GLUTATHIONE S-TRANSFERASE METABOLISM OF THE ANTI NEOPLASTIC PENTAFLUOROPHENYLSULFONAMIDE IN TISSUE CULTURE AND MICE

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ABSTRACT:
The microtubule disrupting agent 2-fluoro-1-methoxy-4-pentafluorophenylsulfonamide (T138067) binds covalently and selectively to β-tubulin and has been shown to evade drug-efflux pumps that confer multidrug resistance to other antimitotic drugs that are used in cancer chemotherapy (Shan et al., 1999). In addition to these resistance mechanisms, eukaryotic cells have developed other protection mechanisms that involve enzymes that modify electrophilic xenobiotics. To determine whether T138067 is a substrate for such enzymatic detoxification pathways, a metabolism study was initiated. GST conjugation was shown to play a major role in T138067 metabolism. T138067-GSH conjugates were isolated from the culture media of T138067-treated cells and the bile of mice treated i.v. with T138067. The major T138067-GSH degradation products were also isolated from these sources. 19F NMR studies of the metabolites showed that metabolic conversions occurred through substitution of the para fluorine atom in the pentafluorophenyl ring of T138067. The T138067-GSH conjugate was also isolated from T138067 incubation buffer that had been exposed to mouse, rat, dog, or human liver slices, suggesting that this mechanism is not species-specific. All three human glutathione S-transferases (α, μ, and π), which are expressed in a wide variety of tissues including human tumors, were shown to metabolize T138067 effectively in vitro. The combined data show that T138067 is being metabolized, in vitro and in vivo, predominantly via a glutathione S-transferase-mediated metabolic pathway.

Microtubules consist of linear polymers of α- and β-tubulin heterodimers (Jordan and Wilson, 1998; Nogales et al., 1998) and are important components of the mitotic apparatus, which is essential for the separation of chromosomes during mitosis (Sorger et al., 1997). Drugs that affect microtubule dynamics represent potent inhibitors of mitotic cell growth and are useful chemotherapeutic agents for the treatment of human cancers (Rowinsky and Donehower, 1991; Rowinsky, 1997). The compound 2-fluoro-1-methoxy-4-pentafluorophenylsulfonamidobenzene (T138067; Fig. 1) is a representative of a new class of antimitotic agents that prevent tubulin from polymerizing into microtubule filaments by binding covalently in a highly selective manner to Cys239 of β-tubulin isotypes β2, β3, and β4 (Shan et al., 1999). A major problem associated with cancer chemotherapy is the emergence of multidrug resistant (MDR) tumor cells. Several cellular mechanisms that confer resistance against such antimitotic agents have been described. These involve the enhanced expression of drug-efflux pumps, including P-glycoprotein and the MDR proteins (Gottesman and Pastam, 1993; Deeley and Cole, 1997). It is well documented that polyclonal microtubule inhibitors such as paclitaxel lose their efficacy against tumor cells that overexpress these proteins (Horwitz et al., 1993; Deeley and Cole, 1997). In contrast, the efficacy of T138067 is not affected by the MDR phenotype (Shan et al., 1999). Thus the covalent modification of β-tubulin by T138067 may provide a mechanism by which T138067 evades (at least partially) these drug-efflux pumps.

It is known that eukaryotic cells have developed certain detoxification mechanisms, which in part also can confer drug resistance. Unlike the drug-efflux pumps, these mechanisms require enzymes that catalyze reactions between a cellular substrate and an electrophilic drug (Jakoby and Ziegler, 1990). One of these mechanisms involves the glutathione S-transferase (GST) family of enzymes. These enzymes catalyze the nucleeophilic attack of the GSH thiol group on molecules with electrophilic character (Pickett and Lu, 1989; Daniel, 1993). Human cells contain at least three major isoforms of GST, referred to as α, μ, and π (Hayes and Pulford, 1995). Each isoform is expressed in a wide variety of tissues, and the relative levels of the various GST isoforms vary among different tissue types. For example, high-level expression of the π isoform has been measured in many

*Abbreviations used are: T138067, 2-fluoro-1-methoxy-4-pentafluorophenylsulfonamidobenzene; ES, electrospray; GST, glutathione S-transferase; MDR, multidrug resistant; MS, mass spectrometry; T138068, 1-methoxy-4-pentafluorophenylsulfonamidobenzene; RT, retention time.

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Fig. 1. Chemical structures of T138067 (R1 = F; R2 = H), [3H]T138067 (R1 = F; R2 = 3H), and T138068 (R1 = H; R2 = H).
types of human tumors, and a deficiency of the $\mu$ isofrom has been associated with a predisposition for some types of cancer. In addition, this thiol detoxification mechanism has been implicated in conferring cellular resistance to anticancer drugs (Tew, 1994; Hayes and Pulford, 1995). Due to the electrophilic character of T138067, it was crucial to determine whether this agent might be a substrate for cellular detoxification pathways. In particular, we sought to determine whether the GSH detoxification mechanism plays a role in the metabolism of this unique antimitotic agent.

**Experimental Procedures**

**Materials.** T138067 and T138068 (1-methoxy-4-pentafluorophenylsulfonamidobenzene) were prepared by methods described previously (Medina et al., 1998). $[^{1}H]$T138067 was prepared by American Radiolabeled Chemicals, Inc. (St. Louis, MO). Its radiochemical (99.3%) and chemical (100%) purity were determined by normal-phase HPLC using $\beta$-emission radioactivity monitoring and UV detection, respectively. $[^{1}H]$[T138067 was obtained as a methanolic solution containing 1.0 mCi/ml (equivalent to 0.05 m$\mu$Ci/ml) of preservation buffer. Cylindrical cores of liver tissue from each species were immediately placed in 4°C V-7 cold preservation buffer. Beagle dogs were housed on Sani-chips, maintained on a 12-h light/dark cycle, and Sprague-Dawley rats (average weight 225 g) were purchased from Harlan Laboratories, Inc., Indianapolis, IN. Animals were housed in groups on irradiated corn cob bedding in HEPA-filtered ventilated rack housing. Irradiated PMI Picolab 5058 rodent diet (PMI Mills, Indianapolis, IN) and autoclaved, hyperchlorinated water were provided ad libitum. The animals were treated in accordance with the “National Institutes of Health Guide for the Care and Use of Laboratory Animals” (National Research Council, National Academy Press, Washington, DC, 1996).

**Animals.** Twenty BALB/c mice, 7 to 8 weeks old and between 14.0 and 19.5 g of body mass were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Animals were housed in groups on irradiated corn cob bedding in HEPA-filtered ventilated rack housing. Irradiated PMI Picolab 5058 rodent diet (PMI Mills, Indianapolis, IN) and autoclaved, hyperchlorinated water were provided ad libitum. The animals were treated in accordance with the “National Institutes of Health Guide for the Care and Use of Laboratory Animals” (National Research Council, National Academy Press, Washington, DC, 1996).

**Liver Slice Preparation.** ICR mice (average weight 20 g) and adult male Sprague-Dawley rats (average weight 225 g) were purchased from Harlan Sprague-Dawley, Inc. The mice and rats were fed Tek-lad (4% mouse/rat, 7001), housed on Sani-chips, maintained on a 12-h light/dark cycle, and allowed free access to drinking water. The animals were sacrificed by cervical dislocation, and the livers were excised through midventral incision and immediately placed in 4°C V-7 cold preservation buffer. Beagle dogs were purchased from Marshall Farms USA, Inc. (North Rose, NY). The dogs were sacrificed by a veterinary surgeon using a standard euthanasia procedure, which consists of an i.v. overdose of pentobarbital. After a dog was pronounced dead, its liver was removed and immediately placed in 4°C cold V-7 preservation buffer. Cylindrical cores of liver tissue from each species were prepared with a sharpened 8-mm stainless steel tube, and 200-$\mu$m precision slices were cut with a Brendel/Vitron tissue slicer (Vitron Inc., Tucson, AZ) submerged in ice-cold V-7 preservation solution. Human liver tissue for this project was procured through the Association of Human Tissue Users (Tucson, AZ) and was handled in strict coherence to the procedures of the Center for Disease Control (CDC, Atlanta, GA). On arrival, the human liver, which was on ice and in ViaSpan, was immediately cored and precision-cut as described above. The viability of the individual liver slices was tested by the standard K$^+$ retention method.

**Determination of the Rate of T138067 Metabolism in Cell Culture.** The cell lines used in this study were: HepG2, human hepatocellular carcinoma cell line; MCF7, human breast adenocarcinoma cell line; MCF7/ADR, MDR subline of MCF7; and T47D, cell line from a human breast ductal carcinoma. For cell culture studies, 2 $\times$ 10$^5$ cells (HepG2, MCF7, MCF7/ADR, and T47D) per experiment were plated in 2 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (v/v) 24 h before treatment with $[^{1}H]$T138067 (25 nM, 1 $\mu$Ci) at 37°C (5% CO$_2$). At 2 min intervals, 500-$\mu$l aliquots were removed from the media and extracted with an equal volume of ethyl acetate. After phase separation (16,000g, 2 min), a 200-$\mu$l aliquot from each organic and aqueous phase was mixed with 5 ml of scintillation cocktail (Protein Ready; Beckman Inc., Palo Alto, CA), and the amount of radioactivity was determined with a Beckman LS 6000 scintillation counter.

**Isolation of T138067 Metabolites from HepG2 Cell Culture Media.** For isolation of T138067 metabolites generated in cell culture, 2.4 $\times$ 10$^6$ HepG2 cells in 240 ml of Cellgro SPINS medium (without fetal calf serum or phenol red) were divided into four 60-ml aliquots. Each aliquot was treated with 42 $\mu$g/ml of T138067. After incubation for 4.5 h at 37°C, 5% CO$_2$, the medium was collected and clarified by centrifugation (26,895g, 15 min). To increase the concentration of the first T138067 metabolite, 6 $\times$ 10$^5$ HepG2 cells were incubated separately for 45 min at 37°C, 5% CO$_2$ in 60 ml of medium containing 150 $\mu$g/ml of T138067.

As an initial purification step, all of the cell culture media (60 ml) was collected from each individual treatment and fractionated on a preparative HPLC system equipped with HPLX solvent delivery pumps (Rainin Instruments Company, Inc., Emeryville, CA) using a reverse-phase C18 Dynamax column (Microsorb 5-$\mu$m particles; 41.4 $\times$ 300 mm, Rainin Instruments Company, Inc.) that had been equilibrated with water. Individual constituents were eluted at a flow rate of 80 ml/min (detection wavelength, 220 nm) with the following solvent system: 100% water for 10 min, followed by a linear gradient from 100% H$_2$O to 100% acetonitrile over 30 min, followed by 100% acetonitrile for 10 min. Putative T138067 metabolites eluting at a retention time (RT) = 22 to 26 min were purified to homogeneity on a Hewlett Packard 1050 diode array HPLC system (Hewlett Packard Inc., Palo Alto, CA) using an analytical size reverse-phase C18 Inertisil column [ODS2 5-$\mu$m particles; 4.6 $\times$ 150 mm; (MetaChem Technologies Inc., Torrance, CA)] at a flow rate of 1.0 ml/min. For isolation of the metabolites, the following solvent was used: A = 10 mM ammonium acetate/98% H$_2$O + 2% methanol and B = 100% acetonitrile. The elution protocol was as follows: a 5-min isocratic elution (100% A), followed by a linear gradient from 100% A to 60% A over 30 min, then a decrease of the concentration of solvent A over 10 min to 0% A, and washing the column for 5 min with solvent B. Three individual T138067 metabolites were isolated in pure form.

**Isolation of T138068 Metabolites from HepG2 Cell Culture Media.** For isolation of T138068 metabolites generated in cell culture, an isolation scheme similar to that described above for T138067 was used. In this case, each 60-ml aliquot was treated with 2.5 mg of T138068 for 4.5 h. Three new uniform and baseline-separated peaks appeared in the preparative HPLC chromatograms (HPLC conditions were identical with those described for T138067) at RT values = 14.8, 16.5, and 18.3 min, representing three putative metabolites of suitable purity for structural evaluation.

**Isolation of T138067 Metabolites from Mouse Bile and Urine.** Twenty BALB/c mice were dosed i.v. with T138067 (30 mg/kg). The animals were sacrificed by cervical dislocation 60 min after injection, then immediately exsanguinated. Gall bladders were ligated before removal from the animals and stored at $\sim$80°C. After the thawed gall bladders were homogenized in 1 ml of deionized water, the homogenate was extracted twice with an equal volume of ethyl acetate. The organic and aqueous phases were lyophilized separately. The dried materials from the aqueous and organic phases were resuspended in 160 and 75 $\mu$l of methanol, respectively. T138067 metabolites in the aqueous and organic phases were purified to homogeneity using the same analytical HPLC method described above. Urine was drawn from the mouse bladders by syringe, and the combined samples were stored at $\sim$80°C. For metabolic analysis, thawed urine aliquots were subjected directly to HPLC/mass spectrometry (MS) analysis without further preparation.

**Identification of T138067 Metabolites Generated in Liver Slices.** Liver slices were floated onto Teflon (mouse, rat, and dog) and titanium (human) rollers, then blotted and placed into glass scintillation vials containing 1.0 ml of Waymouth’s MB 752/1 tissue culture medium fortified with 10% fetal calf serum, 0.35 g/l l-glutamine, 10 ml/l Fungi-Bact, and 84 $\mu$g/ml gentamicin sulfate. The vials were closed with caps that have a central hole 2 mm in diameter to facilitate gas exchange and were placed in a dynamic roller culture incubator at 37°C. The incubators were gassed with 95% oxygen and 5% carbon dioxide. The amounts of T138067 per experiment were 2, 20, and 120 $\mu$g/ml. The incubation times were 30, 90, and 360 min. The experiments were performed in triplicate and included saline and drug-only (no tissue) controls (120 $\mu$g/ml). After incubation, the media was collected and frozen ($\sim$80°C) until analyzed. A 100-$\mu$l aliquot of each individual slice media was analyzed for T138067 metabolites by reverse-phase HPLC using the same analytical equipment, HPLC column, and gradient described above (see Experimental Procedures, Isolation of T138067 Metabolites from HepG2 Cell Culture Media). The UV detection wavelength was 234 nm. The metabolites were identified...
incubated with [3 H]T138067, and sample aliquots were taken from the derived, see Experimental Procedures. The individual cell lines were T47D (for the types of tumors from which these cell lines were including HepG2, MCF7 and its MDR subline (MCF7/ADR), and rate of T138067 metabolism in a variety of human tumor cell lines, rate of T138067 metabolism in various cell lines. We determined the recombinant human GST isoforms [α (A1-1), μ (M1-1), and π (P1-1); Cal-Biochem, La Jolla, CA] were used in place of equine liver GST, the quantity of enzyme in the reaction mixture was 10 μg. Aliquots (100-μl) were removed from the reaction mixtures after the indicated time intervals and extracted with an equal volume of ethyl acetate. After phase separation (16,000g; 2 min), a 50-μl sample of the organic phase was mixed with scintillation cocktail, and the amount of radioactivity was determined in a liquid scintillation counter. The activities of the various GST preparations were as follows: purified equine liver GST, 83 U/mg of protein; human α, 75 U/mg of protein; human π, 95 U/mg of protein; and human μ, 221 U/mg of protein. (Note: The purified equine GST preparation contained a significant amount of GSH; therefore, reaction mixtures containing this enzyme were preincubated for 10 min at 30°C in the presence of unlabeled T138067. After the preincubation period, [1 H]T138067 and a known quantity of GSH were added to the mixture, and incubation was continued for the indicated time period.)

NMR and MS Analysis. Electrospray (ES)/MS data for T138067 and T138068 metabolites I, II, and III isolated from tissue culture medium were obtained with a VG Biotech B-Q mass spectrometer operating in the positive ionspray mode. Sample aliquots of 50 ng/μl were injected into the ionization chamber by flow injection using an acidic mobile phase (50% acetonitrile/49%water/1%acetic acid) at a flow rate of 1.0 ml/min. T138067 metabolite V was identified by chemical ionization/MS (ionization gas/methane) using a HP 5989A mass spectrometer equipped with a HP 5890 Series II gas chromatograph as the delivery system (Hewlett Packard Inc.). Parent T138067 was identified by electron impact/MS using the same instrumentation. The metabolites and the parent compound were characterized further by NMR spectroscopy using a Varian Gemini G400B-8388 NMR spectrometer (Varian Associates Inc., Palo Alto, CA).

1H-Spectra were recorded at 400.12 MHz in methanol-d4 using the residual proton signals of the solvent (83.31 ppm) as an internal standard. 13C-Spectra were recorded at 95.36 MHz without 1H-decoupling in methanol-d4 using hexafluorobenzene (δ63 ppm) as an internal standard.

Results

Metabolism of T138067 in Cell Culture. Determination of the rate of T138067 metabolism in various cell lines. We determined the rate of T138067 metabolism in a variety of human tumor cell lines, including HepG2, MCF7 and its MDR subline (MCF7/ADR), and T47D (for the types of tumors from which these cell lines were derived, see Experimental Procedures). The individual cell lines were incubated with 1H[T138067], and sample aliquots were taken from the media and extracted with ethyl acetate at various time points. After separation of the organic and aqueous layers, we monitored a shift of radioactivity from the ethyl acetate to the aqueous phase, which indicated a conversion of the lipophilic 1H[T138067] to water soluble metabolites. As shown in Fig. 2A, each of the cell lines evaluated exhibited a distinct rate of T138067 conversion. As the radioactivity declined in the organic phase, radioactive T138067 metabolites in the aqueous phase (i.e., the media) increased proportionally (Fig. 2B). For HepG2 cells, the concentration of 1H[T138067] in the organic phase declined over the 10-h incubation period with a half-life of ~3 h.

Exposure of 1H[T138067] to MCF7/ADR cells resulted in a relatively slow conversion of the parent 1H[T138067] compound into water-soluble metabolites, whereas the other two cell lines (MCF7 and T47D) showed intermediate rates of 1H[T138067] conversion.

Isolation of T138067 metabolites generated in HepG2 cell culture. HepG2 cells exhibit the highest rate of metabolism among the cell lines tested. Therefore, we used HepG2 cells in a scaled-up version of the T138067 metabolism experiments shown in Fig. 2 to isolate T138067 metabolites for structural elucidation (see Experimental Procedures). A comparison of HPLC profiles obtained from a control incubation (media plus T138067) and one of culture media from T138067-treated HepG2 cells allowed the identification of the HPLC peaks that represented potential metabolites. Media from cells treated with T138067 gave rise primarily to one new predominant nonuniform peak in the HPLC profile (Fig. 3) at RT = 22 to 26 min. The appearance of this new peak was independent of the amount of T138067 added to the media and the incubation time. Unconverted T138067, the least polar constituent, always eluted last at RT = 33 min. Minor signals around RT = 16 to 22 min could not be related to any metabolic products on spectrosopic analysis. However, further separation of the major peak by analytical HPLC yielded three distinct T138067 metabolites in milligram quantities. T138067 metabolites I, II, and III eluted at RT = 23.5, 25.5, and 24.0 min, respectively. Parent T138067 eluted at RT = 40.8 min under identical analytical HPLC conditions.
To ensure that no major T138067 metabolites had been overlooked, we exposed HepG2 cell cultures to T138067 spiked with [3H]T138067 (data not shown). Aliquots of media taken at various time points (0 min, 45 min, 4.5 h, and 14 h) were analyzed by analytical HPLC, and the collected fractions were tested by scintillation counting. Radioactivity was found only for eluted peaks that corresponded to the parent T138067 compound and the three characterized metabolites. No other peaks of radioactivity were detected.

Identification of T138067 metabolites found in HepG2 cell culture media. The three T138067-metabolites (I, II, and III) were identified to be the γ-Glu-Cys-Gly, Cys-Gly, and Cys conjugates of T138067, respectively. The structures of the three metabolites were determined by first comparing the 1H NMR spectra of the parent T138067 compound and the T138067 metabolites. The spectra showed that none of the nonexchangeable protons of parent T138067 were replaced on metabolism; however, new signals appeared in the 1H NMR spectra of T138067 metabolites I, II, and III, accounting for ten, five, and three new nonexchangeable protons, respectively (data not shown). The mass difference between T138067 metabolite I and II (Δm = 57) accounted for the loss of a glycine. The difference between II and III (Δm = 57) accounted for the loss of a glutamic acid residue, whereas the difference between I and III (Δm = 57) accounted for the loss of a glutamic acid residue, whereas the difference between II and III (Δm = 57) accounted for the loss of a glycine. The mass difference (Δm = 287) between parent T138067 and T138067 metabolite I accounted for the addition of the tripeptide GSH (m/z = 307) minus 20 mass units, which indicated a loss of one fluorine atom during GSH conjugation.

The fluorine displacement was confirmed by analyzing the 19F NMR spectra for all isolated T138067 metabolites. For the parent T138067 compound, four individual fluorine multiplets were found (δ = 132.2 ppm, δ = 136.6 ppm, δ = 146.4 ppm, and δ = 159.8 ppm for positions 2, 2′, 4′, and 3′, respectively). The 19F NMR spectrum for T138067 metabolite I (δ = 131.6 ppm, δ = 133.0 ppm, and δ = 136.8 ppm for positions 3′, 2′, and 2′, respectively) showed only three signal groups (Fig. 1). It was apparent that T138067 metabolite I lacks one of the fluorine atoms that is present in the parent drug. At high resolution, the broad multiplet at δ = 131.6 ppm sharpened and resembled, with its coupled signal multiplet, an AA′XX′ type spin pattern (Günther, 1995). This pattern indicated that a substitution of one of the fluorine atoms at position 2′, 4′, and 3′ (para position of the polyfluorinated benzene ring in parent T138067) occurred during metabolism (compare Fig. 1). Similar results were obtained for the other two isolated T138067 metabolites.

Identification of T138067 metabolites with HPLC chromatography.

A, HPLC chromatogram of culture media after a 4.5-h exposure of HepG2 cells to T138067; B, HPLC chromatogram of culture media spiked with T138067. Chromatograms were recorded at 220 nm. T138067 is indicated with an arrow. The unique peak in A (putative metabolites) is indicated with an asterisk.

### Table 1

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*Electron impact/MS data

**Chemical ionization/MS data (additional signals were found at m/z 400 (M[CH3]2) and 414 (M[C6H4]2)).**
metabolites, which exhibited $^{19}$F NMR signals with essentially identical chemical shifts as those for T138067 metabolite I that corresponded to the remaining five fluorine atoms (data not shown). To rule out any ambiguity about the conjugation site, we repeated our metabolism study in cell culture with T138068, a close analog of T138067. This compound lacks the fluorine atom on the aniline ring (Fig. 1), which simplifies the interpretation of the $^{19}$F NMR data. Similar to T138067, three T138068 metabolites (I, II, and III) were isolated that supported a GSH-based metabolism; their structures were confirmed by MS (Table 1) and $^1$H NMR analysis (data not shown). Here, signals in the $^{19}$F NMR spectra of all three metabolites exhibited a symmetrical AA'XX' spin pattern, unambiguously indicating the displacement of the fluorine at the para position. The $^{19}$F NMR spectrum for T138068 metabolite I is shown in Fig. 4B.

**Metabolism of T138067 in Mice.** To determine whether the same metabolic process observed in cell culture also occurs in vivo, T138067 metabolites were isolated from the bile of mice treated with T138067. The decision to focus on this biological matrix was based on biodistribution studies, which showed that mouse bile contains the largest single fraction of radioactivity after i.v. administration of $[^3]$H]T138067 (data not shown). The bile was partitioned against ethyl acetate and the resulting aqueous and organic phases were subjected separately to analytical HPLC fractionation (see Experimental Procedures). The aqueous phase contained...
T138067 metabolite I as the single major conversion product confirmed by MS and NMR analysis. In contrast, the organic phase yielded two new metabolites not found in our cell culture experiments. The major component, T138067 metabolite IV (RT = 26.0 min), was identified by MS (Table 1) and NMR analysis to be the N-acetylcysteine conjugate of T138067. The minor component, T138067 metabolite V (RT = 39.0 min), was identified by MS (Table 1) and NMR analysis to be the residual thiol breakdown product derived from the T138067-GSH conjugate. No other T138067 metabolites could be isolated from bile, and no parent T138067 was detected. In addition, urine samples were analyzed by LC/MS without prior partitioning. In this matrix, T138067 metabolite III was identified. Again, parent T138067 was absent.

Metabolism of T138067 in Liver Slices. Potential interspecies variability of T138067 metabolism was investigated by incubating T138067 at three concentrations with slices of mouse, rat, dog, and human livers and analyzing the incubation buffer by HPLC/MS after various times of incubation. Representative HPLC chromatograms for the rat and dog liver slice experiments using 20 µg/ml of T138067 are shown in Fig. 5. In all preparations, a time-dependent decrease in the concentrations of T138067 (RT = 41 min) with a concomitant increase of T138067 metabolite I (RT = 23.5 min) was observed. The identity of T138067 metabolite I was confirmed by ES/MS analysis. No other metabolites were detected. Mouse and rat liver slices showed rapid metabolism of T138067. Dog and human liver slices metabolized T138067 more slowly. Apart from random signal variations (noise), two peaks were shown to change in all samples including the media-only controls (media + sliver slices, no T138067). One constituent (RT = 10.5 min) showed a decrease in signal intensity over time and the other constituent (RT = 4.0 min) showed an increase in signal intensity over time. Both variations obviously were not a result of T138067 metabolism.

Chemical Reactivity Profile of T138067. In vitro GST/GSH reactivity. It is possible that T138067, as a potentially reactive compound (as shown through the isolation of metabolites), is merely undergoing a chemical reaction with a sulfur nucleophile like GSH, without the aid of a catalytic support (GST in this case). To differentiate between these two possible mechanisms, in vitro reactions were carried out using purified liver GST, GSH, and [3H]T138067. Aliquots of the reaction mixtures were removed after distinct time intervals, and the ethyl acetate extracts were analyzed by scintillation counting. As shown in Fig. 6A, radioactivity in the organic phase did not decline when [3H]T138067 was incubated with GSH for 12 min in the absence of GST. A similar profile was obtained when [3H]T138067 was incubated with GST in the absence of GSH (control reaction). In contrast, in the presence of GST and GSH, a decline of radioactivity in the organic phase was observed within 2 min, indicating that T138067 was converted into a GSH conjugation product. These data indicate that T138067 is not sufficiently reactive to be converted into a GSH conjugate in the absence of GST under the experimental conditions and reaction times tested.

T138067 is a substrate for GST isoforms α, μ, and π. The three human GST isoforms, α, μ, and π, are expressed in a wide variety of tissues including human tumors (Tew, 1994). Having shown that [3H]T138067 can be a substrate for GST, we next determined whether any of the three GST isoforms can convert [3H]T138067 into its GSH conjugate. Recombinant GST α, GST μ, and GST π that had been purified from Escherichia coli were incubated in the presence of [3H]T138067 as described above. As shown in Fig. 6B, in the presence of GST α, GST μ, and GST π only small amounts of radioac-
known that GSH conjugates of xenobiotic compounds can be further modified primarily via the GST metabolic pathway. The first metabolite, T138067, was identified as a GSH conjugate. It is surprising that liver-derived HepG2 cells induced the highest rate of metabolism in comparison with the other investigated tumor cell lines. However, two of the three isolated in vivo metabolites were not detected in the cell culture experiments. The first product, T138067 metabolite IV, is the N-acetylcysteine conjugate of T138067; the second, T138067 metabolite V, is a degradation product of the initial GSH conjugate to the residual thiol (Fig. 7). N-acetylcysteine conjugates are known to be the excretion products of xenobiotics, and their formation may involve the enzyme N-acetylase. The formation of a residual thiol from a GSH conjugate may involve cysteine conjugate β-lyase enzymes, which occur in several tissues including the gastrointestinal tract, liver, and kidneys (Gibson and Skett, 1994).

The analysis of T138067 samples exposed to mice, rat, dog, and human liver slices in vitro led to the isolation of T138067 metabolite I and thus suggests that the metabolic pathway used by cells to degrade T138067 is not likely to be species-specific. However, as expected, differences in the conversion rates were observed. The varying extents to which the individual T138067 metabolites are found in different test systems are likely the result of differences in the capacities of these systems to carry out the individual steps of the GST metabolic pathway.

Structural studies on T138067 and its isolated metabolites indicated that covalent modification of the parent compound occurs at the para position of the fluorophenyl ring through displacement of the fluorophenyl ring through displacement of the fluorine substituent by the cysteine sulfhydryl group. The postulated reaction mechanism of T138067 reaction with GSH and T138067 was further substantiated through studies of its close relative, T138068, and its metabolites. This series of compounds possesses more easily interpretable 19F NMR spectra than the parent compound T138067 and its metabolic conversion products. The 19F NMR spectra for the T138068 metabolites exhibited symmetrical AA’XX’ spin pattern, confirming a fluorine substitution at position 4’ of the pentafluorophenyl ring in parent T138067.

In vitro experiments showed that T138067 is not converted into its GSH adduct at physiological pH on exposure to GSH alone, but requires the presence of the enzyme GST. Enzymatic reactions carried out in vitro showed that any of the three human GST isoforms (α, μ, and π) can effectively metabolize T138067. These GST isoforms are expressed in a wide variety of tissues, including many human tumors. Despite the fact that T138067 is metabolized when exposed to various tumor cell lines, a portion of the parent compound clearly is available for covalent modification of β-tubulin (Shan et al., 1999). This notion is also consistent with the observation that T138067 displays similar cytotoxic potencies (IC50 values ~150 nM) against all tumor cell types investigated in this study. (data not shown). These findings are consistent with studies showing that T138067 reacts with Cys150 of β-tubulin spontaneously at physiological pH (Shan et al., 1999) whereas GSH conjugation, on the other hand, requires the activity of GST.

Although the mechanisms of T138067 binding to GSH and β-tubulin isotypes β1, β2, and β4 differ, the spectroscopic information
obtained for the major T138067 metabolites were the first indication that a cysteine constituted the binding site of T138067 in \( \beta \)-tubulin. Subsequently, Cys\(_{239} \) was shown to be the only T138067 binding site in \( \beta \)-tubulin (Shan et al., 1999). Furthermore, there is strong evidence that the binding of T138067 to Cys\(_{239} \) also occurs through the establishment of a sulfide bond at the \textit{para} position of the polyfluorinated ring of T138067 (W. P. Frankmoelle, D. Stott, J. Krenisky, E. Santha, L. Huang, J. C. Medina, and H. Beckmann, unpublished results).

T138067 is a representative member of a new class of pentafluorophenylsulfonamidobenzenes (including T138068) that exhibit antineoplastic activity. Members of this class of compounds evade cellular detoxification pathways involved in the development of multidrug resistance, probably because of the covalent and irreversible nature of the interaction between these compounds and \( \beta \)-tubulin. Although T138067 and related compounds experience partial detoxification through the GST/GSH metabolic pathway, these compounds may prove to be useful clinically for the treatment of malignant tumors, including those that possess a MDR phenotype.

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**References**


**Fig. 7.** GST metabolism scheme for T138067. T138067 metabolites isolated from HepG2 cell culture media (I, II, and III) or mouse bile (I, IV, and V) are shown. Amino acids are presented in their standard triple letter code. No other major metabolites were found in either matrix.