

# Fully automatic flow-based device for monitoring of drug permeation across a cell monolayer

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Received: 21 September 2015 / Revised: 10 November 2015 / Accepted: 12 November 2015 / Published online: 28 November 2015  
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**Abstract** A novel flow-programming setup based on the sequential injection principle is herein proposed for on-line monitoring of temporal events in cell permeation studies. The permeation unit consists of a Franz cell with its basolateral compartment mixed under mechanical agitation and thermostated at 37 °C. The apical compartment is replaced by commercially available Transwell inserts with a precultivated cell monolayer. The transport of drug substances across epithelial cells genetically modified with the P-glycoprotein membrane transporter (MDCKII–MDR1) is monitored on-line using rhodamine 123 as a fluorescent marker. The permeation kinetics of the marker is obtained in a fully automated mode by sampling minute volumes of solution from the basolateral compartment in short intervals (10 min) up to 4 h. The effect of a P-glycoprotein transporter inhibitor, verapamil as a model drug, on the efficiency of the marker transport across the cell monolayer is thoroughly investigated.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00216-015-9194-0) contains supplementary material, which is available to authorized users.

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The analytical features of the proposed flow method for cell permeation studies in real time are critically compared against conventional batch-wise procedures and microfluidic devices.

**Keywords** Fully automated system · Permeation study · P-glycoprotein transporter · Rhodamine 123 · Sequential injection analysis

## Introduction

Permeation studies [1–3] are commonly used and recommended by drug regulatory authorities [4, 5] to analyze the transport of tested substances (exogenic compounds) through live epithelial cell monolayers. In the experiment, absorption characteristics of new compounds or their interaction with transport proteins is usually investigated. Elucidation of the function of membrane transporters that might affect the transfer rate of tested substances is undertaken by means of different types of cells expressing transporters (such as CACO-2 or genetically modified MDCKII cell lines). The cell monolayers are cultivated onto plastic inserts with a given membrane type and pore size [1–3, 6]. Intrinsic fluorescent species of such transport are commonly selected as markers in as much as they could be determined with sufficient sensitivity with simple analytical instrumentation, and their behavior in case of interaction with membrane transporters is well known [1, 2, 6]. The combination of tested substances with the marker enables designing a universal system for permeation testing. Evaluation is based on quantitative differences between the transport rate of the marker itself against the marker in combination with the tested substance (potential transporter inhibitor), which could increase/decrease the permeation of the marker. In case

of P-glycoprotein (MDR1/ABCB1) efflux transporter, which is one of the most important transporter controlling trans-epithelial pharmacokinetics of many drugs in the body, rhodamine 123 has been often used as a good model substrate, and verapamil is often used as a strong inhibitor of P-glycoprotein. P-glycoprotein is localized on the apical membrane of an epithelial cell and pumps its substrates out of the cell. In case of an epithelial monolayer, P-glycoprotein restricts trans-epithelial transport in apical-to-basolateral direction; therefore, P-glycoprotein inhibitors increase transport of P-glycoprotein substrates into basolateral side of the cells.

Permeation in dedicated chambers furnished with inserts is usually assessed using end-point measurements with a duration of 4 h [7–9] or, alternatively, a maximum of three samples is taken manually from the apical (AP) and/or basolateral (BL) compartments of the chamber to get further insight into the cell monolayer permeability or for calculation of permeability coefficient or transport rate parameters. The kinetics of such transport is thus only roughly estimated and the final concentration of the marker in the compartments is mostly used for comparison and description of such permeation tests. In general, there is a quest for novel on-line monitoring schemes for real-time investigation of the interaction of target substances with membrane transporters.

Several attempts to automate cultivation steps and/or the permeation studies in bioreactor systems [10] and the design of microfluidic devices for miniaturization of permeation testing were reported over the past couple of years [11, 12]. Unfortunately, the above methods do not follow basic rules for cell-based permeation tests including the lack of temperature control under physiological conditions ( $37.0 \pm 0.5$  °C) [11] or apply continuous displacement of the recipient liquid in the basolateral compartment that is not fully comparable with standard batch-wise permeation studies. Commercially available Transwell membrane inserts have been resorted and allied to microchip systems [11] and thus permeation studies were reproduced from batch-wise standard tests with respect to the membrane material, pore size, and cell monolayer parameters including surface area for mimicking standard assays. However, without temperature control in the course of the permeation test (2–4 h) the viability of cells to keep monolayer intact (with T-junction between adjacent cells) is debatable and the results thus questionable. In our study, the necessity of keeping temperature was proven and if the cell monolayer is damaged only para-cellular transport of the tested substances through the membrane pores would be monitored. The cultivation step always includes also CO<sub>2</sub> atmosphere (5 %) but if the gas exchange is enabled (permeation unit is not completely closed) this parameter does not limit the viability of cells in permeation tests lasting 4 h.

In this work, we present a novel concept based on the second generation of flow analysis (so-called sequential

injection approach) [13] that combines flow programming with automatic sampling from the basolateral compartment in standard cell-monolayer permeation tests at preset intervals and on-line fluorometric determination of the marker (rhodamine 123) [14–19] in the presence and absence of potential membrane transporter inhibitors [20–22]. Verapamil is here used as a model inhibitor of P-glycoprotein transporter [23]. This study was extended to get detailed insight into the time response of the interaction of the P-glycoprotein transporter with inhibitor. Hence, this paper reports for the first time the development of a programmable-flow setup based on the novel generations of flow systems to bring completely new information about kinetics into the field of permeation/bioavailability and toxicological studies that is usually neglected in batch-wise conventional assays.

## Experimental

### Chemicals and material

Rhodamine 123 (Rho123), verapamil hydrochloride (verapamil), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Prague, Czech Republic) and used as received without further purification. Ultra-pure water was obtained from a Milli-Q RG unit (Merck Millipore, Prague, Czech Republic). Opti-MEM Reduced Serum Medium (Gibco) used for cell assays was purchased from Life Technologies, Prague, Czech Republic.

A Rho123 stock solution ( $2.19 \text{ mmol L}^{-1}$ ) was prepared by dissolution of 16.70 mg of Rho123 in 7.0 mL of DMSO and completed to the final volume of 20.0 mL with ultra-pure water. This solution was kept at 4 °C whenever not used. Rho123 working and standard solutions were prepared by dilution of the Rho123 stock solution in Opti-MEM Reduced Serum Medium (Gibco).

A verapamil stock solution ( $1.0 \text{ mmol L}^{-1}$ ) was prepared by dissolution of 2.60 mg of verapamil hydrochloride in ultra-pure water and completed to a final volume of 5.0 mL. A verapamil standard solution ( $50 \text{ } \mu\text{mol L}^{-1}$ ) was prepared by a dilution of the verapamil stock solution by Opti-MEM Reduced Serum Medium (Gibco).

Transwell cell culture inserts with translucent polycarbonate membrane (membrane diameter of 12 mm, pore size of  $3.0 \text{ } \mu\text{m}$ , growth surface area of  $1.12 \text{ cm}^2$ ) from Corning Incorporated, Baria s.r.o., Czech Republic, were used for cell cultivation and permeation studies.

Madin-Darby canine kidney type II live cells (MDCKII) stably transfected with human MDR1 (ABCB1) gene encoding P-glycoprotein (P-gp) under viral promoter control were used for pharmacokinetic experiments. The cell line has been kindly provided by Dr. Alfred Schinkel (The Netherlands Cancer Institute).

## Cell culture

MDCKII-MDR1 cell lines expressing P-glycoprotein transporter were cultivated in high glucose DMEM medium (Dulbecco's Modified Eagle's medium) supplemented with fetal calf serum (10 %). The cell lines were kept at 37 °C in a 5 % carbon dioxide atmosphere and 95 % relative humidity [7–9, 24] with daily cultivation medium replacement. The cells were seeded on Transwell inserts with polycarbonate membrane in 12-well plates (200,000 cells/cm<sup>2</sup>). The cultivation was started 3 d before the permeation tests were performed.

## Standard procedure for cell permeation studies

Transport studies of target species in permeation tests are commonly performed on Transwell plates using cell monolayers cultured on permeable membranes [1]. The integrity of monolayer is verified using the trans epithelial electrical resistance (TEER) value or alternatively by the <sup>3</sup>H-mannitol or Lucifer yellow test [1, 8, 9, 24]. Apical-to-basolateral, basolateral-to-apical, or bidirectional flux can be also examined.

After washing of the cells with Opti-MEM reduced serum medium/buffer (2–3 times for 5–10 min) [7, 8, 24] and pre-incubation with medium/buffer (30 min) [8, 24], tested substances are added into AP or BL chamber (donor part) and blank medium/buffer to the complementary chamber (receiver part) [1]. During experiments with transporter inhibitors, an additional step of pre-incubation with inhibitor can be added [8]. Volumes of AP and BL chambers are 0.5 and 1.5 mL, respectively, in most cases [7, 8]. Sampling during the test length is usually undertaken manually by withdrawing 100 µL of solution from AP and/or BL chambers in time intervals of 30 min. After sampling, the volume of the acceptor compartment is refilled with fresh medium/buffer to assure that cells are moisturized throughout [7, 8, 24]. The plates are incubated at 37 °C for 2–4 h [1, 8, 9, 24]. Collected samples can be analyzed using fluorescent plate readers, radioactivity detectors, or by liquid chromatographic methods [1, 2] depending on the type of marker used for evaluation. Dilutions are taken into account during data evaluation.

## Flow based apparatus

The apparatus for automation of drug permeation tests was composed of a Sequential Injection (SI) setup (MicroSIA system, FIALab Instruments, Bellevue, WA, USA), a Franz diffusion cell connected with a thermostat U1 (VEB Prüfgeräte-Werk, Medingen, Germany) and an external peristaltic pump Minipuls 3 (Gilson, USA). The configuration of the flow system is shown in the Fig. 1.

The miniaturized flow manifold consisted of a 2.5 mL piston pump, a holding coil (volume of 400 µL), and an eight-

port selection valve using fluorinated ethylene propylene (FEP) 1520 XL tubing of 0.75 mm i.d. to build flow conduits. A flow-through fluorometric detector (PMT-FL, FIALab Instruments) with UV light source (D-1000-CE; Analytical Instrument Systems, Inc., USA) was used as a detector with an integration time of 40 ms. The flow-through detection cell consisted of a quartz Hellma cuvette (model 176.751, Helma-Analytics, Müllheim, Germany) with 3 mm optical path and inner volume of 100 µL. The flow system was controlled by flow programming aided by the FIALab software for Windows 5.0 (FIALab<sup>1</sup>).

The liberation unit, so-called Franz diffusion cell (FDC), was composed of a double-wall water-jacketed vessel thermostated at 37 °C throughout the permeation experiments. The basolateral/acceptor compartment of the FDC has an inner volume of 15.0 mL with a height of 6.0 cm, width of 3.6 cm and i.d. of 2.0 cm. The conventional apical/donor FDC compartment was replaced in this work by the Transwell insert with a cell monolayer cultivated onto the membrane.

The circulation loop of the acceptor liquid (see magnified scheme in Fig. 2) was 56 cm long and was made of a 40-cm long peristaltic pump tube (1.02 mm i.d., Gilson) furnished with 16-cm long FEP 1520 XL tubes (0.75 mm i.d.) connecting the FDC inlet on one side and one of the ports of the selection valve and the FDC outlet via a common T-connector (Tee Assembly Tefzel, 0.50 mm i.d. of the inner hole; Upchurch, Germany) on the other side. The peristaltic pump was programmed to operate at 1.3 mL/min. A LabJack U12 (LabJack Corporation, Lakewood, CO, USA) was used to control the on/off actuation of the peristaltic pump. Homogenous mixing of the recipient solution was effected with an electromagnetic stirrer (KMO2 basic; IKA Labortechnik, Staufen, Germany) operating at 600 rpm.

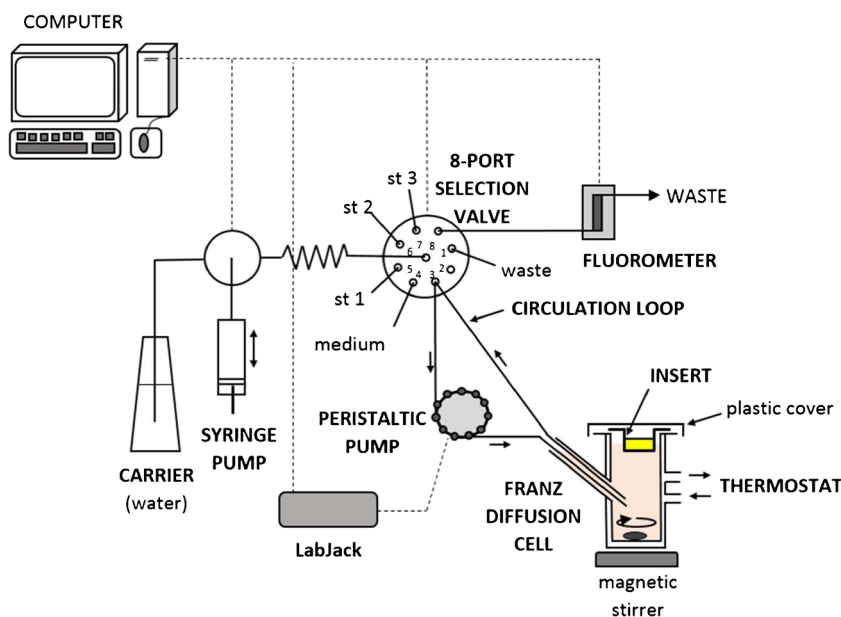
## Analytical Procedure

### *Analysis of Rho123 standards*

Working solutions of Rho123 were prepared daily from the stock solution using cell cultivation medium for dilution followed by recording of the fluorescence readout. To this end, the cleaning step of the whole flow system (aspiration of water as carrier, aspiration of standard Rho123 solution, and emptying to the waste) was performed twice. Afterwards, the measuring step, including the aspiration of the carrier (2.0 mL) followed by aspiration of the standard Rho123 solution (20 µL) from the BL compartment and its fluorometric signal detection, was performed three times using aspiration and detection flow rates of 10 and 60 µL s<sup>-1</sup>, respectively. The linear range of the calibration curve spanned from 5 to 50 µmol L<sup>-1</sup>, with correlation coefficients >0.99 under optimized conditions.

The limits of detection (LOD) and quantitation (LOQ) of Rho123 were evaluated as a ratio of 3- and 10-times,

**Fig. 1** Scheme of the programmable flow system connected to Franz diffusion cell for automated monitoring of permeation tests across a cell monolayer



respectively, of the  $s_{y/x}$  (standard error estimate) and the method sensitivity [25], and were found to be 1.0 and  $3.3 \mu\text{mol L}^{-1}$ , respectively.

The precision of the developed method was evaluated by determination of six Rho123 standard solutions in Opti-MEM medium at the concentration level of  $5 \mu\text{mol L}^{-1}$ . Each sample was measured in triplicate. This concentration level was used as a calibration point in pharmacokinetic studies. Relative standard deviation (RSD, fluorescence signal,  $n=6$ ) was 4.82 %.

The intra-day and inter-day precisions were calculated from calibration curves that were measured every day prior to the pharmacokinetic assay. Rho123 standard solutions in Opti-MEM medium were measured in triplicate at the concentration levels of 1, 5, and  $10 \mu\text{mol L}^{-1}$ , respectively. The RSD

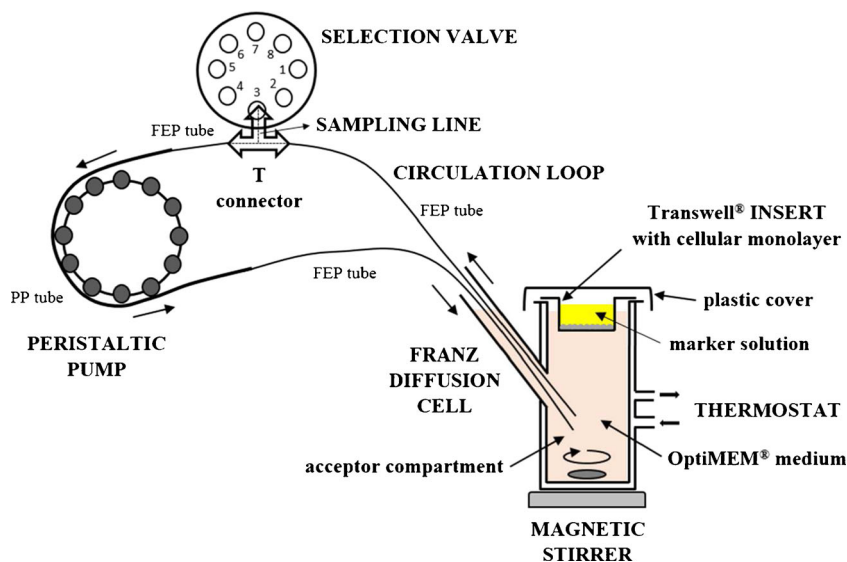
values (fluorescence signal,  $n=3$ ) did not exceed 5 % for both intra-day and inter-day precisions.

The repeatability of the Rho123 standard solutions determination was evaluated by analysis of standard solutions in Opti-MEM medium at the concentration levels of 1, 5, and  $10 \mu\text{mol L}^{-1}$ . Each sample was injected 10 times. The RSD value (fluorescence signal,  $n=10$ ) for all concentration levels did not exceed 5 %.

#### Investigation of experimental variables

Different parameters had to be explored before undertaking pharmacokinetic assays with a cell monolayer. At first the sample volume probed from the BL compartment and the effect of aspiration and detection flow rates on the

**Fig. 2** Scheme of the circulation loop integrated in the automatic flow system and Franz diffusion cell in detail





repeatability of the fluorometric signals, sensitivity of Rho123 determination, and analysis throughput were explored.

Another important parameter is the volume of liquid inside the FDC since it is crucial to keep it fixed and constant throughout the permeation test. The flow conditions in the sampling loop and peristaltic pump operation need also to be explored for ensuring thorough mixing of the sample loop content.

#### *Pharmacokinetic studies and inhibition tests*

Before initializing the automated SI-based assay, the BL compartment was filled up with 11.0 mL of the Opti-MEM medium and thermostated at 37 °C for 20 min. The circulation loop was filled with the same cultivation medium and immersed into the side inlet of the FDC (see Fig. 2). The peristaltic pump was switched on throughout the washing step to get suitable mixing inside the circulation/sampling loop (20 min before start of the permeation test). During all procedure the electromagnetic stirrer and the peristaltic pump were actuated at 600 rpm and 1.3 mL/min, respectively.

The MDCKII-MDR1 live cell monolayer was seeded directly onto the Transwell inserts in DMEM medium as described in section above. Prior to starting the test, the insert was removed from the plate hole, the medium was taken off by a pipette, and the insert was immediately placed onto the FDC. Thereafter, a metered volume of 800 µL of a test solution prepared in medium (Rho123 used as a marker with or without membrane transporter inhibitor) was pipetted into the insert (AP compartment). These steps were performed quickly to ensure that the cells do not get dry and the monolayer remains intact. Tight cover on the top of the AP compartment can cause pressure problems in the sampling and refilling steps. Therefore, the FDC with the insert was not sealed but a plastic cover was used instead to minimize the evaporation of medium and tested solution.

The measurement cycle for assessment of Rho123 permeation through the cell monolayer (Table 1) consists of the cleaning step of the sampling line by aspiration of 100 µL from the circulation loop to overcome void volume and nuisance bubble effects. Then, the aspiration of a well-defined volume of 20 µL of sample from the circulation loop into the holding coil at 10 µL s<sup>-1</sup> was performed followed by the flow reversal of the sample zone to the flow-through detector at 60 µL s<sup>-1</sup>. The final step involved the refilling of the BL compartment, which was carried out directly after each sampling step to prevent the acceptor liquid in the FDC to level off. Samples were analyzed in triplicate, which is 60 µL per cycle, taken in 10-min intervals for 2–4 hours. The total duration of the respective permeation test was chosen on the basis of the observed kinetic profile. In case of experiments without P-gp inhibition, steady-state was reached in about 2 h (12 measuring cycles for 2 h test) whereupon the permeation test

was stopped. This proves the possibility of tailoring the time-consuming permeation studies via proper assessment of assay kinetics. On the other hand, the combination of Rho123 with model inhibitor (verapamil) caused a slow increase of the fluorescence signals. The permeation test was in this case monitored for up to 4 h (24 measuring cycles) for comparison of the permeated amounts with the batch-wise test. The fluorescence peak height of Rho123 was taken as the analytical signal and a matrix match calibration with cell culture medium was used throughout the assays.

#### *Evaluation of pharmacokinetic studies*

The sampling step followed by the refilling of the FDC causes the progressive decrease of the Rho123 amount and its concentration within the FDC BL compartment in every individual analytical cycle. The measured concentration is thus diminishing and for that purpose the expected concentration at time  $t$  needs to be recalculated according to the equation below [26–28]:

$$m_{n,corrected} = m_{n,measured} + \frac{volume_{sample}}{volume_{acceptorFDC}} \times m_{n-1,measured}$$

where  $m_{n,measured}$  is the Rho123 amount (nmol) of sample  $n$  at time  $t$ . The  $volume_{sample}$  was set to 160 µL (including sampling of 20 µL in triplicate+100 µL used for the cleaning step). The  $volume_{acceptorFDC}$  was 11.0 mL. The higher volume (250 µL) sent back to the FDC in the refilling step (see below) was not considered in this recalculation because the evaporation did not cause changes in the amount of Rho123 but merely in the volume of the acceptor liquid.

## **Results and discussion**

### **Investigation of SI system variables**

For the sampling and detection steps exploiting the SI-based FDC manifold, the aspiration volume (10–50 µL), the aspiration flow rate (10, 15, and 20 µL s<sup>-1</sup>), and the flow rate of transport of the sample to the detection cell (40–60 µL s<sup>-1</sup>) were tested on the basis of our previous experiments exploiting FDC-based assays [26–28] to get rapid but sensitive determination of Rho123 within the expected concentration range using just aspiration and detection of Rho123 standards. In fact, about 1–7 % of the nominal concentration (50–200 µmol L<sup>-1</sup> Rho123) in the insert would be in our case detected in the BL compartment of the FDC (taking into account the marker dilution from 800 µL (AP compartment) to 11 mL (BL compartment), corresponding to the range spanning from 1 to 15 µmol L<sup>-1</sup>. Finally, aspiration of 20 µL of

**Table 1** Analytical procedure of pharmacokinetics measurement cycle using programmable SI flow system

Step	Syringe pump	Selection valve port	Flow rate ( $\mu\text{L s}^{-1}$ )	Operation	Description
1	Valve in	-	50	Aspirate 2000 $\mu\text{L}$	Filling the syringe with the carrier (water)
	Valve out	3	10	Aspirate 100 $\mu\text{L}$	Priming the sampling port
	Valve out	1	60	Empty	Cleaning the holding coil
2–4*	Valve in	-	50	Aspirate 2000 $\mu\text{L}$	Filling the syringe with the carrier (water)
	Valve out	3	10	Aspirate 20 $\mu\text{L}$	Aspiration of the sample (Rho123)
	Valve out	8	60	Dispense 2020 $\mu\text{L}$	Detection of Rho123
5	Valve out	4	50	Aspirate 250 $\mu\text{L}$	Aspiration of the acceptor medium
	Valve out	3	50	Dispense 250 $\mu\text{L}$	Refilling up the BL compartment
-	-	-	-	Delay 240 s	Time for recirculation of the acceptor medium

Step 1: washing, steps 2–4: sampling, step 5: re-filling; \*steps 2–4 were repeated in triplicate in each sampling cycle.

sample at  $10 \mu\text{L s}^{-1}$  was used for repeatable sampling without undue sample consumption and its detection at  $60 \mu\text{L s}^{-1}$  that allows sufficient sensitivity without prolonged analysis.

The volume of the BL compartment of FDC should be investigated for the acceptor liquid (medium) to keep the cell monolayer permanently wet but preventing flooding back into the insert. The volume of a test solution added to the insert was also explored to minimize the risk of overflow of the liquid out of the insert. The medium volume in the FDC acceptor compartment was set to 11.0 mL following experimental verification when the insert was immersed into the liquid of the FDC. The volume of a marker solution, Rho123 alone or in combination with verapamil (tested as a P-gp inhibitor) in the Opti-MEM medium, was set to 800  $\mu\text{L}$  so as to use the greatest volume in the AP compartment but without risk of overflow.

In order to maintain the volume of the BL compartment of the FDC invariable in the course of the assays, the flow system was programmed to refill the chamber with the same volume of medium (taken from the appropriate port of the selection valve) as for the sample aspiration (namely, 160  $\mu\text{L}$  per cycle). Despite these measures, the final volume of the FDC acceptor compartment after 2 h decreased significantly. Therefore, larger volumes for FDC refilling were tested (170–250  $\mu\text{L}$  per cycle) additionally while a plastic cover was used to minimize the liquid evaporation occurring at  $37^\circ\text{C}$ . The final volume of medium replacement in each run was set to 250  $\mu\text{L}$ . This is higher than the sample volume probed (160  $\mu\text{L}$ ) and the difference is caused by the slight evaporation of solution from the BL compartment and adherence of a minute volume of medium onto the tube walls of the circulation loop.

Large-bore tubing was selected for the circulation loop of the peristaltic pump (1.02 mm i.d.) because of better flow repeatability and amelioration of flow-backpressure during long-term testing. The effect of the recirculation flow rate was evaluated in the range of 1.1–2.0 mL/min, and 1.3 mL/min was found to be optimal evaluating repeatability (RSD values). The effect of stopping the peristaltic pump (PP) during the sampling step was also tested using a LabJack module

to assess potential differences in recorded fluorescence signals in terms of repeatability. The results showed that both ways are possible. The fluorescence signal was found to be even more stable with the PP turned on (see Electronic Supplementary Material (ESM) Fig. S1) compared with stopped-flow conditions because of the influence of electronic noise on the readout while activating/stopping the PP, and thus the PP was operated during the whole permeation test without deterioration of the measured signal.

Passive diffusion rates of Rho123 across the Transwell insert without seeded cell monolayer were also evaluated as a quality control tool for elucidating potential damage of the cell monolayer in the course of the pharmacokinetic tests.

### Pharmacokinetic assay

The aim of this assay was to monitor the transport of Rho123 in the absence and the presence of verapamil, a P-gp inhibitor, through MDCKII-MDR1 cell monolayer.

At first, the developed set-up was tested with respect to the increase of Rho123 in the acceptor compartment and the feasibility of monitoring changes by the increase of fluorescence signals. Additions of a 100  $\mu\text{L}$  of  $0.46 \text{ mmol L}^{-1}$  Rho123 solution in the BL compartment (without AP compartment) at intervals of 10 min were tested. The results (see ESM Fig. S2) demonstrated that monitoring of Rho123 changes is possible with delay times of less than 10 s.

The passive diffusion of Rho123 through the Transwell inserts without seeded cell monolayer was also tested. The assay was performed by adding  $0.46 \text{ mmol L}^{-1}$  Rho123 solution to the AP compartment followed by monitoring changes in BL compartment during 2 h at the temperature of  $37^\circ\text{C}$ . A fast diffusion of Rho123 across the insert was observed with steady-state concentrations in about 30 min as a result of the large pore size (3.0  $\mu\text{m}$ ) of the polycarbonate membrane (see ESM Fig. S3).

The pharmacokinetic assays with transfected MDCKII cells were performed according to the optimized procedure

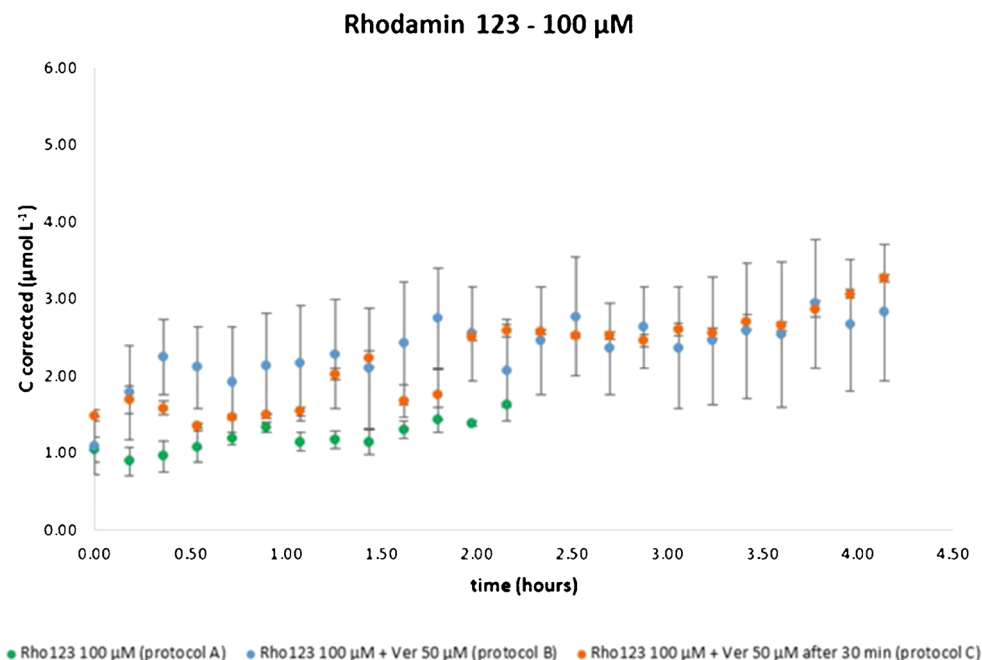
described above. The calibration curve (including three concentration levels – 1, 3, and 5 or 10  $\mu\text{mol L}^{-1}$  Rho123 solution in medium following the tested concentration of Rho123) was measured every day, directly before the permeation test. The permeation experiments were performed with different concentrations of Rho123 alone and in combination with verapamil in the AP compartment to find the appropriate concentration of Rho123 for permeation studies to clearly differentiate the kinetic profiles without and with the P-gp inhibitor.

At first, the assay with Rho123 alone (concentration of 100 and 200  $\mu\text{mol L}^{-1}$ ) for 2 h was performed (results are shown in Figs. 3 and 4 – analytical protocol A). A significant increase in fluorescence signal is not expected when cell monolayer integrity and tight junctions between cells are maintained. Results showed that there was no destruction of the cell monolayer for a 2-h experiment as

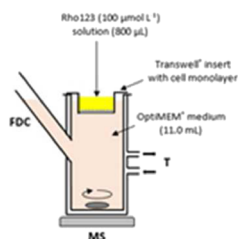
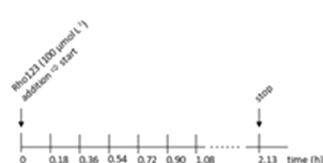
demonstrated by the stable signal recorded, corresponding to 1.5–2.2  $\mu\text{mol L}^{-1}$  concentration level in the basolateral compartment of the FDC.

Secondly, the assay with Rho123 (100 and 200  $\mu\text{mol L}^{-1}$ ) in the presence of verapamil at the 50  $\mu\text{mol L}^{-1}$  level (which is sufficient for inhibition of the P-gp transporter at the cell membrane) was performed. Under these experimental conditions, a slight increase of the fluorescence signal might be expected because of the inhibition of P-gp and, thus, easier permeation of Rho123 used as a marker should be observed. Comparison of profiles of 100  $\mu\text{mol L}^{-1}$  Rho123 alone and with 50  $\mu\text{mol L}^{-1}$  verapamil did not, however, show significant differences in fluorescence intensity and, thus, a higher concentration of Rho123 (200  $\mu\text{mol L}^{-1}$ ) and longer measurements (4 h) were undertaken (results are shown in Figs. 3 and 4 – analytical protocol B). Hence, for 200  $\mu\text{mol L}^{-1}$  Rho123,

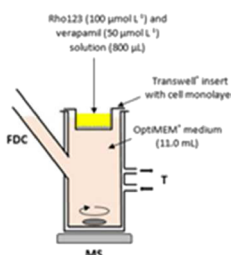
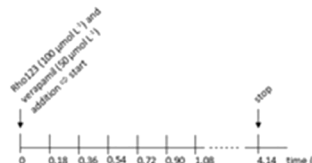
**Fig. 3** Pharmacokinetic assays with Rho123 (100  $\mu\text{mol L}^{-1}$ ) and verapamil (50  $\mu\text{mol L}^{-1}$ ) using automatic flow programming



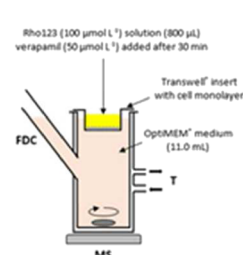
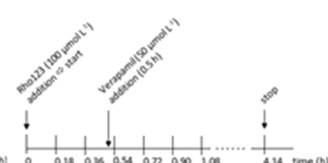
#### Protocol A



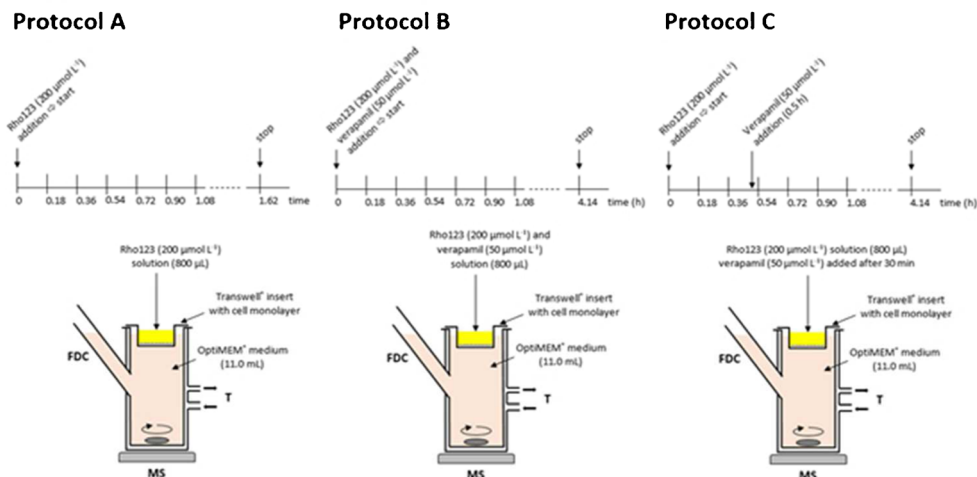
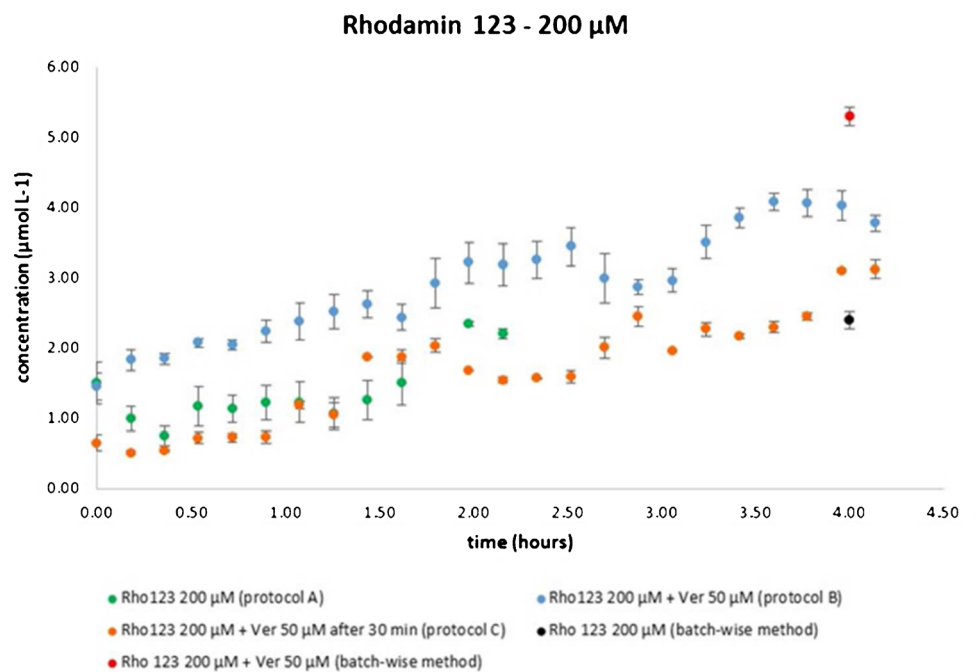
#### Protocol B



#### Protocol C



**Fig. 4** Pharmacokinetic assays with Rho123 ( $200 \mu\text{mol L}^{-1}$ ) and verapamil ( $50 \mu\text{mol L}^{-1}$ ) using automatic flow programming



different profiles in the presence and absence of the model inhibitor were obtained.

The third type of assay was performed with Rho123 (concentration of  $100$  and  $200 \mu\text{mol L}^{-1}$ ) with a delay of 30 min for addition of verapamil at the  $50 \mu\text{mol L}^{-1}$  level, in order to elucidate if the introduction of verapamil could be evaluated with respect to the kinetics of verapamil binding to P-gp transporter in the cell membrane (measurement of Rho123 permeation was carried out from the beginning of the test). Experimental results are shown in Figs. 3 and 4 – analytical protocol C. The inhibition effect of verapamil upon its addition into AP compartment was proven to occur after 30 min or 60 min, respectively, depending on the analytical protocol (simultaneous introduction of Rho123 and verapamil or with a delayed addition of a small amount verapamil and thorough mixing directly onto the insert). Differences between verapamil

inhibition effects are most likely explained by the previous binding of Rho123 to the transporter in case of delayed introduction of verapamil while simultaneous introduction of marker and inhibitor in the AP compartment causes direct competition between both substances. In fact, the rate of Rho123 passage to the acceptor chamber is higher ( $0.734 \pm 0.040 \mu\text{M h}^{-1}$ ) when P-gp inhibitor is added at the same time, compared with its delayed addition (rate of  $0.534 \pm 0.036 \mu\text{M h}^{-1}$ ).

ANOVA results for the first 10 samples (kinetic measurements) showed statistically significant differences (at the 95 % probability level) for protocols A/B and A/C for  $100 \mu\text{mol L}^{-1}$  Rho123 in combination with  $50 \mu\text{mol L}^{-1}$  verapamil. In case of comparison of protocols B/C, the differences were proven not to be significant and, thus, the test was repeated with a higher Rho123 concentration.



Using  $200 \mu\text{mol L}^{-1}$  Rho123 in combination with  $50 \mu\text{mol L}^{-1}$  verapamil statistically significant differences for protocols A/B and B/C were clearly observed. Difference was, however, negligible comparing protocols A/C for the first 10 samples because this observation was influenced by the lack of verapamil (which was added before fourth point in protocol C).

The experimental results proved that the first 10 measurements of the permeation profile could be used for evaluation of inhibition effects in case of application of the marker (or drug) and potential inhibitor at the same time (from the beginning of the permeation test). The delayed addition of inhibitor/test solution is also a good strategy to evaluate the quality of cell monolayer because the initial profile for Rho123 can be ascertained, making sure that cells are confluent and suitable for further testing. It is also clear that our novel approach can detect P-glycoprotein inhibitory effect from the early phase of the experiments. Conversely, in traditional batch-wise setup with one or two sampling points, experiments should be performed for a longer time, and no on-line monitoring of cell viability and integrity of monolayer is possible (Fig. 4 – black circle and rectangle).

### Comparison with microfluidic devices and other flow systems

Previously described microfluidic systems [10–12] were based on the same approach herein described with the combination of commercially available Transwell inserts and flow systems. The main aim was to cultivate cells in the form of monolayer [10] and then carry out the permeation test using slightly different conditions, e.g., dynamic permeation with continuous flow and renewal of the acceptor compartment followed by manual sampling from the insert after 1 h of the permeation test (with off-line LC/MS/MS analysis) [11]. Using this setup, direct comparison with batch-wise assays was not straightforward and the developed system was intended only for specific academic studies. Additionally, the microfluidic system did not keep in view the needed temperature control to maintain cells alive through the whole permeation test.

Another microfluidic device [12] integrated cell culture arrays and cell-based cytotoxicity assays with cells of different lines being exposed to the tested substance (pyocyanine) for 6 h. Cell imaging using fluorescence microscope was used for evaluation of the interaction with six different concentration levels of the tested substance. The same system was applied for evaluation of drugs that were sequentially introduced in the microfluidic system, again at different concentration levels. In this case, paclitaxel and aspirin were tested. The main shortcoming of this fluidic arrangement was the fabrication of a rather complex multi-layer microfluidic platform with diverting valves that is not readily available to interested users.

The similarity was found in previously published work based on automated monitoring dissolution and permeation of propranolol using the SIA system [29], where the Transwell inserts with the monolayer of Caco2 cells were used for the permeation step while the sampling from apical and basolateral compartments was accomplished. The permeation study was carried out in Krebs-Ringer buffer but the temperature and  $\text{CO}_2$  atmosphere were used only for cell cultivation and equilibration steps. The main advantage of the proposed flow system was sampling of small volumes from different sampling points and on-line fluorometric detection of propranolol. Thus, the kinetic profiles of dissolution and permeation were obtained in the same flow system.

Compared with previously published articles, the proposed fully automated flow system capitalizing upon SI-based flow programming allows practitioners to mimic batch-wise assays while keeping identical experimental conditions (in terms of temperature control that was found to be crucial to keep intact cell monolayer) but provides insightful details of permeation kinetics. The detailed pharmacokinetic profiles of permeation tests obtained in SI-based automated monitoring increase the level of understanding of the mechanisms of activation/inhibition of membrane transporters in real time. More importantly, the experimental results demonstrated that significant shortening of the permeation tests is possible by evaluation of initial samples taken in short time periods (in this case 10 measurements obtained in 10-min intervals each). Compared with dynamic studies, there is a problem to evaluate results with respect to common batch permeation studies and, thus, the authors prefer to keep the same conditions.

### Conclusion

A fully automated system for drug transport monitoring is herein described by smart combination of SI-flow programming with on-line FDC based measurements under temperature control that is needed to assure viability of cells and compactness of the cell monolayer. This combination allows the automated sampling and re-filing of the FDC basolateral compartment and fluorometric monitoring of permeated substances at real time with high sampling frequency, sampling repeatability and perfectly controlled experimental conditions exploiting user-friendly software control. In the present configuration, a single permeation test is monitored at a time whereby sampling frequency is not comparable to parallel batch-wise permeation tests. Further research work is underway in our laboratory to set-up multiple real-time monitoring of parallel permeation tests.

Experimental results herein reported corresponded well with the expected behavior of Rho123 transport as a marker of the P-gp function in conventional assays (Fig. 4) [30].

As a result of the high sampling frequency the kinetic profiles are in our system more detailed than those potentially available by end-point measurements. The sampling period of 10 min with triplicate sample measurements corresponds to modern demands of pharmacokinetic studies for which the kinetic profile could bring additional valuable information for fast identification of the interaction of drugs with membrane transporters so as to assess inhibition or activation effects.

**Acknowledgments** The authors gratefully acknowledge the financial support of the Ministry of Education of the Czech Republic, project no. SVV 260184, Grant Agency of the Charles University, project no. 159415, and the Grant Agency of the Czech Republic, project no. 15-10781S/P206. This work received financial support from the European Union (FEDER funds through COMPETE) and Portuguese National Funds (FCT, Fundação para a Ciência e Tecnologia) through project UID/Multi/04378/2013. M.M. is grateful to the Spanish Ministry of Science and Competitiveness for financial support through projects CTM2014-56628-C3-3-R and CTM2014-61553-EXP. Special thanks to Mgr. Lucie Hyršová for cell cultivation and kinetic profiles evaluation.

#### Compliance with ethical standard

**Conflict of interest** The authors declare no conflict of interest.

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