

Potent and Specific Inhibition of the Breast Cancer Resistance Protein Multidrug Transporter *in Vitro* and in Mouse Intestine by a Novel Analogue of Fumitremorgin C¹

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Abstract

Inhibitors of the breast cancer resistance protein (BCRP/ABCG2) multidrug transporter are of interest as chemosensitizers for clinical drug resistance, for improving the pharmacokinetics of substrate chemotherapeutic drugs, and in functional assays of BCRP activity for tailoring chemotherapy. The fungal toxin fumitremorgin C (FTC) is a potent and specific inhibitor of BCRP, but its neurotoxic effects preclude use *in vivo*. We have therefore evaluated a new tetracyclic analogue of FTC, Ko143, as a practical inhibitor of BCRP, comparing it with two other analogues in the same class and with GF120918. All three FTC analogues are effective inhibitors of both mouse Bcrp1 and human BCRP, proving highly active for increasing the intracellular drug accumulation and reversing Bcrp1/BCRP-mediated multidrug resistance. Indeed, Ko143 appears to be the most potent BCRP inhibitor known thus far. In contrast, the compounds have only low activity against P-glycoprotein, the multidrug resistance-associated protein (MRP1), or other known drug transporters. They are nontoxic *in vitro* at useful concentrations and evinced no signs of toxicity in mice at high oral or i.p. doses. Administered p.o. to inhibit intestinal Bcrp1, Ko143 markedly increased the oral availability of topotecan in mice. It is thus the first highly potent and specific BCRP inhibitor applicable *in vivo*. As such, Ko143 and other FTC analogues of this type represent valuable reagents for

analysis of drug resistance mechanisms and may be candidates for development as clinical BCRP inhibitors.

Introduction

The principal reason for failure of anticancer chemotherapy is drug resistance, wherein tumors are either innately refractory to the drug(s) used or else are initially drug sensitive but, after remission, recur in drug-resistant form. Frequently, such tumors are refractory to more than one class of cytostatic drugs. Prominent among possible mechanisms of this MDR³ are the broad specificity drug efflux pumps of the ABC family (1), including P-gp [P-gp/ABCB1 (2)], the MRP [MRP1/ABCC1 (3)], and the BCRP [BCRP/MXR/ABCP/ABCG2 (4–6)]. The potential for such proteins to mediate clinical MDR has generated considerable interest in agents able to inhibit their activity and so reverse innate or acquired drug resistance. Indeed, a variety of proprietary inhibitors of P-gp are presently under trial for improving specific chemotherapy regimes (7). The recently identified BCRP is able to confer resistance to mitoxantrone, topotecan, anthracyclines, and related drugs when expressed in cell line models (4, 6, 8, 9) and is thus an important addition to potential sources of clinical multidrug resistance.

P-gp is present at locations in the body that give it a major influence over the pharmacokinetics of substrate drugs. In the intestinal epithelium, it reduces the uptake of p.o.-administered drugs; in liver canaliculi, it contributes to drug elimination by biliary excretion, and in the placental and blood-brain barriers, it markedly reduces drug penetration to the fetus and central nervous system, respectively (10, 11). Similarly, BCRP is found in the intestinal epithelium, liver canaliculi, the placental trophoblasts, mammary ducts and lobules, and endothelial cells of veins and capillaries (12), a distribution that leads to the expectation that it, too, will have a significant role in the pharmacokinetics of substrate drugs. Indeed, inhibition of Bcrp1 in mice markedly increases the oral availability of topotecan, slows its elimination from the body, and increases its penetration of the placental barrier to the fetus (13). Inhibitors of BCRP therefore have the potential to improve such aspects of chemotherapy, and at least one clinical trial along these lines is presently underway (J. H. M. S.).

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³ The abbreviations used are: MDR, multidrug resistance; MRP, multidrug resistance-associated protein; ABC, ATP-binding cassette; P-gp, P-glycoprotein; BCRP/Bcrp1, breast cancer resistance protein; FTC, fumitremorgin C; IC₅₀, concentration inhibiting cell proliferation 50%; EC₅₀/EC₉₀, effective concentration reversing 50% (or 90%) of drug resistance; HPLC, high-performance liquid chromatography; RF, resistance factor.

Two effective inhibitors of BCRP have been described thus far. The compound GF120918 was developed as a P-gp inhibitor (14), but serendipitously, it also inhibits BCRP (15). Clinically important substrate drugs of BCRP are often also P-gp substrates; therefore, such a dual specificity inhibitor could be advantageous in some applications. However, there may also be cases where dual specificity will prove a handicap, because P-gp plays an important role in protecting certain tissue compartments, as in the blood-brain barrier, from the toxic side effects of cytostatic drugs. Inhibitors specific for BCRP may thus be preferred in some clinical applications.

The mycotoxin FTC, isolated from *Aspergillus fumigatus*, is a specific and potent inhibitor of BCRP (16, 17). Unfortunately, it induces tremors or convulsions in mice and other animals through toxicity to the central nervous system, similar to many other members of the fumitremorgin/verruculogen/tryprostatin class of alkaloids (18–20). Such neurotoxicity precludes its use *in vivo*. Less toxic and synthetically tractable analogues of FTC are thus of interest as specific BCRP inhibitors. Indeed, pentacyclic FTC analogues have recently been reported to inhibit BCRP (21); two of the compounds in question were of similar potency to native FTC but were more cytotoxic, and their activity against other transporters was not evaluated. Previously, we independently screened a combinatorial panel of 42, mostly tetracyclic, indolyl diketopiperazine FTC analogues (22), finding that many showed considerable inhibition of Bcrp1/BCRP-mediated drug efflux (23). Two of the most promising leads, Ko132 and Ko134, were selected for further evaluation herein, and one of these compounds was further improved by structural modification, yielding the new compound Ko143. These three compounds have now been tested *in extenso* as practical inhibitors of Bcrp1/BCRP. They have proved to be potent, specific, and of low toxicity *in vitro* and are effective for inhibiting Bcrp1 activity in the gastrointestinal tract of mice.

Materials and Methods

FTC Analogues. The solution phase synthesis of the single (S,S,S)-diastereoisomers of FTC analogues Ko132 and Ko134 (Fig. 1) from L-tryptophan methyl ester has been described elsewhere (23). The new analogue Ko143 (Fig. 1) was prepared via the same route with modifications, starting with 6-methoxy-L-tryptophan methyl ester prepared from commercially available *N* α -methoxycarbonyl-L-tryptophan methyl ester following literature procedures (24, 25). Further information concerning the syntheses is available from the authors [A. v. L. and G.-J. K. (1)].⁴ Spectral data of the final product Ko143, single (S,S,S)-diastereoisomer: ¹H-NMR (400 MHz, CDCl₃, δ) (ppm): 7.85 (br s, 1H), 7.44 (d, *J* = 8.6 Hz, 1H), 6.89 (d, *J* = 2.1 Hz, 1H), 6.83 (dd, *J* = 8.6 Hz, *J* = 2.2 Hz, 1H), 6.75 (br s, 1H), 5.45 (dd, *J* = 9.2 Hz, *J* = 4.0 Hz,

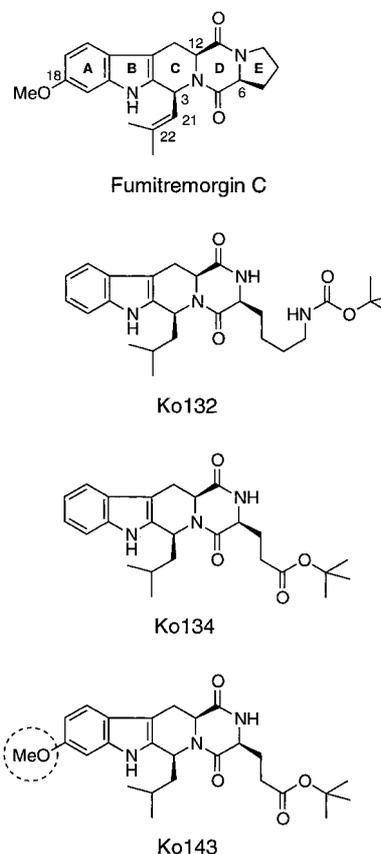


Fig. 1. FTC and analogues. All analogues tested were pure diastereoisomers with the same configuration at the chiral centers C-3, C-6, and C-12 (S,S,S) as FTC. The analogues differ from the native compound at either two or three positions. All lack the E ring derived from L-proline, having Boc-protected L-lysine (Ko132) or *t*-Bu-protected L-glutamic acid (Ko134 and Ko143) side chains as a C-6 substituent. All have an isobutyl substituent at C-3, which is the C-21, C-22 saturated equivalent of the natural side chain. Ko132 and Ko134 lack the methoxy group at C-18 in the indole moiety.

1H), 3.99–4.05 (m, 2H), 3.85 (s, 3H), 3.53 (dd, *J* = 15.8 Hz, *J* = 4.9 Hz, 1H), 3.03 (dd, *J* = 15.7 Hz, *J* = 11.7 Hz, 1H), 2.50 (t-like, 2H), 2.33–2.41 (m, 1H), 2.18–2.25 (m, 1H), 1.72–1.78 (m, 1H), 1.50–1.62 (m, 2H), 1.47 (s, 9H), 1.06 (d, 6.5 Hz, 3H), 0.84 (d, 6.4 Hz, 3H). ¹³C-NMR (APT, 100 MHz, CDCl₃, δ) (ppm): 173.07, 169.82, 168.33, 156.29, 136.46, 132.95, 120.55, 118.61, 109.45, 106.48, 95.12, 81.22, 55.78, 55.64, 54.00, 51.05, 45.74, 31.18, 27.99, 25.02, 24.70, 23.75, 21.73, 21.65. HR-MS (FAB): observed mass 470.2652; calculated mass for C₂₆H₃₅N₃O₅ (M + H) 470.2655. Elemental analysis (C₂₆H₃₅N₃O₅). Found: C, 66.34; H, 7.42; N, 8.78%. Calculated: C, 66.50; H, 7.51; N, 8.95%. Optical rotation: [α]_D = –99.8° (c = 0.6, methanol).

Concentrated stocks of the FTC analogues (20 or 50 mg/ml) were prepared in DMSO and stored at –20°C. Solutions of 1 mg/ml in DMSO or in 20% (v/v) tetrahydrofuran, 80% (v/v) 0.2 M HCl buffer (pH 1.0), were stable at room temperature for at least 16 h, as determined by HPLC (detected at 225 and 254 nm). The compounds are soluble in complete medium up to at least 32 μ M, well above the highest working concentrations used herein.

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Cell Lines. The mouse fibroblast cell lines (except NIH3T3) were derived by us previously and characterized for their drug resistance properties (8, 26). The mouse MEF3.8/Bcrp1 A2 subline was made by transduction of MEF3.8 cells with a *Bcrp1* expression construct (13) containing a full coding sequence (8) and green fluorescent protein marker. Clones were obtained by flow cytometry, sorting single cells positive for green fluorescent protein and, after expansion, screening for low mitoxantrone accumulation. The 77.1/MDR1 clone 5 was obtained by transduction of 77.1 cells lacking functional *Mdr1a* and *Mdr1b* P-gp genes (26) with pHaMDRA1 virus (27), containing a full-length, wild-type human MDR1 cDNA, and selecting clones resistant to 0.8 μM vincristine. The human cell lines expressing transfected drug transporters are described elsewhere: 2008/MRP1 clone 4 and 2008/MRP3 clone 8 (28, 29); MDCKII/MRP2 clone 17 (30); HEK293/MRP4 clone 4.1;⁵ and HEK293/MRP5 clone 5 I (31). The IGROV1 human ovarian carcinoma line and its topotecan-resistant T8 subline have also been described previously (32). NIH3T3, MCF7, CCRF-CEM, WiDr, and A549 cells were obtained from the American Type Culture Collection. Cell lines were grown in complete medium, *i.e.*, DMEM or RPMI 1640, supplemented with 10% FCS, penicillin G, and streptomycin.

Drug Accumulation, Cytotoxicity, and Inhibitor Potency Assays.

Sources of drugs and the drug accumulation assays have been described previously (8, 26). Cytotoxicity assays were also performed as described therein, with modifications. Briefly, cells were plated at 400 or 1000/well in 96-well plates the night before addition of drugs. A concentration series of drug was applied along one plate axis and left for the duration of the assay. Plates were harvested after 4–5 days while untreated wells were still subconfluent. Relative cell proliferation was quantified with CyQuant or Sybr Green I fluorescent nucleic acid stains (Molecular Probes, Eugene, OR). Assays with human cell lines were performed in the presence of 0.1 μM PSC833 to inhibit confounding P-gp activity.

As an index of the potency of BCRP inhibitors, the EC_{90} was defined as the effective concentration of inhibitor that reduces drug resistance (*i.e.*, the IC_{50}) by 90%. EC_{90} s were thus determined by modified cytotoxicity assays in which a concentration series of inhibitor was applied in the presence of the cytotoxic drug at 10% of its IC_{50} concentration, where the IC_{50} had been determined immediately beforehand. The concentration of inhibitor that results in a 50% reduction in cell growth under these conditions has thus reduced the IC_{50} by 90%. In the absence of cytotoxic drug, the maximum applied concentration of inhibitor did not itself affect the growth of the cells. The EC_{50} was defined and determined in an analogous manner.

Mice. Mice were housed and handled, and experiments were conducted according to institutional ethical guidelines complying with Dutch legislation. The mice used were FVB strain wild-type or *Mdr1a/1b* knockouts (33) between 9 and 14 weeks of age. Only males were used in topotecan oral

availability experiments. Where possible, comparisons were made between littermates.

Oral and i.p. Administration of FTC Analogues. Oral toxicity of FTC analogues in mice was tested by mixing 50 mg/ml stocks in DMSO 1:1 with Tween 80 (polyoxyethylene sorbitan mono-oleate) and diluting with 5% w/v glucose such that the final volume administered by oral gavage was 10 $\mu\text{l/g}$ of body weight. Pairs of mice were administered oral doses of 50 mg/kg Ko132, Ko134, Ko143, or vehicle under light methoxyflurane anesthesia. Final tests of 50 mg/kg Ko134 or Ko143 were performed on additional pairs of unanesthetized animals to observe any behavioral effects. Further, another pair of mice received the higher dose of 100 mg/kg Ko134. For i.p. toxicity tests, the FTC analogue stocks in DMSO were dispersed in at least 10 volumes of sterile corn oil such that the injected volume was 5 $\mu\text{l/g}$ of body weight. After pilot tests at lower doses showed no adverse effects, mice (4 per group) were administered vehicle or 10 mg/kg i.p. of Ko132, Ko134, or Ko143. The mice were observed continuously during the first hour after administration and then at increasing intervals for 2 weeks, after which they were sacrificed for histological examination of major organs and structures including brain, salivary glands, heart, lungs, liver, adrenal glands, kidneys, urinary tract, spleen, thymus, bone marrow, pancreas, stomach, intestines, cecum, colon, testes, epididymus, skin, head, trunk, and limbs.

Modulation of Topotecan Oral Availability with FTC Analogues. Ko143 stock in DMSO was diluted to 2 mg/ml with aqueous hydroxypropylmethyl cellulose (10 mg/ml) + 5% v/v Tween 80, producing a micellar suspension. This was diluted 1:1 with 5% glucose so that dosing with 10 $\mu\text{l/g}$ of body weight was equivalent to 10 mg/kg of the suspended compound. For comparison, GF120918 was formulated in the same vehicle. Mice were given oral vehicle with or without inhibitor 30 min before 1 mg/kg oral topotecan (Hycamtin; SmithKline Beecham, Welwyn Garden City, United Kingdom). The latter was administered as 5 $\mu\text{l/g}$ body weight of a 0.2 mg/ml solution in 5% glucose, spiked with [¹⁴C]topotecan (SmithKline Beecham, King of Prussia, PA) to 0.008 $\mu\text{Ci}/\mu\text{l}$, equivalent to ~ 1 $\mu\text{Ci}/\text{mouse}$. Mice were sacrificed 30 or 60 min later, and heparinized blood was collected by axillary bleeding under methoxyflurane anesthesia. Plasma topotecan concentrations were inferred by comparison of scintillation counts of plasma *versus* the oral formulation, because topotecan is hardly metabolized *in vivo* (34) and subsequently verified by HPLC analysis. The latter method yielded plasma concentrations that were $80 \pm 7\%$ of those determined by radioactivity measurements. The difference had no effect on the results of statistical tests or the conclusions drawn.

Results

Synthesis of a Novel FTC Analogue. The earlier synthesis of the combinatorial panel of 42 FTC analogues and demethoxy FTC, their initial screening for inhibition of mouse Bcrp1 and human BCRP, and structure-activity relationships have been described elsewhere (22, 23). These were each mixtures of 2–4 diastereoisomers, of which only one was substantially active in inhibiting Bcrp1/BCRP. Pure active

⁵ G. Reid, *et al.*, manuscript in preparation.

diastereoisomers were prepared for two of the most potent compounds, Ko132 and Ko134 (23). In addition, a novel compound was synthesized, representing a modification of Ko134 that restores the methoxy group present in native FTC on carbon 18 on the indole moiety, and its active diastereoisomer was isolated. This new analogue is denoted Ko143 (Fig. 1 and "Materials and Methods"). The importance of the methoxy group for potent inhibition of BCRP was suggested by the observation by us (23) and others (21) that demethoxy FTC has low activity compared with native FTC.

FTC Analogues Increase Intracellular Drug Accumulation. The three selected FTC analogues were found to be potent inhibitors of both mouse Bcrp1 and human BCRP. We made initial comparisons of potency on the basis of inhibition of Bcrp1-mediated drug efflux, as manifested by increased cellular mitoxantrone accumulation in the mouse MEF3.8/T6400 fibroblast cell line. This line lacks P-gp and Mrp1 (having an *Mdr1a/1b*^{-/-}, *Mrp1*^{-/-} genotype) and has been selected for resistance to topotecan, resulting in high levels of *Bcrp1* expression that also render it highly resistant to mitoxantrone (8). Fig. 2A depicts the effects of various concentrations of the FTC analogues on mitoxantrone accumulation in the T6400 cells, as measured by flow cytometry. Intracellular mitoxantrone could be increased at least 20-fold, *i.e.*, readily restored to levels similar to or greater than those obtained in the parent cell line, MEF3.8. In contrast, Ko143 (for example) had only a small effect on mitoxantrone levels in the parent line, where Bcrp1 expression is very low (8, 26). Very similar results (Fig. 2B) were obtained with human T8 cells, a topotecan resistant subline of the IGROV1 ovarian carcinoma line that has elevated BCRP expression (32). Hence, there does not appear to be a marked difference in either the absolute or relative potencies of the FTC analogues for inhibition of human BCRP *versus* mouse Bcrp1.

By considering the midpoints of the accumulation curves in Fig. 2, the Ko132 and Ko134 compounds are seen to be 15–30-fold more potent than demethoxy FTC, and Ko143, the 18-methoxy variant of Ko134, was 4-fold more potent still. Although native FTC was not available for direct comparison, a previous study (21) indicated that it is ~10-fold more potent than demethoxy FTC for reversing drug resistance in cells overexpressing human BCRP. This would mean that the three analogues considered here are more potent than native FTC. Ko143 is also approximately twice as potent as GF120918 in this context. It should be noted that the cell lines used in the accumulation assays have very high rates of mitoxantrone efflux (8, 32), and that achieving even 50% maximal drug accumulation requires inhibition of nearly all Bcrp1/BCRP activity.

Reversal of Bcrp-mediated Drug Resistance. The effects of the FTC analogues on mitoxantrone accumulation were reflected in their performance as reversal agents for Bcrp1/BCRP-mediated drug resistance. A convenient index of potency in this role is the effective concentration for 90% reversal of Bcrp1-mediated drug resistance (EC₉₀), *i.e.*, the concentration of an inhibitor that renders a cell line 10 times as sensitive to a Bcrp1 substrate drug. This represents a degree of Bcrp1/BCRP inhibition that would be highly attractive in practical applications (but is still considerably milder

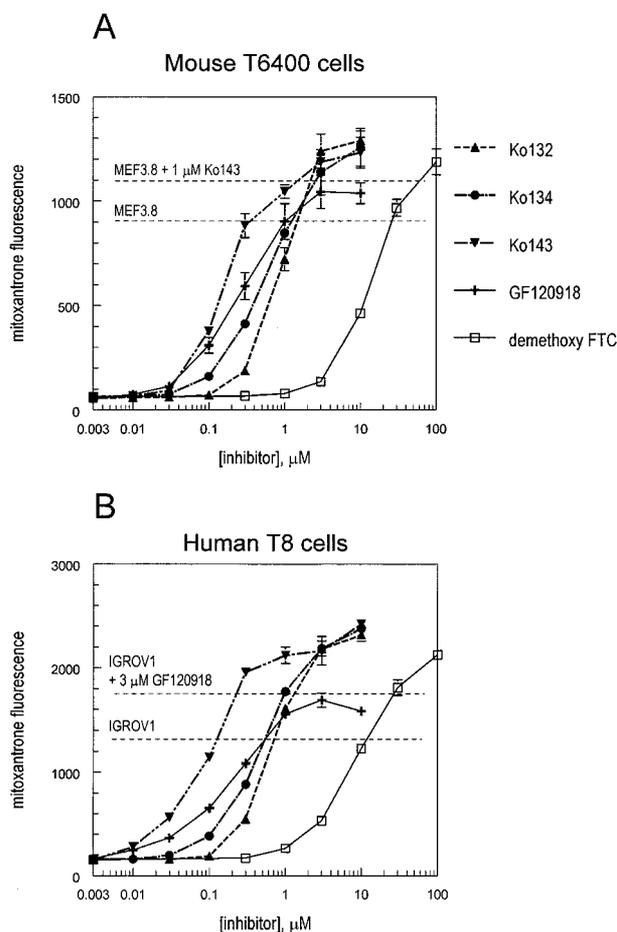


Fig. 2. FTC analogues increase mitoxantrone accumulation in drug-resistant mouse MEF3.8/T6400 cells, which have elevated Bcrp1, and in human IGROV1/T8 cells, which have elevated BCRP. Cells were exposed to 20 μM mitoxantrone in complete medium at 37°C for 80 min in the presence of different concentrations of the analogues. Mitoxantrone fluorescence is in arbitrary units, determined by flow cytometry with 633 nm excitation and emission detected through a 661-nm bandpass filter. Neither the FTC analogues nor GF120918 exhibited detectable fluorescence under these conditions. Data points show means of triplicate repeats; bars, SDs. Horizontal dashed lines, levels of mitoxantrone fluorescence in the drug-sensitive parent line MEF3.8, in the presence or absence of 1 μM Ko143, and likewise in IGROV1, with or without 3 μM GF120918.

inhibition than that needed for 50% maximal mitoxantrone accumulation, as discussed above). EC₉₀s can be determined by modified cytotoxicity assays (see "Materials and Methods"), and *Bcrp1*-transduced MEF3.8 cells were used for the purpose. These cells have an *Mdr1a/1b*^{-/-}, *Mrp1*^{-/-} genotype and very low endogenous *Bcrp1* levels, whereas expression of *Mrp2* and *Mrp3* is undetectable (not shown), making them highly sensitive to both mitoxantrone and topotecan (8, 26), whereas the *Bcrp1*-transduced clone A2 is >50-fold resistant to mitoxantrone and >30-fold resistant to topotecan (Table 1). EC₉₀ determinations for reversal of resistance to mitoxantrone and topotecan by the FTC analogues and GF120918 are compared in Table 1. The analogues are all highly active at submicromolar concentrations, and their relative potencies for reversal of drug resistance

Table 1 Potency of FTC analogues for reversal of Bcrp1-mediated drug resistance

Values shown are EC_{90} s, the concentration reversing 90% of Bcrp1-mediated drug resistance in MEF3.8/Bcrp1 clone A2 cells (IC_{50} for mitoxantrone = 42 ± 4 nM, RF = 58 compared with parent MEF3.8 cells and the IC_{50} for topotecan = 1750 ± 50 nM, RF = 36). The means are derived from eight repeats. All differences in potency between compounds were statistically significant by simple *t* test ($P < 0.001$).

Compound	Mean EC_{90} (nM) \pm SD	
	Mitoxantrone	Topotecan
Ko132	190 \pm 25	270 \pm 20
Ko134	85 \pm 10	110 \pm 10
Ko143	23 \pm 2	26 \pm 5
GF120918	51 \pm 7	61 \pm 8

agree well with the relative effects on mitoxantrone accumulation mentioned above; Ko143 is easily the most potent analogue, with an EC_{90} of ~ 25 nM, ~ 4 -fold lower than Ko134 and 2-fold lower than GF120918.

The EC_{90} concentration of Ko143 was equally effective at reversing Bcrp1/BCRP-mediated resistance in the drug-selected mouse MEF3.8/T6400 and human IGROV1/T8 cell lines, resulting in the expected 10-fold sensitization to topotecan and mitoxantrone (Table 2). Similar results (not shown) were obtained for EC_{90} concentrations of Ko134 or Ko132 (100 and 200 nM, respectively). At higher doses, Ko143 reversed essentially all resistance to mitoxantrone in these cell lines and nearly all topotecan resistance (Table 2). In the latter case, the small residual resistances may be attributable to other cellular responses specific to the selecting drug. No significant effects were seen on drug resistance in the parental cell lines where the Bcrp1/BCRP level is low. Also, as expected, the actions of the FTC analogues were restricted to Bcrp1/BCRP substrate drugs (mitoxantrone and topotecan), having no effect on resistance to (for example) vincristine (Table 2).

Cytotoxicity. It was important to establish whether concentrations of the FTC analogues useful for inhibiting Bcrp1/BCRP *in vitro* are cytotoxic. Accordingly, IC_{50} s were determined for a panel of human cell lines (Table 3) derived from tumor types typically treated with BCRP substrate drugs (ovarian, lung, breast and colon carcinomas, and a T-lymphoblastic leukemia; see "Materials and Methods") and a set of mouse embryo fibroblast lines, the drug resistance properties of which have been characterized by us elsewhere (8, 26) or herein. The IC_{50} s observed for the FTC analogues were all in the range 9 to 34 μ M, *i.e.*, from 50 to 1000 times higher than the corresponding EC_{90} s for reversal of Bcrp1/BCRP-mediated drug resistance noted above. The cytotoxicity of the compounds was not strongly influenced by cell type, because the range of the IC_{50} s obtained was relatively narrow, given the diverse origins and properties of the cell lines tested. In contrast, some cell lines were comparatively sensitive to GF120918, as has been observed previously (14).

As well as being derived from diverse tissues, the cell lines tested differ widely in their levels of P-gp, Mrp1/MRP1, and Bcrp1/BCRP. They include lines lacking P-gp entirely [77.1 (26)] or both P-gp and Mrp1 [MEF3.8, which also has ex-

tremely low Bcrp1 levels (26)], as well as lines transduced with expression constructs for these proteins [77.1/MDR1 and 2008/MRP1 (28)] and lines expressing high levels of human BCRP or mouse Bcrp1 [IGROV1/T8 (32) and MEF3.8/T6400 (8), respectively]. That the cellular toxicity of the FTC analogues (or GF120918) was not influenced to any substantial degree by such differences in MDR transporter expression suggests that the compounds are not effectively transported by these proteins. This inference, however, remains tentative.

Activity on Other Drug Transporters. The activity of the FTC analogues on P-gp was assessed in MDR1-transduced 77.1 cells ("Materials and Methods"), which are >100 -fold resistant to the P-gp substrate paclitaxel (Table 4), compared with the parent cell line 77.1 (that lacks endogenous P-gp). EC_{50} and EC_{90} s for reversal of P-gp-mediated paclitaxel resistance in this system are shown in Table 4. Ko132 and Ko134 were 25–30-fold less active against P-gp than Bcrp1, whereas Ko143 was at least 200-fold less active (compare EC_{90} s with those in Table 1). Comparison with GF120918, a model P-gp inhibitor, is instructive; the FTC analogues are three orders of magnitude less active against P-gp.

Inhibition of MRP1-mediated drug resistance by the FTC analogues was also weak, as assessed in MRP1-transfected 2008 cells (28, 29). The latter are >20 -fold resistant to the MRP1 substrate etoposide, compared with the parent cell line, when confounding P-gp activity is suppressed by 0.1 μ M PSC833. EC_{50} s for reversal of the MRP1-mediated etoposide resistance by the FTC analogues (Table 4) were again much higher than concentrations effective for reversal of Bcrp1-mediated drug resistance. EC_{90} s could not be determined in this case because the required concentrations of FTC analogues are well into the cytotoxic range.

Overall, all three FTC analogues examined had low activity against P-gp and MRP1, and Ko143 the least. Given that it is a more potent Bcrp1/BCRP inhibitor than the other two analogues, it is also easily the most specific of the three. This emphasizes the importance of the methoxy group on carbon 18 (Fig. 1) for specificity as well as potency.

Some other members of the multispecific organic anion transporter family (ABCC), *i.e.*, MRP2, 3, 4, and 5, also transport cytotoxic drugs (29), although their clinical significance in this respect is undetermined. Given that the FTC analogues all had little effect on MRP1, we simply tested the effect of Ko143 on drug resistance mediated by the other MRP transporters in transfected cell line models. As shown above, 0.5 μ M Ko143 is a higher concentration than needed to completely reverse BCRP-mediated drug resistance. However, it had little, if any, effect on the resistance of MDCKII/MRP2 cells (30) to vincristine ($IC_{50} = 13.5 \pm 1.3$ nM, RF = 17, $IC_{50} + Ko143 = 12.8 \pm 0.3$ nM, $P = 0.55$), the resistance of 2008/MRP3 cells (28) to etoposide ($IC_{50} = 530 \pm 90$ nM, RF = 3.1, $IC_{50} + Ko143 = 510 \pm 110$ nM, $P = 0.20$), or resistance to the nucleoside analogue prodrug bis-POM-PMEA in HEK293/MRP4 cells⁵ ($IC_{50} = 11.3 \pm 0.5$ μ M, RF = 8.5, $IC_{50} + Ko143 = 11.6 \pm 0.2$ μ M, $P = 0.30$), or HEK293/MRP5 cells (Ref. 31; $IC_{50} = 6.8 \pm 0.6$ μ M, RF = 5.1, $IC_{50} + Ko143 = 7.1 \pm 0.8$ μ M, $P = 0.39$). Each of these results represents the mean \pm SD of three cytotoxicity as-

Table 2 Reversal of drug resistance in topotecan-selected mouse MEF3.8/T6400 cells and human IGROV1/T8 cells by FTC analogue Ko143

Values shown were derived from quadruplicate repeats. Ko143 was applied at zero, one, or eight times the EC₉₀ concentration of 25 nM. The IGROV1 and IGROV1/T8 lines were assayed in the presence of 0.1 μM PSC833 to suppress confounding P-gp activity. RFs are calculated relative to the parent cell line in the absence of Ko143.

Drug + Inhibitor	Mean IC ₅₀ (nM) ± SD and (resistance factor)			
	MEF3.8 (parent)	MEF3.8/T6400 (resistant)	IGROV1 (parent)	IGROV1/T8 (resistant)
Mitoxantrone	0.52 ± 0.12	100 ± 15 (190) ^a	4.5 ± 0.3	125 ± 20 (28) ^a
+ 25 nM Ko143	1.10 ± 0.20 (2.1)	10.5 ± 1.9 (20) ^a	4.9 ± 0.5 (1.1)	11 ± 3 (2.4) ^b
+ 200 nM Ko143	0.34 ± 0.05 (0.65)	0.35 ± 0.04 (0.67)	3.7 ± 0.6 (0.8)	2.2 ± 0.4 (0.5)
Topotecan	33 ± 3	12,000 ± 1700 (360) ^a	7.7 ± 1.0	370 ± 40 (48) ^a
+ 25 nM Ko143	35 ± 4 (1.1)	1,150 ± 200 (35) ^a	7.0 ± 0.7 (0.9)	45 ± 8 (5.8) ^a
+ 200 nM Ko143	36 ± 5 (1.1)	98 ± 11 (3.0) ^a	6.7 ± 0.6 (0.9)	20 ± 2 (2.6) ^a
Vincristine	0.31 ± 0.07	0.37 ± 0.03 (1.2)	3.4 ± 0.8	1.8 ± 0.2 (0.5)
+ 25 nM Ko143	0.34 ± 0.02 (1.1)	0.37 ± 0.03 (1.2)	3.1 ± 0.6 (0.9)	1.7 ± 0.2 (0.5)
+ 200 nM Ko143	0.26 ± 0.06 (0.84)	0.36 ± 0.02 (1.2)	3.7 ± 0.7 (1.1)	2.0 ± 0.3 (0.6)

^{a,b} Cases where the drug-selected lines retained residual resistance significantly greater than that of the parent cell line (*i.e.*, RF > 1.0): ^a ($P < 0.005$), and ^b ($P < 0.05$).

Table 3 Cytotoxicity of FTC analogues

Values are means of quadruplicate repeats. SDs in no case exceeded 25% of the means and have therefore been omitted for clarity. In an independent repetition of the experiments, only one result differed by >30% from the value shown; it is shown in parentheses. The origins and relevant properties of the cell lines are detailed in "Materials and Methods" and "Results."

Cell line	Type	IC ₅₀ (μM)			
		Ko132	Ko134	Ko143	GF120918
Human					
2008	Ovarian	13	17	25	17
2008/MRP1	Transfected: high MRP1	17	18	26	20
IGROV1	Ovarian	26	22	34	1.3 (0.65)
IGROV1/T8	Topotecan-selected: high BCRP	22	26	27	1.0
A549	Lung	15	9.0	25	4.0
CCRF/CEM	T-lymphoblast	20	22	24	2.7
MCF-7	Mammary	15	15	19	18
WiDr	Colon	18	13	28	1.1
Mouse fibroblast					
NIH3T3	Wild-type	13	18	27	0.85
2ac.1	Wild-type	19	19	30	17
77.1	<i>Mdr1a/1b</i> ^{-/-}	14	16	30	30
77.1/MDR1	Transduced: high P-gp	29	21	25	18
MEF3.8	<i>Mdr1a/1b</i> ^{-/-} <i>Mrp1</i> ^{-/-}	14	15	25	1.4
MEF3.8/T6400	Topotecan-selected: high Bcrp1	12	11.5	18	1.2

says; RFs were computed in comparison with IC₅₀s of the untransfected parent cell lines determined in parallel, and *P*s are the results of paired sample *t* tests.

Toxicity in Mice. Alkaloids of the fumitremorgin/verruculogen/tryprostatin class are typically neurotoxic, producing sustained tremors and tetanic convulsions in mice or other animals within minutes of administration. Verruculogen TR-1 and fumitremorgins A, B, and C, for example, induce tremors at oral doses of 25 mg/kg, or a fraction of 1 mg/kg given *i.p.* (19, 20). The potential neurotoxicity of FTC analogues is thus a concern for their application as BCRP inhibitors *in vivo*. However, neither Ko132 ($n = 2$), Ko134 ($n = 6$), nor Ko143 ($n = 4$) showed any evidence of acute or delayed toxicity in mice at oral doses of 50 mg/kg or higher (see "Materials and Methods" for details). Specifically, no signs of tremors or convulsions were seen, and the mice showed normal breathing, activity, gait, posture, drinking, eating, sleeping and nest-making behaviors compared with vehicle-treated litter-

mate controls. *i.p.* doses of 10 mg/kg Ko132, Ko134, or Ko143 also had no obvious adverse effects ($n = 4$). Subsequent blind histological examination showed no evidence of tissue pathology in any of the major organs or structures (see "Materials and Methods") other than minor granulomatous processes present in the peritoneum of all of the mice, attributable to the corn oil vehicle. Of course, the toxicity of the compounds may depend on their formulation, and at present it is unknown whether higher or repeated doses could produce subtle, long term, or cumulative effects. However, the results above indicated that the compounds are sufficiently well tolerated in mice to allow evaluation of their efficacy as Bcrp1 inhibitors *in vivo*.

Inhibition of Intestinal Bcrp1. The intestinal epithelium is a major site of *Bcrp1/BCRP* expression (12), and it was recently shown (13) that inhibition of intestinal Bcrp1 with 50 mg/kg oral GF120918 increased the oral availability of topotecan 6-fold in *Mdr1a/1b*^{-/-} mice, evidently by reduction of

Table 4 Effect of FTC analogues on P-gp- and MRP1-mediated drug resistance

Shown are the effective concentrations of inhibitor compounds for reversal of 50% (EC₅₀) or 90% (EC₉₀) of the resistance of the cell line for the drug specified. Values shown are means ± SD of quadruplicate repeats; 95% confidence intervals may be obtained by multiplying the SDs by 1.84. Independent repetition of the experiments gave similar results, not differing by >15% from the means shown.

	Ko132	Ko134	Ko143	GF120918	BSO ^a
P-gp-mediated paclitaxel resistance ^b					
EC ₅₀ (μM)	2.3 ± 0.4	0.61 ± 0.07	1.0 ± 0.3	0.0030 ± 0.0004	ND
EC ₉₀ (μM)	5.0 ± 0.4	2.8 ± 0.4	5.5 ± 0.5	0.0045 ± 0.0004	ND
MRP1-mediated etoposide resistance ^c					
EC ₅₀ (μM)	1.6 ± 0.4	1.8 ± 0.5	2.0 ± 0.4	ND	0.65 ± 0.15
EC ₉₀ (μM)	>8	>8	>8	ND	>8

^a L-Buthionine sulfoximine depletes glutathione and is thus an indirect inhibitor of MRP1. ND, not determined.

^b Assessed in MDR1-transduced 77.1 cells (see "Materials and Methods" and Table 3 for properties): IC₅₀ for paclitaxel = 2.4 ± 0.2 μM (n = 3), RF = 200, compared with the parent 77.1 cell line.

^c Assessed in MRP1-transfected 2008 cells (see "Materials and Methods" and Table 3 for properties): IC₅₀ for etoposide = 5.2 ± 0.4 μM (n = 4), RF = 22 compared with the parent 2008 cell line.

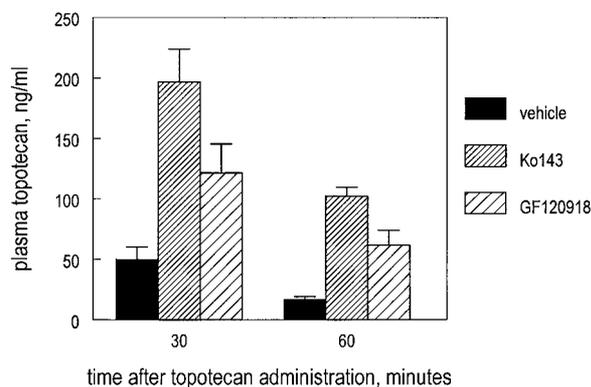


Fig. 3. Ko143 increases the oral availability of topotecan. Vehicle ± 10 mg/kg Ko143 or GF120918 was administered to *Mdr1a/1b*^{-/-} mice by oral gavage 30 min before 1 mg/kg oral topotecan. Plasma topotecan concentrations 30 or 60 min later are shown as the means of four mice (except the 30-min time point for GF120918, where n = 3); bars, SD. At each time point, results for Ko143 and GF120918 were significantly different from vehicle controls and also from each other (for every comparison, P < 0.025 by heteroscedastic t test).

its back-transport to the intestinal lumen. *Mdr1a/1b*^{-/-} mice (33) were used to exclude confounding effects from simultaneous inhibition of P-gp by GF120918. It is desirable to compare the FTC analogues in the same role, because they are of similar or greater potency to GF120918 *in vitro*. Indeed, administration of a comparatively low oral dose of Ko143, 10 mg/kg, increased plasma topotecan concentrations in *Mdr1a/1b*^{-/-} mice 4–6-fold at 30 and 60 min after oral administration of the drug (Fig. 3). These time points bracket the peak of the plasma topotecan curve seen in the earlier study using GF120918 (13) and corroborate the finding that intestinal Bcrp1 limits the oral availability of topotecan. In the present study, GF120918 was somewhat less effective than Ko143 with this dose and formulation. Pilot studies (not shown) indicated that Ko134 can also increase the oral availability of topotecan, although higher doses may be required to achieve quantitatively similar effects.

Discussion

BCRP emerged recently as an effective transporter of several major antineoplastic drugs. It is expressed widely in the body

at locations where it can affect the pharmacokinetics of substrate drugs (12, 13): in the placenta, where it reduces penetration of drugs to the fetus; in the intestinal epithelium and biliary canaliculi of the liver, where it affects drug uptake and elimination; and also in endothelial cells of veins and capillaries, where it may contribute to some blood-tissue barriers. Although very few clinical data are available yet, the widespread expression of BCRP suggests it could also contribute to either innate or acquired resistance of tumors to antineoplastic drugs in a manner analogous to P-gp. Thus, there are several important avenues for clinical application of BCRP inhibitors to modify drug resistance or drug pharmacokinetics, as well as for analysis of drug resistance mechanisms relevant to tailoring chemotherapy, and in the research laboratory.

We have developed and characterized tetracyclic analogues of FTC that are potent inhibitors of both mouse Bcrp1 and human BCRP. The most potent of these, Ko143, is also the most specific, having little effect on the activity of P-gp or MRPs 1–5. The possibility remains that the FTC analogues may have significant activity against other ABC transporters, particularly other members of the ABCG class. However, none of these has been shown to transport cytotoxic drugs. The analogues are not noticeably toxic *in vitro* or in mice at doses effective for inhibition of Bcrp1 activity. Ko143 or Ko134 can markedly improve the oral availability of the substrate drug topotecan in mice. These promising initial results were obtained despite present ignorance of the pharmacokinetics, optimal formulations, or dose-response relationships of the analogues. Further work to address such issues is necessary. It will then be interesting to see how potent the compounds ultimately prove to be for increasing the oral availability of topotecan or other substrate drugs, for increasing their penetration to the fetus, and for reversing BCRP-mediated drug resistance in mouse tumor models.

Previously, analyses of the biological effects of fumitremorgin-type alkaloids have focused on their inhibition of the cell cycle *in vitro* (35) and neurotoxicity in animals (18–20). The cytotoxic effects of such compounds are typically seen at concentrations in the range 10–100 μM, as is also true for the FTC analogues examined herein. As noted in "Results," such concentrations are orders of magnitude

higher than those effective for inhibition of Bcrp1/BCRP. We conclude that cytotoxicity is unlikely to be a problem in realistic applications of these compounds as Bcrp1/BCRP inhibitors. This view is supported by the observation that GF120918, despite being considerably more cytotoxic to some cell lines than the FTC analogues, is extremely well tolerated in mice (13, 14) and humans (36). Low cytotoxicity appears to be a general property of this class of FTC analogues, because the results for the pure diastereoisomers Ko132 and Ko134 are similar to those from earlier tests of the corresponding diastereoisomeric mixtures and 18 others from the original combinatorial library of 42 analogues on the NIH/National Cancer Institute standard tumor panel (23).

Unlike native FTC, our analogues are not obviously toxic to mice at useful doses, although the question of subtle, chronic, or cumulative toxicity at high doses remains open. The neural targets of fumitremorgin-type alkaloids are presently unknown, but there is some evidence that their neurotoxicity arises from stereochemical and other constraints on the conformation of the diketopiperazine D ring (Ref. 18; Fig. 1). The replacement of the proline moiety (E ring) by an acyclic substituent might thus allow the adjacent diketopiperazine ring to assume a different conformation that renders the analogues less neurotoxic than native FTC or related alkaloids.

BCRP and P-gp share a number of clinically relevant substrates, including the antineoplastic drugs topotecan, mitoxantrone, and the anthracyclines. The expression of the two transporters also overlaps, in the intestinal epithelium, placental trophoblasts, liver parenchymal cells forming the biliary canaliculi, the capillaries that form the blood-brain barrier, and other sites (10–12, 37) as well as numerous tumor cell lines. It has rightly been pointed out (15) that dual inhibitors of both BCRP and P-gp, such as GF120918, could therefore be advantageous for reversing resistance to drugs that are substrates of both transporters. However, we caution that it is also possible to foresee situations where such dual specificity could represent an undesirable complication. P-gp is known to be important for protection of the brain and bone marrow against toxic side effects of several antineoplastic and other drugs (38–40). It may thus be that inhibiting P-gp as well as BCRP will expose these or other tissues to unacceptable toxicity. The likelihood of such unfavorable interactions would clearly be increased in multidrug treatment regimens.

If, indeed, there is a place in the clinic for specific inhibitors of BCRP, FTC analogues like those examined here are candidates for this application. We have focused on only two of the better compounds from the original combinatorial panel of 42 (Ko132 and Ko134) and on a newly developed derivative of one of those (Ko143). Yet several other compounds in that modest panel exhibited similar potency for inhibition of Bcrp1/BCRP (23) and might be similarly enhanced by restoration of the methoxy group on carbon 18. Alternative modifications at this or other sites provide clear avenues for further modifications of their biological properties.

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