Preclinical anti-tumor activity of XR5944 in combination with carboplatin or doxorubicin in non-small-cell lung carcinoma

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XR5944 (MLN944) is a novel bis-phenazine currently in phase I clinical trials that has demonstrated potent cytotoxic activity against a variety of tumor models. The combinations of XR5944 with carboplatin or doxorubicin were investigated in COR-L23/P human non-small-cell lung carcinoma (NSCLC) cells *in vitro* and the corresponding xenografts *in vivo*. *In vitro* cytotoxicity was evaluated by the sulforhodamine B assay and the drug interactions following simultaneous or sequential exposure were determined using median-effect analysis to calculate combination indices (CIs). XR5944 demonstrated potent cytotoxicity compared to either carboplatin or doxorubicin in COR-L23/P cells. Simultaneous or sequential exposure of XR5944 followed by carboplatin led to a synergistic response (CI < 1), whereas the reverse order of addition showed an additive or antagonistic response (CI ≤ 1). Sequential administration of doxorubicin followed by XR5944 demonstrated marginally improved cytotoxicity (CI = 1.31–0.77) than other schedules (CI = 1.50–1.22) relative to individual drugs. Anti-tumor activity against COR-L23/P xenografts in nude mice was enhanced by administration of XR5944 (2 or 5 mg/kg) immediately before carboplatin (50 mg/kg) compared to single-agent treatment at the same doses. Improved efficacy was also observed by sequential administration of 7 mg/kg doxorubicin 48 h before 2.5 or 5 mg/kg XR5944. No additional toxicity was observed with combinations compared to single-agent treatment alone as determined by body weights. These data suggest that combinations of XR5944 with carboplatin or doxorubicin are of significant interest for clinical use, and that the schedule of administration may be important for achieving clinical efficacy over single-agent therapy. *Anti-Cancer Drugs* 16:945–951 © 2005 Lippincott Williams & Wilkins.

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Introduction

XR5944 (MLN944) is currently in phase I clinical trials for the treatment of advanced stage solid tumors. XR5944 is a novel bis-phenazine that has shown outstanding cytotoxic activity in a range of human and murine tumor cell lines *in vitro*, as well as significant anti-tumor activity against human tumor xenografts at well-tolerated doses [1]. In *ex vivo* biopsy samples from ovarian and melanoma patients, XR5944 has also demonstrated good potency which was significantly greater than for other known drugs [2]. Initially, the mechanism of action of XR5944 was thought to involve the joint inhibition of topoisomerases I and II [1]. However, recent studies have indicated that the topoisomerase enzymes are not the primary cellular targets for XR5944 and instead they support a novel mechanism of action. For example XR5944 was evaluated against the NCI human cell line panel [3], and COMPARE analysis showed a unique mechanism of action through absence of correlation with known topoisomerase I and II inhibitors (data not shown). Additionally, reduction of functional topoisomerase enzymes did not alter the efficacy of XR5944 in the yeast *Saccharomyces cerevisiae* or in human cell lines [3,4]. Furthermore, gene profiling studies in yeast cells showed that XR5944 treatment induced a unique alteration in gene expression compared with that induced by known topoisomerase inhibitors [5]. Further studies supporting a novel mechanism of action have also been carried out in human tumor cell systems. Human tumor xenografts treated with XR5944 or irinotecan showed distinct gene expression profiles, revealing clusters of differentially regulated genes by the two drugs [4]. In addition, XR5944 does not inhibit the catalytic activity of topoisomerase I or II at active concentrations and does not significantly stimulate DNA scission mediated by either topoisomerase I or II, unlike camptothecin or etoposide [4]. Cell cycle effects of XR5944 also indicate mechanisms of action distinct from known topoisomerase inhibitors. Exposure of HCT116 colon carcinoma cells to XR5944 resulted in both G_1 and G_2 cell cycle arrest [4,6], whereas conventional topoisomerase inhibitors such as irinotecan or doxorubicin demonstrate characteristic G_2 phase arrest [7–9]. Taken together, these data suggest that XR5944 exerts a cytotoxic response predominantly

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via mechanisms other than the inhibition of topoisomerase I and II.

Lung cancer is one of the major causes of cancer-related deaths and is frequently only diagnosed at late stage. The most common type of lung cancer is non-small-cell lung carcinoma (NSCLC), accounting for around 80% of lung cancers [10]. Standard chemotherapy for advanced lung cancer typically consists of combinations of two or more drugs usually involving platinum-based agents [11]. Drug pairings that have shown improved efficacy include carboplatin or cisplatin combined with paclitaxel, vinorelbine or gemcitabine [12–15]. Such combination therapy has been shown to improve the overall response to treatment over single-agent therapy. The principle of combination therapy is that an additive or synergistic response may be achieved by the use of multiple agents with different mechanisms of action. In addition, by combining mechanistically distinct therapies it may be possible to overcome intrinsic drug resistance.

In light of the possible novel mechanism of action of XR5944, we investigated the therapeutic potential of combining XR5944 with either carboplatin or doxorubicin for the treatment of NSCLC. We have previously shown that XR5944 in combination with 5-fluorouracil or irinotecan shows enhanced anti-tumor activity in human colon cancer xenografts and may be of benefit in the treatment of colon cancer [16]. In this study, we show that carboplatin and XR5944 in combination demonstrate schedule-dependent synergistic activity *in vitro*, and significantly improved efficacy over single-agent therapy *in vivo*. Additionally, an additive response was achieved with sequential exposure to doxorubicin followed by XR5944 against the COR-L23/P human NSCLC cells *in vitro* that also translated to *in vivo* activity.

Methods

Drugs

XR5944 (dimesylate salt) (Fig. 1) was synthesized at Auckland Cancer Research Centre and was dissolved in filter-sterilized (0.2 μM) 5% dextrose (w/v). All doses are quoted as free base equivalent and all drugs were made up immediately prior to use. For *in vitro* use, doxorubicin was purchased from Sigma (Poole, UK) and was dissolved in sterile water. For *in vivo* use, doxorubicin (doxorubicin hydrochloride) was obtained from Faulding Pharmaceuticals (Leamington, UK) and diluted in sterile 5% dextrose. Carboplatin (paraplatin solution) was obtained from Bristol-Myers Squibb (New York, USA) for use both *in vitro* and *in vivo*, and was diluted in sterile 5% dextrose.

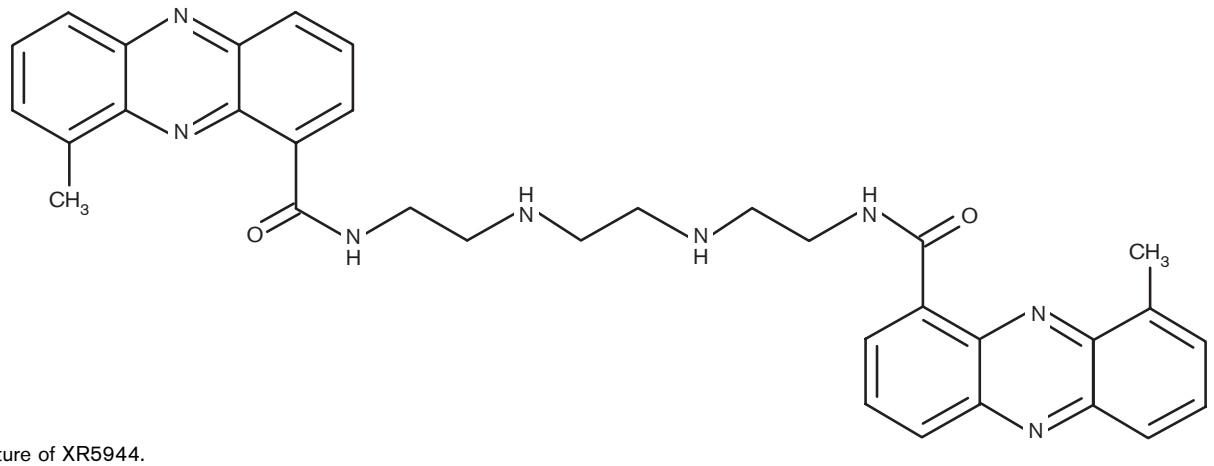
Cell lines

COR-L23/P human NSCLC cells were kindly provided by Dr P. R. Twentyman (MRC Clinical Oncology and Therapeutics Unit, Cambridge, UK). Cells were grown as monolayers in RPMI medium supplemented with 1% L-glutamine and 10% FCS in a humidified atmosphere containing 5% CO_2.

Cytotoxicity assays

Cells were seeded in 96-well plates at 1×10^4 cells/well for 5-day assays and 1×10^3 cells/well for 7-day assays 4 h prior to the addition of 2-fold serial dilutions of the cytotoxic. These cell densities were chosen so that cells were in exponential growth for the duration of the assay. Analysis of cell growth was assessed using the sulforhodamine B (SRB) assay after 5 days of drug exposure [17] and used to calculate the IC_{50} values. For calculation of the molar ratio for sequential combination assays, IC_{50} values were also calculated following a 48-h incubation of cytotoxic drug either from days 0 to 2 or 2 to 4 with analysis of cell growth on day 7. The SRB technique was

Fig. 1



Structure of XR5944.

performed for determination of the IC_{50} values as described by Skehan *et al.* [17]. Briefly, 50 µl ice-cold 50% TCA was added to all wells, and fixed for 1 h at 4°C, washed 3 times with water and air dried. Fixed cells were stained with 50 µl of 0.4% SRB in 1% acetic acid solution for 30 min at room temperature. After washing 3 times in 1% acetic acid and air drying, SRB was solubilized in 100 µl/well 10 mM unbuffered Tris. Optical density was measured at 510 nm and growth inhibitions were determined relative to untreated cells. For *in vitro* combination assays, cytotoxics were incubated with cells both individually and together at the ratio of their IC_{50} values as a series of 2-fold dilutions from 8 to 0.0625 times IC_{50}. Combination assays were performed as a simultaneous schedule (5-day incubation followed by analysis) or sequential schedules (two 48-h incubations followed by analysis on day 7). All assays were carried out in duplicate and data presented are the mean of at least three independent experiments.

Median-effect analysis

The combined effect of XR5944 and doxorubicin or carboplatin treatment was analyzed by median-effect analysis according to the method of Chou and Talalay [18]. Combination index (CI) values were expressed at each fraction affected (F_a) using CalcuSyn software (Biosoft, Cambridge, UK) developed by Chou and Chou. CI < 1 indicates synergism, CI = 1 indicates additivity and CI > 1 indicates antagonism of the interaction. The linear regression coefficient was automatically generated for each assay and was greater than 0.95 in each case.

Animals

All animal experimentation was performed to UK Home Office regulations and the UKCCCR guidelines were adhered to throughout the studies. Female CD1 nude mice were purchased from Charles River (UK). Animals were maintained under constant temperature and humidity, and a 12-h light/dark cycle with food and water available *ad libitum*.

In vivo combination studies

COR-L23/P cells were harvested from *in vitro* incubation and were inoculated s.c. at 1×10^6 per animal in 100 µl PBS into the right flanks of CD1 athymic mice. When tumors had reached a mean diameter of 4–7 mm (day 0), the animals were randomized into groups of six or seven and treated by i.v. or i.p. injection at 10 ml/kg. Body weights and two perpendicular diameters of the tumors were measured at least 3 times per week. Each tumor volume was calculated according to the following equation: $v = 0.5236[(l + w)/2]^3$, where l and w are the largest and smallest perpendicular diameters. Tumor volume and body weights were expressed as mean ± SEM relative to tumor volume or body weight values on day 0 (start of treatment). The T/C% ratio (mean relative tumor volume of the treated tumors/mean relative volume of control group × 100) was calculated each time the tumors were measured. The lowest value is expressed as the optimal T/C% for each group. Statistical analysis was performed using two-way ANOVA with Bonferroni post-tests.

To evaluate the interaction between doxorubicin and XR5944, doxorubicin (7 mg/kg) was administered first on day 0, followed by XR5944 (2.5 or 5 mg/kg) 48 h later (day 2). This cycle was repeated on days 7 and 14. To evaluate the effects of individual drugs, animals were dosed with doxorubicin (5.5, 7 or 8.5 mg/kg) or XR5944 (2.5, 5 or 10 mg/kg) on day 0 and vehicle (5% dextrose) on day 2 or vehicle on day 0 and XR5944 (5 mg/kg) on day 2. Control animals were dosed with vehicle alone on days 0 and 2. All the treatments were administered intravenously, and all cycles were repeated on days 7 and 14.

The interaction between XR5944 (2 or 5 mg/kg) and carboplatin (50 mg/kg) was evaluated by administration of carboplatin (i.p.) immediately following XR5944 (i.v.). To evaluate the effects of individual drugs, animals were dosed with XR5944 (2, 5 or 10 mg/kg) administered i.v. or carboplatin (50 or 100 mg/kg) administered i.p. Control animals were dosed with vehicle alone (5% dextrose i.v. followed immediately by 5% dextrose i.p.). All treatments were repeated on days 7 and 14.

Results

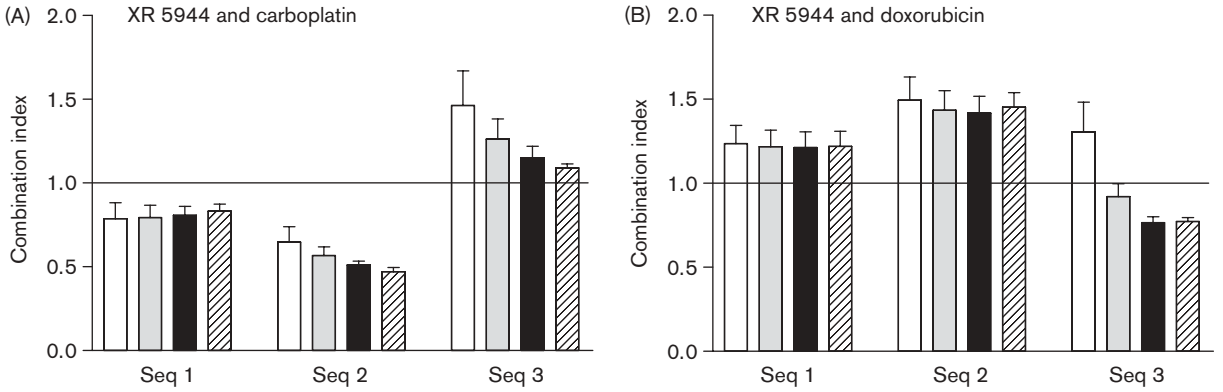
Cytotoxicity of doxorubicin, carboplatin and XR5944 in COR-L23/P cells

IC_{50} values for doxorubicin, carboplatin, and XR5944 in the cytotoxicity assay were 20 ± 0.02 nM, 3.1 ± 0.93 µM and 0.17 ± 0.10 nM, respectively, and were determined by SRB assay following a 5-day incubation with COR-L23/P cells. XR5944 was significantly more potent than either doxorubicin or carboplatin in this cell line. These values were used to generate the fixed ratios for the simultaneous exposure combination studies. Cytotoxicity assays were also performed using either exposures for 0–48 or 48–96 h, followed by analysis on day 7, for the generation of fixed ratios for the sequential exposure combination studies (data not shown).

Median-effect analysis of XR5944 in combination with doxorubicin or carboplatin

Figure 2 shows the median-effect analysis of XR5944 in combination with either doxorubicin or carboplatin in COR-L23/P cells assuming mutual exclusivity of the interactions. Simultaneous exposure of COR-L23/P cells to XR5944 and carboplatin led to slight synergism (CI = 0.79–0.83 at F_a = 0.25–0.9). Sequential exposure to XR5944 for 48 h followed by carboplatin for 48 h demonstrated stronger synergistic activity than simultaneous exposure. The combination index at F_a = 0.9 was 0.47 indicating that the amount of the two drugs required to kill 90% of cells was less than half (0.47 times) as much

Fig. 2



Median-effect analysis of the interaction between XR5944 and carboplatin or doxorubicin in L23/P cells. Sequence 1, simultaneous incubation for 5 days; Sequence 2, XR5944 exposure for 48 h then carboplatin or doxorubicin exposure for 48 h; Sequence 3, carboplatin or doxorubicin exposure for 48 h then XR5944 exposure for 48 h. Open bars: $F_a = 0.25$; grey bars: $F_a = 0.5$; black bars: $F_a = 0.75$; shaded bars: $F_a = 0.9$. CI is plotted as a function of the fraction of cells affected by the cytotoxic effect (F_a). CI > 1.1 indicates antagonism, CI = 0.9–1.1 indicates additivity and CI < 0.9 indicates synergism. Values are the means of at least three independent experiments ± SEM.

as would be required if they demonstrated purely additive behavior. Conversely, the reverse order of addition led to slight antagonism at low F_a or additivity at high F_a (Fig. 2A).

Simultaneous exposure of COR-L23/P cells to XR5944 and doxorubicin demonstrated additive or slightly antagonistic effects (CI = 1.24–1.22 at $F_a = 0.25$–0.9) (Fig. 2B). Sequential exposure to XR5944 (48 h) followed by doxorubicin (48 h) did not significantly alter the response compared to simultaneous exposure ($P > 0.05$, Student's *t*-test at $F_a = 0.5$). Improved efficacy was observed by sequential exposure of doxorubicin followed by XR5944 and some indication of synergistic activity was observed at high fractional effects (CI = 0.77 at $F_a = 0.75$ and 0.9).

Anti-tumor activity of XR5944 and doxorubicin alone, and in combination against the COR-L23/P xenograft in nude mice

To further assess the possible advantage of combining XR5944 and doxorubicin, we studied the activity of this combination against the COR-L23/P xenograft in nude mice using the most favorable *in vitro* schedule (doxorubicin followed by XR5944). XR5944 alone at 2.5, 5 or 10 mg/kg showed a dose-dependent response (Fig. 3A). Tumor doubling times were 7.1, 45.4 and more than 47 days for 2.5, 5 and 10 mg/kg XR5944, respectively, and T/C% ratios were 41.6, 12.7 and 3.7, respectively, at day 16. The highest dose of XR5944 led to complete tumor regression in all seven animals in the group, whereas three of seven and none of seven animals showed complete regression in groups treated with 5 and 2.5 mg/kg XR5944, respectively. All doses of XR5944 were well tolerated as indicated by lack of significant body weight loss compared to vehicle-treated animals (Fig. 3B).

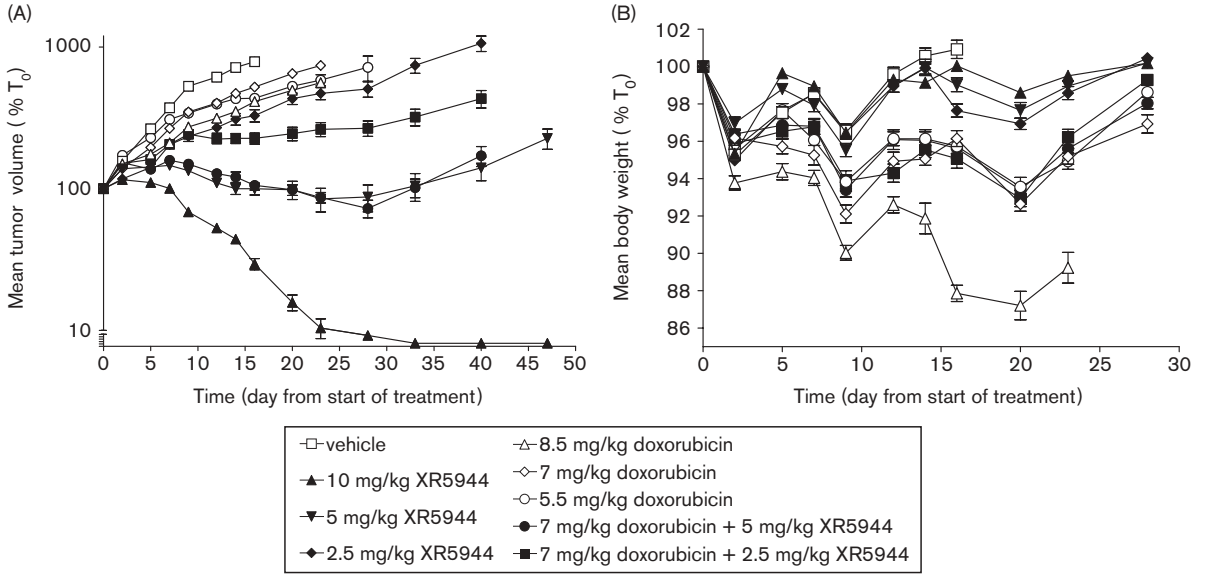
Efficacy was relatively poor with doxorubicin alone compared with XR5944 (Fig. 3A), as shown by the T/C ratios, which were 55.2, 65.4 and 51.2% for 5.5, 7 and 8.5 mg/kg doxorubicin, respectively. In addition, 8.5 mg/kg doxorubicin led to 13% body weight loss on day 20 in this study and was considered to be near to its maximum tolerated dose (Fig. 3B).

Sequential dosing of 7 mg/kg doxorubicin (days 0, 7 and 14) followed by 5 mg/kg XR5944 (days 2, 9 and 16) showed similar efficacy to 5 mg/kg XR5944 alone dosed on days 0, 7 and 14. However, when compared to 5 mg/kg XR5944 alone dosed starting on day 2 (days 2, 9 and 16) (data not shown), the combination showed improved efficacy (T/C% ratio 13.4 compared to 26.0). This difference is likely to be attributable to the larger mean tumor volume at the start of treatment for the group dosed starting on day 2 (156.9% of day 0). Sequential dosing of 7 mg/kg doxorubicin (0 h) followed by 2.5 mg/kg XR5944 (48 h) showed significantly ($P < 0.01$) better anti-tumor activity than either agent alone at these doses. Both combination doses were well tolerated in this study and did not show any more body weight loss than 7 mg/kg doxorubicin alone (Fig. 3B).

Anti-tumor activity of XR5944 and carboplatin alone and in combination against the COR-L23/P xenograft in nude mice

The efficacy of combination therapy with carboplatin and XR5944 was evaluated in COR-L23/P xenografts following a simultaneous dosing schedule. Carboplatin alone (50 mg/kg) did not show significant efficacy ($P < 0.05$) until day 15, at which point some of these tumors had reached their maximum permitted diameter and the group was therefore terminated. At 100 mg/kg, carboplatin showed

Fig. 3



Anti-tumor activity of doxorubicin and XR5944 alone, and in combination against the L23/P human NSCLC xenograft. (A) Tumor volume plotted as a percentage of that on day 0. (B) Animal body weight plotted as a percentage of that on day 0. All solutions were administered i.v. at 10 ml/kg using a q7d $\times$3 dosing schedule starting on day 0. For the combination groups, doxorubicin was administered on days 0, 7 and 14 and XR5944 on days 2, 9 and 16. Data are expressed as means $\pm$ SEM. $n = 6$–7.

significant efficacy by day 7 ($P < 0.001$), but this dose was not well tolerated and the mean body weight dropped to 88% of the starting weight on day 5 (Fig. 4B). By day 7, the animals had not recovered and were not dosed until day 14.

XR5944 alone showed a good dose-dependent response with T/C% ratios of 55.0, 31.5 and 11.5 at 2, 5 and 10 mg/kg, respectively. In addition, all doses were well tolerated. Treatment with 10 mg/kg XR5944 led to complete tumor regression in five of six animals. The remaining animal showed tumor regression to 1.8 mm diameter. None of the tumors in this group had re-grown by day 75.

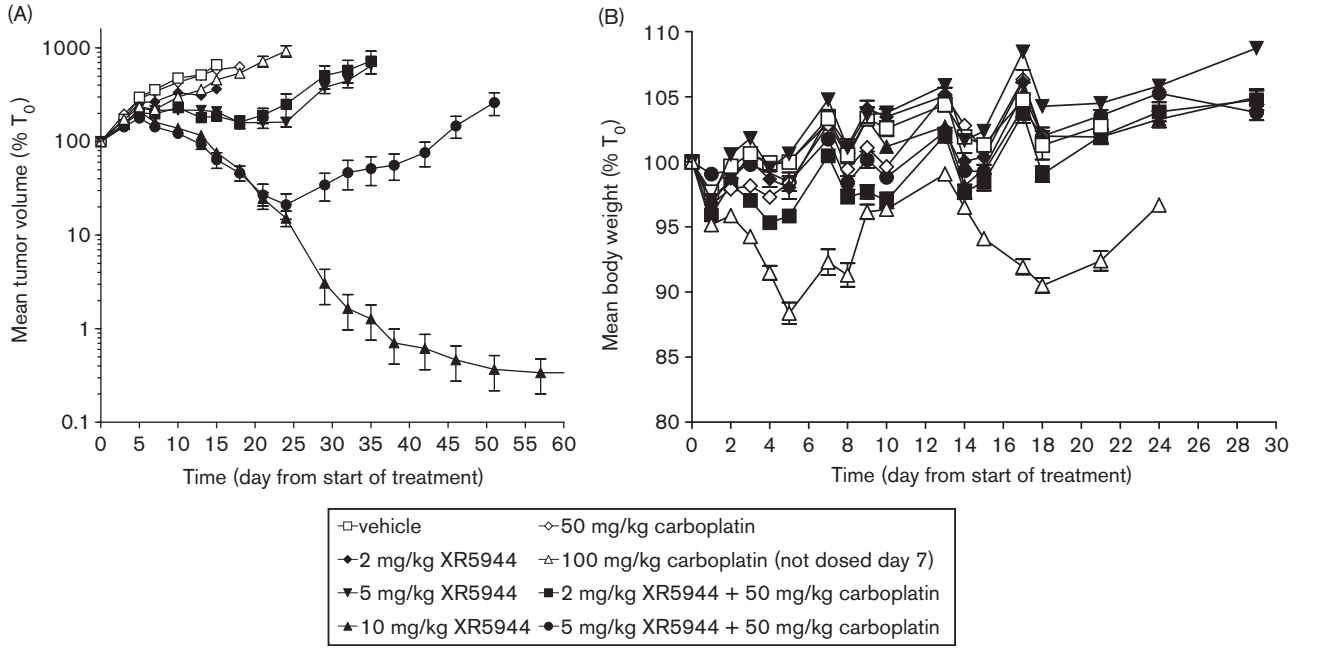
Both of the combination treatment groups showed a significant ($P < 0.001$) difference in efficacy to treatment with either agent alone at the same doses. In addition, 2 mg/kg XR5944 and 50 mg/kg carboplatin in combination showed similar efficacy to 5 mg/kg XR5944 alone (T/C% ratios 28.2 and 31.5, respectively). Both combination doses were well tolerated with a maximum body weight loss of 4.7% (Fig. 4B).

Discussion

The aim of this study was to investigate the potential for the use of XR5944 in combination with the chemotherapeutic agents doxorubicin and carboplatin for the treatment of lung carcinoma. Combination therapy is a viable approach to improving efficacy in the clinic, especially in advanced tumors, by inducing an additive or synergistic response. Multiple drugs with different mechanisms of action may also help overcome the problem of resistance, one of the main problems associated with platinum-based agents [19]. In this study XR5944 was more potent than either carboplatin or doxorubicin *in vitro* against COR-L23/P human NSCLC cells. In addition, optimal combination schedules of XR5944 with these agents demonstrated additive or synergistic responses. Exposure of cells *in vitro* to XR5944 has recently been shown to cause cell cycle arrest predominantly in G_1 phase and to some extent in G_2 of an asynchronous cell population [6]. With this in mind it is not surprising that pre-exposure of cells to XR5944 before carboplatin (and to some extent simultaneous exposure) caused synergistic efficacy, since cells are most sensitive to carboplatin and cisplatin in the G_1 phase of the cell cycle [20]. In contrast, the reverse order of addition only showed at best an additive response ($CI \geq 1$). The response observed with the combination of XR5944 and doxorubicin could also be attributed to cell cycle effects. It is possible that the G_1 and G_2 arrest by simultaneous or pre-exposure of cells to XR5944 to some extent inhibits the activity of doxorubicin which is S phase specific [21].

In agreement with the *in vitro* data, XR5944 showed excellent anti-tumor activity in COR-L23/P NSCLC xenografts in nude mice causing complete regression at

Fig. 4



Anti-tumor activity of carboplatin and XR5944 alone, and in combination against the L23/P human NSCLC xenograf. (A) Tumor volume plotted as a percentage of that on day 0. (B) Animal body weight plotted as a percentage of that on day 0. All solutions were administered at 10 ml/kg using a q7d × 3 dosing schedule. XR5944 was dosed i.v. on days 0, 7 and 14 and carboplatin was dosed i.p. immediately after XR5944. Data are expressed as means ± SEM. $n = 6$.

well-tolerated doses. In contrast, single-agent treatment with doxorubicin or carboplatin dosed close to their maximum tolerated dose caused only a marginal tumor growth delay (T/C% of 51.2 and 61.4, respectively). T/C% below 40% is considered to be biologically relevant as determined by the National Cancer Institute, Division of Cancer Treatment. Previous studies have also shown that XR5944 causes complete cures in H69 small lung carcinoma xenograft-bearing nude mice [1]. Thus, it appears that XR5944 may be particularly useful in treating lung carcinoma.

In general, sequential dosing of doxorubicin followed by XR5944 48 h later, demonstrated improved efficacy over single-agent treatment. This was evident at a low dose of XR5944 and when XR5944 was dosed starting on day 2, as in the combination group. These data suggest that the clinical utility of combining XR5944 with doxorubicin should be considered.

To further investigate the therapeutic potential for XR5944 and carboplatin in combination, the efficacy of simultaneous exposure to these drugs was also explored against COR-L23/P xenografts *in vivo*. Administration of XR5944 followed immediately by carboplatin showed improved efficacy over single-agent treatment. In light of the complementary cell cycle effects of these two agents as described above, it is possible that a sequential schedule of XR5944 followed by carboplatin 48 h later might have shown even better synergistic efficacy in this xenograft model. However, it is not proven how well *in vitro* synergistic activity translates to *in vivo*.

Both combinations were well tolerated in nude mice and did not appear to cause any more body weight loss than treatment with doxorubicin or carboplatin alone. It is clear, however, that in order for these combinations to be of therapeutic benefit in the clinic, the apparent lack of overlapping toxicities would have to be translatable to patients. The toxicity of XR5944 in mice is primarily hematological, including reduced red and white blood cell count, and in dogs the major toxicity is gastrointestinal intolerance (data not shown). In man, the major toxicities of carboplatin and doxorubicin are myelosuppression (severe thrombocytopenia), and cardiotoxicity and myelosuppression respectively [22–26].

Taken together, these data demonstrate that XR5944 is a potent cytotoxic both alone and in combination with doxorubicin or carboplatin. Treatment of NSCLC using these combinations may provide significantly improved clinical efficacy over single-agent treatment. However, the successful combination of any two cytotoxic drugs will depend on the ability to demonstrate acceptable clinical

safety profiles and may also be dependent on sequence of administration.

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