ABCG2 Mediates Differential Resistance to SN-38 (7-Ethyl-10-hydroxycamptothecin) and Homocamptothecins

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Received November 20, 2003; accepted March 16, 2004

ABSTRACT

One activity potentially limiting the efficacy of camptothecin anticancer agents is their cellular efflux by the ATP-binding cassette half-transporter, ABCG2. Homocamptothecins are novel anticancer drugs that inhibit topoisomerase 1 with a greater potency than camptothecins. Homocamptothecins differ from camptothecins by their E-ring, which is seven-membered instead of the six-membered ring of camptothecins. We report herein that, like camptothecins, homocamptothecin and its difluoro derivative BN80915 are substrates for ABCG2. However, the resistance of three selected cell lines overexpressing wild-type or mutant ABCG2 to homocamptothecin or BN80915 was less than resistance to SN-38 (7-ethyl-10-hydroxycamptothecin), indicating that both the seven-membered E-ring present in homocamptothecin and the A- and B-ring modifications present in SN-38 are involved in substrate recognition by ABCG2. HEK-293 cells transfected with vectors encoding wild-type or mutant ABCG2 were found to be less resistant to both homocamptothecins than to SN-38. However, transfectants overexpressing mutant ABCG2 had relative resistance values for homocamptothecin and BN80915 4- to 14-fold higher than cells expressing wild-type ABCG2, suggesting that the gain of function resulting from mutation at amino acid 482, although not affecting SN-38, extends to the homocamptothecins. Resistance was reversed by the ABCG2 inhibitor fumitremorgin C. BN80915 was 17-fold more potent than SN-38 in wild-type ABCG2-transfected cells, suggesting that BN80915 has the potential to overcome ABCG2-related resistance to SN-38, the active metabolite of CPT-11 (irinotecan).

Camptothecins are among the most potent anticancer drugs introduced for cancer treatment in recent years. Two semisynthetic derivatives of camptothecin (CPT) are currently approved by the U.S. Food and Drug Administration, irinotecan for first-line chemotherapy of colon cancers and topotecan for the treatment of refractory ovarian cancers. Both derivatives bear substitutions on the A- and B-rings of CPT (Fig. 1), which provide water solubility and enhanced potency against Top1 (Tanzawa et al., 1994), the selective target of these drugs (Hsiang et al., 1985; Chen and Liu, 1994) (http://discover.nci.nih.gov/pommier/topo1.htm). Human carboxylesterase converts CPT-11 (irinotecan) to its active metabolite SN-38 (Fig. 1).

One of the limitations of camptothecins is the presence of, and the requirement for, an α-hydroxylactone in the E-ring for Top1 inhibition and antitumor activity (Giovanella et al., 1989; Hsiang et al., 1989; Jaxel et al., 1989). Because of this α-hydroxylactone, the E-ring can hydrolyze readily at physiological pH and generate carboxylic acid forms of camptothecins. Carboxylate derivatives are inactive or weakly active against Top1 (Hsiang et al., 1989; Jaxel et al., 1989). They also bind avidly to and are sequestered by human serum albumin, which shifts the equilibrium between lactone and carboxylate away from the active lactone (Burke and Mi, 1994). To overcome the E-ring instability, the group of Bigg and Lavergne (Lavergne et al., 1998) synthesized homocamptothecins, which differ from CPT by an E-ring containing an additional carbon resulting in a seven-membered E-ring. This modification decreases the rates of hydrolysis and conversion to the carboxylate and also prevents the reverse reaction, i.e., the conversion of the homocamptothecin carboxylate to lactone (Lavergne et al., 1998; Lesueur-Ginot et al., 1999). Interestingly, homocamptothecins are more potent Top1 inhibitors than camptothecins and retain potent antitumor activity (Lavergne et al., 1998, 2000; Bailly et al., 1999; Lansiaux et al., 2001; Larsen et al., 2001). A difluoro homocamptothecin derivative, difluoro-
Camptothecin (BN80915), whose Top1 and antitumor activity in model systems are greater than those of homocamptothecin (Lansiaux et al., 2001; Larsen et al., 2001), is being pursued for clinical development.

Another limitation of camptothecins is the resistance of cells overexpressing the ATP-binding cassette half-transporter, BCRP/ABCP/MXR/ABCG2 (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999). ABCG2 mediates resistance to mitoxantrone as well as to the camptothecins topotecan, SN-38, and 9-aminocampthotecin (Brangi et al., 1999; Rajendra et al., 2003). Acquired mutations that replace the wild-type arginine with a glycine or a threonine at amino acid 482 in the 
\[ \text{ABCG2} \]

gene alter the substrate specificity of the resulting protein. Cells overexpressing the mutant ABCG2 proteins exhibit a gain of function and add rhodamine 123 and the anthracyclines to the list of substrates (Honjo et al., 2001; Robey et al., 2001). ABCG2-mediated resistance is effectively inhibited by fumitremorgin C (FTC) (Rabindran et al., 1998). Topotecan has also been shown to be a P-glycoprotein substrate (Hoki et al., 1997).

To determine whether homocamptothecins are ABCG2 substrates, we compared the cytotoxicity of homocamptothecin and its clinical difluoro derivative, BN80915, to CPT and to SN-38 in three ABCG2-overexpressing selected cell lines expressing either wild type (R482) or mutant (R482T, R482G) ABCG2. Cross-resistance studies were also carried out with human embryonic kidney cells (HEK-293) stably transfected with any of the three ABCG2 proteins to exclude bias due to other potential mechanisms of resistance in the selected cell lines. We also examined the ability of FTC to reverse resistance to these compounds in the ABCG2 transfectants. Finally, we attempted to develop drug-resistant cell lines by selecting them in increasing concentrations of BN80915.

**Materials and Methods**

**Cell Lines.** The human colon carcinoma cell line S1 and its ABCG2-overexpressing S1-M1–80 subline were cultured in RPMI with the S1-M1–80 cells maintained in 80 μM mitoxantrone (Rabindran et al., 1998). The MCF-7 AdVp3000 subline was maintained in 3000 ng/ml adriamycin in the presence of 5 μg/ml verapamil (Chen et al., 1990); the MX100 subline was maintained in 100 ng/ml mitoxantrone (Robey et al., 2001). Camptothecin resistance in the S1-M1–80, MCF-7 AdVp3000, and MCF-7 MX100 cell lines has previously been described (Brangi et al., 1999; Litman et al., 2000). The MCF-7 breast carcinoma cell line and its ABCG2-overexpressing sublines were grown in improved minimum essential medium. HEK-293 cells were transfected with either empty pcDNA3.1 vector (Invitrogen, Carlsbad, CA) or pcDNA3.1 vector containing full-length 
\[ \text{ABCG2} \]

encoding either an arginine, threonine, or glycine at amino acid 482. Stable transfectants were maintained in Eagle’s minimum essential medium (American Type Culture Collection, Manassas, VA) supplemented with 10% FCS, penicillin, and streptomycin with G418 at a concentration of 2 mg/ml. The transfected cell lines have previously been described (Robey et al., 2003).

**Top1-Mediated DNA Cleavage Reactions.** Human recombinant Top1 was purified from baculovirus as described previously (Pourquier et al., 1999; Strumberg et al., 1999). The 161-bp fragment from pBlueScript SK(–) phagemid DNA (Stratagene, La Jolla, CA) was cleaved with the restriction endonuclease PvuII and HindIII (New England Biolabs, Beverly, MA) in supplied norepinephrine buffer 2 (50 μl reactions) for 1 h at 37°C and separated by electrophoresis in a 1% agarose gel made in 1× Tris/borate/EDTA buffer. The 161-bp fragment was eluted from the gel slice using the QIAEX II kit (QIAGEN Inc., Valencia, CA). Approximately 200 ng of the fragment were 3'-end labeled at the HindIII site by fill-in reaction.
with [α-32P]dGTP and 0.5 mM dATP, dCTP, and dTTP in React 2 buffer (50 mM Tris-HCl, pH 8.0, 100 mM MgCl2, and 50 mM NaCl) with 0.5 units of DNA polymerase I (Klenow fragment). Unincorporated [32P]dGTP was removed using mini Quick Spin DNA columns (Roche Diagnostics, Indianapolis, IN), and the eluate containing the 161-bp 3’-end-labeled DNA fragment was collected. Aliquots (approximately 50,000 dpm/reaction) were incubated with Top1 at 25°C for the indicated times in the presence of the tested drug. Reactions were terminated by adding SDS (0.5% final concentration). The samples (10 μl) were mixed with 30 μl of loading buffer (80% formamide, 10 mM sodium hydroxide, 1 mM sodium EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue, pH 8.0). Aliquots were separated in a denaturing gel (16% polyacrylamide, 7 M urea). Gels were dried and visualized using a PhosphorImager and ImageQuant software (Amer sham Biosciences, Sunnyvale, CA).

Cytotoxicity Assays. The assays performed were based on those of Skehan et al. (1990). Cells were plated in 96-well plates at a density of 2000 cells/well and allowed to attach overnight at 37°C in 5% CO2. Chemotherapy drugs with or without 5 μM of the ABCG2 inhibitor FTC were added at the desired concentrations and the cells were allowed to incubate for 96 h. Subsequently, cells were fixed in 50% trichloroacetic acid. Plates were then stained with a sulforhodamine B solution (0.4% sulforhodamine B w/v in 1% acetic acid), and optical densities were read on a plate reader at an absorbency of 540 nm. Each concentration was tested in quadruplicate, and controls were performed in replicates of eight.

Flow Cytometry. For studies with the anti-ABCG2 antibody 5D3 (Zhou et al., 2001), cells were trypsinized and resuspended in Dulbecco’s phosphate-buffered saline with 2% bovine serum albumin to which was added phycoerythrin-conjugated 5D3 (eBioscience, San Diego, CA) or phycoerythrin-conjugated mouse IgG according to the manufacturer’s instructions. The cells were incubated with antibody for 30 min at room temperature, washed twice with Dulbecco’s phosphate-buffered saline, and kept in the dark until analyzed. Cells were analyzed on a FACSort flow cytometer equipped with a 488-nm argon laser. For all samples, at least 10,000 events were collected. Debris was eliminated by gating on forward versus side scatter, and dead cells were excluded based on propidium iodide staining.

Results

Topoisomerase 1 Inhibition by BN80915. Figure 2 shows trapping of Top1 cleavage complexes by BN80915 compared with homocamptothecin. Both drugs were comparable in potency, and no noticeable difference was observed in the distribution of drug-induced DNA cleavage sites.

To further compare BN80915 and homocamptothecin, the stability of Top1 cleavage complexes was assessed using a salt-reversal assay. In this assay, addition of 0.35 M NaCl to the Top1 reactions inhibits Top1-mediated DNA cleavage without blocking Top1-mediated DNA religation resulting in a shift of the cleavage-religation equilibrium toward religation. CPT is known to act as a Top1 poison by preventing the religation of Top1 cleavage complexes (Hsiang et al., 1985; Tanizawa et al., 1994) (http://discover.nci.nih.gov/pommier/top1.htm). Figure 3 shows that reversal of Top1 cleavage at many sites was slower for BN80915 than for homocamptothecin and CPT. Therefore, BN80915 is more efficient than homocamptothecin and CPT in trapping Top1 cleavage complexes.

Relative Resistance of ABCG2-Overexpressing Selected Cell Lines to Homocamptothecins and SN-38. Table 1 summarizes the results obtained by cytotoxicity assays in the five cell lines for the four drugs tested. In agreement with previous results (Brangi et al., 1999), we found that resistance to SN-38 was high in all three drug-selected cell lines overexpressing either wild-type 482R (MCF-7 MX100), mutant R482T (MCF-7 AdVp3000), or R482G (S1-M1–80) ABCG2. As previously reported (Brangi et al., 1999), all selected cell lines exhibited high levels of resistance to SN-38 (500-, 991-, and 1485-fold in MCF-7/MX100, MCF-7/AdVp3000, and S1-M1–80 cells, respectively), and resistance to CPT was minimal (Table 1). Relative resistance (RR) values for homocamptothecin and BN80915 were comparable with that for SN-38 in MCF-7 AdVp3000 cells, whereas MCF-7 MX100 and S1-M1–80 cells exhibited a marked reduction in RR values. Differences in IC50 values for resistant versus parental cell lines were significant in all cases (p < 0.05), except for one cell line with BN80915 (MCF-7 MX100, p = 0.12) and one cell line for CPT (MCF-7 MX100, p = 0.13).

Relative Resistance of ABCG2-Transfected Cells to Homocamptothecins and SN-38 and Reversal by FTC. Since variations in RR values in the selected cell lines could be due to other mechanisms of resistance or differing levels of protein expression, we repeated the cytotoxicity assays with HEK-293 cells transfected with wild-type (482R-2) or mutant (482G-2, 482T-10) ABCG2. The transfected cell lines have comparable expression of ABCG2 as evidenced by staining with the 5D3 antibody (Zhou et al., 2001), which recognizes an external epitope of ABCG2 (Fig. 4A). Empty vector-transfected cells do not express the protein. Representative results of 4-day cytotoxicity assays with the drugs tested are shown

![Figure 2](image-url)
ABCG2-transfected cells for each drug were significant with parental cell lines. Values obtained were from at least four separate experiments.

**TABLE 1**

RR to camptothecin and homocamptothecins in selected cell lines overexpressing ABCG2.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Residue</th>
<th>SN-38 (IC50)</th>
<th>CPT (IC50)</th>
<th>hCPT (IC50)</th>
<th>BN80915 (IC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>R</td>
<td>9 ± 2</td>
<td>11 ± 5</td>
<td>3.7 ± 3.7</td>
<td>2 ± 0.1</td>
</tr>
<tr>
<td>MCF-7 AdVp3000</td>
<td>T</td>
<td>891 ± 156*</td>
<td>500</td>
<td>24 ± 50*</td>
<td>170 ± 760*</td>
</tr>
<tr>
<td>MCF-7 MX100</td>
<td>R</td>
<td>4500 ± 707*</td>
<td>187 ± 209</td>
<td>663 ± 488*</td>
<td>127 ± 123</td>
</tr>
<tr>
<td>S1</td>
<td>G</td>
<td>1633 ± 706*</td>
<td>32 ± 9*</td>
<td>243 ± 50*</td>
<td>73 ± 15*</td>
</tr>
<tr>
<td>S1-M1–80</td>
<td>G</td>
<td>1633 ± 706*</td>
<td>32 ± 9*</td>
<td>243 ± 50*</td>
<td>73 ± 15*</td>
</tr>
</tbody>
</table>

* Statistical difference from parental value with p < 0.05.
shown as a positive control. These results suggest BN80915 does not readily increase expression of ABCG2 in tumors.

**Discussion**

The present study extends previous results with camptothecin analogs to include homocamptothecin and BN80915 as substrates for ABCG2. Three drug-selected cell lines overexpressing wild-type (R482) or mutant (R482G, R482T) ABCG2 as well as HEK-293 cells expressing wild-type or mutant ABCG2 were found to be resistant to homocamptothecin and BN80915. However, the difluorocamptothecin 80915 was found to be approximately 16-fold more potent than SN-38 in wild-type ABCG2-transfected cells. Resistance to all camptothecin compounds was reversed by the ABCG2 antagonist FTC.

Camptothecins were among the first drugs identified as substrates for ABCG2. Camptothecins have also been shown to select for ABCG2-mediated drug resistance (Kawabata et al., 2001; Nakatomi et al., 2001), whereas, to the best of our knowledge, there is no cell line described as selected by camptothecins for P-glycoprotein/MDR1- or multidrug resistance-associated protein 1-mediated resistance. Hence, ABCG2 appears to be the major transporter for camptothecins and thus potentially a major factor in drug resistance. Interestingly, camptothecin resistance in yeast is also mediated by the ATP-binding cassette transmembrane transport proteins PDR5, YOR1, and SNQ2 (Reid et al., 1997).

Our data again show that CPT is a poorer substrate for ABCG2 than SN-38, the active metabolite of irinotecan. This observation is consistent with a previous structure-activity study indicating that camptothecin derivatives with potential for glucuronidation are better ABCG2 substrates than CPT itself (Brangi et al., 1999). Because these derivatives bear substitutions on the A- and B-rings of camptothecins (see Fig. 1 for SN-38), it appears that ABCG2 recognizes the A- and B-ring portion of the camptothecins. The present finding that resistance to homocamptothecins in ABCG2-transfected cells was markedly higher than CPT suggests that the stabilized E-ring also contributes to recognition of CPT molecules by the ABCG2 transporter. The finding that BN80915 resistance was comparable with homocamptothecin resistance in HEK-293 cells transfected with wild-type ABCG2 suggests that the two fluoro substitutions on the A-ring do not interfere with the binding of the compound to ABCG2.

The amino acid at position 482 has been previously shown to impact substrate specificity with mutation from arginine to glycine, threonine, or methionine adding the capacity to transport anthracyclines and rhodamine 123 (Allen et al., 2002; Ozvegy et al., 2002). Furthermore, our studies with ABCG2 transfectants indicate that mitoxantrone is more effectively transported by the mutant protein as well (Robey et al., 2003). The present study shows that the amino acid at position 482 also affected the ability of ABCG2 to confer resistance to the camptothecins tested. Resistance to homocamptothecin and BN80915 was highest in cells transfected with mutant (R482G, R482T) compared with wild-type

**Table 2**

Cross-resistance profile of HEK-293 cells transfected with wild-type or mutant ABCG2 protein

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SN-38 (IC50)</th>
<th>RR</th>
<th>CPT (IC50)</th>
<th>RR</th>
<th>hCPT (IC50)</th>
<th>RR</th>
<th>BN80915 (IC50)</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3–10</td>
<td>0.7 ± 0.5</td>
<td>3.8 ± 2.2</td>
<td>1.2 ± 0.4</td>
<td>0.16 ± 0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>482T-10</td>
<td>90 ± 8</td>
<td>129</td>
<td>7.3 ± 2.0</td>
<td>30</td>
<td>9.0 ± 1.1</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>482R-2</td>
<td>94 ± 48</td>
<td>134</td>
<td>7.0 ± 1.6</td>
<td>6.7</td>
<td>1.3 ± 0.5</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>482G-2</td>
<td>123 ± 51</td>
<td>175</td>
<td>7.4 ± 2.2</td>
<td>1.9</td>
<td>57 ± 11</td>
<td>112</td>
<td></td>
<td></td>
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</table>
TABLE 3

DMF values for camptothecin and homocamptothecin derivatives in selected cell lines. IC₅₀ values were calculated by dividing the IC₅₀ value for each drug in the absence of FTC by the IC₅₀ value in the presence of 5 μM FTC. Experiments were performed three times. Reversal of resistance was statistically significant for all ABCG2-transfected cell lines (p < 0.05), except for 482R-2 cells with camptothecin (p = 0.1).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SN-38 + FTC (IC₅₀)</th>
<th>DMF</th>
<th>CPT + FTC (IC₅₀)</th>
<th>DMF</th>
<th>hCPT + FTC (IC₅₀)</th>
<th>DMF</th>
<th>BN80915 + FTC (IC₅₀)</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>PcDNA3-10</td>
<td>1.8 ± 1.8</td>
<td>0.38</td>
<td>5.3 ± 2.3</td>
<td>0.71</td>
<td>0.8 ± 0.1</td>
<td>1.3</td>
<td>0.15 ± 0.13</td>
<td>1.1</td>
</tr>
<tr>
<td>482T-10</td>
<td>0.86 ± 0.11</td>
<td>100</td>
<td>6.7 ± 1.1</td>
<td>1.1</td>
<td>1 ± 0.1</td>
<td>36</td>
<td>0.13 ± 0.06</td>
<td>69</td>
</tr>
<tr>
<td>482R-2</td>
<td>0.9 ± 0.1</td>
<td>104</td>
<td>6.3 ± 0.6</td>
<td>1.1</td>
<td>1 ± 0.1</td>
<td>8</td>
<td>0.13 ± 0.06</td>
<td>10</td>
</tr>
<tr>
<td>482G-2</td>
<td>0.7 ± 0.26</td>
<td>175</td>
<td>5.7 ± 1.1</td>
<td>1.3</td>
<td>0.97 ± 0.1</td>
<td>58</td>
<td>0.72 ± 0.11</td>
<td>25</td>
</tr>
</tbody>
</table>

**Fig. 5.** Selection with BN80915 does not up-regulate ABCG2. Resistant cell lines were incubated with phycoerythrin-labeled negative control antibody (solid line) or anti-ABCG2 antibody 5D3 (dashed line) according to the manufacturer's instructions. MCF-7 MX100 cells are shown as a representative.

(R482) ABCG2. These data would suggest that although homocamptothecin and BN80915 appear less susceptible to ABCG2-mediated resistance, their effectiveness could be reduced by tumors acquiring mutations in ABCG2 at amino acid 482. However, no such mutations have been described clinically, nor have changes at this position been observed in single-nucleotide polymorphism analyses of the ABCG2 gene (Honjo et al., 2002; Imai et al., 2002; Zamber et al., 2003).

From a clinical standpoint, it is not clearly established to what extent ABCG2 contributes to resistance to camptothecins. However, studies have implicated ABCG2 in decreasing the oral absorption of the camptothecins. Bioavailability studies in knockout mice in which the murine ortholog for the human MDR-1 gene has been deleted show increased topotecan absorption after exposure to elacridar (GF120918), an inhibitor of both MDR-1 and ABCG2 (Jonker et al., 2000). Similar findings were reported in a clinical study in which patients were treated with oral topotecan and elacridar, thereby increasing the oral bioavailability of topotecan (Kruijtzar et al., 2002). Although these results do not indicate that ABCG2 has a role in clinical drug resistance, they do show that levels of ABCG2 found in normal tissues can modulate drug retention. It is notable that BN80915, the difluoro derivative of homocamptothecin selected for clinical development, was found to be more active than SN-38 (the active metabolite of irinotecan) in ABCG2-transfected cells. These studies suggest that the circumvention of ABCG2 represented in BN80915 has the potential to bring dual activity to the clinic, efficacy in ABCG2-expressing cancers and oral bioavailability. Further studies will be needed to confirm these activities.

**Acknowledgments**

We thank Beaufour-Ipsen, and particularly Dr. Paola Principe-Nicolás, for providing homocamptothecin and BN80915. We also appreciate the technical assistance of Kenneth Steadman.

**References**


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