PAK-104P, a Pyridine Analogue, Reverses Paclitaxel and Doxorubicin Resistance in Cell Lines and Nude Mice Bearing Xenografts That Overexpress the Multidrug Resistance Protein

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ABSTRACT

Multidrug resistance (MDR) is considered multifactorial and has been associated with overexpression of the multidrug resistance protein (MRP). However, effective compounds for reversal of MRP-related MDR are limited. In the present study, the modulatory activity of the novel pyridine analogue PAK-104P on MRP-mediated resistance to doxorubicin and paclitaxel was investigated in two doxorubicin-selected human tumor cell lines [HT1080/DR4 (sarcoma) and HL60/ADR (leukemia)] and compared with the nonimmunosuppressive cyclosporine analogue PSC-833. In cell lines HT1080/DR4 (MRP lung resistance-related protein phenotype) and HL60/ADR (MRP phenotype), doxorubicin resistance was significantly higher (250-fold and 180-fold, respectively) than that to paclitaxel (6-fold and 9-fold, respectively). With noncytotoxic concentrations of PAK-104P (1 and 5 μM), the reversal of doxorubicin resistance was significant but partial in HT1080/DR4 and HL60/ADR cells (dose-modifying factor for 5.0 μM PAK-104P, 25.0 and 31.2, respectively), whereas complete reversal of paclitaxel resistance was achieved in HL60/ADR cells. In contrast, PSC-833 modulation of doxorubicin and paclitaxel resistance was modest. Cellular drug uptake and retention studies by flow cytometry analysis demonstrated that PAK-104P was effective in restoring cellular doxorubicin concentrations in resistant cells to levels comparable to those obtained in parental cells. In athymic nude mice, PAK-104P significantly potentiated the therapeutic efficacy of doxorubicin and paclitaxel against resistant HT1080/DR4 xenografts. Of significance is that the maximum tolerated doses of doxorubicin and paclitaxel were administered in combination with PAK-104P, documenting improvement in the therapeutic index of these agents.

In addition to reversing P-glycoprotein-mediated MDR, the pyridine analogue PAK-104P provides an example of an effective in vivo modulator of MRP-mediated MDR.

INTRODUCTION

MDR is considered multifactorial and has been associated with overexpression of either the P-170 transmembrane glycoprotein commonly known as Pgp or a M₉₀,₀₀₀ integral membrane phosphoglycoprotein, designated MRP (1). Like Pgp, MRP belongs to the ATP-binding cassette transmembrane transporter superfamily and is believed to function in drug transport processes (1–4). Overexpression of MRP mRNA was demonstrated in several drug-selected MDR cell lines that do not express Pgp (1, 5, 6). Recently, transfection of a MRP cDNA expression vector into sensitive HeLa cells conferred a MDR phenotype (7) accompanied by alterations in cellular accumulation and retention for vincristine (2).

In addition to MRP, the expression of a M₉₀,₀₀₀ membrane protein, termed LRP, has been described in non Pgp-mediated MDR (8). Current data suggest LRP belongs to the vault proteins, which appear to act as multisubunit organelles in nucleocyttoplasmic transport mechanisms (9).

Several agents have been identified as capable of reversing Pgp-associated MDR; however, effective compounds for reversal of MRP-associated MDR are limited (4, 10–12). Recent data suggest that pyridine analogues are effective modulators with a broad spectrum of activity (13) and can modulate non-Pgp-mediated MDR (14).

An important feature in terms of clinical application with pyridine analogues is that these drugs have the advantage over other calcium antagonists to exert MDR modulatory properties with no significant calcium channel-blocking activity (13). In the present study, we investigated the effect of the pyridine analogue PAK-104P on MRP-mediated resistance to doxorubicin and paclitaxel in the doxorubicin-selected multidrug-resistant human fibrosarcoma cell line HT1080/DR4 (MRP/LRP phenotype) and the promyelocyte leukemic cell line HL60/ADR (MRP phenotype).

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4 The abbreviations used are: MDR, multidrug resistance; Pgp, glycoprotein P-170; MRP, multidrug resistance protein; LRP, lung resistance-related protein; MAb, monoclonal antibody; IC₅₀, 50% inhibitory concentration; GST, glutathione S-transferase; GSH, glutathione; MWF, maximum weight loss; MIR, mean maximum inhibitory ratio; TD, tumor doubling time; PR, partial response; CR, complete response.
The MDR modulatory activity of PAK-104P was compared with the nonimmunosuppressive cyclosporine analogue PSC-833, a potent modulator of Pgp-mediated MDR, using comparable noncytotoxic concentrations and drug exposure time. In addition, the in vivo effect of PAK-104P on the therapeutic efficacy of doxorubicin and paclitaxel was evaluated in athymic nude mice bearing parental HT1080 and resistant HT1080/DR4 xenografts.

**MATERIALS AND METHODS**

**Chemicals.** Doxorubicin HCl (USP) was obtained from Farmitalia Carla Erba S. P. A. (Milan, Italy). Paclitaxel was provided by Bristol-Myers Squibb (Princeton, NJ) and dissolved in methanol (stock solution 2 mM). PAK-104P (13) was kindly supplied by Nissan Chemical Ind. Co. Ltd (Chiba, Japan) and dissolved in DMSO (stock solution 10 mM). PSC-833 was kindly provided by Sandoz (East Hanover, NY) and dissolved in an ethanol:Tween 20 (9:1) solution (stock solution 8.23 mM). The highest concentrations of DMSO (0.05%) and ethanol: Tween 20 (9:1) (0.03%) used in the assays were found to be noncytotoxic and without effect on drug resistance.

**Cell Lines.** The characteristics of the human fibrosarcoma cell line HT1080 and the MDR subline HT1080/DR4...
Fig. 2  Modulation of doxorubicin and paclitaxel resistance by PAK-104P and PSC-833 in MRP- and LRP-expressing HT1080/DR4 cells (A) and MRP-expressing HL60/ADR cells (B). Resistant cells without modulator (■) or in the presence of the highest noncytotoxic concentration of 2.5 μM PSC-833 (▲) or 5.0 μM PAK-104P (●); parental cells (□) without modulator. Results are the mean values of at least three independent experiments in quadruplicate; SDs were <10%.

Table 1  In vitro modulation of MRP-associated resistance to doxorubicin and paclitaxel

<table>
<thead>
<tr>
<th>Drug</th>
<th>HT1080</th>
<th>HT1080/DR4</th>
<th>HL-60</th>
<th>HL-60/ADR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No modulator</td>
<td>1.0 μM</td>
<td>1.2</td>
<td>8.4 (28.4)</td>
<td>1.0</td>
</tr>
<tr>
<td>PAK-104P</td>
<td>1.0 μM</td>
<td>2.0</td>
<td>25.0 (9.6)</td>
<td>1.4</td>
</tr>
<tr>
<td>PSC-833</td>
<td>0.5 μM</td>
<td>1.0</td>
<td>1.2 (199)</td>
<td>1.0</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>2.5 μM</td>
<td>1.9</td>
<td>3.6 (66)</td>
<td>1.1</td>
</tr>
<tr>
<td>No modulator</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK-104P</td>
<td>1.0 μM</td>
<td>1.0</td>
<td>3.1 (1.9)</td>
<td>1.0</td>
</tr>
<tr>
<td>PSC-833</td>
<td>5.0 μM</td>
<td>1.0</td>
<td>4.3 (14.4)</td>
<td>1.0</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.5 μM</td>
<td>2.1</td>
<td>1.8 (3.4)</td>
<td>1.0</td>
</tr>
<tr>
<td>PSC-833</td>
<td>2.5 μM</td>
<td>1.9</td>
<td>2.1 (2.8)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>(IC<sub>50</sub> drug)/(IC<sub>50</sub> drug + modulator).
<sup>b</sup>RDR, relative degree of resistance; IC<sub>50</sub> resistant/IC<sub>50</sub> parental cell line.

have been described by Slovak et al. (15). For the studies carried out herein, HT1080 and HT1080/DR4 cells were grown as monolayers in Eagle’s MEM supplemented with Earle’s balanced salt solution and 10% heat-inactivated FCS, nonessential amino acids, and l-glutamine. The resistance of the MDR subline HT1080/DR4 to doxorubicin was found to be stable during the course of all experiments in the absence of continuous drug exposure.

The human promyelocyte leukemic cell line HL60 and the MDR subline HL60/ADR were previously characterized by Marsh et al. (16).
Fig. 3 Modulation of cellular accumulation and retention of doxorubicin in resistant HL60/ADR and HT1080/DR4 cells: A, cells were exposed for 2 h to 2.5 μM doxorubicin with or without modulator. Cellular doxorubicin accumulation (0 h, □) and retention (4 h, □) were measured by flow cytometry after reincubation of cells in drug-free medium with or without modulator. Doxorubicin concentrations are expressed as mean arbitrary fluorescence units. The results are representative of at least two independent experiments. B, parental and resistant cells were exposed for 2 h to the individual IC_{50} of doxorubicin with or without modulator of each cell line, respectively. Cellular drug concentration at 0 h (■) and at 4 h (■) were measured as described above. The highest nontoxic concentration of PAK-104P (5.0 μM) and PSC-833 (2.5 μM) were used with the appropriate IC_{50} of doxorubicin (in parentheses).

HL60 cells were maintained as suspension culture in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% FCS. Cell cultures were kept at exponential growth in a fully humidified atmosphere of 5% CO_{2} in air at 37°C.

Immunohistochemical Characterization of MDR Phenotype. For the detection of Pgp, paraffin sections of parental and resistant HT1080 and HL60 cells were used as described previously using the MDR-1-specific MAb JSB-1 (17). For the detection of LRP and MRP, cytopsins were air dried, fixed in acetone for 10 min, quenched for 30 min in 3% H_{2}O_{2}, and blocked with 0.03% casein/PBS. Immunostaining was performed using the LRP-specific MAb LRP-56 (8) and MRP-specific MAb MRPm6 (18). A polyvalent biotinylated secondary antibody and streptavidin-peroxidase reagent (Shandon-Lipshaw, Pittsburgh, PA) were used to visualize the binding of primary antibodies utilizing 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) as chromogen.

MRP overexpression in HT1080/DR4 xenografts was confirmed using Western blot analysis of a purified membrane fraction. Fifty μg membrane proteins were dissolved on a 7.5% SDS polyacrylamide gel and electroblotted to nitrocellulose. MRP-specific binding of a monoclonal IgG2a antibody (1.66 μg/ml MRPv1; Ref. 18) was visualized with the enhanced chemiluminescence detection (Amersham, Arlington Heights, IL) using a goat horseradish peroxidase-conjugated antirat antibody. An isotype control (rat IgG2a) showed no unspecific staining.

Cytotoxicity Evaluation in Vitro. Drug sensitivity of the HT1080 cell line was assessed using the sulforhodamine B assay (19). Cells in exponential growth were seeded at a density of 1000 cells/well in 96-well microtiter plates (Falcon; Becton Dickinson Labware, Plymouth, United Kingdom) and allowed to attach overnight. Twenty-four h later the monolayers were exposed to doxorubicin (2 h) or paclitaxel (3 h) in the presence or absence of the modulator. Following drug exposure, cells were washed, then incubated in drug-free medium in the continuous presence or absence of the modulator. Previous studies (data not shown) demonstrated that the highest reversal of drug resistance was achieved with continuous exposure to the modulator. Evaluation of treatment was carried out four cell doubling times after drug-treatment (72 h for parental HT1080 and 144 h for HT1080/DR4 cells). Cells were then fixed with trichloroacetic acid, washed, and stained as originally described (19).

Drug sensitivity in HL60 and HL60/ADR cells was determined using a modified 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay (20). HL60 and HL60/ADR were drug-treated as described above except that drug treatment was performed in suspension culture at cell densities of 10^{6} cells/ml.
Fig. 3 (continued)
Cells were seeded at a density of 20,000 cells/well in 96-well plates (Falcon; Becton Dickinson Labware) and incubated for four cell doubling times (96 h).

All experiments were performed at least in quadruplicate and repeated at least three times. The drug concentration that inhibited cell growth by 50% (IC$_{50}$) was obtained from semi-logarithmic dose-response plots.

**Quantitative Flow Cytometric Determination of Cellular Doxorubicin.** A linear relationship between the average cellular fluorescence intensity and cellular doxorubicin concentration has been demonstrated previously (21, 22). Cellular doxorubicin accumulation and retention was performed as described by Minderman et al. (23). In brief, to determine drug uptake and retention, cells in exponential growth were exposed at 37°C for 2 h to 2.5 μM doxorubicin in the presence or absence of the modulator. Samples were taken immediately after doxorubicin exposure (zero time) and after 4 h in drug-free medium in the presence or absence of the modulator. In a second protocol, the cellular retention of doxorubicin was evaluated using the individual IC$_{50}$ of doxorubicin with or without the modulator (equicytotoxic drug exposure). Samples were taken as described above. Cellular doxorubicin concentrations were determined by analyzing cells with a FACScan (Becton Dickinson, San Jose, CA). Excitation wavelength was 488 nm, and emitted light was captured using a 650 long-pass filter. Results were calculated and analyzed with Win List software (Verity Software House, Topsham, ME). Cellular doxorubicin concentrations are expressed as mean arbitrary fluorescence units.

**Enzyme Activity of GSTs (EC 2.5.1.18) and GSH Content.** Cells in exponential growth were harvested with 1 mM EDTA in PBS, washed twice with ice-cold PBS, and lysed by sonication in 5 mM dipotassium hydrogen phosphate buffer. Enzyme activity of GSTs (substrate: 1-chloro-2,4-dinitrobenzene) was determined at 25°C in the 120,000 × g supernatant as described previously (24). Results are expressed as nmol min$^{-1}$/mg extract protein. Cellular GSH content was determined using the GSH reductase recycling method of Griffith (25). Total GSH concentrations are expressed as nmol/mg protein.

All experiments were performed in triplicate.

**Xenografts.** HT1080 and HT1080/DR4 xenografts were initially established in 8-12-week old (body weight, 20–25 g) female athymic nude mice (Sprague-Dawley, Inc., Indianapolis, IN) by implanting 10$^6$ parental HT1080 and 10$^7$ resistant HT1080/DR4 cells s.c. Xenografts were maintained for several generations by s.c. implantation of 50 mg non-necrotic tumor tissue. Treatments were initiated at a tumor size of 150–200 mg (approximately 5–6 days for HT1080 and 10–12 days for HT1080/DR4 xenografts postimplantation).

**Administration of Drugs and Growth Inhibition Studies in Vivo.** Doxorubicin was dissolved in sterile 0.9% saline and injected i.v. at a dose of 10 mg/kg (maximum tolerated dose). Paclitaxel was dissolved in DMSO and diluted in 0.9% saline solution (final concentration of DMSO, 5%) and injected at a dose of 100 mg/kg i.v. 1 h before drug administration, followed every 1 h for three doses thereafter. The maximum tolerated dose was defined as the maximum dose that could be administered to tumor-bearing mice without causing drug-related lethality and a MWL not >20%. Tumor weight was measured with a Vernier caliper and calculated assuming unit density by the following formula: \(V = \frac{L \times W^2}{2}\), where \(L\) is the length and \(W\) is the width in mm. Relative tumor volumes were calculated using the formula: relative tumor volume (%) = \(V_t/V_i \times 100\), where \(V_t\) represents the tumor volume on day \(x\) and \(V_i\) represents the initial tumor volume. Tumor measurements and body weights of the mice were recorded daily. The antitumor activity was assessed by the mean MIR of the relative tumor volume of the treated animals over the untreated controls. TD was defined as the mean time for the tumor to reach twice the initial size. A PR was defined as a reduction in tumor size greater than 50% and a CR as the inability to detect a tumor by palpation at the initial site of the tumor appearance for more than 60 days.

**Statistical Analysis.** The differences between the mean values were analyzed for significance using the unpaired two-tailed Student's \(t\) test for independent samples; \(P\) values < 0.05 were considered to be statistically significant.

**RESULTS**

**Detection of MRP and LRP.** The data in Fig. 1 demonstrate a positive reaction for MRP (A) and LRP (B) in HT1080/DR4 cells. A weak but detectable signal was also observed in the parental HT1080 cells (data not shown). In the HL60 cell line, only HL60/ADR cells were positive for MRP (Fig. 1C) but not for LRP, whereas the parental cell line was negative for both markers. Since in HL60/ADR cells MRP is also present in the endoplasmic reticulum (6), the appearance of nuclear staining for MRP in HL60/ADR cells is likely to be related to the cytoplasmic staining superimposed over the large nuclei in these cells. This is consistent with the results on paraffin sections (5 μm) demonstrating no nuclear staining for MRP in HL60/ADR cells (data not shown).

Both HL60 and HT1080 cell lines were negative for Pgp using the MDR-1-specific JSB-1 MAb (data not shown).

The results of the present immunocytochemical characterization confirmed the MDR phenotypes of these cell lines as reported previously (8, 18).

**Modulation of in Vitro Drug Sensitivity.** Using continuous in vitro exposure of HT1080 and HL60 cells, the highest noncytotoxic concentrations of PAK-104P and PSC-833 were 5.0 and 2.5 μM, respectively. The data in Fig. 2 indicate that the relative degree of resistance to doxorubicin was about 200-fold and to paclitaxel about 6-fold. The effects of the modulators on the in vitro reversal of resistance to doxorubicin and paclitaxel in HT1080 and HL60 cells are shown in Table 1 and Fig. 2. In both cell lines, PK-104P was more effective than PSC-833 in reversal of resistance to doxorubicin and paclitaxel. With PK-104P, the ability of paclitaxel resistance to doxorubicin was partial, complete reversal of paclitaxel resistance was achieved in HL60/ADR cell line.

**Modulation of Cellular Accumulation and Retention of Doxorubicin.** The effect of PAK-104P and PSC-833 at their highest noncytotoxic concentrations, 5.0 μM and 2.5 μM, respectively, on the cellular accumulation of doxorubicin (0 h, immediately after 2-h exposure to doxorubicin) and retention (4 h after drug exposure in the presence or absence of modulator) was evaluated (Fig. 3).
Using 2.5 μM doxorubicin in HL60 and HT1080 cells, lower cellular drug accumulation and retention were observed in both doxorubicin-resistant cell lines compared with the parental cell lines (Fig. 3A). PSC-833 produced limited effects in these parameters, whereas PAK-104P was more effective, restoring drug concentration in resistant cells to a level comparable to those obtained with the parental line (Fig. 3A).

Using the individual IC_{50} of doxorubicin with or without modulators, the cellular retention of doxorubicin in HL60 and HT1080 cells was determined and is shown in Fig. 3B. The results confirmed the drug efflux in resistant HL60/ADR and HT1080/DR4 cells and demonstrate the effectiveness of PAK-104P but not PSC-833 in restoring the cellular concentration of doxorubicin in resistant cell lines. The data in Fig. 3B also indicate that at comparable cytotoxic drug concentrations, the cellular concentrations of doxorubicin in resistant cells were significantly higher than those in parental cells.

Effect of the Modulator on GSTs and GSH Content. Since evidence suggested that the MRP-mediated efflux of glutathione conjugate leukotriene C4 and structurally related anionic amphiphilic conjugates is dependent on conjugation to GSH (26), the effect of PAK-104P on GST activity and GSH content was investigated in HT1080/DR4 cells. The GST activity in control and PAK-104P-treated cells was similar: 11.8 ± 0.82 nmol min^{-1}/mg extract protein in control cells and 11.2 ± 0.61 nmol min^{-1}/mg extract protein for 5 μM PAK-104P-treated cells. However, we found a lower GST activity in HT1080/DR4 cells than reported by Zwelling et al. (Ref. 27; 20.7 ± 0.2 nmol min^{-1}/mg protein). There was no significant effect (P > 0.2) of PAK-104P on the cellular GSH concentration: 47.0 ± 10.8 nmol/mg protein in the control cells and 39.6 ± 12.0 nmol/mg protein for 5 μM PAK-104P-treated cells.

Antitumor Activity in Mice Bearing Xenografts. We have previously demonstrated that up to 150 mg/kg PAK-104P had no antitumor activity and no toxicity in vivo (28). For the present study, the maximum tolerated doses were as follows: PAK-104P, 150 mg/kg (i.v.); doxorubicin, 10 mg/kg (i.v. push); and paclitaxel, 50 mg/kg (3-h i.v. infusion).

The MIR, TD, and MWL were compared for untreated and all drug-treated groups as shown in Table 2. The data demonstrate the therapeutic efficacy of doxorubicin and paclitaxel against HT1080 xenografts and the superiority of paclitaxel over doxorubicin in terms of MIR, TD, PR, and CR. The data in Table 2 also indicate that PAK-104P did not potentiate the toxicity nor the antitumor activity of doxorubicin or paclitaxel in mice bearing parental HT1080 xenografts.

In contrast to HT1080 xenografts, treatment with doxorubicin induced no significant growth inhibition in HT1080/DR4 xenografts, whereas paclitaxel had modest antitumor activity, confirming the results obtained with cultured HT1080/DR4 cells (Table 1 and Fig. 2). When the therapeutic efficacy of doxorubicin and paclitaxel treatment against HT1080/DR4 xenografts was compared with and without PAK-104P, PAK-104P significantly potentiated the antitumor activity of both drugs (overall response rate for doxorubicin with and without PAK-104P was 50% and 0% and for paclitaxel with and without PAK-104P was 80% and 10%, respectively) without increasing toxicity in terms of MWL. No treatment-related deaths occurred (data not shown). It is interesting to note that the antitumor activity of paclitaxel modulated by PAK-104P (CR rate, 40%) in mice bearing HT1080/DR4 xenografts was similar to that observed with paclitaxel alone in sensitive HT1080 xenografts, demonstrating that complete reversal of drug resistance to paclitaxel can be achieved in vivo.

**DISCUSSION**

Overexpression of the MRP has been observed in several resistant cell lines with non-Pgp-mediated MDR (1, 5, 6, 11). Previous studies suggested that MRP-mediated drug resistance was not effectively reversed by selected modulators which are active in Pgp-mediated resistance (4, 10–12). Since recent data demonstrated that the new pyridine analogue PAK-104P is active in Pgp (13) and non-Pgp-mediated MDR (14), we investigated the modulatory effect of PAK-104P on MRP-mediated MDR in drug-selected cell lines and mice bearing HT1080 and HT1080/DR4 xenografts.

In the MRP-expressing cells, the relative degree of resistance to doxorubicin was significantly higher than to paclitaxel. The low degree of resistance to paclitaxel in MRP-mediated MDR is consistent with previously reported results by Cole et al. (2) and Twentyman et al. (29). The results obtained herein document that the pattern of cross-resistance for the MRP phenotype is different

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Table 2 In vivo effect of PAK-104-P on the therapeutic efficacy and toxicity of doxorubicin and paclitaxel in mice bearing HT1080 and HT1080/DR4 xenografts

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIR (%)</th>
<th>TD (days)</th>
<th>CR (%)</th>
<th>PR (%)</th>
<th>MWL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT1080</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No drug</td>
<td>94.6 ± 3.8</td>
<td>2.6 ± 1.0</td>
<td>12.0 ± 1.0</td>
<td>12.0 ± 1.0</td>
<td>12.0 ± 1.0</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>95.5 ± 4.2</td>
<td>10.2 ± 2.0</td>
<td>20.0 ± 2.0</td>
<td>30.0 ± 3.0</td>
<td>5.0 ± 1.8</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>96.5 ± 4.2</td>
<td>10.8 ± 2.1</td>
<td>20.0 ± 2.0</td>
<td>40.0 ± 2.0</td>
<td>9.0 ± 2.0</td>
</tr>
<tr>
<td>Paclitaxel + PAK-104P</td>
<td>95.6 ± 3.5</td>
<td>14.6 ± 1.6</td>
<td>40.0 ± 2.0</td>
<td>50.0 ± 2.0</td>
<td>7.6 ± 2.6</td>
</tr>
</tbody>
</table>

HT1080/DR4

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIR (%)</th>
<th>TD (days)</th>
<th>CR (%)</th>
<th>PR (%)</th>
<th>MWL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>24.0 ± 7.5</td>
<td>4.4 ± 1.1</td>
<td>10.0 ± 1.0</td>
<td>0.0 ± 0.0</td>
<td>3.4 ± 1.2</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>82.6 ± 6.2b</td>
<td>11.8 ± 1.2b</td>
<td>0.0 ± 0.0</td>
<td>50.0 ± 2.0</td>
<td>13.2 ± 1.8</td>
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<tr>
<td>Paclitaxel</td>
<td>48.2 ± 8.8</td>
<td>7.1 ± 1.0</td>
<td>0.0 ± 0.0</td>
<td>10.0 ± 1.0</td>
<td>14.8 ± 3.2</td>
</tr>
<tr>
<td>Paclitaxel + PAK-104P</td>
<td>88.8 ± 8.2b</td>
<td>16.2 ± 1.5b</td>
<td>40.0 ± 2.0</td>
<td>40.0 ± 2.0</td>
<td>14.8 ± 2.2</td>
</tr>
</tbody>
</table>

*Drug treatments were conducted as follows: Paclitaxel, 50 mg/kg i.v. 3-h infusion; doxorubicin, 10 mg/kg i.v. push; PAK-104P, 100 mg/kg i.v.*

× 4 with the first dose 1 h before drug treatment, then every 1 h × 3. Ten mice per group were treated.

bP < 0.001 drug alone versus drug + modulator.

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from the Pgp-mediated MDR, in which the relative degree of resistance to paclitaxel is comparable to or higher than the relative degree of resistance to doxorubicin (30, 31). Thus, it appears that paclitaxel is a poorer substrate for MRP-mediated MDR. In contrast to the cyclosporine analogue PSC-833, PAK-104P is an effective modulator of doxorubicin and paclitaxel resistance in Pgp-5 and MRP-expressing cells. Since the above-mentioned resistant HL60/ADR cells do not overexpress LRP, the modulatory effect of PAK-104P is likely related to MRP. Previous investigations have demonstrated that cyclosporin A (4, 11) and PSC-833 (11, 29) are not active in MRP-mediated MDR. Furthermore, using an achievable human blood level of 2.5 μM, PSC-833 failed to effectively reverse MRP-mediated doxorubicin and paclitaxel resistance in vitro (29). Studies by Cole et al. (10) demonstrated that 10 μM verapamil is not an efficient modulator of doxorubicin resistance in MRP-mediated MDR. Zaman et al. (4) reported reduced drug efflux in MRP cDNA-transfected cells by the isoflavonoid genistein, but not in Pgp-mediated MDR (32). Collectively, these results strongly indicate that mechanisms of MRP-associated MDR may be different from those of Pgp.

In the results reported herein (Fig. 3A), decreased accumulation and retention of doxorubicin in both resistant cell lines was observed, suggesting that alterations in cellular drug concentrations are mechanisms associated with MRP-mediated MDR in HL60/ADR and HT1080/DR4 cells. However, using the individual IC50 of doxorubicin with or without modulator of each cell line (Fig. 3B), significantly higher drug concentrations were detected at equicytotoxic concentrations in resistant cells than in doxorubicin-sensitive cells, suggesting that multiple mechanisms of resistance are present in cells with a high degree of resistance to doxorubicin. These findings are consistent with previously reported data demonstrating that in HT1080/DR4 cells topoisomerase II and several antioxidant enzymes are involved in drug resistance (27). Furthermore, it has been shown that LRP, which is also expressed in HT1080/DR4 cells, is thought to act as a vault protein in nucleocytoplasmic transport mechanisms (9).

In resistant HL60/ADR cells, MRP is also present in the endoplasmic reticulum, suggesting that MRP may possibly contribute to drug resistance by vesicular sequestration mechanisms (6). This possibility is consistent with the immunocytochemical analysis of the present study demonstrating that the staining for MRP is predominantly cytoplasmic in HL60/ADR cells. However, since FACScan analysis only provides information about the total cellular doxorubicin concentration, the subcellular drug distribution has not been elucidated in the present study.

In the MDR cells with relatively low resistance to paclitaxel (6- and 9-fold), PAK-104P was capable of restoring completely the response to paclitaxel. Whether rapid efflux mechanism is solely responsible for resistance to paclitaxel in MRP-mediated MDR needs to be confirmed however.

The GSH pathway has been implicated in MRP efflux mechanisms (26, 33), thus, the effect of PAK-104P on GSTs and cellular GSH content was investigated. No significant differences in enzyme activity of GSTs and GSH content in control and PAK-104P-treated cells were found, suggesting that reversal of drug resistance by PAK-104P was not related to alterations of the GSH pathway.

Since PAK-104P reverses drug resistance to doxorubicin and paclitaxel in HT1080/DR4 and HL60/ADR cells in vitro, we investigated the in vivo effect of the pyridine analogue on the therapeutic efficacy of doxorubicin and paclitaxel in athymic nude mice bearing HT1080 and HT1080/DR4 xenografts. Overexpression of MRP in HT1080/DR4 xenografts was confirmed using immunoblot analysis of a purified membrane fraction (MAb MRP1; Fig. 4). In the results reported herein (Table 2), PAK-104P significantly increased the therapeutic index of paclitaxel and doxorubicin in mice bearing HT1080/DR4 xenografts, restoring drug sensitivity to paclitaxel to a level seen in parental HT1080. The reversal of drug resistance to doxorubicin was limited in HT1080/DR4 xenografts, perhaps due to the expression of a high degree of resistance to doxorubicin which may involve multiple mechanisms of drug resistance.

The new pyridine analogue PAK-104P is an effective modulator of MRP-mediated drug resistance to doxorubicin and paclitaxel. The reversal of doxorubicin resistance by PAK-104P was associated with a restoration of intracellular drug accumulation and retention in both resistant cell lines to levels comparable to those seen in parental cells. Preclinical studies in mice bearing HT1080/DR4 xenografts confirmed the ability of PAK-104P to reverse MRP-associated MDR in vivo.

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REFERENCES


PAK-104P, a pyridine analogue, reverses paclitaxel and doxorubicin resistance in cell lines and nude mice bearing xenografts that overexpress the multidrug resistance protein.

U Vanhoefer, S Cao, H Minderman, et al.