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Studies on the Metabolism of Tolmetin to the Chemically Reactive Acyl-Coenzyme A Thioester Intermediate in Rats

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Abbreviations:

i.p., intraperitoneal; Tol, tolmetin; Tol-*O*-G, tolmetin acyl glucuronide; CoA, Coenzyme A; Tol-CoA, tolmetin acyl coenzyme A thioester; Tol-Gly, tolmetin glycine conjugate; Tol-Tau, tolmetin taurine conjugate; ESI, electrospray ionisation; Tol-Car, tolmetin acyl carnitine ester; Tol-SG, tolmetin acyl-*S*-glutathione conjugate; Tol-OH, tolmetin hydroxy-metabolite; Tol-COOH, tolmetin carboxy-metabolite.

Abstract

Carboxylic acids may be metabolized to acyl glucuronides and acyl-coenzyme A thioesters (acyl-CoAs), which are reactive metabolites capable of reacting with proteins *in vivo*. In this study, the metabolic activation of tolmetin (Tol) to reactive metabolites and the subsequent formation of Tol-protein adducts in the liver were studied in rats. Two hours after dose administration (100 mg/kg, i.p.), tolmetin acyl-CoA (Tol-CoA) was identified by LC-MS/MS in liver homogenates. Similarly, the acyl-CoA dependent metabolites tolmetin-taurine conjugate (Tol-Tau) and tolmetin-acyl carnitine ester (Tol-Car) were identified in rat livers. In a rat bile study (100 mg/kg, i.p.), the *S*-acyl glutathione thioester conjugate was identified, providing further evidence of the formation of reactive metabolites such as Tol-CoA or Tol-acyl glucuronide (Tol-*O*-G), capable of acylating nucleophilic functional groups. Three rats were treated with clofibric acid (150 mg/kg/day, i.p. for 7 days) prior to dose administration of Tol. This resulted in an increase in covalent binding to liver proteins from 0.9 nmol/g liver in control rats to 4.2 nmol/g liver in clofibric acid treated rats. Similarly, levels of Tol-CoA increased from 0.6 nmol/g to 4.4 nmol/g liver following pre-treatment with clofibric acid, whereas the formation of Tol-*O*-G and Tol-Tau formation was unaffected by clofibric acid treatment. However, Tol-Car levels increased from 0.08 to 0.64 nmol/g following clofibric acid treatment. Collectively, these results confirm that Tol-CoA is formed *in vivo* in the rat and that this metabolite can have important consequences in terms of covalent binding to liver proteins.

Introduction

Drugs with a carboxylate functional group can be metabolized to chemically reactive metabolites that react with nucleophilic amino acids of proteins to form drug protein adducts (Boelsterli, 2002; Bailey and Dickinson, 2003). Tolmetin (Tol; 5-[4'-methylbenzoyl]-1-methylpyrrole-2-acetic acid) is an example of a carboxylic acid containing drug, which in humans is partly metabolized to the acyl glucuronide (Hyneck et al., 1987). *In vitro* studies have shown that the tolmetin-acyl glucuronide (Tol-*O*-G) is a reactive metabolite that reacts with the amino acid side chains of lysine, arginine and serine present in human serum albumin (Ding et al., 1993; Ding et al., 1995).

Covalent binding of Tol has also been studied in humans, where Tol-protein adducts have been detected in plasma (Hyneck et al., 1988; Zia-Amirhosseini et al., 1994). Tol-protein adducts in plasma were believed to result from reaction of the acyl glucuronide with plasma proteins, as a good correlation between covalent plasma protein adducts and exposure to Tol-*O*-G was observed (Hyneck et al., 1988). In the liver, other metabolic pathways may contribute to Tol-protein adduct formation. In a recent study, it has been shown that the pyrrole moiety of Tol may undergo bioactivtion to a reactive arene oxide (Chen et al., 2006). Other reports have shown that acyl coenzyme A thioesters (acyl-CoAs) are also reactive electrophilic metabolites (Sallustio et al., 2000; Grillo and Benet, 2002; Sidenius et al., 2004). Acyl-CoAs are intermediates in the amino acid conjugation, carnitine conjugation, fatty acid synthesis and β-oxidation. *In vitro* characterization of the chemical reactivity has shown that the acyl-CoAs are significantly more reactive than the corresponding acyl glucuronides in the spontaneous reaction with glutathione (Grillo and Benet, 2002; Li et al., 2002; Olsen et al., 2002). Therefore, covalent binding of xenobiotic carboxylic acids *in vivo* may occur *via* at least two mechanisms, the importance of which depends on the concentration and the relative reactivity of each reactive metabolite.

In a recent study, the reactivity of tolmetin-acyl-CoA (Tol-CoA) was examined and the Tol-CoA dependent metabolites tolmetin-glycine (Tol-Gly) and tolmetin-taurine conjugates (Tol-Tau) were identified in rat urine (Olsen et al., 2003a). The aim of the present study was to investigate the acyl-CoA dependent metabolism of Tol, and to examine the significance with respect to drug protein adduct formation *in vivo*. Therefore, acyl-CoA formation was induced with clofibric acid to test the hypothesis that increased levels of acyl-CoA would result in a corresponding increase in the levels of drug protein adducts in the liver.

Materials and Methods

Chemicals. Tol (sodium, dihydrate salt), zomepirac (5-[4'-chlorobenzoyl]-1,4-dimethylpyrrole-2-acetic acid, sodium salt), glycine, taurine, DL-carnitine and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *N*, *N*-carbonyldiimidazol was obtained from Fluka Chemie AG (Buchs, Switzerland). *N*-hydroxysuccinimid and anhydrous tetrahydrofuran were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). CoA (tri-lithium, dihydrate salt) was purchased from AppliChem (Darmstadt, Germany). Tol-CoA, Tol-Tau, Tol-Gly, Tol-Car and Tol-SG were synthesized as described below.

Animal experiments and sample preparation procedures. Male Sprague-Dawley rats (250 – 300 g) were purchased from Bantan and Kingman Universal (Livermore, CA) and maintained in a controlled housing environment with a 12-h light/dark cycle and received standard laboratory chow and water ad libitum. Rats were acclimated to the housing conditions for at least three days before use in the experiments. All animal use was approved by the University of California San Francisco Committee on Animal Research.

Three rats were treated with clofibric acid (150 mg/kg/day, i.p.) and three rats received blank vehicle for 7 days. The dose solution of Tol was prepared in a 0.05 M phosphate buffer pH 7.4 (40 mg/mL). The dose solution of clofibric acid (40 mg/mL) was made in a 0.1 M bicarbonate buffer. On day seven, 2h after the last dose of clofibric acid or blank vehicle, the rats received 100 mg/kg tolmetin (i.p.). Two hours after administration of Tol, the rats were sacrificed by cervical dislocation, and the livers were removed and snap frozen in liquid nitrogen. The livers were

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processed essentially as described by Li et al. (2003). Briefly, 1.0 g of liver was homogenized in 3.0 mL of 0.05 M KH_2PO_4 (pH 4.5, 0 °C) to which 200 µL of 20% phosphoric acid was added. Proteins in the liver homogenates were precipitated by addition of one volume of ice-cold acetonitrile, and the samples were then centrifuged at 4 °C (10,000 g for 2 min). The supernatant was stored at - 80 °C until analysis for Tol metabolites, and the pellet was assayed for covalent binding of Tol to proteins.

One rat (250 g) was given a dose of Tol (100 mg/kg, i.p.) and bile, including predose bile, was collected over a 4h period at 1h intervals. Bile was collected over 21% phosphoric acid (200 µl to each container). Bile (100 µl) was diluted with 400 µl of 50% acetonitrile containing 1% formic acid and centrifuged (10,000 g for 2 min) prior to analysis of the supernatant.

Covalent binding of Tol to liver proteins. The protein pellets were washed seven times with 6 mL of methanol/ether (3/1, vol/vol) following the procedure previously reported (Smith et al., 1986). Subsequently, the pellets were washed as described by Bailey and Dickinson (1996) with slight modifications. Briefly, the pellet was resuspended in 1 mL of 10 mM KH_2PO_4 before re-precipitation with 4 mL of acetonitrile followed by centrifugation. This step was repeated nine times. The washed protein pellets were dried overnight and dissolved in 2 mL of 1 N NaOH at 60 °C for 12 h. The protein hydrolysates were cooled to room temperature and 0.2 mL 85% H_3PO_4 was added before the internal standard was added (1 µg of zomepirac). In order to extract hydrolysed Tol and internal standard, 8 mL of dichloromethane was added and the solution was shaken gently for 10 min. After centrifugation to separate the layers, the organic phase was evaporated gently under nitrogen and reconstituted in 200 µl of 10% acetonitrile containing 0.1% TFA. From this mixture, 50 µl was injected onto the HPLC system to quantify the amount of Tol covalently bound

to liver proteins. For construction of standard curves, blank pellets were spiked with Tol and internal standard. Duplicate standards at six levels, covering the concentration range of the samples, underwent the same sample preparation procedure as the samples. Recoveries were 80-100% and the correlation coefficient was 0.997. The repeatability of the extraction procedure, expressed as the relative standard deviation, was 3.9% (1.2 nmol tolmetin/g liver, n = 6). The adequacy of the washing procedure was assessed from a control experiment in which 5 mg of Tol was added to 1 g of liver that was homogenised in 3 mL of 50 mM phosphate buffer (pH 4.5). The control samples (n=3) were precipitated with one volume of cold acetonitrile and subsequently underwent the same washing, hydrolysis and extraction procedures as described above for the samples. No binding could be detected in the control samples. HPLC conditions for quantification of extracted tolmetin were as follows: mobile phases A consisted of 10 % acetonitrile containing 0.1 % trifluoroacetic acid, while mobile phase B was 50 % acetonitrile containing 0.1 % trifluoroacetic acid. A linear gradient from 0% B to 100% B over 20 min was used at a flow rate of 1 mL/min. A Luna C-18 column (15 cm x 4.6 mm i.d.) from Phenomenex (Torrence, CA, USA) was used with UV detection at 320 nm.

LC-MS Analysis. Unless otherwise stated, LC/MS was performed on an Agilent 1100 LC system connected to an ion trap mass spectrometer (MSD trap from Agilent technologies) equipped with an electrospray ionisation (ESI) interface. LC/ESI/MS with accurate mass measurements was performed on a Surveyor HPLC system (ThermoFinnigan) that was connected to a triple quadrupole mass spectrometer (TSQ Quantum Ultra AM from ThermoFinnigan).

Analysis of Tol-CoA was conducted as described recently for analysis of zomepirac acyl-CoA (Olsen et al., 2005). Briefly, HPLC was performed on a Waters Xterra column (3µ, 100 mm x 2.0

mm i.d.), which was maintained at 40 °C and operated at a flow rate of 0.3 mL/min. Mobile phase A consisted of 10 mM ammonium acetate and mobile phase B consisted of 10 mM ammonium acetate in 80% acetonitrile. Gradient elution was performed from 5%-100%B in 20 min. MS/MS fragmentation was performed in positive ionization mode with a spray voltage at 3500 V. Quantification of Tol-CoA was performed by LC/MS/MS of $[M + H]^+$ at *m/z* 1007 with extraction of fragment ions at *m/z* 500, *m/z* 580 and *m/z* 598. Calibration standards were constructed in blank matrix by addition of synthetic Tol-CoA, and the standards then underwent the same preparation as the samples. Determination of Tol-CoA in liver samples was performed in duplicate.

Tol and Tol-*O*-G were analysed using the same HPLC conditions as described for analysis of Tol-CoA. Identification and quantification of Tol-*O*-G was performed as described by Olsen et al. (2003a). Briefly, Tol-*O*-G was identified by LC/MS/MS in positive and negative ionisation modes. Both Tol and Tol-*O*-G were quantified by UV detection at 320 nm using Tol as the calibration standard assuming that the molar absorptivities of Tol and Tol-*O*-G were the same.

HPLC analysis of Tol-Tau and Tol-Gly was performed on a Luna Phenylhexyl column (5µ, 150 x 2.0 mm i.d.) from Phenomenex (Torrence, CA, USA), which was maintained at 40 °C. The composition of mobile phases A and B were: A. 10 mM ammonium formate (pH 3.5) and B. 10 mM ammonium formate (pH 3.5) in 80% acetonitrile. The pH of the ammonium formate solution was adjusted to pH 3.5 with concentrated formic acid. Gradient elution from 30%-100% B in 10 min was performed at a flow rate of 0.3 mL/min. The mass spectrometer was operated in both positive and negative ionisation mode for identification of the conjugates. Quantification was performed in negative ionisation mode where the *m/z* 124 fragment of Tol-Tau ($[M - H]^-$ at *m/z* 363) and the *m/z* 238 fragment of Tol-Gly ($[M - H]^-$ at *m/z* 313) were extracted. Calibration

standards were constructed in the blank matrix using the preparation procedure described for the samples. Analyses of the liver samples were performed in duplicate.

Analysis of Tol-Car was performed on a Luna C-18 column (3µ, 100 mm x 2.0 mm i.d.) from Phenomenex (Torrence, CA, USA). Mobile phase A consisted of 0.2% formic acid and mobile phase B consisted of 0.2% formic acid in acetonitrile. A gradient was performed from 10-50% B in 20 min at a flow rate at 0.3 mL/min. MS detection was performed using ESI in positive ionization mode with a spray voltage at 4000 V. Tol-Car was quantified by UV detection at 320 nm using Tol as the calibration standard assuming the UV contribution from the carnitine moiety was negligible. When blank liver extracts treated as the sample were analyzed, no chromatographic peaks at 320 nm were observed at the retention time observed for Tol-Car. For LC/ESI/MS with accurate mass measurements, gradient elution was performed from 10%-100% B in 10 min at a flow rate of 0.2 mL/min. Accurate mass measurements were performed in selected ion monitoring mode at *m/z* 401.2. A solution of lock masses (*m/z* 520.3328 and *m/z* 564.3590 from a solution of polyethylene glycol 200, 400 and 600) was infused into the HPLC eluent via a T-piece at a flow rate of 5 µL/min.

Identification of Tol-metabolites in rat bile was performed by LC-MS(/MS) on a Luna Phenylhexyl column (150 mm x 2.0 mm i.d.) from Phenomenex (Torrence, CA, USA). The temperature of the HPLC column was maintained at 40 °C and the flow rate was 0.3 ml/min. Mobile phase A consisted of 0.2% formic acid and mobile phase B consisted of 0.2% formic acid in acetonitrile. Gradient elution was performed from 10%-100% B in 30 min. Tol-*O*-G, Tol-Tau and Tol-SG were identified based upon *i*) MS/MS fragmentation as described above, *ii*) comparison of HPLC retention times and MS/MS spectra with those of authentic standards when available. Accurate mass measurement of Tol-SG was performed as described above for analysis of Tol-Car with the exception that Tyr-

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Tyr-Tyr (m/z 508.2080) and reserpine (m/z 609.2812) were used as lock masses. Identification of hydroxy-Tol (Tol-OH) and carboxy-Tol (Tol-COOH) was performed as described previously (Olsen et al., 2003a).

Syntheses. Tol-CoA and Tol-SG were essentially prepared by the method for synthesis of fatty acyl-CoAs (Kawaguchi et al., 1981) with minor modifications (Olsen et al., 2003a). **Tol-CoA**: ESI-MS/MS of $[M + H]^+$ at *m/z* 1007: *m/z* 598 (43%), *m/z* 580 (41%), *m/z* 500 (100%), *m/z* 428 (13%) and *m/z* 398 (16%). Assignment of fragment ions is given in the Results section. **Tol-SG**: ESI-MS/MS of $[M + H]^+$ at *m/z* 547: *m/z* 529 (19%), *m/z* 472 (24%), *m/z* 418 (100%), *m/z* 411 (35%), *m/z* 400 (31%), *m/z* 382 (31%), *m/z* 343 (18%), *m/z* 308 (6%), *m/z* 240 (55%), *m/z* 222 (3%), *m/z* 212 (6%). ESI-MS/MS of $[M - H]^-$ at *m/z* 545 gave an intense ion at m/z 306 (100%). See the Results Section for assignment of the fragment ions. Accurate mass measurements: Tol-SG was added to a solution of lock masses (Tyr-Tyr-Tyr and reserpine) and this solution was infused directly at a flow rate of 3 µl/min into the mass spectrometer. $[M + H]^+$ was determined to be m/z 547.1861, which corresponds to the elemental composition of Tol-SG with an accuracy of +0.73 ppm.

Tol-Gly and Tol-Tau were synthesized as described previously (Olsen et al., 2005). ESI/MS/MS: **Tol-Tau**: $[M + H]^+$ at *m/z* 365: *m/z* 240 (100 %, $[Tol + H - H_2O]^+$), *m/z* 212 (35 %, $[Tol - COOH]^+$), *m/z* 119 (18%, $[CH_3(C_6H_4)CO]^+$). $[M - H]^-$ at *m/z* 363: *m/z* 124 (100%, $[Taurine - H]^-$). **Tol-Gly**: $[M + H]^+$ at *m/z* 315: *m/z* 240.1 (100 %, $[Tol + H - H_2O]^+$), *m/z* 212 (30 %, $[Tol - COOH]^+$), *m/z* 179 (9 %, $[Tol\text{-}Gly + 2H - CH_3(C_6H_4)CO - H_2O]^+$), *m/z* 151 (5 %, $[m/z\ 179 - CO]^+$) and *m/z* 119 (23%), $[CH_3(C_6H_4)CO]^+$). $[M - H]^-$ at *m/z* 313: *m/z* 238 (100%, $[Tol - H - H_2O]^-$).

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The synthesis of Tol-Car was performed following a method previuosly described (Stadtmann, 1957). To a solution of Tol (1.6 mmol as protonated carboxylic acid dissolved in 30 mL anhydrous tetrahydrofuran), 220 µL triethylamine and 160 µL ethylchloroformate were added. After 30 min, the reaction mixture was filtered through a paper filter into a mixture of 750 mg DL-carnitine HCl dissoved in a solution of $NaHCO_3$ (5.3 g/L with the pH was adjusted to pH 10 with NaOH). The mixture was allowed to react for 90 min, after which the reaction was stopped by addition of 0.5 mL of 30% HCl. Tetrahydrofuran was removed using a rotary evaporator and the remaining Tol was removed by extractions with ethyl acetate. The aqueous phase was lyophilized, redissoved in 10% acetonitrile and further purified by solid phase extraction on Oasis^{TM} cartridges (1cc, 30 mg) obtained from Waters Corporation (MA). The elutate was dried under a stream of nitrogen and for NMR analysis, the eluate was reconstituted in CD_3CN. Proton NMR spectra were acquired on a Bruker Advance (600 MHz) at 25 °C using a dual 1H-13C 3 mm cryo probe. The chemical shifts are expressed as parts per million relative to tetramethylsilane. ^{1}H-NMR. δ 7.70 (d, J = 8.0 Hz, $CH_3(C_6H_2\mathit{H}_2)CO$-), δ 7.33 (d, J = 7.8 Hz, $CH_3(C_6\mathit{H}_2H_2)CO$-), δ 6.64 ppm (d, J = 4.0, -1H-pyrrole), δ 6.15 ppm (d, J = 4.0, -1H-pyrrole), δ 5.55 ppm (s, T-*O*-C*H*-), δ 3.89 ppm (m, pyrrole-$C\mathit{H}_2CO$-), δ 3.89 ppm (m, pyrrole-$NC\mathit{H}_3$), δ 3.70 ppm (m, -$C\mathit{H}_2N^+(CH_3)_3$), δ 3.06 ppm (s, -$N^+(C\mathit{H}_3)_3$), δ 2.77 ppm (dd, J = 4.0, 16.5 Hz, -$CHC\mathit{H}_2COOH$), δ 2.60 ppm (dd, J = 9.2, 16.5 Hz, -$CHC\mathit{H}_2COOH$), δ 2.44 ppm (s, $C\mathit{H}_3(C_6H_4)$-). ESI/MS/MS of $[M]^+$ at *m/z* 401: *m/z* 119 (100%), *m/z* 144 (7%), *m/z* 254 (40%), *m/z* 258 (5%), *m/z* 342 (69%). Assignment of fragment ions is given in the Results section.

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Results

Identification of Tol-CoA

Tol-CoA was identified *in vivo* in rat livers using the method described for identification of the zomepirac acyl coenzyme A thioester (Olsen et al., 2005). Tol-CoA eluted at 12.1 min and MS/MS fragmentation of $[M + H]^+$ at *m/z* 1007 resulted in an MS/MS spectrum with characteristic fragment ions at *m/z* 598, 580, 500, 428 and 398 (Figure 1). Assignment of the fragment ions is shown in Figure 2. This fragmentation pattern is similar to that described for other acyl-CoAs (Grillo and Benet, 2002; Li et al., 2002; Olsen et al., 2003b; Sidenius et al., 2004) including that of the structurally related non steroidal anti-inflammatory drug, zomepirac (Olsen et al., 2005). The retention time and MS/MS spectrum of a synthetic standard was similar to those seen in liver samples from rats that had received a dose of 100 mg/kg.

Identification of Tol-Car.

Tol-Car was identified in rat livers following administration of Tol. LC-MS/MS of Tol-Car ($[M]^+$ at *m/z* 401) eluting at 15.5 min gave fragment ions at *m/z* 342, 258, 254, 144 and 119 as shown in Figure 3, in which assignment of the fragment ions is also shown. Further evidence for the proposed fragmentation pattern came from LC-MS^3 analysis of *m/z* 401→342 (Figure 4), which gave fragment ions at *m/z* 258 ($[Tol + H]^+$), *m/z* 240 ($[Tol - H_2O + H]^+$ and *m/z* 119 ($[CH_3(C_6H_4)CO]^+$). This MS^3 spectrum was similar to an MS/MS spectrum of Tol. Accurate mass measurements gave an $[M]^+$ at *m/z* 401.2087 consistent with an elemental composition corresponding to that of Tol-Car (+ 2.7 ppm). Finally, a synthetic standard of Tol-Car co-eluted and showed the same MS/MS characteristics as that observed from analysis of the liver extracts.

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Identification of Tol-*O*-G, Tol-Tau and Tol-Gly

Tol-*O*-G and Tol-Tau were identified as previously described (Olsen et al., 2003a). Briefly, for the most abundant Tol-*O*-G isomer, one intense fragment ion of Tol-*O*-G ($[M + H]^+$ at *m/z* 434) was observed at *m/z* 258 ($[Tol + H]^+$) which by MS^3 (m/z 434 → 258) gave one intense fragment at *m/z* 119 ($[CH_3(C_6H_4)CO]^+$). This isomer was assumed to be the 1-*O*-isomer. The most intense MS/MS fragment for the other isomers was a loss of water in positive and negative ionisation mode. In negative ionization mode, MS/MS of the most abundant isomer ($[M - H]^-$ at *m/z* 432) gave fragment ions at *m/z* 175 (19%, $[Glucuronic\ acid - H_2O - H]^-$) and *m/z* 193 (100%, $[Glucuronic\ acid - H]^-$). Accurate mass measurements showed that the $[M + H]^+$ was 434.1454 Da, which is consistent with the *m/z*-value of protonated Tol-*O*-G with an accuracy of 1.8 ppm.

Tol-Tau was identified in rat livers by LC-MS/MS. The retention time and MS/MS fragmentation patterns in positive and negative ionisation modes of a synthetic standard were in accordance with that obtained from analysis of liver samples. The formation of Tol-Gly was also studied, but this metabolite was only observed at levels below the limit of quantification (0.6 nmol/g liver).

***In vivo* formation of Tol metabolites and covalent binding to liver proteins**

Formation of Tol-*O*-G, Tol-CoA, Tol-Tau, Tol-Car and covalent binding of Tol to liver proteins were determined in liver homogenates of rats (either pre-treated with clofibric acid or control rats) that had received a dose of 100 mg/kg Tol as shown in Table 1. In control rats, the level of Tol-CoA was 0.6 nmol/g liver whereas the level of Tol-*O*-G was approx. 40 times higher. Formation of Tol-*O*-G was unaffected by the clofibric acid pre-treatment whereas the concentration of Tol-CoA in liver homogenates increased significantly to 4.4 nmol/g. Formation of Tol-Car also increased upon treatment with clofibric acid from 0.08 to 0.64 nmol/g liver whereas the levels of Tol and Tol-Tau

were unaffected by the treatment with clofibric acid. Covalent binding of Tol to liver proteins was determined to be 0.9 nmol/g liver in control rats whereas covalent binding increased to 4.2 nmol/g liver in rats pretreated with clofibric acid.

Identification of Tol-metabolites in rat bile

Identification of Tol-SG, resulting from transacylation of Tol-CoA or Tol-*O*-G, was performed by LC-MS/MS ($[M + H]^+$ at *m/z* 547), which resulted in the characteristic loss of *m/z* 129 (pyroglutamyl moiety – H) observed at *m/z* 418 (see Figure 4). Other fragment ions were produced at *m/z* 529 ($[M + H – H_2O]^+$), *m/z* 472 ($[M + H – \text{glycine}]^+$), *m/z* 411 ($[M + H – \text{glycine} – \text{COOH} – NH_2]^+$), *m/z* 400 ($[M + H – \text{pyroglutamic acid}]^+$), *m/z* 382 ($[M + H – \text{pyroglutamic acid} – H_2O]^+$), *m/z* 343 ($[\text{Tol-S-CH}_2\text{(CO)CHNH}_2]^+$) and *m/z* 240 ($[\text{Tol} + H – H_2O]^+$). This fragmentation pattern and the retention time were identical to those observed for a synthetic standard. Tol-SG was not quantified in bile, but based on the UV chromatogram at 320 nm (Figure 4), which is the UV absorption maximum of Tol, Tol-SG (t_R at 13.8 min) was a minor metabolite in bile as compared to Tol-*O*-G (t_R at 15.6 min). In addition, the presence of Tol-SG in rat liver homogenates was investigated, but the metabolite could not be detected. The only acyl-CoA dependent metabolite that was detected in bile was Tol-Tau (t_R ~19.1 min) which was formed at relatively low levels as assessed from the UV chromatogram at 320 nm. Other metabolites that were identified in rat bile were Tol-OH (t_R at 12.1 min) and Tol-COOH (t_R at 13.1 min) for which oxidation occurred on the methyl group on the phenyl-ring, and the sulfate conjugate of Tol-OH (t_R at 16.2 min) as previously described (Olsen et al., 2003a).

Discussion

Carboxylic acids may be bioactivated to reactive metabolites capable of reacting with proteins *in vivo*, and these covalent modification of proteins may be an important mechanism underlying toxicity such as allergy and hepatotoxicity (Boelsterli, 2002). An acyl glucuronide is one example of a reactive carboxylic acid metabolite (Smith et al., 1986), but recent studies on acyl-CoAs and acyl-glutathione conjugates have shown that thioester metabolites react more rapidly with thiols (glutathione/*N*-acetylcysteine) than the corresponding ester glucuronides (Grillo and Benet, 2002; Li et al., 2002; Olsen et al., 2002; Grillo et al., 2003).

It has recently been reported that the pyrrole moiety of Tol undergoes bioactivation to a reactive arene oxide (Chen et al., 2006), which may also contribute to covalent binding to liver proteins. However, the present study focused on reactive phase 2 metabolites, capable of trans-acylating proteins. Covalent binding was determined as the release of Tol by alkaline hydrolysis of Tol-protein adducts and therefore, the metabolism studies focused on addressing metabolic pathways involved in the formation of these protein adducts.

Three novel Tol metabolites, Tol-CoA, Tol-Car and Tol-SG, were identified in this study. Acyl-CoA dependent metabolism of Tol is important to identify, as studies have shown that Tol-CoA reacts spontaneously with the thiol of glutathione indicating that trans-acylating reactions may also occur in liver cells *in vivo* (Olsen et al., 2003a; Sidenius et al., 2004). Tol-Car is formed via the reactive Tol-CoA intermediate by carnitine acyl transferases and its formation is therefore of mechanistic significance showing the formation of the corresponding reactive acyl-CoA. Although Tol-SG could not be detected in rat liver homogenates and appeared to be a quantitatively minor metabolite in bile, it was also important to identify Tol-SG as the formation of this metabolite

indicates that acylating Tol-metabolites, e.g., Tol-CoA or Tol-*O*-G, capable of trans-acylating glutathione to form acyl-*S*-Tol glutathione conjugate were formed in the liver. It is, however, also interesting to note that Tol-SG is a thioester and therefore, Tol-SG is also a reactive metabolite that is probably as reactive as Tol-CoA, i.e. significantly more reactive than Tol-*O*-G. A schematic of the pathways leading to metabolic activation of Tol is shown in Figure 6.

The levels of Tol metabolites were investigated in liver homogenates in an *in vivo* study. These results were in accordance with observations from a study on zomepirac in which the acyl-glucuronide was identified as the major metabolite and the acyl-CoA and the amino acid conjugates were formed at lower levels (Olsen et al., 2005). In the current study, a group of rats were pre-treated with clofibric acid in order to induce acyl-CoA formation. As shown for palmitic acid (Knights et al., 1991) and ibuprofen (Shirley et al., 1994), such pre-treatment leads to an increased formation of acyl-CoA, which was also observed for Tol in this study. Pre-treatment with clofibric acid led to an increase in Tol-CoA from 0.6 nmol/g liver to 4.4 nmol/g liver (see Table 1), but had no apparent effect on the concentration of Tol-*O*-G in liver homogenates as compared to control rats. This is in accordance with reports showing that clofibrate induces UGT1A1 and 1A5, but not the isoforms UGT 2B1 (in rat) and 2B7 (in man), which generally catalyse the formation of acyl glucuronides of non steroidal anti-inflammatory drugs (Ritter, 2000). In general, covalent binding may have been underestimated for both pre-treated animals and control rats as imine-protein adducts are chemically unstable and may have been lost during sample processing. However, this error would be approximately the same for both groups of animals, as Tol-O-G levels were similar. In addition, mainly one isomer, presumably the 1-*O*-isomer, was detected in the liver homogenates, which were treated with acid to avoid isomerisation. Therefore, as the covalent binding to proteins increased upon pre-treatment of rats with clofibric acid, the levels of Tol-CoA appeared to correlate

with the amount of covalent binding of Tol to liver proteins, which supports the hypothesis that the reactive Tol-CoA reacted with hepatic proteins and thus contributed to covalent modification of liver proteins *in vivo*.

As seen for Tol-CoA formation and covalent binding, clofibric acid pre-treatment also led to an increase in Tol-Car. This increase of Tol-Car is in accordance with the reported induction of carnitine acyl transferases (Katsutani et al., 2000) and increases in free carnitine and CoA levels following pre-treatment with fibrates (Gregus et al., 1998). Acyl carnitine esters are rarely observed metabolites of xenobiotic carboxylic acids and to our knowledge, the only xenobiotic carnitine esters that have been previously identified *in vivo* are the pivaloyl (Vickers et al., 1985; Totsuka et al., 1992), valproyl (Melegh et al., 1990) and zomepirac carnitine esters (Olsen et al., 2005).

As seen in Table 1, conjugation of tolmetin with glycine and taurine appeared to be unaffected by clofibric acid pre-treatment, which is consistent with a report on taurine-conjugation of ibuprofen in hepatocytes (Shirley et al., 1994). In general, amino acid conjugation is a high affinity-low capacity metabolic pathway, i.e., xenobiotic carboxylic acids tend to quantitatively form amino acid conjugates at low doses, but as the dose increases, other metabolic pathways – e.g., glucuronidation – become more important (Hutt, 1990).

In conclusion, Tol-CoA and Tol-Car were identified in this study. The presence of Tol-CoA shows that alternative pathways to glucuronidation may lead to formation of other reactive carboxylic acid metabolites capable of reacting with protein nucleophiles or trans-acylate GSH to form Tol-SG. Mechanistically, identification of Tol-Car is important, as this metabolic route is Tol-CoA dependent.

Acknowledgement

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Footnotes

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Legends to figures

Figure 1 LC/MS/MS analysis of Tol-CoA ($[M + H]^+$ at *m/z* 1007) in a liver sample from a rat, pre-treated with clofibric acid, and administered Tol (100 mg/kg i.p.). See Figure 2 for proposed identities of fragment ions.

Figure 2 Proposed identities of the fragment ions of Tol-CoA.

Figure 3 MS/MS spectrum of Tol-Car ($[M]^+$ at *m/z* 401) obtained from LC-MS/MS analysis of a liver sample from a rat pre-treated with clofibric acid and administered Tol.

Figure 4 LC/MS/MS of Tol-SG (*m/z* 547) in a bile sample from a rat which received Tol (100 mg/kg). A. UV trace at 320 nm showing elution of 1. Tol-OH, 2. Tol-COOH, 3. Tol-*O*-G, 4. Sulphate conjugate of Tol-OH, 5. Tol, 6. Tol-Tau. B. Extracted ion chromatogram of *m/z* 418. C. MS/MS spectrum of *m/z* 547 from 13.6-13.9 min. Fragmentation patterns are discussed in the text.

Figure 5 Scheme showing the metabolic activation of tolmetin to reactive metabolites

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Tables

Table 1 Summary of the quantitative data, expressed as nmol/g liver, in rat liver homogenates following administration (i.p.) of 100 mg/kg tolmetin to clofibric acid pre-treated rats (n=3) or control rats (n=3). Values are expressed as the mean ± S.D (n=3).

	Covalent binding	**Tol**	**Tol-CoA**	**Tol-*O*-G**	**Tol-Tau**	**Tol-Car**
Clofibric acid treated[1]	4.2 ± 1.1	530 ± 220	4.4 ± 1.9	19 ± 5.5	4.0 ± 2.0	0.64 ± 0.21
Control	0.9 ± 0.3	550 ± 130	0.6 ± 0.2	25 ± 5.3	7.2 ± 6.0	0.08 ± 0.07

[1]Pre-treatment was performed with clofibric acid for 7 days (150 mg/kg/day, i.p.). Livers were removed 2h after administration of tolmetin.

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Figure 1

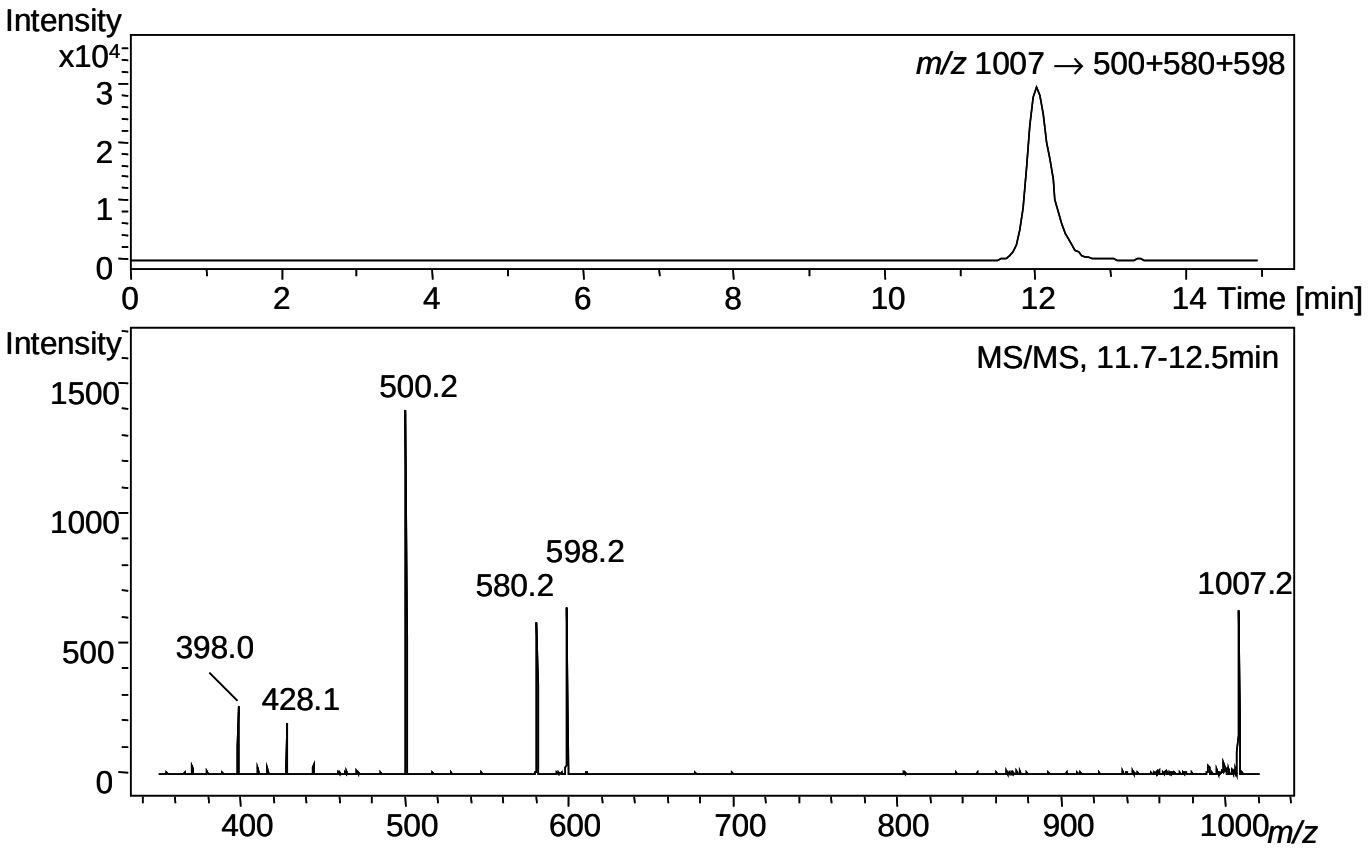

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Figure 2

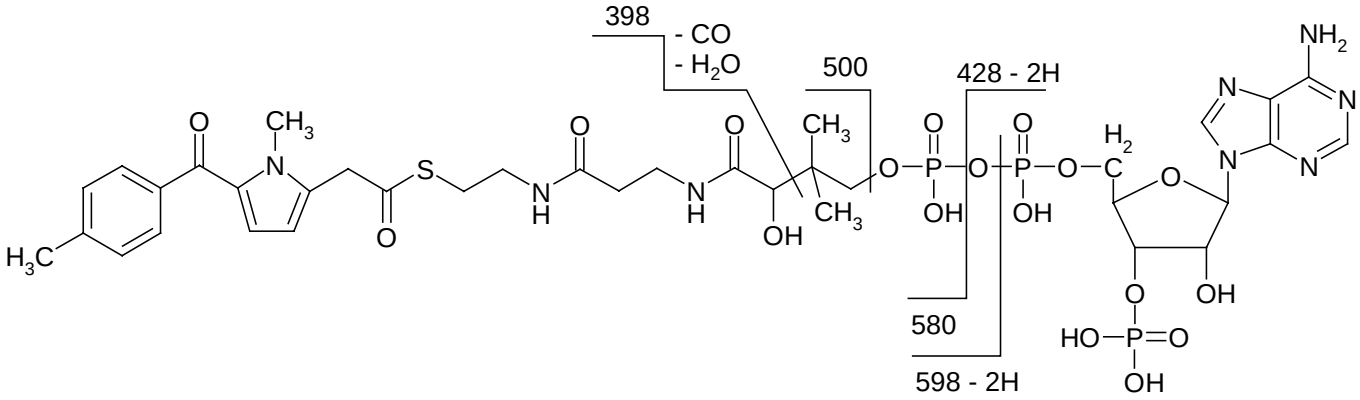

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Figure 3

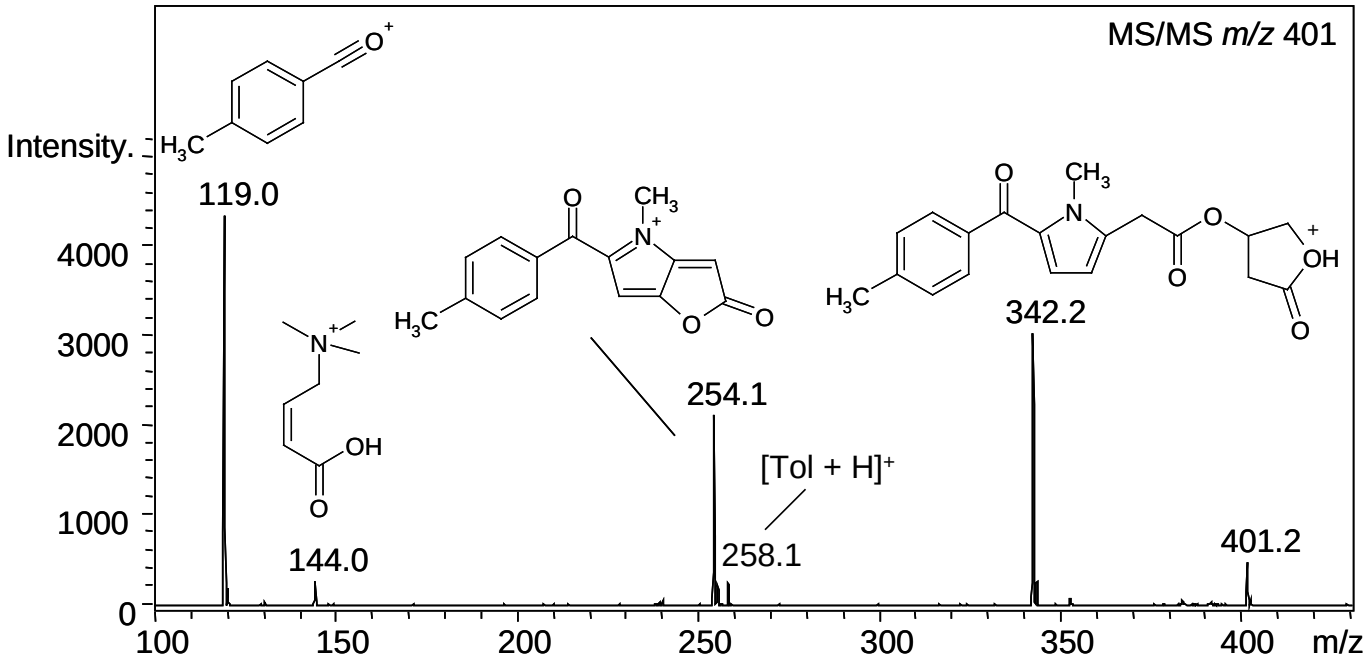

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Figure 4

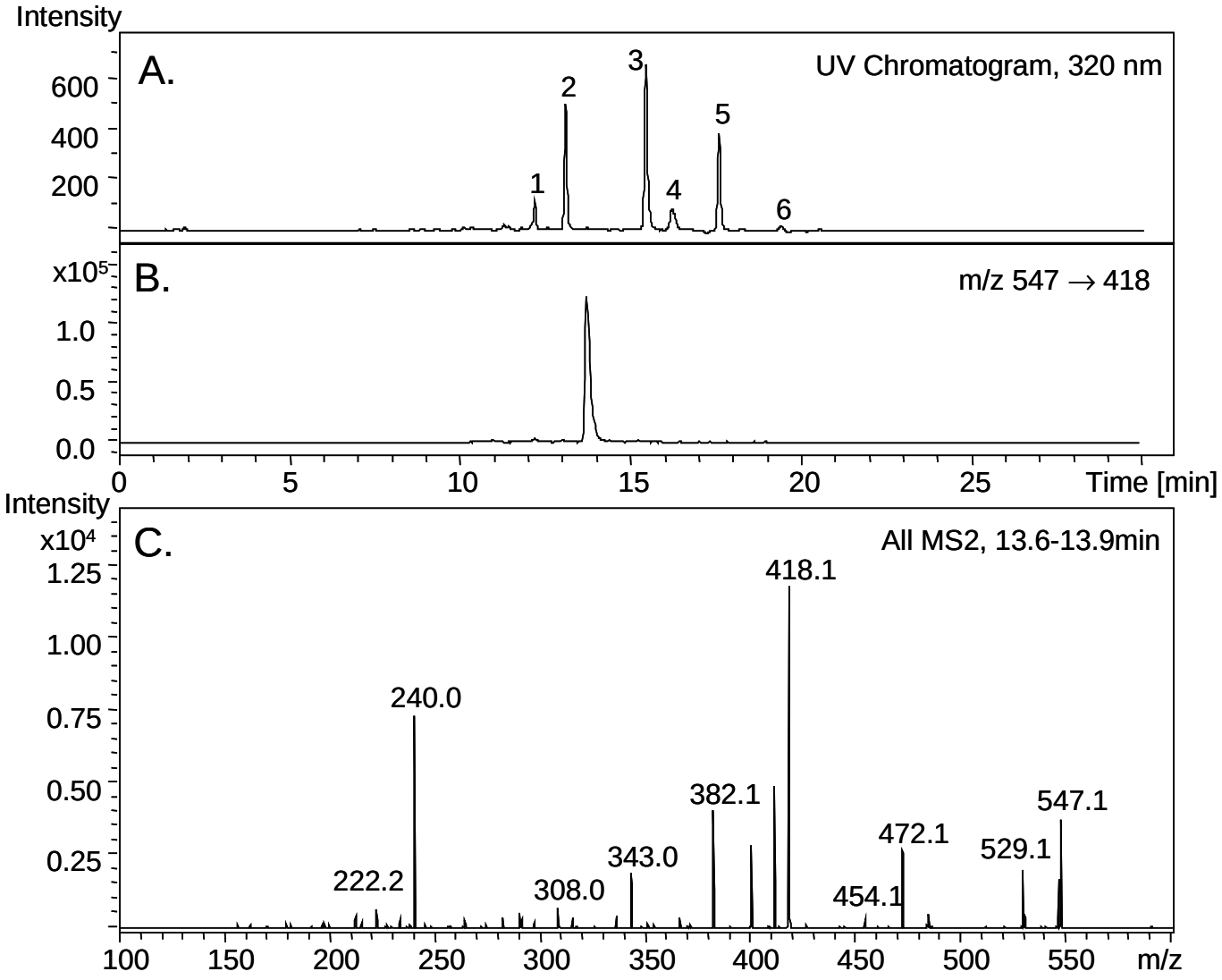

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Figure 5