Delineating Novel Metabolic Pathways of DPC 963, a Non-Nucleoside Reverse Transcriptase Inhibitor, in Rats. Characterization of Glutathione Conjugates of Postulated Oxirene and Benzoquinone Imine Intermediates by LC/MS and LC/NMR

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The metabolic activation of (*S*)-5,6-difluoro-4-cyclopropylethynyl-4-trifluoromethyl-3,4-dihydro-2(1*H*)-quinazolinone, DPC 963, in rats was investigated by identifying and characterizing the GSH and mercapturic acid conjugates excreted in the bile and urine, respectively. The structures of these adducts, which were unequivocally elucidated by LC/MS/MS and NMR experiments, revealed the existence of at least three distinct metabolic pathways leading to these products. One of the pathways, which has been described previously, involves the activation of the acetylene group after an initial hydroxylation on the methine carbon of the cyclopropyl ring. Metabolite **M1** was demonstrated to be formed via this pathway after an enzymatic addition of GSH across the triple bond of the substituted acetylene. The second pathway, also previously described, leads to diastereoisomeric GSH adducts **M3** and **M4** after the formation of a highly reactive oxirene intermediate. This postulated oxirene subsequently rearranges to an α, *β*-unsaturated cyclobutenyl ketone intermediate capable of undergoing a 1,4-Michael addition with a nucleophile such as GSH. In addition to these pathways, DPC 963 was found to undergo a metabolic activation previously undescribed for structural analogues of this compound. It is postulated that an oxidative defluorination mediated by cytochrome P450 leads to the formation of a putative benzoquinone imine intermediate which subsequently reacts with GSH to form two aromatic ring-substituted regioisomeric conjugates, **M5** and **M6**. In addition to forming the GSH adducts, the benzoquinone imine was also found to be reduced to its unreactive hydroquinone metabolite, which was excreted as the glucuronide conjugate in rat bile. Studies with induced rat microsomes, cDNA-expressed rat P450 isozymes, and polyclonal antibodies against rat P450 clearly demonstrated that the rat P450s 3A1/3A2 were responsible for the formation of postulated oxirene and benzoquinone intermediates.

Introduction

One of the objectives in the field of metabolism-mediated toxicity is to elucidate the mechanisms by which xenobiotics undergo metabolic activation to produce reactive intermediates. Knowledge of the different pathways by which xenobiotics can be metabolized to reactive intermediates increases our understanding of the relationship between the chemical nature of xenobiotics and their effects on living systems. Furthermore, such knowledge will be helpful in identifying chemical functional groups in a given molecule, which predispose that compound to a sequence of processes that might eventually lead to toxicity.

DPC 963 (Figure 1), (*S*)-5,6-difluoro-4-cyclopropylethynyl-4-trifluoromethyl-3,4-dihydro-2(1*H*)-quinazolinone, is a specific non-nucleoside human immunodeficiency virus-1 reverse transcriptase inhibitor (NNRTI) and is currently in the early stages of clinical trials. DPC 963 is a structural analogue of efavirenz and DPC 961, the metabolism of which have been described previously (*1*−*5*). DPC 963 differs from DPC 961 and efavirenz in having two fluorine substituents on the aromatic ring (Figure 1). The addition of fluorines to the ring decreases the reactivity of the pi electrons (i.e., decreases the energy level of the highest occupied molecular orbital in the aromatic ring). This subsequently leads to deactivation of the aromatic system toward P450-mediated metabolism such as hydroxylation. However, substitution with fluorine also predisposes the aromatic ring to a potential P450-mediated dehalogenation reaction. There is ample

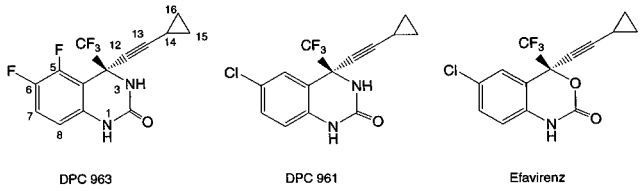

Figure 1. Chemical structures of DPC 963, DPC 961, and efavirenz.

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literature precedence for heme-mediated oxidative dehalogenation, whereby fluorine is lost easily from a compound. One of the aims of having two fluorine substituents on the aromatic ring was to confer some degree of metabolic stability, but at the same time, it was realized that the potential for defluorination did exist. Efavirenz and DPC 961, which had a chlorine substituent, were easily metabolized by aromatic ring hydroxylation. There was no evidence of any dechlorinated metabolites formed from these compounds, consistent with previous observations that chlorine is not as easily lost as fluorine during metabolism of compounds. Efavirenz and DPC 961 were found to be metabolized extensively by rats, cynomolgus monkeys, and humans with the major metabolite being a glucuronide conjugate of the aromatic ring hydroxylated metabolite. There were significant differences in the nature of the metabolites that were excreted in the urine of all three species (*1*, *2*, *5*) after being dosed with either efavirenz or DPC 961. LC/MS and NMR analyses of bile and urine samples from rats dosed with efavirenz showed the presence of glutathione-derived adducts formed by enzymatic addition of glutathione across the triple bond of the acetylene moiety. The formation of such conjugate has been linked to the species-specific nephrotoxicity observed in rats (*3*, *4*). Further studies with the structural analogue, DPC 961, were done in rats to investigate if a similar GSH adduct was produced. However, the structures of the GSH conjugates isolated from the bile of rats dosed with DPC 961 pointed to a totally different mechanism for the formation of the GSH adducts with DPC 961 as compared to that with efavirenz. The GSH conjugate of efavirenz was formed by enzymatic addition of GSH across the triple bond after the initial hydroxylation on the methine carbon of the cyclopropyl ring (*2*). It was postulated that the GSH adducts of DPC 961 found in rat bile were produced as a result of oxidation of the triple bond leading to an unstable oxirene intermediate which subsequently rearranged to an α,*β*-unsaturated cyclobutenyl compound (*5*). It was of considerable interest to us to compare and contrast the metabolites formed from DPC 963 with its previously studied analogues, efavirenz and DPC 961. The metabolism of DPC 963 was investigated in rats, cynomolgus monkeys, and humans (*6*). It was found that DPC 963 was metabolized extensively in these species to produce 8-OH DPC 963 glucuronide and sulfate conjugates as the major metabolites. However, significant differences in the nature of metabolites that were excreted in the urine of all three species were observed (*6*).

The present study was carried out to elucidate the structures of all the GSH conjugates of DPC 963 in rats. An attempt was made to delineate the possible routes of metabolism that led to these metabolites. Furthermore, we aimed to identify the rat P450 enzyme(s) responsible for the metabolic activation of DPC 963 using various techniques. To confirm the isozymes responsible for forming the reactive intermediates, microsomal incubations were performed employing antibodies or selective chemical inhibitors directed against specific P450. The results were confirmed by carrying out incubations with various rat cDNA-expressed P450 isozymes.

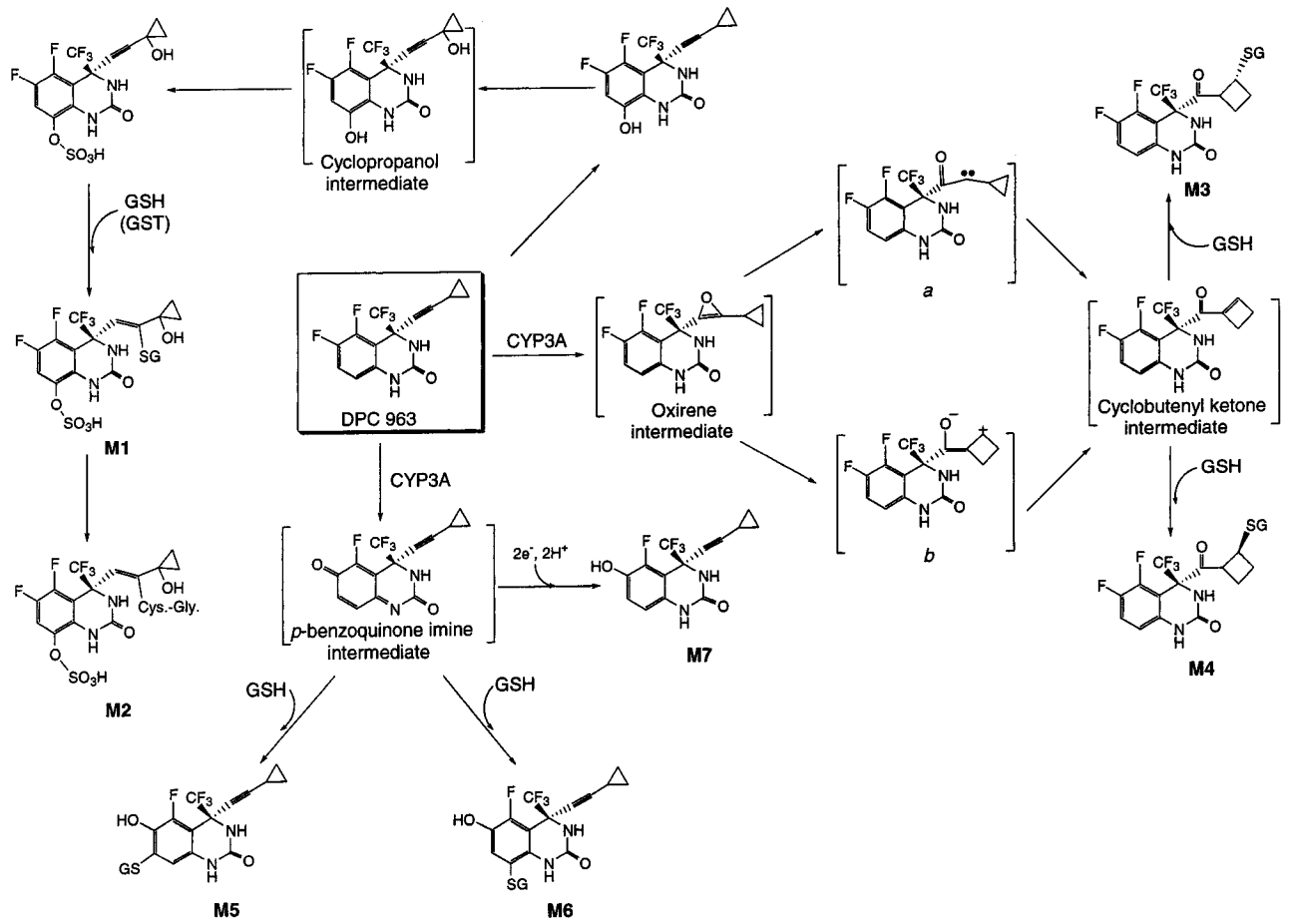
Materials and Methods

Chemicals and Supplies. DPC 963 was synthesized as described before (*7*). Bond-Elut C18 cartridges (10 g/60 cm^3) were purchased from Varian Sample Preparation Products (Harbor City, CA). Waters Symmetry C18 columns (2.1 × 150 and 3.9 × 150 mm, 5 μM) were obtained from Waters Corp. (Milford, MA). Beckman C18 semipreparative and analytical C18 columns (10 × 300, 4.6 × 250 mm, respectively) were obtained from Beckman Instruments Inc. (Fullerton, CA). Control and various induced (dexamethasone, phenobarbital, 3-methylcholanthrene, *β*-naphthoflavone, Aroclor 1254) rat liver microsomes were obtained from In Vitro Technologies (Baltimore, MD). Rat P450 1A1, 1A2, 2A2, 2B1, 2C6, 2C11, 2C12, 2D1, 2D2, 2E1, 3A1, and 3A2 supersomes; polyclonal antibodies against rat P450s 1A2, 2B1/2B2, and 2C11; and normal rabbit serum were purchased from Gentest Corp. (Woburn, MA). Monoclonal antibody against rat P450 3A1 and polyclonal antibody against rat P450 3A1/3A2 were obtained from Xenotech (Kansas City, KS). The chemical inhibitors sulfaphenazole (human P450 2C9), troleandomycin (human P450 3A4), and D609 (*8*, rat P450s 2B1/2) were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI) while furafylline (human P450 1A2) was purchased from Research Biochemicals International (Natick, MA). Ketoconozole (P450 3A) was obtained from ICN Biomedicals Inc. (Aurora, OH). *β*-Glucuronidase (*E. coli*, EC 3.2.1.31) was obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI). All solvents and reagents were of the highest grade commercially available.

Liquid Chromatography/Mass Spectrometry (LC/MS). LC/MS was carried out by coupling an HPLC system to a PE Sciex API 300 mass spectrometer using a turbo ionspray interface held at 400−450 °C. The electrospray needle was maintained at 4500−5000 V with the declustering potential set at 30−40 V. Ultrapure nitrogen was used as the nebulizer gas set at 40 psi. The turbo ionspray gas flow rate was 6 L/min. The mass spectrometer was operated either in the positive or in the negative ion mode (both full scan and MS/MS mode) to achieve optimum sensitivity for GSH adducts. MS/MS analysis was carried out using nitrogen as the collision gas. The collision energy was kept between 30 and 40 eV. The metabolites were separated on a Waters Symmetry C18 column (2.1 × 150 mm) by a gradient solvent system consisting of acetonitrile and 10 mM ammonium formate (pH 3.2). The percentage of acetonitrile was increased from 25 to 80% over 18 min with the solvent flow rate set at 0.4 mL/min. After 18 min, the column was washed with 80% acetonitrile for 2 min before reequilibrating with the initial mobile phase. Aliquots of bile and urine samples were injected directly onto the HPLC column, and the eluent was introduced into the source of the mass spectrometer. To detect metabolites in the fractions from C18 cartridges and from a semipreparative HPLC column, aliquots (60 μL) of samples were introduced to the mass spectrometer using the flow injection analysis method. The mobile phase consisted of a mixture of acetonitrile and 10 mM ammonium formate (pH 3.2) (1:1 v/v) delivered at a flow rate of 0.4 mL/min.

Liquid Chromatography/High-Field NMR. All the spectra were obtained using a Bruker Avance 500 MHz NMR spectrometer equipped with a $^1H/^{13}C$ or ^{19}F LC/NMR flow-probe with a cell volume of 120 μL. Suppression of the residual water and acetonitrile signals was carried out using the WET solvent suppression method in all the LC/NMR experiments. Proton chemical shifts were referenced to DMSO at δ 2.49 and to acetonitrile at δ 2.0, and fluorine chemical shifts were referenced to TFA at δ −78.5. Signal multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (double of doublets), and m (multiplet). An HP1100 LC system was used with a variable-wavelength UV detector set at 240 nm. A Waters Symmetry C18 column (3.9 × 150 mm, 5 μm) was used to separate DPC 963 metabolites. A gradient from 25% acetonitrile-d_3 and 75% D_2O to 80% acetonitrile-d_3 over 20 min at a flow rate of 0.8 mL/min was employed for separation. Both solvents contained 0.05% TFA. The structures of DPC 963 GSH adducts were determined from ^1H and ^{19}F NMR as well as proton−proton (COSY) and total correlated spectroscopy (TOCSY) 2-dimensional NMR.

Scheme 1. Proposed Metabolic Pathways Leading to the Formation of DPC 963 Glutathione Adducts in Rats



Animal Studies. Bile duct-cannulated Sprague−Dawley male rats (300−350 g; Charles River Laboratories, Wilmington, MA) housed in metabolic cages were given an oral DPC 963 suspension twice daily at a dose of 30 mg/kg for 3 days before treatment with a higher dose of DPC 963 at 300 mg/kg/day for the next 3 days. The rats were housed individually in suspended, stainless-steel, wire-mesh cages equipped with an automatic watering system. The study room was environmentally controlled for temperature (72 ± 4 °F), relative humidity (40−70%), and light (a 12 h light/dark cycle). Rats had free access to water and were given a specific amount of certified Purina rodent chow each day. Bile and urine samples were collected over ice on a daily basis and stored at −20 °C until assayed. Aliquots of bile and urine samples were analyzed by LC/MS/MS as described above.

Isolation of GSH-Related Adducts of DPC 963 from Rat Bile and Urine. Bile or urine samples collected from several rats were pooled, diluted 1:1 with distilled water (for bile only), and loaded on a Bond-Elut C18 cartridge (10 g/60 cm^3), which was preconditioned with methanol and water. The sample was allowed to elute under gravity at a rate of less than 1 mL/min. After the sample was loaded, the column bed was washed with 25 mL of deionized water followed by eluting with 25 mL of different percentages of methanol in water (5−100%). The fractions containing the GSH adducts (5−30% methanol fraction) were dried and reextracted on C18 cartridges using different proportions of methanol in 0.2% acetic acid as the eluent. The samples from the second extraction on C18 cartridges (60−70% methanol fraction) were concentrated. The presence of GSH adducts was confirmed by LC/MS analyses. The resultant crude mixture of GSH conjugates was purified on a C18 semipreparative column (Beckman, 10 × 250 mm) using a mixture of either acetonitrile/0.1% acetic acid or acetonitrile/10 mM ammonium formate buffer (pH 3.4) as the mobile phases. The percentage of acetonitrile was increased from 20% to 60% in 20 min with the solvent delivered at a flow rate of 4.7 mL/min. The eluent was monitored at 240 nm. After pooling the fractions containing the GSH adducts, the samples were dried under vacuum and submitted for NMR analyses.

Synthesis (Enzymatic) of Metabolite M7. The glucuronide conjugate of defluorinated metabolite **M7** (Scheme 1) was isolated from the bile of rats by using the similar method as described above. Briefly, the glucuronide conjugate was extracted from the bile samples using C18 cartridges. The crude bile extract was further purified on a Symmetry C18 column (3.9 × 150 mm) using a mixture of acetonitrile/0.05% trifluoroacetic acid as the mobile phases. The percentage of acetonitrile was increased from 10% to 70% in 18 min with the solvent delivered at a flow rate of 1.0 mL/min. The peak eluting at 7 min (t_R) was collected from several injections, pooled, and dried under vacuum to give a white powder (approximately 3.4 mg). The sample was analyzed by LC/MS/MS and shown to have $[M-H]^-$ at *m/z* 489 with characteristic fragment ions at *m/z* 175 (glucuronic acid), 243, and 313 (− glucuronic acid). ^{1}H NMR clearly showed the presence of glucuronic acid and two aromatic proton signals. The existence of an intact cyclopropyl moiety was also confirmed by NMR data. The glucuronide conjugate was dissolved in 1 mL of potassium phosphate buffer (0.1 M, pH 6.5) containing β-glucuronidase (1000 units). The mixture was incubated at 37 °C, and after 3 h, the reaction mixture was analyzed by HPLC using a Symmetry C18 column (3.9 × 150 mm). The column was eluted with a mixture of acetonitrile/0.05% trifluoroacetic acid (40:60, v/v) delivered at a flow rate of 1.0 mL/min. The peak eluting at 3.5 min was collected from several injections and analyzed by LC/MS/MS. The fractions containing metabolite **M7** were pooled and samples dried under vacuum to give approximately 2.6 mg of compound. ^{1}H NMR (DMSO-d_6) δ 0.71 (2H, m, cyclopropyl-CH_2), 0.85 (2H, m,

cyclopropyl-CH_2), 1.35 (1H, m, cyclopropyl-CH), 6.71 (1H, d, J = 8.5 Hz, aromatic 8-H), 7.34 (1H, dd, J = 8.4, 8.5 Hz, aromatic 7-H); ESI-MS m/z 313 ($[M-H]^-$).

Characterization of the Rat P450 Responsible for the Formation of GSH Adducts M3−M6. The formation of metabolites **M3**−**M6** was investigated by incubating DPC 963 with either noninduced or induced rat liver microsomes and with cDNA-expressed rat P450 enzymes. All incubations were performed either in the presence or in the absence of GSH. To further characterize the enzyme(s) involved in a particular reaction, the in vitro formation of the respective GSH adducts was studied using antibodies against the rat P450 isozymes and/or by employing selective chemical inhibitors.

Liver Microsomal Incubations. Unless stated otherwise, all microsomal incubations were carried out using the following protocol: rat liver control and induced microsomes (100 μg), ±NADPH (0.1 mM), ±GSH (1 mM), $MgCl_2$ (3 mM), DPC 963 (50 μM), with the final volume adjusted to 0.5 mL with 0.1 M phosphate buffer (pH 7.4). Incubations were carried out for 40 min and the reactions terminated by the addition of 1 mL of ice-cold acetonitrile. The samples were vortexed and centrifuged at 1600*g* for 10 min. The supernatant was separated and dried under nitrogen. The dried samples were reconstituted in 200 μL of the HPLC mobile phase and aliquots analyzed by LC/MS operated in the selected ion monitoring mode (SIM).

cDNA-Expressed P450 Supersome Incubations. To probe which rat isozyme was responsible for the formation of GSH adducts **M3**−**M6**, incubations were carried out with commercially available rat P450 supersomes including 1A1, 1A2, 2A2, 2B1, 2C6, 2C11, 2D1, 2D2, 3A1, and 3A2. Each of these supersomes consisted of the cytochrome P450, the P450 reductase, and cytochrome b_5. The incubation mixtures consisted of the supersomes (50 pmol of P450), NADPH (0.1 mM), DPC 963 (50 μM), $MgCl_2$ (3 mM), and 0.1 M phosphate buffer. Incubations were performed in the presence or absence of GSH (1 mM). The final volume of incubation was adjusted to 0.5 mL. The mixtures were incubated for 40 min, after which the reactions were terminated, extracted, and analyzed as described above.

Inhibitory Studies with Antibodies. Dexamethasone-induced rat liver microsomes were used in immunoinhibition experiments. The incubations consisted of microsomes (10 μg), anti-rat CYP enzyme polyclonal antiserum or anti-rat CYP antibody (100 μg), $MgCl_2$ (3 mM), NADPH (1 mM), DPC 963 (50 μM), and 0.1 M phosphate buffer (pH 7.4) in the presence of GSH (1 mM) in a final volume of 0.5 mL. Microsomes were preincubated with individual antibodies for 15 min at room temperature, followed by addition of other components. The incubations were carried out for an additional 40 min, after which the reaction was terminated by the addition of 1 mL of ice-cold acetonitrile. The samples were analyzed by LC/MS SIM as described above.

Inhibitory Studies with Chemical Inhibitiors. Inhibition of **M3**−**M6** GSH adduct formation was assessed by known chemical inhibitors for P450s which have been well characterized for human P450s (*9*). We attempted to use these chemical inhibitors so that we could gain information about the rat P450 that might be involved in the production of metabolites **M3**−**M6**. The incubation consisted of dexamethasone-induced rat liver microsomes (100 μg), NADPH (0.1 mM), $MgCl_2$ (3 mM), GSH (1 mM), and various chemical inhibitors used at two concentrations. The following selective inhibitors were examined for their effect: furafylline (10 and 25 μM), D609 (10 and 25 μM), sulfaphenazole (10 and 25 μM), troleandomycin (10 and 25 μM), and ketoconazole (1 and 10 μM). Furafylline, D609, and troleandomycin were preincubated for 20 min with the microsomes and NADPH in the presence or absence of GSH (1 mM) before adding DPC 963 (50 μM). The volume of each incubation was adjusted to 0.5 mL with 0.1 M phosphate buffer (pH 7.4). The incubations were carried out at 37 °C for 40 min. To terminate the reaction, 1 mL of ice-cold acetonitrile was added to the incubation mixtures, and the samples were vortexed and centrifuged. The supernatants were separated and dried under nitrogen. The residues were reconstituted and analyzed by LC/MS as described above.

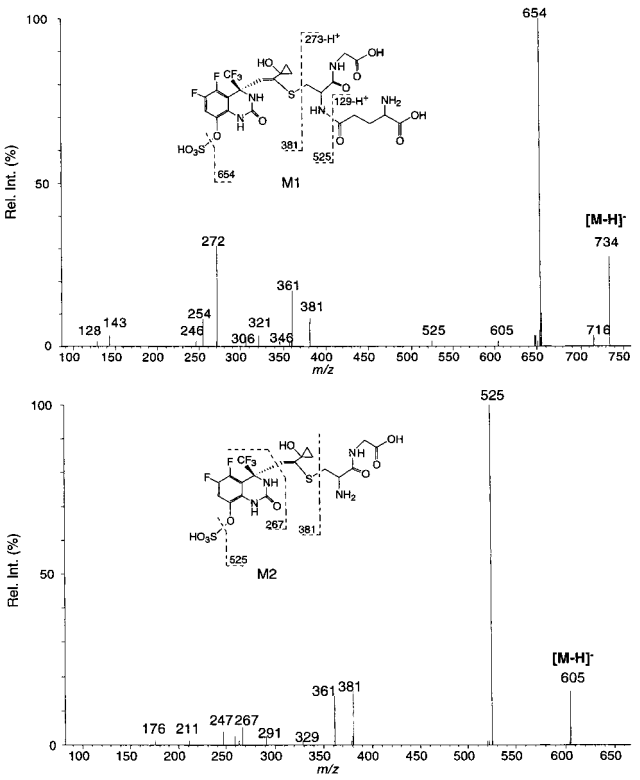


Figure 2. LC-ESI/MS/MS spectra of the glutathione adduct **M1** (top) isolated from rat bile and the cysteinylglycine conjugate **M2** (bottom) isolated from rat urine.

Results

Characterization of GSH-Derived Conjugates M1 and M2. Metabolite **M1** was found to be a GSH-sulfate diconjugate of dihydroxylated DPC 963. LC/MS/MS analyses (Figure 2) of **M1** showed a characteristic loss of a sulfate group (−80 amu) from the parent ion ($[M-H]^-$ at m/z 734). The total addition of 419 amu to the molecule suggested that this metabolite was formed by an initial hydroxylation with subsequent phase II conjugation reactions. Because of the presence of a sulfate group, the mass difference (419 − 80 = 339) suggested the addition of two oxygen atoms (due to phase I oxidation) as well as a GSH moiety (339 − 32 = 307). The ion at m/z 525, a characteristic fragment by a loss of glutamate (129 amu) from the GSH (*10*), confirmed **M1** as a GSH conjugate. The MS/MS spectrum (Figure 2) of metabolite **M1** suggested that the GSH moiety was attached to the cyclopropyl ethynyl side chain as previously observed with efavirenz (*2*). An ion at m/z 381 indicated a fragment produced by cleavage of the cysteinyl C−S bond with the retention of sulfur by the drug molecule (Figure 2). ^{1}H-LC/NMR of metabolite **M1** present in rat bile provided conclusive evidence of a GSH adduct and the possible site of conjugation. The ^{1}H-LC/NMR showed signals for the aromatic proton (δ 7.40, 1H, dd, J = 7.2, 10.3 Hz) which was coupled to the two fluorines (positions 5 and 6). A singlet at δ 6.40 was assigned to the alkene proton, and multiplets at δ 1.01 and 1.05 were assigned to the cyclopropyl protons (Figure 3). The characteristic multiplet for the cyclopropyl methine proton seen in ^{1}H NMR (at approximately δ 1.3) was distinctly absent as was demonstrated by total correlated spectroscopy (TOCSY,

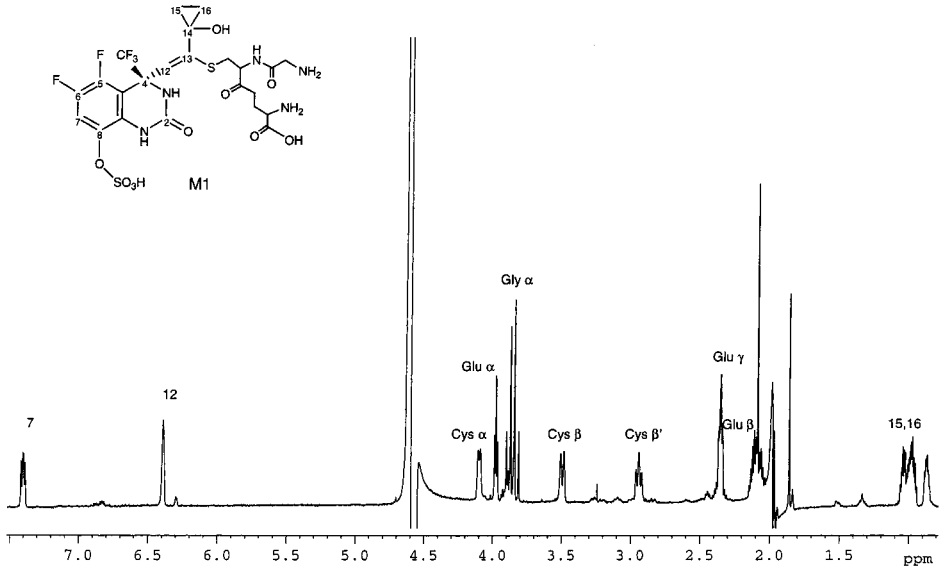


Figure 3. ^{1}H-LC/NMR of the glutathione conjugate **M1** isolated from rat bile.

data not shown). The GSH protons were assigned by TOCSY experiments to be at δ 4.10 (Cys α), 3.98 (Glu α), 3.85 (Gly α), 3.50 (Cys β), 2.94 (Cys β'), 2.35 (Glu γ), and 2.12 (Glu β). The ^{1}H-LC/NMR of the isolated **M1** clearly showed the presence of GSH, the loss of the aromatic proton on C8, and the appearance of a new singlet at 6.40 ppm (Figure 3). The proton at 6.40 ppm was assigned to position 12 (Figure 3) based on ^{1}H−^{19}F long-range couplings. The absence of the cyclopropyl methine proton, which was replaced by a hydroxyl group, was confirmed by TOCSY (data not shown). The hydroxylation at the cyclopropyl methine is consistent with the slight downfield shift of the cyclopropyl methylene protons. Comparisons with reference metabolite (^{1}H NMR of glutathione-sulfate diconjugate of dihydroxylated efavirenz, *2*) suggested that the hydrogen sulfate was at the C8 position and not on C14. The geometry of the substituents on the double bond was not determined. Metabolite **M2**, isolated from rat urine, was identified as a cysteinylglycine-sulfate diconjugate with [M−H]$^-$ at *m*/*z* 605. It showed a similar fragmentation pattern (Figure 2) as metabolite **M1**. However, **M2** showed [M−H]$^-$ which was 129 amu less than that of metabolite **M1**. Fragment ions at *m*/*z* 381 and 267 confirmed that this was an S-linked hydroxylated metabolite probably formed by the hydrolysis of glutamate (−129 amu) from metabolite **M1**. The structure of metabolite **M2** was confirmed by isolating sufficient quantities from rat urine and carrying out one-dimensional proton NMR. The chemical shifts of protons on positions 7, 12, 15, and 16 (Figure 3) were similar to the corresponding protons in metabolite **M1**. The long-range ^{1}H−^{19}F coupling/decoupling experiments confirmed the alkene proton on C12. The major difference between the ^{1}H NMR spectra (data not shown) of **M1** and **M2** was the absence of any glutamic acid proton signals for **M2**.

Characterization of GSH Adducts (M3 and M4) Derived from the Cyclobutenyl Ketone Intermediate. Metabolites **M3** and **M4** were separable on HPLC and confirmed as stereoisomers derived from a putative cyclobutenyl ketone intermediate. The structures of these two GSH adducts isolated from rat bile are shown in Scheme 1. The MS/MS analyses (Figure 4) of these adducts exhibited the same pseudomolecular ion ([M+H]$^+$)

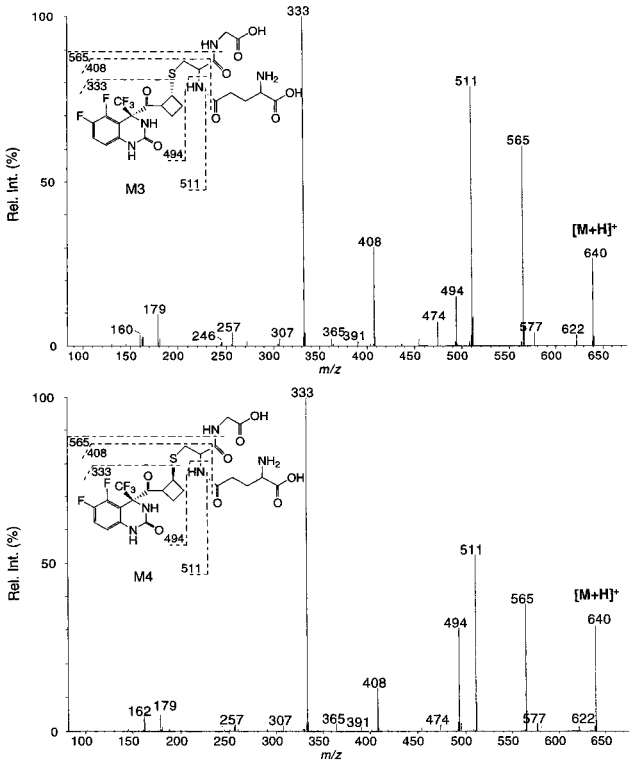


Figure 4. LC-ESI/MS/MS spectra of the glutathione adducts **M3** (top) and **M4** (bottom) isolated from rat bile.

at *m*/*z* 640 with identical fragment ions at *m*/*z* 565, 511, 494, 408, and 333 (the base peak). The ions at *m*/*z* 565 and 511 were characteristic fragments produced by a loss of glycine (75 amu) and glutamate (129 amu), respectively (*10*). The ion at *m*/*z* 408, a characteristic fragment produced from the internal cleavage of aliphatic thioether conjugates (*10*), suggested that the GSH moiety was attached to the cyclopropyl ethynyl side chain. Further tandem mass spectrometry studies of the ion at *m*/*z* 333 (MS/MS/MS, data not shown) showed that the quinazolinone ring of adduct **M3** or **M4** was intact. The structures of these two GSH adducts were elucidated by ^{1}H and TOCSY NMR experiments. The NMR data for one of the adducts (**M3**) are shown in Figures 5 and 6. The ^{1}H NMR spectrum clearly showed the characteristic signals for the

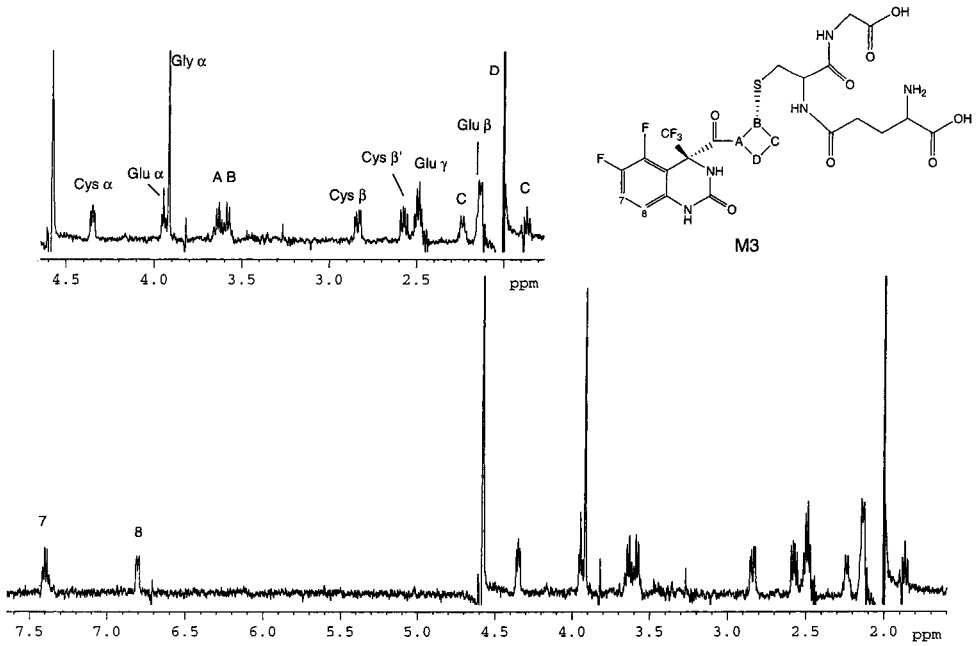


Figure 5. ^{1}H-LC/NMR of the glutathione adduct isomer **M3** isolated from rat bile.

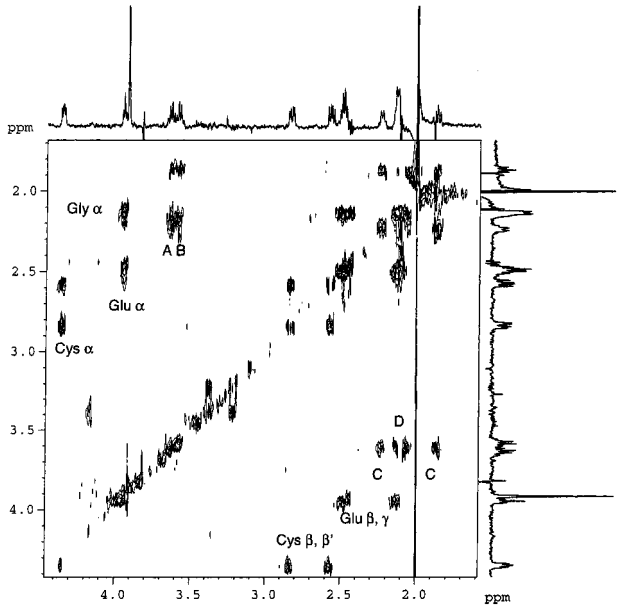


Figure 6. Total correlated spectroscopy (TOCSY) of the glutathione adduct isomer **M3** isolated from rat bile.

GSH protons at δ 4.35 (Cys α), 3.94 (Glu α), 3.92 (Gly α), 2.82 (Cys β), 2.58 (Cys β'), 2.48 (Glu γ), and 2.12 (Glu β). The presence of the aromatic protons on C7 and C8 was clearly demonstrated by ^{1}H NMR (Figure 5). However, the characteristic multiplet for the cyclopropyl protons seen in ^{1}H NMR of the parent compound was absent. Instead, characteristic signals for a cyclobutane ring were present (Figure 5). The protons of the cyclobutane ring were further demonstrated to be part of the same spin system by TOCSY (Figure 6). The ^{1}H NMR analyses of the other GSH adduct **M4** exhibited a similar spectrum with slight differences in the chemical shifts and the split patterns of the cyclobutyl ring protons (data not shown) as compared to **M3**.

The structures of two corresponding *N*-acetylcysteine conjugates, present in rat urine, were confirmed by mass spectral and NMR analyses. Both of these mercapturic acids showed the same pseudomolecular ion ($[M+H]^+$) at *m*/*z* 496 with a mass spectral fragmentation pattern similar to those of the GSH adducts (data not shown). The ^{1}H NMR spectra (data not shown) were similar to those of GSH adducts except for the distinct absence of any proton signals from the glutamic acid and glycine. The following chemical shifts were observed for the protons of one of the isolated *N*-acetylcysteine adducts: δ 1.85 (2H, m, cyclobutyl), δ 2.10 (2H, m, cyclobutyl), δ 2.24 (2H, m, cyclobutyl), δ 2.65 (1H, m, cysteine β), δ 2.87 (1H, m, cysteine β'), δ 3.55 (1H, q, cyclobutyl), δ 3.62 (1H, q, cyclobutyl), δ 4.35 (1H, m, cysteine α), δ 6.80 (1H, d, Ar-8), δ 7.39 (1H, q, Ar-7). The singlet for the methyl group of the *N*-acetyl appeared at 1.90 ppm. The other *N*-acetylcysteine adduct isolated from rat urine was not further characterized.

Characterization of GSH Adducts (M5 and M6) Derived from the Benzoquinone Imine Intermediate. LC/MS/MS analyses of rat bile showed two well-resolved GSH adducts (**M5** and **M6**) with the same pseudomolecular ion ($[M+H]^+$ at *m*/*z* 620). The MS/MS analysis of these adducts demonstrated slightly different fragment ions for **M5** and **M6**. The major fragment ions produced from **M5** were at *m*/*z* 545, 491, 474, 425, 408, 388 (the base peak), and 345; in contrast, the major fragment ions observed with **M6** were at *m*/*z* 602, 557, 545, 491, 371 (the base peak), and 345 (Figure 7). The ions at *m*/*z* 545 and 491 were characteristic fragments produced by a loss of glycine (75 amu) and glutamate (129 amu), respectively. The presence of the ion at *m*/*z* 425 (relatively in high abundance in the **M5** spectrum but fairly low in **M6**) produced by losing the glutamate (129 amu) and the cyclopropyl acetylene group (66 amu) indicated that the conjugation occurred on the aromatic ring. The diagnostic ion at *m*/*z* 345 was derived from cleavage of the cysteinyl C−S bond with the retention of the sulfur on the aromatic ring. Compared to the molecular weight of DPC 963, the addition of 29 amu to the ion at *m*/*z* 345 (316 − 19 + 16 + 32 = 345) suggested that a fluorine was replaced by an oxygen atom. The origin of major fragment ions for both **M5** and **M6** is

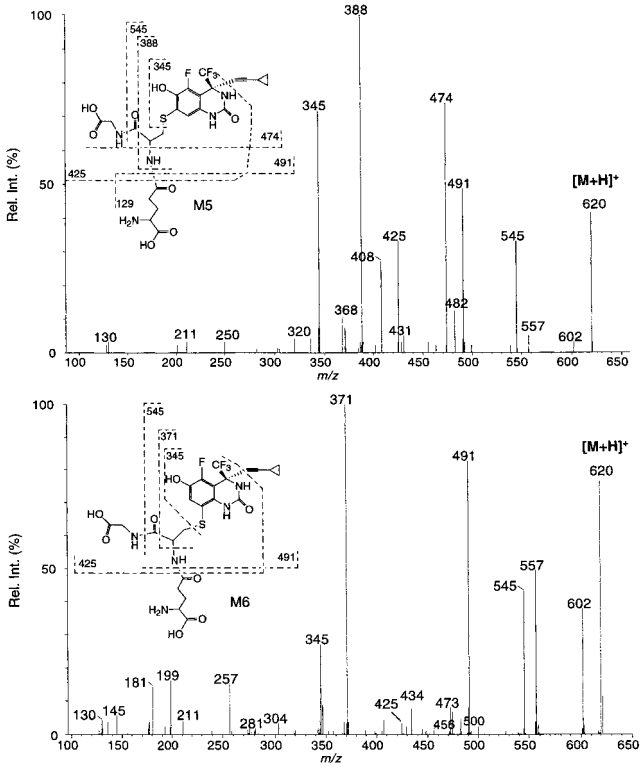


Figure 7. LC-ESI/MS/MS spectra of the glutathione adducts **M5** (top) and **M6** (bottom) isolated from rat bile.

postulated in Figure 7. The structures of these two GSH adducts were further elucidated by ^{1}H and ^{19}F NMR experiments. The NMR data for one of these adducts (**M5**) are shown in Figures 8 and 9. The ^{1}H-LC/NMR spectrum clearly showed the characteristic proton signals for the cyclopropyl group as well as for the GSH moiety (Figure 8). The GSH protons were assigned at δ 4.42 (Cys α), 3.85 (Glu α), 3.74 (Gly α), 3.29 (Cys β), 3.14 (Cys β'), 2.48 (Glu γ), and 2.05 (Glu β). In addition, the presence of the proton at C8 was unequivocally demonstrated as a singlet at δ 6.62 while the proton at C7 was distinctly absent. Further ^{19}F-LC/NMR characterization clearly showed the absence of fluorine at C6 and retention of the fluorine at C5 (δ 134.7, d) as compared to the fluorine signals of DPC 963 (see the inset in Figure 9). As a comparison, the ^{1}H-LC/NMR spectrum of the other GSH adduct (**M6**) is shown in Figure 10. The presence of a signal as a doublet (δ 7.26, d, J = 7.3 Hz) for the proton on C7 is consistent with the expected coupling to the fluorine at C5.

The structures of two corresponding *N*-acetylcysteine conjugates formed from **M5** and **M6** were confirmed by mass spectral and NMR analyses. Both mercapturic acids, found in rat urine, showed the same pseudomolecular ion ($[M+H]^+$) at *m*/*z* 476 with the mass spectral fragmentation patterns similar to the corresponding GSH adduct. The ^{1}H NMR spectra were similar to those of GSH adducts except for the distinct absence of glutamate and glycine proton signals at δ 2.08 (2H, Glu β), 2.42 (2H, Glu γ), 3.84 (1H, Glu α), and 3.74 (2H, Gly). The signals for the protons of *N*-acetylcysteine adducts matched those of the corresponding GSH adduct (see Figures 3 and 5 for the assignment for signals). The following chemical shifts were observed for the protons of one of the isolated *N*-acetylcysteine adducts: δ 0.27 (2H, m, cyclopropyl), 0.46 (2H, m, cyclopropyl), 1.29 (1H, m, cyclopropyl), 3.22 (1H, m, cysteine β), 3.45 (1H, m, cysteine β'), 4.42 (1H, m, cysteine α), 6.64 (1H, s, Ar-H). The singlet for the methyl group of the *N*-acetyl appeared at 1.85 ppm. In addition, ^{19}F NMR clearly showed the presence of signals for the fluorine at C-5 and the absence of fluorine at C-6. The other *N*-acetylcysteine adduct isolated from rat urine was not characterized by ^{1}H and ^{19}F NMR.

LC/MS analyses of bile samples from rats dosed with DPC 963 showed the presence of a number of GSH conjugates including **M1** and **M3**−**M6** (Scheme 1). However, the glucuronide and sulfate conjugates of the aromatic ring hydroxylated metabolite were the major drug-related components in bile and urine.

Characterization of the Rat P450 Responsible for the Formation of GSH Adducts M3−**M6.** Several approaches were used to identify the rat P450 enzyme(s) responsible for the formation of GSH adducts **M3**−**M6**. The studies using control and induced rat liver microsomes demonstrated a marked difference in the formation of respective GSH conjugates. It was found that greater quantities of the GSH adducts **M3**−**M6** were produced in the presence of dexamethasone-induced rat microsomes as compared to control (saline-treated) mi-

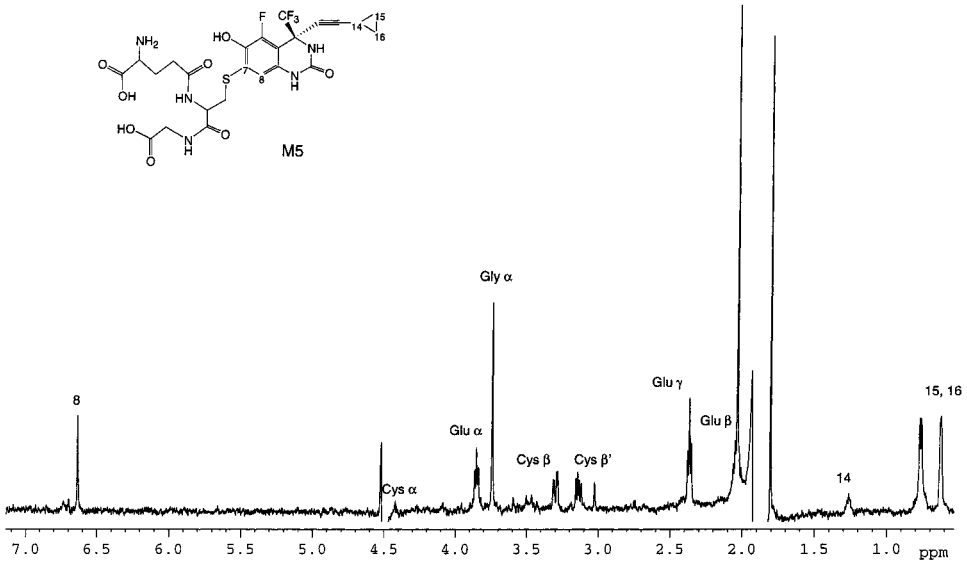


Figure 8. ^{1}H-LC/NMR of the glutathione adduct isomer **M5** isolated from rat bile.

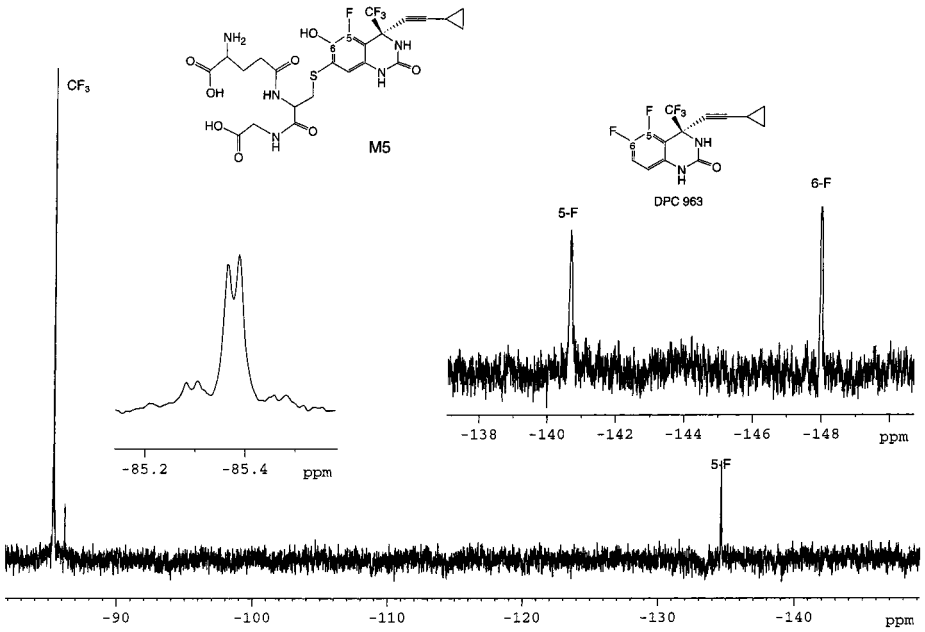


Figure 9. ^{19}F NMR of the glutathione adduct isomer **M5** isolated from rat bile.

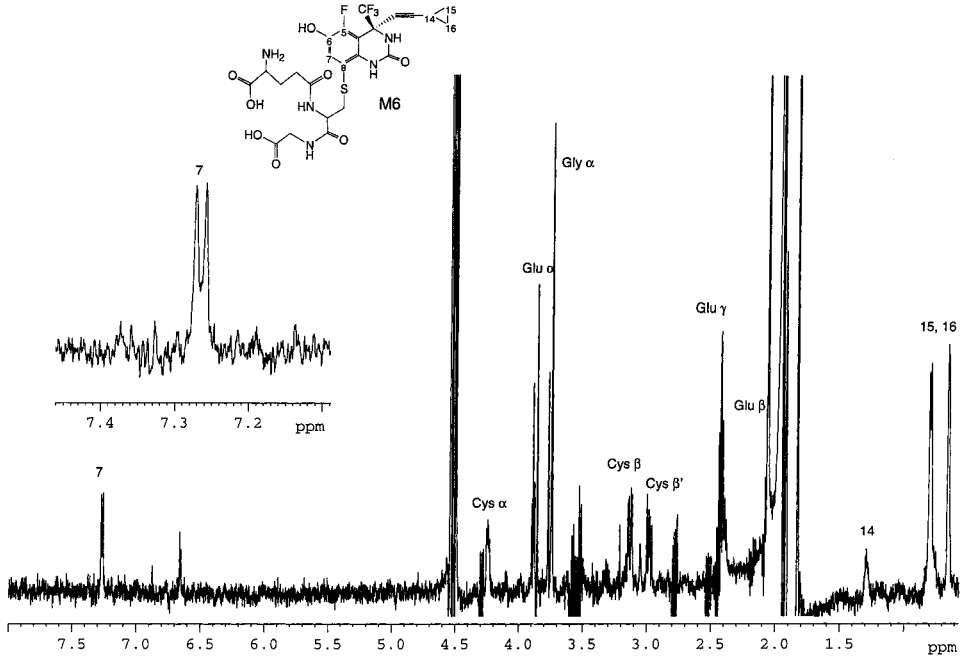


Figure 10. ^{1}H-LC/NMR of the glutathione adduct isomer **M6** isolated from rat bile.

crosomes. In addition to **M5** and **M6**, dexamethasone-induced rat microsomes produced much higher levels of metabolite **M7** as compared to microsomes induced with other chemicals. The cDNA-expressed rat P450 enzymes produced similar GSH adducts as microsomes. The rat P450 3A1 and 3A2 produced the highest levels of metabolites **M3**−**M7** as compared to other P450s, hence confirming the significant role of these isozymes in producing reactive metabolic intermediates of DPC 963. The rat P450 responsible for the formation of **M3**−**M7** was further examined by the inhibitory effects of the mono- and polyclonal antibodies to rat P450 enzymes. The anti-rat P450 3A1/3A2 antibody inhibited production of **M3**−**M6** by about 85−90% as compared to the control. Studies carried out with the antibody against P450 1A2, 2B1/2, 2C11, and 3A1 showed much less extent of inhibition on the formation of these conjugates. The reduction in the formation of GSH adducts in the presence of other anti-rat P450 antibodies could possibly be due to cross-reaction with P450 3A1/3A2 isozymes. Nonetheless, the contribution from other P450 isozymes capable of catalyzing the same metabolic reactions cannot be ruled out. Further evidence for the rat P450 3A1/3A2 being responsible for the production of **M3**−**M6** was obtained by inhibiting microsomes with chemical inhibitors. Studies with chemical inhibitors specific for human P450 isozymes (*9*) supported the involvement of rat P450 3A1/3A2 in forming reactive metabolic intermediates from DPC 961, a close analogue of DPC 963 (*5*). Incubation of microsomes in the presence of TAO or ketoconazole resulted in marked inhibition (>70%) in the formation of each DPC 963 GSH adduct **M3**−**M6**. In contrast, the inhibitors for other P450 enzymes [furafylline (P450 1A2, 10 μM), D609 (P450 2B1/2, 10 μM), and sulfaphenazole

(P450 2C9, 10 μM)] had no effect on the production of these GSH conjugates. In the absence of enzyme kinetic data and the inability to quantitate each of the GSH adducts accurately, it was impossible to obtain the exact contributions each P450 made toward the formation of the reactive intermediates. However, the studies conducted with cDNA-expressed P450s, chemical inhibitors, and P450 antibodies suggested that P450 3A1/3A2 played a dominant role in forming these metabolites.

Discussion

The structural differences between DPC 963 and its analogues, DPC 961 and efavirenz, are shown in Figure 1. The metabolism of DPC 961 and efavirenz has been described previously (*1*, *2*, *5*). Compared to efavirenz, which has a cyclocarbamate fused to a chlorobenzene, DPC 963 consists of a cyclourea coupled to a difluorobenzene. DPC 963 and DPC 961 are both cycloureas, except the latter is fused to a chlorobenzene (see Figure 1). In our previous studies with the structural analogues efavirenz and DPC 961 (Figure 1), a number of unusual GSH adducts were found in the bile of rats (*2*, *5*). Due to slight structural differences between DPC 963 and these two compounds, it was of interest to see if DPC 963 was metabolized to produce similar GSH conjugates.

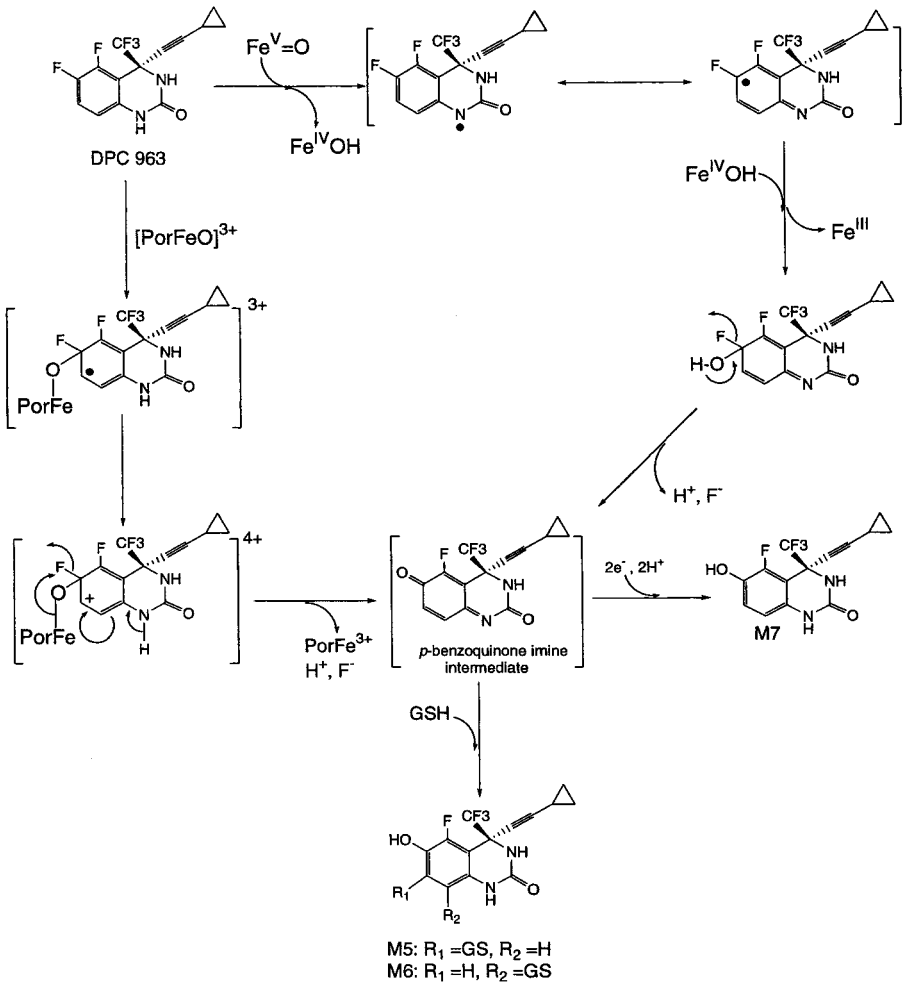
DPC 963 was metabolized via similar metabolic pathways as those described previously for DPC 961 and efavirenz. The glucuronide and sulfate conjugates of the aromatic ring hydroxylated metabolite were the major metabolites found in the urine of various species (*6*). In addition to the glucuronide and sulfate conjugates, a number of glutathione-derived adducts were also found in the bile and urine of rats dosed with DPC 963. The structures of these GSH adducts, isolated from rat bile and urine, were determined by LC/MS/MS and LC/NMR. Characterization of these GSH conjugates, which provided valuable insight into the identity of the reactive intermediates from which they were derived, revealed at least three distinct metabolic pathways leading to these adducts.

Analysis of bile from rats dosed with DPC 963 showed that one of the GSH adducts was similar to that produced by efavirenz. Consistent with the observations made with efavirenz glutathione adduct (*2*), this was truly a diconjugate possessing both a sulfate and GSH moieties. Significant quantities of the corresponding cysteinylglycine conjugate were also present in the urine of rats dosed with DPC 963. The formation of this GSH conjugate (**M1**) of dihydroxylated DPC 963 appeared to follow a similar metabolic pathway to efavirenz (Scheme 1). The compound was hydroxylated on the methine carbon of the cyclopropyl ring before glutathione *S*-transferase-catalyzed addition of GSH across the triple bond could take place, although the exact configuration of the substituted product remains unknown. As with efavirenz, the addition of GSH to the triple bond of the cyclopropanol derivative of DPC 963 was catalyzed by a glutathione transferase, and occurred in a species-specific manner only in rats and did not take place in cynomolgus monkeys and humans (*6*). The structure of metabolite **M1** indicated the existence of multiple steps that involved both phase I and phase II biotransforming enzymes prior to the formation of the final product.

It appears that the oxidation of the DPC 963 triple bond took place to form an unstable oxirene metabolic intermediate, which was responsible for the production of two isomeric GSH conjugates, **M3** and **M4** (Scheme 1). Such a formation of an oxirene intermediate has been postulated and reported before with the close analogue DPC 961 (*5*). As shown in Scheme 1, it is postulated that an initial oxidation of the triple bond, catalyzed by cytochrome P450, results in the formation of a putative oxirene intermediate which undergoes rapid rearrangement with the adjacent cyclopropyl ring to form a reactive cyclobutenyl ketone intermediate. The rearrangement to the cyclobutenyl ketone could take place via the ketocarbene *a* or through the intermediate *b* (*5*). The GSH conjugation with the intermediate occurs via 1,4-Michael addition to produce two isomeric GSH adducts. Such a ring expansion of the cyclopropane via metabolic activation of an adjacent triple bond was observed with DPC 961 and was absent in efavirenz-dosed animals. The ability of DPC 963 to undergo an oxidation of the triple bond perhaps depends on how it is docked at the active site on P450. The hydrogen bonding provided by the cyclourea moiety of DPC 961 and DPC 963 may make it favorable for the triple bond to undergo oxidation. Protein−substrate hydrogen bonding can dictate a catalytically favorable orientation of substrate within the P450 active site (*11*). As discussed previously (*5*, and references cited therein), the postulated occurrence of oxirene as an intermediate during chemical and enzymatic oxidation of alkynes has been previously reported. However, prior to these studies there was no direct evidence for the existence of such oxirenes mainly due to the high reactivity of these intermediates. By introducing a cyclopropyl substituent, which has been widely used to probe enzymatic mechanisms (*12*), and by using a ^{13}C analogue, the transient formation of the oxirene intermediate was demonstrated via ^{13}C NMR studies and by identifying GSH adducts that were derived from the rearranged intermediate, a cyclobutenyl ketone (*5*).

In addition to these GSH adducts formed by two distinct metabolic pathways, structures of additional GSH conjugates, **M5** and **M6** (Scheme 1), found in rat bile pointed to the existence of yet another metabolic pathway. These were characterized as being ring-substituted regioisomers with only one fluorine remaining at the aromatic C5 position. It was also found that glucuronide and sulfate conjugates of the 6-hydroxy derivative (formed after defluorination at this position) were present in the bile of rats. It appears that the defluorination of DPC 963 took place preferentially at the aromatic C6 position. It is postulated that these GSH adducts (**M5** and **M6**) are a consequence of a putative *p*-benzoquinone imine intermediate produced via P450-mediated oxidative defluorination of DPC 963 (Scheme 1). Halogen-containing compounds are considered relatively harmless because of their chemical stability. However, removal of halogen substituents is thought to be a crucial step in the bioactivation to products ultimately responsible for the toxic effects. Various P450 enzymes play a pivotal role in this metabolic activation (*13*−*16*). DPC 963 was found to be defluorinated both in vivo and in vitro, resulting in products that could potentially lead to toxicities. This observation has implications in the search for new chemical entities for pharmaceutical development, since incorporation of a fluorine substituent in a drug has traditionally been viewed as a means of slowing down the metabolism or preventing bioactivation of compounds (*17*−*23*). More

Scheme 2. Postulated Mechanisms of Cytochrome P450-Mediated Oxidative Defluorination of DPC 963 Leading to the Formation of *p*-Benzoquinone Imine Intermediate



importantly, benzoquinone imine, being a "soft" electrophile with high reactivity, may covalently interact with cellular macromolecules and could elicit oxidative stress through redox cycling.

It appears that the *p*-benzoquinone imine derivative represents a likely candidate for the reactive intermediate, based on the structure of the GSH conjugates. The possibility of cytochrome P450-mediated formation of the *p*-benzoquinone imine metabolic intermediate from DPC 963 was demonstrated by rat liver microsomal incubations. Trapping the reactive intermediate with microsomes fortified with GSH produced two GSH conjugates identical to those found in vivo. The proposed metabolic pathway leading to the formation of these GSH adducts is shown in Scheme 1. Rat P450 3A1/3A2 enzymes catalyze the oxidative defluorination of DPC 963 to directly form the reactive *p*-benzoquinone imine intermediate. GSH adds to this intermediate via Michael addition, giving rise to two regioisomeric adducts, **M5** and **M6**. Metabolite **M5** was expected to form in greater quantities, since the anion resulting from GSH addition enjoys greater resonance stabilization. Indeed, it was found that **M5** was present in relatively greater quantities than **M6**, both in vivo and in vitro. It was also found that the addition of GSH to the benzoquinone imine intermediate took place nonenzymatically since both conjugates were formed with cDNA-expressed enzymes that apparently did not have any glutathione transferase activities. The presence of a 6-hydroxy derivative (**M7**) can be explained due to the possible reduction of benzoquinone imine with the two electrons coming from NADPH via P450 reductase. Since P450 can effectively transfer electrons from its reductase to many substrates with high enough oxidation potentials (*24*−*26*), it is not unreasonable to expect that the P450 reaction intermediate might also accept electrons in this fashion. The benzoquinone imine intermediate is an oxidant and a highly reactive electrophile. Nevertheless, in the presence of NAD(P)H or other reducing equivalents, the benzoquinone imine can be reduced to its nonreactive hydroquinone form.

One of the postulated mechanisms of DPC 963 defluorination involves an abstraction of a single electron by the active ferryl species of cytochrome P450, leading to a nitrogen-centered radical intermediate (Scheme 2). Delocalization of the unpaired electron on the nitrogen to the aromatic carbons affords the C6-centered radical. Recombination of the carbon radical with the iron-bound hydroxyl leads to an unstable "σ-complex" that loses a proton and fluoride anion, giving rise to the benzoquinone imine. A similar mechanism had been previously postulated for bioactivation of *para*-fluorinated anilines (*16*). The location of fluorine at the C6 position (*para* to the nitrogen of the cyclourea) on DPC 963 bears resemblance to these *para*-fluorinated anilines. Recent studies on the P450-catalyzed conversion of *para*-fluorinated anilines or phenols have indicated the direct formation of benzoquinone imines or benzoquinones, accompanied by loss

of a halogen anion upon the oxidation of these compounds (*16*, *27*, *28*). Alternatively, the P450 $(FeO)^{3+}$, a high-valent iron−oxo intermediate, adds to the pi electron system of the aromatic ring at C6 directly and forms a σ adduct with a delocalized unpaired electron at C7 and other positions (Scheme 2). Electron transfer within this σ adduct from the aromatic ring to the porphyrin−iron converts it to a σ cation adduct with a positive charge delocalized in the ring (see Scheme 2). Rearrangements of the bonds in this σ cation adduct may create the benzoquinone and the halogen anion. This postulated mechanism is similar to the one proposed previously for the metabolism of hexahalogenated benzenes (*15*).

Recently, a GSH conjugate of a COX-2 inhibitor possessing a fluorobenzene ring was described (*29*). The origin of this GSH conjugate was postulated to occur via an epoxide, which led to the formation of only one GSH adduct. However, with DPC 963, two GSH adducts were formed, indicating the involvement of a benzoquinone imine rather than an epoxide intermediate in the defluorination of the aromatic ring.

The P450 involved in the formation of oxirene and benzoquinone imine intermediate was investigated by using differently induced microsomes, anti-rat P450 antibodies, and selective chemical inhibitors, and by employing cDNA-expressed isozymes. It was demonstrated that dexamethasone-induced microsomes produced much higher levels of GSH conjugates **M3**−**M6** than the noninduced microsomes. These results suggested the involvement of P450 3A enzymes in catalyzing the metabolic reactions capable of producing the GSH adducts. It is known that CYP 3A2 is constitutively expressed in control male rat liver microsomes, making up approximately 5% of the total hepatic P450, while cytochrome CYP 3A1 is not constitutively expressed in either male or female rat liver microsomes (*30*). A significant induction of these enzymes is observed in rats treated with dexamethasone (*30*). The higher levels of GSH adducts produced by dexamethasone-induced rat liver microsomes further supported the involvement of CYP3A1/3A2 in forming reactive intermediates from DPC 963. Results from studies conducted with anti-rat P450 antibodies, chemical inhibitors, and cDNA-expressed P450s confirmed that CYP 3A1/3A2 played a major role in forming these reactive precursors to the GSH adducts **M3**−**M6**.

Overall, the metabolism of DPC 963 was similar to that of efavirenz in being principally converted to the glucuronide or sulfate conjugate of the ring-hydroxylated metabolite. However, changes in the fused cyclic ring of DPC 963, whereby the cyclocarbamate fused with a chlorobenzene was replaced with a cyclourea coupled with a difluorobenzene, led to significant differences in the metabolic activation between these two compounds. The most notable difference was the nature of GSH adducts produced by the two compounds. In rats, an initial hydroxylation on the cyclopropyl ring of efavirenz was found to be a prerequisite for enzymatic addition of GSH across the triple bond. However, in addition to the GSH conjugate described above, rats were able to directly oxidize the triple bond of DPC 963, resulting in an intermediate that was capable of 1,4-Michael addition with GSH. Another novel metabolic reaction observed in rats was the oxidative defluorination which produced a chemically reactive *p*-benzoquinone imine intermediate capable of reacting with a nucleophile such as GSH. The direct triple bond oxidation and oxidative defluorination of DPC 963 was found to be specifically catalyzed by rat P450 3A enzymes.

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