Supplementary material for

Re-use of Caco-2 monolayers for a higher throughput assessment of drug permeability – methodology and validation for passive permeation

Cristiana L. Pires[1], Susana Rosa[2], Ana L. M. Batista de Carvalho[3], Lino Ferreira[2,4], Maria Paula M. Marques[3,5] and Maria João Moreno[1,*]

[1] Coimbra Chemistry Center - Institute of Molecular Sciences (CQC-IMS), Department of Chemistry, University of Coimbra, Coimbra, Portugal; cristiana.lages.pires@gmail.com

[2] CNC-Center for Neuroscience and Cell Biology, CIBB-Centre for Innovative Biomedicine and Biotechnology, University of Coimbra, UC-Biotech Parque Tecnológico de Cantanhede, Coimbra, Portugal; susrosa@gmail.com (S.R); lino@uc-biotech.pt (L.F.)

[3] Molecular Physical-Chemistry R&D Unit, Department of Chemistry, University of Coimbra, Coimbra, Portugal almbc@uc.pt (A.L.M.B.d.C.); PMC@ci.uc.pt (M.P.M.M.)

[4] Faculty of Medicine, University Coimbra, Coimbra, Portugal

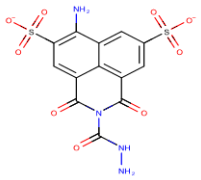
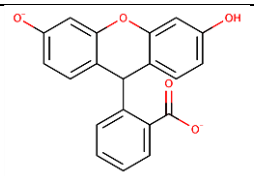
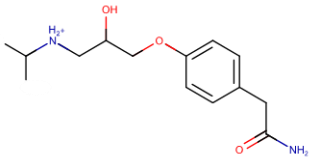
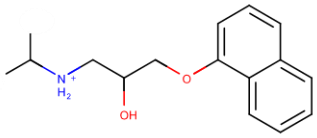
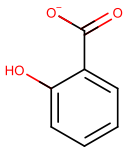
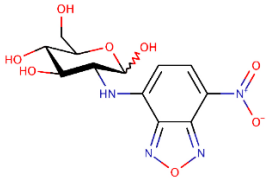
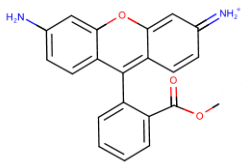
[5] Department of Life Sciences, University of Coimbra, Coimbra, Portugal

*Correspondence: mmoreno@ci.uc.pt; Tel.: +351-239854481

Section **Page**

S1. Structure and general properties of the tested compounds

Table S1. Molecular structure and some molecular descriptors calculated using the Marvin® software version 22.9.0 by ChemAxon (https://www.chemaxon.com) for the solutes used in this work.

Permeation pathway	Name	Structure	Molecular descriptors	
Paracellular	Lucifer Yellow (LY)		MW (g/mol) pK_a Charge at pH 7.4 PSA (Å^2) cLogD$_{7.4}$ cLogP	428 -3, -2, 3, 9.5 -2 250 -6.2 -1.4
Passive permeation, mostly Paracellular	Fluorescein (Flu)		MW (g/mol) pK_a Charge at pH 7.4 PSA (Å^2) cLogD$_{7.4}$ cLogP	332 3.8, 6.1 [a] -2 90 0.16 4.1
	Atenolol		MW (g/mol) pK_a Charge at pH 7.4 PSA (Å^2) cLogD$_{7.4}$ cLogP	266 9.7 +1 89 -1.8 0.4
Passive permeation, mostly Transcellular	Propranolol (PROP)		MW (g/mol) pK_a Charge at pH 7.4 PSA (Å^2) cLogD$_{7.4}$ cLogP	260 9.7 +1 46 0.4 2.6
	Salicylic acid (SA)		MW (g/mol) pK_a Charge at pH 7.4 PSA (Å^2) cLogD$_{7.4}$ cLogP	137 3 -1 60 -1.5 2.0
Active transport influx	2-NBD-Glucose		MW (g/mol) pK_a [a] Charge at pH 7.4 PSA (Å^2) cLogD$_{7.4}$ cLogP	342 - 0 192 -2.3 -2.3
Passive (transcellular) permeation, active efflux by P-gp	Rhodamine 123 (Rho)		MW (g/mol) pK_a Charge at pH 7.4 PSA (Å^2) LogD$_{7.4}$ cLogP	345 6.1 [a] +1 87 0.53 [b] 2.8

[a] Experimentally determined pK_a values (from Ref. [1] and [2] for Flu and Rho, respectively), [b] LogD value from Ref. [2]

S2. Analytical procedures for the quantification of tested compounds

The quantification of the tested compounds (except for sodium fluorescein) was performed by reverse phase HPLC using a a Nucleosil C18 ODS column (150 × 4.6 mm, 5 µm), preceded by a pre-column Zorbax ODS C18 column (12.5 mm × 4.6 mm, 5 µm). The flow rate was maintained constant at 1 mL/min and the columns were equilibrated at 30 °C. The elution of the compounds included both isocratic and gradient elution steps comprising two eluents, eluent A consisted of 0.05 M ammonium formate adjusted with formic acid to pH 3.5, and eluent B was methanol. The quantitative analysis was carried out using a fluorescence detector. The samples from the acceptor compartment of the permeability assays were injected directly on the HPLC. In the case of solutes that permeated very slowly, with a very small amount being expected at the basolateral side, the injected volume was the maximum of the injection loop (900 µL) to increase the sensitivity for their detection. The samples from the donor compartment were previously diluted 1:20 with 950 µL of HBSS and a volume of 100 µL was injected. The chromatograms obtained for the samples in the donor and acceptor compartments at the end of the multi-time sampling permeability assays are presented in Figure S1**.** Due to the setup of the injection system, a larger volume of sample injected leads to a corresponding delay in the elution of the compounds. Apart from this, the comparison of the chromatograms for samples from the donor and acceptor compartments show that the elution profile was not affected by the 1 - 2 h incubation or transport through the Caco-2 monolayers. The optimal chromatographic conditions applied for each solute are detailed in Table S2. The optimized method for the paracellular marker LY consisted of 100 % eluent A (ammonium formate), and leads to a retention time of the major band around 8 min. To allow the analysis of the samples when both the control LY and the test compound were applied on the same cell monolayer, the developed methods for the other solutes always started with 100 % of eluent A (with the exception of Atenolol). Then, after 7 min, the eluent B (methanol) was added to allow the elution of the test compound. The simultaneous quantification of LY and 2-NBDG or Rho123 is illustrated in Figure S1. For each solute, the calibration curves were built from 3 independent set of solutions, for the two injected volumes of 100 and 900 µL. The performance of the analytical methods was validated through the determination of linearity (r^2 and linear range), method detection limit (MDL) and method quantification limit (MQL). The parameters obtained for the calibration curves are provided in Table S2.

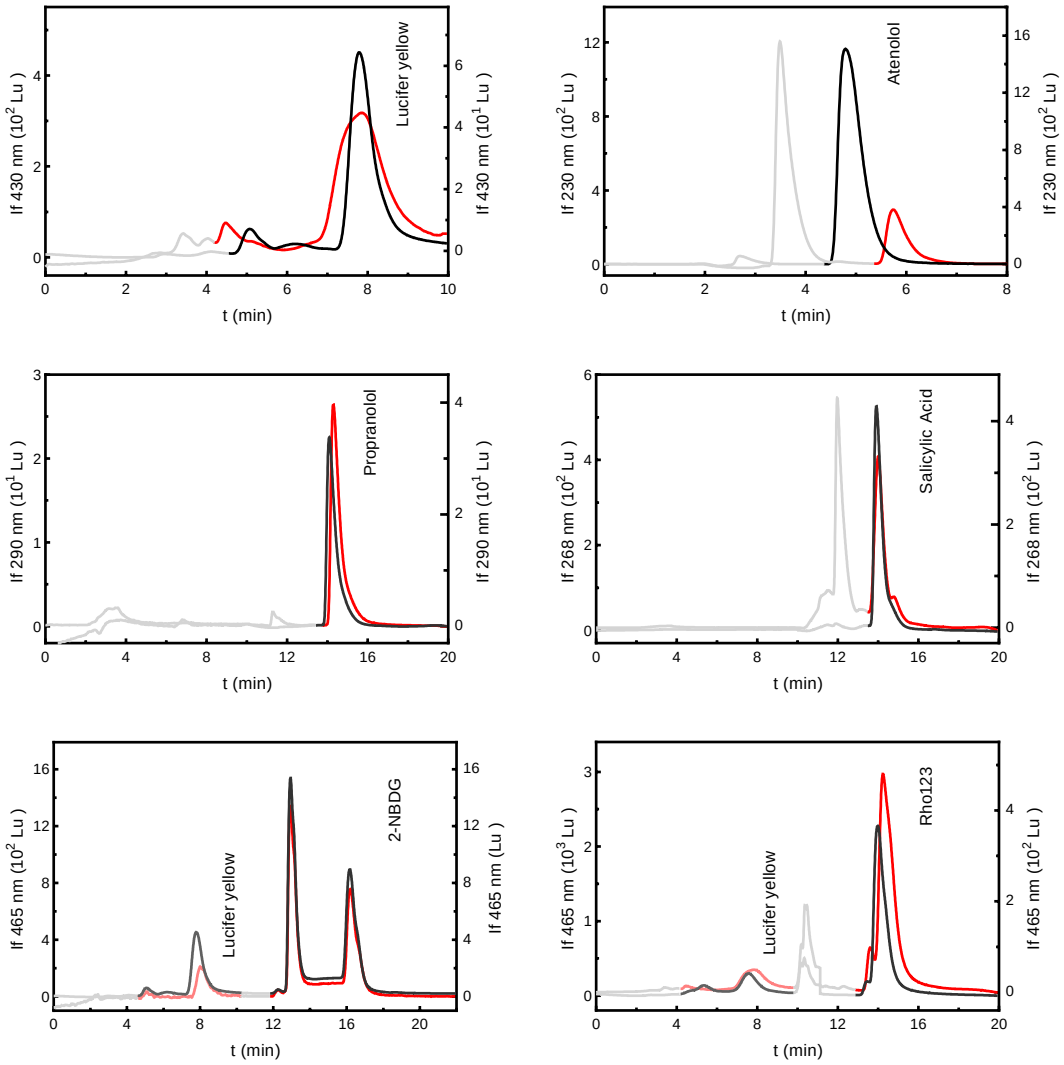


Figure S1. Typical HPLC chromatograms with fluorescence detection, for the compounds used in the Caco-2 permeability assays. The chromatograms of the samples collected from the donor compartment correspond to the left y-axis, and those from the acceptor compartment correspond to the right y-axis. The examples shown correspond to a typical sample at the end points of a multi-time sampling permeability assay. The concentration of the analytes was calculated from the integration of the chromatogram in the regions indicated in black or red, for samples collected from the donor or acceptor compartments, respectively. The chromatogram regions indicated in grey correspond to residual fluorescence from the mobile phase and/or HBBS. When the test compound and LY were in the same sample, LY concentration was calculated from the area between 4 and 10 min (light black and red).

Flu was quantified by its fluorescence intensity using a plate reader. To increase sensitivity, the pH of all samples was increased to 8.5-9 by adding a small aliquot of 10 M NaOH. To guaranty that the samples analyzed are within the linear concentration range, the donor solutions were previously diluted 24-fold with HBSS. The parameters obtained for the calibration curve are also shown in Table S2.

Table S2. Conditions and parameters of the calibration curves for the tested compounds used in the permeability assays.

	Conditions					**Calibration curve** [a]			
HPLC with RP18 stationary phase and fluorescence detection									
Reference compounds (in HBSS)	Mobile phase A : Ammonium formate 50 mM pH 3.5 (adjusted with formic acid) B : Methanol time (min)	% A	Fluorescence detection wavelengths (nm)	Injection volume (µL)	Retention time (min)	r^2	Calibration range (µM)	MDL (µM) [b]	MQL (µM) [c]
Lucifer Yellow (LY)	0 − 10	100	Ex: 430 Em: 530	100	5.1 & 7.8	0.9992	0.13 − 2.1	0.045	0.14
				900	5.1 & 8.0	0.9987	0.0011 − 0.038	0.0016	0.0047
Atenolol (with 1% DMSO)	0 − 8	80	Ex: 230 Em: 302	100	4.8	0.9999	0.043 − 2.7	0.019	0.056
				900	5.8	0.9998	0.0013 − 0.17	0.0023	0.0068
Propranolol (PROP)	0 − 5 5 − 7 7 − 15 15 − 18	100 40 40 100	Ex: 290 Em: 340	100	14.2	0.9998	0.19 − 2.2	0.038	0.11
				900	14.2	0.9967	0.008 − 0.29	0.019	0.056
Salicylic Acid (SA)	0 − 5 5 − 7 7 − 15 15 − 18	100 60 60 100	Ex: 268 Em: 406	100	13.9	0.9989	0.088 − 2.6	0.11	0.33
				900	14.1	0.9991	0.0028 − 0.21	0.0076	0.023
2-NBDG	0 − 7 7 − 7.5 7.5 − 18 18 − 20	100 85 85 100	Ex: 465 Em: 530	100	13.1	0.9999	0.19 − 10.0	0.11	0.33
				100	13.1	0.9999	0.0046 − 0.26	0.0013	0.0039
Rhodamine 123 (Rho)	0 − 5 5 − 7 7 − 15 15 − 18	100 40 40 100	Ex: 465 Em: 530	100	14.2	0.9995	0.012 − 0.20	0.0065	0.020
				900	14.3	0.9978	0.00077 − 0.024	0.0013	0.0040
Fluorescence detection in 96 well plate reader									
		Sample pre-treatment	Fluorescence wavelengths (nm)	Volume (µL)		r^2	Calibration range (µM)	MDL (µM) [b]	MQL (µM) [c]
Sodium Fluorescein (Flu)	pH % NaOH 10 M	8.5-9 0.1 %	Ex: 485 Em: 530	200		0.9998	0.0019 − 0.95	0.0019	0.005

[a] For the calibration curves, three independent samples were analysed for each concentration. [b] method detection limit (MDL) calculated as $3\times S_{y/x}/b$ where $S_{y/x}$ is the standard error of the estimated curve and b is the slope of the calibration curve. [c] method quantitation limit (MQL) calculated as $10\times S_{y/x}/b$.

S3. Effect of Caco-2 monolayer re-use on passive permeation

Low permeation reference compounds – Lucifer yellow, Fluorescein and Atenolol

Table S3. Statistical analysis of LY, Flu and atenolol P_{app} and amount transported into the acceptor compartment Q_A (% of total amount) after single time and multi-time sampling permeability assays through single use (day 22) and re-use (day 25 and 28) Caco-2 monolayers. The average and standard deviation, and the corresponding confidence intervals at 95 % confidence (CI_{95}), were obtained directly from the analysis of Log (P_{app}) or Log (Q_A).

Assay Conditions				Lucifer Yellow				Fluorescein				Atenolol			
				P_{app} (10^{-7} cm/s)		Q_A (%)		P_{app} (10^{-7} cm/s)		Q_A (%)		P_{app} (10^{-7} cm/s)		Q_A (%)	
Day	**Sampling**	**N[a]**	**Monolayer use**	**μ**	**CI_{95}**	**μ**	**CI_{95}**	**μ**	**CI_{95}**	**μ**	**CI_{95}**	**μ**	**CI_{95}**	**μ**	**CI_{95}**
All	all	607 & 60 & 94	Single use & re-use	4.6	0.6; 33.0	0.15	0.03; 0.78	3.7	0.8, 18.3	0.11	0.03, 0.38	6.2	2.9, 13.5	0.21	0.07, 0.59
	all 1st	240 & 24 & 24		3.0	0.4; 22.7	0.12	0.03; 0.55	2.5	0.4, 15.4	0.10	0.03, 0.39	5.3	2.4, 11.9	0.22	0.05, 0.95
	1st 60 min	118 & 12 & 12		1.3	0.3; 5.1	0.13	0.03; 0.51	1.2	0.3, 5.1	0.12	0.03, 0.52	4.5	2.2, 9.1	0.45	0.22, 0.91
	1st 10 min	122 & 12 & 12		6.6	1.2; 35.5	0.11	0.02; 0.59	5.1	1.3, 19.9	0.09	0.02, 0.33	6.3	2.6, 15.1	0.11	0.04, 0.25
	2nd 10 min	124 & 12 & 12		7.4	1.4; 39.2	0.14	0.03; 0.70	6.2	1.8, 21.3	0.10	0.03, 0.36	8.5	4.1, 17.7	0.14	0.07, 0.30
	3rd 10 min	119 & 12 &29		7.9	1.3; 46.9	0.16	0.03; 0.87	6.5	1.8, 22.8	0.11	0.03, 0.38	6.9	3.1, 15.3	0.17	0.08, 0.40
	4th 30 min	124 & 12 & 29		4.0	0.7; 22.2	0.25	0.05; 1.2	3.0	0.9, 9.6	0.15	0.05, 0.49	5.7	2.9, 11.3	0.29	0.14, 0.57
22	all	216 & 20 & 30	Single use	6.0	0.7; 52.8	0.19	0.03; 1.2	3.3	0.8, 14.4	0.10	0.03, 0.29	6.2	2.9, 12.9	0.21	0.08, 0.54
	all 1st	87 & 8 & 8		3.4	0.4; 31.5	0.14	0.03; 0.76	2.2	0.4, 13.3	0.09	0.02, 0.34	5.7	2.5, 12.9	0.23	0.05, 1.2
	1st 60 min	45 & 4 & 4		1.4	0.4; 5.9	0.15	0.04; 0.60	1.1	0.2, 5.8	0.11	0.02, 0.58	4.8	2.2, 10.4	0.48	0.22, 1.1
	1st 10 min	42 & 4 & 4		8.5	1.2; 59.0	0.14	0.02; 0.99	4.2	1.0, 18.2	0.07	0.02, 0.30	6.8	2.4, 19.1	0.11	0.04, 0.32
	2nd 10 min	44 & 4 & 4		10	1.7; 62.0	0.18	0.03; 1.1	5.7	1.5, 21.1	0.09	0.03, 0.35	9.5	3.8, 23.7	0.16	0.06, 0.40
	3rd 10 min	41 & 4 & 9		12	1.7; 84.8	0.22	0.03; 1.5	5.8	1.9, 18.0	0.10	0.03, 0.30	6.2	2.6, 14.9	0.15	0.08, 0.29
	4th 30 min	44 & 4 & 9		5.7	0.9; 37.1	0.32	0.05; 2.0	2.6	0.7, 9.3	0.13	0.04, 0.47	5.5	3.0, 9.9	0.27	0.15, 0.50
25	all	70	Single use	5.8	1.2; 27.9	0.17	0.06; 0.53								
	all 1st	22		4.8	0.9; 25.9	0.13	0.03; 0.51								
	1st 60 min	6		1.8	0.6; 5.4	0.18	0.06; 0.54								
	1st 10 min	16		6.9	1.6; 30.5	0.12	0.03; 0.51								

	2nd 10 min	16		7.4	1.7; 32.6	0.15	0.05; 0.45								
	3rd 10 min	16		7.9	1.7; 38.1	0.18	0.08; 0.40								
	4th 30 min	16		4.1	0.8; 21.7	0.28	0.13; 0.62								
	all	133 & 20 & 32		4.2	0.5; 37.9	0.14	0.02; 0.90	3.9	0.5, 30.7	0.12	0.02, 0.60	6.9	2.7, 17.5	0.23	0.07, 0.74
	all 1st	58 & 8 & 8		2.4	0.3; 22.7	0.11	0.02; 0.57	2.2	0.2, 26.3	0.09	0.02, 0.54	5.5	1.8, 16.5	0.23	0.04, 1.2
	1st 60 min	33 & 4 & 4	Re-use	1.1	0.3; 4.5	0.11	0.03; 0.45	0.9	0.1, 7.6	0.09	0.01, 0.76	4.3	1.7, 10.8	0.44	0.17, 1.1
25	1st 10 min	25 & 4 & 4		6.8	0.9; 51.5	0.11	0.02; 0.82	5.3	0.5, 52.5	0.09	0.01, 0.88	7.0	1.6, 30.6	0.12	0.03, 0.51
	2nd 10 min	25 & 4 & 4		8.0	1.0; 61.5	0.15	0.02; 1.2	6.6	0.7, 61.1	0.11	0.01, 1.0	8.6	2.5, 29.8	0.14	0.04, 0.50
	3rd 10 min	25 & 4 & 10		7.8	1.0; 60.2	0.15	0.02; 1.2	7.5	0.7, 76.5	0.13	0.01, 1.3	8.2	3.0, 22.0	0.21	0.07, 0.65
	4th 30 min	25 & 4 & 10		4.1	0.7; 24.2	0.23	0.03; 1.5	3.6	0.5, 28.6	0.18	0.02, 1.4	6.5	2.7, 15.8	0.33	0.14, 0.79
	all	70		6.0	2.0; 18.7	0.18	0.07; 0.47								
	all 1st	22		5.9	1.6; 20.6	0.16	0.04; 0.60								
	1st 60 min	6	Single use	3.2	1.7; 6.1	0.32	0.17;0.62								
28	1st 10 min	16		7.2	2.0; 25.9	0.12	0.03; 0.43								
	2nd 10 min	16		7.4	2.6; 20.9	0.14	0.07; 032								
	3rd 10 min	16		7.7	3.1; 19.3	0.17	0.09; 0.30								
	4th 30 min	16		4.1	1.3; 13.2	0.28	0.15; 0.53								
	all	118 & 20 & 32		2.4	0.6; 10.1	0.09	0.02; 0.35	4.0	1.0, 15.9	0.12	0.04, 0.33	5.7	2.9, 11.0	0.19	0.07, 0.53
	all 1st	51 & 8 & 8		1.8	0.4; 8.7	0.08	0.03; 0.28	3.1	0.5, 19.5	0.13	0.03, 0.51	4.9	2.3, 10.2	0.20	0.03, 1.1
	1st 60 min	23 & 4 & 4	Re-use	1.0	0.3; 3.9	0.11	0.03; 0.38	1.6	0.3, 9.8	0.16	0.03, 0.99	4.4	1.5, 12.6	0.44	0.15, 1.3
28	1st 10 min	23 & 4 & 4		3.6	1.4; 9.3	0.06	0.02; 0.16	5.9	1.4, 24.2	0.10	0.02, 0.41	5.3	2.6, 10.7	0.09	0.04, 0.18
	2nd 10 min	23 & 4 & 4		3.6	1.3; 10.3	0.07	0.02; 0.25	6.3	2.0, 19.6	0.11	0.03, 0.33	7.5	4.5, 12.7	0.13	0.08, 0.21
	3rd 10 min	23 & 4 & 10		3.5	1.0; 12.8	0.09	0.02; 0.44	6.2	1.9, 20.5	0.10	0.03, 0.34	6.4	3.3, 12.5	0.16	0.07, 0.36
	4th 30 min	23 & 4 & 10		2.0	0.5; 7.7	0.15	0.03; 0.64	2.7	1.0, 7.7	0.14	0.05, 0.39	5.2	2.6, 10.4	0.26	0.13, 0.52

No statistically significant differences are observed between the distinct experimental days.

[a] Number of time points sampled for Lucifer yellow & Flu & Atenolol permeability assays.

High permeation reference compounds – Propranolol and salicylic acid

Table S4. Statistical analysis of P_{app} values of Propranolol and Salicylic Acid after single time and multi-time sampling permeability assays through single use and re-used Caco-2 monolayers from day 22 to 28. The average and standard deviation, and the corresponding confidence intervals at 95 % confidence (CI_{95}), were obtained directly from the analysis of Log (P_{app}).

Assay Conditions			P_{app} (10^{-6} cm/s)			
			Propranolol		Salicylic Acid	
Day	Sampling (N)[a]	Monolayer use	μ	CI_{95}	μ	CI_{95}
All	all (102 & 150)	Single use & re-use	4.1	1.6, 10.8	2.9	1.1, 7.6
	all 1st (39 & 51)		3.0	0.8, 10.8	2.7	0.9, 8.6
	1st 60 min (18 & 18)		1.4	0.9, 2.3	1.2	0.8, 1.8
	1st 10 min (21 & 33)		5.7	3.4, 9.5	4.2	2.3, 7.9
	2nd 10 min (21 & 33)		5.8	4.7, 7.3	3.6	1.9, 7.0
	3rd 10 min (21& 33)		6.2	4.9, 7.7	3.6	1.9, 7.1
	4th 30 min (21 & 33)		3.3	2.1, 5.3	1.9	0.8, 4.9
22	all (34 & 50)	Single use	4.1	1.5, 10.8	2.9	0.9, 9.2
	all 1st (13 & 17)		3.1	0.8, 11.4	2.6	0.7, 10.0
	1st 60 min (6 & 10)		1.5	1.1, 2.2	1.2	0.8, 1.8
	1st 10 min (7 & 11)		5.6	2.7, 11.7	4.1	1.6, 10.7
	2nd 10 min (7 & 11)		5.8	4.4, 7.6	3.8	1.6, 9.1
	3rd 10 min (7 & 11)		6.4	4.9, 8.5	3.6	1.3, 10.1
	4th 30 min (7 & 11)		3.0	2.0, 4.5	2.1	0.6, 7.7
25	all (34 & 50)	Single use & re-use	3.9	1.4, 11.1	2.9	1.1, 7.2
	all 1st (13 & 17)		2.9	0.7, 12.4	2.8	0.9, 9.1
	1st 60 min (6 & 6)		1.3	0.6, 2.7	1.2	0.8, 2.0
	1st 10 min (7 & 11)		5.7	3.2, 9.9	4.4	2.7, 7.2
	2nd 10 min (7 & 11)		5.8	4.6, 7.3	3.3	1.9, 5.8
	3rd 10 min (7 & 11)		5.8	4.7, 7.2	3.9	2.2, 6.8
	4th 30 min (7 & 11)		3.3	2.1, 5.2	1.9	0.8, 4.7
	1st 10 min (4 & 2)	Single use	7.0	5.1, 9.5	5.7	1.5, 21.1
	2nd 10 min (4 & 2)		6.3	5.5, 7.3	5.2	1.5, 17.6
	3rd 10 min (4 & 2)		5.8	4.2, 8.1	4.9	1.9, 12.7
	4th 30 min (4 & 2)		3.0	2.0, 4.5	2.4	2.1, 2.8
	1st 60 min (6 & 6)	Re-use	1.3	0.6, 2.7	1.2	0.8, 2.0
	1st 10 min (3 & 9)		4.3	3.1, 6.0	4.2	2.8, 6.3
	2nd 10 min (3 & 9)		5.1	4.5, 5.9	3.0	2.1, 4.4
	3rd 10 min (3 & 9)		5.7	5.1, 6.4	3.7	2.1, 6.5
	4th 30 min (3 & 9)		3.7	1.8, 7.6	1.8	0.7, 5.0
28	all (34 & 50)	Single use & re-use	4.3	1.6, 11.4	2.9	1.2, 6.9
	all 1st (13 & 17)		3.1	0.8, 12.0	2.9	1.0, 8.4
	1st 60 min (6 & 6)		1.5	0.8, 2.5	1.3	0.9, 2.0
	1st 10 min (7 &11)		5.9	3.8, 9.2	4.3	2.9, 6.5
	2nd 10 min (7 & 11)		5.9	4.6, 7.6	3.8	1.9, 7.5
	3rd 10 min (7 & 11)		6.3	5.1, 7.7	3.4	2.1, 5.4
	4th 30 min (7 & 11)		3.7	2.0, 7.1	1.8	0.9, 3.7
	1st 10 min (4 & 2)	Single use	6.9	5.4, 8.9	4.2	2.6, 6.7
	2nd 10 min (4 & 2)		6.0	4.1, 8.8	4.6	1.8, 11.4
	3rd 10 min (4 & 2)		5.9	5.2, 6.6	4.2	1.1, 15.7

	4th 30 min (4 & 2)		3.5	1.9, 6.7	2.3	0.9, 6.2
	1st 60 min (6 & 6)	Re-use	1.5	0.8, 2.5	1.3	0.9, 2.0
	1st 10 min (3 & 9)		4.8	3.4, 6.8	4.4	2.8, 6.9
	2nd 10 min (3 & 9)		5.8	5.4, 62	3.7	1.8, 7.6
	3rd 10 min (3 & 9)		6.9	5.5, 8.6	3.3	2.2, 4.8
	4th 30 min (3 & 9)		4.0	1.4, 11.3	1.7	0.8, 3.5

No statistically significant differences are observed between the distinct experimental days.

[a] Number of time points sampled for Prop & SA permeability assays.

S4. Effect of Caco-2 monolayer re-use on transport of 2-NBDG

Table S5. Statistical analysis of P_{app} values of 2-NBDG in the absorptive (A→B) and secretory (B→A) directions and the resulting influx ratio. The paracellular marker LY was applied together with 2-NBDG and the resulting P_{app} is also show, as well as the ratio between the P_{app} values of 2-NBDG and LY.

Day	Monolayer use (number of cell monolayers in A→B & B→A))	2-NBDG P_{app} (10^{-7} cm/s)						LY P_{app} (10^{-7} cm/s)				P_{app} 2-NBDG/ P_{app} LY	
		A→B		B→A		Influx Ratio		A→B		B→A		A→B	B→A
		μ	CI_{95}	μ	CI_{955}	μ	CI_{955}	μ	CI_{95}	μ	CI_{95}		
All	Single use (12 & 12)	3.5	1.3, 9.1	7.7	2.4, 24.4	0.5	0.1, 2.0	2.8	0.8, 10.2	8.2	2.8, 24.6	1.2	0.9
	Re-use (11 & 10)	3.5	1.6, 7.9	9.0	3.2, 25.2	0.4	0.2, 1.4	3.4	0.7, 17.5	9.0	3.8, 20.9	1.0	0.9
	Single use & re-use (23 & 22)	3.5	1.4, 8.5	8.1	2.7, 24.4	0.4	0.1, 1.8	3.0	0.7, 12.9	8.5	3.1, 23.1	1.2	0.9
22	Single use (4 & 4)	5.0	2.1, 11.6	14	3.9, 48.4	0.4	0.09, 1.7	4.4	0.9, 21.0	14	4.2, 45.5	1.1	1.0
25	Single use (4 & 4)	2.6	0.9, 7.6	5.6	2.0, 15.7	0.5	0.1, 1.9	1.7	0.6, 4.7	6.2	2.4, 15.9	1.5	0.9
	Re-use (3 & 2)	3.0	1.6, 5.3	11	2.4, 48.1	0.3	0.06, 1.3	4.5	0.4, 56.3	9.3	2.7, 32.1	0.7	1.0
	Single use & re-use (7 & 6)	2.8	1.2, 6.5	7.1	2.0, 24.8	0.4	0.09, 1.7	2.6	0.4, 17.2	7.1	2.5, 19.9	1.1	0.9
28	Single use (4 & 4)	3.2	1.5, 7.1	5.8	3.6, 9.5	0.5	0.2, 1.4	2.8	1.1, 7.1	6.5	3.4, 12.6	1.0	0.7
	Re-use (7 & 4)	3.8	1.6, 9.1	8.2	3.8, 17.7	0.5	0.2, 1.5	3.0	0.9, 10.0	8.8	4.3, 18.0	1.3	0.9
	Single use & re-use (11 & 8)	3.6	1.6, 8.2	6.9	3.5, 13.7	0.5	0.2, 1.5	2.9	1.0, 8.6	7.6	3.8, 15.4	1.2	0.8

No statistically significant differences are observed between the distinct experimental days.

To better understand the interaction of 2-NBDG with the Caco-2 cells, the monolayers were analyzed by confocal microscopy at wavelengths where the NBD group emits fluorescence. Representative images of the projections of maximum intensity of all z-stacks are shown in Figure S2. Images were taken of Caco-2 monolayers not used in permeability assays (panel A) and after the permeability assay with 2-NBDG in the A→B (panel B) and B→A (panel C) directions. A quantitative analysis of the fluorescence intensity was performed considering the total image area (428 × 428 μm^2), leading to mean fluorescence intensity of 16 for (A) 22 for (B) and 24 for (C).

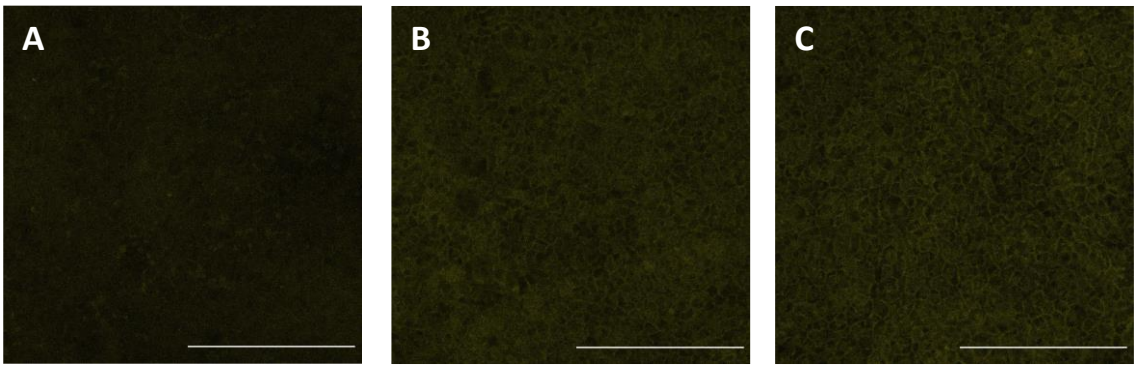


Figure S2. Confocal microscopy images of Caco-2 monolayers obtained with excitation light at 458 nm and emission detection from 459–551 nm. Maximum intensity projections of z-stacks are shown for (A) cell monolayers not used for any permeability assay and for cell monolayers exposed to 250 μM of 2-NBDG in the A→B direction (B) and in the B→A direction (C) during the permeability assays at day 28 post-seeding. Scale bar 200 μm.

S5. Effect of Caco-2 monolayer re-use on transport of Rho

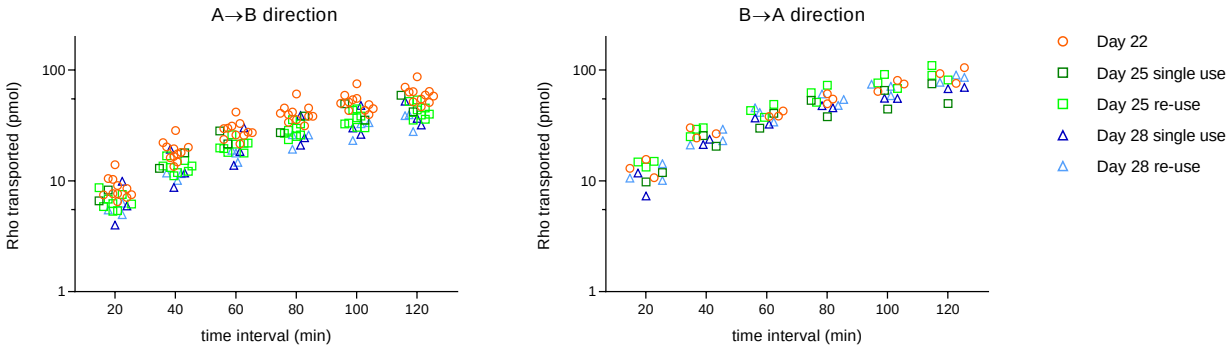


Figure S3. Effect of the re-use and the day post seeding on the cumulative amount of Rho transported as a function of the incubation time, in the A→B direction (left plot) and in the B→A direction (right plot). The cell monolayers were used at day 22 (orange), day 25 (green) and day 28 (blue) post-seeding. On days 25 and 28, the results obtained for cell monolayers used in a single permeability experiment are shown in dark colors, and those obtained for re-used cell monolayers are in light colors. Note the logarithmic scale in the y axis.

Table S6. Statistical analysis of the P_{app} values of Rho in the absorptive (A→B) and secretory (B→A) directions. The resulting efflux ratio and the contribution of passive permeation (P_{app}^{PP}) and active efflux (P_{app}^{AE}) to the measured Rho P_{app} values were determined using equations (4) and (5) in the manuscript. The paracellular marker was applied together with Rho, and the permeability obtained is also shown.

Day	Monolayer use (number of cell monolayers in A→B & B→A)	Rho P_{app} (10^{-6} cm/s)						Rho P_{app} (10^{-6} cm/s)		LY P_{app} (10^{-7} cm/s)			
		A→B		B→A		Efflux Ratio		P_{app}^{PP}	P_{app}^{AE}	A→B		B→A	
		μ	CI_{95}	μ	CI_{95}	μ	CI_{95}	μ	μ	μ	CI_{95}	μ	CI_{95}
All	Single use (16 & 7)	1.6	1.0, 2.8	2.2	1.3, 3.6	1.3	0.6, 2.7	1.9	0.27	6.7	2.7, 16.8	5.9	1.8, 19.4
	Re-use (11 & 6)	1.2	0.8, 2.0	2.5	1.7, 3.7	2.0	1.1, 3.7	1.9	0.63	4.8	2.3, 9.8	7.3	2.6, 20.4
	Single use & re-use (27 & 13)	1.5	0.8, 2.5	2.3	1.5, 3.7	1.6	0.8, 3.3	1.9	0.43	5.8	2.4, 13.6	6.6	2.2, 19.5
22	Single use (11 & 3)	1.9	1.4, 2.6	2.5	1.5, 4.1	1.3	0.7, 2.3	2.2	0.30	9.3	4.9, 17.5	8.6	3.9, 18.9
25	Single use (2 & 2)	1.5	1.0, 2.2	1.9	1.1, 3.3	1.3	0.9, 3.1	1.7	0.22	4.7	2.4, 9.5	7.1	2.1, 23.2
	Re-use (8 & 3)	1.4	1.0, 1.9	2.6	1.8, 3.8	1.9	0.7, 2.5	2.0	0.62	5.6	3.1, 9.9	11	4.2, 28.2
	Single use & re-use (10 & 5)	1.4	1.0, 2.0	2.3	1.4, 3.8	1.7	1.2, 3.2	1.8	0.46	5.3	3.0, 9.4	9.4	3.4, 25.8
28	Single use (3 & 2)	1.0	0.6, 1.8	2.0	1.3, 3.1	2.0	1.2, 4.3	1.5	0.49	5.7	1.9, 17.2	2.4	1.6, 3.6
	Re-use (3 & 3)	0.9	0.6, 1.5	2.4	1.6, 3.6	2.5	1.0, 3.8	1.7	0.72	4.1	1.8, 9.3	4.8	2.8, 8.2
	Single use & re-use (6 & 5)	1.0	0.6, 1.6	2.2	1.5, 3.4	2.3	1.4, 3.7	1.6	0.62	4.9	1.9, 12.6	3.8	1.8, 8.0

No statistically significant differences are observed between the distinct experimental days.

S6. Effect of Caco-2 monolayer re-use on P-gp expression

The effect of re-use and day post-seeding on P-gp expression of Caco-2 monolayers was accessed by flow cytometry after incubation of the cells with a FITC-conjugated antibody to human P-gp. Caco-2 cells from monolayers maintained in culture and that were not used in any permeability assays were labeled to evaluate the P-gp expression at days 22, 25 and 28. Caco-2 cells from monolayers before their re-use for permeability assays with Rho at day 25 (previously used at day 22 in Rho permeability assay) and day 28 (previously used at days 22 and 25 in Rho permeability assays) were also labeled. The green fluorescence due to FITC was recorded using a 530/30 nm band-pass filter (FL1) and plotted as a histogram of FL1 staining. Non-labeled Caco-2 cells were also analyzed for each condition to evaluate the possible contribution from cells autofluorescence to the FL1 signal (negative control sample). The histogram overlays are presented in Figure S4. The mean fluorescence intensity (MFI) was the parameter used for comparison. The P-gp expression values were calculated through the shift in the MFI of the cells in the presence of the antibody in relation to the non-labeled cells.

A small population of Caco-2 cells displayed a relatively high fluorescence intensity for the re-used cell monolayers. Since those cell monolayers were previously used in permeability assays with Rho, we pose the question if all the Rho has been removed from the cells. To answer that we have incubated Caco-2 cells in a 12 well-plate with Rho for 2h. After incubation, one batch of cells was washed with HBSS and prepared for flow cytometry. The other batch was maintained in culture and incubated with DMEM following the cell monolayer regeneration procedure proposed.

It was observed that Rho is efficiently removed from cells after washing with HBSS, the fluorescence intensity becoming essentially equal to that of control cells. No further variation was observed for cells incubated with DMEM. This suggests that the presence of the cell population with higher fluorescence observed for the re-used cell monolayers is not due to an inefficient removal of Rho. The relative abundance of this population was higher when the number of counts was smaller, suggesting that it may be due to an artifact independent on the presence of the cells.

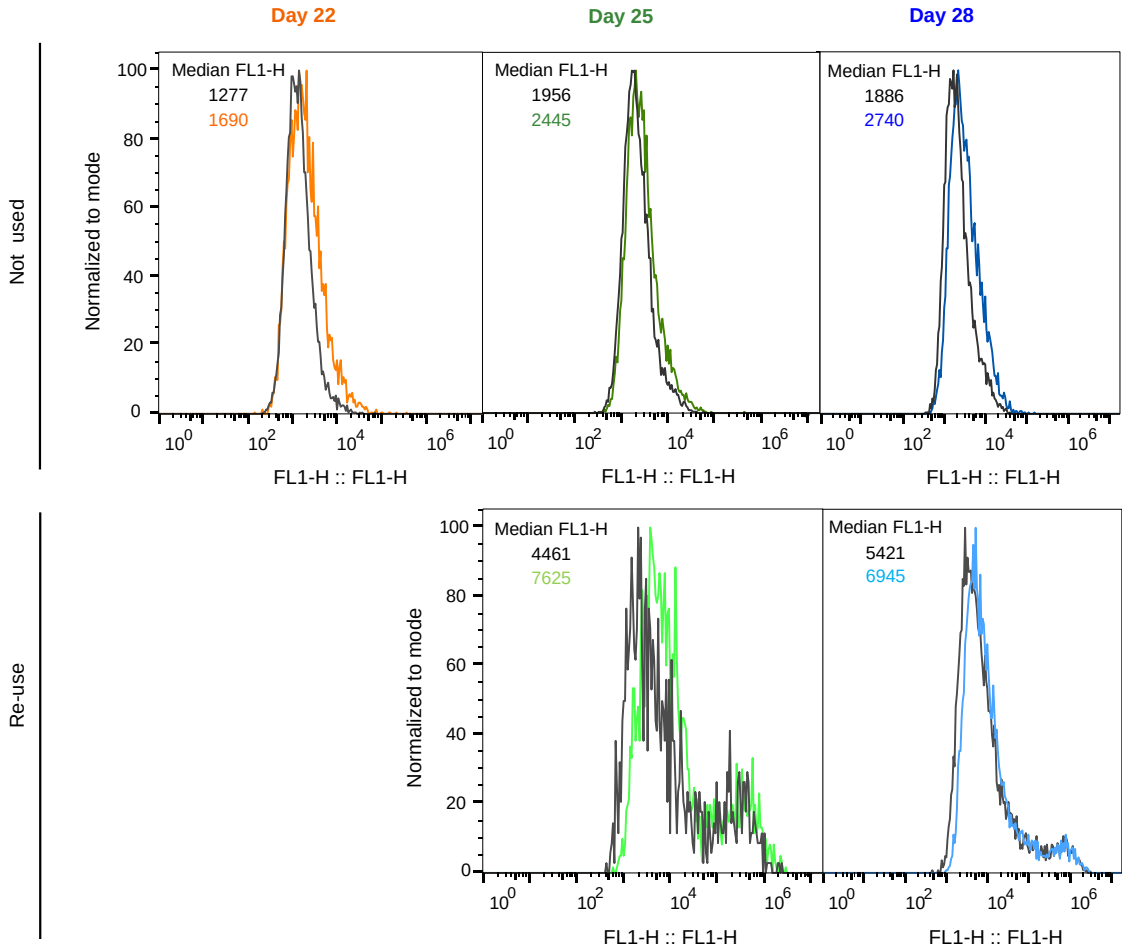


Figure S4. Representative histograms overlay of flow cytometric analysis to determine expression of P-gp in Caco-2 cells. The non-labeled Caco-2 cells (negative control) are displayed in black, and the cells incubated with P-gp antibody are displayed in colors. The expression of P-gp acquired in cells from monolayers at day 22, 25 and 28 are shown in the left, middle and right panels, respectively. The upper plots correspond to cells from monolayers that were not used in permeability assays, while the lower plots correspond to cells from monolayers before their re-use for Rho permeability assays at day 25 and 28. The number of events were normalized by mode. The median fluorescence intensity (MFI) was determined for each histogram. The experiments were performed for two independent cell monolayers and the average MFI is shown in Figure 7B of the manuscript.

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