Fumitremorgin C Reverses Multidrug Resistance in Cells Transfected with the Breast Cancer Resistance Protein

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Abstract

Fumitremorgin C (FTC) is a potent and specific chemosensitizing agent in cell lines selected for resistance to mitoxantrone that do not overexpress P-glycoprotein or multidrug resistance protein. The gene encoding a novel transporter, the breast cancer resistance protein (BCRP), was recently found to be overexpressed in a mitoxantrone-selected human colon cell line, S1-M1–3.2, which was used to identify FTC. Because the drug-selected cell line may contain multiple alterations contributing to the multidrug resistance phenotype, we examined the effect of FTC on MCF-7 cells transfected with the BCRP gene. We report that FTC almost completely reverses resistance mediated by BCRP in vitro and is a pharmacological probe for the expression and molecular action of this transporter.

Introduction

Multidrug resistance (MDR) is a common phenomenon in cancer patients, and it limits the effectiveness of chemotherapy (1). In experimental systems, selection for resistance to a single cancer chemotherapeutic agent often results in cross-resistance to a variety of structurally and functionally diverse molecules (2). Although the mechanistic basis for this phenomenon is complex, the overexpression of ABC transporters is often associated with this phenotype. Among the 33 genes predicted to encode human ABC transporters (3), 5 mediate MDR when transfected into drug-sensitive cells: P-gp/MDR1 (4, 5), the MRP (6–8) and its homologues mMDR/MRP2 (9) and MRP3 (10), and a newly reported transporter, the BCRP (13). These proteins mediate resistance to such commonly used chemotherapeutic drugs as doxorubicin, mitoxantrone, etoposide, paclitaxel, and vincristine. However, the exact cross-resistance profile for each transporter is distinct, although overlapping. These membrane-embedded proteins are thought to act as drug efflux pumps, preventing the cytotoxic agent from reaching lethal levels within the cells.

Reversal of MDR is a major goal in the clinical management of cancer. Pharmacological inhibition of the ABC transporters can be expected to resensitize cells to the action of antitumor agents. Inhibitors of P-gp (2, 14) and MRP (15) have been reported, and P-gp reversal agents are being tested in the clinic (14, 16). These agents have also served as valuable tools for understanding the interaction of small molecules with ABC transporters. Recently, FTC, a novel chemosensitizing agent, was identified and shown to reverse drug resistance in a mitoxantrone-selected human colon carcinoma cell line (S1-M1–3.2) that does not overexpress P-gp or MRP (17). S1-M1–3.2 cells, like other mitoxantrone-selected lines, have recently been shown to overexpress BCRP mRNA (11, 18). In this cell line, FTC reversed resistance to mitoxantrone, doxorubicin, and topotecan, but not to paclitaxel (17). FTC was also highly effective on other drug selected cell lines overexpressing BCRP: MCF-7 breast cancer cells selected in mitoxantrone, MCF-7 cells selected in doxorubicin plus verapamil (17), and 8226 multiple myeloma cells selected in mitoxantrone (19). Reversal was associated with an increase in the amount of drug accumulated by the resistant cells (17, 19). These data suggest that FTC resensitizes drug-resistant cells by inhibiting BCRP-mediated drug transport. However, other mechanisms of resistance also exist in the drug selected cells, including alterations in topoisomerase activity (17, 19, 20), and FTC may overcome resistance through these mechanisms. We therefore evaluated the activity of FTC in cells transfected with BCRP to determine specifically whether FTC can reverse resistance mediated by this transporter.

Materials and Methods

Cell Culture. MCF-7 cells transfected with the pcDNA3 vector (MCF-7/pcDNA3) or the BCRP expression construct (MCF-7/BCRP) clone 8 were obtained as previously reported (13) and were cultured in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS (Life Technologies), 50 μg/ml gentamicin (Life Technologies) and 100 μg/ml G418 (Geneticin, Life Technologies) at 37°C, under 7% CO2. Cells were passaged once per week. MCF-7/BCRP clone 8 cells are resistant to mitoxantrone, daunorubicin, and doxorubicin, compared with vector-transfected cells, but not to etoposide, paclitaxel, or vincristine (13).

Cytotoxicity Assays. Procedures were carried out as described previously (17). Briefly, cells were plated in 96-well microtiter dishes (10,000 cells per well) in medium containing 5% FBS. After 4–6 h, dilutions of the antitumor agent (Sigma Chemical Co., St. Louis, MO) were added, along with FTC. Cell survival after 3 days of growth was determined using the sulforhodamine B assay. EC50 (drug dose causing 50% inhibition of cell growth, compared with untreated cells) were determined from the cytotoxicity curves.

Measurement of Intracellular Drug Accumulation. The intracellular accumulation and retention of BBR 3390 (kindly provided by M. Hacker, Grand Valley State University, Allendale, MI) was determined using flow cytometry. Cells cultured in 24-well tissue culture flasks were exposed to BBR 3390 (5 μM) for up to 120 min at 37°C (accommodation phase) or exposed to the drug for 120 min, washed in ice-cold saline, and then exposed to prewarmed culture medium in the absence of the drug at 37°C (retention phase). At various time intervals, cells were collected by trypsinization, and intracellular drug content was determined by florescence analysis (488-nm excitation, 525-nm emission) in a FACScan instrument (Becton Dickinson, Mountain View, CA). Where FTC was used, accumulation and retention times were 60 and 30 min, respectively, and cells were pretreated with FTC for 15 min at 37°C prior to the addition of BBR 3390. For drug retention analyses with daunorubicin, cells were collected by trypsinization, counted, and incubated in 12 × 75-mm tubes with 1 μM of the drug at 37°C (1 × 106 cells/tube). After 2 h, the cells were washed in ice-cold PBS and incubated in medium without the drug for 1 h at 37°C. Both incubations were carried out in the absence or presence of FTC (1

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3 The abbreviations used are: MDR, multidrug resistance; ABC, ATP binding cassette; BCRP, breast cancer resistance protein; DMF, dose-modifying factor; FTC, fumitremorgin C; MRP, MDR protein; P-gp, P-glycoprotein; FBS, fetal bovine serum.

4 BCRP is also known as the mitoxantrone resistance gene product (MXR; Ref. 11) or placenta-specific ABC transporter (ABCP; Ref. 12).
or 5 μM). Cells were washed three times in PBS containing 5% FBS and analyzed by flow cytometry (FACSort, Becton Dickinson). In all fluorescence assays, parallel samples were stored on ice to control for nonspecific binding of the drug to the plasma membranes. The fluorescence produced by these samples was subtracted from the experimental values. For radioactive drug retention assays, cells were plated in 24-well dishes (2–2.5 × 10^5 cells/well). The following day, the growth medium was replaced with medium containing 1 μM [14C] doxorubicin (66 mCi/mmol, Amersham). Cells were incubated for 2 h at 37°C, washed, and incubated in medium without the drug for 1 h. Where applicable, cells were treated with FTC (1 or 5 μM) during both incubations. The wells were washed twice with PBS, solubilized in 2 N NaOH, and counted in a scintillation counter. Protein content was determined using a modified Lowry assay (Bio-Rad, Richmond, CA).

Results and Discussion

MCF-7 cells overexpressing BCRP are resistant to mitoxantrone, daunorubicin, and doxorubicin, but not to cis-platinum, paclitaxel, or vincristine compared with vector-transfected cells (13). Consistent with the drug resistance profile of the mitoxantrone selected S1-M1–3.2 cells, we observe here that MCF-7/BCRP cells are also cross-resistant to topotecan (Fig. 1). The reversal activity of FTC in MCF-7/BCRP cells was determined by using a fixed dose of the compound in combination with increasing doses of antitumor drugs (Fig. 1 and Table 1). FTC (5 μM) potentiated the toxicity of mitoxantrone (29.4-fold), doxorubicin (6.6-fold), and topotecan (6.5-fold). No activity was detected with paclitaxel (1.1-fold). These findings closely parallel the activity of FTC in three drug-selected cell lines that overexpress BCRP (17). As indicated by the maximum DMF values (Table 1), almost a complete reversal of resistance was obtained for mitoxantrone and doxorubicin. FTC also enhanced the toxicity of mitoxantrone and topotecan in vector-transfected MCF-7 cells (2.5–5.6 fold). As a consequence of this effect, FTC reduced the IC_{50} of topotecan in BCRP-overexpressing cells below that observed in the untreated vector-transfected cells (Fig. 1), and the observed DMF exceeded the expected maximum DMF value. This has been previously observed in MCF-7 cells (17) and in two non-small cell lung carcinoma cell lines (H460 and A549) that were not selected for resistance to chemotherapeutic drugs.5 The activity of FTC on vector-transfected MCF-7 cells is most probably due to the known low level of expression of BCRP in the parental MCF-7 cells (18), and it suggests that intrinsic resistance to mitoxantrone occurs in some cell lines in the absence of drug selection. However, we cannot rule out the possibility that a second, FTC-sensitive, mechanism of drug resistance may exist in MCF-7 cells.

Previously, transfection of BCRP cDNA in MCF-7 cells was shown to result in a reduced accumulation and retention of daunorubicin and an ATP-dependent enhancement of rhodamine 123 efflux (13). To investigate the mechanism of reversal activity by FTC, we measured the intracellular content of BBR 3390, a fluorescent aza-anthrapyra-

Table 1 Activity of FTC in BCRP-transfected cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mitoxantrone</th>
<th>Doxorubicin</th>
<th>Topotecan</th>
<th>Paclitaxel</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7/pcDNA3</td>
<td>2.5</td>
<td>1.0</td>
<td>5.6</td>
<td>0.7</td>
</tr>
<tr>
<td>MCF-7/BCRP</td>
<td>29.4 (35.1)</td>
<td>6.6 (7.6)</td>
<td>6.5 (3.3)</td>
<td>1.1 (2.7)</td>
</tr>
</tbody>
</table>

Fig. 1. Reversal activity of FTC. MCF-7/BCRP cells (■, □) and vector-transfected cells (●, △) were cultured in the absence (●, △) or presence (■, □) of 5 μM FTC, along with increasing doses of four antitumor agents: mitoxantrone (A), doxorubicin (B), topotecan (C), and paclitaxel (D). Cell survival was determined after 3 days using the sulforhodamine B assay. SDs (not shown) were <10%.

5 S. E. Bates, personal communication.
zole, previously shown to be transported out of mitoxantrone-resistant cells (19). For these experiments, cells were incubated with the agent (accumulation phase) or exposed to the drug, washed, and incubated in medium without the drug. Samples were collected at various times and analyzed by flow cytometry. Arrow, the time point at which BBR 3390 was washed from the cells. B, effect of FTC on BBR 3390 accumulation and retention. MCF-7 cells transfected with the vector (left panel) or the BCRP cDNA (right panel) were incubated with 5 μM BBR 3390 in the absence (open bars) or presence (solid bars) of 10 μM FTC. Parallel samples were washed and incubated in medium without BBR 3390 (without or with FTC) for an additional 30 min. The cells were collected and analyzed by flow cytometry. The mean fluorescence of duplicate samples is shown along with the range.

Fig. 2. Measurement of intracellular BBR 3390 content. A, time course of BBR 3390 accumulation and retention. MCF-7 cells transfected with the vector (●) or with the BCRP expression construct (■) were incubated with BBR 3390 or incubated with the drug, washed, and incubated in medium without the drug. Samples were collected at various times and analyzed by flow cytometry. Arrow, the time point at which BBR 3390 was washed from the cells. B, effect of FTC on BBR 3390 accumulation and retention. MCF-7 cells transfected with the vector (left panel) or the BCRP cDNA (right panel) were incubated with 5 μM BBR 3390 in the absence (open bars) or presence (solid bars) of 10 μM FTC. Parallel samples were washed and incubated in medium without BBR 3390 (without or with FTC) for an additional 30 min. The cells were collected and analyzed by flow cytometry. The mean fluorescence of duplicate samples is shown along with the range.

Fig. 3. Effect of FTC on antitumor drug retention. MCF-7/BCRP and vector-transfected cells were incubated in medium containing daunorubicin (A) or [14C] doxorubicin (B), washed, and incubated in the absence of drug. The cells were then washed and analyzed by flow cytometry (daunorubicin) or scintillation counting (doxorubicin). All incubations were done in the absence (open bars) or presence (cross-hatched bars, 1 μM; solid bars, 5 μM) of FTC. For daunorubicin, the data shown represents the mean channel number of duplicate points. For doxorubicin, the results were normalized to the protein content of the samples, and the graph represents the mean of triplicate points. SDs are shown.

Fig. 3. Effect of FTC on antitumor drug retention. MCF-7/BCRP and vector-transfected cells were incubated in medium containing daunorubicin (A) or [14C] doxorubicin (B), washed, and incubated in the absence of drug. The cells were then washed and analyzed by flow cytometry (daunorubicin) or scintillation counting (doxorubicin). All incubations were done in the absence (open bars) or presence (cross-hatched bars, 1 μM; solid bars, 5 μM) of FTC. For daunorubicin, the data shown represents the mean channel number of duplicate points. For doxorubicin, the results were normalized to the protein content of the samples, and the graph represents the mean of triplicate points. SDs are shown.

The reversal agent had a more dramatic effect in the retention phase. Here, BBR 3390 fluorescence was undetectable in untreated MCF-7/BCRP cells, and FTC increased this to 51% of the levels seen in the corresponding vector-control samples. No effect of FTC was observed in vector-transfected MCF-7 cells during the drug accumulation or the retention phases. We also examined the pharmacokinetics of daunorubicin and doxorubicin, two drugs to which the BCRP-overexpressing MCF-7 cells are resistant (13). The intracellular content of the antitumor agents 1 h after removal of the agents from the medium was determined. Daunorubicin retention was measured by fluorescence analysis; [14C] doxorubicin retention was measured by scintillation counting. The use of a radiolabeled drug allowed us to examine the possibility of self-quenching, a problem inherent in fluorescence-based assays at high drug concentrations. MCF-7/BCRP cells retained reduced amounts of both drugs, compared with the vector-transfected cells (45–52%; Fig. 3). FTC treatment increased the amount of daunorubicin (1.8–2.3 fold) and [14C]-doxorubicin...
(1.8-fold) retained by the cells, compared with untreated cells. The increase in drug retention by FTC brought the level close to that seen in the vector controls. No effect of FTC was observed in the cells transfected with the vector alone. We were unable to observe decreased retention of [14C] mitoxantrone in MCF-7/BCRP cells compared with the vector control and, therefore, could not study the effect of FTC on mitoxantrone transport. This may be due to the high concentration of mitoxantrone required for the analysis (1 μM), the relatively low levels of drug resistance of the BCRP-overexpressing cells, and the tendency of mitoxantrone to bind nonspecifically to cellular proteins. Another possible explanation is that complete reconstitution of the mitoxantrone resistance phenotype will require the coexpression of other ABC transporters. Unlike P-gp and MRP, which contain two sets of transmembrane domains and two ATP binding sequences, BCRP structurally resembles a half-transporter because it contains only a single putative transmembrane region and ATP-binding motif. BCRP may, therefore, participate in drug resistance via a multiprotein transporter complex (11–13).

Although these data clearly suggest that FTC inhibits the function of BCRP, we cannot conclude that FTC directly interacts with this protein. However, this is the most likely possibility because FTC is a planar, multi-ring structure like mitoxantrone and doxorubicin and therefore may compete with these molecules for the binding sites on the transporter. This mechanism would be similar to P-gp, where it has been shown that inhibitors and substrates that are transported by P-gp directly interact with the transporter protein (2). Competition studies between FTC and chemotherapeutic drugs in BCRP-containing membrane vesicles or whole cells may resolve this issue. Determination of the structure of FTC bound to BCRP will provide insights into the molecular interactions between other chemotherapeutic drugs and BCRP. For these purposes, the relative simplicity of BCRP compared to the other larger ABC transporters makes it an ideal model into the molecular basis of drug transport.

Acknowledgments

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References


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