Circumvention of Breast Cancer Resistance Protein (BCRP)-mediated Resistance to Camptothecins in Vitro Using Non-Substrate Drugs or the BCRP Inhibitor GF120918


ABSTRACT

This study was aimed at characterizing the role of BCRP/MXR/ABCP (BCRP) in resistance of the human ovarian tumor cell lines T8 and MX3 to camptothecins more extensively and investigating whether resistance can be reversed by inhibiting BCRP by GF120918. Camptothecins studied were topotecan, CPT-11, and its active metabolite SN-38, 9-aminocamptothecin, and the novel experimental camptothecins NX211, DX8951f, and BNP1350. Notably, DX8951f and BNP1350 appeared to be very poor substrates for BCRP, with much lower resistance factors observed both in T8 and MX3 cells than observed for the other camptothecins tested. In the presence of a nontoxic dose level of GF120918, the intracellular accumulation of topotecan in the T8 and MX3 cells was completely restored to the intracellular levels observed in the sensitive IGROV1 parental cell line. This resulted in almost complete reversal of drug resistance to topotecan and to most of the topoisomerase I drugs tested in the T8 cell line and to complete reversal in the MX3 cells. However, incubation of DX8951f or BNP1350 with GF120918 did not affect the cytotoxicity of either of these drugs significantly. From the combined data, we conclude that the affinities of topoisomerase I drugs for BCRP are, in decreasing order: SN-38 > topotecan > 9-aminocamptothecin ~ CPT-11 > NX211 > DX8951f > BNP1350. Furthermore, GF120918 appears to be a potent reversal agent of BCRP-mediated resistance to camptothecins, with almost complete reversal noted at 100 nM. Potential BCRP-mediated resistance to topoisomerase I inhibitors can also be avoided by using the BCRP-insensitive drugs DX8951f or BNP1350. This observation may have important clinical implications for future development of novel camptothecins.

INTRODUCTION

Camptothecins are believed to act at least partly by stabilizing the cleavable complexes that are formed by topo I, which consequentially results in chromosomal fragmentation and inhibition of DNA replication. Members of this class of drugs (Fig. 1) show preclinical and clinical activity against, e.g., ovarian, colorectal, and small cell lung cancer (1–5). Clinically, this class of topo I inhibitor is becoming increasingly important. TPT is now registered as standard treatment against ovarian cancer, whereas irinotecan (CPT-11) is widely used in first- and second-line treatment of advanced colorectal cancer (6, 7).

Intrinsic or acquired tumor-mediated drug resistance is a major clinical obstacle that can result in the lack of tumor responsiveness in patients undergoing treatment. In preclinical models, resistance to topo I drugs has been characterized as reduced topo I content (8, 9), mutations in the topo I gene leading to reduced catalytic activity (10, 11), or decreased cleavable complex-forming ability of the topo I protein (10, 11). Furthermore, reduced accumulation because of overexpression of MDR1 (P-gp), MRP1, or MRP2 can invoke resistance to several topo I inhibitors (12–14). The clinical importance of these laboratory observations is uncertain at this time. Recently, a new multidrug transporter has been identified, named BCRP (15). High expression of BCRP initially was described in tumor cell lines selected with mitoxantrone, but BCRP appeared to be also capable of effluxing several topo I drugs and by that means conferred tumor resistance to this class of drugs (16, 17). BCRP is an efficient transporter of TPT and of other topo I drugs in vitro (3, 17, 18).

This study was aimed at characterizing the role of BCRP in resistance to camptothecin class topo I inhibitors more extensively and investigating whether the resistance can be reversed by inhibiting BCRP by GF120918. This compound, initially developed as a P-gp inhibitor (19), has been demonstrated to be an efficient inhibitor of BCRP, both in human and murine...
systems (20, 21). For this study, we tested whether GF120918 was also capable of inhibiting transport of camptothecins in BCRP-overexpressing human ovarian cancer cell lines, and by that means reducing the resistance of these cells to these drugs. Camptothecins included in this study were TPT, CPT-11, and its active metabolite SN-38, 9-AC, lurtotecan (NX211, the liposomal formulation of the drug formerly also known as GI147211, GG211, and GW211), DX-8951f, and BNP1350, a novel highly lipophilic camptothecin that has extremely high lactone stability in patients’ plasma. The hydrophilic drug DX8951f and the highly lipophilic BNP1350 are new-generation, camptothecin-derived inhibitors of topo I that display promising activity in preclinical and clinical systems (22, 23).

MATERIALS AND METHODS

Chemicals and Drugs. TPT was generously supplied by Smith Kline Beecham Pharmaceuticals (King of Prussia, PA), and CPT-11 and SN-38 by Rhône-Poulenc Rorer (Alfortville, France). 9-AC was obtained from Pharmacia and Upjohn (Woerden, the Netherlands), NX211 was generously supplied by Gilead Sciences, Inc. (Foster City, CA), DX8951f by Daiichi Pharmaceutical Company Ltd. (Tokyo, Japan), and BNP1350 by BioNumerik Pharmaceuticals, Inc. (San Antonio, TX). GF120918 was kindly supplied by Glaxo-Wellcome (Greenford, United Kingdom). RPMI 1640 was obtained from Life Technologies (Breda, the Netherlands). Fetal bovine serum was obtained from Hyclone (Logan, UT).

Cell Lines and Culture Conditions. The IGROV1 human ovarian adenocarcinoma (24) and the IGROV1-derived resistant T8 and MX3 cell lines (17) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 25 mM HEPES, 110 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were kept in continuous logarithmic growth at 37°C in a humidified atmosphere of 5% CO₂ in air. The T8 and MX3 cells are resistant to the topo I drugs TPT, SN-38, 9-AC, as well as to mitoxantrone. However, cells are not resistant to camptothecin, cisplatin, doxorubicin, 5-fluorouracil, and paclitaxel. Resistance of the T8 and MX3 cells is caused by overexpression of BCRP, as described previously (17).

Cytotoxicity Assay. Exponentially growing cells were trypsinized and plated (1500 cells/200 μl per well for the IGROV1 cells, and 2000 cells/200 μl for the T8 and MX3 cells) in 96-well microplates and allowed to attach for 48 h at 37°C under 5% CO₂. After this attachment period, 100 μl of drug solution (diluted with RPMI 1640) were added to the wells at day 2, and cells were incubated for 5 days at 37°C under 5% CO₂. In the case of a combination of topo I inhibitors with GF120918 was tested, GF120918 was added 10 min prior to adding the topo I inhibitor in a 10-μl volume to each well to obtain a final concentration of 2 μM. At day 7, cytotoxicity was evaluated using the SRB method as described (25, 26). Each agent and combination was tested in quadruplicate in at least three independent experiments.

Accumulation of Topotecan. Accumulation of TPT in the IGROV1, T8, and MX3 cells was monitored using a sensitive high-performance liquid chromatography assay as described by Rosing et al. (27). Exponentially growing cells were exposed to 1.90 μM TPT, with or without 2 μM GF120918, for
30 min at 37°C. After this incubation period, flasks were processed, and intracellular TPT levels were determined as described previously (28). Protein concentrations were determined using the Bradford method (29). Accumulation of TPT was determined in at least three independent experiments.

**Accumulation of Paclitaxel.** Accumulation of paclitaxel was monitored using high-performance liquid chromatography analysis, as described by Huizing et al. (30). Exponentially growing cells were exposed to 100 nM paclitaxel (as the clinical formulation Taxol), with or without 2 mM GF120918 for 45 min at 37°C. Cells were washed twice with ice-cold PBS, scraped, spun down, and finally lysed in methanol. Accumulation of paclitaxel was determined in three independent experiments.

**Statistical Analysis.** Statistical evaluation was performed using Student’s t test analysis. P < 0.05 was considered to be significant.

**RESULTS**

GF120918 has been reported recently to be an inhibitor of human BCRP (20). In this study, we evaluated the effect of GF120918 on cytotoxicity and accumulation of various camptothecins in resistant human ovarian T8 and MX3 cancer cell lines. Resistance in these cell lines can be largely explained by overexpression of BCRP (17), as shown for TPT in Fig. 2. Coincubation of the cells with a nontoxic dose of 2 μM GF120918 and TPT resulted in complete reversal of the accumulation defect in the T8 and MX3 cells (Fig. 2). Under identical conditions, coincubation with 40 μM verapamil or 5 μM cyclosporin A (both resulting in inhibition of P-gp), 5 mM probenecid, or glutathione depletion by 50 μM BSO (both yielding inhibition of MRP1) did not affect accumulation of TPT (data not shown). In line with this observation, coincubation with GF120918 resulted in reversal of resistance in the T8 and MX3 cell lines, whereas coincubation did not significantly affect cytotoxicity of TPT in the parental IGROV1 cell line (Fig. 3).

To check whether low-level expression of P-gp affects transport in the T8 and MX3 cells, we incubated the cells with paclitaxel, an established P-gp substrate (31, 32). No significant differences in accumulation of paclitaxel were observed in the IGROV1, T8, and MX3 cells in the absence or presence of 2 μM GF120918 (not shown). Furthermore, cytotoxicity of paclitaxel, as assessed using the SRB assay, was not affected by coincubation with the P-gp inhibitors GF120918 or verapamil (not shown).

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**Fig. 2**  Relative cellular accumulation of TPT in the parental IGROV1 and the resistant T8 and MX3 cell lines in the absence (■) or presence of 2 μM GF120918 (■). Cells were loaded with 1.9 μM TPT for 30 min at 37°C, as described in “Materials and Methods.” Data are means of three independent experiments; bars, SD.

**Fig. 3**  Cytotoxicity of topotecan with (●) or without (○) the BCRP inhibitor GF120918 in parental IGROV1 (a) and the resistant T8 (b) and MX3 (c). Cytotoxicity was assessed using the SRB assay. Representative curves are shown [one determination (means of quadruplicate; bars, SD) of three independent determinations].

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**Figure Captions:**
- **Fig. 2:** Relative cellular accumulation of TPT in the parental IGROV1 and the resistant T8 and MX3 cell lines in the absence (■) or presence of 2 μM GF120918 (■). Cells were loaded with 1.9 μM TPT for 30 min at 37°C, as described in “Materials and Methods.” Data are means of three independent experiments; bars, SD.
- **Fig. 3:** Cytotoxicity of topotecan with (●) or without (○) the BCRP inhibitor GF120918 in parental IGROV1 (a) and the resistant T8 (b) and MX3 (c). Cytotoxicity was assessed using the SRB assay. Representative curves are shown [one determination (means of quadruplicate; bars, SD) of three independent determinations].
The T8 and MX3 cells have been shown to be resistant to the camptothecins TPT, SN-38, and 9-AC (17). In this study, we extended the study to include additional camptothecins and tested CPT-11, NX211, DX8951f, and BNP1350 for their susceptibility to BCRP-mediated drug resistance. Both T8 and MX3 cells were cross-resistant to all of these drugs, although the resistance of the MX3 against DX8951 (Rf, 4.0) and BNP1350 (Rf, 2.1) was only of borderline significance (P < 0.04) and low in comparison with the other drugs tested (Table 1). The effect of GF120918 on the cytotoxicity of these topo I inhibitors was first tested in the parental IGROV1 tumor cell line. Cytotoxicity of the topo I inhibitors in the IGROV1 was not significantly affected by coincubation with a nontoxic dose of 2 μM GF120918, yielding an IC50 ratio with/without GF120918 ranging from 0.62 for BNP1350 to 1.58 for SN-38 (Table 1). In contrast, addition of GF120918 to the resistant T8 cell line yielded significantly reduced IC50s and consequently reduced Rfs for TPT, CPT-11, SN38, MX211, and DX8951. However, it is important to note that no significant reduction was observed for BNP1350 (Table 1). In the resistant MX3 cell line, cytotoxicity of both DX8951f and BNP1350 was not significantly affected by GF120918 (Fig. 4; Table 1). Interestingly, in the T8 cells, a residual resistance to topo I drugs was observed in the presence of GF120918 (with, on average, a Rf of 4.4), whereas resensitization of the MX3 cell line was almost complete (average Rf, 1.6).

In the experiments described above, 2 μM GF120918 was used. We also tested the lowest concentration of GF120918 that was effective in reverting resistance in the T8 and MX3 cell line by incubating the cells with a nontoxic dose of TPT in the presence of serial dilutions of GF120918. For this purpose, a concentration of TPT was used that yielded approximately the IC40 or IC70 doses in the IGROV1 cells, i.e., 7 and 20 nM, respectively. As shown in Fig. 5 partial reversal of resistance was noted at a dose of 30 nM GF120918, whereas almost complete reversal was obtained at a dose of 100 nM. At higher doses of GF120918, no further significant increase in cytotoxicity was observed up to 2 μM, with cytotoxicity of GF120918 itself becoming apparent at doses of 5 μM (not shown).

### DISCUSSION

Resistance to anticancer drug therapy is a major clinical problem often resulting in treatment failure. Therefore, investigations aimed at circumvention of intrinsic tumor-mediated drug resistance are warranted. Recently, a new ABC transporter gene has been characterized, named BCRP (ABCG2, MXR/ABCP; Refs. 15–17, 33, and 34). This transporter gene, originally observed almost exclusively in mitoxantrone-exposed tumor cells, appeared to be up-regulated also after exposure to TPT or SN-38 (17, 35, 36). At this moment, two inhibitors of BCRP are known: the fungal FTC (37, 38) and the acridone carboxamide derivative GF120918 (20, 21). In contrast to FTC, for GF120918 a synthetic route is known, thereby making

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<td>IGROV1 + GF120918 IC50 nm</td>
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<td>BNP1350</td>
<td>0.93</td>
<td>2.1</td>
<td>1.38</td>
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- No significant difference ± GF120918 (P > 0.05).
- IC50 (T8 or MX3):IC50 IGROV1.
- Significant difference ± GF120918 (P < 0.05).
large-scale production of this agent more feasible. Moreover, although FTC may be a useful pharmacological tool in vitro, it may be neurotoxic, potentially hampering its future clinical use. Therefore, in this study we used GF120918. GF120918 was originally developed as a P-gp inhibitor (19). However, recently it was shown that GF120918 was also capable of inhibiting mouse and human BCRP (20, 21).

The accumulation defect of TPT in the BCRP-overexpressing T8 and MX3 cells diminished completely upon coincubation of the cells with GF120918. Consequently, the T8 and MX3 cells were resensitized to TPT. Rfs for TPT in the T8 and MX3 dropped markedly when cells were coincubated with GF120918. Using this panel of cell lines and GF120918, we tested whether other topo I drugs were substrates for BCRP as well. Of the tested topo I drugs, CPT-11, SN-38, 9-AC, and NX211 are clearly substrates for BCRP, as became clear from the resistance data and the reversal of resistance by GF120918. DX8951f, a novel hydrophilic topo I inhibitor, appeared to be a much lesser substrate for BCRP. Finally, BNP1350, a novel highly lipophilic topo I inhibitor, was the poorest substrate for BCRP, yielding no significant drug resistance in the T8 and MX3 cells. Estimated from these results, the substrate affinity of BCRP appears to decrease from SN-38 > TPT > 9-AC ~ CPT-I1 > NX211 > DX8951f > BNP1350. These observations relating to relative sensitivity to BCRP-mediated drug resistance have clinically important implications for the further development of camptothecins and the optimization of drug therapy for patients. The affinity of camptothecins for BCRP does not seem to be directly related to lipophilicity of the drugs, because the two poorest substrates, DX8951f and BNP1350, are hydrophilic and lipophilic, respectively. BNP1350 is known to form a complex by which BNP1350 is delivered to the cell. It is not clear whether this complex is responsible for the poor transport characteristics of BNP1350, because the parent lipophilic drug camptothecin, which does not form such complexes, is also not transported by BCRP (17). These broad substrate characteristics may indicate that, as suggested for P-gp, there may be multiple drug binding sites or that other chemical factors are more important. For P-gp, it is also known that in many cases amino groups, which can be protonated at physiological pH, are present in substrates (39). Because not all camptothecin BCRP substrates have such an amino group, this does not appear to be the case for BCRP.

GF120918 is a much more potent inhibitor of P-gp than inhibitors, such as verapamil and cyclosporin A, and was capable of restoring sensitivity at concentrations as low as 100 nM (19, 40, 41). In this study, we demonstrate that GF120918 is also potent in reverting BCRP-mediated resistance to a number of topo I inhibitors. Both in the T8 and MX3 cells, cells were resensitized to TPT in the presence of 100 nM GF120918. These results indicate that GF120918 has a broader working range than initially anticipated. Although topo I inhibitors such as TPT are substrates for P-gp, the affinity for this transporter is very low (12). Furthermore, by using the excellent P-gp substrate paclitaxel, we demonstrated that P-gp activity in the T8 and MX3 cells most likely is too low to contribute significantly to the resistance to TPT in these cell lines.

Because of the fact that TPT is a good substrate for BCRP,
GF120918 may prevent or revert resistance to this drug. GF120918 is a low-toxicity drug that can be administered to animals as well as patients at high doses (19). Therefore, further clinical testing of GF120918 as a reversal agent is warranted. Furthermore, it is well known that the oral bioavailability of the BCRP substrate TPT is relatively low and variable (30 ± 7.7%) in patients (42). Considering the presence of BCRP in the small intestine and colon of humans (15, 43), this bioavailability can possibly be improved by combining oral TPT with oral GF120918. This concept has been proven in mice and patients for paclitaxel, which has low oral bioavailability because of its high affinity for P-gp (32), when administered p.o. combined with the effective P-gp blocker cyclosporin A (44, 45). Clinical trials testing this approach for the combination of oral TPT with GF120918 are currently ongoing in our institute. Consistent with the foregoing, it has been observed that BNP1350 has similar antitumor activity when administered by the oral or the i.v. route; the estimated oral bioavailability of BNP1350 is approximately 66–90%. This contrasts substantially with other camptothecins. Thus, our findings are consistent that BNP1350 does not appear to be a substrate to BCRP, unlike most of the other camptothecins we have tested.

ACKNOWLEDGMENTS

We thank M. C. Ruevekamp-Helmers for technical assistance and Dr. H. Rosing for analysis of paclitaxel.

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*Clin Cancer Res* 2001;7:935-941.

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