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ORIGINAL ARTICLE

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Evidence of enhanced in vivo activity using tirapazamine with paclitaxel and paraplatin regimens against the MV-522 human lung cancer xenograft

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Abstract *Purpose*: Tirapazamine (3-amino-1,2,4-benzotriazine 1,4-dioxide; SR 4233) is a bioreductive agent that exhibits relatively selective cytotoxicity towards cells under hypoxic conditions and can enhance the antitumor activity of many standard oncolytics. In the present study we examined the interaction between tirapazamine in vivo with paclitaxel and paraplatin in two- and three-way combination studies using the MV-522 human lung carcinoma xenograft model. *Methods*: Agents were administered as a single i.p. bolus, with tirapazamine being given 3 h prior to paclitaxel, paraplatin, or their combination. Tumor growth inhibition (TGI), final tumor weights, partial and complete responses, and time to tumor doubling were determined after drug administration. *Results*: Tirapazamine as a single agent was ineffective against this human lung tumor model. A substantial increase in TGI was seen in animals treated with the triple-agent regimen (tirapazamine-paclitaxel-paraplatin) compared to animals treated with double-agent regimens that did not include tirapazamine. The addition of tirapazamine to paclitaxel-paraplatin therapy resulted in a 50% complete response rate; there were no complete responses seen when only the paclitaxel-paraplatin combination was administered. Time to tumor doubling was also significantly improved with the addition of tirapazamine to the paclitaxel and paraplatin combinations. Tirapazamine did not increase the toxicity of paclitaxel, paraplatin, or their combinations as judged by its minimal impact on body weight and the fact that no toxic deaths were observed with tirapazamine-containing regimens. *Conclusions*: These results are important since recent studies have suggested that the combination of paclitaxel and paraplatin may be particularly active in patients with advanced stage non-small-cell lung cancer. Since tirapazamine can significantly improve efficacy, but does not appear to enhance the toxicity of paclitaxel and paraplatin, its evaluation in future clinical trials in combination with paclitaxel-paraplatin-based therapy appears warranted.

Key words Tirapazamine · Lung cancer · Platinum · Taxol

Introduction

Tirapazamine (3-amino-1,2,4-benzotriazine 1,4-dioxide; SR 4233) is a bioreductive agent that exhibits relatively selective cytotoxicity towards cells under hypoxic conditions [33]. Low oxygen tensions facilitate the one-electron activation of tirapazamine by cellular reductases, such as cytochrome p450 reductase, resulting in the formation of nitroxide radicals [11, 24, 25]. Recent studies have suggested that intranuclear matrix-associated reductases may play the predominant role, compared to cytoplasmic enzymes, in activation of tirapazamine [6, 10]. This active intermediate has been shown to produce single- and double-stranded DNA breaks [32, 33]. Preliminary studies have also suggested that cells exposed to tirapazamine will undergo apoptosis related to genetic instability [22, 31], which may be associated with failure to induce *p*21, but is also independent of p53 expression by the tumor [31].

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While the resultant damage to cellular DNA by tirapazamine may be cytotoxic, it has also been shown to enhance the effects of both radiation therapy and chemotherapy [7, 8, 13, 14, 16, 20, 21, 28, 29, 34]. Preclinical in vivo studies with tirapazamine in combination with several alkylating agents including cisplatin, carboplatin, cyclophosphamide, carmustine, and melphalan, as well as with nonalkylators such as paclitaxel, doxorubicin, 5-fluorouracil, and flavone acetic acid have been conducted [7, 8, 13, 16, 20, 21, 29]. The addition of tirapazamine to these agents produces a greater than additive antitumor effect with 1.6- to 5.3-fold increases in tumor growth delay, usually with little increase in toxicity. This beneficial interaction has been seen in murine fibrosarcoma [14, 16, 29] and human breast, colon, and melanoma [7, 8, 20] tumor models. These superadditive interactions are tumor-specific and highly schedule-dependent, with maximal responses being observed when tirapazamine is administered shortly before the standard cytotoxic agent [7].

The present studies were conducted to evaluate the interaction between tirapazamine with paclitaxel and paraplatin in two- and three-way combination regimens in vivo using the MV-522 human lung carcinoma xenograft model. The MV-522 model was chosen because it appears to be a good predictor of clinical activity against non-small-cell lung cancer [18]. In addition, spontaneous tumor regressions with this model are seldom seen [18]. The results of these extensive combination studies clearly show that tirapazamine can potentiate the antitumor activity of paclitaxel-paraplatin-based therapy in this human lung tumor model without excessive toxicity.

Materials and methods

Cytotoxic drugs

Tirapazamine was obtained from Sanofi Pharmaceuticals (Great Valley, PA). Paclitaxel and paraplatin were obtained from Bristol-Myers Squibb Oncology Division (Princeton, N.J.).

Dose, dose range-finding and scheduling

The maximum tolerated dose (MTD) for each agent was determined using B6D2F1 mice (Harlan Sprague Dawley, Indianapolis, Ind.). The MTD was defined as the dose immediately below the dose associated with >15% weight loss or ≥20% mortality. Preliminary efficacy studies in nude mice were then conducted to determine the dose-response relationship using these agents with the MV-522 tumor model. Paclitaxel and tirapazamine were administered i.p. as a single bolus at doses of 20 mg/kg (67% MTD) and 70 mg/kg (100% MTD), respectively. Paraplatin was given as a single bolus at doses of 100 mg/kg (100% MTD) or 50 mg/kg (50% MTD). The doses of paclitaxel and paraplatin were chosen to provide a tumor growth inhibition (TGI) of approximately 25–50%. Administration of drugs or vehicle (0.9% NaCl) began on day 1. Tirapazamine and paraplatin were administered in 0.9% NaCl, while paclitaxel was delivered in Cremophor/ethanol/saline.

Tirapazamine was administered 3 h prior to paclitaxel and paraplatin. This schedule was based on an earlier study that compared the efficacy of administering tirapazamine before (2–3 h), concurrent with, or after a cytotoxic agent [7]. In that study, reported by Dorie and Brown, administration of tirapazamine before cisplatin was the most efficacious. No other schedules were evaluated in this current study with paclitaxel and paraplatin.

In vivo evaluation using MV-522 tumor model

The MV-522 human lung adenocarcinoma was derived as previously described [18]. Female nude mice (Harlan Sprague Dawley, Indianapolis, Ind.) were implanted s.c. by trocar with fragments of s.c.-grown tumors harvested from nude mouse hosts. When tumors were approximately 5 × 5 mm (usually about 10 days after implantation), the animals were pair-matched into treatment and control groups (day 1). Each group contained eight tumor-bearing mice that were ear-tagged and followed individually throughout the experiment.

Following tumor implantation, mice were weighed twice weekly and tumor measurements were made using calipers twice weekly, beginning on day 1. Tumor measurements were converted to tumor weight (mg) using an established formula:

$$\text{Weight (mg)} = \frac{\text{Width (mm)}^2 \times \text{Length (mm)}}{2}$$

Experiments were terminated when tumors in control animals reached a size of 1–2 g. At termination, all mice were weighed, sacrificed, and their tumors excised. Tumors were then weighed, and mean tumor weights per group were calculated. TGI was calculated for each group as:

$$100\% - \frac{\text{Mean treated tumor weight}}{\text{Mean control tumor weight}} \times 100\%$$

Animals experiencing partial or complete tumor regression were not included in the calculation of TGI.

Partial tumor shrinkage or response was calculated for individual animals if the final tumor weight for a given animal was less than its weight at the start of treatment. The percent shrinkage was derived from the difference divided by the initial tumor weight. A mean percent tumor shrinkage was calculated from the data if more than one mouse in a group experienced tumor regression. A complete response was defined as no palpable tumor at the end of the study, which was then confirmed by histologic examination.

The toxicities of the treatment regimens were determined by following changes in body weight and drug-related deaths.

Statistical analysis

Statistical analysis examined the two outcomes actual tumor weight at study conclusion and time to tumor doubling. Actual tumor weight was analyzed using analysis of variance, after transformation to log (tumor weight + 1) [natural logarithm] as indicated by a Box-Cox analysis [2]. One-way analysis with multiple range tests was used to determine which groups were different from each other. Survival, or time to tumor doubling, was computed using Kaplan-Meier methods and compared using log-rank tests. In the "multiple range" analyses of survival, pair-wise log-rank tests were computed and then the *P*-values were adjusted using the "stepdown" Bonferroni method [15]. In the statistical analysis of tumor weight and tumor doubling time, tirapazamine was found to be inactive. Therefore, synergy was defined as a statistically significantly better antitumor activity with the triple combination of agents (paclitaxel-paraplatin-tirapazamine) than with the double combination (paclitaxel-paraplatin).

Results

Single agent studies

The results of single agent studies are shown in Table 1 and Fig. 1. Following dose range-finding studies to

Table 1 Paclitaxel, paraplatin and tirapazamine against MV-522 Xenografts. Tirapazamine was administered 3 h prior to paclitaxel and paraplatin; agents were administered as a single i.p. bolus ($n = 8$)

Group	Dose (mg/kg)	Final tumor weight (mg) (Mean + SEM)	Tumor growth inhibition (%)	Mice with partial shrinkage	Mean tumor shrinkage (%)	Mice with complete shrinkage
Control	Saline	881.9 ± 93.1	0.0	0 of 8	–	0 of 8
Paraplatin	100	556.5 ± 129.8	39.7	0 of 8	–	0 of 8
Paraplatin	50	698.1 ± 160.5	22.5	0 of 8	–	0 of 8
Paclitaxel	20	552.9 ± 68.6	40.2	0 of 8	–	0 of 8
Tirapazamine	70	835.3 ± 111.2	5.7	0 of 8	–	0 of 8
Paclitaxel + tirapazamine	20 + 70	284.0 ± 51.8	73.0	0 of 8	–	0 of 8
Paclitaxel + paraplatin	20 + 100	241.4 ± 70.3	68.8	2 of 8	53.0	0 of 8
Paclitaxel + paraplatin	20 + 50	474.0 ± 93.5	49.8	0 of 8	–	0 of 8
Tirapazamine + paraplatin	70 + 100	600.5 ± 85.6	34.4	0 of 8	–	0 of 8
Tirapazamine + paraplatin	70 + 50	549.8 ± 95.8	40.3	0 of 8	–	0 of 8
Paraplatin + paclitaxel + tirapazamine	100 + 20 + 70	50.4 ± 30.9*	85.9	2 of 8	77.3	4 of 8
Paraplatin + paclitaxel + tirapazamine	50 + 20 + 70	58.4 ± 39.8*	61.2	4 of 8	54.9	3 of 8

* Significantly different from double combination groups ($P < 0.001$)

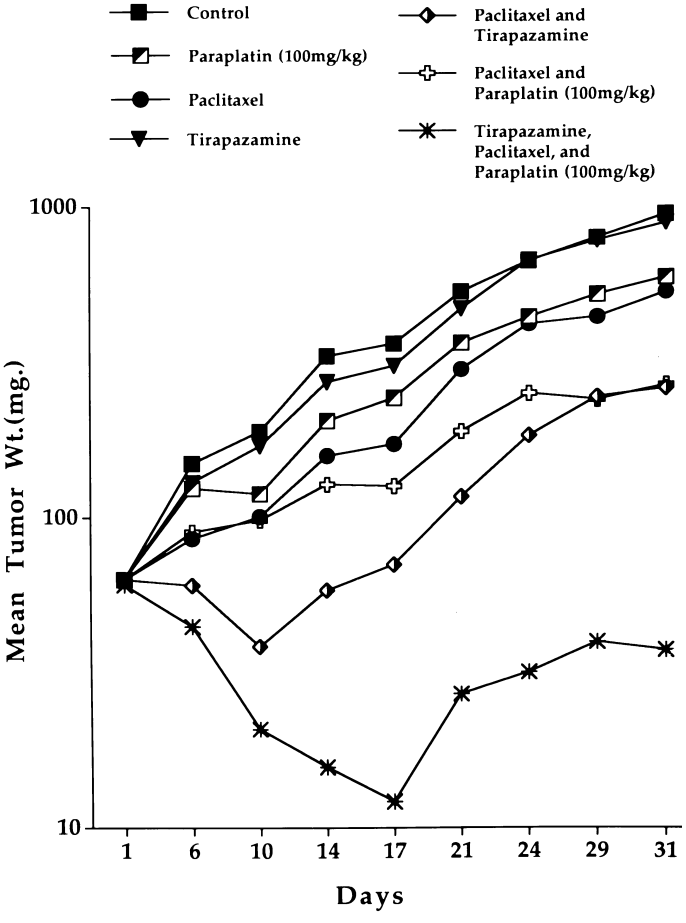

Fig. 1 MV-522 human lung adenocarcinoma fragments were implanted s.c. by trocar into nude mice. Treatment was initiated when the primary tumor had reached a size of approximately 5 × 5 mm (day 1). Paclitaxel and tirapazamine were administered i.p. as a single bolus at doses of 20 mg/kg and 70 mg/kg, respectively. Paraplatin was given as a single bolus at doses of 50 mg/kg or 100 mg/kg. Tirapazamine was administered 3 h prior to paclitaxel and paraplatin. Tumors were measured using calipers twice weekly, and volumes were calculated using the formula weight (mg) = [width $(mm)^2$ × length (mm)]/2. Mean tumor size for each treatment group is plotted ($n = 8$)

determine the MTD of tirapazamine, paclitaxel, and paraplatin, the efficacy of these single agents was evaluated using the MV-522 human lung adenocarcinoma xenograft model. Tirapazamine (70 mg/kg) as a single agent was not effective (TGI 5.7%) against this tumor model. Paclitaxel and paraplatin each caused a modest TGI of approximately 40%. There was no significant difference in final tumor weight compared with controls (881.9 ± 93.1 mg) for animals treated with either dose of paraplatin (556.5 ± 129.8 mg with 100 mg/kg and 698.1 ± 160.5 mg with 50 mg/kg) or paclitaxel (552.9 ± 68.6 mg). In addition, there were no partial or complete responses or tumor shrinkage observed in the single agent studies.

Double combination studies

The combination of paclitaxel and tirapazamine resulted in a TGI of 73% compared with 40.2% and 5.7%, respectively, for these agents administered alone. There was no significant difference in mean final tumor weight for animals treated with paclitaxel and tirapazamine compared with those treated with single agent paclitaxel alone (Table 1). Maximum tumor shrinkage was noted approximately 10 days after drug administration, subsequently followed by tumor regrowth (Fig. 1). There were no partial or complete responses in animals treated with the paclitaxel-tirapazamine combination.

The TGIs for animals treated with low-dose (50 mg/kg) and high-dose (100 mg/kg) paraplatin in combination with tirapazamine were 40.3% and 34.4%, respectively. There was no significant difference in mean final tumor weight between animals treated with either low- or high-dose paraplatin-tirapazamine combinations and those given single agent paraplatin at the corresponding doses (Table 1). There were no partial tumor shrinkage or complete responses in animals treated with these paraplatin combinations.

The mean final tumor weight with the paclitaxel low-dose paraplatin combination was 474.0 ± 93.5 mg and was 241.4 ± 70.3 mg with the paclitaxel high-dose paraplatin combination (Table 1). Two animals given paclitaxel and high-dose paraplatin had a mean partial tumor shrinkage of 53%, with the remaining six animals having a mean TGI of 68.8%. There were no partial or complete responses for animals treated with paclitaxel and low-dose paraplatin.

Triple combination studies

The results of the triple combination studies with paclitaxel, paraplatin (50 and 100 mg/kg), and tirapazamine are shown in Table 1 and Figs. 1 and 2. There were four animals with partial tumor shrinkage (mean 54.9%) and three with complete responses to the low-dose paraplatin triple combination. The remaining mouse had a TGI of 61.2%. The mean final tumor weight was significantly ($P \leq 0.001$) smaller in mice treated with paclitaxel, low-dose paraplatin and tirapazamine (58.4 ± 39.8 mg) than in those treated with the combination without tirapazamine (474.0 ± 93.5 mg). Time to tumor doubling in animals treated with paclitaxel, paraplatin (50 mg/kg) and tirapazamine was also significantly ($P \leq 0.015$) improved compared with the time in animals given paclitaxel and paraplatin alone (Fig. 2a).

Two mice treated with the high-dose paraplatin triple combination showed partial responses (mean tumor shrinkage 77.3%) and four showed complete disappearance of tumor. The remaining two animals had a mean TGI of 85.9%. Tumor shrinkage was most apparent approximately 17 days after treatment (Fig. 1). The mean final tumor weight was 50.4 ± 30.9 mg compared with 241.4 ± 70.3 mg in mice treated with paclitaxel and paraplatin (100 mg/kg) without tirapazamine ($P \leq 0.001$). Time to tumor doubling was significantly ($P \leq 0.015$) improved with the addition of tirapazamine to paclitaxel and high-dose paraplatin (Fig. 2b).

Toxicity

Single, double, and triple combinations studies were well tolerated, with minimal weight loss (maximum 4.2% as seen with tirapazamine and low-dose paraplatin) being observed in all treatment groups. There was one toxic death in the single agent paclitaxel group. There were no other obvious toxicities noted with tirapazamine alone or in combination.

Discussion

Tirapazamine represents an exciting new class of agents that may augment the efficacy of standard cytotoxic agents and radiation therapy, especially in tumors that have been classically semiresistant to classic therapeutic modalities [7, 8, 13, 14, 16, 20, 21, 28, 29, 34]. Several phase I studies have shown that single agent tirapazamine does not have a significant overlapping toxicity profile with most oncolytics [12, 27]. In addition, it appears that tirapazamine does not increase the toxicity of standard antitumor agents when used in combination regimens [17, 26]. Currently, the spectrum of clinical antitumor activity is being defined for tirapazamine in combination with cisplatin, cisplatin-paclitaxel, cisplatin-5-fluorouracil, cyclophosphamide or radiotherapy in several phase I and II clinical trials. Tumor types that have been targeted include glioblastoma multiforme, head and neck cancer, and melanoma. In addition, studies are ongoing to determine the efficacy of cisplatin-tirapazamine and cisplatin-paclitaxel-tirapazamine combinations for patients with non-small-cell lung cancer [5]. In this regard, it should be noted that in recent adult phase I trials, cisplatin-tirapazamine has shown excellent antitumor activity in patients with refractory tumors including patients with non-small-cell lung cancer that had been previously treated with cisplatin [17, 26].

The results of the studies now reported using the MV-522 human lung non-small-cell lung cancer xenograft, a tumor model typically considered relatively resistant to chemotherapy [18], support the further development of tirapazamine in combination with paraplatin and paclitaxel. These studies show that tirapazamine can substantially enhance the activity of the combination of paclitaxel and paraplatin without increasing toxicity. In addition, since both final tumor weight and time to tumor doubling were significantly improved with the addition of tirapazamine, which was inactive as a single agent, it is reasonable to conclude that synergy was present when the triple combination of agents was used. These results confirm previous results that tirapazamine produces a superadditive increase in the antitumor activity of paclitaxel in murine tumor models using a clonogenic assay [8]. It is of interest to note that while tirapazamine increased the activity of paclitaxel-paraplatin-based therapies, it did not appear to enhance the activity of single agent paraplatin. In this regard, previous studies have shown that tirapazamine can increase the antitumor activity of both cisplatin and paraplatin. This apparent discrepancy may reflect the larger dose of paraplatin used in prior in vivo studies and different tumor susceptibilities to paraplatin [8]. The limited impact of tirapazamine on paraplatin efficacy in the MV-522 lung tumor model, compared to other reported studies, may also reflect differences in degree and extent of tumor hypoxia.

The molecular basis by which tirapazamine can potentiate the antitumor activity of cytotoxic agents such as paclitaxel and paraplatin remains unclear. The increase in cytotoxicity of paclitaxel and paraplatin likely reflects the enhanced pharmacological activity of these agents resulting from changes in their pharmacokinetics

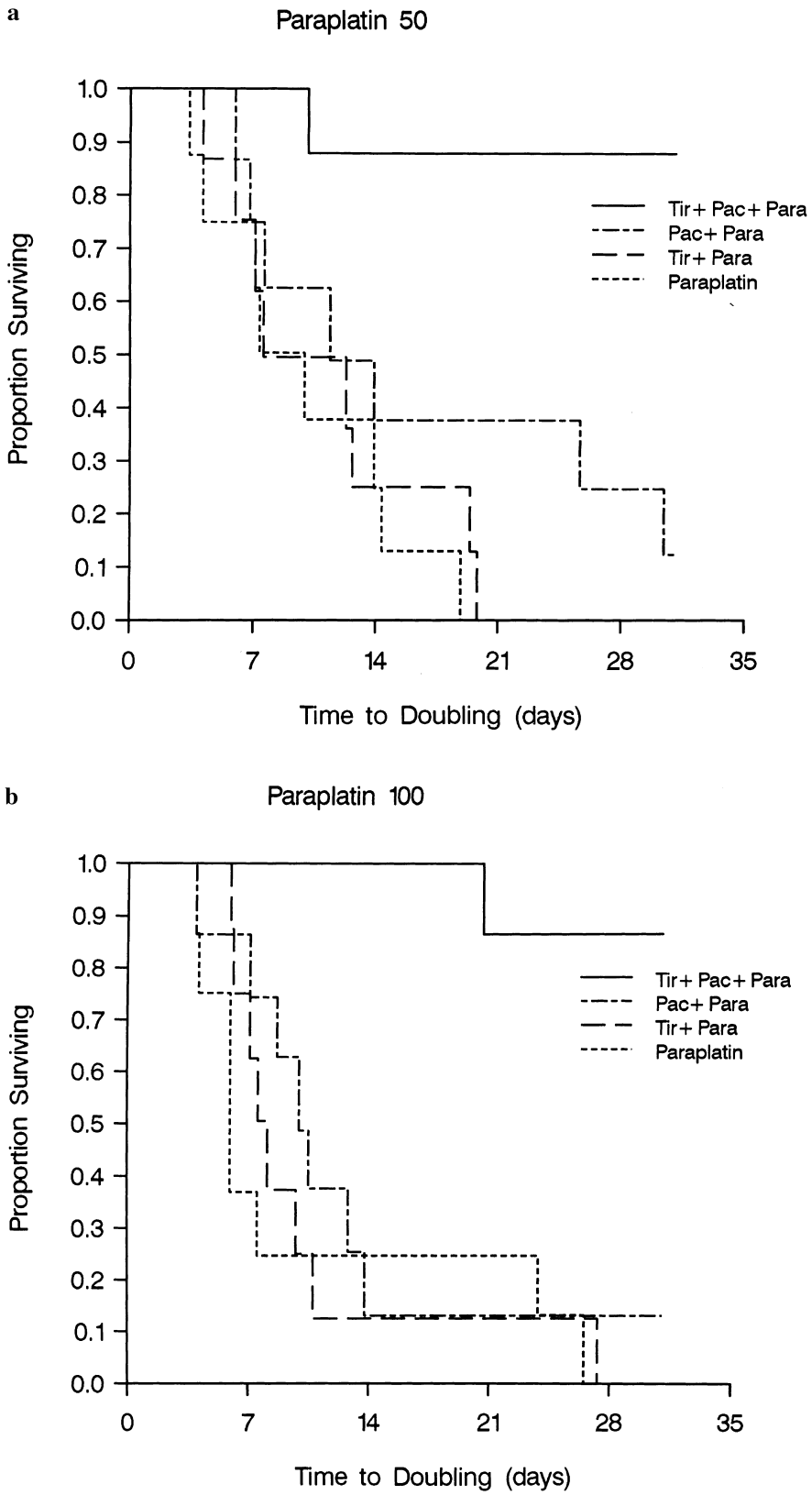

Fig. 2a,b Kaplan-Meier curves showing the time to tumor doubling (**a** studies using paraplatin at a dose of 50 mg/kg alone and in combination with tirapazamine, and paclitaxel; **b** studies using paraplatin at a dose of 100 mg/kg alone and in combination). Time to tumor doubling was significantly ($P \leq 0.015$) prolonged in groups treated with paraplatin (50 and 100 mg/kg), paclitaxel, and tirapazamine compared with those treated with paraplatin and paclitaxel without tirapazamine

or molecular target susceptibility. Recent clinical studies have shown that the clearance of cisplatin is not altered when coadministered with tirapazamine [17]; no similar studies have been reported for carboplatin. Whether tirapazamine affects the pharmacokinetics of paclitaxel is unknown. In this regard, the reduction of tirapazamine to its active moiety appears predominantly mediated by the P-450 cytochrome CYP2B which has not been

implicated in the metabolism of paclitaxel or paraplatin [25].

The schedule-dependent synergism previously reported with tirapazamine is suggestive of compromised DNA repair possibly as a result of DNA damage from this agent [7, 20]. Tirapazamine-induced damage of DNA may also cause the DNA to be more susceptible to induction of apoptosis or damage by other cytotoxic agents. Tumor cells exposed to tirapazamine have been shown to accumulate in G_2/M of the cell cycle [19]. This phase of the cell cycle has also been associated with induction of apoptosis by cisplatin and other agents [1].

The activity of the triple combination of tirapazamine-paraplatin-paclitaxel is particularly pertinent, since patients with non-small-cell lung cancer continue to have a poor outcome, with survival rates of less than 15% at 5 years for patients with stage III or IV disease [23]. While platinum-based therapies remain the mainstay of chemotherapy regimens for non-small-cell lung cancer, new agents (i.e. taxanes, topoisomerase I inhibitors, vinorelbine, and gemcitabine) and combinations are continuously being explored [3, 4, 9, 23, 30]. One of the most promising new regimens includes the paraplatin-paclitaxel combination. In the studies presented here, the paclitaxel-paraplatin combination resulted in better antitumor activity than when either agent was used alone. Response rates for this combination in patients with non-small-cell lung cancer have been reported to be as great as 50–60%, with a median survival of 10 months and 1-year survival values of 30–50% [3, 9]. Based on the results presented here with the MV-522 non-small-cell lung cancer model and the lack of overlapping toxicities seen with this chemosensitizing agent and standard cytotoxics, future clinical trials should consider the addition of tirapazamine to the combination of paclitaxel and paraplatin.

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