Pharmacological Studies on 3-Formylamino-7-methylsulfonylamino-6-phenoxy-4H-1-benzopyran-4-one (T-614), a Novel Antiinflammatory Agent. 3rd Communication: The Involvement of Bradykinin in Its Analgesic Actions

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In order to elucidate the analgesic mechanism of 3-formylamino-7-methylsulfonylamino-6-phenoxy-4H-1-benzopyran-4-one (T-614), its effects on the kinin-forming system were examined both in vivo and in vitro. T-614, at doses more than 10 mg/kg p.o., exhibited a significant inhibitory effect on the increased levels of bradykinin released into the pouch fluid of kaolin-induced inflammation in rats. In the kaolin-induced writhing response in mice, which is shown to be mainly dependent on the action of bradykinin, T-614 reduced not only the writhing frequency but also the peritoneal levels of bradykinin in a dose-dependent manner. Whereas, in the zymosan-induced writhing response in which prostaglandin I$_2$ (PGI$_2$) is shown to be an important mediator, it did not exert an obvious inhibition on either writhing responses or peritoneal PGI$_2$ levels at a highest dose of 100 mg/kg. T-614 did not inhibit the activities of serine proteases, such as trypsin, thrombin, kallikrein and plasmin. Furthermore, it did not affect the kinin-forming enzymes of rat plasma in vitro. The above results suggest that the analgesic effects of T-614 may be partly mediated by the inhibition of bradykinin release in the local inflamed tissue.

Keywords — 3-formylamino-7-methylsulfonylamino-6-phenoxy-4H-1-benzopyran-4-one (T-614); antiinflammatory agent; analgesic activity; bradykinin; prostaglandin; writhing response

Introduction

3-Formylamino-7-methylsulfonylamino-6-phenoxy-4H-1-benzopyran-4-one (T-614; CAS 123663-49-0), a novel class of antiinflammatory compounds, was selected from a series of chromone derivatives, in which a methylsulfonylamino group is introduced, on the basis of its potent antiinflammatory activities without any gastrointestinal ulcerogenicity.\(^1\) Previous studies\(^2\) have shown that although the analgesic activity of T-614 was hardly demonstrated in writhing tests in mice, its potency against the inflammatory pain such as the hyperalgesia induced by yeast or adjuvant injection into rat paw was superior to that of ibuprofen. Moreover, T-614 was found to be a weak cyclooxygenase inhibitor in a prostaglandin (PG) synthetase assay using rabbit renal microsomes, and to be scarcely active in inhibiting the gastric PG production, however, it has effectively reduced the PG levels in inflammatory exudates in rats.\(^3\)

On the other hand, non-steroidal antiinflammatory drugs (NSAIDs) are believed to exert their analgesic effects via the inhibition of PG production.\(^4\) PGs, at physiological concentrations, seem not to be direct mediators of nociception and to elevate the sensitivity of pain nerve endings and receptors to algesic stimuli. The state of hyperalgesia has been considered to be brought about by the release of other chemical mediators with PGs, resulting in intensification of the receptive power of the peripheral pain sense. Kinins including bradykinin are effective candidates for the direct, endogenous mediators of the nociceptive receptors.\(^5\)

Therefore, in order to search the analgesic mechanisms other than the inhibition of PG synthesis, the effects of T-614 on the kinin-forming system were investigated in vivo and in vitro. In particular its effects on bradykinin levels in sites of the inflammatory reaction such as writhing responses were estimated.

Materials and Methods

Animals — The animals used were male Wistar rats (6—7 weeks) and male ICR mice (4—5 weeks) purchased from Japan Slc Inc. (Hamamatsu, Japan). Before use, these animals
were housed under conditions of constant temperature and controlled illumination, and given food and water ad libitum.

**Agents Used** — T-614 was synthesized in our laboratories. Other test compounds used were nimesulide (synthesized in our laboratories), indomethacin (Sigma Chemical Co.), ibuprofen, (Toyama Chemical Co.), and gabexate mesilate (FOY, Ono Pharmaceutical Co.). All test compounds were suspended in 0.5% sodium carboxymethylcellulose (CMCNa) solution for oral administration to animals or dissolved in dimethylsulfoxide (DMSO) for in vitro experiments.

**Kaolin-Induced Air Pouch Inflammation** — A modification of the method of Kumakura and Tsurufuji was employed and 8—10 rats (175—220 g) were used for each dose group. Briefly, air-pouch type inflammation was induced by subcutaneous injection of 4 ml of 1% kaolin (Wako Pure Chemical Co.) suspension in 0.8% CMCNa solution containing 0.2 mg captopril (Sankyo Co.) as a kininase II inhibitor into a preformed air pouch on the dorsum of rats. After 20 min, the animals were sacrificed by exsanguination under ether anesthesia and the pouch fluid was collected. Test compounds were orally administered 2 h before the injection of kaolin suspension. The content of bradykinin in pouch fluid was determined by using an enzymeimmunoassay (ELISA) kit (MARKIT-A bradykinin, Dainippon Pharmaceutical Co.) after the ethanol extraction and prostaglandin E2 (PGE2) levels in these samples were determined by a radioimmunoassay after the extraction according to the method previously reported.

**Kaolin-Induced Writhing Response** — The method of Fujiyoshi et al. was used with a slight modification. Groups of 9—10 mice (4 weeks old) were given test compounds orally. After 30 min, 0.5 ml of 0.5% kaolin suspension in saline containing 0.05 mg of captopril was intraperitoneally injected. The number of writhes was counted for 15 min immediately after the injection. Then, mice were sacrificed using a rising concentration of carbon dioxide and intraperitoneally injected with 1.0 ml of ice-cold saline. After gentle massage, the peritoneal cavity was exposed through a 10—15 mm incision and the lavage fluid was collected by pipette. Each recovered lavage fluid was extracted with ethanol and ELISA of bradykinin was performed as described above.

**Zymosan-Induced Writhing Response** — The method of Berkenkopf and Weichman was employed and groups of 6—10 mice (5 weeks old) were given test compounds orally. After 1 h, 0.5 ml of 0.2% zymosan A (Sigma) suspension in saline was intraperitoneally injected. The number of writhes was counted for 15 min starting 5 min after zymosan injection. The animals were then sacrificed to collect peritoneal fluid samples. The content of 6-keto-prostaglandin F1α (6-keto-PGF1α, a stable metabolite of PGF2α) in each fluid was determined by a radioimmunoassay according to the method previously reported.

**Effect on Activities of Several Serine Proteases** — Bovine pancreatic trypsin (P-L Biochemicals Inc.) was dissolved in 25 mM borate buffer (pH 8.5) containing 10 mM CaCl2. Human thrombin (Green Cross Co.) was dissolved in 1/15 M phosphate buffer (pH 7.4). Porcine pancreatic kallikrein (Sigma) and plasma plasmin (Sigma) were dissolved in 25 mM borate buffer (pH 8.5). The rates of hydrolysis of Nα-tosyl-L-arginine methyl ester (TAME, Peptide Institute Inc.) by above enzymes were determined according to the method of Muramatsu and Fujii at a substrate concentration of 10 mM. For measurement of inhibitory effects, mixtures of enzyme solution and test compound dissolved in DMSO (final concentration of DMSO: 2%) were preincubated at 37 °C for 5 min and then residual enzyme activity was determined.

**Effect on Activation and Activity of Prekallikrein in Rat Plasma** — A blood sample was collected from normal rats (230—260 g) into a polyethylene syringe containing 3.8% sodium citrate (1/10 volume) and centrifuged at 3000 rpm for 10 min. Plasma prekallikrein was assayed according to the modified method of Oh-ishi and Katori. Briefly, 100 µl of rat plasma was mixed with 800 µl acetonitrile–HCl–saline solution (pH 8.0) with or without a test compound and allowed to stand for 10 min at room temperature. Then 100 µl of 1.0% kaolin suspension
was added and mixed vigorously. At 5 min intervals after the addition of kaolin, 50 µl aliquots of the reaction mixture were taken and incubated at 37 °C for 10 min with 1.0 ml of 50 µM carbochromoxy-L-phenylalanyl-L-arginine 4-methylcoumarinyl-7-amide (Z-Phe-Arg-MCA, Peptide Institute) in 10 mM CaCl₂-tris-HCl saline (pH 8.0) in the presence (Tube A) or the absence (Tube B) of 40 µg soybean trypsin inhibitor (SBTI, Sigma). The reaction was terminated by the addition of 2 ml of 17% acetic acid and the fluorescence was read at 460 (emission) and 380 (excitation) nm with a fluorescence spectrophotometer. The difference between the values from Tube A and Tube B was calculated as the prekallikrein activity. The results are expressed in arbitrary units where one unit is defined as the amount of enzyme which releases 1 nmol of 7-amino-4-methylcoumarin (AMC, Peptide Institute)/5 min under the described conditions.

Statistical Evaluation — Student’s t-test was used for statistical analysis of the results.

Results

Effect on Increased Levels of Bradykinin and PGE₂ Induced by Kaolin Injection into the Air Pouch in Rats

T-614 (3—30 mg/kg, p.o.) exhibited a dose-dependent and significant inhibitory effect on the immunoreactive bradykinin levels in the pouch fluid. Nimesulide (30 mg/kg) also showed a significant inhibition but indomethacin (10 mg/kg) did not (Table I). The potency of T-614 was somewhat higher than that of nimesulide. On the other hand, PGE₂ levels were reduced to the same extent by all treatments with the test compounds.

Effect on Writhing Response and Increased Levels of Bradykinin Induced by Intraperitoneal Injection of Kaolin in Mice

T-614 (10—100 mg/kg, p.o.) reduced not only the number of writhes but also the peritoneal levels of bradykinin in a dose-dependent manner. In contrast, ibuprofen (10 and 30 mg/kg) inhibited the writhing frequency without reducing the levels of bradykinin (Fig. 1).

Effect on Writhing Response and Increased Levels of 6-Keto-PGF₁α Induced by Intraperitoneal Injection of Zymosan in Mice

T-614 (100 mg/kg, p.o.) did not exert an obvious inhibition on either writhing frequency or the peritoneal levels of 6-Keto-PGF₁α. Nimesulide, indomethacin and ibuprofen reduced the levels of 6-Keto-PGF₁α at doses effective in reducing the number of writhes (Fig. 2).

Effect on Activities of Serine Proteases

T-614 did not show any inhibitory effects on the esterolysis of trypsin, thrombin, kallikrein and plasmin up to a concentration of 100 µg/ml.

Table I. Effects of T-614 and Other Reference Compounds on Bradykinin and PGE₂ Levels in the Pouch Fluid of Kaolin-Induced Inflammation in Rats

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Dose (mg/kg)</th>
<th>Bradykinin levels (ng/ratᵃ)</th>
<th>PGE₂ levels (ng/ratᵃ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Vehicle)</td>
<td>—</td>
<td>45.3 ± 3.7</td>
<td>1.08 ± 0.14</td>
</tr>
<tr>
<td>T-614</td>
<td>3</td>
<td>39.3 ± 4.3 (13.2)</td>
<td>0.37 ± 0.05ᵃ (65.7)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>35.2 ± 1.5ᵇ (22.3)</td>
<td>0.44 ± 0.06ᵇ (59.3)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>29.3 ± 2.2ᶜ (35.3)</td>
<td>0.38 ± 0.02ᶜ (64.8)</td>
</tr>
<tr>
<td>Control (Vehicle)</td>
<td>—</td>
<td>46.6 ± 5.3</td>
<td>1.96 ± 0.27</td>
</tr>
<tr>
<td>T-614</td>
<td>30</td>
<td>28.1 ± 3.3ᵇ (39.7)</td>
<td>0.52 ± 0.08ᵇ (73.6)</td>
</tr>
<tr>
<td>Nimesulide</td>
<td>30</td>
<td>33.4 ± 2.9ᵇ (28.3)</td>
<td>0.75 ± 0.15ᵇ (61.9)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>36.5 ± 3.5 (21.7)</td>
<td>0.71 ± 0.19ᵇ (64.0)</td>
</tr>
</tbody>
</table>

Test compounds were orally administered 2 h before subcutaneous injection of 4 ml of 1% kaolin suspension in 0.8% CMCNa containing 0.2 mg of captopril into a preformed air pouch on the dorsum of rats. After 20 min, the animals were sacrificed to collect pouch fluid samples. a) Each value represents the mean with S.E. of 8 to 10 rats. Significant difference from vehicle control; b) p<0.05, c) p<0.01. The figures in parentheses represent percent of inhibition vs control.
Nimesulide, indomethacin and ibuprofen also had no effect, while FOY inhibited trypsin, thrombin and plasmin with the IC₅₀ values of 9.1—40 µg/ml (data not shown).

**Effect on Activation and Activity of Prekallikrein of Rat Plasma**

Test compounds were orally administered 0.5 h before intraperitoneal injection of kaolin suspension (2.5 mg in 0.5 ml of saline) containing 0.05 mg of captopril. Writhes were counted for 15 min after kaolin injection and the animals were then sacrificed to collect peritoneal fluid samples. Each column and bar indicates the mean with S.E. of 9 to 10 mice. Significant difference from vehicle control: a) p<0.05, b) p<0.01.

The effect of T-614 on the activity of plasma prekallikrein was examined by its addition to plasma prior to the activation with kaolin and the kallikrein so formed was measured using a specific fluorogenic substrate. T-614 (10 and 100 µg/ml) had no inhibitory effect on the prekallikrein activity and among the test compounds, FOY exhibited a significant inhibition at a concentration of 100 µg/ml (Table II).
TABLE II. Effects of T-614 and Other Reference Compounds on Prekallikrein Activation of Rat Plasma

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concen. (µg/ml)</th>
<th>Prekallikrein activity Arbitrary units(^a)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (DMSO)</td>
<td>---</td>
<td>3.49 ± 0.44</td>
<td>---</td>
</tr>
<tr>
<td>T-614</td>
<td>10</td>
<td>3.94 ± 0.63</td>
<td>-12.9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.74 ± 0.60</td>
<td>-7.2</td>
</tr>
<tr>
<td>Nimesulide</td>
<td>10</td>
<td>4.40 ± 0.50</td>
<td>-26.1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.80 ± 0.82</td>
<td>-8.9</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>3.68 ± 0.42</td>
<td>-5.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.83 ± 0.56</td>
<td>-9.7</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>10</td>
<td>4.07 ± 0.60</td>
<td>-16.6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.97 ± 0.67</td>
<td>-13.8</td>
</tr>
<tr>
<td>FOY</td>
<td>10</td>
<td>2.81 ± 0.63</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.91 ± 0.17(^b)</td>
<td>73.9</td>
</tr>
</tbody>
</table>

\(^a\) Each value represents the mean with S.E. of 4 to 5 separate experiments. Significant difference from control; \(^b\) p < 0.01.

Discussion

We have found that T-614, in spite of its potent analgesic activities against the inflammatory pain, does not exhibit obvious inhibitory effect on writhing responses provoked by either acetic acid, phenylquinone or acetylcholine in mice.\(^2\) In comparison with the antiinflammatory activities, the relative weakness of T-614 in inhibiting the writhing responses has also been shown in rats.\(^2\) Boettcher et al.\(^{13}\) have also reported the effects of CGP28237, which possesses the same acidic group (methylsulfonylamino moiety) as T-614, on the writhing responses, and similar results have been shown. Furthermore, we have found that T-614 and nimesulide, unlike the classical NSAIDs, showed inhibitory effects on the edemas induced by some phlogistic agents other than carrageein, such as dextran and bromelain, in rats.\(^2\) These experiments suggest that this new class of antiinflammatory compounds including T-614 has apparently dissimilar action to classical NSAIDs and, in addition to the inhibition of PG production, other action mechanisms may be involved in its analgesic effects.

The data reported in this paper indicate that T-614 has an inhibitory effect on the release of bradykinin into the inflammatory exudates. It has been clearly shown that kaolin-induced air pouch inflammation in rats is closely related to the kallikrein-kinin system at the early inflammatory stage.\(^6,14\) Bradykinin has been reported to play an essential role for the sudden rise of the vascular permeability observed immediately after the injection of kaolin in this model.\(^14\) T-614 exerted a dose-dependent inhibitory effect on the increased bradykinin levels and its potency appeared to be higher than those of nimesulide and indomethacin. Therefore, its inhibitory effects on the dextran- or bromelain-induced edema\(^2\) may be brought about by the inhibition of the increased vascular permeability due to bradykinin. The bradykinin released incites the production of PGs by surrounding cells, and the produced PGs amplify the bradykinin-induced pain and/or inflammatory responses.\(^15,16\) Accordingly, we attempted the measurement of PGE\(_2\) levels in pouch fluid in this animal model, however, it was likely that this mediator in the exudates had not reached the peak level because of the early inflammatory stage. T-614 also obviously suppressed the PGE\(_2\) production as did indomethacin and nimesulide. This result supports our previous observation that T-614
Reduced effectively the PGE$_2$ contents in the inflammatory exudate of carrageen-in-sponge type inflammation in rats.\(^3\)

In mice, T-614 exhibited a significant inhibitory effect on the increased bradykinin levels in peritoneal fluid provoked by kaolin but no effect on the 6-keto-PGF$_1\alpha$ levels induced with zymosan. Its inhibitory effects on the writhing frequency were observed in agreement with those on contents of the mediators; i.e. T-614 inhibited the writhing response induced with kaolin, but not with zymosan. The kaolin-induced writhing model has been shown to be mainly dependent on the action of bradykinin produced by activation of the kallikrein-kinin system.\(^9,17\)

Further, it has been reported that the activation of the complement system and the subsequent release of PGI$_2$ play a major role in zymosan-induced writhing response.\(^10,18\) Therefore, based on the above results, it was suggested that a highly potent analgesic effect of T-614 on the inflammatory pain was related to the inhibition of release of bradykinin rather than PGs.

In *in vitro* experiments, T-614 had no inhibitory effect on the activities of serine proteases including kallikrein, and did not affect the activation process of prekallikrein in rat plasma. Based on these results, it was inferred that T-614 does not influence the synthesis process of bradykinin in the plasma but it affects a certain stage of its leaking process from the plasma into the inflammatory sites. The precise mechanism of the inhibition of the leaking process by T-614 remains obscure. Recently, it has been proposed that in addition to the plasma kinin-forming system, the tissue kallikrein system is as important as the molecular events occurring during kinin generation.\(^19\) T-614 might, therefore, influence the secretion of the active form of tissue kallikreins which are widely distributed in local tissues. We have indeed observed that T-614 inhibits the production and/or secretion of some other inflammatory mediators such as lysosomal enzymes (personal communication) and cytokines\(^20\) by various inflammatory cells *in vitro*.

In summary, the present studies show that T-614 has the capacity to inhibit the release of bradykinin into the local inflamed tissues in addition to an inhibitory effect on the production of PGs. It is suggested that at least some of the analgesic and antiinflammatory effects of T-614 may be mediated by this capacity and an agent with such property, which is different from the classical NSAIDs, may be useful for the treatment of inflammatory pain.

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