of the DMPK Study for PROTAC Drugs

The preclinical optimization of PROTAC drugs is mainly performed through the cascade optimization of physicochemical and DMPK properties [3].

- In the preliminary screening stage, the *in vivo* and *in vitro* characterization of PROTAC molecules is required, including physicochemical properties, permeability, protein binding, and drug-drug interaction (DDI).
- In the optimization stage, to better understand the DMPK properties of PROTAC, such as absorption and metabolism, the focus should be on improving the metabolic clearance and solubility of PROTAC molecules combined with the PK properties of extravascular drug delivery,. The structure of PROTACs poses a challenge in improving their permeability. Therefore, at this phase, more attention should be paid to solubility and metabolic stability when administered orally.
- At the PCC stage, in to order to obtain a more in-depth exposure–response relationship, PROTAC molecules with potent and better oral bioavailability can be used in further pharmacokinetics/pharmacodynamics (PK/PD) studies.

1.1.6 Summary

Although there is no marketed drug for PROTAC until July 2024, it is attracting more and more biopharmaceutical innovators and entrepreneurs to compete on this brand-new track. Over the course of more than 20 years of development, PROTAC has redefined small molecules with its unique mode of action. It has shattered the conventional rules of drug discovery and ushered in a new era of drug development. We anticipate a growing number of designed and developed PROTAC molecules, which will unlock fresh opportunities in various disease areas and give hope to a greater number of patients.

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1.2 Strategies to Improve the Oral Bioavailability of PROTACs

Liping Ma, Jie Hu, Chengyuan Li, Jing Jin

1.2.1 Introduction

Because of its unique mechanism of action, PROTAC has gradually become one of the most popular small molecules over the last five years. However, there are many obstacles to overcome due to its druggability. One of the most discussed challenges is that, unlike traditional small-molecule drugs, PROTAC does not adhere to the classical Lipinski's Rule of Five. PROTAC is considered to violate the tenets of medicinal chemistry and functions as a redefined small-molecule compound. Consequently, poor oral absorption remains a common issue for PROTAC compounds. Here, we discuss the PROTAC molecules' properties that are closely related to oral absorption and bioavailability, including solubility, lipophilicity, permeability, metabolic stability, etc., to provide ideas for further research and development of PROTAC molecules.

1.2.2 PROTAC Molecules Hardly Meet Lipinski's Rule of Five

Oral small-molecule drugs with high bioavailability and desired pharmacokinetic properties generally meet Lipinski's Rule of Five:

a) The molecular weight is less than 500.
b) No more than 5 hydrogen bond donors.
c) No more than 10 hydrogen bond acceptors.
d) Octanol–water partition coefficient log P not greater than 5.
e) No more than 10 rotatable bonds.

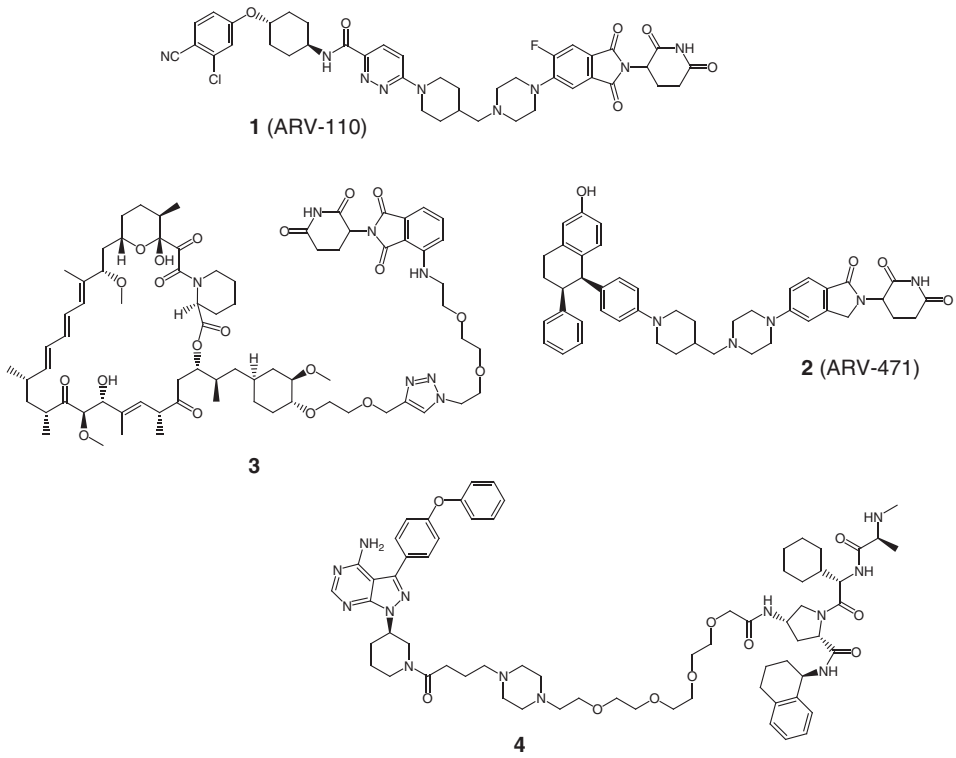
In terms of molecular weight, PROTAC consists of three key structural parts, a ligand binding with the target protein, a ligand binding with the E3 ubiquitin ligases, and a linker connecting the two ligands, which results in a large molecular weight of PROTAC, typically more than 700 Da [1]. PROTAC is also poorly soluble and permeable.

The two PROTAC molecules, ARV-110 and ARV-471, which have entered Phase II clinical trials, exhibit physical and chemical characteristics that are closer to Lipinski's Rule of Five (Figure 1.5). This may indicate that PROTACs that adhere more closely to Lipinski's Rule of Five may possess better pharmacokinetic properties [2].

1.2.3 Strategies to Improve PROTAC's Bioavailability

1.2.3.1 Improve the PROTAC's Solubility

The solubility of compounds in a pH 7.4 phosphate buffer solution (PBS) is typically measured by conventional solubility studies. However, since drug absorption occurs in the gastrointestinal tract, it is important to consider the solubility of PROTAC in physiological media as well. It has been reported that the solubility of PROTAC molecules significantly improves in fasted-state simulated intestinal fluid (FaSSIF) and fed-state simulating intestinal fluid (FeSSIF), with optimal solubility observed in FeSSIF (Figure 1.6). The clinical trial design of ARV-110 and ARV-471 published by Arvinas disclosed that the phase I clinical administration modes of these two PROTAC molecules



	MW (Da)	cLogP	HBD	HBA	TPSA	NRotB
bRo5 outer	1000	10	6	15	250	20
1	812	4.5	2	10	181	11
2	724	6.0	2	5	96	7
3	1426	5.3	4	21	338	23
4	1241	3.5	5	15	262	35

Figure 1.5 Structure and physicochemical properties of ARV-110, ARV-471, and two other PROTAC molecules. *Source:* [2] / with permission of Taylor & Francis Group.

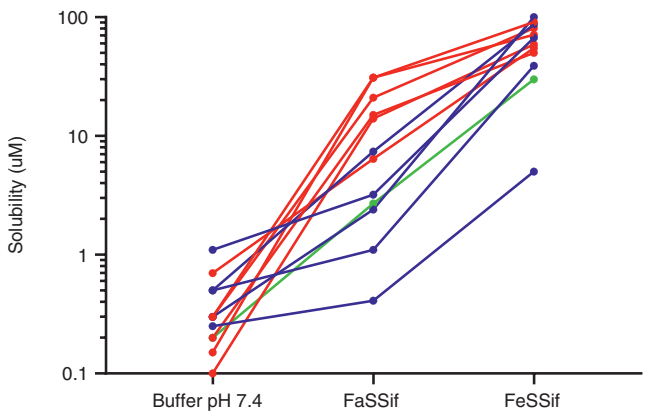


Figure 1.6 Solubility of PROTAC molecules in different buffer solutions. *Source:* [3] / ELSEVIER.

are both "once daily with food." Our study also found that postprandial administration increased the exposure of some PROTAC molecules in animals. This suggests that the *in vivo* pharmacokinetics of PROTAC may improve its *in vivo* drug exposure via postprandial administration.

Another way to solve the solubility problem of the PROTAC is to use optimized formulations. Studies have shown that the in vivo exposure can be increased by employing amorphous solid dispersions (such spray drying and hot melt extrusion), nano delivery systems, self-emulsifying delivery methods, or different solvents to make the formulation clear.

1.2.3.2 Improve the PROTAC's Permeability

Permeability is another crucial factor that affects oral bioavailability. On the one hand, drugs need to pass through the membrane barrier of small intestinal epithelial cells for oral absorption. On the other hand, for intracellular protein degradation, PROTACs also need to enter target cells. It has been demonstrated that the cell permeability of PROTACs can be significantly increased by substituting the PEG linker with a 1,4-disubstituted benzene ring [4].

In the preclinical compound screening stage, it is critical to select an appropriate *in vitro* permeability model for PROTAC. Conventional models for *in vitro* permeation research include Caco-2, MDCK, LLC-PK1, and PAMPA. When researching PROTAC molecules, PAMPA is not recommended as a suitable *in vitro* permeability model because of the limitations of its noncellular structure. Caco-2, MDR1-MDCK, or LLC-PK1 cells can be used to evaluate the *in vitro* permeability of PROTAC, whereas PROTAC molecules showed low permeability in most of the models (Figure 1.7a–d).

In addition to low permeability, the low solubility and high nonspecific binding (NSB) would also lead to the insufficient recovery of PROTAC molecules in permeability models. Low recovery makes it hard to determine whether the permeability data are accurate, thus greatly reducing the credibility of the data. We carried out additional validation studies to solve this problem. The recovery of PROTAC molecules in the Caco-2 cell models can be greatly improved by using a transfer buffer with BSA or a physiological medium as the transfer buffer. The optimized Caco-2 cell systems have been recommended for *in vitro* permeability evaluations of PROTAC.

1.2.3.3 Improve the PROTAC's Metabolic Stability

When absorbed by the intestine, the compound undergoes metabolism by the liver or intestine before it enters the circulatory system. This process is known as "first-pass" metabolism, which restricts the oral absorption of many drugs. One strategy to enhance the oral bioavailability of PROTACs is to improve their metabolic stability and reduce the first-pass metabolism. Various approaches have been explored to achieve this, including altering the length of the linker, modifying the anchor point of the linker, employing cyclic linkers, and changing the attachment site of the linker [5].

1.2.3.4 Choose Smaller E3 Ligand

PROTACs' properties are closely related to the type of E3 ligase ligands. The most commonly utilized E3 ligases for PROTAC mainly include CRBN, VHL, cIAP, and MDM2, with CRBN and VHL being the most frequently employed. As shown in Figure 1.8, CRBN has a smaller volume compared to VHL ligands. It has been reported that VHL ligand-containing PROTACs usually have poor oral absorption due to larger molecular weights [2]. Vasanthanathan et al. compared the chemical properties of PROTAC molecules based on different E3 ligases with oral drugs included in Drugbank and found that the PROTAC based on VHL **(green)** is far away from the oral drug region **(blue)** and it has been reported that the bioavailability of such PROTAC is indeed low. In contrast, the PROTAC based on CRBN E3 ligase **(red)** is closer to the oral drug region [2]. The two PROTAC molecules (ARV-110 and ARV-471) that have entered the clinical phase II are with CRBN E3 ligase. Therefore, searching for new E3 ligands with smaller molecular weights is worth exploring.

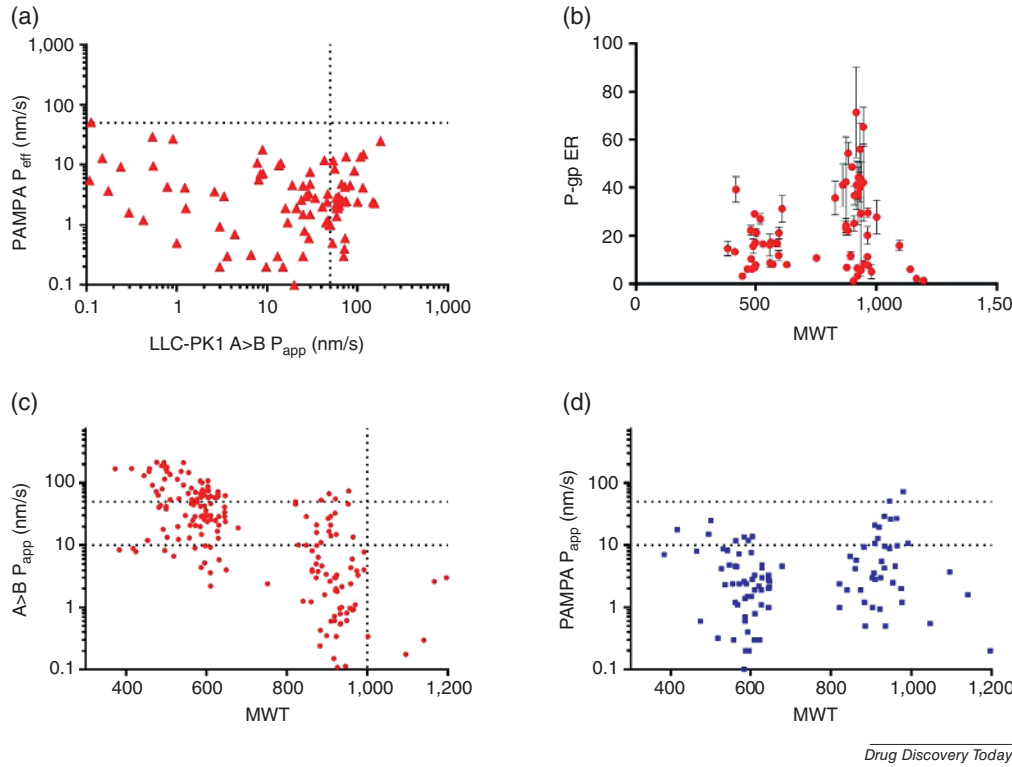


Figure 1.7 Molecular weight and permeability of PROTAC. *Source:* [1] / ELSEVIER. (a) The correlation between PAMPA and LLC-PK1, the area under the dotted line represents low permeability; (b) relations between P-gp efflux ratio and molecular weight; (c) the permeability of compounds with different molecular weights in LLC-PK1 cell model, molecular weight less than 650 is a single ligand, and molecular weight more than 650 is a complete PROTAC; (d) the permeability of compounds with different molecular weights in the PAMPA model, molecular weight less than 650 is an individual ligand, and molecular weight more than 650 is an intact PROTAC.

1.2.3.5 Introduce Intramolecular Hydrogen Bonds

PROTACs usually have high polarity and many rotatable bonds, and these structures generally make it difficult to penetrate the lipid bilayer of the cell membrane. Recent research found that the formation of intramolecular hydrogen bonds can reduce the polar molecular surface area of PROTACs and then improve their permeability. Under the action of intramolecular hydrogen bonds, an original strip-type molecule will be transformed into a "ball" form, which makes it easier to penetrate the lipid bilayer of the cell membrane [6].

1.2.3.6 Select Appropriate Solutions

PROTAC compounds generally have long structures, large molecular weight, high polarity, and a large number of rotatable bonds. Atilaw et al. showed that a PROTAC acts like a chameleon in that its conformation changes with the environment [6]. In solutions that mimic extra- (dimethyl sulfoxide, DMSO) or intracellular (DMSO mixed with water by 10:1), PROTAC molecules present an elongated shape and have a high molecular polarity. However, in the solution that mimics a cell membrane interior (chloroform), PROTAC molecules are folded by forming intramolecular hydrogen bonds and $\pi-\pi$ interaction, thus becoming a molecule with a smaller polar surface area (Figure 1.9). In other words, it shifts from the conformation of a polar molecule to the conformation of a nonpolar molecule. This finding suggests that the permeability of PROTAC may be related to whether it can form a conformation with a small polar surface area during the permeation process.

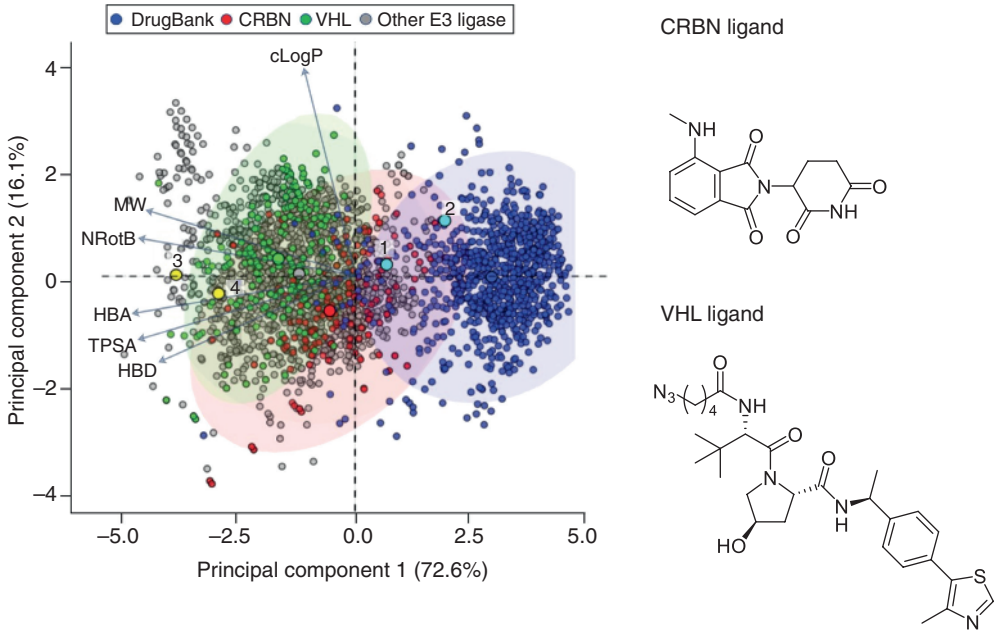


Figure 1.8 Structures of CRBN, VHL ligands, and comparison of chemical properties of PROTAC molecules with oral drugs included in the Drugbank. Blue circles represent oral drugs included in the Drugbank (n = 888), and red, green, and grey circles represent PROTAC of CRBN, VHL, and other E3 ligases (n = 2,082), respectively. *Source:* [2] / with permission of Taylor & Francis Group.

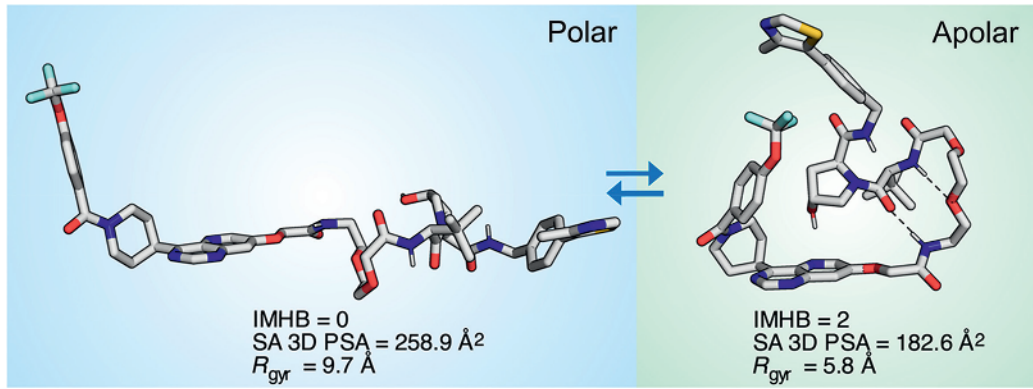


Figure 1.9 Conformation change of PROTAC in different solutions. *Source:* [6] / American Chemical Society.

1.2.3.7 Use Prodrug Strategy

Prodrug is a common approach to improving the oral bioavailability of drugs. A prodrug is obtained by structural modification of pharmacologically active compounds. Prodrugs themselves have little or no activity, and pharmacologically active parent drugs will be released *in vivo* by enzymatic catalysis. Chemists designed a prodrug from a PROTAC by adding a lipophilic group to the CRBN ligand. The results showed that the bioavailability of a PROTAC was significantly increased by prodrug design [7]. To improve the oral bioavailability of different PROTACs with similar E3 ligands, CRBN ligand-based prodrug design can also be used (Figure 1.10). However, increasing the molecular weights of PROTACs is a potential problem when designing prodrugs for PROTACs.

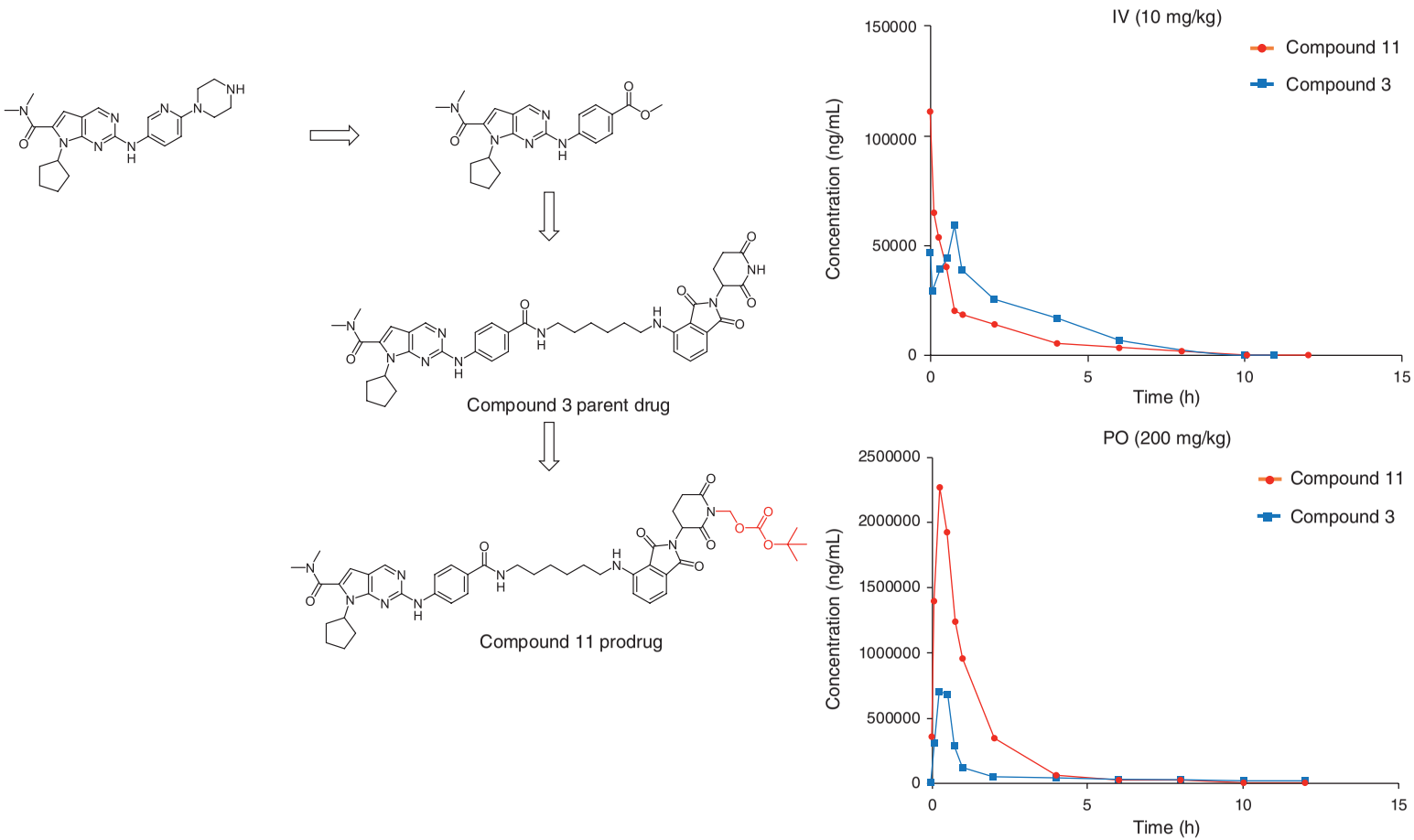


Figure 1.10 Oral bioavailability of compound 3 was significantly improved after its prodrug design as compound 11. *Source:* [7] / ELSEVIER.

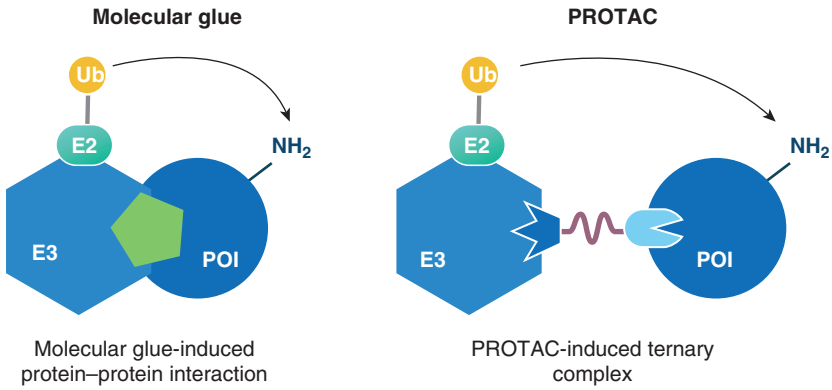


Figure 1.11 Comparison of the mechanism of action between molecular glues and PROTACs.

1.2.3.8 Use Molecular Glues

Because of the large molecular weight of PROTACs, chemists continue to explore new strategies to reduce molecular weight to make PROTACs more "drug-like." Molecular glues are considered to be a more "dense molecule" than PROTACs and they can also trigger a ternary complex like that of PROTACs (Figure 1.11). For these reasons, the molecular glues have better drug-like properties than PROTACs [8].

1.2.4 Summary

The development of PROTAC technology has provided unprecedented therapeutic options for drug development, with the most exciting potential ability to target "undruggable" targets. Despite the industry's optimism toward some late-stage clinical studies, researchers still face challenges in optimizing the pharmacokinetic behavior of PROTACs without compromising their efficacy. By modifying the structure of PROTAC molecules, such as modifying the linker, introducing intramolecular hydrogen bonds, designing as prodrugs, as well as selecting appropriate administration methods, such as administering with meals, the oral bioavailability of PROTAC molecules can be enhanced. It is hoped that academic and industrial researchers in the field of drug development will collaborate to address the challenges in pharmacokinetics faced by PROTACs and unleash their enormous potential.

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1.3 Research on PROTAC Metabolism: Strategies and Main Approaches

Liping Ma, Chengyuan Li, Jing Jin

1.3.1 Introduction

Drug metabolism refers to the process in which a drug undergoes structural changes under the action of various drug-metabolizing enzymes (primarily in the liver), and this process is also named biotransformation (Figure 1.12). Notably, the drugs are metabolized into non-pharmacologically active metabolites or pharmacologically active metabolites, or even toxic metabolites. The activity or safety concerns arising from these metabolites can significantly impact the success or failure of drug development to some extent.

Despite over two decades of research on PROTAC, there are no PROTAC drugs that have been approved for the market, and little literature on PROTAC metabolism. This chapter summarizes the studies on the metabolism of PROTAC molecules according to the literature and the practical experience to assist researchers in studying PROTAC molecules. The main parts of this chapter are as follows: (i) understanding the key metabolic enzymes of PROTACs, (ii) selecting an appropriate *in vitro* metabolism model, and (iii) facilitating structural optimization through studies on metabolic soft spots.

1.3.2 Understanding the Key Metabolic Enzymes of PROTACs

First, it is critical to identify the enzymes that may participate in the PROTACs' metabolism. The molecular weight of PROTAC compounds is about 600–1,200, which is slightly larger than that of conventional small molecules (the molecular weight of compounds meeting Lipinski's Rule is generally less than 500). Its metabolism may be more complex because the PROTAC is a ternary

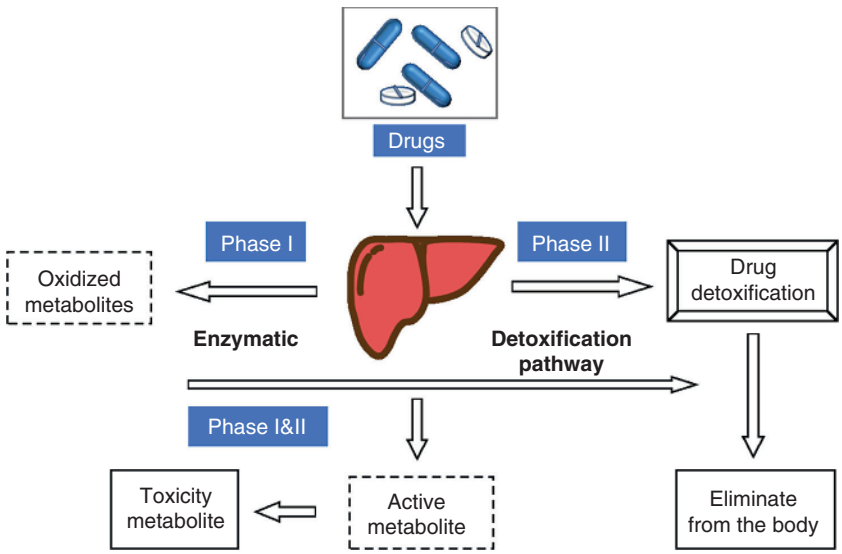

Figure 1.12 General pathway of drug metabolism. *Source:* [1] / Frontiers Media S.A. / CC BY 4.0.

complex made up of two small molecular compounds linked through linkers. Common drug-metabolizing enzymes involved in small-molecule metabolism mainly include phase I metabolizing enzymes (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A, etc.) and phase II metabolizing enzymes (uridine diphosphate (UDP)-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), etc.). The enzyme system of phase I metabolism, represented by cytochrome P450 (CYP450) enzymes, is considered to be involved in the metabolism of about 50% of clinical drugs [2].

Goracci et al. [3] reported that CYP3A4 and human aldehyde oxidase (hAOX) are involved in PROTAC metabolism. CYP3A4 is the most major phase I metabolizing enzyme among the CYP450 enzymes. However, as a cytosolic drug-metabolizing enzyme that is mainly expressed in the liver, aldehyde oxidase (AO) is a non-CYP drug-metabolizing enzyme. It is worth noting that there are marked species differences in the metabolism by AO, with large individual differences in rats, almost no expression in dogs, and the most expression in humans [4]. Therefore, although compounds that are mainly metabolized by AO are metabolically stable in animals, they may be metabolically unstable in humans. As the metabolism by AO is easily neglected in early development, some candidate compounds are terminated due to rapid clearance and low bioavailability in humans. From the perspective of drug screening, the role of AO in the metabolism of PROTACs should be identified as early as possible.

Additionally, *in vitro* plasma or whole blood stability is another important metabolic system of PROTAC screening at an early stage. According to the experimental data, we found that PROTAC molecules have various degrees of stability problems in the plasma or blood. The plasma or blood contains rich hydrolases that can catalyze the hydrolysis of drugs. Compounds with functional groups, such as ester bonds, amide bonds, sulfonamide bonds, and peptide bonds, may be unstable in the blood or plasma. Besides, some PROTACs may be unstable in the linker or linker binding sites.

Overall, the drug-metabolizing enzymes involved in PROTAC metabolism are phase I or phase II metabolizing enzymes involved in the metabolism of conventional small molecules. For the types of metabolizing enzymes, attention needs to be paid to PROTAC metabolism by CYP3A4, aldehyde oxidase, and hydrolase. Aldehyde oxidase, in particular, is not a common metabolizing enzyme. As shown in Figure 1.13c,f, aldehyde oxidase and xanthine oxidase (XO) are only involved in the metabolism of about 3% of the drugs in the market. In addition, further study on identifying particular enzymes that may cause the instability of PROTAC in the plasma or blood needs to be conducted.

1.3.3 Selecting an Appropriate *In Vitro* Metabolic Model

Some metabolic models, including liver microsomes, hepatocytes, and liver S9, are usually used in the *in vitro* metabolism of small molecules and are also applicable to PROTAC molecules (Figure 1.14). The appropriate *in vitro* metabolic model can be selected according to the PROTAC metabolism characteristics. Studies found that the metabolic rates of some PROTAC compounds using liver microsomes or hepatocytes, respectively, may differ significantly. This has also been confirmed in our studies. Liver microsomes mainly contain phase I metabolizing enzymes and their metabolism can better reflect the *in vitro* metabolism when compounds are mainly metabolized by CYP enzymes. However, when there is strong non-phase I metabolism, the hepatocyte system is more appropriate. The mentioned aldehyde oxidase is a drug-metabolizing enzyme in the cytosol, and its content in liver microsomes is low. The hepatocyte contains a membrane barrier and a complete metabolizing enzyme system, so it is an appropriate system for studying the *in vitro* metabolism of PROTAC.

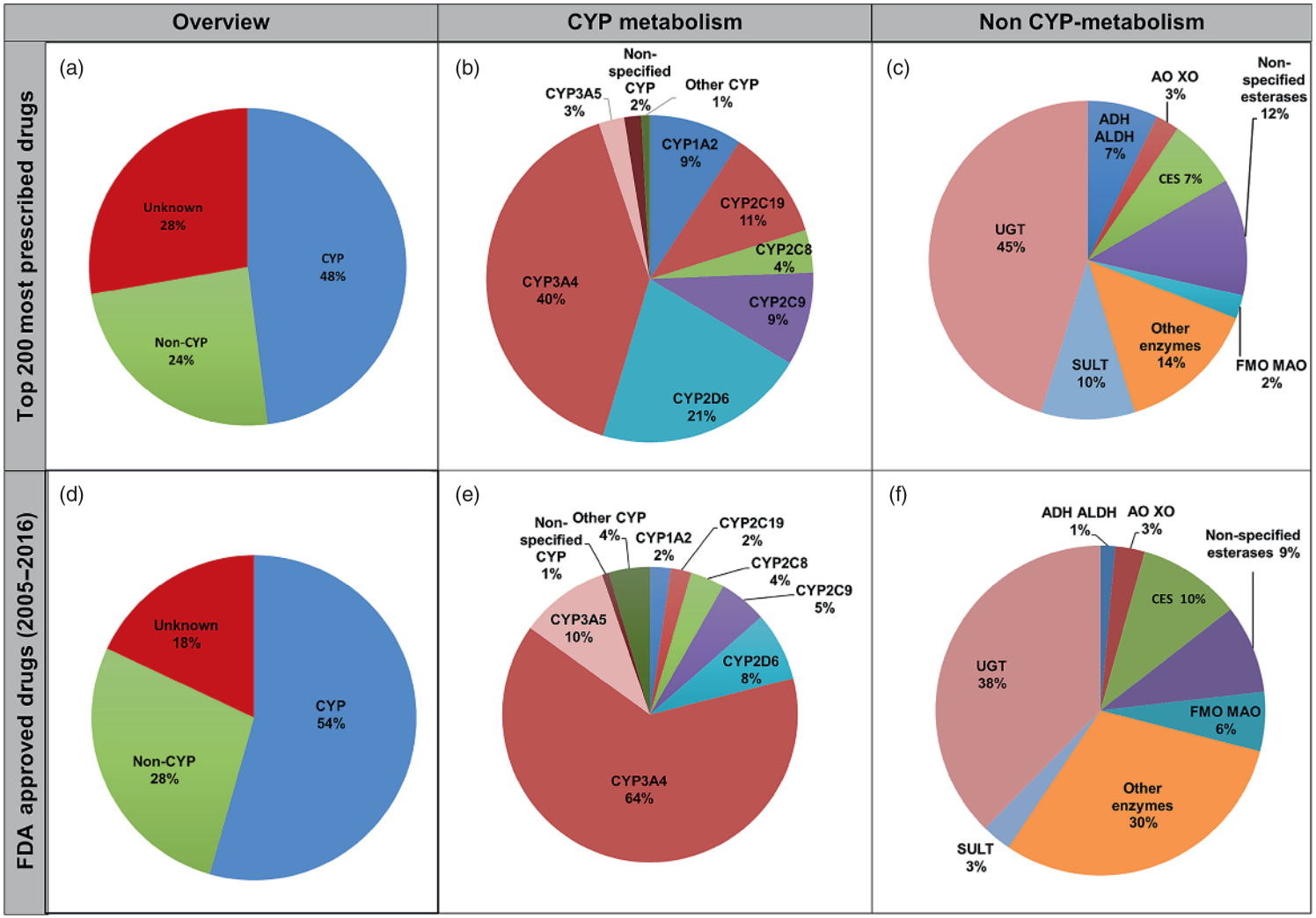

Figure 1.13 Drug metabolism involved in CYP and non-CYP [2]. Note: The contribution of major enzymes (a,d), major cytochrome P450s (CYPs) (b,e), and major non-CYP enzymes (c,f) in the metabolism of "top 200 most prescribed" and "2005–2016" FDA-approved drugs.

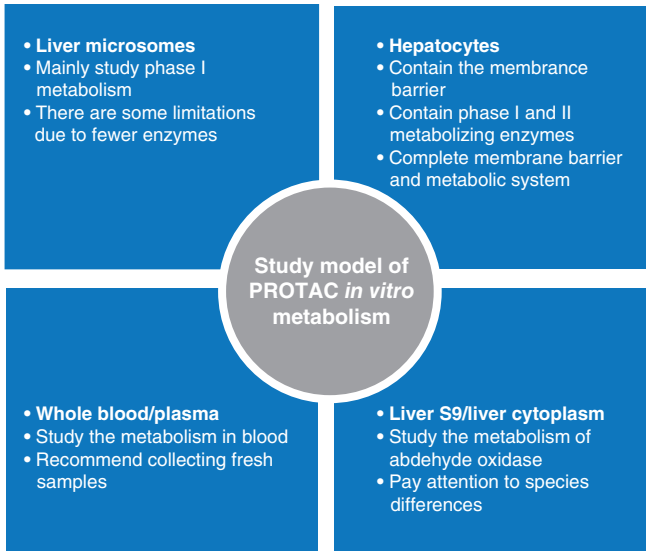

Figure 1.14 Study model of PROTAC *in vitro* metabolism.

It is necessary to study the metabolism of aldehyde oxidase specifically if aldehyde oxidase may be involved in PROTAC metabolism. Because aldehyde oxidase is found in the cytosol, it can be studied via liver S9 or the liver cytosol system. At the same time, the effects on metabolism with or without aldehyde oxidase inhibitors need to be investigated. If aldehyde oxidase participates in metabolism, the metabolic rate with or without aldehyde oxidase inhibitors may be different.

There may be stability problems with PROTAC in the blood or plasma. The most direct assessment method is to investigate the stability of PROTAC compounds in the whole blood/plasma *in vitro*. The conventional incubation time (about two hours at most) could meet the needs of the *in vitro* study of PROTAC molecules. Our study found that the stability data obtained from the whole blood and plasma may be quite different. It is speculated that the enzyme activities of frozen plasma may be different from those of freshly collected whole blood. Therefore, it is recommended to use freshly collected whole blood to study the metabolic stability.

It is worth noting that the protein binding rate of PROTAC molecules is generally high, and the free concentrations in the incubation system need to be fully considered when the interspecies comparison or *in vitro–in vivo* extrapolation (IVIVE) is conducted. It is recommended to conduct IVIVE after the protein binding rate has been accurately determined. It was reported that *in vitro–in vivo* correlation was good by using the *in vitro* hepatocyte data of mice, with the theoretical *in vivo* clearance of most PROTAC molecules within threefold of the actual value (Figure 1.15).

1.3.4 Facilitating Structural Optimization Through Studies on Metabolic Soft Spots

The PROTAC molecule is a ternary complex formed by connecting two ligands via a linker. The cleavage of the linker or the metabolism of the two ligand are both potential metabolic sites. The following conclusions were drawn by Goracci et al. after comparing the metabolic soft spots and metabolic rates of the PROTAC molecule and its ligands [3]:

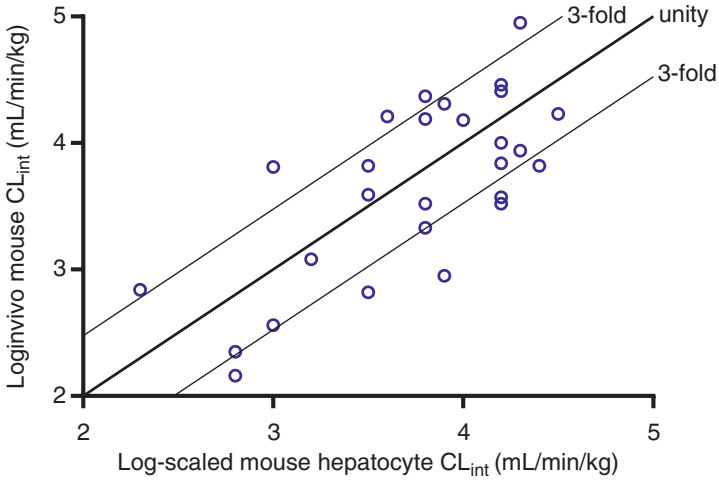

Figure 1.15 Analysis of *in vitro–in vivo* correlation of PROTAC metabolism via hepatocytes. *Source:* [5] / ELSEVIER.

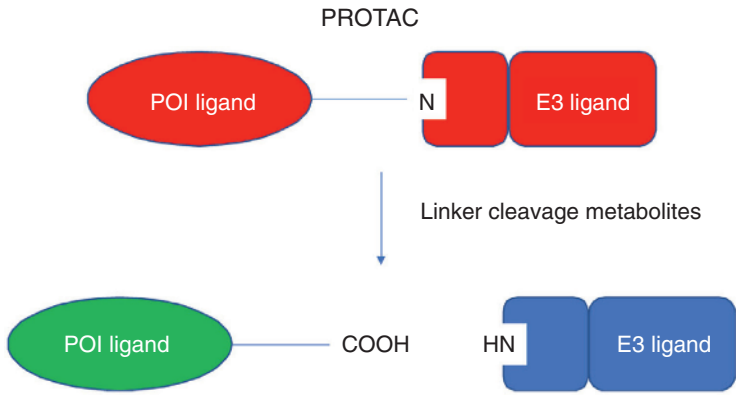

Figure 1.16 Linker cleavage metabolites produced by PROTAC. *Source:* [5] / ELSEVIER.

a) The metabolic soft spot of PROTAC might be completely different from that of ligands.
b) The linker cleavage site of PROTAC is a common metabolic site.
c) The metabolic rate of PROTAC might be completely different from that of ligands.

Therefore, it was difficult to predict the metabolism of PROTAC molecules according to the metabolism of ligands. PROTAC metabolism shall be studied as a whole. Furthermore, the linker cleavage site is a metabolic type of PROTAC that is different from conventional small molecules (Figure 1.16). Linker cleavage can produce two ligand-related metabolites, which may have the following characteristics:

a) The metabolic clearance rates are completely different from that of PROTAC.
b) The protein binding rates are lower than that of PROTAC due to the small molecular weight.
c) Two ligands are intact in their structure so that the target protein or E3 ligase can be bound.

If there are obvious linker cleavage metabolites, the effect of metabolites on drug efficacy needs to be fully considered in the PK/PD study of PROTAC. In addition, possible safety problems associated with metabolites shall be studied as needed.

1.3.5 Summary

PROTAC metabolism can be evaluated and predicted by various *in vitro* metabolic models. At present, it is difficult to identify which models are completely appropriate for PROTAC metabolism. It has been reported that good prediction results can be obtained by IVIVE via hepatocyte metabolic data, but this does not mean that the method is suitable for all PROTAC molecules. During the screening process, it is recommended to summarize the metabolic characteristics of the same series of PROTAC compounds, selectively conduct metabolic studies, and find an *in vitro* metabolic model appropriate for PROTAC molecules to predict *in vivo* clearance or evaluate the early screening of compounds. In view of the study on PROTAC metabolism, we expect more experience to be summarized to gain an in-depth understanding of the metabolic characteristics of PROTAC molecules.

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1.4 Metabolic Characteristics and Research Strategies for Metabolite Identification of PROTACs

Junjie Wang, Hong Zhang, Ruixing Li, Weiqun Cao

1.4.1 Introduction

PROTAC (the same below) is an emerging class of therapeutic agents that has attracted much attention in academia, industry, and investment in the field of biomedicine because it expands the coverage of druggable targets and has the potential to solve the problem of drug resistance of small-molecule inhibitors. In October 2021, the Health and Environmental Sciences Institute (HESI) brought together more than 150 pharmaceutical scientists, regulators, and academic researchers to deeply explore important issues, such as the safety evaluation of targeted protein degradants [1]. Participants generally agreed that the safety challenges of PROTAC are mainly involved in off-target protein degradation, differences in PK/PD from conventional small-molecule drugs, and the selection of appropriate species for safety assessment in nonclinical trials. Therefore, we urgently need to study the metabolites of PROTAC in depth as early as possible, which is essential for analyzing the distribution of drugs in human circulation, assessing their safety, comparing metabolic differences between different species, and selecting appropriate toxicological species. In this section, we initially discuss the importance of metabolite identification (MetID) at various stages of PROTAC drug development. Subsequently, we summarize the structural and metabolic characteristics of PROTAC. Finally, we outline the challenges encountered during PROTAC MetID research and suggest our corresponding strategies according to our experience. We aim to lay a foundation for developing robust MetID research strategies, help medicinal chemists design PROTAC molecules with favorable pharmacokinetic (PK) attributes, and provide effective direction for the development of PROTAC drugs.

1.4.2 Importance of Metabolite Identification Studies in the Development Process of PROTAC Drugs

Given the relatively large molecular weight of PROTACs, enhancing permeability and solubility often necessitates the incorporation of heteroatoms into the structure during the designing of the drug. However, this approach will escalate the risk of more metabolic soft spots being introduced. Specifically, the linker component of PROTACs can undergo metabolic reactions, such as dealkylation or hydrolysis, leading to the production of cleavage products [2]. These metabolites have the potential to competitively bind with the target of PROTACs, thereby inhibiting the pharmacodynamic effects of these drugs or leading to off-target toxicities. For example, E3 ligase ligand fragments obtained by linker cleavage can bind to nontarget proteins as molecular gels and produce off-target effects [1]. Results obtained from *in vitro* MetID studies can help identify the metabolic soft spots of PROTAC and screen linker-cleaved products. This information further guides the structural optimization of PROTAC to obtain more metabolically stable compounds. In addition, because the metabolic characteristics of PROTAC cannot be predicted from one of the ligands used for their design and synthesis, PROTACs represent independent chemical entities [2].

During the preclinical development phase, conducting *in vitro* MetID studies in different species can help assess the metabolic differences of PROTAC between species and select species suitable for toxicological studies. At the same time, the *in vitro* and the *in vivo* metabolic correlation of PROTAC can be assessed by comparative analysis with the results of *in vivo* MetID studies, and then the

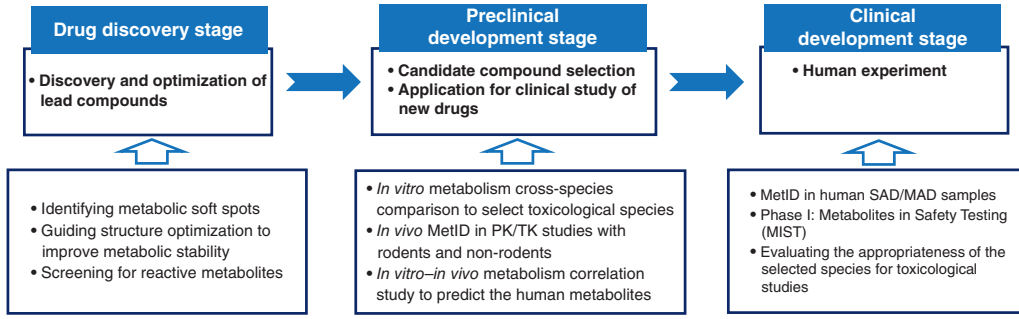


Figure 1.17 The role of metabolite identification studies in various stages of PROTAC drug development.

PROTAC metabolism in humans can be speculated. Additionally, by analyzing the concentration of PROTAC and its metabolites in plasma and tissues such as the liver, kidney, and tumor, we can evaluate the *in vivo* efficacy and toxicity of PROTAC. This is crucial to understand the potential off-target risks associated with PROTAC [3]. In clinical phase I, MetID studies can help analyze the metabolic pathways and clearance of PROTAC in the human bodies and can be used to assess whether the selection of toxicological species for preclinical studies is appropriate (Figure 1.17).

Hence, it is crucial to undertake MetID studies of PROTAC as early as feasible to aid its drug discovery and safety evaluations. The experimental methods and analytical techniques required for PROTAC MetID are more demanding than those for traditional small-molecule drugs. While PROTAC falls under the category of small-molecule compounds, its metabolic characteristics significantly diverge from traditional small-molecule drugs, attributable to its larger molecular weight and complex structural features.

1.4.3 Structural Characteristics of PROTAC Drugs

PROTAC drugs bring significant therapeutic advantages with their unique structure and mechanism of action. However, this particular structure also endows it with unique physicochemical properties as well as different metabolic characteristics from traditional small-molecule drugs. For a deeper understanding of its metabolic profile, first, we will introduce the structural characteristics of PROTAC, providing a base to summarize and investigate its metabolic characteristics. PROTAC primarily consists of three critical structures: one region is the ligand structure that targets the protein of interest (POI); another region is the ligand structure for E3 ligase; and situated between these two is the linker, which connects both ligands (Figure 1.18). In the following sections, we will individually introduce these three components based on their structural characteristics.

1.4.3.1 POI Ligand

Among the reported PROTAC molecules to date, the number of POI ligands exceeds 360 [4]. The target proteins include androgen receptor (AR), estrogen receptor (ER), bromodomain-containing protein (BRD), and Bruton tyrosine kinase (BTK). The POI ligand component of PROTAC typically utilizes active inhibitors that are either marketed or reported in scholarly literature [3]. For instance, Gray's group, in 2019, developed BSJ-03-204 [5], deriving from the CDK4/6 inhibitor Palbociclib. In 2021, Jinlei's team developed B03 [6], which is based on the CDK9 inhibitor BAY-1143572.

PROTAC drugs target not only conventional small-molecule drugs but also extend to non-druggable proteins, scaffold function proteins, protein polymers, drug-resistant mutant proteins,

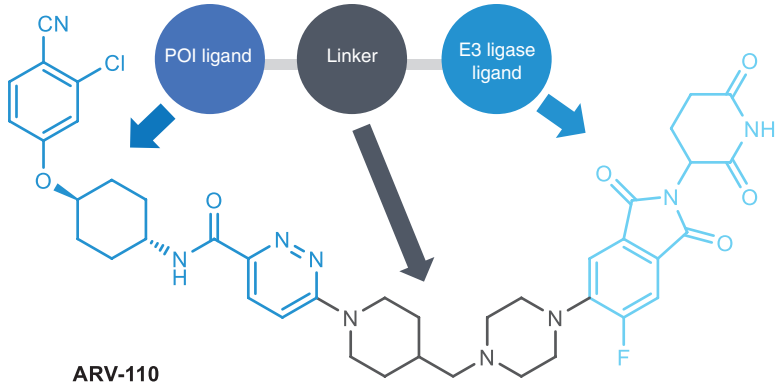
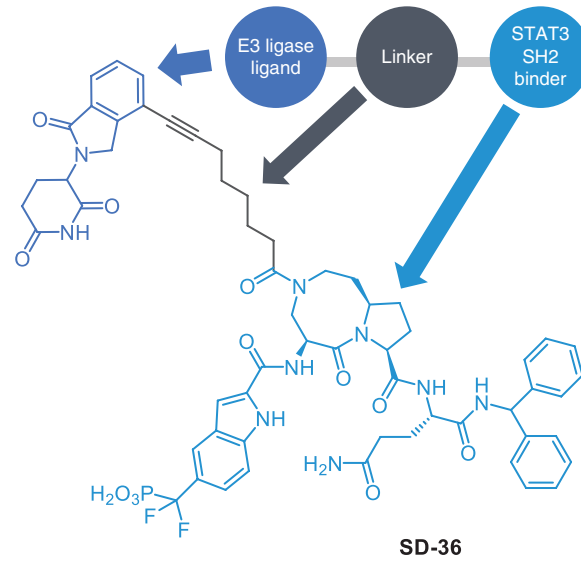


Figure 1.18 Structural diagram of PROTAC and representative structure of PROTAC: ARV-110.

Figure 1.19 STAT3 degradant SD-36.



and specific isoform proteins. For example, signal transducer and activator of transcription 3 (STAT3) is a transcription factor closely related to the development of cancer and is considered to be a non-druggable protein. Based on SI-109, a binder of the SH2 domain in STAT3, Wang's group [7] developed the PROTAC molecule SD-36 which has high degradation activity for STAT3 protein in both *in vitro* and *in vivo* experiments (Figure 1.19). KT-333, also a PROTAC drug targeting STAT3, developed by Kymera, has entered phase I clinical trials to assess its safety in patients with large granular lymphocytic leukemia.

1.4.3.2 E3 Ligand

There are over 600 known types of E3 ligases encoded by the human genome. However, in PROTAC-related drug design, the primary E3 ubiquitin ligases include cereblon (CRBN, with 71 subtypes), von Hippel-Lindau (VHL, with 47 subtypes), an inhibitor of apoptosis protein (cIAP, with 7 subtypes), and murine double minute gene (MDM2, with 4 subtypes) (Figure 1.20) [3].

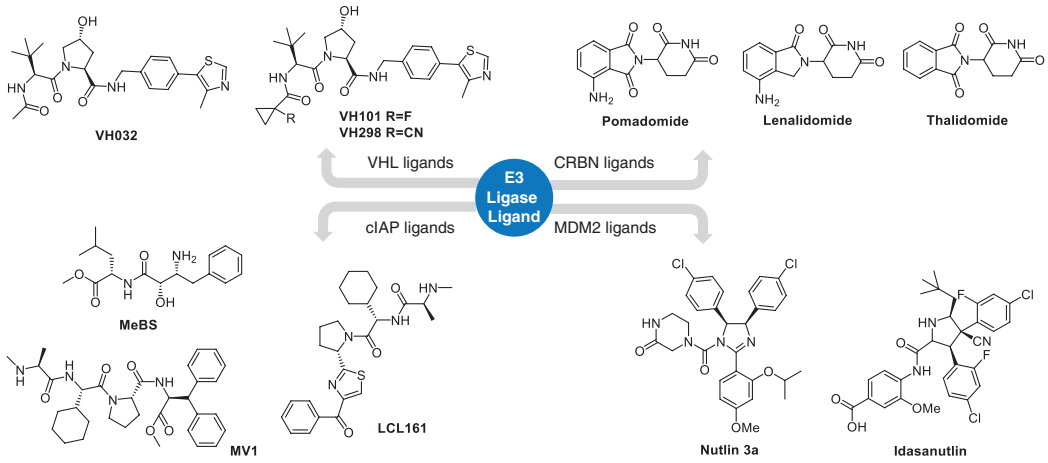


Figure 1.20 Representative E3 ligands.

Among PROTACs which have entered the clinical stage and published structures, more than 39% of the molecules have CRBN ligands, and only DT2216 contains VHL-containing ligand.

Investigating novel E3 ubiquitin ligase targets is a prominent area of interest in PROTAC research. In recent years, several structurally unique E3 ligases have been discovered, including DCAF11, DCAF15, DCAF16, RNF114, RNF4, AhR, FEM1B, and KEAP1. Additionally, over 80 novel E3 ligase ligands have been reported. Moving forward, it is anticipated that researchers will identify a more diverse range of E3 ligases, as well as E3 ligase ligands with smaller molecular weight and higher activity.

1.4.3.3 Linker

The linker, an important part, connects the two active groups of PROTAC drugs, playing an essential role in the activity, metabolic stability, and kinetic properties of PROTAC molecules. Over 1,500 different linker structures have been reported [4], and from a molecular variability perspective, linkers can be divided into two types: flexible and rigid (Figure 1.21). Relevant literature [2]

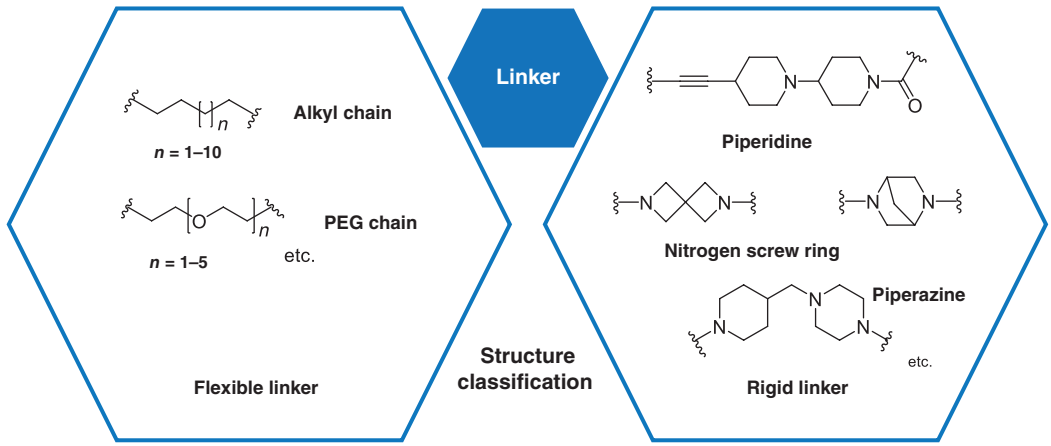


Figure 1.21 Representative structures of linkers used in PROTACs.

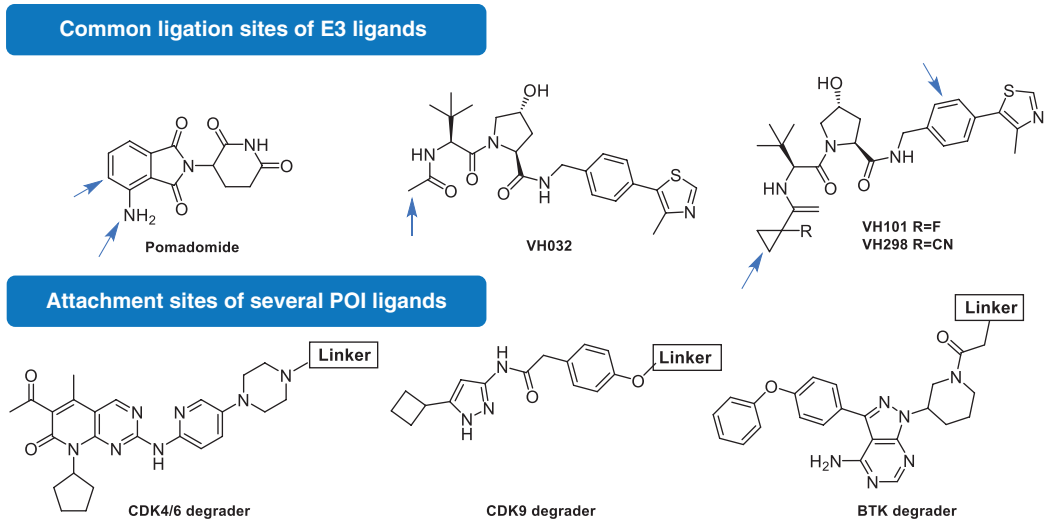


Figure 1.22 Common attachment sites of linkers with E3 and POI ligands.

indicates that neither short nor long linkers are beneficial for the efficacy of PROTAC. If the linker is too short, it can impede the binding of ligands on both sides to the corresponding protein due to steric hindrance, making the formation of the ternary complex difficult. Conversely, when the linker is too long, the E3 ligase cannot sufficiently approach the target protein, preventing the ubiquitination process from occurring, and the excessive molecular weight reduces PROTAC's membrane permeability. Studies suggest that linkers possessing rigid elements (e.g., a piperidine moiety) or polar groups (e.g., polyethylene glycol (PEG)) can enhance the pharmacokinetic properties of PROTAC.

Besides the structure of the linker, its attachment site can also influence the selectivity and activity of PROTAC. Generally, the principles for selecting ligation sites are as follows: (i) do not diminish the binding capacity between E3 ligase ligands or POI ligands and their receptors; (ii) select the solvent-exposed region of the ligand binding pocket; (iii) ensure the integrity of POI ligands as much as possible when attaching the linker to prevent any impact on their binding capacity. Figure 1.22 illustrates the common ligation sites of E3 ligase ligands (indicated by the red arrow section) as well as the ligation sites of several POI ligands (3).

1.4.4 Metabolic Characteristics of PROTAC Drugs

Due to its unique structural characteristics, the molecular weight and physicochemical properties of PROTAC often exceed the limitations of Lipinski's Rule of Five, and its metabolic process is also significantly different from that of traditional small-molecule drugs. An in-depth understanding of their metabolic properties will help researchers to more effectively cope with the challenges encountered in the study of their metabolites. Therefore, this part will thoroughly summarize the metabolic characteristics of PROTAC drugs based on their structural features.

1.4.4.1 The Influence of PROTAC's E3 Ligand on PROTAC Metabolism

Cruciani's team [2] discovered, after comparing three datasets, that for PROTACs targeting BET, CK2, and PARP proteins, when the POI ligands remain constant and linkers are similar, the

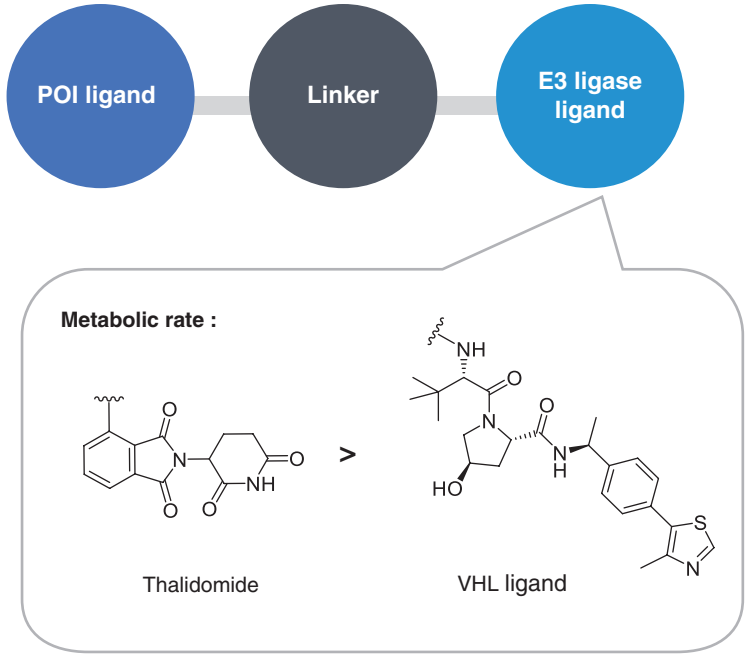


Figure 1.23 Effect of E3 ligands on the metabolic stability of PROTACs.

metabolic stability of PROTACs with thalidomide as the E3 ligase ligand is significantly lower than those with VHL ligand (as depicted in Figure 1.23). The team hypothesizes that the plausible reason could be that thalidomide and its analogs contain multiple amide structures, which might undergo non-enzyme-catalyzed hydrolysis reactions. Another reason is that due to the larger molecular weight of the VHL ligand, the corresponding PROTAC has reduced permeability [3], thereby not providing an accurate estimation of its half-life. Therefore, for metabolic stability studies of PROTACs containing VHL ligands, liver microsomes may be a more appropriate *in vitro* metabolic study system [8].

1.4.4.2 The Influence of PROTAC's POI Ligand on PROTAC Metabolism

Cruciani's team [2] discovered that the metabolic stability of PROTAC molecules changes as the POI ligand varies, keeping the linker and E3 ligase ligand constant. Figure 1.24 illustrates that for PROTACs using the AR receptor antagonist as the POI ligand, their metabolic stability is lower compared to other POI ligands, regardless of changes in the linker and E3 ligase ligand. MetID studies identified that the primary soft metabolic spots of these compounds are on the POI ligand, not on the E3 ligase ligand. Given the fast metabolic rate of the POI ligand itself (a half-life of 18.3 minutes), this suggests that the metabolic stability of the POI ligand needs to be considered when designing PROTAC drugs to avoid significant weaknesses. For example, the PROTAC drug ARV-110 uses an AR receptor antagonist with better metabolic stability than the POI ligand, boasting a half-life of up to 110 hours.

1.4.4.3 The Influence of Linker on PROTAC Metabolism

In PROTAC molecules, the linker is the part that is most likely to be metabolized. The main metabolic sites are where the linker attaches to the ligand. Variations in the linker's length, attachment

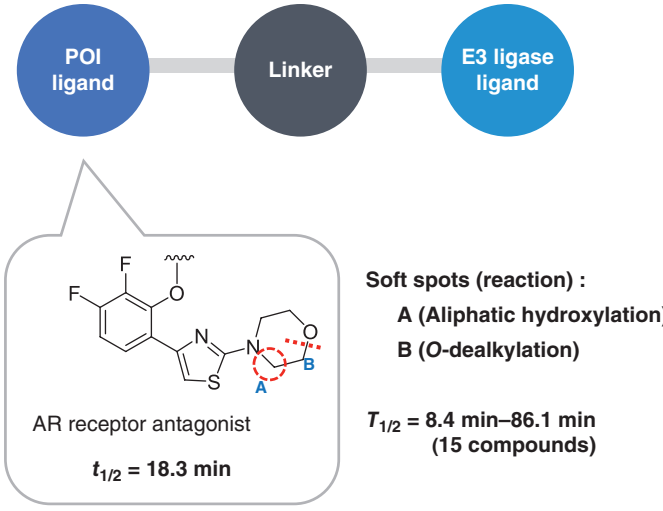
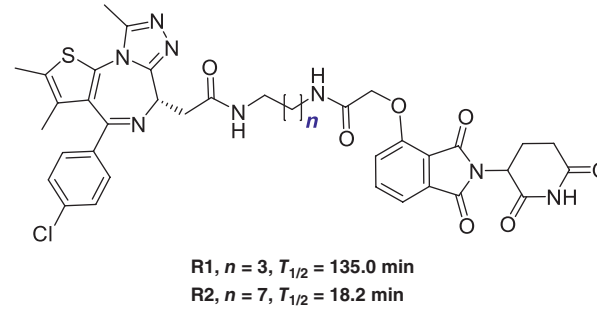


Figure 1.24 Effect of POI ligands on metabolic stability.

Figure 1.25 Effect of linker length on the metabolic stability of PROTACs.



points, and the degree of rigidity or flexibility can significantly influence the overall metabolic stability of the molecule.

1.4.4.3.1 Effect of Linker's Length and Ligation Site on the Metabolic Stability of PROTAC

In the majority of PROTACs, the metabolic stability of the molecule tends to diminish as the linker length extends. As depicted in Figure 1.25, R1 represents a PROTAC, ingeniously designed using JQ1 (a BET inhibitor) and Thalidomide. When the straight-chain alkyl linker in R1 is elongated from four methylene units to eight, forming R2, the half-life dramatically contracts from 135 minutes to just 18.2 minutes. This phenomenon can likely be attributed to the increased steric hindrance of the shorter linker, which effectively prevents the PROTAC from entering the catalytic site of the metabolic enzyme.

It has also been found that the changes in the ligation site of linker and E3 ligase ligand may also affect the metabolic stability of PROTAC, as shown in Figure 1.26.

1.4.4.3.2 Effect of Linker Rigid Flexibility on the Metabolic Stability of PROTAC

Studies have shown that when both the POI ligand and the E3 ligase ligand are kept constant, the assembly of the PROTAC molecule with different linkers can significantly impact its metabolic stability (as depicted in Figure 1.27). Specifically, PROTACs featuring piperidine or triazole ring

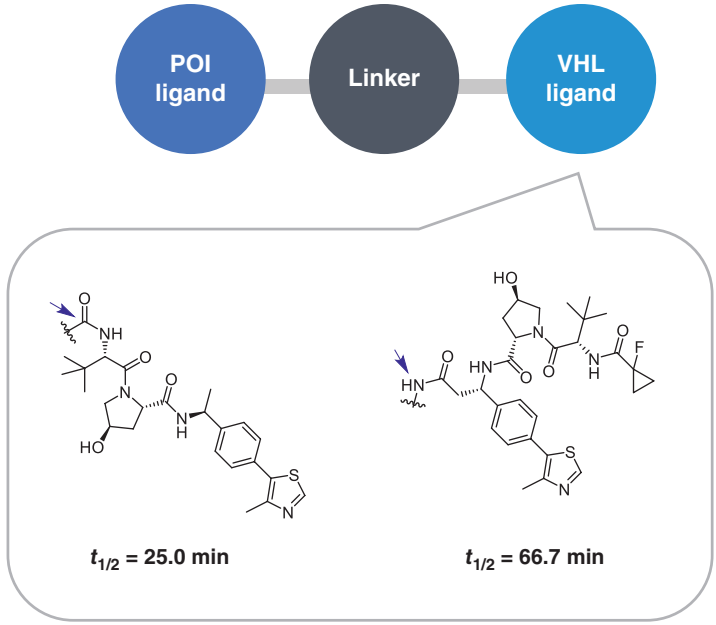


Figure 1.26 Impact of the linker attachment site on the metabolic stability of PROTACs.

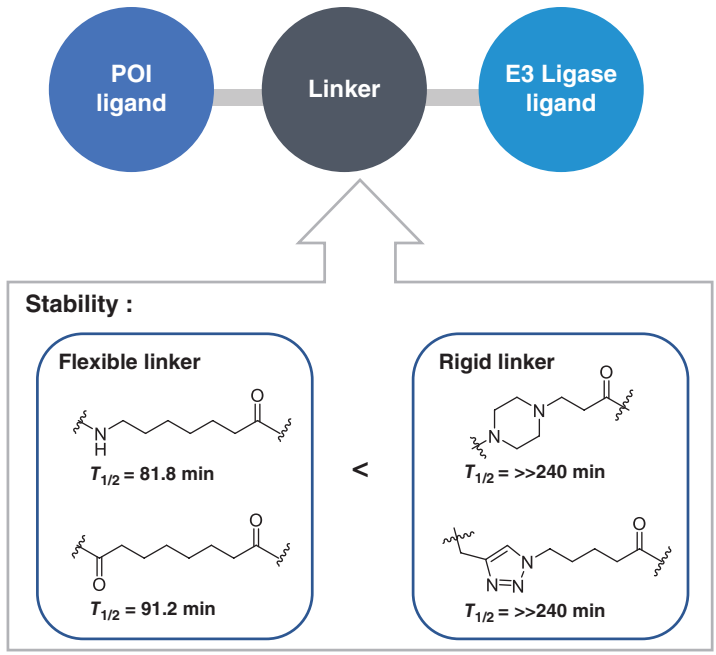


Figure 1.27 Effect of linker rigidity on metabolic stability.

linkers demonstrate a prolonged half-life compared to those with straightforward linear linkers. This evidence suggests that PROTACs constructed with rigid linkers tend to exhibit enhanced metabolic stability.

Incorporating rigid structures into the linker presents a superior approach to bolster metabolic stability. For instance, in the development process of ARV-110 (as depicted in Figure 1.28), the

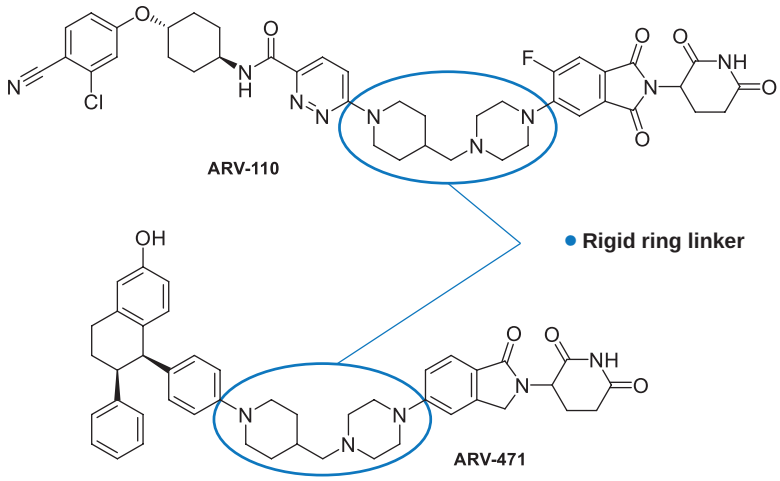


Figure 1.28 Structure of ARV-110 and ARV-471 with rigid cyclic linker.

initial flexible chain-like linker was refined into a stiff cyclic linker, bridged by piperidine and piperazine rings. This optimization notably enhanced its metabolic stability and therapeutic efficacy. Arvinas, the company that developed ARV-110, implemented the same linker design in another PROTAC drug, ARV-471 [2].

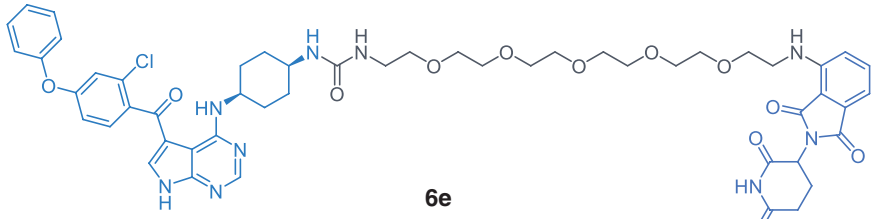
In 2021, Wang Yonghui's research team at Fudan University [9] unveiled the PROTAC molecule 6e. Despite its high degradation activity against BTK protein, 6e demonstrated relatively low metabolic stability, with a half-life of just 1.3 minutes in mouse liver microsomes. However, in 2023, the team significantly enhanced the metabolic stability of the molecule by implementing a strategy to increase the rigidity of its linker. Through conducting structure–activity relationship studies on a series of rigid linkers and CRBN ligands, they replaced the linear PEG linker with a more rigid one, featuring two piperidine rings. This modification led to the creation of molecule 3e, which not only displayed a greatly improved metabolic stability, with a half-life exceeding 145 minutes in mouse liver microsomes, but also outperformed its predecessor, 6e, in terms of activity (as depicted in Figure 1.29).

1.4.4.4 Relationship Between the Metabolic Characteristics of Various PROTAC Components and the Whole Molecule

Considering that a PROTAC molecule is composed of three crucial structures, one might wonder if it is possible to predict the metabolic characteristics of PROTAC based on the metabolic traits of each ligand. To address this, Cruciani's team [2] embarked on a comprehensive study. As demonstrated in Figure 1.30, the POI ligand and CRBN ligand individually undergo metabolic reactions of alkyl hydroxylation and amide hydrolysis, respectively. However, when these ligands are conjugated via a linker into a PROTAC molecule, neither ligand exhibits the respective metabolism, with the primary metabolic activity occurring on the linker itself. Moreover, the metabolic rate of the entire PROTAC molecule varies from that of its separate ligands. Thus, as a novel chemical entity, PROTAC's metabolic characteristics cannot be extrapolated merely from the metabolic attributes of its constituent parts, underscoring the need for a holistic investigation of its metabolites.

1.4.4.5 Summary of Metabolic Characteristics of PROTACs

From the above analysis, it can be seen that the type of E3 ligase, the stability of the POI ligand, and the length, ligation site, and rigidity of the linker all affect the metabolism of PROTAC. The



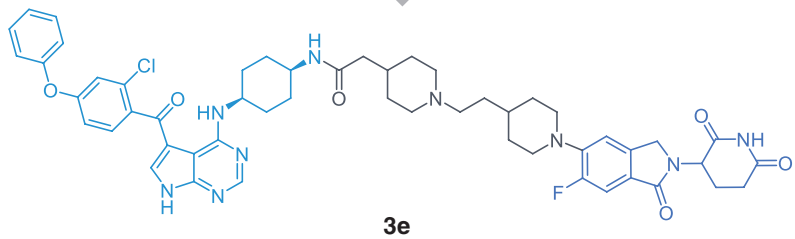
6e

BTK DC_{50} = 20.0 Nm, D_{max} = 93.8%

Microsomal stability : $t_{1/2}$ = 1.3 min

Linker optimization

CRBN ligand optimization



3e

BTK DC_{50} = 7.0 nM, D_{max} = 96.5%

Microsomal stability : $t_{1/2}$ > 145 min

Figure 1.29 Study case: enhancing PROTAC metabolic stability and activity through structural optimization.

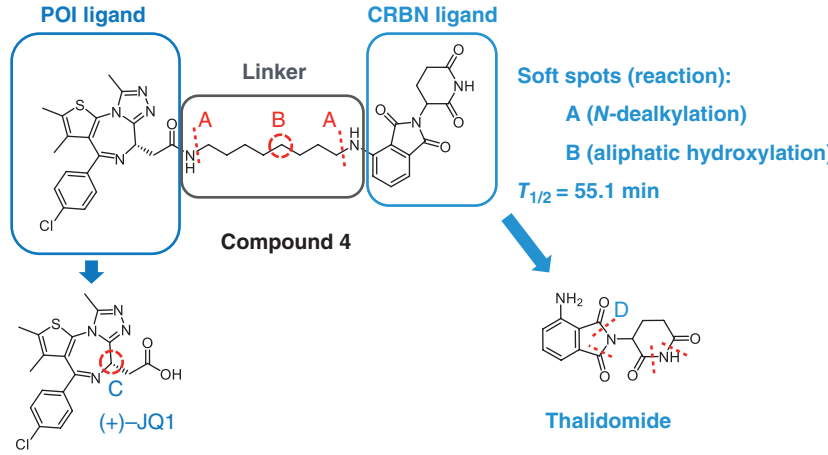


Figure 1.30 Comparison of PROTAC's metabolic soft spots with those of its individual ligands.

metabolic characteristics of PROTAC molecules are different from the sum of metabolic characteristics of their various partitions, and the metabolic rate may also be completely different.

Metabolic pathways of PROTAC include hydroxylation of its ligand's moiety, amide hydrolysis, and *O*-dealkylation and hydroxylation of the linker's moiety, *N*-dealkylation, and amide hydrolysis. If the drug contains a PEG-like linker, there are a large number of *O*-dealkylation reactions. Enzymes involved in the metabolism of PROTAC mainly include phase I metabolizing enzymes (CYP enzymes) and common phase II metabolic enzymes (UGTs, SULTs, etc.). In addition, Cruciani's team [2] found that for PROTACs containing VHL ligands, aldehyde oxidase (hAOX) is involved in metabolism and catalyzes the hydroxylation of its thiazole ring. Since aldehyde oxidase is more expressed in humans, metabolic studies targeting this enzyme need to be paid attention to.

1.4.5 Challenges and Strategies for PROTAC Metabolite Identification Studies

The process of identifying metabolites for a PROTAC molecule aligns closely with that associated with traditional small-molecule drugs (Figure 1.31). Initially, an appropriate metabolic study

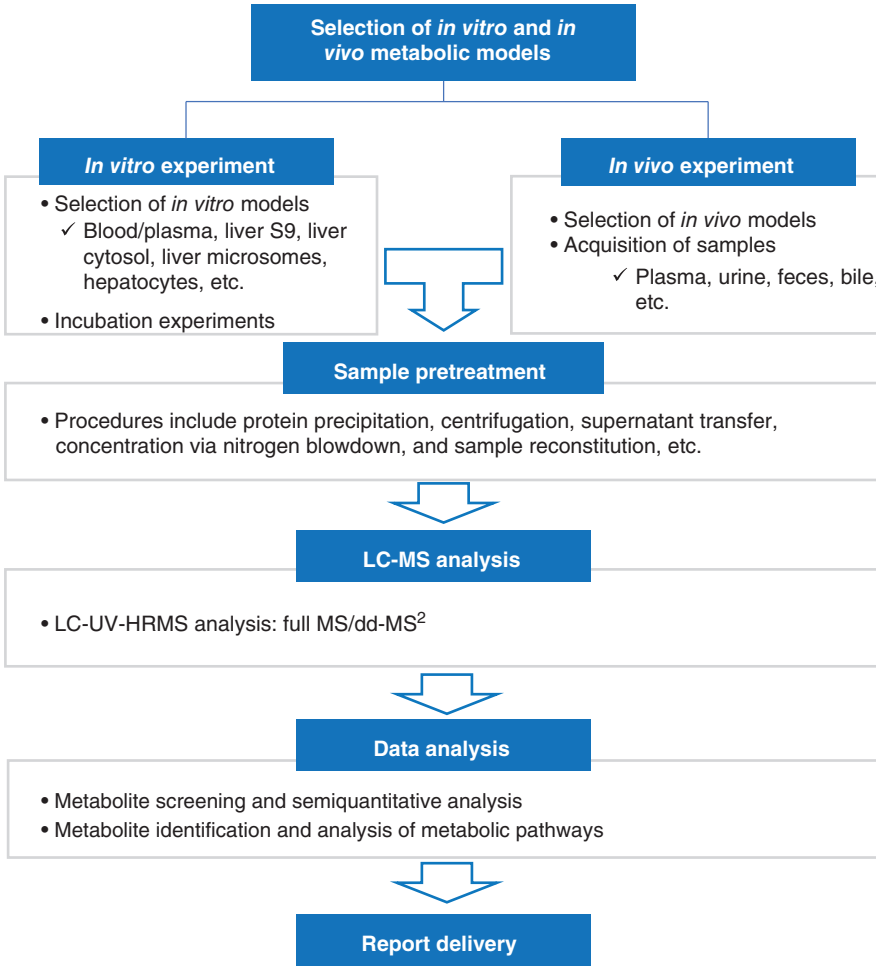


Figure 1.31 Workflow for metabolite identification of PROTACs.

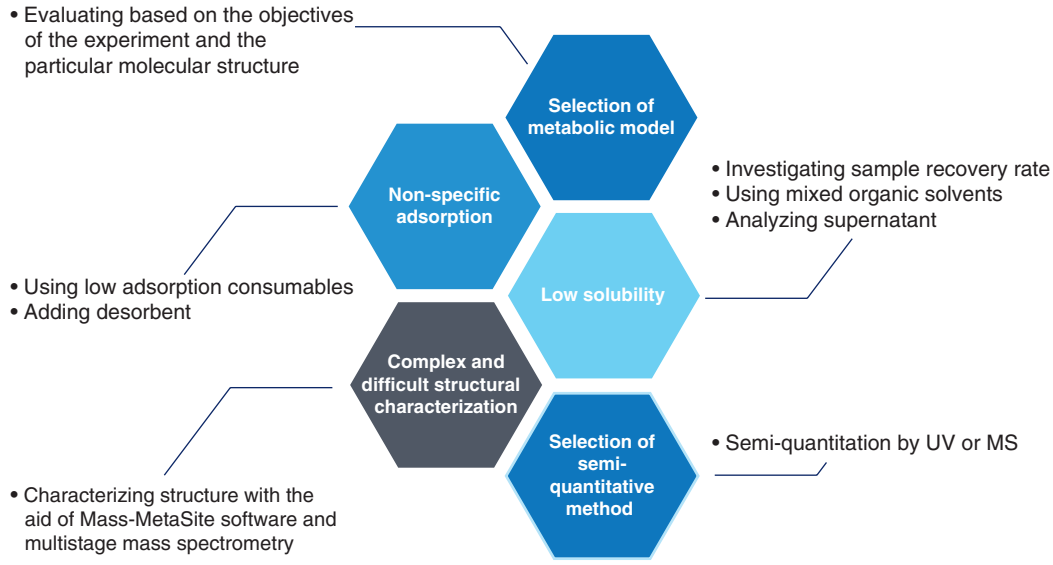


Figure 1.32 Diagram of challenges and strategic solutions for PROTAC metabolite identification studies.

system is selected for either *in vitro* incubation or *in vivo* administration in animals. Following this, samples, either from incubation or collected *in vivo*, undergo a process of liquid chromatography-ultraviolet-high-resolution mass spectrometry (LC-UV-HRMS) analysis after protein precipitation, centrifugation, supernatant transfer, nitrogen blowing, concentration, and redissolution. The parent drug and its metabolites are separated through chromatography, and a comparison of the information between the drug substance and its metabolites based on the MS and MS^2 signals from high-resolution mass spectrometry, the metabolic pathways, and reaction sites of PROTAC is analyzed.

However, due to the distinct structural and metabolic characteristics of PROTAC drugs compared to traditional small molecules, the study of their metabolites presents additional challenges. These include the selection of appropriate *in vitro* and *in vivo* models, addressing issues of nonspecific adsorption and solubility of parent drugs during sample preparation, and efficiently acquiring the relative abundance of metabolites. Furthermore, in the data analysis phase, accurately completing the structural characterization also poses a challenge.

Drawing from the extensive research expertise of the WuXi AppTec DMPK (drug metabolism and pharmacokinetics) MetID team, we have devised a comprehensive research strategy to effectively address the challenges encountered during PROTAC MetID studies (Figure 1.32). Our approach aims to deliver high-quality research reports to our clients, thus expediting the drug development process for PROTAC molecules. We delve into these strategies in greater detail in the following sections.

1.4.5.1 Selection of Metabolic Models

The selection of an appropriate metabolic model is key for successful *in vitro* and *in vivo* metabolite research. For the study of PROTAC molecules, from which dimensions should appropriate metabolic models be selected?

The selection of an appropriate *in vitro* metabolic system for studying PROTAC molecules depends on the experiment's objective and the molecule's structural characteristics (Table 1.3). If

Table 1.3 The *in vitro* and *in vivo* systems for PROTAC metabolism and their respective purposes.

Study subjects	Matrix	Purpose
PROTAC	*In vitro* blood/plasma	Investigating hydrolases-mediated metabolism in blood/plasma
	Liver S9/liver cytosol (with or without aldehyde oxidase inhibitor)	Investigating aldehyde oxidase-mediated metabolism
	Liver microsomes	Investigating CYP enzyme-mediated metabolism
	Hepatocytes	Investigating both phase I and II enzyme-mediated metabolism
	In vivo samples (plasma, blood, urine, feces, and bile)	Investigating the correlation between *in vitro* and *in vivo* metabolism to predict metabolic processes in the human body
	In vivo samples obtained from the human body	Evaluating the exposure of PROTACs in the human body and the appropriateness of species selection for toxicology studies

the study involves the metabolic stability of PROTAC under the action of blood hydrolytic enzymes, *in vitro* plasma or incubation can be utilized. Liver S9 or liver cytosol is selected as the metabolic system when aldehyde oxidase is involved in metabolism. This is particularly critical for PROTACs containing a VHL ligand, as its thiazole ring may be hydroxylated under aldehyde oxidase catalysis [2]. Hepatocyte systems are used to study the effects of CYP enzymes and non-phase I metabolizing enzymes on compounds. For larger molecular weight PROTAC molecules with poor cell permeability, the liver microsomal system is more suitable than the hepatocyte system for studying reactions involving only CYP enzymes [8].

For *in vitro–in vivo* metabolism correlation, plasma, urine, feces, bile, and other *in vivo* samples can be used to evaluate the correlation between *in vitro* and *in vivo* metabolism of PROTAC, helping predict human metabolism. MetID studies of human *in vivo* samples can assess the intra-body exposure scenarios of PROTAC and determine the appropriateness of the selected toxicological species.

1.4.5.2 Method Development

During the method development of PROTAC molecules, nonspecific adsorption and solubility issues are common challenges. Improper handling of these issues can significantly impact the comprehensiveness and accuracy of metabolite analysis results. Hence, it is crucial to develop methods and strategies in advance to effectively address these challenges.

For nonspecific adsorption issues, the large molecular weight of PROTAC can result in easy NSB to the surface of consumables [10]. To mitigate this, it is recommended to use low-adsorption consumables and assess compound adsorption during the method development stage. If a compound is found to bind nonspecifically to consumables, the addition of an appropriate amount of desorbent may be required. For further information on this topic, refer to Section 1.6 PROTAC Bioanalysis: Challenges and Strategies.

To address solubility issues, the solubility can be first evaluated by investigating the recovery of PROTAC and selecting the suitable solvent. If the recovery rate is insufficient, it may result in

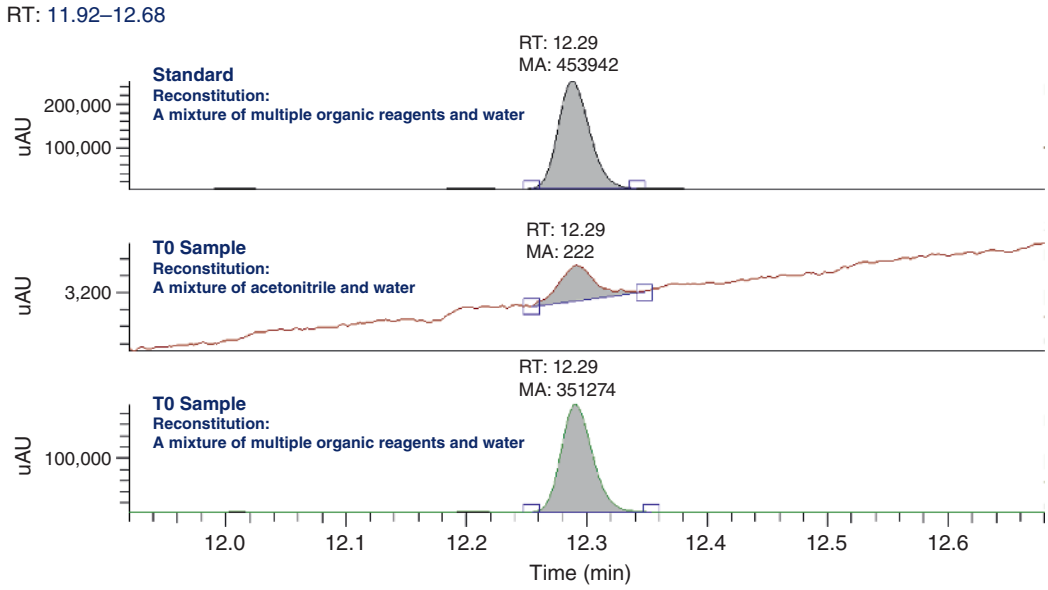


Figure 1.33 LC-UV chromatograms of a PROTAC in different reconstitution solvents.

missed metabolites, inaccurate semiquantitative results, and ultimately unreliable MetID. For instance, a PROTAC molecule in a mixed acetonitrile/water solvent may show low recovery at 0 minutes of hepatocyte incubation (T0 sample) and cause system stratification. Adjusting the proportion of acetonitrile might not improve this situation, suggesting the need for other organic reagents in the reconstitution solution. In one case, the highest recovery rate was achieved when the reconstitution solution was a mixed solvent made up of several organic reagents and water, fulfilling the analytical requirements (as depicted in Figure 1.33).

To solve PROTAC's solubility problem, strategies may include using solutions containing mixed organic reagents for compound dissolution, dilution, and pretreatment, or directly using the supernatant after protein precipitation and centrifugation for LC-UV-HRMS analysis. By adopting these strategies, the problems of nonspecific adsorption and solubility of PROTAC samples can be effectively overcome to ensure the comprehensiveness and accuracy of metabolite analysis and identification results.

1.4.5.3 Structural Characterization of Metabolites

Structural characterization is a critical step in MetID. For PROTAC molecules, their large molecular weight and the presence of multiple reaction sites pose significant challenges for efficient and high-quality structural characterization, a crucial aspect of metabolite research. To solve these challenges, we've developed a set of strategies specifically for PROTAC metabolite profiling and structure identification (Figure 1.34). These strategies, derived from extensive experimental explorations and experiences, leverage the strengths of the MetID software Mass-MetaSite, the expertise of senior scientists, and high-resolution mass spectrometry platforms.

In this strategy, our experienced professionals characterize metabolite structures by interpreting the UV and mass spectral information from LC-UV-HRMS analysis data. This includes elucidating the metabolic pathways and metabolic sites of PROTAC metabolites through chromatographic

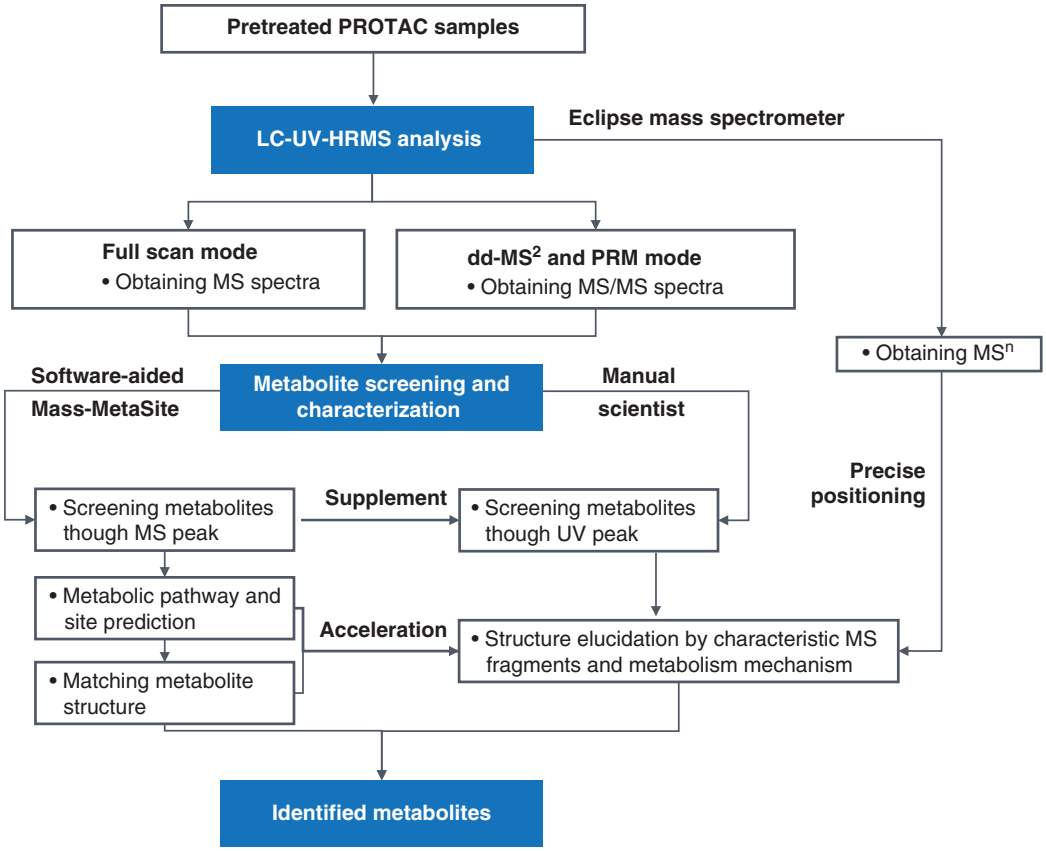


Figure 1.34 Workflow for the metabolite identification of PROTACs.

peak searching and mass spectral fragment information interpretation. Simultaneously, we employ Mass-MetaSite software for automated screening and identification of metabolites, calculating metabolic pathways and potential metabolic sites, and structurally aligning them with experimental results. This integrated approach that combines expert interpretation with automated software analysis speeds up the process of characterizing PROTAC metabolite structures. This assists researchers and developers in their work, making the process more efficient and reliable.

Additionally, WuXi AppTec DMPK platform features an industry-leading Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer capable of multistage (third grade and above) mass spectrometry data. During the structural characterization process, R&D scientists utilize this multistage mass spectrometry data to deeply analyze the fragmentation behavior of drug candidates and their metabolites. This allows for the acquisition of more refined fragment information and precise localization, ensuring high-quality structural characterization.

Through our strategy that combines professional software, experienced expert teams, and industry-leading high-resolution mass spectrometry platforms, structural characterization in MetID can be efficiently carry out. This approach helps us navigate the challenges posed by the complexity of PROTAC molecules, speeds up PROTAC metabolite research, and provides crucial support for the design and development of PROTAC drugs.

1.4.5.4 Semi-quantification of Metabolites

Through semiquantitative analysis of characterized metabolites, we can estimate the relative abundance of the parent drug and its metabolites. This allows for an initial understanding of the proportion of each metabolite and helps identify the main metabolites. This process is crucial for identifying metabolic soft spots, comparing *in vitro* metabolic differences across species, and comparing *in vitro* and *in vivo* metabolic differences. The information generated provides a basis for further structural optimization of subsequent drugs, prediction of drug metabolism in the human body, and safety evaluation of metabolites.

Given the structural properties of PROTAC, it is vital to ensure the accuracy of semiquantitative results [11]. In semi-quantification using MS, the difference in ionization efficiency of the parent drug and its products in the ion source can impact the semiquantitative data accuracy. Moreover, PROTAC and its metabolites often produce not only single-charged ions but also multi-charged ions and the ions cleaved in the ion source. These factors need to be fully considered when performing semi-quantification by mass spectrometry integration. This involves synchronously extracting and integrating single-charged ions, multi-charged ions, and ions cleaved in the source, which enhances the accuracy of semi-quantification. For instance, in a PROTAC study, we integrated the extracted ion current for a metabolite. The peak area after superimposing the extracted single- and multi-charged ions was approximately eight times that of the single-charged extracted ion.

When employing a UV detector for semi-quantification, it's crucial to note that if the E3 ligand of PROTAC has a different UV chromophore compared to the POI ligand, the metabolite of linker breakage might differ from the parent drug in terms of the absorbance coefficient. This discrepancy could significantly reduce the accuracy of UV semiquantitative data. In such scenarios, other detectors, such as charged aerosol detector (CAD), can be considered for more accurate results.

Therefore, the approach to semi-quantifying metabolites should be chosen comprehensively, taking into account their structural characteristics, UV spectra, and mass spectra.

1.4.6 Summary

After over two decades of development, PROTAC has become popular among leading pharmaceutical companies and research institutions worldwide due to its unique mechanism of action and advantages. Metabolite studies are crucial during the discovery, preclinical, and clinical stages of PROTAC drugs, making the experimental data for MetID increasingly critical for new drug applications (NDA). Leveraging the advanced Waters ACQUITY UPLC liquid-phase system and mass spectrometry systems such as Orbitrap Eclipse Tribrid and Q-Exactive™ HF, in conjunction with data processing software like Mass-MetaSite, Compound Discoverer, and Metabolynx, WuXi AppTec DMPK MetID team has developed a research platform for PROTAC metabolite profiling and identification. This platform, based on ultra-performance ultraviolet-liquid chromatography-high-resolution mass spectrometry (UPLC-UV-HRMS) technology, has established a series of robust solutions to overcome the challenges in the experimental process, thereby aiding the metabolic study of PROTAC molecules in numerous drug development companies globally. Looking forward, we anticipate an increasing number of PROTAC drugs transitioning into clinical studies and, ultimately, achieving a successful market launch, bringing hope to more patients.

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1.5 Evaluation of Drug-Drug Interactions of PROTACs with Efflux Transporters

Liping Ma, Chengyuan Li, Jing Jin

1.5.1 Introduction

Drug transporters can be major determinants of drug pharmacokinetics. To evaluate the drug interactions with these drug transporters, suitable *in vitro* methods or models should be applied. The literature has revealed the drug interactions between a PROTAC drug and rosuvastatin. The elevation of aspartate aminotransferase and alanine aminotransferase (AST/ALT) was observed in subjects taking both the rosuvastatin and a PROTAC drug; symptoms alleviated after discontinuation of rosuvastatin, and treatment with the PROTAC drug could be continued. The possible reason is that the PROTAC drug inhibits the breast cancer resistance protein (BCRP) transporter, for which rosuvastatin is a known BCRP transporter substrate [1]. The combination of rosuvastatin with BCRP inhibitors may inhibit its efflux in the intestine and its biliary excretion in the liver, thus resulting in elevating the plasma concentrations of rosuvastatin and triggering corresponding adverse effects [2]. Considering the potential DDI risk in the clinic, it is necessary to assess the DDI of PROTAC with BCRP transporters at the early screening stage. Based on our research experience, *in vitro* transporter studies of PROTAC molecules present some challenges. Improper model selection may lead to erroneous results and mislead the assessment of DDI risk.

1.5.2 What Is a BCRP Transporter?

BCRP is a major ATP-binding cassette (ABC) transporter that plays an important role in drug transport. BCRP is predominantly expressed in the apical membrane of the intestinal epithelia, in liver hepatocytes, and in the basolateral membrane of the blood–brain barrier, which can exert a significant impact on restricting the entry of BCRP substrates into intestinal epithelia, mediating drug biliary excretion and the blood–brain barrier [3]. Many therapeutic agents commonly used in clinical practice are substrates for BCRP, such as the statins (rosuvastatin and pitavastatin) and the antineoplastic agents (axitinib, canatinib, imatinib, lapatinib, sunitinib, irinotecan, topotecan, etoposide, SN-38, and methotrexate) [2]. The latest Food and Drug Administration guidelines for *in vitro* DDI studies also clarify that BCRP is one of the nine drug transporters recommended for evaluation [4].

1.5.3 How to Evaluate the Inhibition of BCRP by PROTACs

The risk of DDI can be assessed at an early stage using *in vitro* transporter models. If *in vitro* studies indicate that a drug is a BCRP inhibitor, the sponsor should consider whether to conduct a clinical drug interaction study. With regard to the selection of transporter models, Caco-2, BCRP-overexpressed cells, or membrane vesicles with a high expression of BCRP can be used to determine if an investigational drug can inhibit BCRP. Of these, Caco-2 cells are common models for evaluation due to their easy access. First, we selected the most frequently used Caco-2 model to evaluate the BCRP inhibition potential of PROTACs. But here's the lesson we learned: improper model selection may lead to erroneous conclusions. We would like to share our experiences on this study and emphasize the model selection for PROTAC molecules.

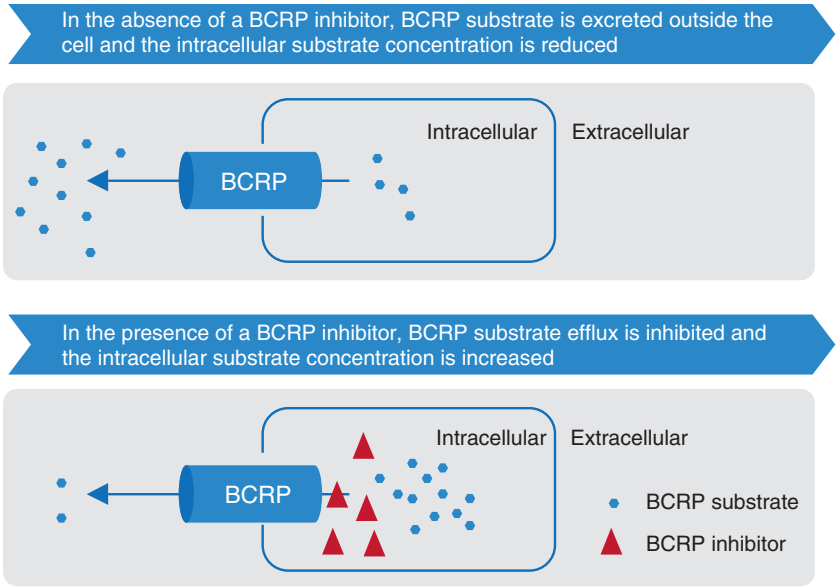

Figure 1.35 Breast cancer resistance protein (BCRP) efflux and transport inhibition.

1.5.4 Case Study and Analysis on the Inhibition of BCRP by PROTACs

The mechanism of the BCRP inhibition assay is shown in Figure 1.35. BCRP substrate can be pumped out of cells via BCRP, and the direction is from inside to outside. In the presence of the BCRP inhibitor, the BCRP-mediated efflux of the substrate can be inhibited. The inhibitory effect of a PROTAC molecule on BCRP was determined by using a Caco-2 cell model. The results of the assay showed no inhibitory effect of the PROTAC molecule on BCRP, which is inconsistent with the literature. We ruled out solubility and adsorption issues and tried to find the underlying reasons.

The molecular mechanisms of BCRP inhibition are relatively complicated: (i) one class of inhibitors, such as fumitremorgin C and Ko143, inhibits the ATPase activity of BCRP; (ii) another class of inhibitors has the same binding site as the substrate, affecting the binding of the substrate to BCRP; and (iii) the third class of inhibitors binds outside of the substrate binding site, causing a conformational change in BCRP and thus affect substrate transport [5]. Reports on the third mechanism are relatively limited, for which the inhibitor binding site may be intracellular or extracellular, whereas the other two inhibition mechanisms require the inhibitor to enter the cell first.

A general characteristic of PROTAC molecules is poor permeability. In the Caco-2 assay, inhibitors in the incubation system need to enter the cells to work. Although the concentration of PROTAC outside Caco-2 cells is high, the intracellular drug concentrations may be low due to poor permeability. Therefore, we speculate that it is likely that the concentration of PROTAC in Caco-2 cells is too low to exert an inhibitory effect (Figure 1.36).

1.5.5 Customized Assay on PROTACs

Based on the analysis of the above influence factors, we switched to a membrane vesicle model to evaluate BCRP inhibition by PROTAC. Membrane vesicles expose the substrate binding sites of ABC transporters to extracellular buffers, which is conducive to studying the interaction of drug

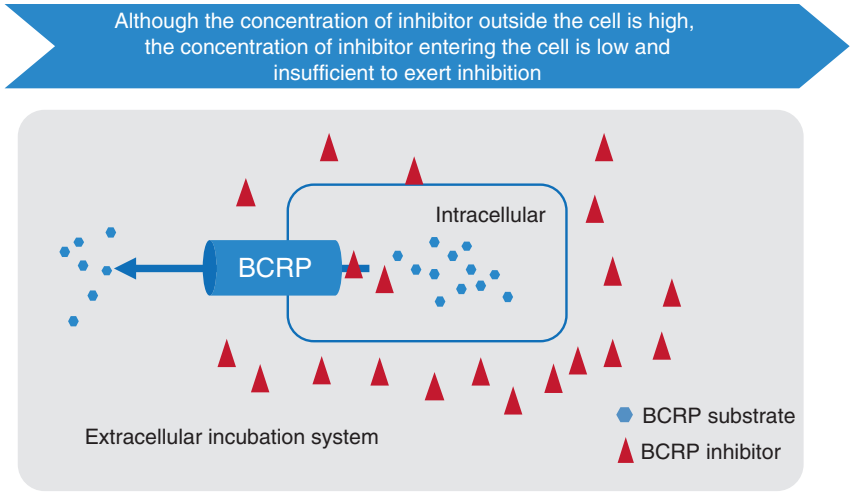

Figure 1.36 A low intracellular inhibitor concentration fails to exert an inhibitory effect.

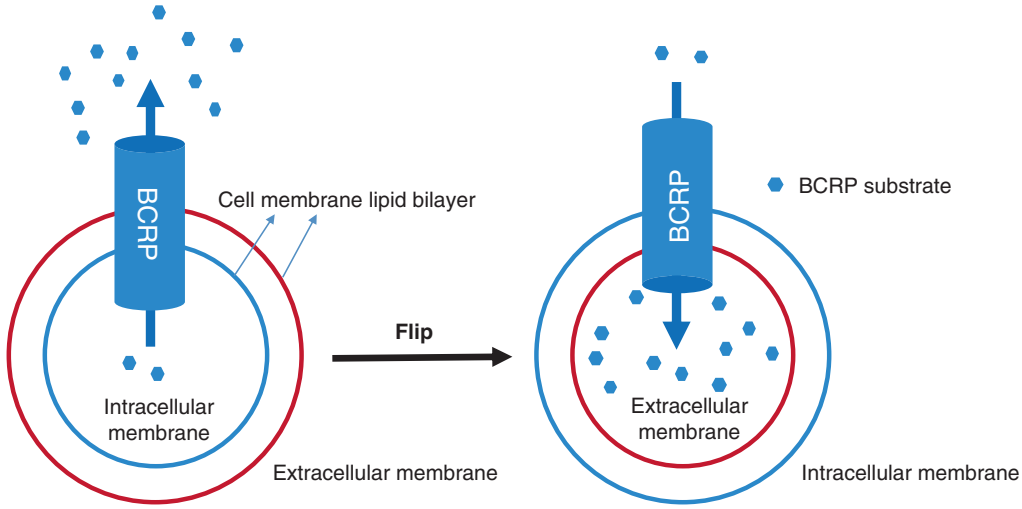

Figure 1.37 Mechanism of eversion of epithelial membrane vesicles.

candidates as substrates and inhibitors with ABC transporters. Figure 1.37 illustrates the mechanism of everted membrane vesicles. Originally, the substrate transport direction mediated by efflux transporters is from intracellular to extracellular, but after eversion, the substrate transport direction is reversed (extracellular to intracellular). In this model, the inhibitor can be in direct contact with the transporter substrate binding site outside the membrane.

1.5.6 Results

The membrane vesicle method showed that the PROTAC molecule had a strong inhibitory effect on BCRP, which was consistent with the previous literature reports.

1.5.7 Summary

The vesicle model is more sensitive in assessing efflux transporter inhibition than conventional methods that fail to obtain inhibition. This case caused us to pay attention to the model selection in the study of the inhibition of efflux transporters by PROTAC molecules. In addition to BCRP, the same problem may exist in the inhibition of other efflux transporters by PROTAC molecules. The poor permeability of PROTAC may lead to an underestimation of the risk of PROTAC inhibition of efflux transporters when assessing the inhibition of efflux transporters by PROTAC molecules using an intact cell model. The membrane vesicle model is more suitable to study the inhibitory effect of low-permeability PROTAC molecules on the efflux transporter.

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1.6 PROTAC Bioanalysis: Challenges and Strategies

Tiantian Dang, Weimin Hu, Weiqun Cao, Lili Xing

1.6.1 Introduction

In order to exert their therapeutic effects, traditional small-molecule drugs or large-molecule antibodies generally must be bound to the active sites of an enzyme or receptor; however, 80% of the targets in human cells lack such sites. As an emerging strategy for new small-molecule drug development, PROTAC significantly combines the advantages of small-molecule compounds and small-molecule nucleic acids. They could capture target proteins through any binding site anywhere in a cell, making it easier to enter cells and exert the desired effect. PROTAC technology has embraced constant iterations and updates and has become a hot research area in recent years.

However, the mechanism involved in PROTAC's crossing of the cell membrane remains unclear. Therefore, to support the research on its absorption, distribution, metabolism, excretion, and toxicity, further theoretical and practical studies are necessary. Despite PROTAC having substantial potential, their complex structures may result in poor druggability, posing huge challenges to clinical trials, bioanalysis, and PK/PD studies. This chapter focuses on some challenges and practical experiences in PROTAC bioanalysis.

1.6.2 Key Points of PROTAC Bioanalysis

According to bioanalysis, a PROTAC molecule consists of a target protein–ligand, linker, and E3 ligand, resulting in a relatively large molecular weight, typically around 1,000 Da. Multi-charged ions in mass spectrometry could cause signal dispersion, potentially leading to a loss of sensitivity. In addition, the linker structure in a PROTAC molecule is fragile and prone to breakage [1]. Based on our practical experience, the optimization of MS parameters is crucial in the LC-MS/MS analysis process to avoid in-source fragmentation. Furthermore, PROTAC compounds often have multiple chiral centers, which can result in peak splitting during liquid chromatography. Therefore, adjusting the liquid-phase method to optimize peak shape is necessary for achieving smoother analysis. The exposed ions may also nonspecifically bind to the experiment material, such as glass or plastic [2]. Hence, it is essential to eliminate nonspecific adsorption to ensure no compound loss during the operation. Although the lyophilized powder of such compounds is stable, some hidden stability issues may arise when they exist in a biological matrix. Therefore, timely detection and preventive measures are necessary to ensure data accuracy. The following are the key points for PROTAC bioanalysis.

1.6.2.1 Optimal MS Parameters

The fragile linker structure in PROTAC makes it prone to in-source fragmentation, which can be reduced by optimizing the low ionizing energy and ion source temperature. In addition, for compounds with high molecular weights, the MS conditions are further optimized to enhance sensitivity by seeking out multiple charges. The current lower limit of quantification (LLOQ) can reach 1 ng/mL.

1.6.2.2 Peak Shape Optimization – Peak Splitting

The existence of multiple chiral centers [3] in the structure of PROTAC leads to peak splitting during liquid chromatography. By optimizing the method of liquid chromatography (such as mobile phase replacement, chromatographic column screening, gradient regulation, and ion source

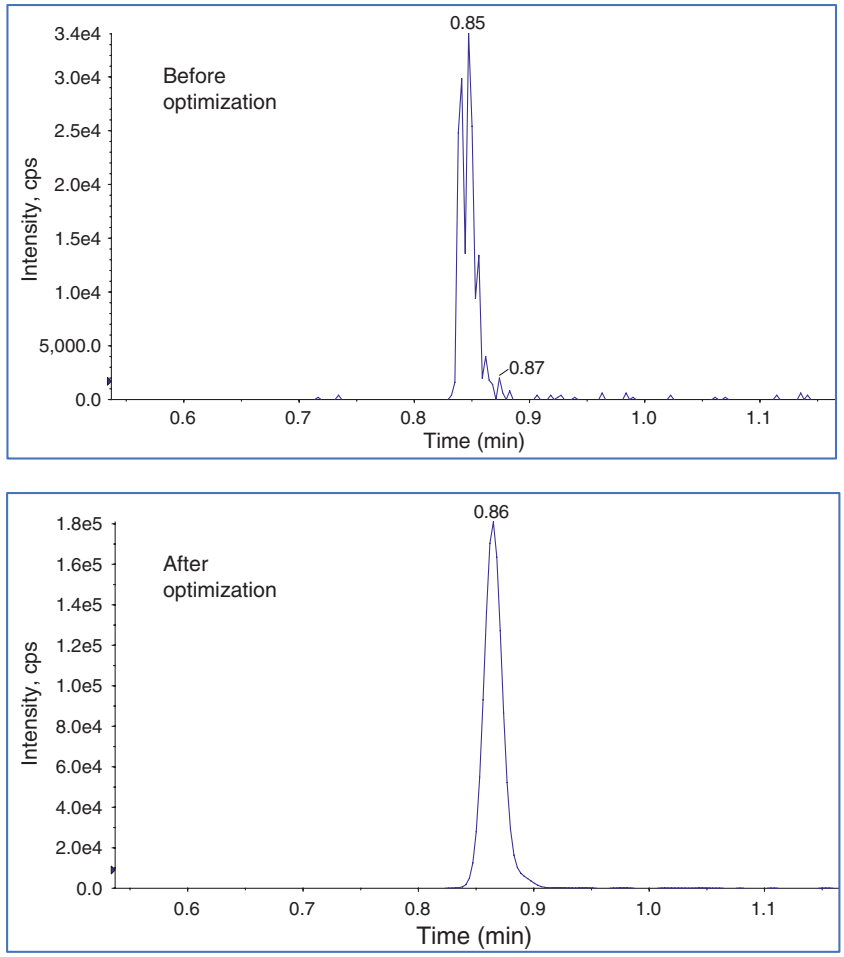


Figure 1.38 Comparison of compound A chromatographic peaks before and after optimization.

temperature reduction), a symmetrical and good peak shape can be achieved, and the compound's signal can be enhanced (Figure 1.38).

1.6.2.3 Elimination of Nonspecific Binding

PROTAC is a small-molecule compound with a large molecular weight (~1,000 Da) and exposed ions tend to nonspecifically bind to the surface of experiment material such as glass or plastic. Therefore, it is necessary to use appropriate container materials to collect a sample after dosing, use low-binding materials as much as possible, and investigate the NSB of compounds during the development of analytical methods. If NSB was found, a certain proportion of desorbent (such as Triton X-100 and Tween 20) should be added to block the NSB sites, ensuring that the compound remains intact during operation (Table 1.4).

1.6.2.4 Controllable Matrix Stability

PROTAC exhibits distinctive and covert plasma stability issues. Typically, enzyme activity in the fresh matrix is higher than that in the frozen matrix, making enzyme-sensitive compounds less

Table 1.4 Comparison of compound B transferred three times in blank plasma before and after adding a desorbent.

Sample	Compound B remaining (before adding the desorbent)	Compound B remaining (after adding the desorbent)
T0	100%	100%
T1	90%	87%
T2	85%	90%
T3	65%	85%

Note: T1: compound B was transferred once in the blank plasma; T2: compound B was transferred twice in the blank plasma; T3: compound B was transferred three times in the blank plasma.

Table 1.5 Comparison of stability of compound C in fresh and frozen plasma at room temperature for two hours.

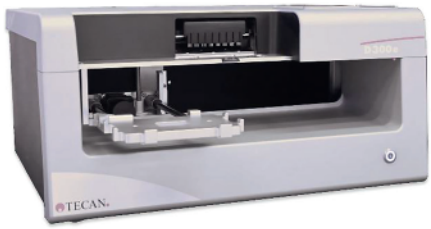
Matrix	Compound C remaining
Fresh plasma	95%
Frozen plasma	35%

stable in the fresh matrix or samples. Some PROTAC compounds are stable when placed at room temperature in the fresh matrix for two hours, but they become highly unstable in the frozen matrix, and no inhibitors have been identified to effectively stabilize them (see Table 1.5). Therefore, a fresh blank matrix should be used to prepare calibration standards and quality control samples.

Furthermore, it is necessary to avoid repetitive freezing of samples to ensure the accuracy of the unknown sample's concentration. After collecting biological samples, protein precipitation is directly carried out to obtain the preserved supernatant for testing. In this case, the stability of the treated supernatant is the main concern. In the case of compound C, a standard solution was quantitatively added to the fresh matrix to simulate the real sample. The supernatant, after protein precipitation, was placed in a sample injector at 4°C and a refrigerator at –40°C for 24 hours, respectively. Next, the supernatant of 4°C and –40°C were injected alongside the 0-hour sample, indicating that the supernatant was stable after storage in the sample injector at 4°C and refrigerator at –40°C for 24 hours (Table 1.6). This shows that it is feasible to obtain and store the supernatant samples for preservation in the analysis of a DMPK project by immediately performing protein precipitation on biological samples after collection.

Table 1.6 Stability of compound C in supernatant of fresh plasma.

Sample	Remaining of the substance to be tested
0-hour sample	100%
Storage in sample injector at 4°C for 24 hours	95.4%
Storage in the refrigerator at –40°C for 24 hours	99%



HP D300e digital dispenser

Hamilton microlab STAR

Sciex triple QuadTM 6500+

Figure 1.39 Biological sample analysis platform.

Each step of the bioanalysis operation is interlinked. During the sample pretreatment stage, the use of HP D300e digital titration equipment allows for swift preparation of calibration standards and quality control samples within eight seconds, eliminating the need for multiple transfers of compound solutions and mitigating NSB. Biological samples are precipitated immediately after collection, and the whole sample pretreatment process is carried out on wet ice conditions to ensure the accuracy and reliability of sample concentration detection, which provides reliable data support for the absorption, distribution, metabolism, and excretion (ADME) experiments of the PROTAC *in vivo* study.

1.6.3 PROTAC Bioanalysis Process

Based on different characteristics of PROTAC and the above-mentioned analysis experience, the PROTAC bioanalysis process is summarized as follows (Figure 1.39):

Optimization of MS parameters: Adjusting ionizing energy and ion source temperature; exploring multiple charges to improve sensitivity.

Optimization of liquid chromatography conditions: Screening for a suitable chromatographic column, mobile phase, and mobile phase gradient to obtain a better peak shape.

Elimination of nonspecific binding: Carry out the method development initially and screen suitable desorbents if adsorption occurs.

Optimization of sample processing procedure: Rapid operation at low temperatures, precipitation immediately after sample collection, and using the fresh matrix to prepare calibration standards and quality control samples.

Method reproducibility: Further optimizing the method to meet analysis requirements (reproducibility, LLOQ, matrix effects, etc.).

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