Reversal of \textit{MDR1}-associated resistance to topotecan by \textit{PAK-200S}, a new dihydropyridine analogue, in human cancer cell lines

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**Summary** Recent data suggest that expression of the membrane \textit{P}_{170}-glycoprotein (P-gp) may confer resistance to the topoisomerase-I-interactive agent topotecan. The present study describes the cellular effects of a new dihydropyridine analogue, \textit{PAK-200S}, on P-gp-mediated resistance to topotecan in human breast and ovarian tumour cells. \textit{PAK-200S} at a non-cytotoxic concentration of 2.0 \textmu M completely reversed resistance to topotecan in P-gp-expressing MCF-7/adr (breast) and A2780/Dx5 (ovarian) tumour cells, respectively, with no effects on parental cells. Cellular pharmacokinetic studies by reversed-phase high-performance liquid chromatography analysis showed significantly lower cellular drug concentrations of the pharmacologically active closed-ring lactone of topotecan in multidrug-resistant cells than in parental cells. \textit{PAK-200S} was effective in restoring the cellular lactone concentrations of topotecan in resistant MCF-7/adr cells to levels comparable to those obtained in parental cells. Furthermore, exposure of MCF-7/adr cells to topotecan in the presence of \textit{PAK-200S} significantly increased the induction of protein-linked DNA breaks. \textit{PAK-200S} did not alter nuclear topoisomerase-I-mediated ex vivo pBR322 DNA plasmid unwinding activity and topoisomerase-I protein expression. These results suggest that reversal of P-gp-mediated resistance to topotecan by \textit{PAK-200S} was related to the restoration of cellular drug concentrations of the active lactone form of topotecan rather than a direct effect on topoisomerase-I function. © 1999 Cancer Research Campaign

**Keywords**: topotecan; \textit{PAK-200S}; multidrug resistance; breast cancer; ovarian cancer

Topotecan (10-hydroxy-9-dimethyl-amino-methyl-camptothecin) is a new topoisomerase-I (TOP-I) targeting agent with significant clinical efficacy against a variety of solid tumours (Slichenmeyer et al, 1993; Rowinsky et al, 1997). Like other TOP-I interactive agents topotecan interacts with the breakage-rejoining reaction between DNA and TOP-I, generating intermediate forms of drug-stabilized covalent DNA–TOP-I complexes, referred to as cleavable complexes (Hsiang et al, 1988; Liu, 1989; Chen et al, 1994; Pommier et al, 1996). The cellular accumulation of such complexes may lead to cell death due to replication arrest and replication fork disassembly as well as chromosomal fragmentation (Covey et al, 1989; Ryan et al, 1991, 1994; Tsao et al, 1993). Like other camptothecin (CPT) analogues, topotecan establishes an equilibrium between the pharmacologically active closed lactone ring and the inactive open ring hydroxy acid form by an equilibrium between the pharmacologically active closed-ring lactone and the inactive open ring hydroxy acid form by reversible pH-dependent cellular hydrolysis (Grochow et al, 1992).

Acquired resistance to TOP-I interactive drugs has been related to down-regulation of TOP-I gene and protein expression (Eng et al, 1990; Kanzawa et al, 1990; Sugimoto et al, 1990; Kapoor et al, 1995; Sorensen et al, 1995) as well as alterations in TOP-I structure and function (e.g. point mutations, deletions and rearrangements of the TOP-I gene) (Andoh et al, 1987; Tan et al, 1989; Benedetti et al, 1993; Gromova et al, 1993). Current data suggest that positively charged CPT derivatives (e.g. topotecan) may be also transported by the \textit{MDR1} gene product \textit{P}_{170}-glycoprotein (P-gp), a \textit{M}_{r} 170 000 energy-dependent transmembrane glycoprotein efflux pump (Hendricks et al, 1992; Mattern et al, 1993; Maliepaard et al, 1996). Although altered cellular drug accumulation has been related to topotecan resistance, so far only few data are available on cellular pharmacokinetics of the pharmacologically active lactone form of topotecan and reversing agents of topotecan resistance in P-gp-expressing multidrug-resistant (MDR) cells (Hendricks et al, 1992).

The dihydropyridine analogue \textit{PAK-200S} (2-[benzyl(phenyl)-amino]ethyl-1,4-dihydro-2,6-dimethyl-5-(5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinan-2-yl)-1-(2-morpholinoethyl)-4-(3-nitrophenyl)-3-pyridinecarboxylate) is an effective chemosensitizing modulator of membrane protein-associated drug resistance (P-gp and multidrug resistance protein [MRP]; U Vanhoefer et al, unpublished data) with a broad spectrum of activity in vitro and in vivo (Niwa et al, 1992). It has been shown that non-cytotoxic concentrations of \textit{PAK-200S} inhibited \textit{[H]}azidopine photo-labelling of P-gp, associated with reversal of drug resistance in MDR KB-C2 and xenografted COK-36LN cells. An important feature in terms of clinical application of the dihydropyridine analogue \textit{PAK-200S} is that this agent has the advantage over other calcium antagonists to exert effective P-gp modulatory properties with no significant calcium channel-blocking activity or other in vivo toxicity.

The present study describes the effects of the new dihydropyridine analogue \textit{PAK-200S} on the cellular determinants of resistance
to topotecan in MDR human ovarian A2780/Dx5 and breast MCF-7/adr tumour cells that express the P-gp phenotype.

**MATERIALS AND METHODS**

**Chemicals**

Topotecan was obtained from SmithKline Beecham (King of Prussia, Philadelphia, PA, USA) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM (stock solution). PAK-200S was supplied by Nissan Chemical Ind. Co. (Chiba, Japan) and dissolved in DMSO (stock solution 10 mM). The highest concentration of DMSO used in all assays was found to be non-cytotoxic and without effect on the biochemical assays.

**Cell lines**

The characteristics of the human ovarian cancer cell line A2780/wt (parental), the MDR subline A2780/2Dx5 (P-gp-positive, MRP-negative), the human breast cancer cell line MCF-7/wt (parental) and the MDR subline MCF-7/adr (P-gp-positive, MRP-negative) have been reported previously (Fairchild et al., 1987; Minderman et al., 1996). Parental and resistant MCF-7 cells were grown as monolayer in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), and 2 mM l-glutamine. Cell cultures were kept in a humidified atmosphere of 5% carbon dioxide in air at 37°C. P-gp expression in A2780/2Dx5 and MCF-7/adr cells was confirmed by flow cytometric analysis using monoclonal antibody (mAb) UIC2 (Müller et al., 1994). Representative histograms of single parameter green fluorescence plots showed a homogenous population of MCF-7/adr cells staining for P-gp, while two subpopulations with different P-gp expression were found in the A2780/2Dx5 cell line (data not shown) as already reported earlier (Minderman et al., 1996). Thus, MCF-7/wt and MCF-7/adr cells were used only for further biochemical analysis.

**In vitro cytotoxicity**

Drug sensitivity was assessed by the sulphorhodamine B (SRB)- assay (Skehan et al., 1990). Exponentially growing human ovarian carcinoma A2780/wt and MDR A2780/Dx5 cells were seeded at a density of 1000 cells well⁻¹ in 96-well microtitre plates (Falcon, Becton-Dickinson Labware, Plymouth, UK) and allowed to attach overnight. Human breast cancer cells MCF-7/wt and MDR MCF-7/adr cells were seeded at a density of 800 cells well⁻¹. After 24 h cells were exposed for 24 h either to topospan or to doxorubicin, respectively, washed twice with phosphate-buffered saline (PBS) and reincubated in drug-free medium. At four cell doubling times after beginning of drug treatment, cells were fixed with trichloric acid, washed and stained with sulphorhodamine B as originally described. For modulating studies the cells were exposed for 24 h to the drug alone or in combination with a non-cytotoxic concentration (2.0 μM) of PAK-200S, washed twice with PBS and reincubated in drug-free medium with or without the modulator. The absorbance was measured at 570 nm using a 96-well plate reader (340 EL BIO Kinetics Reader, Bio-Tek Instruments Inc., Winooski, VT, USA). The drug concentrations which inhibited cell growth by 50% (IC₅₀) were determined from semilogarithmic dose–response plots.

**RP-HPLC-analysis of topotecan concentrations**

Exponentially growing cells were trypsinized, washed twice with PBS and 2 x 10⁶ cells per sample were resuspended in RPMI-1640 medium. Then, cells were exposed to drug-free medium or to medium containing 2.0 μM PAK-200S for 30 min at 37°C. For the measurements of cellular topotecan concentrations, 1.0 μM topotecan was added for the indicated time. For reversed-phase high-performance liquid chromatography (RP-HPLC) analysis cells were centrifuged at 11 000 g at 4°C, washed twice in ice-cold PBS buffer and resuspended in 500 μl of 0.1 M sodium citrate solution, and the extraction procedure was performed as described earlier (Beijnen et al., 1990). In brief, cells were lysed and deproteinized with 3 ml ice-cold methanol–acetone (50:50, v/v), covered with nitrogen and frozen at –20°C for 12 h. After centrifugation at 1000 g for 15 min, supernatants were evaporated in a Speed Vac Plus Concentrator (Savant Instruments Inc., Farmingdale, NY, USA), resuspended in 200 μl of the mobile phase (methanol–phosphate buffer [60:40, v/v] adjusted to pH 5.9), covered with nitrogen and stored at –20°C for a further 2 h. The samples were centrifuged (21 900 g at 4°C for 5 min), and 50 μl of each sample were applied to HPLC. HPLC was performed with reversed-phase columns (4.6 × 250 mm) at 20°C packed with Nucleosil (C18) 5-μm particles (Machery and Nagel, Düren, Germany); sample injections were performed using a Waters 717 Plus Autosampler (Waters Inc., Eschborn, Germany). Absorbance of the column effluent was monitored using a fluorescence detector (Waters 470 Scanning Fluorescence Detector). Excitation was set to 370 nm and emission was adjusted to 525 nm. The peak areas were calculated using a Waters Millennium Chromatography Manager. Linearity was given to r² = 0.999 in the range of 5.0 μM–1.0 μM TPT, the limit of detection was 20 pm, the limit of quantitation was 50 pm (data not shown).

**TOP-I and TOP-II protein expression**

Fifty micrograms of nuclear proteins were dissolved on a 7.5% sodium dodecyl sulphate (SDS) polyacrylamide gel and electroblotted to nitrocellulose (Bio-Rad, Hercules, CA, USA). For TOP-I immunoblotting, membranes were blocked with 5% (w/v) dried non-fat milk in PBS. TOP-I-specific binding of the human mAb against TOP-I (TopoGen Inc., Columbus, OH, USA) was visualized with the enhanced chemiluminescence (ECL) detection (Amersham Co., Arlington Heights, IL) using a protein A–horse-radish peroxidase complex (Amersham Co.). TOP-II-specific binding of a rabbit polyclonal Ab (TopoGen Inc.) was visualized as mentioned above using a horseradish peroxidase complex.

**TOP-I unwinding activity of pBR322 plasmid DNA**

Nuclear extracts were prepared according to the method of Danks et al. (1988). In brief, 10⁶ cells were permeabilized in 5 mM potassium dihydrogen phosphate buffer pH 7.0 containing 2 mM magnesium chloride (MgCl₂), 4 mM dithiothreitol (DTT), 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulphonyl fluoride (PMSF) (buffer A). Then, cells were centrifuged at 400 g for 5 min, and resuspended in buffer A containing 0.25 mM sucrose, overlayed with buffer A containing 0.3 M sucrose and centrifuged at 2000 g for 20 min. The pellet was resuspended in 5 mM potassium dihydrogen phosphate buffer pH 7.0 containing 4 mM DTT, 1 mM EDTA and 1 mM PMSF. Nuclear
proteins were eluted as 0.35 and 1.0 M salt extracts as originally described, washed and stored in aliquots at –80°C. To evaluate the effect of PAK-200S on the topotecan-mediated inhibition of ATP-independent unwinding of supercoiled pBR322 plasmid DNA ex vivo, a reaction mixture containing 0.3 μg ml–1 pBR322 plasmid DNA and different dilutions of nuclear extract protein were incubated at exactly 37°C for 30 min with drug-free medium or with 50 μM topotecan – 2.0 μM PAK-200S. The reaction products were separated on a 1.0% (w/v) agarose gel (4.6 V cm–1) and stained with 0.5 μg ml –1 ethidium bromide. Topotecan, DMSO and PAK-200S at the concentrations used had no effect on the pBR322 plasmid DNA (data not shown).

Quantitation of protein-linked DNA breaks

The DNA of mid-logarithmic phase MCF-7/adr cells was labelled for 24 h with 0.1 μCi ml–1 [14C]dThy (DuPont, Boston, MA, USA; specific activity 62.8 mCi mmol–1). Cells were harvested, washed and reincubated for 30 min at 37°C in [14C]dThy-free medium containing 1.0–100 μM TPT with or without 2.0 μM PAK-200S. After drug exposure cells were washed in pre-warmed PBS (37°C) and the potassium chloride–SDS co-precipitation assay was immediately performed using 1 ml lysis buffer (1.25% SDS, 5 mM EDTA, 0.4 mg ml–1 sheared salmon sperm DNA, adjusted to pH 8.0) per 106 cells as described earlier (Trask et al, 1984; Vanhoefer et al, 1997). The results were calculated as percentage of precipitable [14C]dThy-labelled DNA of drug-treated cells of total [14C]dThy-DNA.

Statistical analysis

The differences between the mean values were analysed 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

Cytotoxicity

The cytotoxicity of a 24-h drug exposure to doxorubicin (Figure 1A) and topotecan (Figure 1B) was investigated in parental and resistant MCF-7 and A2780 cells. MCF-7/adr and A2780/Dx5 cells showed 88-fold and 189-fold resistance to doxorubicin, whereas cross-resistance to topotecan was 5.6-fold and 23.6-fold, respectively. Cellular exposure to a non-cytotoxic concentration of PAK-200S (2 μM) partially reversed resistance to doxorubicin, but almost completely restored sensitivity to topotecan in both MDR cell lines (remaining resistance to topotecan in MCF-7/adr and A2780/Dx5 cells was 1.8-fold (P > 0.05) and 1.6-fold (P > 0.05), respectively) with no effect on parental MCF-7/wt and A2780/wt cells.

Pharmacokinetics of cellular topotecan concentrations

The cellular topotecan concentrations in parental MCF-7/wt and P-gp-expressing MCF-7/adr cells were analysed by RP-HPLC and the results are shown in Figure 2. Cellular topotecan lactone concentrations in MCF-7/wt and MCF-7/adr cells were accumulated with steady-state levels at approximately 20 min. Resistant MCF-7/adr cells showed about 2.5-fold lower cellular lactone concentrations than parental MCF-7/wt cells with peak intracellular concentrations of 19.55 ± 3.19 pmol topotecan lactone 10–6 cells and 48.87 ± 3.92 pmol topotecan lactone 10–6 cells, respectively (P < 0.01). Exposure of MCF-7/adr cells to a non-cytotoxic concentration of 2.0 μM PAK-200S completely restored cellular lactone concentrations with 47.56 ± 2.98 pmol lactone 10–6 cells to the level observed for MCF-7/wt cells. PAK-200S had no effect on the drug concentrations in parental MCF-7/wt lacking P-gp expression (Figure 2). Similar results were observed in...
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parental A2780/wt and resistant A2780/Dx5 cells (data not shown).

**Immunoblot analysis of TOP-I and TOP-II expression**

A representative TOP-I and TOP-II immunoblot of parental MCF-7/wt and resistant MCF-7/adr cells is shown in Figure 3A. Two bands were identified, the intact \( M_r 100\,000 \) TOP-I protein band and a \( M_r 68\,000 \) TOP-I proteolytic fragment (D'Arpa et al, 1988). No differences in TOP-I protein expression were found between topotecan-sensitive and -resistant MCF-7/wt cells. However, using a rabbit polyclonal antibody against TOP-II protein, MCF-7/adr cells revealed a minimally lower expression of TOP-II. Exposure of MCF-7/adr cells to 2\( \mu \)M PAK-200S did not affect either TOP-I or TOP-II expression (Figure 3B).

**Induction of protein-linked DNA breaks**

The maximum induction of protein-linked DNA breaks (PLDBs) by topotecan without or with PAK-200S was investigated in MCF-7/adr cells (Figure 4). After a 30-min exposure to increasing concentrations of topotecan, the induction of PLDBs increased in a dose-dependent manner (demonstrated as maximal induction of PLDBs), followed by a plateau phase. Exposure of MCF-7/adr cells to topotecan in the presence of a non-cytotoxic concentration of 2.0 \( \mu \)M PAK-200S significantly increased the maximum induction of PLDBs (PLDBs for 50 \( \mu \)M topotecan without or with 2.0 \( \mu \)M PAK-200S were 10.5 ± 2.1% vs 16.9 ± 4.4% \( P < 0.01 \), respectively), demonstrating increased binding of topotecan to the nuclear DNA–TOP-I complex. No effect of PAK-200S on the maximum induction of PLDBs was found in parental MCF-7/wt cells (data not shown).

**TOP-I DNA unwinding activity**

The effect of PAK-200S on the topotecan-mediated inhibition of the unwinding activity of TOP-I was investigated ex vivo using nuclear extracts of MCF-7/adr cells. The quantitation of ATP-independent TOP-I DNA unwinding activity was accomplished ex vivo by adding nuclear extracts to a reaction mixture containing supercoiled pBR322-DNA as substrate and the results are shown in Figure 5. Exposure of nuclear extracts to 50 \( \mu \)M of topotecan significantly inhibited TOP-I unwinding activity at 160 ng of nuclear proteins (Figure 5B). PAK-200S at 2.0 \( \mu \)M had no effect on the topotecan-mediated inhibition of TOP-I unwinding activity of pBR322 plasmid DNA (Figure 5C), results which indicate that PAK-200S does not affect the interactions between topotecan and TOP-I ex vivo.

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DISCUSSION

The present study demonstrates that: (a) expression of the membrane transporter P-gp may result in decreased cellular drug concentrations of the pharmacologically active closed-ring lactone of topotecan, (b) dihydropyridine analogue PAK-200S, a new potent MDR-reversing agent, almost completely reverses resistance to topotecan in P-gp-expressing MCF-7/adr and A2780/Dx5 cells, with no effect on parental cells, and (c) reversal of topotecan resistance by PAK-200S is related to restoration of drug concentrations of the closed-ring lactone of topotecan rather than a direct effect on topoisomerases. Non-cytotoxic concentrations of PAK-200S (2.0 μM) completely restored cellular concentrations of the active lactone form of topotecan in MCF-7/adr cells (Figure 2), with no effect on parental cells lacking P-gp expression. Furthermore, restoration of cellular lactone concentrations by PAK-200S resulted in higher steady-state levels of PLDBs, the critical cytotoxic lesions of TOP-I interactive agents (Holm et al, 1989; Hsiang et al, 1989).

PAK-200S had no effect on the cellular TOP-I protein expression and enzyme activity (Figures 3B and 5) and no alterations of TOP-II expression were observed. These results are of importance because DNA topoisomerases I and II perform related functions, and TOP-II has been shown to compensate for TOP-I function (Uemura et al, 1984; Yang et al, 1987; Eng et al, 1990; Sugimoto et al, 1990; Friedman et al, 1994). The level of nuclear TOP-II is linked to cell proliferation, increasing during S phase and dropping to low levels during G1 phase (Markovits et al, 1987). TOP-II expression was slightly decreased in resistant MCF-7/adr cells (Figure 3A), however, no differences in cell doubling time were found between MCF-7/wt and MCF-7/adr cells (U Vanhoefer et al, unpublished data).

The reversible cellular hydrolysis of the pharmacologically active closed-ring lactone to the inactive open-ring hydroxy acid of topotecan has been shown to be pH-dependent (Grochow et al, 1992). PAK-200S at a concentration of 2.0 μM showed no effect on the pH of whole cell lysates of MCF-7/adr cells. In addition, no differences were found between MCF-7/wt and resistant MCF-7/adr cells in terms of cellular pH (data not shown). Based on these data, reversal of topotecan resistance by PAK-200S is likely related to inhibition of P-gp function in MCF-7/adr cells.

Previous studies on drug-selected P-gp-expressing MDR cells (Hendricks et al, 1992; Mattern et al, 1993; Maliepaard et al, 1996) and MDRI cDNA-transfected NIH 3T3 cells (Hoki et al, 1997) showed that expression of P-gp may confer resistance to positively charged TOP-I interactive agents (e.g. topotecan). Furthermore, inhibition of P-gp function by quinidine resulted in an increased accumulation of topotecan in MCF-7/adria and KG1a cells (Hendricks et al, 1992). Of importance is that irinotecan (CPT-11; [7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxy-CPT]) and its active metabolite SN-38 (10-hydroxy-7-ethyl-campto-
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