Reversal of a Novel Multidrug Resistance Mechanism in Human Colon Carcinoma Cells by Fumitremorgin C

Sridhar K. Rabindran, Haiyin He, Maya Singh, Eileen Brown, Karen I. Collins, Tami Annable, and Lee M. Greenberger

ABSTRACT

We selected a human colon carcinoma cell line in increasing concentrations of mitoxantrone to obtain a resistant subline, S1-M1-3.2, with the following characteristics: profound resistance to mitoxantrone; significant cross-resistance to doxorubicin, bisanterine, and topotecan; and very low levels of resistance to Taxol, vinblastine, colchicine, and camptothecin. This multidrug resistance (MDR) phenotype, which was not reversed by verapamil or another potent P-glycoprotein (Pgp) inhibitor, CI. 329,753, was dependent, in part, upon an energy-dependent drug efflux mechanism. Pgp and the multidrug resistance protein (MRP) were not elevated in the resistant cells relative to the drug-sensitive parent, suggesting that resistance was mediated by a novel pathway of drug transport. A cell-based screen with S1-M1-3.2 cells was used to identify agents capable of circumventing this non-Pgp, non-MRP MDR. One of the active agents identified was a mycotoxin, fumitremorgin C. This molecule was extremely effective in reversing resistance to mitoxantrone, doxorubicin, and topotecan in multidrug-selected cell lines showing this novel phenotype. Reversal of resistance was associated with an increase in drug accumulation. The compound did not reverse drug resistance in cells with elevated expression of Pgp or MRP. We suggest that fumitremorgin C is a highly selective chemosensitizing agent for the resistance pathway we have identified and can be used as a specific pharmacological probe to distinguish between the diverse resistance mechanisms that occur in the MDR cell.

INTRODUCTION

MDR is a major problem in cancer chemotherapy (1). The best-characterized mechanism of MDR is mediated by Pgp, a member of the ATP-binding cassette transporter family of proteins (2). Inhibition of this drug efflux pump by pharmacological agents has been shown to reverse resistance and re-rninize resistant cells to antitumor agents in vitro (3), in animal tumor models (4), and in the clinic (5). Although Pgp is expressed in up to 50% of human tumors (6) and is a negative prognostic indicator for chemotherapy outcome in some cancers (7), several lines of evidence in the laboratory (3) and in cancer patients (8) suggest that Pgp alone does not account for all forms of MDR. This phenomenon of MDR that is not mediated by Pgp (non-Pgp MDR) has been studied extensively in vitro. For example, selection of cells in mitoxantrone, doxorubicin, or the podophyllotoxins often results in drug-resistant sublines in which Pgp levels remain low or are absent (3). Here, resistance is associated with decreased drug accumulation, altered drug trafficking within the cell, alterations in the activity/levels of topoisomerase II, or changes in glutathione metabolism (3). In addition, multiple changes may exist together within the drug-resistant cell, and a switch in the resistance mechanism (from non-Pgp to Pgp-mediated MDR) may occur as a cell line is cultured in increasing concentration of the selective agent (e.g., Refs. 9 and 10).

Mitoxantrone is an important anticancer drug with therapeutic efficacy that is similar to that of standard induction and salvage regimens in non-Hodgkin's lymphoma, a variety of leukemias (11, 12), and advanced breast cancer (Ref. 11, but see Ref. 13) and to that of palliative therapy in advanced prostate cancer (14). Like doxorubicin, it is a multiring planar molecule that intercalates with DNA (15). However, it can be distinguished from doxorubicin based on its mechanism of action, its reduced cardiotoxicity compared to anthracyclines (11), and its mechanism of resistance. In particular, resistance to doxorubicin and mitoxantrone involves transporters with overlapping but distinct specificities. For example, cells expressing Pgp are often cross-resistant to mitoxantrone and doxorubicin, and both molecules are efficiently transported by this protein (16). However, a second ATP-binding cassette transporter, the MRP, is induced by and can mediate resistance to doxorubicin but not mitoxantrone (17-19).

Finally, unlike doxorubicin, selection of tumor cells for resistance to mitoxantrone frequently results in sublines with decreased drug accumulation but no overexpression of Pgp or MRP (20-22).

Our goal is to identify and characterize novel agents that can reverse non-Pgp, non-MRP MDR. This will provide us with the pharmacological probes required to assess the incidence and frequency of this resistance mechanism in cancer patients and to study the mechanistic basis of resistance. In addition, newly identified compounds themselves may have clinical utility as reversal agents. We report here the isolation and characterization of a mitoxantrone-selected human colon carcinoma cell line showing a non-Pgp, non-MRP MDR phenotype. Furthermore, we demonstrate the usefulness of this cell line in a screen designed to identify agents that can reverse the cells to antitumor drugs. The properties and mechanism of action of a potent and highly specific reversal agent, FTC, are evaluated.

MATERIALS AND METHODS

Cell Culture. The S1-M1-3.2 cell line was obtained by selection of S1 cells (a clone of LS174T colon carcinoma cells) in increasing concentrations of mitoxantrone, beginning at 6.25 nM and doubling approximately every 3 weeks, up to 3200 nM. S1-B1-20 cells, selected in a similar way with bisanterine, have been described previously (16). MCF-7 human breast cells and its mitoxantrone-selected subline, MCF-7/mtxR, were provided by Dr. Kenneth Cowan (National Cancer Institute, Bethesda, MD; Ref. 20). A second parental MCF-7 line and its drug-selected counterpart MCF-7/AdrVp were provided by Dr. Susan Bates (National Cancer Institute, Bethesda, MD; Ref. 23). Promyelocytic leukemia cells HL-60 and the doxorubicin-resistance HL-60/AR cells were provided by Dr. Alexander Hindenburg (Winthrop Hospital, Mineola, NY; Ref. 24). All cell lines were grown in RPMIX 1640 (Life Technologies, Inc.) at 37°C under 7% CO2, and were passaged once per week. For S1, S1-M1-3.2, and S1-B1-20 cells, the medium was supplemented with 15% FBS (Life Technologies, Inc.), 2 mM additional glutamine (Life Technologies, Inc.), 0.2% insulin-transferrin-selenium mixture (Collaborative Research), and 50 µg/ml gentamycin (Life Technologies, Inc.). The MCF-7 cells
were maintained in medium containing 5% FBS and 50 μg/mL gentamicin, and HL60 cells were cultured in medium containing 10% FBS, 1% nonessential amino acids, 2 mM additional glutamine, and 20 μg/mL gentamicin. Drug-resistant sublines were routinely cultured with appropriate chemotherapeutic drug: 3.2 μM mitoxantrone (Lederle) for S1-M1-3.2, 20 μM bisantrene (Lederle) for S1-B1-20, 250 nM mitoxantrone for MCF-7/mtrX, 1 μM doxorubicin (Sigma Chemical Co.) for HL-60/AR, and 1 μM doxorubicin plus 10 μg/mL verapamil (Sigma) for MCF-7/AdrVp. All antitumor drug and reversal agent stocks were prepared in DMSO.

Fluorescence Analysis. Cells were collected by trypsinization and resuspended in PBS containing 5% FBS (10^6 cells/0.1 mL) in the absence or presence of 1 μg/mL anti-Pgp antibody (4E3) on ice (25). Following extensive washing, the cells were incubated with a fluorescein-labeled secondary antibody [antirabbit (Fab), fragment (EY Laboratories), 1:20 dilution]. Quantitative analysis of Pgp was determined by fluorescence analysis on a FACS 440 (Becton Dickinson, San Jose, CA), as described previously (16).

Protein Immunoblotting. For Pgp and MRP analysis, membrane-enriched fractions were prepared from sensitive and drug-resistant cells according to described procedures (26). Proteins within these fractions were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose under standard conditions. Immunodetection was carried out using specific antibodies for Pgp (Ab-3; Oncogene Sciences), topoisomerase II (16), and MRP (MRP1, a gift of R. Schepers, Free University, Amsterdam, the Netherlands; Ref. 27). Signals were visualized using 125I-labeled protein A (New England Nuclear) or enhanced chemiluminescence (ECL; Amersham).

Northern Blots. Total RNA was prepared using RNAzol reagent (Biotecx) according to the manufacturer’s instructions. Ten to 40 μg of total RNA were fractionated on 1% agarose-formaldehyde gels and transferred onto a solid support [Nytren (Schleicher and Schuell) or Genescreen Plus (New England Nuclear)]. Blots were hybridized with a MRP probe prepared by PCR amplification of cDNA from S1 cells (16), purified by agarose gel electrophoresis, and labeled with [32P]dCTP by random primed labeling (Boehringer Mannheim). The identity of the PCR product was confirmed by sequencing. Blots were hybridized with 15–20 ng of probe in 7–10 mL of 0.25 M Na2HPO4 (pH 7.2), 1% BSA, 7% SDS, and 100 μg/mL denatured, sheared salmon sperm DNA at 65°C, for 16–20 h; washed at high stringency [0.1X SSC and 0.1% SDS at 65°C (1× SSC: 0.15 M NaCl and 0.015 M trisodium citrate)]; and exposed to film. Alternatively, hybridization was carried out in 6× SSC, 5× Denhardt’s solution (1× Denhardt’s solution: 0.1% each of Ficoll, polyvinylpyrrolidone, and BSA), 5% dextran sulfate, 1% SDS, and 100 μg/mL salmon sperm DNA. An oligonucleotide probe for glyceraldehyde-3-phosphate dehydrogenase (Oncogene Sciences) was used to normalize RNA loading. Hybridization and washing were carried out as recommended by the manufacturer. Autoradiograms were scanned and quantified using an Image Scanner (Molecular Dynamics).

Drug Accumulation Assays. Cells were plated in 60-mm dishes (1 × 10^6 cells per dish) or in 24-well clusters (2 × 10^5 cells per well) and allowed to grow for 2–4 h. Cells were washed twice in MEM containing radioactive drug in the absence or presence of test agent, for 2–4 h at 37°C. Drugs used were [14C]mitoxantrone (Amersham; 1 or 5 μM; specific activity, 66 mCi/mmol) or [3H]doxorubicin (Amersham, 0.5 or 3 μM; specific activity, 50–62 mCi/mmol). For experiments with 3 μM doxorubicin, 1.8 μM unlabeled drug was combined with 1.2 μM 14C-labeled drug. Unbound drug was removed by washing twice in ice-cold PBS, and cells were solubilized in 2 N NaOH at 37°C for 1–2 h and analyzed by scintillation counting. Results were normalized to cell protein content measured by a Lowry assay. For drug efflux studies, cells were incubated with the radioactive drug for 4 h, washed twice in prewarmed medium, and incubated in medium lacking the drug for various lengths of time. No attempt was made to equalize loading of mitoxantrone between the sensitive and drug-resistant cells because these treatments might interfere with subsequent drug transport during the efflux period. Cells were processed as described above.

Cytotoxicity Assays for Cell Survival. To screen for reversal agents, cells were plated into 96-well dishes (100 μL/well) in growth medium supplemented with 5% FBS. The number of cells seeded per well varied with the cell line and was chosen to keep the final absorbance values after 3 days of growth within the linear range of the instrument (15,000 cells for S1-M1-3.2 and S1-B1-20). After 4–6 h, 100 μL of the appropriate dose of chemotherapeutic agent (at 2X) were added with the candidate reversal agent. The final concentration of antitumor drug was the same as that used for routine culture of the cells. Reversal agents were tested from 0.1 to 80 μM. In parallel wells, cells were grown in the presence of the reversal agent alone. Following a 3-day growth period, cells were fixed in 10% trichloroacetic acid for 1 h and washed extensively with water, and cell-associated protein was stained using 0.1% SRB (Sigma), a protein binding reagent (28). Excess reagent was removed by washing plates in 5% acetic acid, the dye was solubilized in 10 mM Tris base (Sigma), and absorbance was determined in a UV Max spectrophotometer (Molecular Devices) at 540 nm. Cell survival was determined relative to control wells (no drugs).

For determination of EC_{50} of chemotherapeutic drugs, cells (15,000 per well for S1 cells and 20,000 per well for all others) were plated in increasing concentration of antitumor agent in the absence or presence of a fixed dose of reversal agent. Cell survival was estimated as above, except that 20% trichloroacetic acid was used to fix the HL-60 cells. Graphs of cell survival against dose of antitumor agent were plotted, and EC_{50} were calculated from the graphs.

Topoisomerase II Activity Assay. Decatenation of kinetoplast DNA derived from Crithidia fasciculata, a measure of the strand passing activity of topoisomerase II, was carried out as described previously (16). Nuclear extracts from drug-sensitive and -resistant cells were incubated with radiolabeled kinetoplast DNA at 30°C for 30 min. Following the addition of 0.5% SDS, tubes were centrifuged at 8000 x g for 15 min. Radioactivity in the supernatant was measured by scintillation counting. Background counts (produced in the absence of added nuclear extract) were subtracted from experimental values.

Culture of Aspergillus fumigatus and Purification of FTC. The culture of A. fumigatus was stored frozen at −140°C in potato dextrose broth (FM-3) with 25% glycerol. Cultures were revived by streaking a Bennett’s agar plate and incubating at 22°C for 7–10 days. Seed 1 was prepared by inoculating two to three loopfuls of the culture from the plate into a tube containing 10 ml of FM-3 and incubating at 22°C and 150 rpm for 4 days. Seed 2 was prepared by transferring seed 1 into a 250-ml Erlenmeyer flask containing 50 ml of FM-3 and incubating for an additional 4 days at 22°C and 200 rpm. Seed 2 was homogenized by gentle grinding, and a 2.5-ml volume was inoculated into the fermentation medium FM-5 (15 g of white rice and 5 ml of 0.1% yeast extract in a 250-ml Erlenmeyer flask). After 7–10 days of stationary fermentation at 22°C, the content of the flask was extracted with 30 ml of methanol. The methanolic extract was purified by column chromatography on an LH-20 column with methanol as solvent, followed by high-performance liquid chromatography on a C-18 column (4 cm) with methanol/water as solvent. Related molecules FTA and FTB were separated from FTC at this step. Finally, FTC was crystallized from methanol or ethyl acetate. The purity was estimated to be 90–95%, based on high-performance liquid chromatography analysis. Structures were determined by spectroscopic methods.

RESULTS

Selection of Drug-resistant Cells. A subclone (S1) of a colon carcinoma cell line (LSI74T) was grown in increasing concentration of mitoxantrone. The initial concentration used for selection was 6.25 nm. Surviving cells were collected and exposed to a drug concentration 2-fold higher than the previous step. This procedure was repeated until the cell line S1-M1-3.2, the growth of which was not inhibited by 3.2 μM mitoxantrone, was obtained. The isoenzyme profiles of the resistant and sensitive cells were identical.4 Chromosomal analysis revealed a translocation between chromosomes 4 and 17 ([t(4qter>4q21::17p12>17qter)] in resistant cells but not in the parental line. The translocation was an early event, occurring prior to the selection step in 400 nm mitoxantrone. A deletion in chromosome 10 [del(10q11q22)] was seen in both lines.5 No significant changes in morphology or growth properties were observed between sensitive and drug-resistant cells.4

Resistance Profile of Drug-selected Cells. The sensitivities of the drug-selected S1-M1-3.2 cells to a variety of anticancer agents, 4 S. K. Rabindran and L. M. Greenberger, unpublished observations.
5 M. L. Slovak, personal communication.
compared to the parental cells, are shown in Table 1. The cells show very low levels of resistance to the microtubule-active agents vinblastine (3-fold), Taxol (6-fold), and colchicine (3-fold). Low levels of resistance were also observed for the topoisomerase II poisons 4’-(9-acridinylamino)methanesulfon-m-anisidide (11-fold), etoposide (6-fold), and teniposide (VM-26; 18-fold). The cells were profoundly resistant to mitoxantrone (1435-fold) and significant cross-resistance to doxorubicin (54-fold) and bisantrene (24-fold), but they were not resistant to a structurally related molecule, mitoxantrone-190,000 was observed in S1 cells; S1-M1-3.2 and S1-B1-20 cells had slightly reduced levels of this protein. For the RNA and protein blotting studies, a promyelocytic cell line (HL-60) and the doxorubicin-selected counterpart (HL-60/AR), previously shown to express high levels of MRP message (16), served as controls.

**Determination of Intracellular Drug Accumulation.** To determine the mechanism of resistance, drug-resistant and -sensitive cells were incubated with radioactive analogues of antitumor agents (mitoxantrone and doxorubicin), and cell-associated radioactivity was measured by scintillation counting. S1-M1-3.2 cells accumulated a 1.5-fold increase in Pgp levels in the S1-M1-3.2 cells, relative to S1. S1-B1-20 is a subline of LS174T cells selected in bisantrene. This cell line, alone with KB-V1, expresses high levels of Pgp (16) and serves as a positive control for the immunoblotting procedure. The amount of Pgp on the cell surface was also quantified by fluorescence analysis using an antibody that recognizes an external epitope of Pgp. Consistent with the protein immunoblot data, a low level of specific staining was observed in the S1 cells (data not shown), and S1-M1-3.2 cells show a 1.6-fold increase in Pgp levels relative to the drug-sensitive parent (Table 2).

**Table 2 Fluorescence analysis of Pgp expression**

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<tr>
<th>Cell line</th>
<th>Mean channel no.</th>
<th>Relative Pgp levels</th>
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<tr>
<td>S1</td>
<td>23 ± 3.9</td>
<td>1</td>
</tr>
<tr>
<td>S1-M1-3.2</td>
<td>33 ± 9.4</td>
<td>1.6</td>
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*The surface expression of Pgp was quantified by fluorescence analysis using a Pgp-specific antibody. Relative Pgp levels were calculated as described in "Materials and Methods." S1-B1-20 cells showed a 22-fold increase in Pgp levels in a similar analysis (16).*

**Pgp and MRP Expression in Sensitive and Drug-resistant Cell Lines.** We determined whether the drug-resistant phenotype in the S1-M1-3.2 cells was due to the expression of the two known mediators of MDR, Pgp and MRP. Fig. 1A shows a protein immunoblot of membranes derived from the parental S1 and the drug-resistant cell line S1-M1-3.2, using Pgp-specific antibody. Low levels of Pgp were observed in both the drug-sensitive and the drug-resistant cell lines.
REVERSAL OF DRUG RESISTANCE BY FTC

**Fig. 2.** Drug accumulation analysis. A, effect of FTC on drug accumulation. Drug-sensitive (S1) and drug-resistant cells (S1-M1-3.2 and S1-B1-20) were incubated in medium containing [14C]mitoxantrone (left) or [14C]doxorubicin (right), for 90 min at 37°C in the absence (○) or presence (□) of 1 µM FTC. The cells were then washed, solubilized, and counted in a scintillation counter. Results were normalized to the protein content of samples, determined by a Lowry assay. Columns, means (triplicate samples); bars, SD. B, drug efflux analysis. Parental cells (S1, ■) and drug-resistant cells (□, S1-M1-3.2; ○, S1-B1-20) were loaded with [14C]mitoxantrone for 4 h at 37°C, washed, and incubated in medium without drug. Cell-associated radioactivity was measured at the indicated times. Data points, percentages of the counts obtained at the end of the drug loading period (0 time) for each cell line (triplicate samples); bars, SD. C, effect of energy depleters on drug efflux. During the efflux period, cells were incubated in growth medium (□), PBS (○), PBS supplemented with sodium azide (■), or sodium azide and 2,4-dinitrophenol (△). After 1 h, cells were prepared for analysis by scintillation counting. Columns, amounts of drug remaining at the end of the efflux period for each treatment group, relative to values obtained with cells incubated in growth medium alone (normalized to 100%; means of triplicate determinations); bars, SD.

only 41% of mitoxantrone and 17% of doxorubicin of the parental cells (Fig. 2A; □). A similar decrease was seen in the Pgp-expressing S1-B1-20 cells. The reduction in the amount of mitoxantrone and doxorubicin bound by S1-M1-3.2 cells compared with S1 cells is similar, despite a 27-fold difference in the relative resistance for these two drugs (compare Fig. 2A with Table 1). This is most likely due to the ability of mitoxantrone to associate with various cellular proteins, increasing the background counts. To determine whether reduced accumulation was due to increased clearance of the drug, the cells were loaded with the radioactive mitoxantrone, washed, and incubated in medium lacking the drug (Fig. 2B). The amount of radioactivity remaining in the parental cells decreased marginally from 100 to 86%
over a 4-h period, whereas the amount in the S1-M1-3.2 cells decreased to 39% in the same period. Pgp-expressing S1-B1-20 cells also showed a similar decrease (to 31%). To examine whether this efflux was energy dependent, cells were incubated with sodium azide or a combination of sodium azide and 2,4-dinitrophenol during the efflux period (Fig. 2C). In contrast with the modest increase (1.1–1.4-fold) seen in the S1 cells by these treatments, the S1-M1-3.2 cells showed a 2.1–2.5-fold increase, similar to that seen in the S1-B1-20 cells (2.3–2.6-fold). No significant effect on cell viability (determined by trypan blue exclusion) was observed as a result of these treatments.

**Determination of Topoisomerase II Activity.** Because the drug-resistant cells showed low but detectable resistance to the topoisomerase II poisons 4’-[(9-acridinylamino)methanesulfon-m-anisidide, etoposide, and teniposide (Table 1), we determined the activity of topoisomerase II in nuclear extracts using a decatenation assay. A 12-fold decrease in activity was seen in the S1-M1–3.2 cells compared with the parental cells (Table 3). This decrease in enzyme activity correlated with a decrease in the amount of topoisomerase IIβ message and protein levels. No significant alterations were observed for topoisomerase IIα mRNA or protein.4

**Identification of Reversal Agents.** A variety of pharmacological agents have been shown to resensitize MDR cells to antitumor drugs in vitro. One of the first reported molecules with reversing activity in Pgp-expressing cells was verapamil, a calcium channel blocker (3). This compound was also active in MRP-expressing cells (24). We have recently described a new reversal agent for Pgp-mediated MDR, CL 329,753 (α-(3,4-dimethoxyphenyl)-3,4-dihydro-6,7-dimethoxy-α-[(4-methylphenyl)thio]-2(1H)-isouquinolineheptanenitrile), that is at least 10-fold more potent than verapamil (29). We determined whether verapamil and CL 329,753 had reversal activity in the S1-M1–3.2 cell lines. Cells were incubated with the compound alone or combined with 3.2 μM mitoxantrone, a dose that has no inhibitory effect on the growth of these cells. The cytotoxicity curves for verapamil and CL 329,753 (or its HCl salt) in the absence and presence of mitoxantrone were almost overlapping, suggesting no reversal activity of these compounds in the S1-M1–3.2 cell lines (Fig. 3).

When CL 329,753, a Pgp-specific reversal agent, was used in combination with bisantrene, an excellent substrate for Pgp (16), a small amount of reversal was detected in both S1 and S1-M1 cells (2.5–3.1-fold), consistent with the low expression of Pgp in these cells (Fig. 1A and Table 2).

To identify molecules that can resensitize the mitoxantrone-selected cells to anticancer drugs, we screened a library of extracts derived from a variety of microorganisms (microbial library). Extracts (1:50–1:5000 dilution) that had little or no toxicity by themselves but that, when combined with mitoxantrone, showed substantially increased cell death were analyzed further. Using this screen, an extract from a solid medium fermentation broth of A. fumigatus was found to be highly active. The pharmacologically active ingredient in this broth was purified by chromatographic methods. Fractions at each stage of the purification were screened for activity using a drug accumulation assay. The active ingredient, purified to homogeneity, was shown to be FTC (Fig. 4A). Fig. 4B shows the dose-response curve of S1-M1–3.2 cells to FTC alone and in combination with mitoxantrone. No toxicity of the compound alone was observed in the dose range tested (0.1–80 μM). However, in combination with mitoxantrone, 50% of the cells were killed with 0.35 μM drug. Two analogues of FTC occurring naturally in the same extract, FTA and FTB (Fig. 4A), were more toxic than FTC (20% cell death at 15 μM compared with >80 μM). Both molecules also showed reduced activity in the reversal assay (20- and 14-fold lower, respectively; compare Fig. 4, B and C). To determine the specificity of FTC for non-Pgp MDR, the compound was tested on S1-B1–20 cells, which express high levels of Pgp (Fig. 4D). No resensitization to bisantrene was detected. However, resistance to bisantrene (Fig. 4D) and mitoxantrone (data not shown) was reversible in S1-B1–20 cells by the Pgp reversal agent, CL 329,753.

**Reversal Activity of FTC.** The reversal activity of FTC in S1-M1–3.2 cells was determined by using a fixed dose of the compound in combination with increasing doses of chemotherapeutic drugs (Fig. 5). FTC (5 μM) significantly potentiated the toxicity of mitoxantrone (93-fold; Fig. 5A and Table 4), doxorubicin (26-fold; Fig. 5B and Table 4), and topotecan (24-fold; Fig. 5C), as shown by the shift of the cytotoxicity curves to the left. FTC also reversed bisantrene resistance (25-fold).4 Reversal activity of FTC was not detected with Taxol in the resistant cells (1.1-fold; Fig. 5D and Table 4) or with any antitumor drug in the parental cells (0.7–1.4-fold; Table 4).

To determine the utility of FTC as a reversal agent in other multidrug-resistant cell lines, we tested its activity in two breast cancer cell lines that show a multidrug-resistant phenotype with no overexpression of Pgp or MRP. In MCF-7/mtxR (a mitoxantrone-selected cell line), FTC reversed mitoxantrone resistance (114-fold) and doxorubicin resistance (3-fold; Table 4). No reversal of Taxol resistance was found in these cells (1.1-fold) or in the parental selected cells (0.8–1.7-fold). A second non-Pgp, non-MRP cell line, MCF-7/AdrVP, was obtained by selection in doxorubicin, in the presence of the Pgp reversal agent verapamil (23). This was done to favor the induction of non Pgp forms of resistance. These cells were resistant to doxorubicin (2,600-fold) but were profoundly more resistant to mitoxantrone (333,000-fold). Here, FTC (5 μM) reversed re-

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**Table 3** Topoisomerase II activity in nuclear extracts

<table>
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<tr>
<th>Cell line</th>
<th>50% activity</th>
<th>Relative activity</th>
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<tr>
<td>S1</td>
<td>0.5 ± 0.2</td>
<td>12</td>
</tr>
<tr>
<td>S1-M1-3.2</td>
<td>6.0 ± 1.6</td>
<td>1</td>
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*Topoisomerase II activity was determined by ability of nuclear extracts to decatenate C. fasciculata kinetoplast DNA. The amount of nuclear extract (in μg) for 50% decatenation of kinetoplast DNA is shown. Values shown are the mean ± SE of four independent determinations.
REVERSAL OF DRUG RESISTANCE BY FTC

A

FUMITREMORGIN C

FUMITREMORGIN A

FUMITREMORGIN B

B

Fig. 4. Specificity of FTC activity. A, structures of FTC (M, 379), FTA (M, 579), and FTB (M, 479). B, effect of FTC on S1-M1–3.2 cells. Cells were incubated for 3 days with the indicated doses of FTC alone (○) or in combination with 3.2 μM mitoxantrone (●). Cell survival was measured using the SRB assay. Bars (SD, triplicate determinations) are shown where larger than the data point. C, effects of FTA and FTB on S1-M1–3.2 cells. Cells were incubated for 3 days with the indicated doses of FTA (□ and ○) or FTB (● and ○), either alone (□ and ○) or in combination with 3.2 μM mitoxantrone (● and ○). D, reversal of resistance in Pgp-expressing cells. S1-B1-20 cells were grown in increasing doses of FTC (● and ○) or CL 329,753 (● and ○), either alone (□ and ○) or in combination with 21 μM bisantrene (● and ○). This concentration of bisantrene is not inhibitory to the growth of the cells.

Effect of FTC on Drug Accumulation. To determine the mechanism of reversal activity by FTC, we carried out a drug accumulation analysis on drug-sensitive and -resistant cells using [14C]mitoxantrone and [14C]doxorubicin. In Fig. 2A, 1 μM FTC increased the amount of mitoxantrone (2-fold) and doxorubicin (3.4-fold) retained by the S1-

D

120

% SURVIVAL

100

80

60

40

20

100

.1

1

10

100

FUMITREMORGIN C (μM)

Dose (μM)

% SURVIVAL

120

100

80

60

40

20

100

.1

1

10

100

Dose (μM)

sistance to both doxorubicin (2591-fold) as well as mitoxantrone (5000-fold), with little effect on parental cells (Table 4). No reversal was seen to Taxol.

The activity of FTC was also examined on HL-60/AR cells, which were selected for doxorubicin resistance and which express high levels of MRP message and proteins (see above). FTC had no significant reversal activity in this cell line (2.6–3.3-fold) compared to the parental line, HL-60 (1.0–2.1-fold), although significant resistance to mitoxantrone and doxorubicin was observed in the resistant line (Table 4).
M1-3.2 cells. The increase in mitoxantrone accumulation by FTC brought the level close to that seen in the parental cells. No effect of the compound was seen in the parental cells (S1) or in the Pgp-expressing S1-B1-20 cells.

**DISCUSSION**

S1-M1-3.2, a multidrug-resistant subline of a human colon carcinoma cell line, was obtained by stepwise selection of cells in increasing concentrations of mitoxantrone. Selection of tumor cells for resistance to mitoxantrone frequently results in a characteristic phenotype that has been found in human tumors derived from diverse tissue types, including colon [WiDr (30) and LS174T, this study], breast [MCF-7 (20, 21)], myeloid [8226 (22)], and stomach [EGP85 (31)]. The primary phenotypic feature is profound resistance to mitoxantrone, moderate resistance to related anthracycline molecules (doxorubicin and bisantrene), and little or no cross-resistance to the microtubule-active drugs, vinblastine, Taxol, and colchicine. Overexpression of either of the two putative drug efflux pumps Pgp or MRP is generally not observed. Consistent with this, inhibitors of Pgp (verapamil and cyclosporine A) are unable to overcome mitoxantrone resistance. In most cases, resistance appears to be due to energy-dependent drug efflux. However, in at least one cell line (EGP85-27), altered sequestration of mitoxantrone in cytoplasmic vesicles was found (31). We now propose a new phenotypic feature: reversal of mitoxantrone and doxorubicin resistance with FTC.

The simplest explanation for the phenotype described above is the existence of a novel drug transport pathway in the resistant cells that has distinct substrate specificities from either Pgp or MRP. However, alterations in topoisomerase II or glutathione metabolism may contribute to the phenotype (3). Consistent with this, alterations in topoisomerase II activity were found in S1-M1-3.2 cells and are frequently observed in other cell lines selected in mitoxantrone or doxorubicin...
REVERSAL OF DRUG RESISTANCE BY FTC

Table 4 Activity of FTC in multiple cell lines*

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<tr>
<th>Cell line</th>
<th>Mitoxantrone</th>
<th>Doxorubicin</th>
<th>Taxol</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>1</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>S1-M1-3.2</td>
<td>93 (1435)</td>
<td>25.9 (54)</td>
<td>1.1</td>
</tr>
<tr>
<td>MCF-7</td>
<td>1.7</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>MCF-7/mXR</td>
<td>114 (2090)</td>
<td>3.0 (11.4)</td>
<td>1.1</td>
</tr>
<tr>
<td>MCF-7</td>
<td>7.5</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>MCF-7/AdrVp</td>
<td>5,000 (333,333)</td>
<td>100 (2,591)</td>
<td>0.7 (10.7)</td>
</tr>
<tr>
<td>HL-60</td>
<td>1.3</td>
<td>2.1</td>
<td>1.0</td>
</tr>
<tr>
<td>HL-60/AR</td>
<td>3.3 (53)</td>
<td>2.8 (92.3)</td>
<td>2.6 (5.1)</td>
</tr>
</tbody>
</table>

* Cells were grown for 3 days with 5 μM FTC and increasing doses of the indicated antitumor drugs (triplicate points). Cell survival was determined by the SRB assay, and EC_{50} were determined from toxicity curves. The dose-modifying factor is the ratio of EC_{50} obtained in the absence and presence of reversal agent. Maximum dose-modifying factors (shown in parentheses) are the ratios of EC_{50} of resistant and sensitive cells in the absence of the reversal agent. This value is obtained when resistance is reversed completely and is the equivalent of the relative resistance. The experiments were performed once or twice; representative data are shown. For MCF-7 and HL-60 cells, maximum dose-modifying factors were calculated from the experimental data. For S1-M1-3.2 cells, the values shown were obtained from Table 1 (relative resistance).

(3). Furthermore, HL60 cells selected for resistance to mitoxantrone have reduced topoisomerase II activity, protein levels, and mislocalization of topoisomerase IIα mediated by truncation of the gene product (although no reduced accumulation of mitoxantrone was found; Refs. 32–34). Detoxification of xenobiotics can occur via conjugation to glutathione, and MRP has been implicated in the export of these conjugates from the drug-resistant cell (35, 36). Supporting this observation, elevated levels of glutathione S-transferase and peroxidase are seen in some multidrug-resistant cells selected with doxorubicin (37, 38). We did not detect any reversal activity of buthionine sulfoximine, an agent that depletes endogenous levels of glutathione, nor did we detect any changes in mitoxantrone metabolism or covalent modification. A variety of protein changes have been associated with non-Pgp MDR, but their actual contribution to resistance remain unclear. Among these are a M₉, 110,000 protein first identified in SW-1573 lung cancer cells (39); a Mₓ, 95,000 protein in MCF-7/AdrVp cells (23), and Mₓ, 42,000 and 85,000 species in MCF-7/mXR cells (20). We did not detect overexpression of these or other proteins in S1-M1 cells by immunodetection, two-dimensional gel electrophoresis, or photoaffinity labeling with [³²P]azido ATP. However, FTC reversed resistance in S1-M1-3.2 and the two drug-resistant MCF-7 cell lines suggesting that a common, yet undefined, mechanism underlies the drug resistance phenotype in all three cell lines.

Reversal of MDR is a major goal of cancer chemotherapy. Potent reversal agents for Pgp have been identified, and clinical trials using these agents are ongoing (5, 8). Verapamil (24, 40–42), genistein (43), a 1,4-dihydropyridine analogue (NIK 250; Refs. 44 and 45), and a PKC inhibitor (GF 109203X; Ref. 46) have been reported to reverse Pgp-mediated resistance. We attempted to identify reversal agents that were highly specific for non-Pgp-, non-MRP-mediated MDR in the S1-M1-3.2 cells. Twelve of 11,900 microbial library extracts that were tested were found to be active as reversal agents. One of these (S266), an extract of A. fumigatus grown under solid fermentation, had consistent reversal activity in S1-M1-3.2 cells, with minimal toxicity over a wide dose range. The active agent was purified and shown to be FTC. This compound, and the related molecules, FTA and FTB, belong to a class of diketopiperazines that are potent myotoxins. FTC caused tremors in cockerels at 25 mg/kg (oral; Ref. 47). FTA and FTB cause tremors in mammals, probably due to their effect on the brain stem or spinal cord (48, 49). In vivo, FTC shows little toxicity by itself but can potently resensitize cells to mitoxantrone and doxorubicin. Both FTA and FTB, which differ from FTC at two positions (Fig. 4A), were considerably more toxic than FTC with lower reversal activity. FTC is a highly selective reagent, be-


Reversal of a Novel Multidrug Resistance Mechanism in Human Colon Carcinoma Cells by Fumitremorgin C

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