Synergistic Inhibition by Verapamil and Quinine of P-Glycoprotein–Mediated Multidrug Resistance in a Human Myeloma Cell Line Model

By Manfred Lehnert, William S. Dalton, Denise Roe, Scott Emerson, and Sydney E. Salmon

In an effort to develop a clinically useful approach to overcoming P-glycoprotein–mediated multidrug resistance (MDR1), we evaluated combined chemosensitization with verapamil and quinine in a multidrug-resistant (MDR) human myeloma cell line model. In clonogenic assay, verapamil was used at concentrations from 0.1 to 1.0 μg/mL, bracketing the plasma levels achieved by oral administration and high-dose intravenous (IV) infusion, respectively. The dose of quinine was held constant at 1.0 μg/mL; a plasma concentration readily achieved by oral administration. At each dose level of verapamil tested, the combination with quinine proved more effective than either drug individually in reversing resistance to doxorubicin and vinblastine and synergistic chemosensitizing interaction was observed. Verapamil at 0.1 μg/mL combined with quinine was capable of restoring sensitivity to doxorubicin fully and reduced resistance to vinblastine as effectively as verapamil alone at 1.0 μg/mL. Furthermore, the combination of 1.0 μmol verapamil with 10 μmol quinine increased accumulation and retention of anthracycline in the resistant cells to a greater extent than did either drug individually (P < .001) and inhibited drug efflux as effectively as verapamil alone at 10 μmol. Our findings suggest that combined chemosensitization with verapamil and quinine may prove useful for overcoming MDR1 in patients with drug-refractory B-cell neoplasms such as multiple myeloma or non-Hodgkin’s lymphomas.

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RESISTANCE TO cancer chemotherapy is a major obstacle to successful treatment of disseminated human neoplasms. Clinically, tumors initially sensitive to chemotherapy commonly recur and exhibit resistance to a broad range of cytotoxic agents. A comparable experimental phenomenon, termed multidrug resistance (MDR), has been extensively studied in recent years. After exposure to gradually increasing doses of a single agent such as doxorubicin (DOX) or vinblastine (VBL), tumor cells can develop cross-resistance to a wide spectrum of structurally and functionally unrelated natural product anticancer drugs, e.g., anthracyclines, vinca alkaloids, podophyllotoxins, and actinomycins. The pharmacologic basis of MDR appears to be decreased intracellular drug accumulation due to enhanced efflux. This process is mediated by a 170,000-dalton membrane protein, designated P-glycoprotein or p170, which functions as an energy-dependent multidrug transporter. The human gene encoding p170, termed mdr1, has been isolated, and transfection of cloned mdr1 sequences confers MDR on sensitive cells.

Several lines of evidence have indicated that p170-mediated MDR (MDR1) has clinical significance. Overexpression of p170 in tumor has been detected in a variety of hematologic malignancies as well as solid tumors. Increased expression of p170 in tumor cells has been reported to predict in vitro resistance to DOX in tumor biopsies from patients with myeloma, lymphoma, and breast cancer and has been correlated with treatment failure to chemotherapy with vincristine, DOX, and dexamethasone in patients with multiple myeloma. In addition, in childhood soft tissue sarcoma, immunohistochemical detection of p170 in tumor biopsies correlated with a lower complete response rate to chemotherapy and a significantly decreased probability of disease-free and overall survival.

A number of compounds reverse MDR1 in vitro. These include calcium channel blockers, calmodulin inhibitors, antiarrhythmics, antimalarials, and other lysosomotropic agents, steroids, and antieosinophils, and cyclic peptide antibiotics. Unfortunately, the optimum concentrations for overcoming MDR1 in tissue culture are usually well above the maximally tolerated plasma levels for many of these chemosensitizers. Although evidence shows the clinical efficacy of high-dose infusion verapamil in reversing drug resistance in myeloma and lymphoma, this regimen induces substantial cardiac toxicities. The mean plasma level of verapamil achieved with this intensive approach averaged approximately 1.0 μg/mL, which is less than the concentration required to overcome MDR1 completely in tumor cell lines. Therefore, better approaches are clearly needed to circumvent MDR.

We postulated that chemosensitizers in combination at readily achievable concentrations might be capable of reversing MDR1 as effectively as the individual agents at higher concentrations, thereby increasing the therapeutic index and potential clinical usefulness of such sensitizers. To achieve this goal, the chemosensitizers used in combination must meet two basic requirements: first, they must lack overlapping dose-limiting toxicities; second, and even more essential, they must produce additive or synergistic chemosensitizing interaction on the resistant tumor cells. Because verapamil and quinine meet the first condition, we evaluated the effectiveness of combined chemosensitization with verapamil and quinine in an MDR human myeloma cell line model.
VERAPAMIL PLUS QUININE FOR REVERSING MDR1

MATERIALS AND METHODS

Cells. RPMI 8226 human myeloma cells, obtained from the American Type Culture Collection (Rockville, MD), were selected for resistance to DOX as previously described. The resulting MDR mutants (8226/DOX) exhibited the characteristics of the MDR1 phenotype, eg, overexpression of p170 at a level correlating with the degree of drug resistance. The 8226/DOX6 and 8226/DOX40 cells used in these studies were obtained by gradually exposing the cells to a maximum concentration of $6 \times 10^{-8}$ and $4 \times 10^{-7}$ mol DOX, respectively, a concentration six and four times higher than initially used in the selection process. Once established, resistance has been stable in the MDR sublines for at least 1 year. Cells were grown in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, penicillin, streptomycin, and L-glutamine (1% vol/vol) (GIBCO, Grand Island, NY). Cell cultures were maintained at 37°C in humidified air supplemented with 7.5% CO2. The resistant cells were maintained in DOX-free medium for at least 7 days before use for cytotoxicity or cellular pharmacokinetic studies.

Drugs. Doxorubicin and daunorubicin (DNR) were purchased from Adria Laboratories (Columbus, OH). VLB was purchased from Eli Lilly (Indianapolis, IN), racemic verapamil was purchased from Knoll Pharmaceuticals (Whippany, NJ), and quinine sulfate was purchased from Sigma Chemical (St Louis, MO).

In vitro sensitivity testing. Drug sensitivity testing was performed using a two-layer soft-agar culture system. We continuously exposed cells to drugs by incorporating the agents into the plating layer. The dose of quinine was held constant at 1.0 μg/mL through all experiments. Verapamil was used at concentrations ranging from 0.1 to 1.0 μg/mL. Exponentially growing cells were plated in triplicate at a concentration of $1 \times 10^4$ cells/35-mm tissue culture dish (Falcon Plastics, Division of Becton Dickinson, Oxnard, CA). Tumor cell colonies 60 μm in diameter were enumerated 14 to 18 days after plating using an automated image analysis instrument optimized for tumor colony counting (FAS II Omnicon; Bausch and Lomb, Rochester, NY). The percentage of survival was determined from the plating efficiencies of treated and control cells. The IC50 for DOX and VLB, alone or in combination with chemosensitizer(s), was defined as the drug concentration that reduced colony formation to 50% of untreated control cells. The sensitization factor (SF) was determined by dividing the IC50 for DOX or VLB alone by the IC50 in the presence of the particular chemosensitizer(s).

To analyze the type of drug interaction that occurred between verapamil and quinine, the effect-multiplication criterion was applied. According to this method, the SF of verapamil combined with quinine equals the product of the SF of verapamil and quinine alone, when the drugs do not interact (additive or so-called “expected” effect). When the SF of the combination is higher than the expected effect, the interaction of verapamil and quinine is synergistic. A value lower than the expected effect indicates negative interaction (antagonism).

Drug accumulation and efflux studies. The effect of verapamil and quinine on anthracycline accumulation in 8226/DOX40 cells was analyzed with a fluorescence-activated cell sorter (FACS) cell scanner (FACSscan, Becton Dickinson, San Jose, CA). DNR was used as the index anthracycline in the flow cytometry (FCM) studies because it produces less nongenotoxic background fluorescence and quenching than DOX. Preliminary experiments established that the fluorescence spectra of the chemosensitizers did not interfere with the fluorescence emitted by DNR. Exponentially growing cells at a concentration of $5 \times 10^6$/mL were incubated at 37°C with 3.0 μmol DNR with or without 1.0, 5.0, or 10 μmol verapamil, 10 μmol quinine, or the chemosensitizers in combination. Each treatment was tested in triplicate. After 1-hour drug exposure, cells were pelleted by centrifugation and washed twice with ice-cold phosphate-buffered saline (PBS). Cells were resuspended in ice-cold PBS and kept on ice, and the cellular DNR content was immediately analyzed by FCM. The excitation and emission wavelengths used were 485 and 585 nm, respectively. Ten thousand cells were analyzed for each histogram generated.

The effects of the chemosensitizers on DNR efflux from the resistant cells also was evaluated by FCM. The number of cells exposed to drugs and laser setting were identical to those used in the DNR accumulation studies. Exponentially growing cells were incubated with 6.0 μmol DNR at 37°C for 1 hour. Cells were then washed twice with ice-cold PBS and immediately resuspended in warm medium without or with 1.0 or 10 μmol verapamil, 10 μmol quinine, or the combination of quinine and 1.0 μmol verapamil; they were then reincubated at 37°C. After 10, 30, and 60 minutes, cell aliquots were washed twice with ice-cold PBS and resuspended in cold PBS and kept on ice, and the amount of cellular DNR retained was immediately analyzed by FCM. Each treatment was tested in triplicate. Differences in the increase of cellular DNR accumulation and retention by adding verapamil and quinine alone or in combination were calculated by Tukey’s Studentized range test.

RESULTS

Cytotoxicity studies. Individually, verapamil and quinine enhanced the sensitivity to DOX in the resistant cells two- to fourfold. In contrast, each combination of verapamil and quinine yielded a sensitization factor of approximately 20 and fully restored DOX sensitivity. Analysis of the type of interaction between verapamil and quinine indicated clear synergism according to the effect-multiplication criterion. Table 1 summarizes the effects of verapamil and quinine on DOX cytotoxicity in the 8226/DOX6 cells. Of particular interest was the observation that 0.1 μg/mL verapamil was equivalent in effectiveness to the higher concentrations when combined with quinine and was able to reverse DOX resistance completely (Fig 1).

The effects of verapamil and quinine on VLB resistance are summarized in Table 2. Verapamil alone at 1.0 μg/mL increased the sensitivity to VLB sixfold, whereas quinine or

<p>| Table 1. Effects of VER and Q Alone and in Combination on Resistance to DOX in 8226/DOX Myeloma Cells |</p>
<table>
<thead>
<tr>
<th>Drug(s)</th>
<th>IC50* (μg/mL)</th>
<th>Sensitization Factor†</th>
<th>Type of Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX alone</td>
<td>19.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+VER 1.0</td>
<td>4.2</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>+VER 0.5</td>
<td>5.0</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>+VER 0.1</td>
<td>7.8</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>+Q 1.0</td>
<td>6.0</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>+VER 1.0 + Q</td>
<td>0.9</td>
<td>21.1</td>
<td>Synergistic</td>
</tr>
<tr>
<td>+VER 0.5 + Q</td>
<td>1.0</td>
<td>19.0</td>
<td>Synergistic</td>
</tr>
<tr>
<td>+VER 0.1 + Q</td>
<td>0.9</td>
<td>21.1</td>
<td>Synergistic</td>
</tr>
</tbody>
</table>

Abbreviations: VER, verapamil; Q, quinine.

*The IC50 refers to the DOX concentration that results in 50% reduction in colony formation.
†Sensitization factor and type of interaction between VER and Q were determined as described in the Materials and Methods section.
§All concentrations of VER and Q are in micrograms per milliliter; Q was used at a fixed concentration of 1.0 μg/mL when combined with VER.
0.1 μg/mL verapamil had little effect. The enhanced effectiveness of combined chemosensitization was generally less pronounced with VLB than with DOX; eg, complete restoration of VLB sensitivity was obtained only by combining 1.0 μg/mL verapamil and quinine. Nonetheless, each combination of verapamil and quinine produced synergistic chemosensitizing interaction and was more effective than the individual compounds in reversing VLB resistance. Furthermore, the combination of 0.1 μg/mL verapamil and quinine was as effective as verapamil alone at the 10-fold higher concentration of 1.0 μg/mL.

Figure 2 shows the potentiating effects of verapamil and quinine on subtoxic concentrations of DOX and VLB. At each dose level of verapamil tested, the combination with quinine enhanced cytotoxicity more effectively than either sensitizer alone; eg, 1.0 μg/mL verapamil or quinine enhanced inhibition of colony formation by DOX or VLB by no more than 30%, whereas the combination of the sensitizers suppressed growth by more than 90%. The combination of 0.1 μg/mL verapamil with quinine increased sensitivity to DOX more effectively than 1.0 μg/mL verapamil alone and was equivalent in potentiating VLB cytotoxicity.

At the concentrations tested, verapamil and quinine were nontoxic to the cells, either alone or in combination, and the sensitizers had no effect on the drug-sensitive parent cells.

**Table 2.** Effects of VER and Q Alone and in Combination on Resistance to VLB in 8226/DOX6 Myeloma Cells

<table>
<thead>
<tr>
<th>Drug(s)</th>
<th>IC₅₀** (x 10⁻⁴ mol)</th>
<th>Sensitization Factor†</th>
<th>Type of Interaction Between VER and Q‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLB alone</td>
<td>10.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+VER 1.0</td>
<td>1.65</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>+VER 0.5</td>
<td>4.2</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>+VER 0.1</td>
<td>7.0</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>+Q 1.0</td>
<td>7.0</td>
<td>1.4</td>
<td></td>
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<tr>
<td>+VER 1.0 + Q</td>
<td>0.55</td>
<td>18.2</td>
<td>Synergistic</td>
</tr>
<tr>
<td>+VER 0.5 + Q</td>
<td>0.7</td>
<td>14.2</td>
<td>Synergistic</td>
</tr>
<tr>
<td>+VER 0.1 + Q</td>
<td>1.65</td>
<td>6.1</td>
<td>Synergistic</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1.

*The IC₅₀ refers to the concentration of VLB that results in 50% reduction in colony formation.

†Sensitization factor and type of interaction between VER and Q were determined as described in the Materials and Methods section.

‡All concentrations of VER and Q are in micrograms per milliliter; Q was used at a fixed concentration of 1.0 μg/mL when combined with VER.

**DNR accumulation and efflux.** After observing synergism between verapamil and quinine in clonogenic assay, we examined their effect on cellular anthracycline pharmacokinetics in 8226/DOX40 cells by FCM. In contrast to the cytotoxicity studies, molar concentrations of the chemosensitizers including doses higher than clinically achievable were used in the FCM studies for better definition of the quantitative interaction of verapamil and quinine with p170.
Figure 3 demonstrates the net increase in DNR accumulation by adding 10 μmol quinine or verapamil at 1.0, 5.0, or 10 μmol, either alone or in combination. Each combination of verapamil and quinine increased the intracellular accumulation of DNR significantly relative to the individual compounds at the same dose (P < .0001).

Figure 4 shows the inhibition of DNR efflux by addition of 1.0 or 10 μmol verapamil, 10 μmol quinine, or the combination of quinine and 1.0 μmol verapamil. Verapamil at 1.0 μmol and quinine increased DNR retention to a comparable degree. In comparison, their combination inhibited DNR efflux more effectively (P < .001) and was as effective as verapamil alone at 10 μmol.

**DISCUSSION**

The recently established clinical significance of MDR1 has created the opportunity to develop clinically useful approaches to overcome this type of drug resistance. We previously reported our experience with high-dose infusion of verapamil in patients with drug-refractory multiple myeloma or malignant lymphoma.15-35 These studies have provided initial evidence that the concept of reversing MDR by adding a chemosensitizer to cytotoxic treatment can be successfully translated from the laboratory to the clinic but have also emphasized the problem that the concentration of verapamil needed to inhibit MDR1 maximally in vitro cannot be achieved in patients' plasma owing to the dose-limiting cardiac toxicities.

In an effort to identify a clinically more useful approach to circumventing MDR1, we evaluated combined chemosensitization with verapamil and quinine in an MDR human myeloma cell line model. The 8226/DOX6 cells used in clonogenic assay overexpress p170 at a level similar to myeloma cells from patients with drug-refractory disease.36

For clinical relevance, we used verapamil and quinine in the cytotoxicity studies at concentrations that can be achieved in the patients' plasma. One microgram per milliliter verapamil is the average plasma level observed with high-dose infusion.35 This aggressive therapy induces serious although transient cardiac toxicities. In contrast, plasma levels of 0.1 μg/mL verapamil or 1.0 μg/mL quinine can be readily achieved by oral administration and are well tolerated.44,45

When the chemosensitizers were tested individually at these particular concentrations, they reduced resistance to DOX approximately two- to fourfold. In contrast, each combination of verapamil and quinine yielded a sensitization factor of around 20 and fully restored sensitivity to DOX. Of particular clinical interest was the observation that 0.1 μg/mL verapamil in combination with quinine was capable of circumventing DOX resistance in vitro completely.

Each combination of verapamil and quinine also reversed resistance to VLB more effectively than the individual agents, and verapamil at 0.1 μg/mL combined with quinine was as effective as verapamil alone at the tenfold higher concentration of 1.0 μg/mL. The relative increase in effectiveness by combined chemosensitization was less pronounced with VLB than with DOX, however, and complete reversal of resistance to VLB required treatment with 1.0 μg/mL verapamil plus quinine. These findings are in contrast to the observation that chemosensitizers are more able to circumvent resistance to vinca alkaloids than to anthracyclines when used at higher and thus more potent concentrations.3,38

We selected quinine to combine with verapamil mainly because these two sensitizers lack overlapping dose-limiting...
toxicities. The dose of verapamil has been limited in clinical studies by cardiovascular toxicities, including conduction block, myocardial dysfunction, and systemic hypotension. High doses of quinine can produce the syndrome of so-called "cinchonism," which includes tinnitus, blurred vision, headache, nausea, and other usually transient symptoms.45

Several lines of evidence suggested that verapamil and quinine may have a positive chemosensitizing interaction. The observation that colchicine and dactinomycin, two drugs to which MDR cells are usually cross-resistant, did not inhibit the binding of vinca alkaloids to p170,67 led to the hypothesis that multiple drug-binding sites may exist on this multidrug transporter. Theoretically, therefore, the chemosensitizers could compete with cytotoxic drugs at different binding sites of p170. Both verapamil and quinine have been shown to inhibit binding of vinca alkaloids to p170, but with differing affinities.48,49

Alternatively, verapamil and quinine in combination might act on different subcellular mechanisms involved in the MDR1 phenotype. Several recent studies showed that, in addition to overexpression of p170, the intracellular distribution of anthracyclines is different in sensitive and in MDR cells.51,52 While the anthracyclines rapidly accumulate in the nucleus of the sensitive cells, both DOX and DNR are mainly distributed in the cytoplasm of resistant cells. This altered distribution pattern appeared to contribute significantly to the level of drug resistance and was reversed by addition of a chemosensitizer.50,56 We hypothesize that verapamil predominantly inhibits the p170-mediated efflux of a cytotoxic drug, whereas the lysosomotropic agent quinine might block drug sequestration in acidic subcellular organelles when the sensitizers are used in combination. The synergistic effect observed between verapamil and quinine in clonogenic assay appears to support this hypothesis, but we recognize that there are alternative explanations.

Our studies of the cellular pharmacokinetics of DNR indicate that addition of verapamil and quinine in combination can significantly increase the intracellular retention and accumulation of anthracycline as compared with addition of the sensitizers individually. The relative increase in drug accumulation by the combination appears to be less pronounced than the enhancement of cytotoxicity. Many differences exist between the FCM and cytotoxicity studies, however, including use of DNR rather than DOX for optimum FCM results, timing of drug exposure, the number of cells required, and the drug concentrations used. Therefore, the results of FCM and cytotoxicity studies cannot be correlated directly. Other investigators have reported similar discrepancies between drug content and cell killing.53,54 Nevertheless, the data from the pharmacokinetic experiments suggest that the synergism between verapamil and quinine observed in clonogenic assay results, at least in part, from the functional inhibition of p170.

Results with two other combinations of chemosensitizers were recently reported. Verapamil and cyclosporin A produced synergistic chemosensitizing interaction in human leukemia cell lines,50 whereas verapamil and the thioxanthene trans-flupenthixol showed an additive effect in an MDR breast cancer cell line.50 We have begun to examine various other sensitizers in combination, and drug interaction in these preliminary studies has ranged from antagonistic to synergistic. These observations emphasize the need for evaluating each particular combination of chemosensitizers in relevant model systems before using them in patients.

A major concern in clinical trials has been that concurrent administration of chemosensitizers might enhance the toxicities of cytotoxic agents in normal tissues that express p170 because this might increase the concentration of the cytotoxic drugs in such cells. In addition, the sensitizers could interfere with renal or hepatic elimination of the cytotoxic agents, thereby leading to prolonged serum halflife and/or increased serum levels. In our studies with high-dose infusion verapamil and oral quinine, as well as in other clinical trials, however, the toxicity of the chemotherapeutic protocol was not increased.50,52,57,58 Furthermore, several preclinical studies have demonstrated that suppression of hematopoietic progenitor cells by cytotoxic agents was comparable with or without a chemosensitizer.59,61 In ongoing phase 1-II clinical trials at the Arizona Cancer Center, we are currently administering the combination of verapamil and quinine with combinations of cyclophosphamide, vincristine, DOX, and dexamethasone and have found it to be well tolerated and without increased organ toxicities (S.E. Salmon, unpublished observations, August 1990).

Our findings using a myeloma cell model of MDR suggest that combined chemosensitization with verapamil and quinine is a promising approach for overcoming MDR1 in patients with drug-refractory B-cell neoplasms such as multiple myeloma or non-Hodgkin's lymphomas. Based on our observations, clinical trials with combination chemotherapy supplemented with oral verapamil plus quinine have been initiated at our institution and were recently approved by the National Cancer Institute for implementation in a Southwest Oncology Group phase III myeloma study. We anticipate that these studies will provide useful information on the clinical utility of this combination of chemosensitizers.

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