NEW 4-ARYL-1,4-DIHYDROPYRIDINES AND 4-ARYLPYRIDINES AS P-GLYCOPROTEIN INHIBITORS

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ABSTRACT:
Efflux of cytotoxic agents mediated by P-glycoprotein is believed to be an important mechanism of multidrug resistance, which remains a serious limitation to successful chemotherapy in cancers such as metastatic breast cancer. A series of 4-aryl-1,4-dihydropyridines and corresponding aromatized 4-arylpyridines have been synthesized based on structure modifications of niguldipine to enhance multidrug resistance reversal activity, while minimizing calcium channel binding. Thirty new compounds were characterized. [3H]Vinblastine accumulation studies indicated that at a concentration level of 3 μM, 15 of 18 4-aryl-1,4-dihydropyridines and all 4-arylpyridines can successfully restore intracellular accumulation of vinblastine in a resistant human breast adenocarcinoma cell line, MCF-7/adr, which overexpresses P-glycoprotein. The most potent compounds led to an approximately 15-fold increase of vinblastine accumulation. All of the test compounds that significantly increased vinblastine accumulation in MCF/adr cells were able to substantially reduce IC50 values of daunomycin and increase its cytotoxicity in MCF-7/adr-resistant cells, confirming the results of the vinblastine accumulation studies. Calcium channel binding assays for these newly synthesized compounds were conducted using rat cerebral cortex membrane. All but eight compounds demonstrated negligible calcium channel binding over the concentration range from 15 to 2500 nM. The results demonstrate that the newly synthesized series of 1,4-dihydropyridines and pyridines represent P-glycoprotein modulators with negligible calcium channel blocking activity.

Chemotherapy plays an important role in breast cancer treatment. However, the development of drug resistance is often observed in metastatic breast cancer patients during chemotherapeutic treatment. Broad resistance to multiple structurally and functionally unrelated drugs is often observed after intermittent or prolonged exposure of tumor cells to only a single agent. This “classical” multidrug resistance is typically characterized by cross-resistance to commonly used natural product drugs, the anthracyclines, Vinca alkaloids, taxanes, and epipodophyllotoxins. Among the most extensively studied mechanisms of multidrug resistance is P-glycoprotein-mediated active extrusion of cytotoxic drugs from the tumor cells. P-Glycoprotein, a 170-kDa plasma membrane protein, acts as an ATP-dependent drug efflux pump, thereby decreasing the intracellular drug accumulation (Giaccone et al., 1995). Breast cancer patients with tumors expressing P-glycoprotein were more likely to fail to respond to chemotherapy than patients whose tumors were P-glycoprotein-negative (Trock et al., 1997). P-Glycoprotein is also frequently expressed in lymph node metastases of breast cancer patients (Zochbauer-Muller et al., 2001). Thus, drug resistance remains one of the major obstacles in successful chemotherapy in breast cancer patients with advanced disease that is not curable by surgery and/or radiation therapy.

A diverse range of agents has been identified as multidrug resistance modifiers. These include calcium channel blockers (Tsuruo et al., 1981), calmodulin inhibitors (Kamath et al., 1991), protein kinase C inhibitors (Gekeler et al., 1996), steroids (Aebi et al., 1999), immunosuppressants (Wigler, 1999; List et al., 2002), and various other agents (Bhat et al., 1995; Kruijtsen et al., 2002). Among these agents, calcium channel blockers may be the most extensively investigated class of multidrug resistance modulators.

1,4-Dihydropyridines belong to the important category of Ca2+ channel antagonists that are active on the L-type Ca2+ channels. Dexniguldipine, the (−)-enantiomer of the 1,4-dihydropyridine niguldipine, is approximately 40 times less potent than (+)-niguldipine with respect to their interaction with 1,4-dihydropyridine receptors on L-type calcium channels (Graziadei et al., 1989). Additionally, in comparison with other multidrug resistance reversal agents such as PSC833 (valspodar), cyclosporin A, verapamil, dipyridamole, quinidine, and amiodarone, dexniguldipine represents the most potent P-glycoprotein inhibitor (Boer et al., 1994). This indicates that the multidrug resistance reversal activity is independent of the calcium channel-antagonistic activity of the 1,4-dihydropyridines (Szabo and Molnar, 1998). Based on these findings, structure modifications of 1,4-dihydropyridines have been designed to increase their P-glycoprotein interaction, while at the same time minimizing their Ca2+-antagonistic activity.

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ABBREVIATIONS: TLC, thin-layer chromatography; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein; SAR, structure-activity relationship.
We have synthesized a series of 1,4-dihydropyridines and corresponding aromatized pyridine compounds designed to maximize inhibition of P-glycoprotein-mediated efflux while minimizing Ca^{2+} channel binding, based on the structure modifications of nigrulidine (Table 1). In this study, evaluation of the P-glycoprotein-inhibitory effect of these newly synthesized compounds was performed using the human breast cancer cell line MCF-7. The affinity of 4-aryl-1,4-dihydropyridines and 4-arylpyridines to Ca^{2+} channels was also determined in competition binding experiments using rat cerebral cortex membranes.

**Materials and Methods**

**Chemicals, Cell Lines, and Reagents.** Radiolabeled [3H]vinblastine sulfate was purchased from Moravek Biochemicals (Brea, CA). Cell culture reagents were supplied by Invitrogen (Carlsbad, CA). Cell culture flasks, dishes, and sterile 96-well plates were purchased from Falcon; BD Biosciences Discovery Labware (Bedford, MA). Nonsterile 96-well plates were purchased from Marsh-BioMarket (Rochester, NY). [3Hf (+)-PN200-110 (isradipine) and biodegradable liquid scintillation cocktail were purchased from Amersham Biosciences Inc. (Piscataway, NJ). BCA Protein Assay Kit was purchased from Pierce Chemical (Rockford, IL). MCF-7adr, a variant of the MCF-7 cell line, which overexpresses P-glycoprotein, was obtained from the National Cancer Institute (Bethesda, MD). Daunomycin, niguldipine, and nifedipine were purchased from Sigma-Aldrich (St. Louis, MO).

**Syntheses of Unsymmetrical 1,4-Dihydropyridines.** Symmetrical 1,4-dihydropyridine esters are generally obtained in high yield using either a Hantzsch or Collin protocol (Collin, 1884; Loev et al., 1974). Unsymmetrical 1,4-dihydropyridines are generally obtained in high yield using either a Hantzsch or Collin protocol (Collin, 1884; Loev et al., 1974). A series of 1,4-dihydropyridine esters, however, are often more difficult to prepare due to the formation of symmetric by-products. This problem can be overcome by applying the modified Hantzsch-type condensation (Fig. 1), i.e., the Michael addition of the appropriate methyl 3-benzylidene-acetoacetate (4a–q) with an enamine (5) resulting in the formation of unsymmetrical 1,4-dihydropyridines (Ia–q) in fair to satisfactory yields (Di Stilo et al., 1998). A series of acetooxime esters were synthesized via Knoevenagel condensation of the corresponding benzaldehyde (Ia–q) with methyl acetooxime (2) in the presence of a catalytic amount of piperidine and acetic acid (Zoeller and Sumner, 1990). This enamine is a common intermediate for the syntheses of all the proposed 1,4-dihydropyridines. The enamine (5), as a common intermediate for the syntheses of all the proposed 1,4-dihydropyridines, was yielded by aminolysis of the 4-keto ester (3) with excess amounts of liquid ammonia. The 4-keto ester (3) was readily obtained through the known synthetic sequence (Wetzel et al., 1995). These intermediates and the final products were purified by column chromatography on silica gel. The 1,4-dihydropyridines prepared in this manner are shown in Table 1. All the 1,4-dihydropyridines were prepared and crystallized as their hydrochloride salts for biological screening. Their corresponding 4-arylpyridines (Table 1) were obtained by the oxidation of the 1,4-dihydropyridines using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (Walker and Hiebert, 1967). These pyridines were also converted into their hydrochloride salts for screening. The 1,4-dihydropyridine salts were characterized by their NMR and infrared spectra and elemental analyses. The final products, 1,4-dihydropyridines and their corresponding 4-arylpyridines, were characterized by their proton and carbon NMR spectra, high resolution mass spectra using electron-impact, and elemental analyses.

**General Procedure for the Preparation of Knoevenagel adducts (4a–q).** A solution of glacial acetic acid (7% Eq) and anhydrous piperidine (2% Eq) in anhydrous methanol was stirred at 25°C for 15 min. To this solution was added appropriate benzaldehyde (1) (1.0 Eq) and methyl acetoacetate (2) (1.0 Eq), and the resulting mixture was stirred at 60°C for 2.5 h. After cooling to 25°C, Amberlyst-15 resin was added and stirred for 10 min. Filtration followed by concentration in vacuo afforded a yellow residue, which was purified by silica gel chromatography to yield (4) as a light yellow crystalline solid.

**General Procedure for the Preparation of 1,4-Dihydropyridines (Ia–q).** Equimolar amounts of a methyl 2-benzylidenecetooxime (4) and 3-amino-crotonic acid (5) were dissolved in an appropriate amount of anhydrous alcoholic solvent. The resulting solution was stirred at 65–75°C under an argon atmosphere for 10 min, resulting in the formation of unsymmetrical 1,4-dihydropyridines (Ia–q) in fair to satisfactory yields (Di Stilo et al., 1998). A series of acetooxime esters were synthesized via Knoevenagel condensation of the corresponding benzaldehyde (Ia–q) with methyl acetooxime (2) in the presence of a catalytic amount of piperidine and acetic acid (Zoeller and Sumner, 1990). This enamine is a common intermediate for the syntheses of all the proposed 1,4-dihydropyridines. The enamine (5), as a common intermediate for the syntheses of all the proposed 1,4-dihydropyridines, was yielded by aminolysis of the 4-keto ester (3) with excess amounts of liquid ammonia. The 4-keto ester (3) was readily obtained through the known synthetic sequence (Wetzel et al., 1995). These intermediates and the final products were purified by column chromatography on silica gel. The 1,4-dihydropyridines prepared in this manner are shown in Table 1. All the 1,4-dihydropyridines were prepared and crystallized as their hydrochloride salts for biological screening. Their corresponding 4-arylpyridines (Table 1) were obtained by the oxidation of the 1,4-dihydropyridines using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (Walker and Hiebert, 1967). These pyridines were also converted into their hydrochloride salts for screening. The 1,4-dihydropyridine salts were characterized by their NMR and infrared spectra and elemental analyses. The final products, 1,4-dihydropyridines and their corresponding 4-arylpyridines, were characterized by their proton and carbon NMR spectra, high resolution mass spectra using electron-impact, and elemental analyses.

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atmosphere for 24 to 72 h, monitoring with TLC for the completion of the reaction. The reaction solution was concentrated in vacuo to give a residue, which was purified by silica gel column chromatography to furnish the target compound as a foam-like solid.

**Hydrochloride Salt Formation (Ia-q • HCl).** To a solution of a 4-aryl-1,4-dihydropyridines (Ia-q) in methanol or ethanol was added dropwise a solution of HCl in diethyl ether (1.0 M, 1.3–3.0 Eq) at 0°C. The resulting mixture was stirred for 15 min and concentrated in vacuo to yield a residue, which was dissolved in a minimum amount of anhydrous methylene chloride. This solution was then added dropwise to anhydrous diethyl ether with swirling to afford, after filtration, a crystalline solid. Most of the corresponding hydrochloride salts formed were highly hygroscopic upon exposure to the air.

**General Procedure for 4-Arylpyridines (IIa-q).** A solution of a 4-aryl-1,4-dihydropyridines (Ia-q) (1.0 Eq) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (1.30–1.50 Eq) in anhydrous tetrahydrofuran was refluxed under an argon atmosphere for 4 to 24 h. The completion of the reaction was monitored with TLC. The resulting brown solution was concentrated in vacuo to give a residue, which was purified by silica gel column chromatography followed by preparative TLC to afford the target compound (IIa-q) as a foam-like solid.

**Hydrochloride Salt Formation (IIa-q • HCl).** To a stirred solution of 4-arylpiperidine (IIa-q) in anhydrous methanol or ethanol was added dropwise a solution of HCl in diethyl ether (1.0 M, 1.3–3.0 Eq) at 0°C. The resulting solution was stirred for 15 min and concentrated in vacuo to yield a residue, which was dissolved in a minimum amount of anhydrous methylene chloride. This solution was then added dropwise to anhydrous diethyl ether with swirling to afford, after filtration, a crystalline solid. Most of the corresponding hydrochloride salts formed were highly hygroscopic upon exposure to the air.

All these new compounds were synthesized in the Department of Chemistry, University at Buffalo (Buffalo, NY) and were >98.5% chemically pure as determined by high-performance liquid chromatography analysis.

**Cell Culture.** MCF-7 and MCF-7/adr, used between passages 16 and 24, were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, penicillin (100 units/ml), and streptomycin (10 μg/ml). Cells were incubated at 37°C supplemented with 5% CO2/95% air. Cells were subcultured two to three times a week using 0.05% trypsin-0.53 mM EDTA. Cells were grown in 35-mm2 plastic culture flasks, seeded in 35-mm2 plastic culture dishes for accumulation studies, and seeded in 96-well plates for cytotoxicity studies. Accumulation studies were performed 2 to 3 days after seeding.

**[3H]Vinblastine Accumulation Studies.** Growth medium was removed from monolayer cells and washed twice with sodium buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl2, 1.2 mM MgCl2·6H2O, 10 mM HEPES, pH 7.4). One milliliter of incubation buffer containing 0.05 μM [3H]vinblastine and 3 μM compound (final concentration) was added to the dish and incubated for 2 h. The commercially available (+)-niguldipine was used as a positive control in all studies. The uptake was stopped by aspirating the incubation buffer and washing the cells three times with icd-cold stop solution (137 mM NaCl, 14 mM Tris-base, pH 7.4). One milliliter of 0.5% Triton X-100 or 0.3 N NaOH-1% SDS was added to each dish to lyse the cells. Samples were collected after an hour. Radioactivity was determined by adding 3 ml of scintillation liquid and 150 μl of the lysed sample. A liquid scintillation counter (1900 CA, Tri-Carb liquid scintillation analyzer; PerkinElmer Life and Analytical Sciences, Boston, MA) was used to determine the radioactivity of each sample at 10 min. Protein concentration was determined by the Pierce BCA Protein Assay Kit. Bovine serum albumin was used as the standard.

**Daunomycin Cytotoxicity Assay.** Cells were seeded in 96-well plates at a density of 4000 cells per well. The cells were allowed to attach for 24 h at 37°C, following which additional medium was added to each well containing the desired final concentration of daunomycin (0.003–30 μg/ml) with or without the compounds (3 μM). After a 2-h exposure to the drug, the cells were washed twice with sterile phosphate-buffered saline, and fresh medium was added to each well. The cells were allowed to grow for 4 more days. After 4 days, the total protein was measured by a sulfonfodamine B staining assay (Skehan et al., 1990). Briefly, 10% trichloracetic acid was added to the cells for an hour, rinsed five times with water, and allowed to air dry. Sulfonfodamine B (0.4% w/v in 1% glacial acetic acid) was added to each well for 15 min and washed four times with 1% acetic acid. After drying the plates, protein-bound dye was solubilized in 10 mM Tris-base and quantitated by measuring the absorbance at 570 nm.

**Western Assay for P-Glycoprotein Expression in MCF-7 Cells.** Cells grown in 100 × 15 mm culture dishes were washed with phosphate-buffered saline and harvested using a cell scraper. Total cell lysates were prepared by adding the lysis buffer (20 mM Tris, pH 7.5, 120 mM NaCl, 100 mM sodium fluoride, 1% Nonidet P40 (octylphenoxypolyethoxy ethanol), 200 μM sodium orthovanadate, 50 mM β-glycerol phosphate, 10 mM sodium pyrophosphate, 4 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) to the harvested cells and keeping on an ice bath for 30 min. The soluble extracts were obtained by centrifuging the cell lysate at
13,000g for 20 min. The protein concentrations of the soluble extracts were determined by BCA Protein Assay Kit. Proteins (30 μg) were electrophoresed on 7.5% SDS-polyacrylamide gels and electrophotolysed into nitrocellulose membranes (Invitrogen). Membranes were then blocked overnight at 4°C in Tris-buffered saline containing 0.2% (v/v) Tween 20 and 5% (w/v) fat-free dry milk (Bio-Rad, Hercules, CA). The membranes then were incubated with primary antibody at room temperature for 2 h, followed by incubating with secondary antibody for 1.5 h. C219 (DakoCytomation California Inc., Carpinteria, CA), a monoclonal antibody to P-glycoprotein, was used as primary antibody. Anti-mouse IgG horseradish peroxidase (Amersham Biosciences Inc.) was used as secondary antibody. After incubation with the antibodies, membranes were washed and detected with chemiluminescent ECL detection reagent (Amersham Biosciences Inc.).

Calcium Channel Binding Assay. The calcium channel binding assay was performed based on the method described by Liu et al. (1994). Rat cerebral cortex membranes were prepared by homogenization of rat cerebral cortex using a motor-driven glass-Teflon homogenizer. The homogenate was passed through a double layer of prewetted gauze and centrifuged at 45,000g for 30 min. The resulting pellet was suspended in ice-cold (4°C) Tris buffer (50 mM, pH 7.2–7.4) to yield an approximate concentration of 100 μg/100 μL. Competition binding assays of calcium channel antagonists with [3H]+-PN200-110 (isradipine) were carried out by the method previously established by Bolger et al. (1983). A fixed amount of the membrane protein (~75–100 μg) was incubated with various concentrations of test compounds and a fixed concentration of the radioactive tracer [3H]+-PN200-110 (0.06–0.07 nM) for 4 h at 25°C in 2.5 ml of 50 mM Tris buffer (pH 7.2–7.4). The reaction was terminated by harvesting the membranes on a cell harvester using Whatman GF/B filters (VWR, West Chester, PA), followed by three rapid rinses with 5 ml of ice-cold (4°C) 5 mM Tris buffer. Nonspecific binding was determined in the presence of 10 μM unlabeled nifedipine. Radioactivity trapped on the filters was eluted with scintillation fluid and measured using a liquid scintillation counter. Nifedipine was included as positive control.

Data Analysis. Statistical significance was determined using a one-way analysis of variance followed by Dunnett’s post hoc test. Differences were considered to be significant when p < 0.05. Cell growth inhibition by daunomycin (IC50 value) alone or with the modulators was obtained by fitting the percentage of cell growth (F) by eq. 1 using the computer program WinNonlin (Pharsight, Mountain View, CA).

\[
F = 100 \times \left(1 - \frac{I_{\text{max}} + C}{I_{\text{IC50}} + C}\right)
\]

The F value is the ratio of the cell number after treatment with daunomycin alone or combined with a modulator to the cells that were treated with vehicle as control. C is the concentration of daunomycin. Since most of the test compounds have relatively poor solubility in the incubation buffer, the concentrations of the test compounds required to exert maximum inhibition could not be achieved. Therefore, the whole inhibition profile of the test compounds could not be assessed. Equation 2 was used to calculate the inhibition constant Ki of the test compounds, which represents the competitive calcium channel binding activity of the compounds. The assumption for this equation is that the binding is either competitive or noncompetitive and that the concentration of ligand is much lower than its Ki.

\[
\frac{B}{B_{\text{max}}} = \frac{1}{1 + \frac{I}{K_i}}
\]

B represents the dpm specifically bound in the presence of a test compound; Bmax represents the dpm specifically bound in the absence of a test compound; I is the concentration of an inhibitor (test compound).

Results

Western Blotting Assay for P-Glycoprotein Expression. Both parental human breast cancer MCF/wt (drug-sensitive) cells and resistant MCF/adr cells were analyzed for their P-glycoprotein expression by Western blotting. There was an undetectable amount of P-glycoprotein in MCF/wt cells, whereas high expression in MCF-7/adr cells was observed by loading the same amount of total cell lysate (Fig. 2).

[3H]Vinblastine Accumulation Studies. The 2-h accumulation of 0.05 μM vinblastine in MCF-7/adr cells was measured in the presence of 3 μM 4-aryl-1,4-dihydropyridines (Ia–q) and 4-arylpyridines (IIa–q). As shown in Fig. 3A, all 4-aryl-1,4-dihydropyridine compounds demonstrated a significant increase of intracellular vinblastine concentration either at a significance level of p < 0.001 or p < 0.01 except for compounds Ia, Iq, and Iu. These three compounds demonstrated activity similar to that of the control. The compounds in this series that appear to have the most potent effects are Ia and Iq, leading the series with an approximately 15-fold increase. The synthesized narcemic nigulidine (Iq) displayed the same effect as the commercial sample.

The corresponding 4-arylpyridine analogs (IIa–q) were also evaluated for their inhibitory activities on P-glycoprotein, shown in Fig. 3B. All compounds in this series significantly increased the intracellular vinblastine concentrations in the drug-resistant breast cancer cells. The 4-arylpyridines with effects greater than that of nigulidine were IIa, IIc, IIe, IIo, IIp, IIr, and IIu. Pyridine derivatives IIc, IIp, IIo, and IIu showed stronger inhibitory activity than their precursor 4-aryl-1,4-dihydropyridines, especially IIc, which produced a substantial increase in vinblastine accumulation compared with its corresponding 1,4-dihydropyridine Ia, which had minimal effect. Similarly, IIa and IIp emerged as the most active compounds among the pyridine series of compounds surveyed.

From the above experiments, compounds from both groups (total of seven compounds) were randomly chosen to examine their effects in MCF-7/wt (drug-sensitive) cells. All the compounds chosen had no effect on vinblastine accumulation in MCF-7/wt cells (data not shown).

Daunomycin Cytotoxicity Studies. The effect of increasing concentrations of daunomycin on the inhibition of cell growth in MCF-7/adr cells was examined in the absence and presence of the 4-aryl-1,4-dihydropyridines (Ia–q) and 4-arylpyridines (IIa–q). The IC50 values in MCF-7/adr cells are presented in Table 2. The control studies were performed using the vehicle (0.1% DMSO), whereas nigulidine (3 μM) served as a positive control. All of the compounds that significantly increased vinblastine accumulation were able to significantly decrease the IC50 values of daunomycin. Ia, h1, and h2, which did not affect drug accumulation, also did not lower the IC50 of daunomycin when compared with the control.

The inhibition of cell growth in MCF-7/wt cells (Table 3) was also examined for selected compounds. Selection was based on compounds that had either the greatest or smallest effects on the cellular accumulation of vinblastine. The compounds selected, Ia, Ic, Iq, Ip, Iu, IIc, IIp, and IIu produced IC50 values that were similar to the control value. These data confirm the results of the accumulation studies in the sensitive MCF-7/wt cell line.

Calcium Channel Binding Assay. Thirty new compounds, 18 of them 4-aryl-1,4-dihydropyridines and 12, 4-arylpyridines, were tested for their calcium channel binding activity. Most test compounds...
demonstrated negligible calcium channel binding over the concentration range of 15 nM to 2500 nM using a rat brain cortex membrane preparation. However, dihydropyridine compounds Ie, Ig, Ii, and Ig, and pyridine compounds IIc, IIi, and IIp show some extent of Ca\(^{2+}\)/H\(^{+}\) channel binding. The inhibition constants (\(K_i\)) of test compounds that demonstrated calcium channel binding activity are presented in Fig. 4.

**Discussion**

Despite the discovery of various agents intended to modulate or reverse P-glycoprotein-mediated multidrug resistance, current chemosensitizers in clinical trials are in limited use, largely due to their inherent toxicity or insufficient potency at the tolerated dosage. 1,4-

Dihydropyridines represent the most extensively investigated class of multidrug resistance modulators; these compounds exhibit cardiovascular effects at P-glycoprotein reversal doses due to their calcium channel blocking activities (Tsuruo et al., 1983; Kamiwatari et al., 1989; Futscher et al., 1996). Previous studies have indicated that the calcium channel blocking activity of 1,4-dihydropyridines is independent of their P-glycoprotein-inhibitory effect (Tasaka et al., 2001). The dextro enantiomer of verapamil was reported to be 7- to 10-fold less active on cardiac conduction than the levo enantiomer (Echizen et al., 1985; Ferry et al., 1985); however, the two compounds exhibited comparable potency at reversing P-glycoprotein-mediated drug efflux (Haussermann et al., 1991; Wilson et al., 1995). Dextniguldipine, the
Effect of 4-aryl-1,4-dihydropyridines (series I) and 4-arylpyridines (series II) on daunomycin cytotoxicity in human breast cancer MCF-7/adr (resistant) cells

Studies were performed as described under Materials and Methods. Niguldipine and the test compounds were used at 3 μM concentrations. Significant differences from the control were obtained for all compounds except Ib, Ic, and Ih.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 ± S.E.</th>
<th>Compounds</th>
<th>IC50 ± S.E.</th>
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<tr>
<td>Control (DMSO)</td>
<td>10.55 ± 1.71</td>
<td>Ia</td>
<td>0.99 ± 0.13</td>
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<tr>
<td>Niguldipine</td>
<td>1.09 ± 0.22</td>
<td>Ib</td>
<td>1.00 ± 0.13</td>
</tr>
<tr>
<td>Ia</td>
<td>1.38 ± 0.24</td>
<td>Ic</td>
<td>0.79 ± 0.10</td>
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<tr>
<td>Ib</td>
<td>2.44 ± 0.37</td>
<td>Id</td>
<td>0.92 ± 0.12</td>
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<tr>
<td>Ic</td>
<td>3.28 ± 0.56</td>
<td>Ie</td>
<td>0.81 ± 0.23</td>
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<tr>
<td>Id</td>
<td>3.80 ± 0.64</td>
<td>If</td>
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<tr>
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<td>1.96 ± 0.32</td>
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<td>0.43 ± 2.10</td>
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<tr>
<td>If</td>
<td>14.16 ± 0.97</td>
<td>Ih</td>
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<td>Ig</td>
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<td>13.68 ± 0.68</td>
<td>Ik</td>
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<td>14.76 ± 0.71</td>
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<td>Il</td>
<td>0.58 ± 0.06</td>
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<td>2.18 ± 0.40</td>
<td>In</td>
<td>0.41 ± 0.07</td>
</tr>
<tr>
<td>In</td>
<td>1.33 ± 0.20</td>
<td>Ip</td>
<td>1.18 ± 0.24</td>
</tr>
<tr>
<td>Ip</td>
<td>0.40 ± 0.07</td>
<td>Iq</td>
<td>0.52 ± 0.08</td>
</tr>
<tr>
<td>Iq</td>
<td>0.32 ± 0.05</td>
<td>IIa</td>
<td>0.61 ± 0.77</td>
</tr>
</tbody>
</table>

Effect of 4-aryl-1,4-dihydropyridines (series I) and 4-arylpyridines (series II) on daunomycin cytotoxicity in human breast cancer MCF-7/3Hv (sensitive) cells

Studies were performed as described under Materials and Methods. Niguldipine and the test compounds were used at 3 μM concentrations. No significant differences from the control were obtained.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Estimated IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.29 ± 0.27</td>
</tr>
<tr>
<td>Niguldipine</td>
<td>0.31 ± 0.32</td>
</tr>
<tr>
<td>Ia</td>
<td>0.13 ± 0.09</td>
</tr>
<tr>
<td>Ib</td>
<td>0.13 ± 0.08</td>
</tr>
<tr>
<td>Ic</td>
<td>0.12 ± 0.08</td>
</tr>
<tr>
<td>Id</td>
<td>0.33 ± 0.22</td>
</tr>
<tr>
<td>Ie</td>
<td>0.41 ± 0.38</td>
</tr>
<tr>
<td>If</td>
<td>0.34 ± 0.27</td>
</tr>
<tr>
<td>Ig</td>
<td>0.31 ± 0.21</td>
</tr>
</tbody>
</table>

(−)-enantiomer of niguldipine, has been reported to be a potent chemosensitizer with high binding affinity to P-glycoprotein \( (K_i \approx 10 \text{ nM}) \), whereas its calcium channel-antagonistic activity is negligible (Boer et al., 1989). Additionally, pyridine analogs retain the multidrug resistance-modulatory properties of 1,4-dihydropyridines with no significant calcium channel-channel-blocking activities (Coburn et al., 1989). Furthermore, the pyridine analog PAK-104P provides an example of an effective in vivo modulator of MRPI-mediated multidrug resistance in addition to reversing P-glycoprotein-mediated efflux (Vanhoef et al., 1996; Sumizawa et al., 1997), suggesting that pyridine analogs would be good candidates for multidrug resistance inhibitors.

The purpose of this study was to design and synthesize multidrug resistance inhibitors through systematic modifications of the lead compound, dexniguldipine, which was reported to modulate multidrug resistance by direct interaction with P-glycoprotein (Hofmann et al., 1995). The initial design of the target compounds was therefore focused on the modifications of certain key structural features, including the following two groups of 4-aryl-1,4-dihydropyridines and corresponding analogs as shown in Table 1.

Racemic 4-Aryl-1,4-dihydropyridines (Series I). Various functionalities at the 4-phenyl ring were modified on the basis of systematic structure-activity relationship (SAR) studies among 4-ary1-1,4-dihydropyridines as calcium channel blockers and P-glycoprotein modulators.

Aromatized Series I Products, 4-Arylpyridines (Series II). Modifying the 1,4-dihydropyridine template structures leads to a substantial loss of calcium channel binding activity, and possible enhancement of P-glycoprotein inhibition.

In the present study, the new compounds (series I and II) were synthesized and their P-glycoprotein-inhibitory activities were evaluated in human breast cancer MCF-7 cells. Fifteen of eighteen compounds in series I were able to increase vinblastine accumulation in P-glycoprotein overexpressing MCF-7/adr cells, while having little effect on vinblastine retention in wild-type MCF-7/wt cells. The results suggested that the compounds restored the intracellular drug accumulation by inhibiting P-glycoprotein efflux activity. Compounds Ia and Ib from series I demonstrated a striking 15-fold increase of vinblastine accumulation in P-glycoprotein-overexpressed MCF-7/adr cells. Similar results were obtained in the series II compounds, where compounds IIa and IIb showed the greatest P-glycoprotein inhibition compared with the other test compounds. Interestingly, pyridine derivative IIc demonstrated stronger inhibitory effects than did its corresponding precursor Ic, producing a 7-fold increase of vinblastine accumulation, whereas its 1,4-dihydropyridine precursor Ic had no significant effect on P-glycoprotein in resistant MCF-7/adr cells. The result suggested that oxidation of the 1,4-dihydropyridine ring to the corresponding pyridine template may favor P-glycoprotein interaction.

It might be possible that the observed effects of these new compounds on intracellular vinblastine accumulation could be partly due to inhibition of MRPI or the recently identified BCRP. However, the possibility of significant MRPI or BCRP inhibition can be ruled out by our Western blot results. Neither MRPI nor BCRP protein expression was detected in MCF-7/adr cells (data not shown), whereas a substantial amount of P-glycoprotein was detected in this resistant MCF-7 cell line. In addition, vinblastine is not a substrate for BCRP (Litman et al., 2000). Based on these facts, we conclude that the increase of intracellular retention of vinblastine in resistant MCF-7/adr cells was due to the inhibition of drug efflux mediated by P-glycoprotein.

Results from the daunomycin cytotoxicity assays are in agreement with those from the \([3H]\)vinblastine accumulation assays. Most test compounds were able to substantially lower the IC50 values of daunomycin in MCF/adr cells except compounds Ia and Ib, which exhibited no significant effect on either daunomycin cytotoxicity or vinblastine accumulation.

Several SAR studies have been conducted for 1,4-dihydropyridine analogs (Coburn et al., 1988; Kawashima et al., 1993) with regard to their calcium channel blocking effect. The proposed structural requirement for calcium channel binding may be summarized as follows: 1) The 1,4-dihydropyridine ring is essential, and oxidation of the 1,4-dihydropyridine core structure to the corresponding pyridines leads to a significant loss of activity. 2) An aryl substituent, preferably a substituted phenyl group, appears optimum at the 4-position of 1,4-dihydropyridines. A para substitution in the 4-phenyl ring leads to invariable loss of calcium channel blocking activity regardless of substituent type (electron-releasing groups or electron-withdrawing groups), whereas ortho or meta substitutions in the phenyl ring at the 4-position are requisite for the strong calcium-antagonistic activity.

The results from our calcium channel binding study are generally in accordance with these SAR studies. Compounds Ia, Ib, Ic, and Id demonstrated some calcium channel binding based on the rat brain cortex membrane preparations. Based on the structures of these compounds, it appears that compounds with bulky para substituents in the
4-phenyl ring have relatively higher binding activity compared with smaller group substitution in the same position. Interestingly, both compounds IIi and its precursor Ii demonstrated calcium channel binding, suggesting that bulky electron-deficient groups may not be favored to reduce the calcium channel binding. In this study, the calcium channel binding activity of the compounds was not correlated with their P-glycoprotein modulation effect.

The results from vinblastine accumulation, daunomycin cytotoxicity, and calcium channel binding assays demonstrate that this series of synthesized 1,4-dihydropyridines and pyridines have potent P-glycoprotein-inhibitory effect but negligible or low calcium channel binding. These findings suggest that the synthesized 1,4-dihydropyridines and analogs may have potential as P-glycoprotein reversal agents for clinical chemotherapy.

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References


References


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