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Effects of CI–930, a novel phosphodiesterase Ⅲ inhibitor, on platelet aggregation and arachidonic acid metabolism[1]

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ABSTRACT In the platelet–rich plasma of rabbits, 4,5–dihydro–6–[4–(1*H*–imidazol–1–yl)phenyl]–5–methyl–3(2*H*)–pyridazinone (CI–930) inhibited platelet aggregation triggered by AA, U–46619, ADP, collagen and PAF, with the IC_{50} values of 0.91, 0.73, 2.12, 2.35 and 7.15 μmol / L, respectively. The inhibitory effect of CI–930 on AA–induced aggregation was potentiated by PGE_1, an adenylate cyclase activator, and antagonized by SQ–22536, an adenylate cyclase inhibitor. The contents of cAMP in washed rabbit platelets were increased by CI–930 5–50 μmol / L . In the concentration range of 0.5–500 μmol / L, CI–930 reduced the synthesis of TXB_2 by either washed rat or rabbit platelets or rat pleural neutrophils. At the same time, CI–930 induced a dose–dependent increase of PGE_2, $PGF_{2\alpha}$ and PGD_2 biosynthesis by rat platelets and had no significant influence on the formation of 6–keto–$PGF_{1\alpha}$ by the neutrophils. It is showed that CI–930 is an anti–platelet agent with a wide–spectrum activity and its anti–aggregating action may be exerted by dual mechanisms, both increasing cAMP contents and selectively inhibiting TXA_2 synthesis in platelets.

KEY WORDS phosphodiesterase inhibitors; CI–930; platelet aggregation; thromboxane A_2; prostaglandins; prostaglandins X; adenosine cyclic monophosphate; radioimmunoassay; thin layer chromatography

CI–930 (4,5–dihydro–6–[4–(1*H*–imidazol–1–yl) phenyl]–5–methyl–3(2*H*)–pyridazinone), a specific phosphodiesterase Ⅲ (PDE Ⅲ) inhibitor, has been shown to exert positive inotropic action in a variety of experimental preparations *in vitro* or *in vivo* and patients with severe congestive heart failure[1,2]. The several derivatives of dihydropyridazinone have been found to prevent platelet aggregation and retention[3,4]. Thromboxane A_2 (TXA_2) and prostacyclin (PGI_2), the major cyclooxygenase metabolites of arachidonic acid (AA), are reciprocal regulators of homeostasis in platelet and vascular functions. The agents inhibiting, TXA_2 synthesis, such as selective TXA_2 synthetase inhibitor, may prevent thrombosis–related cardiovascular diseases[5]. The present study was aimed at 1) investigating the effects of CI–930 on the platelet aggregation induced by a few platelet agonists, 2) observing the effect of CI–930 on AA metabolism in washed rat or rabbit platelets and rat neutrophils.

MATERIALS AND METHODS

Platelet aggregation studies Platelet–rich plasma (PRP) and platelet–poor plasma (PPP) were prepared[4] from blood of New Zealand rabbits (♂ , body weight 2.2 ± SD 0.3kg) collected by cardiopunctures at intervals of 8 d. The blood or PRP was exposed only to plastic or siliconized glassware. Each experiment was completed within 3 h after the collection. Platelet aggregation in PRP was measured photometrically[4]. For that purpose, 0.2 ml PRP (4×10^8 platelets / ml) and control liquid or compound solution (2–10 μl) were incubated for 10 min (CI–930, SQ–22536 or normal saline) or 1 min (PGE_1) followed by the observation of 3–5 min at 37℃ before the addition of ADP (2 μl , 9.5 μmol / L), collagen (6 μl, 60 μg / ml), AA(2.5–5 μl, 0.1–0.2 mmol / L), U–46619 (2–4 μl, 1–2 μmol / L) and platelet–activating factor (PAF, 1.8 μl,

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22.3 nmol / L).

Determination of cAMP contents in washed rat platelets The wash of platelets from ♂ New Zealand rabbits weighing 2.4 ± 0.2 kg, the incubation of the washed platelet suspensions with CI–930 and the determination of cAMP by competitive protein–binding assay were performed[4].

Incubation of compounds with suspensions of washed platelets or neutrophils in rats

1 Washed platelets Washed platelets were obtained[6] from the PRP of ♂ Sprague–Dawley rats weighing 285 ± 15 g and made into platelet suspensions (5×10^8 cells / ml) with 50 mmol / L Tris–HCl buffer (pH 7.5). The suspensions (1 ml) were incubated at 37℃ in the presence of CI–930 solution or Tris–HCl buffer (10 μl) with a gentle shaking. After the reaction for 25 min, the incubation was immediately terminated with formic acid (making pH of suspensions into 3.5) and placed in an ice–bath waiting for extraction within 2 h.

2 Neutrophils Neutrophils were prepared[7] from pleural fluid of ♂ Sprague–Dawley rats weighing 251 ± 16 g elicited by carraggeenin and suspended in pH 7.4 phosphate buffer (5×10^6cells / ml). The suspensions were incubated for 20 min at 37℃ in the presence of compounds or phosphate buffer (10 μl) and supplemented with calcium ionophore A–23187 (calcimycin, 2 μmol / L). After the stimulation for 5 min, the suspensions were added into formic acid (final pH 3.5) and placed in an ice–bath. The mixtures were centrifuged for 20 min (4℃, 3500 × g) followed by collecting the supernatants to extract within 2 h.

Radioimmunoassay of AA metabolites The platelet suspensions and neutrophil supernatants mentioned above were extracted twice with purified acetic ether (4 ml) respectively. The organic layers were dried under a stream of nitrogen. The residues were redissolved in radioimmunoassay phosphate buffer containing gelatin (0.1%). TXB_2 or PGE_2 in platelet suspensions and TXB_2 or 6–keto–$PGF_{1\alpha}$ in neutrophil supernatants were determined by radio–immunoassay.

Thin layer chromatography (TLC) and quantification of radioactive products of [^{14}C] AA The wash of platelets of ♂ New Zealand rabbits weighing 2.9 ± 0.5 kg and the TLC analysis of [^{14}C] AA metabolites were performed[4,6]. Briefly. the washed platelets were pre–incubated for 20 min at 37℃ with CI–930 or normal salines and then were in contact with [^{14}C] AA (3.4 μmol / L) for 5 min at 37℃. Various radioactive products of [^{14}C] AA were quantified by liquid scintillation counting after separated by TLC.

Chemicals CI–930 was synthesized by Department of Medicinal Chemistry at our college and dissolved in normal saline with further dilutions in phosphate buffer (pH 7.4). Stock solutions and dilutions of dazoxiben (Shanghai Zhaohui Pharmaceutical Factory), PGE_1 (Pharmaceutical Factory, Bethune Medical Univ.), ADP and sodium arachidonate (Sigma) were prepared[4,6]. Collagen (Sigma) was dissolved in distilled water and stored for 1 wk at 4 ℃. U–46619 (Upjohn), PAF (College of Pharmacy, Beijing Medical Univ.) and A–23187 (Sigma) were dissolved in ethanol, stored at −20 ℃ and diluted in distilled water containing 0.2% Na_2CO_3, normal saline and ethanol respectively just before use. SQ–22536 (Squibb) was freshly dissolved in normal saline. [^{14}C] AA (2.205 GBq / mmol, Amersham), TXB_2, $PGF_{2\alpha}$, PGE_2 and PGD_2 (Upjohn) were conditioned as described[6]. The kits for the radio– immunoassay of TXB_2, PGE_2, and 6–keto–$PGF_{1\alpha}$ and for competitive protein–binding assay of cAMP were purchased from PLA General Hospital and Chinese Academy of Medical Science. respectively.

RESULTS

Inhibition of CI–930 on rabbit platelet aggregation induced by AA, U–46619, ADP,

collagen and PAF The activity profile of CI–930 against aggregation in PRP is summarized in Fig 1. When pre–incubated for 10 min with rabbit PRP, CI–930 inhibited the platelet aggregation triggered by the several inducers used. CI–930 was the most potent inhibitor of platelet response to both AA and U–46619 (IC_{50} : 0.91 and 0.73 μmol / L). In contrast to its effect on AA– or U–46619–induced aggregation, CI–930 showed relatively less inhibition on ADP– or collagen– induced aggregation (IC_{50}: 2.12 and 2.35 μmol / L). Of all inhibitions of aggregation elicited by the agonists used, the effect of CI–930 on PAF–induced aggregation was the least, with the IC_{50} value of 7.15 μmol / L. In the presence of control solutions, the aggregations induced by AA, U–46619, ADP, collagen or PAF were 87.3 ± 7.1, 73.1 ± 13.4 71.6 ± 7.4, 82.6 ± 6.3 and 74.8 ± 11.0%, respectively.

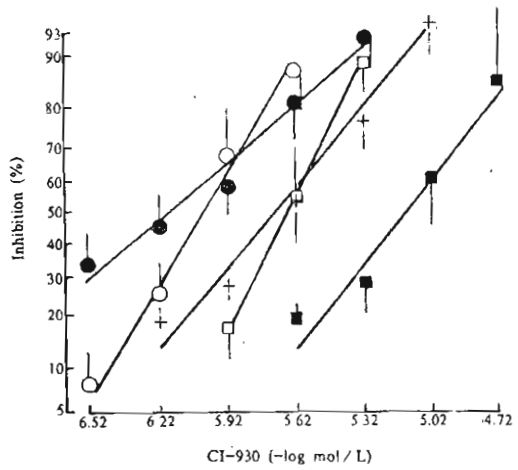

Fig 1. Inhibitory actions of CI–930 on rabbit platelet aggregation *in vitro*. Inducers : AA 0.1–0.2 mmol / L (O); U–46619 1–2 μmol / L (●); ADP 9.5 μmol / L, (×); collagen 60 μg / L (□); PAF 22.3 nmol / L (■). Aggregations were observed for 3–5 min after PRP was incubated for 10 min in presence of CI–930 solution or saline. n = 4–6, $\bar{x}$ ± SD.

Effects of the combinations of CI–930 with PGE_1 or SQ–22536 on AA–induced aggregation of rabbit platelets When PRP was exposed to PGE_1 50 nmol / L, which showed minor inhibition of aggregation, 9 min after the addition of CI–930 0.60 μmol / L under which the aggregation was reduced by 32 and the mixture continued to be incubated 1 min, a synergistic inhibition of AA–induced aggregation was seen and the inhibition reached about 90% (Fig 2 left). On the contrary, SQ–22536, an inhibitor of adenylcyclase[8], at a concentration (100 μmol / which presented a minor stimulation AA–induced aggregation, antagonized the hibition of CI–930 2.4 μmol / L on aggregation (Fig 2 right).

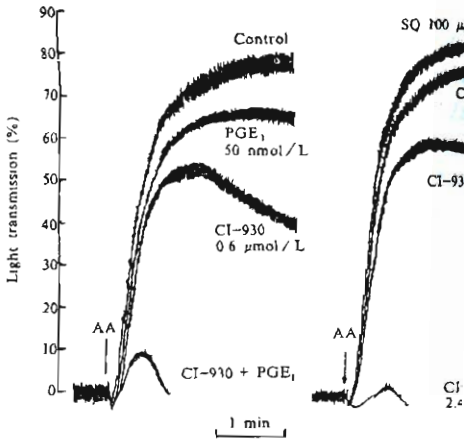

Fig 2. Combination of CI–930 with PGI SQ–22536 (SQ) on AA–induced aggregation fo after PRP was incubated for 10 min with CI–9 or for 1 min with PGE_1.

Effects of CI–930 on cAMP conte washed rabbit platelets After platelet su sions were incubated with normal saline trol) or CI–930 0.5, 5.0 or 50 μmol / L cAMP contents in the platelets were 7.1 (n = 6), 8.6 ± 0.9 (n = 5, $P > 0.05$ *vs* co 12.9 ± 2.9 (n = 5, $P < 0.01$), and 17.7 (n = 5, $P < 0.01$) pmol / L, respectively, enting a dose–dependent increase.

Effects of CI–930 on the product TXB_2 or PGE_2 by washed rat platelet washed rat platelets, CI–930 inhibited concentration–dependent manner, the p

tion of TXB_2 after exposed to the suspension for 25 min, with the IC_{50} value of 8.02 μmol / L (Tab 1). At the same time, the agent induced an overproduction of PGE_2 in the platelets (Tab 1). CI-930 50 μmol / L left the contents of PGE_2 in the suspensions to increase 2.56 times more than the control.

Tab 1 Effects of CI-930 on the production of TXB_2 or PGE_2 by washed rat platelets. $n=4-5$ determinations in duplicate. $\bar{x} \pm$ SD. $^{*}P>0.05$ $^{***}P<0.01$ *vs* controls.

CI-930 (μmol / L)	TXB_2 (ng / 10^9 cells)	PGE_2 (ng / 10^9 cells)
0	101 ± 12	8.0 ± 2.1
0.5	94 ± 17*	12 ± 6*
5.0	57 ± 7**	19 ± 3***
50.0	21.3 ± 2.5**	29 ± 5**
500.0	18 ± 5**	21 ± 8***

Effects of CI-930 on A-23187-stimulated synthesis of TXB_2 or 6-keto-$PGF_{1\alpha}$ by rat pleural neutrophils Tab 2 shows that CI-930 caused a concentration-dependent decrease of A-23187-induced conversion of endogenous AA in neutrophils into TXB_2 (IC_{50} = 4.28 μmol / L). However, the agent had no significant influence on A-23187-induced formation of 6-keto-$PGF_{1\alpha}$ by the neutrophils in 0.5-500 μmol / L. Dazoxiben, a known TAX_2 synthetase inhibitor, resulted in a concentration-dependent increase of 6-keto-$PGF_{1\alpha}$ while the agent strongly inhibited TXB_2 formation (Tab 2).

Tab 2. Effects of CI-930 or dazoxiben on the A-23187 induced synthesis of TXB_2 or 6-keto-$PGF_{1\alpha}$ by rat pleural neutrophils. $n=4-6$ determinations in duplicate. $\bar{x} \pm$ SD. $^{*}P>0.05$, $^{**}P<0.05$ or $^{***}P<0.01$ *vs* controls.

Drugs (μmol / L)	TXB_2 (ng / 10^7 cells)	6-keto-$PGF_{1\alpha}$ (ng / 10^7 cells)
Control	13.9 ± 1.3	12 ± 3
CI-930		
0.5	12.2 ± 0.8**	13.9 ± 1.4*
5.0	5.6 ± 0.6***	12.5 ± 1.2*
50.0	0.8 ± 0.4***	12.6 ± 1.2*
500.0	0.22 ± 0.17**	11.4 ± 1.7*
Dazoxiben		
0.5	7.3 ± 0.7**	24 ± 3***
5.0	3.3 ± 1.0***	30 ± 4***
50.0	3.4 ± 1.0***	28 ± 7***

Effects of CI-930 on the metabolism of exogenous [^{14}C] AA by washed rabbit platelets In washed rabbit platelet suspensions CI-930 inhibited, in a concentration-dependent manner, the conversion of [^{14}C] AA into TXB_2 (IC_{50} = 26.2 μmol / L) and induced an overproduction of PGE_2, $PGF_{2\alpha}$ and PGD_2 (Tab 3).

Tab 3. Effects of CI-930 on [^{14}C]AA metabolism by rabbit platelets. $n=4$. $\bar{x} \pm$ SD. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* controls.

CI-930 μmol/L	$10^{-3}\times$ Radioactivity (dpm / 5×10^8 cells) TXB_2	PGE_2	$PGF_{2\alpha}$	PGD_2
0	22±6*	1.7±0.7*	1.5±0.3*	1.5±0.5
0.5	23±4*	2.1±0.3*	1.9±0.3*	1.9±0.4*
5.0	13.9±1.5**	3.2±0.6**	2.7±0.4***	2.2±0.5*
50.0	10.0±1.8***	7.6±1.5*	5.2±1.2**	3.7±0.5**
500.0	3.5±0.4**	12.1±2.1***	6.9±2.2**	3.9±0.5**

DISCUSSION

The results of study characterize CI-930 as a potent inhibitor of platelet aggregation induced *in vitro* by a variety of agonists. Becuase CI-930 inhibits the aggregations induced by the stimulators including AA, ADP, and PAF, mediators of independent 3 pathways of platelet activation[9], it is evident that CI-930 is a platelet function inhibitor with a wide spectrum of activity. CI-930 inhibits the activity of PDE Ⅲ, a cAMP-PDE, in platelets and in cardiac and smooth muscles[10]. The present results demonstrate that the inhibitory action of CI-930 on AA-induced aggregation was potentiated by PGE_1, an adenylate cyclase stimulator, and antagonized by SQ-22536, an adenylate cyclase inhibitor, showing that cAMP which is known as a mediator strongly depressing platelet function is involved in the effect of CI-930 on platelet aggregation. Besides, CI-930 elevates cAMP

levles in rabbit platelets in a concentration range overlapping with that of its inhibiting aggregation triggered by various inducers. From these data, it is considered that the increase of cAMP contents induced by inhibiting PDE Ⅲ activity[10] may be one of mechanisms by which CI–930 depresses aggregation.

Of all inducer–triggered aggregations, however, the inhibitions of CI–930 on the aggregations induced by AA or U–46619, a substance reported to mimic the actions of TXA_2 / PGH_2[11], is most potent. TXA_2 which AA is converted into by cyclooxygenase and then TXA_2 synthetase has been considered one of mediators promoting platelet aggregation and vasoconstriction. The present findings revealled that CI–930 decreased the synthesis of TXB_2, a more stable metabolite of TXA_2, by either washed platelets or pleural neutrophils in rats, indicating that CI–930 may affect the activity of cyclooxygenase or TXA_2 synthetase. Becuase CI–930 induced a concentration–dependent increase of PGE_2, $PGF_{2\alpha}$ and PGD_2 synthesis by platelets while it inhibited TXB_2 production, it is suggested that CI–930 may selectively inhibit the activity of TXA_2 synthetase in platelets to reorient AA metabolism to other prostanoids, such as PGE_2. Unlike the observations that TXA_2 synthetase inhibitors promoted the formation of 6–keto–$PGF_{1\alpha}$, a more stable metabolite of PGI_2, by whole blood[12], CI–930 did not significantly promote 6–keto–$PGF_{1\alpha}$ synthesis by neutrophils although it did induce a dose–related decreases of TXB_2 synthesis, showing that CI–930 did not reorient AA metabolsim to 6–keto–$PGE_{1\alpha}$ after it inhibited TXA_2 synthesis. Since cAMP prevents the increase of 6–keto–$PGF_{1\alpha}$ synthesis[13], it is supposed that the increase of cAMP contents induced by CI–930 by inhibiting PDE Ⅲ activity may neutralize the reorientation of AA metabolism to PGI_2 caused by inhibiting synthetase. The present finding is accordant with the result obstained by Pattison *et al* with amrinone, another cAMP–PDE inhibitor with positive inotropic effect[14]. However, our results do not rule out the possibility that high concentration of CI–930 may inhibit the cyclooxygenase activity, for CI–930 500 μmol / L did not only inhibit the production of TXB_2 but also present a less increase of PGE_2, a cyclooxygenase product, in rat platelets, compared with CI–930 50 μmol / L.

In accordance with the hypothesis that cAMP may interfere in the TXA_2 formation by affecting the activity of TXA_2 synthetase[15], it is important to clarify that CI–930 inhibits TXA_2 synthetase by acting on TXA_2 synthetase whether directly or indirectly by raising the cAMP levels in cells.

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CI-930,一种新的磷酸二酯酶抑制剂,对血小板聚集和花生四烯酸代谢的影响

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提要 4,5-二氢-[6-4(1*H*-咪唑-1-)苯基]-5-甲基-3(2*H*)-哒嗪酮(CI-930)抑制 AA,U-46619, ADP, 胶原或 PAF 诱导的兔血小板聚集, IC_{50} 分别为 0.91, 0.73, 2.35 和 7.15μmol/l. 抑制 AA 诱导聚集的作用可被 PGE_1 增强, 而被 SQ-22536 减弱, 并可明显升高兔血小板 cAMP 水平. 5-500μmol/L 的 CI-930 对大鼠或兔血小板或大鼠中性白细胞 TXB_2 的生成呈剂量依赖性抑制. 与此同时, CI-930 使血小板 PGE_2, $PGF_{2\alpha}$ 和 PGD_2 的合成增加, 而对白细胞 6-keto-$PGF_{1\alpha}$ 的产生无明显影响. 结果提示, CI-930 是一种广谱的血小板功能抑制剂, 并可能具有升高 cAMP 水平和选择性抑制 TXA_2 生成的双重作用机理.

关键词 磷酸二酯酶抑制剂; CI-930; 血小板聚集; 血栓素 A_2; 前列腺素 X; 前列腺素类; 放射免疫测定; 薄层色谱法; 腺苷环一磷酸