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Protein degradation for drug discovery

Targeted protein degradation *in vivo* with Proteolysis Targeting Chimeras: Current status and future considerations

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Proteolysis Targeting Chimeras (PROTACs) are a rapidly expanding new therapeutic modality inducing selective protein degradation and offering the potential of a differentiated pharmacological profile across multiple therapeutic areas. As the repertoire of protein targets and E3 ligases available for incorporation into PROTACs continues to grow, understanding the drug- and system-dependent parameters for PROTACs will be critical for achieving tissue/cell specific pharmacology. The review discusses the current knowledge and future direction of *in vivo* PROTAC study evaluation. The importance of establishing the quantitative relationship between loss of protein target and biological function *in vivo*, coupled with building mechanistic PK/PD and ultimately PBPK/PD models, is emphasised with the aim to aid translation from preclinical to clinical space.

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Introduction

Targeted protein degradation is a rapidly expanding area with a high level of interest from the Pharma/Biotech. industry. Clinical benefit has been obtained with monovalent protein degraders such as Faslodex® (Fluvestrant, ICI-182, 780), a selective estrogen receptor degrader (SERD), and the phthalimide compound class known as IMiDs (Immunomodulatory drugs) of which Lenalidomide (Revlimid®, CC-5013) and Pomalidomide (Pomalyst®, CC-4047) [1] are examples. The molecular mechanism for Lenalidomide and Pomalidomide has been elucidated [2], binding of IMiDs to the $CUL4^{CRBN}$ (cereblon, CRBN) E3 Ligase alters the substrate specificity of the CRBN E3 ubiquitin ligase complex resulting in degradation of its neosubstrates via the proteasome, whereas Faslodex® is thought to accelerate ERα

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turnover *via* the ubiquitin proteasome system [3]. This review focuses on the latest addition to the field of targeted protein degradation, Proteolysis Targeting Chimeras (PROTACs).

PROTACs are bifunctional molecules, which induce the selective loss of a given cellular protein through simultaneously binding the target protein of interest and an appropriate E3 ubiquitin ligase resulting in the protein being ubiquitinated and subsequently degraded by the proteasome [4–6]. Consequently, this opens up the field of protein degradation in terms of the range of proteins which can be targeted [7]. Currently three E3 ligases (*i.e.* von Hippel Lindau (VHL), Cereblon (CRBN) and Inhibitor of Apoptosis Proteins (IAP)) are routinely employed and the biology associated with each has been described elsewhere [8]. Recently the E3 ligase, murine double minute 2 (MDM2) protein, a cellular inhibitor of the tumour suppressor p53, has also been incorporated into PROTAC molecules and has resulted in potent degradation of the BRD4 bromodomain protein [9]. The applicability of this approach to a wide range of protein targets together with the development of more 'drug-like' properties of PROTACs have resulted in a burgeoning interest in these molecules as a therapeutic modality.

The selective loss of cellular proteins following PROTAC treatment is an attractive therapeutic concept and compliments the focus on the use of human genetics to support the choice and validation of cellular protein targets for drug discovery [10–12]. The biological profile resulting from protein loss may be differentiated compared to pharmacological inhibition/antagonism as illustrated by the comparison with genetics studies. However it is acknowledged that caveats exist with genetic approaches and also that many proteins, such as kinases, have biological roles that extend beyond their enzymatic function or signalling events [13–15]. This is exemplified by the differentiated biological profile demonstrated with P300/CBP-associated factor/general control nonderepressible 5 (PCAF/GCN5) PROTACs [16] and Focal Adhesion Kinase (FAK) PROTACs [17]. The PCAF/GCN5 PROTAC treatment but not pharmacological inhibition recapitulated the phenotype of PCAF-deficient immune cells. The tyrosine kinase, FAK, has been reported to control tumour growth through both kinase -dependent and -independent mechanisms [18] and FAK PROTACs [17] have been reported to display more effective reduction of FAK activation and FAK-mediated cell migration and invasion. Additionally, there are *in vitro* examples where the PROTAC treatment impacts a wider and differentiated gene set compared to inhibitor treatment [19,20] thereby offering a potentially differentiated biological profile. A recent example of a BRD4 PROTAC utilising the E3 ligase MDM2 [9] in oncology cell lines highlights the potential synergy achieved *via* the combined pharmacology of the target protein and E3 ligase. Furthermore, as PROTACs only require a binder and not necessarily a functional inhibitor, this affords an additional opportunity to access unique biological profile associated with so-called 'undruggable' targets.

Targeted protein degradation has been demonstrated *in vitro* for a wide range of targets [7] although a much-limited set of proteins have been degraded *in vivo*. Although their biological activity is the result of 'event-driven' rather than 'occupancy-driven' pharmacology [4], much remains to be understood regarding these molecules, notably building and understanding their pharmacokinetic (PK)/pharmacodynamic (PD) relationships. PK/PD relationships are the cornerstone of drug discovery and development [22] enabling the translation of these molecules into the clinic to explore their human pharmacology and aiding the reduction of drug attrition rates [23,24]. This review summarises the current knowledge of *in vivo* efficacy with PROTACs and highlights potential future direction for development of PK/PD understanding.

In vivo efficacy with PROTACs

In vivo targeted protein degradation with PROTACs was first reported in 2015 by two independent groups [25,26], which targeted the estrogen-related receptor α (ERRα) and Bromodomain and extraterminal (BET) and utilised different E3 ligases, von Hippel Lindau (VHL) and $CUL4^{CRBN}$ (cereblon, CRBN), respectively. Data from a single time-point demonstrated partial knockdown (~40%) of the ERRα in mice bearing MDA-MB-231 tumour cells with equivalent reduction in both tumour and endogenous ERRα levels following intraperitoneal (IP) administration of PROTAC_ERRα at high dose and dose frequency (100 mg/kg three times a day) [25]. PROTAC_ERRα utilised VHL as its E3 ligase and measured blood compound concentrations, which were in the μM range and greater than the concentration required to produce 50% degradation of ERRα in 50% mouse serum *in vitro*, suggesting that the PROTAC distributed well into tissues and tumour. Winter et al. [26] demonstrated a substantial reduction of tumour volume in a mouse xenograft model of human MV4;11 acute myeloid leukaemia (AML) cells following treatment with dBET1, a CRBN based PROTAC for BET proteins, at a dose 50 mg/kg given IP once daily. The reduction in tumour weight and progression was associated with a reduction in BRD4 (>80%) and cMYC (>50%) protein levels, which were determined at a single timepoint of 14days post-treatment initiation. PK data suggested that the compound was readily available (T_{MAX} = 0.292 h) achieving C_{MAX} value of 12,394 ng/mL, which is greater than the concentration required for 50% degradation of BRD4, and the concentrations falling over the next 4 h to ~100 ng/mL. Since these initial reports there have been several examples where PROTAC treatment in preclinical species has degraded the target protein of interest and has resulted in efficacy in mouse xenograph models. Focussing on peer-reviewed literature, the majority of examples utilise CRBN (Table 1) and VHL (Table 2) as the E3 ubiquitin ligase but the E3 ligase, Inhibitor of Apoptosis Proteins (IAP) has also been used *in vivo* [27].

Table 1. *In vivo* efficacy examples with CRBN-based PROTACs.

Target Protein	Compound name	*In vitro* potency	Pre-clinical animal model		Dosing schedule	PK profile	Experimental outcome
			'PK/PD-like'	Efficacy			
BET [26]	dBET1	MOLM13 cells: DC_{50} = 430 nM, D_{MAX} > 95%.		Murine xenograft model of human MV4;11 leukaemia cells.	50 mg/kg QD IP for 14 days	50 mg/kg IP: C_{MAX} = 12394 ng/mL, T_{MAX} = 0.292 h and AUC_{inf} = 15972 h.ng/mL, with >100 ng/ml at 4 hours post-dose.	Reduction in tumour progression (>70%) and tumour weight (>50%) with reduction of tumour BRD4 (>80%) levels and cMYC (>50%) at day 14 post-treatment initiation. BRD4 and cMYC reductions were consistent with data obtained at 4 time-point after one or two doses.
BTK [21]	PROTAC (10)	RAMOS cells: DC_{50} ~1 nM & D_{MAX} ~90%. PROTAC potency is context dependent with reduced potency on THP-1 cells.	Wistar-Hannover rats		0.35, 35, 175 mg/kg SC or 175 mg/kg SC QD for 2 days.	C_{MAX} & AUC values not detailed, and the PK does not appear to be dose proportional.	Dose-dependent degradation of endogenous BTK in spleen but not lung tissues, with more profound degradation obtained with 2175 mg/kg. Plasma and tissue compound concentrations determined. At 24 h post-dose, compound blood concentrations were ~2, ~200, ~1000 ng/mL and tissue concentration 15–50, 500–800, 2000–3000 ng/mL at 0.35, 35 and 175 mg/kg.
BET [33]	BET degrader 23 (BETd-260)	Degrades BRD2/3/4 at 30–100 pM and with >90% degradation at 1 nM. cMYC levels are also reduced. RS4;11 cell growth: IC_{50} = 51pM.	SCID mice bearing RS4;11 xenograft tumours		5 mg/kg IV	Plasma concentrations: 392 ng/mL at 1 h, reducing to 6.4 ng/mL at 6 h post-dose and <1 ng/mL at 24 h timepoint. Tumour levels followed a similar time profile with 166.3 ng/g tumour at 1 h falling to 35.8 ng/g at 6 h post-dose.	A single dose degraded BRD2/3/4 at 1, 3 and 6 h post-dose, with some recovery of levels at 24 h. cMYC, PARP cleavage and caspase-3 levels followed a similar time-profile.
				SCID mice bearing RS4;11 xenograft tumours	5 mg/kg IV 3 times per week for 3 weeks.		Profound reduction in tumour volume (>90%).
BET [32]	ARV-825			NSG mice implanted with MM1.S cells IV.	50 mg/kg QD IP	No PK data reported.	Significant partial reduction in circulating human lambda light chain immunoglobulins from day 28 post-cell transfer with partial preservation of femoral bone volume determined at 30 days post cell transfer.

Table 1 (*Continued*)

Target Protein	Compound name	*In vitro* potency	Pre-clinical animal model		Dosing schedule	PK profile	Experimental outcome
			'PK/PD-like'	Efficacy			
BET [31]	QCA570 (compound 35)	RS4;11 cells: Degradation:>10 pM produced rapid and profound degradation of BRD3/4 (>3 h incubation) and >30 pM significant BRD2 degradation obtained. Cell growth: IC_{50} = 32pM.	CB.17 SCID Mice bearing RS4.11 xenograft tumours		PK time course at 5 mg/kg IV. PD time course at 1 and 5 mg/kg IV.	Plasma concentrations: 1968 (1 h), 155 ng/mL (3 h) and BLQ at 6 h post-dose. A similar time-profile was obtained in liver, heart, kidney, lung and intestine. Tumour concentrations: 524 (1 h), 188 (3 h) and 78 (6 h) ng/g tissue.	PD data showed degradation of BET proteins in tumour at both dose levels over 1–6 h time-points and with full recovery at 1 mg/kg dose and partial recovery at 5 mg/kg. Suppression of cMYC was less profound but followed the same time-profile with partial recovery at 24 h post-dose.
		MV4;11 cells: Degradation:>30 pM produced rapid and profound BRD3/4 degradation (>3 h incubation) and >30pM produced significant BRD2 degradation. BRD3 degradation required >0.3 nM. Cell growth: IC_{50} = 32pM.		CB.17 SCID mice bearing MV4;11 cell xenograft tumours.	1 & 5 mg/kg IV on days 1, 3 & 5 for 2 weeks.		Complete tumour regression obtained with both dose levels with the 5 mg/kg arm producing a long-lasting tumour regression.
MDM2 [35]	MD-224	Rapid degradation within 2 h, and maximal degradation at 3–10 nM with concomitant accumulation of p53 and p21 proteins and PARP cleavage. RS4;11 cell growth: IC_{50} = 1.5 nM,	SCID mice bearing RS4;11 xenograft tumours		25 mg/kg IV	No PK data reported.	A time-dependent reduction in MDM2 achieved with 80% degradation obtained at 24 h time-point. This was accompanied with a time-dependent accumulation of p53 and p21 proteins and cleavage of PARP at the 24 h time-point.
				SCID mice bearing RS4;11 xenograft tumours	Study 1: 10 or 25 mg/kg IV 3 times per week and 25 mg/kg IV QW. Study 2: 25 mg/kg QD for 5 days per week for 2 weeks, 50 mg/kg QW for 3 weeks or 25 and 50 mg/kg Q2D for 3 weeks		Study 1: The 25 mg/kg on Day 1, 3 & 5 produced ~50% tumour regression, whilst the other two treatment regimens were equi-effective producing a lower level efficacy. Study 2: Complete tumour regression was obtained with the two highest dosing regimens (25 mg/kg QD for 5 days per week for 2 weeks and 50 mg/kg Q2D for 3 weeks).
BTK [34]	DD-03-171	RAMOS cells: 100 nM produces complete degradation on BTK.	C57Bl6 mice		50 mg/kg IP BID	C_{MAX} = 192 ng/mL, $AUC_{(0-LAST)}$ = 22238 ng.h/mL, T_{MAX} = 0.25 h with one dose.	Significant BTK degradation obtained at 6 hours post the third compound dose.

Table 1 (*Continued*)

Target Protein	Compound name	*In vitro* potency	Pre-clinical animal model		Dosing schedule	PK profile	Experimental outcome
			'PK/PD-like'	Efficacy			
			PDX model of DLBCL (DFBL-18689 cells) in NSG mice		50 mg/kg QD IP		Incomplete but significant BTK degradation at study termination. Variable IKZF1 protein levels between animals and no profound degradation obtained.
				PDX model of MCL (DFBL-39435, DFBL-44685 and DFBL-98848 cells) in NSG mice.	50 mg/kg QD IP		**DFBL-39435 cells:** Profound degradation of BTK and IKZF1 in spleen after 3 days of treatment. Significant reduction (~80%) in peripheral tumour blood burden after 14 days comparable to that obtained with ibrutinib and lenalidomide alone or in combination. **DFBL-44685 cells:** Degradation of BTK but not IKZF1 after 3 days of treatment. DD-03-171 had no effect on survival as did ibrutinib and lenalidomide alone or in combination. **DFBL-96069 cells:** Profound degradation of BTK in mice treated for 3 days. BTK inhibition upregulated IKZF1/3 protein levels, which were reduced with DD-03-171 treatment, but the levels were not reduced below the vehicle control levels. A significant reduction in peripheral blood tumour burden and extension of survival compared to ibrutinib and lenalidomide treatment alone.

Table 2. *In vivo* efficacy examples with VHL-based PROTACs.

Target Protein	Compound name	In vitro potency	Pre-clinical animal model		Dosing schedule	PK profile	Experimental outcome
			PK/PD-like'	Efficacy			
ERRα [25]	PROTAC_ERRα	MCF-7 cells: DC_{50} ~100 nM, D_{MAX} = 86%. DC_{50} ~0.9 μM (mouse serum).	CD1 mice bearing MDA_MB-231 tumour cells.		100 mg/kg Q3D IP.	At 5 h post-4th dose, [plasma] ~10 μM, [tumour] & [kidney] ~30 μM and [kidney] ~100 μM.	Down regulation of tumour (39%) and endogenous heart and kidney (44%) ERRα.
BET [19]	ARV-771	ARV-885 used in *in vitro*studies. No data presented in HEL92.1.7 cells.			30 mg/kg QD SC for 5 days per week for 3 weeks.	No PK data reported.	Reduction in tumour burden at day 7 post-engraftment. Increased mouse survival with ARV-771 compared to inhibitor treatment, OTX015 (50 mg/kg QD PO).
BET [20]	ARV-771	Z138 cells: IC_{50} = 142 nM (apoptosis). Complete BRD4/3 degradation at 500 nM.		Luciferase-expressing sAML HEL92.1.7 cells were engrafted into NSG mice.	10 mg/kg or 30 mg/kg QD SC for 5 days per week for 3 weeks.	No PK data reported.	Dose-dependent benefit on survival. ARV-771 (30 mg/kg) treated animals had a more profound effect on survival than the OTX015-treated group. ARV-771 degraded BET proteins and reduced cMYC levels in spleen and bone marrow.
BET [36]	ARV-771	cMYC IC_{90} = 100 nM with 50% mouse serum.	Nu/Nu mice implanted with 22Rv1 tumour cells.		10 mg/kg QD SC.	PK data at 10 mg/kg SC: C_{MAX} = 1.7 μM, T_{MAX} = 1 h, AUC = 7.3 μM.h with 8 nM on board at 24 h post dose.	PD readouts: 10 mg/kg for 3 days resulted in a reduction of tumour BRD4 (37%) and cMYC (76%) levels. A dose-dependent reduction (>80%) of BRD4 and cMYC was obtained after 2 weeks of dosing.
				Nu/Nu mice implanted with 22Rv1 tumour cells.	3, 10 and 30 mg/kg QD SC or intermittent dosing regimens (Q3D or 3 on/4 off) with 30 mg/kg SC.		A dose -dependent reduction of tumour size obtained and at the 30 mg/kg dose 2/10 animal had no palpable mass. Clear differentiation in level of efficacy compared to both inactive PROTAC molecule ARV-766 and inhibitor, OTX015. Intermittent dosing regimens produced 50% reduction in tumour growth and >55% BRD4 degradation and >90% suppression of tumour cMYC.
ERα [37]	TD_PROTAC	DC_{50} <20 μM (T47D cells). IC_{50} = 24 μM (T47D cell growth). Similar activity in MCF-7 cells.		CB.17 SCID mice bearing VCaP tumour xenografts.	10 mg/kg QD IP for 42 days.	No PK data reported.	Tumour volume reduced by >75% which is comparable to that produced with Tamoxifen (4 mg/kg IP QD) treatment. Significant reduction of tumour ERα levels were obtained at study termination although levels not quantified.
ALK [38]	TD-004	93% degradation at 1 μM in SU-DHL-1 cells.		Balb/c nude mice implanted with MCF-7 cells.	58 mg/kg QD IP for 14 days.	No PK data reported.	Significant reduction of tumour volume following treatment. Increase in tumour volume from days 16–30 may be due to the removal of drug treatment.

Table 2 (*Continued*)

Target Protein	Compound name	In vitro potency	Pre-clinical animal model		Dosing schedule	PK profile	Experimental outcome
			PK/PD-like'	Efficacy			
FLT-3 [39]	FLT3-PROTAC	MV4-11 cells: DC_{50} <5 nM and D_{MAX} >90%. Similar degradation obtained in MOLM-14 cells.	MV4-11 cells implanted SC into athymic mice.		30 mg/kg QD for 3 days.	PK data at 10 mg/kg IP: C_{MAX} = 1069 ng/ml, T_{MAX} = 2 h, $t_{1/2}$ = 2.31 h. Concentration of compound >5 nM for ~ 22 h of dosing interval	At 5 h post last dose, a significant reduction (>50%) in FLT-3 levels was obtained.

Undoubtedly there are numerous other examples from conferences, for example we have evaluated the biological profile of RIPK2 IAP-based PROTACs [28] and Arvinas has presented on oral administration of androgen and estrogen receptor PROTACs [29,30]. These reports compliment the existing literature.

Several groups describe the activity of CRBN-based PROTACs *in vivo* (Table 1) and many publications focus on targeting BET proteins [26,31–33]. However, more recently BTK [21,34] and MDM2 [35] degradation *in vivo* has been described. *In vivo* efficacy studies [26,31,32,34,35] utilise xenograft models and the *in vitro* activity of the PROTAC determined in the corresponding cellular assays focussing on readouts such as target protein degradation, inhibition of cell growth and apoptosis. In this scenario the *in vivo* efficacy is likely to be dependent on having sufficient exposure of the PROTAC in the relevant tissue compartment. QCA570 [31] and BET degrader 23 [33] were measured in the tumour within 1 h after intravenous (IV) administration. Interestingly QCA570 was detected at appreciable levels in the tumour but not in the blood compartment at 6 h post-administration. This highlights the importance of determining compound concentration-time profiles at the site of action. Both groups investigated the time-course of degradation and noted a consistent picture of rapid degradation within 1 h which was sustained out to the 6-h timepoint but with some recovery in protein levels at 24 h post-dose. The time-course profile was dose-dependent for QCA570 such that the lower dose generated a less profound BET protein degradation over time. The subsequent dosing regimen (3 times per week) for the efficacy studies suggested that complete degradation of BET proteins may not be required for efficacy, but further time-course data from multiple dosing studies would be required to confirm this. The example with MDM2 PROTACs [35] demonstrated a time-dependent reduction in protein levels with ~80% loss at 24 h post-dose, which was accompanied by time-dependent accumulation of p53 and p21 proteins and PARP cleavage following IV dose administration (25 mg/kg) for 5 out of 7 days per week for 2 weeks resulted in complete tumour regression.

BTK_CRBN PROTACs have been described by two groups [21,34]. Zorba et al. investigated the degradation of endogenous BTK in rat spleen and lung tissues at either 24 h post a single (0.35, 35 and 175 mg/kg) or 48 h post two SC doses of 175 mg/kg. Although limited n numbers were used (two animals per timepoint), the data indicated that a high dose was required to degrade BTK in the spleen (33% and 71% following a single or two doses at 175 mg/kg, respectively). Despite a modest increase in plasma compound concentrations with a second dose, there was greater level of degradation and this may suggest that later timepoints may yield improved BTK degradation. Interestingly BTK degradation was not detected in lung tissues despite comparable tissue

compound concentrations (2000–3000 ng/mL). The reason for this difference is unknown and the authors speculate on several potential explanations related to the E3 ligase axis and ubiquitination process. Dobrovolsky et al. [34] describe the development of CRBN-based BTK PROTACs to exploit the combined degradation of BTK and CRBN neosubstrates, IKZF1/3, which would potentially overcome the resistance to BTK inhibition in the clinic. Their lead molecule, DD-03-171, was evaluated for its potential to degrade BTK and IKZF1/3 in naïve mice and in patient derived xenograft (PDX) models. DD-03-171 (50 mg/kg IP BID for 3 days) degraded BTK in mouse spleen tissue but the change in IKZF1 levels was more variable and this was further emphasised in the PDX models in which DD-03-171 (50 mg/kg IP QD for 3 days) consistently degraded BTK but had a differing impact on IKZF1 levels. DD-03-171 (50 mg/kg IP QD) was shown to significantly prolong survival in lymphoma models, but the magnitude of efficacy varied across models and lack of efficacy occurred in models where ibrutinib and lenalidomide were ineffective. Interestingly and in contrast to *in vitro* data, BTK inhibition upregulated the CRBN neosubstrates in the DFBL_96069 engrafted mice, and the PROTAC treatment reduced this upregulation and significantly improved survival compared to ibrutinib and lenalidomide treatment.

Several groups have evaluated VHL-based PROTACs *in vivo* (Table 2) with ARV-771, a VHL-BET PROTAC, being the most common example [19,20,36]. However ERα [37], ALK [38] and FLT-3 [39] have degraded in xenograft models with a VHL-based PROTAC. ARV-771 has been evaluated in a range of xenograft models using a variety of dosing regimens as outlined in Table 2. In essence, dose-dependent reduction of tumour burden was achieved with higher dose levels and/or frequency. This was associated with degradation of BRD4 and reduction in cMYC levels determined generally at study termination. Raina et al. [36] demonstrated superior efficacy compared to an inactive PROTAC molecule (ARV-766) or an inhibitor (OTX015) illustrating that a differentiated *in vivo* efficacy profile may be obtained. This was also noted by Saenz et al. who also employed the inhibitor OTX015 as a comparator. The PK profile of ARV-771 generated at 10 mg/kg SC demonstrated that the compound is a rapidly absorbed and has a good C_{MAX} and AUC consistent with maintaining compound levels greater than 8 nM over 24 h. The exposure is in excess of the *in vitro* IC_{50} value determined in serum for 8–12 h of the dosing interval. PK data was not reported for TD_PROTAC targetting ERα [37] and TD-004 targetting ALK (TD-004) despite both molecules being effective at reducing tumour volume *in vivo* with once daily dosing at 10 or 58 mg/kg IP, respectively. In the case of TD_PROTAC, this would have been particularly valuable as the molecule has high microMolar potency both for degradation and inhibition of cell growth *in vitro*. However, the authors demonstrated a reduction in ERα levels in tumour at study termination. The FLT3_PROTAC [39] was evaluated for its ability to induce protein degradation *in vivo*. The PK profile was evaluated at 10 mg/kg IP and resulted in apprecible exposure in excess of its DC_{50} value *in vitro* for ~22 h. FLT3 degradation was assessed at a single time-point of 5 h post the third daily dose of compound and >50% FLT3 degradation obtained.

IAP-based PROTAC targetting the ERα have been described [40] and investigated in a PK/PD like study and progressed to a xenograft study using MCF-7 cells. SN(ER)-87 degraded ERα and inhibited growth of MCF-7 cells *in vitro* at low nanoMolar concentrations. Following IP administration (10 and 30 mg/kg), endogenous ERα receptor in ovaries was degraded by >75% and 65% at 6 and 24 h post-dose, respectively, whilst >50% degradation of ERα in implanted MCF-7 cells was obtained at 24 h post dose of 30 mg/kg IP. Daily administration of 30 mg/kg resulted in a significant reduction in tumour volume, which was consistent with inhibition of cell proliferation. The PK profile was determined at 10 mg/kg IP and indicated that the systemic concentrations (C_{MAX} = 1970 ng/mL) were greater than its *in vitro* potency.

In summary, the current literature examples illustrate the potential of targeted protein degradation with PROTACs to offer a differential efficacy profile compared to a conventional inhibitor. However, there are few examples of *in vivo* efficacy outside of the oncology area and the use of xenograft models in which profound efficacy (reduction in tumour volumes and increased mouse survival) has been observed. The pharmacokinetic profiles described suggest that the compounds are rapidly absorbed and present in the blood compartment at concentrations sufficient for degradation based on *in vitro* activity profile. In some cases, the PROTAC concentrations in a relevant effect compartment, for example the tumour in xenograft models have also been determined. However, the routine assessment of compound exposure in both plasma and relevant effect compartments is limited and often PD assessment of protein levels is only determined at a single time-point. The majority of PROTACs evaluated *in vivo* are of low nanomolar potency, whilst the efficacious dose range is generally >10 mg/kg. This dose when coupled with the dose frequency appears to be higher than might be expected for 'event-driven' pharmacology and this may reflect the fact that these molecules are unlikely to be fully optimised. The observation of by Zorba et al. [21] of BTK degradation in spleen but not lung tissue highlights the importance of understanding the factors which control protein degradation *in vivo*. This data set provides an excellent starting point, but more thorough *in vivo* studies are required to build a detailed understanding of these molecules in terms of their PK/PD relationship. To the best of our knowledge, a mathematical PK/PD model describing the activity of PROTACs *in vivo* has not been reported and this represents a key future direction.

Building PK/PD understanding for PROTACS in preclinical species

Tuntland et al. [22] describe a guide for the optimal design and conduct of PK/PD studies in preclinical species in the research phase. The authors emphasise that this is a collaborative effort across disciplines and an iterative process with the definition and testing of a PK/PD hypothesis. There are several key components (1) full characterisation of the compound concentration over time and also the modulation of PD over time after dosing to assess temporal delays between drug exposure and effect, (2) choice of PD marker and whether it is linked to the target protein and/or the disease of interest [41] and (3) impact of repeated dosing on both the PK and PD. In so doing the PK profile and evidence of target engagement, pathway modulation and biological effect would be determined. This is consistent with the 'three pillars of survival' [24] which represents an integrated understanding of the PK/PD principle of exposure at the site of action, target binding and the expression of pharmacological activity. Like any small and/or large therapeutic molecules, PROTACs can only induce the desired pharmacological activity associated with targeted protein degradation when an adequate exposure is achieved and of sufficient duration within the target tissue cells (PK) enabling binding of PROTAC to both E3 ligase and target protein to result in ubiquitination and degradation of the target protein within cells (PD). The reduction in the protein of interest is in turn linked to the expression of *in vivo* efficacy.

PK profiles are generally assessed in naïve animals however pharmacodynamic knowledge is built across a range of *in vivo* models depending upon the question being asked. Preclinical animal models generally fall into two categories, PK/PD and efficacy models (Table 3), and these models provide different information. Characterisation of the temporal relationship of compound concentration and modulation of PD, where the readout is target engagement, is generally assessed in naïve animals. The PD readout may require *ex vivo* stimulation of blood/tissue. For PROTACs, assessment of protein levels would be the simplest PD readout and an indication of 'target engagement'. These time course studies should assess multiple dose levels and dose frequencies and the data generated would inform on dosing regimens for efficacy models in addition to understanding any temporal delays between PK and PD. For PROTACs, understanding the relationship between targeted protein degradation and biological function is important as this will determine the dosing regimen required for efficacy and expression of the desired pharmacological phenotype. In Table 3, efficacy models have been split into two categories, mechanistic and disease. Mechanistic models enable the exploration of pathway modulation in a complex multi-cellular system and so PD readouts can be linked to a functional response. Disease models enable the exploration of pathway modulation in a relevant tissue compartment, such as the joint in arthritis models, and often utilise similar readouts to those in human disease. From a PK perspective the blood/plasma concentrations of compounds are often used as a surrogate of tissue (effect compartment) concentrations, but it is important to determine and demonstrate that the PK profile is consistent across the models employed and assess exposure in the relevant tissue compartment whilst linking to the PD readouts. Evaluation of molecules in efficacy models enables the PK driver (C_{MAX}, C_{MIN}, C_{AVE}) for efficacy to be determined through dose fractionation studies [22]. Close consideration of the dosing schedule is key particularly from a dose frequency perspective to avoid large difference between peak to trough compound concentrations

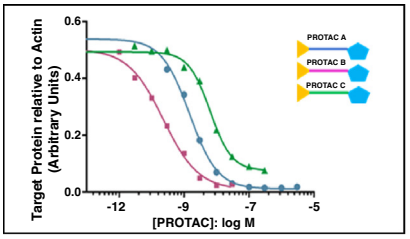
Table 3. Preclinical animal models, their utility and value.

PK/PD model	Efficacy models	
Sampling at fixed time-points post-dose	Mechanistic/short term (up to 1 week duration)	Disease (>1 week duration)
• Target-dependent PD readouts (*ex vivo*) • Endogenous target • Simple robust readouts with good signal: noise ratio	• Target-dependent system in a multi-mediator/ cell system • Endogenous target[a] • Target mechanism in a more complex context	• Ideally disease mechanisms in a relevant tissue compartment • Endogenous target[a] • Similar readouts to man/human disease
What they give you		
• Confirmation of activity with the dosing level/ regimen • Concentration response relationship • Confirmation of pathway modulation in an intact animal • Concentration response relationship and understanding of the PK driver of efficacy	• Symptoms and pathology in a relevant tissue compartment • Concentration/biomarker/efficacy relationship and understanding of the PK driver of efficacy	
What they do not give you		
•Target validation • Relevant effect compartment	• Target validation • A guarantee the molecule will work in patients	

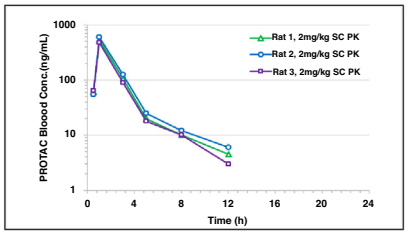
[a] Generally endogenous target but in some cases (e.g. oncology models) this may be exogenous target (e.g. implantation cells in the animal).

and the potential risk of toxicity [42]. PK/PD knowledge is built across different assay systems and *in vivo* EC_{50} values [43] can be generated which may be used to support human dose predictions. This approach was used by Dowty et al. [44] for the Janus Kinase Inhibitor, Tofacitinib, and concluded that C_{AVE} was the driver of efficacy in preclinical studies in mouse collagen-induced arthritis model and in Rheumatoid arthritis patients.

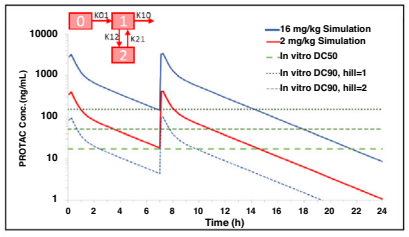
Fig. 1 highlights some basic principles for the *in vitro* to *in vivo* translation of PROTACs and building PK/PD relationships. In a drug discovery project, the *in vitro* activity and ADME properties are characterised, which may identify compounds for further studies such as the evaluation of *in vivo* PK. The *in vitro*activity data is likely to include cell lines, human primary cells and *in vitro* activity in the preclinical species of choice. Dose route, dose level and choice of species are important considerations and depend on the scientific question being addressed. Once suitable compound(s) are identified, studies to evaluate the *in vivo* PK/PD relationship are designed and conducted. Data from these can be utilised to build mechanistic PK/PD models which incorporate a protein turnover component and enable the estimation of *in vivo*



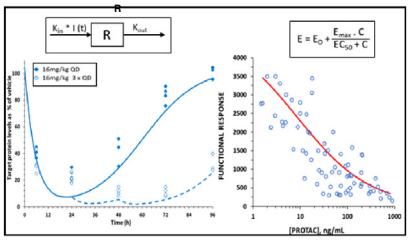
- *In vitro* characterisation (protein levels and function)
- *In vitro* ADME characteristics and molecule optimisation (physicochemical properties)
- Identification of PROTAC molecule(s) to progress for further characterisation



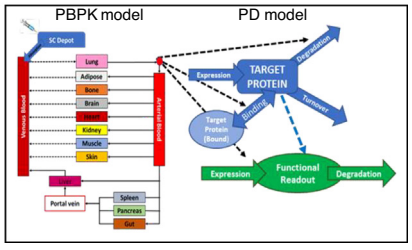
- *In vivo* PK evaluation of PROTAC molecule(s)
  - Dose route and level, species and formulation
  - Consider tissue distribution assessment



- Identify PROTAC molecule(s) for *in vivo* PK/PD evaluation
  - PK modelling to predict appropriate dose levels and dosing regimen
  - Determine appropriate *in vivo* study design and PD readouts based on *in vitro* assessment



- Generate *in vivo* PK/PD experimental data
  - Primary PD readout of protein levels
  - Incorporate functional response readouts
- Build mechanistic PK/PD models
  - Generate *in vivo* DC_{50} and D_{max} values
- Identify dosing regimens for efficacy models
- Learn-confirm-predict cycle to identify knowledge gaps



- Build translational PBPK/PD models
  - Evaluate safety and clinical dose prediction

Drug Discovery Today: Technologies

Fig. 1. In *vitro- in vivo* translation of targeted protein degradation in drug discovery and development. Developing PROTAC targeted therapeutics requires *in vitro* characterisation (pharmacology, ADME and physicochemical properties) and *in vivo* PK evaluation of PROTAC molecules of interest. Through use of mechanistic PK/PD models and data integration, a predict-first and learn-confirm-predict approach to PK/PD study design can be employed. PD models in conjunction with PBPK modelling can lead to more in depth translational models to aid clinical dose prediction.

DC_{50} and D_{MAX} values. Knowledge gaps should be identified and addressed with further experiments.

The 'event-driven' pharmacology for PROTACs is due to triggering the ubiquitination and subsequent degradation of a protein of interest *via* the proteasome and so protein re-synthesis rate is a key factor impacting PK/PD relationships. Analysis of multiple compounds with differing PK profiles or determination of the recovery of protein levels once the PROTAC has been eliminated would be valuable to aid the estimation of the protein half-life *in vivo*. Although protein levels have been shown to partially recover *in vivo* [31,33], protein half-life has not been determined. Additionally, it would be valuable to understand how consistent protein half-life is across tissues and whether variations in E3 ligase concentration and proteasome activity influence the level of target protein degradation. There may be many other questions identified or hypotheses formed which will need further experiments.

Future considerations for building translational PK/PD models

Many aspects of PROTAC ADME (Absorption, Distribution, Metabolism and Excretion), such as non-specific protein binding in plasma and tissue, permeation into blood cells and hepatocytes, catabolism by CYPs and/or non-CYPs, renal excretion and so on, remain to be investigated in future studies. Nevertheless, the linker region of PROTACs can be optimized to improve cell permeability, tissue distribution, metabolism/elimination and potency [6,25,45]. However, it is reasonable to believe that the distribution and metabolism of PROTACs within tissue are limited by the permeability of PROTACs across cell membranes, by virtue of their high molecular weight and complex structure. Therefore, the time course of PROTAC concentrations (total and/or free) driving the binding-ubiquitination-degradation mechanism within the cells may be significantly different from concentrations in the circulatory blood. In addition to the tissue-specific local concentration, PROTAC tissue disposition may not follow the classic pH-partition theory if PROTAC transfer across the cell membranes are mediated (enhanced or inhibited) by active transporters (yet to be identified). Depending on the affinity (K_D) of PROTACs binding to both E3 ligase and target protein, further complexity in modelling PROTAC PK may arise from the binding itself. Given the high molecular weight of PROTACs, another consideration is target-mediated drug disposition (TMDD), a major elimination mechanism affecting the PK of large molecules at low concentration levels [46]. This mechanism may exist for PROTACs, but at present has not been proven. Physiologically-based PK (PBPK) modelling and simulation (Fig. 1) would be a viable and valuable tool to incorporate all these ADME mechanisms together when exploring the local exposure in the target tissues mechanistically.

Undoubtedly, mechanistic PD models need to incorporate a target protein turnover component and also the binding component, both linked to the PROTAC local concentration defined by the PK (or PBPK) models. These two components reflect degradation and direct inhibition of target proteins, respectively, although the impact of the latter may be minimal due to the lower PROTAC concentrations required to elicit protein degradation. Noteworthily, the protein degradation component can be described mathematically using an indirect response PD model with stimulation of dissipation [47], which results in a loss of target protein. Nevertheless, an appropriate PD model should be linked to the PK (or ideally PBPK) model to analyse the *in vivo* observations of protein knockdown with respect to various dose regimens.

Accumulated knowledge on the kinetics of degradation and the biological mechanisms of PROTACs is based on a wide range of *in vitro* and *in vivo* PK and PD studies across species and in healthy and diseased tissues. Translating these data to a clinical application is the goal, though it may introduce extra challenges due to the inevitable species differences that have been experienced in traditional small and large molecule drug discovery and development. To the best of our knowledge, experience of either developing allometry approaches for distribution and elimination or applying mechanistic PBPK models to define human PK/PD based on preclinical PK/PD has not been reported in the literature for PROTACs yet. In this context, a possible strategy is to first establish translation of PROTAC (PB)PK/PD modelling across preclinical species. With confidence in translation, the human dose regimen could be predicted from preclinical PK/PD relationship. This is full of uncertainties until the first PK/PD profile readout from the first-time-in-human (FTiH) study.

Summary

The potential of PROTACs as a new therapeutic modality has been highlighted in the existing literature. The focus to date has been on understanding the preclinical profile of PROTACs that degrade a range of protein targets. Within the pharmaceutical industry there is a strong focus on establishing preclinical PK/PD relationships to aid translation and to reduce drug attrition in later stage clinical development [24,48]. This review discusses *in vivo* efficacy with PROTACs and highlights opportunities for the development, specifically in conjunction with a range of PK/PD modelling. Building confidence in (PB)PK/PD modelling and its translation may be achieved through multiple predict-learn-confirm exercises by developing a unified (PB)PK/PD model using *in vivo* PK/PD data across multiple preclinical species. This relies on the principles outlined in this article. This field will continue to rapidly evolve over coming years particularly as the repertoire of E3 ligase binders expands and tissue restricted pharmacology is explored, and so the clinical evaluation of PROTACs will inform the next steps for this exciting new modality.

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