Mechanisms of Satigrel (E5510), a New Anti-platelet Drug, in Inhibiting Human Platelet Aggregation. Selectivity and Potency against Prostaglandin H Synthases Isozyme Activities and Phosphodiesterase Isoform Activities

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Satigrel (E5510, 4-cyano-5,5-bis(4-methoxyphenyl)-4-pentenoic acid) is a potent inhibitor of platelet aggregation. Like cyclooxygenase/prostaglandin H synthase (PGHS) inhibitors such as aspirin, which suppress platelet aggregation by inhibiting thromboxane A₂ production, satigrel inhibits collagen- and arachidonic acid-induced aggregation of human platelets. In contrast to other PGHS inhibitors, satigrel, like cyclic nucleotide phosphodiesterase (PDE) inhibitors such as cilostazol, shows inhibitory activity against thrombin-induced platelet aggregation. To investigate the mechanism of the anti-platelet activity of satigrel, we examined the selectivity and potency of satigrel against PGHS isozyme activities and PDE isoform activities. Two isozymes of PGHS are known; constitutive enzyme (PGHS1) and inducible enzyme (PGHS2). Satigrel showed inhibitory activity against PGHS1 (IC₅₀: 0.081 μM) and PGHS2 (IC₅₀: 5.9 μM), suggesting the selective inhibition of PGHS1. Indomethacin, which is a selective inhibitor of PGHS1, showed similar selectivity against PGHS isozymes (IC₅₀: 0.12 μM and 1.4 μM, respectively). These results support that satigrel suppresses thromboxane A₂ production by inhibiting PGHS1. It is known that three isozymes of PDE exist in human platelets: Type V, which specifically hydrolyzes guanosine 3',5'-cyclic monophosphate (cGMP), Type III, which mainly hydrolyzes cAMP, and Type II, which hydrolyzes both cGMP and cAMP. We separated these three isozymes from human platelets and examined the inhibitory activity of satigrel against each enzyme. Of the three isozymes, the inhibitory activity of satigrel was the most potent against Type III PDE (IC₅₀: 15.7 μM). The IC₅₀ value for Type III corresponded with that for thrombin-induced platelet aggregation. Type V and Type II were also inhibited by satigrel (IC₅₀: 39.8 and 62.4 μM, respectively). In human platelets, satigrel increased both cAMP and cGMP levels in a dose-dependent manner (100, 300 μM). In conclusion, satigrel inhibits collagen- and arachidonic acid-induced platelet aggregation through preventing thromboxane A₂ synthesis by selective inhibition of the target enzyme, PGHS1, which exists in platelets. The anti-aggregating activity of satigrel against thrombin-induced aggregation may be due to elevation of the cyclic nucleotide levels through the inhibition of PDE isozymes.

Key words satigrel; prostaglandin H synthase; phosphodiesterase; platelet; anti-platelet aggregating agent

Satigrel (E5510, 4-cyano-5,5-bis(4-methoxyphenyl)-4-pentenoic acid) is a potent platelet aggregation inhibitor.¹⁾ This compound inhibits collagen- and arachidonic acid-induced human platelet aggregation through preventing thromboxane A₂ synthesis by inhibiting cyclooxygenase/prostaglandin H synthase (PGHS).²⁾

Recently, two isozymes of PGHS have been reported^{3–8)}; PGHS1 and PGHS2. PGHS1 is constitutively expressed in most tissues, including platelets,^{9,10)} while PGHS2 is an inducible enzyme involved in prostaglandin production in the inflammatory process.^{11,12)} The inhibitors of PGHS are used as non-steroidal anti-inflammatory drugs and anti-platelet drugs. The selectivity and potency of non-steroidal anti-inflammatory drugs to inhibit PGHS1 and PGHS2 have been well studied.^{13–18)} However, the selectivity and potency of the anti-platelet drug, satigrel, for PGHS1 and PGHS2, have not yet been examined.

Unlike inhibitors of PGHS such as aspirin, satigrel dose-dependently inhibits thrombin-induced platelet aggregation through elevating the level of cAMP,²⁾ which acts as an inhibitor of platelet aggregation.¹⁹⁾ The elevation of cAMP in satigrel-treated platelets is caused by the inhibition of phosphodiesterase (PDE(s)).²⁾ In human platelets, three isoforms of PDE have been isolated²⁰⁾; a

guanosine 3',5'-cyclic monophosphate (cGMP)-specific family which is selectively inhibited by zaprinast (Type V),²¹⁾ a cGMP-inhibited family which is selectively inhibited by a large number of positive inotropic agents such as milrinone (Type III), and a cGMP-stimulated family (Type II).²⁰⁾ Type V PDE specifically hydrolyzes cGMP, Type III PDE mainly hydrolyzes cAMP and Type II PDE hydrolyzes both cGMP and cAMP.²²⁾ cGMP, as well as cAMP, acts as an inhibitor of platelet aggregation.²³⁾ However, the specificity of satigrel for the three isoforms of PDE and the level of cGMP in satigrel-treated platelets has not been investigated.

In the present study, we examined the selectivity of satigrel for PGHS and PDE isoforms, and also measured the levels of cAMP and cGMP in satigrel-treated human platelets.

MATERIALS AND METHODS

Materials Satigrel was synthesized by Eisai Chemical Co., Ltd. (Ibaraki). U53059 (4,5-bis(4-methoxyphenyl)-2-(trifluoromethyl)thiazole)²⁴⁾ and zaprinast were synthesized at Eisai Tsukuba Laboratories. Cilostazol was extracted from Pletaal® 100 tablets (Otsuka Pharmaceutical Co., Tokyo) at our laboratories. NS-398, PGHS1

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(isolated from ram seminal vesicles, 30600 U/mg) and PGHS2 (isolated from sheep placenta, 5923 U/mg) were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). Indomethacin, aspirin, hematin, arachidonic acid, cGMP, cAMP, dipyridamole and papaverine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Prostaglandin E₂ (PGE₂) enzyme immunoassay (EIA) kit, ¹²⁵I-cGMP assay kit and ¹²⁵I-cAMP assay kit were purchased from Amersham K.K. (Tokyo). DEAE-Toyopearl 650S was purchased from Toyo Soda Mfg Co., Ltd. (Tokyo). Blue sepharose CL-6B was purchased from Pharmacia (Uppsala, Sweden). AG-1-X2 anion exchange resin was purchased from Bio-rad (Tokyo). BCA protein assay kit was from Pierce (Rockford, IL, U.S.A.). 8,5'-³H-cGMP (1.3 Tbq/mmol) and 2,8-³H-cAMP (1039.7 Gbq/mmol) were purchased from Daiichi Pharmaceutical Co. (Tokyo).

Measurement of PGHS Activity Measurement of PGHS activity was carried out according to the method of Futaki *et al.*, with slight modification.¹⁴⁾ Briefly, 1 unit of PGHS1 or PGHS2 was suspended in 0.5 ml of 100 μM Tris-HCl buffer (pH 8.0) containing 1 μM hematin and 2 mM phenol as co-factors. The reaction mixture was preincubated with or without the tested drug for 2 min at 37 °C, then arachidonic acid was added at 100 μM and the mixture was incubated for 2 min at 37 °C. To terminate the reaction and extract PGE₂, 2 ml of *n*-hexane-ethyl acetate (2:1, v/v) was added to the reaction mixture and the preparation was shaken for 5 min. The mixture was then centrifuged at 2000 × *g* for 10 min at 4 °C. The aqueous phase was frozen and the organic solvent phase was discarded. The extraction procedure was repeated twice, then the contaminating organic solvent phase was removed by blowing it with N₂ gas. PGE₂ was measured by EIA. The drugs used were dissolved in dimethyl sulfoxide (DMSO, final concentration was 1%).

Preparation of Human Washed Platelets and Isolation of Cyclic Nucleotide PDE Isozymes Washed platelets were obtained from human venous blood of three healthy male volunteers who had not received any medication for more than one week. One hundred milliliters of venous blood from each volunteer was anti-coagulated with one-tenth volume of 3.8% sodium citrate and centrifuged at 100 × *g* for 10 min at room temperature to obtain platelet-rich plasma. After the addition of 15% (final concentration) of ACD-A solution (Terumo Co., Tokyo), platelet-rich plasma was centrifuged at 2500 × *g* for 10 min at room temperature. Isolated platelets were suspended with 2.7 mM EDTA saline and pooled. The platelet suspension was centrifuged at 100 × *g* for 10 min at room temperature to remove contaminated erythrocytes. The platelet suspension was re-centrifuged at 2500 × *g* for 10 min at room temperature. Washed platelets thus obtained were stored at -80 °C until used.

PDE isozymes from platelets were prepared as described previously.²¹⁾ Washed platelets (3.2 × 10¹⁰ cells) were resuspended with 30 ml of 20 mM Tris-HCl buffer (pH 7.4) containing 5 mM EDTA, 2 mM Mg acetate, 1 mM dithiothreitol, 0.02 mM leupeptin, 1.3 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 50 mg/l aprotinin and 10 mg/l trypsin inhibitor, and were homogenized by

sonication (20 W) for 10 s. The sonication was repeated 3 times. The homogenate was centrifuged at 105000 × *g* for 60 min at 4 °C. The supernatant was applied to a DEAE-Toyopearl 650S column (2 × 35 cm) pre-equilibrated with an application buffer (20 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM EGTA, 2 mM Mg acetate, 0.2 mM phenylmethylsulfonyl fluoride and 10 mM 2-mercaptoethanol). After application, Type V and Type III were separated by a linear gradient of 0.05–0.3 M NaCl.

All fractions containing Type V activity were pooled and applied to a Blue sepharose CL-6B column (2 × 30 cm) pre-equilibrated with an application buffer. After application, the column was washed with an application buffer containing 0.3 M NaCl. Type V was eluted by an application buffer containing 0.3 M NaCl and 10 mM cGMP. The eluted fraction was used as the enzyme source of Type V in PDE assay. All fractions containing PDE activity, but not Type V and Type II activity, were pooled and used as the enzyme source of Type III in the PDE assay. Type II PDE was isolated according to the method of Martins *et al.* with minor modification.²⁵⁾ Type II fractions contaminated with Type III activity were applied to the cGMP-sepharose affinity column pre-equilibrated with an application buffer. The column was washed with an application buffer, and Type III was eluted with 0.5 M NaCl in an application buffer. Type II was eluted with 10 mM cGMP and 0.3 M NaCl in the application buffer.

Fractions containing each PDE isozyme were concentrated by dialyzation against an application buffer containing 20% polyethylene glycol 20000 and then resuspended in an application buffer containing 30% ethylene glycol and stored at -20 °C.

Measurement of PDE Activity PDE activity was determined by a previously described method,²¹⁾ which was a modification of the two-step radioisotopic procedure of Thompson *et al.*²⁶⁾ Samples were incubated at 30 °C for 10 min in a total volume of 0.2 ml assay buffer (40 mM Tris-HCl buffer (pH 8.0) containing 10 mM MgCl₂, 0.126 mg/ml bovine serum albumin (BSA), 0.6 ml/l 2-mercaptoethanol), 1 mM EGTA and 1 μM ³H-cGMP (100000 dpm/tube) or 1 μM ³H-cAMP (100000 dpm/tube)) with or without drug. For the assay of Type V PDE activity, 1 μM of cGMP was used as a substrate and 1 μM of cAMP was used for the assay of Type II and Type III PDE activities. For the assay of Type II PDE activity, 10 μM cGMP was added to promote the reaction. The reaction was stopped by incubation at 95 °C for 1.5 min, then the assay tube was chilled in ice water. After the addition of 50 μl of 5'-nucleotidase (10 U/ml), the assay mixture was incubated at 30 °C. After 10 min of incubation, 550 μl of AG1-X2 resin slurry (resin:H₂O=1:2) was added, and then the assay tube was allowed to stand in ice water for 10 min. The assay tube was centrifuged at 7800 × *g* for 2.5 min at 4 °C, and radioactivity in 450 μl of the supernatant was measured. The drugs used were dissolved in DMSO and diluted with an assay buffer. The final concentration of DMSO was 0.2% or less.

Protein concentration was determined by the BCA method, after precipitation of the PDE fractions with 10% (final concentration) trichloroacetic acid.

Measurement of Cyclic Nucleotide Content in Plate-

lets Cyclic nucleotide content in the platelets was determined by a protein binding method. Preparation of washed platelets was performed using blood taken from six healthy male volunteers who had not received any medication for more than one week. Washed platelets were resuspended in a Ca^{2+} -free Tyrode solution containing 1% BSA at a concentration of about 5×10^8 platelets/ml. For the assay, 900 μl of platelet suspension was mixed with 100 μl of test drug solution or vehicle and incubated at 37°C . After 10 min, 2 ml of cold ethanol was added, and the assay tube was chilled in ice water. After 10 min, the assay tube was centrifuged at $2500 \times g$ for 20 min at 4°C . The supernatant was collected and the pellet was rinsed with 1 ml of 65% cold ethanol. The supernatant and the rinse were dried by bubbling them with N_2 gas, and the residue was stored at -20°C until the determination of cyclic nucleotide content. cAMP and cGMP content were determined by ^{125}I -cAMP assay kit and ^{125}I -cGMP assay kit, respectively. The drugs used were dissolved in DMSO and diluted 100-fold with saline containing 1% BSA.

Statistical Analysis Data was expressed as the mean $\pm$ S.E. IC_{50} values and 95% confidence limits were calculated by Fieler's linear regression. Statistical analysis was performed by one-way analysis of variance and Dunnett's multiple range test.

RESULTS

Selectivity of E5510 for PGHS1 and PGHS2 The selectivity and potency of satigrel and reference drugs are shown in Fig. 1 and Table 1. Satigrel showed similar selectivity to that of indomethacin, which is well known to be a selective inhibitor of PGHS1.¹³⁾ The IC_{50} values of satigrel and indomethacin for PGHS1 were 0.081 and $0.12 \mu\text{M}$, respectively. Both satigrel and indomethacin were less selective against PGHS2 (IC_{50} : 5.9 and $1.4 \mu\text{M}$). U53059 also showed high specificity against PGHS1. The IC_{50} values of U53059 for PGHS1 and PGHS2 were 0.00044 and $1.5 \mu\text{M}$, respectively. However, aspirin did not show selectivity against either of the isozymes (IC_{50} : 150 and $220 \mu\text{M}$). NS-398, a selective inhibitor of PGHS2,¹⁴⁾ showed inhibitory activity against PGHS2 (IC_{50} : $0.43 \mu\text{M}$) but not PGHS1 at the concentration of 0.001 to $10 \mu\text{M}$.

Isolation of Human Platelet PDE Isoforms Figure 2 shows the elution profiles from DEAE-Toyopearl 650S chromatography of human platelet extracts using two different assay conditions. Fractions 38—55 preferentially hydrolyzed cGMP, while fractions 77—90 weakly hydrolyzed cGMP. Fractions 80—100 showed cAMP hydrolytic activities. Fractions 38—55, except for fraction 46, were pooled. cGMP hydrolytic activity and cAMP hydrolytic activity of the pooled fraction were 3.91×10^3 and 1.00×10^2 (pmol/mg protein/min), respectively. This fraction was re-applied to a Blue sepharose CL-6B column and eluted with 10 mM cGMP. The fraction was used as the enzyme source of Type V in PDE assay. Fractions 77—90 were pooled and re-applied to a cGMP affinity column. cGMP hydrolytic activity and cAMP hydrolytic activity of the pooled fraction were 7.32×10^2 and 3.31×10^3 (pmol/mg protein/min), respectively. The fraction containing Type II PDE activity was eluted with 10 mM cGMP. Fractions

a) PGHS 1

b) PGHS 2

Fig. 1. Inhibitory Effects of Satigrel and Reference Drugs on PGHS1 and PGHS2

Data are shown as mean $\pm$ S.E. of three different experiments. Each experiment was performed in duplicate. PGE_2 concentration in PGHS1 and PGHS2 control groups was 10 ± 2 and 27 ± 1 ng/ml, respectively.

Table 1. Inhibitory Effects of Satigrel and Reference Drugs on PGHS1 and PGHS2

	IC_{50} values (μM) (95% confidence limits)	
	PGHS1	PGHS2
Satigrel	0.081 (0.070—0.093)	5.9 (5.1—6.8)
Indomethacin	0.12 (0.083—0.16)	1.4 (1.3—1.6)
Aspirin	150 (95—250)	220 (160—310)
NS-398	— ^{a)}	0.43 (0.27—0.86)
U53059	0.00044 (0.00019—0.0011)	1.5 (0.69—3.7)

Values were obtained from three different experiments. a) NS-398 did not show inhibitory activity against PGHS1 at the concentration of 0.001 to $10 \mu\text{M}$.

92—100 were collected and concentrated and used as the enzyme source of Type III in PDE assay. Specific activities of the three PDE isozymes are shown in Table 2. Type V preferentially hydrolyzed cGMP. Type III preferably hydrolyzed cAMP, and this hydrolytic activity was inhibited by cGMP (IC_{50} : $1.02 \mu\text{M}$, 95% confidence limit: 0.868 — $1.21 \mu\text{M}$, $n=3$). Type II hydrolyzed both cGMP and cAMP. The cAMP hydrolytic activity of Type II was elevated about 3.7 times in the presence of $10 \mu\text{M}$ cGMP.

Inhibition of Isolated PDE Isozymes by Various PDE Inhibitors The inhibitory effects of satigrel, cilostazol, zaprinast, and dipyridamole are shown in Table 3. Satigrel showed inhibitory activity against Type V, Type II and Type III PDE activities in human platelets, with IC_{50} values of 39.8, 62.4 and $15.7 \mu\text{M}$, respectively. Type V was most potently inhibited by zaprinast (IC_{50} : $0.234 \mu\text{M}$), a

Fig. 2. Elution Profile from DEAE-Toyopearl 650S Chromatography of Human Platelet Extract in the Absence of Ca^{2+}

PDE activity was determined using $1 \mu\text{M}$ cGMP (circles) or $1 \mu\text{M}$ cAMP (triangles) as a substrate, in the presence of 1 mM EGTA. Washed platelets (3.2×10^{10}) were homogenized in 30 ml of 20 mM Tris-HCl buffer (pH 7.4) containing 5 mM EDTA, 2 mM Mg acetate, 1 mM dithiothreitol, 0.02 mM leupeptin, 1.3 mM benzamide, 0.2 mM phenylmethylsulphonyl fluoride, 50 mg/l aprotinin and 10 mg/l trypsin inhibitor. The homogenate was centrifuged at $105000 \times g$ for 60 min . The supernatant was applied to a DEAE-Toyopearl 650S column ($2 \times 35 \text{ cm}$) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM EGTA, 2 mM Mg acetate, 0.2 mM phenylmethylsulphonyl fluoride and 10 mM 2-mercaptoethanol. After application, the column was washed with the same buffer and a linear gradient of $0.05\text{--}0.3 \text{ M}$ NaCl was started.

Table 2. Specific Activities of Three PDE Isozymes

Isozymes of PDE	Specific activities (pmol/mg protein/min)	
	cGMP	cAMP
Type V PDE	1.00×10^4	2.91×10^2
Type II PDE	3.33×10^3	1.90×10^3
Type III PDE	4.67×10^2	3.67×10^3

Table 3. Inhibitory Effects of Satigrel and Reference Drugs on Three Isoforms of PDE

	IC_{50} values (μM) (95% confidence limits)		
	Type V PDE	Type II PDE	Type III PDE
Satigrel	39.8 (32.0–51.3)	62.4 (47.4–86.7)	15.7 (12.5–20.3)
Cilostazol	4.53 (3.59–5.75)	36.4 (26.4–54.0)	0.241 (0.196–0.295)
Zaprinast	0.234 (0.187–0.291)	64.1 (40.9–119)	N.E.
Dipyridamole	0.259 (0.227–0.295)	3.48 (2.90–4.26)	69.2 (42.3–139)

Values were obtained from three different experiments. N.E., not effective.

well-known selective inhibitor of Type V,²¹) Cilostazol, a specific inhibitor of Type III,²⁷) showed selective inhibitory activity against Type III (IC_{50} : $0.241 \mu\text{M}$). Dipyridamole showed inhibitory activity against Type V and Type II, with IC_{50} values of 0.259 and $3.48 \mu\text{M}$, respectively.

Effects of E5510, Cilostazol and Papaverine on Cyclic Nucleotide Content in Human Platelets Effects of satigrel, cilostazol and papaverine, a nonspecific PDE inhibitor,²¹) on the cAMP-content in human platelets, are shown in Fig. 3a. Satigrel caused a significant dose-dependent increase in cAMP-content at 100 and $300 \mu\text{M}$. Both cilostazol and papaverine significantly increased the content of cAMP in a dose-dependent manner.

cGMP content of treated platelets is shown in Fig. 3b. Satigrel significantly increased the content of cGMP at 100 , $300 \mu\text{M}$. The cGMP-content of cilostazol- and

a) cAMP

b) cGMP

Fig. 3. Cyclic Nucleotide Contents of Satigrel-Treated Human Platelets

Data are shown as mean $\pm$ S.E. ($n=6$). Each experiment was performed in duplicate. * $p < 0.05$.

papaverine-treated platelets was significantly and dose-dependently increased.

DISCUSSION

We have previously reported that satigrel inhibited human platelet aggregation induced not only by collagen (IC₅₀: 1.5 μ M), adenosine diphosphate (ADP, IC₅₀: 2.0 μ M), epinephrine (IC₅₀: 1.1 μ M), platelet-activating factor (IC₅₀: 1.6 μ M) and arachidonic acid (IC₅₀: 0.7 μ M), but also by thrombin (IC₅₀: 21.0 μ M).¹⁾ In a clinical study, satigrel showed a benefit over aspirin, a PGHS-inhibitor.²⁸⁾ Aspirin did not inhibit thrombin-induced aggregation, but cilostazol, a specific inhibitor of Type III PDE,²⁷⁾ inhibited thrombin-induced aggregation (IC₅₀: 1.4 μ M).¹⁾ From these results, it is likely that satigrel exerts anti-platelet aggregating activity through two mechanisms *in vitro* and *in vivo*: 1) preventing thromboxane A₂ synthesis by inhibiting PGHS and 2) elevating cAMP by inhibiting PDEs.²⁾ In this study, we examined the selectivity of satigrel towards isozymes of PGHS and PDE. Furthermore, we examined the levels of cAMP and cGMP, which are able to inhibit platelet aggregation,^{19,22)} in satigrel treated platelets.

There are two isoforms of PGHS, termed PGHS1 and PGHS2.³⁻⁸⁾ PGHS1 is constitutively expressed in most tissues, including platelets. PGHS1 is likely involved in "housekeeping" prostanoid biosynthesis.¹⁰⁾ On the other hand, PGHS2 expression is induced by inflammatory stimuli and mediates inflammatory processes.¹²⁾ Some PGHS inhibitors are used as non-steroidal anti-inflammatory drugs and anti-platelet drugs.¹³⁻¹⁸⁾ Most non-steroidal anti-inflammatory drugs show selectivity against PGHS1. Thus, we determined the selectivity of three anti-platelet aggregating agents, satigrel, U53059 and aspirin, which have inhibitory activities against PGHS(s). In this assay system, selective inhibitors of PGHS1 (indomethacin)¹³⁾ and PGHS2 (NS-398)¹⁴⁾ exhibited selectivity for PGHS1 and PGHS2, respectively. The IC₅₀ values of indomethacin were 0.12 μ M for PGHS1 and 1.4 μ M for PGHS2. NS-398 did not show any inhibitory activity against PGHS1 at the concentration of 0.001 to 10 μ M. The IC₅₀ value for PGHS2 was 0.43 μ M. Satigrel, like indomethacin, showed selectivity for PGHS1, with IC₅₀ values of 0.081 μ M for PGHS1 and 5.9 μ M for PGHS2. U53059 also showed selectivity for PGHS1. Aspirin did not show selectivity for either of the two enzymes in this system (IC₅₀: 150 μ M for PGHS1 and 220 μ M for PGHS2). There are some conflicting reports on the selectivity of aspirin for PGHSs.^{15,29)} Our results are consistent with the report of Bhattacharyya *et al.* in which aspirin was suggested to be a non-selective inhibitor.²⁹⁾

It is known that platelets constitutively express PGHS1.¹⁰⁾ Thus, satigrel, a selective inhibitor of PGHS1, is considered to exhibit its anti-platelet activity through preventing thromboxane A₂ synthesis by inhibiting its target enzyme, PGHS1, like other PGHS inhibitors.

Recently, five isozymes of PDE were identified²¹⁾: a Ca²⁺-calmodulin dependent family (Type I); a cGMP-stimulated family, which hydrolyzes both cAMP and cGMP (Type II); a cGMP-inhibited family, which mainly hydrolyzes cAMP and is selectively inhibited by cilostazol (Type III); a cAMP-specific family, which is selectively inhibited by rolipram (Type IV); and cGMP-specific

family, which mainly hydrolyzes cGMP and is selectively inhibited by zaprinast (Type V).

Three isozymes of PDE (Type V, Type III and Type II) were identified in human platelets,²⁰⁾ although type II exists only in small amounts in platelets. In the present study, Type V, Type III and Type II isolated from human platelets were inhibited by zaprinast (IC₅₀: 0.234 μ M), cGMP (IC₅₀: 1.02 μ M) and dipyridamole (IC₅₀: 3.48 μ M), respectively. These results are consistent with our recent report using isozymes of PDE isolated from porcine aorta.²¹⁾ Recently, Umekawa *et al.* reported that cilostazol inhibited Type III (IC₅₀: 0.19 μ M) and Type V PDE (IC₅₀: 7.8 μ M) from human platelets.²⁷⁾ The results obtained in this study are consistent with our former results obtained using porcine aorta²¹⁾ and with the results of Umekawa *et al.* using human platelets.²⁷⁾

Satigrel inhibited the three above isozymes of PDE. Satigrel showed the most marked inhibition of Type III PDE among the three isozymes (IC₅₀: 15.7 μ M). The inhibitory activities of satigrel and cilostazol against Type III PDE (IC₅₀: 15.7 and 0.241 μ M) are close to their anti-aggregating activities against thrombin-induced platelet aggregation (IC₅₀: 21.0 and 1.4 μ M, respectively).¹⁾ Type V PDE was also inhibited by satigrel (IC₅₀: 39.8 μ M) and cilostazol (IC₅₀: 4.53 μ M). Inhibition of Type III and Type V PDE causes the elevation of both cAMP and cGMP levels. So, we determined the effects of satigrel and cilostazol on the levels of cyclic nucleotides in human platelets. Papaverine, a nonspecific inhibitor of PDE, dose-dependently elevated the levels of cyclic nucleotides. Satigrel and cilostazol also elevated the levels of both cAMP and cGMP. The cyclic nucleotide-elevating activities of satigrel and cilostazol were similar to their inhibitory activities against Type III and Type V PDE. It has been reported that the elevation of both cAMP and cGMP showed inhibitory activity against thrombin-induced platelet aggregation.^{19,23)} The inhibitory mechanism of cAMP and cGMP is explained through different pathways, but they act synergistically.^{19,23,30)} cAMP inhibits phosphatidylinositol turnover through inhibiting the activation of phospholipase C, the initial reaction of platelet activation.^{19,29)} On the other hand, cGMP inhibits platelet aggregation indirectly through elevation of the level of cAMP by inhibiting Type III PDE.²³⁾ Thus, satigrel shows anti-aggregating activity against thrombin-induced platelet aggregation, probably by elevating the levels of cAMP and cGMP through the inhibition of Type III and Type V PDE.

In summary, satigrel shows anti-aggregating activity against platelet aggregation induced by collagen, ADP, epinephrine, platelet-activating factor and arachidonic acid through preventing thromboxane A₂ synthesis by inhibiting PGHS1, like other PGHS inhibitors, and it also inhibits thrombin-induced aggregation through the elevation of the level(s) of cyclic nucleotide(s) by inhibiting the isozymes of PDE.

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