



# Antiplatelet action of R-99224, an active metabolite of a novel thienopyridine-type G<sub>i</sub>-linked P2T antagonist, CS-747

<sup>1</sup>Atsuhiko Sugidachi, <sup>\*</sup><sup>1</sup>Fumitoshi Asai, <sup>2</sup>Kenji Yoneda, <sup>2</sup>Ryo Iwamura, <sup>1</sup>Taketoshi Ogawa, <sup>1</sup>Ken-ichi Otsuguro & <sup>1</sup>Hiroyuki Koike

<sup>1</sup>Pharmacology and Molecular Biology Research Laboratories, Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan and <sup>2</sup>Ube Research Laboratory, Ube Industries, Ltd., Kogushi 1978-5, Ube City, Yamaguchi 755-8633, Japan

**1** CS-747 is a novel thienopyridine-type platelet ADP inhibitor which lacks *in vitro* activity. This study examined pharmacological profiles of R-99224, a hepatic metabolite of CS-747.

**2** R-99224 produced a concentration-dependent inhibition of *in vitro* platelet aggregation in washed human platelets (0.03–1  $\mu\text{g ml}^{-1}$ ), which was relatively specific to ADP compared to collagen and thrombin.

**3** R-99224 (0.1–3  $\mu\text{g ml}^{-1}$ ) also elicited a similar inhibition of ADP-induced aggregation in rat platelets. The inhibition by R-99224 (10  $\mu\text{g ml}^{-1}$ ) persisted even after platelets were washed three times. Intravenous injection of R-99224 (0.1–3  $\text{mg kg}^{-1}$ ) to rats resulted in a dose-dependent inhibition of *ex vivo* ADP-induced platelet aggregation.

**4** R-99224 (0.1–100  $\mu\text{M}$ ) decreased binding of [<sup>3</sup>H]-2-methylthio-ADP ([<sup>3</sup>H]-2-MeS-ADP), a stable ligand for platelet ADP receptors, to washed human platelets. The inhibition by R-99224 reached a plateau at a concentration of 3  $\mu\text{M}$  (1.4  $\mu\text{g ml}^{-1}$ ), but complete inhibition was not achieved even at the highest concentration used (100  $\mu\text{M}$ ).

**5** R-99224 (10  $\mu\text{M}$ ) in combination with ARL-66096 (0.3  $\mu\text{M}$ ), an ATP analogue-type G<sub>i</sub>-linked P2T receptor antagonist, produced no additional inhibition of [<sup>3</sup>H]-2-MeS-ADP binding. In contrast, [<sup>3</sup>H]-2-MeS-ADP binding was completely abolished by R-99224 (10  $\mu\text{M}$ ) in combination with A3P5PS (300  $\mu\text{M}$ ), a selective P2Y<sub>1</sub> antagonist, suggesting that R-99224 selectively binds to the G<sub>i</sub>-linked P2T receptor.

**6** R-99224 (0.01–3  $\mu\text{g ml}^{-1}$ ) inhibited ADP-induced [<sup>125</sup>I]-fibrinogen binding to human platelets in a concentration-dependent manner. R-99224 (0.1–1  $\mu\text{g ml}^{-1}$ ) also inhibited the ADP-induced decrease in cyclic AMP levels in PGE<sub>1</sub>-stimulated platelets, whereas the agent did not affect ADP (10  $\mu\text{M}$ )-induced Ca<sup>2+</sup> mobilization.

**7** These findings suggest that R-99224 is a selective and irreversible antagonist of G<sub>i</sub>-linked P2T receptors and that R-99224 is a responsible molecule for *in vivo* actions of CS-747.

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**Keywords:** Platelet aggregation; G<sub>i</sub>-linked P2T receptor; ADP; CS-747; R-99224; active metabolite; thienopyridine

**Abbreviations:** ADP, adenosine 5'-diphosphate; A3P5PS, adenosine 3'-phosphate 5'-phosphosulfate; BSA, bovine serum albumin; EGTA, ethylene glycol-bis ( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; 2-MeS-ADP, 2-methylthio-adenosine 5'-diphosphate; PGE<sub>1</sub>, prostaglandin E<sub>1</sub>; PPP, platelet-poor plasma; PRP, platelet-rich plasma

## Introduction

ADP is one of the most physiologically and pathophysiologically important platelet agonists. By inducing a number of platelet responses, including shape change from disc to sphere, aggregation, and secretion of granule contents, ADP contributes to haemostasis, pathological thrombus formation, and vascular occlusion. Transduction of the ADP-induced intracellular signalling events involves the activation of G proteins, the inhibition of adenylyl cyclase, the activation of phospholipase C, and the elevation of intracellular calcium levels (Hourani & Hall, 1994; Mills, 1996). These responses

are believed to be mediated by the interaction of a series of platelet ADP receptors tentatively designated as P2T receptors (Hourani & Hall, 1994; Gachet & Cazenave, 1991). A three-receptor model has recently been proposed in which the P2T receptors are composed of three distinct receptors, i.e., the P2X<sub>1</sub>, a ligand-gated ion channel receptor and two distinct G-protein coupled ADP receptors (a G<sub>q</sub>-linked P2Y<sub>1</sub> receptor and a G<sub>i</sub>-linked P2T receptor distinct from P2Y<sub>1</sub>) (for review see Kunapuli, 1998a,b).

Several lines of evidence indicate the importance of the G<sub>i</sub>-linked P2T receptor for platelet aggregation, and blockers of these receptors have been the target of drug development in the field of cardiovascular diseases. Up to now, two different types of blockers against G<sub>i</sub>-linked P2T receptors have been reported: thienopyridine analogue- and ATP analogue-type

\*Author for correspondence at: Pharmacology and Molecular Biology Research Laboratories, Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan.  
E-mail: toasai@shina.sankyo.co.jp

inhibitors. Ticlopidine and clopidogrel, both thienopyridine derivatives, are orally active inhibitors of ADP-induced platelet aggregation (Defreyn *et al.*, 1991; Mills *et al.*, 1992; Savi *et al.*, 1994b) which are now used in clinical settings. Since ticlopidine and clopidogrel are essentially inactive *in vitro*, their *in vivo* activities may be due to putative active metabolite(s) the chemical structures of which remains to be determined (Saltiel & Ward, 1987; Savi *et al.*, 1994a; Coukell & Markham, 1997).

We have recently reported that CS-747 (2-acetoxy-5-( $\alpha$ -cyclopropylcarbonyl-2-fluorobenzyl)-4,5,6,7-tetrahydrothieno [3,2-c]pyridine), a novel thienopyridine derivative, is an orally active antiplatelet agent with a fast onset and a high potency in rats (Sugidachi *et al.*, 2000). In the same study we have demonstrated that while CS-747 is inactive *in vitro*, one of its hepatic metabolites R-99224 ((2Z)-[1-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-mercapto-3-piperidinylidene] (Figure 1) inhibits the *in vitro* aggregation of rat platelets. In the present study, we investigated the *in vitro* pharmacological profile of R-99224 using washed human platelets and examined the effects of injected R-99224 on platelet aggregation in rats. We now provide evidence that R-99224 is an active metabolite of CS-747, a thienopyridine-type P2T antagonist.

## Methods

### Volunteers

After obtaining consent, venous blood was collected from healthy male volunteers by clean venepuncture using a 21-gauge butterfly needle and minimal stasis. All subjects avowed that they had not taken any medication in the 1 week preceding sampling.

### Animals

Male Sprague-Dawley rats purchased from Japan SLC (Shizuoka, Japan) were used. The animals were allowed free access to standard rat chow and water. The experimental procedures employed in this study were in accordance with the guidelines of the Institutional Animal Care and Use Committee at Sankyo Research Laboratories (Tokyo, Japan).

### Preparation of platelets

Blood was drawn from the healthy volunteers and rats anaesthetized with sodium pentobarbital (40 mg kg<sup>-1</sup>, i.p.) using 3.8% (w v<sup>-1</sup>) sodium citrate (nine parts blood, one part citrate) as an anticoagulant. Washed platelets were used in experiments with human platelets, and either platelet-rich plasma (PRP) or washed platelets were used in experiments

with rat platelets. PRP was prepared by centrifugation at 180  $\times$  g (human) or 230  $\times$  g (rat) 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 rat PRP were adjusted to  $5 \times 10^8$  platelets ml<sup>-1</sup> by adding PPP.

Washed platelets were prepared as described previously (Sugidachi *et al.*, 1998) with slight modifications. The PRP was centrifuged at 1200  $\times$  g for 6 min, and the resulting platelet pellet was resuspended in washing buffer containing (in mM): NaCl 140, KCl 2.7, NaH<sub>2</sub>PO<sub>4</sub>  $\cdot$  2H<sub>2</sub>O 0.4 NaHCO<sub>3</sub> 12, MgCl<sub>2</sub>  $\cdot$  6H<sub>2</sub>O 1 glucose 5 HEPES 10 and 3.5 mg ml<sup>-1</sup> fatty acid-free bovine serum albumin (BSA), pH 6.7. Next, this platelet suspension was washed two more times and resuspended in the suspension buffer (same composition as the washing buffer, pH 7.4). PGE<sub>1</sub> (100–200 nM) and/or apyrase (0.05–0.5 u ml<sup>-1</sup>) were used to prevent platelet activation while the platelets were being washed.

### Measurement of platelet aggregation

All aggregation studies were performed in Mebanix aggregometers (model PAM-6C and PAM-8C, Tokyo, Japan). In studies on washed platelet aggregation, the washed platelet suspension was supplemented with human fibrinogen (1 mg ml<sup>-1</sup> for human and 0.068 mg ml<sup>-1</sup> for rat) and 1 mM Ca<sup>2+</sup>. The washed platelet suspension ( $3 \times 10^8$  platelets ml<sup>-1</sup>) or PRP ( $5 \times 10^8$  platelets ml<sup>-1</sup>) was incubated at 37°C for 1.5 min in the aggregometer with continuous stirring at 1000 r.p.m. and then stimulated with ADP, collagen, or thrombin. Changes in light transmission were recorded for at least 5 min and the maximum aggregation was estimated. The extent of aggregation was expressed as a percentage of the maximum light transmittance, obtained with the suspension buffer (washed platelet aggregation) or PPP (PRP aggregation). In the experiment to determine the duration of action *in vitro*, rat platelet aggregation was measured before and after each of up to three platelet washings.

### [<sup>3</sup>H]-2-MeS-ADP binding

The washed human platelet suspension ( $3 \times 10^8$  platelets ml<sup>-1</sup>) was incubated with 10 nM [<sup>3</sup>H]-2-MeS-ADP at room temperature. After 60 min, the reaction mixture was layered onto a suspension buffer containing 20% sucrose and the bound ligand was separated by centrifugation at 10,000  $\times$  g for 3 min at room temperature. After careful aspiration of the supernatant, the platelet pellet was dissolved in NCS-II (Amersham, Buckinghamshire, U.K.) and its radioactivity was measured by scintillation counting. Specific binding was defined as the difference between the total binding and nonspecific binding determined by the addition of unlabelled 2-MeS-ADP at 100  $\mu$ M.

### [<sup>125</sup>I]-Fibrinogen binding

The ability of washed platelets to bind fibrinogen in response to ADP was assessed with [<sup>125</sup>I]-fibrinogen according to the method previously described (Sugidachi *et al.*, 1998) with slight modifications. [<sup>125</sup>I]-Fibrinogen (70  $\mu$ g ml<sup>-1</sup>) was added to the washed human platelets in the presence of 1 mM Ca<sup>2+</sup>. The mixtures containing R-99224 or vehicle (total volume of

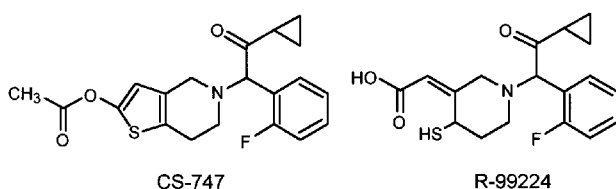


Figure 1 Chemical structures of CS-747 and R-99224.

240  $\mu$ l) were incubated for 1.5 min, and fibrinogen binding was initiated by adding 10  $\mu$ M ADP. Following a 10-min incubation, a 200  $\mu$ l aliquot from each reaction mixture was layered on 400  $\mu$ l of the suspension buffer containing 20% sucrose, and centrifuged at  $10,000 \times g$  for 3 min. The supernatants were aspirated, and the radioactivity was measured using a gamma counter (Riastar, Packard). Specific fibrinogen binding was calculated by subtracting the nonspecific binding measured in the resting platelets from the total binding. The data were expressed as the per cent (%) of binding in ADP (10  $\mu$ M)-stimulated control platelets.

#### Measurement of cyclic AMP concentration

Cyclic AMP levels were determined according to the method of Defrey *et al.* (1991) with slight modifications. A mixture of 1 ml buffer (mM): Tris 15, NaCl 120, KCl 4,  $\text{MgSO}_4$  1.6,  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  2, glucose 10 (0.2% BSA), IBMX 1.5, pH 7.4, and 2 ml PRP ( $3 \times 10^8$  platelets  $\text{ml}^{-1}$ ) was incubated for 1.5 min, and then  $\text{PGE}_1$  (10  $\mu$ M), a stimulator of adenylyl cyclase, was added. Three min after the  $\text{PGE}_1$  stimulation, ADP (10  $\mu$ M) or saline was added to the reaction mixture. Aliquots in a volume of 0.5 ml were taken from the reaction mixture before and 3 and 6 min after the  $\text{PGE}_1$  stimulation. These samples were supplemented with 50  $\mu$ l of 6N HCl and 50  $\mu$ M EDTA solution and boiled for 5 min. After rapid cooling on ice, the samples were centrifuged at  $10,000 \times g$  for 5 min at  $4^\circ\text{C}$ . The supernatants (300  $\mu$ l) were incubated after adding  $\text{CaCO}_3$  (60 mg) at room temperature for 15 min and then centrifuged again at  $10,000 \times g$  for 5 min at  $4^\circ\text{C}$ . The final supernatants were assayed for cyclic AMP concentrations using an EIA kit (Amersham, Buckinghamshire, U.K.).

#### Intracellular $\text{Ca}^{2+}$ concentration ( $[\text{Ca}^{2+}]_i$ )

$[\text{Ca}^{2+}]_i$  was determined after loading human platelets with a fluorescent dye, fura 2-AM. PRP was incubated with 2  $\mu$ M fura 2-AM at  $37^\circ\text{C}$  for 40 min, and this was then washed twice by centrifugation and resuspended at  $3 \times 10^8$  platelets  $\text{ml}^{-1}$  in a suspension buffer containing 0.05 u  $\text{ml}^{-1}$  apyrase. Measurement of fura 2 fluorescence was performed in a Hitachi F-2000 fluorescence spectrophotometer. ADP (10  $\mu$ M) was added to the platelet suspension in the presence of 1 mM  $\text{Ca}^{2+}$ . Fluorescence from fura 2 in platelets was excited with two excitation wavelengths of 340 and 380 nm, and the relative intensities of fluorescence were measured at 510 nm. Maximum fluorescence was achieved by lysing the platelets with 0.1% Triton X-100, and the minimum fluorescence was obtained in the presence of 3 mM EGTA.  $[\text{Ca}^{2+}]_i$  was calculated by the equation of Grynkiewicz *et al.* (1985).

#### Agents and administration

R-99224 ((2Z)-[1-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-mercapto-3-piperidinylidene], acetic acid, trifluoroacetate) and CS-747 (2-acetoxy-5-( $\alpha$ -cyclopropylcarbonyl-2-fluorobenzyl)-4,5,6,7-tetrahydrothieno [3,2-c]pyridine) were synthesized by Ube Industries (Yamaguchi, Japan). ARL-66096 was synthesized by Chemtech Labo., Inc. (Tokyo, Japan). Adenosine 3'-phosphate 5'-phosphosulfate (A3P5PS), ADP (sodium salt), human fibrinogen, fatty-acid-free BSA,

apyrase and gum arabic were purchased from Sigma (St. Louis, MO, U.S.A.).  $\text{PGE}_1$  was from Funakoshi (Tokyo, Japan). [ $^{125}\text{I}$ ]-Fibrinogen (297  $\mu\text{Ci mg}^{-1}$ ) and 2-[methyl- $^3\text{H}$ ]-methylthio-adenosine-5'-diphosphate ( $^3\text{H}$ )-2-MeS-ADP, ammonium salt, specific activity 85 Ci  $\text{mmol}^{-1}$ ) were obtained from Amersham (Buckinghamshire, U.K.), and 2-MeS-ADP (trisodium salt) was obtained from Research Biochemicals International (Natick, MA, U.S.A.). Fura 2-AM was obtained from Dojin (Kumamoto, Japan).

In *ex vivo* studies, R-99224 (0.1–3 mg  $\text{ml}^{-1}$ ) dissolved in saline was injected intravenously to rats anaesthetized with pentobarbital sodium (40 mg  $\text{kg}^{-1}$ , i.p.) in a volume of 1 ml  $\text{kg}^{-1}$ . Blood was drawn from the rats 1 h post-dose, and the ADP (0.3–30  $\mu$ M)-induced platelet aggregation was measured in PRP prepared by the method described above. In the experiment to examine the duration of action, CS-747 (3 mg  $\text{ml}^{-1}$ ) suspended in 5% gum arabic solution was orally administered to non-fasted, conscious rats in a volume of 1 ml  $\text{kg}^{-1}$ . Blood was drawn from the rats 4 h post-dose, and ADP (10  $\mu$ M)-induced platelet aggregation was measured in PRP prepared by the method described above.

#### Statistics

Results are expressed as the mean  $\pm$  s.e.mean unless otherwise stated. Differences between two experimental groups were assessed by the unpaired *t*-test, and differences between multiple groups were assessed by Dunnett's multiple comparison test or Turkey 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

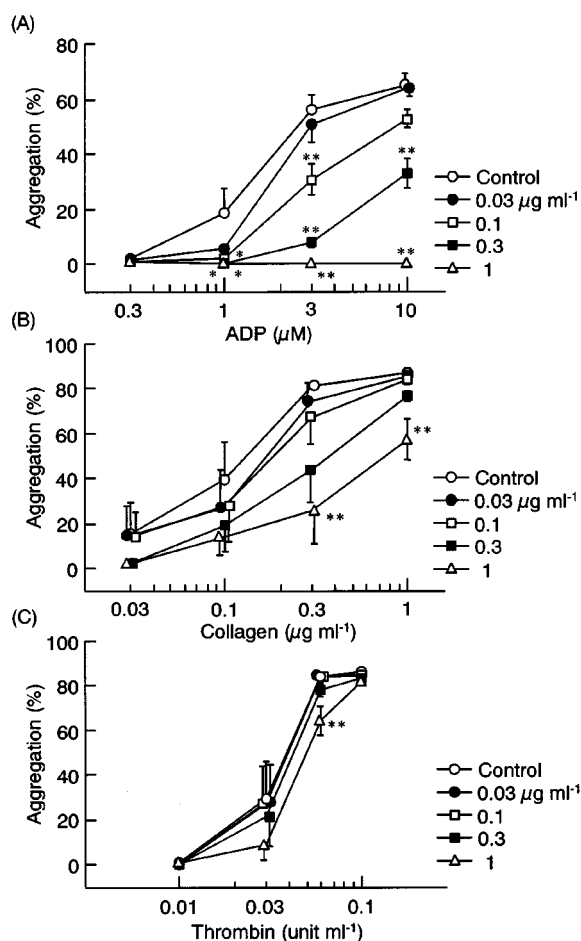
#### In vitro platelet aggregation in human washed platelets

The addition of R-99224 to washed human platelets at concentrations up to 100  $\mu\text{g ml}^{-1}$  did not elicit any aggregating responses, indicating that this agent is devoid of agonist activity (data not shown). To determine the inhibitory potency and specificity of R-99224, we examined the effects of R-99224 on platelet aggregation in washed human platelets. As shown in Figure 2, pretreatment of platelets with R-99224 (0.03–1  $\mu\text{g ml}^{-1}$ ) inhibited ADP-induced platelet aggregation. This inhibition was concentration-dependent, and the  $\text{IC}_{50}$  value against 3  $\mu\text{M}$  ADP was 0.11  $\mu\text{g ml}^{-1}$ . R-99224 did not affect the ADP-induced shape change even at the highest concentration used (1  $\mu\text{g ml}^{-1}$ ) (data not shown).

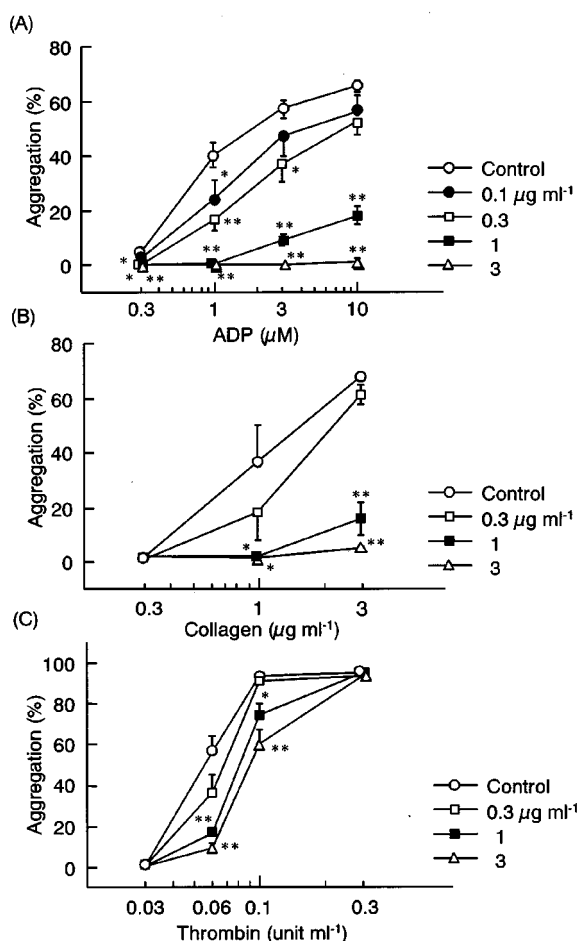
Platelet aggregation induced by low concentrations of collagen and thrombin (0.06 u  $\text{ml}^{-1}$ ) was slightly inhibited at the highest concentration of R-99224 (1  $\mu\text{g ml}^{-1}$ ), but platelet aggregation induced by higher concentrations of thrombin was negligible. Similar results of specificity to ADP were observed for apyrase (data not shown).

#### In vitro and ex vivo platelet aggregation of rat platelets

The *in vitro* effect of R-99224 on platelet aggregation was also examined in washed rat platelets. R-99224 (0.1–



**Figure 2** *In vitro* effects of R-99224 ( $0.03\text{--}1\text{ }\mu\text{g ml}^{-1}$ ) on platelet aggregation induced by ADP (A), collagen (B) and thrombin (C) in washed human platelets. Results are expressed as the mean  $\pm$  s.e.mean ( $n=5\text{--}6$ ). \* $P<0.05$ , \*\* $P<0.01$  vs each control.



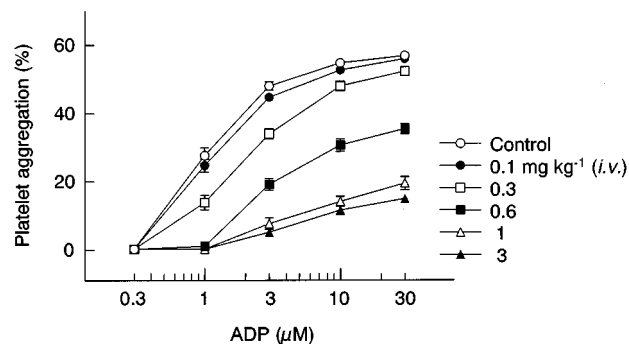
**Figure 3** *In vitro* effects of R-99224 ( $0.1\text{--}3\text{ }\mu\text{g ml}^{-1}$ ) on platelet aggregation induced by ADP (A), collagen (B) and thrombin (C) in washed rat platelets. Results are expressed as the mean  $\pm$  s.e.mean ( $n=5\text{--}6$ ). \* $P<0.05$ , \*\* $P<0.01$  vs each control.

$3\text{ }\mu\text{g ml}^{-1}$ ) inhibited ADP-induced platelet aggregation in a concentration-dependent manner with an  $\text{IC}_{50}$  value of  $0.42\text{ }\mu\text{g ml}^{-1}$  against  $3\text{ }\mu\text{M}$  ADP (Figure 3A). R-99224 also inhibited collagen-induced platelet aggregation in a similar concentration range (Figure 3B). In contrast, the effects of R-99224 on thrombin-induced aggregation were moderate (Figure 3C). Similar specificities to ADP were also observed in the results for apyrase (data not shown).

To investigate the *in vivo* potency of R-99224, *ex vivo* platelet aggregation was determined using platelets collected from rats that received intravenous bolus injection of R-99224 ( $0.1\text{--}3\text{ mg kg}^{-1}$ , i.v.). *Ex vivo* platelet aggregation induced by ADP ( $0.3\text{--}30\text{ }\mu\text{M}$ ) was inhibited by R-99224 ( $0.1\text{--}3\text{ mg kg}^{-1}$ , i.v.) in a dose-dependent manner (Figure 4). The  $\text{ED}_{50}$  value against  $3\text{ }\mu\text{M}$  ADP was approximately  $0.48\text{ mg kg}^{-1}$  (i.v.).

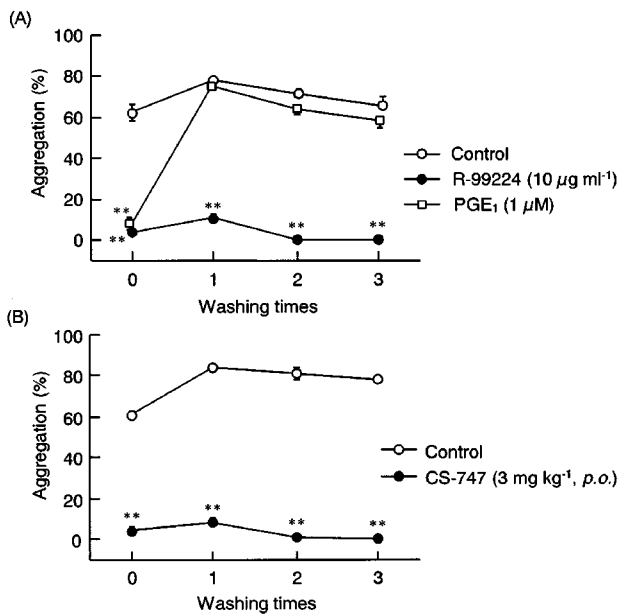
#### Duration of action

The *in vitro* duration of action of R-99224 was investigated in comparison with  $\text{PGE}_1$ , a potent but reversible inhibitor of platelet aggregation. ADP-induced aggregation of rat platelets was completely inhibited in the presence of  $\text{PGE}_1$  ( $1\text{ }\mu\text{M}$ ),



**Figure 4** *Ex vivo* effect of single intravenous administration of R-99224 ( $0.1\text{--}3\text{ mg kg}^{-1}$ ) on ADP ( $0.3\text{--}30\text{ }\mu\text{M}$ )-induced platelet aggregation in rats. R-99224 was intravenously administered to rats 1 h before the blood collection. Results are presented as the mean  $\pm$  s.e.mean ( $n=6$ ).

but this inhibition was not evident after platelets were washed to eliminate  $\text{PGE}_1$  in the plasma (Figure 5A). In contrast, the inhibition of platelet aggregation by R-99224 ( $10\text{ }\mu\text{g ml}^{-1}$ )



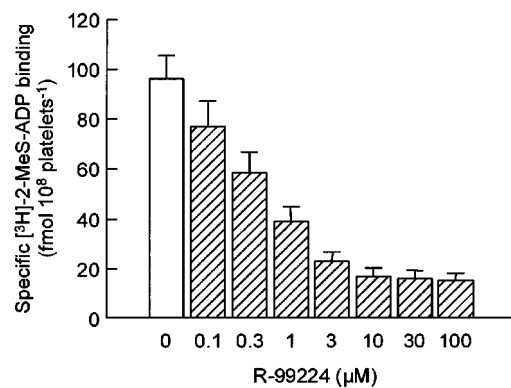
**Figure 5** *In vitro* duration of antiaggregatory effects: (A) Rat platelet-rich plasma was preincubated with R-99224 (10 µg ml<sup>-1</sup>) or PGE<sub>1</sub> (1 µM) for 1 min, and then ADP (10 µM)-induced platelet aggregation was measured before and after washing the platelets; (B) ADP (10 µM)-induced *ex vivo* aggregation of platelets from vehicle- or CS-747 (3 mg kg<sup>-1</sup>, p.o.)-treated rats was measured before and after washing the platelets. Results are presented as the mean ± s.e.mean (*n* = 6). \*\**P* < 0.01 vs each control.

persisted even after the platelets were washed three times (Figure 5A). Likewise, inhibition of *ex vivo* platelet aggregation of rats treated with CS-747 (3 mg kg<sup>-1</sup>, p.o.) was not affected by washing the platelets up to three times (Figure 5B).

#### [<sup>3</sup>H]-2-MeS-ADP binding

To determine the effects of R-99224 on platelet ADP receptors, we examined the effects of R-99224 on the binding of [<sup>3</sup>H]-2-MeS-ADP, a stable ADP analogue, to human platelets. Our preliminary study showed that [<sup>3</sup>H]-2-MeS-ADP binding to human platelets was time-related and saturable. The [<sup>3</sup>H]-2-MeS-ADP (10 nM) binding to vehicle-treated (control) platelets was 96.5 ± 9.5 fmol 10<sup>8</sup> platelets<sup>-1</sup> (*n* = 6). Treatment with R-99224 (0.1–100 µM) produced a concentration-related inhibition of the [<sup>3</sup>H]-2-MeS-ADP binding and the inhibition reached a plateau at 3 µM (1.4 µg ml<sup>-1</sup>) of R-99224 (Figure 6). However, this inhibition by R-99224 was partial (84% of control) even at the highest concentration used (100 µM = 46 µg ml<sup>-1</sup>). Our preliminary experiment showed that CS-747 has no *in vitro* inhibitory activity at concentrations of up to 100 µM (data not shown).

To further characterize the binding properties of R-99224, we used ARL-66096, an ATP-type antagonist for a G<sub>i</sub>-linked P2T (Kunapuli & Daniel, 1998; Daniel *et al.*, 1998), and A3P5PS, a selective P2Y<sub>1</sub> antagonist (Boyer *et al.*, 1996; Kunapuli & Daniel, 1998). ARL-66096 and A3P5PS inhibited [<sup>3</sup>H]-2-MeS-ADP binding in a concentration-dependent manner, with maximal inhibition observed at 0.3 µM (ARL-66096) and 300 µM (A3P5PS), respectively (data not shown).



**Figure 6** *In vitro* effect of R-99224 (0.1–100 µM) on [<sup>3</sup>H]-2-MeS-ADP binding to washed human platelets. Results are expressed as the mean ± s.e.mean (*n* = 6).

As shown in Figure 7A, R-99224 (10 µM = 4.6 µg ml<sup>-1</sup>) in combination with ARL-66096 (0.3 µM) caused no additional inhibition of [<sup>3</sup>H]-2-MeS-ADP binding. In contrast, R-99224 (10 µM) in combination with A3P5PS (300 µM) completely abolished [<sup>3</sup>H]-2-MeS-ADP binding (Figure 7B). Likewise, ARL-66096 (0.3 µM) combined with A3P5PS abolished [<sup>3</sup>H]-2-MeS-ADP binding.

#### [<sup>125</sup>I]-Fibrinogen binding

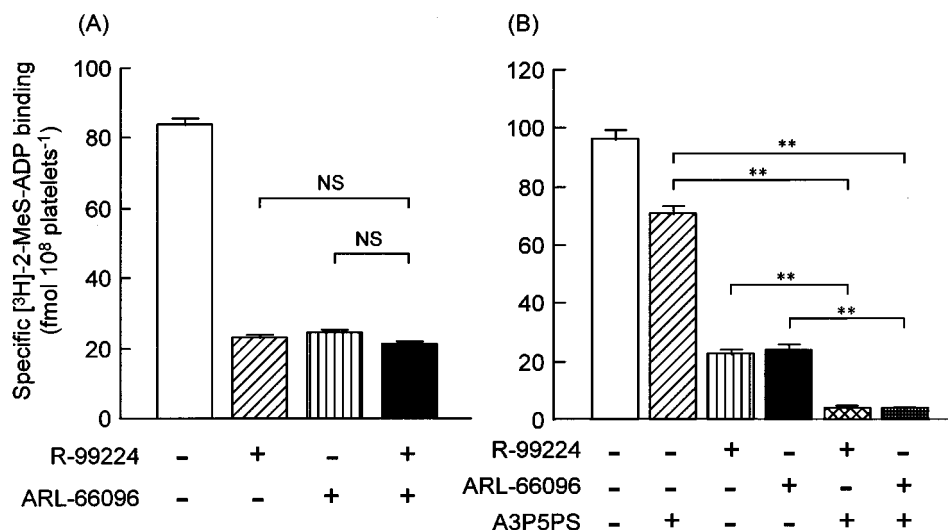
We examined the effects of R-99224 on fibrinogen binding to platelets, the final common step of platelet activation. The ADP-induced [<sup>125</sup>I]-fibrinogen binding to control platelets was 1.097 ± 0.144 pmol 10<sup>8</sup> platelets<sup>-1</sup> (*n* = 6). R-99224 (0.01–3 µg ml<sup>-1</sup>) produced a concentration-related inhibition of the [<sup>125</sup>I]-fibrinogen binding (Figure 8). *In vitro* treatment with CS-747 (30 and 100 µg ml<sup>-1</sup>) had no effect on [<sup>125</sup>I]-fibrinogen binding to the washed human platelets (data not shown).

#### Cyclic AMP levels in platelets

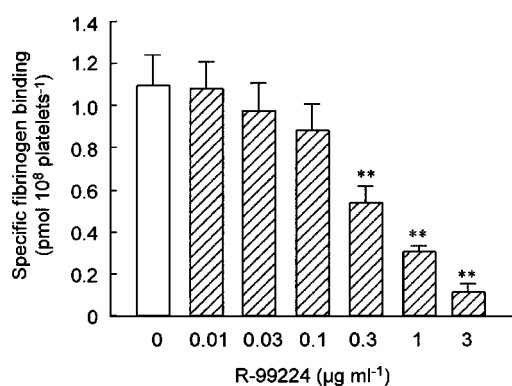
Since ADP inhibits adenylyl cyclase *via* activation of G<sub>i</sub> protein (Defrey *et al.*, 1991), we examined if R-99224 attenuates ADP-mediated suppression of PGE<sub>1</sub>-induced cyclic AMP elevation. An addition of PGE<sub>1</sub> (10 µM) produced a progressive increase of intraplatelet cyclic AMP levels, indicating the activation of adenylyl cyclase. The elevated cyclic AMP levels were suppressed by ADP (10 µM) added 3 min after PGE<sub>1</sub> stimulation. As shown in Figure 9, the inhibitory effect of ADP (10 µM) on elevated cyclic AMP levels was inhibited substantially in R-99224 (0.1–1 µg ml<sup>-1</sup>)-treated platelets in a concentration-related manner. There was no difference in the basal cyclic AMP levels between R-99224-treated and vehicle-treated (control) platelets.

#### Ca<sup>2+</sup> mobilization

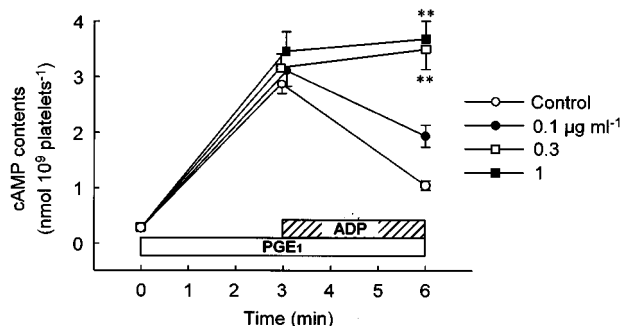
Since ADP causes elevation of intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>), we examined the effects of R-99224 on [Ca<sup>2+</sup>]<sub>i</sub> using fura 2-loaded platelets. There were no differences in basal [Ca<sup>2+</sup>]<sub>i</sub> between control and R-99224 (up to 1 µg ml<sup>-1</sup>)-treated



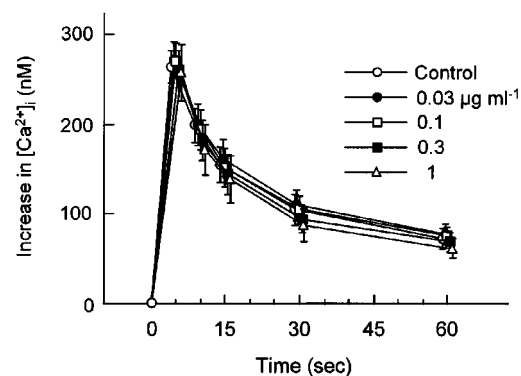
**Figure 7** (A) *In vitro* effect of R-99224 (10  $\mu\text{M}$ ) combined with ARL-66096 (0.3  $\mu\text{M}$ ) on  $[^3\text{H}]\text{-2-MeS-ADP}$  binding to washed human platelets; (B) *In vitro* effects of A3P5PS (300  $\mu\text{M}$ ) combined with R-99224 (10  $\mu\text{M}$ ) or ARL-66096 (0.3  $\mu\text{M}$ ) on  $[^3\text{H}]\text{-2-MeS-ADP}$  binding to washed human platelets. Results are expressed as the mean  $\pm$  s.e. mean ( $n = 6$ ). \*\* $P < 0.01$ , NS, not significant.



**Figure 8** *In vitro* effect of R-99224 (0.01–3  $\mu\text{g ml}^{-1}$ ) on  $[^{125}\text{I}]\text{-fibrinogen}$  binding to washed human platelets. Results are expressed as the mean  $\pm$  s.e. mean ( $n = 6$ ). \*\* $P < 0.01$  vs control.



**Figure 9** *In vitro* effect of R-99224 (0.1–1  $\mu\text{g ml}^{-1}$ ) on ADP (10  $\mu\text{M}$ )-induced cyclic AMP decrease in PGE<sub>1</sub> (10  $\mu\text{M}$ )-stimulated human platelets. ADP was added to the reaction mixture 3 min after PGE<sub>1</sub> stimulation. Results are expressed as the mean  $\pm$  s.e. mean ( $n = 6$ ). \*\* $P < 0.01$  vs control.



**Figure 10** *In vitro* effect of R-99224 (0.03–1  $\mu\text{g ml}^{-1}$ ) on ADP (10  $\mu\text{M}$ )-induced increase in intracellular  $\text{Ca}^{2+}$  concentration in washed human platelets. Results are expressed as the mean  $\pm$  s.e. mean ( $n = 6$ ). There are no statistically significant differences at any points.

platelets (data not shown). In control platelets, ADP (10  $\mu\text{M}$ ) induced a marked increase in  $[\text{Ca}^{2+}]_i$  that peaked 5 s after the addition of ADP ( $264.3 \pm 17.5$  nM,  $n = 6$ ). R-99224 (0.03–1  $\mu\text{g ml}^{-1}$ ) had minimal effects on the ADP-induced increase in  $[\text{Ca}^{2+}]_i$  at all time points (5, 10, 15, 30 and 60 s) (Figure 10).

## Discussion

CS-747, a novel thienopyridine derivative, is an orally active antiplatelet and antithrombotic agent with a potency higher than that of clopidogrel and ticlopidine (Sugidachi *et al.*, 2000). Like clopidogrel and ticlopidine, CS-747 is inactive *in vitro*, but R-99224, a metabolite of CS-747, is active *in vitro* in inhibiting ADP-induced aggregation of rat platelets. The purpose of the present investigation was to identify the

subtype of ADP receptors to which R-99224 binds in human platelets. The results of this study have demonstrated that R-99224 binds to  $G_i$ -linked P2T receptors selectively and irreversibly, and that R-99224 is a responsible metabolite for *in vivo* actions of CS-747.

Several lines of evidence suggest that the *in vivo* antiplatelet effects of the thienopyridine derivatives, clopidogrel and ticlopidine, are due to their active metabolite(s), but no such active metabolites have been identified (Saltiel & Ward, 1987; Savi *et al.*, 1994a; Coukell & Markham, 1997). Recently, one group (Weber *et al.*, 1999) claimed that the antiaggregatory effects of clopidogrel could be due to its direct interaction with human platelets *in vitro*. However, this might be artifactual due to the long period of incubation of the washed platelets with clopidogrel (Herbert & Savi, 1999). R-99224 was originally isolated from the incubation medium of CS-747 and rat hepatocytes. R-99224 has been detected in the plasma of various animals treated with CS-747 (unpublished data). In the present study, intravenous administration of R-99224 to rats potently inhibited ADP-induced platelet aggregation. To our knowledge, therefore, R-99224 is the first thienopyridine-derived metabolite that is active *in vitro* and *in vivo*.

The present study has shown that R-99224 is a potent inhibitor of ADP-induced platelet aggregation both in human and rat platelets. Collagen- and thrombin-induced aggregation was moderately inhibited by R-99224 at high concentrations. This is consistent with *ex vivo* results of CS-747 orally administered to rats (Sugidachi *et al.*, 2000), and also with the *ex vivo* results of ticlopidine on human platelet aggregation (Cattaneo *et al.*, 1991). In addition, apyrase, an ADP scavenger, produced a similar inhibitory profile on human and rat platelet aggregation. Thus, the antiaggregatory effects of R-99224 against collagen- and thrombin-induced aggregation are most likely attributable to its inhibitory effects on ADP released from the dense granules of activated platelets.

Previous study has shown that CS-747 exerts long-lasting *ex vivo* antiaggregatory effects (Sugidachi *et al.*, 2000). The duration of action of CS-747 is comparable to the life span of circulating platelets in the rat (Cattaneo *et al.*, 1985; Jackson *et al.*, 1992) suggesting that CS-747 interacts with platelets in an irreversible manner. Indeed, the present study showed that the inhibition of platelet aggregation in PRP prepared from rats given an oral dose of CS-747 were maintained even after the platelets were washed three times. Likewise, the *in vitro* treatment of platelets with R-99224 was maintained after platelets were washed up to three times to remove R-99224 from the medium. In contrast, the inhibition by  $PGE_1$ , a reversible antiaggregating agent, was eliminated after the first washing. These findings strongly suggest that R-99224 interacts with platelets in an irreversible manner. Our structure-activity relation study showed that the thiol group is essential for achieving antiaggregatory effects (data not shown). Although further studies will be necessary to elucidate the molecular mechanism of the antiaggregatory action of R-99224, its irreversible mode of action may derive from the formation of a disulfur bridge between the reactive thiol group and that of a cysteine residue of the platelet ADP receptor.

Recent studies have demonstrated that the platelet ADP receptor is not homogeneous. In addition to the ligand-gated

ion channel  $P2X_1$  receptor (MacKenzie *et al.*, 1996), two subclasses of G-protein coupled ADP receptors have been proposed to exist on human platelet membranes: the  $G_q$ -linked  $P2Y_1$  receptor and the  $G_i$ -linked P2T receptor (Fagura *et al.*, 1998; Daniel *et al.*, 1998; Jantzen *et al.*, 1999). 2-MeS-ADP is a stable agonist for  $P2Y_1$  and  $G_i$ -linked P2T receptor (Kunapuli, 1998a,b), and radiolabelled 2-MeS-ADP has been widely used for the study of ADP receptors (Léon *et al.*, 1999; Mills *et al.*, 1992; Savi *et al.*, 1994b). In the present study, R-99224 produced a significant inhibition of [ $^3H$ ]-2-MeS-ADP binding to human platelets in a concentration-dependent manner. This finding is consistent with the *ex vivo* effects of CS-747 in rats (Sugidachi *et al.*, 2000) and suggests that R-99224 is a specific platelet ADP receptor antagonist.

However, the inhibition of [ $^3H$ ]-2-MeS-ADP binding by R-99224 was partial: 16% of [ $^3H$ ]-2-MeS-ADP binding still remained even at the highest concentration used ( $100 \mu M = 46 \mu g ml^{-1}$ ). This suggests that there are two binding sites, one of which is resistant to R-99224. Thus, we further characterized binding properties of R-99224 using ARL-66096, a selective  $G_i$ -linked P2T receptor antagonist (Kunapuli & Daniel, 1998; Daniel *et al.*, 1998) and A3P5PS, a selective  $P2Y_1$  receptor antagonist (Boyer *et al.*, 1996; Kunapuli & Daniel, 1998). If the two antagonists bound to two separate receptors, an additive inhibition could be anticipated. As expected, R-99224 abolished [ $^3H$ ]-2-MeS-ADP binding, when it was combined with A3P5PS, but not with ARL-66096. These results are consistent with the contention that R-99224 binds selectively to  $G_i$ -linked P2T receptors.

To further determine if R-99224 inhibits  $G_i$ -linked P2T receptor functions, we investigated the effects of R-99224 on ADP-induced intracellular signal events.  $P2Y_1$  receptors are linked to heterodimeric G proteins that stimulate PLC, which leads to mobilization of  $Ca^{2+}$  ions. Activation of  $P2X_1$  receptors linked to  $Ca^{2+}$  channels also leads to elevation of [ $Ca^{2+}$ ] $_i$ . The present study showed that R-99224 had no effects on ADP-induced  $Ca^{2+}$  mobilization. In contrast, R-99224 neutralized ADP-induced inhibition of cyclic AMP elevation by  $PGE_1$ . Since  $G_i$ -linked P2T receptors are linked G proteins that stimulate adenylyl cyclase, these results are consistent with R-99224 being a selective inhibitor of  $G_i$ -linked P2T receptors. This contention is further supported by the fact that R-99224 had no effects on ADP-induced shape change (Sugidachi *et al.*, 2000), a process mediated *via* the  $P2Y_1$  receptor (Kunapuli, 1998a,b). Taken together, in the present study, inhibitions of [ $^3H$ ]-2-MeS-ADP binding, ADP-induced fibrinogen binding, and cyclic AMP reduction were achieved by treatment with R-99224 at a similar concentration range. These data suggest that R-99224 irreversibly binds to  $G_i$ -linked P2T receptors, thereby inhibiting signaling events and fibrinogen binding, and ultimately resulting in a long-lasting inhibition of ADP-induced platelet aggregation. Our results may partially resolve ongoing controversy regarding the importance of the  $G_i$ -linked P2T receptor (Léon *et al.*, 1999; Jarvis *et al.*, 2000) and  $P2Y_1$  receptor (Fabre *et al.*, 1999; Jarvis *et al.*, 2000) in ADP-induced platelet aggregation, by providing additional evidence that the former is in fact essential in this process (Jin & Kunapuli, 1998).

In summary, the present study demonstrated that R-99224 is an irreversible and selective  $G_i$ -linked P2T receptor

antagonist. The potent antiaggregatory and antithrombotic effects of CS-747 seem to be mediated by the interaction of its hepatic metabolite, R-99224, with circulating platelets *in vivo*. Whether R-99224 is the sole active metabolite of CS-747 remains to be elucidated.

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