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ORIGINAL ARTICLE

Effect of phenazine compounds XR11576 and XR5944 on DNA topoisomerases

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Abstract
Purpose Previous in vitro cleavage data showed that XR11576 and XR5944 stabilised topoisomerase I and topoisomerase II complexes on DNA in a dose-dependent fashion. However, some studies indicated a possible topoisomerase-independent mechanism of action for these drugs.
Methods Three methods, the TARDIS assay, immunoband depletion and the K^+/SDS assay have been used to assess topoisomerase complex formation induced by XR11576 or XR5944 in human leukaemic K562 cells.
Results TARDIS and immunoband depletion assays demonstrated that XR11576 and XR5944 induced complex formation for both topoisomerase I and topoisomerase II (alpha and beta) in a dose- and time-dependent manner, following exposure times of 24 and 48 h at concentrations of 1 or 10 μM. The K^+/SDS assay showed the formation of protein/DNA complexes after a 1 h exposure to 1 or 10 μM XR11576.
Conclusion Our data confirm that XR11576 or XR5944 can form topoisomerase complexes, after long periods of exposure.

Keywords Topoisomerase I · Topoisomerase II · Dual inhibitor · TARDIS assay

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Introduction

DNA topoisomerases (topos) have evolved to solve the topological problems within DNA [39]. Type I topoisomerases (topo I) transiently break only one strand of the DNA whereas, type II topoisomerases (topo II) transiently break a pair of complementary strands and pass a double-stranded segment through the break. The mechanism of action of topo I and topo II makes them attractive targets for chemotherapy. The formation of the transient covalent complex provides a crucial molecular target that can be exploited by compounds to poison the function of topo I or topo II. Indeed, a number of topo I or topo II targeting drugs are currently used in the clinic.

Development of an anticancer drug capable of targeting both topo I and II activity would help circumvent drug resistance due to alterations in levels of expression and catalytic activity of topo I and/or topo II known as 'atypical' resistance [39]. A number of drugs have been reported to target both topo I and topo II activity, including DACA [5, 13, 26], TAS-103 [37] and F11782 (Tafluposide) [28]. However, subsequent investigations have not provided defintive evidence of inhibition of both topo I and topo II [2, 7, 14, 20, 26, 27]. More recently another drug, batracylin, was shown to target both topo I and topo II in tumour cells [30].

Two novel substituted phenazine derivatives, XR11576 (MLN576) and XR5944 (MLN944), were discovered as

part of a drug design program aimed at identifying dual topoisomerase inhibitors (Fig. 1) [23, 36]. XR5944 has entered phase I clinical trials [38]. Combination studies of XR5944 with carboplatin, doxorubicin, 5-fluorouracil or irinotecan have demonstrated synergistic activity over single-agent therapy [17, 18]. The mechanism of anticancer action attributed to XR5944 is not clear. Initial reports showed inhibition of both topoisomerase I and II in vitro and cell lines exhibiting 'atypical' drug resistance were sensitive to XR5944 [36]. Data also showed that, although pumped, XR5944 was still able to overcome multidrug resistance mediated by P-gp and MRP in resistant cell lines and the compound was more potent against three human tumour cell lines (H69 SCLC, COR-L23 NSCLC, HT29 colon) than TAS-103, doxorubicin or Topotecan [36]. In addition, ex vivo tumours were more sensitive to XR5944 than other topoisomerase inhibitors [12]. However, subsequent reports have suggested the primary mechanism of action of XR5944 is independent of topoisomerase and may involve inhibition of estrogen receptors [29], transcription inhibition [6] and DNA binding [33]. The monophenazine, XR11576, has also entered clinical trials [10]. Like XR5944, in vitro data showed that XR11576 mediated both topo I and topo II associated complexes, although it showed different cleavage patterns from camptothecin and etoposide respectively [23]. XR11576 was also unaffected by the presence of overexpressed P-gp or MRP, or 'atypical' drug resistance due to topo II down regulation. Ex vivo tumours have been shown to be more sensitive to XR11576 than other topoisomerase inhibitors [11]. More recently, XR11576 has been shown to induce DNA protein cross-links (DPCs) following long exposures [21]. In addition, an XR11576-resistant cell line showed resistance to topoisomerase inhibitors camptothecin and etoposide, thus suggesting a role for topoisomerase in the cytotoxic mechanism of action of this compound [21].

We sought to further investigate the role of topoisomerase inhibition in the mechanism of action of XR11576 and XR5944. Using two immunological methods TARDIS (Trapped in Agarose DNA-immunoStaining) and immunoband depletion assays, we assessed the effect of XR11576 and XR5944 on topoisomerase complex formation in a dose- and time-dependent manner. In addition, the K^+/SDS assay was utilized, which measures the formation of protein/DNA complexes, but is not specific for topoisomerases. The induction of γ-H2AX foci was also investigated for XR11576 and XR5944. Finally, growth inhibition in K562 cells and DNA repair-deficient paired cell lines were examined.

In this study, we demonstrate that exposure of either X11576 or XR5944 results in accumulation of topo I- and topo II-DNA complexes after long periods of exposure, which coincides with the induction of γ-H2AX foci as a marker for DNA damage. Although structurally related, exposure of these drugs in DNA-repair deficient cell lines indicate that the two drugs possess slightly different mechanisms of actions which may be related to their other proposed cellular effects.

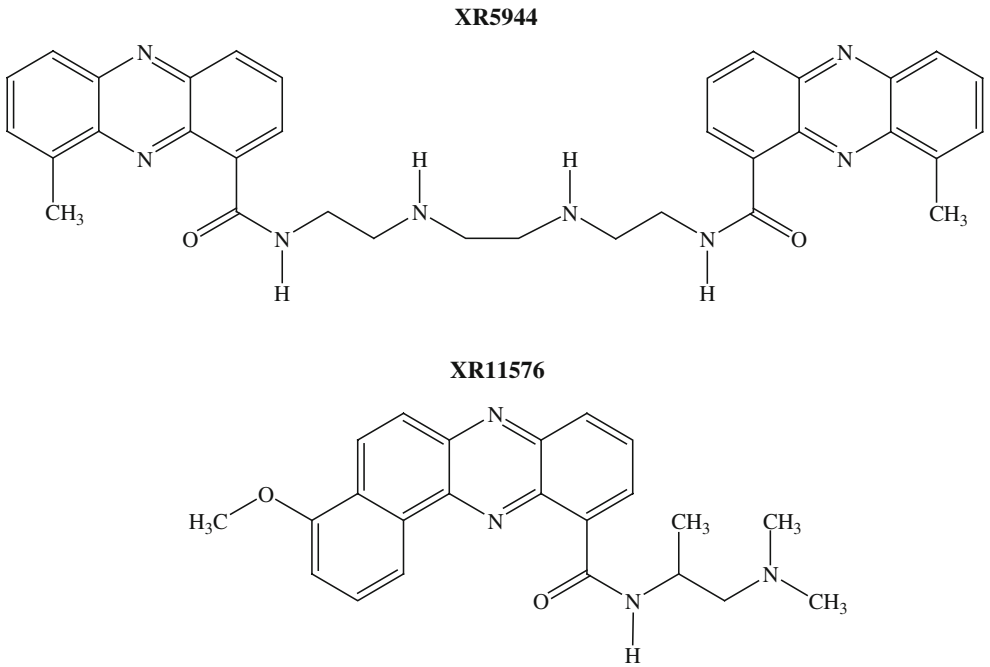

Fig. 1 Structure of the phenazine XR11576 (4-methoxy-benzo[a]phenazine-11-carboxylic acid (2-dimethylamino-1-(*R*)-methyl-ethyl)-amide) and XR5944

Materials and methods

Cell culture and reagents

Human chronic myelogenous leukaemic K562 cells, human colorectal cancer HT29 cells, human breast cancer MCF7 and the Chinese hamster ovary isogenic cell lines AA8, EM9, IRS^{1SF} and V3 were kindly provided by the Northern Institute for Cancer Research (NICR). The AA8 cell line is the parental cell line. EM9 has a deficiency in the DNA ligase III activity via XRCC1 (Involved in the Base excision repair pathway). V3 cells are defective for DNA dependent protein kinase (DNA-PK). IRS^{1SF} cells are deficient in XRCC3.

General reagents were obtained from Merck (Poole, UK), Sigma (Dorset, UK), Boehringer Mannheim, Promega (Southampton), or Amersham Biosciences (Little Chalfont, UK) unless otherwise stated. Etoposide and camptothecin were obtained from Sigma (Dorset, UK). XR11576 and XR5944 were provided by Xenova PLC (Slough, UK). Etoposide was dissolved in ethanol at 10 mM and all the other drugs were dissolved in DMSO at 10 mM and stored at −20°C.

Antibodies

Rabbit 18511(α) polyclonal antibody was raised to full length recombinant topo IIα, the rabbit 18813 (β) polyclonal antibody was raised to a recombinant human topo IIβ C-terminal fragment [40]. The topo I antibody used was a polyclonal antibody (Topogen, USA).

Growth inhibition assays

All cells were seeded in 96-well plates for 24 h before drug exposure. After each exposure period the drug supernatant was removed and the cells washed in fresh media and resuspended in 100 µL of fresh media (K562 cells were centrifuged at 1,000 rpm for 5 min using a microplate adapter to pellet cells prior to each medium removal step). Cells were grown for a total of 96 h from drug addition. The values obtained for each of the six replicates were averaged, and IC_{50} values were defined as the concentrations of drug(s) that inhibited growth by 50% relative to controls. The XTT assay has previously been described [34]. Briefly, 5 mL of the XTT labeling reagent had 0.1 mL of the electron-coupling reagent PMS (*N*-methyl dibenzopyrazine methylsulfate), 0.383 mg/mL (1.25 mM), in PBS added. Fifty micro litres of the above solution was added to each well. The cells were incubated at 37°C for approximately 4 h. The optical density of each individual well was determined at 450 nm using a microplate reader (Biorad). The SRB assay has been previously described [40]. Briefly, 25 µL of ice-cold 50% trichloroacetic acid was added to each well on the microplate and incubated at 4°C for 1 h. The plates were then washed five times under running tap water then dried at 60°C for 2 h. Plates were stored at 4°C until required. To stain the cells, the plates were brought to room temperature before 100 µL of 0.4% (w/v) SRB (in 1% acetic acid) was added to each well and incubated for 30 minutes. The SRB stain was removed and the plates were washed five times in 1% acetic acid. The plates were then dried at 60°C. Following this, 100 µL of 10 mM Tris, pH 10.5, was added to each well. The plates were placed on a plate shaker for 20 min to allow the stain to solubilise. The optical density of each well was determined at 540 nm using a microplate reader (Biorad).

TARDIS assay

This method has been previously described in detail [40]. Briefly, K562 cells were treated with drug at appropriate concentrations. Microscope slides were pre-coated with a thin layer of low melting point agarose, 0.5% in water (Seaprep ultralow gelling, FMC Bioproducts, Rockland, USA) and dried at room temperature. Drug-treated and control (untreated) cells were washed in ice-cold PBS then pelleted by centrifugation (1,000 rpm for 3 min) and re-suspended in PBS (approximately 200 µL) on ice. Fifty micro litres aliquots of cell suspensions were warmed to 37°C and mixed with equal volumes of 2% low melting point agarose in phosphate buffered saline (PBS) kept at 37°C prior to use. The mixture containing the cell suspension and agarose was spread evenly across the pre-coated microscope slides using another microscope slide and then allowed to set on a cold surface. Slides were then placed in lysis buffer containing 1% SDS, 80 mM phosphate buffer (pH 6.8), 10 mM EDTA, and protease inhibitors (2 µg/mL leupeptin, 2 µg/mL pepstatin, 1 mM PMSF, 1 mM benzamidine and 1 mM DTT) for 30 min, at room temperature. Slides were then placed in 1 M NaCl plus protease inhibitors (mentioned above) for 30 min, at room temperature. Slides were then washed three times in PBS (3 × 5 min). Next, slides were incubated with primary specific topo antibody at dilutions in PBS containing 0.1% Tween 20 (PBST) and 1% BSA, topo I-antibody diluted 1:1,000 (topogen), topo II alpha antibody (18511) diluted 1 in 50, topo II beta antibody (18513) diluted 1 in 200 for 1–2 h then washed with PBST. Slides were then stained with a secondary FITC-conjugated anti-rabbit (topo II) or anti-human (topo I) antibody diluted 1 in 100 in PBST and 1% BSA (700 µL/slide) for 1–2 h. Slides were washed twice in PBST (2 × 5 min) and placed at 4°C in PBS plus protease inhibitors (as before) overnight. The slides were then stained with Hoechst 33258 (10 µmol/L in PBS) for 5 min to stain DNA and cover slips were applied and secured with a sealant. Images were

captured using a cooled slow-scan CCD camera and fluorescence levels were quantified as previously described [16].

SDS/K^+ precipitation assay

This assay has been previously described [32]. The DNA in logarithmically growing K562 cells in culture was labeled for 24 h with 0.04 μCi/mL of [2-^{14}C-] thymidine (53 mCi/mmol) (Amersham). Labeled cells were pelleted by centrifugation (1,000 rpm for 10 min), and resuspended in fresh growth media for an additional 3 h at 37°C to chase all the labeled thymidine into high molecular weight DNA. Cells were counted after incubation in radiolabel-free media, and 4×10^5 cells/mL (4 mL total volume per well) were treated in six well tissue culture plates with various concentrations of drug for different exposure times at 37°C. Control cells received the same concentration of DMSO as cells exposed to the drugs. The assay was performed for each drug or control treatment in triplicate.

Following treatment, 2 mL of cell suspension was transferred to 15 mL Falcon tubes (Becton Dickinson) and then pelleted by centrifugation at 1,000 rpm for 5 min. The supernatant media was carefully aspirated, and the cell pellet lysed by the addition of 1 mL of preheated (65°C) lysis solution (1.25% (w/v) SDS, 0.4 mg/mL herring sperm DNA, 5 mM EDTA, pH 8.0). After incubating for 10 min at 65°C, 250 μL of 325 mM KCl was added to each tube to achieve a final concentration of 65 mM KCl. The sample was then vigorously vortexed for exactly 10 s to achieve reproducible fragmentation of DNA. Because the fragment size of DNA influences the sensitivity of the assay, reproducible shearing of nuclear DNA by vortexing was crucial. One milli litre of the lysate was transferred to a 1.5 mL microcentrifuge tube, and cooled on ice for 10 min to allow precipitation. The tube was then centrifuged (10,000*g*) for 10 min at 4°C and the supernatant was removed. The pellet was then resuspended in 1 mL of wash solution (10 mM Tris–HCl, pH 8.0, 0.1 mg/mL herring sperm DNA, 100 mM KCl, 1 mM EDTA). The tube was heated at 65°C for 10 min with periodic mixing, and then placed on ice for an additional 10 min to reprecipitate the covalent topo-DNA complexes. The tube was then centrifuged at 10,000*g* for 10 min to recover the precipitate. The supernatant was removed and the wash procedure repeated. The washed pellet was resuspended in 500 μL H_2O preheated to 65°C followed by 4 mL of Ecolume biodegradable liquid scintillation cocktail. The samples were then counted on a scintillation counter. Subsequent counts disintegrations per minute represent the amount of radiolabeled DNA (covalently bound to protein) that has been precipitated in the assay.

Immunoband depletion

K562 cells seeded in 6-well tissue culture plates were incubated for 48 h at 37°C. Drugs were added at appropriate concentrations for the indicated exposure periods. Following drug (or control) exposure cells were washed in PBS and cell numbers were determined by trypan blue exclusion. Two micro litre aliquots of cells were collected in 15 mL tubes and were pelleted at 1,000 rpm for 3 min and the supernatant was aspirated off. SDS loading buffer was immediately added to lyse the cells such that every 15 μL contained 2×10^5 cells. The samples were then heated in a boiling water bath for 10 min and then aliquoted into smaller volumes and stored at −20°C. Fifteen micro litre aliquots were subjected to western blotting. For each gel, serial dilutions (1:2) of the control samples were loaded along with at least one marker lane. The developed western blots were scanned with an Epson flatbed scanner and analysed using TINA software (Raytest). The degree of band depletion seen compared to control is an indication of the level of topo/DNA complex formation.

γ-H2AX foci assay

The human breast cancer cell line, MCF7 was utilised for this assay. Briefly, cells were seeded into wells of a 6-well tissue culture plate that contained pre-sterilised glass cover-slips in the base of each well. Controls used for these experiments included untreated cells and cells that had been treated with 2 Gy ionising radiation (Nordion Cs source, decay rate 3 Gy/min). After irradiation, cells were incubated for 30 min at 37°C to allow foci formation. Other cells were exposed to drug as indicated in the figure legends. For all treatments, cover-slips were then removed from the plates, washed with ice-cold PBS and fixed for 5 min with ice-cold methanol. Cells were rehydrated twice by incubation with PBS for 10 min. The cells were then blocked for 1 h in 3% bovine serum albumin in PBS (BSA/PBS). The cells were then incubated with an anti-γH2AX antibody (Upstate) overnight in a sealed box at 4°C. The cells were then washed five times in PBS then incubated for 1 h at room temperature in the dark with an Alexa 488-conjugated secondary antibody (molecular probes). After this, cells were then washed five times in PBS and the cover-slips were inverted onto a microscope slide onto a 20 μL droplet of Hoechst solution (10 μmol/L in PBS). Images were captured using a cooled slow-scan CCD digital camera and Leica DMLB fluorescence microscope using 5 s exposures for Hoechst (UV filter), then a 10 s exposure for the same field of view under the green filter to detect the Alexa-488 stained foci. Several fields of view from two cover-slips were acquired, and these were scored for foci as follows. Foci were counted from at least 50 individual nuclei and the mean number was calculated.

Results

Dose- and time-dependent growth inhibition of K562 cells by XR11576 or XR5944

The growth inhibitory effect of increasing exposure times to etoposide, camptothecin, XR11576 and XR5944 was assessed using the XTT assay on K562 cells (Fig. 2a–d). For etoposide and XR11576 increasing exposure time from 1 to 6 h had little effect on growth inhibition, whereas an exposure time of 24 h or longer resulted in significant growth inhibition. There was a clear increase in the growth inhibitory effect of XR11576 when exposure time was increased from 6 to 96 h exposures (about 10.2 fold decrease in IC_{50}). The 96 h IC_{50} value was comparable to that of camptothecin (Table 1). Whereas XR5944 growth inhibition increased in a more linear pattern in relation to exposure time IC_{50} values calculated from growth inhibition data are shown in Table 1.

Formation of topo I, topo II alpha, and II beta complexes using the TARDIS assay

The TARDIS assay immunologically detects covalent adducts on DNA [15]. The TARDIS assay software allows quantification of the immunoflourescence associated with topo II adducts, where the nature of the adduct detected depends upon the antibody used. Moreover, unique to this method, adduct levels in individual cells can be detected. In this study we have analysed complexes formed by DNA topo I [25], topo II alpha, and topo II beta [40] following exposure to XR11576 and XR5944.

DNA within each cell is visualised with Hoechst and the mean intensity levels of Hoechst dye fluorescence

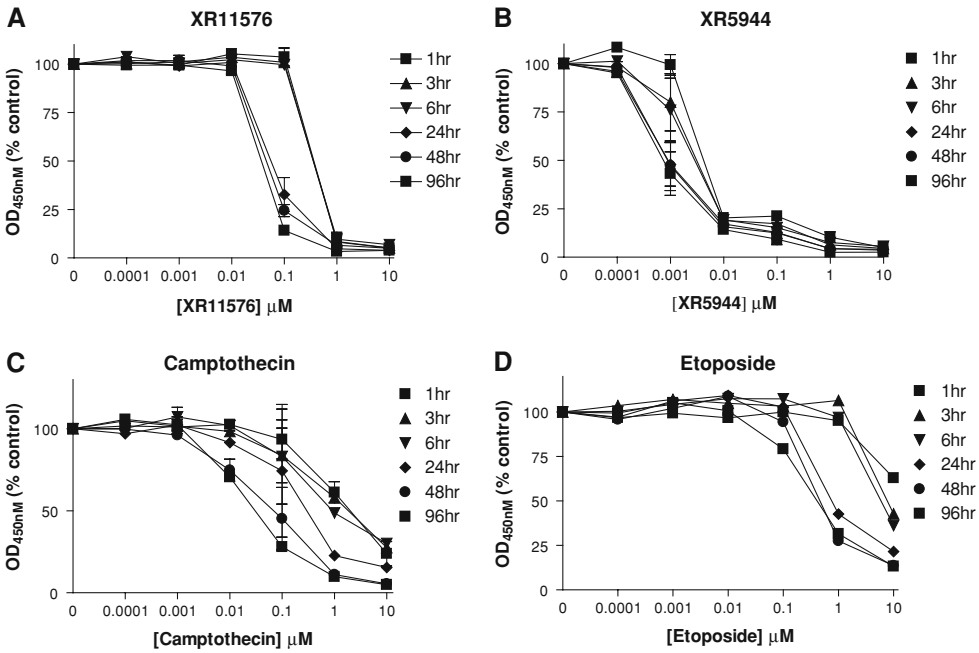

Fig. 2 Effect of XR11576 and XR5944 on growth inhibition of K562 cells following varying exposure times. K562 cells were treated with XR11576 (**a**), XR5944 (**b**), camptothecin (**c**) or etoposide (**d**) for varying exposure times at the indicated concentrations. Cell number was indicated by OD_{450}nM (expressed as % of control wells) following incubation with XTT reagent as described in the methods section. Each point represents the average (±SEM) of three experiments

Table 1 IC_{50} values for K562 cells treated with XR11576, XR5944, camptothecin or etoposide for varying exposure times

Exposure time (h)	XR11576 mean IC_{50} (nM)	XR5944 mean IC_{50} (nM)	Camptothecin mean IC_{50} (nM)	Etoposide mean IC_{50} (nM)
1	371 ± 69	5.4 ± 2.0	2,020	>10,000
3	357 ± 19	3.1 ± 1.5	1,780	7,700
6	358 ± 35	3.1 ± 1.1	880	5,900
24	68 ± 38	1.2 ± 0.7	280	700
48	46 ± 2	1.2 ± 0.7	70	500
96	36 ± 2	0.8 ± 0.4	30	400

K562 cells were treated with XR11576 for varying exposure times at the indicated concentrations. Mean IC_{50} values (nM) are shown. Each value represents the average (±SEM) of three experiments for XR11576 and XR5944 and two experiments for camptothecin and etoposide

were quantified. The levels of Hoechst fluorescence were not markedly affected by XR11576 or XR5944 concentration (data not shown). This suggested neither XR11576 nor XR5944 changed the conformation of the DNA.

The effect of XR11576 on the formation of topo I-mediated complexes is shown in Fig. 3a. There was no significant difference between cells exposed to 0.1 μM and zero time point (untreated cells) cells for any exposure period, however significant increases in mean topo I FITC-immunofluorescence were observed for exposure periods of 24 and 48 h at XR11576 concentrations of 1 and 10 μM ($P = < 0.001$). However, the positive control camptothecin produced comparable levels after exposure for only 1 h (Fig. 3g). The effect of XR11576 on topo II (α and β) levels are shown in Fig. 3b and c. Only exposure times of 48 h resulted in a significant increase in immunofluorescence for both 1 and 10 μM XR11576. Both topo II beta and topo II alpha FITC-immunofluorescence showed significant increases in mean FITC-immunofluorescence but only for 48 h exposure periods with 1 or 10 μM XR11576 (Fig. 3b, c), unlike the positive control etoposide (Fig. 3h, i) which gave a very high signal after only an hour. The effect of XR5944 on topo I and topo II adducts was also analysed by TARDIS. For both topo I and topo II it required a 24 h treatment to see significant signals (Fig. 3d–f) unlike the positive controls camptothecin and etoposide (Fig. 3g–i). Thus, for both XR11576 and XR5944 long exposures were needed to detect topo I and topo II adducts on the DNA with the TARDIS assay.

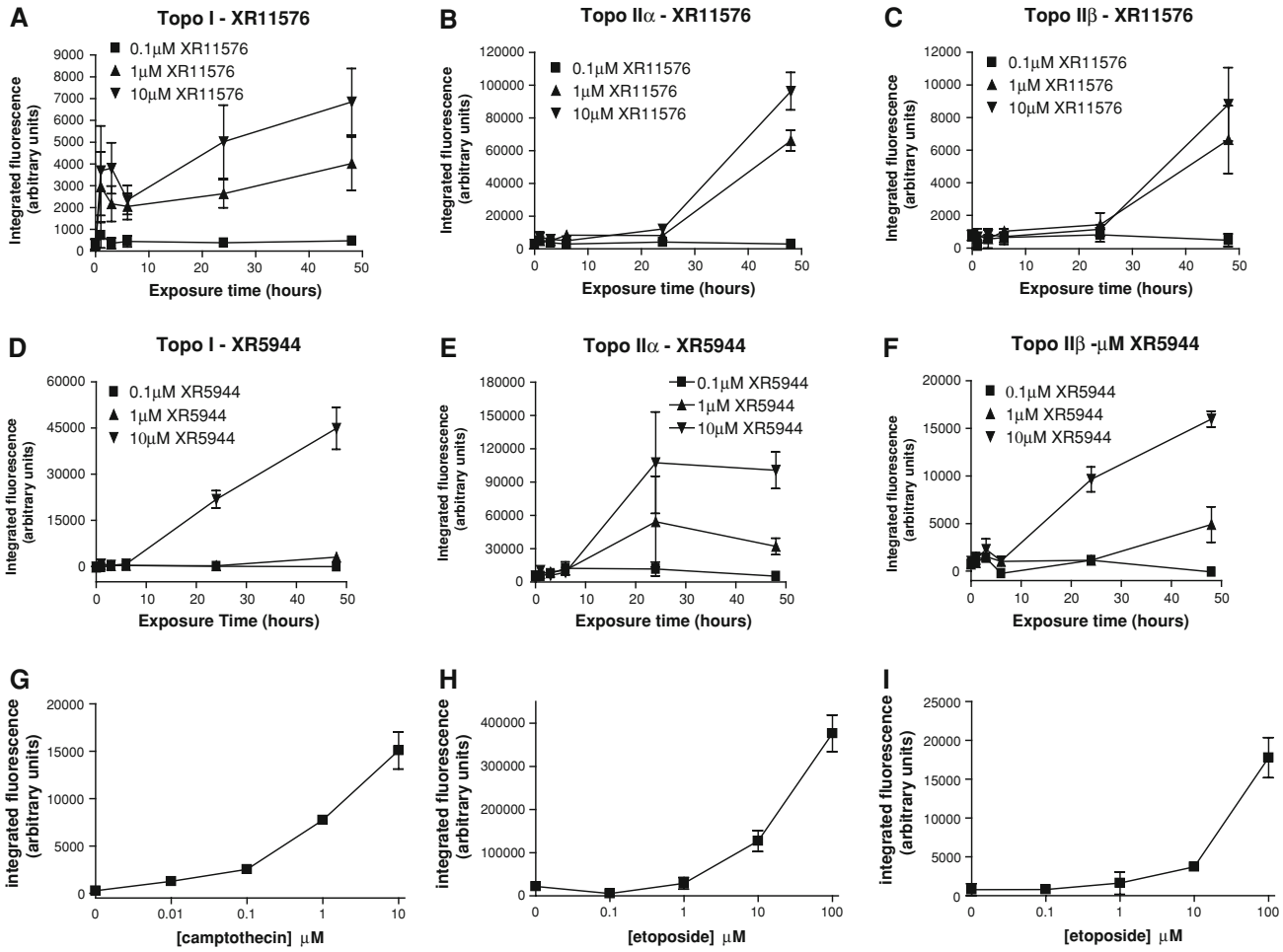

Fig. 3 Levels of topoisomerase I, II alpha and II beta-DNA cleavable complex levels, measured by the TARDIS method for K562 cells treated with XR11576 for varying exposure times. K562 cells were treated with XR11576 for varying exposure times at concentrations of 0.1, 1, and 10 μM. Topo I (**a**), II alpha (**b**) and II beta (**c**) or XR5944 for varying exposure times at concentrations of 0.1, 1 and 10 μM topo I (**d**), topo II alpha (**e**) and topo II beta (**f**). Topo–DNA cleavable complex levels measured by the TARDIS method are represented by the mean FITC-immunofluorescence. K562 cells were treated with varying concentrations of camptothecin (G), etoposide (**h** and **i**) for 1 h and topo I (**g**), II alpha (**h**) and II beta (**i**)-DNA cleavable complex levels measured by the TARDIS method are represented by the mean FITC-immunofluorescence. Each point represents the average (±SEM) of three experiments, in each of which the mean and median integrated immunofluorescence of at least 100 individual cells was determined

Immunoband depletion

In order to confirm the results obtained using the TARDIS assay we employed another technique capable of detecting topo/DNA complexes, immunoband depletion. Under normal cellular conditions the levels of covalently bound topo-DNA complexes are very low due to their transient nature [8]. However, treatment with certain drugs increases the number of these complexes which results in a depletion of extractable topo from a cell. The immunoband depletion assay enables the separation and subsequent detection of extractable topo and covalently bound non-extractable topo-DNA complexes and their detection in an isoform specific manner.

K562 cells exposed to 0.1, 1, 10, and 50 μM XR11576 for 1 h showed no change in quantifiable soluble topo I band intensity (Fig. 4a). This trend was also seen following an exposure period to XR11576 of 6 h (Fig. 4b). However, 50 μM XR11576 for 24 h showed a depletion of soluble topo I and topo II (Fig. 4c, g). K562 cells exposed to XR5944 showed a slight decrease in soluble topo I after 6 h but as with XR11576, 24 h and higher concentrations of drug were required to see a large depletion of soluble topo I (Fig. 4d, e and f).

We performed the same experiment with 1 h exposures to camptothecin and etoposide and found that very high concentrations of camptothecin (25 μM) or etoposide (400 μM) were required to decrease the level of soluble topo following drug treatment (Fig. 4h). This was in agreement with previous studies that demonstrated a topo II alpha-depleting effect using high concentrations of etoposide [41] and depletion of extractable topo I in human cancer cell lines following treatment with camptothecin [19]. Due to the high concentration of drugs needed in the immunoband depletion assay, a more sensitive assay, the K^+/SDS precipitation assay, was used to detect DNA-protein complexes.

K^+/SDS assay

DNA that is complexed to proteins can be readily separated from free DNA using the K^+/SDS precipitation assay [22, 24]. In the presence of high concentrations of KCl, SDS causes precipitation of crystals of potassium dodecyl sulphate (K^+/SDS); this co-precipitates free protein and covalent protein-DNA complexes without causing precipitation of free DNA. When the DNA is radiolabeled, the amount of label in the precipitate can be used as a measure of DNA molecules with covalently bound protein.

K562 cells were treated with varying exposures of XR11576 or XR5944 for 1, 3, 6 and 24 h and were then subjected to the K^+/SDS precipitation assay. Increases in radioactivity associated with the precipitate were observed following 1 h exposures to 0.1, 1, and 10 μM XR11576 (Table 2). This increase was dose dependent with both 1 and 10 μM drug concentrations and statistically significant from control ($P < 0.01$, Dunnet's multiple comparison post test, ANOVA). The levels of radioactivity observed at 10 μM XR11576 were similar to those seen with 10 μM etoposide but lower than those caused by 10 μM camptothecin (Table 2). The levels of radioactivity measured after a 3 or 6 h exposure to XR11576 were similar to those observed following a 1 h exposure to XR11576 where both 1 and 10 μM XR11576 exposures were significantly different from control ($P < 0.01$, Dunnet's multiple comparison test). However, the data obtained from a 24 h exposure of XR11576 showed lower radioactivity levels (Table 2). Only the data for the 10 μM 24 h exposure showed a significant increase over the control using Dunnet's multiple comparison test, ($P < 0.01$). XR5944 exposure produced no more precipitation of radioactivity than control untreated cells (Table 2).

Effect of XR11576 and XR5944 on the induction of γ-H2AX foci in MCF7 cells

H2AX has been shown to be rapidly phosphorylated over large chromosomal regions in response to DNA damage (approx 0.03% of H2AX phosphorylated per DSB) forming visualisable foci at interphase and is commonly used as an indirect measurement of DNA double strand break formation [31]. We investigated if either XR5944 or XR11576 induce γ-H2AX foci. Induction of such foci would indicate these compounds induce DNA damage such as DNA double strand breakage.

MCF7 cells were exposed to 2Gy ionizing radiation and incubated for 30 min following irradiation. Quantification of foci showed a 25-fold increase in mean number of foci per nucleus following irradiation (Fig. 5). A single experiment, where exposure of XR11576 to MCF7 cells for 24 h caused a16-fold increase γ-H2AX foci formation (compared to untreated control), is shown in Fig. 5. Exposures of either 1 or 6 h to 1 μM XR5944 showed there was no detectable γ-H2AX foci formation. However, there was a concentration dependent increase in γ-H2AX foci staining following a 24 h exposure with maximum levels being attained at 1 μM, the highest concentration. The long exposures required for γ-H2AX foci formation suggests that double strand breaks are not the primary mechanism of action for either XR11576 or XR5944, but the data is consistent with the long exposures necessary for topo/DNA complexes to form, as demonstrated from the TARDIS assay data.

Effect of XR11576 and XR5944 on isogenic DNA repair-deficient cell lines

To further investigate the relationship between XR11576- and XR5944-mediated DNA damage formation, as demonstrated with the previous experiments showing topo/DNA

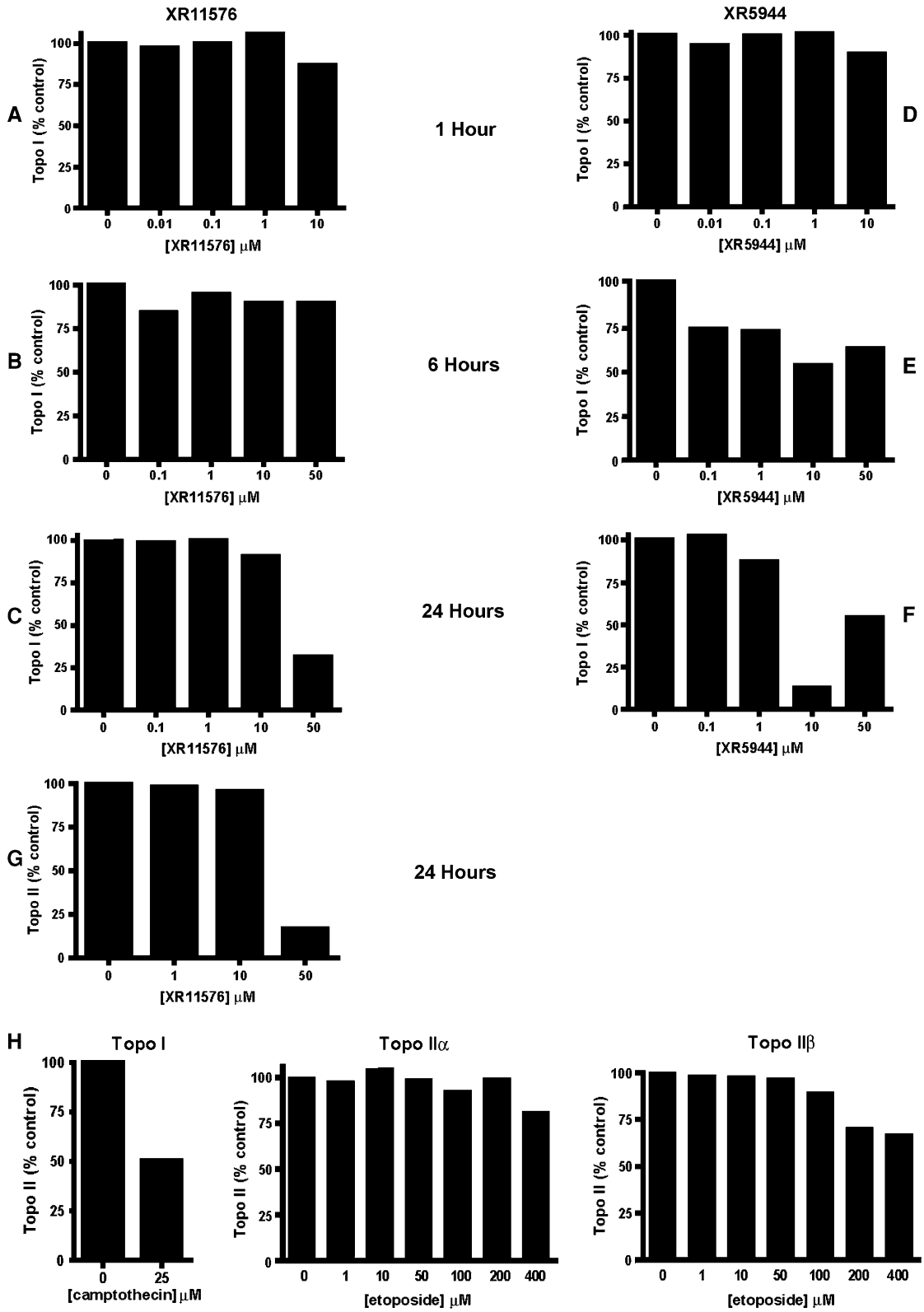

Fig. 4 Immunoband depletion data for K562 cells probed for topo I and topo II (alpha and beta) after being treated with varying concentrations of XR11576 or XR5944 for varying exposure times. K562 cells were treated with XR11576 for 1 (**a**), 6 (**b**) and 24 (**c**) hours at concentrations of 0.1, 1, 10, and 50 μM. Cell extracts were made and probed with topo I antibodies. K562 cells were treated with XR11576 for 1 (**d**), 6 (**e**) and 24 (**f**) hours at concentrations of 0.1, 1, 10, and 50 μM. Cell extracts were made and probed with topo I antibodies. K562 cells were treated with XR11576 for 24 h (**g**) at concentrations of 1, 10 and 50 μM and probed with topo II antibodies. Positive controls, K562 cells treated with etoposide or camptothecin (**h**)

Table 2 K^+/SDS precipitation analysis for K562 cells treated with XR11576 or XR5944 for 1-, 3-, 6-, and 24-h exposures

Hours	CPT (DPM) 10 (μM)	VP16 (DPM) 10 (μM)	XR11576 (DPM)			XR5944 (DPM)		
			0.1 (μM)	1 (μM)	10 (μM)	0.1 (μM)	1 (μM)	10 (μM)
1	24,887	7,653	2,478	7,103	10,521	1,154	903	655
3	–	–	3,022	6,872	9,835	1,366	1,371	900
6	–	–	2,687	6,646	10,091	1,608	983	830
24	–	–	2,332	4,097	6,719	2,782	2,257	1,183

K562 cells labeled with [^{14}C] thymidine were treated with XR11576 or XR5944 for 1, 3, 6, or 24 h at concentrations of 0.1, 1 and 10 μM respectively. The effect of 10 μM camptothecin and etoposide is shown as a comparison. Covalent protein/DNA complex levels, measured by the K^+/SDS precipitation assay, are represented by the mean DPM values

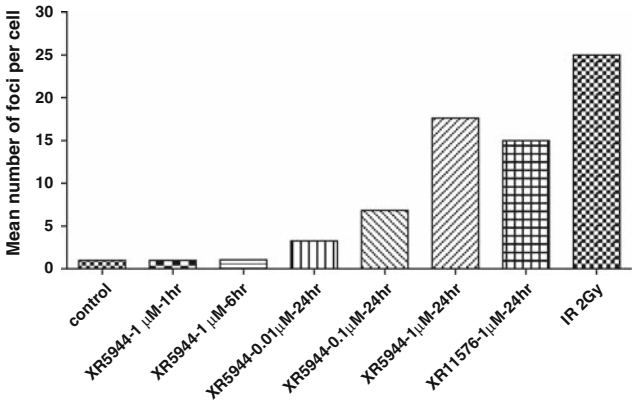

Fig. 5 Formation of of γ-H2AX foci in MCF7 cells following exposure of XR11576 or XR5944. MCF7 cells were exposed to XR5944 at 1 μM for either 1 or 6 h and at 0.01, 0.1 and 1 μM for 24 h. XR11576 exposure was 24 h at 1 μM. MCF7 cells were also exposed to 2 Gy ionizing radiation and incubated for 30 min following irradiation. The cells were then incubated with an anti-γH2AX antibody and DNA was visualized after Hoechst staining. Foci were counted from at least 50 individual nuclei and the mean number was calculated

complexes and γ-H2AX foci formation, we examined the growth inhibitory effect of XR11576 and XR5944 on parental AA8 and DNA repair-deficient cell lines. Growth inhibitory effects were seen in all cell lines following a 1 h exposure, and inhibitory effects increased with increasing XR11576 or XR5944 concentration and exposure time. A general trend in increasing sensitivity of the DNA repair deficient cell lines was observed compared to the parental AA8 cell line.

Table 3 shows the IC_{50} values and sensitivity ratios of the data for the cell lines in response to X11576 exposure. The IRS^{1SF} cell line (defective in homologous recombination) was the most sensitive, with sensitivity ratios of 3.24–3.88. V3 cells (Lacking functional DNA-PKcs) showed no sensitivity for a 1 h exposure, rising to modest sensitivity at 6 and 24 h (sensitivity ratios 1.46–1.45). EM9 cells (defective in BER) showed sensitivity ratios of 1.55–2.04. Table 4 shows the IC_{50} values sensitivity ratios of the data for the cell lines in response to XR5944. All the repair deficient cell lines exhibited lower IC_{50} values than the repair competent AA8 cell line for 1, 3, 6 and 24 h exposure times. The sensitivity ratios for V3 and IRS^{1SF} cells were similar (1.28–2.18). The sensitivity ratios were lowest for the EM9 cell line (1.1–1.75). These differences in IC_{50} were only significant for the 24 h exposure.

The data for XR11576 exposure showed higher sensitivity ratios for EM9 and IRS^{1SF} cells compared to XR5944. This indicated a greater relative importance of XRCC1 (EM9) and XRCC3 (IRS^{1SF}) in relation to XR11576 than to XR5944.

Discussion

Previous in vitro studies led to the hypothesis that both XR11576 and XR5944 may act as dual inhibitors of topo I and topo II [23, 36]. This hypothesis was further enhanced by more recent studies showing the cross-resistance to topoisomerase poisons of an XR11576-resistant cell line and the formation of DNA protein crosslinks following long exposure times to the drugs. Whilst there are reports of mechanisms of action distinct from topoisomerase [6, 9, 29, 33], the primary mechanism of action for both XR11576 and XR5944 remains unclear. For this reason we sought to confirm the original hypothesis of XR11576 and XR5944 being capable of inhibiting topoisomerase using a number of methods designed to specifically detect inhibition of topoisomerase in a cellular environment. In addition, we examined the importance of DNA damage in their mechanisms of action following drug treatment.

Our data first defined the effects of XR11576 and XR5944 on growth of K562 cells, specifically that XR11576 and XR5944 caused significant growth inhibition following short exposures, and that growth inhibition increased with increasing exposure time. The data showed that both drugs were more potent than the classical topoisomerase poisons, VP16 and camptothecin. This observation is in accordance with data reported showing more potent activity of XR11576 and XR5944 compared to doxorubicin,

Table 3 IC_{50} values and sensitivity ratios of the data for parental AA8 and DNA repair-deficient cell lines in response to XR11576 exposure

Exposure time (h)	AA8	EM9		V3		IRS^{1SF}	
	XR11576 IC_{50} (±SEM)	XR11576 IC_{50} (±SEM)	Sensitivity ratio	XR11576 IC_{50} (±SEM)	Sensitivity ratio	XR11576 IC_{50} (± SEM)	Sensitivity ratio
1	183 ± 2	103 ± 27	1.8	197 ± 69	0.9	52 ± 8	3.5
3	138 ± 45	68 ± 20	2.0	106 ± 15	1.3	36 ± 8	3.9
6	113 ± 41	59 ± 11	1.9	77 ± 10	1.5	30 ± 1	3.8
24	55 ± 20	36 ± 3	1.5	38 ± 4	1.5	17 ± 4	3.2

Cells were treated for the indicated time and concentration with XR11576 then subjected to the SRB assay. Data presented are the means of three independent experiments

Table 4 IC_{50} values and sensitivity ratios of the data for parental AA8 and DNA repair-deficient cell lines in response to XR5944 exposure

Exposure time (h)	AA8	EM9		V3		IRS^{1SF}	
	XR5944 IC_{50} (±SEM)	XR5944 IC_{50} (±SEM)	Sensitivity ratio	XR5944 IC_{50} (±SEM)	Sensitivity ratio	XR5944 IC_{50} (±SEM)	Sensitivity ratio
1	304.2 ± 38	293.6 ± 9	1.04	215.9 ± 5	1.41	213.2 ± 10	1.43
3	194.1 ± 22	176.9 ± 7	1.10	140.3 ± 7	1.38	142.3 ± 44	1.36
6	159.8 ± 17	143.3 ± 28	1.12	124.6 ± 22	1.28	109.5 ± 4	1.46
24	87.7 ± 10	50.0 ± 3	1.75	45.7 ± 1	1.92	40.3 ± 1	2.18

Sensitivity ratios were calculated as a ratio between IC_{50} values of parental AA8 cells and the indicated DNA repair-deficient cell line

topotecan, etoposide and camptothecin in ex vivo derived tumours [11, 12].

We then used the TARDIS assay to determine if XR11576 and XR5944 could trap topo-DNA complexes. The data showed that XR11576 and XR5944 did not cause detectable topo-DNA covalent complex formation up to 6 h exposure. This is in contrast to either camptothecin or etoposide which showed the presence of covalent topo I or topo II-DNA complexes respectively following short exposure periods. Following exposures to XR11576 and XR5944 lasting 24 and 48 h, concentration-dependent increases in immunostaining for topo I, topo II alpha or topo II beta were detected. These increases were reproducible and significant, and are consistent with the stabilization of topo I, topo II alpha or topo II beta complexes. Immunostaining following exposure to etoposide or camptothecin showed similar levels in the majority of cells. However, the lack of detectible topo-DNA complexes at short exposure times is in agreement with recent studies showing the lack DPCs formed after a 4 h exposure to XR5944 in tumour cells. However, DPCs were detected following longer exposures to both XR11576 and XR5944, which is in accordance with our data using the TARDIS assay and immunoband depletion. The observation that XR11576 and XR5944 stabilised topoisomerase complexes was further examined using the K^+/SDS precipitation assay. The results indicated that XR11576 caused the precipitation of radiolabeled DNA following a 1 h exposure, and up to 24 h. These data are again in accordance with the generation of DPCs at early time points shown by Lewis et al. [21]. However, the data obtained using longer exposure times were difficult to interpret due to the length of time the radiolabeled thymidine was present in the cell samples. Thus for drugs that form covalent complexes over longer periods of time the data are inconclusive. Exposure of cells to XR5944 did not produce any detectable precipitation of radiolabeled DNA.

The immunoband depletion method was used as an alternative method to determine whether topos were a target of XR11576 and XR5944. The data suggested that, following long exposures of XR11576 at high concentrations, levels of extractable topo I and topo II decreased. This observation is consistent with the stabilisation of topo I- or topo II-DNA complexes. Long exposures of XR5944 also showed levels of extractable topo I decreased. However, because of the extended exposure times for both drugs, we cannot exclude the possibility that the decrease in extractable topo I or topo II was due to protein-DNA adduct formation in response to ROS or apoptosis or due to decreased topo I or topo II synthesis or increased degradation rather than complex formation.

Interestingly, a clear increase in numbers of γ-H2AX nuclear foci per nucleus of MCF7 cells that had been exposed to XR5944 or XR11576 was seen following 24 h exposures. Formation of γ-H2AX foci is indicative of DNA double strand break formation. These data are consistent with the long time points required for topo-DNA com-

plexes seen with the TARDIS assay and immunoband depletion data and hence may indicate a role of toposiomerase in mediating these double strand breaks. However, we cannot exclude the role of other mechanisms in the formation of double strand breaks such as the onset of apoptosis.

The data presented here demonstrate that the formation of detectable topo-DNA complexes occurred only following prolonged drug exposure. However, XR11576 and XR5944 caused significant growth inhibition of K562 cells following short exposures. There are several possible explanations for this apparent discrepancy. It may be that only a small number of topo-DNA complexes induced by XR11576 and XR5944 were sufficient to cause cytotoxicity, while greater accumulation of complexes was necessary to be detectable by the methods used. For example, levels of topoisomerase II-mediated double strand breaks formed by anthracyclines do not always correlate with cytotoxicity [4]. The DNA sequence at which the damage occurs via XR11576 may also be a 'sensitive' region to DNA damage as has been shown for anthracyclines [4]. We also cannot exclude the possibility that XR11576- or XR5944-mediated topo I- or topo II-DNA complex formation, following long exposure periods, is due to reactive oxygen species (ROS) such as those generated during apoptosis [35].

To determine if DNA damage is involved in the mechanism of action of XR11576 and XR5944, cell lines deficient in various DNA repair activities were tested. The effect of drug on a parental CHO cell line, AA8, was compared to that on DNA repair-deficient cell lines EM9, IRS^{1SF} and V3. The results for XR11576 indicated that there was greater drug sensitivity in the IRS^{1SF} cell line after just a 1 h exposure. This indicated a greater relative importance of XRCC3 (IRS^{1SF}) in relation to XR11576 than to DNA-PK (V3). The lower sensitivity of V3 cells to XR11576 treatment suggests that non-homologous end-joining mediated by DNA-PK is less important than other repair pathways for XR11576. In contrast, the sensitivity ratios for V3 and IRS^{1SF} cells after exposure to XR5944 were consistently higher and all IC_{50} values were significantly lower than for AA8 cells. This suggests a role for both DNA-PK and XRCC3 in protecting any damage caused by XR5944. The sensitivity ratios were relatively small, this may be because DNA-PK or XRCC3 pathways can compensate for each other when one is lacking. Interestingly, the IRS^{1SF} cell line was shown to be more sensitive to camptothecin treatment than the V3 cell line where there is a lack of functional non-homologous end-joining [1]. It is therefore interesting that XR11576 shows a similar trend in the data we have shown. The results for XR11576 also indicated that there was greater drug sensitivity in the EM9 cell line after just a 1 h exposure. The role of base excision repair has been previously investigated with topo I poisons in human tumour cell lines. Camptothecin has a greater cytotoxic effect in cell lines deficient in base excision repair (XRCC1) [3]. This suggests that topo I may be a target for XR11576 activity.

Previous studies have suggested that the main cytotoxic effect of XR5944 could have been caused via alternative mechanisms not involving topoisomerase. Indeed, studies of XR5944 have demonstrated a role of transcription inhibition [6], DNA binding [9, 33] and estrogen receptor inhibition [29]. In addition, XR5944 showed a differential cell cycle response and topoisomerase cleavage pattern when compared to known topoisomerase inhibitors [33]. Yeast studies have also indicated a topoisomerase independent mechanism of action of XR5944 [33]. We have also shown the accumulation of topoisomerase-DNA complexes at longer exposure times which coincide with γ-H2AX foci, which suggests a role for topoisomerase in the mechanism of action of XR5944 and XR11576. The possibility for a role of topoisomerase in the mechanism of action of these drugs is augmented by data from Lewis et al. [21] showing the resistance to XR5944, camptothecin and etoposide in an XR11576-resistant cell line.

As noted by Lewis et al. [21] and Sappal et al. [33], although structurally similar, XR11576 and XR5944 exhibit different cellular activities such as DPC formation and cell cycle profiles. This may be due to the differential DNA-binding capacities of the two compounds. Likewise, we have shown that there are differences in the mechanisms by which these drugs work. For instance the fact that XR11576 was able to induce DNA–protein complexes using the SDS/K precipitation assay, while XR5944 did not show any detectable complex formation. However, this difference is particularly evident from the data for cell lines deficient for DNA-repair pathways where we show that XR11576 is more potent in cells lacking functional BER, suggesting topo I as a target for the drug. Conversely, only cells deficient for DNA-PK and XRCC3 were more sensitive to XR5944. These data would indicate that the drugs have distinct mechanisms of action.

In summary, the data suggest that exposure of mammalian cells to XR11576 or XR5944 can lead to formation of DNA damage that includes the accumulation of topo I- and topo II-DNA complexes after long exposure periods. Although the long exposure times required to detect topo-DNA complexes could indicate a non-topo primary cytotoxic mechanism of XR11576 and XR5944, our data suggest that cytotoxicity is still mediated (at least in part) via a DNA-damaging effect.

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