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Effects of TRA-418, a novel TP-receptor antagonist, and IP-receptor agonist, on human platelet activation and aggregation

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1 {4-[2-(1,1-Diphenylethylsulfanyl)-ethyl]-3,4-dihydro-2H-benzo[1,4]oxazin-8-yloxy}-acetic acid *N*-Methyl-D-glucamine salt (TRA-418) has both thromboxane A_2 (TP)-receptor antagonist and prostacyclin (IP)-receptor agonist properties. The present study examined the advantageous effects of TRA-418 based on the dual activities, over an agent having either activity alone and also the difference in the effects of TRA-418 and a glycoprotein αIIb/β3 integrin (GPIIb/IIIa) inhibitor.

2 TRA-418 inhibited platelet GPIIb/IIIa activation as well as P-selectin expression induced by adenosine 5′-diphosphate, thrombin receptor agonist peptide 1–6 (Ser-Phe-Leu-Leu-Arg-Asn-NH_2), and U-46619 in the presence of epinephrine (U-46619 + epinephrine). TRA-418 also inhibited platelet aggregation induced by those platelet-stimulants in Ca^{2+} chelating anticoagulant, citrate and in nonchelating anticoagulant, D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl ketone (PPACK).

3 The TP-receptor antagonist SQ-29548 inhibited only U-46619 + epinephrine-induced GPIIb/IIIa activation, P-selectin expression, and platelet aggregation.

4 The IP-receptor agonist beraprost sodium inhibited platelet activation. Beraprost also inhibited platelet aggregation induced by platelet stimulants we tested in citrate and in PPACK.

5 The GPIIb/IIIa inhibitor abciximab blocked GPIIb/IIIa activation and platelet aggregation. However, abciximab showed slight inhibitory effects on P-selectin expression.

6 TRA-418 is more advantageous as an antiplatelet agent than TP-receptor antagonists or IP-receptor agonists separately used. TRA-418 showed a different inhibitory profile from abciximab in the effects on P-selectin expression.

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Abbreviations: ADP, adenosine 5′-diphosphate; GPIIb/IIIa, glycoprotein αIIb/β3 integrin; PPACK, D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl ketone; PRP, platelet-rich plasma; RGDS, Arg-Gly-Asp-Ser; TRAP, thrombin receptor agonist peptide 1–6 (Ser-Phe-Leu-Leu-Arg-Asn-NH_2)

Introduction

Theoretically, thromboxane A_2 (TP)-receptor antagonists and prostacyclin (IP)-receptor agonists are both expected to be useful as antithrombotic drugs. Despite their potent antithrombotic effects in several animal models (van der Giessen *et al*., 1988; Takiguchi *et al*., 1992; Kotze *et al*., 1993), TP-receptor antagonists have been reported to show only poor antithrombotic effects in the clinical trials (Serruys *et al*., 1991; Norris *et al*., 1996). On the other hand, the clinical application of IP-receptor agonists has been limited to pulmonary hypertension or moderate thrombotic disease because of their potent vasodilating effects (Muller *et al*., 1988; Nagaya *et al*., 1999).

In vitro studies have revealed that remarkable synergistic effects on human platelet aggregation can be observed with TP-receptor antagonists when used concomitantly with an IP-receptor agonist (Bertele & De Gaetano 1982; Parise *et al*., 1982; Sturzebecher & Witt, 1988). In fact, we have confirmed that the antithrombotic effect of aspirin is substantially potentiated by the coadministration of beraprost, an IP-receptor agonist, at a limited dose that causes no severe hypotension (Yamada *et al*., 1993). Aspirin has an inhibitory effect on the production of thromboxane A_2 through its cyclooxygenase inhibitory effect. Therefore, compounds having a relatively potent TP-receptor antagonist activity together with a relatively weak IP-receptor agonist activity would be useful as therapeutic antithrombotic agents.

Recently, we have found TRA-418 ({4-[2-(1,1-diphenylethylsulfanyl)-ethyl]-3,4-dihydro-2H-benzo[1,4]oxazin-8-yloxy}-acetic acid *N*-Methyl-D-glucamine salt, Figure 1) in our screening for compounds having such dual activities. The K_i values are 50 and 430 nM when estimated in reactions to TP-receptors and IP-receptors on human platelet membrane, respectively, and TRA-418 inhibits *in vitro* platelet aggregation in human and experimental animals (Yamada *et al*., 2003). Furthermore, TRA-418 exhibits an inhibitory effect on *ex vivo* platelet aggregation at a dose where the compound induces no significant decrease in blood pressure in monkeys (Yamada *et al*., 2003). These results support our hypothesis that compounds having a relatively potent TP-receptor antagonist

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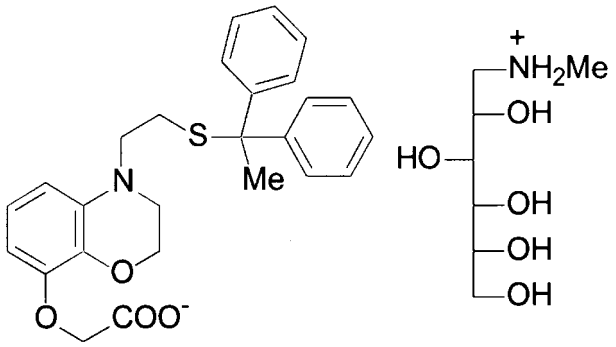

Figure 1 Chemical structure of TRA-418, {4-[2-(1,1-diphenylethylsulfanyl)-ethyl]-3,4- dihydro-2H-benzo[1,4]oxazin-8-yloxy}-acetic acid *N*-methyl-D-glucamine salt.

activity together with a relatively weak IP-receptor agonist activity are useful as antithrombotic agents.

Although our previous study has shown the inhibitory effects of TRA-418 on platelet aggregation, nothing is yet known about the effects of TRA-418 on platelet activation including glycoprotein αIIb/β3 integrin (GPIIb/IIIa) activation and P-selectin expression on the membrane surface and on platelet aggregation in different anticoagulants. The GPIIb/IIIa activation is a crucial event in platelet aggregation (Phillips *et al*., 1988), while P-selectin is mainly involved in platelet–leukocyte adhesion (Larsen *et al*., 1989; Palabrica *et al*., 1992). The IP-receptor agonists prostacyclin and iloprost have been reported to inhibit both GPIIb/IIIa activation and P-selectin expression (Lindemann *et al*., 1999; Sakamaki *et al*., 2000). However, little is known about the effects of a TP-receptor antagonist on these molecules. Therefore, in this study, we have investigated the effects of TRA-418 on human platelet GPIIb/IIIa activation and P-selectin expression induced by various platelet-stimulating agents, compared with the TP-receptor antagonist SQ-29548, the IP-receptor agonist beraprost, and the GPIIb/IIIa inhibitor abciximab. In addition, we have further examined the influence of anticoagulants on the effects of TRA-418 on platelet aggregation, which we have not yet reported.

Methods

Materials

TRA-418 and beraprost were synthesized in Toray Industries, Inc. (Kamakura, Japan). SQ-29548 and U-46619 were purchased from Cayman Chemical (MI, U.S.A.), abciximab from Eli Lilly (IN, U.S.A.), adenosine 5′-diphosphate (ADP) and Arg-Gly-Asp-Ser (RGDS) from Sigma (MO, U.S.A.), thrombin receptor agonist peptide 1–6 (Ser-Phe-Leu-Leu-Arg-Asn-NH_2, TRAP) from Peninsula Laboratories Europe (U.K.), D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl ketone (PPACK) from Calbiochem (CA, U.S.A.), epinephrine from Daiichi Pharmaceutical (Tokyo, Japan). The following monoclonal antibodies were purchased from Becton Dickinson (CA, U.S.A.): peridinin chlorophyll protein (PerCP)-conjugated CD61 antibody (clone RUU-PL 7F12), fluorescein isothiocyanate (FITC)-conjugated PAC-1, phycoerythrin (PE)-conjugated CD62P antibody (clone AC1.2), and PE-conjugated mouse IgG_1 control.

Blood samples

Blood samples used in this study were collected from healthy male human volunteers who had taken no drugs at least within 2 weeks before their participation. The study protocol was reviewed and approved by the Institutional Ethics Committee of the Pharmaceutical Research Laboratories, Toray Industries, Inc., and written informed consent was obtained from each of these volunteers.

Platelet activation

Blood samples were collected in acid citrate dextrose from volunteers by venipuncture *via* a 19-gauge butterfly needle. About the first 2 ml of blood was discarded to minimize the platelet activation during blood collection. Within 10 min after the blood sampling, the blood was then diluted five-fold with modified Tyrode's solution at pH 7.4 containing NaCl 137 mM, KCl 2.8 mM, $MgCl_2$ 1 mM, $NaHCO_3$ 12 mM, Na_2HPO_4 0.4 mM, bovine serum albumin 0.35%, HEPES 10 mM, and glucose 5.5 mM.

Each of the diluted blood samples was treated for 10 min with various concentrations of TRA-418, beraprost, SQ-29548, or abciximab. Of the resulting incubation mixture, 15 μl was further incubated with 30 μl of an antibody mixture containing excessive amounts of FITC-conjugated PAC-1, PE-conjugated CD62P, and PerCP-conjugated CD61 in the presence of ADP (1 μM), TRAP (1 μM), or U46619 (1 μM) + epinephrine (0.5 μM) for 15 min at room temperature.

PAC-1 is an IgM antibody that specifically binds to activated GPIIb/IIIa (Shattil *et al*., 1985). The PAC-1 binding is blocked in the presence of the peptide, RGDS. CD62P is an IgG_1 antibody that is directed against P-selectin. PerCP-conjugated CD61 antibody is an antibody that binds to GPIIIa, but does not interfere with the PAC-1 binding to GPIIb/IIIa. Accordingly, in the negative control experiments, drug-treated platelets were incubated with a mixture of FITC-conjugated PAC-1, RGDS, PE-conjugated mouse IgG_1, and PerCP-conjugated CD61 antibody in place of the antibody mixture of FITC-conjugated PAC-1, PE-conjugated CD62P, and PerCP-conjugated CD61.

All incubation was terminated by the addition of 1 ml of cold 1% paraformaldehyde. The resulting incubation mixtures were kept at 2–8°C in the dark for 2 h and analyzed by cell flow cytometry to determine the fluorescence intensity bound to the platelets.

Platelet flow cytometry

For platelet flow cytometry, a FACScan flow cytometer (Becton Dickinson, CA, U.S.A.) equipped with a 5 W laser operating at a power of 15 mW and a wavelength of 488 nm was used. Calibration of the cytometer was carried out by using the calibration kit CaliBRITE 3 (Becton Dickinson) on a daily basis before the measurement in terms of fluorescence and light scattering with the aid of the software package FACSComp (Becton Dickinson) installed in the cytometer. The logarithmic amplification was used for the forward and side light scattering gains. The voltage and compensation settings were optimized by using human platelet samples with the aid of the software package CELLQuest (Becton Dickinson) installed in the cytometer. The population of platelets

was identified, based on particle size (forward and side scattering) and also on the binding with CD61 antibody. For each analytical sample, a total of 10 000 events were analyzed in the gate with the aid of CELLQuest, and the data were expressed as mean fluorescence intensities (MFI).

The basal MFI values estimated in the absence of any platelet-stimulating agent under the conditions described above were 9.62 ± 0.37 ($n = 11$) for FITC-conjugated PAC-1 binding and 4.8 ± 0.24 ($n = 11$) for PE-conjugated CD62P binding. Platelet activation was induced by ADP (1 μM), TRAP (1 μM), or U-46619 (1 μM) in the presence of epinephrine (0.5 μM), by which more than 70% of cells were activated in terms of GPIIb/IIIa activation and P-selectin expression as monitored by the PAC-1 binding and CD62P binding, respectively.

The effect of each drug was presented as percentage of inhibition based on the determined MFI value by assuming that vehicle alone induced no inhibition.

Platelet aggregation

For measurement of platelet aggregation, blood was collected from a volunteer into a plastic syringe containing trisodium citrate at a final concentration of 0.38%, or PPACK at a final concentration of 40 μM. Blood was centrifuged at $120 \times g$ for 10 min at room temperature to obtain platelet-rich plasma (PRP) as the supernatant. The remaining sediment was further centrifuged at $1400 \times g$ for 10 min at room temperature to obtain platelet-poor plasma (PPP) as the supernatant.

For examining the effects of TRA-418, beraprost, SQ-29548, and abciximab on the platelet aggregation, PRP was pre-incubated with a test drug for 1 min at 37°C. Platelet aggregation was induced by ADP (5 μM), TRAP (3 μM), or U-46619 (2 μM) + epinephrine (0.5 μM). We have previously reported the effect of TRA-418 on U-46619-induced platelet aggregation (Yamada *et al*., 2003), we used the strong stimulant, U-46619 + epinephrine in this study.

Platelet aggregation was monitored for 5 min by recording transmittance on a four-channel light transmission NBS Hematracer Model 601 aggregometer (MC medical, Tokyo, Japan). The aggregometer was calibrated for each PRP sample before the addition of any test drug by adjusting the light transmission to 0% for PRP and to 100% for PPP. The maximum increase in light transmission was read from the recorded transmittance curve. The effects of the test drugs were expressed as percentage of inhibitions of platelet aggregation calculated from the maximum increases observed with the test drugs and that with vehicle alone.

Data processing

All data in the text, tables, and figures are expressed as mean ± their standard error of n determinations. pIC_{50} values were calculated with Excel (Microsoft, ver. 5.0) for Macintosh.

Results

Effects on GPIIb/IIIa activation

Effects of TRA-418 on GPIIb/IIIa activation were examined *in vitro* in human platelets stimulated with ADP, TRAP, or U-46619 + epinephrine. As seen in Figure 2a, TRA-418 showed concentration-dependent inhibitory effects on GPIIb/IIIa activation induced in human platelets *in vitro*, regardless of the platelet-stimulating agent used. TRA-418 inhibited both TRAP- and U-46619 + epinephrine-induced GPIIb/IIIa activation in similar concentration ranges, but required higher concentrations to inhibit ADP-induced activation (Table 1).

In contrast, the IP-receptor agonist beraprost showed concentration-dependent inhibitions of GPIIb/IIIa activation at concentrations much lower than those required for the inhibitions by TRA-418 (Figure 2b). The most potent inhibition by beraprost was observed with TRAP-induced activation, followed in the order by ADP- and U-46619 + epinephrine-induced activation (Table 1).

Although the TP-receptor antagonist SQ-29548 inhibited U-46619 + epinephrine-induced GPIIb/IIIa activation, but not ADP- or TRAP-induced activation (Figure 2c and Table 1), the antibody against GPIIb/IIIa, abciximab, showed similar inhibitory effects on GPIIb/IIIa activation induced with any of the platelet-stimulating agents (Figure 2d and Table 1).

Effects on P-selectin expression

Effects of TRA-418 on P-selectin expression were examined *in vitro* in human platelets stimulated with ADP, TRAP, or U-46619 + epinephrine and compared with those of beraprost, SQ-29548, and abciximab. TRA-418 inhibited P-selectin expression in human platelets in manners similar to those observed with the inhibitions of GPIIb/IIIa activation (Figure 3a and Table 2). Beraprost inhibited TRAP-induced P-selectin expression the most effectively and the least effectively U-46619 + epinephrine-induced P-selectin expression (Figure 3b and Table 2). SQ-29548 inhibited P-selectin expression induced by U-46619 + epinephrine, but not those induced by the other two stimulating agents (Figure 3c and Table 2). In contrast, abciximab exhibited slight inhibitory effect on P-selectin expression, as evidenced with the maximum inhibitions of 20% in platelets stimulated by ADP (Figure 3d). Abciximab even enhanced TRAP-induced P-selectin expression at high concentrations.

Effects on platelet aggregation

TRA-418 inhibited more effectively on U-46619 + epinephrine-induced aggregation than ADP or TRAP-induced aggregation, the differences between IC_{50} values on those agonist-induced aggregation were approximately 1.6-fold in citrate and 2.9-fold in PPACK. In the case of beraprost, inhibitions were more effective on ADP- and TRAP-induced platelet aggregations than U-46619 + epinephrine-induced aggregation, the differences between IC_{50} values were approximately 12-fold in citrate and 15-fold in PPACK. With SQ-29548, clear inhibitions were observed only on U-46619 + epinephrine-induced platelet aggregation. Abciximab inhibited platelet aggregation induced by any of the platelet-stimulating agent, the effect was the strongest in ADP-induced aggregation. In general, these platelet aggregations were more potent inhibited in citrated specimens than in PPACK specimens (Tables 3 and 4).

Discussion

In this study, we have demonstrated *in vitro* that TRA-418 inhibits human platelet GPIIb/IIIa activation, P-selectin expression, and platelet aggregation, using ADP, TRAP, and U-46619 + epinephrine as the platelet-stimulating agents.

When human platelets were activated with ADP, TRA-418 inhibited the GPIIb/IIIa activation, P-selectin expression, and platelet aggregation as well as beraprost, the IP-agonist. In contrast, SQ-29548, the TP-antagonist, did not show any noticeable inhibitory effects on these activations. Therefore, the IP-receptor agonistic activity of TRA-418 was predominantly observed in inhibitions of ADP-induced platelet activation.

Although beraprost has been shown to inhibit platelet aggregation, this report is the first evidence that beraprost inhibits not only the GPIIb/IIIa activation, but also the P-selectin expression. Prostacyclin and its derivative, iloprost, have been reported to inhibit platelet GPIIb/IIIa activation and P-selectin expression *via* induction of cyclic AMP production linked to a decrease in inositol 1,4,5-triphosphate production and Ca^{2+} mobilization (Watson *et al*., 1984; Zavoico & Feinstein, 1984; Cavallini *et al*., 1996). Accordingly, beraprost, a prostacyclin derivative, is reasonably assumed to show its inhibitory effect on platelet activation by binding to IP-receptors to activate adenylate cyclase and thereby to accelerate cyclic AMP production. It seems that TRA-418 also inhibited ADP-induced platelet activation by elevating cyclic AMP through IP-receptors; cyclic AMP production by TRA-418 has been previously reported (Yamada *et al*., 2003).

Essentially similar results were obtained when human platelets were activated with TRAP, a more potent stimulant than ADP. In short, TRA-418 and beraprost inhibited platelet activation and aggregation; however, SQ-29548 inhibited them with little or no effects. These results suggest that the effects of TRA-418 on TRAP-induced platelet activation and aggregation are mediated *via* IP-receptor inducing cyclic AMP elevation.

Data showed that TRA-418 has a different character from beraprost, when human platelets were stimulated by U-46619 + epinephrine. In beraprost, the inhibitory effects on U-46619 + epinephrine-stimulation were weaker than ADP- or TRAP-stimulation. In contrast, TRA-418 inhibited U-46619 + epinephrine-induced platelet activation and aggregation more effective than ADP- or TRAP-stimulation. We also

Table 1 Effects of TRA-418, beraprost, SQ-29548, and abciximab on GPIIb/IIIa activation

Platelet-stimulating agent	*pIC50* (M) *TRA-418*	*Beraprost*	*SQ-29548*	*pIC50* ($g\,ml^{-1}$) *Abciximab*
ADP	5.79±0.06	8.45±0.05	<5	6.64±0.03
TRAP	6.49±0.06	9.02±0.06	<5	6.64±0.08
U-46619 + epinephrine	6.44±0.11	7.89±0.05	8.14±0.06	6.45±0.04

Values are mean±s.e. of three to four determinations. Platelets were stimulated by ADP (1 μM), TRAP (1 μM), or a mixture of U-46619 (1 μM) and epinephrine (0.5 μM).

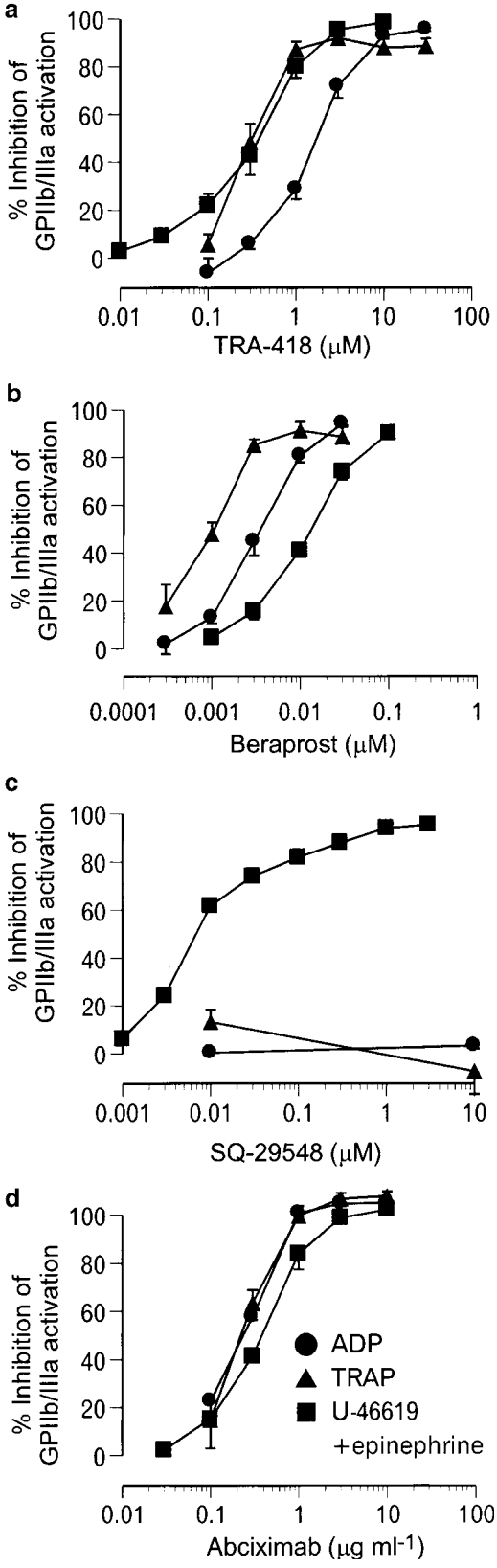

Figure 2 Effects of TRA-418, beraprost, SQ-29548, and abciximab on GPIIb/IIIa activation. GPIIb/IIIa activation was determined by monitoring PAC-1 binding. Platelets were stimulated by ADP (1 μM, $n=4$), TRAP (1 μM, $n=4$), and U-46619 (1 μM, $n=3$) in the presence of epinephrine (0.5 μM). Effects of the drugs are presented as the percentage of inhibition against MFI for ADP-, TRAP-, and U-46619 + epinephrine-stimulation *versus* the vehicle. Values are mean±s.e. of three to four determinations.

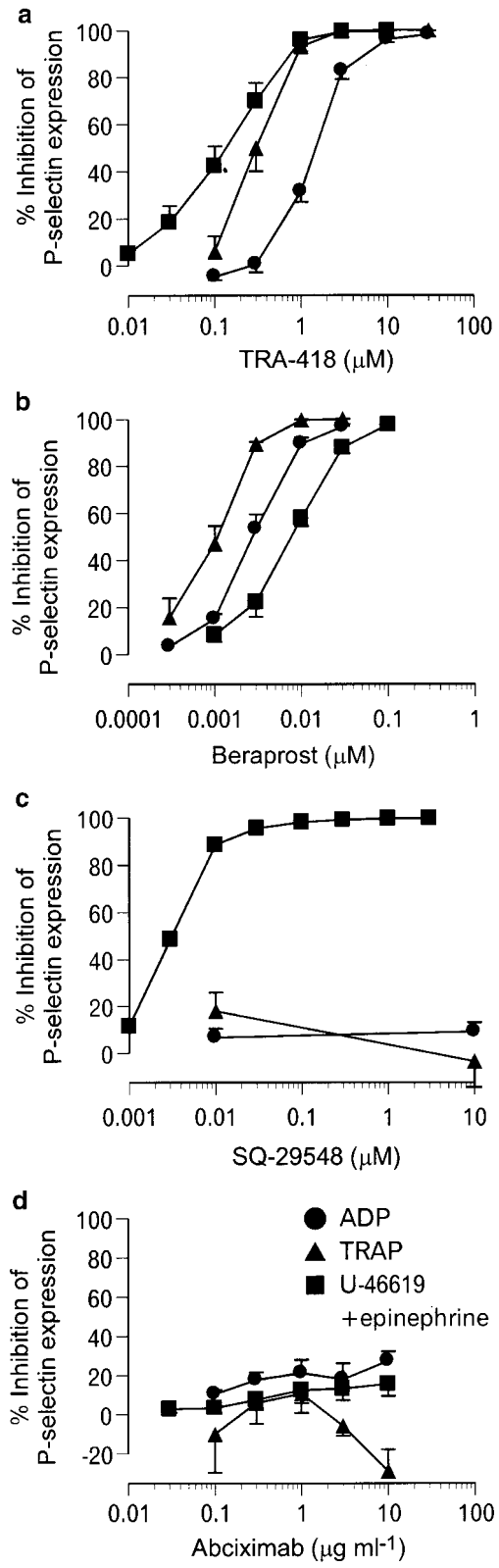

Figure 3 Effects of TRA-418, beraprost, SQ-29548, and abciximab on P-selectin expression. Platelets were stimulated by ADP (1 μM, $n=4$), TRAP (1 μM, $n=4$), and U-46619 (1 μM, $n=3$) in the presence of epinephrine (0.5 μM). Effects of the drugs are presented as the percentage of inhibition against MFI for ADP-, TRAP-, and U-46619 + epinephrine-stimulation *versus* the vehicle. Values are mean ± s.e. of three to four determinations.

Table 2 Effects of TRA-418, beraprost, SQ-29548, and abciximab on P-selectin expression

	pIC_{50} (M)			pIC_{50} (g ml^{-1})
Platelet-stimulating agent	*TRA-418*	*Beraprost*	*SQ-29548*	*Abciximab*
ADP	5.87 ± 0.05	8.56 ± 0.05	< 5	< 5
TRAP	6.50 ± 0.07	9.00 ± 0.07	< 5	< 5
U-46619 + epinephrine	6.88 ± 0.15	8.12 ± 0.07	8.53 ± 0.05	< 5

See Table 1 footnotes.

Table 3 Effects of TRA-418, beraprost, SQ-29548, and abciximab on platelet aggregation in citrate

	pIC_{50} (M)			pIC_{50} (g ml^{-1})
Platelet-stimulating agent	*TRA-418*	*Beraprost*	*SQ-29548*	*Abciximab*
ADP	5.77 ± 0.07	8.26 ± 0.09	< 5	5.60 ± 0.08
TRAP	5.62 ± 0.11	8.16 ± 0.25	< 5	5.33 ± 0.14
U-46619 + epinephrine	5.81 ± 0.06	7.31 ± 0.21	6.99 ± 0.11	5.22 ± 0.02

Values are mean ± s.e. of four determinations. Platelet aggregation was induced by ADP (5 μM), TRAP (3 μM), or a mixture of U-46619 (2 μM) and epinephrine (0.5 μM) in citrate.

Table 4 Effects of TRA-418, beraprost, SQ-29548, and abciximab on platelet aggregation in PPACK

	pIC_{50} (M)			pIC_{50} (g ml^{-1})
Platelet-stimulating agent	*TRA-418*	*Beraprost*	*SQ-29548*	*Abciximab*
ADP	5.40 ± 0.05	8.00 ± 0.08	< 5	5.29 ± 0.02
TRAP	5.28 ± 0.02	7.75 ± 0.01	< 5	4.87 ± 0.04
U-46619 + epinephrine	5.75 ± 0.01	6.79 ± 0.02	7.12 ± 0.12	4.81 ± 0.03

Values are mean ± s.e. of three to four determinations. Platelet aggregation was induced by ADP (5 μM), TRAP (3 μM), or a mixture of U-46619 (2 μM) and epinephrine (0.5 μM) in PPACK.

confirmed SQ-29548, the TP-antagonist, only inhibited U-46619 + epinephrine-induced platelet activation and aggregation. These results suggest that inhibitory effects of TRA-418 are mediated, at least in part, by its TP-receptor antagonistic activity.

As for abciximab, an anti-GPIIb/IIIa antibody, concentration-dependent inhibition of the GPIIb/IIIa activation was observed, regardless of the platelet-stimulating agents used. These results were compatible with the observations reported in paper (Coller *et al*., 1991). Although abciximab is considered to inhibit the platelet activation by blocking the outside–in signal pathway by binding GPIIb/IIIa (Clemetson, 1995), the available data concerning its effects on the P-selectin expression are rather controversial (Scazziota *et al*., 2000; Schneider *et al*., 2000; Dickfeld *et al*., 2001; Rossi *et al*., 2001). In our experiments, abciximab showed limited inhibitory effects on P-selectin expression. Thus, the contribution of the blocking outside–in signal transmission in the P-selectin expression is considered to be limited if at all.

Clinical application of IP-agonists has been limited due to vasodilation, in fact, we previously reported that the IP-

agonist showed obvious inhibition of ADP- and arachidonic acid-induced platelet aggregation at the dose of causing hypotension; however, TRA-418 inhibited both agonists-induced aggregation at the dose of nonaffecting blood pressure because of dual activity of TRA-418 in monkeys (Yamada *et al*., 2003). Therefore, it is speculated that TRA-418 can show the inhibition of U-46619 + epinephrine-induced aggregation at the dose of nonaffecting blood pressure.

In conclusion, the results in this study clearly illustrate the advantage of TRA-418 having both IP-receptor agonistic and TP-receptor antagonistic activities. Moreover, TRA-418 has a different profile from abciximab on inhibitory effects of P-selectin expression *in vitro*, thus TRA-418 might be effective on microembolism or microthrombus including platelet–leukocyte adhesion mediated by P-selectin. However, further studies are needed to demonstrate those effects of TRA-418.

References

BERTELE, V. & DE GAETANO, G. (1982). Potentiation by dazoxiben, a thromboxane synthetase inhibitor, of platelet aggregation inhibitory activity of a thromboxane receptor antagonist and of prostacyclin. *Eur. J. Pharmacol.,* **85,** 331–333.

CAVALLINI, L., COASSIN, M., BOREAN, A. & ALEXANDRE, A. (1996). Prostacyclin and sodium nitroprusside inhibit the activity of the platelet inositol 1,4,5-trisphosphate receptor and promote its phosphorylation. *J. Biol. Chem.,* **271,** 5545–5551.

CLEMETSON, K.J. (1995). Platelet activation: signal transduction *via* membrane receptors. *Thromb. Haemost.,* **74,** 111–116.

COLLER, B.S., SCUDDER, L.E., BEER, J., GOLD, H.K., FOLTS, J.D., CAVAGNARO, J., JORDAN, R., WAGNER, C., IULIUCCI, J., KNIGHT, D., GHRAYEB, J., SMITH, C., WEISMAN, H.F. & BERGER, H. (1991). Monoclonal antibodies to platelet glycoprotein IIb/IIIa as antithrombotic agents. *Ann. N.Y. Acad. Sci.,* **614,** 193–213.

DICKFELD, T., RUF, A., POGATSA-MURRAY, G., MULLER, I., ENGELMANN, B., TAUBITZ, W., FISCHER, J., MEIER, O. & GAWAZ, M. (2001). Differential antiplatelet effects of various glycoprotein IIb–IIIa antagonists. *Thromb. Res.,* **101,** 53–64.

KOTZE, H.F., LAMPRECHT, S., BADENHORST, P.N., VAN WYK, V., ROODT, J.P. & ALEXANDER, K. (1993). *In vivo* inhibition of acute platelet-dependent thrombosis in a baboon model by Bay U3405, a thromboxane A_2-receptor antagonist. *Thromb. Haemost.,* **70,** 672–675.

LARSEN, E., CELI, A., GILBERT, G.E., FURIE, B.C., ERBAN, J.K., BONFANTI, R., WAGNER, D.D. & FURIE, B. (1989). PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell,* **59,** 305–312.

LINDEMANN, S., KLINGEL, B., FISCH, A., MEYER, J. & DARIUS, H. (1999). Increased platelet sensitivity toward platelet inhibitors during physical exercise in patients with coronary artery disease. *Thromb. Res.,* **93,** 51–59.

MULLER, B., KRAIS, T., STURZEBECHER, S., WITT, W., SCHILLINGER, E. & BALDUS, B. (1988). Potential therapeutic mechanisms of stable prostacyclin (PGI_2)-mimetics in severe peripheral vascular disease. *Biomed. Biochim. Acta,* **47,** S40–S44.

NAGAYA, N., UEMATSU, M., OKANO, Y., SATOH, T., KYOTANI, S., SAKAMAKI, F., NAKANISHI, N., MIYATAKE, K. & KUNIEDA, T. (1999). Effect of orally active prostacyclin analogue on survival of outpatients with primary pulmonary hypertension. *J. Am. Coll. Cardiol.,* **34,** 1188–1192.

NORRIS, R.M., WHITE, H.D., HART, H.H. & WILLIAMS, B.F. (1996). Comparison of aspirin with a thromboxane antagonist for patients with prolonged chest pain and ST segment depression. *N.Z. Med. J.,* **109,** 278–280.

PALABRICA, T., LOBB, R., FURIE, B.C., ARONOVITZ, M., BENJAMIN, C., HSU, Y.M., SAJER, S.A. & FURIE, B. (1992). Leukocyte accumulation promoting fibrin deposition is mediated *in vivo* by P-selectin on adherent platelets. *Nature,* **359,** 848–851.

PARISE, L.V., VENTON, D.L. & LE BRETON, G.C. (1982). Prostacyclin potentiates 13-azaprostanoic acid-induced platelet deaggregation. *Thromb. Res.,* **28,** 721–730.

PHILLIPS, D.R., CHARO, I.F., PARISE, L.V. & FITZGERALD, L.A. (1988). The platelet membrane glycoprotein IIb–IIIa complex. *Blood,* **71,** 831–843.

ROSSI, F., ROSSI, E., PARETI, F.I., COLLI, S., TREMOLI, E. & GALLO, L. (2001). *In vitro* measurement of platelet glycoprotein IIb/IIIa receptor blockade by abciximab: interindividual variation and increased platelet secretion. *Haematologica,* **86,** 192–198.

SAKAMAKI, F., KYOTANI, S., NAGAYA, N., SATO, N., OYA, H., SATOH, T. & NAKANISHI, N. (2000). Increased plasma P-selectin and decreased thrombomodulin in pulmonary arterial hypertension were improved by continuous prostacyclin therapy. *Circulation,* **102,** 2720–2725.

SCAZZIOTA, A., ALTMAN, R., ROUVIER, J., GONZALEZ, C., AHMED, Z., JESKE, W.P., WALENGA, J.M. & FAREED, J. (2000). Abciximab treatment *in vitro* after aspirin treatment *in vivo* has additive effects on platelet aggregation, ATP release, and P-selectin expression. *Thromb. Res.,* **100,** 479–488.

SCHNEIDER, D.J., TAATJES, D.J. & SOBEL, B.E. (2000). Paradoxical inhibition of fibrinogen binding and potentiation of alpha-granule release by specific types of inhibitors of glycoprotein IIb–IIIa. *Cardiovasc. Res.,* **45,** 437–446.

SERRUYS, P.W., RUTSCH, W., HEYNDRICKX, G.R., DANCHIN, N., MAST, E.G., WIJNS, W., RENSING, B.J., VOS, J. & STIBBE, J. (1991). Prevention of restenosis after percutaneous transluminal coronary angioplasty with thromboxane A_2-receptor blockade. A randomized, double-blind, placebo-controlled trial. Coronary Artery Restenosis Prevention on Repeated Thromboxane-Antagonism Study (CARPORT). *Circulation,* **84,** 1568–1580.

SHATTIL, S.J., HOXIE, J.A., CUNNINGHAM, M. & BRASS, L.F. (1985). Changes in the platelet membrane glycoprotein IIb–IIIa complex during platelet activation. *J. Biol. Chem.,* **260,** 11107–11114.

STURZEBECHER, S. & WITT, W. (1988). The PGI_2-analogue iloprost and the TXA_2-receptor antagonist sulotroban synergistically inhibit TXA_2-dependent platelet activation. *Prostaglandins,* **36,** 751–760.

TAKIGUCHI, Y., WADA, K. & NAKASHIMA, M. (1992). Comparison of the inhibitory effects of the TXA_2 receptor antagonist, vapiprost, and other antiplatelet drugs on arterial thrombosis in rats: possible role of TXA_2. *Thromb. Haemost.,* **68,** 460–463.

VAN DER GIESSEN, W.J., ZIJLSTRA, F.J., BERK, L. & VERDOUW, P.D. (1988). The effect of the thromboxane receptor antagonist BM 13.177 on experimentally induced coronary artery thrombosis in the pig. *Eur. J. Pharmacol.,* **147,** 241–248.

WATSON, S.P., MCCONNELL, R.T. & LAPETINA, E.G. (1984). The rapid formation of inositol phosphates in human platelets by thrombin is inhibited by prostacyclin. *J. Biol. Chem.,* **259,** 13199–13203.

YAMADA, N., ISOGAYA, M., UENO, Y., KUMAGAI, H., OCHI, Y. & NISHIO, S. (1993). Synergic effect of beraprost sodium, a PGI_2 analogue, and aspirin on canine carotid artery thrombosis model induced by electrical stimulation. *Thromb. Haemost.,* **69** (Suppl.), 591.

YAMADA, N., MIYAMOTO, M., ISOGAYA, M., SUZUKI, M., IKEZAWA, S., OHNO, M., OTAKE, A. & UMEMURA, K. (2003). TRA-418, a novel compound having both thromboxane A_2 receptor antagonistic and prostaglandin I_2 receptor agonistic activities: its antiplatelet effects in human and animal platelets. *J. Thromb. Haemost.*, **1,** 1813–1819.

ZAVOICO, G.B. & FEINSTEIN, M.B. (1984). Cytoplasmic Ca^{2+} in platelets is controlled by cyclic AMP: antagonism between stimulators and inhibitors of adenylate cyclase. *Biochem. Biophys. Res. Commun.,* **120,** 579–585.

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