Assessing PROTAC Permeability and Protein Binding: Challenges and Assay Optimization

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Introduction and Aim

Heterobifunctional degraders such as PROTACs offer a powerful approach to target proteins previously considered “undruggable”. However, their complex physicochemical properties — high molecular weight, large polar surface area, and low solubility/permeability — pose major challenges for DMPK characterization. Therefore, standard assay protocols typically used for small molecules often needs to be adopted for PROTACs.

In this study, we selected several well-known degraders (**dTAG-7, dBET57,** and **ARV-110**) (Fig. 1) and assessed their Caco-2 permeability, experimental polar surface area (EPSA), and PPB using both standard and modified ADME protocols.

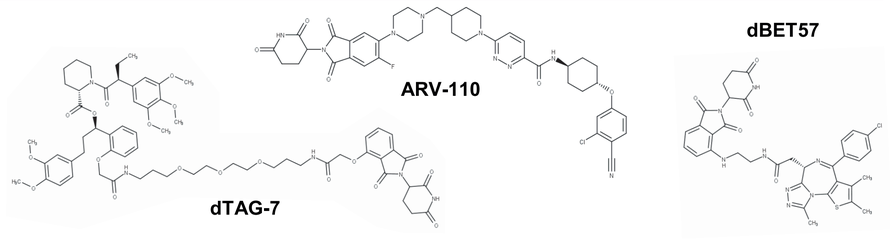

Figure 1. Structures of tested PROTACs

Methods

Caco-2 Permeability

- Concentration: 10 µM
- Protein-free assay buffer (standard) & buffer with 0.25-4% bovine serum albumin (BSA) in both compartments or in the basolateral compartment only
- Temperature: 37º C
- Incubation: 90-120 min
- Analysis: LC-MS/MS
- **Papp, Recovery, Efflux Ratio** (Fig. 2-4)

Experimental Polar Surface Area (EPSA)

- Chirex (S)-VAL and (R)-NEA (50×4.6 mm, 5 mkm)
- Mobile phase: CO_2 and 20 mM ammonium formate in methanol
- Flow rate: 2.0 mL/min
- Temperature: 35 °C
- Injection volume: 1 µL
- **EPSA** values (Tab. 1)

Plasma Protein Binding

- Concentration: 2 µM
- Equilibrium dialysis in multiple-use 96-well dialysis unit (HTD96b dialyzer)
- 10% mouse plasma ± NaF & protease inhibitors
- Temperature: 37º C
- Incubation: 24 h
- Analysis: LC-MS/MS
- % **of Bound compound, Recovery, Stability** (Tab. 2)

Results

Caco-2 Permeability Findings

Standard protocol gave poor recovery for all compounds; extending incubation to 2 h decreased recovery for **dTAG-7** and **ARV-110.**

The presence of 0.5% **BSA** in the transport buffer improved recovery but decreased the efflux ratio for **dTAG-7**, and **dBET57** (Fig. 2).

BSA (0.25–1%) in both compartments increased **ARV-110** recovery, while **dTAG-7** efflux ratio increased at ≥ 0.5% BSA (Fig. 3). High concentrations of BSA may lead to the misidentification of efflux substrates in the assay.

BSA in the **basolateral compartment only** (0.25–2%) decreased permeability of **dTAG-7** and **ARV-110**, and improved recovery for **dTAG-7** at 1% and **ARV-110** at 0.25% BSA (Fig. 4).

Thus, 0.25% **BSA** was selected as the optimal concentration for further PROTAC permeability optimization in the Caco-2 assay.

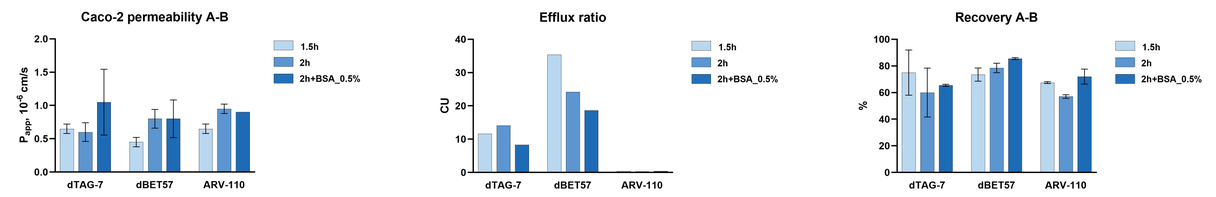

Figure 2. Permeability data: 2h incubation, 0.5% BSA

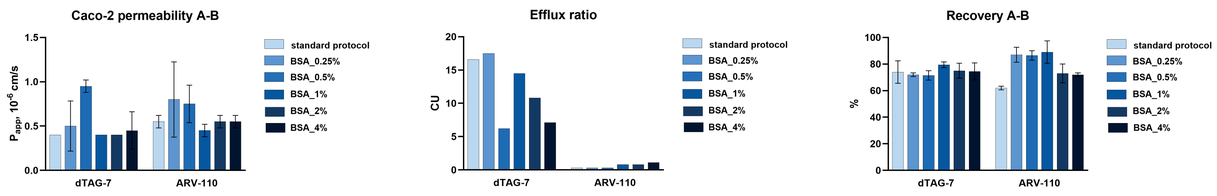

Figure 3. Permeability data: 0.25% - 4% BSA (both compartments)

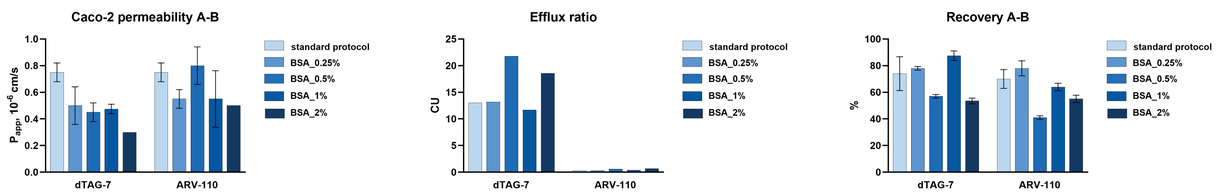

Figure 4. Permeability data: 0.25% - 2% BSA (basolateral compartment)

Test compound	MW	Retention time, min	EPSA, Å²	EPSA, mean, Å²
dTAG-7	1209	5.376	121.6	121.4
		5.365	121.4	
		5.352	121.2	
dBET57	698	5.040	115.7	115.9
		5.047	115.9	
		5.052	116.0	
SIAIS178	1011	7.346	156.0	156.0
		7.347	156.0	
		7.347	156.0	
MZ 1	1002	4.963	114.4	114.3
		4.952	114.2	
		4.961	114.4	
PROTAC ERRα Degrader-3	957	3.847	94.9	94.9
		3.840	94.8	
		3.842	94.9	
PROTAC-O4I2	608	5.545	124.6	124.6
		5.539	124.5	
		5.551	124.7	
PROTAC Sirt2 Degrader-1	853	6.517	141.5	141.5
		6.517	141.5	
		6.517	141.5	
XY028-140	760	7.375	156.5	156.4
		7.365	156.3	
		7.365	156.3	
MS4078	913	5.714	127.5	127.4
		5.704	127.3	
		5.704	127.3	

Table 1. EPSA values for selected PROTACs

Plasma Protein Binding (PPB) of PROTACs

dTAG-7, dBET57, and **ARV-110** were tested by equilibrium dialysis in 10% mouse plasma over 24 h (Tab. 2).

All compounds showed poor plasma stability; adding **NaF** and **protease inhibitors** improved stability for **dTAG-7** and **dBET57**, but **ARV-110** remained unstable.

The additives also affected binding levels, suggesting potential interactions with assay components.

Test compound	10% mouse plasma				10% mouse plasma + NaF 40 mg/ml				10% mouse plasma with protease inhibitors				PBS, pH=7.4
	% of bound compound	Undiluted % of bound compound	Recovery, %	Stability, %	% of bound compound	Undiluted % of bound compound	Recovery, %	Stability, %	% of bound compound	Undiluted % of bound compound	Recovery, %	Stability, %	Stability, %
Verapamil	35	84	86	111	51	91	79	105	32	82	94	98	–
dTAG-7	58	93	105	1	98	99.8	80	57	74	96.6	74	60	20
dBET57	77	97	128	6	97	99.7	80	91	81	97.8	83	114	70
ARV-110	99.0	99.9	73	31	>99.4	>99.9	103	25	97	99.7	93	25	89

Experimental Polar Surface Area (EPSA)

EPSA was measured for 9 known PROTACs using the method described by Goetz et al.[1]; the obtained values were within 94.9-156.4 (Tab. 1).

Conclusions

PROTAC molecules generally exhibit poor recovery and stability in long-incubation assays, but these limitations can be mitigated by adding stabilizers or preventing interactions with assay components. Experimental polar surface area (EPSA) may also serve as a valuable parameter for modelling PROTAC permeability.

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References

1. G.H. Goetz, W. Farrell, M. Shalaeva, S. Sciabola, D. Anderson, J. Yan, L. Philippe, and M.J. Shapiro, *“High Throughput Method for the Indirect Detection of Intramolecular Hydrogen Bonding”, J. Med. Chem.* **2014**, 57 (7), 2920-2929. DOI: 10.1021/jm401859b