INTRODUCTION

CPTs specifically target the enzyme DNA topoisomerase I, resulting in DNA damage and cell death (1), and are effective in a variety of cancers (2). Structure-activity studies indicate that modifications at the 9 or 10 position of the A ring of CPT may enhance activity in vitro and in vivo (3, 4). Irinotecan and TPT are two CPTs that are approved for the treatment of a variety of cancers; TPT contains a hydroxyl at the 10 position of SN-38 (7-ethyl,10-hydroxycamptothecin), which also contains a hydroxyl at the 10 position. 9-NC and 9-AC are two other CPTs that are undergoing clinical evaluation and contain nitro and amino groups at the 9 position, respectively. Although randomized trials have not been performed, responses to 9-NC appear more common than responses to 9-AC in early clinical trials (5–14). This possible difference in activity may relate to differences in susceptibility to tumor resistance mechanisms.

In this regard, several members of the ATP-binding family of drug efflux proteins are implicated in resistance to CPTs, including Pgp (15), MRP1 (16), and MRP2 (17). BCRP is a newly described drug efflux protein that was cloned independently from human placenta (denoted ABCG2; Ref. 18), from a cell line selected for resistance to doxorubicin and verapamil (19), and from a cell line selected for resistance to mitoxantrone (denoted MXR; Ref. 20). BCRP is widely expressed in normal human tissues, including the placenta, brain, small intestine, testis, ovary, colon, and liver (19). In contrast to Pgp, human BCRP contains only one NH2-terminal ATP-binding domain and one COOH-terminal transmembrane domain and is believed to function as a dimer (18). Overexpression of BCRP confers resistance to TPT and SN-38 (21–23). Furthermore, mutations of amino acid 482 (R482) of BCRP can occur in cells selected for resistance to doxorubicin, and this residue has been implicated in substrate interactions (24–27). In this report, we demonstrate that whereas overexpression of Pgp, MRP1, or MRP2 has little effect on the cytotoxicity of either 9-NC or 9-AC, overexpression of either wild-type or mutant R482T BCRP confers resistance to 9-AC, but not to 9-NC.

MATERIALS AND METHODS

Chemicals and Reagents. 9-NC was obtained from SuperGen (Dublin, CA) and prepared as a 10 mM stock solution in DMSO. 9-AC was obtained from Pharmacia (Kalamazoo, MI) and used as a 1 mM stock solution in DMSO. TPT was obtained from GlaxoSmithKline (King of Prussia, PA) and used a 4 mM stock solution in DMSO. SN-38 was a gift from Pharmacia and Upjohn, and it was used as a 1 mM stock solution prepared in DMSO. Flavin adenine di nucleotide, flavin mononucleotide, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, β-NADP, and MgCl2 were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC-grade methanol and acetonitrile were purchased from Fischer Scientific (Fair Lawn, NJ). All other American Chemical Society-grade chemicals were obtained from JT Baker (Phillipsburg, NJ).

Cell Lines and Growth Inhibition Assays. Cell lines used for these studies are listed in Table 1. All cell lines were grown under conditions as specified in the relevant reference. Antiproliferative effects of drugs on cell growth were determined using a MTT assay as described previously (28). For cell lines maintained in media containing Geneticin, cells were grown in the absence of this drug for 48 h before performing MTT assays. Drug concentrations associated with 50% inhibition of growth (IC50) were obtained by curve-fitting analyses of the percentage of control (untreated cells) absorbance at 570 nm versus drug concentrations, using WIN-NONLIN (Version 2; Scientific Consulting Inc.). The data were fitted to a sigmoidal inhibitory effect model as described by the equation $E = E_{\text{max}} \times \frac{1}{1 + \left[ \frac{C}{C_{\text{IC50}}} \right]^n}$, where $E$ is the percentage of control absorbance, $C$ is the drug concentration, $y$ is the curve shape parameter, and $EC_{\text{IC50}} = IC_{50}$. Relative resistance was calculated as the ratio of the IC50 of the resistant cell line to the IC50 of the parental cell.
leupeptin, and 1/100 M EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.5% SDS, 0.1% Triton X-100, and 1% sodium deoxycholate] supplemented with 1 mM precipitation assay buffer [50 mM Tris (pH 7.2) at 25°C]. Differences in mean IC50 values were compared using Student’s t test, with P ≤ 0.05 considered significant.

BCRP Immunoblotting Assays. Cell pellets were lysed in radioimmunoprecipitation assay buffer [50 mM Tris (pH 7.2) at 25°C, 150 mM NaCl, 0.1% SDS, 0.1% Triton X-100, and 1% sodium deoxycholate] supplemented with 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 1 μg/ml pepstatin. Lysates were sonicated and then centrifuged at 10,000 × g for 10 min. After determining supernatant protein concentrations by the Bradford method (29), equal quantities of lysate protein were subjected to SDS-PAGE as described previously (28).

Immunoblotting for BCRP expression was performed using the BXP-21 BCRP antibody (30). Without stripping, the blots were also probed for β-actin expression using a β-actin antibody (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

BCRP cDNA Sequencing. Total RNA was isolated from CPT-K5 cells using a lysis buffer containing a chaotropic salt supplemented with 145 mM β-mercaptoethanol (QIAamp RNA minikit, Qiagen, Valencia, CA). The oligonucleotide primers 5′-GCTGGGTAATCCCCAGGCCTCT and 5′-AGAGATCGATGCCTCGTTAACA were used to amplify the region encompassing bases 1100–1800 of the human BCRP cDNA (numbering according to GenBank accession number AB051855), which includes the region coding for residue 482. Reverse transcription-PCR was performed as described previously (31). The resultant PCR fragments were directly sequenced using an automated sequencer (ABI-3100 Genetic Analyzer; Applied Biosystems, Foster City, CA).

Intracellular Drug Accumulation Studies. Cells were exposed to 9-AC or 9-NC at 37°C for 35 min. Subsequently, the media were aspirated and, and cells were washed twice with ice-cold calcium- and magnesium-free Dulbecco’s PBS and then harvested by scraping. After centrifugation at 1000 × g for 4°C for 10 min, cells were washed twice in ice-cold PBS, lysed by sonication in PBS, and then centrifuged at 1000 × g for 10 min at 4°C. Cell lysates were analyzed for protein content and stored at −80°C until further analysis. Concentrations of 9-NC and 9-AC in the lysates were determined using validated HPLC assays as described previously (32, 33). Measurement of 9-NC (which is not fluorescent) was performed by enzymatic reduction of 9-NC to 9-AC. Briefly, the reaction mixture contained 20 μl of bovine liver S-9 fraction containing 0.8–1 μg protein/reaction, 120 μl of a NADPH-generating system [100 mM phosphate buffer (pH 7.4), 10 mM NADP, 1 mM flavin adenine dinucleotide, 33 mM glucose-6-phosphate, and 0.5 unit of glucose-6-phosphate dehydrogenase], and 200 μl of lysate. Samples were incubated at 37°C for 60 min. The reaction was terminated by addition of 20 mM cold acidified methanol, and the samples were subjected to HPLC analysis.

### RESULTS

Effects of Overexpression of Drug Efflux Proteins on the Antiproliferative Activity of 9-AC and 9-NC. MDCKII cell lines consisting of stable transfectants of various efflux protein expression vectors were used to assess the effects of overexpression of Pgp,
MRP1, or MRP2 on the antiproliferative activities of 9-NC and 9-AC (Table 1). As expected, cells overexpressing Pgp were markedly resistant to the antiproliferative effects of vinblastine (Fig. 1; Table 2). Overexpression of Pgp in MDCKII cells also conferred about 100-fold resistance to TPT (Fig. 1; Table 2). By contrast, overexpression of Pgp had little effect on the growth inhibition associated with exposure to 9-AC or 9-NC (Fig. 1; Table 2). Overexpression of MRP1 or MRP2 conferred significant resistance to vinblastine but had little effect on cellular sensitivity to TPT, 9-AC, or 9-NC (Fig. 1; Table 2).

Similar studies were done using HEK293 cells overexpressing wild-type BCRP or R482T BCRP and with MDA-MB-231 cells overexpressing R482T BCRP (Table 1). Immunoblotting studies confirmed prominent expression of BCRP in the transfectants but not vector controls (Fig. 2). As observed previously (27), expression of the R482T protein was slightly lower than that of the wild-type protein in the stable HEK293 transfectants (Fig. 2).

Similar to previous results (27, 34, 35), overexpression of wild-type or mutant BCRP conferred resistance to both TPT and SN-38 (Fig. 3; Table 3). In addition, overexpression of wild-type or mutant BCRP was associated with resistance to 9-AC compared with TPT or SN-38 (Table 3). Furthermore, in HEK293 cells, overexpression of the R482T BCRP mutant was associated with levels of resistance that were similar to those observed with the wild-type protein for TPT, SN-38, and 9-AC (Fig. 3; Table 3). By contrast, overexpression of either wild-type or R482T BCRP had no effect on cellular sensitivity to 9-NC (Fig. 3; Table 3).

Effects of Overexpression of BCRP on the Cellular Accumulation of 9-AC and 9-NC. To further characterize the effects of BCRP overexpression on 9-AC and 9-NC, we studied the cellular accumulation of these compounds in HEK293 transfectants. After a 30-min

<table>
<thead>
<tr>
<th>Drug</th>
<th>MDCKII</th>
<th>MDCKII Pgp</th>
<th>RR*</th>
<th>MDCKII MRP1</th>
<th>RR</th>
<th>MDCKII MRP2</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>VBL</td>
<td>0.02–0.10</td>
<td>32.7–24.4</td>
<td>1640</td>
<td>49.96–0.03</td>
<td>2500</td>
<td>0.44–0.051</td>
<td>22</td>
</tr>
<tr>
<td>TPT</td>
<td>0.17–0.04</td>
<td>24.3–12.8</td>
<td>145</td>
<td>0.20–0.05</td>
<td>1.2</td>
<td>0.20–0.09</td>
<td>1.17</td>
</tr>
<tr>
<td>9-AC</td>
<td>0.10–0.02</td>
<td>0.13–0.09</td>
<td>1.3</td>
<td>0.24–0.13</td>
<td>2.4</td>
<td>0.17–0.02</td>
<td>1.7</td>
</tr>
<tr>
<td>9-NC</td>
<td>0.22–0.04</td>
<td>0.43–0.02</td>
<td>1.95</td>
<td>0.31–0.07</td>
<td>1.4</td>
<td>0.46–0.09</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*RR, relative resistance of efflux protein-expressing cell line compared with control cell line.

Results are expressed as mean–SDs (µM) for calculated IC₅₀s for six replicate experiments.
Table 3 Antiproliferative effects of camptothecin analogues in the presence and absence of forced expression of wild-type or mutant BCRP

<table>
<thead>
<tr>
<th>Drug</th>
<th>HEK293 HEK293 BCRP RR a</th>
<th>HEK293 HEK293 BCRP R482T RR a</th>
<th>MDA-MB-231 MDA-MB-231 BCRP R482T RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPT</td>
<td>0.034 ± 0.01 b</td>
<td>0.39 ± 0.19 c</td>
<td>11.5</td>
</tr>
<tr>
<td>SN-38</td>
<td>0.022 ± 0.00</td>
<td>0.17 ± 0.08 c</td>
<td>7.7</td>
</tr>
<tr>
<td>9-AC</td>
<td>0.033 ± 0.01 c</td>
<td>0.24 ± 0.13 c</td>
<td>7.3</td>
</tr>
<tr>
<td>9-NC</td>
<td>0.030 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>1</td>
</tr>
</tbody>
</table>

a RR, relative resistance of BCRP-expressing cell line compared with control cell line.
b Results are expressed as mean–SDs (μM) for calculated IC₅₀обр for six replicate experiments.
c Significantly different compared with the parental cell line (P < 0.05). None of the differences between wild-type versus mutant BCRP IC₅₀обр were significantly different.

DISCUSSION

Until recently, it appeared that efflux proteins implicated in clinical multidrug resistance, such as Pgp and MRP1, were not likely to be important in clinical resistance to CPTs. However, results initially obtained in yeast (37) and then in mammalian cells (19, 21, 22, 26, 30, 38–41) identified BCRP orthologues capable of conferring high levels of resistance to certain CPTs.

9-AC and 9-NC are currently under investigation in clinical trials, and little is known regarding the effects of drug efflux proteins on these compounds. Our studies indicate that overexpression of Pgp, MRP1, or MRP2 does not alter cellular sensitivity to 9-AC or 9-NC. Furthermore, neither the antiproliferative effects nor intracellular accumulation of 9-NC is affected by BCRP overexpression. By contrast, overexpression of wild-type or R482T BCRP confers resistance to 9-AC, associated with reduced intracellular accumulation of this drug. Together with data indicating that BCRP confers resistance to TPT and SN-38 but not CPT, these results suggest that polar residues at the 9 or 10 position of CPT enhance interactions with BCRP.

Similar to results reported for TPT (26, 27), we found that overexpression of the R482T mutant yielded resistance to 9-AC that was similar to that observed with overexpression of the wild-type protein. Furthermore, unlike cells selected for resistance to doxorubicin or mitoxantrone, we found that whereas cells selected for resistance to irinotecan up-regulate BCRP expression, they do not express BCRP containing mutations in residue 482. Together, these findings suggest exposure to 10 μM 9-AC, intracellular drug levels were about 2-fold higher in vector controls compared with wild-type or R482T BCRP transfectants (Fig. 4). By contrast, exposure to 10 μM 9-NC yielded intracellular drug concentrations that were similar in the vector controls and BCRP transfectants (Fig. 4). These results are consistent with those obtained from antiproliferative studies and suggest that wild-type and mutant R482T BCRP mediate cellular efflux of 9-AC, but not 9-NC.

Expression of BCRP in Cells Selected for Resistance to CPTs. Previous studies indicated that BCRP expression may be increased in cells selected for resistance to TPT (21); however, relatively little is known regarding BCRP expression in cells selected for resistance to other CPTs. We analyzed BCRP expression in two different U-937 cell lines selected for resistance to 9-NC (28, 31) and in the CPT-K5 cell line, which was selected for resistance to irinotecan (36). We are unaware of cell lines specifically selected for resistance to 9-AC. The results indicate that BCRP expression is not detectable in either parental U-937 leukemia cells or in two 9-NC-resistant sublines (Fig. 5). By contrast, BCRP expression is increased significantly in CPT-K5 leukemia cells compared with the parental line (Fig. 5). These data are consistent with BCRP functioning as an efflux protein for irinotecan/SN-38, but not 9-NC.

Because mutant forms of BCRP containing substitutions at residue 482 were identified in certain drug-resistant cell lines (19, 24), we sequenced this region in BCRP cDNA obtained from CPT-K5 cells. The results indicate that CPT-K5 cells express only BCRP containing wild-type sequence at codon 482 (Fig. 5).
that whereas mutations in residue 482 of BCRP can increase resistance to doxorubicin or mitoxantrone, they do not increase resistance to CPTs. Although a role for BCRP in clinical resistance to CPTs is not yet proven, expression of BCRP was reported in blast cells obtained from leukemia patients (42–44), and increased expression of BCRP was recently implicated in resistance to induction therapy in children with acute myeloid leukemia (45). In addition, in a patient with acute leukemia treated with a combination of TPT and 1-β-arabinofuranosylcytosine, we found that compared with pretreatment levels, BCRP expression increased 25-fold on day 3 in circulating leukemic blasts (data not shown), suggesting that TPT may induce BCRP expression in patient malignancies. It is possible that development of CPT analogues that are not affected by BCRP may improve the clinical use of these drugs. In addition to 9-NC, certain other CPT analogues, such as lurtotecan (NX211), DX-8951F, BNP1350, and STI1481X, do not appear to be BCRP substrates (46, 47). Additional studies are needed to determine the importance of BCRP expression in the clinical response to CPTs and whether use of topoisomerase I-targeting drugs that are not affected by BCRP will improve clinical outcomes in patients treated with these drugs.

REFERENCES


Differential Effects of the Breast Cancer Resistance Protein on the Cellular Accumulation and Cytotoxicity of 9-Aminocamptothecin and 9-Nitrocamptothecin

Rajeev Rajendra, Murugesan K. Gounder, Ahamed Saleem, et al.


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