Synergistic Effect of Prochlorperazine and Dipyridamole on the Cellular Retention and Cytotoxicity of Doxorubicin

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ABSTRACT

Incubation of drug-resistant human tumor cells with a combination of prochlorperazine and dipyridamole has additive/synergistic effect on the cellular retention and cytotoxicity of doxorubicin. In patients administered a fixed dose of doxorubicin and prochlorperazine with escalating doses of dipyridamole, mean plasma levels of dipyridamole and prochlorperazine achieved were as high as 3.01 ± 0.41 µM and 0.94 ± 0.09 µM, respectively. Plasma samples from patients were analyzed in an in vitro assay to monitor the effect on the cellular retention of tritium-labeled daunorubicin in MDR1-transfected P388 cells. In 22 of 49 of the plasma samples analyzed, the daunorubicin in efflux blocking activity was one-half or greater than that of cells incubated with 12.5 µM verapamil, a well-known efflux blocker. These observations suggest that a combination of prochlorperazine and dipyridamole may enhance cellular doxorubicin retention by blocking efflux while reducing normal tissue toxicity and unwanted side effects in vivo.

INTRODUCTION

Tumor cell resistance to doxorubicin may involve altered drug retention and/or biochemical mechanisms such as xenobiotic detoxification (1, 2). The role of drug efflux as a major mechanism for cellular resistance to doxorubicin has been elucidated, and several unrelated drugs (e.g., verapamil, phenothiazines, and cyclosporins) have been used for blocking efflux and enhancing retention and chemosensitivity to doxorubicin (3–5). Clinical trials have been carried out on the use of efflux blockers to enhance drug retention and response in patients with a variety of malignant diseases (6–9). In general, most of these protocols have either failed to achieve plasma efflux blocker concentrations high enough to enhance drug retention or caused major alterations in the pharmacokinetics and elimination of the anti-tumor drug (8, 9).

We have reported earlier that phenothiazines such as prochlorperazine and trifluoperazine enhance cellular retention of doxorubicin in tumor cells, which are insensitive to the efflux blocking activity of verapamil (5, 10). In Phase I clinical trials of prochlorperazine (administered i.v. for 15 min with 60 mg/m² of doxorubicin), we established the MTD of prochlorperazine to be 75 mg/m². In some of the patients on this protocol, plasma concentrations of prochlorperazine achieved were higher than 1 µM. In patients administered an i.v. infusion of prochlorperazine for 2 h (with 60 mg/m² doxorubicin), the MTD of prochlorperazine was 180 mg/m². Prochlorperazine levels >0.6 µM were sustained for 24 h in all patients treated at 135 mg/m² (11, 12).

In a Phase I trial of escalating doses of i.v. dipyridamole administered for 72 h concurrent with the i.v. infusion of 60 mg/m² doxorubicin, the MTD of dipyridamole was 50 mg/kg (13). Most of the patients treated with dipyridamole doses of >17.5 mg/kg had sustained dipyridamole plasma levels of >5 µM.

The pharmacokinetic evaluation of these two doxorubicin efflux blockers suggested that although peak plasma levels of prochlorperazine at the MTD were low, the decay in plasma levels of this efflux blocker was slow. In contrast, peak plasma levels of dipyridamole achieved were high but decayed rapidly at the end of infusion.

Several earlier studies suggest that combinations of certain efflux blockers could have synergistic effects on doxorubicin retention and cytotoxicity (14–17). In contrast to the use of single efflux blockers at high concentrations, the use of additive/synergistic efflux blocker combinations could overcome heterogeneity as well as reduce toxicity to normal tissues.

The present study was designed to determine whether: (a) the additive/synergistic effect of prochlorperazine and dipyridamole on doxorubicin retention and cytotoxicity can be seen in drug-resistant human nonhematological tumor cells; (b) the peak plasma levels and pharmacokinetics of doxorubicin, prochlorperazine, and dipyridamole in patients administered fixed doses of doxorubicin and prochlorperazine with escalating doses of dipyridamole will be sufficient to enhance cellular doxorubicin retention in vivo; and (c) the efflux blocking activity of plasma from patients on the efflux blocker combination protocol can be seen in MDR1-transfected cells in vitro.

The abbreviations used are: MTD, maximum tolerated dose; P-gp, P-glycoprotein; HPLC, high-performance liquid chromatography; CI, combination index; AUC, area under the curve; MDR, multidrug resistance.

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MATERIALS AND METHODS

Cell Lines. P388/R84, a doxorubicin-resistant murine cell line, was cultured in RPMI 1640 containing 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μM 2-mercaptopethanol at 37°C in a humidified atmosphere of 5% CO₂. The P388/R84 cells are ~80-fold more resistant to doxorubicin than the parental P388 cells (2). Doxorubicin resistance in this cell line (IC₅₀, 2.5 μM) is multifactorial and involves efflux, enhanced detoxification, altered topoisomerase activity, and reduced DNA damage and enhanced repair (2, 18). For monitoring the effect of plasma (from patients on efflux blocker protocols) on H₃-labeled daunorubicin (daunomycin) retention, P388 leukemia cells transfected with the human MDR gene were used (17).

The SW620/Ad300 human colon cancer cell line established by stepwise exposure to doxorubicin is ~76-fold more resistant to doxorubicin than the parental line (SW620), and P-gp-related drug efflux seems to be the major mechanism responsible for its doxorubicin resistance (19). The SW620/Ad300 cells were cultured in the RPMI 1640 with serum, antibiotics, and 0.5 μM doxorubicin. Cells were grown in doxorubicin-free medium for 7 days before their use in experiments.

Reagents and Drugs. Doxorubicin (Adriamycin hydrochloride, NSC-123127; Adria Labs, Columbus, OH), prochlorperazine edisylate (Smith Kline and Beecham Laboratories, Philadelphia, PA), dipyridamole (Persantine; Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT), and chlorpromazine hydrochloride (Sigma Chemical Co., St. Louis, MO) were purchased. Daunorubicin was obtained from the Investigational Drug Branch, National Cancer Institute, Bethesda, MD.

To determine the effect of the doxorubicin alone or in combination with the efflux blockers, 10⁷/mL cells from logarithmic cultures were incubated at 37°C with the different drug concentrations in an atmosphere of 95% air and 5% oxygen. After 1 h, cells were centrifuged, washed twice in tissue culture medium, and reincubated for 24 h in 16-well plates. Aliquots were removed and stained with trypan blue, and the number of dye-excluding (viable) cells was counted in a hemocytometer.

Soft Agar Assays. SW620 or SW620/Ad300 tumor cells were incubated with doxorubicin alone or in combination with the efflux blockers for 2 h at 37°C in an atmosphere of 5% carbon dioxide and 95% air. Cells retrieved by centrifugation were washed with tissue culture medium (1×), mixed with 0.3% agar (final cell concentration, 0.25 × 10⁶/ml), and layered on a preformed layer under layer of 0.5% agar in multiwell culture plates (each drug concentration was tested in triplicate). The culture plates were incubated at 37°C for 7 days (for P388 cells) or 14 days (SW620 and Ad300 cells) in an atmosphere of 5% CO₂ and 95% air. Colonies containing more than five cells across (in one dimension) were counted under an inverted microscope.

H₃-Labeled Daunorubicin Retention. Studies on P388 cells transfected with the human MDR gene were carried out in the Biological Chemistry Department of the Hebrew University by Dr. Stein and his colleagues. Transfected P388 cells (2 × 10⁶) grown in RPMI 1640 with serum and antibiotics (17) were incubated with 45 μCi of plasma collected and shipped to Israel from patients on the efflux blocker combination protocol in Miami. Verapamil (12.5 or 25 μM) was added to the control cultures as a daunorubicin efflux blocker. After the addition of 2.5 μl of H₃-labeled daunorubicin, cells were incubated at 37°C for 1 h. The cell pellet retrieved by centrifugation was resuspended in 10% Triton X-100, and radioactivity was determined by liquid scintillation counting in a Beckman liquid scintillation counter.

Laser Flow Cytometric Studies. Excitation from a 488-nm argon laser line was used to analyze cellular doxorubicin or daunorubicin fluorescence of tumor cells. Details of our flow cytometric procedures for the quantitation of cellular anthracycline fluorescence have been reviewed earlier (20).

Protocol Administration and Drug Analysis. Patients selected for administration of the efflux blocker combination with doxorubicin were put on a University of Miami Institutional Review Board-approved protocol. Criteria for eligibility or exclusion from the study were strictly adhered to as described in our earlier publications (11, 12). Informed consent was obtained from all patients prior to the start of therapy. Doxorubicin (60 mg/m²) was administered as a 15-min i.v. infusion, followed immediately by i.v. administration of a fixed dose of prochlorperazine (135 mg/m²) and escalating doses of dipyridamole (0.3–1.5 mg/kg body weight) for 120 min. Peripheral blood (4.5 ml) was collected by venipuncture and centrifuged at 1000 rpm for 8 min in a tabletop clinical centrifuge. Plasma was transferred to polypropylene tubes with a plastic Pasteur pipette and stored at −20°C. Plasma samples were thawed, vortexed, and briefly sonicated before analysis.

For HPLC analysis, 3-ml disposable extraction cartridges (Bakerbond solid phase octadecyl; J. T. Baker, Inc., Philipsberg, NJ) conditioned by sequential washing with column volumes of 100% methanol, 25% methanol in HPLC grade water and 0.05 M phosphate buffer (pH 8.5) were used. After addition of 20 μl of internal standards (100 ng/ml daunorubicin and chlorpromazine), 0.5 ml of the plasma was pipetted into the conditioned cartridge and washed with 2 ml of 10% methanol in HPLC grade water and 2 ml of hexane. A Supelco vacuum manifold was used to control the flow rate at 1–2 ml/min. The cartridge was eluted three times with 1 ml of chloroform:methanol (2:1, v/v), and the eluant was evaporated under nitrogen at 45°C. The residues were reconstituted in 200 μl of methanol, and 20–40 μl of the sample were injected into the HPLC column. Details of our method for simultaneous measurement of plasma doxorubicin and prochlorperazine content were reported earlier (21).

Statistical Analysis. WinNonlin statistics program (Scientific Consulting, Inc., Apex, NC) was used for modeling of the pharmacokinetic parameters and to determine the optimum fit from the diagnostic factors such as the Akaika Information Criterion and the Schwartz Criterion (22). The hybrid coefficients (A, B, and C) and hybrid exponents (α, β, and γ) for the secondary parameters including the initial distribution and terminal elimination half-life, the total volume of distribution at steady state, the total volume of clearance, and the area under the curve were calculated by the WinNonlin program. The method of Gauss-Newton with Levenberg and Hartley modification (22) carried out the minimization modeling of nonlinear regression on pharmacokinetic data. The increment for partial derivatives was 0.001, and convergence criteria was 0.0001, at which it was assumed that the method had converged to the minimum sum of squares of the deviations between the ob-
served values and the values predicted by the model within 50 iterations. Plasma doxorubicin, prochlorperazine, and dipyridamole content values were fitted into the WinNonlin software program using weighted nonlinear least-square estimation regression analysis for the model discrimination and parameter estimation. A three-compartment model of i.v. bolus drug administration provided the best fit for the plasma doxorubicin concentration data. A one-compartment model of i.v. infusion had the best fit for the plasma dipyridamole and prochlorperazine data. CalcuSyn Software (BioSoft, Ferguson, MO) was used to analyze data from clonogenic assays of cells exposed to doxorubicin alone or in combination with the efflux blockers to determine additive or synergistic effects. The CI equation in CalcuSyn is based on the multiple drug-effect equation of Chou and Talalay (24) and defines synergism as a more-than-expected additive effect and antagonism as a less-than-expected additive effect. Chou and Talalay defined a CI of $<1$, $1$, and $>1$ as synergism, additive, or antagonism, respectively.

RESULTS

Effect of Efflux Blocker Combinations on Cellular Retention of Doxorubicin and Daunorubicin. Laser flow cytometric detection of cellular anthracycline fluorescence was used to monitor the effect of efflux blockers (alone or in combination) on the cellular fluorescence of doxorubicin and daunorubicin. Although cellular daunorubicin fluorescence appears more rapidly than that of doxorubicin, the effect of the efflux blockers used alone or in combination on the cellular retention of these two anthracyclines is similar (25, 26). In dot plots of Fig. 1, we have compared the cellular drug fluorescence of P388/R-84 cells incubated with daunorubicin alone (single arrow) or in the presence of efflux blockers (double arrows) with prochlorperazine (10 μM, A), dipyridamole (10 μM, B), or 5 μM each of prochlorperazine and dipyridamole.

Fig. 1 Scattergrams of daunorubicin cellular retention (fluorescence on X axis, four decade log scale) and forward angle scatter (Y scale, linear axis) of drug-resistant P388/R-84 cells incubated with daunorubicin (2 μM) alone (single arrow) or in combination (double arrows) with prochlorperazine (10 μM, A), dipyridamole (10 μM, B), or 5 μM each of prochlorperazine and dipyridamole.
additive and essentially similar to that of 10 μM of the either blockers used alone. Data from direct excitation of the cellular daunorubicin fluorescence by laser excitation shown in Fig. 1 was confirmed by HPLC analysis of cellular daunorubicin content, as shown in Fig. 2.

Cytotoxicity of Doxorubicin, Prochlorperazine, and Dipyridamole. The IC_{50} of doxorubicin in SW620 cells was ~0.1 μM, whereas the SW620/Ad300 cells were ~40-fold more resistant with IC_{50} of 4 μM (Fig. 3, A and B). In contrast to doxorubicin (Fig. 3, A and B), there was no significant difference in the cytotoxicity of prochlorperazine or dipyridamole in SW620 versus SW620/Ad300 cells (Fig. 3, C and D). Concentrations of >20 μM prochlorperazine were toxic to both the cell lines, whereas dipyridamole concentrations of <80 μM were relatively nontoxic.

Effect of Efflux Blocker Combinations on the Cytotoxicity of Doxorubicin. In P388 cells, prochlorperazine (5–12 μM) or dipyridamole (5–10 μM), used alone or in combination, did not alter doxorubicin cytotoxicity (data not shown). In contrast, coincubation of the doxorubicin-resistant P388/R84 cells with a combination of prochlorperazine and dipyridamole had a highly additive/synergistic effect on the cytotoxicity of doxorubicin. Data from soft agar clonogenic assays were analyzed by CalcuSyn software (24) for the determination of additive, antagonistic, or synergistic effects of the various combinations of doxorubicin (1.0–4.0 μM) with prochlorperazine (1.5–7.5 μM) and dipyridamole (0.5–10 μM). The dose effect (Fig. 4A) and the CI plots (Fig. 4B) show that most of the prochlorperazine and dipyridamole combinations tested had highly synergistic effects on the cytotoxicity of doxorubicin, as indicated by the CI values of <1.0 (Fig. 4B).

SW620 Human Colon Cancer Cells. Dose effect and CI plots in Fig. 4, C and D, show that in this parental drug-sensitive human solid tumor cell line, coincubation with the efflux blocker combinations did not significantly alter doxorubicin.

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**Fig. 3** Data from soft agar clonogenic assay of human SW620 (A and C) and the doxorubicin-resistant SW620/Ad300 (B and D) cells. SW620/Ad300 cells were ~40-fold more resistant to doxorubicin (A and B) than the parental cell line. However, SW620/Ad300 cells were as sensitive as SW620 cells to dipyridamole and prochlorperazine (C and D). Bars, SE.
Fig. 4  Soft agar clonogenic assay data plotted and analyzed by CalcuSyn software. A, C, and E, dose effect plots of doxorubicin, alone or in combination with dipyridamole or prochlorperazine alone or their combination. In plots B, D, and F, data were analyzed for determination of CI. A CI index of <1 is additive/synergistic, and >1 is antagonistic. Strong synergism is indicated by CIs of 0.1–0.3, and values of 0.7–0.85 are considered to be of moderate synergism. A majority of prochlorperazine and dipyridamole combinations tested with doxorubicin had moderate to high synergism (B) in P388/R-84 cells. In SW620 cells, only two of the combinations tested had moderate synergism, whereas other combinations were additive (C and D). In contrast, most of the combination of doxorubicin with the two efflux blockers in SW620/Ad300 cells were highly synergistic (E and F).
cytotoxicity. Except for two combinations with CI values of 0.5 (Fig. 4D), most of the other combinations tested had CI values of 0.5–1.0 (moderate synergism to nearly additive effects).

**SW620/Ad300 Human Colon and MCF7-AdR Cancer Cells.** Dose effect and CI plots generated by the CalcuSyn software and shown in Fig. 4, E and F, are based on mean values from four individual sets of soft agar clonogenic assays. The drug concentrations tested were doxorubicin (0.4–5 μM), prochlorperazine (1.5–40 μM), and dipyridamole (2.3–20 μM). The data plotted are for a ratio of 1:5:3.3 of doxorubicin:dipyridamole:prochlorperazine. Data in these plots show that in SW620/Ad300 doxorubicin-resistant cells, blockers used alone or in combination had highly synergistic effects on the cytotoxicity of doxorubicin (CI values of <0.5; Fig. 4F). In paired human breast tumor drug-sensitive (MCF-7) and doxorubicin-resistant (MCF-7/Adr) cells, the synergistic effects of the two efflux blockers on the cytotoxicity of doxorubicin were similar to those seen in the SW620 and SW620/Ad300 cells described above (data not shown).

CI simulation of data from the *in vitro* clonogenic assays analyzed by the CalcuSyn software indicated that doxorubicin concentrations of 0.4–0.97 μM in combination with dipyridamole (2.3–4.8 μM) and prochlorperazine (1.5–3.2 μM) will have highly synergistic effects (CI of <0.5) on the cytotoxicity of doxorubicin in the drug-resistant tumor cells.

**Plasma Pharmacokinetics.** In the following section (Fig. 5 and Tables 1–3), we describe the plasma pharmacokinetics of doxorubicin, prochlorperazine, and dipyridamole in patients administered doxorubicin alone (60 mg/m²) for 15 min or doxorubicin with a fixed dose of prochlorperazine (135 mg/m²) and escalating doses of dipyridamole (0.6–1.5 mg/kg/m) for 120 min. Tables 1–3 list the pharmacokinetic parameters of doxorubicin, dipyridamole, and prochlorperazine from patients on this protocol. A dipyridamole dose-related increase in AUC of doxorubicin was seen in patients administered 0.9–1.5 mg/kg of dipyridamole with a fixed dose of prochlorperazine. The SE of doxorubicin AUC increased from 4.3% to 19, 29, and 35% with increase in the dipyridamole dose, thus indicating large interpatient variation in plasma doxorubicin content. A corresponding decrease in plasma clearance accompanied the increase in doxorubicin AUC. The maximum plasma level of doxorubicin achieved in patients on this protocol was 3.78 ± 0.37 μM (Table 1).

The mean plasma dipyridamole level (Table 2; Fig. 6) achieved was from 0.84 ± 0.20 μM to as high as 3.01 ± 0.41 μM in patients administered prochlorperazine and dipyridamole. In two patients, the peak dipyridamole levels reached were as high as 32.7 and 7.0 μM. The peak plasma level of prochlorperazine was between 0.46 ± 0.07 to 0.94 ± 0.09 (Table 3) and in two patients, peak levels achieved were 1.35 and 1.55 μM, respectively (Table 3).

**In Vitro Efflux Blocking Activity of Patient Plasma.** Plasma samples from patients on the Phase I protocol of doxorubicin with prochlorperazine and dipyridamole were tested *in vitro* for their effect on the retention of radiolabeled daunorubicin in MDR₁-transfected P388 cells. In control cultures, verapamil (12.5, 25.0 μM) was added to block [³H]daunorubicin efflux. Data in Fig. 7 show the effect on labeled daunorubicin retention. Each point records the mean of two measurements from each plasma sample. Fig. 7B plots data on the 22 plasma samples, which had 50% or greater daunorubicin efflux blocking activity than that of cells incubated with 12.5 μM of verapamil. The dipyridamole and prochlorperazine content in these 22 samples ranged from 0.01–1.69 and 0.2–1.36 μM, respectively. In Fig. 7C, daunorubicin efflux blocking activity of 12 samples, which had dipyridamole and prochlorperazine content of greater than 1.2 and 0.5 μM, respectively, is plotted. The daunorubicin efflux blocking activity of these plasma samples was 6.9–59% that of cells incubated with 12.5 μM of verapamil. In Fig. 8, we have plotted the daunorubicin efflux blocking activity versus the dipyridamole, prochlorperazine, and doxorubicin content of all of the samples analyzed in the present study. Lack of a strong correlation between individual dipyridamole, prochlorperazine, and doxorubicin plasma content and the *in vitro* daunorubicin efflux blocking activity in the MDR₁-transfected cells was indicated by a weak correlation coefficient (0.008–0.197).

**DISCUSSION**

Observations in the present study show that: (a) combinations of prochlorperazine and dipyridamole have synergistic/additive effects on the cellular retention and cytotoxicity of doxorubicin/daunorubicin in drug-resistant human solid tumor cells; (b) some of the plasma samples from patients on the efflux blocker combination protocol had significant daunorubicin efflux blocking activity in a MDR₁-transfected cell line; and (c) a strong correlation between plasma content of the efflux blockers
in Adriamycin-resistant P388 or combinations for their effect on drug retention and cytotoxicity and his colleagues (15–17, 27) have studied efflux blocker more effective than either drug alone in reversing resistance to a multidrug-resistant multiple myeloma cell line, Lehnert (15–17, 27) have suggested that a combination of drug efflux may involve multiple P-gp regions (26). Several earlier studies by the use of an individual efflux blocker at high concentrations. One of the possible alternatives could be the use of synergistic high concentrations of the efflux blockers must be maintained in P-gp pump. To achieve the desired drug efflux blocking effects, efflux blocker in combination with drugs that are effluxed by the demonstrated.

Pharmacokinetic parameters of doxorubicin (mean ± SE)*

<table>
<thead>
<tr>
<th></th>
<th>DOX alone</th>
<th>DOX + PCZ + DPD (0.6 mg)</th>
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<th>DOX + PCZ + DPD (1.5 mg)</th>
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<tbody>
<tr>
<td>AUCb</td>
<td>64.29 ± 2.8</td>
<td>66.87 ± 12.7</td>
<td>98.23 ± 28.2</td>
<td>110.8 ± 39.32</td>
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<tr>
<td>α-halff</td>
<td>4.00 ± 0.16</td>
<td>4.25 ± 0.34</td>
<td>3.77 ± 5.74</td>
<td>2.48 ± 1.26</td>
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<tr>
<td>β-halff</td>
<td>26.09 ± 6.12</td>
<td>43.19 ± 14.93</td>
<td>16.28 ± 12.4</td>
<td>23.32 ± 8.21</td>
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<tr>
<td>γ-halff</td>
<td>451.07 ± 50.6</td>
<td>549.50 ± 272.2</td>
<td>501.26 ± 330.1</td>
<td>784.68 ± 496.2</td>
</tr>
<tr>
<td>Cmax</td>
<td>2.57 ± 0.07</td>
<td>2.35 ± 0.16</td>
<td>2.61 ± 0.49</td>
<td>3.78 ± 0.37</td>
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<tr>
<td>CLf</td>
<td>0.93 ± 0.04</td>
<td>0.89 ± 0.17</td>
<td>0.61 ± 0.17</td>
<td>0.54 ± 0.19</td>
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</table>

* Three-compartment with bolus input, first-order output, and macro constants as primary parameters for doxorubicin. $C(t) = A \times e^{-\alpha t} + B \times e^{-\beta t} + C \times e^{-\gamma t} + D$, DOX, doxorubicin; PCZ, prochlorperazine; DPD, dipyridamole. 

Table 2  Pharmacokinetic parameters of dipyridamole (mean ± SE)∗

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<tr>
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<tr>
<td>AUCf</td>
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<td>K10-halff</td>
<td>79.1 ± 13.2</td>
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<tr>
<td>CLf</td>
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<td>Vss</td>
<td>0.44 ± 0.13</td>
<td>0.19 ± 0.03</td>
<td>0.63 ± 0.14</td>
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* One-compartment with constant i.v. input and first-order output model for dipyridamole as efflux blocker. DPD, dipyridamole.

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or doxorubicin and efflux blocking activity in vitro could not be demonstrated.

Most of the reported clinical trials involved use of a single efflux blocker in combination with drugs that are effluxed by the P-gp pump. To achieve the desired drug efflux blocking effects, high concentrations of the efflux blockers must be maintained in the plasma, which in turn can result in normal tissue toxicity. One of the possible alternatives could be the use of synergistic combinations of the efflux blockers, which could have additive/synergistic effects on efflux without the toxicity, perhaps caused by the use of an individual efflux blocker at high concentrations.

Recent studies suggest that binding of drugs to the P-gp may involve multiple P-gp regions (26). Several earlier studies (15–17, 27) have suggested that a combination of drug efflux blockers may be better at enhancing cellular drug retention in refractory tumors and reducing cytotoxicity in normal tissues. In a multidrug-resistant multiple myeloma cell line, Lehnert et al. (15) described that a combination of verapamil and quinine was more effective than either drug alone in reversing resistance to doxorubicin or vinblastine. In a series of recent studies, Stein and his colleagues (15–17, 27) have studied efflux blocker combinations for their effect on drug retention and cytotoxicity in Adriamycin-resistant P388 or MDR-transfected P388 cell lines. In combinations (using one-fifth or one-tenth the concentration of individual efflux blockers), they reported that activity was additive. Ayesh et al. (17) have shown that whereas verapamil, cyclosporin, and trifluoperazine interact with P-gp as a single entity, vinblastine, dipyridamole, and tamoxifen act as pairs of modulators for MDR reversal. When efflux blockers (in pairs) were incubated with the P388/MDR cells to block efflux and enhance cytotoxicity, both competitive and noncompetitive activity was noted. Thus, verapamil was competitive with trifluoperazine and dipyridamole but had noncompetitive activity when paired with tamoxifen or vinblastine. Stein and his colleagues have monitored the effect of different efflux blocker combinations on the P-gp ATPase activity. Their studies indicate that P-gp has more than one binding site, and different drugs binding to different sites could enhance or decrease P-gp activity (27). To our knowledge, there are no reported clinical trials of efflux blocker combinations that would use the appropriate pair of the efflux blockers to obtain synergistic or additive effects. The use of efflux blocker combinations is further warranted by the observation that human tumors have extensive heterogeneity in drug retention, and subpopulations differ in their response to different efflux blockers (10, 20). Thus, the concept of using efflux blocker combinations should be attractive for reducing toxicity, obtaining synergistic efflux blocking effects, as well as to overcome heterogeneity in the response of tumor subpopulations to individual blockers.

In tissue culture medium containing 20–30% of fetal bovine serum, the efflux blocking effect of dipyridamole or prochlorperazine on doxorubicin retention are seen at concentrations of >5 μM. Data in the present study show that a combination of prochlorperazine and dipyridamole has additive effects on doxorubicin retention and synergistic effects on cytotoxicity. Concentrations as low as 2 μM of dipyridamole and prochlorperazine in combination enhanced doxorubicin retention in drug-resistant cells. CI simulation of data from the in vitro clonogenic assays analyzed by the CalcuSyn software indicates that dipyridamole concentrations of 2.3–4.8 μM and prochlorperazine levels of 1.5–3.2 μM could have highly synergistic effects (CI value of <0.5) on the cytotoxicity of doxorubicin concentrations of 0.4–0.97 μM.

One of the major concerns about dipyridamole is related to bioavailability because of its avid binding to plasma protein (28). Our flow cytometric studies show that in P388/R-84 doxo-
rubicin-resistant cells growing in medium containing 25% fetal bovine serum, concentrations of 0.5 \( \text{mM} \) dipyridamole are needed to block doxorubicin efflux. Parallel studies in doxorubicin-resistant (SW520/Ad300) human colon cells show that coincubation with 0.5 \( \text{mM} \) dipyridamole decreased the ED50 of doxorubicin from 5.7 to 1.43 \( \text{mM} \). Plasma levels of prochlorperazine as high as 2 \( \text{ mM} \) (administered as a 15- or 120-min infusion in combination with doxorubicin) can be achieved without any major toxicity (11, 12). Similarly, in patients administered 17.5 mg/kg of dipyridamole for 72 h, high plasma concentrations (\( \geq 60 \text{ mM} \)) were achieved (13). As shown in the present study, peak plasma levels of prochlorperazine and dipyridamole achieved in patients on our protocol were 0.94 \( \pm 0.09 \) and 3.01 \( \pm 0.41 \) \( \text{mM} \), respectively. In some of the patients, plasma levels of the two efflux blockers achieved were as high as 5 \( \text{mM} \).

The present Phase I clinical trial was carried out in Miami, and plasma samples (after pharmacokinetic analysis) stored in a freezer were sent for in vitro analysis in Dr. Stein’s laboratory. Because the in vitro assay based on the use of MDR1-transfected P388 cells, labeled daunorubicin, and verapamil as an efflux blocker has been validated and standardized by Dr. Stein and his colleagues, we expected a fair amount of correlation between the plasma efflux blocker content and the efflux blocking effect. Data in Figs. 7 and 8 did not show any significant correlation between plasma drug concentration (of either the efflux blockers individually or doxorubicin) and the ability to block daunorubicin efflux in vitro. Because the in vitro studies were not initially planned as part of our protocol but were added on later, we cannot explain the lack of a positive correlation. Our ongoing studies are focused on concurrent analyses of patient plasma

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Pharmacokinetic parameters of prochlorperazine (mean ( \pm ) SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCZ + DPD (0.6 mg/kg)</td>
</tr>
<tr>
<td>AUC</td>
<td>210.8 ( \pm 43.98 )</td>
</tr>
<tr>
<td>K10-half</td>
<td>272.7 ( \pm 80.81 )</td>
</tr>
<tr>
<td>( C_{\text{max}} )d</td>
<td>0.46 ( \pm 0.07 )</td>
</tr>
<tr>
<td>( CL )f</td>
<td>0.28 ( \pm 0.06 )</td>
</tr>
<tr>
<td>( V_{ss} )f</td>
<td>112.0 ( \pm 20.3 )</td>
</tr>
</tbody>
</table>

* One-compartment with constant i.v. input and first-order output model for dipyridamole as efflux blocker. PCZ, prochlorperazine; DPD, dipyridamole.

a AUC, area under the \( C(t) \) curve. The area under the curve was calculated from the experimental data by the trapezoidal rule and the terminal elimination (\( \mu \text{M} \times \text{h} \)).

b K10-half, the half life time associated with the elimination rate constant.

c \( C_{\text{max}} \), peak plasma concentration (\( \mu \text{M} \)).

d \( CL \), total body clearance (l/h).

e \( V_{ss} \), volume of distribution at steady state (l/kg).

rubricin-resistant cells growing in medium containing 25% fetal bovine serum, concentrations of \( >5 \mu \text{M} \) dipyridamole are needed to block doxorubicin efflux. Parallel studies in doxorubicin-resistant (SW520/Ad300) human colon cells show that coincubation with \( >5 \mu \text{M} \) dipyridamole decreased the ED50 of doxorubicin from 5.7 to 1.43 \( \mu \text{M} \). Plasma levels of prochlorperazine as high as 2 \( \mu \text{M} \) (administered as a 15- or 120-min infusion in combination with doxorubicin) can be achieved without any major toxicity (11, 12). Similarly, in patients administered 17.5 mg/kg of dipyridamole for 72 h, high plasma concentrations (\( \geq 60 \mu \text{M} \)) were achieved (13). As shown in the present study, peak plasma levels of prochlorperazine and dipyridamole achieved in patients on our protocol were 0.94 \( \pm 0.09 \) and 3.01 \( \pm 0.41 \mu \text{M} \), respectively. In some of the patients, plasma levels of the two efflux blockers achieved were as high as 5 \( \mu \text{M} \).

The present Phase I clinical trial was carried out in Miami, and plasma samples (after pharmacokinetic analysis) stored in a freezer were sent for in vitro analysis in Dr. Stein’s laboratory. Because the in vitro assay based on the use of MDR1-transfected P388 cells, labeled daunorubicin, and verapamil as an efflux blocker has been validated and standardized by Dr. Stein and his colleagues, we expected a fair amount of correlation between the plasma efflux blocker content and the efflux blocking effect. Data in Figs. 7 and 8 did not show any significant correlation between plasma drug concentration (of either the efflux blockers individually or doxorubicin) and the ability to block daunorubicin efflux in vitro. Because the in vitro studies were not initially planned as part of our protocol but were added on later, we cannot explain the lack of a positive correlation. Our ongoing studies are focused on concurrent analyses of patient plasma
samples by both HPLC (for the determination of efflux blocker and their major metabolite content) and in vitro analyses.

Although translation of the in vitro studies into effective clinical protocols to overcome chemoresistance has been slow because of problems of pharmacokinetics, bioavailability, and possibly the multifactorial nature of drug resistance in advanced tumors, our understanding of the problems and opportunities has been advanced by the work of numerous investigators (6–9). Sikic et al. (9) has listed the following four reasons that provide a rationale for modulation of drug resistance by efflux blockers: (a) in most human tumors, P-gp expression and drug efflux are seen either at diagnosis or after failure of chemotherapy; (b) several studies have shown a strong association between P-gp expression and poor prognosis in some tumor types; (c) P-gp and drug efflux-related resistance could be overcome by the use of efflux blockers that increase cellular drug retention and cytotoxicity both in vitro and in vivo models; and more importantly (d) coadministration of the efflux blockers with the cytotoxins can prevent the emergence of de novo drug resistance.

Our preliminary observations on patients entered on prochlorperazine and dipyridamole with doxorubicin Phase I trial suggest that this combination is less toxic and better tolerated than either of the efflux blockers used alone. We expect that the combination of doxorubicin with prochlorperazine and dipyri-
doxorubicin alone or doxorubicin in combination with other MDR drugs. The rationale for the use of combination blockers is further strengthened by the fact that two different efflux blockers may have different pharmacokinetic profiles, and thus, although one of them may reach peak plasma levels rapidly, the second blocker may have lower peak plasma levels but slower plasma clearance. We may expect the initial high plasma level of dipiridamole to be complemented by the longer plasma life of prochloperazine, which could result in better efflux blocking. Another advantage to be gained by use of blocker combinations may be that their individual cytotoxicities may not overlap, and thus one can reduce side effects by using lower concentrations of the two efflux blockers. A good example of this may be that nausea and vomiting induced by dipiridamole can be reduced by the antiemetic properties of prochloperazine.

REFERENCES


Synergistic Effect of Prochlorperazine and Dipyridamole on the Cellular Retention and Cytotoxicity of Doxorubicin


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