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Report

In vitro and *in vivo* characterization of XR11576, a novel, orally active, dual inhibitor of topoisomerase I and II

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XR11576, a novel phenazine, was developed as an inhibitor of both topoisomerase I and II. This study characterized the ability of XR11576 to inhibit both enzymes, and determined its *in vitro* and *in vivo* antitumor efficacy against a number of murine and human tumor models. XR11576 was a potent inhibitor of purified topoisomerase I and IIα, and exhibited similar potency for both enzymes. The compound stabilized enzyme–DNA cleavable complexes indicating that it acted as a topoisomerase poison. The DNA cleavage patterns obtained with XR11576 were different from those induced by camptothecin and etoposide, which are topoisomerase I and II poisons, respectively. XR11576 demonstrated potent cytotoxic activity against a variety of human and murine tumor cell lines (IC_{50}=6–47 nM). Its activity profile was comparable to or better than that of many widely used anticancer drugs. Moreover, XR11576 was unaffected by multidrug resistance (MDR) mediated by overexpression of either P-glycoprotein or MDR-associated protein, or by down-regulation of topoisomerase II. The latter property supports the dual inhibitory mechanism of action of the compound. XR11576 exhibited a similar pharmacokinetic profile in mice and rats after either i.v. or p.o. administration. *In vivo* XR11576 showed marked efficacy against a number of tumors including sensitive (H69/P) and multidrug-resistant (H69/LX4) small cell lung cancer and the relatively refractory MC26 and HT29 colon carcinomas following i.v. and p.o. administration. The efficacy of XR11576 was at least comparable to that of TAS-103, originally proposed as a dual inhibitor of topoisomerase I and II. These results suggest that XR11576 is a promising new antitumor agent with oral and i.v. activity, and warrants further development.

Key words: Antitumor agent, dual topoisomerase I and II poison, pharmacokinetics, XR11576.

Introduction

There is currently a great need for more effective treatment of common cancers such as those of the colon, ovary and lung. One important class of antitumor agents with the potential for treating such tumors comprises of agents which inhibit the action of DNA topoisomerases. Two types of topoisomerase enzymes, type I and type II (which exists as two isoforms, α and β), are well characterized. Both topoisomerase I and II are involved in the maintenance of DNA structure, and in the relief of torsional stress occurring during DNA transcription and replication. Topoisomerase II also appears to be essential for chromosome condensation and separation during mitosis, and plays an important role in the organization of chromatin. Topoisomerase I transiently breaks one strand of the duplex DNA, whereas the topoisomerase II enzymes transiently breaks both strands of DNA, allowing passage of single or duplex DNA strands, respectively, and relief of torsional stress. During this process transient covalent links are formed between the enzymes and the DNA, referred to as 'cleavable complexes'. The expression of topoisomerase I and IIβ does not vary significantly during the cell cycle, whereas topoisomerase IIα expression increases during S phase and reaches a peak at G_2/M phase.[1–4]

Clinical agents such as doxorubicin and etoposide, which target topoisomerase II, and derivatives of the plant product camptothecin such as topotecan and CPT-11, which target topoisomerase I, have established an important role in the treatment of a variety of human tumors.[5,6] The majority of these agents including the camptothecin derivatives, doxorubicin and etoposide, act as topoisomerase poisons, stabilizing the normally transient cleavable complexes, which in turn results in double-strand breaks and cell death.[5,7] However, some compounds such as aclarubicin, bis(dioxopiperazines) and F11782 act as catalytic inhibitors suppressing the formation of

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cleavable complexes.[8,9] Given the differing roles and expression of the two types of enzymes within the cell cycle,[1–4,10] inhibitors of both topoisomerase I and II ('dual inhibitors') would be expected to have significant therapeutic advantage over agents targeting one type of topoisomerase alone. In addition, dual inhibitors may circumvent mechanisms of drug resistance attributable to alteration (e.g. by mutation or down-regulation) of a single target enzyme ['atypical' multidrug resistance (MDR)].[11–13] Dual inhibitors may also be expected to have a broader spectrum of activity as expression levels of the two enzymes are variable between different types of cancers.[14,15] Several dual inhibitors of topoisomerases have been identified including saintopin,[16] intoplicin,[17] XR5000 (DACA),[13] TAS-103,[18] aclarubicin[8,19] and F11782.[9] Recently, however, it has been reported that TAS-103 predominantly inhibits topoisomerase IIα.[20] All of these dual inhibitors are DNA intercalators with the exception of F11782, which acts as a catalytic inhibitor of both enzymes without binding to DNA (9). In addition these compounds have varied potency, tolerability at efficacious doses and ability to surmount drug resistance. Moreover, the majority of these compounds are orally inactive.

The dual inhibitor XR5000 is an acridine derivative that acts as a DNA intercalating agent, and the steric and electronic effects that determine its cytotoxic activity have been well characterized.[21] In order to find a novel second-generation dual topoisomerase I/II inhibitor, with greater potency than XR5000 and the advantage of oral activity, but still avoiding MDR, a research programme was initiated around a series of substituted mono benzophenazine carboxamides. The phenazines are chemically and structurally different from the acridines, but have all the physicochemical properties for DNA intercalation.[22] This work resulted in the discovery of XR11576 (4-methoxy-benzo[*a*]phenazine-11-carboxylic acid (2-dimethylamino-1-(*R*)-methyl-ethyl)-amide), a novel phenazine, which satisfies all the above criteria (Figure 1). Here we describe the ability of XR11576 to inhibit topoisomerase I and II activity, and its cytotoxicity in a panel of human and murine cell lines. This includes cell lines which exhibit MDR as result of overexpression of P-glycoprotein (P-gp) or MDR-associated protein (MRP) or down-regulation of topoisomerase II. In addition, the pharmacokinetics and *in vivo* efficacy of XR11576 against murine and human tumor xenografts are presented.

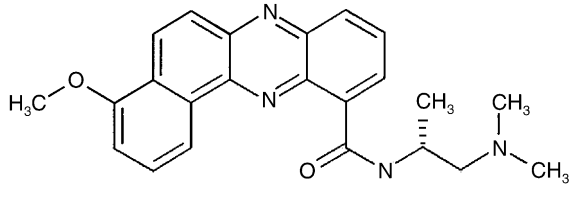

Figure 1. Structure of XR11576.

Materials and methods

Cell lines

COR-L23/P non-small cell lung carcinoma (NSCLC) and H69/P small cell lung carcinoma (SCLC) cell lines, and their respective drug-resistant sublines COR-L23/R and H69/LX4, were kindly provided by Dr PR Twentyman (MRC Clinical Oncology and Therapeutics Unit, Cambridge, UK). The intrinsically resistant MC26 murine colon carcinoma cell line, in which resistance is at least partly attributable to P-gp expression,[23] was obtained from Dr AS Watson (University Hospital, Nottingham, UK). Ovarian carcinoma 1847 cell line was provided by Dr TC Hamilton (Fox Chase Centre, Philadelphia, PA).

Experiments using the Jurkat cell lines, P388 and Lewis lung (LLTC) were carried out at the Auckland Cancer Society Research Centre. These cell lines have been described previously.[13] The HT29 colon carcinoma and MCF-7 breast carcinoma cell lines were obtained from the ATCC (Rockville, MD).

Drugs

XR11576 (base and monohydrochloride salt) and TAS-103 were synthesized by the Department of Medicinal Chemistry at Xenova. All other drugs were obtained from Sigma (Dorset, UK). Etoposide (Vespid) and CPT-11 (irinotecan hydrochloride trihydrate) were purchased from Bristol-Myers (Middlesex, UK) and Rhone-Poulenc Rorer (West Malling, UK), respectively. Drugs were dissolved in DMSO for *in vitro* experiments. For *in vivo* use XR11576 base was dissolved at the required concentration by adding 0.05 M HCl (60% of the required final volume) and heating to about 60°C before making up the final volume by adding 0.05 M NaOH. XR11576 salt and other drugs were dissolved in 5% (w/v) D-(+)-glucose (dextrose) solution or saline for *in vivo* administration.

Animals

All animal experimentation was performed to UK Home Office regulations and the UKCCCR guidelines were adhered to throughout the studies. Female

BALB/c mice, male Wistar rats and female CD1 nude mice were purchased from Charles River. All the animals were maintained under constant temperature and humidity, and 12 h light and dark cycle. Food and water was available *ad libitum.*

Topoisomerase-dependent cleavable complex formation assays

The formation of topoisomerase-dependent cleavable complexes in the presence of XR11576 and other drugs was determined using the method of Finlay *et al.*[13]. Briefly, 4×10^6 c.p.m. ^{32}P-labeled pBR322 DNA and 15 U of calf thymus topoisomerase I (Life Technologies, Paisley, UK) or 20 U of human topoisomerase IIα (Topogen, Columbus, OH) and various concentrations of drugs or DMSO (3.3% final concentration) were incubated for 30 min at 37°C. After digestion of the enzyme/drug incubation with proteinase K and SDS, the samples were electrophoresed on a 1% agarose gel. The gel was then dried and exposed to X-Omat film (Sigma, Poole, UK) for a number of days prior to analysis

Cytotoxicity assays

Cytotoxicity of XR11576 was examined in a number of human and murine cell lines, and the activity was compared with a number of standard agents used in the clinic or in clinical trial. Cells were seeded 4 h prior to addition of various concentrations of drugs. After 4–6 days continuous exposure, cell growth was assessed colorimetrically[24] or by thymidine incorporation.[25] IC_{50} values were determined as the drug concentration required to reduce cell growth by 50%. All points were carried out in quadruplicate and data are presented as the mean of two or more experiments. The resistance factors are calculated as (the IC_{50} in drug resistant cells)/(the IC_{50} in parental cells) and are used to gauge how significantly a compound is affected by a particular mechanism of drug resistance. A value close to 1.0 indicates that the compound is unaffected by a specific mechanism.

Pharmacokinetic studies

Female BALB/c mice and CD1 nude mice were deprived of food 5 h before and 1 h after dosing. XR11576 was administered as an i.v. bolus injection at 20 or 50 mg/kg via the tail vein or as a p.o. dose of 50 mg/kg. Three and four animals per time point were used for i.v. and p.o. administration, respectively. The dosing volume was 10 ml/kg body weight. At various times after dosing (at 2, 15, 30, 60 and 90 min, and 2, 4, 6, 7 and 24 h after i.v., and at 15, 30, 60 and 90 min, and at 2, 3, 4, 6, 7 and 24 h after p.o. administration) the animals were anesthetized with halothane and blood was collected using heparin-treated syringes via heart puncture. The animals were killed by cervical dislocation to collect heart, liver and tumor. All samples were stored immediately at −40°C until analysis. Blood samples were centrifuged at 4°C to isolate plasma and this was stored as 100 μl aliquots at −40°C until analysis. Tissue samples were washed in phosphate-buffered saline (PBS), dried and weighed before homogenization in 3 volumes PBS (all procedures were conducted at 4°C). XR11576 was extracted from plasma and homogenate samples (100 μl) using methanol at –40°C (300 μl) and quantified by reverse-phase HPLC. A 100 μl injection was made of the supernatant and separation achieved on a LiChrospher 100 RP8 column [(250 mm × 4 mm, 5 μm) Merck, Poole, UK; part no. 50832] with triethylammonium phosphate buffer/acetonitrile gradient containing acetic acid and sodium heptane sulfonate at 60°C. XR11576 was detected by its absorbance at 295 nm.

Pharmacokinetic data analysis

For plasma samples taken after i.v. administration, areas under the concentration–time curves (AUC) were calculated using PC Modfit (Gamms, Hertfordshire, UK) using the logarithmic trapezoidal rule and including all values above the limit of quantification (LOQ) up to the last detected time point. For plasma samples taken after p.o. administration and for tissue samples, AUC was calculated using the linear trapezoidal rule up to the peak concentration and the logarithmic trapezoidal rule for post peak concentration.

The mean plasma concentration profiles were fitted to two- and three-compartment models with linear kinetics and compared by the Akaike Criterion using PC Modifit (Gamms). The two-compartment model provided the best fit data with the least errors in the parameter estimations to predict C_0. These predictions were then used in conjunction with non-compartment analysis and/or the appropriate standard equations to calculate pharmacokinetic parameters.

In vivo efficacy studies

MC26 mouse colon carcinoma. MC26 tumors maintained in female BALB/c mice were excised,

tumor slurry prepared and implanted s.c. into flanks of mice (day 0) as described previously.[26] The following day animals were randomized into groups of 13–14 and treated with various regimens of XR11576 and other control drugs. Animals were, weighed at least 3 times per week, and on day 14 they were killed by cervical dislocation and the tumors excised and weighed. The T/C % ratio (mean tumor weight of the treated tumors/mean weight of control group $\times$ 100) was calculated and data were analyzed by Student's *t*-test.

Human carcinoma xenografts. H69/P and H69/LX4 SCLC cells (7×10^6) or HT29 colon carcinoma cells (5×10^6) harvested from *in vitro* incubations in PBS (100 μl) were inoculated s.c. into the right flanks of female CD1 nude mice (20–28 g body weight). The animals were monitored regularly and when the tumors had reached a mean diameter of 0.3–0.7 cm the animals were randomized into groups of at least seven such that the mean tumor volume in each group was similar. Animals were treated with XR11576 and other control drugs, including TAS-103. The start of treatment was day 0 and the drugs were administered i.v. and/or p.o. at various schedules. Relative tumor volume was calculated using the tumor volume on the first day of treatment (day 0). The T/C% ratio (mean tumor volume of the treated tumors/mean volume of control group $\times$ 100) was calculated each time the tumors were measured. The lowest value is expressed as the optimal T/C % for each group. Specific growth delay (SGD) was calculated as $(T_{\text{dtreated}}–T_{\text{dcontrol}})/T_{\text{dcontrol}}$, where T_{d} is the time taken for the relative tumor volume to double (to reach 200% of the starting volume). Statistical analysis was performed using analysis of variance (ANOVA).

Results

Stabilization of topoisomerase I and II induced cleavable complexes by XR11576

The poisoning of topoisomerase I and IIα activity by XR11576 was evaluated using purified enzymes and linearized ^{32}P-labeled pBR322 plasmid DNA as the substrate. The drug-induced cleavable complexes resulting from the poisoning of the enzymes were visualized by electrophoresis of the labeled DNA after digestion with proteinase K. The effects of XR11576 were compared with those of camptothecin (topoisomerase I standard) and etoposide (topoisomerase II standard), which were used as controls (Figure 2).

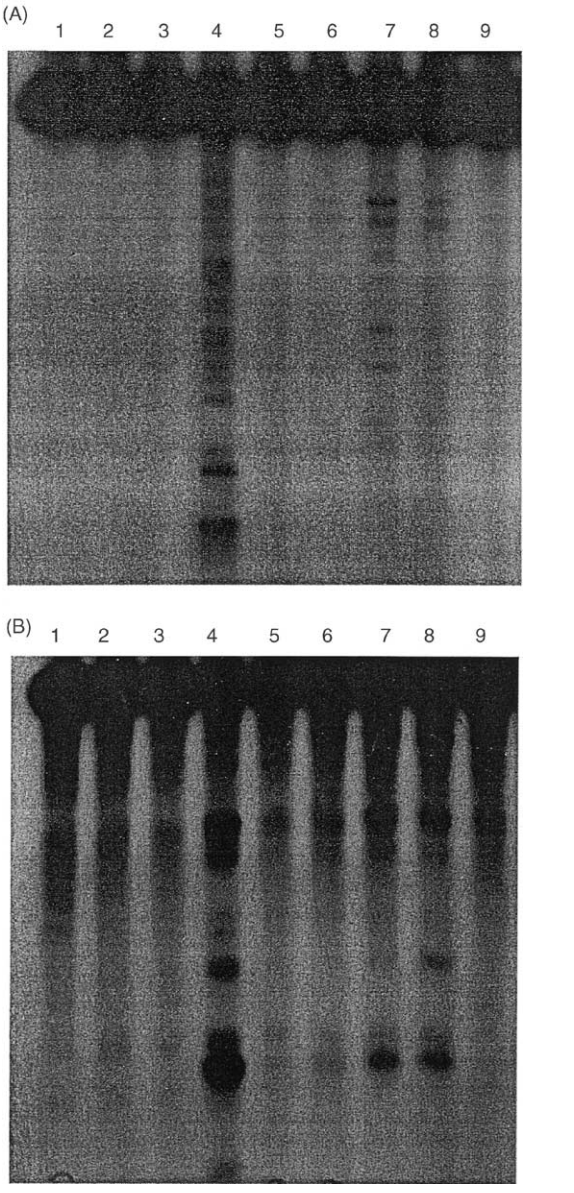

Figure 2. Stabilization of topoisomerase I- and topoisomerase II-associated cleavable complexes by XR11576. ^{32}P-labeled linearized pBR322 DNA was incubated with topoisomerase I or topoisomerase IIα in the presence or absence of the drugs XR11576, camptothecin or etoposide as described in Materials and methods. (A) Lane 1 contains DNA alone; lane 2, DNA and topoisomerase I; lane 3, DNA, topoisomerase I and DMSO (solvent control). Lanes 4–9 all contained DNA, topoisomerase I, DMSO and lane 4, 1.0 μM CPT-11; lane 5, 0.03 μM XR11576; lane 6, 0.1 μM XR11576; lane 7, 0.33 μM XR11576, lane 8, 1.0 μM XR11576; lane 9, 3.3 μM XR11576. (B) Lane 1 contains DNA alone; lane 2, DNA and topoisomerase IIα; lane 3, DNA, topoisomerase IIα and DMSO (solvent control). Lanes 4–9 all contained DNA, topoisomerase IIα, DMSO and lane 4, 1.0 μM etoposide; lane 5, 0.03 μM XR11576; lane 6, 0.1 μM XR11576; lane 7, 0.33 μM XR11576; lane 8, 1.0 μM XR11576; lane 9 3.3 μM XR11576.

The DNA cleavage patterns induced by XR11576 were qualitatively and quantitatively different from that induced by camptothecin or etoposide. XR11576 induced clear dose-dependent poisoning of topoisomerase I and IIα between 0.03–0.33 μM (Figure 2A) and 0.03–1.0 μM (Figure 2B), respectively. Hence poisoning occurred over a similar concentration range for both enzymes with effects visible at concentrations of XR11576 as low as 0.03 μM. At concentrations of 1.0 μM or above the intensity of the bands decreased indicating reduced activity for both topoisomerase I and II. Such a bell-shaped dose–response curve has been described for other topoisomerase inhibitors which either intercalate or bind to DNA.[27] These data provide a clear demonstration of the ability of XR11576 to poison both topoisomerase I and IIα, and suggest that XR11576 is able to bind or intercalate into DNA.

Cytotoxic activity in a panel of human and murine tumor cell lines

Cytotoxicity of XR11576 was evaluated alongside a number of other topoisomerase inhibitors currently used in the clinic or in clinical trials. The activity of XR11576 was similar to or better than that of these benchmark agents in a panel of murine and human cell lines (Table 1). XR11576 exhibited IC_{50} values in the range of 6–47 nM and its activity was similar in cell lines derived from either solid tumors or leukemias.

Activity in drug-resistant human tumor cell lines

The activity of XR11576 was evaluated in a panel of human MDR cell lines expressing elevated levels of P-gp or MRP or reduced levels of topoisomerase II (atypical drug resistance) (Table 2). The results are expressed as resistance factors, which were calculated as the ratio of IC_{50} in the resistant and sensitive paired cell lines (e.g. IC_{50} in the P-gp expressing H69/LX4 cells/IC_{50} in the parental H69/P cells). The results showed that doxorubicin, a known substrate for P-gp and MRP has resistance factors significantly greater than 1 in cell lines expressing these proteins. In contrast the resistance factors for XR11576 in these cell lines was close to 1.0 indicating that it is not a substrate for either P-gp or MRP drug efflux pumps. Similarly XR5000 and TAS-103 are unaffected by either pump. Topotecan appears to be a substrate for P-gp but not for MRP. In addition, cell lines that

Table 1. Cytotoxicity [IC_{50} (nM)] of XR11576 in human and murine cell lines

	H69/P (SCLC)	L23/P (NSCLC)	MCF-7 (breast)	A1847 (ovarian)	HT29	Jurkat (leukemia)	P388 (leukemia)	LLTC (Lewis lung)	MC26 (colon)
XR11576	23 ± 5.9[a]	10.1 ± 4.2	46.7 ± 21.6	16.5 ± 9.2	24.1 ± 1.5	21[b]	10[b]	5.6[b]	22.9 ± 9.5[a]
XR5000	311.2[b]	478.8[b]	ND	ND	433[b]	384[b]	98[b]	189[b]	98.3[b]
TAS-103	21.0 ± 7.7	17.3 ± 4.2	123.1 ± 48.1	9.2 ± 3.5	501 ± 247	5.4[b]	6[b]	1.5[b]	1.3 ± 1.2
Doxorubicin	27.4 ± 30.6	20.1 ± 6.8	104.5 ± 42.3	29.2 ± 5.9	ND	9.6 ± 1.1	15[b]	22 ± 4	26.7 ± 10.2
Topotecan	15.9 ± 5.3	13.1 ± 3.6	112 ± 81.9	11.9 ± 4.2	26.9 ± 10.0	4	ND	ND	ND

Cytotoxicity of the test drugs in human (H69/P, L23/P, MCF-7, A1847, HT29 and Jurkat) and murine (P388, LLTC and MC26) cell lines was evaluated as detailed in Materials and methods. The IC_{50} is calculated as the drug concentration required to produce 50% cell kill relative to vehicle-treated cells.

[a] Values are mean ± SD where $n \geqslant 3$ experiments.

[b] Mean of two experiments.

ND, not determined.

Table 2. Resistance factors for XR11576 in cells expressing P-gp, MRP and reduced topoisomerase II

	P-gp resistance factor	MRP resistance factor	Atypical resistance JL_D/JL_C ratio	Atypical resistance JL_A/JL_C ratio
XR11576	1.2	1.2	0.8	0.7
XR5000	2.3	1.2	2.4	1.8
TAS-103	1.1	2.5	70.4	55.6
Doxorubicin	141	15.9	12.5	4.4
Topotecan	3.9	1.5	ND	ND
Amsacrine	ND	ND	73	83.8
Camptothecin	ND	ND	1.4	2.0

Cytotoxicity of the test drugs was determined in sensitive and resistant paired cell lines as detailed in Materials and methods. The resistance factors were calculated as follows: P-gp factor=IC_{50} in H69/LX4:IC_{50} in H69/P; MRP factor=IC_{50} in L23/R:IC_{50} in L23/R; atypical factor=IC_{50} in JL_{dox} or $JL_{amsacrine}$:IC_{50} in $JL_{control}$. ND, not determined.

have developed resistance to amsacrine (JL_A) or doxorubicin (JL_D) as result of down-regulation of topoisomerase II do not show cross-resistance to either XR11576 or the topoisomerase I inhibitor, camptothecin (Table 2). In contrast the activity of TAS-103, was significantly attenuated in the JL_A and JL_D resistant sublines (Table 2). These data support the results of a recent study which showed using yeast expression system that the primary cellular target for TAS-103 is topoisomerase IIα.[20]

Intravenous and oral pharmacokinetics in rodents

The mean plasma concentration–time profiles of XR11576 in the BALB/c mouse plasma after i.v. administration exhibited biphasic kinetics (Figure 3). The compound showed high systemic clearance, large volume of distribution and a terminal half-life of approximately 2.5–4 h after i.v. and 6 h after p.o. administration (Table 3). There was a linear correlation between i.v. dose and AUC ($y=2.44\times R^2=0.999$) indicating that the kinetics in mice are independent of the dose between 20 and 50 mg/kg. The bioavailability of XR11576 after oral dosing to fasted and fed mice was 72 and 34%, respectively. Pharmacokinetic parameters after 20 mg/kg i.v. dose were similar in BALB/c and H69 SCLC tumor bearing CD1 nude mice (Table 3). The large volume of distribution was consistent with the high tissue levels after i.v. and p.o. administration (Figure 4A and B). After i.v. administration normal tissue levels also displayed biphasic kinetics and the elimination half-life (2.6–3.6 h) was similar to that in plasma (Figure 4A). Tumor XR11576 levels decreased at a much slower rate than in other tissues, and in fact the $AUC_{0-\infty}$ values in tumors after i.v. (20 mg/kg) and p.o. (50 mg/kg) administration (150 and 100 μg · h/ml) were higher than in the liver (60 and 82 μg · h/ml) or heart

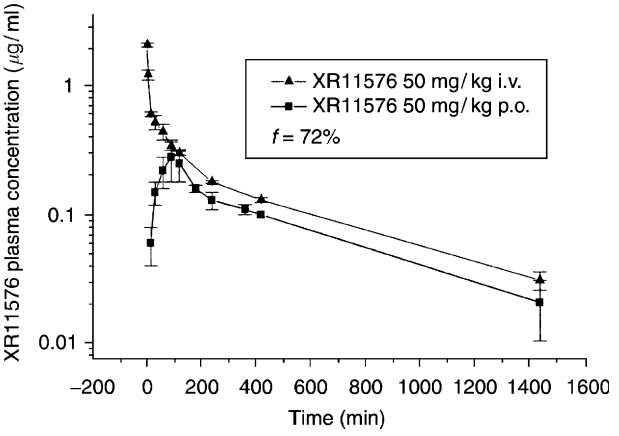

Figure 3. Plasma concentration–time profiles of XR11576 in BALB/c mice after i.v. (50 mg/kg) and p.o. (50 mg/kg) administration. The values are presented as mean $\pm$ SE where n=3–4 animals per time point.

(47 and 27 μg · h/ml), respectively. Similar plasma and tissue pharmacokinetic profiles were observed in male Wistar rats; following a 20 mg/kg i.v. dose the C_{max} was 2.2 ± 0.9 μg/ml, the volume of distribution was 1.5 ± 0.6 l, clearance was 23 ± 5 ml/min and the terminal half-life was 2.5 ± 0.5 h. The oral bioavailability in fasted rats was 54%.

In vivo antitumor activities of XR11576

Efficacy of XR11576 has been evaluated using several models including human SCLC (H69/P, H69/LX4), colon carcinoma (HT29) and murine colon carcinoma (MC26).

Antitumor activity against MC26 murine colon carcinoma. XR11576 administered i.v. and p.o. demonstrated marked antitumor activity against the

Table 3. Plasma pharmacokinetics following intravenous and oral administration of XR11576 in mice

	BALB/c mice			Nude mice
	20 mg/kg i.v.	50 mg/kg i.v.	50 mg/kg p.o.	20 mg/kg i.v.
C_{max} (μg/ml)	0.8 ± 0.3	2.9 ± 0.8	0.35 ± 0.29	0.74 ± 0.09
$AUC_{0-\infty}$ (μg · min/ml)	64 ± 6	156 ± 9	114 ± 30	70 ± 15
Cl (ml/min)	6.3 ± 0.6	6.4 ± 0.4	NA	7.4 ± 1.6
K_{el} (min−1)	0.0044 ± 0.0002	0.0030 ± 0.0004	0.002 ± 0.001	0.0043 ± 0.0007
V_{ss} (l)	1.3 ± 0.4	1.9 ± 0.6	NA	1.6 ± 1.3
F%	NA	NA	72 ± 25	NA

Pharmacokinetic parameters were determined using non-compartmental analysis as outlined in Materials and methods. The values are presented as mean ± 90% confidence limits where n=2–4 animals per time point.

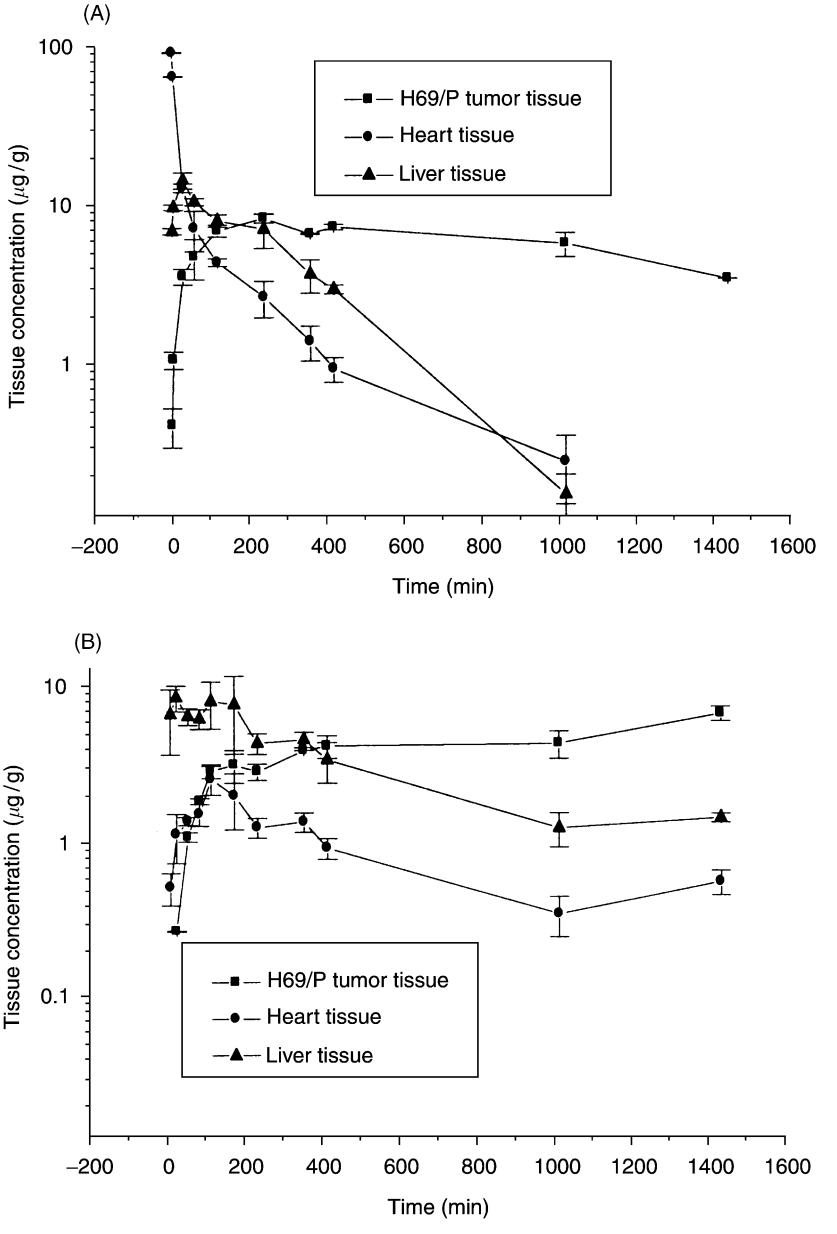

Figure 4. Tumor and tissue levels of XR11576 in CD1 nude mice after (A) 20 mg/kg i.v. and (B) 50 mg/kg p.o. administration of the drug. The data are presented as mean ± SE where n=2–4 animals per time point.

relatively refractory MC26 colon tumors implanted s.c. in BALB/c mice. Comparison of antitumor activity and body weight loss between different dosing schedules shown in Figure 5(A and B) indicated that XR11576 administered i.v. at q7d × 2, q4d × 3 and q2d × 5 schedules at equitoxic doses would have similar antitumor activity in this model. The optimum T/C value was 27% in the experiment shown in Figure 5. The total dose of XR11576 administrable at the three i.v. schedules was similar. The optimum schedules for p.o. administration in fed animals were q7d × 2 and q4d × 3 and at maximum tolerated doses

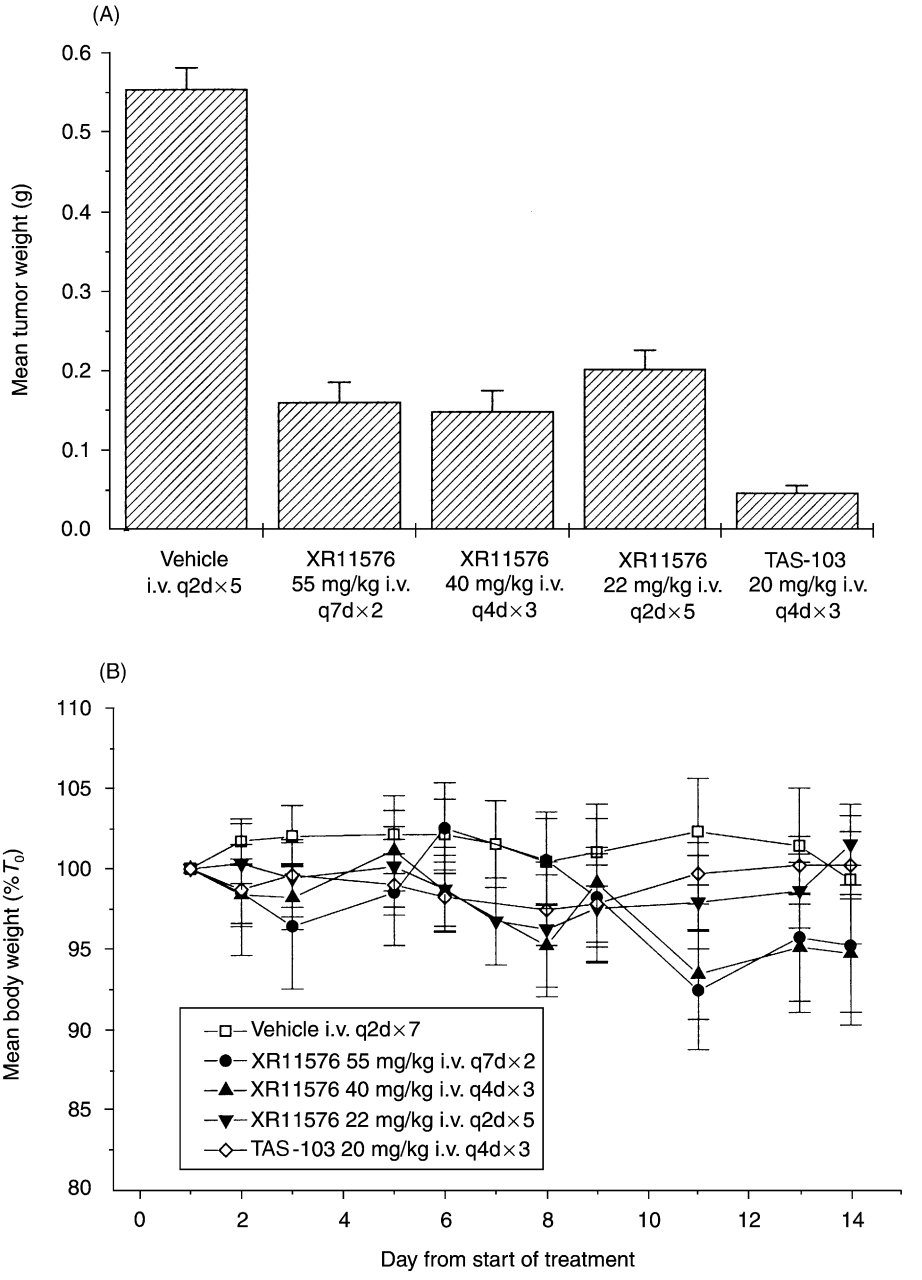

Figure 5. Effects of different i.v. schedules of XR11576 and TAS-103 on (A) MC26 colon tumor growth and (B) body weights of tumor-bearing mice. MC26 tumor slurry was implanted s.c. (day 0) into BALB/c mice and drug treatment was initiated 1 day later. Tumor weights were determined 14 days after tumor implant and the data are presented as mean ± SE where $n = 14$ animals per group. The body weights are presented as percentage of the weight on day 1, the start of treatment (mean ± SE). Vehicle was 5% dextrose and all solutions were administered at 10 ml/kg as described in Materials and methods.

(MTD) the T/C values were approximately 58% for both schedules (data not shown). An additional study at q7d × 2 schedule showed that at equal p.o. doses antitumor activity was greater in fasted than in fed animals, which is consistent with greater oral bioavailability in fasted than in fed animals (data not shown). The control drug, TAS-103, was exceptionally active *in vitro* against the MC26 murine cell line compared with human solid tumor cell lines (see Table 1)[18,28] and this *in vitro* activity translated into significantly greater tumor growth inhibition (T/C 8%) at 20 mg/kg i.v. q4d × 3 schedule compared to XR11576 (Figure 5A).

Antitumor activity in human carcinoma xenografts. A number of studies were carried out in the H69/P and the MDR H69/LX4 SCLC xenograft models in order to examine efficacy after oral and i.v.

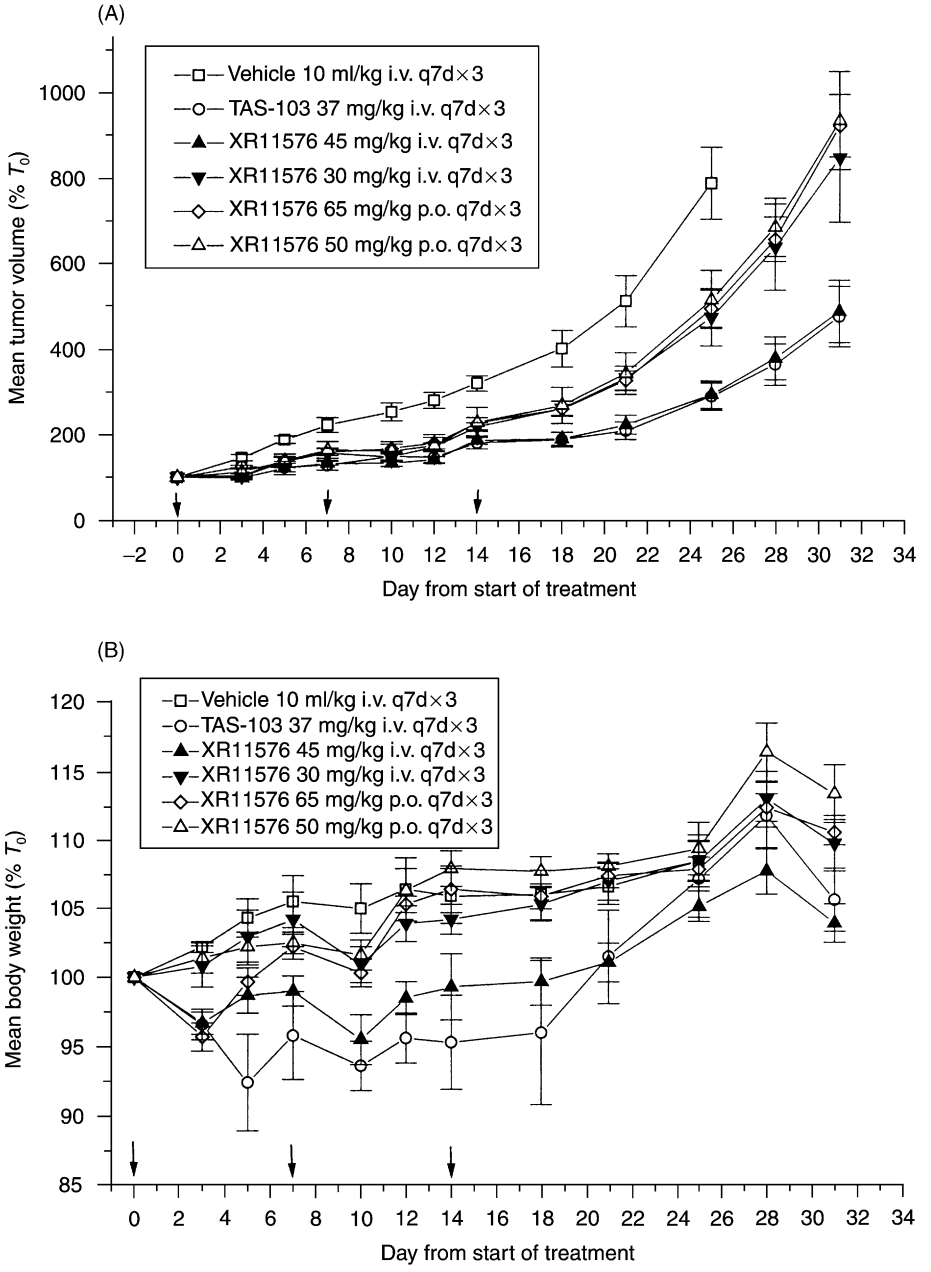

Figure 6. The effect of XR11576 and TAS-103 on (A) the growth of s.c. H69/P SCLC xenografts in nude mice and (B) on the body weights of tumor-bearing animals. The drugs were administered to mice bearing established tumors as described in Materials and methods. Arrows indicate day of dosing. The values are mean ± SE relative to day 0, where n=8 per group.

administration at varying doses and using different dosing schedules. Comparison of antitumor activity and body weight loss between different dosing schedules of XR11576 indicated that activity was similar following equitoxic i.v. doses at q7d × 3, q4d × 3 and (qd × 4)2 schedules in this model. Preliminary results indicated that for oral administration the q7d × 3 schedule may be more efficacious than q4d × 3 or qd × 4 schedules (data not shown). Thus the optimum i.v. and p.o. schedules in the H69 SCLC models were similar to those obtained with the MC26 model.

In another study with the H69 SCLC xenograft the results showed that treatment with XR11576 at 45 mg/kg i.v. inhibited the growth of the H69 tumors significantly (T/C=37%; SGD=2.2) (Figure 6A). At this dose XR11576 was well tolerated as indicated by the maximum mean body weight decrease of 4.5% on day 10. In addition the compound showed a dose related effect with the 30 mg/kg dose showing a T/C value of 58% (Figure 6B). The two oral doses of XR11576, 65 and 50 mg/kg also caused a substantial decrease in growth of the H69 tumors (T/C=63 and 64%, respectively), but there was no clear dose-related effect. This may be due to the variability in absorption between the animals counteracting the small difference in the two doses. All the doses of XR11576 were well tolerated as indicated by changes in body weights compared with the control vehicle treated group (Figure 6B). The growth inhibition observed with XR11576 (at 45 mg/kg i.v.) was similar to that observed with an equimolar dose of TAS-103. However, XR11576 exhibits a better therapeutic index relative to TAS-103; all the animals dosed with XR11576 survived compared with only six out of the eight animals dosed with TAS-103.

In the P-gp overexpressing MDR H69/LX4 xenografts the results showed that treatment with XR11576 at 40 mg/kg i.v. (q7d × 4) inhibited the growth of the H69/LX4 tumors significantly (T/C=39%; SGD=0.8) (Figure 7A). The compound at this dose was well tolerated as indicated by the maximum mean body weight decrease of 7% (Figure 7B). TAS-103 (30 mg/kg i.v. q7d × 4) caused significant growth delay (T/C=40%; growth delay of >15 days; SGD=0.6). However, one animal in this group did not tolerate the TAS-103 dose schedule. Taking into consideration the tumor growth delay and toxicity, these results indicate that the antitumor activity of i.v. XR11576 is greater than or equal to that of i.v. TAS-103 in this model. However, CPT-11 at 35 mg/kg i.v. q4d × 3 exhibited better activity than XR11576. In contrast etoposide, a known substrate of P-gp, had no effect on the growth of these tumors at the maximum administrable dose (30 mg/kg i.v. q5d × 3). This clearly demonstrates that XR11576 is effective against tumors exhibiting P-gp mediated MDR *in vivo*, thus confirming the *in vitro* results.

Studies in mice bearing the HT29 colon carcinoma xenografts showed that i.v. and p.o. administration of XR11576 can significantly inhibit the growth of this relatively refractory tumor (Figure 8A). Treatment with XR11576 (52.5 mg/kg i.v. q7d × 3) inhibited the growth of established tumors significantly (T/C=36%; SGD=1.3). The compound at this dose was tolerated as indicated by the maximum mean body weight loss of 12%, which recovered fully by day 28 (Figure 8B). The antitumor effect of i.v. XR11576 was the same as that observed with an equimolar dose of TAS-103 administered at an optimized schedule (45 mg/kg, q7d × 3) (T/C=39%; SGD=1.3). However, the dose of TAS-103 caused greater toxicity leading to higher and more prolonged loss in mean body weight loss (15%). Thus i.v. XR11576 appears to exhibit a better therapeutic index than i.v. TAS-103. Oral administration of XR11576 at a dose schedule near its MTD (75 mg/kg, q7d × 3) produced an antitumor effect (T/C=39%; SGD=0.9) comparable to the i.v. dose.

Discussion

Our results demonstrate that XR11576 is able to block the action of both topoisomerase I and IIα and the potency is similar for both enzymes. In addition, the demonstration of cleavable complex formation in the presence of XR11576 puts the compound into the class termed 'topoisomerase poisons'. The majority of topoisomerase inhibitors including the camptothecin derivatives, doxorubicin and etoposide belong to this class of inhibitors.[5–7] The bell-shaped dose–response curve observed with XR11576 in the complex formation assay has been reported for other agents known to poison topoisomerases through their initial interaction with DNA.[27] This and the fact that other phenazines have been shown to bind to DNA[22] suggests that the inhibition of topoisomerase action by XR11576 involve binding to DNA.

The potency observed in a range of murine and human tumor cell lines means that XR11576 has a competitive profile relative to many widely used chemotherapeutic agents including those that act on topoisomerases. Furthermore, XR11576 is unaffected by clinically relevant mechanisms of MDR such as

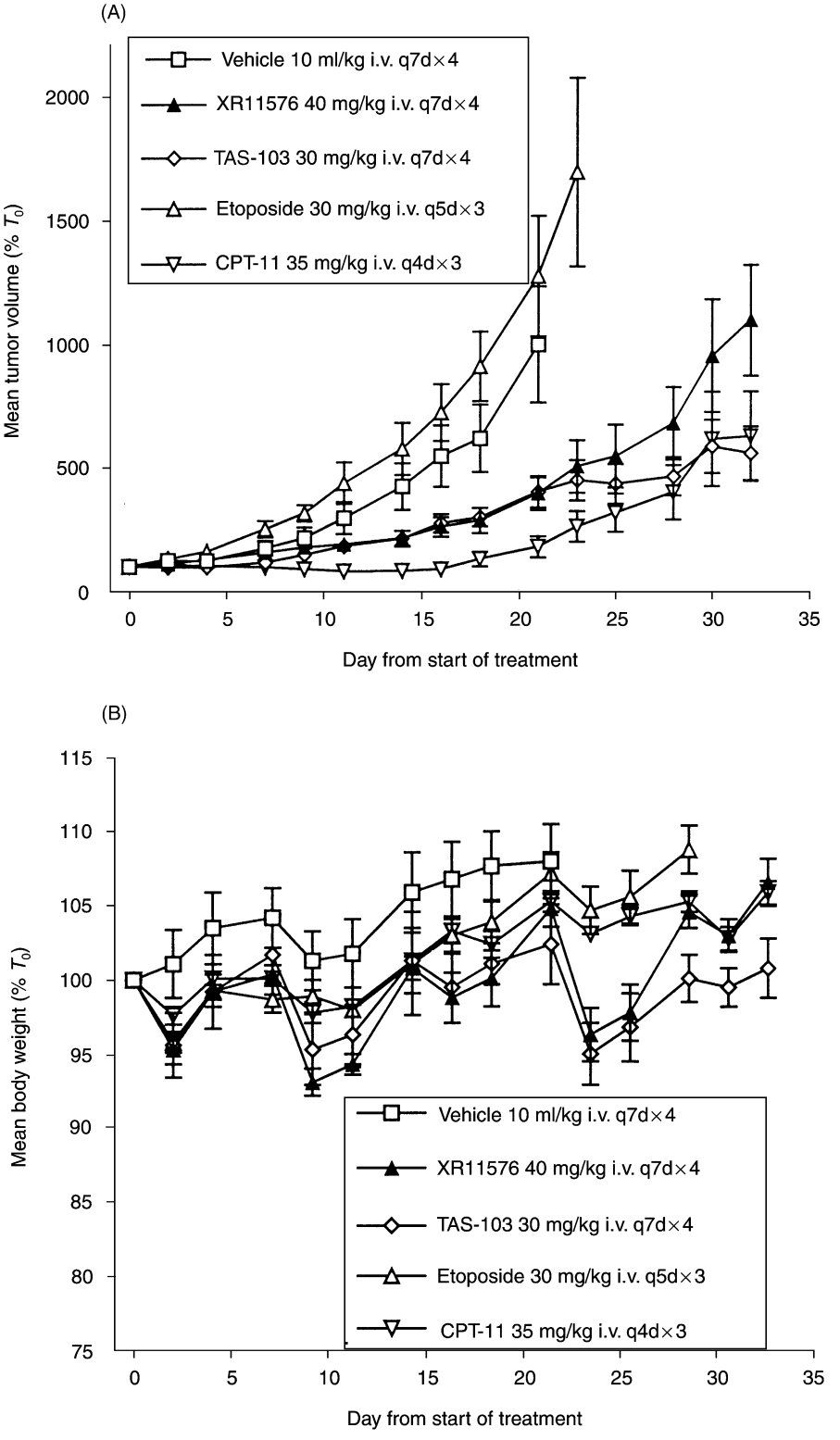

Figure 7. (A) Antitumor activity of XR11576 and other drugs against the MDR H69/LX4 SCLC xenografts in nude mice. (B) Effects of treatment on body weights of tumor-bearing mice. The drugs were administered to mice bearing established tumors at the schedules indicated as described in Materials and methods. The values are mean ± SE relative to day 0, where n=7–8 per group.

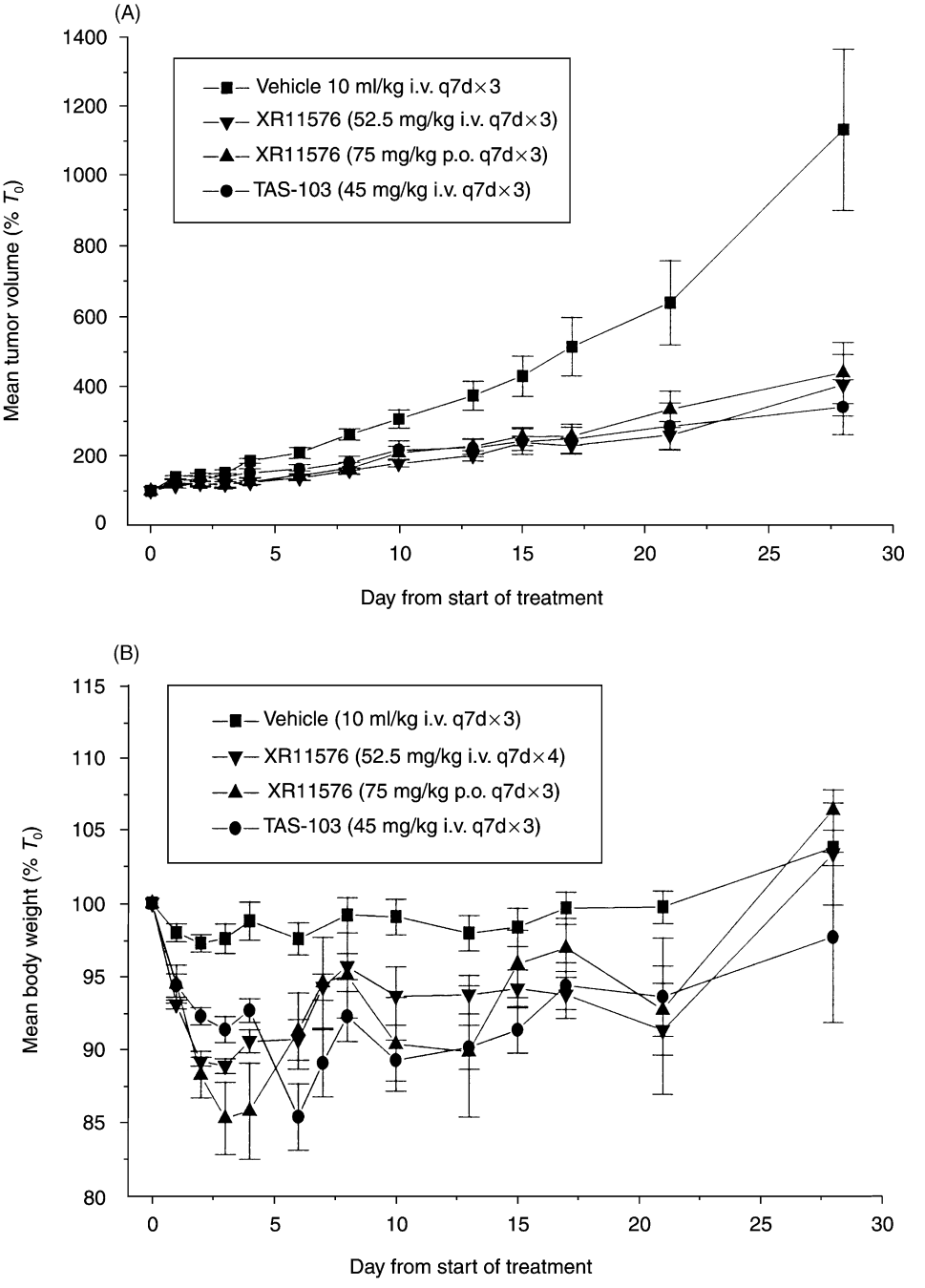

Figure 8. The effect of XR11576 and TAS-103 on (A) the growth of s.c. HT29 colon carcinoma xenografts in nude mice and (B) on the body weights of tumor-bearing animals. The drugs were administered to mice bearing established tumors as described in Materials and methods. Arrows indicate day of dosing. The values are mean ± SE relative to day 0, where n=8 per group.

P-gp and MRP overexpression and down-regulation of topoisomerase II. This gives XR11576 an advantage over other drugs whose activity is severely attenuated by similar mechanisms of MDR. Of mechanistic interest is the fact that XR11576 is equipotent against topoisomerase I and II, suggesting that when topoisomerase II is down-regulated cell kill occurs through a parallel mechanism, which is most likely to be inhibition of topoisomerase I. TAS-103 was significantly less potent in such atypical

drug resistant cells with reduced topoisomerase II suggesting a preference for topoisomerase II. This is in contrast to XR11576 and also contradicts previously reported data for TAS-103.[29] However, a recent study using a yeast expression system found that topoisomerase IIα is the primary cellular target for TAS-103 and that it kills cells through this mechanism [20] which is in accord with our own data.

XR11576 has shown significant oral bioavailability in both mice and rats; in fasted animals the oral bioavailability was 72 and 54%, respectively. The compound exhibited similar pharmacokinetic profile after i.v. and p.o. administration in both species with the terminal half-life greater than 2.5 h and a large volume of distribution. The concentrations of XR11576 reached in tumors and plasma after i.v. and p.o. administration are predicted to be active as judged by its *in vitro* potency (IC_{50}=6–47 nM= 2–19 ng/ml).

The potent *in vitro* activity translated well *in vivo*, where XR11576 has demonstrated marked efficacy at tolerated doses against murine and human xenograft models of solid tumors. This included the sensitive (H69/P) and resistant (H69/LX4) SCLC models, and the refractory MC26 and HT29 colon carcinoma models, which are typically difficult to treat with standard agents. Furthermore, XR11576 has demonstrated activity after i.v. and p.o. administration that was comparable to i.v. TAS-103, but with less toxicity. The marked activity against the MDR H69/LX4 tumors, in which etoposide and vincristine[30] showed no activity at MTDs, demonstrated that XR11576 is able to overcome MDR *in vivo* as well as *in vitro*. Scheduling studies in s.c. implanted H69/P SCLC and MC26 colon carcinoma models demonstrated that several i.v. schedules [intermittent q7d × 3 and q4d × 3 and more frequent q2d × 5 and (qd × 4) × 3] are equally effective, thus allowing more flexibility of dosing in the clinic. The total dose administrable in the various schedules was similar, suggesting that toxicity is related to the total dose. For the oral route, the intermittent schedules were more effective than the frequent schedules. However, oral scheduling studies were performed in fed animals because of the difficulty in frequent fasting of animals and may not reflect the efficacy in fasted animals as these have higher (72%) oral bioavailability than fed ones (32%). In a preliminary study, the higher oral bioavailability in fasted animals correlated with higher antitumor activity compared to fed animals at equal doses on a q7d × 3 schedule.

In conclusion, our results show that XR11576, a novel phenazine, has significant antitumor activity *in vitro* and *in vivo* against a range of murine and human tumor cell lines. Moreover, it retains activity in MDR cells overexpressing P-gp or MRP or reduced levels of topoisomerase II. Mechanistically, XR11576 appears to be a joint topoisomerase I and II inhibitor, stabilizing the enzyme–DNA cleavable complexes. This proposed mechanism is supported by the observation that XR11576 retains activity in topoisomerase II down-regulated cells. Overall the results demonstrate that XR11576 warrants further development as a novel i.v. and p.o. therapy for solid tumors.

References

1. Wang JC. DNA topoisomerases. *Annu Rev Biochem* 1996; **65**: 635–92.
2. Heck MMS, Hittelman WN, Earnshaw WC. Differential expression of DNA topoisomerase I and II during the eukaryotic cell cycle. *Proc Natl Acad Sci USA* 1988; **85**: 1086–90.
3. Adachi Y, Luke M, Laemmli UK. Chromosome assembly *in vitro*: topoisomerase II is required for condensation. *Cell* 1991; **64**: 137–48.
4. Watt PM, Hickson ID. Structure and function of type II DNA topoisomerases. *Biochem J* 1994; **303**: 681–95.
5. D'Incali M. DNA-topoisomerase inhibitors. *Curr Opin Oncol* 1993; **5**: 1023–8.
6. Rothenberg ML. Topoisomerase I inhibitors: review and update. *Semin Oncol* 1997; **8**: 837–55.
7. Fortune JM, Osheroff N. Topoisomerase II as a target for anticancer drugs: when enzymes stop being nice. *Prog Nucleic Acid Res Mol Biol* 2000; **64**: 221–53.
8. Andoh T, Ishida R. Catalytic inhibitors of DNA topoisomerase II. *Biochim Biophys Acta* 1998; **1400**: 155–71.
9. Perrin D, van Hille B, Barret J-M, *et al*. F11782, a novel epipodophylloid non-intercalating dual catalytic inhibitor of topoisomerases I and II with an original mechanism of action. *Biochem Pharmacol* 2000; **59**: 807–19.
10. Woessner RD, Mattern MR, Mirabelli CK, Johnson RK, Drake FH. Proliferation- and cell-dependent differences in expression of the 170 kDa and 180 kDa forms of topoisomerase II in NIH-3T3 cells. *Cell Growth Different* 1991; **2**: 209–14.
11. Beck WT, Cirtain MC, Danks MK, *et al*. Pharmacological, molecular and cytogenetic analysis of 'atypical' multidrug-resistance human leukaemic cells. *Cancer Res* 1987; **47**: 5455–60.
12. Tanizawa A, Pommiery Y. Topoisomerase-1 alteration in a camptothecin-resistant cell line derived from Chinese hamster DC3F cells in culture. *Cancer Res* 1992; **52**: 1848–54.
13. Finlay GJ, Riou J-F, Baguley BC. From Amsacrine to DACA (*N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide): selectivity for topoisomerase I and II among acridine derivatives. *Eur J Cancer* 1996; **32A**: 708–14.

14. Husain I, Mohler JL, Seigler HF, Besterman J. Elevation of topoisomerase I messenger RNA, protein, and catalytic activity in human tumor: demonstration of tumor-type specificity and implications for cancer chemotherapy. *Cancer Res* 1994; **54**: 539–46.
15. McLeod HL, Douglas F, Oates M, *et al*. Topoisomerase I and II activity in human breast, cervix, lung and colon cancer. *Int J Cancer* 1994; **59**: 607–11.
16. Yamashita Y, Kawada SY, Fuji N, Nakano H. Induction of mammalian topoisomerase I and topoisomerase II mediated DNA cleavage by saintopin, a new antitumor agent from fungus. *Biochemistry* 1991; **30**: 5838–45.
17. Riou J-F, Fosse P, Nguyen CH, *et al*. Intoplicine (RP 60475) and its derivatives, a new class of antitumor agents inhibiting both topoisomerase I and II activities. *Cancer Res* 1993; **53**: 5987–93.
18. Utsugi T, Aoyagi K, Asao T, *et al*. Antitumor activity of a novel quinoline derivative, TAS-103, with inhibitory effects on topoisomerase I and II. *Jpn J Cancer Res* 1997; **88**: 992–1002.
19. Bridewell DJA, Finlay GJ, Baguley BC. Differential actions of aclarubicin and doxorubicin: the role of topoisomerase I. *Oncol Res* 1997; **9**: 535–42.
20. Byl JAW, Fortune JM, Burden JA, Nitiss JL, Utsugi T, Yamada Y. DNA topoisomerases as targets for the anticancer drug TAS-103: primary cellular target and DNA cleavage enhancement. *Biochemistry* 1999; **38**: 15573–9.
21. Spicer JA, Gammage SA, Atwell GJ, Finlay GJ, Baguley BC, Denny WA. Structure–activity relationships for acridine analogues of mixed topoisomerase I/II inhibitor *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide. *J Med Chem* 1997; **40**: 1919–29.
22. Rewcastle GW, Denny WA, Baguley BC. Potential antitumor agents. 51. Synthesis and antitumor activity of substituted phenazine-1-carboximides. *J Med Chem* 1987; **30**: 843–51.
23. Spoelstra EC, Dekker H, Schuurhuis GJ, Broxterman HJ, Lankelma J. P-glycoprotein efflux pump involved in the mechanism of intrinsic drug resistance in various colon cancer cell lines. Evidence for a saturation of active daunorubicin transport. *Biochem Pharmacol* 1991; **41**: 349–59.
24. Dale IL, Tuffley W, Callaghan R, *et al*. Reversal of P-glycoprotein-mediated multidrug resistance by XR9051, a novel diketopiperazine derivative. *Br J Cancer* 1998; **78**: 885–92.
25. Finlay GJ, Marshall E, Matthews JHL, Paull KD, Baguley BC. *In vitro* assessment of *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide, a DNA-intercalating antitumuor drug with reduced sensitivity to MDR. *Cancer Chemother Pharmacol* 1993; **31**: 401–6.
26. Mistry P, Plumb J, Eccles S, *et al*. *In vivo* efficacy of XR9051, a potent modulator of P-glycoprotein mediated multidrug resistance. *Br J Cancer* 1999; **79**: 1672–8.
27. Bridewell DJ, Finlay GJ, Baguley BC. Mechanism of cytotoxicity of *N*-[2-(dimethylamino)ethyl] acridine-4-carboxamide and of its 7-chloro derivative: the roles of topoisomerases I and II. *Cancer Chemother Pharmacol* 1999; **43**: 302–8.
28. Etievant C, Kruczynski A, Barret J-M, *et al*. F11782, a dual inhibitor of topoisomerases I and II with an original mechanism of action *in vitro*, and markedly superior *in vivo* antitumor activity, relative to three other dual topoisomerase inhibitors, intoplicin, aclarubicin and TAS-103. *Cancer Chemother Pharmacol* 2000; **46**: 101–13.
29. Aoyagi Y, Kobunai T, Utsugi T, Oh-hara T, Yamada Y. In vitro antitumor activity of TAS-103, a novel quinoline derivative that targets topoisomerase I and II. *Jpn. J Cancer Res* 1999; **90**: 578–87.
30. Mistry P, Stewart AJ, Dangerfield W, *et al*. *In vitro* and *in vivo* reversal of P-glycoprotein-mediated multidrug resistance by a novel potent modulator, XR9576. *Cancer Res* 2001; **61**: 749–58.

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