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Report

Antitumor activity of XR5944, a novel and potent topoisomerase poison

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Inhibitors of topoisomerases are widely used in the treatment of cancer, including inhibitors of topoisomerase I (camptothecin analogs such as irinotecan and topotecan) and topoisomerase II (etoposide and doxorubicin). The novel bis-phenazine, XR5944, is a joint inhibitor of topoisomerase I and II as shown by the stabilization of topoisomerase-dependent cleavable complexes. XR5944 demonstrated exceptional activity against human and murine tumor cells *in vitro* and *in vivo*. In a range of cell lines XR5944 (IC_{50} 0.04–0.4 nM) was significantly more potent than TAS-103, originally proposed as a joint topoisomerase I and II inhibitor, as well as agents specific for topoisomerase I or II (topotecan, doxorubicin and etoposide). In addition, XR5944 was unaffected by atypical drug resistance and retained significant activity in cells overexpressing P-glycoprotein or multidrug resistance-associated protein. Antitumor efficacy of XR5944 was demonstrated in human carcinoma xenograft models (H69 small cell lung cancer and HT29 colon). In the HT29 model, which is relatively unresponsive to chemotherapy, XR5944 (15 mg/kg i.v., q4d × 3) induced tumor regression in the majority of animals (six of eight), whereas TAS-103, dosed at its maximum tolerated dose (45 mg/kg i.v., q7d × 3), only induced a delay in tumor growth compared with control animals. In the H69 model, low doses of XR5944 (5 mg/kg i.v., qd × 5/week for 2 weeks or 10–15 mg/kg i.v., q4d × 3), induced complete tumor regression in the majority of animals. In contrast, topotecan (20 mg/kg i.v., q4d × 3) or etoposide (30 mg/kg i.v., q5d × 5) only slowed the tumor growth rate. These studies show that XR5944 is a highly active novel anticancer agent that is well tolerated at efficacious doses.

Key words: Anti-cancer, poison, topoisomerase I, topoisomerase II, xenografts.

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Introduction

Topoisomerases are important cellular targets for a number of successful chemotherapeutic agents.[1] Drugs that target topoisomerase II (doxorubicin and etoposide) have been widely used for many years,[2] while those that specifically target topoisomerase I (principally the camptothecin analogs) have made an important impact more recently (e.g. irinotecan in colon cancer).[3] In addition to those compounds that specifically target topoisomerase I or II, several joint I/II inhibitors have been identified and may also be beneficial in the treatment of solid tumors. These compounds include DACA/XR5000,[4] intoplicine,[5] F11782[6] and TAS-103,[7] which are at various stages of preclinical or clinical evaluation.

There are several advantages of simultaneously hitting both topoisomerase I and II, the first of which relates to mechanisms of drug resistance. Drug resistance is a major clinical problem with many tumors treated with topoisomerase-active agents developing resistance due to alteration in levels of expression or catalytic activity of topoisomerase I or II.[8–10] By simultaneously inhibiting topoisomerase I and II, compounds such as DACA[4] are able to circumvent this resistance, presumably by targeting the enzyme which has not been altered by the selecting agent.

Secondly, qualitatively different cell cycle disruption has been obtained with inhibitors of topoisomerase I or II,[11] suggesting that they are acting at different parts of the cell cycle. Joint inhibitors of topoisomerase I and II appear to combine the properties of the individual specific inhibitors and act across the cell cycle,[12] hitting a larger population of cells in any asynchronous population and resulting in a greater antitumor activity.[5] Furthermore, a joint inhibitor of topoisomerase I and II may have a broader spectrum of

activity as the expression levels of the two enzymes varies between different types of cancer.[13] These properties of joint inhibitors of topoisomerase I and II make this an attractive strategy for the development of novel chemotherapeutic agents for the treatment of cancer.

In this report we show that XR5944, a novel bisphenazine (Figure 1), is a potent poison of both topoisomerase I and II, and is a highly active anticancer agent, both *in vitro* and *in vivo*. XR5944 produces complete tumor regression in nude mice bearing the H69 human small cell lung carcinoma (SCLC) xenograft and it also causes tumor regression in the relatively refractory HT29 colon carcinoma xenograft at well-tolerated doses.

Materials and methods

Drugs

XR5944 (dimesylate salt) was synthesized at the Auckland Cancer Research Centre. Topotecan and TAS-103 were synthesized by the Department of Medicinal Chemistry at Xenova Ltd. All other drugs were obtained from Sigma (Dorset, UK). Drugs were dissolved in DMSO for *in vitro* experiments and in 5% (w/v) D-(+)-glucose (dextrose) solution or saline for use *in vivo*.

Cell lines

COR-L23/P non-small cell lung carcinoma (NSCLC) and H69/P SCLC cell lines, and their respective drug-resistant sublines COR-L23/R and H69/LX4,[14] were kindly provided by Dr PR Twentyman (MRC Clinical Oncology and Therapeutics Unit, Cambridge, UK).

Experiments using the Jurkat cell lines and the Chinese hamster cell lines were carried out at the Auckland Cancer Society Research Centre. These cell lines have been described previously.[4] The HT29 colon carcinoma cell line was obtained from the ATCC (Rockville, MD).

Cytotoxicity assays

Cells were seeded 4 h prior to addition of serial dilutions of the indicated cytotoxics. After 4–6 days, cell growth was assessed colorimetrically[15] or by thymidine incorporation.[16] IC_{50} values were determined as the drug concentration required to reduce cell growth by 50%. All assays were carried out in quadruplicate and data presented are the mean of at least two experiments.

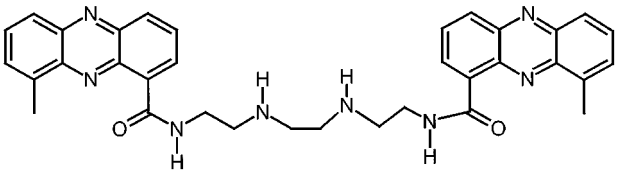

Figure 1. Structure of XR5944.

Cleavable complex formation assays

Drug-induced formation of cleavable complexes were assessed using 4×10^6 c.p.m. of ^{32}P-labeled linear pBR322 DNA and 15 U of calf thymus topoisomerase I (Life Technologies, Paisley, UK) or 20 U of human topoisomerase IIα (Topogen, Columbus, OH) as previously published.[4]

In vivo antitumor activity

All animal experimentation was performed to UK Home Office regulations and the UKCCCR guidelines were adhered to throughout. H69/P SCLC cells (8×10^6) or HT29 cells (5×10^6), harvested from *in vitro* incubations, in PBS (100 μl) were inoculated s.c. into the right flanks of female CDI nude mice (Charles River, Margate, UK). The animals were monitored regularly and when the tumors had reached a mean diameter of 0.4–0.6 cm the animals were randomized into groups of at least seven mice per group. XR5944 and other control agents were administered i.v. (etoposide, topotecan and TAS-103) or i.p. (doxorubicin). The tumor volume and body weights were measured at least twice per week as described previously.[17] The data were analyzed using a one-way analysis of variation.

Results

Formation of cleavable complexes with topoisomerase I and II

Direct demonstration of the effects of XR5944 on topoisomerase activity was evaluated using linearized ^{32}P-labeled pBR322 plasmid DNA as the substrate, and purified topoisomerase I and IIα. Drug-dependent poisoning of the enzyme was visualized by electrophoresis of the labeled DNA after digestion with proteinase K. In this way, XR5944-induced cleavable complexes between DNA and topoisomerases appear as an increase in the number and intensity of bands migrating ahead of the linear substrate DNA (Figure 2). Increasing concentrations of XR5944 induced a clear dose-dependent increase in the number of cleavable complexes in the presence of both topoisomerase I

(Figure 2A) and IIα (Figure 2B) with a very different pattern of cleavable complex formation from the positive controls camptothecin and etoposide. Poisoning occurred over a similar concentration range for both enzymes, with effects visible at concentrations as low as 0.1 μM and increasing further up to 1.0 μM. These data demonstrate the ability of XR5944 to poison both topoisomerase I and IIα.

Cytotoxicity in tumor cell lines

The cytotoxic profile of XR5944 was tested in a number of different human tumor cell lines of both leukemic and solid tumor origin. The data presented in Tables 1 and 2 demonstrate that XR5944 is an exceptionally potent cytotoxic agent. The activity of XR5944 against parental cell lines ranged from an IC_{50} of 0.04 nM in the COR-L23/P NSCLC line to 0.4 nM in the H69/P SCLC line. XR5944 was tested alongside a number of other agents either currently used in the clinic or in clinical trial (TAS-103) and was found to be the most potent compound by far.

The extremely high potency of XR5944 was attenuated by the expression of P-glycoprotein (P-gp) (380-fold) or multidrug resistance-associated protein (MRP) (7.5-fold). This indicates that XR5944 is probably a substrate for both of these efflux pumps. However, even in these cells which hyperexpress P-gp

Table 1. Cytotoxicity [IC_{50} (nM)] in sensitive and multidrug-resistant human cell lines

	H69/P parental SCLC	H69/LX4 P-gp resistance	COR-L23/P parental NSCLC	COR-L23/R MRP resistance
XR5944	0.4±0.3 (*n*=19)	151.8±102 (*n*=19)	0.04±0.01 (*n*=2)	0.3±0.15 (*n*=2)
Topotecan	15.9±5.3 (*n*=20)	62.9±30.0 (*n*=20)	13.2±3.6 (*n*=20)	20.0±4.1 (*n*=20)
Doxorubicin	27.3±30.6 (*n*=22)	3874±2791 (*n*=22)	20.1±7.0 (*n*=19)	319.3±145 (*n*=16)
TAS-103	21.0±7.7 (*n*=45)	22.6±8.3 (*n*=45)	17.3±4.2 (*n*=18)	42.9±22 (*n*=17)
Paclitaxel	1.7±0.9 (*n*=9)	2048±996 (*n*=7)	1.8±0.9 (*n*=4)	4.68±2.4 (*n*=4)

The indicated test drugs were assayed for cytotoxicity in human cell lines as detailed in Methods. The H69/P SCLC line and the COR-L23/P were studied alongside their multidrug-resistant counterparts H69/LX4 and COR-L23/R which overexpress P-gp and MRP, respectively. The IC_{50} is calculated as the drug concentration required to give 50% cell kill and values quoted represent the mean (±SD).

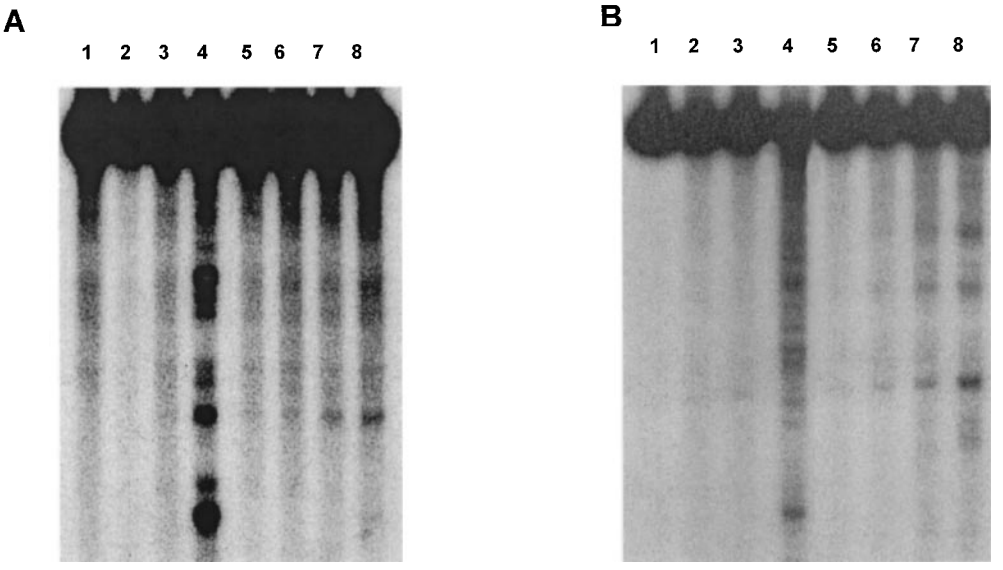

Figure 2. Stabilization of topoisomerase I- and II-associated cleavable complexes by XR5944. ^{32}P-labeled linearized pBR322 DNA was incubated with topoisomerase I or IIα in the presence or absence of the drugs XR5944, etoposide or camptothecin as detailed in Methods. (A) Lane 1 contains DNA alone; lane 2, DNA and topoisomerase I; lane 3, DNA, topoisomerase I and DMSO (solvent control). Lanes 4–8 all contained DNA, topoisomerase I, DMSO and lane 4, 1.0 μM camptothecin; lane 5, 0.03 μM XR5944; lane 6, 0.1 μM XR5944; lane 7, 0.3 μM XR5944; lane 8, 1.0 μM XR5944. (B) Lane 1 contains DNA alone; lane 2, DNA and topoisomerase IIα; lane 3, DNA, topoisomerase I and DMSO (solvent control). Lanes 4–8 all contained DNA, topoisomerase IIα, DMSO and lane 4, 1.0 μM etoposide; lane 5, 0.01 μM XR5944; lane 6, 0.03 μM XR5944; lane 7, 0.1 μM XR5944; lane 8, 0.3 μM XR5944.

Table 2. Cytotoxicity [IC_{50} (nM)] in cell lines expressing resistance to topoisomerase inhibitors

	JL leukemia	JL_A amascrine resistant	JL_D doxorubicin resistant	D3F chinese hamster	D3F/C10 camptothein resistant
	IC_{50} (nM)	IC_{50} (nM)	IC_{50} (nM)	IC_{50} (nM)	IC_{50} (nM)
XR5944	0.09	0.054	0.063	4.4	4.0
TAS-103	5.4	302	384	ND	ND
Amsacrine	17	2125	1815	15.7	10.3
Doxorubicin	7	26	109	ND	ND
Camptothecin	4	7	4	45	15500

The indicated test drugs were assayed for cytotoxicity in cell lines as detailed in Methods. The Jurkat leukemia line and the D3F Chinese hamster ovary line were tested alongside their drug-resistant counterparts which express resistance to topoisomerase II and I active agents, respectively. The IC_{50} is calculated as the drug concentration required to give 50% cell kill and values quoted represent the mean of two or more independent evaluations.

or MRP, XR5944 remains a potent cytotoxic agent with IC_{50} values similar to or better than compounds such as topotecan and paclitaxel (Table 1).

Importantly cell lines that have developed resistance to topoisomerase-active agents through down-regulation of topoisomerase II (JL_A and JL_D)[16] or a point mutation of topoisomerase I (D3F/C10 CPT)[10] do not show cross-resistance to XR5944 (Table 2). By contrast, the selecting agents, amsacrine, doxorubicin and camptothecin, all had their activity severely reduced in the appropriate cell lines. The activity of TAS-103, which has been reported to be unaffected by down-regulation of topoisomerase II,[18] was significantly attenuated (about 60-fold) in these resistant Jurkat lines. These data demonstrate that XR5944, unlike TAS-103, is unaffected by these topoisomerase-associated resistance mechanisms, and supports the proposal that XR5944 targets both topoisomerase I and II.

Antitumor activity in human xenografts

The *in vivo* activity of XR5944 was examined in the H69/P SCLC and HT29 colon carcinoma xenografts in nude mice. The activity against established H69 xenografts appeared to be dose dependent (Figure 3A). At the lowest dose of XR5944 (5 mg/kg i.v., q4d × 3) tumor stasis was observed for approximately 3 weeks. At the two higher doses (10 and 15 mg/kg i.v. q4d × 3) tumor regression was achieved. In the experiment shown, six out of eight mice in both higher dose treatments groups appeared to be completely cured of their tumors. In contrast, topotecan administered at a previously used dose of 20 mg/kg (q4d × 3) or etoposide administered at a dose of 30 mg/kg on a q5d × 3 schedule caused tumor stasis for approximately 10 days before regrowth of the tumor. The mean body weight loss of 10 mg/kg XR5944 was similar to that obtained with a less effective dose of topotecan (20 mg/kg) or etoposide (30 mg/kg) (Figure 3B). In a separate experiment in mice bearing H69/P xenografts, administration of XR5944 at 5 mg/kg i.v. on a daily schedule ([qd × 5/week] × 2) caused complete tumor regression in eight of eight animals and these remained tumor free for more than 60 days. The activity of XR5944 at this daily schedule was comparable to that observed with 15 mg/kg dose on a q4d × 3 schedule (Figure 4A). Both schedules were well tolerated with maximum weight loss of 4 and 5%, respectively, with complete recovery of weight between days 16 and 20 after the start of treatment (Figure 4B). In contrast to XR5944, TAS-103 caused only transient tumor regression in the majority of the animals with only one of eight animals showing complete regression following either a daily (5 mg/kg, qd × 5) or intermittent (40 mg/kg, q4d × 2) schedule. Moreover, the doses of TAS-103 used caused significant toxicity with the loss of two of eight and five of eight animals in the two groups, respectively.

XR5944 was also significantly more active than TAS-103 in the relatively refractory HT29 colon carcinoma xenograft (Figure 5A). TAS-103 dosed at its maximum tolerated dose (MTD) and optimum schedule (45 mg/kg i.v. q7d × 3)[7] resulted in a significant growth delay compared to control animals (Figure 5A). In contrast, XR5944 (15 mg/kg i.v., q4d × 3) resulted in tumor regression in six of eight animals and was significantly more active ($p < 0.05$) than TAS-103. The antitumor effects of XR5944 were seen at well-tolerated doses as indicated by small changes in mean body weights (about 5%) even at the highest dose of XR5944 (Figure 5B). These data also demonstrate that XR5944 was much better tolerated than TAS-103 (Figure 5B). In a separate experiment mice treated with doxorubicin at its MTD (7 mg/kg single i.p. dose) showed far less antitumor activity than XR5944 and significantly more toxicity with animals continuing to lose weight for the duration of the experiment (data not shown).

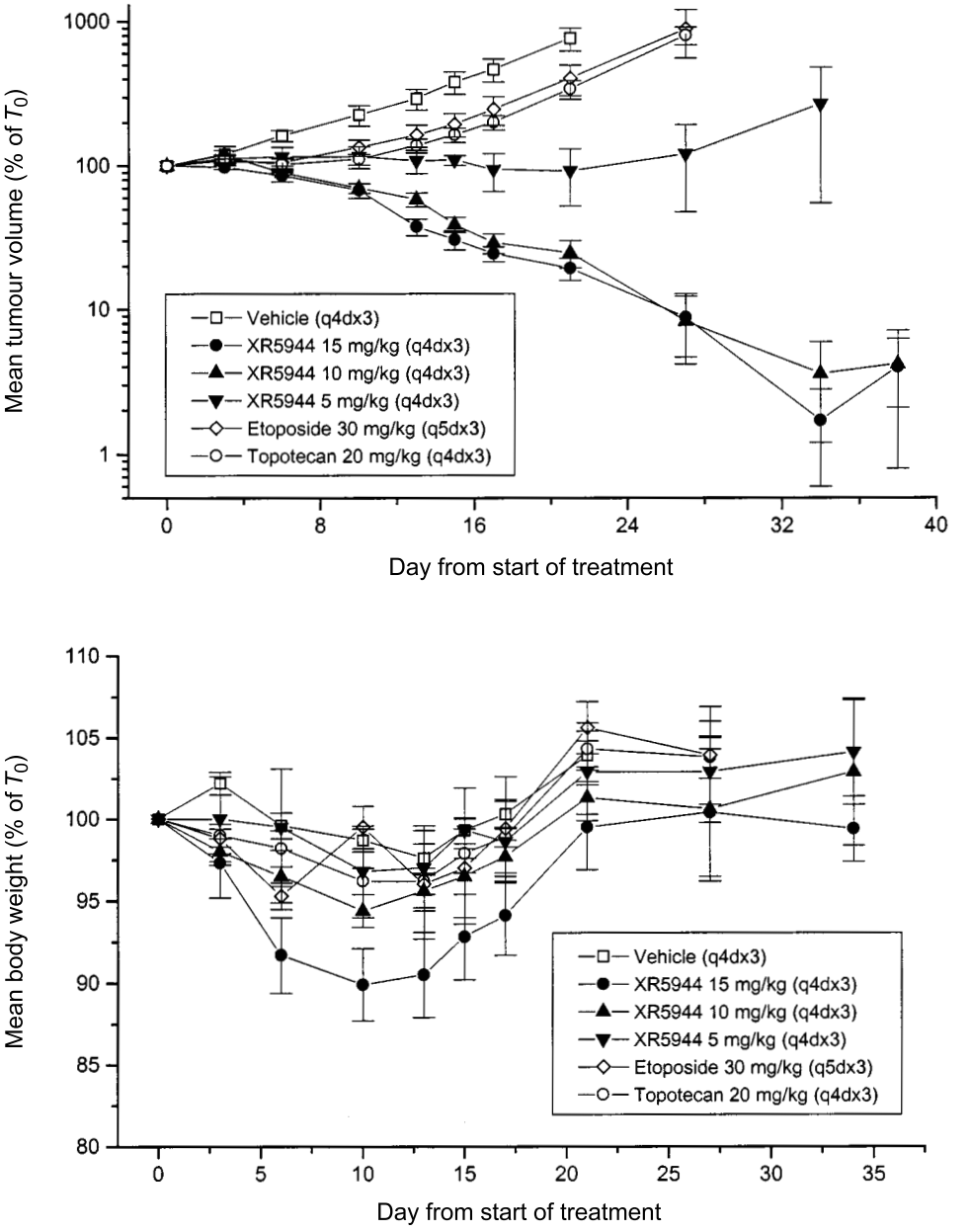

Figure 3. Antitumor activity of XR5944 in the H69 SCLC xenograft. XR5944 was administered to mice bearing established tumors as detailed in Methods. (A) Tumor volume plotted as a percentage of that seen on day 0. (B) Animal body weight plotted as a percentage of that seen on day 0. Vehicle (5% dextrose) (□); topotecan 20 mg/kg (○); etoposide 30 mg/kg (◇); XR5944 15 mg/kg (●); XR5944 10 mg/kg (▲); XR5944 5 mg/kg (▼). All solutions were administered i.v. at 10 ml/kg using either a q4d × 3 schedule (vehicle, topotecan, XR5944) or a q5d × 3 schedule (etoposide) starting on day 0.

Discussion

Agents that simultaneously inhibit both topoisomerase I and II may show benefit due to their ability to avoid topoisomerase-dependent drug resistance and to target the two enzymes which act at different points in the cell cycle. This possible therapeutic advantage has been recognized by a number of investigators. One approach has been to use specific inhibitors of topoisomerase I and II in combination as part of a single schedule.[19–21] Such combinations, including etoposide and camptothecin analogs (irinotecan or topotecan), have given encouraging results in preclinical models; however, clinical trials have suffered from problems of additive toxicities. This has precluded any meaningful efficacy data being collected.

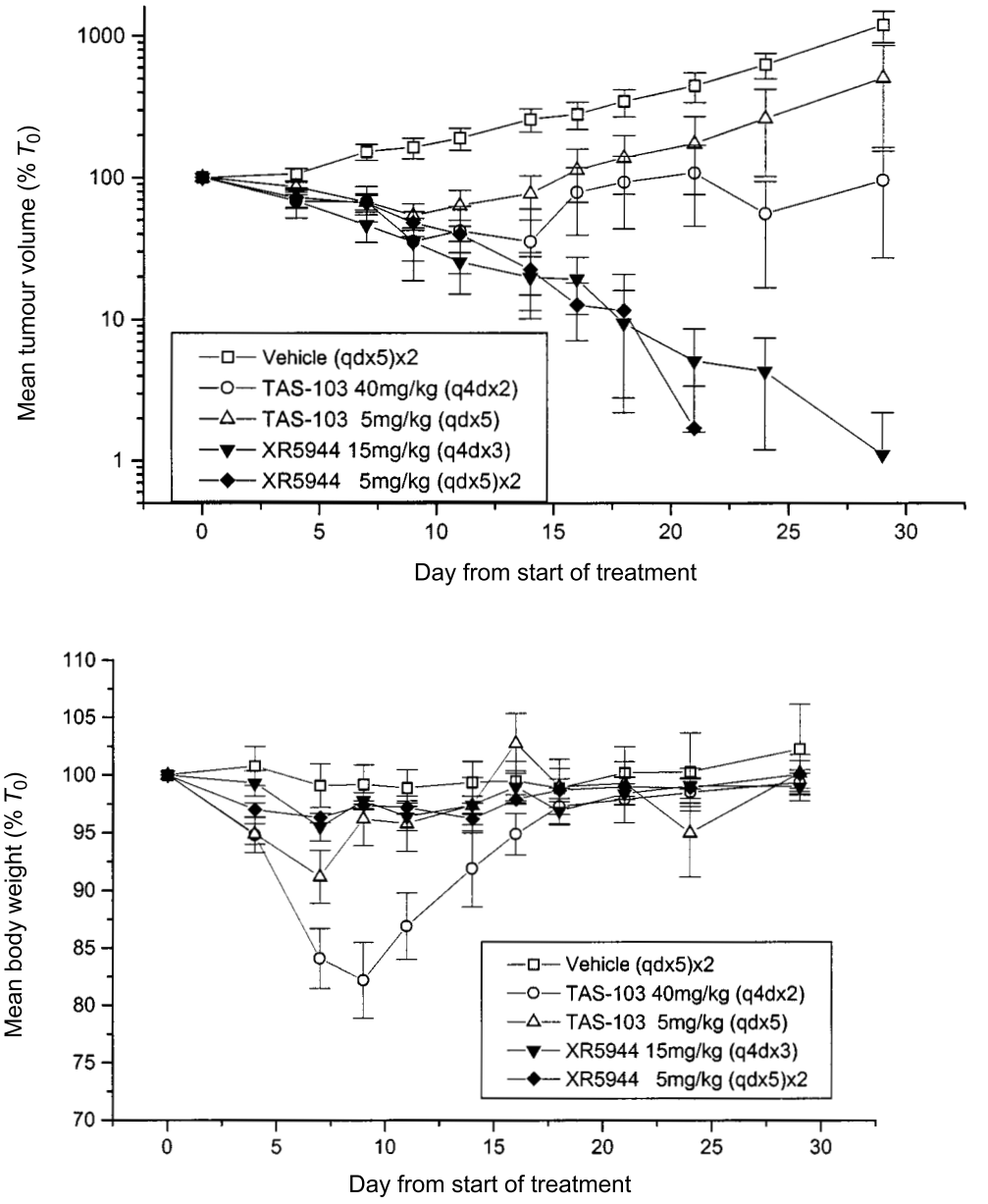

Figure 4. Antitumor activity of XR5944 and TAS-103 in the H69 SCLC xenograft. XR5944 was administered in mice bearing established tumors as detailed in Methods. (A) Effect of treatment on tumor volume plotted as a percentage of that seen on day 0 the start of treatment. (B) Effect of treatment on animal body weight plotted as a percentage of that seen on day 0. Vehicle (5% dextrose) (□); TAS-103 40 mg/kg (○); TAS-103 5 mg/kg (△); XR5944 15 mg/kg (▼); XR5944 5 mg/kg (◆) All solutions were administered i.v. at 10 ml/kg using the schedules indicated starting on day 0.

The second approach has been to develop a single molecule that is able to target both topoisomerase I and II. This strategy is exemplified by DACA/XR5000,[4] intoplicine,[5] F11782[6] and TAS-103.[7] Although TAS-103 was originally proposed as a joint topoisomerase I and II inhibitor, and was thus selected as a control for our studies, recent data suggest that TAS-103 is not a dual inhibitor but targets topoisomerase IIα.[19–21] With the exception of F11782, joint inhibitors are DNA-binding agents that have been shown to be intercalators. F11782 is reported to be a catalytic dual inhibitor that does not bind DNA.[6] These compounds are at various stages of preclinical or clinical development.

XR5944 is able to poison both topoisomerase I and II, presumably through its ability to intercalate into

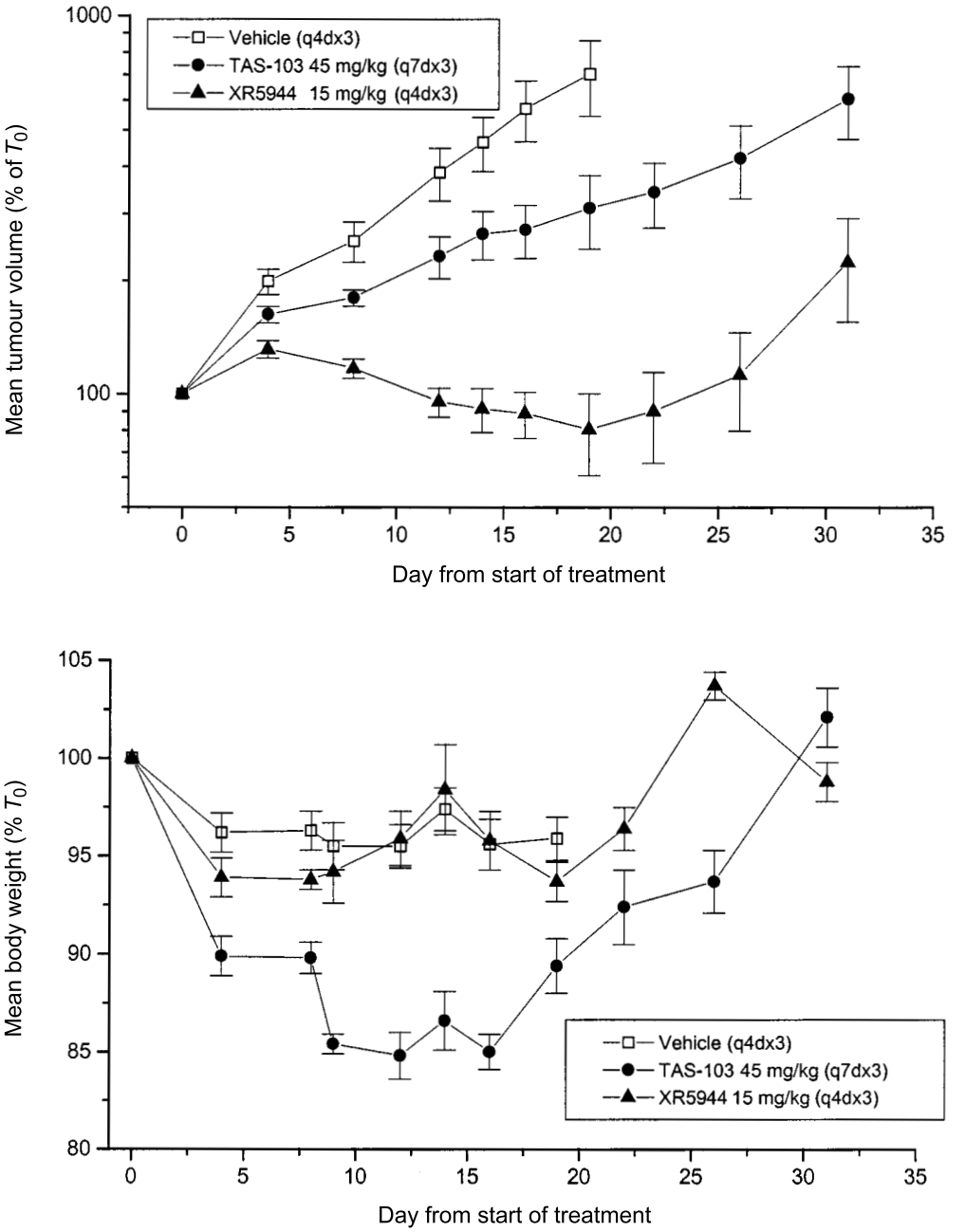

Figure 5. Antitumor activity of XR5944 in the HT29 colon carcinoma xenograft. XR5944 was administered to mice bearing established HT29 colon carcinoma tumors as detailed in Methods. (A) Tumor volume plotted as a percentage of that seen on day 0. (B) Animal body weight plotted as a percentage of that seen on day 0. Vehicle (5% dextrose) (□); TAS-103 (q7d × 3): 45 mg/kg (●); XR5944 (q4d × 3): 15 mg/kg (●). All solutions were administered i.v. at 10 ml/kg.

DNA,[22] although the exact mechanism is currently unknown. The fact that XR5944 has two planar phenazine chromophores allows for the possibility of bis-intercalation, as has been suggested for LU79553.[23] This additional interaction may be partly responsible for the outstanding potency of XR5944 in poisoning topoisomerase I and II and killing cells.

The evidence for XR5944 being a joint inhibitor of topoisomerase I and II comes from the ability to stabilize cleavable complexes with both enzymes, and also from the work with cells expressing alterations in either enzymes (atypical drug resistance). These experiments clearly showed that XR5944 is equally active upon down-regulation of topoisomerase II or a

point mutation in topoisomerase I, suggesting a parallel mechanism. The fact that XR5944 is equipotent against topoisomerase I and II supports the possibility that when topoisomerase II is down-regulated cell kill occurs through topoisomerase I. Interestingly, TAS-103 was significantly less active in these cells with reduced topoisomerase II, suggesting a preference for topoisomerase II. This is in conflict with previous reports showing only a small loss of activity in such cells.[18] 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,[19,20] which is in accord with the data presented here.

The *in vitro* profile of XR5944 in parental human cell lines shows it to be an exceptionally potent cytotoxic agent. In all human cell lines tested, XR5944 was several-fold more active than all other compounds tested, including topotecan and paclitaxel. These *in vitro* studies also showed that, although XR5944 was a substrate for the MDR transporters P-gp and MRP, significant potency was retained despite hyperexpression of these resistance mechanisms. Even in cells overexpressing the MDR transporters, XR5944 showed competitive or better activity than topotecan, doxorubicin and pactitaxel.

This potent activity of XR5944 *in vitro* translated well to human xenograft models *in vivo* where clear tumor regression was observed when mice were dosed with XR5944. In addition, very little change in body weight was observed at efficacious doses, suggesting that XR5944 was well tolerated. Importantly, complete tumor regression was induced by XR5944 at low doses (5–15 mg/kg) in the H69 SCLC model where other well-used cytotoxics, topotecan and etoposide, gave relatively limited delays in tumor growth. Similar data were obtained in the HT-29 xenograft in which XR5944 again induced tumor regression in the majority of animals and was significantly more effective than TAS-103. These data demonstrate XR5944 is an extremely efficacious new anticancer agent *in vivo*, even in tumors such as the HT29 colon carcinoma, which are relatively refractory to chemotherapy.

In summary, our studies show that the bisphenazine, XR5944, has exceptional *in vitro* and *in vivo* activity against both human and murine cell lines. Mechanistically, XR5944 appears to be a joint topoisomerase I/II inhibitor as it showed stabilization of both topoisomerase I- and II-dependent cleavable complexes. This proposed mechanism is supported by the observation that XR5944 is unaffected by atypical drug resistance. Finally, although the activity of XR5944 is attenuated in cells hyperexpressing the MDR transporters P-gp and MRP, the compound retained good potency in these MDR cell lines, which was comparable or superior to several currently used agents such as topotecan and paclitaxel. XR5944 therefore shows significant potential as a novel anticancer agent capable of targeting both topoisomerase I and II.

Acknowledgments

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