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ORIGINAL ARTICLE

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Novel tetrahydroisoquinolin-ethyl-phenylamine based multidrug resistance inhibitors with broad-spectrum modulating properties

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Abstract *Purpose*: The ATP-binding cassette transporters P-glycoprotein (Pgp) and BCRP are implicated in multidrug resistance (MDR) of many tumors. Multi-targeted inhibitors such as cyclosporin A, have been shown to circumvent MDR in clinical trials. Here, we present the characterization of a novel class of effective and multi-targeted tetrahydroisoquinolin-ethyl-phenylamine based MDR inhibitors. *Methods*: The novel MDR inhibitors, XR9577, WK-X-34, WK-X-50 and WK-X-84 were examined for cellular toxicity in several cell lines. Chemosensitivity and inhibition of BCRP-mediated mitoxantrone efflux were analyzed in BCRP-overexpressing MCF7/mx cells. Chemosensitivity towards daunorubicin and inhibition of Pgp-mediated efflux of ^{99m}Tc-Sestamibi were examined in Pgp-overexpressing A2780/Adr cells. Potential MRP-interactions were evaluated with 5-CFDA efflux assays in selectively transfected MRP-1, -2 and -3 cell lines. *Results*: All WK-X-compounds showed significant BCRP inhibition in the MCF7/mx cells resulting in significant increases in mitoxantrone intracellular accumulation and 200–300 fold increases in mitroxantrone cytotoxicity. WK-X-34 and XR9577 were also potent inhibitors of Pgp, increasing ^{99m}Tc-Sestamibi accumulation with IC_{50} values in the nM range. Daunorubicin cytotoxicity was also increased seven to eight-fold in cells co-treated with XR9577 or WK-X-34 (10 μM). These compounds did not appear to interact with the MRP transporters. As compared to cyclosporin A, these compounds showed reduced cellular toxicity and increased potency of BCRP and Pgp inhibition. *Conclusion*: The novel MDR inhibitors WK-X-34 and XR9577 demonstrate superior effectiveness in Pgp and BCRP inhibition, in vitro tolerance and specificity over cyclosporin A. The novel compounds might be the promising candidates for a broad-spectrum based approach to the circumvention of MDR in resistant tumors.

Keywords Multidrug resistance · Ovarian cancer · ABC transporters · P-glycoprotein · BCRP · Mitoxantrone · Daunorubicin

Abbreviation MDR: Multidrug resistance · ABC: ATP-binding cassette · Pgp: P-glycoprotein · BCRP: Breast cancer resistance protein · MRP: Multidrug-resistance associated protein · LRP: Lung resistance protein · FITC: Fluorescein isothiocyanate · MTT: Methylthiazolyldiphenyl-tetrazoliumbromide

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Introduction

Multidrug-resistance (MDR) is a major cause of failure of chemotherapeutic treatment for cancer. Despite extensive research, effective strategies to circumvent multidrug-resistance in patients, are yet to be developed. MDR emerges frequently as a multifactoral, complex problem, as various members of the ATP-binding cassette (ABC)-family of transport proteins can be simultaneously overexpressed: P-glycoprotein (Pgp, ABCB1), the breast cancer-resistance protein (BCRP, ABCG2) and the multidrug-resistance associated proteins (MRPs, ABCC family) [7, 12, 16].

Pgp, which is encoded by the human *mdr1* gene, is frequently overexpressed in tumor cells and to date, has been the most extensively studied ABC-transporter [19]. Pgp is also expressed in the excreting organs and epithelial membranes and is thought to play an important role in xenobiotic protection. In MDR resistant tumor cells,

Pgp actively effluxes a broad range of antineoplastic drugs including the anthracyclines, *Vinca* alkaloids and taxanes, thereby decreasing their intracellular levels and therapeutic efficacy. Likewise, the more recently discovered ABC-half transporter BCRP is expressed in many types of tumors as well as normal epithelial tissues of excreting organs, placenta and breast. BCRP is also expressed in haematopoitic stem cells, where it likely offers survival advantages, particularly under hypoxic conditions and in malignant cells [2]. An extensive overlap in substrate and tissue distribution exists between Pgp and BCRP, suggesting similar involvement in the protective and excretatory cellular mechanisms. Cytotoxic substrates of BCRP include the anthracyclines, mitoxantrone and topotecan [1]. Among the MRP family, another distinct class of ABC drug efflux transporters, the members MRP1, MRP2 and MRP3 are implicated in the MDR phenotype of resistant tumors [3, 17]. MRP1 and MRP2 have similar drug resistance profiles and mainly transport organic anions and chemotherapeutics such as anthracyclines, mitoxantrone as glutathione-S-conjugates. The glucuronate-conjugate transporter MRP3 was found to offer low resistance to some cytotoxic agents like etoposide.

Earlier attempts to reverse multidrug resistance have mainly directed their efforts towards the inhibition of the Pgp-mediated MDR phenotype [33]. However, disappointing results were seen in clinical trials with the first and second generation inhibitors of Pgp such as verapamil and PSC 833 [16, 25]. These compounds were often non-effective in vivo or resulted in unacceptable toxicities and pharmacokinetics when administered in higher doses. Third and fourth generation Pgp inhibitors were developed to increase the specificity for Pgp and to prevent in vivo toxicities and pharmacokinetic interactions [6, 14, 23]. The Xenova compound XR9576, a highly specific and potent third generation Pgp inhibitor [23], emerged as the most promising candidate; however, pivotal phase III clinical trials were discontinued in 2003 due to safety concerns [21]. Only cyclosporin A demonstrated efficacy and sufficient safety in clinical trials reversing MDR in acute myelomic lymphoma AML [20, 32]. Interestingly, cyclosporin A was later found to not only inhibit Pgp but also BCRP, MRP1 and the lung resistance protein (LRP) [27]. Thus, the concept of broad-spectrum MDR modulation has recently attracted much attention. It is believed that many drug resistant tumors and critical cancer stem cell subpopulations such as those seen in acute leukemia, are protected by multiple and redundant cellular mechanisms of resistance [9, 30, 34]. Therefore, modulation of more than one MDR-reversing mechanism appears to be a promising approach.

XR9576, as well as another third generation Pgp inhibitor GF120918 consist of a tetrahydroisoquinolin-ethyl-phenylamine partial structure which is connected to a highly hydrophobic rest via an amide bond [14, 23]. In this study, we developed several novel tetrahydroisoquinolin-ethyl-phenylamine based MDR inhibitors, tested their interaction with multiple MDR transporters and compared potency and toxicity to cyclosporin A, a broad-spectrum inhibitor with successful clinical application [27].

Materials and methods

Chemicals and cell lines

The novel tetrahydroisoquinolin-ethyl-phenylamine based MDR inhibitors WK-X-34, WK-X-50 and WK-X-84 (Fig. 1) as well as XX9577, were synthesized and stock solutions (10 mM) were prepared in DMSO. WK-X-34 has recently been characterized in our laboratory [15]. Other inhibitors were obtained from Sigma (Oakville, ON) and stock solutions (10 mM) were prepared in methanol. All stock solutions were further diluted in PBS. ^{99m}Tc-Sestamibi was freshly prepared by reconstituting kits (Cardiolite$^{(R)}$, Bristol-Myers Squibb, MA, USA) and radiochemical purity was determined by thin layer chromatography to be greater than 93%.

Human ovarian cancer cell lines A2780/wt, its Pgp-overexpressing counterpart A2780/Adr (ECACC, UK) and BCRP-overexpressing MCF7/mx cells (kindly provided by Dr. E. Schneider, Wadsworth Center, Albany, NY,USA) were cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum and 50 μg/ml penicillin and streptomycin. *Mdr1*/Pgp expression was confirmed in A2780/Adr cells by RT-PCR analysis [18] (Fig. 2a) and by protein surface analysis using a FITC-labeled monoclonal Pgp antibody (BD Biosciences, San Diego, CA, USA) with flow cytometry detection. A2780/Adr displayed no detectable levels of BCRP and only very low levels of MRP1. BCRP overexpression in MCF7/mx cells was verified by RT-PCR (Fig. 2b) and using the anti-BCRP monoclonal BXP-21 antibody (Abcam, Cambridge, MA, USA) and anti-mouse Ig fluorescein-linked whole antibody with flow cytometry detection [22]. Transfected MRP1- HeLa cells (kindly provided by Dr. S. Cole, Queen's University, Kingston, ON Canada) and MRP2- and MRP3- transfected MDCK cells

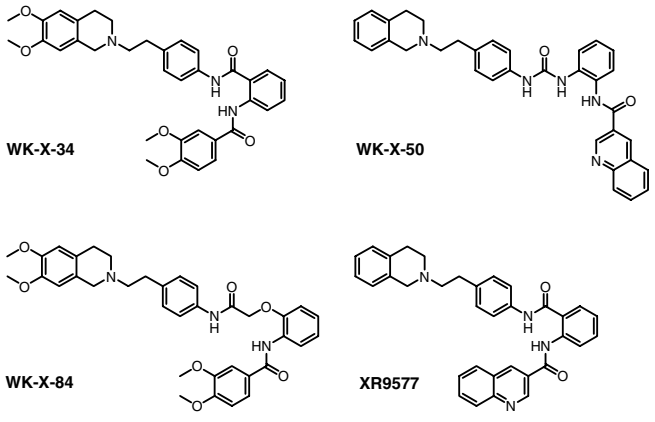

Fig. 1 Chemical structures of WK-X-compounds and XR9577

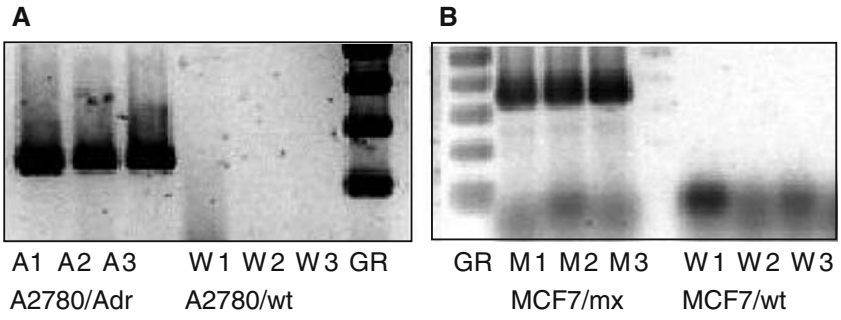

Fig. 2 RT-PCR analysis of resistant and sensitive cell lines. Representative PCR gels depicting RT-PCR analysis of *MDR1* (Pgp) and BCRP in (A) A2780/Adr and (B) MCF7/mx cells. Bands corresponding to MDR1 (237 bp) and BCRP (342) were visible in A2780/Adr and MCF7/mx cells, respectively

(kindly provided by Dr. P Borst, Netherlands Cancer Institute, Amsterdam, Netherlands) were maintained in Dulbecco's medium eagle medium supplemented with 10% fetal calf serum and 50 μg/ml penicillin/streptomycin. Expression of MRP1, MRP2 and MRP3 was verified by RT-PCR analysis (data not shown). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cellular toxicity of all inhibitors was analyzed in A2780/Adr, A2780/wt, MCF7/wt and MCF7/mx cells using the methylthiazolyldiphenyl-tetrazoliumbromide (MTT) cell viability assay. Briefly, 3×10^4cells/well were plated onto 96-well plates and incubated for 72 h with increasing concentrations (100 nM–10 mM) of the inhibitor. Cells were incubated with 20 μl of a 5 mg/ml MTT solution for 1 h, solubilized with isopropanol-HCl (1:300) and the absorption of solubilized formazan was measured at 595 nm using a BMG Fluostar (BMG LABTECH GmbH, Offenburg, Germany). Values were corrected for background absorbance at 690 nm, cell viability was calculated as a percentage of controls and IC_{50} values were derived.

Pgp transport assays

The interaction with Pgp activity was examined in the Pgp-overexpressing A2780/Adr and the parental drug sensitive A2780/wt cell lines using well-established Pgp transport assays with ^{99m}Tc-Sestamibi and daunorubicin. ^{99m}Tc-Sestamibi efflux assays were carried out as previously described [35]. Briefly, 15 μl of a ^{99m}Tc-Sestamibi solution (0.55 MBq/ml in PBS) was added to confluent cells which had been treated for 1 h with or without specific inhibitors (10 μM). At various times (0–60 min), ^{99m}Tc-Sestamibi uptake was terminated by washing and lysing the cells with 200 μl trypsin. For IC_{50} determinations, cells were preincubated with different concentrations (10 nM–1 mM) of WK-X-34, XR9577 or cyclosporin A and ^{99m}Tc-Sestamibi incubation was terminated at 60 min. Cell media and lysates were collected and counted using a gamma-counter (Packard Cobra II® Series Auto-Gamma® Counting Systems, Model 5003, Packard Instrument Company, Meridien, CT, USA). Relative cellular accumulation of ^{99m}Tc-Sestamibi was determined in each well of confluent cells (cpm cells *100/cpm medium), normalized to protein content and IC_{50}s were derived. The analysis was repeated one or two times to confirm the reproducibility of the obtained value. ^{99m}Tc-Sestamibi accumulation studies in the BCRP overexpressing MCF7/mx cells confirmed no involvement of BCRP in the cellular efflux of ^{99m}Tc-Sestamibi (data not shown).

The daunorubicin efflux assay was performed in A2780/Adr according to previously described methods [4]. Briefly, 10^6 A2780/Adr or A2780/wt cells were preincubated with daunorubicin (3 μM) for 30 min at 37°C in a shaking water bath, cells were washed and incubated in daunorubicin-free medium in the presence of 0.1, 1 and 10 μM of each inhibitor. Efflux was terminated after 90 min. Intracellular levels of daunorubicin in samples obtained at 0 and 90 min were measured on a Becton Dickinson FACSCalibur using an excitation wavelength of 488 nm (argon laser) with detection in the FL2 channel (575 nm). Each data point represented an average of the viable cell population of a total of 5,000 cells. Three aliquots were measured per sample and experiments were performed on three different occasions.

BCRP efflux assay

Inhibition of BCRP activity was examined using a previously described mitoxantrone efflux assay in the BCRP-overexpressing MCF7/mx cell line [22]. Briefly, 10^6 cells were preincubated with 3 μM mitoxantrone for 30 min. Cells were washed, aliquots collected and medium was replaced with mitoxantrone-free medium in the presence of 10 μM of inhibitors. Efflux was terminated at 90 min. Novobiocin was used as a positive control and had no significant effects on the sensitive MCF7/wt cell line. Intracellular levels of mitoxantrone at 0 and 90 min was measured on a Becton Dickinson FACSCalibur using an excitation wavelength of 488 nm (argon laser) with detection in the FL4 channel (633 nm). Each data point represents an average of the viable cell population of a total of 5,000 cells. The amount of mitoxantrone effluxed after 90 min was calculated as the difference between the intracellular levels of mitoxantrone in between 0 and 90 min. The results were expressed as the percent inhibition of mitoxantrone efflux. Three aliquots were detected per sample and experiments were performed on three different occasions.

MRP efflux assays

Inhibition of MRP-activity was examined using a 5-CFDA efflux assay in MRP1- transfected HeLa cells and

MRP2- and 3-transfected MDCK cells, as previously described [15, 18]. Briefly, confluent cells were preloaded with 4 μM of 5-CFDA for 30 min. Media were replaced with 5-CFDA-free medium in the presence and absence of the inhibitors (10 or 200 μM). Indomethacin and verapamil were used as positive controls. Efflux was terminated after 5 min, cells were washed and lysed with 1% Triton X. Intracellular 5-carboxy-fluorescein (5-CF) fluorescence intensity was measured using a Spectra MAX Gemini XS spectrofluorometer (Molecular Devices, Sunnyvale, CA,USA) at an excitation wavelength of 490 nm and emission wavelength of 540 nm. Values were normalized for protein content and background fluorescence.

Daunorubicin and mitoxantrone cytotoxicity

Daunorubicin and mitoxantrone cytotoxicity assays were carried out in A2780/Adr and A2780/wt and in MCF7/mx and MCF7/wt cells. Briefly, 3×10^4 cells/well were plated onto 96-well plates and incubated with increasing concentrations of daunorubicin or mitoxantrone (10 nM–1 mM) in the presence or absence of each inhibitor (10 μM) . After 72 h, cytotoxicity was assessed in treated cells using the MTT assay and IC_{50} values obtained. A sensitization factor (S.F.) was calculated for each cell line by dividing IC_{50} values obtained in the absence (control) or presence of inhibitor. In order to distinguish for Pgp- or BCRP-specific effects, ratios of the sensitization factors were further calculated by dividing the S.F. of the Pgp (A2780/Adr) or BCRP (MCF7/mx) -overexpressing cell line by the S.F. of the wild type (wt) cell line. All experiments were performed at least in triplicates on three independent occasions.

Statistical analysis

In vitro cell accumulation, efflux and cytotoxicity studies were performed at least in triplicate on three separate occasions. Data are reported as mean values ± standard deviation (SD). IC_{50} is the concentration required to reduce the measured effect (e.g. Pgp inhibition, cell viability) by 50%. IC_{50}s were derived by nonlinear regression analysis, assuming a sigmoidal dose-response curve using GraphPad Prism Software (San Diego, CA, USA). A two-tailed, unpaired Student's *t*-test with unequal variance was used for statistical comparison between the treated group and control. A difference in mean values of $P \leq 0.05$ and $P \leq 0.01$ was considered statistically significant and is indicated as follows: *P* levels: * < 0.05; ** < 0.01.

Results

Discovery and synthesis of the anthranilic acid based MDR inhibitors

Small molecule MDR inhibitors were initially identified by high-throughput cellular assays based on the restoration of doxorubicin-mediated cytotoxicity and calcein-AM influx assays using A2780/Adr and A2780/wt cell lines. Screening studies resulted in the identification of initial leads, aminobenzoeic acid structured compounds, which were then optimized via structure-activity relationship studies [26], guided by in vitro potency and stability. A selection of the optimized lead structures, WK-X-34 ($C_{35}H_{37}N_3O_6$), WK-X-50 ($C_{34}H_{31}N_5O_2$) and WK-X-84 ($C_{36}H_{39}N_3O_7$) (Fig. 1) were then chosen for further characterization. The Xenova-compound XR9577, formerly described [29] and never characterized, was included. All compounds exhibited very low water solubility but were well-tolerated in the various cell lines. Cell viability MTT assays demonstrated LC_{50} values well above 10 μM for all novel compounds in the A2780/Adr, A2780/wt, MCF7/mx and MCF7/wt cell lines (Table 1). On the other hand, cyclosporin A demonstrated greater cytotoxicity with observed IC_{50} values of 1 μM to 10 μM seen in each cell lines.

Pgp transport assays

The interaction of the novel MDR modulators on the cellular accumulation of ^{99m}Tc-Sestamibi in the Pgp-overexpressing human ovarian cancer cell line A2780/Adr and its sensitive counterpart A2780/wt are depicted in Fig. 3a. Whereas 10 μM of WK-X-34 and XR9577 fully inhibited Pgp activity, thereby increasing intracellular levels of ^{99m}Tc-Sestamibi to levels seen in A2780/wt cells, much smaller effects were seen for cyclosporin A. Dose-response studies demonstrated potent Pgp inhibition with IC_{50} values in the nanomolar range for XR9577 and WK-X-34 and within the lower micromolar range for cyclosporin A (Fig. 3b). WK-X-50 and WK-X-84 had no significant effect on Pgp activity.

Likewise, WK-X-34, XR9577 and cyclosporin A significantly inhibited daunorubicin efflux (Fig. 4). Interestingly, WK-X-84, which did not affect ^{99m}Tc-Sestamibi, significantly inhibited daunorubicin efflux. Dissimilarities in ^{99m}Tc-Sestamibi and anthracyclines transport assays have been noted by others [24] and are thought to stem from involvement of other MDR transporters. However, according to Muzzammil et al. [24] more

Table 1 Effect of inhibitors on cell viability

	IC_{50} (μM)			
	A2780/wt	A2780/Adr	MCF7/wt	MCF7/mx
XR9577	48.6 ± 12	77 ± 12	25 ± 0.3	444 ± 84
WK-X-34	124 ± 28	221 ± 37	24 ± 3	50 ± 6
WK-X-50	36 ± 19	256 ± 303	26 ± 4	76 ± 6
WK-X-84	53 ± 18	50.6 ± 19	77 ± 23	43 ± 15
Cyclosporin A	7 ± 0.6	27 ± 7	4 ± 0.3	2 ± 0.8

The cytoxicity of WK-X-compounds, XR 9577 and cyclosporin A was evaluated in different cell lines using MTT cell viability assays. Cells were incubated for 72 h with different concentrations (10 nM-1 mM) of inhibitors and IC_{50}s were calculated. Experiments were performed in triplicates on three separate occasions and results are shown as mean ± S.D

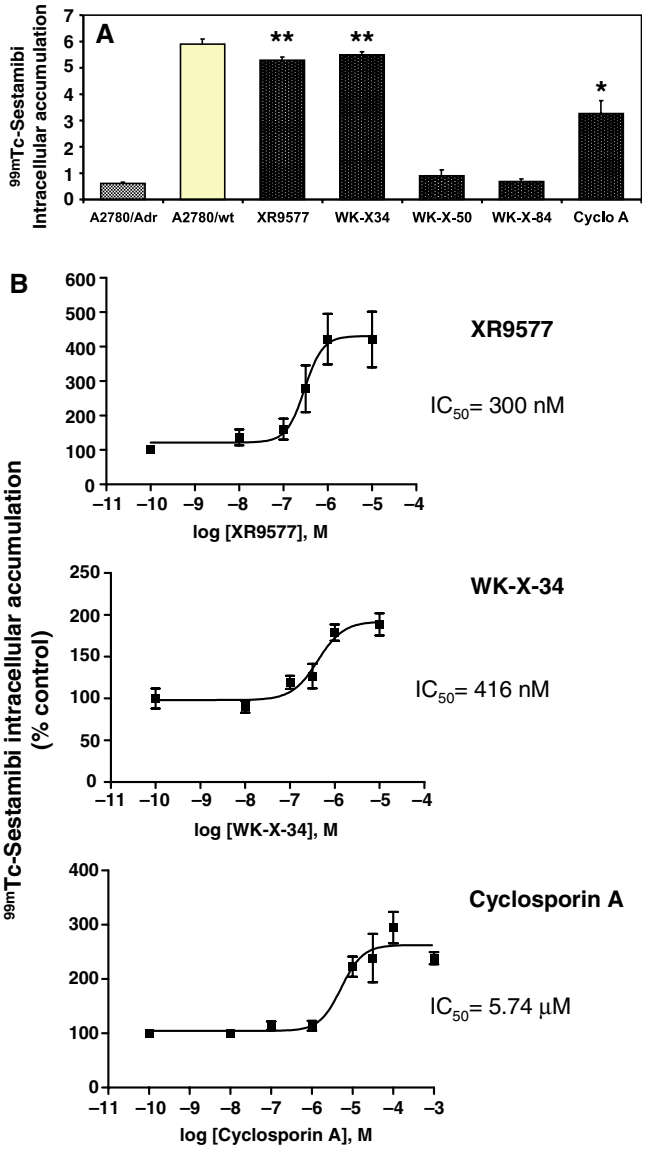

Fig. 3 Intracellular ^{99m}Tc-Sestamibi accumulation in: **a** Pgp overexpressing A2780/Adr and parental A2780/wt cells treated with inhibitors (10 µM). Results represent means of three samples ± SD. Similar results were obtained in three independent experiments. **b** Pgp overexpressing A2780/Adr cell treated with various concentrations (10 nM–100 µM) of WK-X-34, XR9577 and cyclosporin A. ^{99m}Tc-Sestamibi accumulation levels were determined after 60 min of uptake and plotted against molar Log concentrations. IC_{50} values were determined as described in methods

reliable conclusions on Pgp activity are generally seen with the ^{99m}Tc-Sestamibi-transport assays.

BCRP efflux assay

Inhibition of BCRP-mediated efflux of mitoxantrone was examined in MCF7/mx cells. Overexpression of BCRP and lack of detectable expression of Pgp were confirmed in MCF7/mx cells by surface protein analysis using selective Pgp or BCRP antibodies with flow cytometry detection (data not shown). The WK-X-compounds

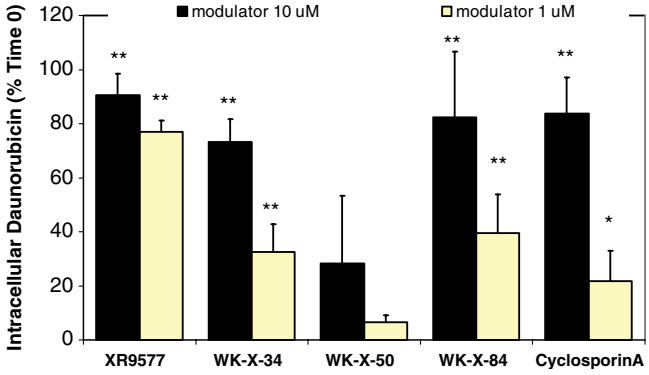

Fig. 4 Intracellular accumulation of daunorubicin in A2780/Adr cells. Effects of WK-X-compounds, XR9577 and cyclosporin A (1 and 10 µM) on Pgp-mediated daunorubicin efflux was determined in A2780/Adr cells. The amount of intracellular retained daunorubicin after 90 min of efflux was measured on a FACSCalibur® and is expressed as a % of total uptake at time 0. Values close to 100% indicate complete Pgp-inhibition. Results represent mean of three samples ± SD. Similar results were obtained in three independent experiments

as well as XR9577 were found to be potent inhibitors of BCRP-mediated efflux of mitoxantrone. FACS histograms show potent inhibition of BCRP-mediated mitoxantrone efflux in the presence of XR9577, WK-X-34, WK-X-50, WK-X-84 and cyclosporin A (Fig. 5a). Indeed, inhibition with XR9577, WK-X-34 and WK-X-50 was more pronounced than that seen with novobiocin (Fig. 5b). As compared to novobiocin, a 2.5 to 3 fold higher accumulation of mitoxantrone is seen in cells treated with WK-X-34 or WK-X-50 Likewise a greater intracellular accumulation of mitoxantrone was seen with these compounds as compared to cyclosporin A.

MRP efflux assays

Inhibition of MRP-mediated efflux of 5-CF was examined in MRP1, MRP2 and MRP3 stably transfected cell lines. As MRP inhibitory effects of indomethacin require concentrations of 200 µM, we also examined the potential impact of similar concentrations (200 µM) of the novel inhibitors on MRP activity. Results generated from these studies are depicted in Fig. 6. Significant inhibition of MRP1 was seen with all of the inhibitors although this was only seen at higher (200 µM) concentrations of XR9577, WK-X-34, WK-X-50 and verapamil. Cyclosporin A inhibited MRP1 activity at concentrations of 10 µM and both MRP1 and MRP3 at higher concentrations. Potent inhibition of MRP1 has previously been reported for cyclosporin A [27]. As expected, verapamil showed unspecific interactions with all three MRP transporters. MRP2-mediated efflux was significantly inhibited by WK-X-50 whereas significant effect on MRP2 or MRP3 were not seen with either WK-X-34 or WK-X-84. These results identify WK-X-50 as an MRP2 inhibitor and demonstrated an enhanced specificity of WK-X-34 and 80 and XR9577 for Pgp and BCRP inhibition, as compared to cyclosporin A.

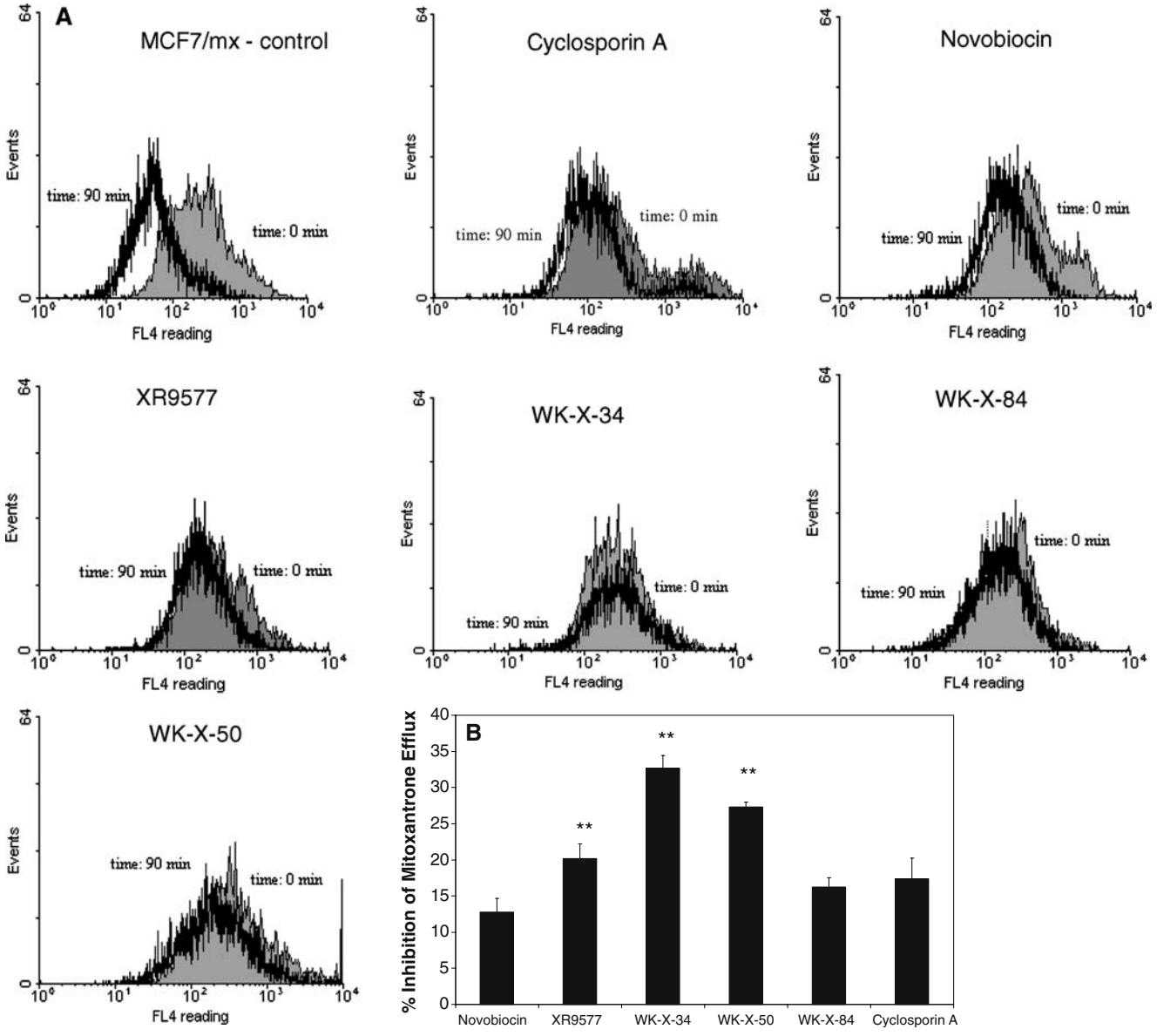

Fig. 5 Mitoxantrone Efflux in BCRP-overexpressing MCF7/mx cells. **a** Representative *FACS histograms.* The effect of inhibitors (10 μM) on BCRP–mediated mitoxantrone efflux are depicted as representative FASC histograms showing intracellular mitoxantrone levels at time 0 and after 90 min of efflux. Overlap of both histograms indicates inhibition of BCRP-mediated mitoxantrone efflux. **b** BCRP inhibition as measured by the % inhibition of mitoxantrone efflux in mitoxantrone-preloaded MCF7/mx cells. Cells were treated with 10 μM of inhibitors. Mitoxantrone levels at 0 and 90 minutes were measured using flow cytometry and % total inhibition of the 90 min efflux was normalized to MCF7/wt cells which do not express BCRP. Results represent mean ± SD of three different samples. Significance of inhibition, as compared to novobiocin are shown $*P < 0.05$ and $**P < 0.01$

Daunorubicin and mitoxantrone chemosensitivity

As shown in Table 2, daunorubicin chemosensitivity was examined in the presence or absence of the novel inhibitors in the Pgp overexpressing A2780/Adr cells. Likewise the impact of these inhibitors on mitoxantrone chemosensitivity was examined in the BCRP-overexpressing MCF7/mx cells. The sensitization factor (S.F.) describes the percentage fold increase of daunorubicin or mitoxantrone cytotoxicity in the presence of 10 μM of inhibitor. In order to control for non-specific effects due to toxicity of the inhibitor or interaction with other MDR factors, cytotoxicity was also examined in the wild-type cells which did not overexpress either Pgp or BCRP. For example, an increased cytotoxicity of daunorubicin was detected in verapamil treated non-Pgp expressing A2780/wt (S.F. 6.8), likely due to cytotoxicity of verapamil itself and/or inhibition of the MRP transporters. On the other hand, verapamil did not impact chemosensitivity of either MCF7/wt or MCF7/mx towards mitoxantrone. Hence, ratios of S.F. values determined in the overexpressing cells versus values in wild-type cell lines are also shown.

A pronounced increase in daunorubicin cytotoxicity was seen in the WK-X-34 and 84 and XR9577 treated

Table 2 Effect of inhibitors on daunorubicin/ mitoxantrone chemosensitivity

	Daunorubicin			Mitoxantrone		
	A2780/wt	A2780/Adr	S.F. ratio	MCF7/wt	MCF7/mx	S.F. ratio
	IC_{50} (μM)	IC_{50} (μM)	Adr/wt	IC_{50} (nM)	IC_{50} (nM)	mx/wt
Control	1.29 ± 0.1	40.4 ± 9.8	30	104 ± 13	22000 ± 4000	210
XR957	0.99 ± 0.1 (1.3)	3.8 ± 0.9 (10.5)*	8.1	85 ± 6 (1.2)	62 ± 40 (357)*	210
WK-X-34	1.06 ± 0.1 (1.2)	4.5 ± 0.7 (8.9)*	7.4	81 ± 8 (1.3)	44 ± 8 (506)*	390
WK-X-50	1.13 ± 0.06 (1.1)	18.5 ± 0.9 (2.2)	2	83 ± 3 (1.3)	460 ± 300 (48)*	40
WK-X-84	1.18 ± 0.03 (1.1)	5.1 ± 12.1 (7.9)*	7.1	87 ± 11 (1.2)	202 ± 190 (109)*	90
Verapamil	0.19 ± 0.08 (6.8)**	1.5 ± 0.2 (28)*	4.1	83 ± 5 (1.3)	22500 ± 14000 (0,9)	0.7
Cyclosporin	0.18 ± 0.16 (7.3)*	1.7 ± 0.3 (24)*	3.2	98 ± 6 (1.1)	86000 ± 21000 (0.26)	0.2
Novobiocin A						
Novobiocin				81 ± 7 (1.3)	40 ± 18 (550)*	420

Increasing concentrations of daunorubicin and mitoxantrone were added in the presence or absence of inhibitors as indicated. Cell viability was examined after 72 h with MTT assays and IC_{50} calculated. The sensitization factor (S.F.) describes the percentage fold increase of daunorubicin or mitoxantrone cytotoxicity in the presence of inhibitor (10 μM) and is shown in brackets for each cell line. The ratio of S.F. between resistant and wild-type cells was calculated as described in methods. Experiments were performed in triplicates on three individual occasions. IC_{50} results are shown as mean ± SD and *P* levels (* < 0.05, ** 0.01) were determined between drug treatment and controls within each cell line

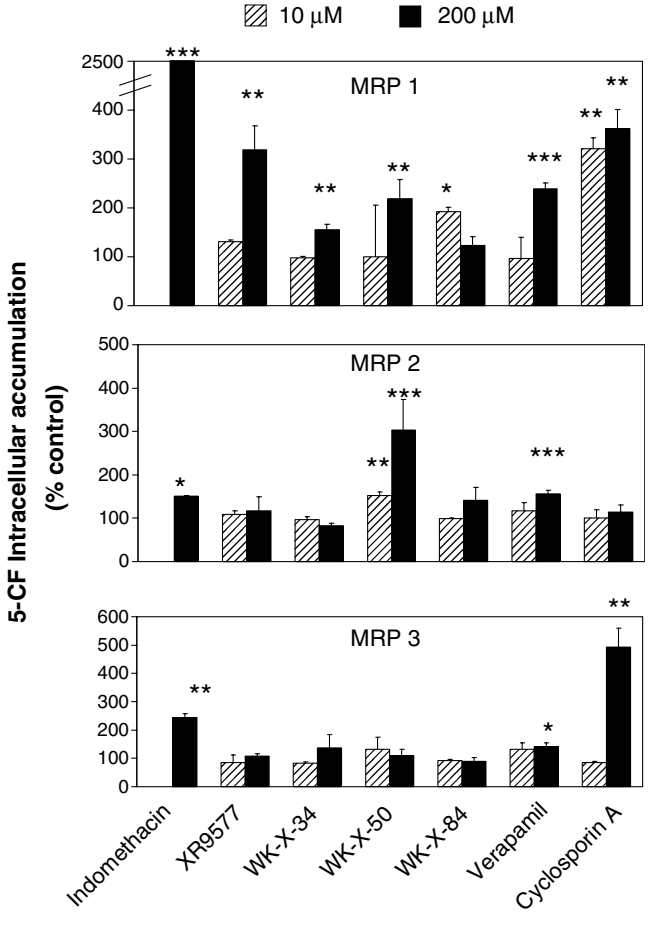

Fig. 6 Intracellular accumulation of 5-CF in MRP Transfected Cell Lines. Effects of 10 μM and 200 μM of inhibitors on **a** MRP1; **b** MRP2 or; **c** MRP3 -mediated efflux of 5-CF in selectively transfected HeLa-MRP1 and MDCK-MRP2/3 cells. Results are calculated as % of vehicle controls (control = 100%) and are shown as mean ± SD. Similar results were obtained in three separate experiments. Statistical differences(P = *<0.05;** <0.01; *** < 0.001) were determined using an unpaired *t* test

A2780 cell lines resulting in a S.F. of > 7 (S.F.: A2780 Adr/wt). Increased cytotoxicity of daunorubicin was seen in both A2780 Adr and A2780/wt cells in the presence of cyclosporin A, suggesting non-specific effects; likely stemming from cytotoxicity of cyclosporine A itself. Even more pronounced or dramatic differences were seen with mitoxantrone chemosensitivity in the presence of these inhibitors. Mitoxantrone cytotoxicity was substantially higher in MCF7/wt (IC_{50} 107 ± 13 nM) than in MCF7/mx (IC_{50} 22 ± 4 μM). In MCF7/wt, the inhibitors did not significantly improve cytotoxicity towards mitoxantrone and all S.F. control/treatment factors were close to 1. However, treatment of the BCRP overexpressing MCF7/mx cells with WK-X-34, XR9577 or novobiocin (10 μM) restored mitoxantrone cytotoxicity to levels seen in MCF7/wt cells with observed 210- 420 fold increases in S.F. values. WK-X-50 and WK-X-84 also increased mitoxantrone cytotoxicity in MCF7/mx cells by 37-fold and 91-fold, respectively.

Discussion

In this study we characterized four novel broad-spectrum MDR inhibitors, which were synthesized in our laboratory. Overall, WK-X-34 and XR9577 were the most effective Pgp and BCRP inhibitors. These compounds significantly increased intracellular accumulation and chemosensitivity towards mitoxantrone and daunorubicin in multiple cell lines. Pgp inhibition as measured in both, ^{99m}Tc-Sestamibi and daunorubicin transport assays was superior to that of cyclosporin A. Likewise, daunorubicin chemosensitivity was more effectively increased by WK-X-34 and XR9577 compared to cyclosporin A. Mitoxantrone chemosensitivity was dramatically increased by 200–400 fold with XR9577 and WK-X- 34 treatments, whereas cyclospo-

rin A did not show any effects. Cyclosporin A appears to be an unspecific broad-spectrum inhibitor with moderate effects on Pgp and MRPs. Furthermore effects on sensitive A2780/wt cells were also detected with cyclosporin-A. In comparison, the two compounds WK-X-34 and XR9577 demonstrate potent and specific inhibition of BCRP and Pgp-mediated transport and functionality with no effects in the corresponding wild type cell lines A2780/wt or MCF7/wt. For potential clinical applications, an increased target specificity and reduced cellular toxicity is required. Both were considerably improved for WK-X-34 and XR9577 as compared to cyclosporin A.

Thus far, inhibition of single MDR mechanisms like Pgp alone has not successfully reversed MDR in clinical applications. With recent discoveries that many MDR resistant tumors and important cancer stem cell populations express multiple MDR transporters, the importance of MDR inhibitors with broad-spectrum properties has been recognized [9, 27, 30]. The use of one effective broad-spectrum inhibitor versus single-spectrum inhibitors in combination may offer advantages such as preventing cumulative toxicities and interactions and ameliorating the pharmaceutical compliance of the therapy. To date, cyclosporin A has been one of the few MDR inhibitors to demonstrate satisfying performance in clinical applications [20, 32]. The fact that cyclosporin A is actually a broad-spectrum inhibitor with effects on Pgp, BCRP, MRP1 as well as LRP [27], underlines the importance of the broad-spectrum approach. One can argue, that the more effective and more potent 3rd generation Pgp inhibitors such as GF120918 [14] or XR9576 [23] are the current state of the art as Pgp inhibitors. However, despite extensive research and clinical development, these compounds have not yet received market authorization. Recent phase III trials using XR9576 in patients with lung cancer closed prematurely due to toxicity concerns. As successful clinical applications are the main motivation for the development of MDR inhibitors, Cyclosporin A was used as the reference compound.

Overexpression of MRP1 and LRP could be associated with MDR in AML and other malignancies. Their prognostic relevance however, remains unclear as studies have been published showing that there is no correlation between high MRP1 [11] and LRP [8] expression and clinical response in AML. In contrast, BCRP has received much attention since high expression levels were found in leukemic stem cell subpopulations, rendering them highly resistant towards chemotherapeutic treatment [28, 11]. BCRP-expression can imply incomplete eradication of cancer stem cells, which results in disease relapse with highly BCRP protected secondary tumors. Therefore, treatment strategies directed against Pgp and BCRP in combination might target the tumor together with its self-renewing cancer stem cell subpopulation [9]. It is plausible that clinical effectiveness of cyclosporin A may stem from potent Pgp inhibition combined with less potent BCRP inhibitory effects [31]. The WK-X-compounds show a strong similarity to the structure of GF120918 [14], which inhibits both BCRP and Pgp. Therefore, these common structures within the molecules might contain the BCRP binding domain. This is of particular interest as no BCRP pharmacophore model has been developed to date. In comparison to cyclosporin A, our novel WK-X- compounds, particularly WK-X-34 and XR9577 demonstrate stronger BCRP and Pgp modulating properties. They effectively inhibited BCRP-mediated efflux and chemosensitivity towards mitoxantrone in a way that was superior to that of the well-established BCRP inhibitor novobiocin [36].

Cyclosporin A, despite convincing performance in clinical trials, is associated with some toxicities particularly neurotoxicities [5, 13]. As clinical toxicities are a major limitation to the successful therapeutic application of MDR inhibitors, this presents a major disadvantage of cyclosporin A. The novel WK-X-compounds and XR9577 demonstrated significantly lower *in vitro* toxicities compared to cyclosporin A. Whether the reductions in *in vitro* toxicities of the WK-X-compounds and XR9577 can be translated into reduced clinical toxicities needs to be investigated in animal studies.

In summary we have identified and *in vitro* characterized novel broad-spectrum inhibitors among which the most effective, WK-X-34 and XR9577 were found to be very potent, specific and non-toxic inhibitors of Pgp and BCRP mediated MDR in different cell lines. Our compounds demonstrate advantages in the effectiveness and cellular tolerance over cyclosporin A, which thus far is the only broad-spectrum MDR inhibitor with convincing results from clinical trials in AML. Additional *in vivo* testing has already been initiated for WK-X-34 [15] to further examine these compounds for potential applications in Pgp and BCRP overexpressing tumors.

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