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Identification of the active metabolite of ticlopidine from rat *in vitro* metabolites

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1 Ticlopidine is a well-known anti-platelet agent, but is not active by itself *in vitro*. We identified a metabolite with anti-platelet activity, which was generated after incubation of 2-oxo-ticlopidine with phenobarbital-induced rat liver homogenate *in vitro*.

2 An active moiety (UR-4501) was isolated by high-performance liquid chromatography after large-scale preparation of metabolites.

3 The chemical structure of UR-4501 was determined by a combination of liquid chromatography mass/mass spectrometry (LC/MS/MS) and nuclear magnetic resonance (NMR) analysis.

4 UR-4501 produced a concentration-dependent inhibition (3–100 μM) of ADP (10 μM)-induced human platelet aggregation, whereas 2-oxo-ticlopidine (3–100 μM) did not elicit inhibitory responses.

5 UR-4501 (10–100 μM) strongly inhibited ADP- and collagen-induced aggregation and slightly inhibited thrombin-induced aggregation.

6 The inhibition of rat washed platelet aggregation by UR-4501 (100 μM) persisted, even after the platelets had been washed twice.

7 These results suggest that UR-4501 is the molecule responsible for the *in vivo* activities of ticlopidine.

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Keywords: Ticlopidine; platelet aggregation; ADP; active metabolite; thienopyridine; rat; UR-4501

Abbreviations: ADP, adenosine 5′-diphosphate; BSA, bovine serum albumin; CYP, cytochrome *P*-450; HEPES, *N*-2-hydroxylethylpiperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; LC/MS/MS, liquid chromatography mass/mass spectrometry; PBS, phosphate-buffered saline; PGE_1, prostaglandin E_1; PPP, platelet-poor plasma; PRP, platelet-rich plasma; S9, supernatant fraction (9000 × *g*); Tris, tris(hydromethyl)aminoethane

Introduction

Platelet activation and subsequent aggregation play a central role in coronary artery diseases such as myocardial infarction and unstable angina. Since aspirin is widely used to prevent these vascular events, an understanding of platelet aggregation and the development of effective anti-platelet agents are important.

Ticlopidine is a well-known anti-platelet agent (Cattaneo *et al*., 1985; Quinn & Fitzgerald, 1999) and the results of two large-scale clinical trials, CATS (Gent *et al*., 1989) and TASS (Hass *et al*., 1989), have demonstrated that ticlopidine reduces the risk of thrombotic events in patients with atherosclerotic diseases. Previous reports have shown that the inhibitory effect of ticlopidine reaches a maximum after 3–5 days administration and the recovery to normal levels of platelet aggregation takes 4–8 days after stopping the medication (Di Minno *et al*., 1985; Saltiel & Ward, 1987). Although the mode of action is unclear, it is speculated that the active metabolite of ticlopidine inhibits platelet aggregation irreversibly.

Ticlopidine inhibits platelet aggregation *in vivo*, but not *in vitro* (Picard-Fraire, 1983). Since clopidogrel, one of the thienopyridine-type anti-platelet agents, requires hepatic metabolism to elicit the inhibitory effect on platelet aggregation (Savi *et al*., 1992), it is believed that ticlopidine is metabolized to produce its active form in the liver.

Recently, two groups have identified the active metabolites of the thienopyridine-type anti-platelet drugs (CS-747, clopidogrel). In previous reports, we described the identification and characterization of the active metabolite of CS-747 (Sugidachi *et al*., 2000; 2001). Another group (Sanofi-Synthelabo) identified the active metabolite of clopidogrel as 2-oxo-clopidogrel (Savi *et al*., 2000). From these studies, the metabolic pathways of CS-747 and clopidogrel gradually became clear and significant progress was made towards understanding their modes of action.

The objective of this study was to identify the metabolite of ticlopidine that is effective on human platelets. An earlier metabolic study of ticlopidine showed that 2-oxo-ticlopidine, but not ticlopidine itself (Figure 1), was the only metabolite that had anti-platelet activity *in vivo* (Picard-Fraire, 1983).

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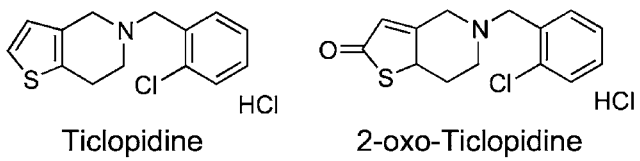

Figure 1 Chemical structure of ticlopidine and 2-oxo-ticlopidine.

Therefore, in our search for the identity of the active metabolite of ticlopidine, we started with 2-oxo-ticlopidine. We used human platelets for the screening of the active metabolite. In addition, we compared the pharmacological features observed with those seen in *ex vivo* experiments on ticlopidine.

Methods

Volunteers

After obtaining informed consent, venous blood was collected from healthy male volunteers. All subjects affirmed that they had not taken any medication in the week preceding sampling.

Animals

Male Wistar rats (8 weeks) were purchased from Charles River Japan (Kanagawa, Japan) and maintained on a light/dark cycle before being used. The animals were allowed free access to a standard rodent diet (CE-2; CLEA Japan, Tokyo, Japan) and water. The experimental procedures employed in this study were in accordance with the guidelines of the Animal Care and Use Committee at Yamaguchi University (Yamaguchi, Japan).

Agents

2-Oxo-ticlopidine (racemic, 5-(2-chlorobenzyl)-5,6,7,7a-tetrahydrothieno[3,2-*c*] pyridin-2(4*H*)-one) was chemically synthesized by Ube Industries (Yamaguchi, Japan). Ticlopidine, ADP (sodium salt), human fibrinogen, HEPES and fatty-acid-free bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO, U.S.A.). Phenobarbital Sodium (PB), tris(hydromethyl)aminoethane (Tris), trifluoroacetic acid (TFA) and acetonitrile were purchased from Wako Pure Chemical (Osaka, Japan). PGE_1 was purchased from Funakoshi (Tokyo, Japan). NADPH was purchased from Oriental Yeast (Tokyo, Japan).

Metabolite preparation

PB-induced rat liver supernatant fraction $9000 \times g$ (S9) was prepared as follows. To induce drug-metabolizing enzymes, the rats were pretreated with a daily intraperitoneal injection of PB at a dose of $40\,mg\,kg^{-1}$ body weight for 4 days. The animals were killed 24 h after the last injection. The livers were immediately perfused with ice-cold 1.15% KCl solution, and then homogenized in $4\times$ volumes of the KCl solution. The PB-induced rat liver S9 was obtained from the homogenate by centrifugation at $9000 \times g$ for 20 min and stored at $-80^{\circ}C$ before use.

The rat liver S9 was adjusted to $4\,mg\,protein\,ml^{-1}$ in 10 mM Tris–HCl buffer (pH 7.4). 2-Oxo-ticlopidine was added at a final concentration of 0.3 mM, and the reaction was initiated with 0.6 mM NADPH. The reaction mixture (40 ml) was incubated at $37^{\circ}C$ for 30 min with continuous stirring (60 r.p.m.). Subsequently, the incubation mixture was cooled at $4^{\circ}C$ and immediately extracted on a solid phase extraction column (Bondelute C18HF 500 mg). The column was washed twice with distilled water and the elution was carried out with 1 ml of 100% acetonitrile at $4^{\circ}C$. The eluate was added to 3 ml of ice-cold distilled water and immediately frozen on dry ice. The mixture containing the metabolites was lyophilized in a freeze dryer and stored at $-80^{\circ}C$.

HPLC

Fractionation of the metabolite mixture was performed by reverse-phase HPLC using a Shimazu 6A-system (Kyoto, Japan), which consisted of type LC-6A-high-pressure pumps, a type SPD-6A UV detector and a type SCL-6A controller. The mobile phase consisted of component A (0.02% ($v\,v^{-1}$) aqueous TFA] and component B (0.02 % ($v\,v^{-1}$) TFA in acetonitrile). A linear gradient of acetonitrile from 15 to 45% was used over 30 min at a flow rate of $0.7\,ml\,min^{-1}$. The separation was performed at room temperature on an Inertsil ODS-2 4.0×150 mm column (GL science, Japan).

Preparation of human platelet-rich plasma (PRP)

Blood was drawn from the healthy volunteers using 3.8%. ($w\,v^{-1}$) sodium citrate (nine parts of blood, one part of citrate) as an anticoagulant. PRP was prepared by centrifugation at $180 \times g$ for 15 min at room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation of the remaining blood at $2000 \times g$ for 10 min. Platelet counts in PRP were adjusted to 3×10^8 platelets ml^{-1} by adding PPP.

Preparation of rat washed platelets

Washed platelets were prepared as described previously (Sugidachi *et al*., 2001), with slight modifications. Blood was drawn from rats anesthetized with sodium pentobarbital ($40\,mg\,kg^{-1}$, i.p.) using 3.8% ($w\,v^{-1}$) sodium citrate (nine parts of blood, one part of citrate) as an anticoagulant. PRP was prepared by centrifugation at $230 \times g$ for 15 min at room temperature. The PRP was centrifuged at $1200 \times g$ for 6 min, and the resulting platelet pellet was resuspended in the washing buffer (NaCl 140 mM, KCl 2.7 mM, $NaH_2PO_4 \cdot 2H_2O$ 0.4 mM, $NaHCO_3$ 12 mM, $MgCl_2 \cdot 6H_2O$ 1 mM, glucose 5 mM, HEPES 10 mM, PGE_1 100 nM and fatty acid-free BSA $3.5\,mg\,ml^{-1}$, pH 6.7). The platelet suspension was then washed at least twice and resuspended in the suspension buffer (NaCl 140 mM, KCl 2.7 mM, $NaH_2PO_4 \cdot 2H_2O$ 0.4 mM, $NaHCO_3$ 12 mM, $MgCl_2 \cdot 6H_2O$ 1 mM, glucose 5 mM, HEPES 10 mM and BSA $3.5\,mg\,ml^{-1}$, pH 7.4). Platelet counts in washed platelet suspensions were adjusted to 3×10^8 platelets ml^{-1} by adding the suspension buffer.

Measurement of platelet aggregation

All aggregation studies were performed using Mebanix aggregometers (model Hematracer VI, Tokyo, Japan). In

these studies, the rat washed platelet suspension was supplemented with human fibrinogen (0.068 mg ml^{-1}) and 1 mM Ca^{2+}. The washed platelet suspension or PRP was incubated at 37°C for 5 min in the aggregometer with continuous stirring at 1000 r.p.m and then stimulated with ADP, collagen and thrombin. Changes in light transmission were recorded for at least 10 min and the maximum aggregation was estimated. The extent of aggregation was expressed as a percentage of the maximum light transmittance, which was obtained with PPP (PRP aggregation) or the suspension buffer (washed platelet aggregation). In the experiment to examine the duration of action of the drug metabolite *in vitro*, rat platelet aggregation was measured before and after each of up to two platelet washings.

Separation of metabolites and assessment of the anti-platelet activity of each fraction

The lyophilized mixture containing metabolites was dissolved in 1 ml of 15% acetonitrile containing 0.02 % (v v^{-1}) TFA and 0.1 ml of the solution was immediately injected into the HPLC system. While monitoring the eluent absorbance at 240 nm, 1-min fractions were collected in test tubes. After pH neutralization with 30 mM ammonium acetate, each fraction was lyophilized in a freeze dryer and stored at −80°C. The lyophilized fractions were dissolved in 0.05 ml of PBS. To assess the anti-platelet activity of each fraction, 0.18 ml of human PRP was incubated at 37°C for 5 min in the aggregometer with 0.01 ml of PBS solution containing each fraction and then stimulated with 0.01 ml of 0.2 mM ADP (final concentration 10 μM). Changes in light transmission were recorded for at least 10 min and the maximum aggregation was estimated.

Large-scale isolation of the active metabolite

To separate the active metabolite, the same HPLC system was used. The mobile phase consisted of 15% acetonitrile containing 0.02% TFA. An isocratic method was used over 30 min at a flow rate 4.2 ml min^{-1}. The separation was performed at room temperature on an Inertsil ODS-2 10.0 × 250 mm column (GL science, Japan). The 4 ml sample of the metabolite mixture was passed through a 0.22 μM filter and injected into the HPLC system. The fraction containing the active metabolite was neutralized with 30 mM ammonium acetate and immediately frozen on dry ice. Acetonitrile in the pooled fraction was removed in a rotary evaporator. Finally, a solid-phase extraction column (Bondelute C18 200 mg; GL science, Japan) was used to purify the fraction. The active metabolite was eluted with 1 ml of acetonitrile and the eluate was added to 4 ml of distilled water. The solution was lyophilized in a freeze dryer and stored at −80°C for later analysis.

Determination of the chemical structure of the active metabolite

The chemical structure of the active metabolite was determined by mass spectrometry (LC/MS/MS) and ^{1}H-NMR. The active metabolite was injected onto an Inertsil ODS-2 4.0 × 150 mm column using an L-7000 liquid chromatograph system (Hitachi, Japan). Isocratic elution was performed with a mixture of 0.1% formic acid/acetonitrile (13:87 v v^{-1}) at 0.7 ml min^{-1}, and the UV signal was followed at 240 nm. MS data were acquired on a Finnigan LCQ instrument from Thermo Quest (San Jose, CA, U.S.A.) in positive electro spray ionization (ESI+) mode. The mass range was scanned between 100 and 900 amu. In MS/MS mode (28% of collision energy), the two parent ions obtained at m/z values of 298 and 300 (with 1.0 amu peak width) correspond, respectively, to the quasi-molecular ions of metabolites containing the isotopes ^{35}Cl and ^{37}Cl.

^{1}H (400 MHz)-NMR spectra were recorded on an FT-NMR spectrometer GSX-400 from JOEL (Tokyo, Japan). The spectra were recorded in CD_3CN solvent. Sample concentration was less than 2 mg in 0.9 ml.

Statistics

Results are expressed as the mean ± s.e.m. unless otherwise stated. Differences between multiple groups were assessed by Dunnett's multiple comparison test (SAS statistical computer package, SAS Institute Inc., Cary, NC, U.S.A.). A P-value of less than 0.05 was considered statistically significant.

Results

Isolation of the active metabolite

The separation of the metabolites obtained from the PB-induced rat liver S9 was performed by reverse-phase HPLC with a linear gradient of acetonitrile from 15 to 45%. Subsequently, the inhibitory effect of each fraction on human platelet aggregation was assessed. The inhibitory effect was detected in a peak eluted at 9 min (immediately after 2-oxo-ticlopidine at 8 min). Although an inhibitory effect was also detected in a peak eluted at 28 min, a preliminarily LC/MS/MS experiment showed that this peak contained no fragments derived from 2-oxo-ticlopidine (data not shown). Therefore we concluded that the peak eluted at 9 min was the active metabolite of ticlopidine (Figure 2).

Large-scale preparation and determination of the chemical structure of the active metabolite

To determine the chemical structure of the active metabolite, a large-scale preparation was carried out. The active metabolite was only separated in acidic conditions; however, it was highly unstable in 0.1% acetic acid or 0.02% TFA (data not shown). For that reason, the online neutralization method was used. When 100 mg of 2-oxo-ticlopidine was metabolized with PB-induced rat liver S9, about 5 mg of active metabolite (UR-4501) was obtained.

In the preliminary LC/MS experiment, a molecular ion MH^+ at m/z 298 was obtained. Therefore, the molecular weight of UR-4501 was 18 amu greater than 2-oxo-ticlopidine. To determine the chemical structure of the active metabolite, isotopes of chlorine proved very useful. The MS/MS data obtained on quasi-molecular ions MH^+ 298 (Figure 3a) and 300 (Figure 3b) containing the isotopes ^{35}Cl or ^{37}Cl, respectively, allowed the detection of fragments bearing a chlorine atom. Thus, if two daughter ions obtained from MH^+ 298 and 300 had the same value, for example if both were 172 amu, these ions would not contain chlorine atoms. On the

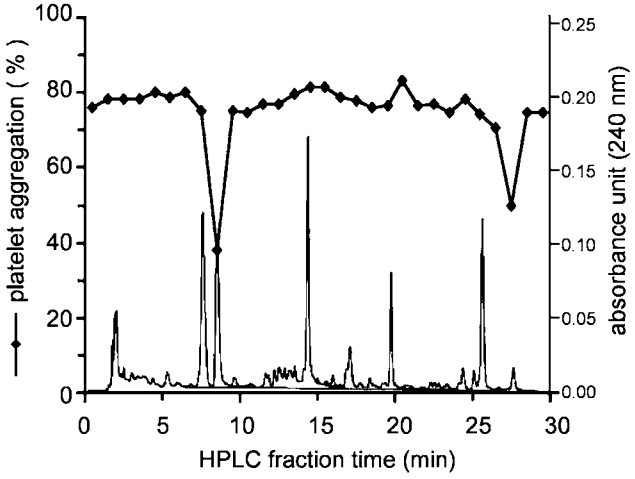

Figure 2 HPLC chromatogram and anti-platelet activity of 2-oxo-ticlopidine metabolites. The lyophilized mixture containing metabolites of 2-oxo-ticlopidine was fractionated by HPLC. While monitoring the absorbance at 240 nm, 1-min fractions were collected and lyophilized. To assess the anti-platelet activity of the metabolites, human PRP was pre-incubated with each fraction solution for 5 min and aggregation was induced by ADP (10 μM).

other hand, if there was a 2 amu difference between the daughter ions, as, for example, if one was 154 amu and the other was 156 amu, these ions would contain chlorine atoms in their chemical structures. Based on these MS/MS analyses, the chemical structure of the active metabolite was proposed (Figure 3c).

Effect of UR-4501 on human PRP and rat washed platelet aggregation

To determine the inhibitory potency and specificity of UR-4501, we examined the effects of UR-4501 on human PRP and rat washed platelet aggregation. For the analysis of the inhibitory effect on agonist-induced platelet aggregation, we used rat platelets, because there are more background data for studies using rat platelets and they are well established compared to studies using human platelets. Pretreatment of human PRP with UR-4501 (3–100 μM) inhibited ADP-induced aggregation in a concentration-dependent manner, whereas 2-oxo-ticlopidine and a low dose of ticlopidine (3–30 μM) did not elicit any aggregating responses, while a high dose (100 μM) of ticlopidine showed a small but statistically significant inhibition (Figure 4). In experiments with rat washed platelets, UR-4501 strongly inhibited ADP- (Figure 5a) and collagen- (Figure 5b) induced aggregation and slightly inhibited thrombin-induced aggregation (Figure 5c).

Duration of action

The *in vitro* duration of the action of UR-4501 was investigated in comparison with PGE_1, a potent reversible inhibitor of platelet aggregation. ADP-induced aggregation of rat platelets was completely inhibited in the presence of PGE_1 (2 μM), but this inhibition was not evident after the platelets were washed to eliminate PGE_1 in the plasma. In contrast, the inhibition of platelet aggregation by UR-4501 (100 μM) persisted even after the platelets were washed twice (Figure 6).

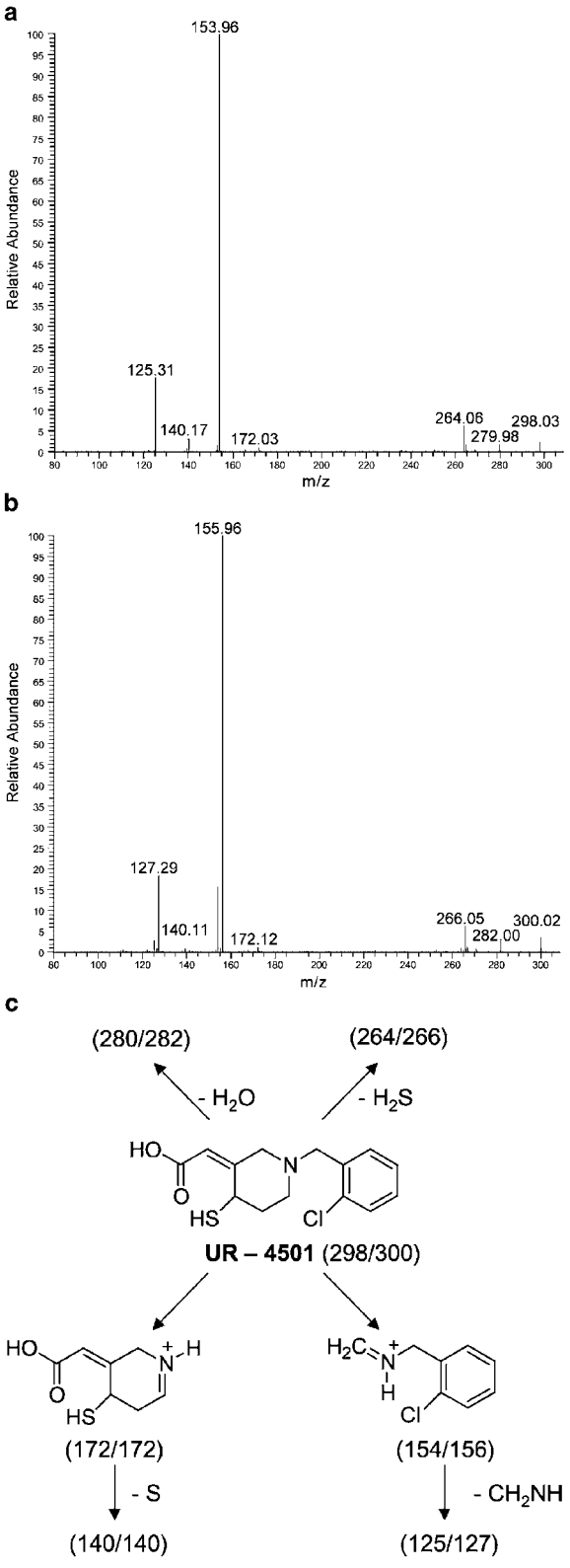

Figure 3 LC/MS/MS spectra of active metabolite. (a) LC-ESI(+)/MS/MS spectrum of m/z 298 ion (MH^+ containing ^{35}Cl isotope). (b) LC-ESI(+)/MS/MS spectrum of m/z 300 ion (MH^+ containing ^{37}Cl isotope). (c) Primary structure of the active metabolite of ticlopidine (UR-4501) and MS fragmentation pathway. The numbers in the parenthesis indicate the m/z measured by LC-ESI(+)/MS/MS.

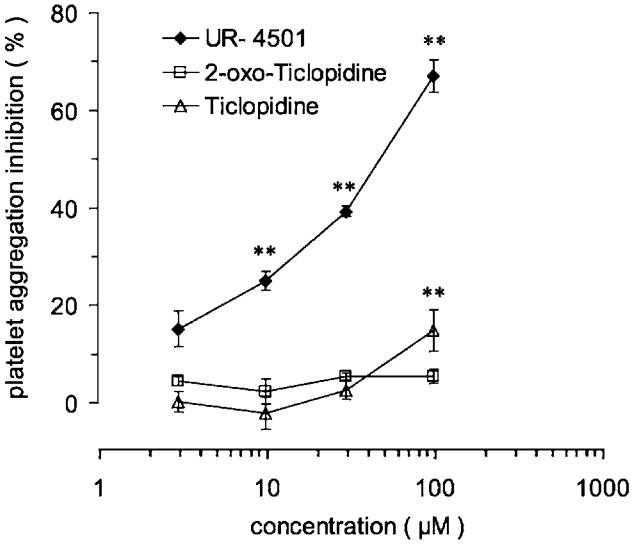

Figure 4 *In vitro* effects of the active metabolite (UR-4501) of ticlopidine on human platelet aggregation. Human platelets were pre-incubated with UR-4501, 2-oxo-ticlopidine, ticlopidine and vehicle for 1 h and aggregation was induced by ADP (10 μM). Results were expressed as the percentage of aggregation inhibition relative to vehicle control (100%). Each point is the mean $\pm$s.e.m. ($n=4$). $^{**}P<0.01$ vs control.

Discussion

In this study, we identified the active metabolite (UR-4501) of ticlopidine from 2-oxo-ticlopidine, which is known to be the only metabolite that has shown anti-platelet activity *ex vivo* (Picard-Fraire, 1983). UR-4501 was a potent inhibitor of ADP-induced platelet aggregation and a moderate inhibitor of thrombin-induced aggregation. This characteristic of inhibition of platelet aggregation agents was consistent with the *ex vivo* results of ticlopidine (Saltiel & Ward, 1987; Cattaneo *et al*., 1991).

From the structural analysis, we have ascertained that UR-4501 has a carboxylic acid and a thiol group as a result of 2-oxo-thiophene ring opening. Previous reports have shown that the active metabolites of other thienopyridine-type anti-thrombotic drugs such as clopidogrel (Savi *et al*., 2000) and CS-747 (Sugidachi *et al*., 2001) have a carboxylic acid function and a thiol group. These results suggest that the mode of action and the target molecule of these drugs are identical.

Since NADPH was essential and PB treatment, which induces some cytochrome *P*-450 (CYP) drug-metabolizing enzymes, was effective at generating UR-4501, we suggest that 2-oxo-ticlopidine is metabolized into the active metabolite through a CYP-dependent pathway.

In this study, we used PB-induced rat liver S9 to obtain UR-4501. However, anti-platelet activity was not observed in metabolites treated with PB-induced rat liver microsomes (data not shown). We speculate that soluble co-factors in S9 may stabilize the active metabolite of thienopyridine-type anti-platelet drugs, since the active metabolite of clopidogrel was prepared from human liver microsomes, in a reaction mixture containing glutathione (Pereillo *et al*., 2002).

We are currently examining which CYP is involved in the metabolic activation of ticlopidine. It has been reported that the activation of clopidogrel involves CYP1A (Savi *et al*., 1994), CYP3A4 and CYP3A5 (Clarke & Waskell, 2003). Ticlopidine and clopidogrel must undergo a two-step metabolism to exert

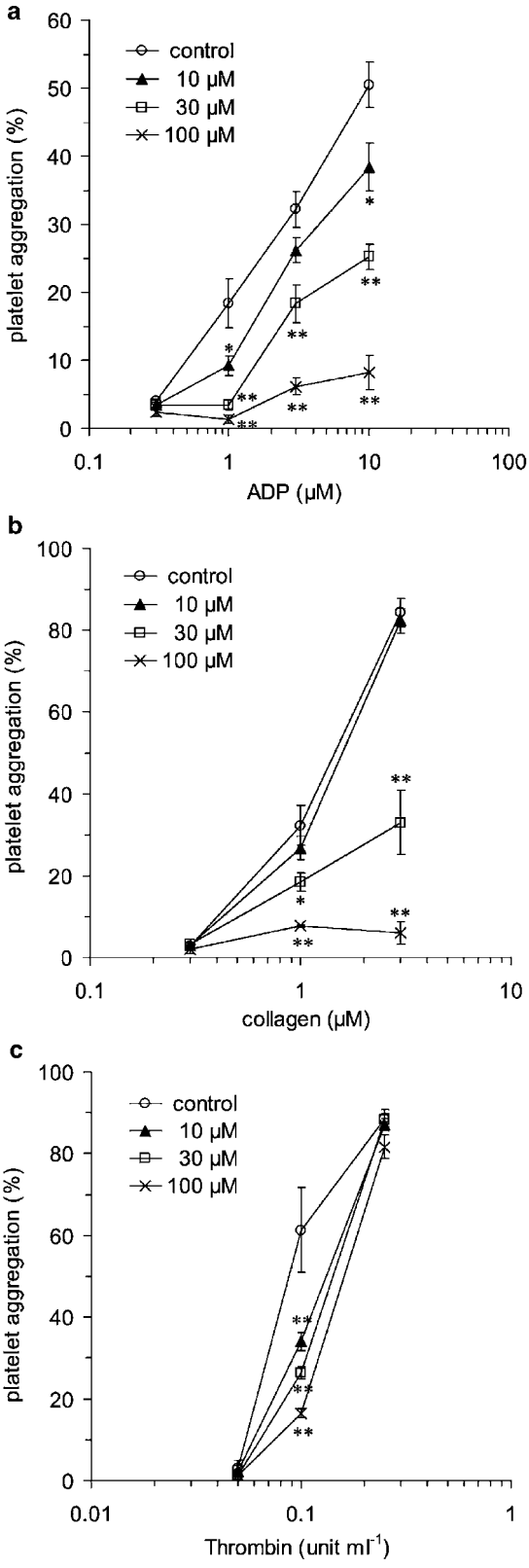

Figure 5 *In vitro* effects of the active metabolite of ticlopidine (UR-4501) on platelet aggregation agonists. Rat washed platelets were pre-incubated with UR-4501 for 5 min and aggregation was induced by ADP (a), collagen (b) and thrombin (c). Results were expressed as the mean $\pm$s.e.m. ($n=4$). $^{*}P<0.05$, $^{**}P<0.01$ vs each control.

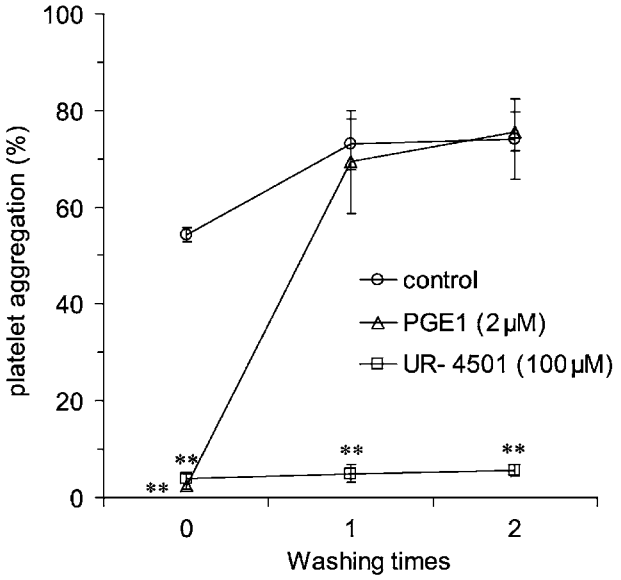

Figure 6 *In vitro* duration of anti-aggregation effects. Rat platelet-rich plasma was pre-incubated with UR-4501 (100 μM) or PGE_1 (2 μM) for 1 min, and then ADP (10 μM)-induced platelet aggregation was measured before and after washing the platelets. Results are presented as the mean $\pm$s.e.m. ($n=4$). $^{**}P<0.01$ vs each control.

anti-platelet activity. First, the aromatic thiophene ring is oxidized to generate 2-hydroxy thiophene and isomerized to the 2-oxo form. Second, the 2-oxo-thiophene ring is opened. It is well known that the actions of CYPs differ between individuals. The metabolic complexity exhibited by CYPs could explain not only pharmacodynamic individual differences but also the adverse effects of ticlopidine.

It has been shown that ticlopidine has long-lasting effects on platelet aggregation *ex vivo* (Nunn & Lindsay, 1980), and the duration of the action of ticlopidine is equal to the lifespan of platelets in the rat (Tomikawa *et al*., 1978). These results suggest that ticlopidine interacts with platelets in an irreversible manner. In the present study, we demonstrated that the inhibitory effects of UR-4501 on platelet aggregation were maintained, even after the platelets were washed. The inhibitory effects against platelet aggregation agonists and the irreversible mode of action strongly suggest that UR-4501 is the molecule responsible for the *in vivo* action of ticlopidine.

In our study of the effects of ticlopidine metabolites on platelet aggregation, we noticed that the thiol group is essential for achieving anti-platelet effects (data not shown). Pharmacological studies have suggested the existence of ADP receptors on platelets (Daniel *et al*., 1998; Jantzen *et al*., 1999). Recently, it was reported that the inactivation of a platelet ADP receptor by thiol reagents requires interaction with extracellular cysteine residues (Ding *et al*., 2003). We speculate that a disulfide bridge is formed between the thiol group of UR-4501 and that of the cysteine residues of platelet ADP receptors, which makes the anti-platelet effect of UR-4501 irreversible.

Recently, the platelet ADP receptor involved in thienopyridine-type anti-platelet drugs has been cloned and called P2Y12 (Foster *et al*., 2001; Hollopeter *et al*., 2001; Zhang *et al*., 2001). Currently, the ADP receptors of platelets are classified into P2Y1 and P2Y12 subtypes, both of which couple with heterotrimeric G proteins and are required for ADP-induced platelet aggregation (Jin & Kunapuli, 1998; Jarvis *et al*., 2000). The P2Y12 receptor, coupled to adenylyl cyclase inhibition, mediates the effect of ADP and P2Y1 activates phospholipase C (Gachet, 2001). Since previous studies have demonstrated that the active metabolites of CS-747 and clopidogrel antagonize the binding of ^{3}H-2-MeS-ADP, a specific ADP ligand (Savi *et al*., 2001; Sugidachi *et al*., 2001), we speculate that UR-4501 antagonizes the P2Y12 receptor.

In these experiments, we have identified UR-4501, the active metabolite of ticlopidine, and investigated its effect on platelet aggregation. UR-4501 will be useful not only for the understanding of platelet aggregation but also for discovering novel anti-platelet agents.

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