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Inhibition of BCRP-Mediated Drug Efflux by Fumitremorgin-Type Indolyl Diketopiperazines

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Abstract—A library of 42 diastereoisomeric mixtures of fumitremorgin-type indolyl diketopiperazines, prepared by parallel solid-phase synthesis, was screened for Breast Cancer Resistance Protein inhibitory activity and compared with GF120918. Demethoxy-fumitremorgin C was synthesized by solid-phase techniques and tested as well. Structure–activity relationship studies have identified several potent analogues, both in assays using the T6400 mouse and the T8 human cell line, whereas low cytotoxicity was seen at effective concentrations.

Among the members of the ATP-binding cassette transport protein superfamily (ABC transporters),[1] there are several plasma membrane proteins associated with multidrug resistance (MDR) of cancer cells when overexpressed. Clinical resistance of cells to chemotherapeutics, a major problem in the treatment of cancer, may in part be due to enhanced activity of these broad specificity drug efflux pumps, like P-glycoprotein (P-gp) and the multidrug resistance protein (MRP1).[2] Inhibitors of these cell surface pumps thus are potential agents of clinical interest for resensitization of certain resistant cancer cells by maintaining lethal intracellular antineoplastic drug levels. Furthermore, inhibition of transporters present at various tissue barriers may open ways to alter the pharmacokinetics of certain drugs.

Recently, a new ABC transporter has been identified: the breast cancer resistance protein (BCRP),[3] also known as placenta-specific ABC transporter (ABCP)[4] or mitoxantrone-resistance gene (MXR)[5] product. Elevated expression of BCRP results in resistance of various cancer cell lines to drugs including topotecan (TPT), doxorubicin, daunorubicin and mitoxantrone (MX).[6] Increased BCRP-mediated efflux of mitoxantrone can be reversed by the compound GF120918 (**1**)[7] (Fig. 1). In addition, GF120918 (**1**) is known to be a modulator of P-gp,[8] which confers a cross-resistance profile overlapping with BCRP for doxorubicin, mitoxantrone and other drugs.[9] Selective inhibitors of BCRP would be useful tools in revealing what mechanisms contribute most to MDR in certain types of tumors.

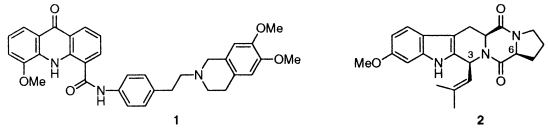

Figure 1. GF120918 (**1**) and fumitremorgin C (FTC, **2**).

Fumitremorgin C (FTC, **2**) (Fig. 1) was recently identified as a specific reversal agent for the BCRP transporter.[10–12] However, since this natural product of fungal origin also has tremor-inducing activity[13] and causes cell cycle arrest at the G_2/M transition,[14] less toxic compounds with more selective action may be found among FTC analogues. Recently, we published a solid-phase synthesis of fumitremorgin-type indolyl diketopiperazines[15] based on a cyclization/cleavage[16] strategy. A library of 42 compounds (Fig. 2) was prepared by parallel synthesis and analyzed with LC–MS. The target compounds were formed as a mixture of diastereoisomers in moderate to high overall yield and high purity.

Because of our interest in the role of the double bond in the substituent at C-3 of the FTC-skeleton, we also

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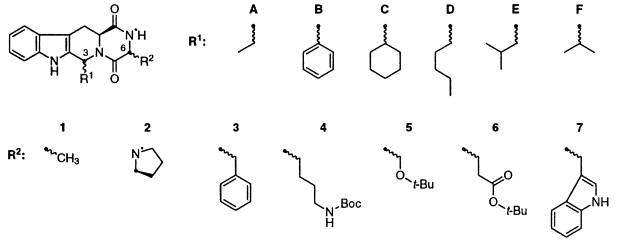
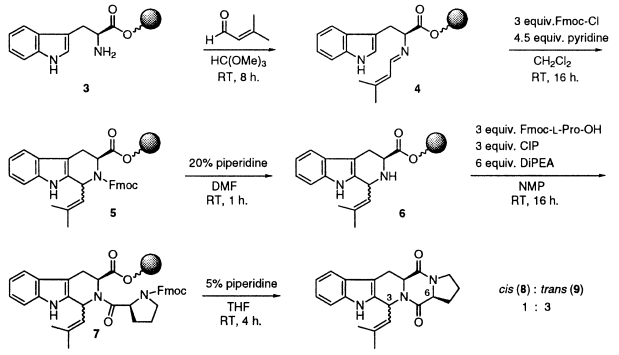


Figure 2. Substituents of the 42-member FTC analogue library.

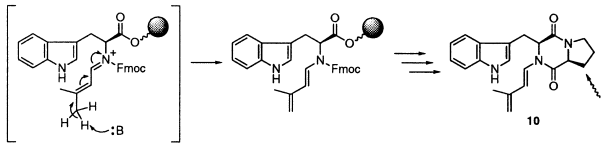
developed solid-phase methodology for the introduction of this type of substituent. In our original route,[15] C-3 substituents were introduced via Pictet–Spengler condensation using aromatic or aliphatic aldehyde building blocks. However, it is known that α,β-unsaturated aldehydes, which would lead to the desired structures, are not compatible with this reaction.[17] At the time we started our synthesis, a solution-phase example[18] was known in which this problem had efficiently been overcome by generating a highly reactive *N*-acyliminium ion from an α,β-unsaturated imine and a protected amino acid chloride. Besides translating this strategy to the solid support, we also wanted to make it adaptable to wider diversification in future applications, making use of only commercially available building blocks and reagents. Thus, the imine (**4**), prepared by treatment of hydroxyethyl polystyrene bound L-tryptophan (**3**) and 3-methylcrotonaldehyde in pure trimethyl orthoformate,[19] was reacted with Fmoc-chloride in dichloromethane in the presence of pyridine to effect *N*-acyliminium ion mediated Pictet–Spengler type cyclization. Removal of the Fmoc-group from product **5** gave the corresponding tetrahydro-β-carboline (**6**) bearing the unsaturated sidechain, which could be elaborated further, in 26% overall yield based on resin loading, to a 1:3 mixture of demethoxy-FTC (**8**) and its C-3 epimer (**9**) using our procedure developed before[15] (Scheme 1).



Scheme 1. Solid-phase synthesis of demethoxy-FTC (**8**) and C-3 epimer (**9**). CIP = 2-chloro-1,3-dimethylimidazolidinium hexafluorophosphate.

Pure demethoxy-FTC (**8**) was obtained after column chromatography followed by crystallization from methanol. Spectral data of **8**[20] and **9**[13] were in agreement with literature reports.

Initially, we carried out the key *N*-acyliminium ion step in the presence of DiPEA. However, after final cyclo-release, a significant part of the material—up to 25% relative to targets **8** and **9**—turned out to have undergone base-catalyzed double bond migration instead of Pictet–Spengler type cyclization in the *N*-acyliminium ion stage, due to the stronger character of this base (Scheme 2). The formed *N*-substituted tryptophan-proline diketopiperazine side product (**10**) is easily recognized by a remarkable upfield shift ($\delta = -0.38$ ppm) of one of the indicated proline-residue protons in the ^{1}H NMR spectrum. During our studies, solid-phase adaptation of the previously mentioned solution-phase route, with direct employment of protected amino acid chlorides, was published.[21]



Scheme 2. Proton abstraction from the *N*-acyliminium ion intermediate leads to formation of *N*-substituted Trp-Pro diketopiperazine (**10**).

Biological Results

The panel of 42 mixtures of diastereoisomers[22] (**A1**–**F7**) (Fig. 2), together with demethoxy-FTC (**8**), was screened for BCRP inhibitory activity and compared with GF120918 (**1**). To illustrate functional comparability of human BCRP and the mouse homologue Bcrp1, assays were performed both on the T6400[6] mouse cell line (Table 1) and the human T8[23] cell line (Table 2). T6400 is a topotecan-selected subline of the embryo fibroblast line MEF3.8, lacking functional genes encoding mouse P-gp and Mrp1. It is more than 100-fold resistant to the selecting drug, and is highly cross-resistant to mitoxantrone. T8 is a topotecan-selected cell line developed from the IGROV1 human ovarian carcinoma cell line with a marked cross-resistance to mitoxantrone. It shows pronounced overexpression of BCRP, but not of the MDR-associated P-gp and MRP pumps. Both the T6400 and the T8 lines showed high rates of mitoxantrone efflux, and consequently low levels of mitoxantrone accumulation compared to their parental cell lines.

Mitoxantrone accumulation assays were performed, analogous to procedures described previously.[6,23] All compounds were tested in triplicate at a 2.5 μM concentration, with a mitoxantrone concentration of 10 μM and an accumulation time of 60 min. Relative cellular accumulation of mitoxantrone was determined by flow cytometry using excitation at 633 nm and a 661 nm band pass filter to detect emission. Though not in a linear relationship, the higher the mitoxantrone fluorescence measured, the better is the inhibition of the transporter protein. Controls included in the assays were the parental cell lines (MEF3.8 in the T6400 assay and IGROV1 in the T8 assay) and the selected cell lines, both without mitoxantrone and without inhibitor. Mitoxantrone fluorescence levels in the T6400 assay are

Table 1. Results of the mitoxantrone accumulation assay on the T6400 mouse cell line, which lacks endogenous P-gp and Mrp1. Compounds were tested at 2.5 μM. Values[a] are in arbitrary units of fluorescence and reflect relative intracellular mitoxantrone concentration

	R^2								
R^1	**1**	**2**	**3**	**4**	**5**	**6**	**7**	Controls[b]	
A	43 (±1)	42 (±3)	63 (±4)	74 (±5)	71 (±7)	80 (±5)	96 (±3)		
B	53 (±4)	54 (±2)	112 (±10)	85 (±2)	106 (±1)	100 (±12)	125 (±19)	MEF3.8, no mitoxantrone	4 (±0)
C	74 (±9)	85 (±5)	91 (±7)	278 (±12)	276 (±9)	252 (±7)	158 (±25)	MEF3.8, no inhibitor	625 (±51)
D	175 (±12)	119 (±11)	199 (±14)	302 (±7)[c]	275 (±11)	283 (±23)	242 (±3)	T6400, no mitoxantrone	4 (±0)
E	80 (±4)	56 (±2)	191 (±10)	305 (±15)	216 (±2)	348 (±42)	279 (±10)	T6400, no inhibitor	40 (±2)
F	49 (±3)	45 (±1)	104 (±7)	133 (±12)	93 (±8)	149 (±7)	96 (±2)	T6400 + GF120918 (**1**)	438 (±17)
								T6400 + demethoxy-FTC (**8**)	80 (±18)

[a]Values are means of three repeats ($n = 3$), standard deviation is given in parentheses.
[b]Control on T6400 starting from a pure DMSO stock to 0.25% final concentration [42 (±3); $n = 3$] showed no relevant effect of this working stock solvent.
[c]For compound **D4**, $n = 2$.

Table 2. Results of the mitoxantrone accumulation assay on the T8 human cell line. Compounds were tested at 2.5 μM, in the presence of 200 ng/mL PSC833 to inhibit confounding P-gp activity. Values[a] are in arbitrary units of fluorescence and reflect relative intracellular mitoxantrone concentration

	R^2								
R^1	**1**	**2**	**3**	**4**	**5**	**6**	**7**	Controls[b]	
A	114 (± 6)	119 (± 2)	228 (±10)	313 (±16)	225 (± 9)	313 (±21)	354 (±19)		
B	181 (±19)	176 (±20)	376 (±20)	305 (±34)	339 (±22)	313 (±21)	376 (± 9)	IGROV1, no mitoxantrone	3 (± 0)
C	268 (± 7)	313 (±16)	nd[c]	629 (±38)	672 (±24)	638 (±30)	410 (±35)	IGROV1, no inhibitor	394 (± 3)
D	436 (±45)	370 (±18)	514 (±21)	599 (±19)	607 (±36)	592 (±11)	601 (±35)	T8, no mitoxantrone	3 (± 0)
E	333 (±22)	187 (±25)	431 (±42)	649 (±58)	522 (±21)	711 (±32)	535 (±27)	T8, no inhibitor	103 (± 4)
F	156 (±19)	136 (±10)	359 (±14)	452 (± 9)	334 (±24)	381 (±40)	310 (±24)	T8 + GF120918 (**1**)	722 (±27)
								T8 + demethoxy-FTC (**8**)	302 (±11)

[a]Values are means of three repeats ($n = 3$), standard deviation is given in parentheses.
[b]Control on T8 starting from a pure DMSO stock to 0.25% final concentration [120 (±5); $n = 2$] showed no relevant effect of this working stock solvent.
[c]nd = no data.

lower because this mouse cell line has a Bcrp1 activity that is higher than the BCRP activity in the human T8 cells. Nevertheless, the pattern for the tested compounds is qualitatively very similar in both assays, illustrating that in this regard mouse Bcrp1 is functionally comparable with human BCRP as a multidrug transporter. Concerning substituent effects, it becomes clear that sidechains of lipophilic nature in the $\mathbf{R^1}$ (C-3) and the $\mathbf{R^2}$ (C-6) positions are of benefit for inhibition of the transporter. Particularly, compounds bearing the **C**, **D** or **E** substituent in the $\mathbf{R^1}$ position and the **4**, **5** or **6** substituent in the $\mathbf{R^2}$ position, show efficient reversal of mitoxantrone efflux. Some of those agents approach the efficacy of GF120918 (**1**). Since these FTC-analogues are still mixtures of four diastereoisomers, there is a reasonable chance of finding a better inhibitor when diastereomerically pure compounds are being used (vide infra). Interestingly, the compounds with the pentacyclic skeleton of natural FTC ($\mathbf{R^2 = 2}$) are not among the best reversal agents. Furthermore, comparing the results for demethoxy-FTC (**8**, single isomer in all-natural configuration) and **E2** (mixture of two diastereoisomers), the naturally occurring double bond in the $\mathbf{R^1}$ substituent at C-3 seems to be of little or no relevance for inhibitory activity.

During our studies, a paper was published comprising reversal of BCRP-mediated MDR by a series of pentacyclic FTC analogues.[24] Though no compounds more active than natural FTC (**2**) were found, these cell survival studies have revealed that compounds having the all-natural stereochemistry (all three stereocenters *S*) were preferred. Substituents at C-3 had to be hydrophobic and of suitable length (e.g. isobutyl) for retaining activity. Though more potent, compounds having longer lipophilic sidechains at this position showed a clear increase in cytotoxicity. Comparison of FTC and demethoxy-FTC revealed the 6-methoxy substituent on the indole moiety to be beneficial, whereas the unsaturated demethoxy-FTC was less active than its saturated sidechain analogue.

With regard to the ability of FTC to inhibit cell cycle progression,[14] cytotoxicity tests of 20 out of the 42 mixtures in the library were performed in an earlier stage in the NIH/NCI in vitro disease-oriented primary antitumor screen.[25] Typically, cell growth inhibition of the samples, including the potent **E4** and **E6** mixtures, is seen at concentrations ≥ 10 μM, whereas short-term cell killing required concentrations in the 100 μM range (data not shown). The findings that, with compound mixtures from our panel, BCRP-mediated MDR can be effectively reversed at concentrations lower than those inducing cytotoxicity, prompted us to continue with more detailed SAR and toxicity studies. Two of the most potent analogues, **E4** and **E6** (Fig. 3), were resynthesized

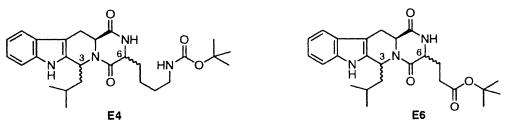


Figure 3. Compounds **E4** and **E6**, selected for further studies.

Table 3. Mitoxantrone accumulation assay on the T6400 cell line using purified diastereoisomers at 1.0 μM concentration. Values[a] are in arbitrary units of fluorescence

Compound	Config.	Activity	Controls	
E4	(3*R*,6*R*/*S*)	48 (±1)		
E4	(3*S*,6*R*)	65 (±1)	MEF3.8, no mitox.	4 (±0)
E4 (Ko132)	(3*S*,6*S*)	500 (±14)	MEF3.8, no inhib.	636 (±21)
E6	(3*R*,6*R*/*S*)	129 (±9)	T6400, no mitox.	4 (±0)
E6	(3*S*,6*R*)	78 (±4)	T6400, no inhib.	43 (±1)
E6 (Ko134)	(3*S*,6*S*)	528 (±5)	T6400 + GF120918 (**1**)	493 (±21)

[a]Values are means of three repeats ($n = 3$), standard deviation is given in parentheses.

in solution phase starting from L-tryptophan methyl ester. In a stepwise deconvolution process, mitoxantrone accumulation studies on the T6400 cells of mixtures of two isomers at 1.0 μM ($\mathbf{R^1}$ determined after flash chromatography in the tetrahydro-β-carboline stage, $\mathbf{R^2}$ racemic) clearly showed the major activity to be in the samples with the isobutyl $\mathbf{R^1}$ substituent at C-3 in the *S* configuration (up). These mixtures, being of similar potency to GF120918 (**1**), were purified to single diastereoisomers by preparative reversed phase HPLC. Final evaluation (Table 3) proved the all-natural (3*S*,6*S*)-configuration of **E4** (Ko132) and **E6** (Ko134) to be the most active.

In conclusion, interesting leads for potent and selective BCRP inhibition have been identified. Further synthetic studies, as well as dose–response and detailed toxicological investigations, are currently in progress and will be reported elsewhere.

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25. Compounds were tested against a panel of 60 cell lines at a minimum of five concentrations. A 48-h continuous drug exposure protocol was used, and a sulforhodamine B protein assay was used to estimate cell viability or growth.