Anti-inflammatory Activity of Bis(3-aryl-3-oxo-propyl)methylamine Hydrochloride in Rat

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In this study the effects of compound B1, bis(3-aryl-3-oxo-propyl)methylamine hydrochloride, and an anti-inflammatory drug, indomethacin, were tested by carrageenan-induced paw edema and cotton pellet granuloma tests, for effects on acute and chronic phases of inflammation, respectively. Their effects on vascular permeability were also determined by hyaluronidase-induced capillary permeability test. Anti-inflammatory activity of B1 was compared with indomethacin. B1 decreased the carrageenan-induced paw edema by 49%, 35%, and 47% at 50, 100, and 200 mg kg\(^{-1}\) doses, respectively, while this decrease was 82% by indomethacin at 20 mg kg\(^{-1}\) dose. Antiproliferative effects in cotton pellet test of B1 at 50 mg kg\(^{-1}\) and indomethacin at 20 mg kg\(^{-1}\) doses were 44% and 43%, respectively. Indomethacin but not B1 inhibited the hyaluronidase-induced increase in capillary permeability. Our results suggest that B1 inhibits both acute and chronic phases of inflammation probably by an effect not mediated by prevention of increased capillary permeability. Especially, its anti-inflammatory activity against chronic phase of inflammation was comparable with that of indomethacin. Further detailed studies are needed to clarify the mechanism(s) of action responsible for the anti-inflammatory activity of B1.

Key words anti-inflammatory activity; hyaluronidase activity; indomethacin; bis-Mannich base

Bis(3-aryl-3-oxo-propyl)methylamine hydrochloride (B1) is a bis-Mannich base. Mannich bases have various biological activities such as antimicrobial,\(^1\)—\(^4\) cytotoxic,\(^5\)—\(^7\) anticancer,\(^8\) analgesic,\(^9\) and anticonvulsant activities.\(^10\),\(^11\) It has been reported that Mannich bases such as 1-diphenylaminomethyl-3-(1-naphthylimino)-1,3-dihydroindol-3-one, 1-diphenylaminomethyl-3-(4-methylphenylimino)-1,3-dihydroindol-3-one, and 1,3-dicyclohexyl-5-alkyl-5-aminomethyl-barbituric acids have very high levels of anti-inflammatory activity. In addition, 8 compounds out of the 18 Mannich bases with structure of 2-substituted-2-dimethylaminomethyl-5-(E)-arylidene cyclopentanone have demonstrated remarkable anti-inflammatory activity.\(^14\) Mannich bases with structure of 2-(E)-(un)substituted benzyldiene-5-dimethylaminomethyl cyclopentanone have been synthesized as dual inhibitors of cyclooxygenase and lipoxygenase. These compounds have inhibited carrageenan-induced paw edema.\(^15\) Anti-inflammatory activity of 3-benzoyl-1-methyl-4-phenyl-4-piperidinol hydrochloride (C1, Fig. 1), which is a semi-cyclic Mannich base and structural and nonclassic isomer of compound B1 (Fig. 2), has been shown in rats in our previous study.\(^16\)

Acute anti-inflammatory activities of compounds or drugs are generally investigated using acute aseptic arthritis model (paw edema) that has been induced by histamine, formalin, dextran, or carrageenan.\(^17\) Cotton pellet granuloma test is widely used to investigate their effects on the chronic phase of inflammation.\(^18\) The observation, that Mannich bases with various chemical structures including compound C1 have anti-inflammatory effects led us to investigate the anti-inflammatory effect of compound B1, which is a bis-Mannich base and also structural and nonclassic isomer of compound C1.

The aim of this study was to investigate the anti-inflammatory effects of compound B1, on acute and chronic phases of inflammation by carrageenan-induced paw edema and cotton pellet granuloma tests, respectively. In addition, its effect on hyaluronidase-induced capillary vascular permeability was tested to give an insight into its mechanism of action.

MATERIALS AND METHODS

Materials Carrageenan and trypan blue stain were obtained from Sigma Chemical Co. (St. Louis, U.S.A.), thiopenthal sodium was from Abbott (Camopoverde di Aprilia (LT), Italy), and indomethacin was from Deva (Istanbul, Turkey), hyaluronidase was from Kiyevskoye predpriyatiye po proizvodstvu Bakteriynih preparatov (Kiev, Ukraine). Hyaluronidase (hyaluronoglucosaminidase, EC 3.2.1.35) was obtained from the bovine testicles. Its specific activity is 0.64 U/mg.

Synthesis of Bis(3-aryl-3-oxo-propyl)methylamine Hydrochloride (B1, Fig. 2) A mixture of methylamine hy-
dichloride (0.08 mol), acetylphophene (0.24 mol), and paraformaldehyde (0.16 mol) was stirred and heated to 82 °C. At this temperature a vigorous reaction occurred. Then, the heating source of the reaction was removed. Temperature in the reaction flask increased spontaneously to over 100 °C. Stirring was discontinued. A solid mass of crystals was formed. After cooling to 60 °C, 40 ml of ethanol was added into the reaction flask and reaction mixture was stirred for 2 h. The crystals were filtered, dried, and re-crystallized from ethanol (mp 163.5, yield 80%). Spectral analysis data obtained by 1H-, 13C-NMR, UV, IR, and ESI-MS spectroscopies and elemental analysis results have been reported in our previous study.10)

**Anti-inflammatory Activity Tests.** Animals Male Wistar albino rats (n=75, 230—240 g) used in this study were fed with standard laboratory Chow as groups at 22 °C. In addition, 18 rabbits (weighing 3—3.5 kg) were used for hyaluronidase-induced capillary permeability test. All animals used were obtained from the Experimental Animal Laboratory, Department of Pharmacology, Faculty of Medicine, Ataturk University. Animal experiments were carried out in an ethically proper way by following guidelines as set by the Ethical Committee of Ataturk University.

**Carrageenan-Induced Paw Edema in Rat** Anti-inflammatory activity of B1 and indomethacin was determined by carrageenan-induced hind paw edema.17,19) B1 at 50, 100, and 200 mg kg⁻¹ doses and indomethacin at 20 mg kg⁻¹ dose were given to rats orally by feeding tube once daily for 2 d. The same volume of distilled water used as vehicle was given to the control group. One hour after the last treatment, carrageenan 0.1 ml (1%, w/v) solution in distilled water was subcutaneously injected into the plantar surface of the right hind paw of all rats. The paw volume until the knee joint was measured by plethysmometer before injection of carrageenan. Carrageenan-induced paw edema was measured at 1 h intervals for 5 h. Anti-inflammatory activities of B1 and indomethacin were determined by comparing their results with those obtained in the control group.

**Cotton Pellet Granuloma Test** In this series of experiments, the effect of B1 and indomethacin on the proliferation phase of inflammation was investigated by cotton pellet model.16,17) B1 at 50 mg kg⁻¹ dose and indomethacin at 20 mg kg⁻¹ dose were given to two different groups of rats orally by feeding tube. The same volume of distilled water used as vehicle was given to the control group. After 30 min of drug administration, the animals were anesthetized by thiopental sodium 25 mg kg⁻¹ intraperitoneally. Then, cotton pellets, which were prepared before weighing 7±1 mg each, were implanted in interscapular distance under the skin under sterile conditions. B1 and indomethacin were administered by the same way once a day for a period of 7 d. The rats were killed by a high dose of thiopental sodium anesthesia (50 mg kg⁻¹) on the eighth day, and the pellets surrounded by granuloma tissues dissected out. The antiproliferative effects of B1 and indomethacin were evaluated by comparison with the control group.

**Hyaluronidase-Induced Capillary Permeability Test** In this series of experiments, the effects of B1 and indomethacin on hyaluronidase-induced capillary permeability were investigated.16) Rabbits (n=18, weighing 3—3.5 kg) were divided into 3 equal groups and hair in their abdominal area was shaved. The first group received B1 (50 mg kg⁻¹), while the second group received indomethacin (10 mg kg⁻¹) by per oral catheter. The third, control group received only the same amount of vehicle (NaCl, 0.9%).

Hyaluronidase (128 unit) was dissolved in 1 ml isotonic NaCl. Trypan blue (0.8 ml of 0.75%) was added to this hyaluronidase solution (0.5 ml). The last mixture (0.1 ml) was injected subcutaneously in the abdominal region after 1 h of per oral drug administration. The appearance of the blue area was measured after 20 s and 5 and 30 min of injection as mm². The size of the blue area corresponds the activity of the hyaluronidase enzyme and capillary permeability, i.e., the smaller the appearance of the blue area, the lower the activity of the hyaluronidase enzyme and capillary permeability.26)

The results obtained were compared with the control group.

**Test for Acute Toxicity of B1** To test the acute toxicity, B1 was administered by oral catheter to four different groups of rats (n=6 for each group) at 250, 500, 1000, and 1500 mg kg⁻¹ doses and survival of the animals was followed for 24 h. Toxicity was evaluated according to the number of animals died during the follow-up period.

**Statistical Analysis** Results are presented as mean± S.D. Data were analysed by using one-way ANOVA with post-hoc LSD test. p<0.05 was accepted as the level of statistical significance.

## RESULTS

**Carrageenan-Induced Paw Edema** As shown in Table 1, B1 inhibited carrageenan-induced paw edema at doses of 50, 100 and 200 mg kg⁻¹ by 49%, 35%, and 47%, respectively, at 3 h. This inhibition was 82% by indomethacin at the dose of 20 mg kg⁻¹.

**Cotton Pellet Granuloma Test** The average weight of the cotton pellets in control group was 489.2±15.8, and

### Table 1. The Effects of Bis(3-aryl-3-oxo-propyl)methylamine Hydrochloride (B1) and Indomethacin on Carrageenan-Induced Rat Paw Edema

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>Dose (mg/kg)</th>
<th>Normal paw volume (ml)</th>
<th>Paw volume 3 h after inflammation (ml)</th>
<th>The increase in volume of paw with inflammation compared with control (ml)</th>
<th>Anti-inflammatory effect %</th>
<th>p value (vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>6</td>
<td>25</td>
<td>0.60±0.06</td>
<td>1.06±0.07</td>
<td>0.46±0.03</td>
<td>6.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>B1</td>
<td>6</td>
<td>50</td>
<td>0.67±0.03</td>
<td>0.92±0.04</td>
<td>0.25±0.01</td>
<td>49</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>B1</td>
<td>6</td>
<td>100</td>
<td>0.65±0.04</td>
<td>0.97±0.06</td>
<td>0.32±0.02</td>
<td>35</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>B1</td>
<td>6</td>
<td>200</td>
<td>0.63±0.05</td>
<td>0.89±0.07</td>
<td>0.26±0.02</td>
<td>47</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>6</td>
<td>20</td>
<td>0.69±0.02</td>
<td>0.78±0.02</td>
<td>0.09±0.02</td>
<td>82</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>—</td>
<td>0.62±0.03</td>
<td>1.11±0.05</td>
<td>0.49±0.02</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

The results given are mean± S.D. n: Number of rats used.
272.2±34 and 281.1±20 mg in rats receiving B1 50 mg kg⁻¹, and indomethacin 20 mg kg⁻¹, respectively (Table 2). Anti-inflammatory activities of B1 and indomethacin in cotton pellet granuloma test were 44% and 43%, respectively.

**Hyaluronidase-Induced Capillary Permeability Test**

As shown in Table 3, the subcutaneous spreading area of trypan blue after 20 s of subcutaneous injection of hyaluronidase was 115±17 mm² the control group, while it was 119.2±19 mm² and 90.1±12 mm² in B1 and indomethacin groups, respectively. The spreading areas of trypan blue after 5 and 30 min are also shown in Table 3.

**Acute Toxicity** None of the rats receiving per oral B1 (dose range starting with 250 mg kg⁻¹ and increased until 1500 mg kg⁻¹) died during the 24 h after ingestion of the compound.

**DISCUSSION**

In this study, the effects of B1 on acute and chronic phases of inflammation were investigated by carrageenan-induced paw edema and cotton pellet granuloma tests, respectively. In addition, the effect of B1 on capillary permeability was investigated by hyaluronidase-induced capillary permeability test. B1 was found effective against carrageenan-induced paw edema in this study. B1 significantly inhibited carrageenan-induced paw edema in rats at 50 and 200 mg kg⁻¹ doses. Fifty and 200 mg kg⁻¹ doses of B1 showed similar anti-inflammatory activity, while the 100 mg kg⁻¹ dose of B1 showed weaker anti-inflammatory activity compared with 50 and 200 mg kg⁻¹ doses. The most effective dose of B1 was 50 mg kg⁻¹ according to the percentage of anti-inflammatory activity. The power of anti-inflammatory activity of B1 was less than indomethacin’s at all doses used. Fifty mg kg⁻¹ dose of B1 showed 32.7% less anti-inflammatory activity than indomethacin. Increases in B1 doses did not increase its anti-inflammatory activity. Indomethacin was more effective than B1 at 20 mg kg⁻¹ dose. This result shows that the power of gravimetric effect of indomethacin was higher than B1.

We had previously reported that semi-cyclic Mannich base C1 at 200 mg kg⁻¹ and indomethacin at 20 mg kg⁻¹ inhibited carrageenan-induced inflammation almost equally. Anti-inflammatory activity of B1 was lower than that of C1. When the chemical structures of the compounds are considered, C1 has a piperidine ring, a tertiary alcohol, a ketone, and a tertiary amine salt functional group (Fig. 1), while B1 has two ketone groups and a tertiary amine salt as functional groups (Fig. 2). As can be noticed easily, the difference between these chemical structures is the presence of the piperidine ring and alcohol groups in C1, which are absent in B1. The presence of these two functional groups in C1 may be responsible for the increased anti-inflammatory activity. Since C1 has a piperidine ring, which is a relatively rigid structure, it has limited conformational arrangements, while B1 may have many conformations because of its aliphatic chain structure. This means that C1 can provide necessary conformational arrangement for interaction with the related receptor. In addition, the alcohol group in C1 may have importance to bind the active site of the related receptor by hydrogen bond. The ketone group may also have importance in bioactivity. Both compounds have ketone group(s). While B1 has two ketone groups, C1 has only one, which causes steric hindrance in chemical environment compared with B1. This suggests that even if a ketone group is important, its position or its chemical environment may also be important. The most important point in the significant and more potent activity of compound C1 might be its chiral structure. Compound C1 having piperidine ring has chiral centers at the 3 and 4 positions of the piperidine ring. This is why it can generate suitable diastereomeric couple, which is responsible for its bioactivity at the chiral receptor site. Although B1 is a non-classical structural isomer of C1, it may have lower anti-inflammatory activity because of its achiral structure and absence of piperidine ring and alcohol functional groups in B1.

There are two phases of carrageenan-induced inflammatory reaction: early or first phase and late or second phase. It has been proposed that the early phase results from histamine, serotonin, and bradykinin liberation, while the late phase is associated with formation of prostaglandins. In addition, neutrophil infiltration, as well as free radicals released from neutrophils such as hydrogen peroxide, superoxide, and hydroxyl radicals, play a role in the late phase of...
carrageenan-induced inflammation. The anti-inflammatory effect of B1 at 50, 100, and 200 mg kg⁻¹ doses on carrageenan-induced inflammation started at the 2nd h and reached peak level at the 3rd h. Reaching the peak level of its anti-inflammatory effect at the 3rd h means that it probably inhibits prostaglandin synthesis. This period is known as the late or second phase of carrageenan-induced inflammation. Late phase of carrageenan-induced inflammation starts 1 h later after the injection and continues for 3 h. The period until this time is known as the early or first phase of inflammation. The anti-inflammatory effect (6.2%) of B1 at 25 mg kg⁻¹ dose was followed at the 3rd h only, and it was non-significant and negligible. Not to be effective at early phase of carrageenan-induced inflammation suggests that B1 does not interact with mediators of inflammation such as histamine, serotonin, and bradykinin. Carrageenan activates macrophages and polymorphonuclear cells (PMNL). These activated cells secrete tumour necrosis factor-α (TNF-α). TNF-α is one of the cytokines taking part in the formation of inflammation. It causes the release of IL-8 from the tissue cells, which increases the attachment of leukocytes to endothelial cells, and degranulation and release of reactive oxygen species from them causing injury in the endothelial cells. TNF-α can also induce inductive nitric oxide synthase (iNOS), which also takes part in inflammation. It is possible that B1 can prevent the release and/or effects of TNF-alpha in the beginning but not after the 3rd h of inflammation according to our results.

In the second series of our experiments, the effect of B1 on the chronic phase of inflammation was investigated by cotton pellet granuloma test. Cotton pellet granuloma test is a chronic inflammation model commonly used to evaluate the anti-proliferative activities of drugs. B1 has significantly decreased the weight of the cotton pellets (44%) inserted under the skin of rats in comparison with the control group. The power of antiproliferative effect of B1 was found almost equal to C1. C1 and indomethacin significantly decreased the weight of implanted cotton pellets 46.1% and 43.1% in comparison with the control group, respectively.

The effect of B1 at 50 mg kg⁻¹ was almost equal to the effect of C1 at 100 mg kg⁻¹ in cotton pellet granuloma test. The dose (50 mg kg⁻¹) used to investigate the effect of B1 on the chronic phase of inflammation was the most effective dose in carrageenan-induced inflammation. The most effective dose of C1 was 200 mg kg⁻¹ in carrageenan-induced inflammation. Acute inflammation continues with the formation of proliferative cells and becomes chronic. These cells may spread or form granuloma. Antiproliferative effect can be followed in the form of preventing generation of collagen fibers and suppressing mucopolysaccharides by the drugs. The volumes of cotton pellets removed from the rats receiving compound B1 were much smaller compared with the control group macroscopically. Pus was observed between cotton pellet and granuloma tissue under the skin in the control group. Monocyte infiltration and fibroblast proliferation rather than neutrophil infiltration and exudation take place in chronic inflammation. Activated monocytes-macrophages are blood cells showing antitumor, antimicrobial properties and have phagocytic function against pathogens. The finding that the effectiveness of B1 on carrageenan-induced inflammation and also decreasing the weight of cotton pellets by about 43% compared with the control group in cotton pellet granuloma test shows that B1 is effective in both acute and chronic phases of inflammation.

At its most effective dose (50 mg kg⁻¹) B1 did not cause a decrease in increased capillary permeability induced by hyaluronidase. Furthermore, the blue area, which is formed by subcutaneous injection of trypan blue given with hyaluronidase enzyme, in animals receiving B1 was 4.2 mm² bigger compared with that measured in the control group. The result that B1 could not decrease the hyaluronidase-induced capillary permeability may explain lower anti-inflammatory activity of B1 against carrageenan-induced paw edema compared with C1. Indomethacin (10 mg kg⁻¹) significantly decreased the increase in hyaluronidase-induced capillary permeability. The blue area was 24.9 mm² smaller in the animals receiving indomethacin. Small blue area shows decreased hyaluronidase enzyme activity and therefore decreased capillary permeability. C1 and indomethacin also decreased the blue area remarkably at 30 min. However, the blue areas at 5 and 30 min in B1-receiving animals increased slightly compared with the control group.

Our results suggest that B1 inhibits both acute and chronic phases of inflammation by a mechanism probably not mediated by prevention of increased capillary permeability. Especially, its anti-inflammatory activity against chronic phase of inflammation was comparable with that of indomethacin. This effect might not result from prevention of the increase in vascular permeability. As NSAID, B1 might have suppressed the acute and chronic inflammation by inhibiting the synthesis of histamine, serotonin, bradykinin, and prostaglandins (PG), decreasing monocyte infiltration and fibroblast proliferation, preventing generation of collagen fibers, and suppressing mucopolysaccharides. Its low toxicity and higher anti-proliferative activity may provide B1 advantage compared with indomethacin in the treatment of chronic inflammation. Further detailed studies are needed to clarify the mechanism(s) of action responsible for the anti-inflammatory activity of B1.

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