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Assessing the Cell Permeability of Bivalent Chemical Degraders Using the Chloroalkane Penetration Assay

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Abstract

Bivalent chemical degraders provide a catalytic route to selectively degrade disease-associated proteins. By linking target-specific ligands with E3 ubiquitin ligase recruiting ligands, these compounds facilitate targeted protein ubiquitination and degradation by the proteasome. Due to the complexity of this multistep mechanism, the development of effective degrader molecules remains a difficult, lengthy, and unpredictable process. Since degraders are large heterobifunctional molecules, the efficacy of these compounds may be limited by poor cell permeability, and an efficient and reliable method to quantify the cell permeability of these compounds is lacking. Herein, we demonstrate that by the addition of a chloroalkane tag on the BRD4 specific degrader, MZ1, cell permeability can be quantified via the Chloroalkane Penetration Assay. By extending this analysis to individual components of the degrader molecule, we have obtained structure-permeability relationships that will be informative for future degrader development, particularly as degraders move into the clinic as potential therapeutics.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Methods for the Chloroalkane Penetration Assay including data from independent experimental replicates, Caco-2 assay methods and results, western blot analysis, and synthetic procedures and compound characterization (PDF)

The authors declare no competing financial interests.

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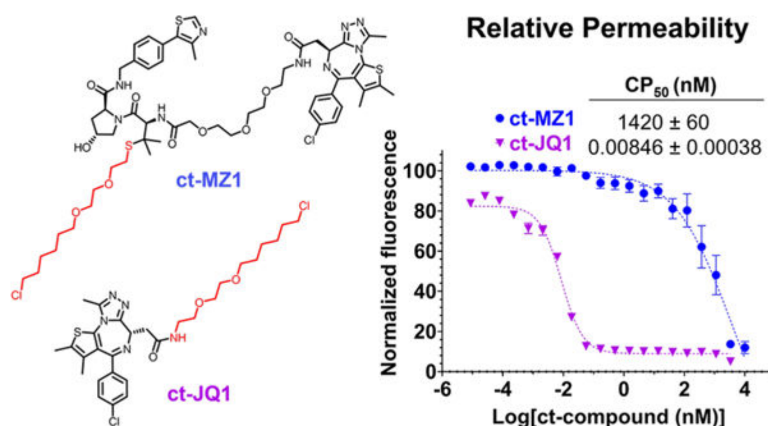

Heterobifunctional small molecule degraders offer an alternative mechanism-of-action to their traditional inhibitor counterparts and hold considerable therapeutic promise in terms of enhanced selectivity and efficacy.[1–6] The modular design of these compounds in theory allows any target-specific ligand to be linked to an E3 ligase ligand, assuming there is an exit vector on the target ligand suitable for functionalization, with the overall goal of degradation of the targeted protein. To induce effective degradation, the degrader must simultaneously bind the protein-of-interest (POI) and an E3 ligase and thereby promote ubiquitination of the target and subsequent degradation by the proteasome. Traditionally, a form of western blot analysis is performed to assess target protein levels in order to identify successful degraders. However, when POI degradation is not observed, this method provides no information as to why a degrader failed to elicit the desired outcome.[7] Protein degraders may be ineffective if they are not cell permeable or do not promote a stable ternary complex, as well as the correct ubiquitination pattern necessary to induce degradation. As a result, degrader development often involves synthesizing and testing multiple iterations of compounds without a clear understanding of what exactly needs to be improved. To more thoroughly characterize protein degraders and guide the rational design of more effective degraders, target engagement assays investigating ternary complex formation and target ubiquitination have been developed.[7–11] By comparison, the cell permeability of degraders has been underexplored despite examples where optimization of the physiochemical properties that influence permeability has produced successful degraders.[12,13]

Due to the high molecular weight and total polar surface area of degraders, we hypothesize that cell permeability is a major limitation to degrader efficacy.[14,15] NanoBRET target engagement studies measuring the binding affinity of degrader molecules to their E3 ligase targets have shown a discrepancy between measurements taken in live cells versus cell lysate, alluding to the limited cell permeability of these molecules.[8] However, since this assay does not measure cell permeability directly, permeability-limited degradation is an assumption as there may be many other factors that contribute to this observed difference in lysate and cellular environments. Similarly, a lack of target engagement in other assays such as the dual-luciferase assay does not confirm a lack of permeability or refute the possibility of a permeable molecule that simply does not engage its intended target.[16] Both of these assays result in a lack of understanding as to what property needs to be improved in the degrader, namely its cell permeability or cellular target engagement. Furthermore, since

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these functional assays cannot assess the cell permeability of degraders that have not yet been optimized to engage their protein targets, cell permeability optimization can only be performed indirectly after target engagement has been achieved, which is unproductive for efficient compound development.

Current methods to assess the cell permeability of small molecules are limited to indirect artificial membrane assays including PAMPA or assays employing cell monolayers such as the Caco-2 assay.[17–19] By sampling compound concentration on either side of a permeable support, these assays determine apparent permeability coefficients to classify compounds into categories of high, moderate, or low permeability. Assays that use LC-MS/MS to detect compound extracted from treated cells allow label-free assessments of permeability, albeit these assays do not distinguish cell-associated (membrane-bound/endosomally-trapped) compounds from those freely available in the cytosol.[20–22] Therefore, to accurately rank-order degraders, a more quantitative assay that estimates free cytosolic compound concentration is required. Here we show that the Chloroalkane Penetration Assay (CAPA) can be employed to assess the cell permeability of degraders and rank-order compounds quantitatively by relative permeability. Our results indicate that CAPA has a lower limit of quantification than the Caco-2 assay, rendering it a more useful assay to assess compounds with inherently low permeability such as degrader molecules. CAPA can provide a better understanding of how to improve the cell permeability of degraders and may help to fine-tune the properties of these molecules, particularly as they are being optimized as potential therapeutics.

CAPA is a novel cell penetration assay that utilizes the HaloTag system to covalently trap permeable chloroalkane-tagged molecules-of-interest in the cytosol.[23,24] The assay uses a cell line that stably expresses a HaloTag-GFP fusion protein that is anchored to the outer mitochondrial membrane facing the cytosol. Following treatment with molecules modified with a chloroalkane tag (ct), the cells are washed and then chased with a chloroalkane-tagged dye molecule that reacts with any remaining unreacted HaloTag-GFP fusion proteins. Flow cytometry is then employed to quantify the resulting fluorescence intensity which is inversely proportional to the permeability of the ct-molecule. To quantify cell permeability, the normalized fluorescence intensity is plotted as a function of ct-molecule concentration and fit with sigmoidal curves to determine the CP_{50} value or the concentration at which 50% of the maximal cell penetration is observed.[23] In this process, GFP levels are also assessed to ensure HaloTag-GFP protein concentrations have remained constant across samples. Since CAPA involves an irreversible step, it does not account for cases where cellular compound concentration is limited by efflux processes, but this is not relevant when initially rank ordering compounds for permeability, which is our intent. Likewise, while this assay requires derivatization of compounds-of-interest with a tag, and is therefore not 'label-free,' it enables quantitative rank ordering of compounds for this critical aspect of degrader efficacy.[23,25–27]

For this proof-of-concept study, we chose to apply this approach to the well-characterized BRD4 degrader, MZ1.[28] In part, this compound was chosen because of the available crystal structure of MZ1 bound to its target protein, BRD4, and to the E3 ligase, von Hippel-Lindau (VHL) (PDB 5T35).[29] In this structure, a solvent exposed *tert*-butyl group offers an

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attractive location for functionalization without disruption of the ternary complex, as shown with the related degrader AT1 (Figure S1).[29] Thus, the synthesis of ct-MZ1 was inspired by that of AT1, in which a modified VHL ligand was coupled to pencillamine to allow chloro-tagging off a free thiol.[29] Likewise, the final step of the ct-MZ1 synthesis was an *S*-alkylation reaction between an MZ1 analog containing a thiol handle and a chloroalkane tosylate species to append the ct (Scheme 1). This design allowed us to test both the cell permeability of ct-MZ1 as well as the capability of ct-MZ1 to degrade BRD4. By additionally comparing the relative degradation efficiencies of ct-MZ1 and MZ1, the impact of the ct on the permeability of the parent drug molecule could be estimated.

To further investigate how each component of MZ1—including the VHL ligand, the parental BRD4 inhibitor, (+)-JQ1, and the polyethylene glycol (PEG) linker—affect the overall permeability of the degrader, a series of 'truncated' MZ1 ct-compounds were synthesized (Figure 1a). To determine CP_{50} values of each, CAPA was performed in a 384-well plate containing twenty 3-fold dilutions beginning at a dose of 10 μM of the respective ct-compounds, ct-MZ1, ct-S-VHL, ct-VHL, ct-PEG_3-JQ1, and ct-JQ1 (Figure 1b). Not surprisingly, the largest molecule, ct-MZ1, had the highest CP_{50} value, while the smallest molecule, ct-JQ1, had the lowest CP_{50} value. Most striking though was the >165,000-fold difference in CP_{50} value between the tagged degrader, ct-MZ1 (CP_{50} = 1420 nM), and parental inhibitor, ct-JQ1 (CP_{50} = 8.46 pM). Furthermore, the addition of a linker containing three ethylene glycol units (PEG_3) on ct-JQ1 also decreased its CP_{50} by >16,500-fold. The two VHL ligand derivatives, ct-VHL and ct-S-VHL, which are functionalized on different portions of the molecule and through different linkages showed distinct permeability profiles as well. Together, these results demonstrate the importance of optimizing linker length and functionality to improve the cell permeability of these large heterobifunctional molecules.

To compare these results to a standard permeability assessment, the Caco-2 assay was performed with ct-MZ1, ct-S-VHL, and ct-JQ1. To ensure that the chloroalkane tag did not have a drastic effect on cell permeability, untagged MZ1, S-VHL, and (+)-JQ1 were also tested. In this assay, the apparent permeability (P_{app}) of each compound at 10 μM was determined by using LC-MS/MS to monitor the transport of compound across cell monolayers over the course of two hours. Both passive (apical to basolateral, A-B) and active transport (B-A) processes were studied. Although A-B permeability coefficients were determined for (+)-JQ1 and ct-JQ1, the other four compounds, S-VHL, ct-S-VHL, MZ1, and ct-MZ1, exhibited low to no A-B permeability with P_{app} values below the limit of quantification (BLQ, <0.4 X10^{-6} cm/sec) (Table 1). Interestingly, B-A movement could be measured for all six compounds (Table 1). These results demonstrate that the ct does not significantly alter cell permeability, with both (+)-JQ1 and ct-JQ1 displaying moderate permeability. However, limited conclusions regarding the relative cell permeability of the overall series of molecules can be drawn due to their similarly low permeability (BLQ). By contrast, although CAPA uses a different cell type than Caco-2, it could detect differences in cell penetration for these low permeability compounds and provided a quantitative ranking of cell permeability, thus demonstrating the utility of this assay for characterizing degraders.

In order to assess further whether the ct significantly perturbs the permeability of the parent molecule, we performed western blot analysis measuring BRD4 degradation in HeLa cells

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treated with either MZ1 or ct-MZ1 at varying concentrations for 24 h (Figure 2). As envisioned, the addition of the ct to the solvent-exposed *tert*-butyl group of MZ1 did not interfere with degradation of BRD4, with ct-MZ1 showing comparable degradation to MZ1. Comparing this result with that of CAPA reveals that although ct-MZ1 is >165,000-fold less permeable than its tagged parental inhibitor ct-JQ1, ct-MZ1 is still an effective BRD4 degrader. These results support prior work showing that degraders are catalytic and, therefore, can drive protein degradation even at low intracellular concentrations.[30] Although only a small amount of compound needs to penetrate the cell to be effective, non-zero cell permeability is still a critical parameter for effective degraders, since despite their catalytic mechanism, early degraders were ineffective when utilizing peptidic E3 ligase ligands and even relied on the appendage of polyArg permeabilizing groups for efficacy.[2,31–33] Thus, in order to maximize the potential of degraders in the clinic, it will become increasingly important to optimize their physiochemical properties that influence permeability as well as other pharmacokinetic properties during lead discovery and development.

The drastic effect of the PEG linker on the cell permeability of ct-JQ1 inspired the design and synthesis of a second set of ct-compounds, in which linker composition and length were varied (Figure 1c). In this case, we chose to append the linkers off the VHL ligand in order to obtain structure-permeability relationships that can more readily be applied to general degrader development. The longest linker incorporated consisted of six ethylene glycol units (PEG_6), while the shortest contained two ethylene glycol units (PEG_2). An alkyl linker was also synthesized that contained the same number of atoms as PEG_2 and therefore, is referred to as $alkyl_2$. Again, CAPA was performed in a 384-well plate containing twenty 3-fold dilutions, this time beginning at a dose of 100 μM in order to accurately quantify CP_{50} values of ct-PEG_6-VHL, ct-PEG_2-VHL, ct-$alkyl_2$-VHL, as well as ct-VHL containing no linker (Figure 1d).

Distinct trends in permeability corresponding to linker length and composition were revealed with permeability increasing accordingly: ct-PEG_6-VHL = ct-PEG_2-VHL < ct-$alkyl_2$-VHL = ct-VHL. The gradual increase in permeability between these compounds can be attributed to a decrease in molecular weight and total polar surface area. These results demonstrate that shorter alkyl linkers are more cell permeable than longer PEG linkers and thus, highlight the importance of limiting linker length and polar surface area when possible. Multiple successful degrader molecules incorporating (+)-JQ1 and various linkers have been reported which suggests there may be room for optimizing linker permeability while still maintaining effective degradation.[28,29,34–36] Importantly, although qualitative trends in permeability can be estimated based on polar surface area and molecular weight, we have shown that CAPA allows quantification of the impact that linker length and composition can have on overall permeability. It is well known that linker length and composition impact ternary complex formation but the effect on cell permeability has been unexplored until now.[35] These results reveal that even minor chemical modifications (*e.g.* a PEG_2 linker containing two oxygen atoms substituted for an alkyl linker) can significantly alter this critical parameter.[12]

Currently, due to the limited availability of small molecules that recruit E3 ligase proteins, the majority of degraders incorporate either VHL or cereblon (CRBN) ligands.[1] During degrader development, the best choice of an E3 ligase recruiting ligand is difficult to predict

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but can be critical to achieve effective degradation by facilitating a stable ternary complex with the corresponding E3 ligase and POI, as well as productive POI ubiquitination.[7] In the case of BRD4, effective degraders have been developed that incorporate both VHL and CRBN ligands.[1,2,28,36] Additionally, different linkages off these E3 ligase ligands can lead to successful degradation, including O and N linked pomalidomide derivatives which bind CRBN.[2, 29, 37–40] We therefore modified pomalidomide (POM)-based ligands with a ct to investigate the difference in cell permeability between CRBN and VHL ligands (Figure 1c). According to our CAPA data, ct-O-Pom and ct-N-Pom are more cell permeable than ct-VHL in agreement with predictions based on molecular weight. Interestingly though, despite offering an additional hydrogen bond donor which is often assumed to decrease permeability, ct-N-Pom displayed improved permeability compared to ct-O-Pom (Figure 1d). Understanding these structure-permeability relationships, in particular regarding VHL and CRBN ligands, is likely to be critical to improve future degrader development. These results suggest that when possible, incorporation of pomalidomide-based CRBN ligands may enhance degrader efficiency by promoting greater cell permeability relative to VHL ligands.

The development of degraders and our ability to optimize degraders in a rational way is currently limited by the cellular assays available for their characterization. In order to facilitate rapid screening of degrader compound libraries, high-throughput assays to monitor ternary complex formation, protein ubiquitination, and degradation have recently been developed.[7–11] However, each of these assays relies on the degrader being cell penetrant, yet there is no highly quantitative assay to assess degrader permeability. Here we have demonstrated that CAPA can be utilized to quantitate the permeability of degraders. Although CAPA is not a tag-free assay and only measures relative permeability, we have shown that structure-permeability relationships among closely related compounds can be obtained with medium-throughput. In comparison, the more standard and tag-free Caco-2 assay was unable to detect and rank-order compounds with similarly low permeability. Using CAPA, we have gained a deeper understanding on how to improve the physiochemical properties of degraders. By expanding this study, there is the potential to develop a more complete understanding of the structure-permeability relationships of degraders in order to enhance their cell permeability and overall degradation efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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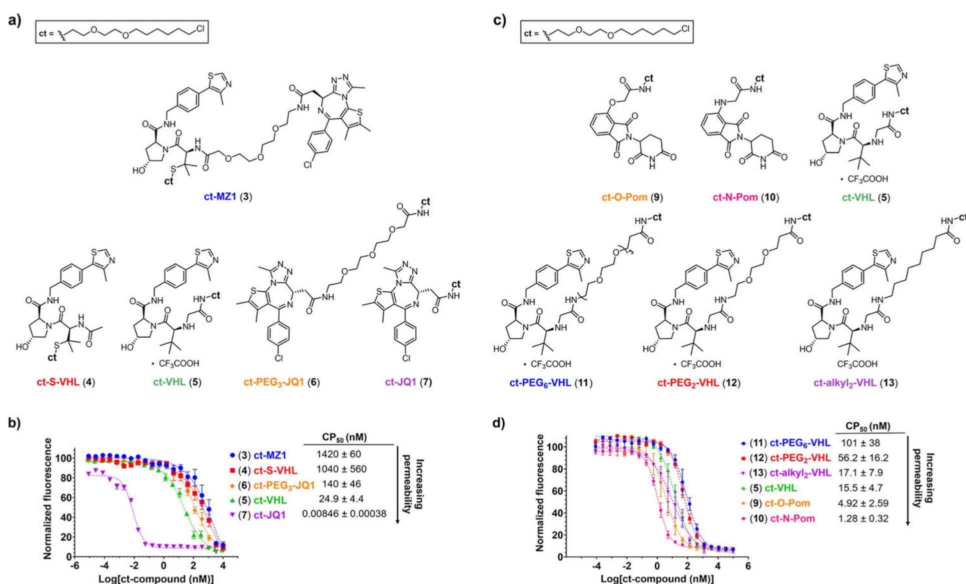

Figure 1.

Cell penetration profiling results. (a) Chemical structures of ct-compounds representing components of the overall structure of ct-MZ1. (b) Cell penetration dose response curves for ct-MZ1, ct-S-VHL, ct-PEG_3-JQ1, ct-VHL, and ct-JQ1. CP_{50} averages and standard error are from five independent curve fits from five independent experiments. Error bars show the standard error from the independent experiments. (c) Chemical structures of ct-compounds varying in either linker length or composition of the E3 ligase ligand. (d) Cell penetration dose response curves for ct-PEG_6-VHL, ct-PEG_2-VHL, ct-$alkyl_2$-VHL, ct-VHL, ct-O-Pom, and ct-N-Pom. CP_{50} averages and standard error are from three independent curve fits from three independent experiments. Error bars show the standard error from the independent experiments. VHL refers to the VHL ligand. Pom refers to the Cereblon ligand pomalidomide.

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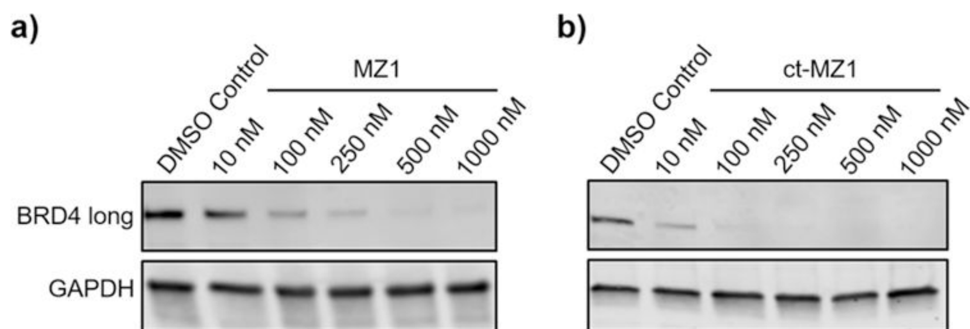
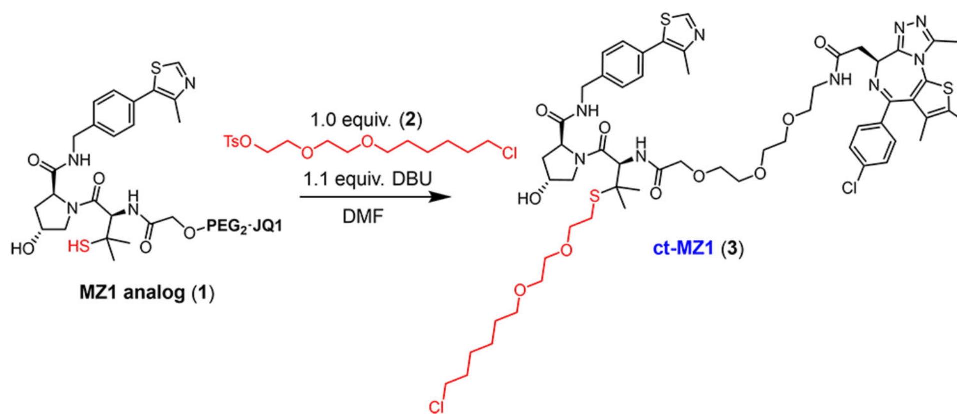


Figure 2.

MZ1 and ct-MZ1 dose dependent degradation of BRD4. (a) HeLa cells were treated for 24 h with varying concentrations of MZ1. (b) HeLa cells were treated for 24 h with varying concentrations of ct-MZ1. The resulting BRD4 protein levels were analyzed by western blot and GAPDH levels were assessed as a loading control.



Scheme 1.
Synthesis of ct-MZ1 (**3**).

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Table 1.

Bidirectional Caco-2 permeability results.

Compounds	**$P_{app, A-B}$ (10^{-6} cm/sec)**[a]	**$P_{app, B-A}$ (10^{-6} cm/sec)**[a]
(+)-JQ1[b]	12.6 ± 0.0	13.3 ± 0.3
ct-JQ1 (**7**)	5.4 ± 0.0	11.2 ± 0.1
S-VHL (**8**)	BLQ	6.8 ± 0.4
ct-S-VHL (**4**)	BLQ	14.1 ± 1.0
MZ1[b]	BLQ	5.6 ± 0.3
ct-MZ1 (**3**)	BLQ	0.9 ± 0.0

[a]P_{app} values are averages of two independent experiments testing compounds at 10 μM. In general, compound permeability is classified as low (3×10^{-6} cm/sec), moderate ($3 - 15 \times 10^{-6}$ cm/sec), or high ($>15 \times 10^{-6}$ cm/sec) depending on the P_{app} value of the compound. BLQ indicates compound quantification was below the limit of quantification (<0.4 cm/sec). For full data sets including efflux ratios, see Table S5 in the Supporting Information.

[b](+)-JQ1 and MZ1 were purchased and tested without further purification.