

# MEMTRANS

Membrane transporters:  
*in vivo* models for the study  
of their role in drug fate

**memtrans**

Contract number:	LSHB-CT-2006-518246
Project type:	Specific Targeted Research Project (FP6)
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## Objectives

The MEMTRANS project focuses on refining and improving *in vitro* methodologies to reduce animal experimentation during the study of the biopharmaceutical and pharmacological properties of drugs with respect to their interaction with transporters. It could lead to the progression of better drug candidates and therefore it could contribute to making drug discovery and development more successful. The general goal of the project is to optimise and prevalidate existing *in vitro* models for the study of the impact, mechanism and regulation of drug and xenobiotic efflux transporters. Specifically, we wish to identify their influence on concentration-time profiles of xenobiotics in the circulation.

The first objective of the study is the characterisation and assessment of the functionality and biological characteristics of each *in vitro* model as well as the characterisation of the functional MDR status of the cell lines.

These studies were developed according to previously determined parameters and written common Standard Operation Procedures (SOP's) for cell culture, transport experiments and transference among laboratories using the cell lines Caco-2, MDCKII and MDCKII-MDR. This pre-validation study of the selected cell lines will enable the further evaluation of the culturing methods and permeation experiment protocols along with the interlab and intralab reproducibility of the results. The validation of the cell lines will lead to the better correlation of the results between the laboratories in the future.

## Experimental design

Name of the test method	<i>In vitro</i> model of intestinal absorption			
Clinical endpoint	Oral route of administration, drug-transporter, drug-drug and drug-food interactions.			
Cell (line)	Caco-2, MDCKII-WT, MDCKII-MDR.			
Method description	<p>Different cell cultures with varying levels of secretion carriers were tested with a group of model drugs. The assays were developed with common standard operation procedures to diminish the interlaboratory variability and to permit a better comparison of the models performance. The <i>in vitro</i> permeability values of model drugs were compared/correlated with the available literature data of pharmacokinetic parameters and bioavailability in humans to check the predictability of each culture model and to characterise the systems parameters.</p> <p>The <i>in vitro</i> permeability was determined by growing the cells monolayers in Transwell™ chambers. The solution of the drug at different concentration was added to either apical or basolateral side while free medium is added to the opposite side. The amount of drug appearing in the acceptor chamber was measured by liquid chromatography. The permeability value is obtained from the slope of the linear regression of amount of drug in acceptor chamber versus time, divided by initial concentration in donor and area of transport. The permeability values obtained at different concentrations were used to obtain Km (Michaelis-Menten constant) and Vm (Maximal velocity of transport) parameters by non-linear regression of a combined passive and Michaelis-Menten equation.</p>			
SOP	Yes.			
Endpoints	Develop reliable <i>in vitro</i> models with improved predictability of <i>in vivo</i> biopharmaceutical characteristics.			
How is a positive result defined?	Cell monolayer integrity	Electrical measurement	Trans-Epithelial Electrical resistance (TEER) measurement, depending on the filter area, can reveal toxicity or an opening of tight junctions induced by the drug.	
		Permeability measurement of test compounds	Mannitol, Lucifer Yellow.	Low paracellular permeability.
			Metoprolol.	High transcellular permeability.
	P-glycoprotein (P-gp) expression	Western blotting.		
		Rhodamine 123 permeability.		
		Calcein Assay.		

Name of the test method	<i>In vitro</i> model of intestinal absorption
How is a positive result expressed?	Absolute values.
Applicability	Compounds tested: Celiprolol, Fexofenadine, Quinidine, Loperamide, Talinolol, Saquinavir, Paclitaxel.
Positive control	Demonstration of low paracellular permeability with either <sup>14</sup> C-Mannitol or Lucifer Yellow. Demonstration of high transcellular permeability with Metoprolol or <sup>3</sup> H-Metoprolol. Demonstration of Pgp efflux using Rhodamine 123.
Negative control	Permeability value in the absence of monolayer.
Performance	Statistical differences between High-Low permeability values of High-Low permeability markers.
Can the test method be used in a regulatory safety context?	No.
Which R would the test method impact?	Reduce and Replace <i>in vivo</i> testing, Refining and improving <i>in vitro</i> methodologies.
How can the test be used?	As a part of an alternative/integrated testing strategy.

## Results

The following tables and figures show the experiments done in Caco-2, MDCKII and MDCKII-MDR cells.

## CACO-2 CELLS

### *Demonstration of low paracellular permeability*

Experiments to demonstrate the appropriateness of Caco-2 cells for the low paracellular permeability were done using <sup>14</sup>C-Mannitol in ACB and Lucifer yellow (LY) in UVEG and SOLVO. The experiments were conducted in the apical to basolateral (ab) direction and in the absence and presence of shaking (orbital shaker set at 100 r.p.m.).

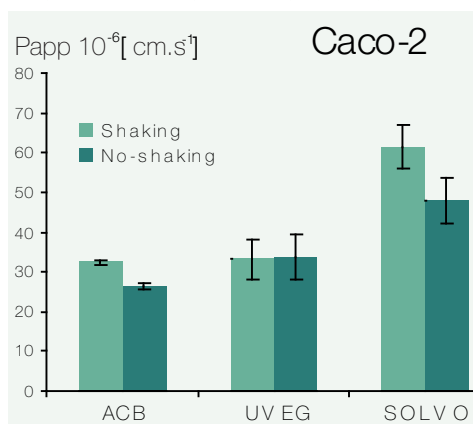
*Table 1. Comparison of the results of the demonstration of the appropriateness of monolayers for the low paracellular permeability gathered in different laboratories. SD denotes standard deviation; RSD denotes relative standard deviation.*

Caco-2 cells								
Partner	Compound	Passage Number	n	Day	Mode	Papp x 10 <sup>-6</sup> [cm/s]		
						Ab	SD	RSD%
UVEG	LY	37	6	14	shaking	0.28	0.21	62,89
		38	6	14	no-shaking	0.11	0.05	47,95
SOLVO	LY	24	3	14	shaking	0.51	0.32	62,75
		24	3	14	no-shaking	0.42	0.25	59,52
ACB	<sup>14</sup> C-Mannitol	43	3	25	shaking	1.89	0.25	13,23
		44	3	25	no shaking	1.36	0.1	7,35

### *Demonstration of high transcellular permeability with Metoprolol*

Experiments to demonstrate the appropriateness of Caco-2 cells for the high transcellular permeability were done using Metoprolol. The experiments were conducted in the apical to basolateral (ab) direction and in the absence and presence of shaking.

Figure 1. Comparison of the results of the demonstration of the appropriateness of monolayers for the high transcellular permeability gathered in different laboratories (n=3-6).



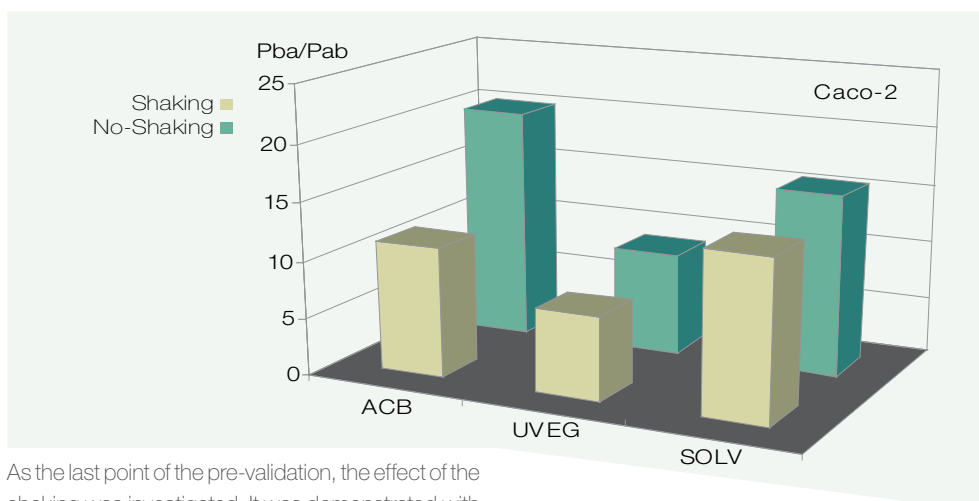
### Demonstration of Pgp efflux with Rhodamine 123

Experiments to demonstrate the presence of the Pgp efflux were done using Rhodamine 123. The experiments were conducted in the apical to basolateral (ab) direction, basolateral to apical (ba) direction and in the absence and presence of shaking.

The ability of the Caco-2 cell monolayers for the separation of low and high permeability were demonstrated using Mannitol, Lucifer yellow and Metoprolol. The pre-validation method was able to determine the Pgp expression of the Caco-2 cells. In the experiments that were conducted with a selective Pgp substrate Rhodamine 123 it was demonstrated that efflux ratios that were determined by all the partners were higher than seven in any case.

Table 2. Comparison of the results of the demonstration of Pgp efflux with Rhodamine 123 gathered in different laboratories.

Caco-2 cells										
Partner	Passage Number	n	Day	Mode	Papp x 10 <sup>-6</sup> [cm/s]					
					ab	SD	RSD%	ba	SD	RSD%
UVEG	37	3	14	shaking	1.40	0.30	21.03	10.08	2.13	21.39
	38	3	14	no-shaking	1.33	0.10	7.69	11.92	1.67	14.25
SOLVO	24	3	14	shaking	1.28	0.02	1.56	17.9	1.23	6.87
	24	3	14	no-shaking	0.93	0.23	24.73	14.6	2.1	14.38
ACB	43	3	25	shaking	0.51	0.04	7.84	5.77	0.6	10.40
	44	3	24	no shaking	0.22	0.01	4.55	4.42	0.51	11.54



As the last point of the pre-validation, the effect of the shaking was investigated. It was demonstrated with the above results that shaking can be used without any sacrifice from the monolayer properties.

Figure 2. Comparison of the efflux ratios of Rhodamine 123 in Caco-2 gathered in different laboratories.

## MDCKII and MDCKII-MDR CELLS

Pre-validation of the MDCKII and MDCKII-MDR cells were carried out by the partners UVEG and ACB. The quality control markers used and permeability experiment conditions were the same as for Caco-2 cell line validation.

Calcein AM assay was carried out by SOLVO for the determination of the Pgp activity of both cell lines.

### *Demonstration of low paracellular permeability*

*Table 3. Comparison of the results of the demonstration of the appropriateness of monolayers for the low paracellular permeability gathered in different laboratories.*

MDCKII cells								
Partner	Compound	Passage Number	n	Day	Mode	Papp x 10 <sup>-6</sup> [cm/s]		
						Ab	SD	RSD%
UVEG	LY	51	6	4	shaking	0.25	0.07	26.74
		58	6	4	no-shaking	0.21	0.05	26.63
ACB	<sup>14</sup> C-Mannitol	12	3	4	shaking	0.88	0.13	14.55
		16	3	4	no shaking	2.32	0.51	21.95

*Table 4. Comparison of the results of the demonstration of the appropriateness of monolayers for the low paracellular permeability gathered in different laboratories.*

MDCKII-MDR cells								
Partner	Compound	Passage Number	n	Day	Mode	Papp x 10 <sup>-6</sup> [cm/s]		
						Ab	SD	RSD%
UVEG	LY	56	6	3	shaking	0.32	0.04	13.21
		57	6	3	no-shaking	0.63	0.19	30.56
ACB	<sup>14</sup> C-Mannitol	12	3	4	shaking	1.19	0.12	9.94
		16	3	4	no shaking	2.28	0.23	10.15

*Demonstration of high transcellular permeability with Metoprolol*

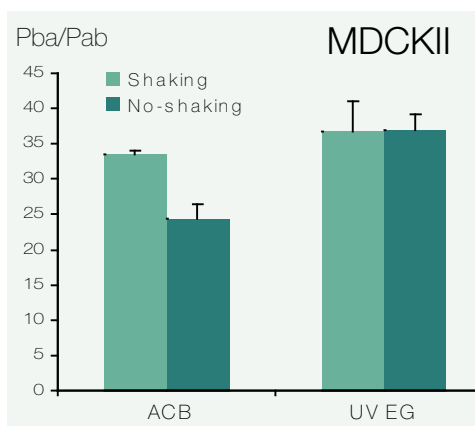


Figure 3. Comparison of the results of the demonstration of the appropriateness of monolayers for the high transcellular permeability gathered in different laboratories (n=3).

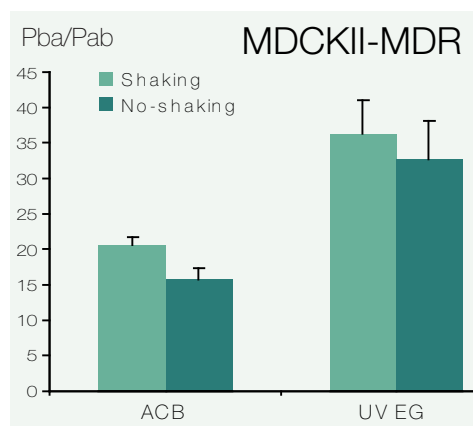


Figure 4. Comparison of the results of the demonstration of the appropriateness of monolayers for the high transcellular permeability gathered in different laboratories (n=3).

*Demonstration of Pgp efflux with Rhodamine 123*

Table 5. Comparison of the results of the demonstration of Pgp efflux with Rhodamine 123 gathered in different laboratories.

MDCKII cells										
Partner	Passage Number	n	Day	Mode	Papp x 10 <sup>-6</sup> [cm/s]					
					ab	SD	RSD%	ba	SD	RSD%
wUVEG	51	3	4	shaking	2.83	0.61	21.00	10.17	2.84	28.00
	52	3	3	no-shaking	1.41	0.27	19.00	8.19	0.49	6.00
ACB	12	3	4	shaking	0.75	0.09	12.00	3.06	0.43	14.05
	16	3	4	no shaking	0.46	0.11	23.91	1.53	0.19	12.42

Table 6. Comparison of the results of the demonstration of Pgp efflux with Rhodamine 123 gathered in different laboratories.

MDCKII-MDR cells										
Partner	Passage Number	n	Day	Mode	Papp x 10 <sup>-6</sup> [cm/s]					
					ab	SD	RSD%	ba	SD	RSD%
UVEG	56	3	4	shaking	1.88	0.36	19.00	12.22	0.88	7.00
	57	3	3	no-shaking	2.32	0.99	42.00	11.69	0.69	6.00
ACB	12	3	4	shaking	0.65	0.13	20.00	3.48	0.32	9.19
	16	3	4	no shaking	0.35	0.04	11.42	2.26	0.30	13.27

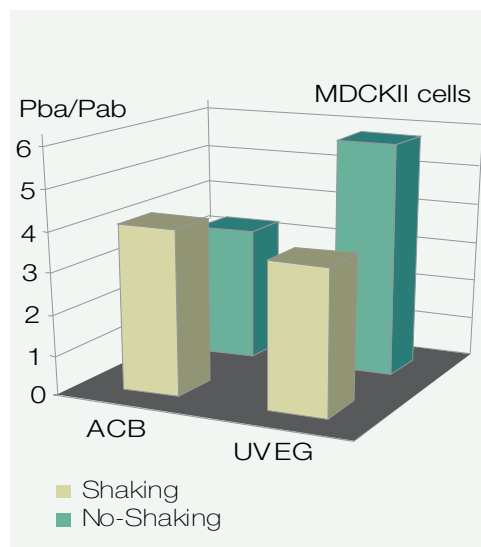


Figure 5. Comparison of the efflux ratios of Rhodamine 123 in MDCKII cells gathered in different laboratories.

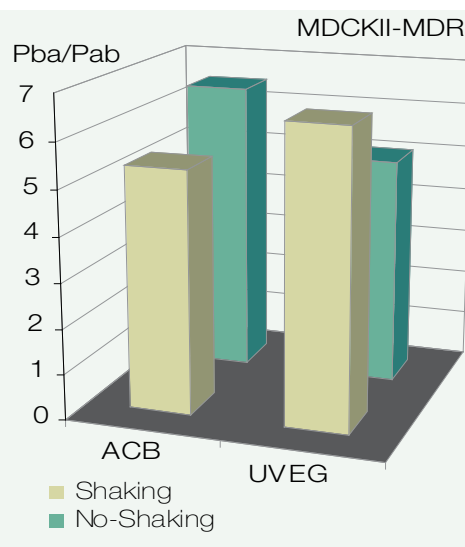


Figure 6. Comparison of the efflux ratios of Rhodamine 123 in MDCKII-MDR cells gathered in different laboratories.

## Calcein AM assay

The functions of the multidrug resistance protein (Pgp) were assessed by measuring the accumulation of a fluorescent dye, Calcein, in tumour cells e.g. by flow cytometry. The quantitative MDR1 activity factors (MAFMDR) are calculated from the Calcein AM extrusion assay by using efficient inhibitors of the multidrug resistance protein. This relatively simple and rapid *in vitro* functional assay provides a reliable quantitative measure for cellular multidrug resistance and the activity of the MDR1 protein.

Calcein AM assay was carried in the presence and absence of the inhibitors Verapamil and LY335979 to determine the different Pgp activity between MDCKII and MDCKII-MDR cells.

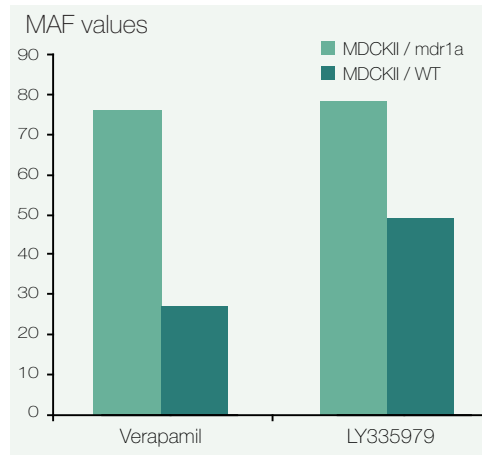


Figure 7. MAF values of the MDCKII/WT and MDCKII/mdr1a cell found using Calcein AM assay.

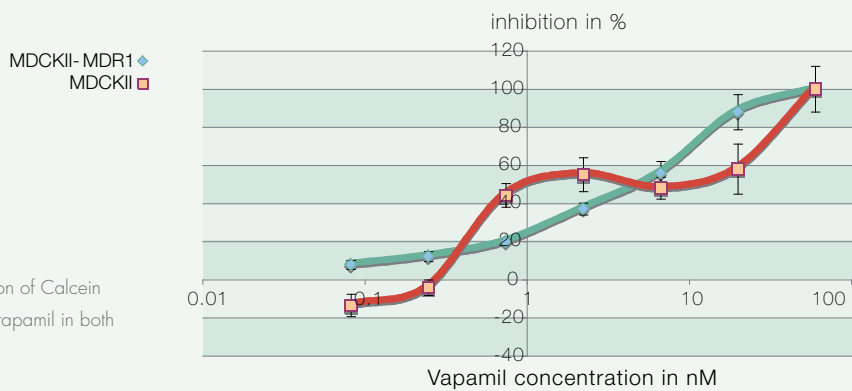


Figure 8. Inhibition of Calcein AM efflux by Verapamil in both cell types.

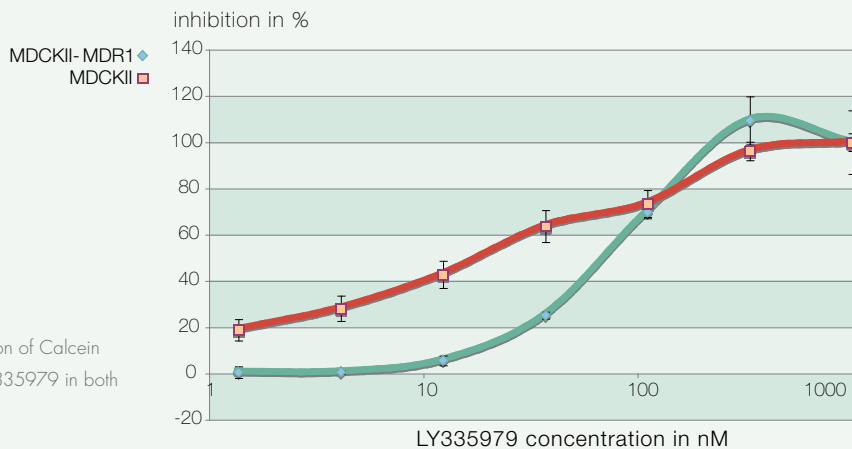


Figure 9. Inhibition of Calcein AM efflux by LY335979 in both cell types.

## Conclusion

The ability of the Caco-2 cell monolayers for the separation of low and high permeability were demonstrated using Mannitol, Lucifer yellow and Metoprolol. The pre-validation method was able to determine the Pgp expression of the Caco-2 cells. In the experiments that were conducted with a selective Pgp substrate Rhodamine 123 it was demonstrated that efflux ratios that were determined by all the partners were higher than seven in any case.

The ability of the MDCKII and MDCKII-MDR cell monolayers for the separation of low and high permeability were demonstrated using Mannitol, Lucifer yellow and Metoprolol. In the experiments conducted with Rhodamine, minor differences were observed between the MDCKII and MDCKII-MDR cells in terms of efflux ratios which is further proved with Calcein AM assay.

MDCKII cells express a transporter that transports Calcein. The transport process can be inhibited by LY335979 and Verapamil. MDCKII-MDR1 cells show higher rate of Calcein AM transport. This transport could be also inhibited by LY335979 and Verapamil, but with different inhibition profiles and IC50 values.

These results show that the MDCKII parental cell line expresses a (most probably canine) transporter that has biochemical characteristics similar to human MDR1 (Calcein AM transporter can be inhibited by LY335979 or Verapamil). The MDCKII-MDR1 cells express the human MDR1 transporter resulting in higher MAF values and different inhibition profiles and IC50 values for the two compounds.

As the last point of the pre-validation the effect of the shaking was investigated. It was demonstrated with the above results that shaking can be used without any sacrifice from the monolayer properties.

Finally, the results obtained by different laboratories were comparable with each other which will enable further correlations and the analyses of the repeatability of the further experiments using previously selected substances.

## Next steps

Permeability studies have been conducted in the three cell lines at four different concentrations and in the absence and presence of 1  $\mu$ M zosuquidar (P-gp inhibitor).

## Project ongoing tasks

From the data generated the transport parameters of each drug will be characterised in each cell line. The correlation analysis and generation of prediction models includes the analysis of the relationships between physicochemical characteristics and affinity for the carrier and the analysis of the correlation between *in vitro* and *in vivo* results. The biophysical models for drug transport (transport location, binding site, apical-basolateral resistances) will be characterised in each *in vitro* model by using a computer modelling and simulation approach. That will allow obtaining the transport parameters for the correlation with physicochemical variables which eventually could be also included as model co-variables.

## Partners

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