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Research Paper

Novel *in vitro* transport method for screening the reversibility of P-glycoprotein inhibitors

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ABSTRACT

The purpose of this study was to establish a novel *in vitro* method for screening reversibility of P-glycoprotein (P-gp) inhibitors. Caco-2 cells with 21 days of cultivation were used as an *in vitro* model. Transport of rhodamine 123 in the presence of various inhibitors and after removing of inhibitors was determined. Transport of rhodamine 123 at 4 °C and in the secretory direction assured that Caco-2 cells exhibited P-gp function at all time of experiment. The apparent permeability coefficient (P_{app}) of rhodamine 123 in the presence of verapamil, cyclosporin A, ritonavir, quinidine, N-ethylmaleimide, Cremophor® EL, Tween 80 and poly(acrylic acid)-cysteine-2-mercaptonicotinic acid (PAA-cys-2MNA) was 2.3-, 3.8-, 2.3-, 3.1, 7.5-, 2.1-, 2.9- and 2.5-fold higher than P_{app} of rhodamine 123 alone. After removing of the inhibitors, P_{app} decreased to the same range of control except in the case of N-ethylmaleimide which was 2.4-fold higher than the control. These results revealed a reversible inhibition of verapamil, cyclosporin A, ritonavir, quinidine, Cremophor® EL, Tween 80 and PAA-cys-2MNA and an irreversible inhibition of N-ethylmaleimide for P-gp. Thus, this novel established that *in vitro* method might be an effective tool for screening the reversibility of inhibition of P-gp inhibitors.

1. Introduction

P-glycoprotein (P-gp) is a 170 kDa protein expressed on the surface of many cell types, including cancer cells [1]. P-gp is expressed at low levels in most tissues, but is found in higher amount at the apical surface of epithelial cells lining the small intestine, colon, pancreatic ductules, bile ductules, kidney proximal tubules and adrenal glands [2]. The presence of P-gp on the surface of those excretory functioned tissues is likely to protect the entering of toxic compounds into the organs and systemic circulation. However, the presence of P-gp especially on apical surface of the intestine prevents the accumulation of substrates in cells by limiting absorption in cells and increasing efflux out of cells resulting in substrates remain in the intestinal lumen [3,4].

Large varieties of compounds have been identified as P-gp substrates although representing unrelated chemical structures. However, common chemical structures of P-gp substrates are amphipathic and relatively hydrophobic [2]. In addition, molecules bearing two or three electron donor units arranged in a fixed spatial separation are predicted to be P-gp substrates and the binding affinity is predicted to increase with hydrogen bond strength [5].

Co-administration of P-gp substrate with inhibitor can overcome the efflux of substrate out of the cells, resulting in intended therapeutic benefits of the molecules. P-gp inhibitors have been classified into three generations. The first generation inhibitors are identified among therapeutics molecules such as verapamil, trifluoperazine and doxorubicin [6]. However, the co-administration of first generation P-gp inhibitor with substrate is limited due to undesired pharmacological effect of the inhibitor [7,8]. Thus the later generations of P-gp inhibitors have been investigated with absent or less of pharmacological effects and higher efficacy than the previous generation.

Reversibility of an inhibition mechanism is crucial for further development of P-gp inhibitors. The use of irreversible inhibitors may result in a permanent inhibition of P-gp transporters. Irreversible inhibition may lead to the loss of the protective function to organs. Thus it is of interest to develop a valid *in vitro* transport method for screening the reversibility of inhibitors.

P-gp function can be inhibited biochemically or physically. The mechanism of biochemical P-gp inhibition is assumed to involve modulation of the membrane transporter by interacting with the binding site or interfering with the source energy of P-gp, ATP [9]. In addition, physical perturbation of cell membrane fluidity also results in the inhibition of P-glycoprotein [10,11]. Thus,

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reversibility of P-gp inhibitors that inhibit P-gp via various mechanisms was investigated.

In this study, an *in vitro* transport method for screening the reversibility of P-gp inhibitors was established. Human Caucasian colon adenocarcinoma (Caco-2) cell line with 21 days of cultivation was used as an *in vitro* model. In addition, a comparison effect of conventional pre-treatment and co-treatment with P-gp inhibitor on rhodamine 123 accumulation in Caco-2 cells was used to ensure validity of the newly established method. Well-known reversible, competitive P-gp inhibitors including verapamil, cyclosporin A, ritonavir and quinidine were used in this study. An irreversible P-gp inhibitor N-ethylmaleimide that permanently inhibits ATPase activity was also evaluated. In addition, surface active agents that interfere with cell membrane fluidity, and thus physically inhibit P-gp and a newly synthesized preactivated thiomer were also used to prove validity of the novel test system. Rhodamine 123 was used as P-gp substrate.

2. Materials and methods

2.1. Materials

Verapamil hydrochloride, rhodamine 123, quinidine, cyclosporin A, Cremophor® EL, Tween 80, resazurin reagent, N-ethylmaleimide, Hanks' Balanced salt solution (HBSS), HEPES and Triton™ X-100 were purchased from Sigma–Aldrich. Ritonavir was obtained from Chemos GmbH, Germany. Poly(acrylic acid)-cysteine-2-mercaptonicotinic acid (PAA-cys-2MNA) with the polymer backbone exhibiting a molecular mass of 250 kDa was synthesized and characterized as described previously [12]. All cell culture media and supplements were purchased from Biochrom AG, Germany. Multiwell plates, transwell inserts and tissue culture flasks were purchased from Greiner bio-one, Austria. Caco-2 cells being intestinal permeability characteristics tested were obtained from European Collection of Cell Culture (ECACC), England.

2.2. Methods

2.2.1. Cell culture

Caco-2 cells passage 50–70 were used in this study. Cells were cultivated in a humidified incubator with 5% CO_2 at 37 °C. Cells were grown in the minimal essential medium (MEM) Earle's with 2.2 g/l $NaHCO_3$ and stable glutamine supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. Caco-2 cells were subcultured at 70–80% confluence and seeded to the plate.

2.2.2. Cytotoxicity study

Caco-2 cells were seeded into 24-well culture plates at a concentration of 2.5×10^4 cells/well. Cells were cultivated for 21 days with the medium changed every other day. The complete medium was changed 12–24 h before the experiment. Resazurin assay was performed on Caco-2 cells to determine the cytotoxicity of the test substances during the period of the experiment. Briefly, on the experiment day, cultivated cells were washed twice with pre-warmed HBSS/HEPES. Test solutions of verapamil (100 μM), cyclosporin A (5 μM), ritonavir (20 μM), quinidine (100 μM), N-ethylmaleimide (50 μM), Cremophor® EL (0.05% w/v), Tween 80 (0.1% w/v), PAA-cys-2MNA (0.5% w/v), negative control (serum-free MEM without phenol red) and positive control (0.1% v/v Triton™ X-100) were added to the cell culture, 500 μl each well. The cells were incubated for 4 h in a humidified incubator with 5% CO_2 at 37 °C. At the end of incubation time, test solutions were removed. Cells were washed with PBS. An aliquot of 250 μl resazurin solution was added to each well and cells were further incubated at 37 °C with 5% CO_2 for 3 h. The fluorescent intensity of the supernatant was measured at excitation/emission wavelength of 540/590 nm (Tecan infinite M200, Grödig, Austria). Cell viability was calculated according to the following equation:

$$\text{Cell viability } (\%) = \frac{A_S}{A_C} \times 100$$

where A_S = fluorescent intensity of sample, A_C = fluorescent intensity of negative control.

2.2.3. Reversibility studies

2.2.3.1. Uptake studies. In uptake studies, Caco-2 cells were seeded into 24-well plates as described in Section 2.2.2. Cells were cultivated for 21 days with the medium changed every other day. On the experiment day, Caco-2 cells monolayers were washed with pre-warmed HBSS/HEPES. Cells were pre-treated for 30 min at 37 °C with 500 μl of either HBSS/HEPES or HBSS/HEPES with P-gp inhibitor (verapamil, cyclosporin A, ritonavir, quinidine, Cremophor® EL, Tween 80, N-ethylmaleimide or PAA-cys-2MNA). At the end of incubation time, all media were removed. Rhodamine (0.001% w/v) 123 solution with or without P-gp inhibitor was added in the respective wells. Cells were further incubated for 120 min at 37 °C. Then, all media were removed and cells were washed with ice-cold HBSS/HEPES. Triton™ X-100 (0.1% v/v) in HBSS/HEPES was used to lyse cells. After lysing, cell suspensions were centrifuged. The amount of rhodamine 123 in supernatant was quantified by fluorometric method at excitation/emission wavelength of 485/535 nm.

2.2.3.2. Transport studies. Caco-2 cells were seeded into 12-well transwell plates at a concentration of 6×10^4 cells/cm^2 and cultivated for 21 days. The volume of complete medium in the insert and in the receiving chamber was 500 and 1200 μl, respectively. To verify integrity of cell monolayers, transepithelial electric resistance (TEER) was measured in each well before and after the experiment. Caco-2 cells with TEER values over 500 Ω cm^2 were used for the experiment.

For transport experiment, culturing medium was changed 12–24 h before the experiment. On the experiment day, Caco-2 cells were washed with pre-warmed transport medium, HBSS/HEPES (pH 7.4), in both donor and receiving chambers. Then, cells were pre-incubated with transport medium with or without P-gp inhibitor (100 μM verapamil, 5 μM cyclosporin A, 20 μM ritonavir, 100 μM quinidine, 50 μM N-ethylmaleimide, 0.05% w/v Cremophor, 0.1% w/v Tween 80 or 0.5% w/v PAA-cys-2MNA). After 30 min of pre-incubation time, transport medium was removed entirely. Solution of rhodamine 123 (0.001%) with or without inhibitor was added to donor chambers (500 μl) in each respective well. HBSS/HEPES (1200 μl) was added to the receiving chambers. Caco-2 cells were further incubated for 120 min. The sampling was done every 20 min by taking 200 μl of the solution from each receiving chamber. HBSS/HEPES was added to the receiving chamber in the same amount after each sampling.

For the reversibility test, after 120 min of experiment Caco-2 cells were washed and further incubated with HBSS/HEPES for 30 min. Then, transport of rhodamine 123 without inhibitor was carried out via the same process as described above.

P-gp function of Caco-2 cells used in the experiment was also verified. The transport experiment of rhodamine 123 in both absorptive (apical to basolateral, A-to-B) and secretory (B-to-A) directions was carried out. In addition, permeated amount of rhodamine 123 at experiment temperature of 4 °C and 37 °C was compared.

The apparent permeability coefficient (P_{app}) of rhodamine 123 was calculated by the following equation: