Original Article

Saiboku-to, a Kampo herbal medicine, inhibits LTC$_4$ release from eosinophils

Koichi Hirai,[1] Yasuko Asada,[2] Toshiaki Takaishi,[2] Toshiharu Nakajima,[1] Yutaka Morita,[2] Jong-Chol Cyong[1] and Koji Ito[2]

[1]Department of Bioregulatory Function and [2]Department of Medicine and Physical Therapy, University of Tokyo School of Medicine, Tokyo, Japan

ABSTRACT

Saiboku-to (TJ-96), a traditional Kampo herbal formation, has been used in the treatment of bronchial asthma in Japan as an anti-allergy herbal medicine. We investigated the effect of TJ-96 on leukotriene (LT)C$_4$ release from eosinophils and basophils isolated from healthy volunteers. Pre-incubation of eosinophils with TJ-96 inhibited ionophore- or formyl-methionyl-leucyl-phenylalanine (FMLP)-induced LTC$_4$ generation by eosinophils in a dose-dependent fashion. The TJ-96 was more potent in the release by ionophore (IC$_{50}$ = 60 mg/mL) than the release induced by FMLP (IC$_{50}$ = 300 mg/mL). Maximal inhibition was observed when eosinophils were pretreated with TJ-96 for 5 min. Although TJ-96 at high concentrations inhibited IgE-mediated histamine release from human basophils, inhibition of IgE-mediated LTC$_4$ release was not statistically significant. The potent inhibitory activity was found in the extract of Glycyrrhiza root, one of the herbal components of TJ-96, but the inhibitory effects were not due to either glycyrrhizin or liquiritin, the main elements of the Glycyrrhiza root. These results raise the possibility that the clinical efficacy of TJ-96 is derived, at least in part, from its potent inhibitory effect on LTC$_4$ release from eosinophils.

Key words: basophil, bronchial asthma, eosinophil, Glycyrrhiza root, LTC$_4$, *Saiboku-to*.

INTRODUCTION

Accumulation of eosinophils and basophils at the sites of inflammation is a characteristic feature of allergic diseases. Both eosinophils and basophils are recognized as active participants in mediating allergic inflammation by virtue of their capacity to generate and release a wide array of preformed as well as newly generated chemical mediators.[1] Leukotriene (LT)C$_4$, one of the newly generated mediators, is a product of the 5-lipoxygenase pathway of the arachidonate metabolism. The pivotal roles of LTC$_4$ in the pathogenesis of allergic inflammation have been well established; LTC$_4$ causes rapid and prolonged constriction of smooth muscle as well as alterations in the vascular tone and permeability.

Saiboku-to (TJ-96, *Formula magnoliae et Bupleuri* in Latin), a traditional oriental herbal medicine, has been used with some success in the treatment of patients with bronchial asthma in Japan. Daily doses of TJ-96 consist of 10 herbs (Table 1), and the clinical efficacy of TJ-96 in patients with steroid-dependent bronchial asthma was proven by a multicenter randomized and controlled clinical trial.[2] Although several *in vitro* as well as *in vivo* studies have been performed to investigate the mechanisms of TJ-96's efficacy on asthma,[3–7] the direct modulatory effect of TJ-96 on the secretion of 5-lipoxygenase products in human eosinophils and basophils has remained obscure. Given the potential importance of pro-inflammatory mediators in the pathogenesis of allergic inflammation together with the therapeutic efficacy of TJ-96 in allergic disorders, we decided to conduct an analysis of the modulatory effect of TJ-96 on the secretion of LTC$_4$ from human eosinophils and basophils. Our results show that TJ-96 exerts a potent inhibitory effect on LTC$_4$ release from eosinophils.

Table 1. Components of TJ-96

English name	Japanese name	Latin name	Weight (g)
Bupleurum root	saiko	*Bupleuri Radix*	7.0
Pinellia tuber	hange	*Pinelliae Tuber*	5.0
Hoelen	bukuryou	*Hoelen*	5.0
Scutellaria root	ogon	*Scutellariae Radix*	3.0
Magnolia bark	koboku	*Magnoliae Cortex*	3.0
Jujube fruit	taiso	*Zyzyphi Fructus*	3.0
Ginseng root	ninjin	*Ginseng Radix*	3.0
Glycyrrhiza root	kanzo	*Glycyrrhizae Radix*	2.0
Perilla herb	soyo	*Perillae Herba*	2.0
Ginger rhizome	shokyo	*Zingiberis Rhizoma*	1.0

MATERIALS AND METHODS

Reagents

Goat anti-human IgE antibody (concentration: 36.04 mg/mL), Percoll, ionophore A23187 and formyl-methionyl-leucyl-phenylalanine (FMLP) were purchased as described previously.[8] The TJ-96 and dried decoctem of medical herbs were provided by Tsumura Co. (Tokyo, Japan). Glycyrrhizin and liquiritin were purchased from Wako (Osaka, Japan).

Buffer

Pipes A buffer contained 25 mmol Pipes, 119 mmol NaCl, 5 mmol KCl, and 0.03% human serum albumin (pH 7.4). For mediator release reactions, Pipes A containing 2 mmol Ca^{2+} and 0.5 mmol Mg^{2+} (Pipes ACM) was used.

Cell preparation

Venous blood was obtained from healthy volunteers with no history of atopic diseases such as bronchial asthma, atopic dermatitis or chronic urticaria. All volunteers gave informed consent. Because we intended to determine the pharmacological efficacy of TJ-96, we did not select donors according to Kampo diagnosis.

Eosinophils were isolated by Percoll gradient centrifugation as previously described.[9] In some experiments, eosinophils were further purified by negative selection using anti-CD16 bound micromagnetic beads (Miltenyi BioTech, Bergisch-Gladbach, Germany) and a magnetic-activated cell sorter column (Miltenyi BioTech) as described previously.[9] Eosinophils were counted in a hemocytometer (Tatai; Towa Kagaku, Tokyo, Japan) after staining with Randolph's stain. The purity of eosinophils after Percoll gradient centrifugation was approximately 70%, and the viability was consistently greater than 95%.

For basophil mediator release, leukocytes were prepared by dextran sedimentation as previously described.[10]

Mediator release

Histamine and LTC_4 release were performed as previously described.[11] In brief, leukocytes were incubated with a test reagent or control buffer for indicated time periods at 37°C. Mediator release reactions were evoked by the addition of secretagogues and incubation was carried out for an additional 45 min at 37°C. The tubes were then centrifuged, and the supernatants were stored until assay.

Histamine was measured using an automated fluorometric technique. The released LTC_4 was quantitated using an ELISA kit ($LTC_4/D_4/E_4$ enzyme immunoassay system, Amersham, Buckinghamshire, UK). Each experiment was performed at least in duplicate.

RESULTS

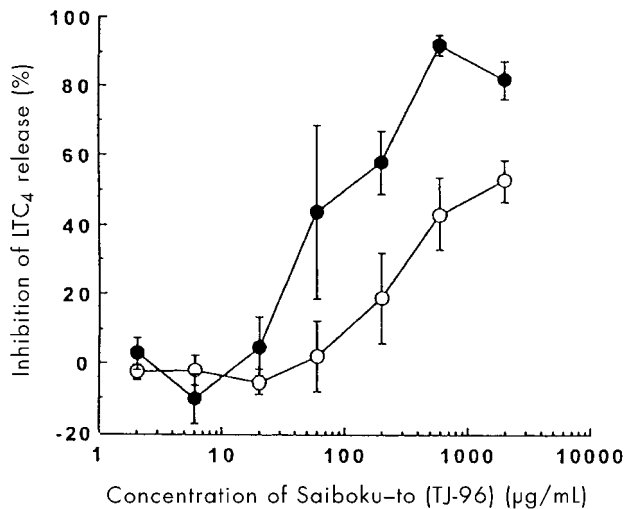### Effect of TJ-96 on LTC_4 release from eosinophils

The effect of TJ-96 on the release of LTC_4 from eosinophils was determined by incubating eosinophils with increasing concentrations of TJ-96 for 15 min before stimulating with ionophore A23187 or FMLP plus cytochalasin B (CB), and then assaying released LTC_4 in the supernatants by enzyme immunoassay. As shown in Fig. 1, TJ-96 inhibited LTC_4 release initiated by both ionophore and FMLP in a concentration-dependent fashion. The maximal inhibition of TJ-96 was greater in ionophore-induced release than in FMLP-induced release. Although the slope and the shape of the concentration-response curve of TJ-96 were similar with each of the stimuli, TJ-96 was more potent in inhibiting the release induced by ionophore ($IC_{50} = 60$ μg/mL) than the release induced by FMLP ($IC_{50} = 300$ μg/mL). The viability of eosinophils treated with TJ-96 (1 mg/mL) for 1 h was not significantly different compared with the medium control as determined by trypan blue dye exclusion (data not shown). In addition, the inhibition of LTC_4 release caused by TJ-96 was a rapid process; maximal

inhibition was observed when eosinophils were pretreated with TJ-96 for 5 min (data not shown).

To the extent that the cellular preparations in the aforementioned experiments contained contaminating neutrophils (≈ 30%), we purified the eosinophils by negative selection and tested the inhibitory effect of TJ-96 on these highly purified eosinophils. The TJ-96 inhibited FMLP-induced LTC_4 release from purified eosinophil preparations (data not shown), indicating that the inhibitory action on eosinophil's LTC_4 secretion is not mediated indirectly such as by products derived from contaminating neutrophils, but rather through direct interaction of TJ-96 with eosinophils.

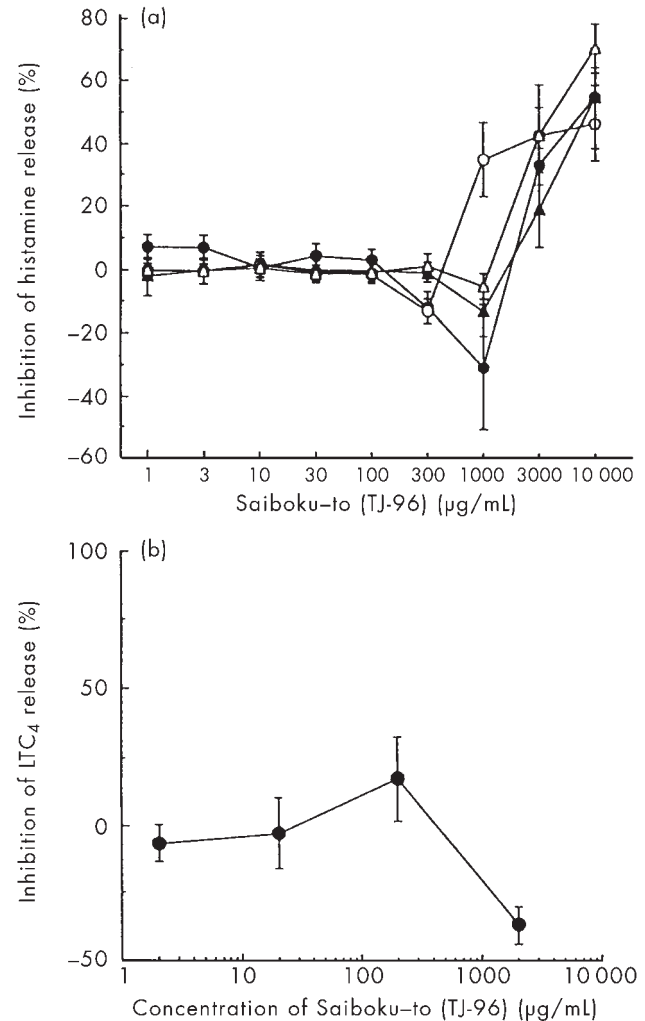## Effect of TJ-96 on mediator release from basophils

In the next series of experiments we studied the effect of TJ-96 on mediator release from basophils. The TJ-96 at high concentrations inhibited IgE-mediated histamine release from human basophils (Fig. 2a), but no statistically significant inhibition of IgE-mediated LTC_4 release was observed (Fig. 2b). In addition, TJ-96 was less potent in inhibiting basophil histamine release compared with eosinophil LTC_4 secretion.
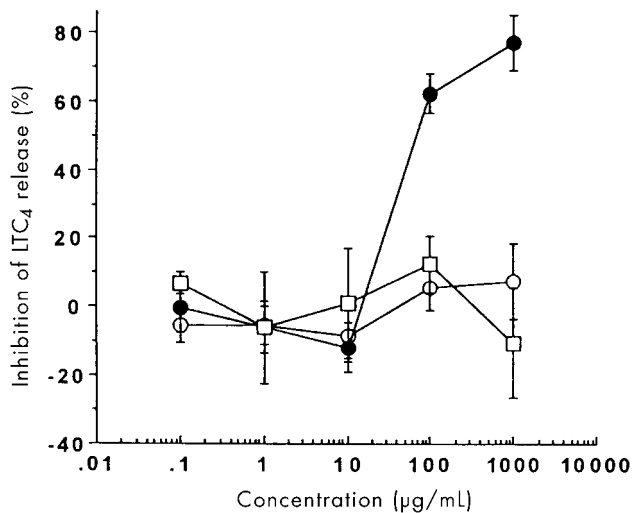
Effect of Glycyrrhiza root, Bupleurum root and Scutellaria root on LTC_4 release from eosinophils

Saiboku-to is a herbal compound mixture of 10 common herbal extracts. The pharmacological effects of TJ-96 are considered to be derived from three major herbs:

Fig. 2   Effects of TJ-96 on basophil mediator release. (a) Leukocytes were pre-incubated with serially diluted TJ-96 for 15 min and then stimulated with anti-IgE (●), 12-o-tetradecanoylphorbol-13-acetate (○), formyl-methionyl-leucycl-phenylalanine (▲), or ionophore A23187 (△) for 45 min. The supernatants were obtained by centrifugation, and histamine was determined by an automated fluorometric technique. (b) Basophils were partially purified by Percoll density centrifugation, and pre-incubated with serially diluted TJ-96 for 15 min. LTC_4 release was initiated by anti-IgE. After 45 min, the supernatants were obtained, and LTC_4 was measured by ELISA. Each value represents the mean ± SEM.

Fig. 1   Dose-dependent inhibition of eosinophil LTC_4 release by TJ-96. Eosinophils were pre-incubated with serially diluted TJ-96 for 15 min and then stimulated with ionophore A23187 (●, 0.1 μg/mL) or FMLP (○, 10^{-6} mol) plus CB (5 mg/mL). After 45 min, the supernatants were obtained by centrifugation, and LTC_4 was measured by ELISA. Each value represents the mean ± SEM.

Fig. 3 Effect of Glycyrrhiza root, (*kanzo*), Bupleurum root (*saiko*) and Scutellaria root (*ohgon*) on formyl-methionyl-leucycl-phenylalanine-induced LTC_4 release. Eosinophils were pre incubated with serially diluted extracts of Glycyrrhiza root (●), Bupleurum root (○) or Scutellaria root (□) for 15 min. LTC_4 release was indued by addition of formyl-methionyl-leucycl-phenylalanine. After 45 min, the supernatants were obtained, and LTC_4 was measured by ELISA. Each value represents the mean ± SEM.

Glycyrrhiza root (*kanzo*), Bupleurum root (*saiko*) and Scutellaria root (*ohgon*). In our final series of experiments, we evaluated the effects of these three components on LTC_4 release from eosinophils. As shown in Fig. 3, Glycyrrhiza root at high concentrations showed a concentration-dependent inhibition of LTC_4 release initiated by FMLP plus CB, whereas Bupleurum root and Scutellaria root failed to exert a significant inhibitory effect. Glycyrrhiza root contains large amounts of glycyrrhizin (≈ 5 w/w%) and liquiritin (≈ 1 w/w%). Thus, we examined the effects of these main elements of Glycyrrhiza root on FMLP-induced LTC_4 release from eosinophils. However, high concentrations of glycyrrhizin, for example 100 mg/mL, showed only marginal inhibition, and liquiritin (100 mg/mL) exerted no significant inhibitory effect (data not shown). These results indicate that the inhibitory effect of Glycyrrhiza root was due to elements other than glycyrrhizin and liquiritin.

DISCUSSION

The clinical effectiveness of Kampo herbal medicines in the treatment of some pathologic conditions has been established by extensive experience in Japan. For over 1500 years, *Hange-koboku-to* and *Sho-saiko-to* have been used to treat patients with a feeling of laryngeal obstruction and common respiratory disorders, respectively. *Saiboku-to* (TJ-96), a combined remedy of *Hange-koboku-to* and *Sho-saiko-to*, was introduced in Japan 50 years ago as a remedy for wheezing, and it has recently been applied in the treatment of bronchial asthma. By acute and chronic toxicity tests in rats, the toxic dose levels of TJ-96 were determined to be above 2000 mg/kg/day, indicating low toxicity of the compound.

To date, there have been several studies designed to evaluate the mechanisms by which TJ-96 achieves its effects. It has been shown that TJ-96 modifies allergic events through inhibition of Type I reactions *in vivo*,[3,12] and through preventing the down-regulation of glucocorticoid and β-adrenergic receptors.[4] *Ex vivo* studies reveal that TJ-96 stimulates Na absorption by airway epithelial cells, probably through nitric oxide generation.[7] However, there have been no prior investigations of the effects of TJ-96 on mediator release from human eosinophils and basophils.

In this study we provided several lines of evidence that TJ-96 modulates the secretion of 5-lipoxygenase products in eosinophils. We found that short-term pretreatment with TJ-96 resulted in marked attenuation of eosinophil LTC_4 release initiated by both ionophore and FMLP in a concentration-dependent fashion. Our next endeavor was focused on identifying the active herbs and components in TJ-96. Because Glycyrrhiza root,[13] Bupleurum root and Scutellaria root,[14,15] among the 10 herbs contained in TJ-96, were observed to show anti-allergic actions, we tested the individual inhibitory effects of these herbs. Potent inhibitory activity was shown by Glycyrrhiza root, whereas inhibitory effect was not shown by its principal known components, glycyrrhizin and liquiritin. Licochalcones, isolated from Glycyrrhiza root, has been shown to exert an inhibitory effect on ionophore-induced LTB_4 and LTC_4 formation in human neutrophils.[13] We observed that TJ-96 failed to inhibit IgE-mediated generation of LTC_4 by basophils, although high concentrations of TJ-96 inhibited degranulation of these cells, indicating that the effect of TJ-96 on eicosanoid release may be cell- and/or stimulus-specific. Further study to identify the active molecule(s) in Glycyrrhiza root may help us to answer these issues.

It can be seen that TJ-96 exerted a potent inhibitory effect on the secretion of eicosanoids by human peripheral blood eosinophils stimulated with FMLP or ionophore. Our data suggest that the clinical efficacy of TJ-96 in the treatment of

bronchial asthma may be due in part to the inhibition of eosinophil activation by this compound. The inhibitory activity was found in the extract of Glycyrrhiza root. Because the concentration of Glycyrrhiza root to inhibit LTC$_4$ release was rather high, further fractionation of Glycyrrhiza root will be helpful to identify the active molecule(s).

REFERENCES

1   Hirai K, Miyamasu M, Takaishi T, Morita Y. Regulation of the function of eosinophils and basophils. *Crit. Rev. Immunol.* 1997; **17**: 325–52.

2   Egashira Y, Nagano H. A multicenter clinical trial of TJ-96 in patients with steroid-dependent bronchial asthma. A comparison of groups allocated by the envelope method. *Ann. N. Y. Acad. Sci.* 1993; **685**: 580–3.

3   Koda A. Inhibition of hypersensitivity reactions by saiboku-to (TJ-96), particularly on type IV reaction. *Ann. N. Y. Acad. Sci.* 1993; **685**: 543–8.

4   Nakajima S, Tohda Y, Ohkawa K, Chihara J, Nagasaka Y. Effect of saiboku-to (TJ-96) on bronchial asthma. Induction of glucocorticoid receptor, beta-adrenaline receptor, IgE-Fc epsilon receptor expression and its effect on experimental immediate and late asthmatic reaction. *Ann. N. Y. Acad. Sci.* 1993; **685**: 549–60.

5   Ondrias K, Stasko A, Gergel D, Hromadova M, Benes L. Formation of stable free radicals from kampo medicines TJ-9, TJ-15, TJ-23, TJ-96, TJ-114 and their antioxidant effect on low density lipoproteins. *Free Radic. Res.* 1992; **16**: 227–37.

6   Tamaoki J, Takeyama K, Chiyotani A, Sakai N, Takizawa T, Konno K. Stimulation of Na absorption by the antiasthmatic kampo drug Saiboku-to in cultured airway epithelium. *Jpn. J. Pharmacol.* 1992; **58**: 47–53.

7   Tamaoki J, Takemura H, Kondo M, Chiyotani A, Sakai A, Konno K. Effect of TJ-96, an anti-allergic herbal medicine, on tracheal transepithelial potential difference *in vivo. Res. Commun. Mol. Pathol. Pharmacol.* 1995; **88**: 39–50.

8   Yamaguchi M, Hirai K, Ohta K *et al.* Nonreleasing basophils convert to releasing basophils by culturing with IL-3. *J. Allergy Clin. Immunol.* 1996; **97**: 1279–87.

9   Miyamasu M, Hirai K, Takahashi Y *et al.* Chemotactic agonists induce cytokine generation in eosinophils. *J. Immunol.* 1995; **154**: 1339–49.

10 Hirai K, Morita Y, Misaki Y *et al.* Modulation of human basophil histamine release by hemopoietic growth factors. *J. Immunol.* 1988; **141**: 3958–64.

11 Hirai K, Miyamasu M, Yamaguchi M *et al.* Modulation of human basophil histamine release by insulin-like growth factors. *J. Immunol.* 1993; **150**: 1503–8.

12 Iikura Y, Saito H, Umesato Y, Shichijo K, Ebisawa M, Nagakura T. Bronchial asthma and traditional Japanese herbal medicine: *In vitro* and *in vivo* studies (in Japanese with English abstract). *J. Med. Pharm. Soc. Wakan. Yaku.* 1989; **6**: 220–3.

13 Kimura Y, Okuda H. Effects of chalcones isolated from Licorice roots on leukotriene biosynthesis in human polymorphonuclear neutrophils. *Phytother. Res.* 1988; **2**: 140–5.

14 Sekiya K, Okuda H. Selective inhibition of platelet lipoxygenase by baicalein. *Biochem. Biophys. Res. Commun.* 1982; **105**: 1090–5.

15 Kimura Y, Okuda H, Arichi S. Effects of baicalein on leukotriene C4 biosynthesis in human leukocyte. *Chem. Pharm. Bull.* 1986; **34**: 2279–81.