Biochemical Pharmacology, Vol. 28, pp. 1959–1961.

0006-2952/79/0615–1959 $02.00/0

A NEW APPROACH TO ANTI-INFLAMMATORY DRUGS

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(*Received* 27 *September* 1978)

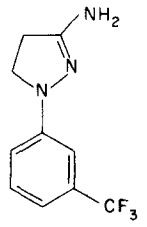
Abstract—All aspirin-like drugs so far tested inhibit prostaglandin biosynthesis but do not prevent the generation of the hydroxy acid 12-L-hydroxyeicosatetraenoic acid (HETE) by arachidonate lipoxygenase. HETE is chemotactic for polymorphonuclear leukocytes, and the failure to inhibit lipoxygenase may explain why the aspirin-like drugs have little or no effect on leukocyte migration at doses which are both anti-inflammatory and inhibit prostaglandin synthesis *in vivo*. 3-Amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline (BW755C) inhibits both pathways of arachidonic acid metabolism *in vitro* and causes a dose-dependent reduction in carrageenin-induced oedema in the rat paw. BW755C also reduces prostaglandin concentrations in inflammatory exudates and has a significantly greater effect on leukocyte migration than indomethacin. The dual inhibition of arachidonate cyclo-oxygenase (prostaglandin synthetase) and lipoxygenase could lead, therefore, to increased anti-inflammatory activity.

Since the demonstration of prostaglandin activity in carrageenin-induced inflammatory exudates [1], overwhelming evidence has accumulated to support the theory that prostaglandins are important mediators of the vascular events in inflammation. Aspirin-like drugs inhibit the biosynthesis of prostaglandins [2–4], and this explains their anti-inflammatory activity [2, 5]. The anti-oedema and anti-erythema actions of the aspirin-like drugs correlate closely with their ability to inhibit the production of vasodilator prostaglandins in experimental inflammation [6]. There is, however, only indirect evidence that prostaglandins are involved in the cellular component of the inflammatory response. Furthermore, the effects of aspirin-like drugs on leukocytes in inflammation is unclear.

Indomethacin reduces the concentration of leukocytes in carrageenin-induced inflammatory exudates by up to 35 per cent [6–9], and the reduction of total numbers of leukocytes by cyclo-oxygenase (prostaglandin synthetase) inhibitors is proportional to the reduction in oedema volume [9, 10]. At doses of indomethacin which completely inhibit prostaglandin synthesis, there is no reduction of leukocyte migration into polyester sponges implanted subcutaneously in rats [11]. These results indicate that aspirin-like drugs have a relatively small effect on leukocyte chemotaxis *in vivo* at doses which are both anti-inflammatory and inhibit prostaglandin biosynthesis.

The fatty acid precursors of the prostaglandins can also be oxygenated by a lipoxygenase, which converts arachidonic acid to a hydroxy acid, 12-L-hydroxyeicosatetraenoic acid or HETE [12, 13]. HETE is a potent chemotactic agent for polymorphonuclear leukocytes [14, 15] and has been detected in inflamed skin [16]. The aspirin-like drugs do not prevent the generation of HETE from arachidonic acid [12], and this may explain their failure to prevent the accumulation of leukocytes in inflammation.

Anti-inflammatory corticosteroids reduce prostaglandin concentrations in inflammation [6, 17, 18], prevent the release of prostaglandins from fat tissue [19], and reduce the availability of prostaglandin precursors [20–22]. Corticosteroids do not inhibit cyclo-oxygenase [23] but prevent the release of fatty acids from phospholipids, thus resulting in a reduction in both cyclo-oxygenase and lipoxygenase products. If HETE is an important mediator of chemotaxis *in vivo*, a reduction of lipoxygenase products by anti-inflammatory steroids may explain why these drugs suppress cell migration [24].



BW 755C

Fig. 1. 3-Amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline (BW755C).

Two types of compound have been shown to inhibit both pathways of arachidonate metabolism; these are arachidonic acid analogues such as 5,8,11,14-eicosatetraynoic acid (TYA) [12] and phenidone (1-phenyl-3-pyrazolidone) [25]. We have shown that BW755C (Fig. 1), a structural analogue of phenidone, is a potent inhibitor of lipoxygenase and cyclo-oxygenase and has anti-inflammatory activity *in vivo*. We have compared the effects of BW755C, indomethacin and dexamethasone on leukocyte migration and prostaglandin production *in vivo*. Some of this work was presented at the Seventh International Congress of Pharmacology [26].

MATERIALS AND METHODS

3-Amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline (BW755C) was synthesized by Dr. C. V. Denyer of the Wellcome Research Laboratories. Indomethacin and dexamethasone sodium phosphate (decadron) were obtained from Merck, Sharpe & Dohme and [1-

[^{14}C]arachidonic acid (batch 17, 61 mCi/m-mole) was purchased from the Radiochemical Centre, Amersham.

Preparation of platelet enzymes. Fresh citrated horse blood was centrifuged at 300 *g* for 20 min to sediment red blood cells. The platelet-rich plasma was decanted and re-centrifuged at 2000 *g* for 30 min. The resultant platelet pellet was resuspended in Tris buffer (100 mM, pH 7.5, 4°) and freeze-thawed four times in a round-bottomed glass flask. This procedure causes complete rupture of platelet membranes. The resultant preparation was diluted to a protein concentration of 25 mg/ml.

Enzyme assays [25]. One ml of the above preparation was incubated for 10 min at 37° in a shaking water bath with 1 μg unlabeled and 0.1 μCi [1-^{14}C]arachidonic acid. BW755C, indomethacin and dexamethasone were added to give concentrations of 0.01 to 100 μg/ml. Reactions were terminated by boiling for 1 min, and the products were extracted into ethyl acetate following acidification of the aqueous phase. The extracts were then taken to dryness in a vacuum dessicator, redissolved in 50 μl chloroform–methanol (1:1), quantitatively applied onto silica gel t.l.c. plates and developed in ethyl acetate, iso-octane, water and acetic acid (11:5:10:2, v/v, upper phase). Radioactive zones were located by autoradiography and quantitated by standard liquid scintillation counting procedures.

Anti-inflammatory activity. Oedema induced by the sub-plantar injection of carrageenin [27] in the hind paws of rats was measured by the mercury displacement method [28]. Groups of five rats were used, and the increase in paw volume was estimated over 4 hr by subtracting the volume of the contralateral paw which received an equal volume of saline. Anti-inflammatory effects were measured as the degree of inhibition of oedema.

Induction and collection of inflammatory exudates. Inflammatory exudates were induced and collected by the subcutaneous implantation of polyester sponges impregnated with carrageenin (20 mg/ml of sterile saline) in male rats (200 g) [6]. The sponges were removed after 24 hr, immersed in 5 ml of heparinized saline and squeezed until dry. Total leukocyte numbers in sponge exudates were estimated using "Improved Neubauer" counting chambers and phase contrast microscopy.

Prostaglandin assays. Prostaglandin activity in acid-lipid extracts of sponge exudates was estimated by bioassay on the superfused rat stomach strip [29] treated with various antagonists [30] and was expressed as prostaglandin E_2 equivalents.

Dosing regime. Doses of drugs were given three times during each experiment. The first dose was given at least 24 hr before (a) the final measurement of paw oedema, or (b) the removal of the sponge. The second dose was given 6–8 hr after the first, and the third, 3–4 hr before the final oedema reading or removal of the sponge. Each drug was given orally in aqueous vehicle to at least five animals, and in each experiment the vehicle was given to a similar group of control rats. In some experiments dexamethasone was given intramuscularly (i.m.).

The mean concentration of prostaglandin E_2 equivalents and the mean number of leukocytes/ml of inflammatory exudate were calculated for each group of treated animals and expressed as a percentage of control values for each experiment.

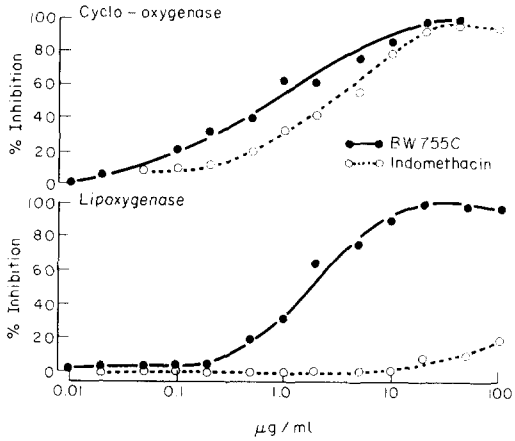


Fig. 2. Inhibition of platelet cyclo-oxygenase and lipoxygenase by indomethacin and BW755C. Each point is the mean of two observations.

RESULTS

Zones of radioactivity on autoradiograms were identified by comparison with authentic standards. When arachidonic acid was incubated with untreated platelet homogenates, a number of products were formed, the most prominent of which co-chromatographed with authentic HETE, 12-L-hydroxy heptadecatrienoic acid (HHT) and thromboxane B_2 (TXB_2). HHT and TXB_2 are products of cyclo-oxygenase [12]. When arachidonic acid was incubated with boiled platelet homogenates, only the unreacted substrate was seen. Indomethacin (10 μg/ml) prevented the production of TXB_2 and HHT but not HETE, while BW755C (20 μg/ml) prevented the formation of both lipoxygenase and cyclo-oxygenase products. Dexamethasone (100 μg/ml) was inactive or caused a slight increase in all products.

Indomethacin and BW755C were tested over a range of concentrations. BW755C gave a concentration-dependent inhibition of lipoxygenase (IC_{50} = 1.70 μg/ml) and cyclo-oxygenase (IC_{50} = 0.72 μg/ml), while indomethacin inhibited cyclo-oxygenase (IC_{50} = 2.80 μg/ml) but reduced the production of HETE by less than 20 per cent at 100 μg/ml (Fig. 2).

Each drug caused a dose-dependent reduction in carrageenin-induced oedema, and their effects on prostaglandin production and leukocyte migration in sponge exudates were examined at a dose which inhibited paw oedema by 45–55 per cent. Indomethacin (4 mg/kg) inhibited leukocyte migration by 26 ± 7 per cent (S.E.M.) (n = 13) and reduced prostaglandin concentrations by over 98 per cent. BW755C (50 mg/kg) reduced leukocytes by 73 ± 9 per cent (S.E.M.) (n = 8) and prostaglandins by 69 ± 7 per cent (S.E.M.). Dexamethasone (0.1 mg/kg) reduced leukocytes and prostaglandins by about 30 per cent when given orally, but was more active when given intramuscularly (0.1 mg/kg), inhibiting prostaglandin synthesis by 62 ± 17 per cent (S.E.M.) (n = 5) and leukocyte migration by 64 ± 6 per cent (S.E.M.) (Fig. 3). Dexamethasone and

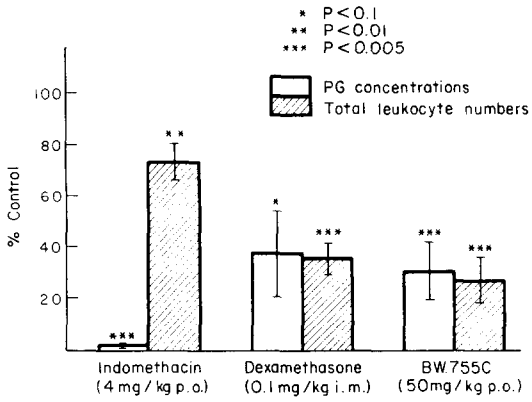


Fig. 3. Effects of indomethacin, dexamethasone and BW755C on prostaglandin concentrations and leukocyte numbers in inflammatory exudates. Each value is the mean of 5–13 experiments, and the bars represent ± 1 S.E.M. Each drug reduced carrageenin-induced oedema by 45–55 per cent at the dose indicated.

BW755C had a significantly greater effect ($P = < 0.0025$ in both cases) on leukocyte migration than did indomethacin.

DISCUSSION

These results suggest that the anti-inflammatory activities of dexamethasone, indomethacin and BW755C are due to interference with three different enzymes. Indomethacin reduces prostaglandin concentrations *in vivo* by selective inhibition of cyclo-oxygenase but has relatively small effects on leukocyte migration at anti-inflammatory doses. The reduction of leukocyte migration by the aspirin-like drugs could be related to a reduction in oedema, rather than a direct effect on chemotaxis [9, 10]. BW755C inhibits both pathways of arachidonic acid oxygenation, reduces prostaglandin production *in vivo*, and has a significantly greater effect on leukocyte migration than indomethacin. A reduction in the number of migrating cells by BW755C may be due to inhibition of production of the chemotactic hydroxy acid HETE. Dexamethasone, which does not inhibit cyclo-oxygenase or lipoxygenase *in vitro*, has an effect similar to that of BW755C on leukocyte migration and prostaglandin production (Fig. 3). This activity may be explained by a reduction in the availability of arachidonic acid due to a reduction in phospholipase A_2 activity [20–22], which would also account for the fall in cyclo-oxygenase and lipoxygenase products [16].

BW755C, therefore, could represent an important new type of anti-inflammatory drug. The simultaneous inhibition of both pathways of arachidonic acid oxygenation may lead to a more selective type of anti-inflammatory action, without the complicating side-effects of the corticosteroids. Such a drug would also have advantages over the aspirin-like drugs in the treatment of chronic inflammation, by having a greater effect on leukocyte migration.

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