Metabolism of Ticlopidine in Rats: Identification of the Main Biliary Metabolite as a Glutathione Conjugate of Ticlopidine S-Oxide

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ABSTRACT:
We have identified several novel metabolites of ticlopidine, a well known antiplatelet agent and have revealed its metabolic route in rats. The main biliary metabolite of ticlopidine was characterized as a glutathione (GSH) conjugate of ticlopidine S-oxide, in which conjugation had occurred at carbon 7α in the thiienopyridine moiety. Quantitative analysis revealed that 29% of the dose was subjected to the formation of reactive intermediates followed by conjugation with GSH after oral administration of ticlopidine (22 mg/kg) to rats. In vitro incubation of ticlopidine with rat liver 9000g supernatant fraction (S9) fractions led to the formation of multiple metabolites, including 2-oxo-ticlopidine, the precursor for the pharmacologically active ticlopidine metabolite, [1-(2-chlorobenzyl)-4-mercaptopiperidin-(3Z)-yliden]-acetic acid. A novel thioephene ring-opened metabolite with a thioether group and a carboxylic acid moiety has also been detected after incubation of 2-oxo-ticlopidine with rat liver microsomes or upon incubation of ticlopidine with rat liver S9 fractions.

Materials and Methods

Chemicals. Ticlopidine (5-(2-chloro-benzyl)-4,5,6,7-tetrahydro-thieno[3,2-c]pyridine), ticlopidine N-oxide [5-(2-chloro-benzyl)-6,7-dihydro-4H-thieno[3,2-c]pyridine 5-oxide], ticlopidine pharmacologically active metabolite ([1-(2-chloro-benzyl)-4-mercaptopiperidin-(3Z)-yliden]-acetic acid, 4,5,6,7-tetrahydrothieno-[3,2-c]pyridine, and 2-oxo-ticlopidine (5-(2-chloro-benzyl)-5,6,7,7a-tetrahydro-4H-thieno[3,2-c]pyridin-2-one) were chemically synthesized. [14C]Ticlopidine (>99% by HPLC and 97.3% by thin-layer chromatography) was synthesized at Daiichi Pure Chemicals Co., Ltd. (Ibaraki, Japan) (Fig. 1). Glucose 6-phosphate (G6P) and glutathione (GSH) were obtained from Sigma-Aldrich (Tokyo, Japan). Glucose-6-phosphate dehydrogenase (G6PDH) and β-nicotinamide adenine dinucleotide phosphate, oxidized form, and monosodium salt (NADP+) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). MgCl2·6H2O and β-naphthoflavone were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Phenobarbital sodium salt was obtained from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). Acetonitrile (Kanto Chemical Co., Inc., Tokyo, Japan) was HPLC grade, and all other chemical reagents were of the highest grade commercially available.

Animals. Six-week-old Sprague-Dawley male rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). The rats were housed at a constant temperature (23 ± 2°C) and humidity (55 ± 20%) under a 12-h light/dark cycle. The rats were given food (Funahashi F-2; Funahashi Farm, Chiba, Japan) and water ad libitum.

Administration and Sample Collection. Animal surgery for the collection of bile and urine was performed as reported previously (Ito et al., 2007) with slight modification. In brief, each rat was anesthetized with diethyl ether in a restraining cage (Bollman cage; Sugiyama-gen Iriki Co., Ltd., Tokyo, Japan).
After recovery from the anesthesia (approximately 30 min after completion of the surgery), the animals were treated orally with \[^{14}C\]ticlopidine (22 mg/9.88 MBq/kg) in distilled water (2.5 ml/kg) by gavage tube. Bile and urine were collected during the periods of 0 to 15 and 15 to 20 h after administration; 5.15 to 12.9 ml of bile was collected during 0 to 20 h for each rat. (The bile flow rate was 0.258–0.645 ml/h.)

Measurement of Radioactivity. A 20-μl aliquot of each bile or urine sample was placed into separate liquid scintillation vials. Then, 10 ml of a liquid scintillation solution (Aquasol-2; PerkinElmer Life and Analytical Sciences, Waltham, MA) was added before measurement of the radioactivity using a liquid scintillation counter (LSC-3500; Aloka, Tokyo, Japan).

Radio-HPLC Analysis. After filtering through an Ultrafree-MC 0.22-μm filter unit (Millipore Corporation, Billerica, MA), 30- to 40-μl aliquots of bile or urine samples were loaded onto an HPLC column (Symmetry C18, 5 μm, 150 mm × 4.6 mm i.d.; Waters, Milford, MA). The column temperature was 40°C. The mobile phase consisted of a linear gradient of solvent A (10 mM CH₃COONH₄) and solvent B (CH₃CN) according to the following program: B, 10% (0–5 min) to 75% (23–29 min) to 10% (30 min). The HPLC system consisted of an L-4000 UV detector (Hitachi, Tokyo, Japan) and an L-6200 Intelligent Pump (Hitachi) set at a flow rate of 1 ml/min. A radio-HPLC system, β-RAM model 3 radioactivity detector (IN/US Systems, Tampa, FL), was used for the radioactive detection. Metabolites were quantified by integration of the peaks found in the radiochromatogram during the radio-HPLC analysis, assuming that all of the radioactivity in bile and urine could be analyzed on the radiochromatogram, because the extraction efficiencies of radioactivity from the bile and urine sample were determined to be more than 99% in our preliminary experiment, in which one bile sample and one urine sample were drawn as representative to ascertain the extraction efficiency of radioactivity.

Preparation of Rat Liver S9 and Microsomes. Rat liver rat liver 9000 g supernatant fraction (S9) and microsomes were prepared from 7-week-old male Wistar rats (Japan SLC, Inc.) treated with β-naphthoflavone (80 mg/kg, 2 ml/kg, once daily, suspended in corn oil) for 2 days followed by treatment with phenobarbital [60 mg/kg, 2 ml/kg, once daily, in 0.9% (w/v) sodium chloride] for 3 days. The rats were sacrificed by cervical dislocation, and the liver of each rat was immediately removed. The livers were perfused with an ice-cold 1.15% potassium chloride solution. The homogenates were pooled. The pooled liver tissues (139 g in total) were homogenized in 240 ml of ice-cold 1.15% potassium chloride solution. The homogenates were centrifuged (Avanti HP-25; Beckman Coulter, Fullerton, CA) at 9000g for 20 min at 4°C, and a part of the supernatant was then stored as liver S9 at −80°C. The remaining supernatant fractions were further centrifuged (SCP70H2 ultracentrifuge; Hitachi) at 105,000 g for 1 h at 4°C. Each supernatant was recentrifuged for 20 min at 4°C. The resulting microsomal pellets were washed with 0.1 M sodium phosphate buffer (pH 7.4) and resuspended at 105,000 g for 1 h at 4°C. The pellets were then resuspended in a small volume of 0.1 M sodium phosphate buffer (pH 7.4) and stored at −80°C. A standard Lowry assay kit (DC Protein Assay Kit 2; Bio-Rad Laboratories, Hercules, CA) was used to measure the rat liver S9 and microsomal protein concentrations.

In Vitro Metabolic Study. The reaction mixtures contained the following components: 100 mM sodium phosphate buffer (pH 7.4), S9 or rat liver microsomal fraction (2 mg of protein/ml), ticlopidine or 2-oxo-ticlopidine (1 mM), G6P (10 mM), G6PDH (1 unit/ml), MgCl₂ (4 mM), GSH (5 mM), and NADP⁺ (1 mM). Each reaction was started by the addition of S9 or microsomes after a 2-min preincubation at 37°C. After 60 or 120 min of incubation, the reaction was terminated by adding a 2-fold volume of ice-cold CH₃CN. The samples were mixed with a vortex mixer and centrifuged (MX-300; Tomy, Tokyo, Japan) at 9100g for 20 min at 4°C. Each supernatant was recentlyrifuged at 9100g for 5 min at 4°C. Each resulting supernatant was collected separately and analyzed by LC-MS and LC-MS/MS.

LC-MS and LC-MS/MS Analysis. The in vitro reaction mixtures were analyzed by LC-MS and LC-MS/MS on an Alliance 2690 HPLC system (Waters) coupled to a Finnigan LCQ ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) in positive ion electrospray mode. The analytical conditions (column and mobile phase) were the same as those described above in the radio-HPLC system. The capillary heater was set to
250°C. For the MS/MS experiments the relative collision energy was set at 20 to 40%.

Preparation of the Sample for Isolation of the Main Biliary Metabolite. Isolation was performed using the bile sample from 10 rats administered nonradioabeled ticlopidine (26 mg/kg) in distilled water (2.5 ml/kg). Bile samples were collected for 16 h after administration. Each biliary sample was pooled and applied (10 ml each for 7 applications) to solid-phase extraction columns (Mega Bond Elut C18 60CC/10GRM; Varian, Inc., Palo Alto, CA). After washing with 5% CH3CN-10 mM CH3COONH4 (75 ml), the metabolites were eluted with 20% CH3CN-10 mM CH3COONH4 (75 ml). This solution was freeze-dried, and the residue was dissolved in distilled water (20 ml) and subjected to the preparative HPLC described below.

Preparation of the Sample for Isolation of in Vitro Metabolites. For isolation of in vitro ticlopidine metabolites, a large-scale incubation using rat

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**Fig. 4.** MS spectrum of isolated metabolite B1 (A) and its product ion spectrum (B).
liver microsomes was conducted. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), rat microsomal protein (1 mg of protein/ml), ticlopidine (666 μM), G6P (10 mM), G6PDH (1 unit/ml), MgCl₂ (4 mM), and NADP⁺ (1 mM). The total volume of the reaction mixture was 500 ml (1 ml for 500 tubes). The reactions were started by the addition of NADP⁺ after a 3-min preincubation at 37°C. After 30 min of incubation, the reactions were terminated by treatment with an equal volume of ice-cold CH₃COCH₂CH₃. Each mixture was pooled and vigorously shaken to extract the metabolites. After evaporation of the organic solvent under reduced pressure, the resulting residue was dissolved in CH₃CN. The fractions containing metabolites were subjected to the preparative HPLC described below.

**Preparative HPLC.** Isolation of metabolites was performed with a Hitachi D-7000 HPLC system, which consists of an L-4000 UV detector and L-7100 pump (Hitachi) set at a flow rate of 2.8 ml/min. A Symmetry C₁₈ column (7 μm, 300 mm × 7.8 mm i.d.; Waters) was used for the separation. The mobile phase consisted of solvent A (10 mM CH₃COONH₄) and solvent B (CH₃CN).

To separate the biliary metabolite, the following linear gradient programs were used: B 15% (0–2 min) to 50% (18–20 min) to 15% (23 min) for rough separation and then 15% (0–20 min) to 50% (25–27 min) to 15% (29 min) for complete separation. To isolate the in vitro metabolites, the following linear gradient program was used for rough fractionation: B 40% (0 min) to 75% (10–25 min) to 40% (28 min). The resulting fraction containing in vitro metabolites was subjected to isocratic elution (45% B) for complete separation.

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*: ¹H NMR signals of 4β, α-Gly, and α-Glu were overlapped.
Characterization of Isolated Metabolites. ¹H-NMR, ¹H-¹H COSY NMR, ¹³C-NMR, and ¹H-¹³C COSY NMR analyses were performed using a JNM-ECP500 NMR spectrometer (JEOL, Tokyo, Japan). Deuterated water (D₂O) was used as a solvent for the biliary metabolite, and chemical shifts (δ) are expressed as parts per million downfield from the internal standard of sodium 3-trimethylsilylpropionate-2,2,3,3-d₄. For the in vitro metabolites, deuterium methanol (CD₃OD) was used as a solvent. Chemical shifts (δ) are expressed as parts per million downfield from the internal standard of tetramethylsilane. MS and MS/MS analyses were conducted using a Finnigan LCQ ion trap mass spectrometer. MS conditions were the same as those described for the LC-MS and LC-MS/MS analyses.

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![13C-NMR spectrum of isolated metabolite B1 in D₂O.](image-url)
**Results**

**Biliary and Urinary Excretion of $[^{14}C]$Ticlopidine.** The cumulative biliary excretion of radioactivity during the 0 to 15 h collection period was 46.6 ± 10.1% of the dose given ($n = 9$) and increased slightly to 48.1 ± 10.1% up to 20 h ($n = 9$). The cumulative urinary excretion of radioactivity during the 0 to 15 h collection period was 12.9 ± 0.3% of the dose given ($n = 3$) and increased slightly to 14.1% up to 20 h ($n = 2$). (Only two or three rats could be used for the measurement of urinary excretion because the penis tubes to collect urine were detached in six of nine animals during 0 to 15 h after administration, and one more rat lost its tube during 15 to 20 h after administration.)

**Radio-HPLC Profiles of Biliary and Urinary Metabolites.** Figure 2A shows a representative radiochromatogram of bile collected 0 to 15 h after $[^{14}C]$ticlopidine administration. Two main peaks, metabolite B1 and metabolite B2, were detected at retention times (RTs) of 12.0 and 13.2 min, respectively. The metabolite (RT 16.4 min) was identified as ticlopidine N-oxide by comparison with an authentic standard.

Figure 2B shows a representative radiochromatogram of urine...
collected 0 to 15 h after [14C]ticlopidine administration. One of the main peaks, metabolite U1, with an RT of 10.1 min, exhibited an [MH]/H ion at m/z 443. The metabolite (RT 16.4 min) was identified as ticlopidine N-oxide by comparison with an authentic standard. Unchanged ticlopidine was detected at an RT of 31.5 min.

**Quantitation of Biliary and Urinary Metabolites.** Metabolites were quantified by integration of the peaks of radioactivity found during the radio-HPLC analysis. During the 0 to 15 h period after administration, biliary excretion of metabolite B1 represented 15.4% of the dose given, metabolite B2 represented 12.5% of the dose given, and the N-oxide represented 5.2% of the dose given (Fig. 3A). In addition, during the 0 to 15 h period after administration, urinary excretion of metabolite U1 represented 1.1% of the dose given, the N-oxide represented 1.0% of the dose given, and less than 0.1% of the dose was detected as ticlopidine (Fig. 3B).

**Identification of the Main Biliary Metabolite of Ticlopidine.** The MS spectrum of isolated metabolite B1 exhibited [MH]+ ions at m/z 587 and m/z 589 (Fig. 4A), which represent the 35Cl and 37Cl isotopes. The MS/MS analysis showed fragment ions at m/z 539 [MH-SO] resulting from the elimination of sulfoxide, at m/z 458 [MH-129] resulting from neutral loss of pyroglutamate, at m/z 410 [MH-SO-129]+, and at m/z 280 [MH-(GSH-S)]+ resulting from cleavage between the cysteinyl C-S bond (Fig. 4B), suggesting that metabolite B1 includes a sulfoxide function and GSH moiety.

The 1H-NMR spectrum of isolated metabolite B1 (Fig. 5) consisted of the expected signals for the GSH moiety (H-Cys, H-Glu, H-Gly, H-Cys, H-Glu, and H-Cys). The 1H-1H COSY NMR spectrum (Fig. 5) showed the marked correlation between the H3 signal at 6.21 ppm and the H2 signal at 3.60 ppm. Another correlation between the H3 signal and the H2 signal at 4.30 ppm was also observed. The two signals of H2 and H3 were assigned by a correlation in the COSY spectrum and their characteristic geminal coupling constant of 18 Hz.

The 13C-NMR spectrum of B1 (Fig. 6) showed the expected signals for the GSH moiety (C-Glu, C-Cys, C-Gly, C-Glu, C-Cys, and C-Cys). Complete proton and carbon chemical shift assignments were accomplished using the 1H-1H COSY and 1H-13C COSY NMR spectra. These MS, 1H-NMR, and 13C-NMR data on metabolite B1 strongly indicated that GSH was conjugated at the carbon 7a of the S-oxide. (The systematic nomenclature for this novel metabolite B1 is 2-amino-4-{1-(carboxymethyl-carbamoyl)-2-[5-(2-chloro-benzyl)-1-oxo-1,2,4,5,6,7-hexahydro-1H*4*-thieno[3,2-c]pyridin-7a-ylsulfanyl]-ethylcarbamoyl}-butyric acid.)

The product ion spectrum of metabolite B2 (Fig. 7) included ions at m/z 569 [MH-H2O]+, m/z 458 [MH-129]+, and m/z 280 [MH-(GSH-S)]+ as observed in that of metabolite B1. On the other hand, its
spectrum pattern was slightly different from that of metabolite B1; for example, the relative intensity of the fragment ion at \( m/z \) 539 (possible loss of sulf oxide) was weaker than that at \( m/z \) 569 (loss of water), whereas \( m/z \) 539 was major and \( m/z \) 569 was minor in the spectrum of metabolite B1. From the present data, metabolite B2 is thought to be a GSH conjugate, but the substituent positions of GSH and oxygen within the thiophene ring could not be definitely determined.

The product ion spectrum of U1 (having an [MH]⁺ ion at \( m/z \) 443) exhibited a major daughter ion at \( m/z \) 395 [MH-SO]⁺, representing a loss of sulfoxide, and also had minor ions at \( m/z \) 425 [MH-H₂O]⁺ resulting from loss of water and at \( m/z \) 280 [MH-(N-acetylcysteine-S)]⁺ attributed to cleavage in the cysteinyl C–S bond. A GSH conjugate loses glycine and glutamic acid to yield the cysteine conjugate, which is acetylated to mercapturic acid (Boyland and Chasseaud, 1969). Therefore, urinary metabolite U1 is thought to be mercapturic acid derived from the GSH conjugates detected in bile. The product ion spectra of U1 and B1 commonly showed major daughter ions at [MH-SO]⁺, which was not observed in the spectrum pattern of metabolite B2, where the ion at [MH-H₂O]⁺ was favored over the minor ion at [MH-SO]⁺. Accordingly, metabolite U1 would be generated from metabolite B1 rather than from metabolite B2, through physiological translation to an N-acetylcysteine conjugate (Fig. 8).

**In Vitro Metabolism of Ticlopidine.** Figure 9 shows an HPLC/UV chromatogram from the incubation of ticlopidine with rat liver S9 for 120 min. In addition to the unchanged ticlopidine (RT 26.5 min), several metabolites were formed. Two metabolites (RTs 9.9 and 11.2 min), which yielded an [MH]⁺ ion at \( m/z \) 587, were assigned as GSH conjugates corresponding to metabolite B1 and metabolite B2 found in rat bile, respectively. The metabolites (RTs 14.2 and 21.9 min) were identified as ticlopidine N-oxide and 2-oxo-ticlopidine, respectively, by comparison with authentic standard substances. The metabolites (RTs 20.2 and 20.9 min) exhibiting [MH]⁺ ions at \( m/z \) 278 and
280 (data not shown) were the lactam forms of ticlopidine and hydroxyticlopidine, respectively, as described previously (Dalvie and O’Connell, 2004). The peak of M1 at an RT of 18.8 min was an unknown metabolite. The peak at an RT of 22.4 min was assigned as ticlopidine S-oxide dimer (TSOD) after complete purification followed by MS and NMR analyses, the formation of which has been reported previously (Ha-Duong et al., 2001).

**Identification of in Vitro Metabolite of Ticlopidine.** In the MS spectrum of the isolated M1 (Fig. 10A), ions [MH]$^+$ at m/z 296 and m/z 298, representing the $^{35}\text{Cl}$ and $^{37}\text{Cl}$ isotopes, were observed. The product ion spectrum implied that M1 contains carboxylic acid and a thioketone group (Fig. 10B).

Figure 11 shows the $^1\text{H}$-$^1\text{H}$ COSY NMR spectrum of the metabolite M1. Signals corresponding to the benzene ring protons (HPh3-HPh6) were found at 7.24 to 7.49 ppm for four protons. Each proton assignment on the piperidine ring was conducted based on the correlation pattern in the COSY spectrum. The signal for one of the methylene protons at 3.27 to 3.34 ppm seems to be overlapped with the signal derived from deuterium methanol. This was supported by a correlation between the signal at 3.27 to 3.34 ppm and the signal for another methylene proton at 3.69 ppm in the spectrum. A signal characteristic of benzyl protons was also observed at 3.79 to 3.80 ppm. A singlet corresponding to the olefin proton was observed at 5.96 ppm, suggesting that metabolism had occurred at the thiophene ring of ticlopidine.

From the evidence provided by the MS and NMR spectra, the chemical structure of M1 was assigned as a thiophene ring-opened thioketone metabolite ([1-(2-chloro-benzyl)-4-thioxo-piperidin-(3Z)ylidene]-acetic acid (ROT) in systematic nomenclature).

**In Vitro Metabolism of 2-Oxo-ticlopidine.** To elucidate the metabolic route on the pharmacologically active metabolite responsible for antiplatelet activity, another in vitro oxidation was performed.
using rat liver microsomes. In this oxidation, 2-oxo-ticlopidine was used as a substrate to produce an amount of the active form sufficient for detection. Figure 12 shows reconstructed ion (\([\text{MH}]^+\) at \(m/z\) 298) and UV chromatograms from incubation of 2-oxo-ticlopidine with rat liver microsomes for 60 min. The production of the pharmacologically active metabolite (RT 13.1 min) was confirmed by cochromatography (chromatographic analysis of that incubation spiked with an authentic standard). ROT was also detected as a metabolite of 2-oxo-ticlopidine (Fig. 12).

**Discussion**

We studied the metabolism of ticlopidine in rats and identified two GSH conjugates of ticlopidine (B1 and B2) in bile and mercapturate in urine (U1) after the administration of ticlopidine. One of the GSH conjugates (B1) was isolated, and its structure was completely characterized as a GSH conjugate of \(S\)-oxide, in which conjugation had occurred at carbon 7a in the thienopyridine moiety. It has been reported that thiophene compounds such as tienilic acid and thiophene itself form highly reactive \(S\)-oxides during hepatic metabolism, which are either trapped by GSH and eventually excreted in urine as mercapturate (Dansette et al., 1992; Valadon et al., 1996) or irreversibly bind to macromolecules such as hepatic microsomal proteins (López-Garcia et al., 1994; Koenigs et al., 1999). Ha-Duong et al. (2001) reported the generation of ticlopidine \(S\)-oxide dimer derived from the Diels-Alder type dimerization of this reactive \(S\)-oxide in vitro. These reports are completely in agreement with our findings on the in vivo metabolites of ticlopidine, in which the structure contains a thiophene moiety.

The location of the glutathionyl moiety in the GSH conjugate of \(S\)-oxide (B1) was determined to be the carbon 7a position in the thienopyridine moiety by MS/MS and NMR analysis. As shown in Fig. 4, the product ion spectrum suggests that B1 contains a sulfoxide function and a GSH moiety. As illustrated in Fig. 5, the \(H_3\) signal was coupled with two geminal proton signals of \(H_2\) and \(H_9\). These MS/MS and NMR data can only be explained by the attachment of GSH at carbon 7a of ticlopidine \(S\)-oxide. In our \(1^H\) NMR spectrum, the \(H_3\) signal was broadened, although it could be spirited by the adjacent \(H_2\) and \(H_9\). However, the corresponding proton signal at the dihydrothiophene ring was also broadened in the \(1^H\) NMR spectrum of the mercaptoethanol adduct of the thiophene \(S\)-oxide, in which the \(S(CH_2)OH\) group is substituted at the corresponding carbon adjacent to the sulfoxide function (Valadon et al., 1996). Although we could not definitely characterize the incorporated position of GSH and oxygen at the thiophene ring, another GSH conjugate (B2, isomer of B1) was also detected. Dansette et al. (2005) reported interesting MS/MS fragment analyses on the two GSH conjugates of 2-phenylthiophene, in which the loss of 48 amu (sulfoxide) was favored over the loss of 18 amu (water) in the case of the GSH conjugate of the thiophene \(S\)-oxide, whereas the loss of 18 amu was dominant in the case of 5-hydroxy-4-glutathionyl-4,5-dihydrothiophene resulting from the addition of GSH to the thiophene epoxide. However, another thiophene GSH adduct of \(S\)-oxide showed no loss of 48 amu but did show a loss of 18 amu in the product ion spectrum (Medower et al., 2008). More recently, GSH adducts derived from both thiophene \(S\)-oxide and thiophene epoxide were identified in the microsomal incubation of 2-phenylthiophene, demonstrating that thiophene derivatives could simultaneously afford two intermediates, \(S\)-oxide and epoxide, during hepatic metabolism (Dansette et al., 2005). Therefore, B2 could be generated by the addition of GSH to either a thiophene \(S\)-oxide or a thiophene epoxide. Further structural analyses, including
an NMR study, are necessary to determine the substituted positions of GSH and oxygen in B2.

During the first 15 h after administration, the total amount of the GSH conjugates and mercapturic acid was 29% of the administered dose (Fig. 3, A and B). This is the first report that quantitatively reveals the metabolic activation (including both S-oxidation and putative epoxidation) of ticlopidine in vivo. N-Oxide found in rat bile and urine was a total of 6% of the dose in our study (Fig. 3, A and B). Tuong et al. (1981) reported that 16% of administered ticlopidine was excreted in rat urine as N-dealkylated metabolites. These observations indicate that the formation of reactive intermediates followed by conjugation with GSH is the major metabolic route of ticlopidine in rats. Ticlopidine has been reported to induce agranulocytosis (Ono et al., 1991), thrombotic thrombocytopenic purpura (Muszkat et al., 1998; Steinthubl et al., 1999), aplastic anemia (Mataix et al., 1992), and hepatotoxicity (Takikawa, 2005). Immune reactions seem to be involved in at least some serious adverse events, such as agranulocytosis (Ono et al., 1991) and hepatic injury (van Zanten and McCormick, 1996; Tsai et al., 2000). It has recently been suggested that reactive metabolites followed by covalent binding are associated with idiosyncratic toxicity through immune mechanisms. Thus, although additional studies are needed to clarify the metabolic pathways of ticlopidine in humans, our quantitative analyses on metabolic activation pathways in rats could support the hypothesis that reactive metabolites such as S-oxide are responsible for ticlopidine-induced adverse events in humans.

Incubation of ticlopidine with rat liver S9 resulted in several products including known metabolites (N-oxide, lactam form, hydroxylated form, TSOD, and 2-oxo-ticlopidine) and novel metabolites (GSH conjugates and ROT) (Fig. 9). TSOD was detected in vitro but not in vivo, whereas GSH conjugate(s) of the reactive S-oxide were formed both in vitro and in vivo, presumably because the concentration of the S-oxide formed in vitro is high enough to dimerize. In contrast, the concentration in vivo is too low for S-oxide molecules to access another one and results in conjugation with hepatic GSH. The pharmacologically active metabolite was generated by incubation of 2-oxo-ticlopidine with rat liver microsomes (Fig. 12) as reported previously (Yoneda et al., 2004). ROT, a novel thiophene ring-opened metabolite with a thioketone group and a carboxylic function, was also found during in vitro oxidation from 2-oxo-ticlopidine (Fig. 12) as well as from ticlopidine itself (Fig. 9), and this metabolite is likely to be formed by oxidation at the thiol group of the pharmacologically active metabolite.

From the results of the present in vivo and in vitro studies, the metabolic pathways of ticlopidine are postulated as depicted in Fig. 13. Oxidation of the thiophene sulfur atom yields a reactive S-oxide, which undergoes either dimerization to form TSOD or a reaction with GSH to give GSH conjugate(s). The resulting GSH conjugate loses
glycine and glutamic acid to yield a cysteine conjugate that is acetylated to the corresponding mercapturic acid. An alternative putative oxidative activation route is epoxidation, which can also yield the GSH conjugate. The total amount of GSH conjugates and mercapturic acid was 29% of the administered dose, which demonstrates that the activation pathways are the major in vivo metabolic routes of ticlopidine in rats. The other main routes appear to be N-dealkylation and N-oxidation. Lactam and hydroxylated metabolites were also detected in vitro. An S-oxide intermediate could also undergo a rearrangement to 2-oxo-ticlopidine, although it is possible for the epoxide intermediate to yield 2-oxo-ticlopidine through the nucleophilic addition of water followed by dehydration (Ha-Duong et al., 2001). This 2-oxo form is biotransformed into the pharmacologically active metabolite of ticlopidine responsible for platelet inhibition. The resulting active metabolite is further oxidized at the thiol group to yield ROT.

In conclusion, we have identified several novel metabolites and revealed the metabolic pathways of ticlopidine. The main biliary metabolite of ticlopidine was the GSH conjugate of 2-oxo-ticlopidine, in which conjugation had occurred at the carbon 7a in the thienopyridine moiety. Furthermore, 29% of the administered ticlopidine was subjected to the formation of reactive intermediates followed by conjugation with GSH in vivo in rats. In vitro incubation of ticlopidine with rat liver S9 fractions led to the formation of multiple metabolites, including 2-oxo-ticlopidine, the precursor for the pharmacologically active ticlopidine metabolite. A novel thiophene ring-opened metabolite with a thioether group and a carboxylic acid moiety has also been detected after incubation of 2-oxo-ticlopidine with rat liver microsomes or upon incubation of ticlopidine with rat liver S9 fractions.

References


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