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Synthesis and structure–activity relationships of phenothiazine carboxylic acids having pyrimidine-dione as novel histamine H_1 antagonists

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ABSTRACT

A series of phenothiazine carboxylic acid derivatives, having 6-amino-pyrimidine-2,4(1*H*,3*H*)-dione moiety via a appropriate linker, were synthesized and evaluated for their affinity toward human histamine H_1 receptor and Caco-2 cell permeability. Selected compounds were further evaluated for their oral anti-histaminic activity in mice and bioavailability in rats. Finally, promising compounds were examined for their anti-inflammatory potential in mice OVA-induced biphasic cutaneous reaction model. Among the compounds tested, phenothiazineacetic acid compound **27** showed both histamine H_1-receptor antagonistic activity and anti-inflammatory activity in vivo model.

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Allergic rhinitis (AR) is a high-prevalence disease, affecting 10–15% of the general population in Japan. AR is sustained by an IgE-mediated reaction and a complex allergic inflammatory network of cells, mediators, and cytokines based on Th2 immune response following persistent exposure to allergen. Current therapy for AR is mainly based on treatment with anti-histamines, which are overall effective in controlling IgE mediated symptoms, such as sneezing and hyper-secretion of nasal mucus, but do not modify immune response that leads to allergic inflammation, such as nasal obstruction. Accordingly, anti-histamines, which possess anti-inflammatory effects, are considered to be next generation anti-histamines with the ability to control Th2 immunity.[1] Takeda group has reported TAK-427 (**1**) as a novel anti-histamine with anti-inflammatory effects in several experimental animal models.[2] This compound proceeded to clinical evaluation, but its development has recently been suspended. It has also been reported that CX-659S (**2**), a pyrimidine-2, 4(1*H*,3*H*)-dione derivative, shows anti-inflammatory effects in various experimental models, including mice contact hypersensitivity reaction model[3] and OVA-induced biphasic cutaneous reaction model.[4] Based on these findings, we hypothesized that a hybrid compound with an anti-histaminic part and a pyrimidine-2, 4(1*H*,3*H*)-dione, might possess not only anti-histaminic activity but also anti-inflammatory effects and that such a compound would be a next generation anti-histamine.

To verify this hypothesis, we prepared several compounds as shown in Figure 1 and evaluated their biological properties.

First of all, we synthesized **3a**, a compound that combines the anti-histaminic dichlorophenoxypiperidine and the 6-amino-pyrimidine-2,4(1*H*,3*H*)-dione. The IC_{50} value of **3a** toward the H_1 receptor was determined as 58 nM, which is equivalent to that of Fexofenadine (**4**), well-known H_1 receptor antagonist (IC_{50} = 78 nM).[5] In addition, **3a** showed potent inhibitory activity for both ITR (Immediate type reaction) and LTR (Late type reaction) in OVA-induced biphasic cutaneous reaction model (vide post, Table 1). In this model, LTR is characterized by local accumulation of activated inflammatory cells, including eosinophils, monocytes, and T lymphocytes.[6] This indicates that compound **3a** possesses anti-inflammatory effect. On the other hand, ITR was primarily caused by IgE-mediated activation of mast cells/basophils to release chemical mediators such as histamine,[4,7] and anti-histamines (Fexofenadine and Ketotifen fumarate, vide post, Table 1) and CX-659S[4] inhibited ITR. Therefore the inhibitory effects of compound **3a** on ITR might be combined effects of anti-histaminic activity and other activity CX-659S having.[8]

With such profile, compound **3a** was considered as a suitable lead for further studies. However, due to its high lipophilicity, it was feared that compound **3a** might cause sedation, one of the main side effects of anti-histamines, because of its potential to cross the blood–brain barrier. It is well-known that introduction of a carboxylate is effective in reducing compounds potential to penetrate the brain. Therefore we directed our synthetic efforts towards the introduction of a carboxylate into compound **3a** without

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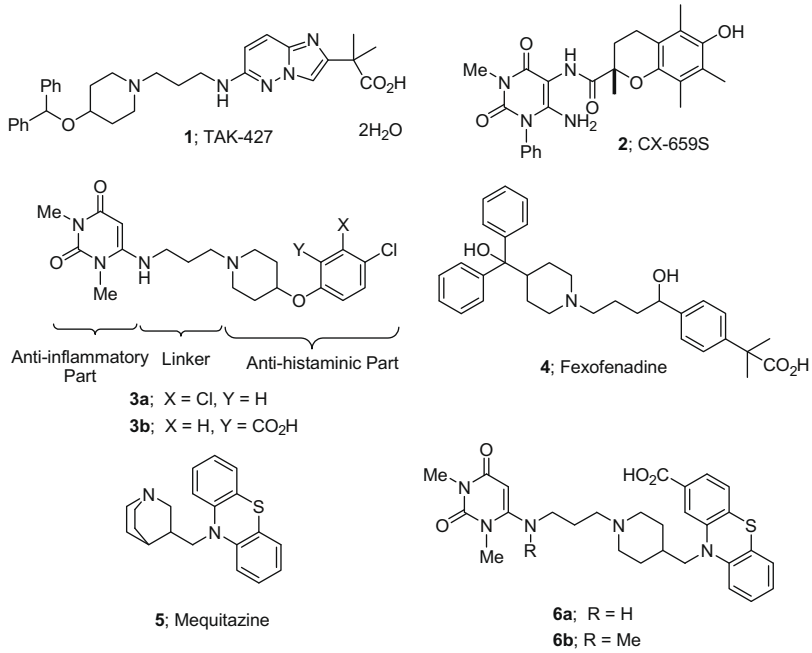


Figure 1. Chemical Structures of **1**, **2**, **4**, **5**, and hybrid compounds (**3**, **6**).

canceling H_1 binding affinity. Compound **3b**, containing a carboxylate at the anti-histaminic phenyl group showed diminished H_1 binding affinity (IC_{50}; >1000 nM). When the anti-histaminic part was replaced with phenothiazine, the core part of Mequitazine (**5**),[9] introduction of the carboxylate was tolerated, and IC_{50} of the resulting **6a** was 46 nM. This result encouraged us to examine the SAR of phenothiazine carboxylate compounds in details.

Phenothiazine carboxylate derivatives were typically synthesized according to the methods described in Schemes 1–3.

Reaction of **7** with an appropriate alkylamine gave **8** in good yield. Coupling reaction with the piperidine **9** followed by hydrolysis afforded compounds **3a**, **3b**, **6a**, and **6b**.

Compounds **21**–**35** were synthesized as shown in Scheme 2. Transformation of the nitrile, acetyl, or methoxy group in the commercially available phenothiazine derivatives (**10a**, **10b**, and **10c)** to an ester group, and introduction of proper linker parts gave the intermediate **12**. We here used the acetylphenothiazine **10b** as a masking phenothiazine acetate to prevent α-methylene from C-alkylation in the N-alkylation step. N-Alkylation of **10b** gave **15**, which was easily converted into the phenothiazine acetate **16** by Willgerodt–Kindler reaction.[10] Mesylation of **16a** gave **12d**. On the other hand, protection of hydroxyl group in alcohol **16a** with *tert*-butyldimethylsilyl group gave silyl ether **16b**. Alkylation of **16b** gave **17**. Hydrolysis of **17**, followed by mesylation gave **12e**, **12f**, and **12g**. Coupling reaction of **12** with the piperazinyl pyrimidine-dione **19**, followed by hydrolysis provided the phenothiazine carboxylate **21**–**35**.

The *C*-junction compound **36** was prepared as shown in Scheme 3. Suzuki coupling reaction of the 6-chloro-pyrimidine-dione **7** with 4-pyridineboronic acid, followed by benzylation, reduction, and deprotection gave the tetrahydropyridine **20**. Finally, coupling of **20** with **12d** followed by hydrolysis afforded **36**.

All compounds were evaluated for their affinity toward human histamine H_1 receptor using radioligand binding assay[11] and for their Caco-2 cell permeability.[12] Compounds that exhibited high affinity toward the H_1 receptor (IC_{50}; <100 nM) as well as high Caco-2 cell permeability (>30 nm/s) were further evaluated for their oral anti-histaminic activity in mice with histamine-induced skin vascular permeability,[13] and their bioavailability in rats. Finally, promising compounds were examined for their anti-inflammatory potential in mice OVA-induced biphasic cutaneous reaction model.[14]

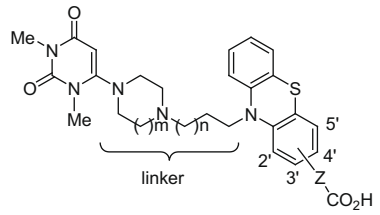
Although compound **6a** showed potent H_1-receptor binding affinity, its Caco-2 cell permeability was poor (4 nm/s, Table 1). Our efforts were then focused on improvement of Caco-2 cell permeability of the compound. Compound **6b**, in which a hydrogen atom of **6a** was replaced with a methyl group, had similar properties to those of **6a**, indicating that the hydrogen atom in **6a** is not essential for the potent anti-histaminic activity and low Caco-2 cell permeability. Next we tried to prepare the piperazine compound **21** by incorporating the nitrogen atom in **6a** into the piperidine ring. Compound **21** showed not only a potent H_1 receptor binding affinity (IC_{50}; 37 nM), but also a significantly improved Caco-2 cell permeability (99 nm/s) compared to **6a**. The bioavailability of **21,** calculated from PK studies in rats, was estimated as 17%. Compound **21** also showed anti-histaminic activity in vivo model (39% inhibition at 10 mg/kg). Furthermore, **21** inhibited both ITR and LTR in OVA-induced biphasic cutaneous reaction model (47% and 65% inhibition at 10 mg/kg, respectively), and the inhibitory effect on LTR indicated that compound **21** possessed anti-inflammatory activity. Therefore compound **21** was considered as suitable for further investigation.

In order to clarify the SAR of **21**, we focused on the length of methylene chain and modification of the carboxylic acid part. As shown in Table 1, the permeability of the compound **21** slightly decreased, when the piperazine was replaced with a homopiperazine giving compound **22**. Although the bioavailability of **22** was lower than that of **21**, probably due to the decrease in Caco-2 permeability, **22** still possessed inhibitory activity on ITR and LAR. Therefore introduction of a homopiperazine unit into **21** did not affect the anti-inflammatory activity.

Biological evaluation of **23** and **24** indicated that the position of the carboxylate is crucial for both H_1 binding activity and Caco-2 permeability and that the 3′-position is preferable. Intramolecular ionic interaction between carboxylic group and basic nitrogen of piperazine assumed to be important for H_1 binding activity.

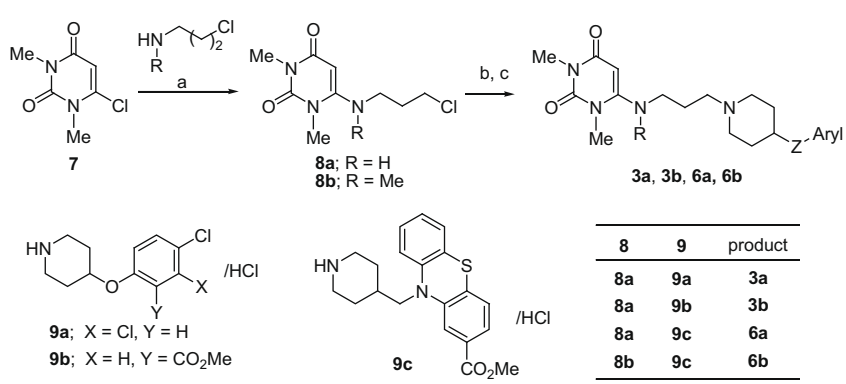
With regard to the methylene chain length between the phenothiazine and the piperazine ring, the propylene **21** or butylene **26**

Table 1
Pharmacological profile derivatives



No.	Linker		Z position	Z	H_1 binding (nM)	Caco-2[a] (nm/s)	H_1[b] (vivo)	PB[c] (%)	BA[d] (%)	OVA[e]	
	m	*n*								ITR (%)	LTR (%)
3a					28	42	(69)[f]	83.4	25	(72)[g]	(61)[g]
6a			3′	Bond	46	4	–	–	–	–	–
6b			3′	Bond	53	6	–	–	–	–	–
21	1	1	3′	Bond	37	99	(39)[f]	99.4	17	47	65
22	2	1	3′	Bond	23	47	14	98.8	7	50	53
23	2	1	2′	Bond	>500	5	–	–	–	–	–
24	2	1	5′	Bond	>500	5	–	–	–	–	–
25	1	0	3′	Bond	124	49	–	–	–	–	–
26	1	2	3′	Bond	35	203	(43)[f]	99.9	16	–	–
27	1	1	3′	CH_2	40	123	61	97.8	27	73	64
28	2	1	3′	CH_2	23	55	53	94.9	19	16	12
29	1	1	3′	CH_2CH_2	105	51	51	99.9	51	–	–
30	2	1	3′	CH_2CH_2	69	77	51	98.8	7	79	49
31	2	1	3′	CHMe	72	69	79	97.8	10	–	–
32	2	1	3′	CMe_2	123	99	–	95.5	–	–	–
33	2	1	3′	CHEt	41	223	51	–	14	–	–
34	2	1	3′	OCH_2	88	5	–	95.3	–	–	–
35	2	1	3′	CH=CH	323	71	–	–	–	–	–
36			3′	CH2	41	47	82	–	30	86	12
37	Fexofenadine				78	4	70	–	–	61	6
38	Ketotifen fumarate				1	111	98	–	–	119[h]	3[i]
39	Prednisolone									22	81

[a] Caco-2 cell permeability.
[b] Histamine induced skin vascular permeability. % inhibition at 3 mg/kg, po.
[c] Protein binding affinity (%) was determined by equilibrium dialysis.
[d] Bioavailability in rat.
[e] OVA induced biphasic cutaneous reaction model. % inhibition at 10 mg/kg, po.
[f] 10 mg/kg, po.
[g] 30 mg/kg, po.
[h] 106% inhibition at 30 mg/kg, po.
[i] −4% inhibition at 30 mg/kg, po.

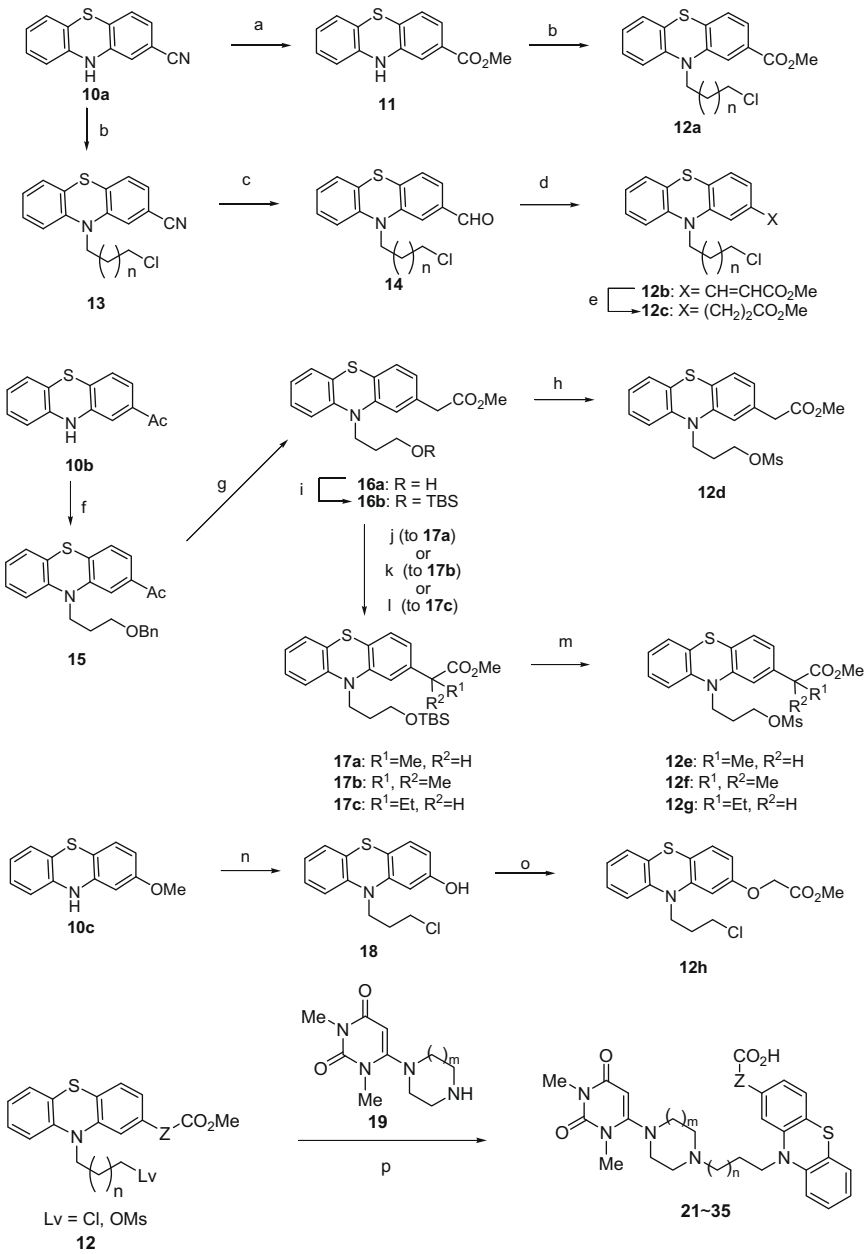


8	9	product
8a	**9a**	**3a**
8a	**9b**	**3b**
8a	**9c**	**6a**
8b	**9c**	**6b**

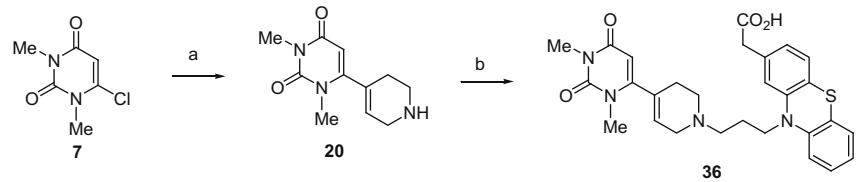
Scheme 1. Reagents and conditions: (a) $^{i}Pr_2NEt$, 100 °C, 40–60%; (b) **9**, KI, $^{i}Pr_2NEt$, 85 °C, 40–75%; (c) aq NaOH, rt, 60–85%.

showed more potent binding affinity toward H_1 receptor than the ethylene **25**.

Compounds **21** and **26** did not show significant inhibitory activity in vivo at 10 mg/kg, and compound **22** did not show significant inhibitory activity in vivo at 3 mg/kg (less than 50%). In order to clarify the reason for these unexpected results, we evaluated compounds **21**, **22**, and **26** protein binding affinity and oral bioavailability. Protein binding affinity (PB) of **21**, **22**, and **26** was 99.4%, 98.8%, and 99.9%, respectively, and their bioavailability was of 17%, 7% and 16%, respectively. These results indicate that compounds high PB affinity might be the reason for their poor in vivo anti-histaminic activity. Oshima et al. have reported that Olopatadine, a phenyacetate compound, shows stronger activity in PCA model than the corresponding benzoate compound, even though the two compounds have similar in vitro H_1 binding affinity.[15] According to these findings, we next prepared the phenylac-



Scheme 2. Reagents and conditions: (a) (i) Concd HCl/MeOH, 100 °C; (ii) K_2CO_3, MeI, rt, 75%; (b) NaH, $Cl(CH_2)_{n+2}Br$, 0 °C to rt, 30–70%; (c) (i) DIBAL, −78 °C; (ii) HCl, −78 °C, 89%; (d) $PPh_3{=}CHCO_2Me$, 50 °C, 84%; (e) H_2, Pd/C, rt, 75%; (f) $BnO(CH_2)_3Br$, NaH, 0 °C, 86%; (g) (i) TsOH/H_2O, morphorine, S_8, 50 °C; (ii) AcOH, concd HCl, 100 °C; (iii) MeOH, H_2SO_4, 80 °C, 45%; (h) MsCl, pyridine, 0 °C to rt, 67%; (i) TBSCl, imidazole, 0 °C to rt, 83%; (j) NaH (1 equiv), MeI, 0 °C to rt, 75%; (k) NaH (2 equiv), MeI, 0 °C to rt, 44%; (l) NaH (1 equiv), EtI, 0 °C to rt, 63%; (m) (i) HCl, 56–85%; (ii) MsCl, pyridine, 0 °C to rt, quant; (n) (i) NaH, $Cl(CH_2)_3Br$, 0 °C; (ii) BBr_3, 0 °C to rt, 87%; (o) K_2CO_3, methyl bromoacetate, 80 °C, 77%; (p) (i) KI, $^iPr_2NEt$, 85 °C; (ii) aq NaOH, rt to 45 °C, 22–76%.



Scheme 3. Reagents and conditions: (a) (i) 4-Pyridineboronic acid, $Pd(PPh_3)_4$, K_2CO_3, 110 °C; (ii) benzyl bromide, 65 °C; (iii) $NaBH_4$, 0 °C; (iv) Troc-Cl, 100 °C; (v) Zn, AcOH, 60 °C, 14%; (b) (i) **12d**, KI, $^iPr_2NEt$, 85 °C; (ii) aq NaOH, rt, 68%.

etate **27**[16] and examined its pharmacological profile. Compound **27** exhibited not only good in vitro H_1 receptor binding affinity, but also good in vivo anti-histaminic activity at 3 mg/kg as well as good oral availability. Compound **27** had a PB affinity of 97.8%, suggesting that PB is an important factor for potent in vivo anti-histaminic activity. Similarly, the homopiperazine **28** showed im-

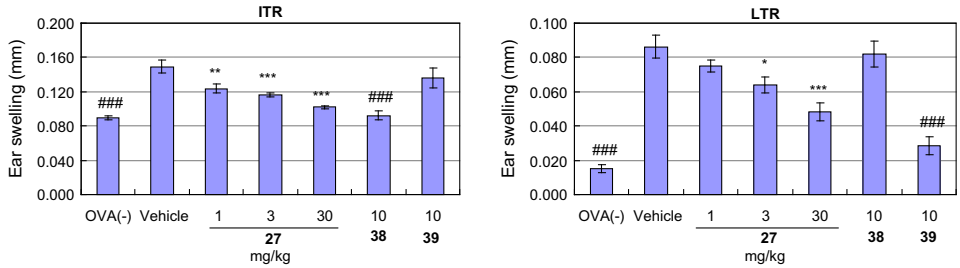


Figure 2. Effect of **27** on OVA-induced biphasic reaction model. Each column with vertical bar represents the mean ± SE of 6-7 mice. *: P <0.05, **: P <0.01 and ***: P <0.001 when compared with the vehicle (0.5% MC)—treated group.[Dunnett's test] . ###: P <0.001 when compared with the vehicle (0.5% MC)—treated group.[t-test]. **38** is Ketotifen fumarate. **39** is Prednisolone.

proved PB affinity (94.9%) compared to **22**, and had good in vivo anti-histaminic activity. Homopiperazine compound **28** showed more potent H_1 receptor binding affinity than piperazine compound **27**, and this result showed good correlation with the result of benzoate (**21** and **22**) and propionate (**29** and **30**).

Our efforts were next directed towards modification of the phenylacetate part. The propionate **29** with piperazine unit showed high PB (99.1%), although corresponding homopiperazine compound **30** showed low PB (98.8%) with proper H_1 binding affinity, so further modification was carried out with homopiperazine compounds. Introduction of an alkyl group onto the α-position of the carboxylate was effective in improving Caco-2 cell permeability with a slight loss in H_1 binding affinity. However, the improvement in Caco-2 cell permeability did not translate into improvement of oral bioavailability (**31**, **32**, and **33**). The phenoxyacetate **34** had a quite poor Caco-2 cell permeability with low PB (95.3%), and the acrylate **35** showed weak H_1 binding affinity. The *C*-junction derivative **36** was also prepared for comparison with the *N*-junction derivatives **21**. Compound **36** exhibited in general good properties, including favorable oral bioavailability.

Next we evaluated the anti-inflammatory effects of the phenylacetate derivatives in OVA-induced biphasic reaction model. As expected, compound **27** showed inhibitory activity on both ITR and LTR. Homopiperazine compound **30** also showed inhibitory activity on both ITR and LTR, even though its bioavailability was low. However, compound **28** suppressed neither ITR nor LTR. This result did not correlate with the result obtained with compound **22**. It is difficult to explain the reason clearly, but one reason might be the difference of distribution to the skin. To explain this discrepancy, clarification of the anti-inflammatory mechanism is required. The *C*-junction derivative **36** showed no inhibitory activity on both of ITR and LTR.

Finally we selected compound **27**, which met all criteria, and evaluated its inhibition of ITR and LTR in OVA-induced biphasic reaction model. As shown in Figure 2, **27** inhibited both ITR and LTR in a dose dependent manner. Ketotifen (**38**), a well known launched anti-histamine, inhibited only ITR, while Prednisolon (**39**) potently inhibited LTR. The biological properties of **27** are different from those of known anti-histamines, and its potential to induce sedation is minimal due to the presence of a carboxylate. It is therefore believed that compound **27** could be a next generation anti-histamine.

In future studies, we will continue further structural optimization of compound **27** and will try to clarify the mechanism of its anti-inflammatory effect.

We synthesized a series of phenothiazine carboxylic acid derivatives, having 6-amino-pyrimidine-2,4(1*H*,3*H*)-dione moiety and evaluated their affinity toward human histamine H_1 receptor and Caco-2 cell permeability. Selected compounds were further evaluated for their oral anti-histaminic activity in mice and bioavailability in rats. Finally, promising compounds were examined for their anti-inflammatory potential in mice OVA-induced biphasic cutaneous reaction model. Among the compounds tested, compound **27** showed potent histamine H_1-receptor antagonistic activity and remarkable anti-inflammatory activity in vivo model. It is therefore believed that compound **27** is a promising next generation anti-histamine.

Acknowledgments

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11. Membrane of stable recombinant CHO-K1 cells expressing the human histamine H_1 receptor (Euroscreen) was incubated with various concentrations of compounds and 1.25 nM [^{3}H]-pyrilamine in a total of 200 μl of binding buffer (50 mM Tris–HCl, pH 7.5). Nonspecific binding was determined in the presence of 1.25 μM triprolidine (Sigma). The assay mixture was incubated for 3 h at room temperature and then filtered through GF/B filters. These were washed and counted by a scintillation counter. Specific binding was calculated as the difference between total and nonspecific bindings.
12. The apical side of Caco-2 cell line monolayer was incubated with the compounds at 37 °C for 120 min. The compound concentration of an apical side and a basal side was measured. The permeability coefficient (*P*app) was calculated by following scheme.
$Papp = dC/dt \times VR/(A \times C_0)$; C_0: initial donor concentration (μM), *VR*: volume of reserver well (mL), *A*: membrane surface area (cm^2), dC/dt: slope of the cumulative reserver concentration (μM/s).
13. Eight-week old female mice (ddY, SLC) was administered orally with various doses of compounds suspended in 0.5% methylcellulose 1 h before the

intradermal injection of histamine (4 μg/site) to back skin following intravenous injections of Evans' blue solution. Thirty minutes after the injection of histamine, mice were sacrificed and the area of dye was measured.

14. Eight-week old female mice (Balb/c, SLC) were sensitized by intraperitoneal injection of OVA with alum. After two weeks, compounds suspended in a 0.5% MC solution was po administered 60 min before OVA injection to the ear in OVA-sensitized mice. Ear swellings were determined at 1 h (ITR) and 24 h (LTR) after elicitation. Each column with vertical bar represents the mean ± SE of 6–7 mice.
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16. Compound **27**; mp 79–82 °C (dec.); ^{1}H NMR ($CDCl_3$, δ ppm): 7.13–7.18 (3H, m), 7.06 (1H, d, J = 7.5 Hz), 6.85–6.94 (3H, m), 5.45 (1H, s), 3.90–3.97 (2H, m), 3.54 (2H, s), 3.31 (3H, s), 3.29 (3H, s), 3.19–3.24 (4H, m), 2.86–2.93 (6H, m), 2.07–2.15 (2H, m), 1.91–1.98 (2H, m). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 192.9, 173.0, 162.4, 158.6, 152.6, 144.9, 144.7, 135.2, 128.1, 127.6, 127.3, 124.4, 124.3, 123.1, 122.5, 117.8, 116.3, 87.6, 53.6, 50.5 (2C), 46.9 (2C), 44.3, 40.7, 32.8, 27.7.; IR (ATR) ν 3354, 2947, 1693, 1643, 1458, 1433, 758 cm^{-1}; MS (ESI) m/z calcd for $C_{27}H_{31}N_5O_4S$ 521; found 522 (M+H).; Anal. Calcd for $C_{27}H_{31}N_5O_4S{\cdot}HCl{\cdot}2.5H_2O$: C, 53.77; H, 6.18; N, 11.61; S, 5.31. Found: C, 53.38; H, 5.93; N, 11.34; S, 5.31.