Inhibitory Potencies of 1,4-Dihydropyridine Calcium Antagonists to P-Glycoprotein-Mediated Transport: Comparison with the Effects on CYP3A4

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Purpose. Recently, we clarified the inhibitory effects of 13 kinds of 1,4-dihydropyridine calcium antagonists on human cytochrome P450 (CYP) 3A4. It has been reported that the substrates and/or inhibitors are overlapped between CYP3A4 and P-glycoprotein (P-gp). The purpose of this study was to investigate the inhibitory effects of 13 kinds of 1,4-dihydropyridine calcium antagonists on P-gp-mediated transport in order to evaluate the overlapping specificity of the inhibitors between P-gp and CYP3A4.

Methods. The transcellular transports of [^{3}H]daunorubicin or [^{3}H]digoxin by monolayers of LLC-GA5-COL150 cells in which P-gp was overexpressed were measured in the presence or absence of the 1,4-dihydropyridine calcium antagonists.

Results. The transport of [^{3}H]daunorubicin was strongly inhibited by manidipine, barnidipine, benidipine, (−)-efonidipine, nicardipine, (+)-efonidipine, and amlodipine with the IC_{50} values of 4.6, 8.6, 9.5, 17.3, 17.5, 20.6, and 22.0 μM, respectively. The transport of [^{3}H]digoxin was strongly inhibited by benidipine, nicardipine, barnidipine, and manidipine.

Conclusions. It was clarified that 13 kinds of 1,4-dihydropyridine calcium antagonists have different inhibitory potencies and substrate specificities to the transport of [^{3}H]daunorubicin or [^{3}H]digoxin. Some compounds did not demonstrate the overlapping specificity for inhibition between P-gp and CYP3A4. It was also clarified that nicardipine, benidipine, manidipine, and barnidipine were strong inhibitors of P-gp as well as CYP3A4.

KEY WORDS: P-glycoprotein; 1,4-dihydropyridine calcium antagonists; inhibition; cytochrome P450 3A4.

INTRODUCTION

The multidrug transporter P-glycoprotein (P-gp) can transport a variety of compounds, including anticancer drugs, peptides, steroids, and antihistamines (1). P-gp-mediated efflux reduces the intracellular accumulation of these compounds, thereby diminishing the drug efficacy. Compounds that interact with P-gp are structurally and mechanistically diverse. However, they tend to be large, amphipathic and aromatic. The overlapping substrate specificity of P-gp and cytochrome P450 (CYP) 3A4 has been reported for drugs such as cyclosporin A and verapamil (2). However, it has been reported that the overlap in substrate and inhibitor specificities of CYP3A4 and P-gp appeared to be fortuitous rather than indicative of a more fundamental relationship (3). It was also suggested that, although many P-gp inhibitors were potent inhibitors of CYP3A4, a varying degree of selectivity was present (4).

It is commonly agreed that 1,4-dihydropyridine calcium antagonists are substrates of CYP3A4. Recently, we investigated the selectivity of inhibition of 13 kinds of 1,4-dihydropyridine calcium antagonists to the human CYP3A4 enzyme (5). These compounds included nicardipine, nilvadipine, nifedipine, benidipine, nisoldipine, nitrendipine, manidipine, barnidipine, efonidipine, amlodipine, felodipine, cilnidipine, and aranidipine, which are now clinically used in Japan. Testosterone 6β-hydroxylase activity catalyzed by CYP3A4 was strongly inhibited by nicardipine, benidipine, manidipine, and barnidipine, but nilvadipine, nifedipine, nitrendipine, and amlodipine exhibited a weak inhibition. Thus, it was clarified that 13 kinds of 1,4-dihydropyridine calcium antagonists have different inhibitory potencies on human CYP3A4 activity. Concerning 1,4-dihydropyridine analogues, several studies on P-gp have been reported. Nicardipine fully reversed vincristine resistance in P388/VCR and K562/VCR cells (6). In contrast, it was reported that nifedipine had no ability to restore the sensitivity of P388/ADR cells to doxorubicin (7) and was not a substrate of P-gp using L-MDR1 cells (3). Nitrendipine has been reported not to be a substrate for the P-gp-mediated transport (8). It has also been reported that felodipine was a less potent drug for restoring the cellular accumulation of vinblastine (9). It is difficult to compare the inhibitory effects quantitatively, since these studies were performed with different experimental systems. Therefore, in the present study, we investigated the inhibitory effects of these 13 kinds of 1,4-dihydropyridine calcium antagonists on the P-gp-mediated transcellular transport in the same condition. The experiments were performed using an LLC-GA5-COL150 cell culture monolayer in which human P-gp is overexpressed as a result of transfection with human *MDR1* cDNA (10,11). Moreover, we investigated the inhibitory effects using two different substrates of P-gp. Then, we compared the inhibitory effects on P-gp-mediated transport to those on human CYP3A4 activity in order to evaluate the overlapping specificity of the inhibitors for P-gp and CYP3A4.

MATERIALS AND METHODS

Chemicals

The 1,4-dihydropyridine calcium antagonists described below were kindly provided by the companies shown in parentheses.

1. Nicardipine hydrochloride, (±)-2-[benzyl(methyl)amino]ethyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate hydrochloride (Yamanouchi Pharmaceutical, Tokyo, Japan).

2. (+)-Nilvadipine and 3. (−)-Nilvadipine, 5-isopropyl-3-methyl-2-cyano-1,4-dihydro-6-methyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (Fujisawa Pharmaceutical, Osaka, Japan).

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ABBREVIATIONS: CYP, Cytochrome P450; P-gp, P-glycoprotein.

4. Nifedipine, dimethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylate, was purchased from Wako Pure Chemical Industries (Osaka, Japan).

5. Benidipine hydrochloride, (±)-(*R*)-3-[(*R*)-1-benzyl-3-piperidyl] methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate hydrochloride (Kyowa Hakko Kogyo, Tokyo, Japan).

6. Nisoldipine, (±)-isobutyl methyl 1,4-dihydro-2,6-dimetyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylate (Bayer Yakuhin, Osaka, Japan).

7. Nitrendipine, (±)-ethyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (Yoshitomi Pharmaceutical Industries, Osaka, Japan).

8. Manidipine hydrochloride, (±)-2-[4-(diphenylmethyl)-1-piperazinyl]ethyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate dihydrochloride (Takeda Chemical Industries, Osaka, Japan).

9. Barnidipine hydrochloride, (+)-(3′*S*,4*S*)-3-(1′-benzyl-3′-pyrrolidinyl) methyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate hydrochloride (Yamanouchi Pharmaceutical, Tokyo, Japan).

10. (+)-Efonidipine hydrochloride and 11. (−)-Efonidipine hydrochloride, 2-[benzyl(phenyl)amino]ethyl 1,4-dihydro-2,6-dimethyl-5-(5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinan-2-yl)-4-(3-nitrophenyl)-3-pyridinecarboxylate hydrochloride ethanol (Nissan Chemical Industries, Tokyo, Japan).

12. Amlodipine besilate, (±)-3-ethyl-5-methyl-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate benzenesulfonate (Sumitomo Pharmaceuticals, Osaka, Japan).

13. Felodipine, (±)-ethyl methyl 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate (Hoechst Marion Roussel, Tokyo, Japan).

14. Cilnidipine, (±)-2-methoxyethyl 3-phenyl-2(E)-propenyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (Nippon Boehringer Ingelheim, Hyogo, Japan).

15. (+)-Aranidipine, 16. (−)-Aranidipine, and 17. (±)-Aranidipine, methyl 2-oxopropyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylate (Taiho Pharmaceutical, Tokyo, Japan).

[^{3}H]Daunorubicin (162.8 GBq/mmol), [^{3}H]digoxin (592.0 GBq/mmol), and [^{14}C]inulin (161.3 MBq/g) were purchased from Du Pont-New England Nuclear Research Products (Boston, MA). Colchicine was obtained from Wako Pure Chemical Industries. All other chemicals were of the highest purity available.

Cell Culture

LLC-PK$_1$ cells were obtained from American Type Culture Collection (Rockville, Maryland). LLC-GA5-COL150 cells were purchased from Riken Gene Bank (Tsukuba, Japan). LLC-PK$_1$ cells derived from porcine kidney and LLC-GA5-COL150 cells established by transfection of human *MDR1* cDNA into LLC-PK$_1$ cells (10,11) were maintained by serial passages in plastic culture dishes. LLC-PK$_1$ cells were incubated in complete medium consisting of Medium 199 (Nissui Pharmaceutical, Tokyo, Japan) with 10% fetal bovine serum (Bio Whittaker, Walkersville, Maryland). For LLC-GA5-COL150 cells, 150 ng/ml of colchicine was added in the medium used for LLC-PK$_1$ cells. LLC-PK$_1$ and LLC-GA5-COL150 cells were seeded in plastic dishes in each complete culture medium. Monolayer cultures were grown in an atmosphere of 5% CO_2-95% air at 37°C.

Transcellular Transport

LLC-PK$_1$ and LLC-GA5-COL150 cells were seeded on microporous polycarbonate membrane filters (Transwell,™3402, Costar, Cambridge, Massachussettes) at a cell density of 5.0×10^5 and 7.5×10^5 cells/cm^2, respectively. Cells were cultured on the membrane filter with 1.5 and 0.5 ml of complete medium without colchicine in the outside and inside of the chamber, respectively, in an atmosphere of 5% CO_2-95% air at 37°C for 4 days. Fresh medium was replaced on the second day and third day after seeding. In our study, LLC-PK$_1$ and LLC-GA5-COL150 were used between passages 129 and 131, and between 7 and 19, respectively. The experiments

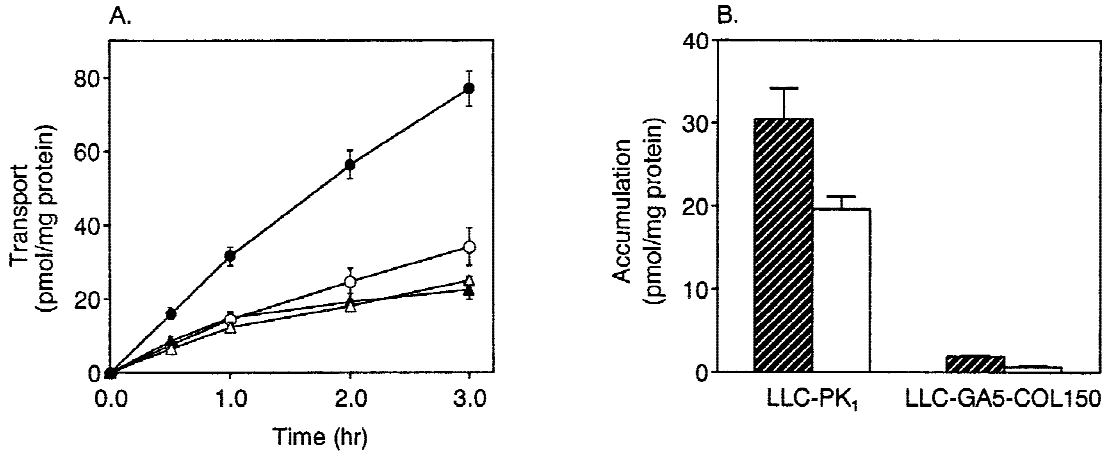

Fig. 1. Transcellular transport (A) and intracellular accumulation at 3.0 hr (B) of [^{3}H]daunorubicin (35 nM) across monolayers of LLC-PK$_1$ (○,△) and LLC-GA5-COL150 (●,▲) cells. A. ○, ●, the basolateral-to-apical transport ; △, ▲, the apical-to-basolateral transport. B. Hatched bar, [^{3}H]daunorubicin was added to the medium of the basolateral side; open bar, [^{3}H]daunorubicin was added to the medium of the apical side. Data represent the mean ± SE of three or more independent measurements.

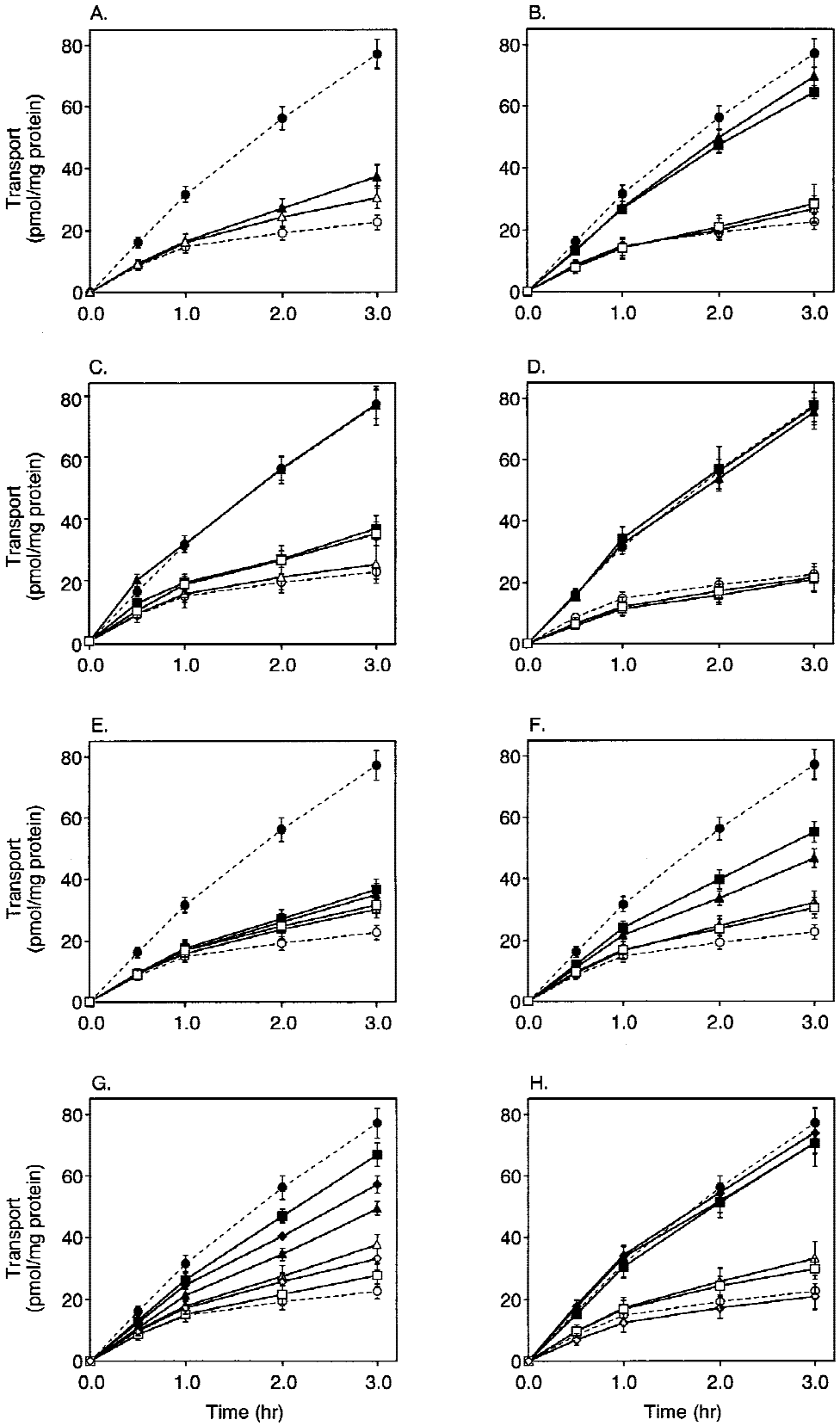

Fig. 2. Transcellular transports of [^{3}H]daunorubicin across LLC-GA5-COL150 monolayers in the presence of 50 μM 1,4-dihydropyridine calcium antagonists. ●, ▲, ■, ◆, the basolateral-to-apical transport ; ○, △, □, ◇, the apical-to-basolateral transport; ●, ○ none (without inhibitors, 0.5% DMSO). (A) ▲, △ , 1. nicardipine. (B) ▲, △ , 2. (+)-nilvadipine, ■, □, 3. (−)-nilvadipine. (C) ▲, △, 4. nifedipine, ■, □, 5. benidipine. (D) ▲, △ , 6. nisoldipine, ■, □, 7. nitredipine. (E) ▲, △ , 8. manidipine, ■, □, 9. barnidipine. (F) ▲, △ , 10. (+)-efonidipine, ■, □, 11. (−)-efonidipine. (G) ▲, △ , 12. amlodipine, ■, □, 13. felodipine, ◆, ◇, 14. cilnidipine. (H) ▲, △ , 15. (+)-aranidipine, ■, □, 16. (−)-aranidipine, ◆, ◇, 17. (±)-aranidipine. Data represent the mean ± SE of three or more independent measurements.

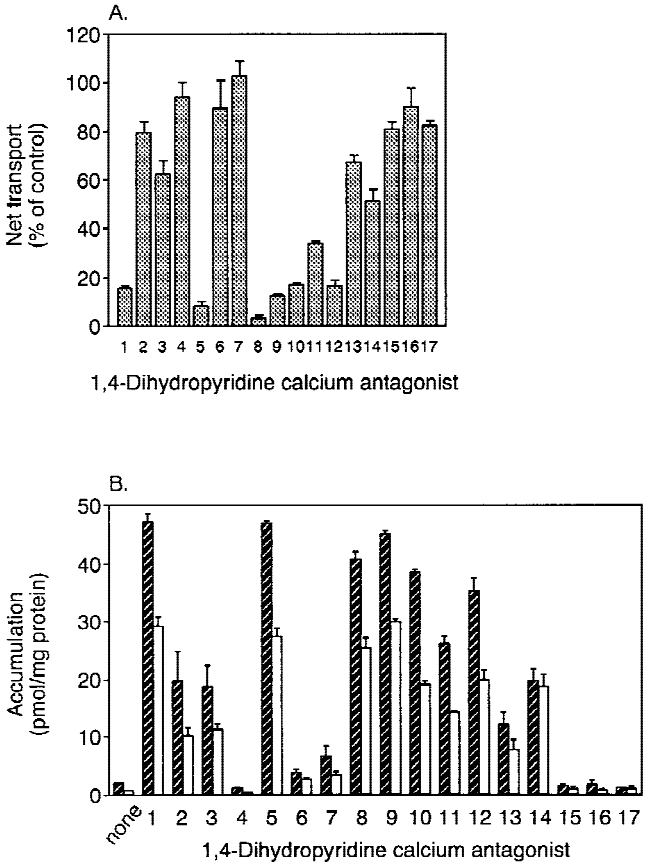

Fig. 3. Inhibitory effects of the 1,4-dihydropyridine calcium antagonists (50 μM) on the transport (A) and intracellular accumulation (B) of 35 nM [^{3}H]daunorubicin at 3.0 hr in LLC-GA5-COL150 cells. A. Net transport was calculated by subtracting the apical-to-basolateral transport from the basolateral-to-apical transport. Net transport without inhibitors (0.5% DMSO) was used as control (54.4 pmol/mg protein). B. Hatched bar, [^{3}H]daunorubicin was added to the medium of the basolateral side; open bar, [^{3}H]daunorubicin was added to the medium of the apical side. None represents the accumulation without inhibitors (0.5% DMSO). Data represent the mean ± SE of three or more independent measurements. 1. nicardipine; 2. (+)-nilvadipine; 3. (−)-nilvadipine; 4. nifedipine; 5. benidipine; 6. nisoldipine; 7. nitrendipine; 8. manidipine; 9. barnidipine; 10. (+)-efonidipine; 11. (−)-efonidipine; 12. amlodipine; 13. felodipine; 14. cilnidipine; 15. (+)-aranidipine; 16. (−)-aranidipine; 17. (±)-aranidipine.

were performed according to the procedure described previously (10,11) with slight modifications. Three hours before the start of the experiments, all culture media were replaced with fresh medium without colchicine. The medium on the donor side of the monolayers was replaced with medium containing 35 nM [^{3}H]daunorubicin (5.7 kBq/ml) or 35 nM [^{3}H]digoxin (20.7 kBq/ml), and the monolayers were then incubated in 5% CO_2-95% air at 37°C. Aliquots from the receiver side (50 μl of inside or 150 μl of outside) were taken at 0.5, 1.0, 2.0, and 3.0 hr. The radioactivity was measured using a liquid scintillation counter (LSC 5100, Aloka, Tokyo, Japan), after the addition of 4 ml scintillation cocktail, Clearsol I (Nacalai tesque, Kyoto, Japan). For the intracellular accumulation study, the cells were washed with ice-cold phosphate-buffered saline immediately after the last sampling. The cells were solubilized with 0.5 ml of 0.3 M NaOH overnight, and were neutralized with 0.5 ml of 0.3 M HCl. The radioactivity in the 200 μl portion of the aliquots was counted. The protein contents in LLC-PK_1 and LLC-GA5-COL150 cells were measured by the method of Lowry *et al.* (12) using bovine serum albumin as a standard. The protein content of LLC-PK_1 and LLC-GA5-COL150 monolayers ranged 0.23 to 0.28 and 0.15 to 0.19 mg/filter, respectively.

To examine the inhibitory effects of the 1,4-dihydropyridine calcium antagonists on the P-gp-mediated transport in LLC-GA5-COL150 cells, these compounds were added into the medium on both sides of the cell monolayer 1.5 hr before adding the substrate. Initially, the inhibitory potencies to the P-gp-mediated transport were investigated with 50 μM 1,4-dihydropyridine calcium antagonists. For the compounds which demonstrated potent inhibition, the inhibitors ranged from 5.0 to 50 μM to obtain the 50%-inhibitory concentration (IC_{50}). The 1,4-dihydropyridine calcium antagonists were dissolved in dimethylsulfoxide so that the final concentration of the solvent in the media was <0.5% (v/v). The paracellular leakage in the presence of the inhibitor (50 μM) was monitored by the appearance of radioactivity on the receiver side after the addition of 14.5 μM [^{14}C]inulin (11.7 kBq/mL) to the donor side.

RESULTS

It has been reported that P-gp overexpressed in LLC-GA5-COL150 cells is localized in the apical membrane (11).

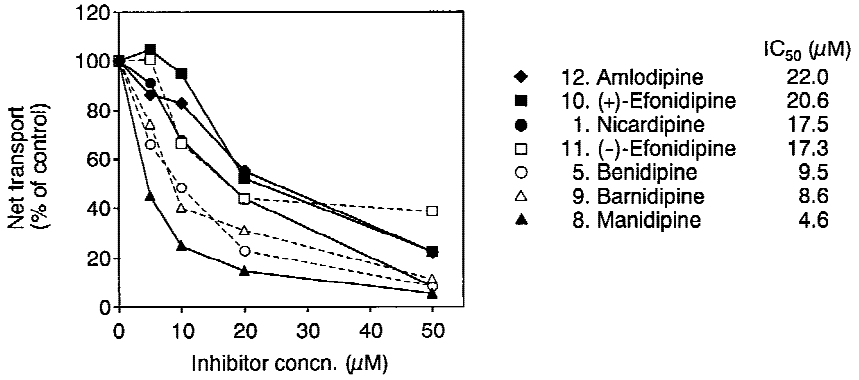

Fig. 4. Concentration-dependent inhibition of the net transport of 35 nM [^{3}H]daunorubicin by the 1,4-dihydropyridine calcium antagonists at 3.0 hr in LLC-GA5-COL150 cells. ●, 1. nicardipine; ○, 5. benidipine; ▲, 8. manidipine; △, 9. barnidipine; ■, 10. (+)-efonidipine; □, 11. (−)-efonidipine; ♦, 12. amlodipine. The inhibitors ranged from 5.0-50 μM. The IC_{50} values of 2. (+)-nilvadipine, 3. (−)-nilvadipine, 4. nifedipine, 6. nisoldipine, 7. nitrendipine, 13. felodipine, 14. cilnidipine, 15. (+)-aranidipine, 16. (−)-aranidipine, and 17. (±)-aranidipine were, >50 μM. Data represent the mean of three independent measurements.

Therefore, a substrate that is expelled by P-gp would show greater basolateral-to-apical transport in LLC-GA5-COL150 cells than in LLC-PK_1 cells. The transcellular transport of [^{3}H]daunorubicin in LLC-PK_1 and LLC-GA5-COL150 cells are shown in Fig. 1A. The basolateral-to-apical transport in LLC-GA5-COL150 cells greatly exceeded that in LLC-PK_1 cells. This phenomenon was accompanied by a marked decrease in the intracellular accumulation of [^{3}H]daunorubicin in LLC-GA5-COL150 cells compared to LLC-PK_1 cells (Fig. 1B).

The transports of [^{3}H]daunorubicin in the presence of 50 μM 1,4-dihydryopyridine calcium antagonists are shown in Fig. 2. The 17 compounds had different inhibitory effects on the transport of [^{3}H]daunorubicin. The net transports of [^{3}H]daunorubicin at 3.0 hr in the presence of 50 μM 1,4-dihydropyridine calcium antagonists are shown in Fig. 3A. The net transport was strongly inhibited (<50% of control) by nicardipine, benidipine, manidipine, barnidipine, (+)-efonidipine, (−)-efonidipine, and amlodipine. (+)-Nilvadipine, (−)-nilvadipine, felodipine, and cilnidipine exhibited weak inhibition (50–80% of control). The effects of 1,4-dihydropyridine calcium antagonists on the intracellular accumulation of [^{3}H]daunorubicin are shown in Fig. 3B. All compounds except nifedipine, (+)-aranidipine, (−)-aranidipine, and (±)-aranidipine increased the intracellular accumulation of [^{3}H]daunorubicin. Particularly, the enhancement of the intracellular accumulation by nicardipine, benidipine, manidipine, barnidipine, (+)-efonidipine, and amlodipine were remarkable.

Subsequently, we determined the apparent IC_{50} values of the transport of [^{3}H]daunorubicin at 3.0 hr for the compounds that exhibited strong inhibition. Nicardipine, benidipine, manidipine, barnidipine, (+)-efonidipine, and amlodipine inhibited the P-gp-mediated transport of [^{3}H]daunorubicin in a dose-dependent manner (Fig. 4). (−)-Efonidipine exhibited almost the same inhibition at concentrations of 20 and 50 μM. The apparent IC_{50} values were as follows: manidipine (4.6 μM), barnidipine (8.6 μM), benidipine (9.5 μM), (−)-efonidipine (17.3 μM), nicardipine (17.5 μM), (+)-efonidipine (20.6 μM), and amlodipine (22.0 μM). For these compounds, the intracellular accumulation of [^{3}H]daunorubicin in LLC-GA5-COL150 cells was increased in response to the attenuation of the transport activities (data not shown).

To compare the inhibitory effects of 1,4-dihydropyridine calcium antagonists on the P-gp-mediated transport of another substrate, [^{3}H]digoxin was used. The transcellular transport of [^{3}H]digoxin in LLC-PK_1 and LLC-GA5-COL150 cells are shown in Fig. 5A. The basolateral-to-apical transport in LLC-GA5-COL150 cells greatly exceeded that in LLC-PK_1 cells. However, this phenomenon was not accompanied by a marked decrease in the intracellular accumulation of [^{3}H]digoxin in LLC-GA5-COL150 cells as occurred in LLC-PK_1 cells (Fig. 5B), thereby differing from [^{3}H]daunorubicin. The transport of [^{3}H]digoxin in the presence of 50 μM 1,4-dihydropyridine calcium antagonists are shown in Fig. 6. The 17 compounds had different inhibitory effects on the transport of [^{3}H]digoxin. The net transports of [^{3}H]digoxin at 3.0 hr in the presence of 50 μM 1,4-dihydropyridine calcium antagonists are shown in Fig. 7A. The net transport was strongly inhibited (<50% of control) by nicardipine, benidipine, manidipine, and barnidipine. (−)-Nilvadipine and efonidipine exhibited weak inhibition (50–80% of control). Although amlodipine, felodipine, and cilnidipine also showed weak inhibition of the net transport of [^{3}H]digoxin, these phenomena were caused by the increase of the apical-to-basolateral transport. None of the 1,4-dihydropyridine calcium antagonists affected the intracellular accumulation of [^{3}H]digoxin (Fig. 7B).

DISCUSSION

P-gp is co-localized in a polarized fashion at the apical membrane of cells in which CYP3A4 is also expressed, such as epithelial cells in the small intestine, hepatocytes, and proximal tubular cells in the kidney (1). It is reasonable to expect that, in epithelial cells in the small intestine, metabolism by CYP3A4 and efflux by P-gp into the lumen limit the oral bioavailability of substrates for these proteins. It has been proposed that a substrate of P-gp repeatedly circulates between the lumen and epithelial cells (13,14), leading to increased metabolism due to prolonged exposure to the metabolic enzyme and reduced absorption of the drug into the blood (15). For the compounds which are recognized to be

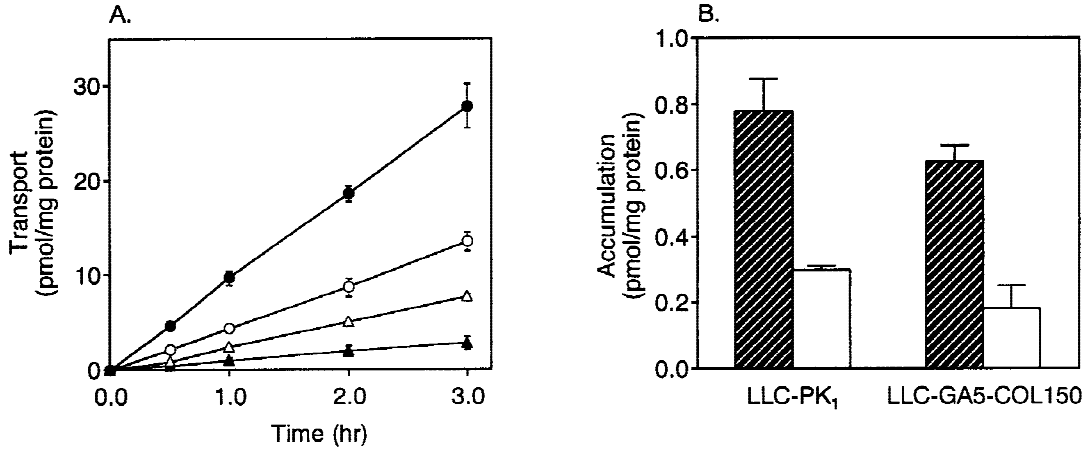

Fig. 5. Transcellular transport (A) and intracellular accumulation at 3.0 hr (B) of [^{3}H]digoxin (35 nM) across monolayers of LLC-PK_1 (○, △) and LLC-GA5-COL150 (●, ▲) cells. A. ○, ●, the basolateral-to-apical transport; △, ▲, the apical-to-basolateral transport. B. Hatched bar, [^{3}H]digoxin was added to the medium of the basolateral side; open bar, [^{3}H]digoxin was added to the medium of the apical side. Data represent the mean ± SE of three independent measurements.

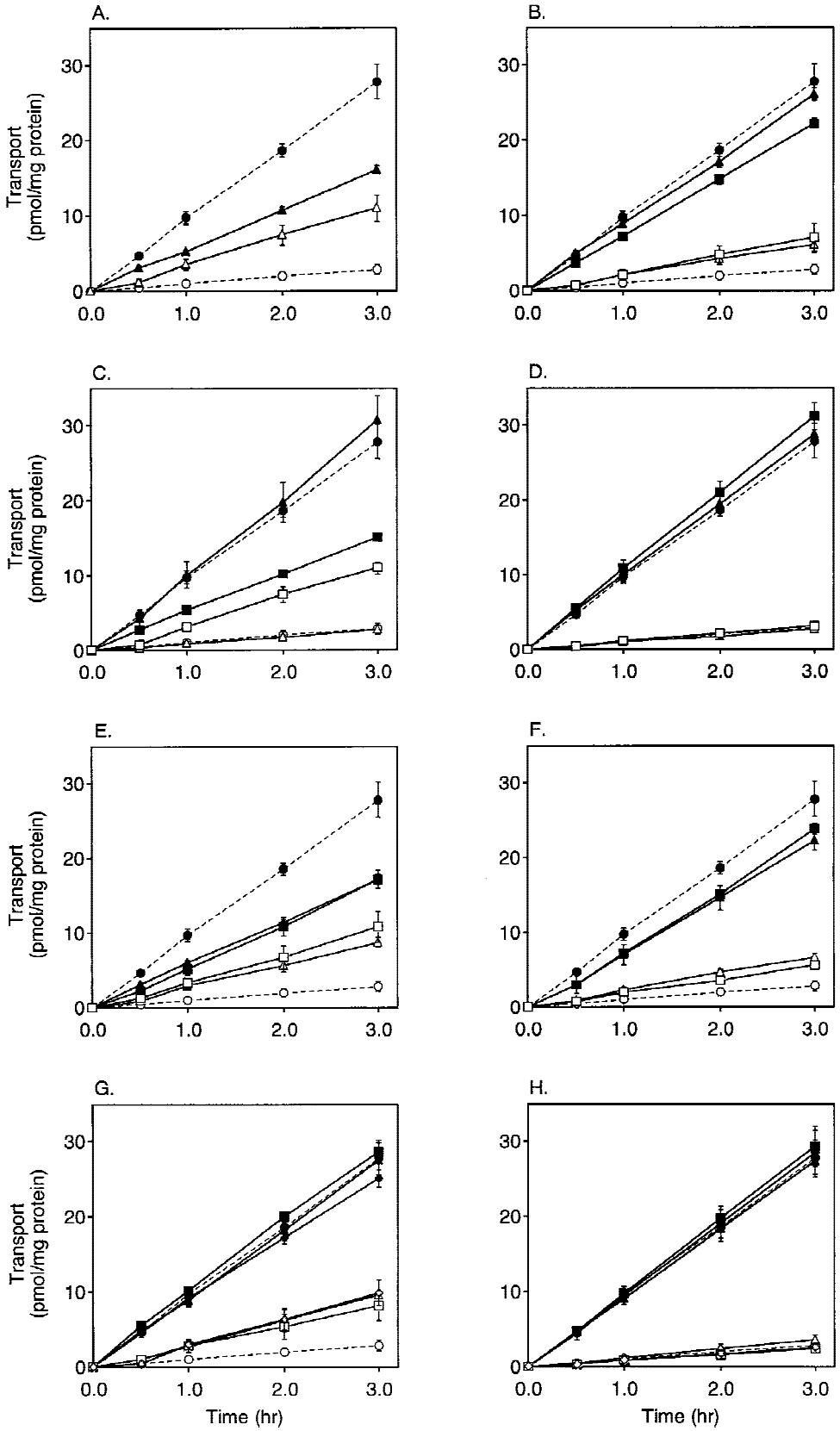

Fig. 6. Transcellular transports of [^{3}H]digoxin across LLC-GA5-COL150 monolayers in the presence of 50 μM 1,4-dihydropyridine calcium antagonists. ●, ▲, ■, ◆, the basolateral-to-apical transport ; ○, △, □, ◇, the apical-to-basolateral transport; ●, ○ none (without inhibitors, 0.5% DMSO). (A) ▲, △, 1. nicardipine. (B) ▲, △, 2. (+)-nilvadipine, ■, □, 3. (−)-nilvadipine. (C) ▲, △, 4. nifedipine, ■, □, 5. benidipine. (D) ▲, △, 6. nisoldipine, ■, □, 7. nitredipine. (E) ▲, △, 8. manidipine, ■, □, 9. barnidipine. (F) ▲, △, 10. (+)-efonidipine, ■, □, 11. (−)-efonidipine. (G) ▲, △, 12. amlodipine, ■, □, 13. felodipine, ◆, ◇, 14. cilnidipine. (H) ▲, △, 15. (+)-aranidipine, ■, □, 16. (−)-aranidipine, ◆, ◇, 17. (±)-aranidipine. Data represent the mean ± SE of three independent measurements.

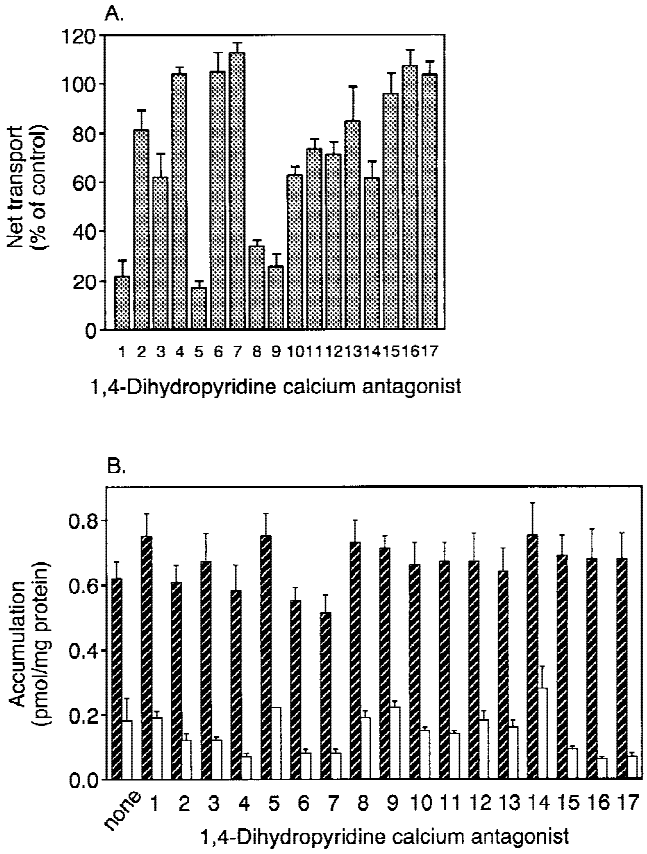

Fig. 7. Inhibitory effects of the 1,4-dihydropyridine calcium antagonists (50 μM) on the transport (A) and intracellular accumulation (B) of 35 nM [^{3}H]digoxin at 3.0 hr in LLC-GA5-COL150 cells. A. Net transport without inhibitors (0.5% DMSO) was used as control (25.0 pmol/mg protein). B. Hatched bar, [^{3}H]digoxin was added to the medium of the basolateral side; open bar, [^{3}H]digoxin was added to the medium of the apical side. None represents the accumulation without inhibitors (0.5% DMSO). Data represent the mean ± SE of three independent measurements. The numbers of the compounds were the same as those in the legend of Fig. 3.

substrates and/or inhibitors of CYP3A4 and P-gp, we should pay attention to drug–drug interactions in the intestine as well as in the liver.

In the current study, we investigated the inhibitory effects of 1,4-dihydropyridine calcium antagonists on the P-gp-mediated transports of [^{3}H]daunorubicin and [^{3}H]digoxin. Nicardipine, benidipine, manidipine, and barnidipine exhibited strong inhibition on the transports of both [^{3}H]daunorubicin and [^{3}H]digoxin. On the other hand, efonidipine and amlodipine strongly inhibited the transport of [^{3}H]daunorubicin, but did not inhibit the transport of [^{3}H]digoxin. It has been clarified that 13 kinds of 1,4-dihydropyridine calcium antagonists have different inhibitory potencies on the P-gp-mediated transport. For nicardipine, the present finding was consistent with a previous report in which nicardipine was found to be a potent antagonist of P-gp (6). Although it has been reported that 10 μM nifedipine exhibits weak inhibition (36 ± 5.9%) on the transport of 5 μM [^{3}H]digoxin in Caco-2 cells (3), nifedipine did not inhibit the P-gp-mediated transport in our study. LLC-GA5-COL150 cells used in this study were established by the transfection of human *MDR1* cDNA into LLC-PK$_1$ cells. Caco-2 cells are from human colon adenocarcinoma. Therefore, the discrepancy may be caused by the difference of cell lines such as the expression level of P-gp and/or the presence of other transporters and the difference of the substrate concentration.

For the transport of [^{3}H]daunorubicin, the compounds with a bulky structure including a nitrogen atom in the side chain of carboxylic acid ester exhibited strong inhibition. The structures in the side chain might affect the inhibitory effects. Further investigations are needed to clarify the relationship between the inhibition potency and the chemical structure as well as the stereochemical differences.

It has been reported that the cyclosporin analogues exhibit different inhibition on the transport of [^{3}H]daunorubicin and [^{3}H]vinblastine (16). Moreover, Kusunoki *et al.* (17) also reported that the inhibitory effects of cyclosporin and SDZ PSC 833 on [^{3}H]vinblastine and [^{14}C]doxorubicin were different. In these reports, it was surmised that the differences in inhibition specificity might be due to the affinity of the substrates to P-gp. In the present study, it also appeared that the inhibitory effects on the P-gp-mediated transport were different depending on the different substrates in the same cell line, supporting the previous reports. Investigations with several substrates of P-gp would be needed to estimate accurately the inhibitory effects on the transport via P-gp. Cyclosporin A has been used as a substrate as well as an inhibitor of P-gp-mediated transport (2). However, we did not use it in the present study, because cyclosporin A is directly toxic to LLC-PK$_1$ cells with reduced DNA synthesis and cell cycle blockade (18).

From our data, the effects of amlodipine, felodipine, and cilnidipine on the transport of [^{3}H]digoxin were due to the increase of the apical-to-basolateral transport. It has also been reported that the apical-to-basolateral transport of [^{14}C]docetaxel (19) and [^{3}H]vinblastine (20) are increased by itraconazole and verapamil, respectively. As was suspected, these phenomena might be explained by the increased non-P-gp-mediated transport. Although the intracellular accumulation of [^{3}H]daunorubicin inversely correlated with the net transport, while that of [^{3}H]digoxin did not. This phenomenon might be caused by the low efficiency of inflow of [^{3}H]digoxin into the cells. The intracellular accumulation of [^{3}H]digoxin without inhibitors was about 1/40 that of [^{3}H]daunorubicin. This finding indicated that the intracellular accumulation was not a determinant of the transporting function of P-gp.

In our previous study (5), the inhibitory effects of these 13 kinds of 1,4-dihydropyridine calcium antagonists on CYP3A4 were compared in detail. As shown in Fig. 8, nicardipine, benidipine, manidipine, and barnidipine strongly inhibited the CYP3A4 activity. These compounds also exhibited strong inhibition on the P-gp-mediated transports of [^{3}H]daunorubicin and [^{3}H]digoxin. However, (+)-efonidipine, (–)-efonidipine, and amlodipine exhibited weak or no inhibition on the CYP3A4 activity, although these compounds strongly inhibited the transport of [^{3}H]daunorubicin. The strong inhibitors of CYP3A4 activity such as nicardipine, benidipine, manidipine, and barnidipine exhibited strong inhibition of the P-gp-mediated transport. Nicardipine has been reported to increase the plasma digoxin concentrations 1.2-fold (21). On the other hand, it has been reported that nifedipine coadministration does not alter the digoxin pharmacokinetics (22). The difference in the *in vivo* drug–drug inter-

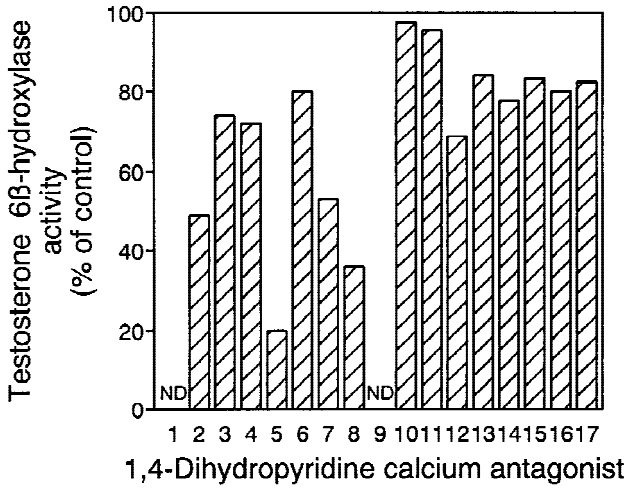

Fig. 8. Inhibitory effects of the 1,4-dihydrpyridine calcium antagonists (10 μM) on testosterone 6β-hydroxylase activity catalyzed by recombinant CYP3A4 expressed in human B-lymphoblast cells. ND: not detected (below the limit of detection). The activity was determined at a testosterone concentration of 100 μM. The control activity was 12.1 pmol/min/pmol CYP. Data represent the mean of duplicate determinations. This figure was redrawn from our previous report (5). The numbers of the compounds were the same as those in the legend of Fig. 3.

actions of digoxin with nicardipine or nifedipine was in accordance with the inhibitory potency to the P-gp-mediated transport of [^{3}H]digoxin and the CYP3A4 activity in our studies.

To understand the overlapping specificity of substrates and inhibitors for CYP3A4 and P-gp, we should determine the 3-D structures of CYP3A4 and P-gp under the physiological condition. Since CYP3A4 and P-gp are membrane-bound proteins, the crystallization of the proteins is very difficult and not reported yet. The facts prevent the study of the overlapping specificity. However, concerning the modeling CYP, the crystallization of soluble CYPs from bacteria could recently develop the modeling study of membrane-bound CYP by homology analyses (23,24). In our laboratory, the analysis of the 3-D structure of CYP3A4 as well as thirteen 1,4-dihydropyridine calcium antagonists is underway using computer programs. Furthermore, their docking structures will also be analyzed. At present, the analysis of 3-D structure of P-gp is difficult and may take time because of the limitations of the information. When all of the structures of chemicals, CYP3A4 and P-gp are defined in detail, the fundamental relationship of CYP3A4 and P-gp will finally be clarified.

In conclusion, the present study revealed that the 13 kinds of 1,4-dihydropyridine calcium antagonists have different inhibitory potencies on the P-gp-mediated transport. It was confirmed that there was an overlapping specificity of inhibitors between P-gp and CYP3A4 for strong CYP3A4 inhibitors, however it might not be strict. It is important to consider the drug–drug interactions between these 1,4-dihydropyridine calcium antagonists and other drugs via both CYP and P-gp in the liver and the intestine.

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