FORMATION OF UNUSUAL GLUTAMATE CONJUGATES OF 1-[3-(AMINOMETHYL)PHENYL]-N-[3-FLUORO-2‘-(METHYLSULFONYL)-[1,1’-BIPHENYL]-4-YL]-3-(TRIFLUOROMETHYL)-1H-PYRAZOLE-5-CARBOXAMIDE (DPC 423) AND ITS ANALOGS: THE ROLE OF γ-GLUTAMYLTANSPSEPTIDASE IN THE BIOTRANSFORMATION OF BENZYLAMINES

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ABSTRACT:

The role of γ-glutamyltranspeptidase (GGT) in transferring glutamate from endogenous glutathione (GSH) to the benzylamine moiety of a compound, such as 1-[3-(aminomethyl)phenyl]-N-[3-fluoro-2’-(methylsulfonyl) -[1,1’-biphenyl]-4-yl]-3-(trifluoromethyl)-1H-pyrazole-5-carboxamide (DPC 423), is described. Studies were performed with structurally related analogs of DPC 423 to demonstrate that this type of reaction was common to compounds possessing a benzylamine group. Synthesizing appropriate standards and confirming by liquid chromatography/mass spectrometry (LC/MS) and LC/NMR made unambiguous assignments of the structures of glutamate conjugates of DPC 423. The use of stable isotope-labeled GSH for metabolism studies has not been described before. In the present study, we report the novel use of deuterated GSH in conjunction with mass spectral analysis to demonstrate the glutamate transfer to the benzylamines in the presence of GGT. To further demonstrate that the α protons on the benzylamines and glutamate (as part of glutathione) were unaffected during the transpeptidation, these protons were replaced with deuterium. Acivicin (AT-125), a potent and selective inhibitor of GGT, was used to abolish the formation of the glutamate conjugates of DPC 423 in vitro and in vivo. This provided further evidence of the role of GGT in forming the glutamate conjugates of benzylamines. This study demonstrated conclusively that GGT was responsible for mediating the transfer of glutamic acid from GSH to the benzylamine moiety of a series of structurally related compounds.

The ability to characterize minor and unusual metabolites has been greatly accelerated with the introduction of versatile analytical techniques, such as liquid chromatography/mass spectrometry (LC/MS) and LC/NMR. Recently, we described the isolation and characterizing the transfer of glutamic acid from GSH to the benzylamine moiety of a compound, such as 1-[3-(aminomethyl)phenyl]-N-[3-fluoro-2’-(methylsulfonyl) -[1,1’-biphenyl]-4-yl]-3-(trifluoromethyl)-1H-pyrazole-5-carboxamide (DPC 423), is described. Studies were performed with structurally related analogs of DPC 423 to demonstrate that this type of reaction was common to compounds possessing a benzylamine group. Synthesizing appropriate standards and confirming by liquid chromatography/mass spectrometry (LC/MS) and LC/NMR made unambiguous assignments of the structures of glutamate conjugates of DPC 423. The use of stable isotope-labeled GSH for metabolism studies has not been described before. In the present study, we report the novel use of deuterated GSH in conjunction with mass spectral analysis to demonstrate the glutamate transfer to the benzylamines in the presence of GGT. To further demonstrate that the α protons on the benzylamines and glutamate (as part of glutathione) were unaffected during the transpeptidation, these protons were replaced with deuterium. Acivicin (AT-125), a potent and selective inhibitor of GGT, was used to abolish the formation of the glutamate conjugates of DPC 423 in vitro and in vivo. This provided further evidence of the role of GGT in forming the glutamate conjugates of benzylamines. This study demonstrated conclusively that GGT was responsible for mediating the transfer of glutamic acid from GSH to the benzylamine moiety of a series of structurally related compounds.

The ability to characterize minor and unusual metabolites has been greatly accelerated with the introduction of versatile analytical techniques, such as liquid chromatography/mass spectrometry (LC/MS) and LC/NMR. Recently, we described the isolation and characterization of unique acetaminophen peptide conjugates using these techniques (Mutlib et al., 2000b). The coupling of these acetaminophen peptide conjugates with glutamic acid was described. The elucidating of these unusual metabolites prompted us to investigate the nature of the enzyme(s) involved in such metabolic reactions. The involvement of γ-glutamyltranspeptidase (GGT) was proposed but not confirmed. The postulated role of GGT in forming some of these unusual metabolites of acetaminophen led us to investigate whether this enzyme plays an even greater role in disposing xenobiotics than we had previously envisioned.

GGT was first identified in kidney tissue and later shown to be present in serum and in all cells except muscle cells (Hanigan and Pitot, 1985). Evidence to date has demonstrated that the GGT is involved in the catabolism of GSH-conjugates of xenobiotics (Curthoys and Hughey, 1979). GGT cleaves GSH and GSH-conjugates extracellularly, leading to catabolites that can be reabsorbed into cells. GGT plays an important role in disposing xenobiotics than we had previously envisioned.

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GGT has also been postulated to be involved in transporting amino acids into cells via this glutamyl cycle (Meister, 1973; Tate and Meister, 1981; Meister and Anderson, 1983; Smith et al., 1991; Coomes, 1997; Griffith and Mulcahy, 1999). This translocation mechanism is mediated through the concerted action of several enzymes, one of them being GGT, which is located on the external part of the cell membrane. Here it forms γ-glutamyl amino acids from extracellular amino acids and intracellular GSH. The γ-glutamyl amino acids are translocated into the cell, where the intracellular enzyme γ-glutamyltranspeptidase (GGT) plays an important role in disposing xenobiotics than we had previously envisioned.
Dicyclohexylcarbodiimide (8 mg, 0.04 mmol) was added to a solution of N-acetylglutamate (8 mg, 0.04 mmol) in DMF (1 ml). The solution was stirred at room temperature for 30 min. To another vial, DPC 423 (22 mg, 0.04 mmol) and dimethylaminopyridine (6 mg, 0.05 mmol) were added in 1 ml of DMF and stirred at room temperature for 30 min. The entire solution in the first vial was added to the second vial, and the mixture was subsequently stirred for 30 min. At the end of the reaction, the organic solvents were removed under a stream of nitrogen, and the residue was reconstituted in 1 ml of 1:4 mixture of acetonitrile and 0.1% acetic acid. Aliquots of 100 μl were injected onto a semipreparative HPLC column (Beckman C18, 250 × 10 mm) (Beckman Instruments, Inc., Fullerton, CA). The separation of products was achieved using an isocratic mobile phase consisting of 1:1 mixture of acetonitrile and 0.1% acetic acid delivered at 3.5 ml/min. The products were monitored using a variable wavelength detector set at 254 nm. Two products, showing retention times at 7.3 and 8.6 min, were collected and submitted for LC/MS and NMR analyses. Furthermore, to confirm the identity of the metabolite present in rat bile, LC/MS/MS of the pseudomolecular ion at m/z 704 was done for both the standards and for the metabolite present in the bile. The retention times and mass spectral fragmentation patterns were then compared. The two isomers were spiked in bile samples containing the N-acetylglutamate conjugate of DPC 423 and the retention times compared.

**Synthesis of Deuterated Glutathione, D3-GSH** The deuterated glutathione was made using custom synthesized Fmoc-d8-Glu-α-O-t-butyler ester obtained from Resinatech (Beckman Instruments, Inc., Fullerton, CA). The peptide was synthesized on solid phase using Fmoc protection and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation on an ABI 433A peptide synthesizer from Applied Biosystems. During the first step, the Fmoc-Gly-resin (0.13 g, 0.1 mmol) by treatment with 20% piperidine/DMF; the resin was then washed with DMF and dichloromethane. The deprotection was followed by the conductance of the solution. After deprotection was completed, the next amino acid in the sequence (Fmoc-Cys, 1 mmol) was preactivated using a solution of HBTU (1 mmol) in DMF/H2O/hydroxybenzotriazole and disopropylethylamine. The activated amino acid solution was added to the resin. When the coupling was completed, the resin was washed, and the instrument was started on another cycle. The last coupling was carried out manually using Fmoc-d8-Glu-α-O-t-butyler ester (0.171 g, 0.4 mmol), HBTU (0.152 g, 0.4 mmol) and disopropylethylamine (14 μl, 0.8 mmol). The reaction was shaken for 2 h. Completion of the reaction was determined by a negative ninhydrin test. The resin was deprotected using 20% piperidine/DMF and was washed. TFA-catalyzed cleavage of the peptide from the resin was done using reagent K (King et al., 1990) [TFA (4 ml), H2O (0.2 ml), thioanisole (0.2 ml), ethanedithiol (0.1 ml), and melted phenol (0.28 ml)]. This was followed by precipitation from ethyl ether and freeze drying, which afforded the crude peptide that was purified by reverse phase HPLC (see below) to yield 3.8 mg of D3-GSH. Electrospray ionization-mass spectrometry (ESI-MS) showed MH+ at m/z 311.1, as expected.

HPLC purification was performed on a C18 semipreparative Vydac column (250 × 22 mm, 10 μm; Vydac, Hesperia, CA). The solvent system consisted of two components (solvent A, 0.1% TFA in water; solvent B, 90% aqueous acetonitrile containing 0.1% TFA). HPLC was performed using isocratic elution for 10 min with solvent A followed by a gradient from 0 to 30% B in 20 min. The solvent flow rate was 18 ml/min, and the components were detected by a UV detector with the wavelength set at 220 nm. The peak was collected from several injections and dried under vacuum before analyses by mass spectrometry.

**Synthesis of [13C3]Benzylamine, B** The synthesis of compound B, labeled with 13C and deuterium on aminomethyl group, is depicted in Fig. 2. Cyclization of 2-bromophenylhydrazine (1) with 4,4,4-trifluoro-1-(2-furyl)-1,3-butanedione (2) provided 3. Refluxing 3 with potassium cyanide (13C-labeled) and catalytic amount of cuprous iodide in N-methyl pyrrolidone (Carr et al., 1994) yielded 4. Lithium aluminum deuteride reduction of 4 followed by treatment with trifluoroacetic anhydride afforded 5. Oxidation of 5 with sodium chlorite led to carboxylic acid, the acid chloride of which was coupled with bipyrenil aniline to provide 8. Careful alkaline hydrolysis of 8 followed by acidification yielded the desired hydrochloride 9 (Galemmo et al., 1999). 1H NMR (500 MHz, dimethyl sulfoxide-d6) showed the absence of proton signals from the aminomethyl side chain as expected, with the rest of the 1H NMR spectrum being identical to benzylamine B. The ESI-MS mass spectrum showed an increment of 3 amu (two deuterium molecules and one 13C) giving the spectrum being identical to benzylamine.
fractions from C18 cartridges and from semipreparative HPLC column, aliquots (20–50 μl) were introduced to the mass spectrometer using the flow injection analyses method. The mobile phase consisted of a mixture of acetonitrile and 10 mM ammonium formate (pH 3.5) (1:1, v/v) delivered at a rate of 0.35 ml/min. LC/MS was carried out by coupling a Hewlett-Packard HPLC system (HP1100) to a Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). LC-ESI/MS was performed on the mass spectrometer operated in the positive ion mode. The glutamate conjugates were detected by operating the mass spectrometer either in the full-scan mode or by selected ion monitoring of the pseudomolecular ions. MS/MS of fragment ions on the LCQ mass spectrometer were obtained with 20 to 25% relative collision energy.

**Accurate Mass Measurement of the Glutamate Conjugate.** Accurate mass of the glutamate conjugate present in the bile of a rat dosed with 100 mg/kg of DPC 423 (compound A) was obtained on the QSTAR hybrid (quadrupole/time-of-flight) LC/MS/MS instrument (PE Sciex, Toronto, Canada) equipped with an electrospray ion source. An aliquot of bile was injected onto the mass spectrometer using the same chromatographic conditions as described above. A two-point internal mass calibration was carried out during the analyses. An accurate mass of the protonated glutamate conjugate was obtained, and an MS/MS experiment was performed to obtain the fragment ions.

**High-Field NMR.** All the spectra were obtained on a Bruker Avance 500 MHz NMR spectrometer (Bruker, Analytische, Karlsruhe, Germany), equipped with either a 2.5-mm 1H/13C inverse LC/NMR flow-probe (cell volume, 120 μl) or a 2.5-mm 1H/13C inverse conventional NMR probe. The structures of glutamate conjugates were determined from proton- and carbon-1-dimensional NMR and proton-proton total correlated spectroscopy, proton-carbon heteronuclear single quantum correlation, and long-range proton-carbon heteronuclear multiple bond correlation (HMBC) two-dimensional NMR experiments.

**In Vivo Studies in Dogs.** Bile duct-cannulated male beagle dogs (weighing between 10–12 kg) were administered with A (8 mg/kg, i.v.), and bile and urine were collected over 24 h. Compound A was prepared in a mixture of polyethylene glycol/water (10:90, v/v) and administered as an i.v. bolus. Predose bile and urine samples were also obtained. In another study, male and female beagle dogs (n = 2 animals per sex) were administered A at 30 mg/kg/day for 1 month. Compound A was prepared as a suspension in 0.5% methocel and administered orally at 10 ml/kg. Urine samples were collected on day 30 and stored frozen until analyzed.

**In Vivo Studies in Rats.** Male Sprague-Dawley rats (weighing between 250–350 g) with cannulated bile ducts were administered a single oral dose of either A, B or C at 100 mg/kg, and urine and bile were collected over ice. 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 (Ralston Purina, St. Louis, MO). The urine and bile samples were collected at 0- to 8- and 8- to 24-h time intervals and stored at −20°C until analyzed. The dosing volume was 5 ml/kg. In another study, male Sprague-Dawley rats (n = 2) were administered acivicin (10 mg/kg, i.v.) 1 h before dosing with A (100 mg/kg, p.o.). Control group of rats was dosed with normal saline instead of acivicin 1 h before dosing with A (100 mg/kg, p.o.). Urine samples (0–24 h) were collected over ice and stored at −20°C until analyzed. In another study carried out as part of safety assessment, male and female Sprague-Dawley rats (n = 9 animals per sex) were administered A at 300 mg/kg/day for 1 month. Urine samples were collected over ice after the first dose (day 1) and after the last dose (day 30). Samples were stored frozen at −20°C until analyzed.

**In Vivo Studies in Mice.** Male BALB-C mice (n = 10 per group) weighing between 20 to 25 g were dosed with either A (600 mg/kg, p.o.) or A (600 mg/kg, PO) with acivicin (10 mg/kg, i.v.). Acivicin (prepared in normal saline) was administered via the tail vein at a dosing volume of 5 ml/kg 1 h before dosing with A. Compound A was prepared as a suspension in 0.5% methocel and administered orally at 10 ml/kg. Mice were housed in metabolism cages, and urine was collected over dry ice 0 to 24 h postdose. The urine samples were kept frozen at −20°C until analyzed.

**Measurement of Total GSH Levels in Kidneys of Mice Given DPC 423 (A).** Kidneys were removed from groups of BALB-C mice that were orally administered with either compound A (600 mg/kg/day) or given the dosing vehicle (control group) over 7 days. After the last dose (day 7), mice (n = 2) were sacrificed at 0, 1, 2, 4, 6, and 8 h, and kidneys were removed from each animal and snap-frozen over dry ice. Kidneys were removed from the control group to correct for the diurnal variation in tissue GSH concentrations.

The total glutathione content (GSH + oxidized glutathione) of kidneys were measured as described in the literature (Griffith, 1980). Kidneys were weighed and homogenized rapidly (less than 2 min) in 10 ml of distilled water. An aliquot of the homogenate (1 ml) was added to 0.5 ml of 10% 5-sulfosalicylic acid to precipitate the proteins. An aliquot (100 μl) of the supernatant was taken, and 24 μl of 25% triethanolamine (v/v) was added to neutralize the sample. An aliquot of the neutralized sample (25 μl) was mixed with 175 μl of NADPH followed by the addition of 25 μl of 5,5′-dithiobis-2-nitrobenzoic acid. The samples were incubated at 30°C for 5 min after which the UV absorbance at 412 nm was read. The standard curves were prepared using phosphate buffer (instead of the kidney homogenate) and treating the samples in the same manner as described above. A statistical difference in the levels of GSH was tested with an unpaired t test and judged significant if p < 0.05.

**In Vitro Studies.** Compounds A, B, B′, and C were incubated with GSH in the presence of the rat kidney S9 subcellular fraction. The incubation mixture consisted of rat kidney S9 (1.4 mg), substrate (100 μM), GSH (3 mM), MgCl₂ (3 mM), and 0.1 M phosphate buffer to a final volume of 1 ml. In a separate experiment, acivicin (a specific inhibitor of GGT) was added to the incubation medium at a concentration of 0.25 to 5 mM, and the samples were preincubated for 40 min before the addition of the substrates (Reed et al., 1980). To demonstrate the transfer of glutamate from GSH, deuterium-labeled GSH was used instead. To rule out the possibility of direct conjugation between endogenous glutamic acid and the benzylamines, 14C-labeled glutamic acid was included in some of the incubation mixtures instead of glutathione. The mixtures were incubated for 1 h, after which 2 ml of cold acetonitrile were added and the proteins precipitated. After centrifuging the samples at 3500g for 5 min, the supernatants were transferred to clean culture tubes and dried under a stream of nitrogen at 25°C. The dried extracts were reconstituted in the HPLC mobile phase (15% acetonitrile/85% ammonium formate, pH 4.0) before analyzing by LC/MS (see above) or by radiochemical detector.

**HPLC with Radiochemical Detector.** Analysis of in vitro extracts were conducted by HPLC using a Radiomatic FLO-ONE/Beta Model A500 radioactivity detector (Packard Instruments, Meriden, CT). The dried extracts were reconstituted with water/acetonitrile (90:10 v/v) and then injected onto Waters...
**Fig. 4.** LC/MS/MS of the glutamate conjugate excreted in the urine of a rat dosed with 100 mg/kg of the benzylamine A.

**Fig. 5.** $^1$H NMR of the synthetic standard of N-acetylglutamate conjugate of DPC 423 (A).

The metabolite isolated from rat bile showed identical spectrum to the standard. The structure of the conjugate with the assigned protons is shown in Fig. 6.
Symmetry C\textsubscript{18} column (150 × 2.1 mm), and the components were resolved using the same gradient HPLC system as described above. The scintillant was introduced at a rate of 1.2 ml/min and mixed with the HPLC eluent before being introduced into the flow cell of the radiochemical detector.

Results

Metabolism of DPC 423 and its analogs to Glutamate Conjugates. The in vivo and in vitro metabolism of DPC 423 and its analogs B, B', and C (Fig. 1) were found to be similar and are discussed in detail elsewhere (Mutlib et al., 2000a). Compounds A and C are regioisomers with the former being an ortho-substituted analog and the later a para-substituted benzylamine. Compounds B and B' are identical to C with the exception of a sulfonamide moiety instead of a methylsulfone group. Compounds B and B' differ from each other, with the later being labeled with \textsuperscript{13}C and deuterium on the amino-methyl side chain. The glutamate conjugates of A, B, B', and C detected in urine and bile of samples of rats showed an addition of 129 amu to the molecular weights of the parent compounds. The acetylated glutamate conjugates showed a further addition of 42 amu, giving a net increase of 171 amu in the molecular weights of the benzylamines. The glutamate conjugates that were produced by A, B, B', and C showed \textit{MH}^+ at \textit{m/z} 662, 663, 666, and 662, respectively. The MS/MS spectra for each of these pseudomolecular ions showed major fragment ions (corresponding to the aglycones) at \textit{m/z} 533, 534, 537, and 533, respectively. The corresponding acetylated glutamate conjugates showed the protonated parent ions at \textit{m/z} 704, 705, 708, and 704, respectively. These conjugates were easily detected in the rodents (bile and urine of rats and in urine of mice), whereas studies conducted in dogs showed a total absence of these conjugates in either bile or urine. The excretion pattern of these glutamate conjugates in rat urine remained unchanged after 1 month of daily dosing with 300 mg/kg/day of DPC 423 (as compared with day 1 urine samples).

Characterization of the Glutamate Conjugate of DPC 423 (A). The LC/MS analyses of bile and urine from rats dosed with DPC 423 (A) showed the presence of both the glutamate and its acetylated derivative (Fig. 3). These two conjugates could be easily detected in the urine and bile of rats by operating the mass spectrometer in the selected ion monitoring mode. The limit of detection for the glutamate conjugates was estimated at 1 to 5 ng/ml. The MS/MS spectrum of the glutamate conjugate of A analyzed on the LCQ ion trap mass spe-
trometer is shown in Fig. 4. The accurate measurement of the glutamate conjugate of DPC 423 present in the bile of rats showed MH\(^+\) at \(m/z\) 662.1672 with an elemental composition of C\(_{30}\)H\(_{28}\)N\(_{5}\)O\(_{6}\)F\(_{4}\)S (calculated \(m/z\) 662.1696). The MS/MS spectrum from the time-of-flight mass spectrometer showed fragment ions similar to those obtained from the ion trap mass spectrometer. The masses of fragment ions observed were at \(m/z\) 645.1449, 599.1318, 533.1285, 516.1084, and 437.1067. The calculated values for these ions were at \(m/z\) 645.1431, 599.1376, 533.1270, 516.1005, and 437.1151, respectively. The accurate masses of the fragment ions confirmed the fragmentation pattern and supported the postulated structure depicted in Fig. 4. The structure of this conjugate was unequivocally confirmed by synthesizing the appropriate standards (acetylated glutamates) and comparing the LC/NMR data with that obtained for the isolated metabolite.

Since there were two possible ways that glutamic acid could be linked to the benzylamine (i.e., via the \(\gamma\)- or \(\alpha\)-carboxyl groups), the NMR data for both of standards (isomers \(X\) and \(Y\), respectively) were obtained. The NMR data clearly suggested that the glutamic acid was coupled to the benzylamine moiety via the \(\gamma\)-carboxyl group. The \(^1\)H NMR of one of the two synthetic standards (N-acetylated glutamate conjugates of A, isomer \(X\)) is shown in Fig. 5. The \(^1\)H NMR of the N-acetylglutamate conjugate of A showed the characteristic signals of intact A and those of the N-acetylglutamate moiety: \(\delta\) at 7.95 (1H, NH, d), 4.10 (1H, CH-CH\(_2\), m), 2.20 (2H, CH\(_2\)-CO, m), 1.95 (1H, CH\(_2\)-CH\(_2\)-CH\(_2\), m), 1.80 (3H, CH\(_3\)-CO, s), 1.78 (1H, CH\(_2\)-CH\(_2\)-CH\(_2\), m). It was shown that the structure of the acetylated glutamate conjugate corresponded to the one shown in Fig. 6. To determine the position of the attachment of N-acetylglutamate moiety, \(^1\)H\(^{13}\)C HMBC experiments were performed on both synthetic standards (Figs. 6 and 7). The \(^{13}\)C chemical shifts of the two glutamate carbonyl carbons were determined in this experiment. The \(\gamma\)-carbonyl of the glutamate moiety (position 35) shows connectivity to protons at positions 26, 27, 33, and 34. Likewise, the \(\alpha\)-carbonyl (position 28) shows connectivity to protons at positions 29 and 33. In the HMBC spectrum of isomer \(X\) of the N-acetylglutamate conjugate of DPC 423 (Fig. 6), correlations between the \(\gamma\)-carbonyl (position 35) and protons at positions 26 and 27 were observed. This indicates that the N-acetylglutamate moiety in isomer \(X\) is attached at the \(\gamma\)-position. The HMBC spectrum of the isomer \(Y\) (Fig. 7) shows correlations between the \(\alpha\)-carbonyl (position 28) and protons 26 and 27, indicating that, in
this case, the attachment is through the α-position. Hence, the structure of this isomer was unambiguously identified as a glutamate conjugate formed through the coupling of α-carboxylic acid of the glutamate with the benzylamine.

The glutamate conjugate of A isolated from rat bile and urine was also acetylated (acetic anhydride/pyridine) and found to match in its retention times and mass spectral fragmentation pattern with the isomer X. Rat bile was also spiked with both of the synthetic standards, and it was found that the isomer X matched in its retention time and mass spectral fragmentation with the metabolite.

**Evidence for the Transfer of Glutamate from Glutathione. In vitro studies.** The formation of the glutamate and N-acetylglutamate conjugates of the benzylamines represent an unprecedented metabolic route. After an unambiguous structural assignment of the N-acetylglutamate conjugate of A, an attempt was made to confirm the nature of the enzyme mediating this metabolic pathway. Furthermore, the origin of the glutamic acid linked to the benzylamine was sought. HPLC of the rat kidney S9 incubated with 14C-labeled glutamic acid showed an absence of any radiolabeled peak that corresponded to the glutamate conjugate. The results demonstrated that direct coupling of glutamic acid with the benzylamine moiety of DPC 423 did not take place. Experiments performed in vitro showed that the formation of the glutamate conjugate by rat kidney preparation was reduced significantly in the presence of various concentrations of acivicin (0.25–5 mM) (Fig. 8). A semiquantitative analysis of the in vitro extracts showed that acivicin (at 0.25–5 mM) reduced the levels of the conjugate to less than 5% of the control values. Furthermore, omitting NADPH from the incubation mixtures had no effect on the formation of these glutamate conjugates. However, the level of the glutamate conjugates formed by these benzylamines was significantly increased if the incubations were fortified with GSH. Control incubations not fortified with GSH produced the glutamate conjugates, although at much lower levels.

**In vivo studies.** Acivicin is a potent and selective inhibitor of GGT, and a 10-mg/kg i.v. dose has been shown to reduce the activity of this enzyme by almost 90% in rats (Elfarra et al., 1984). Studies conducted in mice showed that if the animals were predosed with acivicin (10 mg/kg, i.v.) before administering A, the formation of the glutamate conjugate was reduced significantly. DPC 423 is eliminated rapidly from mice and rats with a short elimination half-life of less than 2 h (data not shown). With such a short half-life of DPC 423 and potent inhibition of GGT by acivicin, the in vivo formation of the DPC 423 glutamate conjugate was reduced markedly. Selected ion monitoring of the glutamate conjugate (m/z 662) in the urine of mice predosed with acivicin showed significantly lower levels (90% reduction) of the glutamate conjugate compared with the control group. Similar reduction in the glutamate conjugate levels was found in rats administered acivicin before dosing with DPC 423.

**Discussion**

Species differences exist in the expression of GGT (Hinchman and Ballatori, 1990). The presence of glutamate conjugates of DPC 423 in urine could be attributed to the high levels of GGT present in the kidneys of rats and mice (Bartoli et al., 1978; McIntyre and Curthroy, 1980; Hinchman and Ballatori, 1990; Commandeur et al., 1995). Rodents (rats and mice) show the greatest expression of this enzyme in kidneys compared with other species. In addition to the species differences in the expression of this enzyme, there is ample evidence in the literature documenting the differences in the tissue distribution of this enzyme. For example, in rats the highest activity of GGT is
localized in the kidney, whereas liver and epithelial cells of the jejunum and bile duct show lower expression of this enzyme (Tate and Meister, 1981; Hinchman and Ballatori, 1990; Commandeur et al., 1995 and the literature cited therein). Hence, the GGT present in bile and bile ducts may explain the presence of the glutamate conjugates in the bile of rats administered compounds A to C. The absence of glutamate conjugates in dog bile and urine may be due to the lower expression of GGT in the kidneys, bile, and bile ducts of dogs compared with rats. To confirm that GGT played a role in forming these glutamate conjugates, a number of in vitro and in vivo studies were conducted. In vitro studies performed with rat kidney S9 showed the formation of glutamate conjugates of DPC 423 and its analogs. Furthermore, the formation of these conjugates was increased significantly when the incubation mixtures were fortified with GSH. Additional experiments employing acivicin, a potent in vitro inhibitor of GGT (Reed et al., 1980), showed a dramatic reduction in the levels of glutamate conjugates formed by DPC 423 and its analogs. The role of GGT and glutathione in forming these unique glutamate conjugates led us to investigate the possible mechanisms leading to such products.

The role of GGT in the synthesis of γ-glutamyl compounds, including γ-glutamyl-glutathione, is well documented (Abbott et al., 1986). It has also been postulated that GGT may play a role in the glutamyl cycle by acting as a transporter for amino acids (Meister and Anderson, 1983; Griffith and Friedman, 1991; Smith et al., 1991; Coomes, 1997). The mechanism involves the transfer of an element of glutamic acid from glutathione to an amino acid that is being transported across the membrane. The glutamate-amino acid complex is then hydrolyzed in the cell to liberate the amino acid. Glutamate is released as 5-oxoproline, which is converted back to glutamate by an ATP-dependent reaction. Glutathione is subsequently resynthesized from its three component parts: glutamic acid, cysteine, and glycine. However, in the presence of the benzylamines such as A and B, the glutamate transferred from endogenous glutathione is not released as oxoproline. It appears that the benzylamine compounds are mistakenly recognized as amino acids and subsequently transported by GGT into the cell as the glutamate conjugates. The glutamate is covalently bound to the benzylamines, and the complex does not appear to be a substrate for the enzyme (5-oxoprolinase) responsible for its hydrolysis. Rather, the glutamate conjugate is released in the urine or is further catabolized by the N-acetyltransferase to the N-acetylglutamate conjugate (Fig. 9). The interaction of these benzylamines with the endogenous pool of glutathione, especially in kidney, may lead to depletion of intracellular GSH. Studies conducted in mice given A at a high dose of 600 mg/kg led to reduction in total glutathione levels to almost 50% of control values at two time points (2 and 6 h postdose, data not shown). Microsomes prepared from kidneys of mice given either saline (control) or A showed no appreciable metabolic activity toward the formation of reactive metabolites (Mutlib et al., 2000a). Hence, it is postulated that the depletion of the GSH levels in kidneys of mice given A was probably due to the direct interaction of the compound with glutathione via the glutamyl cycle.

The use of deuterated glutathione and B' (deuterium and 13C-labeled B) demonstrated unequivocally the transfer of glutamate from glutathione to the benzylamine (Fig. 9). The LC/MS analyses of kidney incubation extracts showed that the glutamate conjugate of B' that was produced in the presence of D₂-GSH had a pseudomolecular ion at m/z 669 (all five deuterium molecules retained—two from the benzylamine and three from GSH, Fig. 10). As a comparison, when B (nonlabeled) was incubated with D₂-GSH the pseudomolecular ion at m/z 666 was observed (three deuterium molecules from D₂-GSH retained, Fig. 10). The retention of deuterium on both the glutamate
and B' indicated that the α protons were not involved in the transfer mechanism. The use of deuterium labeled GSH to study its interaction with the benzylamines was novel because it provided conclusive evidence for the transfer of glutamate moiety from glutathione. Stable isotope-labeled GSH has not been previously used in metabolism studies. In the present study, we have described the novel use of deuterated GSH in conjunction with mass spectral analysis to elucidate the mechanism of glutamate transfer to benzylamines. The deuterium-labeled GSH could also be used to study the propensity of compounds to form reactive metabolites in vitro. By using a 1:1 mixture of nonlabeled and labeled GSH, one would be able to detect the various GSH adducts, as determined by the appearance of twin ions (separated by 3 amu in this case) in the mass spectra. Consequently, this procedure will greatly accelerate the identification of GSH adducts present in biological extracts and, thus, provide a rapid screen for reactive intermediates formed from compounds in early discovery stages.

Most of the major metabolites formed from xenobiotics have been attributed to the P450 enzymes (Schenkman and Kupfer, 1982; Ortiz de Montellano, 1986; Guengerich, 1987) or non-P450 oxidases (Ziegler, 1988; Woolf, 1999 and the literature cited therein). However, often during the investigation of xenobiotic metabolism, small quantities of uncharacterized metabolites have been found. These metabolites, perhaps due to their unusual nature and scarce abundance, have largely been ignored or overlooked. Because the structures of these metabolites are unknown, the metabolic pathways or enzymes leading to these products largely remain undiscovered. Determining the structures of these minor metabolites has been difficult in the past. Recently, with the application of LC/NMR techniques (Spraul et al., 1993; Mutlib et al., 1995; Shockcor et al., 1996; Lindon et al., 1997), the characterization of novel metabolites (Mutlib et al., 2000b) that were once difficult to identify has become more facile. Identification of metabolites such as glutamate conjugates often prompt further investigation into the biochemical mechanisms or pathways leading to such products. In this study, the presence of unusual glutamate conjugates of benzylamines led us to conclude that GGT was one of the enzymes responsible for the biotransformation of benzylamines.

Glutamate conjugates formed by coupling of the γ-carboxyl group of glutamic acid with an amine moiety of a drug molecule has not been described before. However, a few reports have appeared in the literature describing the existence of such conjugates of endogenous compounds in invertebrates and in rat brain. Glutamate conjugates of histamine, dopamine, and 5-hydroxytryptamine have been found in the brain of the gastropod *Aplysia californica* (Stein and Weinreich, 1982; McCaman et al., 1985). The formation of these glutamate conjugates in gastropods is believed to be responsible for the inactivation of chemical messengers, such as dopamine in the brain. It was shown that the formation of the γ-glutamyl conjugate of histamine was mediated by γ-glutamylhistamine synthetase and not by GGT (Stein and Weinreich, 1982). Furthermore, it was shown that this enzyme was able to form the glutamate conjugate of histamine specifically using L-glutamate. Studies done in rat brains have demonstrated an existence of similar glutamate conjugates of dopamine and 5-hydroxytryptamine, present in very low quantities (Tsujii et al., 1977). The formation of these γ-glutamyl amines was found to be

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**FIG. 10.** A, LC-ESI/MS of the glutamate formed from d3-GSH and benzylamine B; B, LC-ESI/MS of the glutamate formed from d3-GSH and 13C/deuterium-labeled benzylamine B'.
mediated by GGT present in rat brain. In this study, we have unambiguously demonstrated that xenobiotics possessing a benzylamine moiety, such as DPC 423, are converted to γ-glutamyl products by mammalian GGT. Furthermore, it was shown that these glutamate conjugates were formed by GGT using glutathione as a donor of glutamic acid.

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References


