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 mdrl 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 M'tlT assay. Cefazolin, cefotetan, cephradine, and ceftriaxone were 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 mdrl 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 mdrl gene (1-6). 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 mdrl 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 mdrl gene (1-6). The demonstration of increased mdrl 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).

INTRODUCTION

MDR can be reversed or modulated by many drugs, including verapamil, trifluoperazine, quinidine, 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 Pharmaceuticals, Whippany, NJ; cephradine (Velosef), E. R. Squibb and Sons Inc., Princeton, NJ: cefoperazone (Cefobid), Roerig, New York, NY; cefotetan (Cefotan), Stuart Pharmaceuticals, Wilmington, DE; ceftriaxone (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 μg/ml), streptomycin (100 μg/ml), insulin (5 μg/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 DxS (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 104 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^-4 to 3.0 x 10^-8 M and the cephalosporin antibiotics at concentrations ranging from 0.25 to 1.0...
multidrug resistance and cefoperazone

Curves (controls) was determined directly from semilogarithmic dose-response curves. Controls were dissolved in 0.1 N HCI in 2-propanol (100 µl per well). Cells were incubated for 72 h with the cytotoxic agent (DOX, VBL, or VP-16) and the modulating drug. Then the medium was removed from each well, and 100 µl of fresh medium and 10 µl of MTT dye (5 mg/ml in phosphate-buffered saline stock solution) were added to each well. Cells were incubated with MTT for 6 h, after which blue formazan was assayed in quadruplicate. The IC₅₀ (the drug concentration resulting in 50% inhibition of MTT dye formation, compared to untreated controls) was determined directly from semilogarithmic dose-response curves.

Octanol-Water Partitioning Studies. Five of the cephalosporin antibiotics (cefoxazone, ceftriaxone, cephradine, cefazolin, and cefotetan) were initially reconstituted in phosphate-buffered saline stock solution and added to each well. Cephalosporins were incubated with MTT for 6 h, after which blue formazan crystals were dissolved in 0.1 N HCl in 2-propanol (100 µl per well). Within 30 min after dissolving the crystals, each plate was read on a Dynatech MR580 Microelisa reader with a test wavelength of 570 nm (34-36). Each drug concentration was assayed in quadruplicate. The IC₅₀ (the drug concentration resulting in 50% inhibition of MTT dye formation, compared to untreated controls) was determined directly from semilogarithmic dose-response curves.

Protein Binding Studies. Three ml of each cephalosporin antibiotic (cefoxazone, ceftriaxone, cephradine, cefazolin, and cefotetan) was determined directly from semilogarithmic dose-response curves. Verapamil was used as a positive control for modulation at 6.0 µM. Cells were incubated for 72 h with the cytotoxic agent (DOX, VBL, or VP-16) and the modulating drug. Then the medium was removed from each well, and 100 µl of fresh medium and 10 µl of MTT dye (5 mg/ml in phosphate-buffered saline stock solution) were added to each well. Cells were incubated with MTT for 6 h, after which blue formazan crystals were dissolved in 0.1 N HCl in 2-propanol (100 µl per well). Within 30 min after dissolving the crystals, each plate was read on a Dynatech MR580 Microelisa reader with a test wavelength of 570 nm and a reference wavelength of 630 nm (34-36). Each drug concentration was assayed in quadruplicate. The IC₅₀ (the drug concentration resulting in 50% inhibition of MTT dye formation, compared to untreated controls) was determined directly from semilogarithmic dose-response curves.

Octanol-Water Partitioning Studies. Five of the cephalosporin antibiotics (cefoxazone, ceftriaxone, cephradine, cefazolin, and cefotetan) were initially reconstituted in phosphate-buffered saline (pH 7.4) to a concentration of 1 x 10⁻⁵ M. Then 3 ml of this solution was added to 50% inhibition of MTT dye formation, compared to untreated controls) was determined directly from semilogarithmic dose-response curves.

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 vincristine. As a positive control for modulation of MDR, verapamil (6 µM) lowered the IC₅₀ of DOX from 3 x 10⁻⁷ M to 1.3 x 10⁻⁸ M in Dx5 cells, nearly complete reversal of resistance. The IC₅₀ of DOX in MES-SA cells was 1.0 x 10⁻⁴ M.

The structures of the six cephalosporins used in these experiments are shown in Fig. 1. Among these, cefoperazone (1.0 mM) was the most effective modulator of MDR, lowering the IC₅₀ of VP-16 from 10 x 10⁻⁶ M to 3.4 x 10⁻⁷ M (modulation ratio of 29x); the IC₅₀ for DOX from 3 x 10⁻⁷ M to 2.2 x 10⁻⁸ M (14x); and the IC₅₀ for VBL from 5.3 x 10⁻⁸ M to 3.3 x 10⁻⁹ M (16x), Tables 1-3. Cefoperazone completely reversed resistance 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

<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>1</td>
</tr>
<tr>
<td>Cefoperazone (1.0)</td>
<td>22 ± 9</td>
<td>14</td>
</tr>
<tr>
<td>Cefotetan (0.5)</td>
<td>50 ± 12</td>
<td>6</td>
</tr>
<tr>
<td>Ceftriaxone (0.25)</td>
<td>75 ± 7</td>
<td>4</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>Cefazolin (1.0)</td>
<td>110 ± 15</td>
<td>3</td>
</tr>
<tr>
<td>Cephradine (1.0)</td>
<td>120 ± 20</td>
<td>3</td>
</tr>
<tr>
<td>Cefoperazone (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.
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*²</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>29</td>
</tr>
<tr>
<td>Cefoperazone (0.5)</td>
<td>0.86 ± 0.04</td>
<td>12</td>
</tr>
<tr>
<td>Cefotetan (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*²</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>Ceftriaxone (1.0)</td>
<td>15 ± 7.0</td>
<td>4</td>
</tr>
<tr>
<td>Ceftriaxone 0.5</td>
<td>26 ± 1.4</td>
<td>2</td>
</tr>
<tr>
<td>Ceftriaxone 0.25</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
Table 5 Effect of cefoperazone (CFP) on vinblastine accumulation in drug-sensitive MES-SA versus multidrug resistant Ds5 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>(−) CFP</th>
<th>(+) CFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES-SA</td>
<td>43.0 ± 1.4*</td>
<td>42.0 ± 0.7</td>
</tr>
<tr>
<td>Ds5</td>
<td>20.1 ± 0.5</td>
<td>43.2 ± 3.5*</td>
</tr>
</tbody>
</table>

* Expressed as nanomoles of [3H]vinblastine per 106 cells. Values represent the mean ± standard deviation, n = 3.

* P < 0.05, (−) CFP versus (+) CFP.

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 thioridazine, 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 cinnarzine, 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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