Multidrug-Resistant Myeloma: Laboratory and Clinical Effects of Verapamil as a Chemosensitizer

By Sydney E. Salmon, William S. Dalton, Thomas M. Grogan, Patricia Plezia, Manfred Lehnert, Denise J. Roe, and Thomas P. Miller

Verapamil was evaluated as a chemosensitizer for reversing multidrug resistance in multiple myeloma both in vitro and in clinical trials. Bone marrows from 59 myeloma patients in relapse were evaluated for several resistance parameters: expression of p-glycoprotein (MDR1), doxorubicin (Adriamycin) and vincristine sensitivity, and the ability of added verapamil to reduce resistance to the cytotoxic agents. We found that verapamil was capable of sensitizing myeloma cells that exhibited resistance to doxorubicin and vincristine in vitro, but did not enhance sensitivity of cells that were drug sensitive (P < .001). Myeloma cells expressing MDR1 immunohistochemically tended to be more doxorubicin resistant in vitro than MDR1-negative cells. In the clinical trials, 22 patients with myeloma refractory to vincristine-Adriamycin-dexamethasone (VAD) were treated with VAD plus high-dose intravenous verapamil (Ve). Among the 22 patients treated with VAD/Ve, five achieved a partial remission (23%). The median relapse-free survival for the VAD/Ve responders was 5.4 months and their overall survival from the start of VAD/Ve was better than that of the nonresponders. Among the subset of 10 patients whose myeloma cells were MDR1 positive, four responded clinically (40%), whereas none of five patients with MDR1-negative myeloma cells achieved remission with VAD/Ve. We also observed that myeloma cells from three of four VAD/Ve clinical responders exhibited in vitro chemosensitization with verapamil, whereas in vitro verapamil chemosensitization was seen in only one of six clinical nonresponders. Our observations demonstrate that clinical reversal of multidrug resistance can be achieved in some patients with VAD-refractory myeloma with the use of verapamil. In addition to their value in drug development, in vitro tests of MDR1 expression and of chemosensitizers plus cytotoxic drugs on the patients’ bone marrow myeloma cells may identify patients who will respond clinically to chemosensitizer-containing regimens. We anticipate that chemosensitizer regimens capable of inhibiting multidrug resistance will play an increasing role in the treatment of hematologic malignancies, including B-cell neoplasms such as multiple myeloma and the non-Hodgkin’s lymphomas.

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MULTIPLE MYELOMA is a disseminated plasma cell neoplasm that exhibits initial sensitivity to alkylating agent-based systemic chemotherapy in most patients. Subsequently, drug resistance usually develops. The VAD regimen, comprised of vincristine (VCR), doxorubicin (DOX), Adriamycin, and dexamethasone, has been useful to reinroduce remission in patients who relapse after initial alkylating-agent therapy.1 While the VAD regimen is active in up to 70% of myeloma patients refractory to, or relapsing after, initial therapy, drug resistance subsequently appears and patients go on to die of VAD-refractory myeloma.

We have focused on mechanisms of drug resistance in myeloma and related B-cell neoplasms to develop alternative therapeutic strategies to circumvent drug resistance. One mechanism of drug resistance that appears to be relevant to patients with myeloma is multidrug resistance (involving the MDR1 gene product) as described in experimental systems.2,3 It is believed to be mediated by a drug-transporter, active efflux pump called the P-glycoprotein.4,5 The P-glycoprotein is a 170-Kd glycoprotein that mediates multidrug resistance (MDR1) and is predominantly distributed in the cell membrane.4 MDR1 is capable of reducing intracellular drug concentrations by increasing the efflux of a variety of different heterocyclic anticancer drugs from neoplastic cells, thereby reducing their cytotoxic potential. Molecular studies in which the MDR1 gene is transfected into sensitive cells in culture renders them multidrug resistant, thus confirming the role of the MDR1 gene in drug resistance.6,7 In our studies to date, we have found MDR1 to be commonly expressed in tumor cells from patients with refractory myeloma, whereas its expression is distinctly uncommon in previously untreated patients.8,9,10 Moreover, in a recent correlative study, we found an association between MDR1 expression and in vitro resistance to DOX in patients with myeloma, lymphoma, and breast cancer in the human tumor colony assay (HTCA).11 Inasmuch as resistance in HTCA has been correlated with clinical treatment failure with cancer chemotherapy, the predictive potential of MDR1 expression can be anticipated.12

The initial identification of verapamil (Ve) as an agent capable of reversing vinblastine resistance in a murine lymphoid leukemia-lymphoma in vivo was reported by Tsuruo et al.12 At the time of his original observation, the relationship of this Ve effect to MDR1 expression had yet to be identified. Subsequently, a number of investigators have reported that Ve and other nontoxic agents can serve as chemosensitizers in vitro and block the drug transport function of the P-glycoprotein.13,14 Several agents, including Ve, quinidine, and quinine have been determined by photoaffinity labeling studies to bind directly to MDR1 and presumably block cytotoxic drug binding and efflux through a competitive inhibition mechanism. We have...
previously reported our initial pilot clinical studies in which six patients with myeloma and one with lymphoma in relapse after multiple chemotherapy combinations including VAD were treated with VAD plus high-dose intravenous (IV) Ve, with re-establishment of remission in several of the patients. Of the six myeloma patients included in that report, VAD resistance was circumvented in two of the patients who went back into remission when Ve was added to VAD. The ability of Ve to increase the intracellular accumulation of vincristine or DOX within MDR1-positive myeloma cells was demonstrated in vitro for several patients in the pilot trial. In the current report, we examined in vitro chemosensitization with Ve and correlated this effect and MDR1 expression with clinical response to VAD/Ve in VAD-refractory myeloma patients. These studies provide both laboratory and clinical evidence for the potential utility of chemosensitizers in the treatment of patients with drug-resistant multiple myeloma.

**MATERIALS AND METHODS**

**Myeloma cell collection.** Bone marrow aspirates from 59 patients with multiple myeloma were collected with a portion of the aspirate obtained in a syringe containing nontoxic heparin. The portion of the marrow aspirate obtained without heparin was sent for standard histopathologic examination and the heparinized portion sent for specialized testing. The heparinized portion was delivered promptly to the research laboratory wherein tumor cell suspensions were prepared under aseptic conditions. An aliquot of the myeloma cell suspensions was used to prepare cytopsin slides, which were provided to the immunopathology laboratory for immunohistochemical determination of MDR1 expression, and the remainder used for drug sensitivity testing in HTCA. Not all patients’ bone marrow aspirates were referred for all tests performed, and some samples were inadequate to yield results. Therefore, reports for specific determinations are reported on subsets of the 59 patients from whom bone marrow aspirates were obtained.

**Immunohistochemical studies.** For determination of p-glycoprotein expression, specific monoclonal antibodies (MoAbs) to MDR1 were used, including the JSB-1 antibody developed at the Free University of Amsterdam (now available commercially from Sanbio (Amsterdam, Netherlands) and the C219 antibody that was originally developed by Gerlach et al and obtained commercially from Centocor (Malvern, PA). Details of our immunohistochemical methods have been reported previously.

In these studies, the staining reaction was calibrated with simultaneously stained positive and negative control myeloma cell lines with known degrees of DOX resistance and MDR1 expression (as determined in total cellular RNA expression), and MoAb staining. All slides were carefully reviewed by one of us, who is an immunopathologist (T.M.G.), and scored independently of any knowledge of the patient’s clinical condition or of results from in vitro drug sensitivity testing. To be called positive, myeloma cells in the patient’s cytopsin exhibited 1+ to 3+ positivity in at least 30% of the tumor cells. More infrequent staining (<30%) was not observed in this study set. In each instance, the staining intensity for p-170 on patient cells was compared with a graded staining reaction in control slides prepared simultaneously with myeloma cell lines expressing either DOX sensitivity, or 6-fold, 10-fold, or 40-fold DOX resistance.

**In vitro sensitivity testing.** Drug sensitivity testing for DOX and VCR and the effects of the chemosensitizing effect of Ve were determined on the fresh myeloma cell suspensions. These tests were conducted in a human tumor colony assay according to standardized procedures for plating tumor cells in semisolid agarose in the presence of complete medium containing 10% fetal calf serum. Sensitivity to DOX and VCR was assessed by adding these drugs to the cultures over a several-log range. Ve was also tested separately for possible cytotoxicity in the absence and in the presence of DOX or VCR. Ve was tested at a fixed concentration of 1.0 μg/mL. This Ve concentration was selected for in vitro testing of the sensitizers because it was noncytotoxic in vitro and was clinically achievable in vivo in the plasma of lymphoma patients treated with chemotherapy plus Ve. All drugs were tested by continuous exposure in the soft agarose cultures. Triplicate 0.5-mL cultures containing 100,000 cells were prepared for control and drug-containing cultures at each dosage level and incubated for 96 hours. Using a modification of the HTCA procedure reported by Tanigawa et al, tritiated thymidine was added at a final concentration of 10 μCi/mL to each culture for an additional 48 hours, at which time the cultures were harvested for liquid scintillation counting of tritiated thymidine incorporation into acid-precipitable DNA.

Results of drug sensitivity testing on each patient’s cells were expressed by determining the IC50 for DOX and VCR (concentration of the drug reducing clonal proliferation to 50% of control) from the drug-inhibition curves. Sensitivity to DOX was defined as an IC50 of less than 0.1 μg/mL, and to VCR as an IC50 of less than 0.001 μg/mL. To evaluate chemosensitizing effects of Ve, the dose-modifying factor (DMF) attributable to the sensitizer was determined by dividing the IC50 for the cytotoxic drug alone by the IC50 of the cytotoxic drug plus the Ve. DMF values >4 were operationally defined to indicate significant chemosensitization.

**Clinical trials.** Consent ing patients with VAD-refractory multiple myeloma who were evaluated at the Arizona Cancer Center (Tucson) were eligible for investigational therapy with VAD plus Ve in accord with protocols approved by the Human Subjects Committee of the University of Arizona. VAD-refractoriness was defined as tumor progression after one course of the VAD regimen, or as a lack of clinical response to VAD or relapse after at least two successive courses of VAD at 3- to 4-week intervals.

The VAD regimen was administered as described by Barlogie et al, with the exception that the additional pulses of dexamethasone between courses of VAD were sometimes omitted, particularly in patients with intolerance to high-dose dexamethasone. VCR (0.4 mg/d) and DOX (9 mg/m2/d) were both administered for 4 days by continuous IV infusion through a central venous access line, along with oral dexamethasone at a dose of 40 mg/d for the 4-day period. The IV formulation of Ve (Isoptin) used in this study was kindly provided by Knoll Pharmaceuticals (Whippany, NJ). As described previously, Ve was administered by continuous IV infusion in the cardiac monitoring unit at University Hospital starting 12 hours before the initiation of VAD therapy and continuing for a total of 5 days of Ve administration. The Ve dosage was started at a dose rate of 0.15 mg/kg/h and escalated at 24-hour intervals based on individualized patient tolerance, usually up to a maximum of 0.45 mg/kg/h. Plasma samples for Ve and verapamil concentrations were obtained on day 4 of the infusion and determined by high-pressure liquid chromatography. Of the 22 patients whose treatment results with VAD/Ve are included in the current report, data on six of them were reported previously. Ve was discontinued if the patient’s blood pressure decreased to less than 90 mm Hg or if second- or third-degree heart block developed. Once the toxicity had resolved, Ve therapy was resumed at a dose that was 50% of the prior dose rate.

Clinical response to therapy was determined in accord with standardized myeloma response criteria based on serial quantitative determinations of the patient’s myeloma Ig (M-protein) in the
serum and/or urine by laser nephelometry or protein electrophoresis, respectively. Typing of M-proteins was determined with immunofixation methodology. Tumor mass regression with therapy was defined in accord with current criteria of the Southwest Oncology Group. Response was defined as a 75% reduction in the rate of serum M-protein production with a serum M-protein, and reduction to less than 200 mg/24 hours or complete clearing of urinary Bence Jones proteins (BJP). Additionally, other manifestations of myeloma, including bone pain, hypercalcemia, and anemia, had to show at least stability or improvement. The development of new osteolytic lesions was considered to represent progression, but the development of compression fractures without evidence of new osteolytic lesions was not. Partial response (PR) to therapy was defined as a 50% reduction in the production rate of serum M-protein and urinary BJP. Minor reductions (MR) in M-protein production (25% to 50% of pretreatment levels) were recorded, but not classified as responses. Relapse-free and overall survival for all patients and responsive patients was determined from the time that treatment was initiated.

Statistical analysis. The correlation between IC50 values for DOX in the presence and absence of Ve was evaluated using the Pearson correlation coefficient. The Spearman rank correlation coefficient was also used because it is less sensitive to extreme values. Comparisons of in vitro results and of some in vitro and in vivo results were evaluated in 2 × 2 contingency tables using Fisher's exact test. Survival curves for patients treated with VAD/Ve were generated using the Kaplan-Meier estimator. Comparisons between curves were evaluated using the log-rank test. All P values reported are two-tailed.

RESULTS

P-glycoprotein expression and in vitro drug resistance. MDR1 expression by myeloma cells had a characteristic cell membrane distribution and occasionally also had a Golgi apparatus distribution. However, membrane staining was specifically required for the staining reaction to be called positive. As assessed by comparison to simultaneously stained myeloma cell lines with graded degrees of DOX resistance, MDR1 staining on fresh specimens usually approximated with about sixfold resistance on the cell lines, or a + staining reaction. The expression of MDR1 by myeloma cells and its relation to in vitro DOX resistance was evaluated on bone marrow aspirates from 30 patients. Among the 21 patients whose myeloma cells were MDR1-negative, 15 (71%) were sensitive to DOX in vitro. Among the nine patients whose myeloma cells were positive for MDR1 expression, only four (44%) were sensitive to DOX (P = .22).

In vitro effects of Ve on drug-sensitive and drug-resistant myeloma cells. The chemosensitizing effects of Ve on DOX- and VCR-treated cells and controls were assessed in HTCA and classified in accord with drug sensitivity or resistance of the myeloma cells to the cytotoxic agents. Figure 1 depicts a log-log plot of data on DOX sensitivity in 40 myeloma patients for whom the IC50 was determined for DOX alone and in the presence of 1.0 µg/mL of Ve. The effect of Ve as a chemosensitizer was the greatest in myeloma cells that showed the greatest degree of DOX resistance. In contrast, there was little or no effect of Ve on myeloma cells that were quite sensitive to DOX alone. When analyzed with correlation techniques, this relationship was statistically significant because the Pearson correlation coefficient was .52 (P < .001) and the Spearman rank correlation coefficient was .35 (P = .026).

An alternative method of depicting the in vitro effects of Ve on DOX- and VCR-treated cells is shown in Fig 2. In this analysis, the frequency of significant chemosensitization (DMF > 4) is related to myeloma cell sensitivity or resistance to DOX or VCR alone. As can be appreciated in Fig 2, the chemosensitizing effects of Ve were observed frequently in myeloma cells resistant to DOX or VCR alone as compared with cells that were sensitive to either of these cytotoxic drugs. For the comparison of DOX and Ve (for which there was the largest sample size), this relationship was statistically significant (P < .001). The same trend was seen with VCR (Fig 2). For all experimental comparisons combined with either DOX or VCR, the addition of Ve produced a highly significant sensitizing effect on the drug-resistant tumor cells (P < .001). Whereas 15 of 19 (78.9%) of the resistant tumor specimens had a DMF > 4 with Ve, only 3 of 31 (9.7%) of the sensitive tumors had a DMF > 4.

Clinical trials of chemosensitizers. A total of 22 refractory myeloma patients who were treated with VAD plus IV Ve (after failing on VAD alone) were evaluable for therapeutic response. Patient characteristics and response information are summarized in Table 1.

VAD plus high-dose Ve. All 22 patients who received high-dose Ve were hospitalized and treated in cardiac telemetry units at University Medical Center. The maxi-
MDR AND VAD/VERAPAMIL IN MYELOMA

Fig 2. Relation of Ve effects to intrinsic sensitivity or resistance of myeloma cells to DOX or VCR. Sensitivity to DOX was defined as an IC50 < .01 µg/mL and to VCR as an IC50 < .001 µg/mL. Ve was added at the noncytotoxic concentration of 1.0 µg/mL. Drug sensitivity to the cytotoxics is depicted with the solid bars, and resistance with the hatched bars. The total number of patient specimens tested and showing sensitivity or resistance to the cytotoxic drug alone is shown above the bars. The percentage of specimens tested that showed a significant sensitizing factor (DMF > 4.0) is shown on the ordinate. For myeloma cells exhibiting sensitivity to DOX, Ve had a significant sensitizing effect in only 3 of 26 instances (12%). In contrast, for myeloma cells showing DOX resistance, Ve had a significant sensitizing effect in 12 of 15 biopsies tested (80%) (P < .001). The same trend was seen with VCR plus Ve. Combining all results, the effect of the sensitizers on drug-resistant cells was significantly greater than on drug-sensitive cells (P < .001).

Dose limiting toxicity of Ve was given based on observed cardiac toxicity. Toxicities of VAD plus IV Ve are summarized in Table 2. The primary toxicity of high-dose IV Ve was cardiac in nature and consisted of hypotension, fluid retention, and EKG abnormalities. These cardiovascular effects normally subsided within 24 hours of cessation of IV Ve.

Hypotension was the dose-limiting toxicity of Ve and was observed in 16 of 22 patients. Patients were generally asymptomatic and no patient required pressor support. Nine patients complained of shortness of breath or had symptoms of mild congestive heart failure. The average weight gain for this group of patients was 4.0 kg. Symptoms were relieved by giving modest doses of furosemide and decreasing the dose of Ve. First-degree heart block was induced in all but four patients in the course of IV Ve therapy. Ten of 22 patients had second-degree heart block or transient AV nodal dissociation, which in all cases were asymptomatic. Discontinuation of Ve resulted in normalization of the EKG within 4 hours.

The only noncardiac toxicity observed was an occasional tremor or headache associated with irritability suggestive of central nervous system (CNS) effects. Plasma Ve assays (generally obtained on day 4 of the infusion) were obtained in 20 myeloma patients and showed the median serum concentration of Ve to be 295 ng/mL (range 129 to 978 ng/mL). For 11 patients tested for levels of the metabolite norverapamil, the median level was 146 µg/mL (range of 47 to 264 µg/mL) and the median Ve/norverapamil ratio was 2.49.

Therapeutic results with VAD/Ve in relation to MDR1 expression are summarized in Table 1. Of the 22 patients treated with VAD plus Ve, five patients (23%) achieved a partial response. Among the remaining 17 patients, six had minor reductions in M-protein production and 11 showed no change or overt progression during therapy.

P-glycoprotein was determined immunohistochemically on bone marrow aspirates from 15 of the patients (68%) before treatment with VAD plus Ve (Table 1). Of these, 10 were MDR1-positive (67%). Among the 10 MDR1-positive myeloma patients, four (40%) achieved PRs with the addition of Ve to the VAD regimen. On the other hand, none of the five MDR1-negative patients achieved PR status with VAD/Ve (P = .23). Of the seven MDR1 unknown patients, one achieved a PR on VAD/Pe. In vitro drug testing and determination of the DMF was performed before VAD/Pe therapy in a total of 10 patients. DMF values > 4 were obtained in three of four patients who achieved PRs on therapy, and in only one of six of the clinical nonresponders (P = .19).

The median survival from the start of VAD/Ve for all 22 patients was only 5.1 months. The median relapse-free interval for the five patients who were responsive to VAD/Ve was 5.4 months. The median survival for the five responders was 13.5 months in comparison with 3.4 months for the nonresponders (P = .11).

DISCUSSION

In the current study we extended our clinical experience with VAD/Ve as well as performing relevant in vitro observations on MDR1 expression and chemosensitization. The frequency of response to VAD/Ve (5 of 22 patients or 23%) is consistent with the response of two of six patients in our original pilot study. The original pilot study also reported an objective response in a single lymphoma patient. More recently we performed a phase II evaluation of cyclophosphamide plus Ve (CVAD) along with high-dose IV Ve (as used in myeloma) in 18 patients with heavily pretreated and drug-resistant lymphoma.14 Thirteen of these patients (73%) were brought back into at least partial remission with the use of the same Ve regimen, with five of the patients (28%) achieving complete remissions. As was observed in the current myeloma study, the responses were somewhat more frequent in lymphoma patients whose biopsies were immunohistochemically positive for MDR1. The objective response rate of lymphoma patients whom we treated with CVAD/Pe was significantly higher than was obtained in myeloma with VAD/Ve. While we recognize that many factors, including tumor type and cyclophosphamide administration, may account for this difference in responsiveness, Ve tolerance may also be an important factor. Perhaps in part because the lymphoma patients were significantly younger, they tolerated significantly higher dosages of IV Ve than did the patients with myeloma. Tol-
Table 1. Patient Characteristics and Response to VAD Plus Ve in Relation to P-glycoprotein Expression

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<th>Patient No.</th>
<th>Sex</th>
<th>Age</th>
<th>M-Component Type</th>
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P-glycoprotein-negative

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P-glycoprotein unknown

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At least 50% reduction in M-protein synthesis and tumor burden.

Abbreviations: PR, partial response; MR, minor response; NR, no response. See Materials and Methods for response criteria.

*Patient included in pilot study (Ref 21).

†Patient had neurotoxicity from prior vincristine, and only received Adriamycin by infusion plus decadron until started on VAD/Ve.


crance was reflected in part by the median plasma concentrations of Ve, which were normally determined on day 4 of the infusions. Among the 18 lymphoma patients tested, the median plasma Ve concentration was 1,022 ng/mL, whereas among the 20 myeloma patients tested in the current study the median Ve concentration was 295 ng/mL.

MDR1 expression has been reported to be present constitutively or is acquired after drug exposure in various forms of human cancer. Our selection of B-cell neoplasms such as myeloma and lymphoma for clinical trials of chemosensitizers was based not only on evidence of P-glycoprotein expression, but also on the original observation by Tsuruo et al. that Ve could reverse vinca alkaloid resistance in a murine lymphoma leukemia model.

In this report, several new laboratory observations on other variables. The level of sensitivity that we currently have in the immunohistochemical detection of MDR1 expression is in the range of a fourfold to sixfold expression of DOX resistance as determined in studies with MDR-expressing myeloma cell lines. Based on our in vitro comparisons, we suspect that in vitro chemosensitization with Ve in HTCA or by flow cytometry may be a somewhat more sensitive test for the presence of functional P-glycoprotein than is immunohistochemistry. On the other hand, the immunohistochemical procedure for MDR1 expression is far simpler to perform and therefore may be more clinically applicable. Unfortunately, some specimens prove unsatisfac-
tory for immunohistochemical evaluation, and this was the case in the current study so that correlations were not available in all patients.

We have closed our local studies of VAD plus Ve and have initiated a prospective randomized trial in refractory multiple myeloma of VAD alone versus VAD plus oral Ve in the Southwest Oncology Group. This large-scale evaluation will critically evaluate the activity of the Ve as well as of VAD.

Ve was selected as the initial chemosensitizers to add to the VAD regimen because it could block multidrug resistance in DOX-resistant, MDR1-expressing human myeloma cell lines derived from the 8226 myeloma line as tested in our laboratories.

Chemosensitizers such as Ve are at best first-generation agents. Ve as marketed is a racemic mixture of s-verapamil and r-verapamil. While s-verapamil is the component predominantly mediating the drug's calcium-channel blocking cardiac effects, the r-enantiomer has approximately 80% less cardiovascular effect in preclinical assays. In contrast to this marked difference in cardiac effects, r- and s-verapamil as well as the racemic mixture appear to have similar potency in inhibiting the function of P-glycoprotein. Accordingly, we have recently initiated clinical studies of r-verapamil. Drugs with differing structures are now under evaluation alone and in combination as potential chemosensitizers to inhibit MDR1 in various neoplasms including myeloma (eg, ref 39).

Under optimal circumstances, the use of chemosensitizers in cancer therapy will not be limited to reversing established drug resistance but might also be able to prevent the development of drug resistance when added to initial chemotherapy. We recognize that clinical drug resistance is multifactorial and mechanisms other than MDR1 may prove important in multiple myeloma and lymphoma. Nonetheless, our findings suggest that we may be approaching a new era in clinical oncology wherein drug resistance can be effectively circumvented.

ACKNOWLEDGMENT

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