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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Anti-inflammatory activity of 2,5-dihydroxycyclohepta-2,4,6-trienone in rats

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Abstract In this study, the anti-inflammatory effects of a tropolone derivative, 2,5-dihydroxycyclohepta-2,4,6-trienone (AD-4), were investigated. The anti-inflammatory potency of AD-4 was compared with that of indomethacin in carrageenan-induced inflammation models in rats. The effect on vascular permeability was also determined by hyaluronidase-induced capillary permeability. AD-4 decreased carrageenan-induced paw edema at doses of 3.62×10^2, 7.24×10^2, and 14.48×10^2 μmol/kg by 45% ($p < 0.001$), 79% ($p < 0.001$), and 83% ($p < 0.001$), respectively, compared with the value of 49% ($p < 0.001$) for indomethacin (69.8 μmol/kg). Additionally, AD-4 decreased hyaluronidase-induced capillary permeability significantly. In conclusion, AD-4 was determined to have anti-inflammatory effects with lower toxicity than indomethacin. Anti-inflammatory effect of AD-4 may be related to its effects on vascular permeability.

Keywords Hyaluronidase · Tropolone · Anti-inflammatory · Capillary permeability · Toxicity

Introduction

2,5-Dihydroxycyclohepta-2,4,6-trienone (AD-4) (Dastan and Balci, 2006; Mori *et al.*, 1993) is a tropolone derivative (Fig. 1). Tropolones have an aromatic seven-membered ring system (Ogata *et al.*, 1999). Tropolones are isolated from plants and

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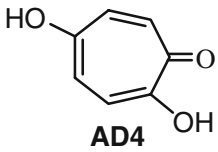


Fig. 1 Chemical structure of AD-4

AD4

2,5-dihydroxycyclohepta-2,4,6-trienone

bacteria or obtained by synthesis (Arima *et al.*, 2003; Budihas *et al.*, 2005; Choi *et al.*, 2006; Kitamura *et al.*, 1986; Korth *et al.*, 1982; Morita *et al.*, 2002, 2004a) and these compounds have chelating capacity with metal ions such as Fe, Cu, and Zn (Doulias *et al.*, 2004; Miyamato *et al.*, 1998). Tropolones exert various biological activities due to their interesting ring structure and chelation properties (Diouf *et al.*, 2002; Doulias *et al.*, 2004). These compounds have been used as deodorants and natural food additives (food preservatives) (Ogata *et al.*, 1999; Nakano *et al.*, 2005). Recently, the biological activities of many compounds derived from tropone and tropolone, used as a starting material have been shown to include antibacterial, antiviral, antifungal, insecticidal, acaricidal, antitumoral, antiallergic, antithyroidal, and antioxidant activities (Bagli *et al.*, 1979; Lee *et al.*, 1979; Miyamato *et al.*, 1998; Morita *et al.*, 2004b). In addition, it was reported that, β-thujaplicin demonstrated an anti-inflammatory effect (Nakano *et al.*, 2005). Azulen derivatives, synthesized from tropolone, were shown to inhibit lipid peroxidation (Rekka *et al.*, 2002). The effects of tropolone derivatives on inhibition of metaloprotease were also reported (Inamori *et al.*, 1999). The role of metaloprotease enzyme in inflammation is known. It is known that vascular dilatation and increased permeability occur in inflammatory area. Role of hyaluronidase enzyme is known in increased vascular permeability (Houck and Chang, 1979). It has been determined that there is a parallelism between increased hyaluronidase enzyme activity and severity of inflammation (Procide *et al.*, 1971). Carrageenan-induced inflammation model is commonly used to determine anti-inflammatory effects of new compounds (Demirezer *et al.*, 2006). Vascular permeability increases in the inflammatory region that occurs after carrageenan application (Nacife *et al.*, 2004). So the aim of this study is to investigate the anti-inflammatory effect of AD-4 on carrageenan-induced paw volume test and also investigate the effect of AD-4 on increased vascular permeability with hyaluronidase test.

Materials and methods

Animals

In this study, male albino Wistar rats ($n = 84$, 220–230 g) and albino rabbits ($n = 24$, 3.5–4.0 kg) were used. These animals were obtained from the Medical Experimental Research Centre, Ataturk University. Prior to conducting the experiments, rats and rabbits were housed and fed as separated groups under

standard conditions at 22°C in the laboratory. Animal experiments were performed in accordance with the national guidelines for the use and care of laboratory animals, and were approved by the local animal care committee of Ataturk University.

Chemicals

Carrageenan and trypan blue stain were obtained from Sigma Chemical Co. (St. Louis, USA), thiopental 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 bovine testicles. Its specific activity was 0.64 U/mg.

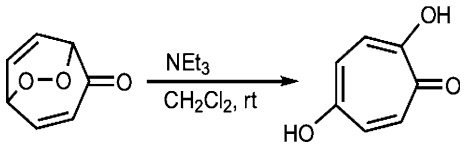
Synthesis of 2,5-dihydroxycyclohepta-2,4,6-trienone (AD-4)

AD-4 was synthesized in the light of previous literature (Coskun *et al.*, 2007; Dastan and Balci, 2006; Mori *et al.*, 1993). The synthesis steps are summarized in Fig. 2. By base-catalyzed rearrangement of the tropone endoperoxide, AD-4 and additive end products have been synthesized.

The effects of AD-4 on carrageenan-induced inflammatory paw edema in rats

In this series of experiments, anti-inflammatory effect of AD-4 on carrageenan-induced paw edema was investigated in a total of 30 rats (Suleyman *et al.*, 2004). The rats were divided into five groups ($n = 6$ for each group) prior to initiating the experiment. AD-4 was administrated by oral gavage to the first, second, and third groups at doses of 3.62×10^2, 7.24×10^2, and 14.48×10^2 μmol/kg, respectively. Indomethacin (69.8 μmol/kg) was given to the fourth group by the same method. The anti-inflammatory effect potency of AD-4 was compared with that of indomethacin. The control group received an equal volume of distilled water. (The mentioned amounts of drugs were administered in 1 ml distilled water.) One hour after administration of drugs, carrageenan solution (0.1 ml, 1% w/v in distilled water) was injected subcutaneously into the plantar surface of the hind paw of all rats. Before administration of carrageenan, the paw volumes of each animal were measured with a plethysmometer up to the knee joint. Carrageenan-induced paw edema was measured five times at 1-h intervals. The anti-inflammatory effects of AD-4 and indomethacin were determined by comparing with the results of the control.

Fig. 2 Synthesis of AD-4



Hyaluronidase-induced capillary permeability test

In this series of experiments, the effects of AD-4 and indomethacin on hyaluronidase-induced capillary vascular permeability were investigated (Suleyman *et al.*, 2007a). Twenty-four albino rabbits weighing 3.5–4.0 kg were used for the experiment. Rabbits were divided into four equal groups and the bilateral abdominal area of each animal was shaved. The first two groups received 3.62×10^2 and 7.24×10^2 μmol/kg AD-4 orally with the aid of gavage, while 27.9 μmol/kg indomethacin was given to the third group by identical route. The last group (control) received the same volume of distilled water.

Hyaluronidase (128 units) was dissolved in 1 ml isotonic NaCl solution and 0.8 ml trypan blue (0.75%) was added to 0.5 ml hyaluronidase solution. One hour after oral drug administration, 0.1 ml of this mixture was injected subcutaneously into the shaved area. The appearance of the blue area was measured in mm^2, 5 and 30 min after injection. Smaller size of blue area indicates decreased hyaluronidase enzyme activity and capillary vascular permeability.

Acute toxicity test

Six rats were allocated for each dose to test acute toxicity. AD-4 was administered at 18.1×10^2, 36.2×10^2, 54.3×10^2, 57.9×10^2, 61.5×10^2, 65.2×10^2, and 68.78×10^2 μmol/kg and indomethacin was administered at 69.8 and 139.6 μmol/kg to different groups of animals using an oral catheter. Survival of the animals was monitored for 24 h. Acute toxicity was evaluated according to the number of deaths during this period (Suleyman *et al.*, 2007a).

Statistical analysis

All results were shown as means ± standard error (SE). One-way analysis of variance with post hoc least significant difference (LSD) test was used to evaluate the results; $p < 0.05$ was accepted as the level of statistical significance.

Results

Carrageenan-induced inflammation test

As seen in Fig. 3, AD-4 decreased carrageenan-induced paw edema at doses of 3.62×10^2, 7.24×10^2, and 14.48×10^2 μmol/kg by 45% ($p < 0.001$), 79% ($p < 0.001$), and 83% ($p < 0.001$), respectively, at the third hour after carrageenan injection. This value was 49% ($p < 0.001$) for indomethacin. The anti-inflammatory effect of 3.62×10^2, 7.24×10^2, and 14.48×10^2 μmol/kg AD-4 was 48% ($p < 0.001$), 41% ($p < 0.001$), and 76% ($p < 0.001$), respectively, at the first hour after carrageenan injection, and was 52% ($p < 0.001$), 72% ($p < 0.001$), and 75% ($p < 0.001$) at the second hour after carrageenan injection. At the fourth hour, anti-inflammatory effect of the same doses of AD-4 was 42% ($p < 0.001$), 67%

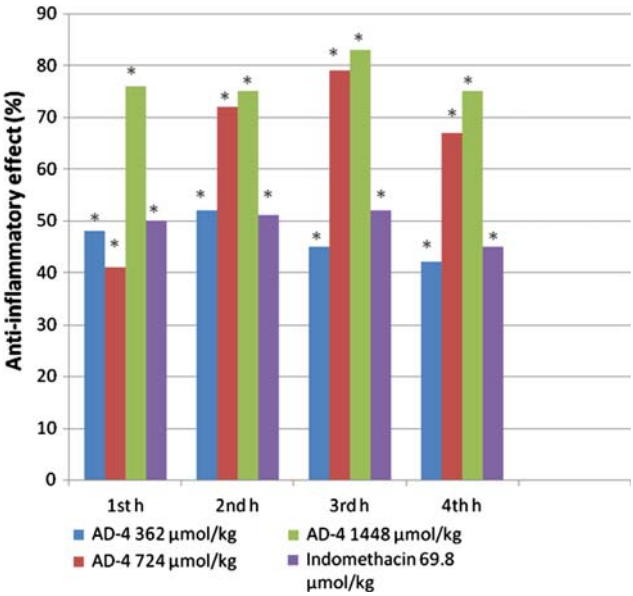


Fig. 3 Anti-inflammatory effects of AD-4 and indomethacin on carrageenan-induced paw edema in rats. *Significant at $p < 0.05$

($p < 0.001$), and 75% ($p < 0.001$). Anti-inflammatory effect of indomethacin at the first, second, and fourth hours was 50% ($p < 0.001$), 51% ($p < 0.001$), and 45% ($p < 0.001$).

Hyaluronidase test

Five and 30 min after subcutaneous injection of hyaluronidase enzyme, the areas of subcutaneous spreading of trypan blue were 288.5 and 498.8 mm^2, respectively, in the control group. In the rabbits receiving indomethacin (27.9 µmol/kg) the corresponding values were 227 ($p < 0.05$) and 279.3 mm^2 ($p < 0.01$) while the values in rabbits receiving AD-4 (3.62×10^2 and 7.24×10^2 µmol/kg) were 212.5 ($p < 0.05$), 196 ($p < 0.05$), 256 ($p < 0.01$), and 267.5 mm^2 ($p < 0.01$) at 5 and 30 min, respectively (Fig. 4).

Acute toxicity

None of the rats receiving AD-4 orally at the doses of 18.1×10^2, 36.2×10^2, and 54.3×10^2 µmol/kg died during the 24-h time period. However, some of the animals receiving AD-4 at the doses of 57.92×10^2, 61.54×10^2, and 65.16×10^2 µmol/kg died. Sixteen percent of rats that received the 57.92×10^2 µmol/kg dose of AD-4 died. While death was observed in 33% of the 61.54×10^2 µmol/kg group, this rate was 66% in the 65.16×10^2 µmol/kg group. All animals that received 68.78×10^2 µmol/kg AD-4 died. There was no observable functional difference in

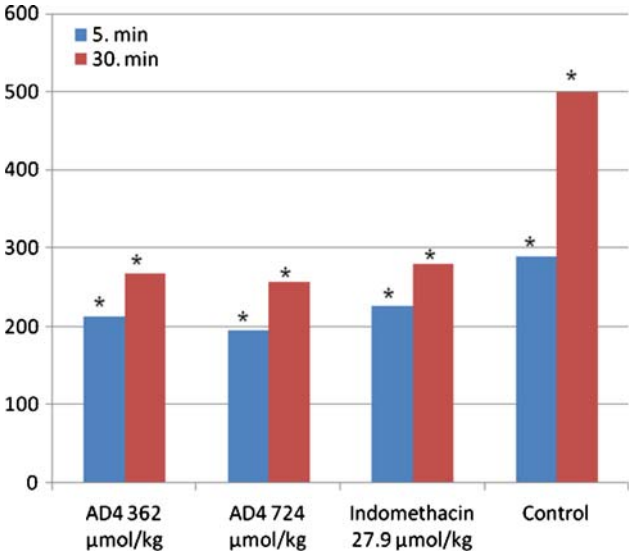


Fig. 4 Effects of AD-4 and indomethacin on hyaluronidase-induced capillary permeability in rabbits. *Significant at $p < 0.05$

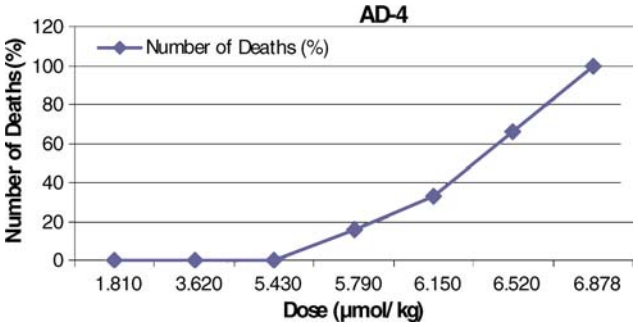


Fig. 5 Acute toxic effects of AD-4 on rats. LD_{50} value for AD-4 determined as 64.44×10^2 µmol/kg

the 18.1×10^2 and 36.2×10^2 µmol/kg AD-4 groups. However, the rats in the 54.3×10^2 µmol/kg AD-4 group were more inactive when compared with those in the 18.1×10^2 and 36.2×10^2 µmol/kg AD-4 groups. The rats in this group drank water between times (did not eat). Also they were positioned close to one another. The rats that received 57.92×10^2 µmol/kg AD-4 were much more inactive and silent. Their drinking frequency was lower than the rats in the 54.30×10^2 µmol/kg group. The posture of the rats in the 61.54×10^2 µmol/kg AD-4 group was worse when compared with others. Twelve hours after drug administration death was observed among the rats of the 65.16×10^2 and 68.78×10^2 µmol/kg AD-4 groups. In the group of rats that received a dose of 69.8 µmol/kg of indomethacin, no deaths were observed, while all of the rats receiving indomethacin at a dose of 139.6 µmol/kg died. It was determined

that the 50% lethal dose (LD_{50}) value of AD-4 is 64.44 $\times$ 10^2 μmol/kg (Fig. 5). These results demonstrate that toxicity of indomethacin is higher than that of AD-4.

Discussion

In the present study, the effect of AD-4 on inflammation was investigated by observation of carrageenan-induced paw edema. In addition, the effect of AD-4 on capillary permeability was investigated using a hyaluronidase-induced test in rabbits.

The results of our experiments showed that AD-4 significantly inhibited carrageenan-induced paw edema at all doses used. The anti-inflammatory effect of AD-4 increased in a dose-dependent manner. The anti-inflammatory effect of AD-4 in all doses studied was accepted as statistically significant. Doses of 7.24 $\times$ 10^2 and 14.48 $\times$ 10^2 μmol/kg of AD-4 were more effective than the 69.8 μmol/kg dose of indomethacin. Doses of 7.24 $\times$ 10^2 and 14.48 $\times$ 10^2 μmol/kg of AD-4 significantly inhibited carrageenan-induced paw edema in rats during the first 3 h following administration, while the 3.62 $\times$ 10^2 μmol/kg dose of AD-4 was only found to be effective at the third hour of the test. The anti-inflammatory effect of indomethacin was found to be effective after the second hour of carrageenan inflammation. These results demonstrated that AD-4 inhibited both early and late phases of carrageenan inflammation at doses of 7.24 $\times$ 10^2 and 14.48 $\times$ 10^2 μmol/kg. A carrageenan-induced inflammatory reaction is known to exhibit an early and a late phase (Gupta *et al.*, 2003). The early phase is associated with histamine, serotonin, and bradykinin, while occurrence of the late phase depends on prostaglandin release (Marzocco *et al.*, 2004). All phases of inflammation have been inhibited by AD-4 administration.

The anti-inflammatory effect of AD-4 (at doses of 7.24 $\times$ 10^2 and 14.48 $\times$ 10^2 μmol/kg) on carrageenan-induced inflammation in both phases of the inflammatory reaction may result from its inhibitory effect on histamine, serotonin, bradykinin, and prostaglandin release. In addition, the ineffectiveness of AD-4 (at 3.62 $\times$ 10^2 μmol/kg dose) in the early phase may result from the lack of inhibition of early-phase mediators. Indomethacin was used in the experiment at a 69.8 μmol/kg dose because this dose of indomethacin is more potent against carrageenan-induced inflammation when compared with lower doses (Suleyman *et al.*, 2004). Indomethacin exhibits an anti-inflammatory effect by the inhibition of histamine, serotonin, bradykinin, and prostaglandin synthesis like other nonsteroidal anti-inflammatory drugs (Suleyman *et al.*, 1999, 2007b).

The mechanism of the anti-inflammatory effect of AD-4 has not been reported in the literature. However, tropolone derivatives have been reported to inhibit lipoxygenase, which has a role in inflammation in vitro (Suzuki *et al.*, 2000). The results of our experiments and others published in the literature show that AD-4 possesses anti-inflammatory effect via inflammation mediators.

In a second series of experiments, effects of AD-4 and indomethacin on hyaluronidase-induced capillary permeability were investigated in rabbits. AD-4 (3.62 $\times$ 10^2 μmol/kg) decreased the hyaluronidase-induced capillary vascular

permeability as effectively as indomethacin (27.9 μmol/kg). In previous studies, this dose of indomethacin demonstrated a significant reduction of hyaluronidase-induced capillary permeability (Suleyman *et al.*, 2001, 2003, 2007a). AD-4 and indomethacin significantly decreased the spread area of hyaluronidase at 5 and 30 min when compared with the control. An increase in vascular dilatation and permeability occurred in the inflamed area. The role of the hyaluronidase enzyme on vascular permeability has been reported (Houck and Chang, 1979). The acute phase of inflammatory response, which shows a classic inflammatory response including calor, dolor, rubor, and tumor, is characterized by transient local vasodilatation and increased capillary permeability (Maslinska and Gajewski, 1998). The activity of the hyaluronidase enzyme increases during the inflammation and decreases as the inflammation reduces (Procide *et al.*, 1971; Houck and Chang, 1979). The role of the hyaluronidase enzyme is also termed the "spreading factor," as the increase of vascular permeability is known (Houck and Chang, 1979). Capillary permeability increases with the effect of inflammation, for many reasons. When inflammation occurs, the activity of hyaluronidase increases and inflammation decreases in parallel with the activity of the hyaluronidase enzyme (Procide *et al.*, 1971).

In the literature, no information exists concerning the effects of tropolone derivatives on vascular permeability. However, it was reported that hydroxytropolones inhibited enzymes such as inositol monophosphate, alkaline phosphates (ALP), and β-monooxygenase by competitive or uncompetitive antagonism (Piettre *et al.*, 1997). Arachidonic acid is also converted to metabolites, including hydroxyeicosatetraenoic acid (HETE), epoxyeicosatrienoic acid (EETA), and 19,20-hydroxyarachidonates, by monooxygenase enzymes. The metabolites (involving the epoxy group) of arachidonic acid (11, 12-EETA) have a potent vasodilatory effect (Kayaalp, 1998). Inhibition of the monooxygenase enzyme by tropolones may result in reduction in the production of epoxy metabolites, which are potent vasodilators, from arachidonic acid. The reduced production of epoxy metabolites may participate in reduction of increased vascular permeability. Also it is important to point out that AD-4 is an α,β-unsaturated carbonyl compound. So it can act as a Michael's acceptor. However in this study we performed only activity experiments and did not pay attention to AD-4's mechanistic properties. A mechanistic approach requires a new investigation of its own.

In conclusion, the results of the present study demonstrated that AD-4 is an anti-inflammatory agent, and exhibited lower toxicity than indomethacin. However, detailed studies are needed to clarify the mechanisms of anti-inflammatory effects of AD-4.

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