



## Effects of Long-term Drug Exposure on P-glycoprotein Function and Expression

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### Introduction

P-glycoprotein (P-gp) is a major player in the multidrug resistance in cancer cells limiting the effectiveness of chemotherapy in cancer, and also related to the drug excretion in the normal cells, particularly intestine, liver, brain, and other epithelial tissues, influencing the pharmacokinetic profile of a vast number of clinically prescribed drugs and contributing to significant drug interactions.<sup>1,2</sup>

Chronic noncommunicable diseases (NCDs)—mainly cardiovascular disease, cancer, chronic respiratory diseases, and diabetes are increasing dramatically in developing countries and have been a burden for premature deaths and disabilities worldwide.<sup>3</sup> Cardiovascular diseases account for most NCD deaths globally, which high blood pressure and high cholesterol are two major risk factors contributing to the development of cardiovascular disorder.<sup>4</sup> Usually, preventions and treatments of NCDs involve the uses of specific drug therapy over a long-term period. The NCD patient receiving multiple medications may take a high risk of multi-drug interaction. The uses of thiazides, beta blockers and statins have proven cost-effectiveness to reduce cardiovascular risks in high risk patients. Propranolol is a beta blocker used to treat high blood pressure and various types of irregular heart rate. The study performed in LS180 colonic epithelial cells exhibited that propranolol (50-300  $\mu$ M) induced MDR1 mRNA and P-gp protein expression, as well as transporter activity.<sup>5</sup> Simvastatin is a lipid-lowering medication used to treat high cholesterol and reduce risk of heart disease. The investigation in a murine monocytic leukemia cell line that over-expresses P-gp showed that simvastatin inhibited transport of R123, a P-gp substrate, in a concentration-dependent manner.<sup>6</sup> Simvastatin (0.1-30  $\mu$ M) exhibited a strong inductive effect on the mRNA expression of MDR1 in LS180 cells.<sup>7</sup> On the contrary, the study in HEK293 cells revealed that simvastatin reduced P-gp expression.<sup>8</sup> However, the concentration used in these previous studies were higher than the drug concentration detected in the human plasma.

This study was carried out to investigate the effects of the drug on P-gp function and expression in Caco-2 when these cells were persistently exposed to the steady-state plasma concentration of the drug. Propranolol and simvastatin were used as the model drugs in this investigation.

### Methods

Caco-2 cell line obtained from ATCC (sale order no. S0249019) was routinely maintained in culture medium (DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids, 1% penicillin-streptomycin, and 2 mM glutamine) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Wild type cells (passage no. 39-63) were used throughout the study. The cells grown in the culture medium supplemented with 10 nM vinblastine were used as a positive control for P-gp expression. For experiments, Caco-2 cells were continuously grown in culture medium containing 0.07-7  $\mu$ M DL-propranolol hydrochloride (Sigma-Aldrich, lot no. 97H0311) or 0.2-20 nM simvastatin (Nangjing Health Herb Bio-tech, lot No. 15020014) for several passages as stated. Cells grown in culture medium containing 0.1% DMSO was used as the control. The toxicity of the drug to Caco-2 cells was tested by trypan blue dye exclusion method and WST-1 assay (Roche). The final concentration of DMSO used to dissolve each drug in culture medium was < 0.1%.

For calcein-AM (CAM) uptake assay, the cells (80,000 cells/cm<sup>2</sup>) were seeded onto 24-well plates and cultured for 21 days. The cells were preincubated in Hank's Balanced Salt Solution (HBSS, Invitrogen) for 30 min. To initiate the experiment, 1  $\mu$ M final concentration of CAM (Sigma-Aldrich) was added to the cells and incubated at 37°C for 30 min. The reaction was stopped by adding ice-cold HBSS. The cells were carefully washed 3 times and then lysed with 0.1% Triton X-100. The amount of the fluorescent calcein, product of CAM after the cleavage by cellular esterases, was determined by reading the fluorescence intensity on Packard Fusion® Universal Microplate Reader at the excitation and emission wavelength of 485 and 535 nm,

respectively. The protein content was analyzed using Qubit® protein assay kit (Thermo Fisher Scientific). One hundred  $\mu\text{M}$  verapamil was used a positive control for P-gp function. The uptake results were calculated from the content of intracellular calcein per mg protein of treatment groups compared to that of the control group.

For extracellular P-gp staining, Caco-2 cells were gently removed from a T25 flask with 0.25% trypsin/1 mM EDTA solution, and then washed twice with ice-cold 1% FBS in phosphate buffer saline (PBS). Notably, the cells were not permeabilized to prevent intracellular staining of P-gp. The cells ( $10^6$  cells/100  $\mu\text{l}$ ) were incubated with 10  $\mu\text{l}$  FITC-conjugated P-gp antibody (Abcam, lot no. GR9207-24) as described in the company instruction manual. After 30-min incubation on ice with light protection, the ice-cold 1% FBS in PBS was added. The cells were centrifuged and washed twice with ice-cold 1% FBS in PBS, and then resuspended in ice-cold 1% FBS in PBS for flow cytometric analysis. FITC-conjugated P-gp were detected with the flow cytometer (Cytoflex, Beckman Coulter), and 10,000 events were acquired for every sample. Unstained cells and/or cells mapped with mouse IgG2a were used as negative controls to correct for background.

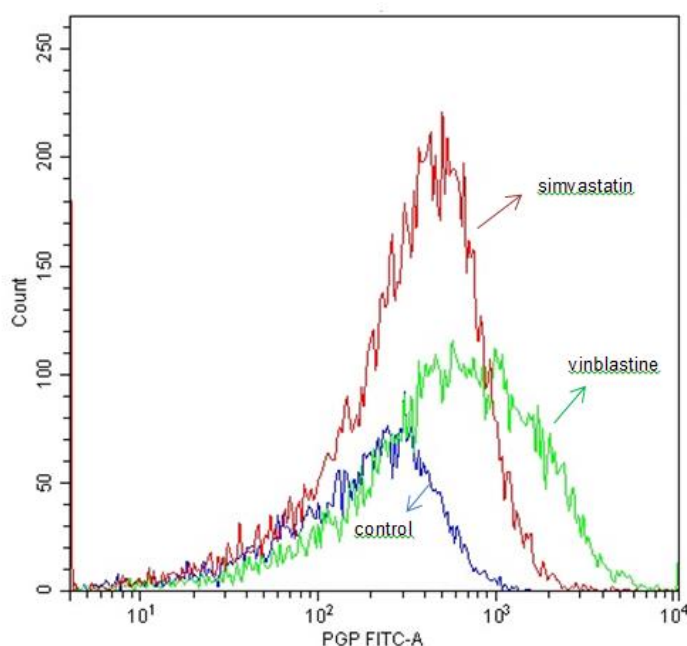
## Results

The study was performed in Caco-2 cell line which is derived from human colorectal carcinoma and the expression of P-gp has been demonstrated.<sup>9</sup> To verify P-gp function of cells grown in the laboratory, CAM uptake assays in the absence and presence of a P-gp inhibitor verapamil were investigated. To validate the expression of P-gp, vinblastine is used for inducing P-gp expression.<sup>10</sup> Table 1 shows that CAM uptakes in the presence of verapamil both in wild type and vinblastine-induced cells respectively increased about 20% and 60%, indicating that P-gp functions in these cell lines still prevailed. The lower values of CAM uptake in vinblastine-induced Caco-2 cells compared with those of wild type cells suggest the higher P-gp expression in vinblastine-induced cells, demonstrating that the expression of P-gp could be modulated. Higher P-gp expression in vinblastine-induced cells was also confirmed by flow cytometry analysis (Figure 1). These data substantiated the suitability of the culture system for further investigation.

**Table 1** CAM uptake assay performed in wild type and vinblastine-induced Caco-2 cells in the absence and presence of verapamil (mean $\pm$ S.D.)

Treatment	CAM uptake (% of control) <sup>a</sup>
Wild type Caco-2	
(+) verapamil	121 $\pm$ 10
Vinblastine-induced Caco-2	
(-) verapamil	80 $\pm$ 15
(+) verapamil	143 $\pm$ 9

<sup>a</sup>Compared to the CAM uptake in the absence of verapamil ( $= 19.8 \pm 3.2$  RFU/mg protein)

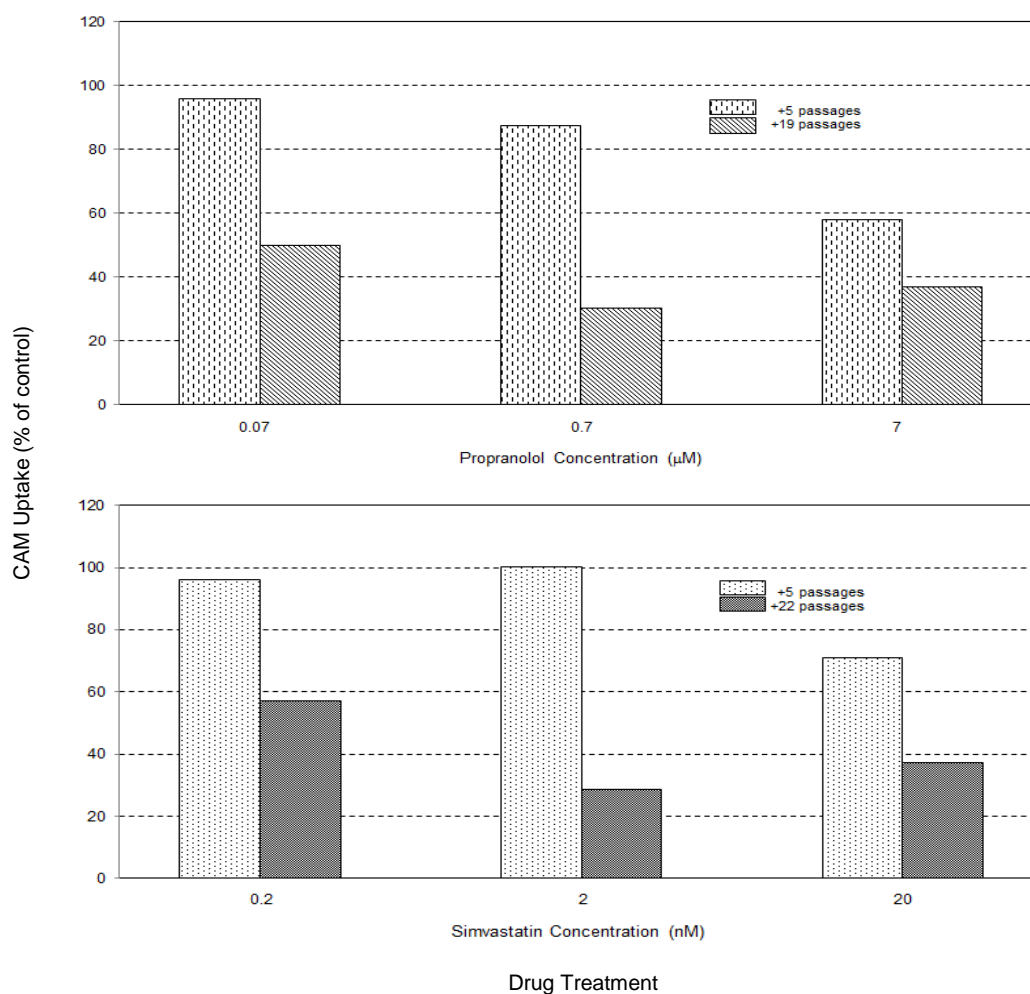


**Figure 1** Flow cytometry analysis of P-gp expression in Caco-2 cells exposed to 10 nM vinblastine for 14 passages and to 20 nM simvastatin for 24 passages. Right-shift histogram represents a higher protein expression.

To study the effects of long-term drug therapy on function and expression of P-gp, Caco-2 cells were continuously exposed to the various concentration of the selected drug for several passages as stated. The toxicity of the drug to Caco-2 cells was evaluated by trypan blue dye exclusion method and WST-1 assay. The

results from both toxicity assays revealed that all concentration used for both drugs did not significantly affected the viability of Caco-2 cells (81-128% of control).

Figure 2 demonstrates CAM uptakes in cells treated with the drug whose 3 different concentrations were applied compared to the uptake in cells exposed to 0.1% DMSO (vehicle control). As can be seen, CAM uptake was obviously lowered for cells continuously treated with propranolol (0.07-7  $\mu\text{M}$ ) in a concentration-dependent manner. The effects of long-term exposure to simvastatin are illustrated in the lower panel. As shown, Caco-2 cells exposed to simvastatin (0.2-20 nM) apparently exhibited less CAM uptakes than those of the control when cells were treated for 22 passages. Longer exposure of cells to the drug demonstrated lower CAM uptakes into the cells. Flow cytometry measurement demonstrate the right shift of the histogram (Figure 1), indicating that simvastatin could increase the P-gp expression while propranolol did not change the histogram.



**Figure 2** P-gp activity evaluated through CAM uptake in Caco-2 cells exposed to the drug for a number of passages as indicated in the legend.

## Discussion

NCDs are recently one of the major concerned health problems in most people. These chronic diseases usually require the long term drug therapy, which may probably cause drug interaction when the patient receives multiple medications. P-gp, the major transporter involving in multidrug resistance, is one of the major concern for transporter-based drug interactions. The present study aimed to investigate the long-term drug use on P-gp function and expression. Propranolol and simvastatin were selected for this study. There have been a number of researches exhibiting the effects of both drugs on P-gp, however the concentrations used were higher than those used in this study. The concentrations of the drug prepared in the methodology were estimated from their pharmacokinetic data.<sup>11,12</sup> The concentrations of drugs tested in this study were designed to mimic the steady-state plasma concentrations. Three different concentrations of the drug were selected to cope with inter-patient variability in drug's plasma concentration commonly observed for most drugs. The study demonstrated that both drug concentration and exposure time affected the amount of CAM uptakes. The results in this study exhibited that P-gp function was increased in cells treated with

propranolol, which is in accordance with the previous study,<sup>5</sup> however the effect on P-gp expression was not observed. Simvastatin increased P-gp expression and reduced the P-gp substrate uptake which may be due to higher protein expression and/or increasing its function. From previous studies, cells treated with simvastatin revealed the different effects in the different cell lines.<sup>6-8</sup> The investigation in intestinal human colon adenocarcinoma cell line LS180 and P-gp over-expressed murine monocytic leukemia cell line showed the inductive effect on the mRNA expression of *MDR1* and enhancement of P-gp function. On the contrary, the study in HEK293 cells revealed that simvastatin reduced P-gp expression. The results from our study exhibited that simvastatin induced P-gp expression and/or its function, which is relevant to that found in LS180.

The histograms from flow cytometry of cells exposed to vinblastine and simvastatin have higher peak than that of the control, suggesting that the cells may exhibit smaller size than the control. The differences in cell size were also apparently seen under the microscope in this present study. Pasquier et al<sup>13</sup> showed the evidence that P-gp play a role in cell volume regulation. Their study demonstrated that P-gp overexpressing cell exhibits smaller size than wild type cells. Consequently, the further investigation should be performed to evaluate the effect of long-term drug exposure on Caco-2 cell morphology.

## Conclusion

In conclusion, the present findings exhibit that Caco-2 cells exposed to propranolol and simvastatin at low drug concentrations with a long exposure time could affect P-gp function and/or expression. These results imply that a patient taking NCD medicines such as propranolol, simvastatin should be considered for the possibility of P-gp mediated drug interaction when taking with a P-gp substrate and/or inhibitor. However, an *in vivo* study should be performed to substantiate these effects.

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