Ex vivo characterization of XR11576 (MLN576) against ovarian cancer and other solid tumors

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XR11576 (MLN576) is a novel monophenazine with a mechanism of action that includes interaction with both topoisomerase (Topo) I and II. The aim of this study was to evaluate its cytotoxicity against fresh tumor cells taken from patients with a variety of solid tumors. Cells were obtained from 89 patients and exposed for 6 days to XR11576 alone, or in combination with doxorubicin, cisplatin, treosulfan, paclitaxel or vinorelbine. Cell survival was measured using the ATP-Tumor Chemosensitivity Assay (ATP-TCA). Immunohistochemical staining of Topo I, Topo IIα and MDR1 was performed on paraffin-embedded blocks in those tumors for which tissue was available ($n=49$). Overall, the median IC_{90} and IC_{50} values of XR11576 in tumor-derived cells were 242 and 110 nM, respectively. In all samples XR11576 was more potent than the other cytotoxics tested. Breast and gynecological malignancies were most sensitive to XR11576, while the potency of this compound was slightly attenuated in gastrointestinal tumors, in which the median IC_{90} and IC_{50} values were 308 and 212 nM, respectively. Cases of synergism were identified when combining XR11576 with vinorelbine (nine of 30 samples) and doxorubicin (12 of 38 samples), while the addition of paclitaxel resulted in an antagonistic effect ($CI_{50}>1.2$) in 38 of 42 tumors. A very modest correlation by linear regression analysis was found between the intensity of MDR1 staining and the IC_{50} of XR11576 ($r=0.311$, $p=0.0312$), but not with the IC_{90} ($r=0.247$, NS). These data support the rapid introduction of XR11576 to clinical trials and suggest that it may be effective against a broad spectrum of tumor types. *Anti-Cancer Drugs* 15:849–860 © 2004 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2004, **15**:849–860

Keywords: ATP, chemosensitivity, MLN576, ovarian cancer, P-glycoprotein, resistance, topoisomerase, XR11576, XR5000

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Sponsorship: This project was funded by Xenova Ltd, UK.

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Received 26 April 2004 **Accepted** 12 July 2004

Introduction

Several joint inhibitors of topoisomerase (Topo) I and II have been recently described (reviewed in [1]): DACA/XR5000 [2], intoplicine [3], F 11782 [4], XR5944 [5], XR11576 [6], XR11612 [7] and TAS-103 [8], although the latter predominantly inhibits Topo IIα [9]. XR11576 (MLN576) is a monophenazine that recently entered clinical evaluation. XR11576 demonstrated potent cytotoxic activity against a panel of human and murine tumor cell lines ($IC_{50}=6$–47 nM) and this activity was unaffected by multidrug resistance (MDR) mediated by overexpression of either P-glycoprotein or MDR-associated protein (MRP), or by downregulation of Topo II. Importantly, XR11576 also showed marked efficacy against a number of human tumors including sensitive (H69/P) and multidrug-resistant (H69/LX4) small cell lung cancer and HT29 colon carcinoma xenografts [6]. XR11576 was proposed as a dual inhibitor of Topo I and II based on its ability to stabilize cleavable complexes for both Topo I and II *in vitro* in a dose-dependent fashion [6]. Cleavable complex formation by XR11576 was also analyzed in human leukemic K562 cells using the TARDIS assay [10,11], which demonstrated drug-induced cleavable complex formation for Topo I, IIα and IIβ in a dose- and time-dependent manner. These observations, however, do not exclude additional or alternative mechanisms of action for the cytotoxic activity of XR11576. In a very recent study, XR11576 was shown to bind to DNA [12].

XR11576 has been selected for further development and thus it is important to demonstrate that the compound is effective against cells derived from clinical tumor samples. A broad *ex vivo* activity profile would strengthen further clinical development and generate information to help design phase II trials. It has been proposed that the ATP-Tumor Chemosensitivity Assay (ATP-TCA) can be used in the development of new agents and combinations for use in cancer patients [13]. As an example, this method has been previously employed to assess the

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ex vivo activity of a novel dual inhibitor of Topo I and II inhibitor, XR5000 [14]: the assay showed that this new drug was effective against melanoma as well as ovarian cancer, but at concentrations which were unlikely to be achieved in patients [15].

In the present study we aimed to determine the *ex vivo* activity of XR11576 in a variety of solid tumors and compare its activity with other dual Topo I/II inhibitors as well as other cytotoxics currently in clinical use.

Materials and methods

Patients and samples

A total of 89 tumors (44 solid tumors, 37 ascites and eight pleural effusions) were tested with XR11576, with local ethics committee approval for the use of tissue or cells not required for diagnosis. The median age of the patients was 60 years (range 35–88; 17M:72F; Table 1). The ovarian cancer patients sub-group were all previously treated with carboplatin alone or carboplatin plus taxanes first line, followed in four cases by an anthracycline-containing regimen, in four cases by the combination treosulfan plus gemcitabine, in three cases by carboplatin plus gemcitabine and in three cases by etoposide. Briefly, tumor tissue that was not required for diagnosis was taken by a histopathologist or surgeon under sterile conditions, with patient consent, and transported to the laboratory in cell culture medium of Dulbecco's modified Eagle's medium (DMEM; Sigma, Poole, UK; cat. no. D5671) with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Sigma; cat. no. P0781) at 4°C.

Drugs

XR11576 hydrochloride salt, XR5000 and TAS-103 were supplied by Xenova (Slough, UK) as powder. They were dissolved in DMSO to give a stock solution of 1 mg/ml and aliquots were stored at −20°C. Cisplatin, treosulfan, vinorelbine (Navelbine), paclitaxel (Taxol), doxorubicin, etoposide (Vepesid) and topotecan (Hycamptin) were obtained from the pharmacy at Queen Alexandra Hospital. Cisplatin and paclitaxel were stored at room temperature, vinorelbine was kept in the refrigerator, and all other drugs were stored at −20°C, as previously reported [16]. XR11576 was tested in every case and six or more other cytotoxics were tested concurrently for each sample. We evaluated the activity of XR11576 in combination with paclitaxel or vinorelbine in a subgroup of tumors ($n = 43$). These samples consisted of 22 ovarian carcinomas (of which 12 had previous taxane exposure), seven unknown primaries, five skin melanomas, four colorectal carcinomas, three breast carcinomas, one esophageal carcinoma and one sarcoma.

ATP-TCA

Cells were obtained from solid tumors by gentle enzymatic dissociation, usually 0.75 mg/ml collagenase (Sigma; cat. no. C8051) overnight. Viable tumor-derived cells were separated from dead cells and debris by density centrifugation (Histopaque 1077-1; Sigma), washed, counted and resuspended to 100 000 cells/ml in the case of ascitic specimens or 200 000 cells/ml for solid biopsies. The cells were used to set up ATP-TCA plates according to the protocol previously described [17].

Briefly, cells were seeded in 96 well polypropylene microplates (Corning-Costar, High Wycombe, UK; cat. no. 3790) at 10 000–20 000 cells/well with each drug/combination at six doubling dilutions in triplicate from 200% test drug concentration (TDC) to 6.25% TDC. TDCs were 150 nM for XR11576, 2.9 μM for XR5000, 1.45 μM for TAS-103, 10.0 μM for cisplatin, 71.9 μM for treosulfan, 11.1 μM for vinorelbine, 15.9 μM for paclitaxel, 2.5 μM for doxorubicin, 81.6 μM for etoposide and 1.64 μM for topotecan, respectively. Combinations were made up by adding both drugs concurrently at their 200% TDC to the wells at the beginning of the assay and diluted in a constant ratio: sequential studies were not performed.

Table 1 Tumors tested with XR11576, showing the average age (range), sex ratio and previous treatment

Tumor type	*n*	Age	Sex	Previous treatment
Ovarian carcinoma	42	58 (38–78)	0M:42F	primary: platinum + taxane ($n=25$), platinum ($n=17$) second-line: anthracycline ($n=5$), treosulfan + gemcitabine ($n=4$), etoposide ($n=3$), carboplatin + gemcitabine ($n=3$), mitoxantrone + paclitaxel ($n=1$)
Cutaneous melanoma	14	58 (35–78)	7M:7F	primary: melphalan ($n=3$), vindesine ($n=2$), cyclophosphamide ($n=1$)
Unknown primary	10	63 (45–78)	3M:7F	primary: carboplatin ($n=1$)
Colorectal carcinoma	9	71 (39–88)	4M:5F	primary: irinotecan ($n=1$)
Breast carcinoma	5	57 (39–61)	0M:5F	primary: cyclophosphamide + anthracycline ($n=3$), antihormonal agent ($n=2$)
Esophageal carcinoma	3	55 (52–72)	3M:0F	primary: epirubicin + cisplatin + 5-FU ($n=2$)
Endometrial carcinoma	2	60 (55–66)	0M:2F	primary: carboplatin ($n=2$) second-line: paclitaxel ($n=1$)
Sarcoma	2	70 (57–82)	0M:2F	none
Lung (non-small cell lung cancer)	1	58	0M:1F	primary: cisplatin + vinorelbine ($n=1$)
Uveal melanoma	1	67	0M:1F	none
Total	89	60 (35–88)	17M:72F	

The plates were then incubated at 37°C in 5% CO_2 for 6 days. The degree of cell inhibition at the end of this period was assessed by measurement of the remaining ATP in comparison with negative control (no drug, M0) and positive control (maximum inhibitor, MI) rows of 12 wells each. ATP was extracted from the cells and measured by light output in a microplate luminometer (Berthold Diagnostic Systems, Hamburg, Germany) following addition of luciferin–luciferase.

Immunohistochemistry

Of the 89 tumor samples studied, material for immunohistochemistry was available for 40 tumors (12 skin melanomas, 10 ovarian carcinomas, nine colorectal carcinomas, three esophageal carcinomas, three unknown primaries, two breast carcinoma and one sarcoma). The monoclonal antibodies P-gp (NCL-JSB1), Topo I, clone 1D6, (NCL-TOPO1) and Topo IIα, clone 3F6 (NCL-Topo IIA) from Novacastra (Newcastle upon Tyne, UK) were detected using the Vectastain Universal Alkaline Phosphatase Kit (Vector laboratories, Peterborough. UK). Sections were cut on to Surgipath positively charged slides (Surgipath Europe, Peterborough, UK) and dried at 60°C for 40 min. Wax was removed from the sections prior to staining by immersion in several changes of solvent followed by several changes of alcohol and rinsed in running tap water.

High-temperature antigen retrieval (pressure cooking), using sodium citrate buffer pH 6.0 for 2 min was used to reveal the antigen-presenting sites blocked by formalin fixation [18]. The sections were rapidly cooled and washed in running tap water.

The sections were then incubated for 20 min in normal blocking serum (in the universal kit used this was horse serum). This was followed by incubation in the primary antibody at room temperature for 30 min for the Topo I and II.

To block endogenous avidin-binding sections for the MDR-1 antibody were treated with separate blocking stages of 20 min in avidin and 20 min in biotin, prior to overnight incubation in the primary antibody at 4°C in a humid incubation chamber.

Following incubation in the primary antibody and rinsing in Tris-buffered saline (TBS), the slides were incubated for 30 min with diluted biotinylated universal secondary solution, rinsed and then incubated for 30 min with Vectastain ABC-AP reagent. To visualize the reaction the slides were incubated for 20 min in Vector Red alkaline phosphate substrate kit (plus levamisole to inhibit endogenous alkaline phosphatase activity).

The slides were counterstained with Gills Hematoxylin dehydrated and cleared using the Leica XL slide staining machine. The sections were mounted in Vector Mount, which produces a permanently mounted section and has an optimal refractive index to retain the color intensity of Vector Red substrate reaction product.

The concentration of the antibody was determined by titration on positive control material and was made up to its optimal dilution in TBS, pH 7.6. A positive control section was run with each batch of staining (renal proximal tubules for P-gp, tonsil or appendix for Topos). A duplicate of each test section was included as a negative control by omitting the antibody and replacing with TBS.

Data analysis

The percentage inhibition for each drug concentration was calculated as $1-[(\text{Test}-\text{MI})/(\text{M0}-\text{MI})]\times 100$ using an Excel 2000 spreadsheet (Microsoft). For each drug concentration curve, the 50% inhibitory concentration (IC_{50}) and the 90% inhibitory concentration (IC_{90}) were calculated as previously described [19]. Non-parametrical statistical methods were used. The calculated and descriptive data were entered into an Access 2000 database (Microsoft) and analyzed using a Wilcoxon two-tailed paired rank sum test or the Mann-Whitney U test for unpaired data, as appropriate (Statsdirect). IC_{50} and IC_{90} values for XR11576 were compared to those for other drugs using Spearman's rank correlation coefficient, with a Bonferroni correction: statistical significance was taken as $p < 0.005$. Combination effects were assessed by the method of Poch *et al.* [20], as previously used with the ATP-TCA in other studies [21–23]. We also performed a Chou and Talalay analysis [24] where the combination indices (CI) were determined at 50 and 90% cell death, and were defined as follows:

$$\begin{aligned}\mathrm{CI}_{A+B} = {} & [(D_{A/A+B})/D_A] + [(D_{B/A+B})/D_B] \\ & + [\alpha(D_{A/A+B}\times D_{B/A+B})/D_A D_B]\end{aligned}$$

where CI_{A+B} = CI for a fixed effect ($F = 50$ or 90%) for the combination of cytotoxic A and cytotoxic B; $D_{A/A+B}$ = concentration of cytotoxic A in the combination A + B giving an effect F; $D_{B/A+B}$ = concentration of cytotoxic B in the combination A + B giving an effect F; D_A = concentration of cytotoxic A alone giving an effect F; D_B = concentration of cytotoxic B alone giving an effect F. α = parameter with value 0 when A and B are mutually exclusive and 1 when A and B are mutually non-exclusive.

The combination index CI indicated: synergism < 0.8, additivity 0.8–1.2 and antagonism > 1.2; slight synergistic and additive cytotoxic activity for values of 0.8 and 1.2, respectively [25].

Immunohistochemistry assessment was performed using the *H*-score. Staining intensity (none = 0 points; weak = 1 point; moderate = 2 points; strong = 3 points) and percentage of positive tumor cells were multiplied to achieve a score between 0 and 300. A *H*-score of 100 or more was regarded as positive. The correlation coefficients were calculated by the method of the least squares, and the correlation between the IC_{90} and IC_{50} values and immunohistochemistry indices was assessed using univariate linear regression (Statsdirect).

Results

Activity of XR11576 against tumor-derived cells

XR11576 showed a steep concentration–response curve in most tumors as exemplified in Fig. 1.

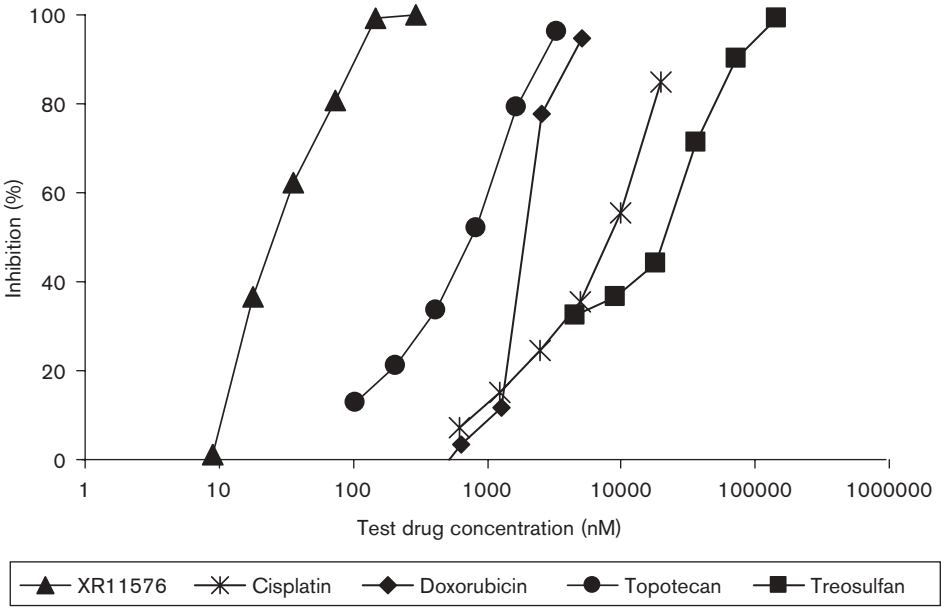
The effect of XR11576 varied both between tumor types and markedly between patients within the same tumor type (Fig. 2). Overall, XR11576 had median IC_{50} and IC_{90} values of 110 and 242 nM, respectively. The median IC_{50} and IC_{90} values calculated for each tumor type are shown in Table 2. The IC_{90} values well correlated with the IC_{50} values ($r = 0.893$, $p < 0.0001$, non-parametric Spearman correlation). In all samples XR11576 was more potent than other experimental or clinical chemotherapeutic agents, such as XR5000, TAS-103, paclitaxel, doxorubicin and topotecan (Table 3).

XR11576 demonstrated its activity on a wide variety of different tumors. Unknown primaries, breast and gynecological malignancies proved highly sensitive to this new agent, though gastrointestinal tumors were less sensitive (Fig. 2). In particular, 37 of 42 (88%) ovarian cancer samples had an IC_{50} value below 200 nM and an IC_{90} below 300 nM. When compared to other agents used in second-line for ovarian cancer, XR11576 was at least 10-fold more potent than doxorubicin or topotecan (Fig. 1 and Table 3). The median IC_{90} value for XR11576 in those samples from ovarian cancer patients who had received a taxane-based regimen ($n = 26$) was 242 nM compared to 155 nM of samples from the small number of patients who had not been pre-exposed to taxanes ($n = 16$), but this difference was not statistical significant ($p = 0.108$, Mann–Whitney *U*-test).

The best activity (e.g. the lowest median IC_{90} value) was observed in the small group of breast tumors tested ($n = 5$). In these tumors, XR11576 had an IC_{90} of 173 nM, which was 170-fold more potent than the test results for paclitaxel. XR11576 showed remarkable activity against skin melanoma, a tumor type that is usually poorly sensitive to chemotherapy. In 11 of 14 (79%) melanoma samples XR11576 had an IC_{50} value below 200 nM and an IC_{90} value below 300 nM.

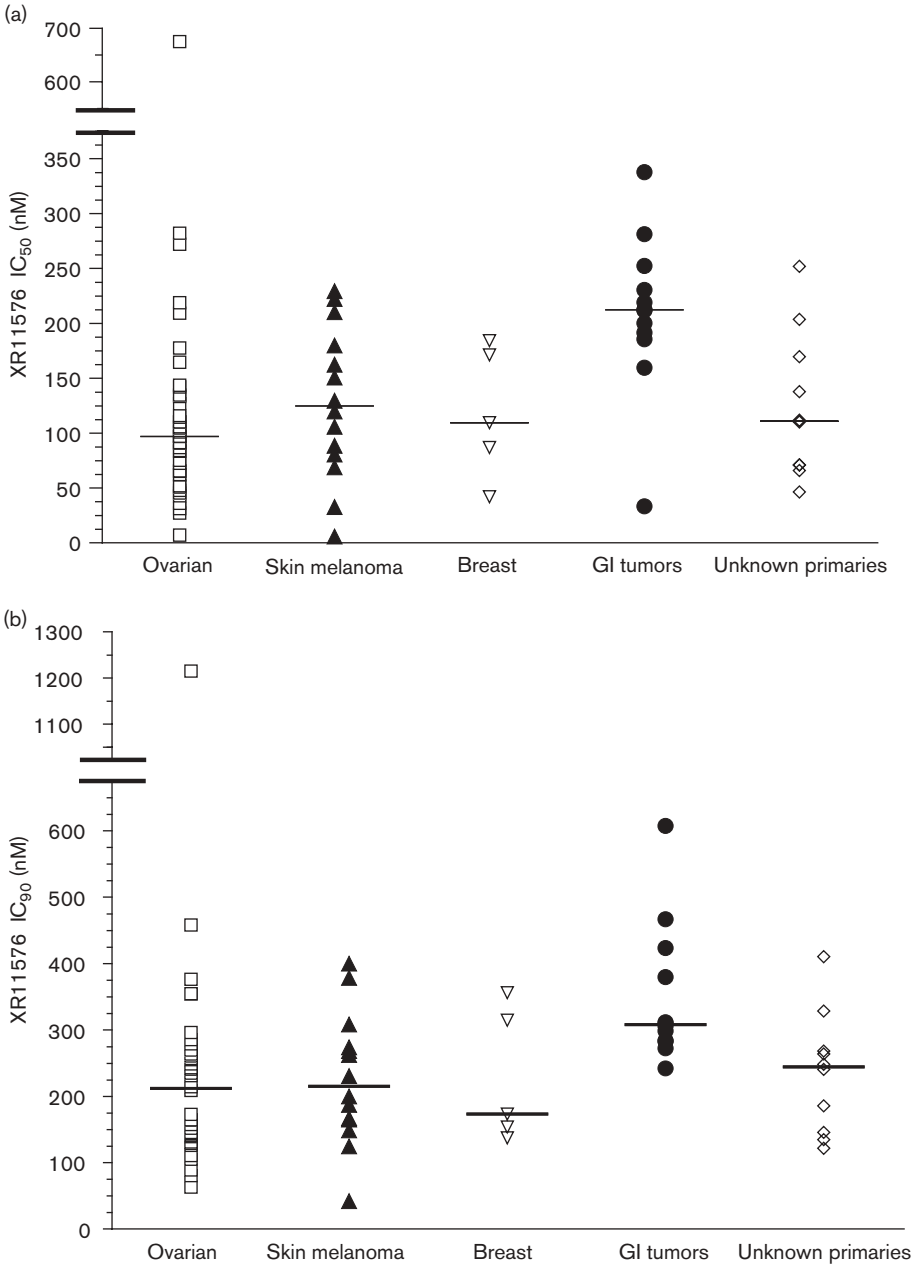
XR11576 showed decreased potency in colorectal and esophageal samples. When all 12 gastrointestinal tumors were analyzed together, the median IC_{90} and IC_{50} values were 308 and 212 nM (Fig. 2), and these values were significantly higher than the corresponding values in ovarian samples ($p < 0.0005$, Mann–Whitney test). Five of nine (56%) colorectal samples had IC_{50} values below

Fig. 1



Example of ATP-TCA results in an ovarian tumor.

Fig. 2



Heterogeneity of XR11576 sensitivity in ovarian samples ($n=42$), skin melanomas ($n=14$), breast samples ($n=5$), gastrointestinal tumors ($n=12$) and tumors of unknown origin ($n=10$). Each dot represents the IC_{50} (a) or IC_{90} (b) value for an individual patient. The lines represent median values.

200 nM, while all three esophageal specimens had an IC_{50} slightly over 200 nM. However, gastrointestinal samples were also very resistant to other drugs: in this tumor type we recorded the highest median IC_{50} and IC_{90} values for most drugs.

Comparison of XR11576 with other anticancer agents

When IC_{50} values were analyzed using Spearman's rank correlation coefficient (non-parametric method for paired data), cross-resistance was seen between XR11576 and the other experimental agents XR5000 ($r=0.609$, $p<0.0001$) and TAS-103 ($r=0.724$, $p<0.0001$). A significant correlation was also found with the other Topo II inhibitors doxorubicin ($r=0.407$, $p<0.0005$) and etoposide ($r=0.428$, $p<0.005$). Some cross-resistance was seen with paclitaxel ($r=0.330$, $p<0.005$), while no correlation was observed between the IC_{90} values for XR11576 and vinorelbine ($r=0.285$, NS), treosulfan

Table 2 Median IC_{90} and IC_{50} values (range) for XR11576 tested in the ATP-TCA in *n*=number of samples for each tumor type

Tumor type	*n*	IC_{50} μM	IC_{90} μM
Ovarian carcinoma	42	0.097 (0.007–0.675)	0.212 (0.064–1.215)
Skin melanoma	14	0.125 (0.006–0.229)	0.215 (0.042–0.400)
Unknown primary	10	0.111 (0.046–0.252)	0.244 (0.122–0.410)
Colorectal carcinoma	9	0.200 (0.033–0.338)	0.306 (0.242–0.608)
Breast carcinoma	5	0.109 (0.042–0.184)	0.173 (0.137–0.356)
Esophageal carcinoma	3	0.213 (0.212–0.231)	0.310 (0.299–0.380)
Sarcoma	2	0.118 (0.015–0.222)	0.176 (0.065–0.287)
Endometrial carcinoma	2	0.125 (0.083–0.167)	0.237 (0.175–0.300)
Lung (non-small cell lung cancer)	1	0.097	0.137
Uveal melanoma	1	0.181	0.325
Total	89	0.110 (0.006–0.675)	0.242 (0.042–1.215)

Table 3 Median IC_{90} and IC_{50} values (range) for the cytotoxics tested in the ATP-TCA in *n*=number of samples

Drug	*n*	IC_{50} μM	IC_{90} μM
XR11576 (MLN576)	89	0.110 (0.006–0.675)	0.242 (0.042–1.215)
XR5000	73	1.94 (0.17–5.13)	5.05 (0.87–7.26)
TAS-103	27	0.99 (0.06–5.54)	2.79 (1.38–9.98)
Cisplatin	79	13.3 (0.4–80.4)	25.9 (7.6–144.8)
Treosulfan	76	39.4 (2.9–390.8)	116.3 (23.9–703.4)
Etoposide	47	74.2 (3.3–932.7)	177.2 (16.8–1679.3)
Topotecan	51	1.02 (0.07–10.02)	3.60 (0.29–18.04)
Doxorubicin	73	1.10 (0.10–45.74)	2.47 (0.30–82.32)
Paclitaxel	82	8.78 (0.55–32.79)	25.35 (3.50–59.03)
Vinorelbine	58	2.17 (0.37–34.49)	16.85 (0.67–62.08)

($r = 0.258$, NS) and topotecan ($r = 0.250$, NS) and cisplatin ($r = 0.148$, NS).

The IC_{90} values confirmed the cross-resistance pattern described above. A significant correlation was found between XR11576 and the Topo inhibitors XR5000 ($r = 0.582$, $p < 0.0001$), TAS-103 ($r = 0.556$, $p < 0.005$), doxorubicin ($r = 0.441$, $p < 0.0001$) and etoposide ($r = 0.389$, $p < 0.005$). Some cross-resistance was still seen with paclitaxel ($r = 0.325$, $p < 0.005$), while no correlation was observed between the IC_{90} values for XR11576 and vinorelbine ($r = 0.240$, NS), cisplatin ($r = 0.135$, NS), treosulfan ($r = 0.106$, NS) or topotecan ($r = 0.100$, NS).

Activity of XR11576 in combination with other anticancer agents

XR11576 plus doxorubicin

The combination of XR11576 with doxorubicin showed a median CI_{50} value of 1.03, therefore suggesting an additive effect (Fig. 3). Considerable heterogeneity was found among samples as shown in Table 4. Synergism ($CI < 0.8$) was identified in six of 38 (16%) and in 12 of 38 (32%) samples when CI values were calculated for 90% (CI_{90}) and 50% (CI_{50}) cytotoxicity, respectively; while antagonism ($CI_{50} > 1.2$) was found in 13 of 38 (34%) samples tested with the combination of XR11576 with doxorubicin, although this percentage increased to 47% when CI_{90} values were calculated. Additivity was identified in 13 of 38 (34%) samples on the basis of the CI_{50} and in 14 of 38 (37%) samples on the basis on the CI_{90}.

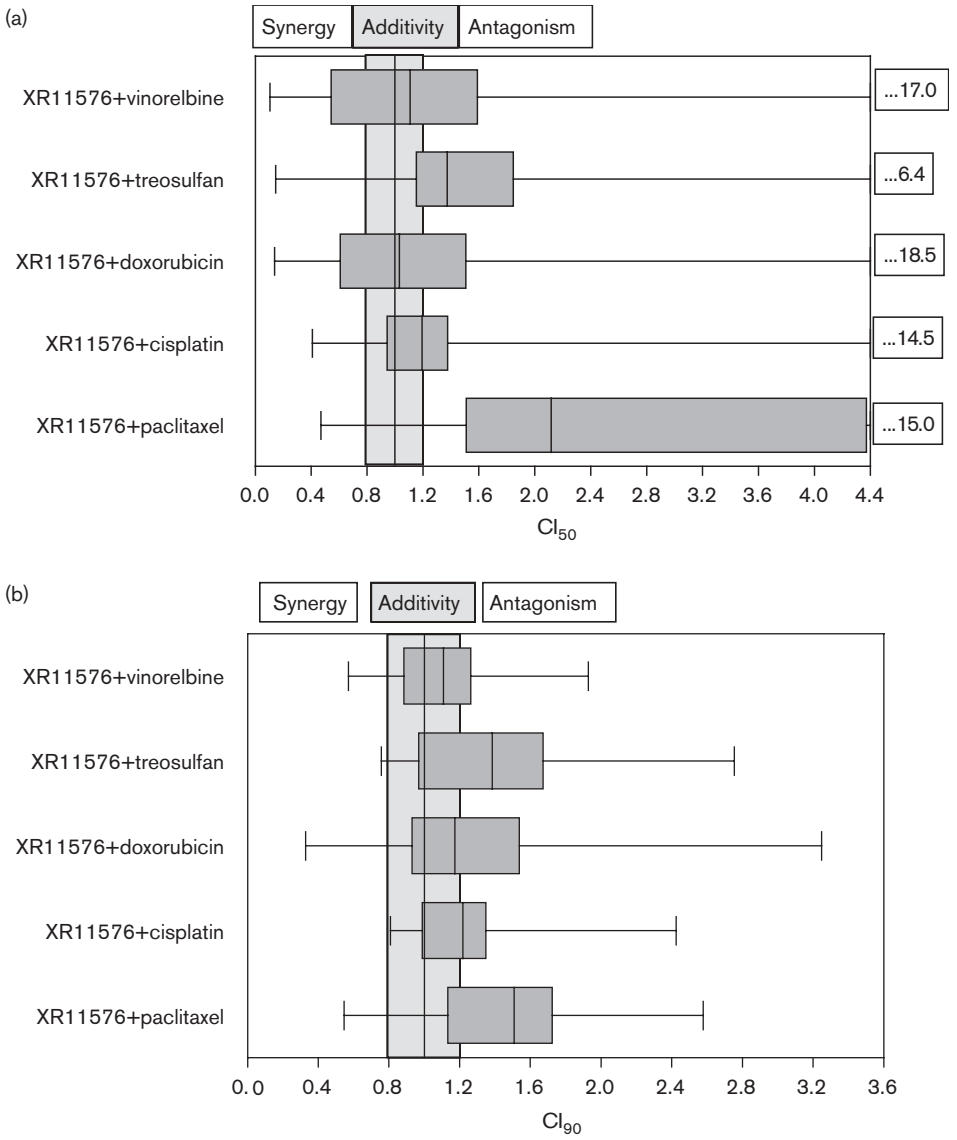
XR11576 plus DNA-damaging agents

The median CI_{50} values for the combinations of XR11576 with cisplatin or treosulfan were 1.19 and 1.37, while the median CI_{90} values were 1.22 and 1.38, respectively. As suggested by these high median CI values, antagonism between XR11576 and these two DNA damaging agents was identified in a large proportion of cases: CI_{50} values > 1.2 were calculated in 28 of 40 (70%) and in 20 of 42 (48%) samples for the combinations with treosulfan or cisplatin, respectively. Synergism was found only in a minority of cases. When CI_{90} values for each individual tumor were considered, the combination of XR11576 with treosulfan showed synergism in only one of 41 (2%) samples ($CI_{90} = 0.76$), while the addition of cisplatin to XR11576 did not produce any synergistic effect. On the basis of the CI_{50} values, the combinations of XR11576 with treosulfan or cisplatin showed synergism in three of 40 (8%) and in five of 42 (12%) samples, respectively.

XR11576 plus microtubule-interfering agents

The median CI_{50} values for the combinations of XR11576 with vinorelbine or paclitaxel were 1.11 and 2.12, while the median CI_{90} values were 1.11 and 1.51, respectively. The best effect was obtained with the combination of XR11576 + vinorelbine, which was better than XR11576 alone in 30 of 31 paired observations ($p < 0.0001$ on IC_{50} or IC_{90}, Wilcoxon matched-pairs signed-ranks test). Figure 4(a) shows the advantage of the combination XR11576 + vinorelbine over the individual agents in terms of inhibition; when analyzed by the Poch method [20], in which the observed effect at each concentration tested is compared with the expected effect, some

Fig. 3



Summary of the CI values calculated for 50% (a) and 90% (b) cytotoxicity in tumor derived cells treated with the combinations shown. For each combinations the interquartile range (grey histograms), the median and the range are shown.

synergism was seen at the lower concentrations, while additivity was observed at the highest dose (Fig. 4b). This is reflected by the combination indices calculated by the Chou and Talalay method [24] on the median dose–response curve, which were 0.45 (synergy) and 0.86 (additivity) for an effect of 50 and 90% inhibition, respectively.

CI_{50} and CI_{90} values > 1.2 (antagonism) were found, respectively, in 38 of 42 (90%) and in 31 of 43 (72%) samples tested with the combination of XR11576 with paclitaxel.

According to Poch, a median antagonistic effect at the lower concentrations between these two drugs was also found (Fig. 5). Some additivity was found at the higher concentrations as shown by the Poch method (Fig. 5) and by the analysis of the CI_{90} values (Table 4b).

Correlation of XR11576 activity with immunohistochemistry

Immunostaining for P-gp was positive in 14 of 48 (29%) samples tested with XR11576. The P-gp-expressing samples consisted of four ovarian tumors, three skin melanomas, three esophageal cancers, two colorectal tumors and two unknown primary carcinomas. The

Table 4 Effect of combining XR11576 with other cytotoxics for *n* = number of samples tested using the Chou and Talalay CI calculated on the IC_{50} (a) and on the IC_{90} (b)

Tumor type	XR11576 and	No. samples tested	CI (*n*)		
			<0.8	0.8–1.2	<1.2
(a) IC_{50}					
all tumors	paclitaxel	42	3	1	38
	vinorelbine	30	9	9	12
	treosulfan	40	3	9	28
	cisplatin	42	5	17	20
	doxorubicin	38	12	13	13
ovarian	paclitaxel	22	0	1	21
	vinorelbine	17	4	5	7
	treosulfan	21	1	2	18
	cisplatin	22	2	6	14
	doxorubicin	22	7	8	7
non-ovarian	paclitaxel	20	3	0	17
	vinorelbine	14	5	4	5
	treosulfan	19	2	7	10
	cisplatin	20	3	11	6
	doxorubicin	16	5	5	6
(b) IC_{90}					
all tumors	paclitaxel	43	2	10	31
	vinorelbine	31	7	15	9
	treosulfan	41	1	17	23
	cisplatin	42	0	19	23
	doxorubicin	38	6	14	18
ovarian	paclitaxel	22	1	4	17
	vinorelbine	17	5	9	3
	treosulfan	22	0	7	15
	cisplatin	22	0	8	14
	doxorubicin	22	5	6	11
non-ovarian	paclitaxel	21	1	6	14
	vinorelbine	14	2	6	6
	treosulfan	19	1	10	8
	cisplatin	20	0	11	9
	doxorubicin	16	1	8	7

median XR11576 IC_{50} values for P-gp-negative and -positive samples were 131 and 210 nM, respectively ($p = 0.0178$, Mann–Whitney *U*-test), while the median IC_{90} values were 262 and 309 nM, respectively ($p = 0.038$, Mann–Whitney *U*-test). A very modest correlation by linear regression analysis was found between the IC_{50} of XR11576 and the intensity of P-gp staining ($r = 0.311$, $p = 0.0312$), but not with the IC_{90} ($r = 0.247$, NS, $p = 0.091$).

Immunostaining for Topo I and IIα was positive in 35 of 46 (76%) and nine of 44 (20%) samples tested with XR11576, respectively. Those samples that were positive for Topo I included 13 ovarian tumors, eight skin melanomas, six colorectal tumors, three unknown primary carcinomas, two esophageal cancers, two breast tumors and one sarcoma. The Topo IIα-expressing samples consisted of six colorectal tumors, two ovarian tumors and one skin melanoma. The median XR11576 IC_{50} values for Topo I-negative and -positive samples were 162 and 135 nM, respectively ($p = 0.7185$, NS, Mann–Whitney *U*-test), while the median IC_{90} values were 274 and 262 nM, respectively ($p = 0.8368$, NS, Mann–Whitney *U*-test).

The median XR11576 IC_{50} values for Topo IIα-negative and -positive samples were 133 and 191 nM, respectively ($p = 0.154$, NS, Mann–Whitney *U*-test), while the median IC_{90} values were 261 and 283 nM, respectively ($p = 0.3226$, NS, Mann–Whitney *U*-test). No correlation by linear regression analysis was found between the IC_{50} or IC_{90} values for XR11576 and either the Topo I or the Topo IIα immunohistochemistry indices (p = NS).

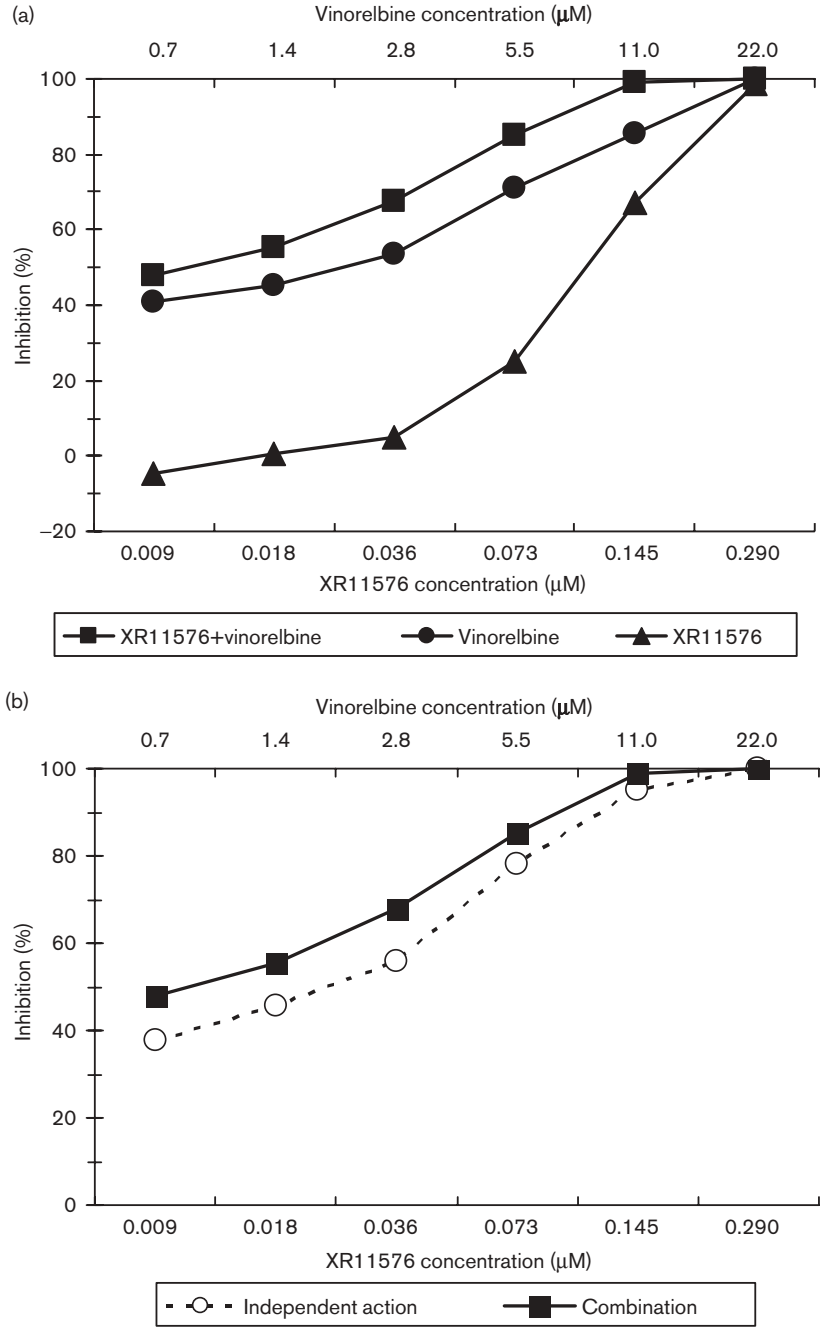
Discussion

The *ex vivo* profile of XR11576 in human solid tumors has confirmed it to be an exceptionally potent cytotoxic agent. In all samples tested, XR11576 was more active than all other compounds tested, including topotecan, doxorubicin, paclitaxel, and the experimental agents XR5000 and TAS-103. As expected, the potency of XR11576 is less in tumor-derived cells compared to cell lines, which probably reflects the homogeneity and the fast-growing status of the cell lines used in previous studies [6]. This is true of most cytotoxic drugs [26]. The effect of XR11576 varied considerably between tumor types as well as against samples from the same tumor type derived from different patients. This is consistent with both the situation found in the clinic and also our previous findings testing conventional cytotoxics [27–29].

XR11576 showed great considerable activity in the subset of ovarian cancer samples. As all the ovarian patients had relapsed after receiving a platinum-based regimen and were resistant in the ATP-TCA, enhancement of drug resistance mechanisms to DNA-damaging agents is expected. Platinum treatment may cause upregulation of drug detoxifying enzymes, such as the glutathione-S-transferases (GST) [30], and loss of mismatch repair mechanisms (MMR) in a proportion of patients [31], therefore rendering the cells more resistant to several chemotherapeutic agents [32]. Our data may therefore suggest that XR11576 activity is not susceptible to these resistance mechanisms, although this should be tested in further studies. The fact that a large proportion of samples obtained from heavily pre-treated ovarian cancer patients do not appear resistant to XR11576 augurs well for its use as second-line therapy in this clinical condition.

It should be noted that even in those samples over-expressing P-gp, XR11576 showed better greater activity than the other cytotoxic agents tested. Furthermore, XR11576 activity is only slightly reduced in gastrointestinal tumors that are well known to overexpress MDR1 and other pump proteins, and are generally refractory to chemotherapy. This is in accordance with previous studies that have shown XR11576 is not a substrate for either P-gp or MRP efflux pumps [6]. Data from this study and our marginal data from this study suggest that P-gp plays little, if any, role in resistance to XR11576.

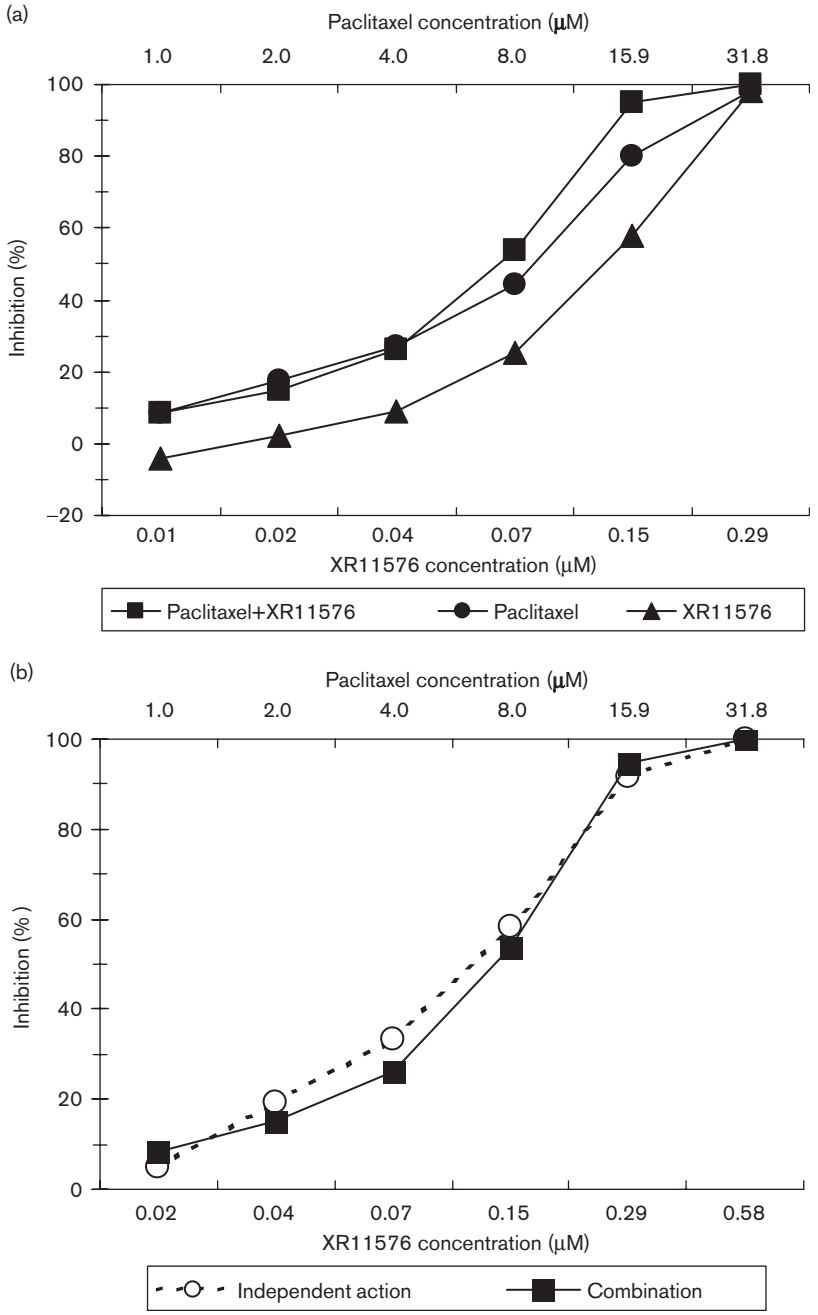
Fig. 4



The combination XR11576 + vinorelbine shows greater than additive effect when analyzed by the Poch method, while a $CI_{50} = 0.45$ (synergy) and a $CI_{90} = 0.86$ (additivity) when analyzed by the Chou and Talalay method. (a) Median inhibition–concentration curve for $n = 31$ samples. (b) When analyzed by the method of Poch, at which the observed effect (combination) at each concentration tested is compared with that expected (independent action), the median effect is greater than additive.

Although some *in vitro* and *in vivo* reports have reported reduced levels of Topo IIα to correlate with resistance to Topo inhibitors such as doxorubicin and etoposide [33–35], our data showed that XR11576 activity does not correlate with the expression of either Topo I or IIα. On the other hand, there is cross-resistance with Topo inhibitors, including doxorubicin, TAS-103 and XR5000. These findings are therefore consistent with inhibition of both enzymes, although we cannot exclude an alternative mechanism of action, as recently suggested [36].

Fig. 5



The combination XR11576 + paclitaxel shows a slightly antagonistic effect at the lower concentrations when analyzed by the Poch method, while a $CI_{50} = 2.26$ (antagonism) and a $CI_{90} = 1.09$ (additivity) when analyzed by the Chou and Talalay method. (a) Median inhibition–concentration curve for $n = 43$ samples. (b) When analyzed by the method of Poch, at which the observed effect (combination) at each concentration tested is compared with that expected (independent action), the median effect is slightly antagonistic.

Previous *in vitro* and *ex vivo* studies have reported enhanced cell kill when Topo inhibitors are combined with vinca alkaloids [23,37,38]. While an increased effect was not found for all samples tested, individual cases that displayed true synergism between XR11576 and vinorelbine were apparent in our series. Despite some cross-resistance between XR11576 and doxorubicin, we found synergism between these two drugs in a proportion of samples, suggesting that non-Topo-mediated effects of the anthracycline may be important for cytotoxicity. We

found, unexpectedly, no synergism between XR11576 and cisplatin or treosulfan; this is in contrast with other *ex vivo* studies that reported some synergism between cisplatin and Topo inhibitors [14,39,40]. Some antagonism was noted between XR11576 and paclitaxel. These results are consistent with previous *in vitro* studies that have shown an antagonistic effect when paclitaxel is combined with certain non-anthracycline Topo inhibitors, such as etoposide, topotecan, SN38 and tafluposide [38,41–45], although others reported additive or synergistic effects in different cell lines [37,39,46].

Chemosensitivity testing using fresh tumor cells taken directly from patients is not commonly used for preclinical studies at present. We believe, nevertheless, that it provides useful information on the cellular sensitivity of novel compounds before they reach more expensive phase I/II trials [15]. The results of this study may assist the further clinical development of XR11576. If phase I studies prove the safety of this compound, then our results suggest that phase II trials should target those clinical conditions (breast, ovarian cancer and cutaneous melanoma) where this new agent is likely to produce the greatest benefit.

Acknowledgments

We are grateful to Mrs Penny Johnson, Mrs Alison Parker and Mrs Lisa Mills for their technical assistance with immunohistochemistry. We wish to thank all of the oncologists and surgeons who submitted material for testing, particularly Drs Lamont, Hindley, Osborne, Allerton, Khoury and Weaver.

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