A Novel 7-modified Camptothecin Analog Overcomes Breast Cancer Resistance Protein-associated Resistance in a Mitoxantrone-selected Colon Carcinoma Cell Line

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Abstract

We selected a mitoxantrone-resistant HT29 colon carcinoma cell line (HT29/MIT) that exhibited a very high degree of resistance to the selecting agent and marked resistance to topotecan and SN38, but limited resistance to doxorubicin. The development of drug resistance was independent of expression of P-glycoprotein or multidrug resistance-associated protein but was associated with high up-regulation of the breast carcinoma resistance protein (BCRP) as shown by Western blot analysis. BCRP overexpression was associated with a reduced intracellular accumulation of topotecan, a known substrate for BCRP. Conversely, a lipophilic 7-modified camptothecin analogue (ST1481) displayed a complete lack of cross-resistance in HT29/MIT cells, suggesting that the drug was not a substrate for BCRP because no defects in intracellular accumulation were found. This conclusion is consistent with the antitumor efficacy of ST1481 against a BCRP-expressing tumor. These results may have therapeutic implications because the antitumor efficacy of ST1481 is in part related to a good bioavailability after oral administration, and the drug is currently under Phase I clinical evaluation.

Introduction

Various mechanisms may contribute to the cellular resistance to camptothecins. As with other topoisomerase inhibitors, cellular resistance to camptothecins may involve defects in cellular drug accumulation and alterations in the target or in the cellular response to the drug-induced DNA damage. Tumor cell lines and yeast models have provided information on potentially relevant mechanisms involved in the development of resistance to camptothecins (1). In particular, resistance to several cytotoxic agents of natural origin including camptothecins has been associated with overexpression of energy-dependent transport systems responsible for drug efflux, leading to reduced intracellular drug accumulation. Clinically relevant camptothecins (e.g., TPT and SN38, the active metabolite of irinotecan) are substrates for P-gp and also for the MRP1 (2, 3). Recently (4), marked resistance to TPT and SN38 has been observed in tumor cell lines that were selected for resistance to mitoxantrone and exhibited a non-P-gp, non-MRP MDR phenotype. In these cell lines, the mechanism of resistance is associated with increased drug efflux and ascribed to overexpression of a novel transporter BCRP, which also belongs to the ATP-binding cassette transporter family (5, 6). The pattern of cross-resistance of the mitoxantrone-resistant phenotype was unusual, because marginal or no cross-resistance was detected with paclitaxel and doxorubicin, which are substrates for P-gp (7).

We have described recently (8, 9) the promising features of novel 7-substituted lipophilic camptothecins (Fig. 1) that exhibit potent cytotoxic and antitumor activity. In addition, the analogues are characterized by a complete lack of cross-resistance in cells with a typical MDR phenotype. Because the primary focus in the clinical development of camptothecins is colorectal cancer, a colon carcinoma cell line HT29 was exposed to increasing concentrations of mitoxantrone in an attempt to select a subline overexpressing BCRP. In this study, we report that the selected mitoxantrone-resistant variant (HT29/MIT), which is cross-resistant to TPT, exhibits marked expression of BCRP. The results provide evidence that a novel 7-substituted camptothecin (ST1481) is able to overcome drug resistance in vitro and in vivo. Our study also shows that the efficacy of ST1481 against BCRP-expressing tumor cells is attributable to the lack of recognition by the transporter and provides additional support to the therapeutic interest of camptothecins with a proper substituent at the position 7.

Materials and Methods

Drugs. Mitoxantrone was provided by Boehringer Mannheim Italia. The camptothecin analogue, ST1481, was provided by Sigma-Tau. The synthesis of ST1481 (7-t-butoxymimomethylcamptothecin) has been described elsewhere (9). The drug was dissolved in DMSO and diluted in sterile distilled water. TPT, kindly supplied by Smith-Kline Beecham Pharmaceuticals (King of Prussia, PA), and SN38, provided by Prof. Luci Merlini (University of Milan, Milan, Italy), were dissolved in sterile distilled water and DMSO, respectively.

Cell Lines and Growth Conditions. The human colon carcinoma cell line HT29 and the mitoxantrone-resistant HT29/MIT subline were used in this study. HT29/MIT cells were generated by exposure to increasing drug concentrations up to 0.3 μg/ml. The growth characteristics of sensitive and resistant cells were similar, the doubling times being 22 and 26 h for sensitive and resistant cells, respectively. Both cell lines were maintained as monolayers in RPMI 1640 medium supplemented with 10% FCS.

Cellular Sensitivity to Drugs. Cellular sensitivity to drugs was evaluated by growth-inhibition assay after 1-h drug exposure. Cells in the logarithmic phase of growth were seeded in duplicates into 6-well plates. Twenty-four h after seeding, the drug was added to the medium. Cells were harvested 72 h after drug exposure and counted with a cell counter. IC50 is defined as the drug concentration causing a 50% reduction of cell number compared with that of untreated control.

Alkaline Elution. Single-strand DNA breaks were determined by the alkaline elution method (10). Briefly, cellular DNA was labeled with 0.08 μCi/ml [3-3H]thymidine (Amersham Pharmacia Biotech, Cologno Monzese, Italy) for 24 h, and the nucleoside-labeled precursor was removed 24 h before...
was transferred into a tube. This procedure was repeated twice. The clear TPT-containing solution was then used for HPLC determination. Quantitative HPLC analysis was performed at room temperature using a C18 reverse-phase column (5 mm; 150 × 4.6 mm; Agilent, Palo Alto, CA). The flow rate was 1.0 ml/min. For ST1481, the eluent was a water/acetonitrile mixture (1:1 w/w), and the drug was detected fluorometrically (excitation 340 nm, emission 510 nm). For TPT, the eluent was a 10:90 w/w mixture of acetonitrile and aqueous 2% triethylamine acetate buffer (pH 5.5). Fluorometric detection was performed also in this case (excitation 382 nm, emission 527 nm). Quantitative determination was performed in quadruplicate by graphic interpolation of the reference curve obtained by plotting the peak areas versus known amounts of the test drugs. A linear relationship was found in the 0–150 ng range for both ST1481 and TPT. To evaluate the extent of nonextractable drug in the various systems, blank experiments were performed adding known amounts (50–100 ng) of each drug to a cell pellet not previously treated with drug. The sample was then centrifuged and washed, and the drug was determined in the supernatant. Then the pellet was extracted as reported before, and the amount of drug was determined. The sums of drug amounts in the supernatant and in the pellet should correspond to the total drug added to the system. Using both mitoxantrone-sensitive and -resistant cell lines with ST1481 or TPT, we always found between 80 and 85% recovery of the theoretical amount of drugs. Therefore, the figures presented in Table 3 should be increased by a factor of about 1.2. Of course, the relative uptake ratios are not affected by these findings.

**Western Blot Analysis.** Cell lysates from exponentially growing cells were prepared as described previously (15) with minor modifications. Samples (80 µg/lane) were fractionated by SDS-PAGE and blotted on nitrocellulose sheets. Blots were preblocked for 1 h at room temperature in PBS containing 5% (w/v) dried nonfat milk. Filters were incubated overnight at 4°C with monoclonal antibodies to BCRP (BXP-21; Ref. 16). A rabbit anti-actin antibody (Sigma Chemical Co., St. Louis, MO) was used as control for loading. Antibody binding to nitrocellulose blots was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Results**

**Cellular Sensitivity Studies.** A human colon carcinoma cell line (HT29/MIT) was selected by exposure of the HT29 cell line to increasing concentrations of mitoxantrone. The HT29/MIT cells exhibited a very high degree of resistance to the selecting agent (66-fold), a low level of cross-resistance to doxorubicin (around 2-fold), and no cross-resistance to paclitaxel (Table 1). Marked cross-resistance to the clinically relevant camptothecins (TPT and SN38) was also observed (around 13-fold and 17-fold, respectively). However, no cross-resistance to the novel 7-modified camptothecin ST1481 was found. Thus, it appears that the mechanism responsible for the drug-resistant phenotype of HT29/MIT is overcome by the novel lipophilic camptothecin.

A markedly reduced level of DNA single-strand breaks (as detected by alkaline elution assay) was observed in the HT29/MIT cell line after 1-h exposure to mitoxantrone. In fact, a high concentration (100 µg/ml) produced a low extent of DNA damage (59 rad-equivalents) in HT29/WT as compared with that found in parental cells exposed to

<table>
<thead>
<tr>
<th>Drug</th>
<th>HT29</th>
<th>HT29/MIT</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitoxantrone</td>
<td>0.072 ± 0.01</td>
<td>4.75 ± 1.2</td>
<td>65.9</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.77 ± 0.3</td>
<td>1.35 ± 0.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Topotecan</td>
<td>1.7 ± 0.3</td>
<td>22.5 ± 5.4</td>
<td>13.2</td>
</tr>
<tr>
<td>SN38</td>
<td>0.26 ± 0.14</td>
<td>4.51 ± 1.05</td>
<td>17</td>
</tr>
<tr>
<td>ST1481</td>
<td>0.0245 ± 0.01</td>
<td>0.0315 ± 0.001</td>
<td>1.2</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.0033 ± 0.0001</td>
<td>0.0022 ± 0.0001</td>
<td>0.7</td>
</tr>
</tbody>
</table>

a Drug sensitivity was measured by growth inhibition assay after 72-h (paclitaxel) or 1-h (all other drugs) exposure.

b IC50, drug concentration causing a 50% decrease in cell growth.

c RI, resistance index, ratio between the IC50 of mitoxantrone-resistant and -sensitive cells.
1 µg/ml (384 rad-equivalents). Reduced drug-induced DNA damage appears to be the consequence of accumulation defects: a 10-fold reduction of intracellular drug content was found in HT29/MIT cells compared with the parental HT29 cells (data not shown).

In Vivo Antitumor Activity Studies. The parental HT29 tumor model was only marginally responsive to mitoxantrone, whereas the HT29/MIT model was completely resistant (tumor growth inhibition ~50% versus <20%, respectively). The results of the in vivo comparative studies with camptothecins are reported in Table 2. TPT exhibited moderate efficacy against the parental model (TVI, 67%) but did not affect the growth of the resistant model. ST1481 was effective against the HT29 and HT29/MIT tumor models, inducing a TVI of 91% and 82%, respectively.

Expression of Resistance-related Genes. In an attempt to identify the molecular basis of the observed MDR phenotype and the mechanism(s) that ST1481 was capable of overcoming, we examined the expression of several genes potentially contributing to the phenotype of HT29/MIT cells. An analysis of the expression of proteins usually associated with resistance to multiple drugs (MDR1 and MRP) showed lack of expression (Fig. 2A). Because quantitative or qualitative alterations in DNA topoisomerases have been related to resistance to their inhibitors (atypical MDR; Ref. 17), we investigated whether DNA topoisomerase expression was altered in the resistant cell line. Northern blot analysis indicated no significant changes in the expression of resistant and sensitive cells was 0.9 ± 0.1 for the α and 0.5 for the β isom. In view of the observed cross-resistance to topoisomerase I inhibitors, we also examined topoisomerase I expression. However, no relevant difference was found between the mitoxantrone-sensitive and the mitoxantrone-resistant cell line, the ratio between the expression level of resistant and sensitive cells being 1.25 ± 0.3.

Table 2 Antitumor activity of orally administered ST1481 and TPT on human tumor xenografts.a

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>TV (mean ± SD)</th>
<th>TVI percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HT29/MIT</td>
<td>HT29/MIT</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>184.5 ± 116</td>
<td>598 ± 217</td>
</tr>
<tr>
<td>ST1481</td>
<td>2</td>
<td>12 ± 12</td>
<td>108 ± 102</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>67 ± 39</td>
<td>603 ± 148</td>
</tr>
<tr>
<td>TPT</td>
<td>2</td>
<td>20</td>
<td>195 ± 20</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>22 ± 4</td>
<td>34 ± 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>250 ± 22</td>
<td>390 ± 18</td>
</tr>
</tbody>
</table>

a Tumors were implanted s.c., and treatments were started when tumors were visible and not measurable. Drugs were delivered every fourth day for four times.

b Tumor volume (TV) in mm3 assessed 10–11 days after the last treatment; 8–10 tumors/group.

c Tumor volume inhibition (TVI) percentage in treated versus control mice.

d P < 0.01 versus TPT-treated group by Student’s t test.

e P < 0.001 versus TPT-treated group by Student’s t test.

BCRP Expression and Cellular Camptothecin Accumulation. Because decreased cellular accumulation of mitoxantrone was not related to expression of MDR or MRP, we performed a Western blot analysis to determine whether BCRP was expressed in the HT29 and HT29/MIT cells. BCRP expression was highly up-regulated in the resistant variant (Fig. 3). To establish whether BCRP was indeed responsible for the peculiar pattern of drug response of HT29/MIT cells, drug accumulation studies were carried out. A markedly reduced drug content was observed in HT29/MIT cells after 1-h exposure to TPT concentrations corresponding to the IC50 of either mitoxantrone-sensitive (2 µg/ml) or mitoxantrone-resistant (20 µg/ml) cells (Table 3). By contrast, ST1481 accumulation in HT29/MIT cells was not lower than in HT29 cells. The extent of drug accumulation observed in both sensitive and resistant cells after exposure to 2 µg/ml ST1481 was much higher (up to 100-fold) than that found after exposure to the same concentration of TPT.

Discussion

The efficacy and the clinical pharmacology of a large number of antitumor drugs are affected by the expression of drug transporters in a wide variety of tumor types and normal tissues. This phenomenon has been extensively studied for the protective and excretory functions of the P-gp, encoded by the MDR-1 gene (18). In addition to P-gp, other transporters potentially implicated in the MDR phenotype have been described (5, 6, 19). The BCRP identified recently (16, 20) shares various features with P-gp in terms of the range of drugs recognized by the transporters and the pattern of expression by normal tissues. Although the physiological function of BCRP is still unknown, the high level of expression found in tumor cells upon exposure to mitoxantrone or TPT suggests that BCRP may have clinical relevance for patients treated with these agents. The high level of resistance found in BCRP-overexpressing cells suggests that clinically relevant camptothecins, including TPT and SN38, are good substrates for this transport system.

In our mitoxantrone-resistant HT29 cell model, we demonstrated...
that the complete lack of efficacy of TPT in vivo reflected the resistance observed in vitro, thus supporting the pharmacological relevance of the BCRP-associated resistant phenotype. In contrast to TPT, the novel 7-substituted analogue ST1481 was not recognized by resistance observed in vitro in vivo it retained an activity comparable with that found in the parental HT29 cell line. In addition, there was no evidence of alterations in intracellular accumulation of ST1481 by HT29/MIT. The basis for the quite different behavior of the lipophilic 7-substituted analogue and other camptothecins remains to be elucidated because details about the molecular interactions between the transporter and these drugs are lacking. BCRP and related transporters cause marked resistance to mitoxantrone, epirubicin, and a variety of camptothecins but a low and variable degree of resistance to doxorubicin and unmodified camptothecin itself. No cross-resistance to taxol was detected in this phenotype (Table 1; Ref. 7). A tentative explanation for the peculiar pattern of cross-resistance in BCRP-expressing cells is that the presence of specific functional groups in rings A or B of the camptothecin (e.g., hydroxyl or amino groups) could result in affinity for the drug transporter (Fig. 1).

Colon carcinoma cells may have a capacity for glucuronidation that could contribute to drug resistance (4). Indeed, camptothecins containing a hydroxyl group at the 10 position are susceptible to glucuronidation (4). Studies (20) using mRNA indicated expression of BCRP by small and large intestine, suggesting that BCRP is involved in the uptake of exogenous compounds by the gastrointestinal tract, as already documented for P-gp (21). If such is the case, the lack of recognition of ST1481 by this transporter and by P-gp could result in the good bioavailability and efficacy of oral treatment (9).

Overall, these results may have implications for (a) the development of novel camptothecins not recognized by BCRP and (b) the therapeutic use of ST1481, an agent selected for clinical evaluation by oral administration on the basis of its promising preclinical profile.

References
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