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Optimization of Experimental Conditions for *In vitro* P-glycoprotein Assay Using LLC-GA5 Cells

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ABSTRACT – Identification of compounds that function as P-glycoprotein (P-gp) substrates or inhibitors can facilitate the selection and optimization of new drug candidates. The purpose of this study is to optimize the experimental conditions for *in vitro* P-gp assay using LLC-GA5 cells, which is a well-known transformant cell line derived by transfecting LLC-PK1 with human *MDR1*. The amount of rhodamine123 transported by the LLC-GA5 and LLC-PK1 cells was evaluated under the following experimental conditions: 3 different types of transport media, colchicine pretreatment or nontreatment of the cells in the culture media, and with and without poly-L-lysine coating of the culture plates. The assay sensitivity was found to considerably differ depending on the diluents used in the transport media. P-gp-mediated transport in LLC-GA5 cells was most clearly characterized in the Hanks' balanced salt solution based transport media. The sensitivity of P-gp-mediated transport was not changed by colchicine pretreatment or poly-L-lysine coating of the culture plates.

Key words – P-glycoprotein, LLC-GA5, Rhodamine 123, Efflux transporter

P-glycoprotein (P-gp), encoded by the multidrug-resistance gene *MDR1*, is an ATP-dependent efflux transporter that affects the absorption, distribution, and excretion of a number of drugs; for example, P-gp is considered to regulate the intestinal absorption of paclitaxel, etoposide, and cyclosporine and CNS penetration of digoxin, quinidine, and HIV inhibitors (Lown et al. 1997; Sarkadi et al. 2006; Schinkel et al. 1995). Because of the significance of P-gp in *in vivo* pharmacokinetics, the identification of compounds that function as P-gp substrates or inhibitors may facilitate the optimization and selection of new drug candidates (Balimane et al. 2006).

LLC-GA5 cells are produced by transfection of *hMDR1* cDNA into the porcine kidney epithelial cell line (LLC-PK1); they selectively overexpress human P-gp on their apical membranes and have been used for the assessment of P-gp-mediated drug transport (Tanigawara et al. 1992; Ueda et al. 1992). However, the sensitivity of these kinds of P-gp assays varies with conditions such as media composition and pH and the plate used for culture (Balimane et al. 2006). In addition, culture condition of LLC-GA5 is a little bit specific. For example, culture medium containing colchicine is usually used for the culture of LLC-GA5 in order to maintain P-gp activity (Ueda et al. 1992). However, use of culture medium containing colchicine has a possibility of an up-regulation of other transporters (Vollrath et al. 1994). In addition, poly-L-lysine coated cell culture plate has been used often in transport studies using LLC-PK1 cells, in order to enhance cell attachment and adhesion (Takada et al. 2005). However, little attention has been given to the optimal experimental condition of P-gp assay using LLC-GA5 cells.

Therefore, the purpose of this study was to optimize the experimental conditions (transport media [buffer system that used in transport experiment], colchicine pretreatment, and coating of the culture plate with poly-L-lysine) for *in vitro* P-gp assay using LLC-GA5 cells.

Materials and Methods

Materials

Rhodamine123 (R123), verapamil, quinidine, colchicine, and poly-L-lysine were purchased from Sigma-Aldrich Corporation (St. Louis, MO). LLC-PK1 cells (No. 3616374) and LLC-GA5 cells (No. RCB0871) were obtained from ATCC (Manassas, VA) and RIKEN BRC cell bank (Ibaraki, Japan), respectively. Fetal bovine serum (FBS), medium 199, and penicillin/streptomycin were purchased from Cellgro (Mediatech, Herndon, VA).

Cell uptake study

LLC-PK1 cells were grown and maintained in medium 199

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containing 10% (v/v) FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2; the cells were subcultured every 2 or 3 days using 0.25% trypsin and 2.21 mM ethylenediaminetetraacetic acid (Tanigawara et al. 1992; Ueda et al. 1992). LLC-GA5 cells were grown under identical conditions, except that 150 ng/mL colchicine was added to the culture medium. Cells at a density of 1×10^5 cells/mL were seeded in 12-well plates, and the R123 uptake was studied at 70-80% confluency.

Nonadherent cells were removed by washing twice with phosphate-buffered saline (PBS), and the adhered cells were then preincubated in transport media for 30 min. Subsequently, the cells were incubated in 0.5 mL transport media containing 10 μM R123 with or without 100 μM verapamil or quinidine. After incubation for 10, 20, 40, and 90 min, the experiment was terminated by removing the transport media and washing the cells with 2.0 mL ice-cold PBS solution. The cells were then solubilized by overnight incubation in 0.5 mL of 0.2 N NaOH. After neutralization of the cell lysate with 0.1 mL of 1 N HCl, the concentration of R123 in each sample was determined by fluorescence spectrophotometry (excitation, 480 nm; emission, 530 nm). The amount of protein in each sample was determined by the Lowry method. The difference in P-gp-mediated R123 uptake was expressed as the R123 uptake ratio at 90 min (Mean (S.D.); ** indicates a significant difference in the R123 uptake ratio compared to the controls [$P < 0.01$]).

R123 uptake ratio = (R123 uptake in LLC-GA5 cells) / (R123 uptake in LLC-PK1 cells)

Effect of transport media

The effect of the type of transport media on R123 uptake was investigated using the following 3 base diluents that are frequently used in the P-gp assays with LLC-GA5 cells: PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), PBS with 5 mM glucose (PBS-G, pH 7.4), and Hanks' balanced salt solution (HBSS) (137 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 16 mM glucose, 4.2 mM $NaHCO_3$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4).

Effect of coating and colchicine treatment

To study the effect of poly-L-lysine-coating of the culture plate on R123 uptake, the LLC-PK1 and LLC-GA5 cells were grown in culture plates with and without poly-L-lysine coating. For coating a 2.5-cm^2 culture plate, 0.5 mL poly-L-lysine solution (0.1 mg/mL) prepared in double distilled water was used. Culture plate was in the open air for 5 min and the aliquot of poly-L-lysine solution was aspirated and washed using PBS.

To study the effect of colchicine pretreatment on R123 uptake, the LLC-PK1 and LLC-GA5 cells were cultured with or without colchicine (150 ng/mL) (Ueda et al. 1992). Uptake study was proceeded using HBSS as transport medium.

Results and Discussion

Transport media with PBS and HBSS as the base diluents were compared. Of these 2 diluents–PBS and HBSS–that are widely used buffer systems in studies on the transport systems in cells, PBS is more cost-effective; HBSS has limited application because of its high cost. We also tested the use of PBS with glucose (PBS-G) as the energy source because P-gp uses energy from ATP hydrolysis to pump drug molecules out of cells (Yokota et al. 2007).

Colchicine is usually added to media for the culture of LLC-GA5 cells in order to maintain P-gp activity (Ueda et al. 1992). However, it may also upregulate other transporters; therefore, we tested whether cell culture without colchicine is feasible. In studies on transport systems in LLC-PK1 cells, poly-L-lysine is coated on the surface of the plasticware used for culture in order to enhance cell attachment and adhesion (Takada et al. 2005).

Fig. 1 depicts the uptake of R123, which is a typical fluorescent substrate of P-gp, into the LLC-GA5 and LLC-PK1 cells cultured in media with PBS, PBS-G, and HBSS. The amount of R123 in the LLC-PK1 cells was significantly greater than that in the LLC-GA5 cells, irrespective of the medium used, which suggested that the P-gp activity in the LLC-GA5 cells was adequate for effective R123 effluxing (Fig. 2A, $P<0.05$, ANOVA). The R123 uptake in the cells cultured in PBS-based media did not differ significantly from that in the cells cultured in PBS-G-based media, suggesting that the energy required by P-gp is derived from an endogenous source (Gatlik-Landwojtowicz et al. 2004). Verapamil and quinidine in PBS- or PBS-G-based media could not inhibit R123 transport mediated by P-gp; however, P-gp mediated R123 transport was inhibited effectively in presence of verapamil and quinidine in HBSS-based media ($P < 0.01$, ANOVA). The difference in the Mg^{2+} and/or Ca^{2+} contents between PBS and HBSS may underlie this difference in the activities of the P-gp inhibitors. Al-Shawi et al. (2005) reported that the ATPase activity associated with P-gp differed between the drug-activated and drug-inhibited phases; further, Shapiro and Ling (1994) reported that excessive amount of Mg^{2+} is needed to attain the maximum ATPase activity required by P-gp. And also, we cannot exclude the possibility that different concentration of glucose (5 mM vs. 16 mM) may affect P-gp-

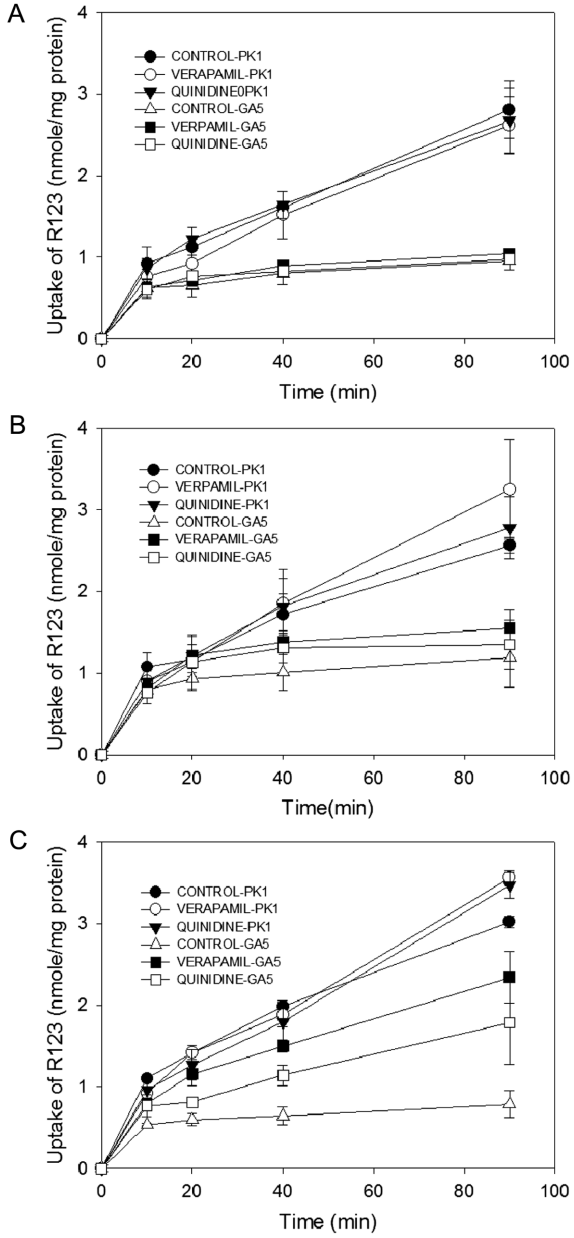

Figure 1. Effects of transport media on R123 accumulation in LLC-PK1 and LLC-GA5 cells. The cells were incubated in (A) PBS-, (B) PBS-G-, and (C) HBSS-based media containing R123 (10 μM) with or without inhibitors (verapamil or quinidine) for 10, 20, 40 and 90 min.

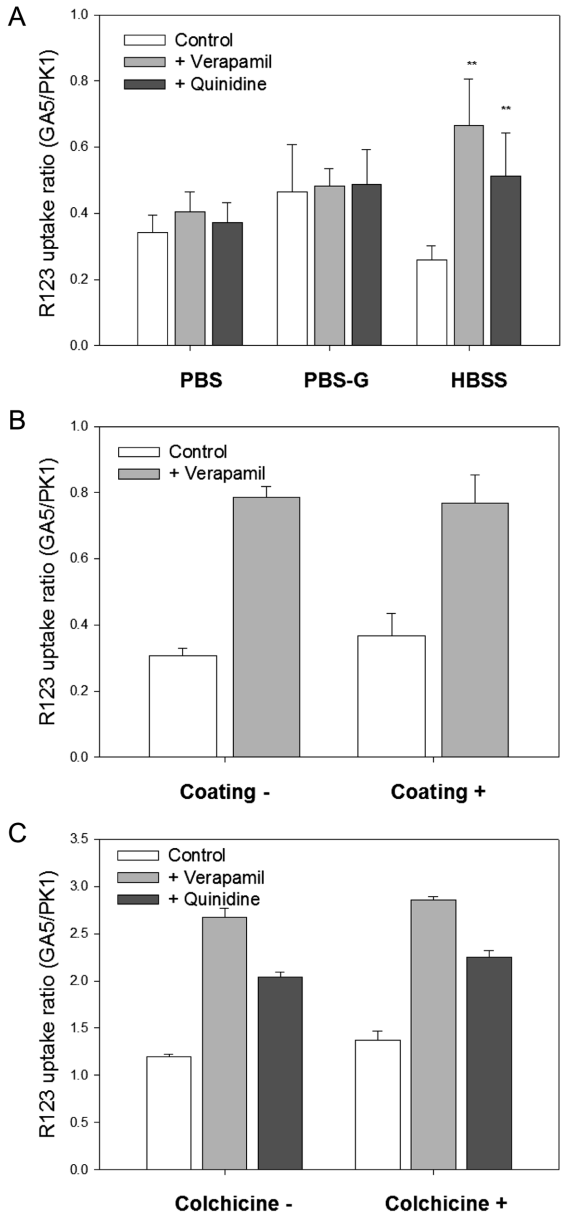

Figure 2. (A) Effect of the type of base media (PBS, PBS-G, and HBSS) on R123 uptake in LLC-PK1 and LLC-GA5 cells. The cells were incubated in PBS-, PBS-G-, and HBSS-based media containing R123 (10 μM) with or without inhibitors (verapamil or quinidine). (B) Effect of poly-L-lysine-coating on R123 uptake in LLC-PK1 and LLC-GA5 cells. (C) Effect of colchicine pretreatment (150 ng/mL) on R123 uptake in the LLC-PK1 and LLC-GA5 cells. Results are expressed as the R123 uptake ratio at 90 min (n=6, Mean (S.D.); ** indicates a significant difference compared to the controls, $P < 0.01$).

mediated transport of R123. The exact mechanism underlying the difference in P-gp-mediated transport needs to be elucidated in future studies.

Poly-L-lysine coating did not affect R123 transport in both cell lines when the cells were cultured in a 12-well plate (Fig. 2B). It did not affect the uptake ratio of R123 or the reversal of P-gp-mediated R123 efflux in the presence of verapamil. Therefore, coating with poly-L-lysine may not be necessary for the P-gp assay with LLC-GA5 cell lines because poly-L-lysine also may enhance unexpected cell proliferation (Foster et al. 1987).

Even pretreatment of the cells with colchicine in the culture media had negligible effects in the P-gp assay (Fig. 2C). In

addition, P-gp-mediated transport and its reversal by verapamil in the LLC-GA5 cells were not affected by colchicine pretreatment. Therefore, considering the unaffected efficacy of the experiment and the potential regulation of other transporters by colchicine (Vollrath et al. 1994), pretreatment with this agent may not be essential for the P-gp assay using LLC-GA5 cell line.

In summary, P-gp-mediated transport in LLC-GA5 cells was most clearly characterized in the HBSS based transport media. The use of colchicine in culture media and poly-L-lysine coating of the culture plates may not essential for the P-gp assay using LLC-GA5 cells.

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