

## EFFECTS OF R-ENANTIOMER (GR66234A) AND L-ENANTIOMER (GR66235A) OF TELUPIDINE, A NEW DIHYDROPYRIDINE DERIVATIVE, ON CELL LINES DISPLAYING THE MULTIDRUG RESISTANT PHENOTYPE

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### ABSTRACT

**Background.** Many dihydropyridine analogues with calcium channel blocker activity are able to reverse multidrug resistance (MDR). We studied the daunorubicin resistance reversing activity of the R enantiomer (GR66234A) and the L-enantiomer (GR66235 A) of telupidine, a new lipophilic calcium channel blocker synthesized by Glaxo.

**Methods.** The daunorubicin resistance reversing activity of the enantiomers of telupidine was evaluated in two MDR cell lines: ARNII, an erythroleukemia cell line which expresses p-glycoprotein, and MCF 7/R, a breast cancer cell line with p-glycoprotein and high levels of glutathione S transferase (GST) and glutathione peroxidase (GSH Px).

**Results.** GR66234A and GR66235A show the same activity in reversing daunorubicin resistance and are more effective than verapamil. The difference in activity between verapamil and the enantiomers of telupidine is greater in ARNII cells than in MCF 7/R cells. Nevertheless, there are no significant differences in cellular daunorubicin accumulation between ARNII and MCF 7/R following exposure to telupidine, nor are there differences in intracellular daunorubicin distribution in the presence of either MDR reversing agent.

**Conclusions.** The low calcium channel antagonistic activity of GR66234A suggests that this compound may be useful in combination with chemotherapy in MDR malignancies.

*Key words:* multidrug resistance, p-glycoprotein, dihydropyridine analogues

The resistance of cells to chemotherapeutic drugs is a major obstacle to successful cancer chemotherapy. Multidrug resistance (MDR) is a phenomenon in which cells selected for resistance to a single compound show cross-resistance to a variety of structurally unrelated lipophilic drugs.<sup>1,2</sup>

The MDR phenotype in neoplastic cells is the result of the expression of the *mdr1* gene,<sup>3</sup> which encodes a membrane glycoprotein called p-glycoprotein or p-170.<sup>4</sup> P-glycoprotein acts as an ATP-dependent efflux pump, transporting

drugs out of MDR cells and thus preventing intracellular drug accumulation.

A wide variety of unrelated agents have been shown to reverse MDR and increase cytotoxic drug accumulation in MDR cells.<sup>5</sup> Recent studies have shown that there are at least two different drug binding sites on p-glycoprotein: one for Vinca alkaloids, verapamil and cyclosporin A, and a second for azidopine, a dihydropyridine calcium channel blocker.<sup>6</sup>

Several dihydropyridine compounds are able to reverse MDR. There does not appear to be a

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direct relationship between the calcium channel blocking activity of these compounds and their ability to reverse drug resistance, but a correlation between the presence of a cationic nitrogen atom in the molecules and MDR reversing activity seems to be important.<sup>7</sup> Some dihydropyridine derivatives are more effective than verapamil in enhancing the antitumoral activities of anthracyclines and Vinca alkaloids *in vitro*. Also, in terms of possible side effects *in vivo*, some specific molecular types of dihydropyridine derivatives with low calcium channel blocker activity could have less toxic effects than verapamil at plasmatic concentrations able to reverse MDR.<sup>8</sup>

In this paper we report data on the MDR reversing activity of the R-enantiomer (GR-66234A) and the L-enantiomer (GR66235A) of a dihydropyridine derivative called *teludipine*.

Teludipine is less cardiotoxic than verapamil and contains a butyl group in the aromatic ring that makes the molecule more lipophilic than other dihydropyridine derivatives (Figure 1). R-enantiomer GR66234A has 100 times lower calcium channel blocking activity than GR66Z35A.

## Materials and Methods

### Cells

Mouse erythroleukemic-sensitive cells FLC<sup>9</sup> and mouse erythroleukemic-resistant cells ARNII<sup>10</sup> were grown in suspension culture using RPMI 1640 medium containing 10% fetal calf serum (FCS), 1% penicillin and streptomycin. WT MCF-7 is a human sensitive breast

cancer cell line (MCF-7/S), and Adr<sup>R</sup> MCF-7 (MCF-7/R) is a MDR subline derived from WT MCF7 by escalating adriamycin exposure.<sup>11</sup> Both parent and resistant MCF-7 cell lines were maintained in RPMI 1640 supplemented with 10% FCS and fed 3 times a week to maintain logarithmic growth (monolayers were trypsinized once a week). The resistant sublines were grown for a minimum of 6 passages in drug-free medium before testing.

### Cytotoxicity assays

Tumor cell cytotoxicity assays were conducted by seeding  $2 \times 10^5$  cells (ARNII) or  $4 \times 10^4$  cells (MCF-7/R) in 1 mL of complete medium in 16 mm wells (tissue culture cluster 24 wells, Costar). Drugs were added immediately after, and cells were incubated at 37°C in a CO<sub>2</sub> atmosphere. After 48 hours for ARNII and 96 hours for MCF-7/R the number of cells was evaluated (after trypsinization for MCF-7/R cells) using an Ortho ELT -8/WS counter (Ortho Diagnostic System Inc.). The percent of cells which excluded trypan blue was also determined. Cell growth inhibition was expressed as percent of control proliferation.

### Flow cytometric quantification of p-glycoprotein

Cells ( $10^6$ ) were suspended in 70% cold methanol and incubated at -20°C for 10 minutes to permeabilize the cell membrane. The efficiency of fixation was determined by staining with trypan blue. Fixed cells were washed twice in PBS by centrifugation at 200 g and -4°C for 5 minutes, and they were resuspended in PBS containing 20% FCS at a concentration of  $10^6$  cells/mL. Aliquots (200 uL) of each cell suspension were incubated with 10 uL of fluorescein-labelled C-219 murine monoclonal antibody (P-glycoCHEK<sup>TM</sup> C-219) and in a separate tube with 10uL of negative antibody (P-glycoCHEK<sup>TM</sup> negative antibody) at 4°C for 60 minutes.

After 2 washings in PBS/FCS the cells were resuspended in PBS for flow cytometry assay.

### Cellular daunorubicin incorporation studies

Cells ( $1 \times 10^6$ ) were incubated with 2 ug/mL daunorubicin with or without verapamil or

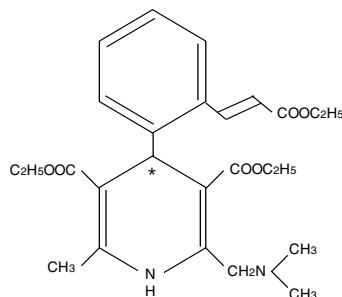


Figure 1. Chemical structure of teludipine.

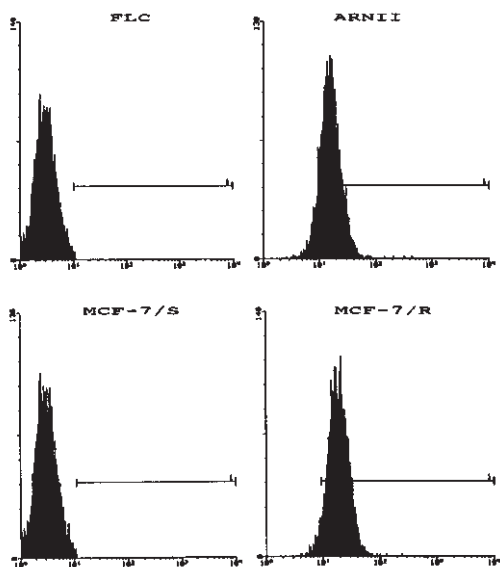


Figure 2. p-glycoprotein expression in FLC, ARNII, MCF-7/S, and MCF-7/R cells using flow cytometric analysis with murine monoclonal antibody C-219.

teludipine at concentrations of 1.25, 2.5, 5, and 10  $\mu\text{M}$ . After 60 minutes of incubation, cells were washed twice and resuspended in 2 mL of culture medium. Intracellular daunorubicin accumulation was measured by flow cytometric analysis and expressed as mean fluorescence. A FACscan (Becton Dickinson) equipped with an argon laser using a 428 nm line operating at 15 mW was used. Daunorubicin distribution in the nucleus and cytoplasm was evaluated with a fluorescence microscope (Leitz Orthoplan) equipped with a superpressure mercury lamp and a 570 nm filter.

#### Chemicals

GR66234A and GR66235A were synthesized by Glaxo Research Center (Verona, Italy). Daunorubicin was obtained from Farmitalia (Milano, Italy), and verapamil from Knoll AG-Liestal (Switzerland). C-219 monoclonal antibody was purchased from CIS, Centocor (Malvern, USA).

#### Statistics

Points reported in all figures represent the

mean of three independent experiments, and bars represents the SE.

#### Results

Figure 2 shows that ARNII and MCF-7/R cells express p-glycoprotein. No p-glycoprotein-related fluorescence was detected in the parental FLC and MCF-7/S cells. Background fluorescence was determined by incubation with the control monoclonal p-glycoCHEK™ negative antibody.

The percentage of cells expressing p-glycoprotein was 90% for ARNII and 97% for MCF-

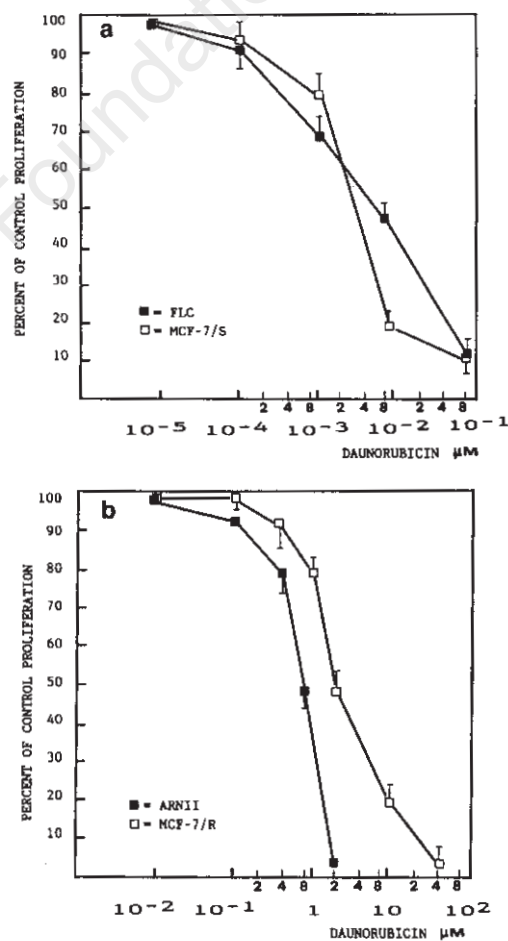


Figure 3. Comparative cytotoxicity of daunorubicin in FLC (a), ARNII and MCF-7/R cells (b). The number of cells was determined after 48 hours of continuous exposure to daunorubicin in leukemic cells and after 96 hours in breast tumor cells.

7/R cells. IC<sub>50</sub> values for daunorubicin by continuous exposure were 0.8  $\mu$ M for ARNII cells, and 2  $\mu$ M for MCF-7/R cells (Figure 3).

Figure 4 shows the effects of verapamil, GR66234A and GR66235A in combination with daunorubicin at concentrations able to inhibit cell growth by 20% (IC<sub>20</sub>). The IC<sub>20</sub> values for daunorubicin were 0.4  $\mu$ M and 1  $\mu$ M for MCF-7/R cells. The concentrations of verapamil, GR66234A and GR66235A used were 0.125  $\mu$ M, 0.25  $\mu$ M, 0.5  $\mu$ M and 1  $\mu$ M.

GR66234A and GR66235A show identical activity in reversing daunorubicin resistance and are more active than verapamil. In ARNII

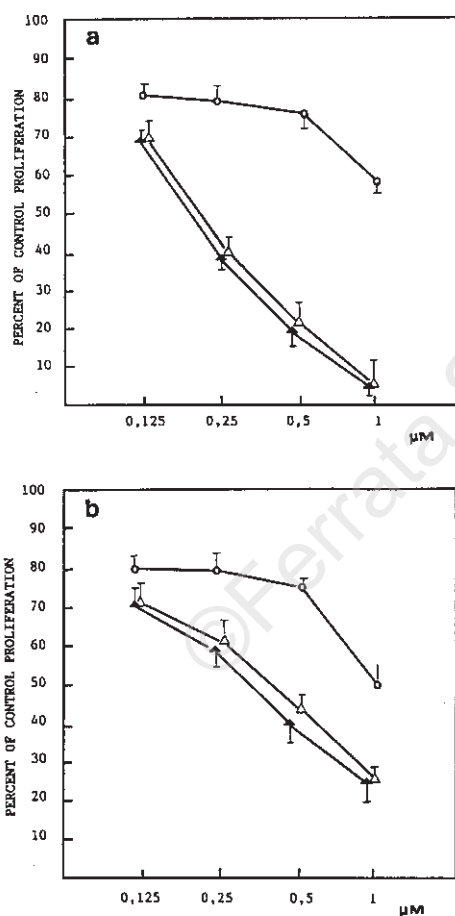


Figure 4. Growth inhibition of ARNII (a) and MCF-7/R cells (b) by daunorubicin (IC<sub>20</sub>) in the presence of verapamil (O), GR66234A ( $\Delta$ ) and GR66235A ( $\blacktriangle$ ). IC<sub>20</sub> daunorubicin was 0.4  $\mu$ M for the ARNII cells and 1  $\mu$ M for the MCF-7/R cells.

cells, 0.15  $\mu$ M GR66234A shows daunorubicin reversing activity similar to 1  $\mu$ M verapamil. In MCF-7/R cells the difference is less marked, and 0.4  $\mu$ M GR66234A have about the same activity as 1  $\mu$ M verapamil. Verapamil and teludipine, used without daunorubicin, do not show cytotoxic effects in sensitive and resistant cells (Figure 5). Daunorubicin accumulation is correlated to the concentration of each MDR reversing agent used (Figure 6). In ARNII cells, 1.25  $\mu$ M teludipine determine a cellular daunorubicin accumulation similar to that obtained with 10  $\mu$ M verapamil. We observed analogous values of daunorubicin accumulation in MCF-7/R cells. Intracellular accumulation of daunorubicin in ARNII and MCF-7/R cells incubated without MDR reversing agents is very low, and the distribution of daunorubicin is prevalently in the cytoplasm. When resistant cells were simultaneously incubated with daunorubicin and verapamil or teludipine, daunorubicin accumulation was more evident and mostly localized in the nucleus, with a distribution pattern similar to that observed in sensitive FLC and MCF-7/S cells. There were no significant differences in intracellular daunorubicin distribution between ARNII and MCF-7/R cells.

### Discussion

Several calcium channel blockers, such as verapamil, niludipine and diltiazem, have MDR reversing activity.<sup>12</sup> They increase antitumor drug concentration in MDR cells and consequently restore the drug sensitivity of these cells.

Verapamil is a calcium antagonist used in several combination therapies with anthracyclines or Vinca alkaloids.<sup>13</sup> However, the concentrations of verapamil needed to overcome MDR *in vivo* cause serious cardiovascular side effects due to its calcium antagonistic activity. Recently, the R-enantiomer of verapamil attracted much attention as a chemosensitizer on MDR tumor cells. R-verapamil has low calcium antagonistic activity and presents MDR reversing activity similar to the racemic compound.<sup>14</sup>

Yoshinari et al. have synthesized a series of new dihydropyridines called BS compounds.<sup>8</sup>

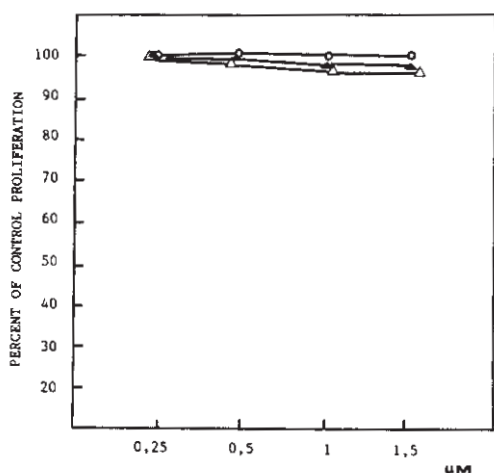


Figure 5. Effects of verapamil (O), GR66234A (Δ) and GR66235A (▲) in ARNII cells.

At equimolar concentrations BS300, -304, and -309 enhanced the antitumor activity of adriamycin on MDR cells more strongly than verapamil, whereas their relaxation effects on arteries were weaker than that of verapamil.

Safa et al.<sup>15</sup> and Yang et al.<sup>16</sup> reported that the dihydropyridine derivative <sup>3</sup>H-azidopine photolabels p-glycoprotein and that the labeling is inhibited by vinblastine and some calcium channel blockers.

Kamiwatari et al.<sup>7</sup> screened a series of newly synthesized dihydropyridine analogues (PAK) for their ability to reverse MDR in human KB cells. PAK-1 has weaker calcium blocking activity than PAK-5 and nifedipine, but completely reverses drug resistance. PAK-5 and nifedipine are better at blocking calcium channels than PAK-1, but they only partially reverse the resistance. Therefore calcium channel blocking activity does not seem to be necessary to allow reversing agents to overcome MDR.

In the present work we studied the R- and L-enantiomers of teludipine, a dihydropyridine compound with higher lipophilia and lower cardiac toxicity than verapamil. The R-enantiomer GR66234A has about 100 times less calcium channel blocking activity than the L-enantiomer GR66235A. In the cell lines studied,

GR66234A and GR66235A showed higher daunorubicin reversing activity than verapamil. The enhanced cytotoxic effect of daunorubicin on MDR cells in the presence of GR66234A and GR66235A is correlated with greater cellular drug accumulation. There is also a correlation between the concentrations of each MDR reversing agent and daunorubicin accumulation. However, we observed a discrepancy between cytotoxicity data and daunorubicin accumulation with respect to ARNII and MCF-7/R cells: to obtain the same daunorubicin cytotoxic effect, it was necessary to use a concentration of verapamil 6.6 times higher than teludipine in ARNII cells and 2.5 times greater in MCF-7/R cells. Conversely, to obtain the same daunorubicin accumulation it was necessary to use a concentration of verapamil about 8 times higher than teludipine in both ARNII and MCF-7/R cells. This phenomenon is not correlated with a different intracellular distribution of daunorubicin.

Schisselbauer et al.<sup>10</sup> observed that ARNII cells have levels of GSH and GSH reductase that are essentially unchanged, and total GSH-Px and GST activities that are slightly elevated with respect to the FLC line.

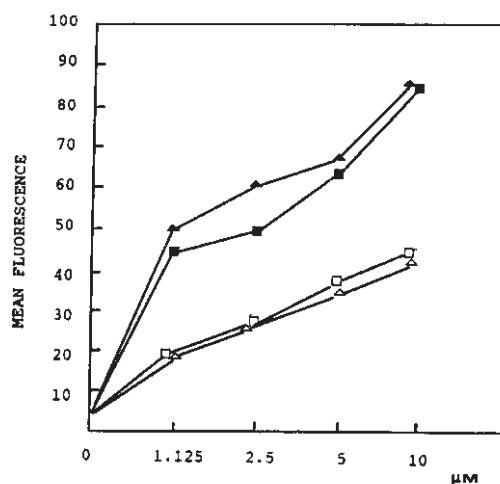


Figure 6. Daunorubicin accumulation as measured by flow cytometric analysis after simultaneous incubation with daunorubicin+verapamil and daunorubicin+teludipine. There are no differences between GR66234A and GR66235A. □=DNR+VER in ARNII; Δ=DNR+VER in MCF-7/R; ■=DNR+TLD in ARNII; ▲=DNR+TLD in MCF-7/R.



Batist et al.<sup>11</sup> demonstrated that MCF-7/R cells present higher levels of GST and selenium-dependent GSH-Px than MCF-7/S, as well as an enhanced capacity to reduce GSH after the peroxide challenge. GST is an important enzyme implicated in resistance to chemotherapeutic agents.<sup>17</sup> These data could explain, at least partially, the discrepancy between cytotoxicity data and the daunorubicin accumulation observed in ARNII and MCF-7/R cells.

Another interesting dihydropyridine calcium channel blocker with MDR reversing activity is the (-) isomer of niguldipine (B859-35). It has 40 times lower calcium channel blocker activity than niguldipine and shows about 10 times more MDR reversing activity than verapamil.<sup>19</sup> There are also preliminary *in vivo* results with B859-35 associated with polychemotherapy in acute myelogenous leukemia (AML) in relapse,<sup>20</sup> and in breast cancer.<sup>21</sup> The enantiomers of teludipine show MDR reversing activity in ARNII cells similar to that observed with B859-35 in other cell lines. B859-35 also shows cytotoxic activity when used without other cytotoxic drugs; the enantiomers of teludipine do not show cytotoxic activity.

In conclusion, our results show that the R- and L-enantiomers of teludipine are more effective than verapamil in reversing MDR. The low calcium channel antagonistic activity of GR 66234A suggests that this compound may be useful in combination with chemotherapy in MDR malignancies. We are now investigating the MDR reversing activity of GR66234A in mice bearing tumors.

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