

Optimization of the Assay Conditions to Increase Recovery in Caco-2 Permeability Assay for PROTACs

Y. Holota, A. Rodnichenko, N. Chyhrynova, M. Pavlichenko, Y. Kheylik, P. Borysko

Enamine Ltd., 78 Winston Churchill Street, 02094 Kyiv, Ukraine

Introduction and Aim

The emergence of new drug modalities raises challenges for DMPK scientists. Heterobifunctional degraders, proteolysis-targeting chimeras (PROTACs) (Fig. 1), have shown encouraging efficacy for cancer treatment, which attracted high interest in academia and industry. However, due to high molecular weight, polar surface area, number of rotatable bonds, and poor solubility and permeability, preclinical profiling of these molecules is more challenging compared to traditional small molecules. Low solubility and high non-specific binding may lead to the insufficient recovery of most PROTAC from *in vitro* test systems. Thus, the standard *in vitro* cell-based assay permeability protocol (Tab. 1) which is applied for small molecules needs to be adapted for PROTACs. The present study determined the impact of adding BSA to the assay buffer on the permeability, recovery and efflux ratio of a few known degraders (dTAG-7, dBET57, and ARV-110) (Fig. 2).

Figure 2. Structures of tested PROTACs

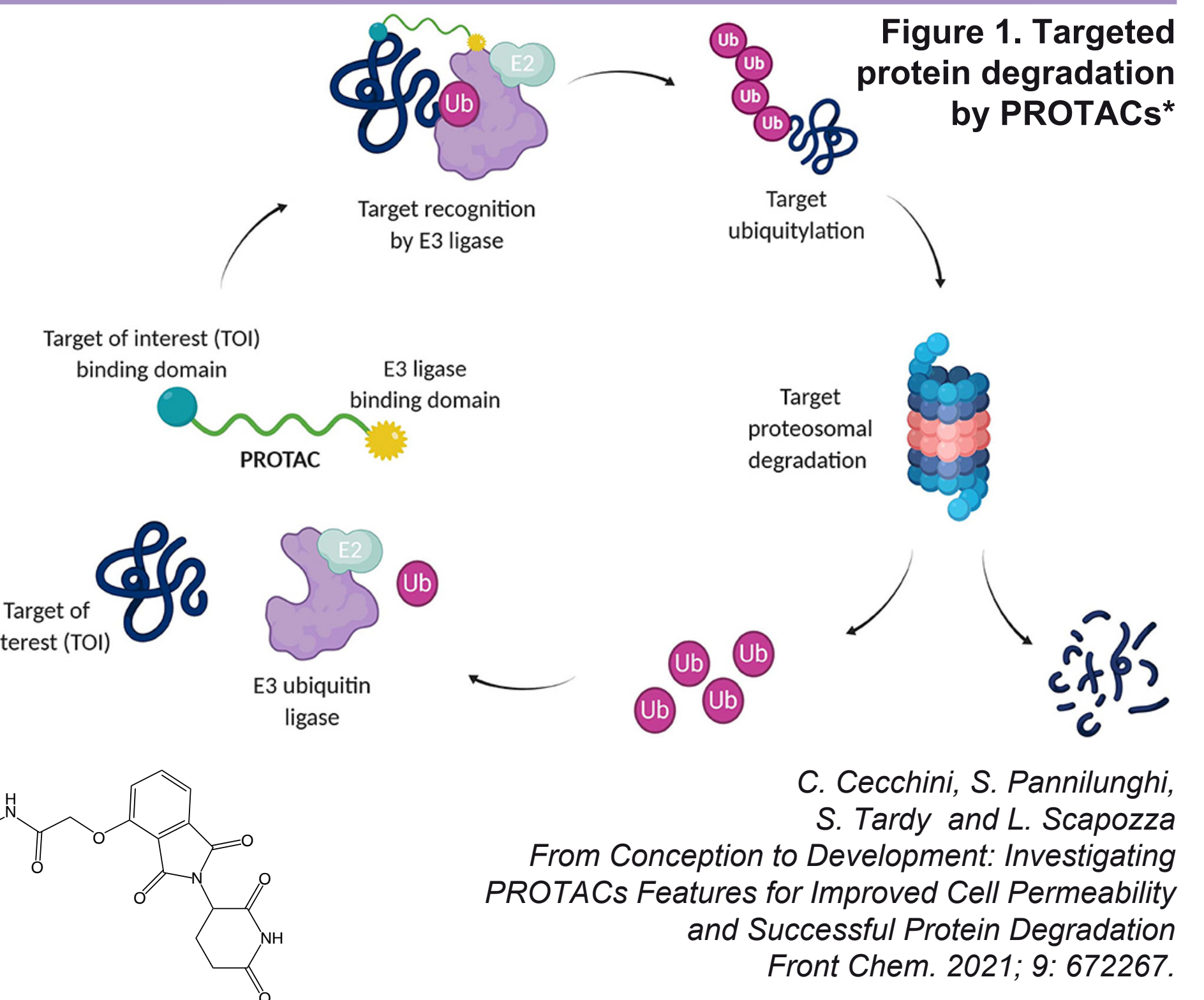
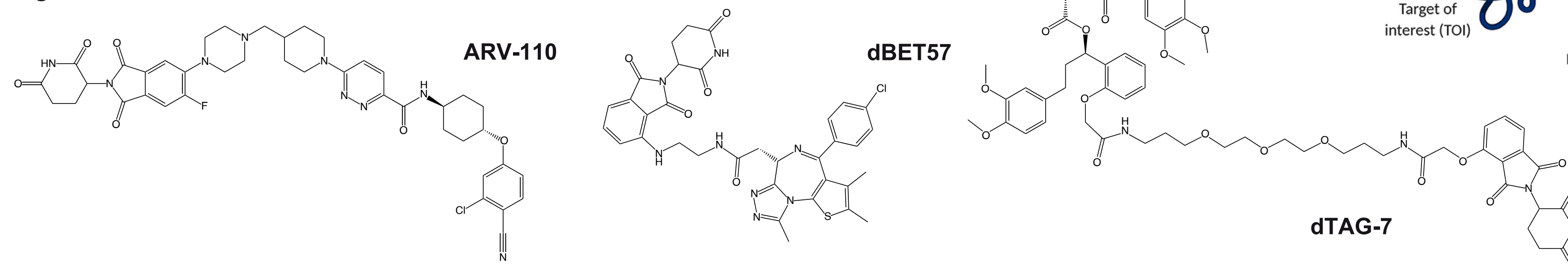


Table 1. Standard and protocol with modifications

	Caco-2 permeability assay standard protocol			Caco-2 permeability assay protocol with modifications								
Article concentration	10 μM			10 μM	10 μM	10 μM	10 μM	10 μM	10 μM	10 μM		
Number of replicates	2			2	2	2	2	2	2	2		
Incubation time	90min			90min	90min/or 120 min	90min	90min	90min	90min	90min		
Temperature	37° C			37° C	37° C	37° C	37° C	37° C	37° C	37° C		
Control compounds	Propranolol, and Ketoprofen (high permeability)			Propranolol, and Ketoprofen (high permeability)	Propranolol, and Ketoprofen (high permeability)	Propranolol, and Ketoprofen (high permeability)	Propranolol, and Ketoprofen (high permeability)	Propranolol, and Ketoprofen (high permeability)	Propranolol, and Ketoprofen (high permeability)	Propranolol, and Ketoprofen (high permeability)		
Control compounds for model P-gp substrates	Quinidine (moderate-high permeability) Digoxin (low permeability)			Quinidine (moderate-high permeability) Digoxin (low permeability)	Quinidine (moderate-high permeability) Digoxin (low permeability)	Quinidine (moderate-high permeability) Digoxin (low permeability)	Quinidine (moderate-high permeability) Digoxin (low permeability)	Quinidine (moderate-high permeability) Digoxin (low permeability)	Quinidine (moderate-high permeability) Digoxin (low permeability)	Quinidine (moderate-high permeability) Digoxin (low permeability)		
P-gp inhibitor	Verapamil			-	-	-	-	-	-	-		
Analysis Method	LC-MS/MS			LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS		
Data	Papp	Efflux ratio	% Recovery	Papp	Efflux ratio	% Recovery	Papp	Efflux ratio	% Recovery	Papp	Efflux ratio	% Recovery
BSA, %	-			0.25 %	0.5 %	1 %	2 %	4 %				
Buffer supplemented with BSA	-			In both compartments(AP and BL)/or basolateral compartment	In both compartments(AP and BL)/or basolateral compartment	In both compartments(AP and BL)/or basolateral compartment	In both compartments(AP and BL)/or basolateral compartment	In both compartments(AP and BL)/or basolateral compartment	In both compartments(AP and BL)/or basolateral compartment	In both compartments(AP and BL)/or basolateral compartment		

Methods

Bidirectional Caco-2 permeability assay was performed using the following conditions: protein-free assay buffer (standard protocol) & buffer with 0.25 - 4 % bovine serum albumin (BSA) in both compartments or the basolateral compartment only; incubation time from 90 min to 120 min. Compounds were tested at 10μM. Samples analysis was performed by LC-MS/MS; Papp, recovery, and efflux were calculated and compared.

Results

Table 2. Permeability data (standard assay protocol)

Test compound	P _{app} (A-B), 10 ⁻⁶ cm/s				P _{app} (B-A), 10 ⁻⁶ cm/s				Efflux ratio	% recovery (A-B)			% recovery (B-A)		
	1	2	Mean	SD	1	2	Mean	SD		1	2	Mean	1	2	Mean
Propranolol	17.3	15.4	16.4	1.3	10.1	10.7	10.4	0.4	0.6	62	56	59	84	95	89
Digoxin	0.2	0.2	0.2	0.0	12.6	11.2	11.9	1.0	54.2	84	97	91	86	85	86
dTAG-7	0.8	0.5	0.5	0.2	5.9	5.1	5.5	0.6	9.0	65	71	68	46	64	55
dBET57	0.5	0.4	0.4	0.1	13.3	12.3	12.8	0.7	29.0	82	77	79	78	44	61
ARV-110	0.7	0.6	0.7	0.1	0.3	0.2	0.3	0.0	0.4	39	40	40	50	42	46

Firstly, we evaluated the permeability of a few known degraders (dTAG-7, dBET57, and ARV-110) in the bidirectional Caco-2 permeability assay using a standard protocol which is usually applied for small molecules. All tested PROTACs exhibit low permeability in the A-B direction. dTAG-7 and dBET57 undergo active efflux. The recovery values for compounds are low (Tab. 2).

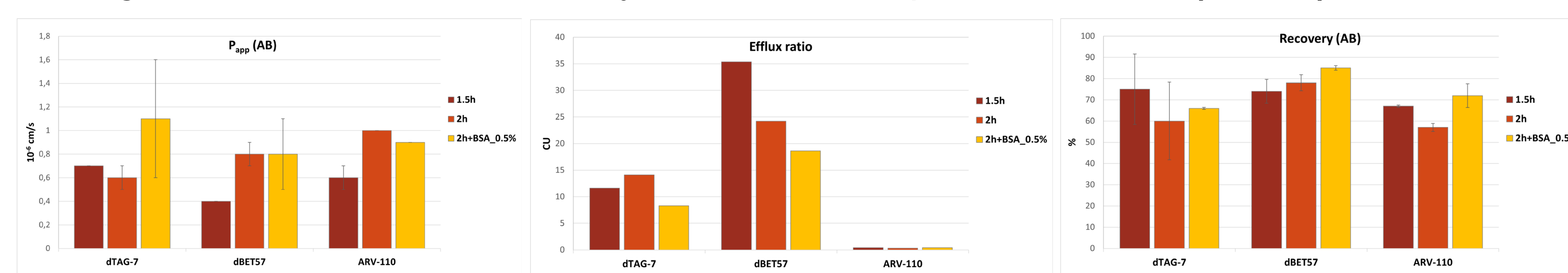


Figure 3. Permeability data: 2h incubation, 0.5% BSA

Next, we investigated how different incubation conditions might influence the Caco-2 permeability assessment for PROTACs. Increasing the incubation time to 2h resulted in decreased recovery values for dTAG-7 and ARV-110. The presence of 0.5% BSA in apical and basolateral compartments increased the recovery values for dBET57 and ARV-110, but decreased the efflux ratio for dTAG-7, and dBET57 (Fig. 3).

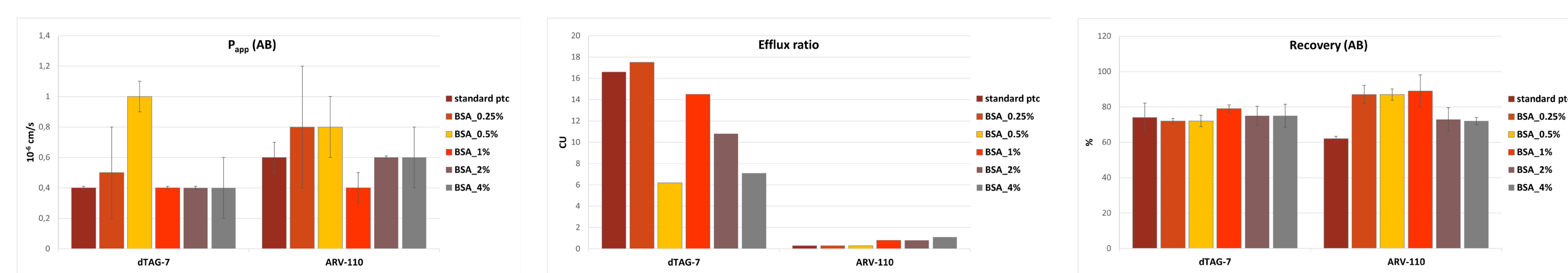


Figure 4. Permeability data: 0.25% - 4% BSA (both compartments)

Adding BSA at different concentrations to both compartments resulted in the increased recovery for ARV-110 in the presence of 0.25% - 1% BSA, while the efflux ratio for dTAG-7 changed in the presence of 0.5% BSA and more (Fig. 4).

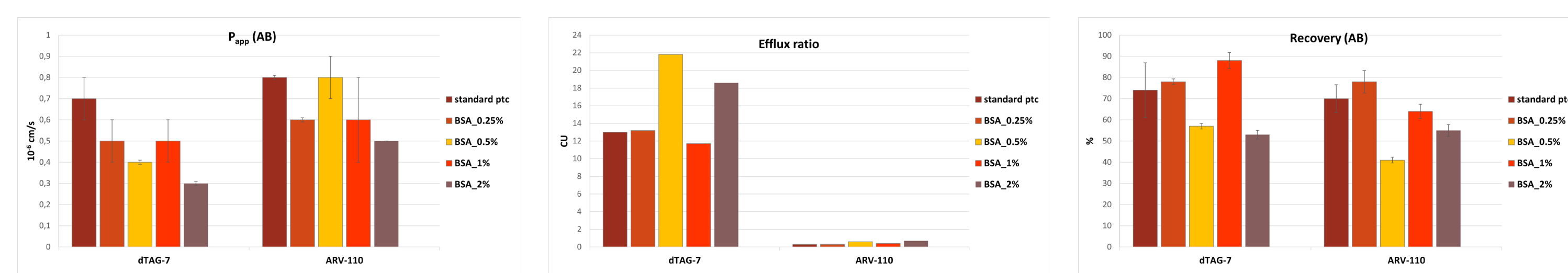


Figure 5. Permeability data: 0.25% - 2% BSA (basolateral compartment)

After adding 0.25%, 1%, and 2% of BSA to the basolateral compartment only, the permeability values for dTAG-7 and ARV-110 decreased, while the efflux ratio for dTAG-7 was increased (0.5% and 2% BSA). The recovery values for dTAG-7 increased in the presence of 1% BSA in the basolateral compartment, for ARV-110 – in the presence of 0.25% BSA in the basolateral compartment (Fig. 5).

Conclusions

Adding proteins to the assay buffer is a frequently used approach to overcome non-specific binding, improve solubility, and mimic better the physiological conditions in the Caco-2 assay. However, it is necessary to pay attention to possible risks during the interpretation of the obtained data. Poorly soluble compounds may not achieve effective concentrations in the transport buffer to generate meaningful permeability data. High concentrations of BSA may cause the misidentification of efflux substrates in the assay; compounds with high protein binding may exhibit decreased Papp values, etc. Thus 0.25% BSA was selected as the optimal concentration for the next steps of the assay optimization for PROTACs permeability measurements in the Caco-2 test system.

Contact

Yuliia Holota, PhD
y.holota@enamine.net
Bienta/Enamine Ltd, www.bienta.net
78 Winston Churchill Street, 02094 Kyiv, Ukraine