Effects of KW-3635, a Novel Dibenzoxepin Derivative of a Selective Thromboxane A_2 Antagonist, on Human, Guinea Pig and Rat Platelets

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ABSTRACT — We examined the binding of [^{3}H]U-46619, a thromboxane A_2 agonist, to human and guinea pig platelets and the binding of [^{3}H]SQ 29,548, a thromboxane A_2 antagonist, to human, rat and guinea pig platelets. KW-3635 (sodium (*E*)-11-[2-(5,6-dimethyl-1-benzimidazolyl)ethylidene]-6,11-dihydrodibenz[*b,e*]oxepin-2-carboxylate monohydrate) concentration-dependently inhibited the [^{3}H]U-46619 binding to human and guinea pig platelets with inhibition constants of 1.2 nM and 2.7 nM, respectively. KW-3635 also potently inhibited the [^{3}H]SQ 29,548 binding to human and guinea pig platelets with inhibition constants of 1.9 nM and 3.2 nM, respectively. In contrast, KW-3635 was less active against thromboxane A_2/prostaglandin H_2 receptors in rat platelets with an inhibition constant of 97 nM. KW-3635 at 10^{-5} M did not antagonize various receptors including prostaglandin E_2, prostaglandin I_2 and neurotransmitters. In addition, 10^{-5} M KW-3635 did not alter the prostaglandin D_2-induced cAMP accumulation in EBTr cells. KW-3635 was inactive towards thromboxane synthase, cyclooxygenase and prostaglandin I_2 synthase up to 10^{-5} M. KW-3635 slightly inhibited 5-lipoxygenase with an IC_{50} value of 71 μM. These data indicate that KW-3635 is a potent and selective non-prostanoic thromboxane A_2 antagonist, and it can recognize the species differences in thromboxane A_2/prostaglandin H_2 receptors.

Keywords: Thromboxane A_2 antagonist, Thromboxane A_2/prostaglandin H_2 receptors, KW-3635, Platelets

Thromboxane (TX) A_2 is synthesized from arachidonic acid via prostaglandin (PG) H_2 and evokes platelet aggregation and vasoconstriction (1 - 3). TXA_2 and PGH_2 are known to stimulate platelet activation and smooth muscle contraction through a TXA_2/PGH_2-receptor-mediated process (4). TXA_2 is implicated in the etiology and pathology of many cardiovascular and pulmonary diseases such as myocardial ischemia, coronary vasospasm and asthma (5, 6). Although many thromboxane synthase inhibitors have been developed, these have generally failed to provide the anticipated beneficial effects (7). One possible reason for this lack of efficacy may have been the accumulation of PGH_2 that accompanies thromboxane synthase inhibition and acts on TXA_2/PGH_2 receptors (8). TXA_2 antagonists should be promising candidates for potent antithrombotic and antiasthmatic drugs (2, 5).

KW-3635, (sodium (*E*)-11-[2-(5,6-dimethyl-1-benzimidazolyl)ethylidene]-6,11-dihydrodibenz[*b,e*]oxepin-2-carboxylate monohydrate) (Fig. 1), a novel dibenzox-

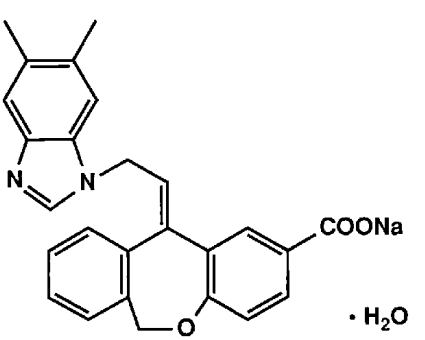

Fig. 1. Chemical structure of KW-3635.

epin derivative, inhibited both collagen and U-46619 induced aggregation in human platelets and suppressed U-46619-induced contraction of vascular smooth muscle cells (9–11). KW-3635 significantly improves the circulatory status of rats in traumatic shock (12), in which TXA_2 appears to contribute to the pathogenesis of the shock state (13).

To characterize TXA_2/PGH_2 receptors, radiolabeled prostanoic TXA_2 antagonists such as SQ 29,548 and S-145, and agonists such as U-46619 and I-BOP, were used for the receptor binding assay (14–16). Heterogeneity of TXA_2/PGH_2 receptors in human platelets has been well-characterized by the receptor binding assay (17, 18), and some work has been reported on the species differences in rabbits, cats, humans and other species (19, 20). Recently, TXA_2/PGH_2 receptor in human placenta was cloned and expressed (21), and they reported the presence of the same type of receptor in the vascular tissue and platelets. The subtype of the receptors has not been clarified at this time. In this study, we characterized TXA_2/PGH_2 receptors in human, guinea pig and rat platelets. We demonstrated that KW-3635 is a novel selective non-prostanoic TXA_2 antagonist, and it has different activities on TXA_2/PGH_2 receptors in human, guinea pig and rat platelets.

MATERIALS AND METHODS

Materials

Commercial sources of materials and reagents were as follows: [^{3}H]SQ 29,548 (1,110 GBq/mmol) and [^{3}H]U-46619 (828.8 GBq/mmol) from Du Pont (Wilmington, DE); [^{14}C]arachidonic acid (2.07 GBq/mmol), [^{3}H]TXB_2 (8,100 GBq/mmol), [^{3}H]6-keto-$PGF_{1\alpha}$ (5,550 GBq/mmol) from Amersham (Arlington Heights, IL); other radioactive compounds from Amersham or Du Pont; carbocyclic thromboxane A_2 (CTA_2), pinane thromboxane A_2 (PTA_2), TXB_2, PGs from Funakoshi (Tokyo); and Scintisol EX-H from Wako Pure Chemical (Osaka). SQ 29,548 was a kind gift from Dr. M.L. Ogletree of the Squibb Institute for Medical Research. KW-3635, BM-13177 (4-[2-[benzenesulphonamido)-ethyl]-phenoxyacetic acid) (22) and BM-13505 (4-2-(chlorobenzenesulfonylamino)-ethyl-benzene acetic acid) (23) were synthesized in our laboratories. All of the other reagents were commercially available.

Binding assay of TXA_2/PGH_2 receptors in platelets

Binding assay was performed according to the previously described methods with some modifications (14, 15). Male Hartley guinea pig blood or male Wistar rat blood was drawn from the abdominal vein and anti-coagulated with 1/10 volume of 77 mM EDTA (pH 7.0) containing 100 μM indomethacin. Human blood was collected into syringes containing 3.8% sodium citrate and then added with 1/10 volume of 77 mM EDTA (pH 7.0) containing 100 μM indomethacin. Blood was centrifuged at 120 $\times$ g for 12 min at room temperature. The platelet-rich plasma was centrifuged at 900 $\times$ g for 10 min, and the platelets were then washed with 25 mM Tris-HCl buffer (pH 7.5) containing 138 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA and 10 μM indomethacin and resuspended in the same buffer. The binding study was performed by incubating 1×10^8 platelets with 5 nM [^{3}H]SQ 29,548 or 10 nM [^{3}H]U-46619 in a total volume of 200 μl at 25°C for 60 min or 37°C for 20 min, respectively. Nonspecific binding was measured in the presence of 100 μM BM-13505 for the [^{3}H]SQ 29,548 binding assay or 10 μM U-46619 for the [^{3}H]U-46619 binding assay. Ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl was added to terminate the reaction, and the content was immediately passed through a Whatman GF/C glass filter using a Brandel MT-24 cell harvester (Gaithersburg, MD). The filter was washed three times, each time with 3 ml of ice-cold buffer, dried and 8 ml Scintisol EX-H was added. The radioactivity on the filter was counted in a liquid scintillation counter.

Various receptors binding studies

The [^{3}H]ligands and tissues used in the various receptors binding assays were as follows: [^{3}H]cyclohexyladenosine (CHA) and guinea pig forebrain for adenosine A_1 receptors (24), [^{3}H]5'-*N*-ethylcarboxamidoadenosine (NECA) and rat striatum for adenosine A_2 receptors (24), [^{3}H]WB-4101 and rat forebrain for adrenaline α_1-receptors (24), [^{3}H]clonidine and rat cerebral cortex for adrenaline α_2-receptors (24), [^{3}H]dihydroalprenolol and rat cerebral cortex for adrenaline β-receptors (24), [^{3}H]flunitrazepam and rat brain for central benzodiazepine receptors (24), [^{3}H]SCH 23390 and rat striatum for dopamine D_1-receptors (25), [^{3}H]spiperone and rat striatum for dopamine D_2-receptors (24), [^{3}H]muscimol and rat brain for γ-aminobutyric acid-A ($GABA_A$) receptors (26), [^{3}H]pyrilamine and guinea pig cerebellum for histamine H_1-receptors (24), [^{3}H]tiotidine and guinea pig cerebral cortex for histamine H_2-receptors (27), [^{3}H]-quinuclidinylbenzilate and rat cerebral cortex or heart for muscarinic acetylcholine M_1- or M_2-receptors (28), [^{3}H]MK-801 and rat cerebral cortex for *N*-methyl-D-aspartate (NMDA) receptors (29), [^{3}H]8-hydroxy-DPAT and rat hippocampus for serotonin-1A receptors (30), [^{3}H]ketanserin and rat frontal cortex for serotonin-2 receptors (24), [^{3}H]C_{16}-platelet activating factor (PAF) and rabbit platelets for PAF receptors

(31), [^{3}H]leukotriene D_4 and guinea pig lung for leukotriene D_4 receptors (32), [^{3}H]PGE_2 and porcine cerebral cortex for PGE_2 receptors (33), [^{3}H]iloprost and human platelets for PGI_2 receptors (34, 35), [^{3}H]nitrendipine and rat heart for dihydropyridine binding sites (24), and [^{3}H]imipramine and rat cerebral cortex for imipramine binding sites (36). Drugs, [^{3}H]ligand and receptor membrane fractions were incubated. Reaction was stopped by rapid vacuum filtration through a Whatman GF/B or GF/C glass fiber filter and then washed with an ice-cold buffer solution. Each experiment was performed in duplicate and repeated 2 times.

PGD_2-stimulated cAMP increase in EBTr cells

EBTr (ATCC CCL 44) cells, the fibroblastic cell line derived from bovine embryonic trachea, were stimulated by 10^{-6} M PGD_2 according to the methods of Sugama et al. (37). The formation of cAMP in the presence or absence of 10^{-5} M KW-3635 was measured by a specific radioimmunoassay kit (Yamasa, Chiba).

Enzyme inhibition

Bovine platelet microsomes or aortic microsomes (Funakoshi, Tokyo) and PGH_2 as substrate were used to determine the TX synthase (38) or PGI_2 synthase (39) activity, respectively. The inhibition of TX synthase was determined by measuring TXB_2, a stable hydrolysis product of TXA_2, using a specific radioimmunoassay (3). The inhibition of PGI_2 synthase was determined by measuring 6-keto-$PGF_{1\alpha}$, a stable hydrolysis product of PGI_2, using a specific radioimmunoassay. The cross-reactivity of anti-6-keto-$PGF_{1\alpha}$-antiserum was 100% for 6-keto-$PGF_{1\alpha}$, 1.02% for 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$, 1.6% for PGE_2, 0.45% $PGF_{2\alpha}$, 0.15% for $PGF_{1\alpha}$ and $< 0.1\%$ for PGD_2 and TXB_2, respectively. [^{14}C]Arachidonic acid was incubated with sheep vesicular gland microsomes (Funakoshi, Tokyo) for cyclooxygenase (40), guinea pig peritoneal leukocyte cytosol for 5-lipoxygenase (41) or bovine platelet microsomes for 12-lipoxygenase (42); and the enzymatic metabolites were analyzed by TLC.

Miscellaneous

The protein concentration was determined by the method of Lowry et al. with bovine serum albumin as a standard (43). Computer analysis (EBDA and LIGAND) (44) was used to evaluate the dissociation constant (K_d value) and the receptor density (B_{max} value). The inhibition constant (K_i value) was obtained from the IC_{50} value of drug according to the Cheng-Prusoff's equation (45).

RESULTS

TXA_2/PGH_2 receptor binding in various species

TXA_2/PGH_2 receptors in human, guinea pig and rat platelets were characterized by the receptor binding assay. The binding of [^{3}H]U-46619 to both human and guinea pig platelets and [^{3}H]SQ 29,548 binding to both rat and guinea pig platelets were saturable, displaceable, and dependent on protein concentration. Scatchard analyses of equilibrium binding showed a single class of high affinity binding sites in each assay. The K_d values of [^{3}H]U-46619 in human and guinea pig platelets were 40 ± 7.1 nM (mean ± S.E.M.) and 24 ± 1.5 nM, respectively. The K_d value of [^{3}H]SQ 29,548 in guinea pig platelets was 3.0 ± 0.22 nM, which was quite similar to the value in human platelets (4.9 ± 0.43 nM) that we previously reported (35). The K_d value of [^{3}H]SQ 29,548 in guinea pig platelets (3.0 ± 0.22 nM) was 4.7-fold smaller than that in rat platelets (14 ± 3.5 nM). The K_d value of [^{3}H]SQ 29,548 in rat platelets was about 5-fold decreased by the addition of 0.1% (w/v) of bovine serum albumin (2.9 ± 0.15 nM). We usually characterized TXA_2/PGH_2 receptors in rat platelets without albumin, because it was preferable to compare the various TXA_2/PGH_2 receptors under the same conditions.

Inhibition of TXA_2/PGH_2 receptors by TXA_2 antagonists and agonists

The specific binding of [^{3}H]U-46619 to human platelets was concentration-dependently inhibited by TXA_2 agonists or antagonists (Fig. 2). The concentration of compounds required to reduce receptor specific [^{3}H]U-46619 binding by 50% (IC_{50}) in human platelets was determined from Fig. 2 and used to calculate the K_i value. The rank order of the K_i values of antagonists from high to low affinity was found to be KW-3635 > SQ 29,548 > BM-13505 > CTA_2 > BM-13177 > PTA_2 in human platelets (Table 1). Linear regression analyses of the log of the K_i value of compounds in guinea pig platelets and human platelets were correlated to each other ($r = 0.945$). Furthermore, the log of the K_i values of compounds for TXA_2 agonist ([^{3}H]U-46619) binding and for TXA_2 antagonist ([^{3}H]SQ 29,548) binding in guinea pig platelets also were well-correlated ($r = 0.948$). In rat platelets, the rank order of K_i values of the antagonists was SQ 29,548 > BM-13505 > KW-3635 > PTA_2 > CTA_2 > BM-13177. The log of the K_i value of compounds for [^{3}H]SQ 29,548 binding in rat platelets and in guinea pig platelets showed a poor correlation ($r = 0.754$).

KW-3635 concentration-dependently inhibited the [^{3}H]U-46619 binding to human (Fig. 2) and guinea pig

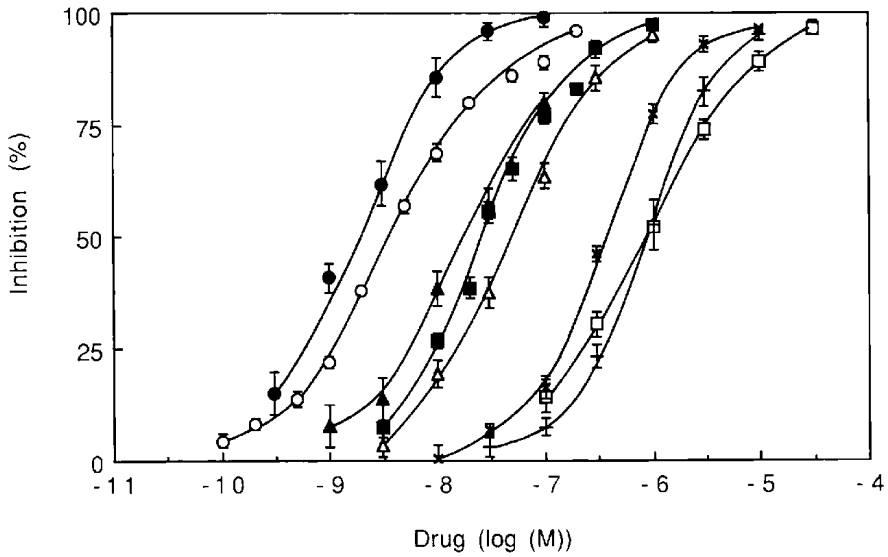

Fig. 2. Displacement of the specific binding of [^{3}H]U-46619 in human platelets. Human platelets were incubated with [^{3}H]U-46619 in the presence of various concentrations of KW-3635 (●), SQ 29,548 (○), CTA_2 (×), PTA_2 (+), BM-13505 (■), BM-13177 (□), U-46619 (△) and U-44069 (▲) in a total volume of 100 μl at 37°C for 20 min. Each data point represents the mean ± S.E.M. of three different experiments.

Table 1. K_i values for the drugs on TXA_2/PGH_2 receptors in human, guinea pig and rat platelets

	Species			
	human	guinea pig	guinea pig	rat
	[^{3}H]Ligands			
Drugs	U-46619	U-46619	SQ 29,548	SQ 29,548
KW-3635	1.2 ± 0.14	2.7 ± 0.22	3.2 ± 0.59	97 ± 19
SQ 29,548	4.0 ± 1.1	2.6 ± 0.50	1.7 ± 0.09	9.1 ± 2.4
U-44069	16 ± 2.2	11 ± 1.1	4.4 ± 0.23	110 ± 10
BM-13505	19 ± 0.3	63 ± 5.3	39 ± 1.3	24 ± 3.2
U-46619	39 ± 4.7	26 ± 2.2	7.7 ± 0.03	150 ± 20
CTA_2	290 ± 16	120 ± 16	490 ± 15	800 ± 260
BM-13177	680 ± 9	1,300 ± 140	780 ± 30	2,400 ± 420
PTA_2	710 ± 140	360 ± 42	340 ± 35	170 ± 110

K_i values for the tested compounds were calculated from the IC_{50} values using the Cheng-Prusoff's equation (45). Data indicated are means ± S.E.M. (n = 3).

platelets with the K_i values of 1.2 ± 0.14 nM and 2.7 ± 0.22 nM, respectively. KW-3635 also inhibited the [^{3}H]SQ 29,548 binding to guinea pig and rat platelets with K_i values of 3.2 ± 0.59 nM and 97 ± 19 nM, respectively. The K_i values in human platelets was also determined by the [^{3}H]SQ 29,548 binding assay. The K_i values of KW-3635, BM-13505 and U-46619 in human platelets were 1.9, 25 and 90 nM, respectively (n = 1 to 2). KW-3635 had similar activity in human and guinea pig platelets in each assay. In contrast, the K_i value of KW-3635 in rat platelets was 30- to 51-fold higher than those in human and guinea pig platelets.

Scatchard analyses were performed in the presence of various concentrations of KW-3635 (Fig. 3). Specific binding sites of [^{3}H]SQ 29,548 in guinea pig platelets were a single class with a K_d value of 2.2 nM and a B_{max} value of 180 fmol/10^8 platelets, respectively. The K_d values were 3.8 nM, 7.2 nM and 20.6 nM in the presence of 1 nM, 3 nM and 10 nM KW-3635, respectively. The K_d values of [^{3}H]SQ 29,548 in guinea pig platelets increased in the presence of KW-3635, concentration-dependently. In contrast, the B_{max} value

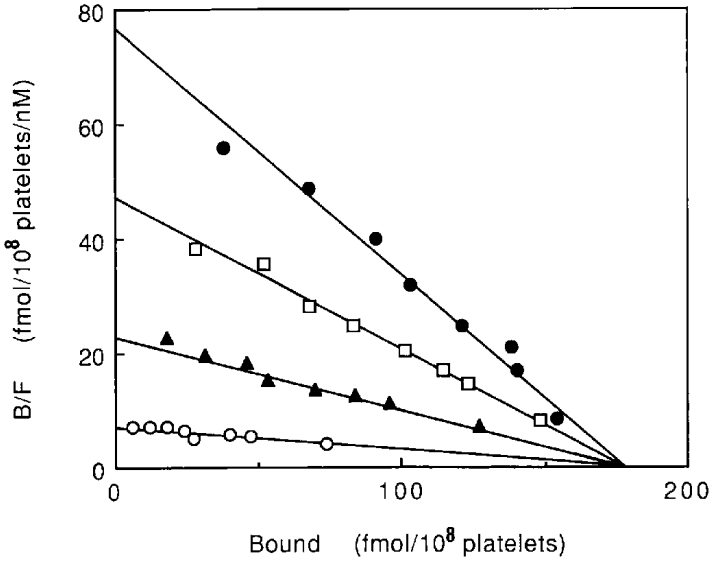

Fig. 3. Scatchard analysis of [^{3}H]SQ 29,548 binding in guinea pig platelets. Guinea pig platelets were incubated with [^{3}H]SQ 29,548 in the absence (●) or presence of KW-3635 at 1 nM (□), 3 nM (▲) or 10 nM (○). The reaction was performed in a total volume of 100 μl at 25°C for 60 min. Specific binding was defined as the difference between binding in the presence and absence of 100 μM BM-13505. Each data point represents the mean of duplicate experiments.

did not change in the presence of KW-3635. These data indicated that KW-3635 competitively antagonized the [^{3}H]SQ 29,548 binding to guinea pig platelets.

Activities of KW-3635 on various receptors

We tested the effect of KW-3635 on various receptors in the arachidonic acid pathway. KW-3635 did not antagonize the receptors of PGE_2, PGI_2, leukotriene D_4 and PAF up to 10^{-5} M. KW-3635 did not affect the various receptors of neurotransmitters (adenosine A_1 and A_2; adrenaline α_1, α_2 and β; benzodiazepine; dopamine D_1 and D_2; $GABA_A$; histamine H_1 and H_2; muscarinic acetylcholine M_1 and M_2; NMDA; serotonin-1A and 2), and did not bind to the nitrendipine or imipramine binding sites at concentrations up to 10^{-5} M. We tested the activities of KW-3635 on PGD_2 receptors using EBTr cells. PGD_2 at 10^{-6} M induced a rise in the concentration of intracellular cAMP in EBTr cells from the basal level of 2.7 ± 0.8 pmol/10^5 cells to 71.5 ± 12.2 pmol/10^5 cells. KW-3635 at 10^{-5} M did not increase the cAMP concentration by itself (3.3 ± 1.2 pmol/10^5 cells) and did not inhibit the PGD_2-induced cAMP production (53.0 ± 12.0 pmol/10^5 cells).

Activities of KW-3635 on various enzymes in the arachidonic acid cascade

KW-3635 at 10^{-4} M did not inhibit TX synthase, cyclooxygenase, PGI_2 synthase and arachidonate 12-lipoxygenase. KW-3635 slightly inhibited 5-lipoxygenase with the IC_{50} value of 71 ± 8.4 μM.

DISCUSSION

We characterized the high affinity binding sites of [^{3}H]U-46619 and [^{3}H]SQ 29,548 in rat, guinea pig and human platelets. The binding of [^{3}H]U-46619 to both human and guinea pig platelets and [^{3}H]SQ 29,548 binding to human, guinea pig and rat platelets were saturable and displaceable. The K_d value of both [^{3}H]U-46619 and [^{3}H]SQ 29,548 in human platelets was close to that in guinea pig platelets. In contrast, the K_d value of [^{3}H]SQ 29,548 in rat platelets was about 3- to 5-fold higher than that in guinea pig and human platelets. In rat platelets, the K_d value of [^{3}H]SQ 29,548 in the presence of bovine serum albumin decreased about 5-fold, approaching the value in guinea pig platelets. Therefore, we have to consider the effect of serum protein to evaluate the potency of compounds, because the affinity of drugs might be changed under this experimental condition. At present, it is uncertain if rat and guinea pig platelet TXA_2/PGH_2 receptors are the same or different by the comparison of K_d values.

KW-3635, a novel non-prostanoic dibenzoxepin derivative, potently inhibited the [^{3}H]U-46619 binding to human platelets with a K_d value of 1.2 ± 0.14 nM. It also inhibited [^{3}H]SQ 29,548 binding in guinea pig platelets in a competitive manner. The K_i value in rat platelets was 51-fold higher than that in human platelets. This ratio seems to be the highest among those of various compounds, which were given in pre-

vious reports (46).

In guinea pig platelets, linear regression analyses of the K_i value of compounds for [^{3}H]U-46619 binding correlated well with those for [^{3}H]SQ 29,548 binding ($r = 0.948$). The K_i values of compounds for [^{3}H]U-46619 binding in human and guinea pig platelets also were well-correlated ($r = 0.945$). On the other hand, poor correlation of the log of the K_i values for [^{3}H]SQ 29,548 binding was observed between rat platelets and guinea pig platelets ($r = 0.754$). The log of K_i values for [^{3}H]SQ 29,548 binding in rat platelets also did not correlate with those for [^{3}H]U-46619 binding in human platelets ($r = 0.722$). Masuda et al. (47) also reported that the IC_{50} value of SQ 29,548 in washed human platelets is lower than that in washed rat platelets. In contrast to our experiment, the K_i value of BM-13177 in rat platelets (200 nM) was reported to be lower than that of human platelets (1,000 nM) (46). This discrepancy was presumed to be derived from the use of the different ligands, SQ 29,548 or U-46619. Another possibility is that the difference was due to the different experimental conditions, because they used buffer containing 0.035% bovine serum albumin in their binding assay (46). In our results, U-46619 and U-44069 had weaker activities in rat platelets than in human and guinea pig platelets. These results suggest that affinities of the stable analogues of PGH_2, U-46619 and U-44069, to TXA_2/PGH_2 receptors in rats are different from those in humans and guinea pigs. On the other hand, guinea pig platelets may be useful for characterizing the compounds before clinical studies.

Many of the metabolites of arachidonic acid have various biological activities. TXA_2, LTD_4 and PAF have potent spasmogenic activity on the airway (5, 32), and PGI_2 is a platelet inhibitor and vasodilator (34). We tested the properties of KW-3635 on various receptors and enzymes. KW-3635 did not affect the various receptors of PGE_2, PGI_2, leukotriene D_4 and PAF. Though PGD_2 weakly acts on TXA_2/PGH_2 receptors (46), KW-3635 did not reduce PGD_2-induced accumulation of cAMP in cultured cells. These results indicated that KW-3635 did not have any agonistic and antagonistic activity on PGD_2 receptors. KW-3635 also did not possess any activities on the receptors of various neurotransmitters. Previously, ridogrel and picotamide were reported to act as a TX synthase inhibitor and a TXA_2 antagonist (8). In contrast to these drugs, KW-3635 did not affect TX synthase, cyclooxygenase, PGI_2 synthase and 12-lipoxygenase at concentrations up to 10^{-4} M. KW-3635 slightly inhibited 5-lipoxygenase, but the effect was negligible compared to the activity of the TXA_2 antagonist. These results indicate that KW-3635 is a potent and selective antagonist on TXA_2/PGH_2 receptors and recognized the species differences between rat and other species without affecting the various sites described above.

In conclusion, the present studies provide evidence for different activities of TXA_2 agonists and antagonists in rat, guinea pig and human platelets. KW-3635 is a novel TXA_2 antagonist which discriminated the species differences. A highly selective non-prostanoic antagonist for human TXA_2/PGH_2 receptors will permit a more comprehensive understanding of the ligand binding sites in the receptors.

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