The recent developments in understanding the biochemical mechanisms in the multistep processes of carcinogenesis have disclosed numerous molecular targets for anticancer drug therapy. Angiogenesis, a process involving the new blood vessel formation needed for tumor growth and metastasis, has become a very important target (Folkman, 1995; Zetter, 1998). Vascular endothelial growth factor (VEGF) and VEGF receptors Flk-1 and flt-1 have been implicated in tumor growth and metastasis, has become a very important target (Folkman, 1995; Zetter, 1998). Vascular endothelial growth factor (VEGF) and VEGF receptors Flk-1 and flt-1 have been implicated in angiogenesis that occurs in many solid tumors (Myoken et al., 1991). Recent studies have linked high VEGF levels with unfavorable prognosis of cancer patients [for review see Cherrington et al. (2000)]. Flk-1/KDR (fetal liver kinase 1/kinase insert domain-containing receptor), also known as VEGF receptor 2 (VEGFR2) is in advanced clinical trials for treatment of AIDS-related Kaposi’s sarcoma and colorectal and nonsmall cell lung cancers. Since this chemical class has not been studied previously with therapeutic intent, the present study was designed to investigate the in vitro metabolism of SU5416 by mouse, rat, dog, monkey, and human liver microsomes and to identify the major metabolites of SU5416. An HPLC procedure was developed and validated to resolve and quantify SU5416 and its metabolites. To evaluate the in vitro metabolism of SU5416, pooled liver microsomes from mice, rats, dogs, monkeys, and humans were incubated with SU5416 (25 μM) in the presence of an NADPH-generating system. In the presence of NADPH, mouse, rat, dog, monkey, and human liver microsomes converted SU5416 to at least 12, 9, 7, and 6 polar metabolites, respectively. Microsomal metabolism of SU5416 showed marked species differences in the levels of different metabolites formed. The overall rate of SU5416 metabolism by liver microsomes from the species examined followed the rank order: monkey > mouse ~ rat > dog > human. Two major metabolites of SU5416 were identified, a hydroxymethyl derivative of SU5416 (M12) and a carboxylic acid derivative of SU5416 (M6), by spectroscopic methods and comparison with authentic compounds. Both of these oxidative metabolites were further metabolized in vivo through gluconoridation. The metabolic fate of SU5416 in microsomes from various species as well as data from in vivo biotransformation in the rat are discussed.

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2 Abbreviations used are: VEGF, vascular endothelial growth factor; KDR, kinase insert domain-containing receptor; Flk-1, fetal liver kinase 1; flt-1, fms-like tyrosine kinase; SU5416, 3-(3,5-dimethyl-1H-pyrrol-2-ylmethylene)-1,3-dihydro-indol-2-one; PTK, protein-tyrosine kinase; AUC, area under the curve; ESI, electrospray ionization; RSD, relative standard deviation; LC/MS/MS, liquid chromatography-tandem mass spectrometry; API, atmospheric pressure ionization; ACN, acetonitrile; DMSO, dimethyl sulfoxide; β-o-glucuronidase, β-β-glucuronide glucuronosohydrolase; ppm, parts per million.

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of tumor cells, including those of epidermoid, melanoma, glioma, lung, and prostate origin, were all above 20 μM (Fong et al., 1999). SU5416, therefore, acts through a highly specific mechanism of action and is not cytotoxic to endothelial cells. Consistent with its in vitro effects on endothelial cells, SU5416 also displays antiangiogenic properties in vivo (Vajkoczy et al., 1999) as well as broad antitumor properties in animal models (Fong et al., 1999; Shaheen et al., 1999).

SU5416 was the first synthetic small molecule with the Flk-1/KDR signaling inhibition mechanism to enter clinical trials for anticancer therapy. A phase I study of SU5416 has been completed, and the results show that SU5416 is well tolerated for chronic administration with indications of biological activity (Cropp et al., 1999; Rosen et al., 1999). Lesion shrinkage and disease stabilization have been observed in patients with terminal cancers such as Kaposi’s sarcoma, colorectal, and nonsmall cell lung cancers (Rosen et al., 1999).

In support of preclinical and clinical studies, the biotransformation of SU5416 was studied in mouse, rat, dog, monkey, and human liver microsomes. These studies showed marked qualitative and quantitative differences in the in vitro metabolism of SU5416 by liver microsomes from these species. The liver microsomes converted SU5416 to hydroxy metabolite mainly through hydroxylation of 5-methyl group on the pyrrole ring, which was transformed further to a corresponding carboxylic acid. Both of these oxidative products are further metabolized in vivo through glucuronidation. Here we compare and contrast the metabolic fate of SU5416 in microsomes from various species as well as data from in vivo biotransformation of SU5416 in the rat.

**Materials and Methods**

**Test System.** Pooled liver microsomes from male CD-1 mice (n > 100), male Sprague-Dawley rats (n = 52), male Beagle dogs (n = 6), male Cyno-
A. **Mouse liver microsomes + SU5416 (25 μM) + NADPH, incubated for 0 min.**

B. **Mouse liver microsomes + SU5416 (25 μM) + NADPH, incubated for 15 min.**

**Fig. 1.** Representative HPLC chromatogram of SU5416 incubated with mouse liver microsomes.

A pool (n = 100) of mouse liver microsomes (1 mg/ml) was incubated with SU5416 (25 μM) and 1 mM NADPH-generating system for 0 or 15 min at 37°C. The reaction was stopped with an equal volume of ice-cold methanol, and the precipitated protein was removed by centrifugation. An aliquot (200 μl) of supernatant fraction was analyzed by reverse-phase HPLC.

**molgus monkeys (n = 6),** and human liver microsomes (n = 15) were used for this study. These microsomes were prepared according to the method described by Lu and Levin (1972). In addition, a bank of human liver microsomes from individual livers was used. Pooled mouse, rat, dog, and monkey liver microsomes have been characterized with respect to P450, b, and NADPH-P450 reductase activities. A pool (n = 15) of human liver microsomes has been extensively characterized with regard to the activities of the major P450 enzymes (Pearce et al., 1996).

**HPLC Setup and Validation.** The HPLC procedures were carried out using an LC-10A binary gradient HPLC system equipped with an SIL-10A autosampler and an SPD-10A variable wavelength UV-visible detector (Shimadzu Scientific Instruments, Columbia, MD). A Hypersil ODS column (200 × 4.6 mm, 5-μm particle size; Hewlett-Packard, Germany) was maintained at 40 ± 1°C with a CH-30 column heater (Eppendorf Inc., Madison, WI). Mobile phase A consisted of 60% (v/v) 50 mM KH₂PO₄ containing 0.1% triethylamine and 40% (v/v) methanol, and mobile phase B consisted of 28% (v/v) 50 mM KH₂PO₄ containing 0.1% triethylamine and 72% (v/v) methanol. The following gradient program with a flow rate of 1.5 ml/min was used to separate SU5416 from its metabolites: 0 to 2 min, mobile phase A, 75%; 2 to 5 min, mobile phase A, 25%; 5 to 22 min, mobile phase A, 0%; 22 to 25 min, mobile phase A, 75%. SU5416 and its metabolites were detected using a UV-visible detector (λ = 440 nm), and SU5416 was quantified by comparing the detector response [peak area under the curve (AUC)] using a standard solution. The HPLC peaks were integrated with CLASS-VP (Version 4.1) computer software from Shimadzu Scientific Instruments.

A reverse-phase HPLC method was developed to separate SU5416 from its in vitro metabolites. The HPLC method was validated with respect to linearity, intraday precision, interday precision, accuracy, sensitivity, and selectivity for SU5416. Briefly, the detector response was found to be linear over a range of 20 to 4000 pmol of SU5416. The analytical procedure was shown to be accurately precise on intra- and interday analyses. The precision (RSD) of 0.3% at 1000 pmol of injected SU5416 (n = 10) was determined. An interday RSD of 0.78% at 1000 pmol of injected SU5416 (n = 5) was measured. Accuracy was determined by spiking mock incubation mixtures (n = 5) with 5 μM SU5416 (500 pmol injected), which was incubated with human liver microsomes (0.25 mg of protein/ml), MgCl₂ (3 mM), potassium...
phosphate buffer (50 mM, pH 7.4), EDTA (1 mM), and an equal volume of ice-cold methanol. The chromatographic data yielded repeatable peak AUCs for SU5416 (RSD of 3.7%). At a minimum, the analytical procedure was able to quantify 25 pmol of SU5416 applied on the column. Recovery was assumed to be 100%, because the analytical procedure did not involve an extraction step. The results of these experiments also established that the analytical procedure adequately resolved SU5416 from its metabolites formed by human liver microsomes.

**In Vitro Metabolism of SU5416 by Mouse, Rat, Dog, Monkey, and Human Liver Microsomes.** Liver microsomes (1 mg/ml) from mice, rats, dogs, monkeys, and humans were incubated at 37°C in 0.5-ml incubation mixtures (final volume) containing potassium phosphate buffer (50 mM, pH 7.4), MgCl₂ (3 mM), EDTA (1 mM), and SU5416 (25 μM, final concentration) with and without an NADPH-generating system, at the final concentrations indicated. The NADPH-generating system contained NADP (1 mM), glucose 6-phosphate (5 mM), and glucose-6-phosphate dehydrogenase (1 U/ml). Reactions were started by the addition of the NADPH-generating system and stopped after 5, 15, or 60 min by the addition of 0.5 ml of ice-cold methanol. Zero time, zero protein, zero NADPH, and zero substrate incubations served as blanks. Precipitated protein was removed by centrifugation (1000–1500 g for 10–15 min at 4°C). The clear supernatant fractions were transferred to amber-colored HPLC vials. An aliquot (200 μl) of the clear supernatant fraction was analyzed on reverse-phase HPLC. All incubations were carried out under dim or yellow light.

**Identification of SU5416 Metabolites M12, M6, and M9.** M12 was generated from in vitro incubation of monkey liver microsomes with SU5416 and NADPH-generating system for 15 min at 37°C. The reaction mixture was extracted with dichloromethane, and the organic layer was evaporated to dryness under a stream of N₂. Zero time, zero protein, zero NADPH, and zero substrate incubations served as blanks. Precipitated protein was removed by centrifugation (1000–1500 g for 10–15 min at 4°C). The clear supernatant fractions were transferred to amber-colored HPLC vials. An aliquot (200 μl) of the clear supernatant fraction was analyzed on reverse-phase HPLC. All incubations were carried out under dim or yellow light.

**Identification of SU5416 Glucuronides in Rat Plasma.** Plasma samples were collected at 3 h postdose from Sprague-Dawley rats that had been dosed with i.v. SU5416 at 16 mg/kg i.v. The organic layer was dried under a stream of N₂ and the dried material was reconstituted with a solution containing 45% acetonitrile (ACN), 22% dimethyl sulfoxide (DMSO), and 33% 10 mM NH₄COOH (v/v). The solution was analyzed with LC/MS/MS on a PE Sciex API 365 triple quadrupole mass spectrometer directly coupled to an HPLC system via a PE Sciex API source operated in the positive ESI mode (turbo ion spray). The ion spray interface was operated at 6000 V, and the mass spectrometer was operated in the positive ion mode. The HPLC system consisted of two Shimadzu LC-10 AD pumps and a PE autosampler. The chromatography was performed on a YMC (Wilmington, NC) ODS-AM, 3.0-μm 3 mm column.

**Identification of SU5416 Glucuronides in Rat Plasma.** Plasma samples were collected at 3 h postdose from Sprague-Dawley rats that had been dosed with i.v. SU5416. The plasma samples (200 μl) were processed by protein precipitation with 1.5 ml of ACN. After centrifugation, the supernatant was evaporated under a stream of N₂ until dry. The samples were reconstituted with 50 μl of a solution that contains 45% ACN, 22% DMSO, and 33% 10 mM NH₄COOH (v/v). The solution was analyzed with LC/MS/MS on a PE Sciex API 365 triple quadrupole mass spectrometer directly coupled to an HPLC system via a PE Sciex API source operated in the positive ESI mode (turbo ion spray). A portion of the above plasma sample (200 μl) was treated with β-o-
glucuronidase (β-D-glucuronidase glucuronosohydrolase; EC 3.2.1.31. Sigma G 8162) to further confirm the identity of SU5416 glucuronides. The plasma sample (200 μl) was diluted with 0.4 ml of 10 mM PBS, pH 6.8, plus 5 mM MgCl₂. After adding about 300 U of β-D-glucuronidase in 5 μl 50% glycerol, the mixture was incubated at 37°C for 2 h. The sample was then processed by protein precipitation with 1.5 ml of ACN. After centrifugation, the supernatant was evaporated under a stream of N₂ until dry, followed by reconstitution with 50 μl of a solution containing 45% ACN, 22% DMSO, and 33% 10 mM NH₄COOH (v/v). The solution was analyzed for SU5416 glucuronides with LC/MS/MS as described above.

Effect of Substrate Concentration on SU5416 Metabolism. Incubations were carried out to ascertain the effects of substrate concentration on the formation of SU5416 metabolites, from which Michaelis-Menten kinetic constants (Kₘ and Vₘₐₓ) were determined. Various concentrations (1, 2, 4, 6, 8, 10, 12.5, 16, 20, 40, 70, and 100 μM) of SU5416 were incubated with a pool of human liver microsomes (0.5 mg/ml) at 37 ± 1°C for 30 min as described previously. The experimental conditions were selected such that the metabolism of SU5416 did not exceed 20%. The incubations were carried out in duplicate. Precipitated protein was removed by centrifugation (1000–1500 g for 10–15 min at 5–15°C). The clear supernatant fractions were transferred to amber-colored HPLC vials. An aliquot (200 μl) of the clear supernatant fraction was analyzed on reverse-phase HPLC.

Results and Discussion

In Vitro Metabolism of SU5416 by Mouse, Rat, Dog, Monkey, and Human Liver Microsomes. SU5416 was incubated with mouse, rat, dog, monkey, and human liver microsomes in the presence of an NADPH-generating system. After a 15-min incubation, the metabolites of SU5416 were analyzed on reverse-phase HPLC. The chromatography peaks were named in an increasing order of their relative retention times, with M1 being most polar and M15 the least. These
liver microsomes formed metabolites in the following rank order:

formed metabolites in the following rank order: M12

presence of NADPH, mouse, rat, and dog liver microsomes converted

matically]. Additionally, two other metabolites, namely, M3 and M15,

HPLC, which suggests that none of the metabolites formed nonenzy-

these samples no metabolite peaks were detected by reverse-phase

substrate (SU5416) and zero time incubations served as blanks. In

matched those formed by human liver microsomes (M12, M6, M7,

SU5416. This assumption was supported by comparing the UV re-

su5416. The kinetic parameters were deter-

microsomes (0.5 mg/ml) and 1 mM NADPH at 37°C for 30 min. Samples were

SU5416 to seven metabolites (M12 >

M6 > M4 ≥ M7 > M2 ≥ M1), but only four metabolites matched those formed by human liver microsomes (M12, M6, M7, and M8). [Incubations devoid of microsomal protein, NADPH, or

zero time incubations served as blanks. In these samples no metabolite peaks were detected by reverse-phase

HPLC, which suggests that none of the metabolites formed nonenzy-

matically]. Additionally, two other metabolites, namely, M3 and M15,

were formed at trace levels and were detected only under conditions of high protein concentration and long incubation time.

Among different metabolites formed by these animal liver microsomes in vitro, M12 was the major metabolite of SU5416 in these

species. There was no discernable delay in M12 formation from

SU5416 by liver microsomes from these species, which suggests that

M12 is a primary metabolite of SU5416. The rates of M12 formation

from SU5416 by mouse, rat, dog, monkey, and human liver microsomes were 273, 402, 123, 695, and 35 pmol/min/mg, respectively. In

addition to the formation of metabolite M12, mouse, rat, dog, monkey,

and human liver microsomes formed M6 as another major metabolite of

SU5416. The rates of M6 formation in mouse, rat, dog, monkey,

and human liver microsomes were 52, 30, 27, 98, and 16 pmol/min/

mg, respectively.

**Structural Identification of the Major Metabolites M6, M12, and M9.** Since M12 was the major metabolite formed by animal liver microsomes, it was isolated to homogeneity from in vitro incubations of monkey liver microsomes with SU5416 and the NADPH-generat-

ing system. LC/MS of M12 gave a strong deprotonated molecular ion

(M + H) + at m/z 255 in the positive ion mode. The molecular ion is 16 mass units higher than that of parent drug SU5416. Subsequent

LC/MS/MS analysis of M12 at m/z 255 gave product ions at m/z 237

and 225, which are consistent with loss of a water molecule and the

hydroxymethyl group, respectively.

The 1H NMR spectrum of M12 displayed signals assigned to four

adjacent aromatic protons on a disubstituted benzene ring (6.70,

6.96, 7.09, and 6.85 ppm), a vinyl proton (6.75 ppm), an aromatic

proton due to the pyrrole ring (6.60 ppm), and two broad proton peaks resulting from NH (6.17 and 13.4 ppm). Compared with the 1H NMR spectrum of the parent compound SU5416, one of two methyl

signals at 62.3 was absent. However, a singlet of two protons at 64.51

indicated an oxygenated methylene group, and a broad peak of one proton at 65.38 indicated a hydroxyl group. The above data of M12 showed that one methyl group on the pyrrole ring was oxidized to a hydroxymethyl. Therefore, M12 was compared with the synthetic standard SU9838, a hydroxymethyl analog of SU5416. M12 coeluted with SU9838 on reverse-phase HPLC. In addition, the 1H NMR spectrum of M12 showed the same chemical shifts of proton signals as that of SU9838, and the LC/MS and LC/MS/MS analyses of M12 displayed the same molecular ion and fragmentation pattern as those of SU9838.

M6 was also a major metabolite of SU5416 in the microsomal incubation. LC/MS analysis of M6 indicated a molecular weight of 268 Da, which represents a shift of 30 mass units compared with parent SU5416 (molecular mass = 238 Da). This increase in molecular weight is in accordance with hydroxylation of the parent compound, followed by further oxidation to yield a carboxylic acid. This proposed structure is supported by the MS/MS fragmentation pattern in which the product ion at m/z 251 occurred due to the loss of a neutral molecule of water. It is believed that loss of 18 mass units gives rise to an acylium ion. The subsequent loss of CO produced the

ion at m/z 223, in which the positive charge was delocalized on the

pyrrole ring. The facile loss of 44 mass units (CO2) is typically observed for carboxylic acids and gave the product ion at m/z 225. The structure of M6 was identified as the carboxylic acid derivative of SU5416. Two metabolite standards SU6595 and SU10742 were synthesized to compare with M6. SU6595 and M6 exhibited identical retention times in reverse-phase HPLC/UV, whereas SU10742 eluted much earlier than M6. In addition, SU6595 has also shown the same fragmentation pattern as seen for M6 in MS/MS analysis. M6 was identified as a carboxylic acid metabolite of SU5416. This metabolite was derived from enzymatic oxidation of M12.
M9 was formed in mouse, rat, dog, and human microsomes when SU5416 was incubated with NADPH. LC/MS analysis of M9 gave (M+H)⁺ at m/z 255, which is 16 mass units higher than the parent compound SU5416. The molecular ion of M9 is indicative of a hydroxyl metabolite. The LC/MS/MS product spectrum of m/z 254 showed two major peaks at m/z 240 and 238 for the loss of a methyl and a hydroxyl group, respectively. No peak corresponding to the loss of water was observed, indicating the hydroxyl group is located on the aromatic benzene ring. M9 showed the same LC retention time and similar mass fragmentation pattern as the synthetic standard, SU6689.

Two SU5416 glucuronides were detected in rat plasma. Reverse-phase chromatography showed a metabolite that was more polar than M6, the carboxylic acid metabolite of SU5416. This metabolite gave an LC/MS/MS product spectrum signal at m/z 445.0, which was 176 mass units greater than M6. The mass fragments at m/z 427 and 409 correspond to the loss of one and two water molecules, respectively, from the molecule at m/z 445.0. The fragments at m/z 269, 251, and 225 correspond to the loss of the glucuronic acid group, a further loss of a hydroxyl group, and the loss the carboxylic acid group, respectively, from the molecule at m/z 445.0. These results are consistent with an acyl glucuronide of SU5416. Another polar compound showed an MS signal at m/z 431.2, which is 176 mass units higher than M12, the hydroxymethyl metabolite of SU5416. LC/MS/MS analysis showed mass fragments at m/z 431.2 and 255.1, which correspond to the loss of one water molecule and the loss of the glucuronic acid group, respectively, from the molecule at m/z 431.2.

The mass fragment at m/z 237.1 corresponds to the further loss of the hydroxyl group from m/z 255.1. These results are consistent with a hydroxyl glucuronide of SU5416. Further confirmation of the SU5416 glucuronides were obtained by β-D-glucuronidase treatment of the plasma samples. After treating the plasma samples with β-D-glucuronidase, the two polar compounds disappeared from the plasma extracts in reverse-phase HPLC and LC/MS/MS analysis, with concurrent increase of signals associated with M6 and M12.

Kinetics of Formation of M12 from SU5416 by Human Liver Microsomes. M12 was the major metabolite in all species, including human. Therefore, experiments were performed to further characterize the enzyme kinetics (i.e., effect of substrate concentration) of M12 formation by a pool of human liver microsomes. The data were analyzed by Eadie-Hofstee plot to determine the kinetic parameters, Vₘₐₓ and Kₘ, where Kₘ is the concentration of substrate at which the rate of metabolite formation is equal to half-Vₘₐₓ, and where Vₘₐₓ is the rate of metabolite formation in the presence of saturating substrate concentration. As shown in Fig. 4, the plot of substrate concentration versus the rate of formation of M12 was biphasic, suggesting that the reaction is catalyzed by two or more kinetically distinct enzymes, one with high affinity but low capacity (Kₘ = 3.24 μM, Vₘₐₓ = 68.4 pmol/min/mg) and one with low affinity but high capacity (Kₘ = 64.0 μM, Vₘₐₓ = 159 pmol/min/mg). The Vₘₐₓ/Kₘ (in vitro intrinsic clearance) value for the high affinity enzyme (21.1 μl/min/mg) was nearly 10 times greater than the Vₘₐₓ/Kₘ value for low affinity
enzyme (2.4 μL/min/mg), which suggests that the formation of M12 from SU5416 in vivo will be primarily catalyzed by the high affinity enzyme(s). Identification of the relative role of the high and low affinity P450 enzymes in the formation of M12 will be important for predicting clinically relevant drug interaction potential.

The major pathways of SU5416 metabolism are shown in Fig. 5. M12 and M6 were the two major in vitro metabolites of SU5416. In microsomes, M12 is a primary metabolite of SU5416 (formed by methyl hydroxylation) and M6 is a secondary metabolite (a carboxylic acid formed by further oxidation of M12). Overall, the results of in vitro metabolism of SU5416 by mouse, rat, dog, monkey, and human suggest there were marked differences in the levels of different metabolites formed by these animal liver microsomes. The rate of SU5416 metabolism by liver microsomes from the species examined followed the rank order: monkey > mouse > rat > dog > human. Although rat, monkey, and dog converted SU5416 to metabolites that did not appear to be formed by human liver microsomes, the converse was not true. The presence of “nonhuman” metabolites in animal species does not present a problem, unless the metabolism through those metabolites is so extensive that it becomes difficult to achieve adequate exposure of the parent compound in the toxicological animal species. Since the major metabolite formed by all species was M12, the appearance of unique, low level metabolites in animal species is not expected to be problematic. However, the converse can be problematic, because when a unique metabolite is found in humans but not other species, it raises concerns regarding interpretation and extrapolation of toxicity data from laboratory animals to humans. In other words, it is possible that such a unique metabolite may be toxic or perhaps pharmacologically active, and such an observation would not be predicted from animal data. The results of the present study suggest that SU5416 is not converted to any unique “human” metabolites.

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