Mode of action of the novel phenazine anticancer agents XR11576 and XR5944

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The substituted phenazines XR11576 and XR5944 were originally described as dual topoisomerase-I/II poisons. Subsequent reports, however, indicated that the association of their cytotoxicity with cellular topoisomerases was not clear. We set out to study this further using human tumour cell lines, PEO1 ovarian cancer, MDA-MB-231 breast cancer and variants with acquired resistance to VP-16 and XR11576: PEO1VPR, MB-231VPR, MB-231-11576R and camptothecin: PEO1CamR. Cytotoxicity testing [3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide assay], DNA–protein crosslink formation, cell cycle analysis (flow cytometry) for DNA content, apoptosis (flow cytometry) for Annexin V and Western blotting for apoptotic factors. Cytotoxicity testing showed potent cytotoxicity with no cross-resistance to XR11576 or XR5944 in VP-16 or camptothecin-resistant lines. Importantly, we have shown for the first time that the activities of XR5944 and XR11576 are similar as MB-231-11576R cells were resistant to both agents and to a similar extent. XR5944 showed the greatest, albeit slower, interaction with DNA with high levels of DNA–protein crosslinks. Levels of apoptosis in XR5944-treated cells were significantly less than those in VP-16 or XR11576 treatments, suggestive of a more cytostatic rather than cytotoxic mode of action. Interestingly, XR5944 failed to give rise to a G_2/M blockade, in contrast to VP-16 or XR11576. XR5944 and XR11576, in line with a dual topoisomerase-I/II-directed mechanism of action, retain potent activity in tumour cells with acquired resistance to VP-16 and camptothecin. Although these agents appear to behave differently from each other according to experimental conditions, this study suggests a substantial overlap in their mechanism(s) of action. *Anti-Cancer Drugs* **18:139–148 © 2007 Lippincott Williams & Wilkins.**

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Introduction

Anticancer agents that target the DNA topoisomerases have a well-defined place in the current armamentarium of chemotherapeutic agents. Resistance to these compounds has, however, been demonstrated and may be attributable to expression of ABC-transporters such as P-glycoprotein (Pgp; ABCB1), the multidrug resistance protein (MRP1; ABCC1) and also to decreased expression of the target enzyme.

It has been demonstrated that inhibition of one type of topoisomerase can lead to alterations in the expression of another type. The concept that the inhibition of both topoisomerase enzymes would be superior over inhibition of either enzyme alone has been supported by several studies. The timed sequential treatment with a topoisomerase-I directed agent followed by a topoisomerase-II directed agent has proven supra-additive in a number of in-vitro and in-vivo studies [1,2]. Clinical trials have supported the observation that sequential treatment is beneficial with topotecan being given first, causing an increase in topoisomerase-IIα protein levels, followed by dosing with VP-16 [3,4]. A phase II study of sequential topotecan and oral etoposide in small cell lung cancer patients also showed increased efficacy [5].

Although combining topoisomerase-I- and topoisomerase-II- directed agents can show improved efficacy, the toxicity with combined use of topoisomerase-I- and topoisomerase-II- directed agents has been reported to be unacceptably high [6]. To address this issue, several compounds have recently been developed and are designed to act as dual inhibitors of topoisomerase-I and topoisomerase-II. These include DACA [7,8], intoplicine, Tas-103 [9] and the dual catalytic inhibitor F11872 [10].

XR11576 (MLN576) and also XR5944 (MLN944) (see Fig. 1) originated from a drug development programme designed to target both topoisomerase-I and

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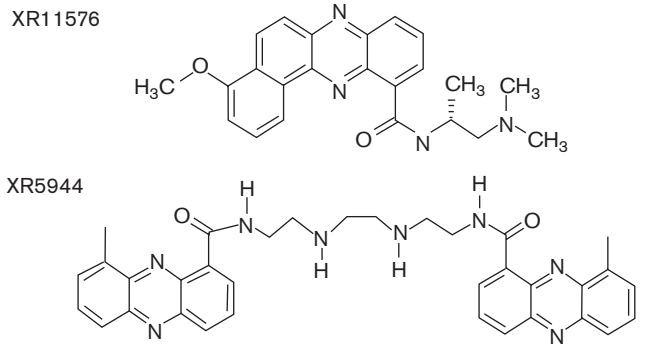
Fig. 1



Structures of XR5944 and XR11576.

topoisomerase-II. These agents showed potent cytotoxic activity in a variety of in-vitro and in-vivo tumour models [11,12]. Data obtained demonstrated that both compounds could stabilize topoisomerase-I- and topoisomerase-II-mediated cleavable complexes as dual topoisomerase I/II inhibitory drugs [13]. Furthermore, XR11576 and related DACA family of acridine carboxamide anticancer agents are DNA intercalators, which act as dual DNA topoisomerase I/II inhibitors [14]. The bisphenazine XR5944 shows some structural similarity to these agents but there is evidence in the literature that its mechanism of action is distinct from that of XR11576 [15]. Work by Dai *et al.* [16] suggested that the DNA-binding mode for the two compounds dictated differing mechanisms of action. Furthermore, XR5944 treatment has been shown to result in upregulation of RNA polymerase subunits and also of genes involved in rRNA processing, suggestive of alternative mechanisms of action in a yeast model system [17].

We set out to further study the mode of action of the two phenazine compounds using a panel of drug-resistant cell lines. Importantly, our more recent studies involved the generation of a cell line with acquired resistance to the agent XR11576, enabling us to identify any association between the mechanisms of action of the two test compounds.

In the present study, we describe the efficacy of XR5944 and XR11576 in cell lines with altered topoisomerase levels as a consequence of acquired resistance to single topoisomerase poisons VP-16 and camptothecin. In addition, to compare and contrast the in-vitro behaviour of these two agents, we looked at their interaction with DNA and also examined their ability to induce apoptosis.

Methods

Reagents and chemicals

All chemical reagents were of the most pure analytical grade possible and obtained from Sigma Aldrich (Poole, UK), unless stated otherwise. XR5944, XR11576 and XR9576 (tariquidar), as a specific modulator of Pgp (as described in [18]) supplied by Xenova (Slough, UK) were dissolved in dimethyl sulphoxide as concentrated stock solutions and stored at −20°C. Doxorubicin (DOX) hydrochloride (Sigma Aldrich) was dissolved in sterile water, whereas VP-16 and camptothecin (Sigma Aldrich) were dissolved in dimethyl sulphoxide and stored at −20°C as concentrated stock solutions.

Cell lines and culture conditions

All cell culture reagents were obtained from Sigma Aldrich, unless stated otherwise. PEO1 human ovarian cancer cells (previously obtained from ICRF, London, UK) were cultured in RPMI-1640 and MDA-MB-231 human breast cancer cells (obtained from ECAAC, Salisbury, Wiltshire, UK) in Dulbeccos' modified Eagle's medium at 37°C/5% CO_2. Media was supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, Paisley, UK), 2 mmol/l glutamine, penicillin and streptomycin. Cells were routinely tested for mycoplasma using a polymerase chain reaction-based method. Drug-resistant PEO1 and MDA-MB-231 cells were generated by growth in step-wise increments of the appropriate inducing agent over a number of months (less than 12 months) until a stable resistance phenotype was achieved. The stability of the resistance phenotypes was monitored over a period of 1 year following their derivation and found to be stable with the given maintenance dose. Moreover, all cell line variants showed stability of their drug resistance for at least 1 month following withdrawal of their maintenance dose. Cell lines were grown up routinely in the presence of a maintenance dose of drug, which was withdrawn for at least one passage of the cells prior to use in experiments. These were for PEO1CamR cells 3 nmol/l camptothecin, PEO1VP-16R cells 0.2 µmol/l VP-16, MB-231VP-16R 1 µmol/l VP-16, MB-231-11576R 40 nM.

3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay

Cytotoxicity testing was determined by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as described previously, [19] using continuous drug exposure for 72 h. IC_{50} values were determined as the drug concentration necessary to cause a 50% reduction in cell viability compared with untreated controls. To look at the effects of Pgp modulation in the presence of either XR5944 or XR11576, the modulating agent XR9576 was added simultaneously with the appropriate test agent to give a final concentration of 100 nmol/l per well in the MTT assay at the beginning of the drug incubation period, as described above.

Western immunoblotting

Crude cell membrane preparations were made from cells by lysis with buffer [150 mmol/l NaCl, in 10 mmol/l Tris buffer (pH 7.4) and protease inhibitors: 1 mmol/l

phenylmethylsulphonyl fluoride, $NaVO_4$, aprotinin and leupeptin] at 0°C followed by ultracentrifugation (30 000 r.p.m, 60 min, 4°C) and the pellet is finally resuspended in buffer containing 0.2% sodium dodecyl sulphate (SDS) and 1% NP-40. For nuclear preparations, trypsinized cells were washed in phosphate-buffered saline (PBS) and lysed in nuclear buffer (30 mmol/l Tris pH 7.5, 1.5 mmol/l $MgCl_2$, 10 mmol/l KCl, and protease inhibitors, 1% Triton X-100, v/v). Samples were vortex mixed, cooled and centrifuged (16 000 *g*, 2 min). The supernatant (cytosolic fraction) was incubated with 0.25 mg/ml RNase, 0.2% SDS and 1.2 mmol/l dithiothreitol. Pelleted nuclei were resuspended in 0.2% SDS, 10 mmol/l dithiothreitol, 50 mmol/l Tris (pH 7.5), and 5 mmol/l $MgCl_2$ and protease inhibitors. Both fractions were incubated at 4°C for 60 min with frequent agitation. Preparations were subjected to SDS–polyacrylamide gel electrophoresis and membranes reprobed with β-actin antibody (Ab-1 rabbit polyclonal antibody; (CN Biosciences, Beeston, UK) to check for uniformity of sample loading.

Cell cycle analysis using propidium iodide

PEO1 human ovarian cancer cells at a density of 4×10^4 cells/ml were left to adhere in flasks for 4–6 h and then processed as indicated in the figure legend of Fig. 5.

DNA protein crosslinks

The method used was modified from that described by Zhitkovich and Costa [20]. MDA-MB-231 cells were seeded at 3×10^4/ml into 80-cm^2 flasks and left for 4 h at 37°C. DNA was labelled with 0.5 mCi/ml of media of tritiated thymidine (specific activity 20 Ci/mmol) for 48 h and then replenished with fresh medium. Cells were drug treated and left at 37°C for the designated time period. Samples for the 4-h time point were treated with the IC_{50} concentration of drug obtained after 24-h acute drug exposure using the MTT assay (VP-16 24 µmol/l, XR5944 150 nmol/l or XR11576 300 nmol/l). It was necessary to adopt a different dosing schedule for 24-, 48- and 72-h samples; otherwise, there was too much cellular fragmentation and measurement of DNA–protein crosslinks (DPC) was compromised. For these extended time points, cells were treated with VP-16 0.5 µmol/l, XR5944 7 nmol/l or XR11576 30 nmol/l, which were approximately 25% of the IC_{50} concentration obtained after 72 h of continuous drug exposure (or less in the case of VP-16, as a higher dose was shown to give rise to excessive cell fragmentation at later time points), using the MTT assay. Control flasks were treated with drug solvent alone. To harvest, adherent cells were removed by scraping, the resulting slurry centrifuged and washed in PBS, and an aliquot taken to determine cell number using a counting chamber. Cells were processed as in [20] and finally resuspended in water and added to Optiphase Safe scintillant (Fisher Scientific, Loughborough, UK). Disintegrations per minute were counted over 1 min and treated samples were compared with control untreated samples.

Assays for detection of apoptosis

PEO1 human ovarian cancer cells were treated for 48 or 72 h with IC_{80} doses of drug, based on MTT assay data obtained using continuous drug exposure. The doses were for VP-16 5 µmol/l, XR5944 100 nmol/l and XR11576 250 nmol/l.

Annexin V apoptosis assay

An Annexin V-fluorescein isothiocyanate (FITC)-conjugated apoptosis detection kit was used as described by the manufacturer's protocol (Oncogene, supplied by CN Biosciences). 1×10^6 PEO1 cells were harvested by trypsinization, washed twice in cold PBS and processed according to the manufacturer's instructions. Samples were analysed by flow cytometry, using the FL1 and FL3 settings, each reading using collection of 10 000 events.

Activated caspase-9

Analyses were carried out using the CaspaTag caspase-9 activity kit (Intergen, supplied by Flowgen, Leicester, UK). This methodology uses a fluorogenic substrate FAM-LEHD-FMK (with high specificity for caspase-9), which becomes fluorescent upon being cleaved within the cell and covalently bound to the active caspase. Cells that were bound to the substrate were analysed by flow cytometry on the FL1 setting detecting FITC-associated fluorescence.

Western immuno-blotting for BCL_{xl}

Western blotting experiments were carried out using whole-cell lysates, as described above. Forty to fifty micrograms of cellular protein were harvested from monolayers of cells treated in flasks for the designated time periods.

Results

Cytotoxicity testing

Table 1 shows cytotoxicity data obtained for the parental and MDR cell lines HL-60AR (MRP1 expressing) and K562AR (Pgp expressing) treated with DOX, XR5944 and XR11576. The levels of DOX resistance were approximately 8-fold for HL-60AR and 7-fold for K562AR, in line with previously established findings [21]. As shown by the IC_{50} values, XR5944 is recognized by the ABC transporters Pgp and MRP, whereas only residual resistance was seen for XR11576. K562AR showed 42- and 2-fold resistance to XR5944 and XR11576, respectively. The values for HL-60AR in the same experiments were 25- and $\leq$ 1-fold, respectively.

Table 1 also shows the reversal of XR5944 resistance in the Pgp-expressing K562AR line. The Pgp-specific modulator XR9576 reversed resistance to XR5944 with

Table 1 IC_{50} values (nmol/l) with chronic 72-h exposure, obtained using the MTT assay for K562 and HL-60 parental and MDR lines (SD of >3 replicate analyses, shown in parenthesis)

Cell line	Doxorubicin	XR5944 + XR9576		XR11576 + XR9576	
HL-60	40.0 (10.1)	6.0 (4.0)	ND	65.0 (10.0)	ND
HL-60AR	300.0 (50.0)	150.0 (50.0)	ND	50.0 (15.0)	ND
K562	1205 (200.0)	0.8 (0.1)	0.5 (0.1)	22.4 (10.1)	27.1 (13.9)
K562AR	8000 (110.0)	33.8 (13.1)	2.8 (0.2)	48.8 (2.9)	32.8 (17.2)

MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; ND, no determined.

Table 2 IC_{50} values (nmol/l) with chronic 72-h exposure, obtained using the MTT assay with drug-resistant human breast and ovarian cancer cell lines models. (SD for >3 replicate analyses shown in parenthesis)

Cell line (fold-resistance)	Camptothecin	VP-16	XR5944	XR11576
PEO1	5.3 (0.8)	160.0 (70.0)	17.0 (4.1)	45.3 (2.0)
PEO1CamR (3-fold)	15.3 (0.5)	650.0 (250.0)	12.7 (5.7)	66.2 (12.1)
PEO1VP16R (10-fold)	7.8 (0.9)	1700.0 (190.0)	17.1 (2.6)	48.8 (5.5)
MB-231	610.0 (40.0)	11 000.0 (270.0)	28.0 (2.4)	126.0 (13.7)
MB-231VPR (6-fold)	1290.0 (200.0)	65 000.0 (5000.0)	30.0 (4.1)	135.0 (8.7)

MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide.

a shift in IC_{50} values from a mean of 33.8 to 2.8 nmol/l ($P \leq 0.020$). The effect of XR9576 on sensitivity to XR11576 in the same experiments was modest with a shift in mean IC_{50} of 48.8–32.8 nmol/l ($P \geq 0.05$).

Cytotoxicity of VP-16, camptothecin, XR5944 and XR11576 on drug-resistant cell lines

Data shown in Table 2 demonstrate the levels of drug resistance of the PEO1 and MDA-MB-231 lines to the respective inducing agent used, and the sensitivity of these lines to XR5944 and XR11576. For the camptothecin-resistant line PEO1CamR, there was no apparent cross-resistance to VP-16, XR5944 or XR11576. Likewise, for both the VP-16-resistant lines, there was no cross-resistance seen to either XR5944 or XR11576.

Cross-resistance profile of the XR11576-resistant cell line

As shown in Fig. 2, the level of resistance of MB-231-11576R to XR5944 was similar to that seen for XR11576: IC_{50} values: XR11576 27.8 and 90.4 nmol/l; XR5944 29.7 and 86.2 nmol/l, for MB-231 parent and MB-231-11576R, respectively. Interestingly, resistance to VP-16 in this line was some 11-fold (IC_{50} values 5.9 and 67.7 μmol/l, MB-231 and MB-231-11576R, respectively) with somewhat lower resistance to camptothecin at 2.9-fold (IC_{50} values 104 and 295 nmol/l, MB-231 and MB-231-11576R, respectively). There is evidence, therefore, for resistance to topoisomerase-I- and topoisomerase-II-directed mechanisms in the MB-231-11576R line, and this also corresponds to cross-resistance to the closely related agent XR5944. These data provide evidence that the mechanism of action of XR5944 is similar to that for XR11576. In addition, if cells are repeatedly exposed to XR11576, they appear to modify their sensitivity to topoisomerase-I- and topoisomerase-II- directed agents.

Expression of ABC transporters and topoisomerase-I and topoisomerase-II proteins in drug-resistant cell lines

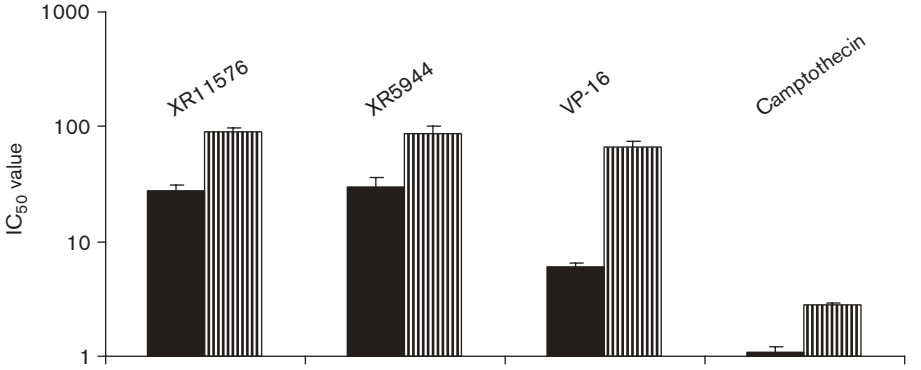
Western immunoblotting for the detection of ABC transporter proteins was carried out using the K562AR, HL60AR and T8 human tumour cell lines as controls for Pgp, MRP1 and BCRP, respectively, [22] Western blotting showed that there was no over expression of these ABC transporter proteins in either camptothecin VP-16 or XR11576-resistant cell lines, as shown in Fig. 3.

The drug resistance profiles of VP-16 and camptothecin-resistant cell lines were further characterized by analysis of their topoisomerase expression levels by Western blotting. Cell fractions were assessed for expression of topoisomerases that were clearly seen in the nuclear preparations, but were absent from the cytosolic fractions (Fig. 4). Camptothecin-resistant PEO1CamR cells showed a marked decrease in topoisomerase-I and -IIα and -β protein expression. VP-16-resistant PEO1VPR cells showed a decrease in both topoisomerases-IIα and topoisomerase-IIβ, but no reduction in the expression of topoisomerase-I when compared with parental PEO1 cells. MB231VPR cells showed a combined very marked decrease in topoisomerase-IIα and topoisomerase-IIβ proteins, and unchanged levels of topoisomerase-I.

DNA cell cycle analysis

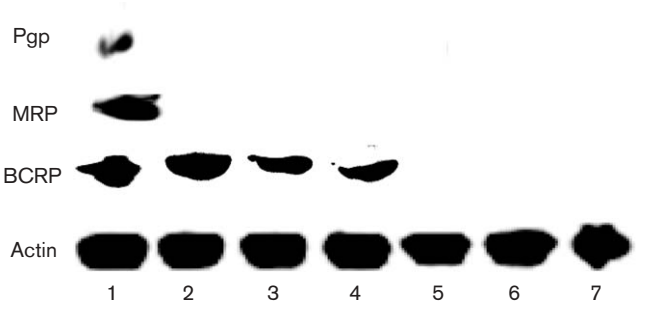
Treatment of PEO1 cells with a sublethal dose of VP-16 showed a substantial accumulation of cells in G_2 and a reduction in G_1 phase of the cell cycle, as indicated in Fig. 5. An equicytotoxic dose of XR11576 treatment also gave rise to a similar effect on cell cycle perturbation as the classic topoisomerase-II poison with a large accumulation of cells in the G_2/M phase. In contrast, the cell cycle analysis of cells treated with an equicytotoxic dose of XR5944 revealed no significant changes in the phases

Fig. 2



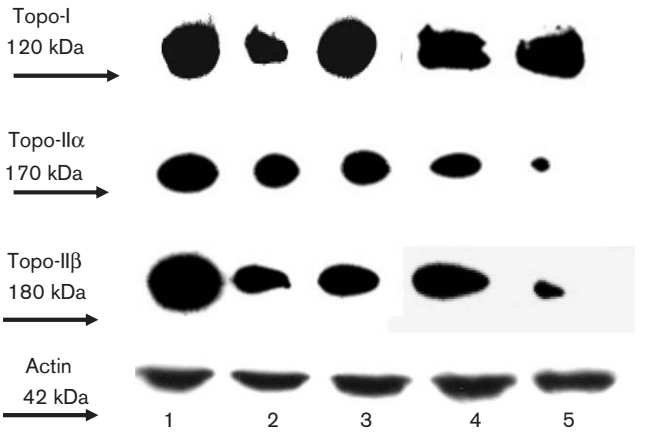
Bar graph showing drug resistance profile of MB-231 (solid black bar) and MB-231-11576R cells (striped bar). Data shown are the mean and SD. Shown are the error bars for more than three repeat experiments.

Fig. 3



Western immunoblotting data showing the lack of expression of ABC transporters P-glycoprotein (Pgp), multidrug resistant protein (MRP) or BCRP in cell lines with acquired resistance to topoisomerase-directed agents. Cell lines used as controls were K562/AR (Pgp-expressing human myelogenous leukaemia cell line) [21] and HL-60 (MRP-expressing human promyelocytic leukaemia line) (both obtained from Jean Sargent, Pembury Hospital, Kent, UK); BCRP-overexpressing cell line T8 was used as previously described [22] and was provided kindly by Professor Jan Schellens, Netherlands Cancer Institute (Amsterdam, Netherlands). Forty to fifty micrograms of protein were run on a 4–12% acrylamide sodium dodecyl sulphate–polyacrylamide gel electrophoresis gel using the Novex system (Invitrogen). Proteins were then transfer blotted for 2 h on to nitrocellulose membranes and blocked for at least 1 h in 1% skimmed milk w/v, 1% bovine serum albumin w/v and 0.1% Tween 20 in phosphate-buffered saline. Membranes were incubated overnight at 4°C with primary antibody. After washing, membranes were incubated with a secondary horseradish peroxidase-linked appropriate species antibody preparation. Visualization of results was achieved using chemiluminescence (Pierce Supersignal) with Biomax X-ray film (Sigma). Primary antibodies used were for MRP C-20 antibody, MDR1 H241antibody and BCRP Ab-1 (CN Biosciences). Forty micrograms of crude membrane preparations were loaded onto sodium dodecyl sulphate–polyacrylamide gel electrophoresis gels and subjected to immunoblotting for the detection of Pgp, MRP and BCRP.
Lane (1) MDR control cell line; (2) MB-231 parent; (3) MB-231VPR; (4) MB-231-11576R; (5) PEO1 parent; (6) PEO1VPR; (7) PEO1CamR.

Fig. 4



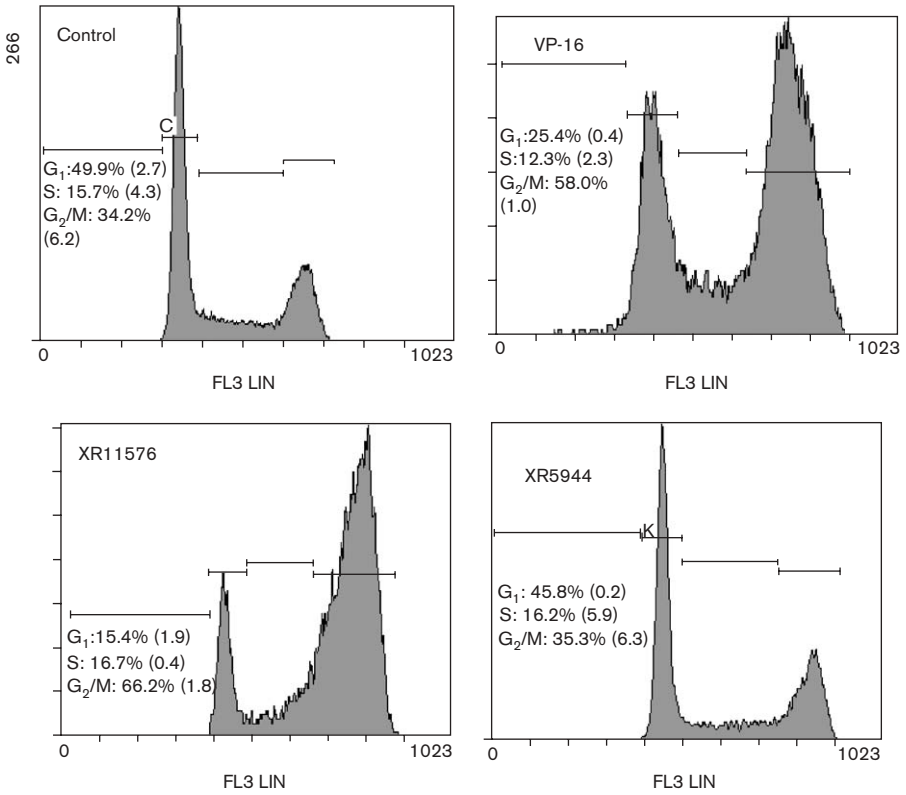
Western immunoblotting result showing levels of topoisomerase (Topo) expression in drug-resistant cell line models using 20 μg of nuclear preparations. Antibodies used were for: topoisomerase-I C-15 antibody, topoisomerase-IIα H-231 antibody, topoisomerase-IIβ H-286 antibody (all obtained from Autogen Bioclear, Calne, UK). Lane (1) PEO1 parent; (2) PEO1CamR; (3) PEO1VPR; (4) MB-231 parent; (5) MB-231VPR.

of the cell cycle as compared with control untreated cells and under the same experimental conditions. This unusual finding was confirmed in a number of repeat analyses and was suggestive of a growth inhibitory effect as a sub-G_1 peak did not form even at later time points (data not shown). There was a reduction in cell growth for the duration of the experiments, when cells were treated with XR5944 and at even higher concentrations.

DNA–protein crosslink formation owing to XR11576 and XR5944

The peak of DPC formation in MDA-MB-231 drug-treated tumour cells varied between the agents XR11576 and XR5944, as seen in Fig. 6. Although both agents were shown to induce appreciable levels of DPC, XR5944 treatment gave rise to the highest levels of these adducts,

Fig. 5



Data showing flow cytometrically generated DNA histograms of drug-treated PEO1 cells using propidium iodide staining. Cells were treated with drug at approximately twice the IC_{50} dose (obtained from the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay data with a continuous exposure over 72 h) and harvested 48 h later. Drug doses corresponded to VP-16 300 nmol/l, XR5944 35 nmol/l and XR11576 80 nmol/l. Cells were harvested and washed in phosphate-buffered saline before resuspension in 70% ethanol in phosphate-buffered saline, added during vortex mixing and left at 4°C for at least 24 h. After washing in phosphate-buffered saline, cells were stained in 10 μg/ml propidium iodide (Sigma) and 1 mg/ml ribonuclease A (Sigma) for at least 30 min at 37°C in the dark. Fluorescence >575 nm versus light scatter was measured with excitation of 488 nm on a Beckman-Coulter Epics-XL. Data shown on each histogram are representative of more than three separate experiments with the mean and standard deviation for each phase of the cell cycle quoted for the individual drug treatments.

which were highest at 72 h. In contrast, XR11576 treatment gave rise to DPC formation peaking at 24 h. These data are suggestive of differences in the temporal nature of DNA interaction for the two experimental agents. Although the levels of DPCs formed in XR11576-treated cells were always significantly increased compared with control cells ($P \leq 0.001$), the levels in XR5944-treated cells did not reach levels of significance until 48 h ($P \leq 0.001$) and rose considerably up to the 72 h time point.

Effects of XR11576 and XR5944 on induction of apoptosis

Annexin V

The data in Fig. 7 indicate that XR5944 at a high dose is capable of inducing apoptosis even though the Annexin V data suggested that, at the same time point of 48 h, the extent of apoptosis was more modest than that seen with XR11576. Data show the mean and standard deviation obtained for three replicate analyses.

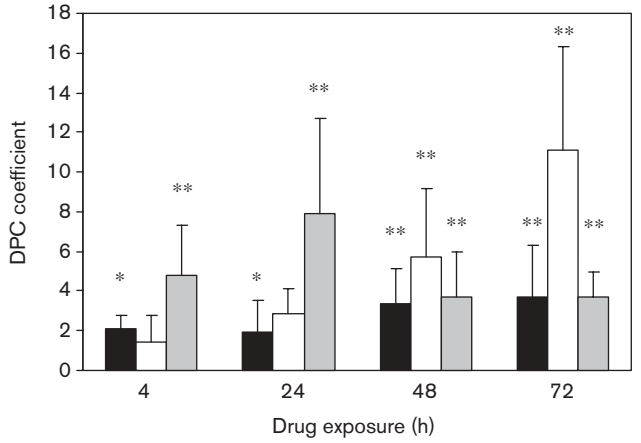
Caspase-9 activation

Figure 8(a) shows the data obtained using the caspase-9 activation assay for PEO1 cells treated with VP-16 as a reference compound alongside XR11576 and XR5944. At the 48-h time point, a similar extent of caspase-9 activation was seen for the two test compounds, as indicated by the gated regions. Caspase-9 activation is expected to be an event associated with early mid stage apoptosis. The data also indicate that both XR11576 and XR5944 are capable of inducing the mitochondrial pathway of apoptosis.

BCL_{xl} levels

VP-16 treatment of PEO1 cells gave rise to a reduction in the anti-apoptotic factor BCL_{xl}: at 48 h. The effects of XR5944 and XR11576 were more marked with levels being substantially reduced at both 24 and 48 h (Fig. 8b).

Fig. 6



Bar graph showing levels of DNA protein crosslinks (DPCs) measured as DPC coefficient versus time of drug exposure in MB-231 cells treated with topoisomerase-directed agents. *Indicates a level of statistical significance of <0.05, relative to untreated controls. **Indicates a level of statistical significance of <0.001, relative to untreated controls. The concentrations of drug were as follows: at 4 h VP-16 24 μmol/l, XR5944 150 nmol/l and XR11576 300 nmol/l. At 24–72 h, the doses were VP-16 0.5 μmol/l, XR5944 7 nmol/l, XR11576 30 nmol/l. Key: VP-16, solid black bars; XR5944, white bar; XR11576, grey bar.

Discussion

We present data on two new cytotoxic agents XR11576 and XR5944 which were originally proposed to inhibit topoisomerases types I and II [11,12]. With any developmental antitumour agent, it is important to understand their mechanism of action, as this can inform and guide the design of clinical trials and our studies in line with those of others have set out to address this. Importantly, it has remained uncertain whether altered expression of cellular topoisomerases was, indeed, relevant to the apparent cytotoxicity of the substituted phenazines. We attempted to address this using tumour cell lines with varying levels of topoisomerase-I/II expression. We showed data that suggests XR5944 and XR11576 may act as dual topoisomerase-directed agents as they retained their activity in the drug-resistant cell lines irrespective of the large variation in levels of topoisomerase expression.

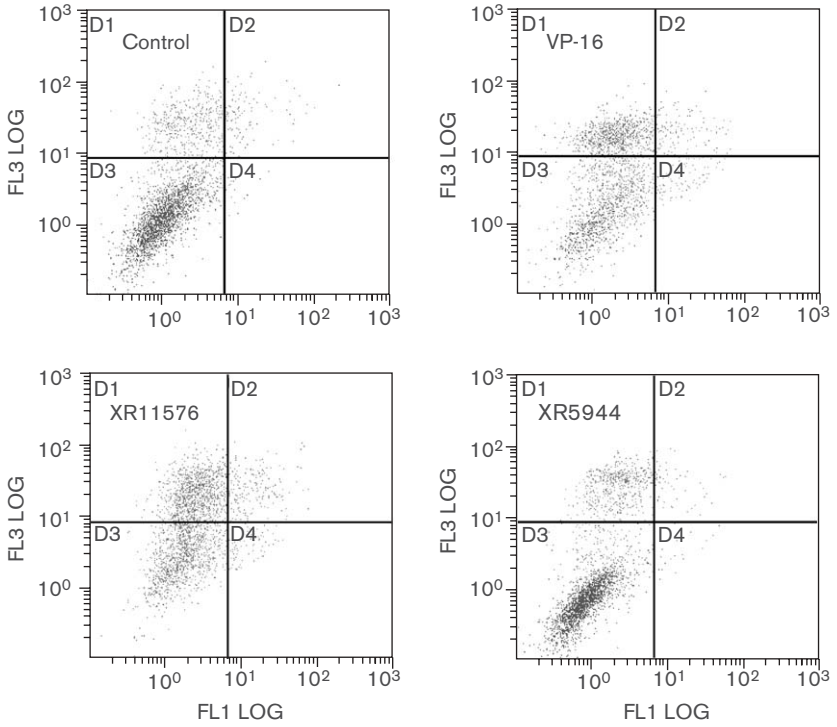
We have used models of breast and ovarian cancers, as these are recognized as tumours with a high incidence of drug resistance that are sometimes associated with elevated topoisomerase levels. Ovarian cancers have been shown to express high levels of both topoisomerase-I and topoisomerase-II [23]. There are reports in the literature of coamplification of *erbB2* and *topoIIα* in breast cancer cell lines and cases of breast cancers [24,25]. Our choice of breast and ovarian tumour cell lines was supported by the findings of Di Nicolantonio *et al.* [26], who reported the ex-vivo activity of XR11576 being highest in fresh tumour cells from breast and ovarian cancers. Hence, the development of agents such as XR11576 and XR5944 may lead to more effective treatments for breast cancers, with possibility for combination with established chemotherapeutic agents and also with new targeting agents such as herceptin, particularly, if they show no evidence of cardiotoxicity.

In Pgp-expressing K562AR cells, we demonstrated resistance to XR5944 but not XR11576, in line with previous findings [11]. This was circumvented by the addition of Pgp-specific modulator XR9576. In spite of the fact that XR5944 appeared to be recognized by ABC transporters, as shown *in vitro*, it is a very potent new antitumour agent. Moreover, this observation has not compromised its entry into clinical development. Importantly, MB-231-11576R cells showing cross-resistance to XR5944 in the absence of P-gp overexpression suggests that there is substantial overlap in the activities of the two phenazine agents in this study (as cross-resistance to XR5944 could merely be a consequence of Pgp overexpression).

Both XR11576 and XR5944 are highly DNA interactive agents, as seen by the production of DPC in drug-treated human PEO1 ovarian cancer cells. The production of these adducts is very characteristic of cleavable complex formation by agents such as VP-16, but not a finding exclusive to topoisomerase-interactive agents [27]. In addition, other laboratory studies demonstrated that the cytotoxicity of both XR5944 and XR11576 was similarly increased by reducing glutathione levels by using buthionine sulphoximide (unpublished data), as described for other topoisomerase-interactive agents such as CPT-11 [28].

The events that culminate in the apoptotic response have been shown to be a consequence of several mitochondrial events, including cytochrome *c* release, and subsequent activation of caspase-9 and caspase-3. VP-16 is known to promote apoptosis either in cancer or in normal cells as a side effect. Although the data obtained for XR5944 showed a more modest, but measurable level of apoptosis at 48 h, this factor does not restrict its in-vivo efficacy [11]. The activation of caspase-9 (Fig. 8) is indicative of a role for the mitochondrial-mediated pathway of apoptosis and both agents showed an ability to activate this caspase. We performed additional experiments that provide evidence for the activation of the mitochondrial pathway of apoptosis. Enhanced pro-apoptotic t-BID and Bax expression was seen in PEO1 cells treated with either XR5944 or XR11576 (data not shown), providing evidence of mitochondrial pore formation. Furthermore, a degradation of the anti-apoptotic factor BCL_{xl} and also BCl2 (data not shown) was seen with reduced levels in drug-treated cells.

Fig. 7



Data showing percentage of cells in apoptosis using PI (FL3) versus Annexin V–FITC (FL1)

Sample	D1	D2	D3	D4
Control	13.7 (1.9)	2.7 (1.2)	82.7 (1.2)	1.3 (1.5)
VP-16	45.0 (5.7)	11.3 (4.9)	40.0 (9.6)	3.7 (0.5)
XR11576	40.8 (6.3)	7.8 (3.8)	47.8 (8.2)	3.5 (1.8)
XR5944	11.0 (5.5)	2.8 (2.1)	85.3 (6.4)	1.3 (0.8)

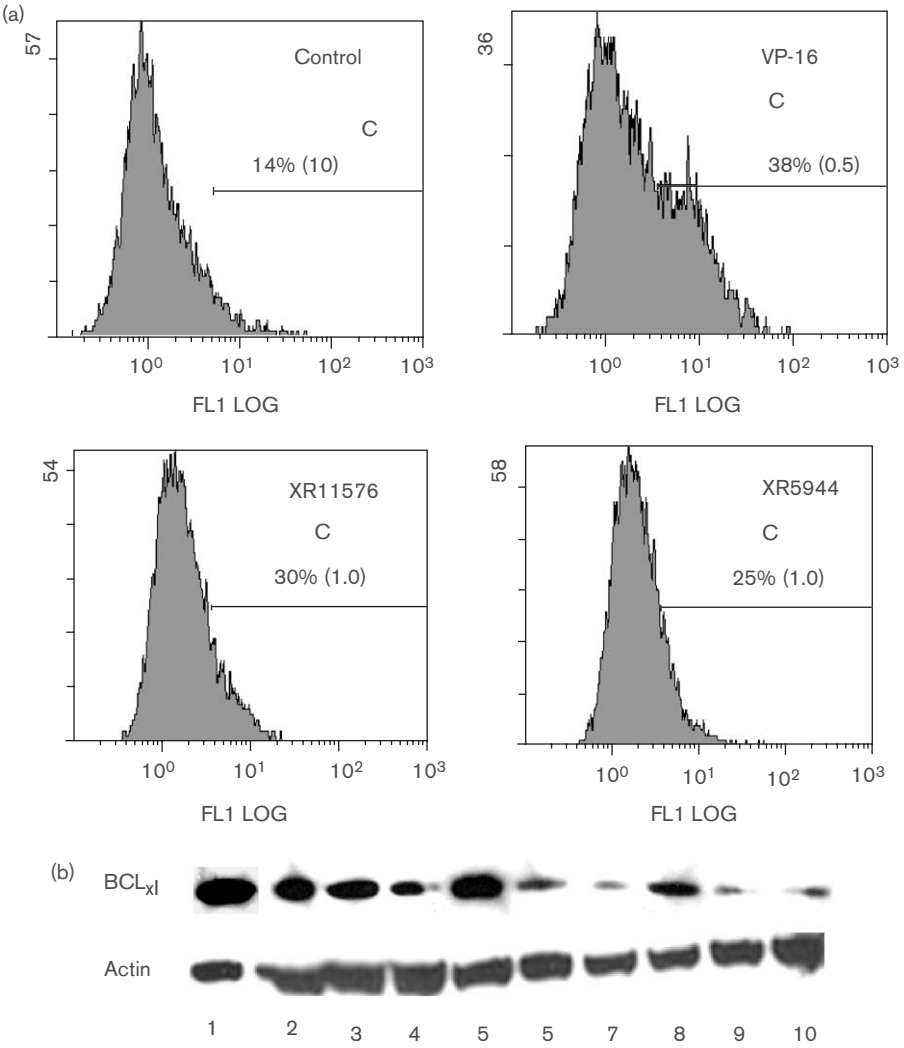
Data shown are mean percentage from more than three repeat experiments with the standard deviation shown in brackets.

Cytograms generated using the Annexin V assay showing extent of propidium iodide staining (*y*-axis) versus fluorescein isothiocyanate (FITC) staining (*x*-axis) for PEO1 cells treated with topoisomerase-directed agents. The quadrants indicate the relative populations (as a percentage) of cells showing characteristics of apoptosis: lower left: healthy, live cells; lower right: early-stage apoptosis, Annexin V positive, propidium iodide negative; upper left: dead, necrotic/very-late-stage apoptosis propidium iodide-positive, Annexin V weakly positive; upper right: intermediate/late-stage apoptosis, Annexin V-positive, propidium iodide-positive. The entire data set are summarized in the adjacent table. The concentrations of drug were as follows: VP-16 5 μmol/l, XR5944 100 nmol/l and XR11576 250 nmol/l.

The data we have shown in the present study have suggested many similarities between the activities of XR5944 and XR11576 at the cellular and molecular level. The timing of the cytotoxicity, profile of DPC formation and cell cycle changes, however, were clearly different for the two compounds. It is not clear whether the cell cycle effects seen are a consequence of the mutant p53 status of the PEO1 cell line. Furthermore, like XR5944 acting as a substrate for Pgp, there are structural differences between the two compounds relating to their binding to DNA that could dictate differing mechanisms of action. The *bis*-phenazine structure of XR5944 and its capability of bis-intercalation should allow more π-orbital interactions with DNA [16] than the *mono*-phenazine XR11576. In addition, differences in side-chains should allow further interactions and greater hydrogen bonding in the case of XR5944 rather than XR11576. Along with its high potency, the greater number of potential DNA interactions with XR5944 may be reflected in the substantial levels of DPCs seen in drug-treated cells. Recently, it has been shown that XR5944 has a specific binding site on DNA, which is recognized by a number of transcription factors [16]. Sappal *et al.* [15] showed that the type of cleavable complex formed by XR5944 was markedly different from those seen with either VP-16 or camptothecin, using naked plasmid DNA. In addition, they suggested that neither topoisomerase-I nor topoisomerase-IIα were the cellular targets for XR5944. The predominant cell cycle perturbation was, however, shown to be G_2/M in the same yeast model system. In contrast to those studies, we have used a whole-cell tumour cell system, which may account for some of the data we have generated in the present study that does not agree with that of Sappal *et al.* [15].

Other data provided in the present study are suggestive of a similarity in the mechanism of action between XR5944 and XR11576. We have shown that XR11576 shares many similarities with established topoisomerase-interactive agents and certainly resembles a topoisomerase-directed agent more than XR5944. Moreover, if cells are repeatedly treated with XR11576, as in the case of drug-resistant MB-231-11576R, it is clear that this attenuates sensitivity to topoisomerase-directed agents (i.e. MB-231-11576R has 11-fold resistance to VP-16) and in the absence of MDR proteins. The most conclusive

Fig. 8



(a) Flow cytometric data showing channel number versus level of fluorescein isothiocyanate-associated fluorescence (on FL1) indicative of the extent of caspase-9 activation using an LEHD-*fmk* fluorescently tagged peptide. The region containing the population of cells showing activation of caspase-9 was clearly defined and gated according to their increased level of fluorescein isothiocyanate-associated fluorescence. Each analysis involved the collection of 10 000 events. The fluorescence signal show a right shift indicative of caspase-9 activation in PEO1 human ovarian cancer cells, with a quantitation of the region given relative to the control, untreated cell population. Data shown are the mean percentage obtained from the populations of cells showing increase in FITC-associated caspase-9 activation and standard deviation for three separate experiments given in parentheses. Drug concentrations were as for Fig. 7. (b) Western blot showing response of the anti-apoptotic protein BCl_{xl} in PEO1 cells treated with topoisomerase-inhibitory agents. Lane (1): control untreated cells; (2) VP-16-treated cells (5 μmol/l) at 12 h, (3) 24 h and (4) 48 h; (5) XR5944-treated cells (100 nmol/l) at 12 h, (6) 24 h and (7) 48 h; (8) XR11576-treated cells (250 nm) at 12 h, (9) 24 h and (10) 48 h. The BCl_{xl} antibody used (H-5) was obtained from Autogen Bioclear, Calne, Wiltshire, UK.

finding from this part of the study is that there is considerable similarity between the mechanism of action for XR11576 and XR5944 based on the cross-resistance profile of MB-231-11576R. This also, provides support evidence that agents XR11576 and XR5944 are associated with topoisomerase interaction as sensitivity to topoisomerase-directed agents is significantly attenuated in our whole-cell system. Although we show indirect evidence for topoisomerase-directed activity by XR5944 and XR11576, we acknowledge that this could be secondary to their principal mode of action. Certainly for XR11576, a non-topoisomerase-directed mode of action has not been inferred by any previous studies.

Using the analysis of whole-cell systems that has encompassed a number of different cell line models, we conclude that there is a substantial overlap in the mode of action of XR5944 and XR11576, but, clearly, they do vary in their in-vitro behaviour. Both agents are in early-phase clinical trial in Europe, the results of which are eagerly

awaited. We provide evidence that the phenazine agents XR11576 and XR5944 are potent new cytotoxic agents with substantial activity in tumour cells with resistance to VP-16 and to camptothecin.

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