In Vitro Prevention of the Emergence of Multidrug Resistance in a Pediatric Rhabdomyosarcoma Cell Line

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ABSTRACT

We have established preclinical models for the development of drug resistance to vincristine (a major drug used in the treatment of pediatric rhabdomyosarcoma) using cell lines. The RD cell line has a mutant P53 phenotype and does not have detectable P-glycoprotein (P-gp) or multidrug resistance-related protein (MRP) despite expressing low levels of mdr-1 mRNA, which encodes P-gp and mrp1 mRNA. Resistant variants of RD were derived by exposure to increasing concentrations of vincristine. This was repeated on six occasions, resulting in three cell lines which could tolerate 64 × the IC_{50} concentration. Six independent agents were tested for their ability to prevent the development of resistance in this model. Despite at least 10 attempts, resistance did not develop in the presence of the multidrug resistance (MDR) modulators PSC833, VX710, and XR9576. This strongly suggests that these agents may delay or even prevent the development of resistance to vincristine. This was also confirmed in a second rhabdomyosarcoma cell line, Rh30. In contrast, the agents indomethacin (MRP1 modulator), CGP41251 (protein kinase C inhibitor), and dexrazoxane (putative MDR prevention agent) did not affect the development of resistance in the RD model. Characterization of the resistant cell lines indicated the presence of increased mdr-1 and P-gp expression, which resulted in resistance to the agents doxorubicin, etoposide, and vincristine but not cisplatin. The resistance could be modulated using PSC833 or VX710, confirming that functional P-gp is present. No apparent differences were seen between the resistant cell lines derived in the absence and presence of the various agents. These experiments strongly suggest that the development of MDR may be preventable using modulators of MDR and merit clinical studies to test this hypothesis.

INTRODUCTION

RMS is a highly malignant tumor which accounts for over half of all soft tissue sarcomas in children (1). The disease is commonly treated using chemotherapeutic agents, including Vinca alkaloids, anthracyclines, etoposide, and cyclophosphamide. Initial treatment is often successful, but relapse because of drug resistance remains a major obstacle to survival. In some, but not all, studies, the expression of P-gp, encoded by the mdr-1 gene, has been associated with adverse prognosis (2, 3). Our recent in vitro studies have indicated that relatively low expression of P-gp and MRP may contribute to resistance in RMS (4).

Historically, studies of acquired resistance have involved the development of a resistant cell line in vitro after exposure to increasing drug concentrations (5, 6). When used with MDR (2) efflux substrates, this method has probably led to a disproportionate number of resistant cell lines which express P-gp or MRP1, as this appears to be the most common mechanism of resistance. Evidence that this is also true in the clinic is limited, and clones surviving a single high dose of drug frequently show other mechanisms of resistance (7). The long-term exposure method has also led to cell lines which overexpress very high levels of P-gp and MRP1, and this degree of resistance is rarely seen in clinical samples. Despite these caveats, cell lines over-expressing P-gp and MRP1 have been extremely useful in understanding the biology of drug resistance.

Expression of mdr-1 may be rapidly up-regulated in response to chemotherapeutic agents both in vitro (8–10) and in vivo (11). These observations may explain the lack of correlation between P-gp expression at the time of diagnosis (before treatment) and eventual outcome in many studies.

At least three modulators of MDR, and P-gp in particular, are currently undergoing clinical trials. They include agents such as the nonimmunosuppressive cyclosporin A analogue, PSC833 (Valspodar; Ref. 12), the nonmacrocyclic pipicolinate derivative VX710 (Biricodar; Ref. 13), and the novel compound XR9576 (14). These compounds are usually administered after relapse, and their effectiveness in the clinic to date is debatable.

An interesting therapeutic strategy is to try to prevent the development of MDR by treating with an MDR modulator at the onset of therapy. If resistance is intrinsic, the modulator sensitizes the tumor, whereas if resistance is acquired, multidrug-resistant cells no longer have a selective advantage. However,

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Received 4/24/01; revised 7/20/01; accepted 7/23/01.

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3 The abbreviations used are: P-gp, P-glycoprotein; RMS, rhabdomyosarcoma; PKC, protein kinase C; MRP, multidrug resistance protein; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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there are several reasons why this strategy may fail. The tumor may initially possess multiple mechanisms of resistance, or alternative mechanisms of resistance may arise as frequently and as quickly as MDR. Despite this, there may be a subpopulation of tumors for which this strategy is beneficial and results in longer disease-free survival or even a cure. There is limited clinical evidence that this is the case (15).

This study has developed an in vitro model for the development of resistance to vincristine in pediatric RMS. Vincristine was selected because it is widely used in treatment and is one of the most effective agents. This model has been used to test the ability of six agents to prevent resistance, including the modulators PSC833, VX710, and XR9576. In addition, the MRP1 modulator indomethacin (16) was included, and because of the postulated role of PKC in MDR (17), the inhibitor CGP41251 was also included (18). Finally, a recent study suggested that dexrazoxane, a compound used to minimize the cardiotoxicity of doxorubicin, may also prevent the development of MDR in vitro (19), and this compound has also been tested.

These studies have been used to investigate whether these agents prevent or delay development of MDR and whether alternative mechanisms of resistance arise. Previous studies have shown that resistance can arise in the presence of modulators (20, 21), so this study investigates how frequently this occurs and whether alternative mechanisms of resistance develop as quickly.

**MATERIALS AND METHODS**

**Cell Lines.** This study uses the cell lines RD and Rh30, which have been described previously (4). The cell line RD was obtained from the American Type Culture Collection (Manassas, VA), and Rh30 was a gift from Dr. P. J. Houghton (St. Jude’s Children’s Research Hospital). The human ovarian carcinoma cell lines CH1 and its multidrug-resistant variant CH1DoxR were used as negative and positive controls for mdr-1 and P-gp expression (22). The human non-small cell lung carcinoma cell line CORL23 and its multidrug-resistant variant CORL23/R were used as negative and positive controls for mrp1 expression (23).

All cell lines were maintained in RPMI 1640 (Sigma Chemical Co., Poole, Dorset, United Kingdom) supplemented with 10% fetal bovine serum (Life Technologies, Inc., Scotland, United Kingdom) and 2 mM l-glutamine (Sigma Chemical Co.). Cells were grown as attached monolayers and were incubated at 37°C in a humidified atmosphere with 5% CO₂. All cell lines were routinely screened for Mycoplasma.

**Drugs and Chemicals.** Vincristine (David Bull Laboratories, Dublin, Ireland) and Etoposide (Bristol Myers Squibb Pharmaceuticals, Hounslow, United Kingdom) stock solutions were obtained as pharmacy preparations at concentrations of 1 and 34 mM, respectively. Doxorubicin (Sigma Chemical Co.) was dissolved in absolute ethanol to give stock solutions of 1 and 50 mM, respectively. Dexrazoxane (Chiron, Harefield, United Kingdom) was dissolved in water to give a stock solution of 20 mg ml⁻¹. XR9576 (Xenova, Slough, United Kingdom) and CGP41251 (Ciba Geigy, Basle, Switzerland) were dissolved in DMSO to give stock solutions of 5 and 1 mM, respectively. All these drugs were stored at −20°C, with the exception of vincristine, which was stored at 4°C. Immediately before use, the drugs were diluted with RPMI 1640 prepared as for cell culture, with the exception of vincristine, which was diluted in RPMI 1640 without any supplements.

MTT (Sigma Chemical Co.) was dissolved in PBS to produce a stock solution of 5 mg ml⁻¹, which was stored at 4°C.

**Development of Resistant Cell Lines.** Cells (1 × 10⁶) were seeded in a 75-cm² tissue culture flask. After 24 h, vincristine was added at the IC₅₀ concentration (10 nM, as determined using the MTT assay; see below). The growth medium and vincristine were replaced weekly if necessary. When the cells grew to confluence, they were harvested by trypsinization, 1 × 10⁶ cells were reseeded, and the vincristine dose was doubled. In this manner, cell lines were developed, which were tolerant of 64 × the IC₅₀ concentration. These cell lines were grown in drug-free medium for 1 week before experiments.

Various agents were tested for prevention of development of resistance. The growth of cell lines in the presence of these agents was monitored for 4 weeks to ensure no effects on growth when used alone. The concentrations used were 2 μM PSC833, 2 μM VX710, 10 μM indomethacin, 2 μM XR9576, 10 μM CGP41251, and 200 μM dexrazoxane. These agents were diluted in tissue culture medium and added to the cells with the vincristine.

**Real-time PCR.** Relative expression of the mdr-1 and mrp1 genes was measured using TaqMan Real-time PCR (PE Biosystems, Foster City, CA), using the Applied Biosystems Prism 7700 sequence detection system. Total RNA (1 μg) was reverse transcribed in a 20-μl volume using Superscript reverse transcriptase (Life Technologies, Inc.) and random hexanucleotides (Amersham Pharmacia Biotech United Kingdom, Ltd., Bucks, United Kingdom), according to the manufacturer’s instructions. Real-time PCR for mdr-1 was performed in a final volume of 25 μl containing 1 × TaqMan Universal PCR Master Mix (PE Biosystems), 900 nM forward primer (5’-AAAAATG-GAAAAAGATAAGAAGGAAAGAAA-3’), 900 nM reverse primer (5’-CACCATATAACACTTGCAAGCCAA-3’), and 150 nM FAM/TAMRA dual-labeled oligonucleotide probe (5’-FAM-TTGAATAGCGAACATTTGAAAATACACTGAC-GTTG-TAMRA-3’). Real-time PCR for mrp1 was performed in a final volume of 25 μl containing 1 × TaqMan Universal PCR Master Mix, 300 nM forward primer (5’-AGATGACACCTCTCCACAAAACCA-3’), 300 nM reverse primer (5’-AGGTCTGC-CCACGACACG-3’), and 50 nM FAM/TAMRA dual-labeled oligonucleotide probe (5’-FAM-AACTGCCTGGATTTCGGTCGTTG-TAMRA-3’). Both primer and probe sets amplified across an exon/exon boundary to prevent amplification of genomic DNA. cDNA (0.1 μl) was used in each reaction. To measure the total amount of sample RNA present, levels of 18S rRNA were quantitated in each reaction using TaqMan rRNA Control Reagents with a VIC/TAMRA oligonucleotide probe.
Relative expression of mdr-1 and mrp1 was calculated from standard curves generated from cell lines CH1DoxR and CORL23/R, respectively.

Immunoblotting. Immunoblotting was carried out as described previously (4). The antibody C219 (Centocor Diagnostics, Malvern, PA) was used to detect P-gp, the antibody MRPm6 (Monosan, Uden, the Netherlands) was used to detect MRP1, and B-1-5-2 (Sigma Chemical Co.) was used to detect α-tubulin to ensure equal loading of wells in all experiments.

Growth Inhibition Assay. The MTT assay (24), using 4 days continuous exposure, was used to measure growth inhibition, as described previously (4). The effects of all of the modulators PSC833 and VX710 on growth inhibition were also tested by this method. The modulators were added immediately before addition of the cytotoxic agent at a concentration which kills <10% of cells (2 μM).

Statistical Analysis. Where appropriate, statistical significance was tested using a two-tailed Student’s t test.

RESULTS

The first aim of this study was to develop an in vitro model for the development of resistance to vincristine in RMS. Previous publications have suggested that expression of mdr-1 may be rapidly expressed after exposure to chemotherapeutic agents (8–10). Despite this, we did not observe rapid (≤24 h) up-regulation of mdr-1 or mrp1 after treatment with vincristine in three RMS cell lines: RD, SCMC, and Rh30 (data not shown).

The cell line RD was used to produce a model for the development of resistance after long-term exposure. This cell line has a mutation in the P53 gene, which leads to a loss of function (4). It expresses low levels of the mdr-1 and mrp1 genes, but the P-gp and MRP1 proteins are not detectable by immunoblotting. Exposure to vincristine was carried out according to a set protocol.

Three independent experiments were established simultaneously, and this resulted in three resistant cell lines from a total of six attempts (Table 1). Resistance developed rapidly on all three occasions (Fig. 1), and the resulting cell lines are designated RD(1), RD(2), and RD(3). Resistance appeared to stabilize over a period of 3 months; the lines were not maintained under selective pressure.

This model was then used to test various agents for their ability to prevent the development of resistance. All agents were used at concentrations which are nontoxic in a 28-day growth assay. In the presence of the MDR modulators PSC833, VX710, and XR9576, resistance did not develop despite at least 10 attempts (Table 1 and Fig. 2A). In contrast, the use of the agents indomethacin (MRP1 modulator), CGP41251 (PKC inhibitor), and dexrazoxane (putative MDR prevention agent) did not prevent the development of resistance to vincristine. This occurred after comparatively few attempts (Table 1) and developed as rapidly as in the absence of any agents (Fig. 2B).

Table 1  Attempts to derive vincristine-resistant derivatives of the cell line RD in the absence and presence of various agents. Duration of attempt represents the time between seeding the first flask and discarding the last flask.

<table>
<thead>
<tr>
<th>Agent present</th>
<th>No. of attempts</th>
<th>Average duration of attempts (days)</th>
<th>No. of resistant cell lines obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6</td>
<td>52</td>
<td>3</td>
</tr>
<tr>
<td>PSC833</td>
<td>25</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>VX710</td>
<td>18</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>XR9576</td>
<td>11</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>CGP41251</td>
<td>5</td>
<td>47</td>
<td>1</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>4</td>
<td>31</td>
<td>1</td>
</tr>
<tr>
<td>Dexrazoxane</td>
<td>2</td>
<td>43</td>
<td>1</td>
</tr>
</tbody>
</table>
In Vitro expression were included in each experiment. The increased expression of P-gp (2-fold) and probably reflects the selection process of a clonal population. The total PKC activity, which may be linked to MDR, remained unchanged (data not shown).

Growth inhibition assays were performed to measure the sensitivity of the resistant cell lines to a variety of chemotherapeutic agents. Changes in sensitivity to cisplatin, which is not a P-gp substrate, were comparatively small and showed no consistent patterns (Fig. 4A), suggesting they are attributable to clonal variation. In contrast, sensitivity to the MDR substrates doxorubicin, etoposide, and vincristine was significantly reduced in all of the resistant cell lines (Fig. 4, B–D, respectively). This strongly suggests that the increased expression of P-gp is responsible for the functional resistance. To confirm this, the P-gp modulators PSC833 and VX710 were tested in the resistant cell lines. The sensitivity of all of the resistant cell lines to vincristine could be modulated between 85-fold and 1191-fold using these agents, confirming that P-gp is the major mechanism of resistance (Fig. 4E).

A second RMS cell line, Rh30, was used to test the hypothesis that P-gp modulators may prevent the development of resistance. The first attempt to develop a vincristine-resistant variant of Rh30 led to a resistant cell line (Fig. 5). In contrast, vincristine-resistant cell lines did not develop in the presence of PSC83 or VX710 despite six and four attempts, respectively.

DISCUSSION

The mechanisms by which RMS cells become resistant to chemotherapeutic agents are relatively unclear. Recent studies have indicated that rapid up-regulation (≤24 h) of mdr-1 after doxorubicin treatment may contribute to resistance both in vitro (10) and in vivo (11). This hypothesis was tested using three RMS cell lines with varying vincristine treatment dose and duration, but up-regulation of mdr-1 or mrp1 was not found. The cell line RD was then used to test whether exposure to vincristine for at least three weeks would lead to drug resistance. The first dose given to the cells was the IC50 concentration (10 nM), and this was doubled until 64 × IC50 was reached. In this experiment, resistance to vincristine developed rapidly on three independent occasions.

The use of modulating agents in the clinic is usually limited to patients who have relapsed after prior chemotherapy. In these patients the tumor may already have acquired diverse mechanisms of resistance after exposure to cytotoxic drugs. A strategy to overcome this problem is to use modulators at the onset of treatment to prevent the survival of a multidrug-resistant clone. However, it is unclear how frequently, and how quickly, alternative mechanisms of resistance would arise. Previous studies have reported that it is possible to develop resistant cell lines in the presence of modulators (20, 21) but do not describe whether alternative mechanisms of resistance were readily expressed.

To test this in vitro, RD was exposed to vincristine as before but in the presence of either PSC833, VX710, or XR9576. Despite at least 10 attempts, in each case no resistant cell lines developed in the absence of these agents. This is probably because the main mechanism of vincristine resistance in these lines is P-gp overexpression. However, it is interesting that alternative mechanisms of resistance did not develop, and if this were true in the clinic, it would represent an increase in the time to relapse and possibly cure rate. We have also confirmed these observations in a second RMS cell line, Rh30.

Three agents, which are not conventional P-gp modulators, were also tested. These were CGP41251 (PKC inhibitor), indomethacin (MRP1 modulator), and dextrazoxane, which is normally used to prevent the cardiotoxicity of doxorubicin but which may also prevent resistance (19). Dextrazoxane was used at the same concentration as in previous studies but was added weekly rather than every 3 days, according to the standard

These experiments led to the development of six resistant cell lines which could tolerate 640 nM vincristine in the growth medium. Three cell lines were derived in the absence of any agents, and three were derived in the presence of indomethacin, CGP41251, or dextrazoxane. These cell lines were characterized to determine the mechanisms of resistance. All of the resistant cell lines expressed significantly higher levels of mdr-1 than the parent cell line (Fig. 3A). Three resistant cell lines expressed significantly lower mrp1 than the parent line, but the fold change was small (~2-fold) and probably reflects the selection of a clonal population. The increased expression of mdr-1 leads to overexpression of P-gp in all six resistant lines, but no changes in MRP1 expression were observed (Fig. 3B). The total PKC activity, which may be linked to MDR, remained unchanged (data not shown).
protocol. None of these agents prevented the development of resistance to vincristine, and resistance developed as quickly as in the absence of any agents. This indicates that none of the pathways affected by these agents (e.g., PKC and MRP1) are involved in the development of resistance in this cell line, although dexrazoxane may be effective if added more frequently. It also indicates that the ability to prevent resistance is specific to modulators of P-gp and not a general finding.

The resistant cell lines resulting from these experiments were characterized, and all were found to express mdr-1 and P-gp at higher levels than the parental cell line. This led to resistance to vincristine, etoposide, and doxorubicin but not cisplatin, which is not a substrate for P-gp. In all cases, the sensitivity to vincristine could be modulated using PSC833 and VX710, confirming the presence of functional P-gp. There were no apparent differences between cell lines developed in the presence and absence of various agents, confirming that these agents do not affect the development of resistance.

In conclusion, these studies demonstrate that in vitro resistance to vincristine does not develop rapidly in RMS cell lines but emerges after a long continuous exposure. The development of resistance can be prevented using modulating agents, such as PSC833, VX710, and XR9576, but not agents which affect other pathways, such as indomethacin and CGP41251.
This supports the clinical use of modulating agents at the onset of chemotherapy to prevent the emergence of resistance and test this hypothesis.

ACKNOWLEDGMENTS

We thank Vertex Pharmaceuticals, Inc., Novartis Pharmaceuticals, Xenova, Ciba Geigy, and Chiron for the donation of VX710, PSC833, XR9576, CGP41251, and dexrazoxane, respectively.

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