Effects of KW-3635, a Novel Dibenzoepin Derivative of a Selective Thromboxane A2 Antagonist, on Human, Guinea Pig and Rat Platelets

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ABSTRACT—We examined the binding of [3H]U-46619, a thromboxane A2 agonist, to human and guinea pig platelets and the binding of [3H]SQ 29,548, a thromboxane A2 antagonist, to human, rat and guinea pig platelets. KW-3635 (sodium (E)-11-[2-(5,6-dimethyl-1-benzimidazolyl)ethylidene]-6,11-dihydrodibenzo[b,e]oxepin-2-carboxylate monohydrate) concentration-dependently inhibited the [3H]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 [3H]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 A2/prostaglandin H2 receptors in rat platelets with an inhibition constant of 97 nM. KW-3635 at 10^{-5} M did not antagonize various receptors including prostaglandin E2, prostaglandin I2 and neurotransmitters. In addition, 10^{-5} M KW-3635 did not alter the prostaglandin D2-induced cAMP accumulation in EBTr cells. KW-3635 was inactive towards thromboxane synthase, cyclooxygenase and prostaglandin I2 synthase up to 10^{-5} M. KW-3635 slightly inhibited 5-lipoxygenase with an IC_{50} value of 71 \mu M. These data indicate that KW-3635 is a potent and selective non-prostanoid thromboxane A2 antagonist, and it can recognize the species differences in thromboxane A2/prostaglandin H2 receptors.

Keywords: Thromboxane A2 antagonist, Thromboxane A2/prostaglandin H2 receptors, KW-3635, Platelets

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 TXA₂ appears to contribute to the pathogenesis of the shock state (13).

To characterize TXA₂/PGH₂ receptors, radiolabeled prostanoic TXA₂ 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 TXA₂/PGH₂ 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, TXA₂/PGH₂ 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 TXA₂/PGH₂ receptors in human, guinea pig and rat platelets. We demonstrated that KW-3635 is a novel selective non-prostanoic TXA₂ antagonist, and it has different activities on TXA₂/PGH₂ receptors in human, guinea pig and rat platelets.

MATERIALS AND METHODS

Materials

Commercial sources of materials and reagents were as follows: [³H]SQ 29,548 (1,110 GBq/mmol) and [³H]U-46619 (828.8 GBq/mmol) from Du Pont (Wilmington, DE); [¹⁴C]arachidonic acid (2.07 GBq/mmol), [³H]TXB₂ (8,100 GBq/mmol), [³H]6-keto-PGF₁α (5,550 GBq/mmol) from Amersham (Arlington Heights, IL); other radioactive compounds were commercially available.

Various receptors binding studies

The [³H]ligands and tissues used in the various receptors binding assays were as follows: [³H]cyclohexyladenosine (CHA) and guinea pig forebrain for adenosine A₁ receptors (24), [³H]S'-N-ethylcarboxamido-adenosine (NECA) and rat striatum for adenosine A₂ receptors (24), [³H]WB-4101 and rat forebrain for adrenaline α₁-receptors (24), [³H]clonidine and rat cerebral cortex for adrenaline α₂-receptors (24), [³H]dihydroalprenolol and rat cerebral cortex for adrenaline β-receptors (24), [³H]flunitrazepam and rat brain for central benzodiazepine receptors (24), [³H]OCH₁ 23390 and rat striatum for dopamine D₁-receptors (24), [³H]piperoxane and rat striatum for dopamine D₂-receptors (24), [³H]muscarin and rat brain for γ-aminobutyric acid-A (GABAₐ) receptors (26), [³H]pyrilarine and guinea pig cerebellum for histamine H₁-receptors (24), [³H]tioridazine and guinea pig cerebral cortex for histamine H₂-receptors (27), [³H]-quinuclidinylbenzilate and rat cerebral cortex or heart for muscarinic acetylcholine M₁- or M₂-receptors (28), [³H]MK-801 and rat cerebral cortex for N-methyl-D-aspartate (NMDA) receptors (29), [³H]8-hydroxy-DPAT and rat hippocampus for serotonin-1A receptors (30), [³H]ketanserin and rat frontal cortex for serotonin-2 receptors (24), [³H]Cl₁₈-platelet activating factor (PAF) and rabbit platelets for PAF receptors.
RESULTS

TXA2/PGH2 receptor binding in various species

TXA2/PGH2 receptors in human, guinea pig and rat platelets were characterized by the receptor binding assay. The binding of [3H]U-46619 to both human and guinea pig platelets and [3H]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 Kd values of [3H]U-46619 in human and guinea pig platelets were 40 ± 7.1 nM (mean ± S.E.M.) and 24 ± 1.5 nM, respectively. The Kd value of [3H]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 Kd value of [3H]SQ 29,548 in guinea pig platelets was 3.0 ± 0.22 nM was 4.7-fold smaller than that in rat platelets (14 ± 3.5 nM). The Kd value of [3H]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 TXA2/PGH2 receptors in rat platelets without albumin, because it was preferable to compare the various TXA2/PGH2 receptors under the same conditions.

Inhibition of TXA2/PGH2 receptors by TXA2 antagonists and agonists

The specific binding of [3H]U-46619 to human platelets was concentration-dependently inhibited by TXA2 agonists or antagonists (Fig. 2). The concentration of compounds required to reduce receptor specific [3H]U-46619 binding by 50% (IC50) in human platelets was determined from Fig. 2 and used to calculate the K; value. The rank order of the K; values of antagonists from high to low affinity was found to be KW-3635 > SO 29,548 > BM-13505 > CTA2 > BM-13177 > PTA2 in human platelets (Table 1). Linear regression analyses of the log of the K; value of compounds in guinea pig platelets and in human platelets showed a poor correlation (r = 0.754).

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 (Kd value) and the receptor density (Bmax value). The inhibition constant (Ki value) was obtained from the IC50 value of drug according to the Cheng-Prusoff’s equation (45).
platelets with the $K_i$ values of $1.2 \pm 0.14$ nM and $2.7 \pm 0.22$ nM, respectively. KW-3635 also inhibited the $[^3H]{\text{U-46619}}$ binding to guinea pig and rat platelets with $K_i$ values of $3.2 \pm 0.59$ nM and $97 \pm 19$ nM, respectively. The $K_i$ values in human platelets was also determined by the $[^3H]{\text{SQ 29,548}}$ binding assay. The $K_i$ values of KW-3635, BM-13505 and U-46619 in human platelets were $1.2 \pm 0.14$, $4.0 \pm 1.1$ and $19 \pm 0.3$ nM, respectively. The $K_i$ values 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 $[^3H]{\text{SQ 29,548}}$ in guinea pig platelets were a single class with a $K_d$ value of $2.2$ nM and a $B_{\text{max}}$ 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 $[^3H]{\text{SQ 29,548}}$ in guinea pig platelets increased in the presence of KW-3635, concentration-dependently. In contrast, the $B_{\text{max}}$ value

| Drugs   | $[^3H]{\text{U-46619}}$ | $[^3H]{\text{U-46619}}$ | $[^3H]{\text{SQ 29,548}}$ | $[^3H]{\text{SQ 29,548}}$ |
|---------|-------------------------|-------------------------|-----------------------------|-----------------------------|
| KW-3635 | $1.2 \pm 0.14$          | $2.7 \pm 0.22$          | $3.2 \pm 0.59$              | $97 \pm 19$                |
| SQ 29,548 | $4.0 \pm 1.1$         | $2.6 \pm 0.50$          | $1.7 \pm 0.09$              | $9.1 \pm 2.4$              |
| U-44069   | $16 \pm 2.2$            | $11 \pm 1.1$            | $4.4 \pm 0.23$              | $110 \pm 10$               |
| BM-13505  | $19 \pm 0.3$            | $63 \pm 5.3$            | $39 \pm 1.3$                | $24 \pm 3.2$               |
| U-46619   | $39 \pm 4.7$            | $26 \pm 2.2$            | $7.7 \pm 0.03$              | $150 \pm 20$               |
| CTA$_2$   | $290 \pm 16$           | $120 \pm 16$            | $490 \pm 15$                | $800 \pm 260$              |
| BM-13177  | $680 \pm 9$             | $1,300 \pm 140$         | $780 \pm 30$                | $2,400 \pm 420$            |
| PTA$_2$   | $710 \pm 140$           | $360 \pm 42$            | $340 \pm 35$                | $170 \pm 110$              |

$K_i$ 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).
did not change in the presence of KW-3635. These data indicated that KW-3635 competitively antagonized the [3H]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 PGE2, PGI2, leukotriene D4 and PAF up to 10^-5 M. KW-3635 did not affect the various receptors of neurotransmitters (adenosine A1 and A2; adrenaline α1, α2 and β; benzodiazepine; dopamine D1 and D2; GABA_A; histamine H1 and H2; muscarinic acetylcholine M1 and M2; 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 PGD2 receptors using EBTr cells. PGD2 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 PGD2-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, PGI2 synthase and arachidonate 12-lipoxygenase. KW-3635 slightly inhibited 5-lipoxygenase with the IC_{50} value of 71 ± 8.4 μM.

**Fig. 3.** Scatchard analysis of [3H]SQ 29,548 binding in guinea pig platelets. Guinea pig platelets were incubated with [3H]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.

**DISCUSSION**

We characterized the high affinity binding sites of [3H]U-46619 and [3H]SQ 29,548 in rat, guinea pig and human platelets. The binding of [3H]U-46619 to both human and guinea pig platelets and [3H]SQ 29,548 binding to human, guinea pig and rat platelets were saturable and displaceable. The K_d value of both [3H]U-46619 and [3H]SQ 29,548 in human platelets was close to that in guinea pig platelets. In contrast, the K_d value of [3H]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 [3H]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 TXA2/PGH2 receptors are the same or different by the comparison of K_d values.

KW-3635, a novel non-prostanoic dibenzoxepin derivative, potently inhibited the [3H]U-46619 binding to human platelets with a K_d value of 1.2 ± 0.14 nM. It also inhibited [3H]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-
In guinea pig platelets, linear regression analyses of the Ki value of compounds for [3H]U-46619 binding correlated well with those for [3H]SQ 29,548 binding (r = 0.948). The Ki values of compounds for [3H]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 Ki values for [3H]SQ 29,548 binding was observed between rat platelets and guinea pig platelets (r = 0.754). The log of Ki values for [3H]SQ 29,548 binding in rat platelets also did not correlate with those for [3H]U-46619 binding in human platelets (r = 0.722). Masuda et al. (47) also reported that the IC50 value of SQ 29,548 in washed human platelets is lower than that in washed rat platelets. In contrast to our experiment, the Ki 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 PGH2, U-46619 and U-44069, to TXA2/PGH2 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. TXA2, LTD4 and PAF have potent spasmodic activity on the airway (5, 32), and PGI2 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 PGE2, PGlt, leukotriene D4 and PAF. Though PGD2 weakly acts on TXA2/PGH2 receptors (46), KW-3635 did not reduce PGD2-induced accumulation of cAMP in cultured cells. These results indicated that KW-3635 did not have any agonistic and antagonistic activity on PGD2 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 TXA2 antagonist (8). In contrast to these drugs, KW-3635 did not affect TX synthase, cyclooxygenase, PGlt 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 TXA2 antagonist. These results indicate that KW-3635 is a potent and selective antagonist on TXA2/PGH2 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 TXA2 agonists and antagonists in rat, guinea pig and human platelets. KW-3635 is a novel TXA2 antagonist which discriminated the species differences. A highly selective non-prostanoid antagonist for human TXA2/PGH2 receptors will permit a more comprehensive understanding of the ligand binding sites in the receptors.

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