Reversal by Cefoperazone of Resistance to Etoposide, Doxorubicin, and Vinblastine in Multidrug Resistant Human Sarcoma Cells

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ABSTRACT

The cephalosporins are a family of semisynthetic antibiotics, some of which have structural features associated with substrates for the multidrug transporter, P-glycoprotein. The activity of a series of six cephalosporins in reversing multidrug resistance (MDR) was examined in MDR variants (Dx5 cells) of the human sarcoma line MES-SA. Dx5 cells express high levels of the mdr1 gene product P-glycoprotein and are 25- to 30-fold resistant to doxorubicin (DOX), etoposide (VP-16), and vinblastine (VBL). Cytotoxicity was measured by the MTT assay. Reversal by Cefoperazone of Resistance to Etoposide, Doxorubicin, and Vinblastine

INTRODUCTION

The typical MDR phenotype includes cross-resistance to anthracyclines, Vinca alkaloids, podophyllotoxins, and other cytotoxic compounds, and increased expression of a membrane protein termed P-gp (1-3). The proposed mechanism of resistance is a decrease in the accumulation and retention of the xenobiotics by P-gp, functioning as an active efflux pump. Evidence supporting this mechanism includes the association of MDR with increased expression of the mdr1 gene which encodes P-gp, the demonstration of direct binding of transport substrates to P-gp by photoaffinity labelling, and transfer of the MDR phenotype by transfection of the mdr1 gene (1-6). The demonstration of increased mdr1 expression in some normal tissues and human cancers supports the hypotheses that P-gp is involved both in normal detoxification of various cytotoxins and in resistance of some cancers to chemotherapy (7).

MDR can be reversed or modulated by many drugs, including verapamil, trifluoperazine, quindine, reserpine, and cyclosporine A (8-18). These agents share some or all of the features of known substrates of P-gp, including lipophilicity, a planar polycyclic stereochemistry, and weak basicity. The mechanism of modulation in most cases is thought to be competitive inhibition of drug efflux.

Verapamil and trifluoperazine have been used in clinical trials to reverse MDR (19-24). A major problem with these trials has been toxicity of the modulators (heart block by verapamil and depression of the central nervous system by trifluoperazine), at drug concentrations below those which reverse MDR in vitro. The ability of these agents to modulate MDR is unrelated to their primary pharmacological action, since their ability to reverse drug resistance does not correlate with their properties of calcium channel blocking or calmodulin inhibition (25-31).

Some of the cephalosporin antibiotics (Fig. 1) share structural characteristics with agents known to modulate MDR, and are considerably less toxic in vivo. In this study, we assessed the ability of a series of cephalosporin antibiotics to modulate MDR and enhance drug accumulation in human sarcoma cells, and identified important physicochemical characteristics for modulating activity.

MATERIALS AND METHODS

Reagents. The sources of the drugs used in these experiments are as follows: DOX (Adriamycin), Adria Laboratories, Columbus, OH; VBL (Velban), Eli Lilly Co., Indianapolis, IN; VP-16 (Vepesid), Bristol Myers Co., Wallingford, CT; verapamil (Isoptin), Knoll Pharmaceutical Co., Whippany, NJ; cefoxazone (Velocef), E. R. Squibb and Sons Inc., Princeton, NJ; cepofoxin (Cefobid), Roerig, New York, NY; cefotetan (Cefotan), Stuart Pharmaceuticals, Wilmington, DE; cefotaxime (Rocephin), Roche Laboratories, Nutley, NJ; cefazidime (Tazidime) and cefazolin (Kezfol), Eli Lilly Co., Indianapolis, IN. The MTT salt and other chemicals unless specified were obtained from Sigma Chemical Co. (St. Louis, MO). All reagents were initially reconstituted in Dulbecco’s phosphate-buffered saline (GIBCO, Grand Island, NY) and final dilutions were made in McCoy’s 5A medium (GIBCO) supplemented with penicillin (100 units/ml), streptomycin (100 //g/ml), insulin (5 //g/ml), and 10% newborn calf serum (GIBCO). High-performance liquid chromatography grade 1-octanol was purchased from Aldrich Chemical Co., Milwaukee, WI.

Cell Culture. The cells used in these experiments were the human sarcoma cell line MES-SA and its doxorubicin-selected MDR variant Dx5 (32, 33). Monolayer cultures of MES-SA and Dx5 cells were grown in tissue culture flasks (Corning Glass Works, Corning, NY) containing McCoy’s 5A medium and 10% newborn calf serum. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2 and subcultured every 5 to 7 days.

MTT Cytotoxicity Assay. Cells grown in 75-cm2 tissue flasks were harvested with 0.06 M EDTA, and cell number and viability was determined by a hemocytometer and trypan blue dye exclusion. Cells were seeded in 96-well plates (Falcon, Becton Dickinson Co., Lincoln Park, NJ) at 8 x 103 cells per well and allowed to grow for 24 h at 37°C. The cells were then treated with drugs. DOX, VBL, and VP-16 were used at concentrations from 3.0 x 10-5 to 3.0 x 10-4 M and the cephalosporin antibiotics at concentrations ranging from 0.25 to 1.0

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The abbreviations used are: MDR, multidrug resistance; DOX, doxorubicin; IC50, the drug concentration resulting in 50% inhibition of MTT dye formation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; logP, the partition coefficient or log ratio of the concentration of drug in octanol versus aqueous medium; P-gp, P-glycoprotein; VBL, vinblastine; VP-16, etoposide.
curves. The percentage of drug protein bound was determined by the
formula: 100 × (total drug concentration-free drug concentration)/total
drug concentration.

Accumulation Studies with [3H]Vinblastine. MES-SA and Dx5 cells were
harvested with 0.06 M EDTA, washed twice in Hank's balanced
salt solution with 10% newborn calf serum, and resuspended at a cell
concentration of 2.0 × 10^6 cells/ml in McCoy's 5A medium with 10%
newborn calf serum and 20 mm of HEPES buffer (Sigma Chemical
Company, St. Louis, MO). Both the MES-SA and Dx5 cells were
exposed to [3H]Vinblastine (specific activity, 23 Ci/mmol; Amersham
International, UK) at a concentration of 1.25 μM alone and in the
presence of 1.0 mM of cefoperazone. Cells were incubated for 60 min
in a water bath at 37°C with shaking. Then 0.5-ml aliquots (containing
10^6 cells) were removed layered on silicone oil (Versilube F50; General
Electric Co., Waterford, NY) in microcentrifuge tubes, and centrifuged
at 12,000 × g for 1 min to remove the cells from drug-containing
medium (2). Following the aspiration of the medium and oil, the tube
tips containing the cell pellets were cut off and the cells were solubilized
in 0.2 N NaOH. The tube contents were then neutralized, scintillation
fluor was added, and radioactivity was counted.

Statistical Analyses. Correlations of lipid solubility and protein bind-
ing with MDR modulation were performed by linear regression. A
coefficient of variation was determined for each drug concentration
on each plate (four wells per drug concentration) and standard deviations
were determined from each IC50 of DOX with the various modulators.
Each experiment was performed at least three times.

RESULTS

Modulating of Cytotoxicity. The strain of Dx5 cells in these
experiments was 30-fold resistant to DOX compared to the
parental drug-sensitive MES-SA cell line. They were also
approximately 30-fold cross-resistant to both etoposide and vin-
blastine. As a positive control for modulation of MDR, verap-
amil (6 μM) lowered the IC50 of DOX from 3 × 10^{-7} M to 1.3
× 10^{-8} M in Dx5 cells, nearly complete reversal of resistance.
The IC50 of DOX in MES-SA cells was 1.0 × 10^{-8} M.

The structures of the six cephalosporins used in these exper-
iments are shown in Fig. 1. Among these, cefoperazone (1.0 mM)
was the most effective modulator of MDR, lowering the IC50 for VP-16 from 10 × 10^{-6} M to 3.4 × 10^{-7} M (modulation
ratio of 29x); the IC50 for DOX from 3 × 10^{-7} M to 2.2 × 10^{-8} M (14x); and the IC50 for VBL from 5.3 × 10^{-8} M to 3.3 × 10^{-9} M (16x), Tables 1-3. Cefoperazone completely reversed resis-
tance to VP-16 (Table 2), and reduced the IC50 of VBL and
DOX in Dx5 cells to a level only two-fold higher than the
sensitive parental line.

Ceftriaxone at 1.0 mM produced 10x modulation of VP-16
cytotoxicity, 8x for DOX, and 2x for VBL. The reversal of

<p>| Table 1 Modulation of resistance to DOX in Dx5 cells by cephalosporin antibiotics |
|-------------------------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Cephalosporin (mm)</th>
<th>IC50, DOX, (nM)</th>
<th>Modulation ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, DOX alone</td>
<td>300 ± 82</td>
<td>-</td>
</tr>
<tr>
<td>Cefoperazone (1.0)</td>
<td>22 ± 9</td>
<td>14</td>
</tr>
<tr>
<td>Cefoperazone (0.5)</td>
<td>50 ± 12</td>
<td>6</td>
</tr>
<tr>
<td>Cefoperazone (0.25)</td>
<td>75 ± 7</td>
<td>6</td>
</tr>
<tr>
<td>Ceftriaxone (1.0)</td>
<td>38 ± 10</td>
<td>8</td>
</tr>
<tr>
<td>Ceftriaxone (0.5)</td>
<td>45 ± 10</td>
<td>7</td>
</tr>
<tr>
<td>Ceftriaxone (0.25)</td>
<td>55 ± 20</td>
<td>5</td>
</tr>
<tr>
<td>Cefazolin (1.0)</td>
<td>85 ± 10</td>
<td>4</td>
</tr>
<tr>
<td>Cefazidime (1.0)</td>
<td>110 ± 15</td>
<td>3</td>
</tr>
<tr>
<td>Cephadine (1.0)</td>
<td>120 ± 20</td>
<td>3</td>
</tr>
<tr>
<td>Cefotetan (1.0)</td>
<td>130 ± 30</td>
<td>2</td>
</tr>
</tbody>
</table>

* Each value represents the mean ± standard deviation of at least three determinations.
* The modulation ratio is defined as the ratio of the IC50 for DOX alone versus the IC50 in the presence of the modulating agent.

Fig. 1. Chemical structures of the cephalosporins.
Table 2 Modulation of resistance to VP-16 in Dx5 cells by cefoperazone and ceftriaxone

<table>
<thead>
<tr>
<th>Cephalosporin (mM)</th>
<th>IC₅₀ (μM) *</th>
<th>Modulation ratio *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, VP-16 alone</td>
<td>10.0 ± 1.0x</td>
<td>1</td>
</tr>
<tr>
<td>Cefoperazone (1.0)</td>
<td>0.34 ± 0.05</td>
<td>30</td>
</tr>
<tr>
<td>Cefoperazone (0.5)</td>
<td>0.86 ± 0.04</td>
<td>12</td>
</tr>
<tr>
<td>Cefoperazone (0.25)</td>
<td>2.40 ± 0.14</td>
<td>4</td>
</tr>
<tr>
<td>Ceftriaxone (1.0)</td>
<td>1.04 ± 0.09</td>
<td>10</td>
</tr>
<tr>
<td>Ceftriaxone 0.5</td>
<td>2.15 ± 0.07</td>
<td>5</td>
</tr>
<tr>
<td>Ceftriaxone 0.25</td>
<td>7.85 ± 0.49</td>
<td>1</td>
</tr>
</tbody>
</table>

* Each value represents the mean ± standard deviation of at least three determinations.

* The modulation ratio is defined as the ratio of the IC₅₀ for VP-16 alone versus the IC₅₀ in the presence of the modulating agent.

Table 3 Modulation of resistance to VBL in Dx5 cells by cefoperazone and ceftriaxone

<table>
<thead>
<tr>
<th>Cephalosporin (mM)</th>
<th>IC₅₀ (μM) *</th>
<th>Modulation ratio *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, VBL alone</td>
<td>53 ± 4.3</td>
<td>1</td>
</tr>
<tr>
<td>Cefoperazone (1.0)</td>
<td>3.3 ± 0.2</td>
<td>16</td>
</tr>
<tr>
<td>Cefoperazone (0.5)</td>
<td>7.9 ± 0.2</td>
<td>7</td>
</tr>
<tr>
<td>Cefoperazone (0.25)</td>
<td>15 ± 7.0</td>
<td>4</td>
</tr>
<tr>
<td>Ceftriaxone (1.0)</td>
<td>26 ± 1.4</td>
<td>2</td>
</tr>
<tr>
<td>Ceftriaxone 0.5</td>
<td>29 ± 1.4</td>
<td>2</td>
</tr>
<tr>
<td>Ceftriaxone 0.25</td>
<td>34 ± 1.5</td>
<td>2</td>
</tr>
</tbody>
</table>

* Each value represents the mean ± standard deviation of at least three determinations.

* The modulation ratio is defined as the ratio of the IC₅₀ for VBL alone versus the IC₅₀ in the presence of the modulating agent.

resistance was dependent on the concentrations of cefoperazone and ceftriaxone over the range of 0.25-1.0 mM, Tables 1-3, and Figs. 2-4. No modulation of cytotoxicity was observed in the parental MES-SA cells, which do not express mdr1 (data not shown). Cefazolin, cephradine, cefotetan, and ceftazidime were relatively ineffective as modulators for DOX cytotoxicity, and were not tested with VBL and VP-16 (Table 1).

Octanol-Water Partitioning Studies. Cefoperazone was the most lipid soluble of the cephalosporins, with a partition coefficient (log₁₀P) of -0.49 at pH 7.4, Table 4. Ceftriaxone was the next most lipid soluble agent with a log₁₀P of -0.60. There was a highly significant correlation between lipid solubility and MDR modulation of these five agents (r² = 0.881).

Protein Binding and Effects of Serum Concentration on Modulation of MDR. Cefoperazone and ceftriaxone were also the most highly protein bound agents in medium with 10% newborn calf serum, Table 4. Ceftriaxone was 52% protein bound, whereas cefoperazone was 30% bound. The correlation of percentage protein binding in medium by the various agents and their ability to modulate MDR was poor (r² = 0.181).

Changing the protein concentration in the medium did not markedly alter modulating activity. Increasing the protein concentration in the medium from 10% to 50% only slightly decreased modulation of DOX by ceftriaxone, with the IC₅₀ increasing from 3 x 10⁻⁸ to 5 x 10⁻⁸ M.

Cellular Accumulation of Vinblastine. The effect of 1.0 mM cefoperazone on the accumulation of [³H]vinblastine in MES-SA and Dx5 cells is presented in Table 5. Cefoperazone did not...
concentration used in our experiments (43-45). Cefoperazone concentrations on the order of 1 μM, which is the highest cefoperazone with P-gp are currently in progress. The concentration dependence of the modulation, and the restoration of intracellular vinblastine levels at high cefoperazone concentrations required to modulate MDR in patients is not surprising, since these drugs are primarily used for their actions in cardiac conduction and the central nervous system, respectively. The search for modulators with a safer therapeutic ratio might rationally focus on agents which are not targeted at mammalian physiology per se. The antibiotics represent such a group of drugs of diverse chemical classes, targeted primarily at microbial metabolism, but which may also be substrates for mammalian P-glycoproteins.

We chose to study the cephalosporins because they contain structural features associated with some modulators of MDR (Fig. 1), and they are relatively nontoxic to humans even at concentrations on the order of 1 mM, which is the highest concentration used in our experiments (43-45). Cefoperazone was the most effective modulator of MDR, reducing resistance to DOX in Ds5 cells from 30-fold to 1.8-fold when compared to sensitive MES-SA cells. Modulation was most marked for VP-16, with complete reversal of resistance to this agent. The concentration dependence of the modulation, and the restoration of intracellular vinblastine levels at high cefoperazone concentrations, is consistent with competitive inhibition of transport of the chemotherapeutic agents. Experiments to confirm this hypothesis regarding the nature of the interaction of cefoperazone with P-gp are currently in progress.

Ceftriaxone was also capable of modulating MDR, but to a lesser degree than cefoperazone for all three of the cytotoxins. These two agents share the physicochemical properties of higher protein binding and greater lipid solubility compared to the other antibiotics tested. Lipid solubility correlated better with modulation of MDR than did protein binding. Lipid solubility has also been shown to be important in the modulation of MDR by other classes of drugs such as analogues of reserpine, quinoline, and dipyrine (29-31). Because of concern that plasma protein binding in vivo may lessen the efficacy of modulation, we varied the serum concentration from 5 to 50%. Only a modest (two-fold) decrease in modulation was seen with the 10-fold increase in protein concentration.

The relative efficacy of cefoperazone as a modulator of MDR may be related to its N-ethylpiperazine group, which is unique among these cephalosporin antibiotics (Fig. 1). This structural characteristic is shared with trifluoperazine and prochlorperazine, two phenothiazines which modulate MDR (15, 25, 31, 38). Other phenothiazines which lack this chemical moiety, such as thiordazine, were not effective modulators (25, 31). The importance of this piperazine group is also suggested by its presence in dilazep, a verapamil analogue, which is a more potent modulator than its parent compound (40). In addition, the antihistamine prenylamine does not modulate MDR, unlike cinnarazine, a compound different only in the addition of a piperazine group (30). These examples support an important role for the piperazine moiety in binding to P-gp.

In summary, we have shown that the cephalosporin antibiotic cefoperazone is an effective modulator of MDR in vitro. This modulation occurs at drug concentrations which may be achievable without major toxicity in patients (43-45). P-gp has been shown to be present at high levels in several normal tissues, and is likely to be important in the normal disposition of MDR-related cytotoxins (7). Further studies should be performed on the efficacy of cefoperazone in vivo in preclinical models as well as its effects on the pharmacokinetics and toxicity of anticancer agents involved in MDR.

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REFERENCES

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