

METABOLIC DRUG INTERACTIONS BETWEEN ANGIOGENIC INHIBITOR, TNP-470 AND ANTICANCER AGENTS IN PRIMARY CULTURED HEPATOCYTES AND MICROSOMES

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ABSTRACT:

The potential metabolic drug interactions between TNP-470, a potent inhibitor of angiogenesis, and several commonly used anticancer agents, such as cyclophosphamide, taxol, and minocycline, were investigated in vitro using primary cultured hepatocytes and microsomes of rhesus monkeys. After incubation of hepatocytes with 5 μM [^3H]TNP-470, rapid and extensive formation of six metabolites was observed, with M-II and M-IV being the predominant metabolites. After 30 min of incubation in the presence of 250 μM cyclophosphamide, concentrations of unchanged TNP-470 and M-IV were increased with values of 1.00 ± 0.02 and 1.49 ± 0.01 μM compared with control values of 0.67 ± 0.09 ($p = .02$), 1.39 ± 0.03 μM ($p < .01$), respectively. In contrast, the concentration of M-II was substantially decreased from 1.69 ± 0.86 to 1.02 ± 0.16

μM ($p = .01$). Combination of taxol with TNP-470 led to a 50% decrease of M-II levels ($p < .01$), whereas unchanged TNP-470 and M-IV levels were increased by at least 2.5-fold compared with control ($p = .08$ and 0.01). Exposure of cells to TNP-470 with 250 μM minocycline had no effect on TNP-470 metabolism in monkey hepatocytes. In vitro studies with isolated monkey liver microsomes confirmed these drug-drug metabolic interactions detected at the cellular level. A detailed understanding of the potential drug interactions in TNP-470 metabolism occurring with taxol or cyclophosphamide is critical to fully elucidate the potentiation of the antitumor activity observed in vivo after coadministration of these two agents with TNP-470.

Angiogenesis, a process also called neovascularization, is a physiological component of reproductive functions, normal growth, and development, as well as wound healing (Brem et al., 1991). Angiogenesis is also observed in a variety of diseases such as diabetic retinopathy, arthritis, and inflammation (Ben Sira et al., 1988; Folkman, 1985, 1990; Oliver et al., 1995). In addition, angiogenesis has been demonstrated to play important roles in the progression of cancer (Folkman, 1972; Folkman and Klagsbrun, 1987) by allowing tumor growth and facilitating formation of metastases (Brem et al., 1993; Konno et al., 1995; Mori et al., 1995; Tanaka et al., 1995; Weidner et al., 1991) leading to the suggestion that inhibition of angiogenesis may represent a potent and selective therapeutic approach for metastatic cancers.

TNP-470 was demonstrated to possess potent in vitro and in vivo antitumor and antimetastatic activities leading to initiation of Phase I and II clinical trials in cancer patients (Levy et al., 1996; Zukiwski et al., 1994), as well as for treatment in HIV-associated Kaposi's sarcoma (Figg et al., 1997; Saville et al., 1993). Furthermore, the com-

bination of TNP-470 with other anticancer drugs was shown to be quite useful for the treatment of solid tumors. Combination of TNP-470 and minocycline resulted in an important tumor growth delay in animals bearing i.c. implanted 9L gliosarcoma (Teicher et al., 1995). Additionally, the combination of TNP-470 with cyclophosphamide resulted in an increase in the efficacy of tumor-cell killing by the alkylating drug in the murine Lewis-lung carcinoma (Teicher et al., 1994). In both cases, long-term survivors in treated animals were increased by approximately 40 to 50% as compared with treatment with cyclophosphamide alone (Teicher, 1994). Furthermore, S.J. Oliver et al. (1994) reported that coadministration of TNP-470 and taxol (paclitaxel) produced a significant reduction of arthritis severity (Oliver et al., 1994). It is well established that metabolism is a major determinant of the variability in drug response and toxicity. Because TNP-470 was demonstrated to be extensively metabolized both in vivo and in vitro (Placidi et al., 1995, 1997; Cretton-Scott et al., 1996) the purpose of the present study was to investigate whether taxol, cyclophosphamide, and minocycline may play a role in the metabolic fate of TNP-470 and the observed synergistic pharmacodynamic effects. Experiments were performed using monkey liver in vitro systems, including hepatocytes in primary culture and microsomal fractions isolated from monkey liver, two in vitro models that were shown to be predictive of the TNP-470 metabolism and biodisposition in humans (Placidi et al., 1997).

Materials and Methods

Chemicals. (3R,4S,5S,6R)-5-methoxy-4-[(2R,3R)-2-methyl-2-butenyl]oxiranyl]-1-[6(^3H)oxaspiro-[2.5]-oct-6-yl-(chloroacetyl)carbamate (^3H]TNP-470; 2.6 Ci/mmol or 6.5 mCi/mg) and authentic standards of unlabeled

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¹ Abbreviations used are: [^3H]TNP-470, (3R,4S,5S,6R)-5-methoxy-4-[(2R,3R)-2-methyl-2-butenyl]oxiranyl]-1-[6(^3H)oxaspiro-[2.5]-oct-6-yl-(chloroacetyl)carbamate; FCS, fetal calf serum.

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TNP-470 were provided by Takeda Chemical Industries, Ltd. (Osaka, Japan) and were >96% pure as ascertained by the HPLC method described below. The structure of this compound was confirmed by proton NMR, ^{13}C NMR, and liquid chromatography-mass spectrometry.

Paclitaxel (Taxol), cyclophosphamide, minocycline, and β -NADPH (reduced form) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent or analytical grade.

Isolation and Cryopreservation of Monkey Hepatocytes Monkey hepatocytes in primary culture were freshly isolated from rhesus monkeys. Monkey liver samples were obtained through the Liver Center at the University of Alabama at Birmingham and had a normal histology. Livers were washed with Eurocollins buffer at 4°C supplemented with heparin to remove blood from the vessels then with Leibovitz medium (L15) supplemented with antibiotics. The liver samples were then perfused with previously oxygenated calcium-free HEPES buffer, pH 7.4, followed by a 0.05% (w/v) collagenase solution containing calcium under recirculation and continuous oxygenation. After 15 to 20 min perfusion, which was necessary for the disruption of the Glisson's capsule, hepatocytes were suspended in L15 containing 5% fetal calf serum (FCS). The freshly isolated cells were then washed three times and centrifuged at 40g at 4°C for 10 min in L15 supplemented with 10% FCS to remove cellular debris, nonparenchymal and damaged cells. After the final wash, cells were immediately cryopreserved. Cell number and viability as determined by erythrosin B exclusion test was higher than 80%.

Cryopreservation and Thawing of Monkey Hepatocytes. Monkey hepatocytes were cryopreserved in L15 medium containing 25 g/liter BSA, 20

g/liter polyvinylpyrrolidone, 10% dimethyl sulfoxide, and 20% FCS as previously described by our group (Placidi et al., 1997).

Primary Hepatocyte Incubation Conditions. After thawing and seeding at a density of 0.4×10^6 cells/ml in 12-well plates previously coated with type 1 rat tail collagen, primary cultured hepatocytes in L15 medium were incubated with 5 μM [^3H]TNP-470 (specific activity 115 dpm/pmol) alone or in combination with either 50 μM taxol, 250 μM cyclophosphamide, or 250 μM minocycline for specified times between 15 min and 6 h at 37°C. The extracellular medium was then removed and 100- μl aliquots were analyzed without further processing by HPLC as described below.

Preparation and Incubation Conditions of Monkey Liver Microsomal Fractions. Microsomal fractions were prepared as previously described (Placidi et al., 1995). All assay mixtures contained 0.5 mg/ml of microsomal protein, 100 mM Tris HCl, 5 mM NADPH, 10 or 50 μM [^3H]TNP-470, and various concentrations of either taxol or cyclophosphamide in a final volume of 250 μl . Reactions were initiated by adding microsomal protein and samples were incubated at 37°C for 30 min. Reactions were terminated by addition of an equal volume of acetonitrile. Proteins were then removed by centrifugation at 14,000g for 5 min in a 5314 Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY) and aliquots (100 μl) were then analyzed by HPLC.

Analysis of [^3H]TNP-470 and Its Metabolites by HPLC. Samples were analyzed by HPLC using a Hewlett-Packard model 1090 liquid chromatograph equipped with an automatic injector, a fixed wavelength spectrophotometer and a Hewlett-Packard 85B chromatographic terminal (Hewlett-Packard Co.,

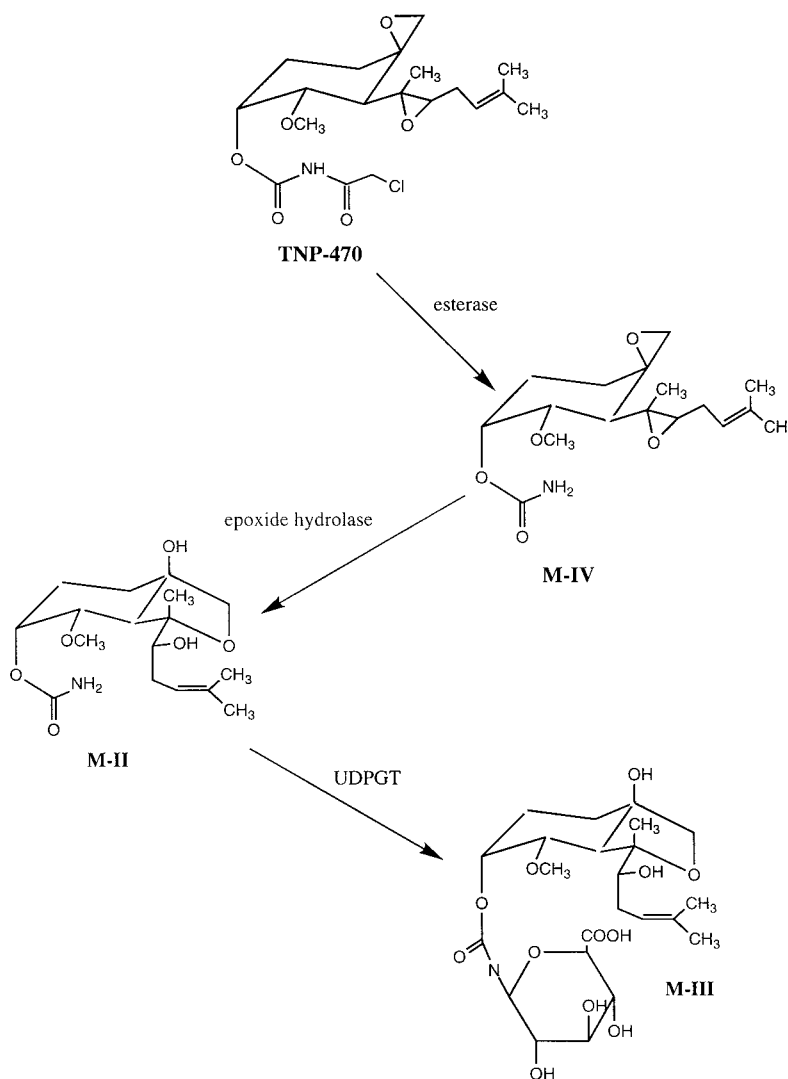


FIG. 1. Proposed metabolic pathway of TNP-470.

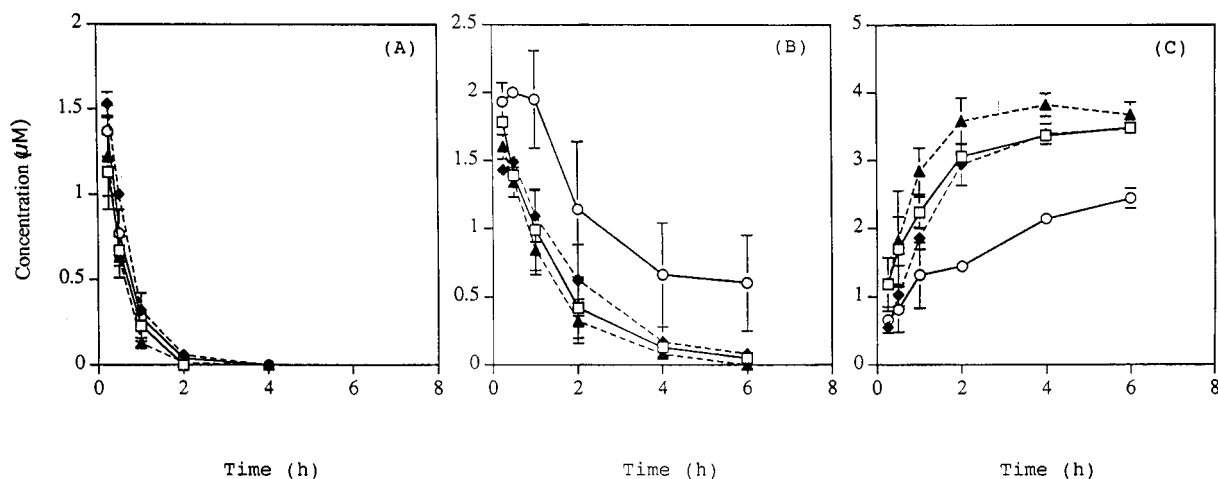


Fig. 2. Effects of coadministration of 25 μM taxol (\circ), 250 μM cyclophosphamide (\blacklozenge), and 250 μM minocycline (\blacktriangle) as compared with control (\square) on levels of unchanged TNP-470 (A) and its two major metabolites **M-IV** (B) and **M-II** (C) after exposure of primary cultured monkey hepatocytes to 5 μM [^3H]TNP-470.

Results represent mean of three experiments with duplicate incubations.

Palo Alto, CA). Reversed-phase chromatography was performed with a Hypersil ODS 5- μm column (Jones chromatography, Littleton, CO) and elution was performed at 1 ml/min with a 50 mM phosphate buffer (pH 3) and a 40-min linear gradient of acetonitrile from 0 to 25% starting at the time of injection followed by an increase to 50% of acetonitrile between 40 and 50 min. Column temperature was maintained at 25°C and absorbance was monitored at 210 nm. Eluent from the column was directed by way of a low dead volume connection line into a 500 TR Radiomatic FLO-ONE radiochromatography analyzer (Packard Instrument Company, Meriden, CT). Under the above conditions, retention times of TNP-470 and its metabolites **M-IV**, **M-II**, and **M-III** were 58, 52, 44, and 38 min, respectively. Three unidentified chromatographic peaks labeled **M-I**, **M-V**, and **M-VI** eluted at 48, 28, and 26 min respectively. Intra- and interday coefficients of variation were less than 5% for all analysis. The standard curve for TNP-470 was linear with $r > 0.99$. However the unavailability of radiolabeled metabolites did not permit the establishment of standard curves for these derivatives.

Results and Discussion

Pharmacokinetic interactions and particularly drug–drug interactions are very difficult to predict, because they can involve several distinct processes, including absorption, distribution, protein binding, metabolism, and excretion. However, modifications of hepatic metabolism have been demonstrated to be the major source of drug interactions (Gibaldi, 1992). Thus being able to predict potential metabolic drug interactions is of particular importance in the early stages of drug development to improve efficacy and/or toxicity of therapies. The purpose of this study was to investigate the potential metabolic drug interactions occurring between the angiogenesis inhibitor TNP-470 and some routinely coadministered anticancer drugs such as taxol, cyclophosphamide, and minocycline, and whether metabolic drug interactions occurred and were possibly responsible, in part, for the increased therapeutic effects observed in combination treatment (Teicher et al., 1994, 1995; Oliver et al., 1995).

Hepatocytes isolated from different species and particularly from monkey have been increasingly used over the past few years for pharmacological studies (Nicolas et al., 1995) and our group has previously demonstrated their value in predicting TNP-470 metabolism and biodisposition (Placidi et al., 1997). Figure 1 illustrates the metabolic pathway of TNP-470 that have thus far been identified in both human and monkey hepatocytes and microsomal fractions. TNP-470 is primarily metabolized to **M-IV** through an ester cleavage, with subsequent conversion of **M-IV** to **M-II** by epoxide hydrolase. **M-II**

is then glucuronidated by uridine-5'-diphospho glucuronyl transferase, leading to the formation of **M-III**. Three other, as yet unidentified, metabolites labeled **M-I**, **M-V**, and **M-VI** were also detected at lower concentrations.

Drug Interactions in Primary Cultured Monkey Hepatocytes. Incubations were performed at a final concentration of TNP-470 of 5 μM based on results obtained in our *in vivo* study using rhesus monkeys, in which the primary metabolite **M-IV** was shown to exhibit a C_{max} value of $3.54 \pm 3.19 \mu\text{M}$ (Cretton-Scott et al., 1996). Figure 2 shows that coadministration of 25 μM taxol and 250 μM cyclophosphamide significantly reduced **M-II** formation within cells, with taxol presenting the most important inhibitory effect. After only 15 min of incubation, **M-II** levels decreased from $1.18 \pm 0.39 \mu\text{M}$ to 0.55 ± 0.06 and $0.66 \pm 0.19 \mu\text{M}$ with coadministration of cyclophosphamide and taxol, respectively. This effect was attenuated over time with cyclophosphamide, whereas it was more important in the presence of taxol, with **M-II** levels declining from 3.06 ± 0.07 to $1.44 \pm 0.03 \mu\text{M}$ at 2 h. In contrast, levels of unchanged parent drug and **M-IV** were increased from undetectable to 0.06 ± 0.04 and $0.02 \pm 0.02 \mu\text{M}$ for TNP-470 and $0.42 \pm 0.22 \mu\text{M}$ to 0.62 ± 0.26 and $1.14 \pm 0.50 \mu\text{M}$ for **M-IV** at 2 h with cyclophosphamide and taxol, respectively. In contrast, minocycline had no effect on the levels of unchanged TNP-470 or its metabolites.

Inhibition of M-II Formation in Monkey Liver Microsomes. When 1 μM TNP-470 was incubated for 15 min with 500 $\mu\text{g/ml}$ monkey liver microsomal proteins in the presence of taxol or cyclophosphamide, a linear increase of the levels of **M-IV** associated with a reduction of the rate of formation of **M-II** was observed (Table 1). Coadministration of 5 μM taxol resulted in a significant increase of **M-IV** levels from 2.36 ± 0.11 to $4.34 \pm 0.20 \mu\text{M}$ associated with a reduction of **M-II** concentrations from 7.39 ± 0.11 to $5.01 \pm 0.14 \mu\text{M}$. Similarly, addition of 25 μM cyclophosphamide to TNP-470 decreased **M-II** formation to $6.48 \pm 0.02 \mu\text{M}$ and increased **M-IV** levels to $3.41 \pm 0.02 \mu\text{M}$. Furthermore, similar results were obtained when TNP-470 was incubated at a final concentration of 50 μM for 30 min with 500 $\mu\text{g/ml}$ monkey liver microsomal proteins and taxol or cyclophosphamide. In the presence of 500 μM taxol or cyclophosphamide, **M-IV** levels represented 176.3 ± 2.3 and $171.5 \pm 0.9\%$ of the control value, respectively, whereas **M-II** levels accounted only for 90.0 ± 0.4 and $90.2 \pm 0.4\%$ of control value, respectively. These results strongly suggest that the drug–drug interaction occurring be-

TABLE 1

Effects of increasing concentrations of taxol and cyclophosphamide on concentrations of metabolites **M-IV** and **M-II** in monkey liver microsomal fractions

Each value of **M-IV** and **M-II** represents mean \pm S.E. of three independent experiments with duplicate incubation.

Drug and Concentration	Concentration		
	M-IV	M-II	
Control	2.36 \pm 0.11	7.39 \pm 0.08	
Taxol			
1 μ M	2.71 \pm 0.13	6.93 \pm 0.11	$p = 0.07$
5 μ M	4.34 \pm 0.20	5.01 \pm 0.14	$p < 0.001$
Cyclophosphamide			
10 μ M	2.80 \pm 0.05	6.80 \pm 0.01	$p < 0.05$
25 μ M	3.41 \pm 0.02	6.48 \pm 0.02	$p < 0.01$
50 μ M	4.11 \pm 0.37	5.45 \pm 0.21	$p < 0.01$

p value represents statistical significance of differences in **M-IV** and **M-II** concentrations in absence (control) or presence of taxol or cyclophosphamide.

tween TNP-470 and taxol or cyclophosphamide is not dependent on the particular concentration of TNP-470 nor of that of the inhibitors, and that coadministration of taxol and cyclophosphamide can modify the ability of epoxide hydrolase to catalyze the formation of **M-II** from **M-IV** as previously reported by our laboratory using primary cultured hepatocytes and microsomal fractions isolated from different organs (Placidi et al., 1995). The present data are of particular importance because **M-IV** also exhibits potent antiangiogenic properties, whereas the metabolite **M-II** is eventually pharmacodynamically inactive (Takeda Chemical Industries, Ltd., unpublished data). Enhancement of the concentration of pharmacologically active drug by cyclophosphamide and taxol may in part explain the synergistic anticancer activity observed in vivo in several animal models.

In conclusion, this study provides the first detailed analysis of the metabolic drug interactions between the angiogenesis inhibitor TNP-470 and two anticancer drugs commonly used in combination therapy and demonstrates the need to better define the pharmacokinetic basis for combination therapy between angioinhibins and other antineoplastic drugs.

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