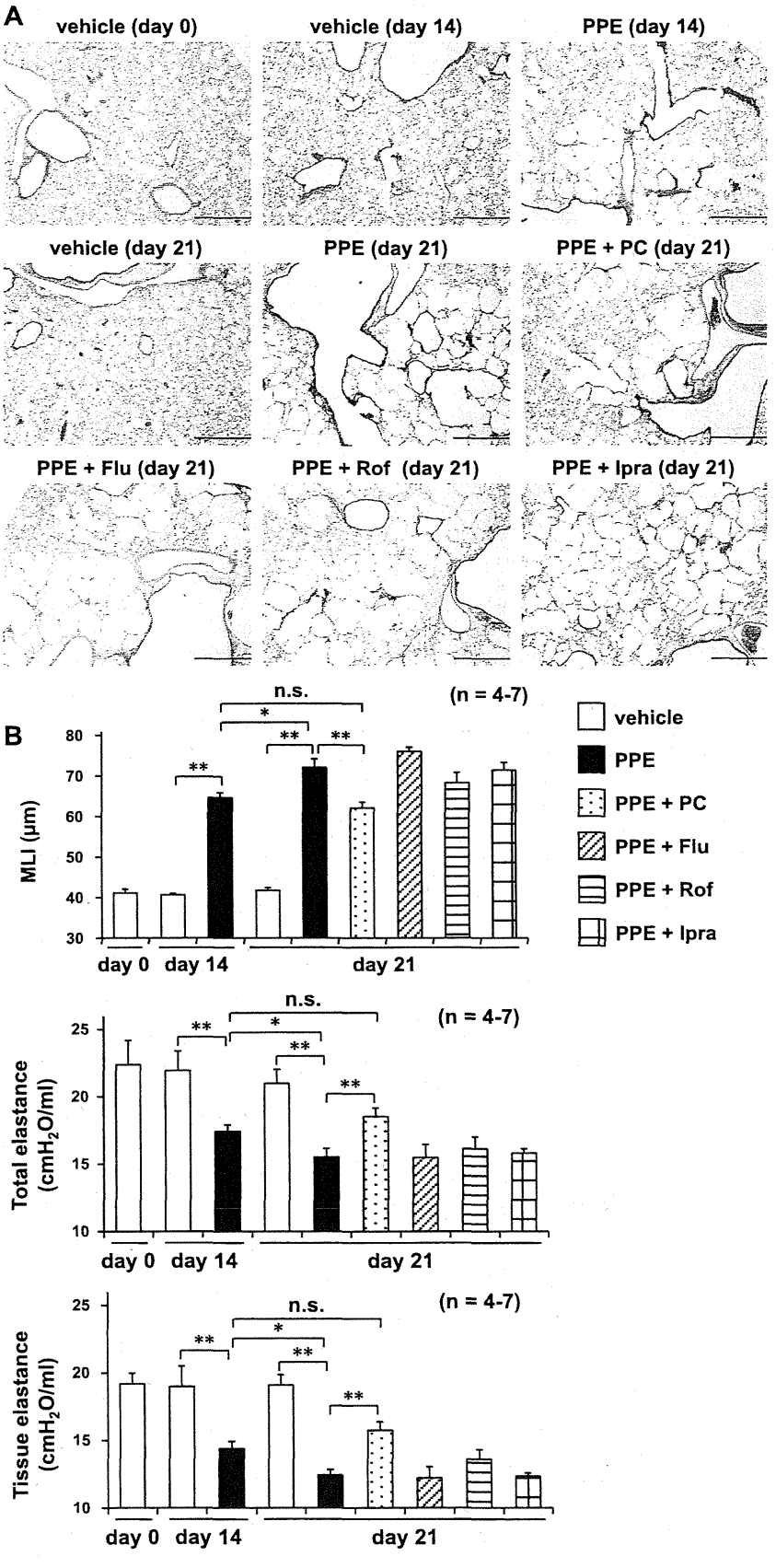

Fig. 7. Effect of different drugs on predeveloped pulmonary emphysema. Mice were treated with or without (vehicle only) PPE (100 μg/mouse) once only on *day 0*. PC-SOD (PC; 60 kU/chamber), fluticasone propionate (Flu; 167 μg/kg), ipratropium bromide (Ipra; 26.7 μg/kg), or roflumilast (Rof; 5 mg/kg) were administered by inhalation, intratracheally, or orally, respectively, once daily from *day 14* to *day 20*. Histopathological examination (scale bar = 500 μm) (*A*), determination of the MLI (*B*), and measurement of total respiratory system elastance (total elastance) and tissue elastance (*B*) were determined as described in Fig. legend 1. Values are means ± SE. $*P < 0.05$; $**P < 0.01$.

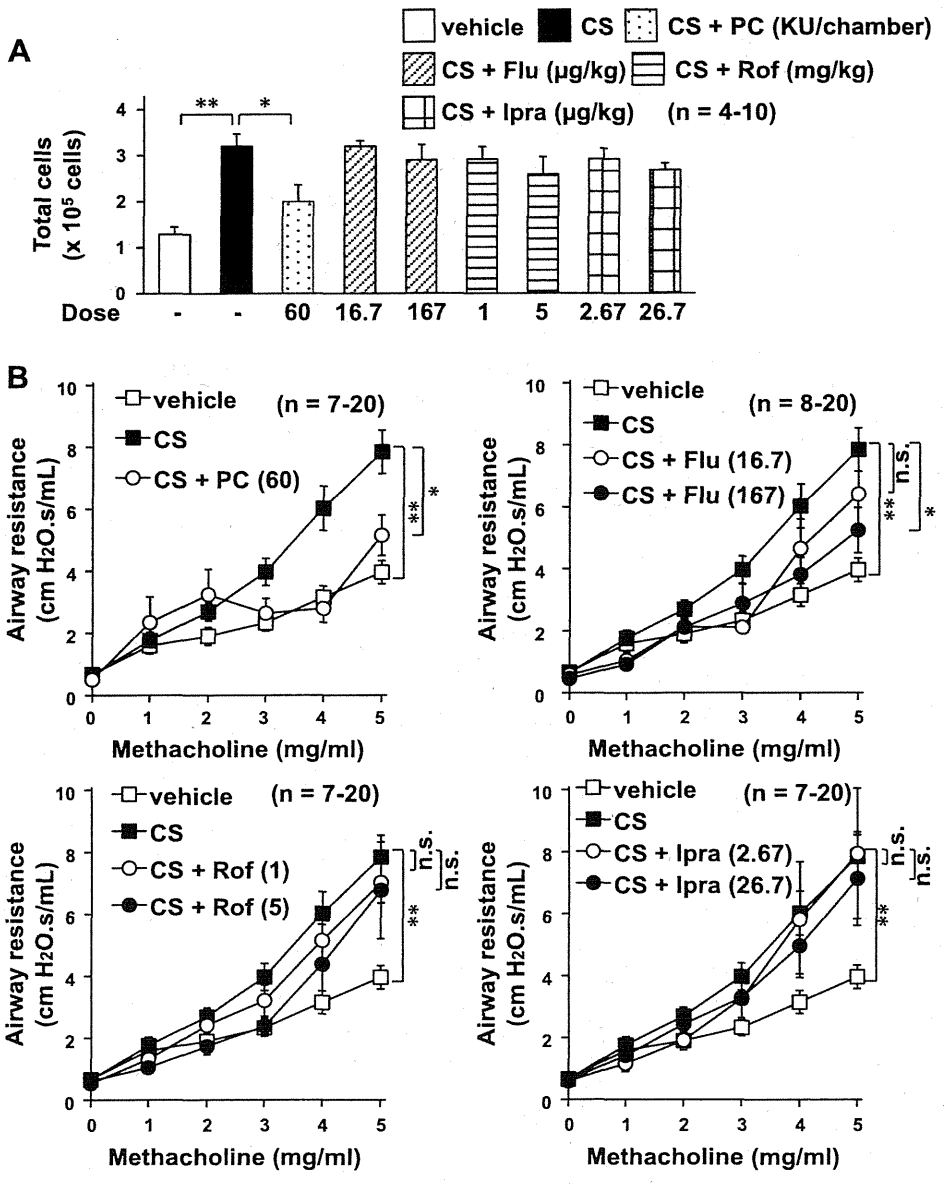

Fig. 8. Effect of different drugs on the CS-induced inflammatory response and hyperresponsiveness to methacholine. Mice were exposed to cigarette smoke (CS) for 3 days as described in MATERIALS AND METHODS. PC-SOD (PC; kU/chamber), fluticasone propionate (Flu; μg/kg), ipratropium bromide (Ipra; μg/kg), or roflumilast (Rof; mg/kg) were administered by inhalation, intratracheally, or orally, respectively, once daily for 3 days (from *day 0* to *day 2*). Inflammatory response (*A*) and airway resistance (*B*) were assessed on *day 3* (26 h after the final administration of each drug) as described in Fig. legends 3 and 5 (*A*). Same data for vehicle and CS are used in all panels in *B*. Values are means ± SE. $*P < 0.05$; $**P < 0.01$; n.s., not significant.

11, 25, 43), we suggested that PC-SOD may be superior to these drugs for the treatment of COPD (39). In this study, we compared the protective and therapeutic effects of various drugs, including PC-SOD, under the same conditions. We used not only anti-inflammatory drugs (fluticasone propionate and roflumilast) but also ipratropium bromide, given the recent report concerning the anti-inflammatory effects of bronchodilators (43). For fluticasone propionate and ipratropium bromide, we used both clinical and higher doses (16.7 μg/kg and 167 μg/kg, respectively, for fluticasone propionate and 2.67 μg/kg and 26.7 μg/kg, respectively, for ipratropium bromide). In most previous animal studies, each drug was used within these dose ranges (24, 29, 41). For roflumilast, although the clinical dose is 500 μg/body (8.3 μg/kg), doses of 1–5 mg/kg have been used in previous animal studies (25, 43). Thus we used roflumilast doses of 1 and 5 mg/kg in this study. Under these conditions, neither fluticasone propionate nor ipratropium bromide showed any protective or therapeutic effects with respect to PPE-induced pulmonary emphysema, alterations in lung mechanics or respiratory dysfunction (decrease in $FEV_{0.05}$%). On the other hand, roflumilast showed a protective effect against PPE-induced pulmonary emphysema and alteration of lung mechanics; however, the degree of protection was less than that afforded by PC-SOD. Furthermore, roflumilast did not exhibit any protective effect against PPE-induced respiratory dysfunction or any therapeutic effect against PPE-induced pulmonary damage. At present, it is not clear why roflumilast is positive for some indexes but not for other ones in this animal model. These results suggest that inhaled PC-SOD could be superior to these other drugs for the treatment of patients with COPD. We also found that the PPE-induced pulmonary inflammatory response and the production of superoxide anions were suppressed more clearly in mice concomitantly treated with PC-SOD compared with those treated with other drugs, suggesting that the antioxidant activity provided by PC-SOD is responsible for its superior therapeutic effects in this animal model. We also found that inhaled PC-SOD suppressed the CS-induced airway hyperresponsive-

ness to methacholine, which was previously suggested to involve the infiltration of leucocytes into the lung (5, 27). These results also suggest the clinical benefit of this treatment method.

In conclusion, we consider that a combination regime of administration of a bronchodilator along with inhaled PC-SOD may be therapeutically beneficial for patients with COPD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: K.-I.T. performed experiments; K.-I.T. and K.S. analyzed data; K.-I.T. and T.M. interpreted results of experiments; K.-I.T. prepared figures; K.-I.T., K.S., K.A., A.A., and T.M. edited and revised manuscript; K.-I.T., K.S., K.A., A.A., and T.M. approved final version of manuscript; T.M. conception and design of research; T.M. drafted manuscript.

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Synthesis and Biological Evaluation of Derivatives of 2-{2-Fluoro-4-[(2-oxocyclopentyl)-methyl]phenyl}propanoic Acid: Nonsteroidal Anti-Inflammatory Drugs with Low Gastric Ulcerogenic Activity

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Synthesis and Biological Evaluation of Derivatives of 2-{2-Fluoro-4-[(2-oxocyclopentyl)methyl]phenyl}propanoic Acid: Nonsteroidal Anti-Inflammatory Drugs with Low Gastric Ulcerogenic Activity

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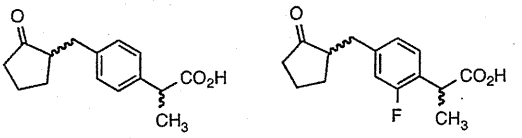
Supporting Information

ABSTRACT: We previously reported that 2-fluoroloxoprofen has lower gastric ulcerogenic activity than loxoprofen, a nonsteroidal anti-inflammatory drug (NSAID) without selectivity for COX-2. We synthesized derivatives of 2-fluoroloxoprofen and studied their properties. Compared to 2-fluoroloxoprofen, one derivative, **11a**, exhibited higher anti-inflammatory activity and an equivalent ulcerogenic effect. These results suggest that **11a** could be therapeutically beneficial for use as an NSAID.



Loxoprofen (1) **2-Fluoroloxoprofen (2)**

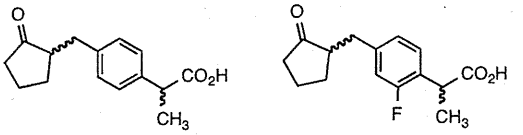
INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are an important family of therapeutic agents, accounting for nearly 5% of all prescribed medications.[1] An inhibitory effect of NSAIDs on cyclooxygenase (COX) activity and a resulting decrease in prostaglandins (PGs) such as PGE_2 have been shown to be responsible for their anti-inflammatory actions. One downside of NSAID use is associated with gastrointestinal side effects.[2] Since PGE_2 has a strong protective effect on the gastrointestinal mucosa, it was considered that the adverse gastrointestinal effects of NSAIDs could also be due to their inhibitory action on COX activity.

COX has two main subtypes, COX-1 and COX-2, that are responsible for the majority of COX activity at the gastrointestinal mucosa and in tissue inflammation, respectively.[3,4] Thus, it stands to reason that a greatly reduced incidence of gastroduodenal lesions has been reported for selective COX-2 inhibitors.[5] However, a recently raised issue concerning the use of selective COX-2 inhibitors is their potential risk for cardiovascular thrombotic events.[6,7] This may be due to the fact that prostacyclin, a potent antiaggregator of platelets and a vasodilator, is mainly produced by the action of COX-2.[8,9] Thus, it is evident that NSAIDs other than COX-2-selective inhibitors that do not cause gastrointestinal problems need to be developed.

We recently suggested that COX-independent NSAID-induced cell death is also involved in NSAID-induced gastric lesions and that this direct cytotoxicity of NSAIDs is due to their membrane permeabilization activity.[10,11] Thus, we proposed that NSAIDs with lower membrane permeabilization activity would be safer on stomach tissue even if they had a reduced selectivity for COX-2.[11]

Loxoprofen (**1**) (Figure 1), an NSAID without selectivity for COX-2, has been used clinically for many years as a standard



Loxoprofen (1) **2-Fluoroloxoprofen (2)**

Figure 1. Structures of loxoprofen and 2-fluoroloxoprofen.

NSAID in Japan, and clinical studies have suggested that it is safer to use than other NSAIDs, such as indomethacin.[12] Compound **1** is a prodrug that is converted to its active metabolite (the trans-alcohol form) after absorption in the gastrointestinal tract.[13] We recently reported that **1** has relatively lower membrane permeabilization activity than other NSAIDs[14] and considered that it could be used as a lead compound to obtain NSAIDs with even lower gastric ulcerogenic activity. We synthesized a series of its derivatives and obtained 2-fluoroloxoprofen (**2**) (Figure 1), which has lower gastric ulcerogenic activity but equivalent anti-inflammatory activity compared with **1**.[15] In order to obtain more clinically beneficial NSAIDs (higher anti-inflammatory activity and/or lower gastric ulcerogenic activity), we describe here details of the synthesis of a series of derivatives of **2** and the results of experiments

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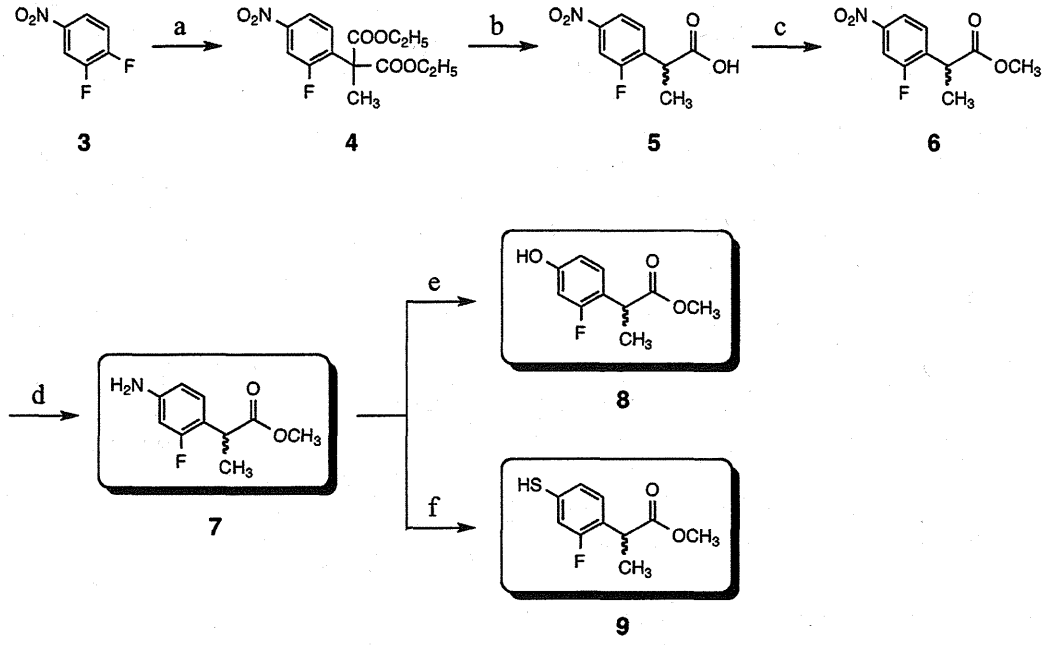
Scheme 1. Synthesis of Key Intermediates 7–9 for the Target Compounds[a]



[a]Reagents and conditions: (a) diethyl methylmalonate, NaOH, DMF; (b) conc H_2SO_4, AcOH, reflux; (c) MeOH, conc HCl, reflux; (d) H_2, 10% Pd/C, MeOH; (e) (i) 6 M H_2SO_4, $NaNO_2$, H_2O, (ii) 3 M H_2SO_4, reflux, (iii) MeOH, conc HCl, reflux; (f) (i) conc HCl, $NaNO_2$, H_2O, (ii) EtOCSSK, H_2O.

carried out to examine their ulcerogenic and anti-inflammatory activities.

■ CHEMISTRY

Compounds **1** and **2** were synthesized as described previously.[15,16]

The synthetic route for key intermediates **7–9** is outlined in Scheme 1. The commercially available 1, 2-difluoro-4-nitrobenzene (**3**) was converted to propanoic acid **5** via methylmalonate **4** as described previously.[17] Methyl esterification of **5** under acidic conditions gave methyl propanoate **6** that was subsequently treated with palladium on carbon under atmospheric H_2 pressure to provide key intermediate **7**. Another key intermediate **8** was obtained by hydrolyzing the diazonium salt that was formed by treatment of **7** with sodium nitrite ($NaNO_2$). On the other hand, the diazonium salt formed by treatment of **7** with $NaNO_2$ was reacted with potassium ethyl xanthate (EtOCSSK)[18] to yield key intermediate **9**.

The synthetic route for type-A target compounds (**10a**, **10b**, **11a**, **11b**, and **12–17**) having a heteroatom (O, N or S) bridge between two rings (Figure 2) is outlined in Scheme 2. The terminal ring is a cycloketone, cycloalkanol, or cycloalkane. The methyl ester precursor of final compounds **10a**, **10b**, **11a**, **11b**, and **12–17** were prepared from the corresponding intermediates **7–9**. Finally, these precursors were hydrolyzed with base to give the final compounds.

The synthetic route for type-B target compounds (**21a**, **21b**, **22a**, **22b**, **23**, and **24**) having an aromatic heteroring or an acyclic ketone (Figure 2) is outlined in Scheme 3. Treatment of **8** with trifluoromethanesulfonic anhydride ($(CF_3SO_2)_2O$)[19] provided **18**, which was then reacted with dimethylzinc ($Zn(CH_3)_2$)[20] to yield **19**. The methyl group of **19** was transformed into an active methylene group by treatment with *N*-bromosuccinimide (NBS) to yield the key intermediate **20**. Compound **20** was reacted with four kinds of boronic acid by the Suzuki–Miyaura coupling method[21] to yield the precursors for **21a**, **21b**, **22a**, and **22b**. Finally, these methyl ester precursors were hydrolyzed with base to give the final compounds. On the other hand, treatment of **20** with two kinds of acetoacetic ester derivatives provided the corresponding precursors for **23** and **24**. Finally, these precursors were subjected to decarboxylation and hydrolysis with acid to give the final compounds.

The synthetic route for type-C target compounds (**33a**, **33b**, **39**, and **40**), which were modified at the 5- or 6-position of the phenyl ring of **2** by halogen (F or Br) or para-phenol (Figure 2), is outlined in Schemes 4 and 5. Compounds **33a** and **33b** were synthesized from the corresponding commercially available starting materials **25a** and **25b**, respectively, in a manner similar to that described in Scheme 1. As part of the process, the amino group of **29a** and **29b** was transformed into a bromo group via a Sandmeyer reaction[22] and further converted into a methyl group by treatment with $Zn(CH_3)_2$ to yield the intermediates **31a** and **31b**.

On the other hand, **39** was synthesized from **34** according to a previously described method.[15] Compound **40** was synthesized via the Suzuki-Miyaura cross-coupling reaction

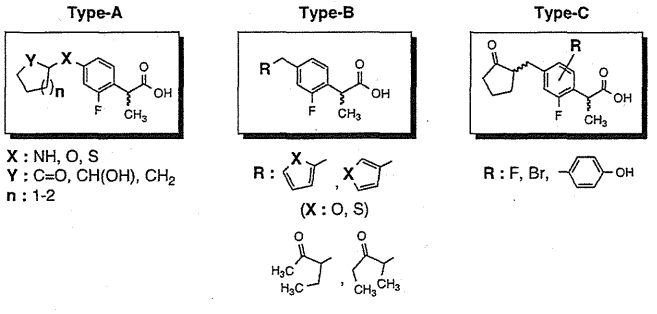


Figure 2. Structure of three types of derivative of 2-fluoroloxoprofen.

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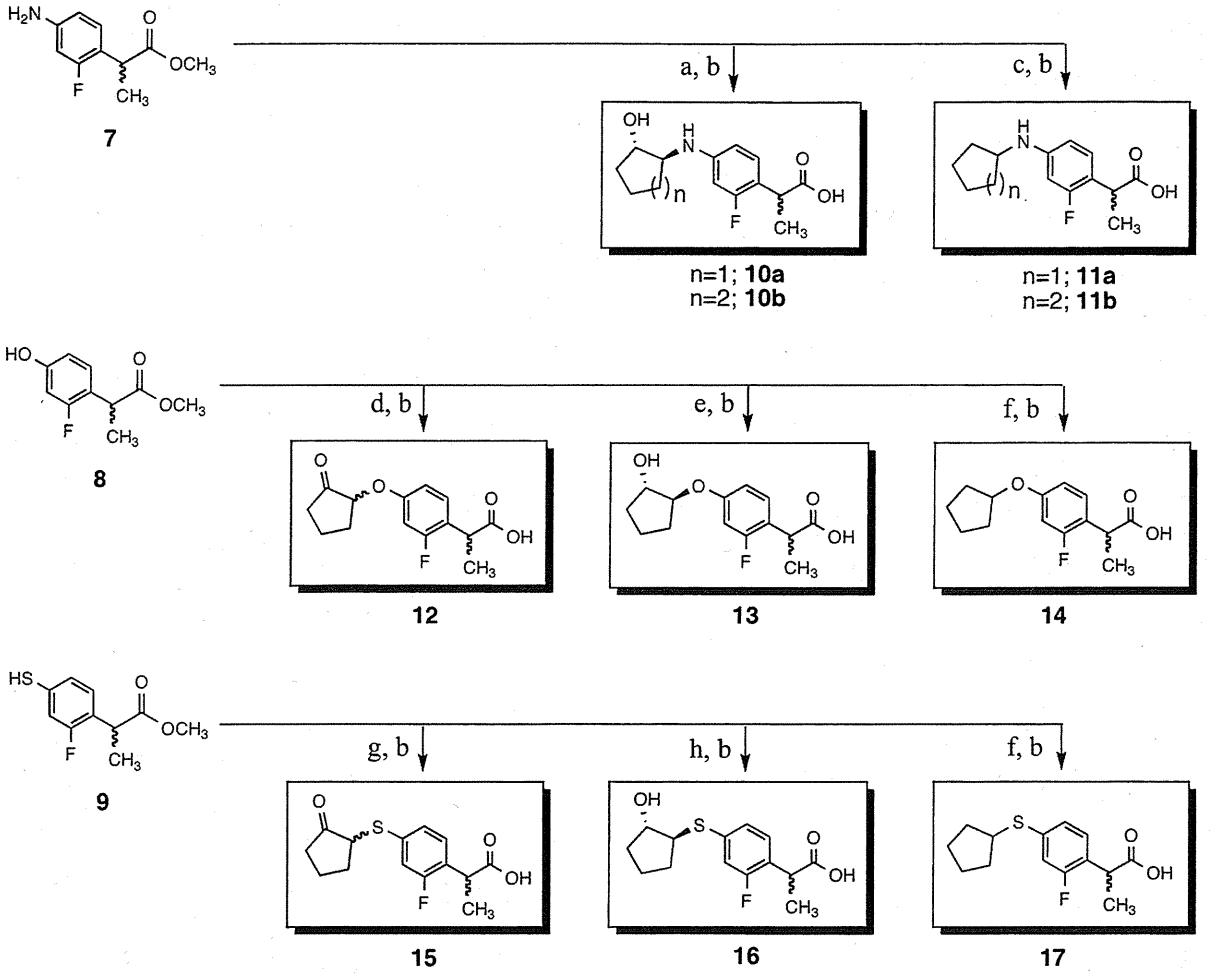
Scheme 2. Synthesis of the Type-A Target Compounds (10a, 10b, 11a, 11b, and 12–17)[a]



[a]Reagents and conditions: (a) 1,2-epoxycyclopentane or 1,2-epoxycyclohexane, LiBr, CH_2Cl_2; (b) NaOH, H_2O, MeOH, reflux; (c) cyclopentanone or cyclohexanone, $NaBH_3CN$, AcOH, MeOH; (d) chlorocyclopentanone, K_2CO_3, DMF; (e) 1,2-epoxycyclopentane, NaH, DMF; (f) bromocyclopentane, K_2CO_3,DMF; (g) cyclopentanone, NBS, CH_2Cl_2; (h) 1,2-epoxycyclopentane, borax, CH_2Cl_2.

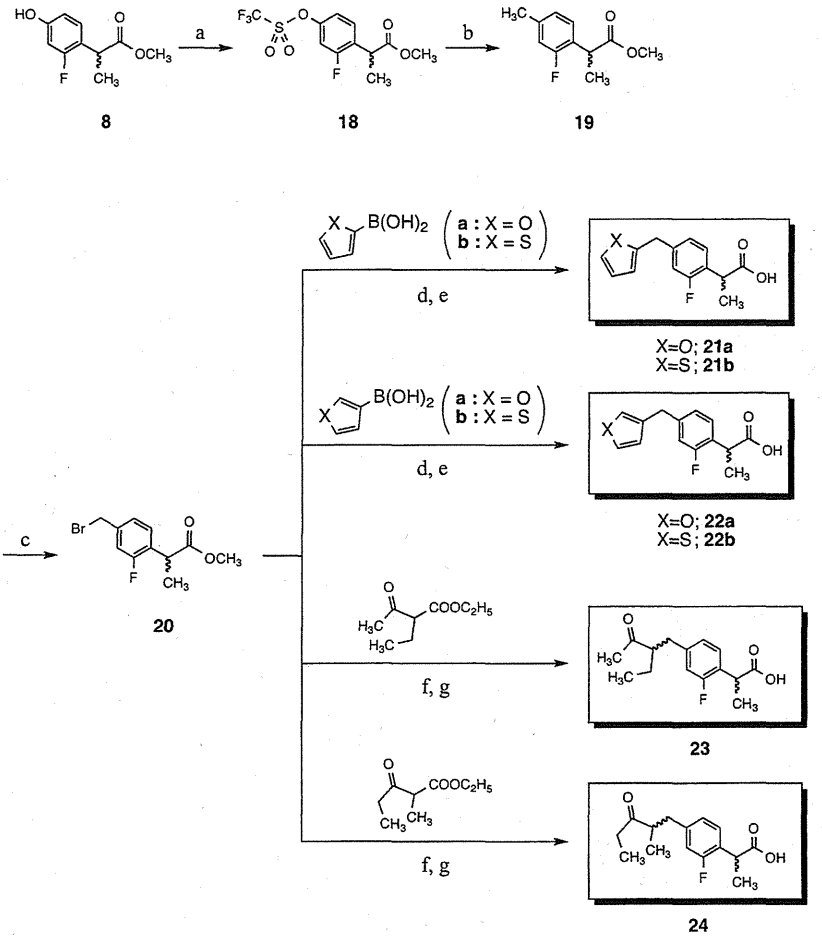
between the methyl ester of **39** and 4-hydroxyphenylboronic acid as described previously.[16]

■ RESULTS AND DISCUSSION

We examined the inhibitory effects of synthesized derivatives of **2** on COX-1 and COX-2 activity using a human whole blood COX assay. To begin with, we determined COX inhibition at derivative concentrations of 10 and 100 μM and eliminated those derivatives that did not have an inhibitory action on either COX-1 or COX-2 activity when employed at 10 μM (Table 1). We also examined the anti-inflammatory effects of all derivatives by employing a rat carrageenan-induced footpad edema assay; derivatives were administered at a dose of 37.3 μM/kg (corresponding to 10 mg/kg for **1**), and those that did not show any anti-inflammatory effect (decrease in the volume of carrageenan-induced footpad edema) were not considered as candidates for further analysis (Table 1). We then examined the inhibitory effects on COX-1 or COX-2 of the remaining derivatives at various concentrations to determine IC_{50} values (concentration of each compound required for 50% inhibition of COX-1 or COX-2 activity) and selectivity for COX-2 (Table 1). Among the derivatives, compounds **10a**, **12**, and **21b** were eliminated as candidates for further analysis because of their low anti-inflammatory activity in the carrageenan-induced footpad edema assay. We also carried out a preliminary examination of the gastric ulcerogenic activity of the derivatives and found that the oral administration of **22b** produced more gastric lesions than **2** (data not shown). This compound was therefore also discounted from further analysis. The results in Table 1 also highlight the fact that, as well as **1** and **2**, some derivatives (**11a**, **14**, **21a**, and **22a**) did not exhibit any apparent selectvity for COX-2 (the COX-1/COX-2 value of COX-2-selective inhibitor celecoxib was found to be 22.7, measured using the same methodology as that used to generate the results presented in Table 1[23]).

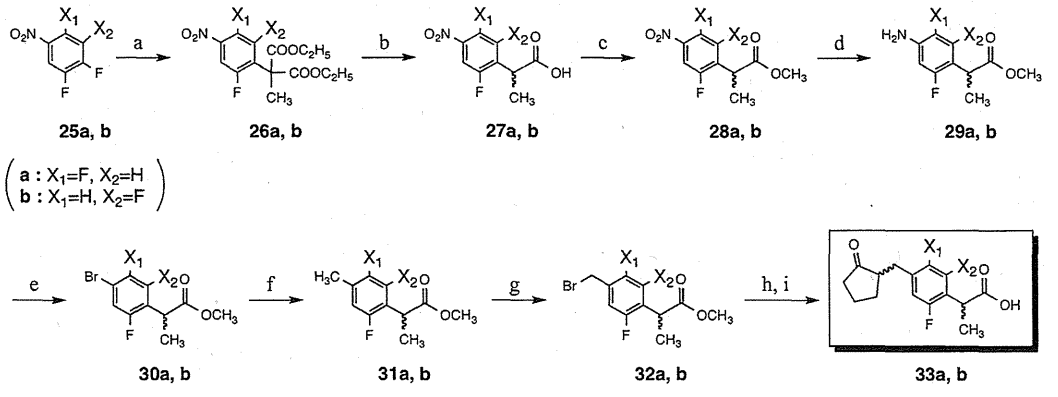
We then evaluated the anti-inflammatory effects of selected derivatives (**11a**, **14**, **21a**, and **22a**) at various doses. As shown in Figure 3, the volume of carrageenan-induced footpad edema was significantly decreased after oral administration of **1** or **2**, confirming their previously described anti-inflammatory activities.[13,15,24] Of the selected derivatives, only **11a** showed a significantly more potent anti-inflammatory activity than **2** for various doses and at different time-points after the challenge with carrageenan (Figure 3). This result suggests that **11a** could be more effective than **2** for use as an NSAID. On the other hand, **21a** showed less anti-inflammatory activity than **2** at the lowest dose, 3 h after the administration of carrageenan (Figure 3).

Scheme 3. Synthesis of the Type-B Target Compounds (21a, 21b, 22a, 22b, 23, and 24)[a]



[a]Reagents and conditions: (a) $(CF_3SO_2)_2O$, Et_3N, CH_2Cl_2; (b) $Zn(CH_3)_2$, Pd(dppe)Cl_2, 1,4-dioxane, reflux; (c) NBS, AIBN, CCl_4, reflux; (d) 3 M Na_2CO_3, *trans*-PdBr(*N*-Succ)$(PPh_3)_2$, THF, reflux; (e) KOH, H_2O, EtOH, reflux; (f) dry Na_2CO_3, dry acetone, reflux; (g) conc HCl, AcOH, reflux.

Scheme 4. Synthesis of the Type-C Target Compounds with Modification at the 2 and 5 Positions or 2 and 6 Positions of the Phenyl Ring by Fluorine (33a and 33b)[a]



[a]Reagents and conditions: (a) diethyl methylmalonate, NaOH, DMF; (b) conc H_2SO_4, AcOH, reflux; (c) MeOH, conc HCl, reflux; (d) H_2, 10% Pd/C, MeOH; (e) (i) 40% HBr, $NaNO_2$, CuBr, H_2O, (ii) MeOH, conc HCl, reflux; (f) $Zn(CH_3)_2$, Pd(dppe)Cl_2, 1,4-dioxane, reflux; (g) NBS, AIBN, CCl_4, reflux; (h) dry Na_2CO_3, methyl 2-oxocyclopentanecarboxylate, dry acetone, reflux; (i) conc HCl, AcOH, reflux.

We then evaluated the gastric ulcerogenic activity of selected derivatives. Oral administration of **2** produced fewer gastric lesions in rats than **1** (Figure 4), as described previously.[15] All of the selected derivatives showed significantly lower ulcerogenic activity than **1** (Figure 4). Furthermore, compared with **2**, compounds **11a** and **14** showed significantly lower ulcerogenic activity at the dose of 7.45 mM/kg while **21a** and **22a** had significantly lower activity at 0.37 and 0.75 mM/kg, suggesting that these compounds could be more effective than **2** as potential NSAIDs. The mechanism for this lower ulcerogenic activity of **11a** and **14**, compared to **2** is unknown at present.

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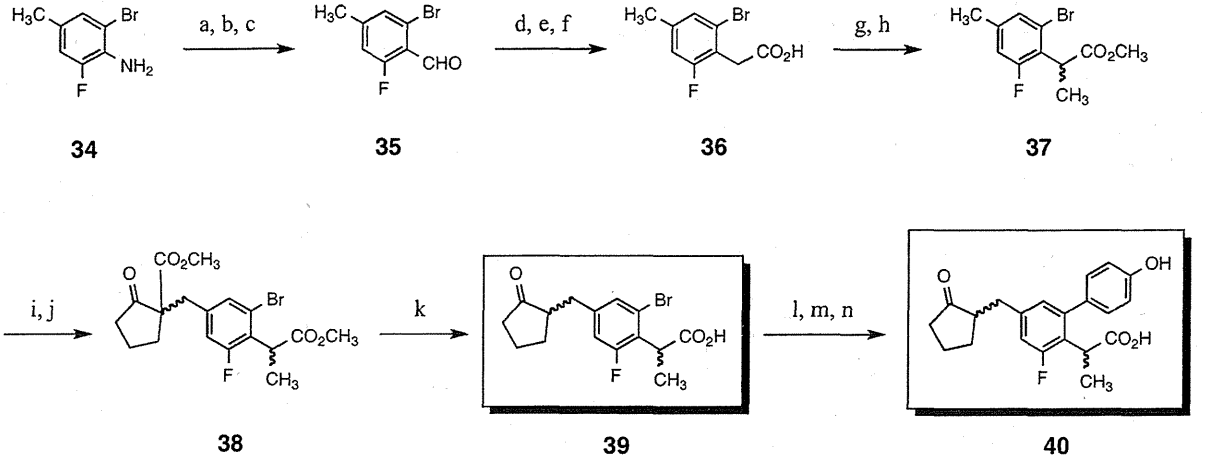
Scheme 5. Synthesis of the Type-C Target Compounds with Modification at the 2 and 6 Positions of the Phenyl Ring by Fluorine, Bromine, or the 4-Hydroxyphenyl Group (39 and 40)[a]



[a]Reagents and conditions: (a) 3 M HCl aq, $NaNO_2$, $CuSO_4$, Na_2SO_3, AcONa, H_2O, 0 °C; (b) NH_2OH·HCl, $(HCHO)_n$, AcONa, H_2O; (c) conc HCl, reflux; (d) $MeOCH_2P(Ph_3)Cl$, $C_6H_{18}KNSi_2$, toluene; (e) 3 M HCl aq, acetone, reflux; (f) PFC (2.0 mol %), H_5IO_6, acetonitrile; (g) conc HCl, CH_3OH, reflux; (h) 2.0 M LDA, CH_3I, dry THF, −78 to −40 °C; (i) NBS, AIBN, CCl_4, reflux; (j) dry Na_2CO_3, methyl 2-oxocyclopentanecarboxylate, dry acetone, reflux; (k) conc HCl, AcOH, reflux; (l) 4-DMAP, EDC, CH_3OH; (m) HO-  -$B(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, THF, reflux; (n) KOH, C_2H_5OH, H_2O, reflux.

Table 1. Experimental Results of in Vitro Human Whole Blood Assay for Inhibition of COX-1- and COX-2-Derived PG Biosynthesis, And in Vivo Anti-Inflammatory Assay by Carrageenan-Induced Rat Paw Edema[a]

	COX-1 inhibition (%)		COX-2 inhibition (%)		IC_{50} (μM)			reduction in paw edema (%)	
compd	10 μM	100 μM	10 μM	100 μM	COX-1	COX-2	COX-1/COX-2	3 h	6 h
1	35.0 ± 5.9	87.5 ± 2.4	53.2 ± 5.9	97.4 ± 1.2	23.5[b] ± 4.8[b]	10.1[b] ± 1.3[b]	2.3	44.0 ± 0.2	53.1 ± 2.5
2	32.7 ± 5.1	85.7 ± 2.2	44.2 ± 2.4	81.8 ± 7.0	24.2[b] ± 8.6[b]	14.3[b] ± 6.8[b]	1.7	41.8 ± 0.4	54.1 ± 0.4
10a	54.6 ± 0.9	83.4 ± 1.1	45.3 ± 1.7	77.6 ± 1.7	9.0 ± 0.8	14.0 ± 0.4	0.7	20.6 ± 9.3	5.7 ± 0.3
10b	0	0	28.3 ± 3.7	76.7 ± 1.9				<0	<0
11a	34.7 ± 0.6	92.0 ± 2.3	40.7 ± 2.8	91.9 ± 1.6	15.6 ± 0.5	21.3 ± 2.8	0.7	45.5 ± 1.0	60.1 ± 1.9
11b	0	0	0	30.0 ± 7.2				<0	19.2 ± 4.3
12	80.6 ± 2.8	82.3 ± 1.5	27.6 ± 3.3	82.5 ± 0.5	1.5 ± 0.1	24.1 ± 6.2	0.1	30.3 ± 0.3	25.0 ± 4.2
13	10.4 ± 0.7	78.2 ± 2.2	25.4 ± 8.3	80.2 ± 7.0				<0	13.3 ± 4.8
14	65.0 ± 5.5	81.4 ± 0.9	39.7 ± 2.0	96.4 ± 1.1	3.0 ± 0.2	26.3 ± 8.8	0.1	40.4 ± 1.9	51.9 ± 6.1
15	0	60.7 ± 1.7	55.5 ± 7.4	93.1 ± 3.0				38.0 ± 1.1	27.2 ± 7.0
16	0	47.6 ± 4.6	0	64.4 ± 5.1				<0	<0
17	4.7 ± 0.7	87.9 ± 0.2	15.6 ± 3.0	80.1 ± 1.8				<0	9.5 ± 0.6
21a	26.3 ± 2.6	79.9 ± 6.0	64.1 ± 1.8	96.1 ± 2.1	21.6 ± 7.5	4.1 ± 2.8	5.3	38.9 ± 6.6	45.8 ± 3.5
21b	27.4 ± 1.1	76.5 ± 1.6	45.8 ± 0.6	89.2 ± 2.8	19.9 ± 5.7	13.0 ± 1.9	1.5	12.5 ± 8.1	28.0 ± 7.3
22a	30.9 ± 2.0	57.5 ± 5.6	58.7 ± 6.2	90.9 ± 2.2	30.1 ± 8.6	4.0 ± 1.1	7.6	32.7 ± 2.5	45.2 ± 0.01
22b	53.9 ± 2.0	87.4 ± 3.4	60.2 ± 4.4	99.5 ± 0.5	9.5 ± 1.4	8.3 ± 2.8	1.2	40.2 ± 3.1	33.4 ± 0.2
23	78.0 ± 6.5	98.1 ± 0.3	0	82.2 ± 2.9				20.9 ± 6.4	20.2 ± 6.7
24	0	13.5 ± 1.9	0	0				<0	<0
33a	0	81.0 ± 0.4	15.0 ± 4.9	84.8 ± 4.1				<0	<0
33b	11.3 ± 1.0	79.3 ± 5.6	0	70.9 ± 6.7				21.6 ± 2.6	17.7 ± 9.6
39	13.8 ± 2.6	49.7 ± 6.6	0	0				<0	<0
40	0	7.2 ± 1.0	15.4 ± 0.3	38.0 ± 3.2				<0	<0

[a]The inhibitory effect of each compound on COX-1- and COX-2-derived PG biosynthesis was measured. The relative inhibition of COX-1 or COX-2 (%) at 10 and 100 μM, IC_{50} values (concentration of each compound required for 50% inhibition of COX-1 or COX-2), and the COX-1/COX-2 ratio of IC_{50} values are shown. The values of IC_{50} were estimated from the sigmoid-like dose−response curve (four-parameter logistic curve model) drawn with the aid of logistic-curve fitting software (ImageJ, version 1.43u; National Institutes of Health, U.S.). Values are the mean ± SEM (n = 3−6). For the in vivo anti-inflammatory assay, rats were orally administered 37.3 μM/kg test compound and 1 h later received an intradermal injection of carrageenan (1%) into the left hindpaw. Footpad edema was measured 3 and 6 h after the administration of carrageenan, and the relative inhibition of the increase in edema volume by each compound was determined. Values are the mean ± SEM (n = 3−6). [b]Data from our previous report.[15]

Finally, the orientation of the selected derivatives in COX-2 and the interaction between these derivatives and amino acid residues in the active site of COX-2 were examined by molecular modeling and docking studies. Since **2** is a prodrug,[15] the

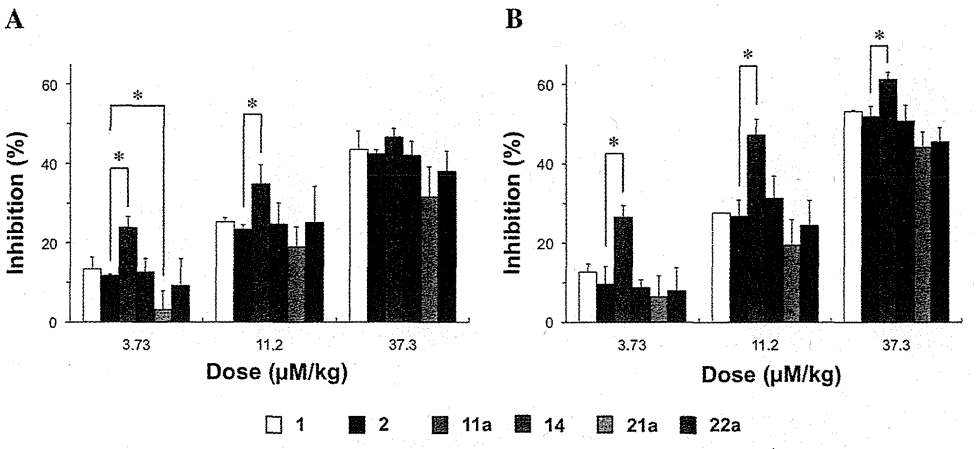


Figure 3. Anti-inflammatory activities of loxoprofen (**1**), 2-fluoroloxoprofen (**2**), and the latter's derivatives (**11a**, **14**, **21a**, and **22a**). Rats were orally administered 3.73, 11.2, or 37.3 μM/kg test compound and 1 h later received an intradermal injection of carrageenan (1%) into the left hindpaw. Footpad edema was measured 3 h (A) and 6 h (B) after the administration of carrageenan, and the relative inhibition of the increase in edema volume by each compound was determined. Values are the mean ± SEM (n = 3–6): (*) $P < 0.05$.

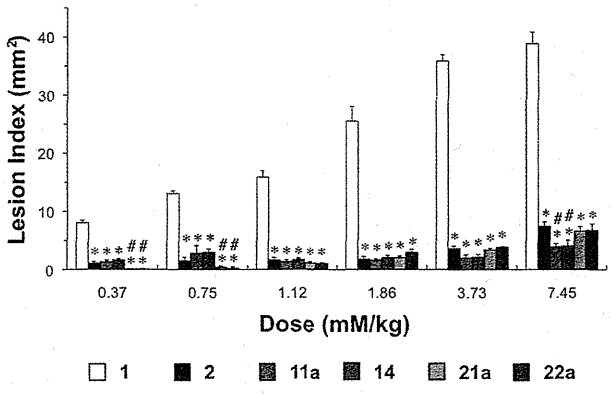


Figure 4. Production of gastric lesions in the presence of loxoprofen (**1**), 2-fluoroloxoprofen (**2**), and the latter's derivatives (**11a**, **14**, **21a**, and **22a**). Rats were orally administered 0.37, 0.75, 1.12, 1.86, 3.73, and 7.45 mM/kg test compound, and their stomachs were removed after 8 h. Stomachs were scored for hemorrhagic damage. Values are the mean ± SEM (n = 3–6): (*) $P < 0.05$ (vs **1**); (#) $P < 0.05$ (vs **2**).

active metabolite (2-[2-fluoro-4-{(2-hydroxycyclopentyl)-methyl}phenyl]propanoic acid) was subjected to the analysis. We recently reported results for **1** by this analysis, which showed that the cyclopentanone ring interacts with Y385 and S530, whereas propanoic acid interacts with R120 and Y355.[16] All of these amino acids were reported to be important for the interaction between COXs and NSAIDs.[25,26] Similar orientation and interactions were observed for **2** and its selected derivatives in this study (Figure 5).

We have thus not only identified here interesting and beneficial NSAIDs (see Conclusion) but also suggested structure–activity relationships of **2** for COX inhibition and anti-inflammatory effects, as follows:

For type-A derivatives, as described above, **2** is a prodrug and the trans-alcohol form of **2** showed a more potent inhibitory effect on both COX-1 and COX-2 activity than **2**.[15] However, **13** and **16**, corresponding to the trans-alcohol form of **12** and **15**, respectively, showed a weaker inhibitory effect on both COX-1 and COX-2 than **12** and **15**, respectively (Table 1). Thus, the alteration in bridge heteroatom (O or S) between the two rings may result in the disappearance of **2**'s property as a prodrug.

For type-B derivatives, **21a** and **22a** showed COX-inhibition and anti-inflammatory effects equivalent to **2**, suggesting that the furan ring can become a bioisostere of the cyclopentanone ring of **2**. On the other hand, **23** and **24** showed very weak COX-inhibition and anti-inflammatory effects, suggesting that the closed circular ring in **2** is important for its COX-inhibition and anti-inflammatory effects.

We previously reported that, as well as **2**, oral administration into rats of 2-bromoloxoprofen or 2-*p*-hydroxyphenylloxoprofen produced fewer gastric lesions but showed an equivalent anti-inflammatory effect compared to **1**.[15,16] Therefore, we examined here the effect of a similar modification of the aromatic ring of **2** by F, Br, or *p*-phenol on the anti-inflammatory effect of **2** (type-C derivatives). However, **33a**, **33b**, **39**, and **40** showed weaker COX inhibition and anti-inflammatory effects than **2**, suggesting that the introduction of a substituted group into the aromatic ring of **1** should be restricted to one position in order to maintain its anti-inflammatory activity.

■ CONCLUSION

Compound **11a** was found to have a more potent anti-inflammatory effect and an equivalent gastric ulcerogenic activity compared with **2**. Furthermore, as for **2**, **11a** has no apparent selectivity for COX-2. Thus, we consider that **11a** could be therapeutically beneficial for clinical use as an NSAID.

■ EXPERIMENTAL SECTION

The purity of the final compounds was greater than 95% as judged by HPLC (for details, see Supporting Information).

2-[4-(Cyclopentylamino)-2-fluorophenyl]propanoic Acid (11a). To a solution of **7** (1.50 g, 7.6 mmol) in a mixture of MeOH (10 mL) and AcOH (0.2 mL) were added cyclopentanone (1.4 mL, 15.2 mmol) and sodium cyanoborohydride ($NaBH_3CN$) (0.96 g, 15.2 mmol). The solution was stirred at room temperature for 12 h. The reaction mixture was evaporated to dryness, extracted with CH_2Cl_2, dried over anhydrous Na_2SO_4, and filtered. The filtrate was evaporated to dryness and the residue was purified on silica gel chromatography (*n*-hexane/AcOEt, 3:2) to afford the methyl ester precursor of **11a**. Hydrolysis with NaOH was done to give final compound **11a** as a brown powder solid (1.09 g, 54%). ^{1}H NMR ($CDCl_3$) δ: 1.28 (6H, d, J = 7.0 Hz), 1.33–1.68 (6H, m), 1.79–1.91 (2H, m), 3.56–3.64 (1H, m), 3.70 (1H, q, J = 7.3 Hz), 6.17–6.30 (2H, m), 6.90 (1H, t, J = 8.8 Hz). ^{13}C NMR ($CDCl_3$) δ: 18.1, 25.0, 33.9, 39.0, 55.7, 100.3, 110.4, 116.1, 116.3, 129.8, 130.0, 150.8, 161.2

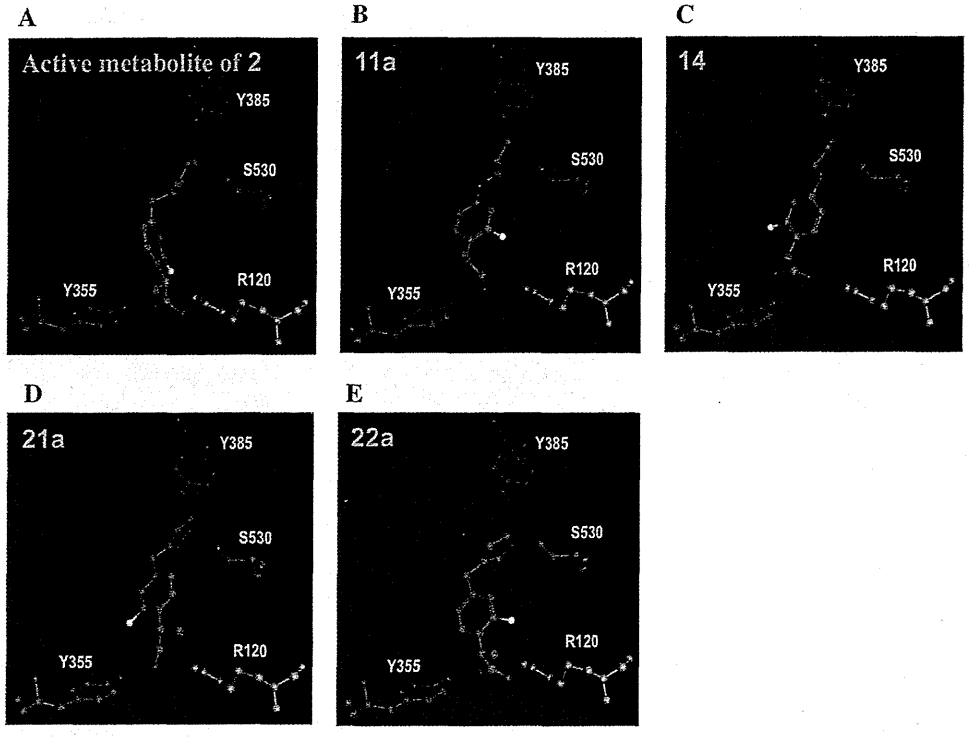


Figure 5. Potential binding mode of the active metabolite of **2** (A), **11a** (B), **14** (C), **21a** (D), and **22a** (E) to the active site of murine COX-2. Hydrogen atoms of the amino acid residues and the ligand have been removed.

(d, J_{C-F} = 242 Hz), 178.4. HR-FAB-MS (*m/z*): 251.1324 (M^+, calcd for $C_{14}H_{18}FNO_2$, 251.1322). Anal. Calcd for $C_{14}H_{18}FNO_2$: C, 66.91; H, 7.22; N, 5,57. Found: C, 67.05; H, 7.24; N, 5,46.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PG, prostaglandin; NBS, *N*-bromosuccinimide; DMF, *N,N*-dimethylformamide; THF, tetrahydrofuran

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Development of NSAIDs with Lower Gastric Side Effect

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Abstract

The anti-inflammatory action of nonsteroidal anti-inflammatory drugs (NSAIDs) is mediated through their inhibitory effects on cyclooxygenase (COX) activity. On the other hand, NSAIDs use is associated with gastrointestinal complications. The inhibition of COX by NSAIDs is not the sole explanation for the gastrointestinal side effects of NSAIDs. In this article, I review our recent work on the COX-independent mechanism involved in NSAID-induced gastric lesions. Using DNA microarray analysis, we found that NSAIDs affect expression of various genes in a COX-independent manner and found that membrane permeabilization activity of NSAIDs and resulting NSAID-induced apoptosis are involved in NSAID-induced gastric lesions. These results suggest that NSAIDs with lower membrane permeabilization activity would be safer on stomach tissue and we found that loxoprofen, a clinically used NSAID, has relatively lower membrane permeabilization activity than other NSAIDs. We synthesized derivatives of loxoprofen and found that fluoro-loxoprofen has lower membrane permeabilization activity and their oral administration produced fewer gastric lesions but showed an equivalent anti-inflammatory effect. These results suggest that fluoro-loxoprofen is likely to be therapeutically beneficial as safer NSAIDs.

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Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most frequently used classes of medicines in the world. An inhibitory effect of NSAIDs on cyclooxygenase (COX) activity is responsible for their anti-inflammatory actions because COX is an enzyme essential for the synthesis of prostaglandins (PGs), such as PGE_2, which have a strong capacity to induce inflammation. However, NSAIDs administration is associated with gastrointestinal complications, such as gastric ulcers and bleeding, which sometimes become life-threatening diseases [1]. Therefore, the molecular mechanism governing NSAID-induced gastrointestinal damage needs to be elucidated in order to develop new NSAIDs that do not have these side effects.

Inhibition of COX by NSAIDs was previously thought to be fully responsible for their gastrointestinal side effects [2], because PGE_2 has a strong protective effect

on gastrointestinal mucosa [2]. There are at least two subtypes of COX, COX-1 and COX-2, which are responsible for the majority of COX activity at the gastric mucosa and tissues with inflammation, respectively [3, 4]. Therefore, it is reasonable to speculate that selective COX-2 inhibitors have anti-inflammatory activity without gastrointestinal side effects. In fact, a greatly reduced incidence of gastroduodenal lesions was reported for selective COX-2 inhibitors (such as celecoxib and rofecoxib) both in animal and clinical data [5]. However, the increased incidence of gastrointestinal lesions and the decrease in PG levels induced by NSAIDs are not always linked with each other. For example, higher doses of NSAIDs were required for producing gastric lesions than were required for inhibiting COX at the gastric mucosa [6]. Understanding the additional mechanisms is necessary in order to establish an alternative method for development of gastrointestinally safe NSAIDs other than simply increasing their COX-2 selectivity. This new class of NSAIDs may be clinically beneficial because clinical disadvantages (i.e. risk of cardiovascular thrombotic disease) of selective COX-2 inhibitors were recently suggested [7, 8].

In this study, I review our recent work on COX-independent actions of NSAIDs in order to understand the molecular mechanism for NSAID-associated gastrointestinal complications. Results suggest that NSAIDs affect expression of various genes in a COX-independent manner, which is involved in NSAID-associated gastrointestinal complications. These studies also provide useful information about development of new types of NSAIDs with lower gastrointestinal side effects.

Results and Discussion

Direct Cytotoxic Effect of NSAIDs

A number of previous reports suggested that NSAIDs are cytotoxic. Thus, using the primary culture of guinea pig gastric mucosal cells that well mimic the gastric mucosal cells in vivo, we examined effect of NSAIDs on cell death. We found that short-term or long-term treatments of gastric mucosal cells with NSAIDs induce necrosis or apoptosis, respectively [9, 10]. In order to test whether the cytotoxic effect of NSAIDs (necrosis and apoptosis) is dependent of their ability to inhibit COX, we examined the effect of exogenously added PGE_2 on necrosis and apoptosis induced by NSAIDs. Exogenously added PGE_2 did not affect the extent of NSAID-induced necrosis or apoptosis even at higher concentrations of PGE_2 than is present endogenously in medium [11]. Results suggest that the cytotoxic effect of NSAIDs (necrosis and apoptosis) is independent of their ability to inhibit COX [11].

Membrane Permeabilization Activity of NSAIDs

Based on structure of NSAIDs, we hypothesized that NSAIDs have membrane permeabilization activity and this activity is involved in the cytotoxic effect of NSAIDs, in

other words, the primary target of NSAIDs is the membrane. We examined membrane permeabilization activity of more than 10 NSAIDs (nimesulide, celecoxib, mefenamic acid, flufenamic acid, flurbiprofen, indomethacin, diclofenac, etodolac, ibuprofen and ketoprofen) using calcein-loaded liposomes [12, 13]. Calcein fluoresces very weakly at high concentrations due to self-quenching, so the addition of membrane permeabilizing drugs to a medium containing calcein-loaded liposomes should cause an increase in fluorescence by diluting out the calcein [12]. All of the NSAIDs tested increased the calcein fluorescence, suggesting that they have membrane permeabilization activity [12, 13]. We also examined the necrosis- and apoptosis-inducing ability of these NSAIDs. To examine the relationship between NSAID-induced necrosis or apoptosis and membrane permeabilization, we determined ED_{50} values of the 10 NSAIDs for necrosis or apoptosis (concentrations of NSAIDs required for 50% inhibition of cell viability by necrosis or apoptosis) and ED_{20} values for membrane permeabilization (concentration of NSAIDs required for 20% release of calcein). Plotting ED_{50} values for necrosis or apoptosis versus ED_{20} values for membrane permeabilization (calcein release) yielded an r^2 value of 0.94 or 0.93, respectively, which suggests that NSAID-induced necrosis and apoptosis is mediated by their ability to permeabilize membranes.

DNA Microarray Analysis

It is believed that necrosis is induced by drastic permeabilization of cytoplasmic membranes; however, the mechanism how membrane permeabilization activity of NSAIDs induces apoptosis remains unclear. In order to understand the molecular mechanism governing this apoptosis, we searched for genes whose expression is induced by indomethacin using DNA microarray analysis and found that CHOP, a transcription factor with apoptosis-inducing ability is induced by various NSAIDs [14–16]. In order to test whether the induction of CHOP by indomethacin is involved in indomethacin-induced apoptosis, we used CHOP-deficient mice. Indomethacin-induced chromatin condensation was observed in peritoneal macrophages from wild-type mice but not so apparently in those from CHOP-deficient mice [15]. This result strongly suggests that the induction of CHOP is involved in NSAIDs-induced apoptosis.

Contribution of the Increase in Intracellular Ca^{2+} Level and Mitochondrial Dysfunction to NSAID-Induced Apoptosis

Permeabilization of cytoplasmic membranes causes an increase in intracellular Ca^{2+} levels by stimulating Ca^{2+} influx across the cytoplasmic membrane and we showed that all of the NSAIDs tested increase the intracellular Ca^{2+} level [13]. We used BAPTA-AM, an intracellular Ca^{2+} chelator that is permeable for cytoplasmic membranes to test the contribution of the increase to NSAID-induced apoptosis. BAPTA-AM inhibited NSAID-induced cell death, apoptotic chromatin condensation and induction of CHOP [13], suggesting that the increase in intracellular

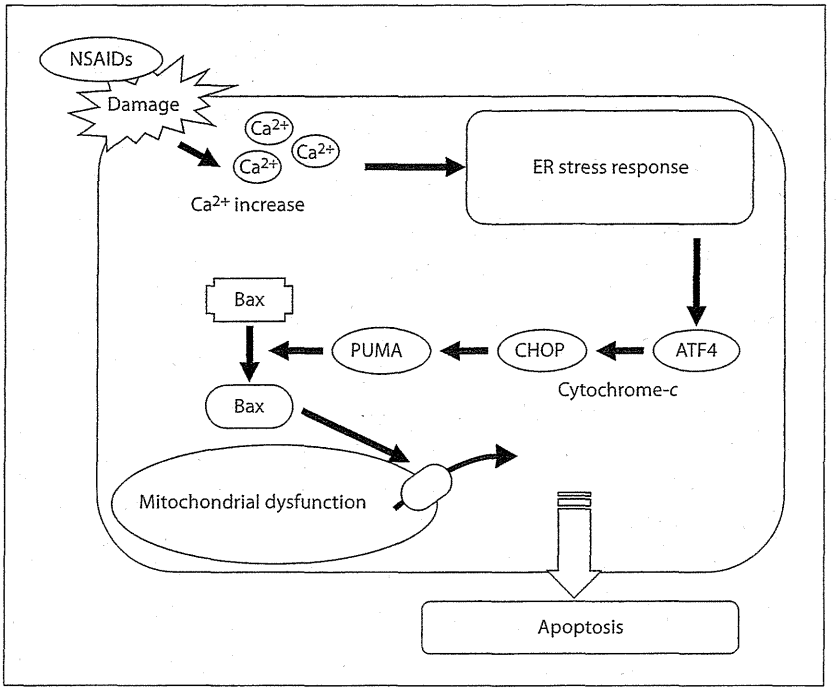

Fig. 1. Molecular mechanism for NSAID-induced apoptosis.

Ca^{2+} levels caused by NSAIDs is involved in NSAID-induced CHOP induction and resulting apoptosis.

CHOP is induced by endoplasmic reticulum (ER) stress response. Accumulation of unfolded protein in the ER induces the ER stress response. On the other hand, PUMA (p53 upregulated modulator of apoptosis) is a BH3 only domain protein with potent apoptosis-inducing activity [17, 18]. PUMA stimulates conformational change, translocation and multimerization of Bax that permeabilizes the mitochondrial outer membrane, resulting in mitochondrial dysfunction and apoptosis [19]. We found that various NSAIDs upregulate PUMA [20]. Furthermore, we suggested that NSAID-induced upregulation of PUMA is mediated through an increase in the intracellular Ca^{2+} level, upregulation of ATF4 and CHOP [20].

Based on these results, we proposed the following pathway for NSAID-induced apoptosis (fig. 1). Permeabilization of cytoplasmic membranes by NSAIDs stimulates Ca^{2+} influx and increases intracellular Ca^{2+} levels, which in turn induces the ER stress response. In the ER stress response, expression of CHOP and ATF4 are induced to induce the expression PUMA and the resulting translocation and activation of Bax. Bax plays an important role in NSAID-induced mitochondrial dysfunction, activation of caspases and apoptosis.

Contribution of Mucosal Cell Death to NSAID-Induced Gastric Lesions

We considered that not only COX inhibition (inhibition of PG synthesis) but also the COX-independent direct cytotoxic effect of NSAIDs is involved in the development of gastrointestinal lesions in vivo. For testing this idea by pharmacological experiments, it is necessary to separate these two properties of NSAIDs (i.e. COX inhibition and direct cytotoxicity) in the model of NSAID-induced gastric lesions in vivo. We tried to achieve this by employing intravenous administration of a nonselective NSAID (indomethacin) and oral administration of cytotoxic selective COX-2 inhibitors (such as celecoxib) in rats. Intravenous administration of nonselective NSAIDs may cause inhibition of both COX-1 and COX-2 (thus inhibition of PG synthesis) at the gastric mucosa without any direct cytotoxicity to the gastric mucosa, because the concentration of NSAIDs at the gastric mucosa following intravenous administration is much lower compared to when NSAIDs are orally administered. On the other hand, oral administration of celecoxib may cause direct cytotoxicity to the gastric mucosa without inhibition of COX-1 and thus PG synthesis may be maintained.

Intravenously administered indomethacin, which completely inhibited COX activity at the gastric mucosa, did not produce gastric lesions. Orally administered celecoxib did not also produce gastric lesions. Interestingly, a combination of the oral administration of celecoxib with the intravenous administration of indomethacin clearly produced gastric lesions [11]. These results suggest that in addition to COX-inhibition by NSAIDs, direct cytotoxicity of NSAIDs is involved in NSAID-induced gastric lesions.

Development of NSAIDs with Lower Gastrointestinal Side Effects

The results described above suggest that NSAIDs without membrane permeabilizing activity have reduced gastrointestinal side effects. Loxoprofen (LOX) has been used clinically for a long time as a standard NSAID in Japan, and clinical studies have suggested that it is safer than other NSAIDs, such as indomethacin [21, 22]. We found that LOX has relatively lower membrane permeabilization activity than other NSAIDs [23].

We then synthesized a series of LOX derivatives, examined their membrane permeabilization activities and selected fluoro-loxoprofen (F-LOX). Membrane permeabilization assay by use of calcein-loaded liposomes revealed that F-LOX has much lower membrane permeabilization activity than LOX [24].

The COX inhibition assay in vitro revealed that F-LOX showed IC_{50} values for COX-1- or COX-2-derived PG biosynthesis that are similar to LOX and that as for LOX, F-LOX did not exhibit apparent selectivity for COX-2 [24].

We then evaluated the activities of F-LOX in vivo. LOX (40 or 50 mg/kg) and equivalent molar amounts of F-LOX were orally administered to rats and the lesion index was calculated. Administration of LOX produced gastric lesions in a dose-dependent manner (fig. 2a). F-LOX produced fewer gastric lesions than

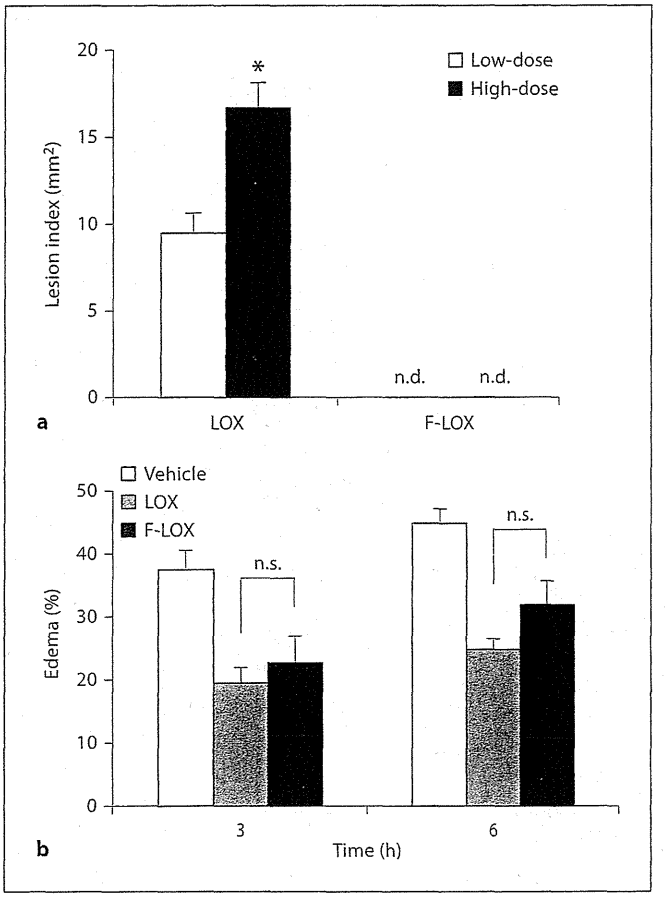

Fig. 2. a,b Ulcer-inducing and anti-inflammatory effects of LOX and F-LOX.

LOX (fig. 2a). This is chemical evidence in support of our proposal that the membrane permeabilization activity of NSAIDs is involved in their induction of gastric lesions.

Finally, we compared the anti-inflammatory effects of F-LOX to LOX by employing a rat carrageenan-induced footpad edema assay. As shown in figure 2b, the volume of carrageenan-induced footpad edema was significantly decreased after oral administration of LOX. The effects of LOX were much the same as that of LOX, showing that F-LOX has anti-inflammatory activity equivalent to LOX.

Conclusion

The results of our recent studies reviewed in this article suggest that both COX-dependent and COX-independent mechanisms are involved in NSAID-induced gastrointestinal complications. We also found that F-LOX showed very low gastric

lesion-inducing activity in rats, although it has no apparent selectivity for COX-2. Thus, we consider that F-LOX has likely to be therapeutically beneficial NSAID in terms of gastrointestinal and cardiovascular safety.

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