Supplementary Information

Table of Contents

Materials

Table S1. Overview of materials

Reagent	**Provider**
Cryopreserved female CD-1 mouse hepatocytes in suspension, pools of 48 to 63 donors, lots QPT, TJH, RQU, DKL	BioIVT
Krebs-Henseleit buffer	BioIVT
Thawing medium for hepatocytes	BioIVT
DMSO	Millipore Sigma
Trypan blue	Millipore Sigma
Acetonitrile	Millipore Sigma
Ammonium formate	Fluka
Formic acid	Millipore Sigma

Methods

Exposed Polar Surface Area (ePSA)

ePSA is a high throughput method used as a permeability surrogate introduced by Goetz et al.[1] The method is sensitive of 3D conformation effect changes of small and large molecules hence provide useful insights on conformers behavior[2] and chameleonicity character.[3]

The experimental measurement of lipophilicity using ePSA for bRo5 compounds is a valuable tool for guiding early-stage R&D, MedChem prioritization, and pharmacokinetic studies. Additionally, it served as a powerful assay for predicting human permeability.[4]

The ePSA determination is conducted by correlating the retention times of known reference compounds using supercritical fluid chromatography (SFC) on a Phenomenex Chirex column, with a dielectric constant that mimics the cell membrane bilayer. This enables for robust correlation analysis and prediction of human permeation.[1,4]

Mix Reference preparation: references stock solutions in 10mM DMSO of antipyrine, chloropromazine hydrochloride, desipramine hydrochloride, diclofenac acid, 4-nitrobenzoic acid, bumetanide are prepared. Subsequently combined to yield mix references, diluted in methanol to an appropriate concentrated to provide similar response factor for the measurement. The reference solution mix is measured before and after the sample solutions to verify optimal operating system. Retention time of the reference compound is measured in double (or triple) determination.

Sample Preparation: the stock solution of the substance 10 mM in DMSO is diluted to circa 1:3 in MeOH. The substance is measured in single determination.
SFC condition: Column: Phenomenex Chirex 00G-3014-EO (s)-VAL and (R)-NEA 250x4.6mm Art.-No.H21-031487. Eluents: A: CO2, B: Methanol for analysis (Merck 1.06009 Lot. -No.: I1263409302) +20 mmol/L ammonium formate (BDH 84884.260 CAS:540-69-2 Lot. -No.: AU751461). Gradient: 0.0 min: 5 % B, 9.0 min: 50 % B, 15.0 min: 50 % B, 15.1 min: 5 % B, 16.0 min: 5 % B. Flow: 4.0mL/min; UV wavelength: 270 nm

Analysis: The compound respective ePSA is determined *via* the calibration curve of references mixture substances and their respective retention time (R2> 0.999).

Chromatographic LogD (ChromLogD)

The ChromLogD is a high throughput system for determining the hydrophobicity of a substance. The determination is carried out through correlation of retention times using a reversed-phase HPLC isocratic to gradient method. The sequence is described below.

The determination is based on Chromatographic Hydrophobicity Index (CHI = Φ). The Φ value is in the range between 0 – 100 and estimates the percentage (per volume) of acetonitrile, which is needed to get an equal distribution of the substance between mobile – and stationary phase of the column. Φ is an Index that characterizes the compound hydrophobicity.[5]

Name Information Distributor Eluent A 50 mM Ammonia-acetate in DI water Sigma Aldrich

Eluent B Acetonitrile – MS Grade Sigma Aldrich

Sample Preparation

The substance is measured in double determination. The stock solution of the substance (10 mM in DMSO) is diluted 1:50 in Eluent A/ACN (1:1).

Reference Mixture

The determination of the Φ will be indirect determined with a reference mixture with known Φ values (Table S2). The reference mixture will be diluted in Eluent A / ACN (1:1). All substances of the reference mixture are combined in equal volume.

Table S2. ChromLogD reference substances with known Φ

Substance	**Φ**	**Concentration**
Theophylline	15.76	2 mM
Benzimidazole	30.71	2 mM
Colchicine	40.00	2 mM
Phenyltheophylline	52.04	0.39 mM*
Acetophenone	64.90	2 mM
Indole	69.15	2 mM
Propiophenone	78.41	2 mM
Butyrophenone	88.49	2 mM
Valerophenone	97.67	2 mM

* poorly soluble compound

Analysis

The experimental determination of Φ of a substance is done via a gradient RP-HPLC method (Table S3) by a calibration curve of the Φ of the reference substances and the retention time.

Table S3: Gradient method for HPLC analysis

Time [min]	Eluent A [%]	Eluent B [%]
0.00	100	0
1.50	100	0
10.5	0	100
11.5	0	100
12	100	0
15	100	0

The intercept (n) and the slope (m) of this calibration line will put into the equation:

$CHI = m * tR\ (sample) + n$

The ChromLogD value is an alignment with the following equation:

$ChromLogD = 0.0857 * \Phi sample - 2$

Pharmacokinetic studies in mice

Pharmacokinetic studies were carried out after approval by the internal animal welfare board and in compliance with the local and international animal welfare regulations. Mice were either dosed intravenously (0.2 mg/kg; 0.5 mg/kg or 1 mg/kg) or orally (10 mg/kg). For both routes of administration solution vehicles were used, such as 40 % Kleptose or 20 % Kolliphor in water or buffer, depending on feasibility in the vehicle screen and the predicted pKa of the compound. Three female CD-1 mice were dosed for each study and blood samples were taken retro-orbitally 0.25, 0.5, 1, 2, 4, 6, and 24 h after oral administration. After intravenous administration, a datapoint at 0.1 h was added. Plasma was obtained by centrifugation (10,000 g; 4 °C; 5 min) and stored at -20 °C until UHPLC-MS/MS analysis. By addition of acetonitrile, the proteins were precipitated and dissevered by centrifugation. The concentration of the respective molecules studied was determined by a standard UHPLC-MS/MS method (generic method similar to the method described for determination of CL_{int} in mouse hepatocytes using experimentally determined compound MS tuning parameters).

Plasma concentrations were determined for each time point sampled. The maximum plasma concentration (c_{max}) and time to reach the maximum plasma concentration (t_{max}) were obtained from the observed data. WinNonlin® (Princeton, NJ, USA), was used to calculate the area under the plasma concentration-time curve (AUC), clearance (CL), volume of distribution at steady state (V_{ss}), and half-life (t1/2). The AUC was obtained by non-compartmental analysis with linear up/log down trapezoidal method. Oral bioavailability was calculated by relating dose-normalized oral and iv AUCs up to the last time point (AUC_{last}).

The fraction escaping hepatic metabolism fh can by estimated by calculating the extraction ratio (clearance as proportion of the liver blood flow):

$$f_h = 1 - \frac{CL}{LBF}$$

Ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) method used for determination of hepatocyte intrinsic clearance (CL_{int})

UHPLC method:
Instrument: Waters i-class UHPLC
Eluent A: Water with 0.1 % (*v/v*) formic acid, 10 mM ammonium formate
Eluent B: Acetonitrile
Gradient:

Table S4: Gradient for UHPLC analysis

Time	**Flow Rate**	**A [%]**	**B [%]**	**Curve**
Initial	0.8	100	0	6
0.1	0.8	100	0	6
0.8	0.8	0	100	6
1	0.8	0	100	6
1.02	0.8	100	0	6
1.4	0.8	100	0	6

Column: Acquity UPLC BEH C18 Column (2.1 x 50 mm)
Temperature column oven: 50 °C
Temperature sample manager: 10 °C
Temperature sample organizer: 10 °C
Injection volume: 4 µL

MS/MS method:

Instrument: Sciex API 6500, software Analyst
Ionization mode: electrospray, positive (ESI+)
Scan type: multiple reaction monitoring (MRM)
Ion source temperature: 600 °C

Mass transitions and tuning parameters were determined experimentally for each compound.

Kinetic solubility method:

The kinetic solubility (KSOL) determination is carried out in a 96-well plate filtration format where the sample (2 µL of sample stock solution 10mM DMSO) is combined to 98 µL of 20 mM buffer phosphate pH 7.4. The filtration plate is then incubated and agitated at 250 rpm for 120 minutes. After stirring, the plate is centrifuged at 2500 rpm for 3 minutes, allowing all solvent to be collected in the lower microplate. Next to avoid precipitation, 50 µL of the filtered solution is diluted with 50 µL of 2% DMSO in pH 7.4 buffer solution to yield a 100 µM sample solution ready for H(U)PLC analytics.
For the standard, each compound is prepared by combining 2 µL of the sample stock solution 10mM DMSO to 198 µL Acetonitrile/Methanol/Eluant A (1:1:1:2 v/v/v).
For the evaluation, wavelength with maximum sensitivity is to be selected. The kinetic solubility is determined according to following formulas, whereas S is the targeted value for KSOL.

$$L = \frac{area_{sample} \times concentration_{standard} \times dilution\ factor_{sample}}{area_{standard} \times dilution\ factor_{standard}} \quad (1)$$

(2) $$S = \frac{L \times molar\ weight}{1000}$$

with: L= Concentration of solved compound in buffer [µg/ml] and S= Molar concentration of solved compound in buffer [mol/L]

Chromatographic conditions: Column Waters XBridge Column C8 3.5µm (Waters Cat No 186003053); Column temperature 37°C; Autosampler Room temperature (approx. 25°C); Injection volume 10 µL (HPLC) / 3µL (UPLC); Wavelength 220 nm, 254 nm, 290 nm. Eluant A: 1 mL Formic Acid + 999 mL Ultrapure Water; Eluant B: 1 mL Formic Acid + 999 mL Acetonitrile.

Table S5: Gradient HPLC

Time	Eluent A (%)	Eluent B (%)	Flow (ml/min)
0.0	90	10	1.7
0.3	90	10	1.7
2.0	10	90	1.7
2.75	10	90	1.7
2.76	90	10	2.5
4.0	90	10	2.5

Reagents and Materials: Phosphate Buffer 20mM pH 7.4: EMS, Art No. 11601-40; DMSO Merck Art. No. 102950; Acetonitrile Merck, Art. No. 100030, Methanol Merck Art. No. 106007; Formic Acid Merck Art.No.100264; Acetonitrile Merck Art. No.10030; Filtration Plate Millipore MultiScreen HTS HV 0.45uM; 96 Well Millipore Art. No. MSHVN4510; Column Waters XBridge Column C8 3.5µm (Waters Cat. No. 186003053); HPLC vials VWR Cat. No. 5480028; Micro-Inserts VWR Cat. No. 548-0006; Screw caps VWR Cat. No. 548-0788.

Table S6. Table summarizing the physicochemical properties of the PROTACs used in the Caco-2 Transwell assay

ABN denotes ion class (A = acid; B = base; N = neutral); MW was rounded for all internal molecules.

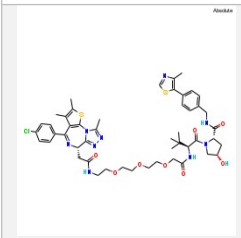
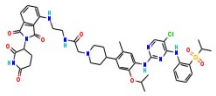
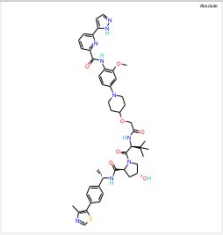
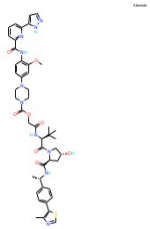
MW	calculated LogD (pH 7.4)	ChromLogD (measured)	ePSA (measured)	Ion class	Linker	E3 ligase
1190	5.785	n.m.	n.m.	N	PEG	VHL
1080	3.638	5.135	113.536	N	PEG	CRBN
1020	5.588	n.m.	n.m.	N	PEG	VHL
890	5.657	n.m.	n.m.	N	PEG	CRBN
1010	1.188	2.494	n.m.	B	PEG	CRBN
1090	4.343	n.m.	n.m.	N	PEG	VHL
820	1.866	3.706	114.335	N	PEG	CRBN
1130	4.634	5.068	107.730	N	PEG	VHL
820	4.813	6.497	122.930	N	Alkyl	CRBN
790	3.184	3.794	98.720	N	Alkyl	CRBN
1000	4.327	4.313	98.995	N	PEG	VHL

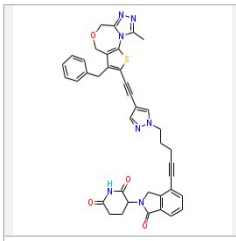
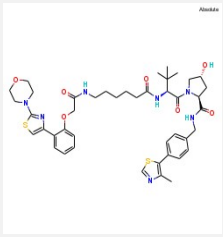
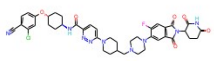
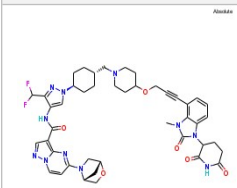
910	2.799	7.367	112.156	B	rigid	CRBN
810	1.636	n.m.	106.263	N	PEG	CRBN
820	2.671	n.m.	98.294	N	PEG	CRBN
950	5.745	7.283	130.440	N	rigid	CRBN
920	5.399	n.m.	135.480	N	rigid	CRBN
920	5.319	n.m.	123.978	N	rigid	CRBN
830	5.154	7.261	n.m.	N	Alkyl	CRBN
1080	5.308	n.m.	n.m.	B	PEG	CRBN
850	3.902	6.110	n.m.	N	PEG	CRBN
960	2.931	6.024	109.220	N	PEG	CRBN
1010	3.205	5.547	n.m.	N	PEG	CRBN
770	1.913	2.114	n.m.	N	rigid	CRBN
780	1.918	4.017	103.343	N	PEG	CRBN
840	4.165	n.m.	n.m.	N	Alkyl	CRBN
1030	2.687	4.689	n.m.	B	PEG	CRBN
920	5.423	5.932	113.480	N	PEG	CRBN
1000	4.646	6.065	111.794	N	Alkyl	VHL
1030	5.133	5.809	109.772	N	PEG	VHL
990	4.789	4.950	95.400	N	PEG	VHL
1020	2.033	5.860	n.m.	B	Alkyl	VHL
860	4.294	7.363	111.333	N	Alkyl	CRBN
890	2.138	4.533	n.m.	N	Alkyl	CRBN
940	5.062	7.109	n.m.	N	Alkyl	VHL
1010	6.043	8.060	101.913	N	Alkyl	VHL
880	3.314	4.809	115.220	N	rigid	VHL
910	3.346	4.384	116.960	N	rigid	VHL
910	3.597	4.725	111.160	N	rigid	VHL
1050	5.375	4.931	n.m.	N	PEG	VHL
1010	4.710	4.958	n.m.	N	PEG	VHL
700	3.936	4.471	108.250	N	rigid	CRBN
910	1.669	2.455	193.143	B	rigid	CRBN
790	2.742	6.283	113.533	B	rigid	CRBN
930	1.785	3.135	183.280	B	rigid	CRBN
810	2.719	3.996	n.m.	Z	rigid	CRBN
1030	4.670	7.171	n.m.	B	PEG	VHL
770	1.099	1.793	197.485	B	rigid	CRBN
860	1.799	n.m.	n.m.	B	rigid	CRBN
720	4.169	6.761	144.686	B	rigid	CRBN
810	2.636	6.723	115.043	B	rigid	CRBN
750	3.999	5.080	121.946	N	Alkyl	CRBN
830	3.381	5.279	141.203	B	rigid	CRBN

1080	4.683	4.144	n.m.	N	PEG	VHL
980	3.646	3.939	n.m.	B	PEG	other
720	4.169	6.778	141.930	B	rigid	CRBN
720	4.169	6.785	145.727	B	rigid	CRBN
920	4.023	5.465	111.320	N	rigid	VHL

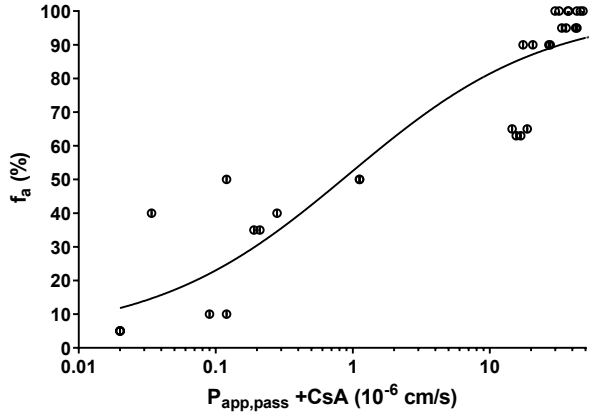
Table S7. Table summarizing the physicochemical properties of the PROTACs including structures of publicly disclosed compounds for the CL_{int} IVIVE analysis

ABN denotes ion class (A = acid; B = base; N = neutral); MW was rounded for all internal molecules. n.m. not measured

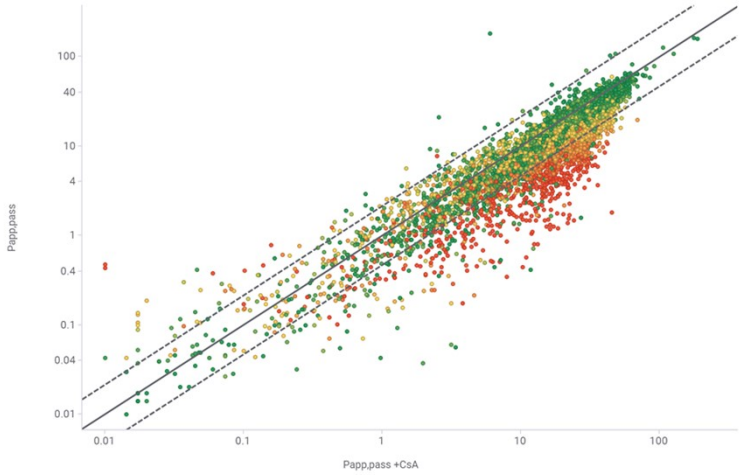
Structure	MW [Da]	calc. LogD (pH 7.4)	Chrom LogD (measured)	ePSA (measured)	Ion class	Linker	E3 ligase	Name
	1030	2.687	4.689	n.m.	B	PEG	CRBN	
	1000	4.646	6.065	111.794	N	Alkyl	VHL	
	1003	4.327	4.313	98.995	N	PEG	VHL	MZ-1
	914	2.799	7.367	112.156	B	rigid	CRBN	MS4078[6]
	878	3.314	4.809	115.220	N	rigid	VHL	JNJ-1013[7]
	907	3.346	4.384	116.960	N	rigid	VHL	Jannssen IRAK1 PROTAC, Cpd 77[8]
	696	3.936	4.471	108.250	N	rigid	CRBN	QCA570[9]

								
	910	1.669	2.455	193.143	B	rigid	CRBN	
	846	3.810	4.474	100.310	N	Alkyl	VHL	MTX-23[10]
	812	2.636	6.723	115.043	B	rigid	CRBN	ARV-110
	820	1.698	2.589	193.693	B	rigid	CRBN	
	960	4.716	5.201	135.675	N	PEG	CRBN	
	900	1.717	3.894	169.576	B	rigid	CRBN	
	910	2.238	4.584	162.052	B	rigid	CRBN	
	940	3.811	4.312	139.974	B	rigid	CRBN	
	866	1.270	3.388	123.882	B	rigid	CRBN	KT-474
	1060	3.294	4.811	146.075	N	rigid	CRBN	
	800	3.048	3.323	143.668	B	rigid	CRBN	
	980	3.420	5.188	144.745	B	rigid	CRBN	
	800	2.958	3.038	143.008	N	rigid	CRBN	
	950	3.901	4.558	136.091	N	rigid	CRBN	
	980	3.685	5.712	149.549	N	rigid	CRBN	
	710	3.780	3.447	134.420	B	rigid	CRBN	
	1000	3.151	5.320	132.442	B	rigid	CRBN	
	940	3.547	3.848	128.200	N	rigid	CRBN	

Caco-2 adaptions



A



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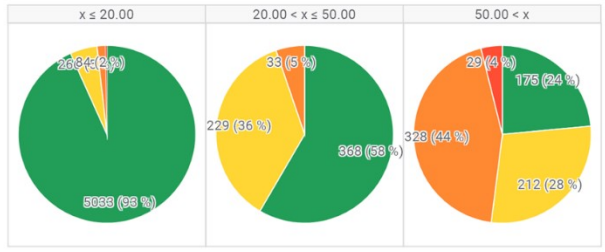
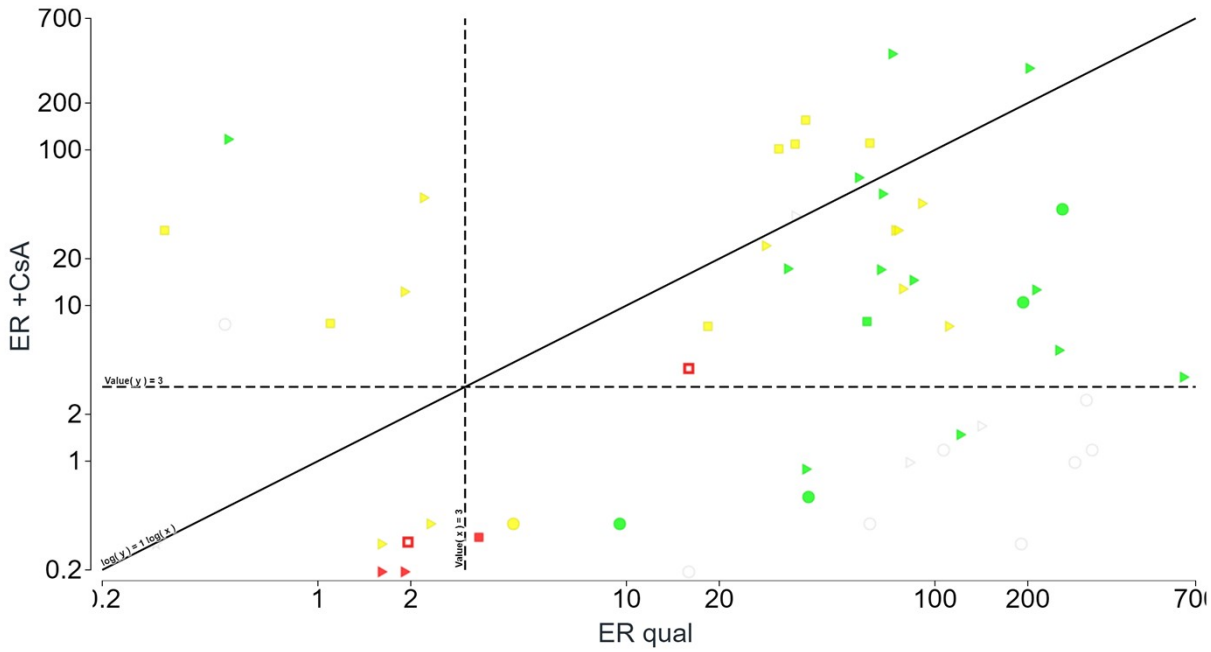
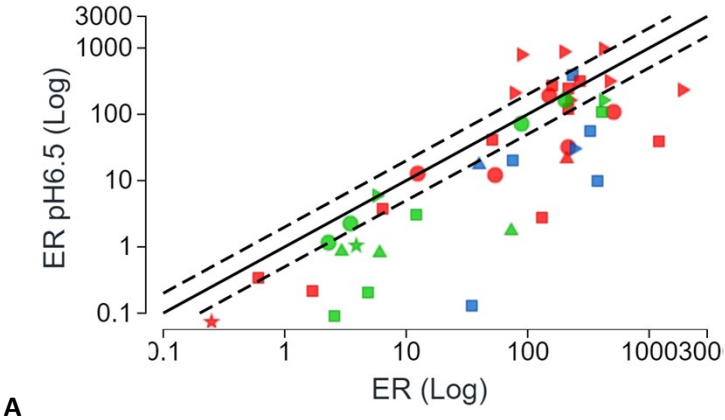


C

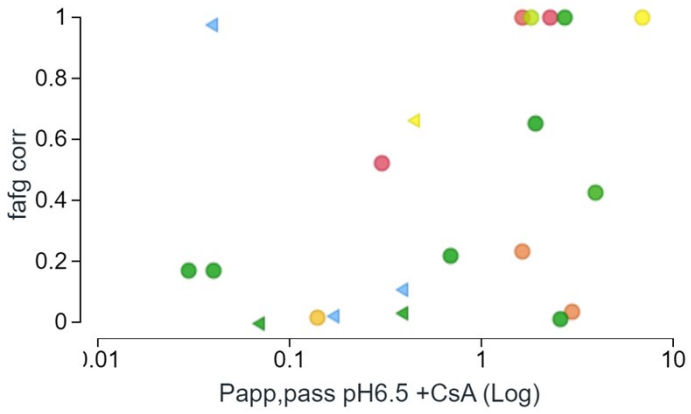
Suppl. Figure 1 A) $P_{app,pass}$ values of small molecule reference compounds (cpds) with known human f_a. Passive permeability typically describe oral bioavailability well, as compound concentrations in the GI tract at therapeutically relevant doses saturate active efflux processes. In our Caco-2 assay, $P_{app,pass}$ >10 · 10^{-6} cm/s indicates a high probability for good oral absorption, whereas a $P_{app,pass}$ <1 · 10^{-6} cm/s indicate a high probability for suboptimal oral absorption. B) Correlation of $P_{app,pass}$ values with and without inhibitors such as CsA. $P_{app,pass}$ determined under active efflux conditions are a good surrogate for estimating true passive permeability (determined with an efflux inhibitor such as CsA) Colours represent ER on a continuous scale with green ER <3, via yellow till red ER >50. C) For compounds with an ER <20, there is a 95 % probability that the $P_{app,pass}$ without inhibitors is within 2-fold of the $P_{app,pass}$ value obtained in the presence of 10 µM Cyclosporine A (CsA). With an ER between 20-50, there is a 95 % probability of it being within 3-fold, whereas at higher ER values, there is a 75 % probability that not using an inhibitor will results in a significantly different $P_{app,pass}$ value.



Suppl. Figure 2 Efflux ratios of PROTACs with and without inhibitor (10 µM CsA). Most PROTACs still exhibit significant efflux even under inhibited conditions. In combination with an occasionally low permeability, this resulted in the vast majority of PROTACs being undetectable in the receiver wells in the A-to-B direction as the levels dropped below the lower limit of quantification (LLOQ). Therefore, the passive permeability could not be determined with confidence. Color-coding indicates $P_{app,pass}$ + CsA (qualified) from <0.1 (red) to <1 (yellow) and between 1 and 10 (green). Triangles represent qualified data, with the tip of the triangle pointing towards its respective directional bias.



A



B

Suppl. Figure 3 A) ER of PROTACs and some small molecule reference compounds with pH 6.5 on the apical and pH 7.4 on the basolateral side vs pH 7.4 on both sides. ER decreased upon introducing this pH gradient. Data points colour-coded, with red representing compounds with a pKa <5.5, blue pKa >8.4, and green ranging in between, which should be affected most by the pH change as they would experience a change of net charge. Triangles represent qualified data, with the tip of the triangle pointing towards its respective directional bias. Squares represent qualified ER values in both conditions. B) Correlation of $f_a f_g$ from mouse PK studies vs $P_{app,pass}$ when with an apical/basolateral pH gradient 6.5/7.4. Even with the pH gradient, the Caco-2 assay was still not predictive of oral absorption. Triangles represent qualified data, with the tip of the triangle pointing towards its respective directional bias.

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