Anti-Inflammatory and Analgesic Potency of Carboxyamidotriazole, a Tumorostatic Agent

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ABSTRACT

Carboxyamidotriazole (CAI) is a calcium influx inhibitor that is undergoing clinical trials for the treatment of various human cancers following the identification of its antiproliferative and antimetastatic activities. The exact mechanism of its action is not clearly understood, and whether it has other functions besides the established antitumor activity has not been reported either. In the present study, we demonstrate for the first time that CAI possesses anti-inflammatory and analgesic activities using a variety of animal models, including croton oil-induced ear edema, cotton-induced granuloma, rat adjuvant-induced arthritis, acetic acid-induced writhing, and the formalin test. We also show that CAI significantly inhibits local vascular permeability stimulated by vascular endothelial growth factor or histamine and decreases tumor necrosis factor-α and interleukin-1β levels at the site of inflammation and in sera, which may contribute to the anti-inflammatory effect. These data suggest that CAI is a promising anti-inflammatory and analgesic agent, and they provide new insight into the biological activity of the drug.

Carboxyamidotriazole (CAI), initially developed as a cocciostatic agent and then identified for its anti-invasive capacity, has been shown to inhibit tumor and endothelial cell proliferation by inhibition of calcium uptake and calcium-mediated signal transduction. Up to now, many studies have been conducted mainly focusing on the cancer fighting properties of CAI, which inhibits the proliferation and invasive characteristics of several tumor cell lines in vitro (Wasilenko et al., 1996; Jacobs et al., 1997; Lambert et al., 1997; Moody et al., 2003; Enfissi et al., 2004; Perabo et al., 2004).

In addition, the antiangiogenic effect of CAI has also been well established. In vitro studies have shown that CAI reduces proliferation, adhesion, motility, and vascular tube formation of human umbilical vein endothelial cells (Kohn et al., 1995). Local administration of CAI inhibits capillary expansion in the chick chorioallantoic membrane assay. Furthermore, inhibition of angiogenesis by orally administered CAI has been observed in animal models and in tumor xenografts (Luzzi et al., 1998). Herein, CAI is also described as a new nonendothelial cell-specific inhibitor of angiogenesis (antiangiopreventive agent). In an earlier study (Felder et al., 1991), CAI was shown to have an immediate inhibitory effect on carbachol-stimulated release of arachidonic acid in muscarinic acetylcholine receptor m2-transfected Chinese hamster ovary cells. Another noticeable finding is that pretreatment of human T cells with CAI inhibits the nuclear accumulation of c-Rel and p65, causing a selective repression of nuclear factor-κB DNA binding and a near complete inhibition of calcium-regulated mitogen-induced transcription from the human immunodeficiency virus long terminal repeat (Yasui et al., 1997). According to our current knowledge, the above-mentioned events, including angiogenesis, release of arachidonic acid, and nuclear factor-κB activation, modulated by CAI are involved in many pathological processes, such as cancer and chronic inflammation (Griffioen and Molema, 2000; Lu et al., 2006). Thus, we put forward the hypothesis that CAI, currently recognized as a tumorostatic and antiangiogenic agent, may have more pharmacological activities in addition to what it already presents.

In the present work, we evaluate the possible beneficial effect of CAI in the treatment of acute and chronic inflammatory processes, such as croton oil-induced ear edema, cot-
tion-induced granuloma, and rat adjuvant-induced arthritis. Local vascular permeability in mouse modulated by CAI has also been determined. In addition, we have assessed the effect of CAI on the levels of proinflammatory cytokines TNF-α and IL-1β at the site of inflammation and in sera. Moreover, antinoceptive effect of CAI on inflammatory pain has been assessed using acetic acid-induced writhing model and the formalin test. This study is, to our knowledge, the first showing that CAI possesses anti-inflammatory and analgesic activities.

Materials and Methods

Materials. CAI was synthesized by the Institute of Materia Medica, Chinese Academy of Medical Sciences. Polyethylene glycol (PEG) 400, acetic acid, and formaldehyde solution 37% (w/v) were provided by Beijing Chemical Reagents Company (Beijing, China). Dexamethasone sodium phosphate injection was from Tianjin Jinyao Amino Acid Co., Ltd. (Tianjin, China). Morphine hydrochloride was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α and IL-1β were from R&D Systems (Minneapolis, MN).

Animals. Female Wistar rats (180–200 g) or male ICR mice (18–22 g; 6–8 weeks old) were obtained from Beijing Vital River Laboratory Animal Co., Ltd. (Beijing, China). They were housed in an air-conditioned room (22 ± 2°C and 40–70% humidity), with a controlled 12-h light/dark cycle (lights on 8:00 AM). Animals had free access to standard chow and water. All animal studies and procedures were approved by the Institutional Animal Care and Use Committee of Peking Union Medical College.

Croton Oil-Induced Ear Edema. The croton oil ear test was performed as described previously (Olajide et al., 2000). Male ICR mice were randomly divided into five groups, with 15 animals in one group. CAI (10 and 20 mg/kg), dexamethasone (0.8 mg/kg), or vehicle (PEG 400 and 0.9% NaCl) was administered once daily (orally for CAI and PEG 400; i.p. for dexamethasone and saline). One hour after the third administration, 100 μl of croton oil solution (croton oil/ethanol/distilled water/ether, 2:20:5:73) was applied to both sides of the right ear of each mouse. The left ear remained untreated. The animals were sacrificed 4 h later, and a plug (6.5 mm in diameter) was removed from both the treated and untreated ear. The difference in weight between the two plugs was taken as a measure of edema intensity. The anti-inflammatory activity was expressed as percentage of the edema reduction in treated mice compared with control mice.

Induction of Granulomatous Tissue. Subacute inflammation was produced in female Wistar rats (180–200 g) by cotton pellet-induced granuloma (Swingle and Shideman, 1972). Sterile cotton (20 ± 1 mg) soaked in 0.4 ml of 5% ampicillin solution was implanted s.c. bilaterally in groin under anesthesia. The following was administered daily: 0.9% NaCl (NS; i.p.), 0.8 mg/kg dexamethasone (i.p.), PEG 400 (p.o.), and 10 and 20 mg/kg CAI (p.o.). On the 7th day, the animals were sacrificed. The granulomatous tissue with cotton pellet was removed and dried at 60°C to constant weight. Increment in the dry weight of the pellets was taken as measure of granuloma formation.

Inhibition of Adjuvant-Induced Arthritis. Female Wistar rats (180–200 g) were divided into five groups. On day 0, animals in groups 1 to 4 were induced adjuvant arthritis by a single intradermal injection of 0.1 ml of Freund’s complete adjuvant into the plantar region of the right hind paw. NS (i.p.), 0.5 mg/kg dexamethasone (i.p.), PEG 400 (p.o.), and 20 mg/kg CAI (p.o.) were administered once a day from day 0 to day 27. As a nonarthritic control, animals in group 5 were free of Freund’s complete adjuvant injection, and they received injections with 0.9% NaCl daily. Right hind paw volume was measured before adjuvant injection and on days 1 to 14 with a water displacement volume meter to assess the primary inflammatory response, and left hind paw volume was measured on days 21, 24, and 27 to assess the secondary inflammatory response. The anti-inflammatory effect was expressed as the difference in paw edema compared with that of saline-treated arthritic rats (Newbould, 1963; Walz et al., 1971).

Histopathology Evaluation. Rat synovial membrane samples were obtained from the knee joint 14 days after adjuvant injection. The specimens were fixed in buffered 10% formalin and embedded in paraffin. They were serially sectioned onto microscope slides at a thickness of 4 μm and then deparaffinized, stained with hematoxylin and eosin, and evaluated for morphological changes and cellular infiltration.

Vascular Permeability Evaluation. Male ICR mice (18–22 g; 6–8 weeks old) were pretreated orally with 0.9% NaCl, PEG 400, or varying doses of CAI for 3 days. Miles assay was then performed (Miles and Miles, 1952; Claffey et al., 1996). In brief, Evans blue solution (dissolved in saline) was injected into mice via the tail vein at a dose of 40 mg/kg. After 10 min, 100 μl of vascular endothelial growth factor (VEGF) (1 μg/ml) or histamine (1 mg/ml) was injected intradermally into the preshaved back skin. After 20 min, the animals were euthanized, and an area of skin that included the entire injection site was removed. Evans blue dye was extracted from the skin by incubation with formamide for 48 h at 37°C, and the absorbance of extracted dye was measured by a Beckman spectrophotometer at 620 nm.

Determination of IL-1β and TNF-α in Rat Serum and Paw Tissue. For arthritic rats, serum was collected on the last day of the experiment (day 27). After animals were sacrificed, the s.c. tissue of the right hind paw and that surrounding the ankle joints was removed and placed in 5 ml of saline. The tissues were homogenized at 4°C, and then it was centrifuged at 10,000g for 15 min. The supernatants were stored at −80°C until further analysis. IL-1β and TNF-α levels in serum and paw tissue supernatant were measured using specific rat ELISA kits according to the manufacturer’s instructions. The sensitivities of the assays for IL-1β and TNF-α were 7 and 4 pg/ml, respectively.

Analogic Activity against Acetic Acid-Induced Writhing. The analgesic activity of CAI was assessed using writhing test (abdominal constriction test) (Collier et al., 1968). Male ICR mice were pretreated with CAI (10 and 20 mg/kg p.o.) for 3 days. Morphine hydrochloride (2 mg/kg i.p.) was used as the reference analgesic drug, and vehicles (PEG 400 and NS) were also administered once daily. One hour after the third administration, 0.1 ml of 0.6% (w/v) acetic acid solution was injected i.p., and the writhing behavior (contraction of abdominal muscles together with stretching of the hind limbs) was cumulatively counted in various groups of animals over a period of 15 min beginning 3 min after acetic acid injection. Analgesic activity was expressed as percentage of inhibition of writhing.

Formalin Test in Mice. The formalin test was carried out as described by Hunskaar and Hole (1987). Mice received injections with 20 μl of 2.5% formalin (formaldehyde solution 37% (w/v) diluted in NS) into the subplantar space of the right hind paw after pretreatment with CAI (20 mg/kg p.o., once daily for 3 days), morphine hydrochloride (2 mg/kg i.p., 10 min before formalin injection), or both. Responses were measured 0 to 5 min (early phase) and 10 to 25 min (late phase) after formalin injection. The animals were placed in a glass cylinder (20 cm in diameter), and the amount of time spent in each one of four behavioral categories was recorded, which were further scored as follows: 0, the injected paw is not favored; 1, the injected paw has little or no weight on it; 2, the injected paw is elevated and is not in contact with any other surface; and 3, the injected paw is licked, bitten, or shaken. An average pain intensity score was calculated, according to the weighed-scores technique (Dubuisson and Dennis, 1977), by multiplying the amount of time spent in each category by its assigned category score, adding these products and dividing by the total time of observation (i.e., 300 and 900 s).

Statistical Analysis. The results are expressed as the mean ± S.E.M.; n represents the number of experiments or animals. Statis-
Results

Effect of CAI on Mouse Ear Edema Induced by Croton Oil. Both CAI and dexamethasone profoundly affected ear edema induced by croton oil in mice. Dexamethasone caused 72.75% inhibition of edema at the dose of 0.8 mg/kg compared with saline-treated animals, and CAI inhibited this inflammatory response at 10 and 20 mg/kg by 18.95 and 44.61%, respectively (Fig. 1). Both the reference drug and the test drug exhibited significant anti-inflammatory properties, although the latter presented a lower effect. PEG 400 vehicle had no effect.

Effect of CAI on Cotton Pellet-Induced Granuloma. Cotton pellet-induced chronic inflammatory response characterized with granuloma formation, fluid infiltration, and undifferentiated connective tissue was measured by weighing the dried pellets after 6 days of implantation and treatment. The effect of CAI on cotton pellet-induced granuloma in rats is shown in Fig. 2. The weight of the granuloma for the control group of animals was found to be 26.77 ± 3.56 mg/100 g wt. Treatment with CAI at 10 and 20 mg/kg p.o. decreased the granuloma weight to 19.69 ± 4.70 and 17.90 ± 2.80 mg/100g wt, respectively. Treatment with dexamethasone (0.8 mg/kg i.p.) produced a granuloma weight of 11.94 ± 2.96 mg/100 g wt. Both CAI and dexamethasone inhibited the granuloma tissue formation significantly.

Effect of CAI on Adjuvant Arthritis. Figure 3 shows the anti-inflammatory effects of CAI in another inflammatory disease model, the rat adjuvant-induced arthritis. In vehicle-treated animals, definite edema developed in adjuvant-injected right paw and slight swelling on the contralateral side. Primary paw edema was diminished by CAI throughout a 14-day period, and more dominant inhibition was observed in dexamethasone-treated group, with both drugs showing significant anti-inflammatory effect compared with vehicle (Fig. 3A). From day 21, paw swelling in the adjuvant noninjected left hind paw became obvious. The edema in saline-treated arthritic rat left paws on day 21 was 0.79 ± 0.08 ml, being comparable with that of right paws. This secondary edema was also significantly suppressed by CAI (p < 0.01) and dexamethasone (p < 0.01) (Fig. 3B).

CAI Reduced the Severity of Synovial Histopathological Changes in Arthritic Rats. The synovial lining was discerned at 40× magnification (Fig. 4, A–D). In naive animals (Fig. 4A), the synovial cells formed a thin layer, and they were flat and quiescent. No leukocyte infiltration was observed. In animals treated with vehicle PEG 400, the synovial membrane cells became hyperplastic, and it formed a thick, multicelled layer, suggesting active proliferation. In addition, the synovial membrane showed infiltration by leukocytes and hyperanemia with dilated blood microvessels (Fig. 4B). In the synovial tissues of CAI- or dexamethasone-treated rats (Fig. 4, C and D), cell hyperplasia and hyper trophy were significantly inhibited, few or no leukocytes were present, and fewer blood microvessels were seen.

CAI Reduced VEGF or Histamine-Induced Vascular Permeability. The Miles assay was used to determine whether CAI affected vascular permeability, a prominent feature of inflammatory pathological process. Evans blue dye was injected i.v., and immediately thereafter, two vascular permeability-inducing agents, VEGF and histamine, were injected into the shaved flank skin of ICR mice. Evans blue dye binds to plasma proteins; therefore, it extravasates along with them at sites of increased permeability (Miles and Miles, 1952). CAI treatment at 20 mg/kg for 3 days strikingly inhibited VEGF- or histamine-induced extravasation of Evans blue dye in mice by 35 and 44%, respectively (Fig. 5A). Furthermore, the antipermeabilizing effect of CAI was dose-dependent. As shown in Fig. 5B, oral administration of CAI at doses of 5, 10, and 20 mg/kg reduced
histamine-induced Evans blue dye accumulation by 7.2, 27.5, and 46.4%, respectively.

Effect of CAI on TNF-α and IL-1β Levels in Paw Homogenates and Sera of Arthritic Rats. Because proinflammatory cytokines play critical role in the pathogenesis of autoimmune and inflammatory diseases such as arthritis, the effect of CAI (20 mg/kg p.o.) and dexamethasone (0.5 mg/kg i.p.) on TNF-α and IL-1β levels in paw homogenates and sera of arthritic rats was determined. CAI reduced tissue TNF-α and IL-1β levels by 54.1 and 26.3%, respectively, which was comparable with the inhibitive effect of dexamethasone (Fig. 6, A and B). In serum, TNF-α and IL-1β levels were also significantly reduced by CAI and dexamethasone (Fig. 6, C and D).

Effect of CAI on Acetic Acid-Induced Writhing. Acetic acid (0.1 ml; 0.6%) produced an average of 20.29 writhings per animal; oral administration of CAI at doses of 10 and 20 mg/kg decreased the number of abdominal constrictions by 44.01 and 60.92%, respectively. These changes were very significant. The vehicle PEG 400 had no analgesic effect. Morphine (2 mg/kg), the reference drug, inhibited the writhing by 93.10%, demonstrating the most potent analgesic effect (Table 1).

Fig. 3. Effect of CAI on the development of adjuvant-induced arthritis in female Wistar rats. Animals received injections with Freund’s complete adjuvant on day 0. A, drugs and vehicles were administered once daily, and the edema in right hind paw was measured every 1 to 2 days to assess the magnitude of the primary inflammatory response. B, effect of CAI on the secondary paw edema in arthritic rats was measured on days 21, 24, and 27. N.A., nonarthritic animals. Data points or columns and bars represent mean ± S.E.M. (n = 15). **, p < 0.01, compared with saline-treated arthritis rats.

Discussion
CAI, which has been well documented for its antiproliferative, antiangiogenic, and antimetastatic properties, is undergoing clinical trials for the treatment of various human cancers (Berlin et al., 1997, 2002; Kohn et al., 1997, 2001; Bauer et al., 1999). Here, we reported for the first time that CAI has potential anti-inflammatory and analgesic effects in addition to its antitumor activity.

In this study, the effects of CAI on acute and chronic phases of inflammation were investigated using models of croton oil-induced ear edema, cotton pellet granuloma, and adjuvant-induced rat arthritis. In addition, the effect of CAI on capillary permeability was investigated by Evans blue leakage assay. Oral administration of CAI was found protective against croton oil-induced ear edema; however, the power of anti-inflammatory activity of CAI was less than that of dexamethasone at both doses (10 and 20 mg/kg) used. In cotton pellet granuloma test, CAI significantly decreased the...
final dry weight of the cotton pellets that correlates very well with the amount of granulomatous tissue (Swingle and Shideman, 1972), suggesting that CAI has the capability in reducing the synthesis of mucopolysaccharide and collagen and the number of fibroblasts, which are natural proliferative events of granulation in tissue formation.

CAI was shown to reduce the vascular permeability by reduction of VEGF- or histamine-induced Evans blue dye leakage. Previous studies demonstrated that VEGF induces venular hyperpermeability through a kinase insert domain-containing receptor-mediated activation of phospholipase C (PLC), subsequent protein kinase C activation and cytosolic Ca\(^{2+}\) elevation, as well as endothelial constitutive nitric-oxide synthase activation (Wu et al., 1999). Regarding histamine-mediated vascular hyperpermeability, the production of inositol triphosphate via PLC and an internal release and further influx of calcium serve important functions (Rotrosen and Gallin, 1986). It has been well established that CAI preferentially blocks calcium influx and inhibits downstream phosphorylation events involving PLC\(\gamma\) and inositol triphosphate (Kohn and Liotta, 1990; Hupe et al., 1991). The modulation of Ca\(^{2+}\)-mediated signal transduction by CAI might disturb the above-mentioned pathways involved in VEGF- or histamine-induced vascular hyperpermeability, resulting in decreased capillary leakage.

CAI exposure has been shown to inhibit vascular tube formation in Matrigel and aortic ring assays and to inhibit capillary expansion in the chick chorioallantoic membrane assay (Kohn et al., 1995; Bauer et al., 1999). Furthermore, inhibition of angiogenesis by orally administered CAI has been observed in animal models and in tumor xenografts (Luzzi et al., 1998). The relation between angiogenesis and chronic inflammation has attracted a lot of attention in recent years. In the first acute
phase of inflammation, functional changes in the vasculature such as dilatation, increase in permeability, and endothelial activation occur. In the second subacute phase, capillaries and venules remodel with extensive endothelial mitotic activity (Majno, 1998). Upon chronic stimulation, both increases in capillary density and vascular dilatation can be observed (Thurston et al., 1998). In rheumatoid arthritis, neovascularization, which is one of the earliest histopathological findings, is thought to be required for pannus development. Rheumatoid synovial endothelium is constantly subjected to remodeling. Besides nurturing the pannus, the blood vessels also play an active role in the inflammation by being a source of cytokines, chemokines, and proteases (Storgard et al., 1999). So, it can be expected that CAI, a known antiangiogenic agent, may have the potential for treatment of arthritis and other chronic inflammatory diseases accompanied by overt neoangiogenesis.

To confirm the hypothesis mentioned above, the model of adjuvant-induced rat arthritis was used. In this model, rats develop a chronic swelling in multiple joints, with influx of inflammatory cells, erosion of joint cartilage, and bone destruction of joint integrity and loss of function. This model of chronic inflammation is a complex response involving different mediators; therefore, there is a possibility of multiple interactions (Stefanovic-Racic et al., 1993; Kollias et al., 1999). Our data indicate that CAI is effective, by oral route, in the treatment of experimental chronic inflammation and that the inhibition of joint inflammation was accompanied by reduction of TNF-α and IL-1β levels in both arthritic paws and in sera. It has been well established that proinflammatory cytokines such as TNF-α and IL-1β promote cartilage and bone resorption by inhibiting proteoglycan and collagen synthesis and induce the expression of collagen degrading enzymes, leading to bone and cartilage destruction in the rheumatoid synovium (Feldmann et al., 2002). They also induce the expression of other proinflammatory mediators, such as IL-8, granulocyte-macrophage colony-stimulating factor, and IL-6, and facilitate inflammatory cell infiltration by enhancing neutrophil and T cell adhesion to endothelium (Arend and Dayer, 1995). In addition, it should be noted that proinflammatory cytokines, including TNF-α and IL-1β, are also involved in the progression of tumors and increasingly recognized to be key molecular players in linking inflammation to cancer (Coussens and Werb, 2002; Lu et al., 2006). Therefore, it may be reasonable to declare the correlation between anti-inflammatory and anticancer properties of CAI, and new research strategies for unraveling the mechanism of CAI based on the correlation might be promising.

Our study also revealed that CAI has potent antinoceptive action in the acetic acid and formalin tests, two highly sensitive and useful tests for analgesic screening. The abdominal constrictions produced after administration of acetic acid or formalin is a sensitive and useful test for analgesic screening. The abdominal constrictions produced after administration of acetic acid or formalin is a sensitive and useful tests for analgesic screening. The ab-

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