Bronchial Anti-spasmogenic Effects and Selectivity of T-440, Phosphodiesterase Type 4 Inhibitor, in the Dog

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We investigated the selectivity of T-440 for the inhibition of phosphodiesterases (PDEs) *in vitro* and for bronchial anti-spasmogenic effects *in vivo*. Using a fast protein liquid chromatography system, five PDE isozymes, PDE 1, PDE 2, PDE 3, PDE 4 and PDE 5 were prepared from guinea pig and dog tissues. T-440 selectively inhibited PDE 4 with an IC_{50} of 0.071 μ M and 0.13 μ M for guinea pig lung and dog trachea, respectively. The IC_{50} values for all other PDE isozymes were over 20 μ M. In contrast, theophylline nonselectively inhibited all the tested PDE isozymes, and the inhibition did not exceed 50%, even at 100 μ M. T-440 inhibited the histamine-induced bronchoconstriction of anesthetized dogs in a dose-dependent manner with an ED_{50} of 0.029 mg/kg, indicating that T-440 is 600 times more potent than theophylline. Both T-440 and theophylline increased LV dp/dt/P (LVP; left ventricular pressure) in anesthetized dogs with ED_{50} values of 3.6 mg/kg and 4.4 mg/kg, respectively. This potency was 1/125 times the bronchial anti-spasmogenic effects for T-440 and 4.2 times that of theophylline. Rolipram, a PDE 4 inhibitor, also showed selective bronchial anti-spasmogenic effects in anesthetized dogs. These results suggest that T-440, which specifically inhibits PDE 4 activity, has potent and selective bronchial anti-spasmogenic effects.

Key words phosphodiesterase inhibitor; bronchoconstriction; cardiotonic effect; theophylline

Asthma is a chronic disease characterized by airway obstruction and inflammation.¹⁾ cAMP and cGMP play an important role in the regulation of airway smooth muscle tone and the activation of inflammatory cells.²⁾ Cyclic nucleotide phosphodiesterases (PDEs) regulate the intracellular concentration of these nucleotides by hydrolyzing them. There are at least seven mammalian PDE isozyme families.^{3,4)} PDE 4 exists in airway smooth muscle and regulates the airway smooth muscle tone.⁵⁾ Recent studies showed that PDE 4 is also located in many types of inflammatory cells and regulates the activities of the cells.²⁾ Inhibition of PDE 4 *in vitro* resulted in increased levels of cAMP which leads to the functional inhibition of eosinophils,^{6,7)} monocytes,⁸⁾ and lymphocytes.⁹⁾ PDE 3 is present in airway smooth muscle,¹⁰⁾ cardiac and vascular tissues and controls cardiac contractility and vascular tone.¹¹⁾ Thus, the inhibition of PDE 3 causes myocardial inotropic and chronotropic action as well as bronchial anti-spasmogenic action. Theophylline, a non-selective PDE inhibitor, has been used for the treatment of asthma for many years.¹²⁾ However, it has a narrow therapeutic index and easily causes side effects, especially on the cardiovascular system.¹³⁾ Therefore, for the purpose of treating asthma, a selective PDE 4 inhibitor seems to be useful due to its action on airway tone with fewer cardiovascular side effects. Indeed, the potential use for several selective PDE 4 inhibitors as therapeutic agents for the treatment of asthma and atopic dermatitis is currently being explored.^{14,15)}

We have developed a selective PDE 4 inhibitor, T-440 (1-[1-(2-methoxyethyl)pyrid-2-one-4-yl]-2,3-bis(hydroxymethyl)-6,7-diethoxynaphthalene, which is a derivative of naphthalene lignan (Fig. 1). We have demonstrated that T-440 shows strong inhibitory effects on antigen- and chemical mediator-induced bronchoconstriction in guinea pigs *in vivo*.^{16,17)} In the present study, we further in-

vestigated the inhibitory effects of T-440 on PDE isozymes *in vitro* and bronchial anti-spasmogenic effects *in vivo*.

MATERIALS AND METHODS

Materials The sources of materials used in this work were as follows: T-440, rolipram and CI-930 were synthesized in the Lead Optimization Research Laboratory Tanabe Seiyaku (Osaka, Japan), theophylline and histamine·2HCl came from Nacalai Tesque (Kyoto, Japan), sodium pentobarbital from Tokyo Kasei (Tokyo, Japan), leupeptin from Peptide Research Foundation (Osaka, Japan), phenylmethanesulphonyl fluoride (PMSF) from Wako Pure Chemicals (Osaka, Japan), cGMP from Sigma (St. Louis, MO, U.S.A.), cAMP from Boehringer-Mannheim Biochemicals (Mannheim, Germany), [2,8-³H]-cAMP and [8-³H]cGMP from Amersham International (Bucks, UK), Mono-Q HR 5/5 column and Q-sepharose column from Pharmacia (Uppsala, Sweden). Other reagents used were of the best quality commercially available.

Preparation of PDE Isozymes The method of Lavan *et al.*¹⁸⁾ was modified to isolate PDE isozymes. Briefly, male guinea pigs were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and hearts and lungs were per-

T - 440

Fig. 1. Chemical Structure of T-440

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fused with heparinized saline (0.9% NaCl). Then the cardiac ventricles, lungs and adrenal glands were excised and rinsed in ice-cold saline. Canine tracheae and ventricle tissue were extracted from euthanized dogs. Tissue samples were stored at -80°C until use. These samples were then minced and homogenized in three vol. of ice-cold homogenization buffer (50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 5 mM benzamidine, $2\text{ }\mu\text{M}$ leupeptin, 0.1 mM EGTA, 5 mM 2-mercaptoethanol, 0.2 mM PMSF) using a Polytron PT-20. The homogenate was then centrifuged for 30 min at $25000\times g$ and the resulting supernatant was filtered ($0.45\text{ }\mu\text{m}$) and applied on a Mono-Q HR 5/5 column which was pre-equilibrated with an elution buffer (50 mM Tris-HCl (pH 7.5), 2 mM benzamidine, 0.1 mM EGTA, 5 mM 2-mercaptoethanol, 0.2 mM PMSF). A Q-sepharose column was used instead of a Mono-Q column for the dog cardiac ventricle. The column was attached to a fast protein liquid chromatography (FPLC) system. After the column was washed with an elution buffer, bound proteins were eluted from the column by using a continuous 0–1 M NaCl gradient. Each fraction was collected and assayed for PDE activity. In guinea pig tissues, fractions containing high levels of PDE 1 or PDE 3 activity from cardiac ventricle, PDE 4 or PDE 5 activity from lung, and PDE 2 activity from adrenal glands were pooled. In the case of the dog tissue, the PDE 3 activity from the cardiac ventricle and the PDE 4 activity from the tracheae were pooled. The combined PDE fractions were diluted to 70% with ethylene glycol and stored at -20°C .

Determination of PDE Activity PDE activity was determined by a modification of the method of Thompson *et al.*¹⁹⁾ The reaction mixture contained 50 mM Tris-HCl, pH 8.0, 5 mM MgCl_2 , and 4 mM 2-mercaptoethanol. In evaluating the inhibitory effects of the test compounds on PDE 1, PDE 2, PDE 3, PDE 4 and PDE 5, the protein concentration in the assay was adjusted to ensure that the hydrolysis of the substrate ($[\text{}^3\text{H}]\text{cAMP}$ or $[\text{}^3\text{H}]\text{cGMP}$) did not exceed 15% of the available substrate in the absence of an inhibitor. The concentration of the substrate was $1.0\text{ }\mu\text{M}$ for these studies. All compounds examined were dissolved in dimethyl sulfoxide (DMSO). Following the addition of the substrate, the contents were incubated for 30 min at 30°C . Inhibition assays were performed in triplicate at three to four different inhibitor concentrations, and IC_{50} values for the compounds were determined from concentration–response curves.

Effects on Histamine-induced Bronchoconstriction

Mongrel dogs of either sex, weighing 7.3–18.0 kg, were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). These dogs were cannulated in the trachea, artificially ventilated with 15–20 ml/kg per stroke of air at a rate of 20 strokes/min (Takashima, model 100 or Acoma, AR-300), and maintained by i.v. infusion of sodium pentobarbital (4–5 mg/kg/h) during the experiments. Changes in the pulmonary mechanics were measured by the method of Konzett and Rössler²⁰⁾ using a differential pressure transducer connected to the tracheal cannula, and the ventilation overflow volume (VOFV) was calculated by an integrator (Nihon Kohden, EI-601G). Bronchoconstriction was induced by the intravenous injection of histamine $\cdot 2\text{HCl}$ (2–3 $\mu\text{g/kg}$). Each test compound was

dissolved in 10% NIKKOL HCO-60 (Nikko Chemicals) and suspended in saline, then intravenously administered 2 min before the histamine injection. The effects of the test compounds were shown as the % inhibition of the histamine-induced increase in VOFV.

Effects on Cardiovascular Functions Mongrel dogs of either sex, weighing 11.6–15.0 kg, were anesthetized with sodium pentobarbital (30 mg/kg, i.v.) which was maintained by an infusion of sodium pentobarbital (4–5 mg/kg/h) and artificially ventilated *via* a tracheal cannula, as noted above. Millar's catheter was introduced into the left ventricle *via* a carotid artery. Left ventricular pressure (LVP), LV dp/dt and LV dp/dt/P were continuously recorded. Mean blood pressure (MBP) and heart rate (HR) were measured in the usual way. Each test compound was dissolved in 10% NIKKOL HCO-60 and suspended in saline, then administered *via* the femoral vein.

RESULTS

Preparation of PDE by FPLC System PDE activities from the supernatants of guinea pig cardiac ventricles, lungs and adrenal gland homogenates were separated by a Mono-Q column. Five main peaks were obtained from guinea pig cardiac ventricles. According to Beavo *et al.*,³⁾ the peaks were characterized as follows. Peak 1 was a Ca^{2+} /calmodulin stimulated PDE (PDE 1), peak 2 was a cGMP stimulated PDE (PDE 2), peak 3 was a cAMP specific PDE (PDE 4), and peaks 4 and 5 were cGMP inhibited PDE (PDE 3). From guinea pig cardiac ventricles, only the PDE 1 and PDE 3 containing fractions were used for the subsequent study. Similarly, two major peaks from guinea pig lungs and one major peak from guinea pig adrenal glands were characterized as cGMP specific PDE (PDE 5), PDE 4 and PDE 2, respectively. PDE activities from the supernatants of dog cardiac ventricle and trachea homogenates were separated by Q-sepharose and a Mono-Q column, respectively. PDE 3 from dog cardiac ventricles and PDE 4 from trachea were used for the subsequent study.

Effects of T-440 on PDE Activities The potencies of T-440, rolipram, CI-930 and theophylline required for the inhibition of PDE 1, PDE 2, PDE 3, PDE 4 and PDE 5 isolated from the guinea pig are summarized in Table 1. Rolipram and CI-930 showed selective inhibitory effects on PDE 4 and PDE 3, respectively. The % inhibition of PDE 1, 2, 3, 4 and 5 by theophylline at $100\text{ }\mu\text{M}$ was 35, 42, 25, 22 and 2%, respectively. Thus, theophylline is a weak (IC_{50} ; $>100\text{ }\mu\text{M}$) and relatively nonselective inhibitor of PDE. T-440 inhibited PDE 4 with an IC_{50} of $0.071\text{ }\mu\text{M}$, and was more potent than rolipram. T-440 was at least 300-fold selective for PDE 4 relative to the inhibition of PDE isozymes PDE 1, PDE 2, PDE 3 and PDE 5. The inhibitory effects of T-440 and the other compounds on PDE 3 and PDE 4 were almost the same between the guinea pig and the dog (Table 1).

Effect on Histamine-induced Bronchoconstriction The effects of T-440, rolipram and theophylline on the histamine-induced bronchoconstriction in anesthetized dogs are shown in Fig. 2. T-440 inhibited the bronchoconstriction in a dose-dependent manner, and the ED_{50} was

Table 1. Inhibitory Effects of T-440, Rolipram, CI-930 and Theophylline on Guinea Pig and Dog PDE Isozymes

PDE	Source	IC ₅₀ μ M (n)			
		T-440	Rolipram	CL-930	Theophylline
Guinea pig					
1	Heart	> 100 (2)	> 100 (1)	> 100 (1)	> 100 (1)
2	Adrenal gland	23 (1)	> 100 (1)	> 100 (1)	> 100 (1)
3	Heart	49 $\pm$ 7 (5)	> 100 (1)	0.88 (2)	> 100 (2)
4	Lung	0.071 $\pm$ 0.002 (9)	0.71 $\pm$ 0.06 (8)	> 100 (2)	> 100 (2)
5	Lung	67 (1)	> 100 (1)	> 100 (1)	> 100 (1)
Dog					
3	Heart	28 (1)	> 100 (1)	0.44 (1)	> 100 (1)
4	Trachea	0.13 (1)	2.1 (1)	> 100 (1)	> 100 (1)

Mean $\pm$ S.E. of IC₅₀ generated from individual enzyme preparations. Each *n* value represents the number of enzyme preparation in which each compound was tested. The mean percent inhibition of each concentration of the compound was determined from triplicate determinations.

Fig. 2. Effects of T-440, Rolipram and Theophylline on Histamine-induced Bronchoconstriction in Anesthetized Dogs

T-440 (●), rolipram (□) and theophylline (△) were intravenously administered 2 min before the histamine injection. Each point represents the mean $\pm$ S.E. of 4 to 5 animals.

0.029 mg/kg (*n*=4). The ED₅₀ of rolipram and theophylline for inhibition of the bronchoconstriction was 0.0077 mg/kg (*n*=4) and 18 mg/kg (*n*=5), respectively, so the effect of T-440 was 4 times less potent than that of rolipram and 600 times more potent than theophylline.

Effects on Cardiovascular Functions The effects of T-440, rolipram and theophylline on the cardiovascular system are shown as the changes in MBP, HR and LV dp/dt/P, an index of cardiac contractility which is increased by cardiac stimulation (Fig. 3). T-440 increased the LV dp/dt/P from 0.3 mg/kg, increased the HR from 0.1 mg/kg and decreased the MBP from 0.1 mg/kg in a dose-dependent manner. These cardiovascular effects of T-440 were exerted at similar doses of theophylline. Rolipram showed little effect on the MBP, but moderately increased the LV dp/dt/P and the HR, from 0.003 to 0.3 mg/kg. The relationship between the cardiovascular effects and bronchial anti-spasmodic effects of T-440, rolipram and theophylline is shown in Fig. 4. T-440 was more selective for bronchial effects than rolipram or theophylline. The bronchial anti-spasmodic effects of T-440 were elicited at doses without the cardiovascular effect, while theophylline stimulated the cardiovascular system at any dose at which it induces bronchial anti-spasmodic effects. MBP₃₀ (the dose that decreased MBP

Fig. 3. Effects of T-440, Rolipram and Theophylline on Mean Blood Pressure, Heart Rate and LV dp/dt/P in Anesthetized Dogs

T-440 (●), rolipram (□) and theophylline (△) were intravenously administered. Each point represents the mean $\pm$ S.E. of 4 to 5 animals.

by 30 mmHg), HR₃₀ (the dose that increased HR by 30 beats/min) and LV dp/dt/P₅₀ (the dose that increased LV dp/dt/P by 50%) are listed in Table 2. The ratios of ED₅₀ to MBP₃₀, HR₃₀ and LV dp/dt/P₅₀ for T-440 were 225, 90 and 125, respectively, while those for theophylline were 0.38, 0.14 and 0.24, respectively. Consequently, in terms of the dose ratio of bronchial and cardiac effects, T-440 is about 600 times more selective for bronchial anti-spasmodic effects than theophylline.

DISCUSSION

In the present study, five distinct PDE isozymes were used to evaluate the selectivity of T-440 among PDEs. To shorten the time and increase the reproducibility, we

Fig. 4. Relationship between Cardiac Effects and Bronchial Anti-spasmodic Effects of T-440, Rolipram and Theophylline in Anesthetized Dogs

T-440 (●), rolipram (□) and theophylline (Δ) were intravenously administered. Each point represents the mean $\pm$ S.E. of 4 to 5 animals.

Table 2. Effects on Mean Blood Pressure, Heart Rate, LV dp/dt/P and Histamine-induced Bronchoconstriction in Dogs

		T-440	Rolipram	Theophylline
Bronchoconstriction	ED ₅₀ (mg/kg)	0.029	0.0077	18
Blood pressure	MBP ₃₀ (mg/kg)	6.5	>0.30	7.1
Heart rate	HR ₃₀ (mg/kg)	2.6	0.020	2.5
Inotropic action	LV dp/dt P ₅₀ (mg/kg)	3.6	>0.30	4.4
Ratio	MBP ₃₀ /ED ₅₀	225	>39	0.38
	HR ₃₀ /ED ₅₀	90	2.6	0.14
	LV dp/dt/P ₅₀ /ED ₅₀	125	>39	0.24

MBP₃₀, the dose that decreased MBP by 30 mmHg; HR₃₀, the dose that increased HR by 30 beats/min; LV dp/dt/P₅₀, the dose that increased LV dp/dt/P by 50%.

adopted an FPLC system to separate the PDE isozymes. There have been few studies on the preparation of guinea pig or dog PDEs using the FPLC system so far. However, with respect to guinea pig cardiac ventricles and lungs, and dog cardiac ventricle and trachea, the elution profiles were similar to those of DEAE ion exchange chromatography previously described.^{10,21-23)}

We previously reported that T-440 inhibited PDE 4 from guinea pig lungs.¹⁶⁾ In the present study, we found that T-440 inhibited PDE 2 secondary to PDE 4, but the effect of T-440 on PDE 4 was 300 times more potent than that on PDE 2 in guinea pigs. The relative potencies of

T-440 for the inhibition of guinea pig PDE isozymes were PDE 4 > PDE 2 > PDE 3 > PDE 5 > PDE 1. In contrast, theophylline inhibited all PDE isozymes with an IC₅₀ of >100 μ M, and so T-440 was at least 2 times more potent in PDE 3 inhibition and at least 800 times more potent in PDE 4 inhibition than theophylline. The PDE 4 vs. PDE 3 selectivity ratio of T-440, rolipram and CI-930 was 690, >140 and <1/110, respectively, in the guinea pig (220, >50 and <1/230 in the dog), indicating that T-440 is a highly PDE 4 selective inhibitor.

In this study, we found that T-440 inhibited histamine-induced bronchoconstriction in dogs in a dose-dependent manner. T-440 has been reported to inhibit the bronchoconstriction induced by all of the following: histamine, leukotriene D₄, U-46619, acetylcholine, neurokinin A and endothelin-1 in guinea pigs *in vivo*,¹⁷⁾ suggesting that T-440 showed bronchial anti-spasmodic effects as a PDE inhibitor instead of as a histamine antagonist. It is to be noted that T-440 was more potent than rolipram in the inhibition of PDE 4, whereas it was less potent than rolipram in the inhibition of histamine-induced bronchoconstriction. One possible explanation for this reverse order is that T-440 and rolipram may be different in terms of membrane permeability and distribution in tissues or cells. Recently, molecular cloning has revealed that PDE 4 consists of 4 different subtypes (PDE 4A to PDE 4D) which are the products of 4 independent genes.²⁴⁾ It is still not clear which subtype is most important in the function of bronchi; however, it is possible that the PDE 4 subtype selectivity of T-440 and rolipram may be different. The selectivity of T-440 for the PDE 4 subtype is thus of interest in order to explain the selective bronchial effects more precisely.

The selectivity of PDE inhibitors for bronchial anti-spasmodic effects compared to cardiac effects have usually been tested by *in vitro* experiments using tracheal smooth muscle strips, papillary muscle, a right atrium preparation, *etc.*²⁵⁻²⁷⁾ It seems likely that these experiments are useful to compare the selectivity of PDE inhibitors, but there is a limitation in predicting the selectivity *in vivo*. Heaslip *et al.*²⁸⁾ compared the relative bronchial and cardiovascular effects of PDE inhibitors following i.v. administration in anesthetized β -blocked dogs, and showed that respiratory muscle tension is regulated by both PDE 3 and PDE 4. We also used anesthetized dogs to compare the effects of T-440 on histamine-induced bronchoconstriction and the cardiovascular system. At 0.1 mg/kg, T-440 inhibited histamine-induced bronchoconstriction about 65% without any significant change in the MBP, HR and LV dp/dt/P. This result demonstrates that T-440 produces sufficient bronchial anti-spasmodic effects with minimal effects on the cardiovascular system. Slight inotropic and chronotropic effects were induced by T-440 at a higher dose range. This may reflect the inhibition of PDE 3, because PDE 3, as well as PDE 4, is inhibited by T-440 at a high concentration *in vitro*.

Adverse effects on the cardiovascular and central nervous systems often limit the clinical use of theophylline, a nonselective PDE inhibitor, for the treatment of asthma. In this study, theophylline showed obvious cardiovascular effects from doses that did not cause significant bronchial

anti-spasmogenic effects. In terms of the effective dose ratio of bronchospasmolytic and cardiostimulant effects, T-440 is about 600 times more selective than theophylline in bronchial anti-spasmogenic effects (Table 2). Since the inhibition of PDE 3 *in vivo* results in cardiotonic activities,²⁹⁾ these suggest that the selective bronchospasmolytic action of T-440 may be accounted for by the selectivity of PDE 4 inhibition. Although rolipram also showed bronchial anti-spasmogenic effects in preference to cardiovascular effects, its selectivity was inferior to that of T-440. This difference may be related to the *in vitro* PDE 4 vs. PDE 3 selectivity. To further clarify the relation between the *in vivo* effects in broncho/cardiovascular activities and *in vitro* effects in PDE 4/PDE 3 activities, examination of the *in vivo* effect of CI-930 is an effective way, and such a study is currently underway.

PDE 4 is prevalent in many types of inflammatory cells, including basophils, mast cells, neutrophils, eosinophils, monocytes, and lymphocytes, and it regulates the activities of the cells.²⁾ There is also considerable evidence that elevated cAMP prevents the activity of inflammatory cells.²⁾ Therefore, for the purpose of treating asthma, a selective PDE 4 inhibitor would seem to be useful due to its action on the inflammatory process as well as the airway tone. It has been shown that T-440 suppresses the production of interleukin-5 in mononuclear cells of asthmatic patients.³⁰⁾ The investigation of T-440 for its effects on inflammatory cell function remains to be determined.

In conclusion, T-440 inhibited histamine-induced bronchoconstriction in anesthetized dogs without producing significant cardiovascular effects. This selectivity for bronchial anti-spasmogenic effects of T-440 was much higher than that of theophylline and may reflect the selective inhibition of PDE 4 in comparison with PDE 3.

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