Inhibition of human neutrophils by auranofin: chemotaxis and metabolism of arachidonate via the 5-lipoxygenase pathway

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SUMMARY The effect of auranofin on human neutrophil (PMN) 5-lipoxygenase activity and leucotriene B₄ (LTB₄) chemotaxis was investigated. [1-¹⁴C]Arachidonic acid was incorporated into the purified cells until steady state conditions were obtained. After preincubations with serial dilutions of auranofin arachidonic acid release and metabolism were stimulated with calcium ionophore A23187. The radioactive eicosanoids released were extracted and separated by thin layer chromatography, followed by autoradiography and quantitative laser densitometry. Chemotaxis of PMNs towards LTB₄ was measured in a modified Boyden chamber. Auranofin showed dose dependent inhibition of both the 5-lipoxygenase pathway (IC₅₀ 17.4 × 10⁻⁶ mol/l) and of chemotaxis (IC₅₀ 45 × 10⁻⁶ mol/l). The release of arachidonic acid from phospholipids was unaffected in the concentration range tested (1–1000 μmol/l). Inhibition of both neutrophil motility and cellular synthesis of proinflammatory eicosanoids may thus contribute to the beneficial clinical effects of auranofin in rheumatoid arthritis.

Key words: inflammation, leucotrienes, rheumatoid arthritis.

In rheumatoid arthritis the polymorphonuclear neutrophil granulocyte (PMN) is of potential importance for modulation of the inflammatory process.¹ This cell type may constitute more than 90% of the cellular exudate in synovial fluid² and is abundant in the inflamed synovial membrane and at the interface of cartilage with pannus.³ Local secretion of arachidonic acid metabolites, formed mainly via the 5-lipoxygenase pathway,^{4,5} oxygen free radicals,⁶ and lysosomal enzymes, especially natural proteases, may be essential for perpetuation of inflammation and for tissue destruction.⁷

PMNs from patients with rheumatoid arthritis show an enhanced capacity for metabolism of endogenous arachidonic acid, with increased release of leucotriene B₄ (LTB₄) during activation *in vitro*.⁸ Accordingly, high concentrations of LTB₄ are found in the synovial fluid from patients with active

rheumatoid arthritis.^{4,5,9} LTB₄ is a proinflammatory mediator, which activates human PMNs with respect to chemotaxis and aggregation,^{10,11} and, further, it is a complete secretagogue in PMNs, showing a physiological profile similar to that of calcium ionophore A23187.¹² Thus inhibitors of LTB₄ synthesis and LTB₄ actions may show anti-inflammatory properties.

Auranofin, a new gold compound for oral treatment of rheumatoid arthritis,¹³ has been shown to affect many of the activities of PMNs in acute inflammation.¹⁴ The aim of the present work was to discover whether auranofin affected two essential functions of PMNs in relation to chronic non-specific inflammation—namely, metabolism of endogenous arachidonic acid mainly via the 5-lipoxygenase pathway to LTB₄ and 5-hydroxy-eicosatetraenoic acid (5-HETE), and chemotaxis to LTB₄ itself, which is elicited via specific surface receptors.

Materials and methods

Blood was drawn in 10 mM edetic acid from 20

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healthy volunteers who had taken no drugs, including salicylates, for at least four weeks.

Neutrophils were isolated by a modification of Böyum's method¹⁵ including: sedimentation of erythrocytes with methylcellulose (0.8%), gradient centrifugation of 'buffy coat' leucocytes on Lymphoprep (Nygaard and Co, Oslo, Norway), and hypotonic lysis of residual erythrocytes. The final cell suspensions contained more than 95% PMNs, with a median recovery of 44%. The viability was 97%, as shown by the trypan blue exclusion test.

DRUGS AND CHEMOATTRACTANT

Shortly before use serial dilutions were made in Gey's solution (pH 7.2–7.4) of auranofin (1–1000 $\mu\text{mol/l}$) (mol. wt 678.5) (Smith, Kline and French, Solna, Sweden) dissolved in 0.1% ethanol. Nordihydroguaiaretic acid (10 $\mu\text{mol/l}$) (Sigma Inc, St Louis, MO, USA), a well established inhibitor of 5-lipoxygenase activity, was included as a control. The amount and purity of LTB_4 (Paesel GmbH, Frankfurt am Main, FRG) was checked by an ultraviolet spectrum and by high pressure liquid chromatography, which showed a purity of more than 95%.

ARACHIDONATE METABOLISM

Isolated PMNs were incubated with [^{14}C]arachidonic acid (37 000 $\times 10^3$ Bq/ 5×10^6 cells, 2.2×10^9 Bq/mmol) (Amersham International, Buckinghamshire, UK) for five hours at 37°C to obtain steady state labelling of intracellular pools of arachidonic acid.¹⁶ Non-incorporated extracellular arachidonic acid was removed by washing. Test drug was then added to the cells 1–30 minutes before stimulation with calcium ionophore A23187 (Calbiochem, La Jolla, CA, USA) (15 minutes) in an optimal concentration of 10 $\mu\text{mol/l}$.¹⁶ Extracellular fluid containing radiolabelled metabolites was prepared by instantaneous removal of the cells by centrifugation (8000 g, one minute) through dibutyl phthalate:dinonyl phthalate 3:1 (density 1.033 g/ml) before extraction of eicosanoids.^{16 17}

The radioactive metabolites were then separated by thin layer chromatography and measured by autoradiography and laser densitometry as previously described.¹⁶ Fractions more lipophilic than arachidonic acid were not included in the calculations.

Identification of arachidonic acid and metabolites was carried out with cochromatography using pure standards (Paesel GmbH, Frankfurt am Main, FRG), and evaluation of specific activities by high pressure liquid chromatography has been described in detail previously.¹⁶ The concentration of auranofin necessary for 50% inhibition of arachidonic acid catabolism (IC_{50}) via the 5-lipoxygenase pathway was calculated by intrapolation from the logarithmic dose-response curves.

REVERSIBILITY AND VIABILITY

In a separate series of six experiments [^{14}C]arachidonic acid labelled PMNs were incubated with auranofin (31 $\mu\text{mol/l}$) for 15 minutes. One half of the aliquots was then challenged with A23187, whereas the other half was washed three times in Gey's medium before challenge. The percentage viability of the PMNs was assessed before and after the incubation.

CHEMOTAXIS OF NEUTROPHILS

Cells ($2 \times 10^6/\text{ml}$) were added to the cellular compartment of modified Boyden chambers and migration proceeded in 3 μm pore size filters (Sartorius Inc, Göttingen, FRG) for 45 minutes at 37°C.¹¹ An optimal concentration of LTB_4 (10 nmol/l), which earlier had been shown to be a potent chemoattractant, was chosen as activating agent.¹¹ Serial dilutions of test drugs were added to the cell compartments. Results were based on analyses of five randomly selected fields in each of two replicate filters by the leading front technique.¹⁸ Chemotaxis was corrected for spontaneous migration towards Gey's solution.

Inhibition of PMN migration by auranofin was expressed as an IC_{50} value (the drug concentration needed to suppress chemotaxis by 50%). 'Spontaneous' PMN migration towards Gey's solution was subtracted before analysis of the logarithmic dose-response curves by intrapolation. Casein (5 g/l)¹⁹ was included as a further positive control in all experiments.

Results

METABOLISM OF ENDOGENOUS ARACHIDONIC ACID

During controlled conditions, when incubation occurred without test drugs, LTB_4 constituted 8.3% (6.9–12.0), 5-HETE 12.3% (10.8–13.8), and unmetabolised arachidonic acid 58.8% (54.0–78.1) of the released radioactivity. The remaining radioactivity consisted of other mono-HETEs and catabolites of LTB_4 (20-OH- LTB_4 and 20-COOH- LTB_4), whereas only about 2% was cyclo-oxygenase products, tentatively defined as 12-hydroxyheptadecatrienoic acid. Release of LTB_4 and 5-HETE from unstimulated cells was below the detection limit (0.2%). Preliminary time-response experiments showed maximal inhibition by auranofin after an exposure time of 15 minutes before activation. Auranofin was an inhibitor of the 5-lipoxygenase pathway as the release of both 5-HETE and LTB_4 was depressed markedly with a median IC_{50} value for LTB_4 and 5-HETE of 17.4 $\mu\text{mol/l}$ (range 12.3–23.8) (Fig. 1).

Auranofin did not inhibit the arachidonic acid release from phospholipids, indicating that it did not have a steroid-like effect on the phospholipase system (Table 1). The spontaneous release during control conditions was 1.7×10^2 Bq/ml (1.2–2.3). The established lipoxygenase inhibitor nordihydroguaiaretic acid (10 μ mol/l) included to validate the assay nearly abolished (>95% inhibition) the formation of the two 5-lipoxygenase metabolites, LTB₄ and 5-HETE ($p < 0.01$).

REVERSIBILITY AND VIABILITY
The 5-lipoxygenase inhibitory effect of auranofin

Fig. 1 Effect of auranofin at varying concentrations on release of leucotriene B₄ from activated neutrophils. Percentage inhibition as compared with control conditions given as medians. (n=10).

(31 μ mol/l) was almost reversible. Thus an inhibition of LTB₄ of 93% was obtained with this concentration, and after washing four times a minor inhibition of 18% remained (range 2–33%). Incubation of cells with auranofin for 15 minutes in the concentration range selected did not affect the viability as more than 97% of the PMNs excluded trypan blue under these conditions.

CHEMOTAXIS
Auranofin caused complete inhibition of neutrophil chemotaxis to LTB₄ at a concentration of 316 μ mol/l, the median IC₅₀ value being 45 μ mol/l.

Fig. 2 Inhibition of auranofin on neutrophil chemotaxis to leucotriene B₄ (10 nmol/l). Medians of percentage inhibition are given for various concentrations of auranofin (ordinate). Maximum migration corrected for spontaneous migration is set to 0% of inhibition (abscissa). (n=10).

Table 1 Lack of effect of auranofin on the total release of radioactivity indicating arachidonic acid and its metabolites ($\times 10^2$ Bq/ 5×10^6 cells) during activation of neutrophils with A23187. Medians of 10 experiments are given with ranges in brackets

	Concentration of auranofin (mol/l)				
	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Radioactivity released	6.7 (2.4–9.7)	6.8 (2.6–8.9)	6.8 (2.2–9.3)	6.5 (2.4–9.6)	6.8 (2.9–9.3)

(22–81) in 10 individual experiments (Fig. 2). Migration towards LTB₄ (10 nmol/l) and casein (5 g/l), constituting the positive control, was 102 µm/45 min (58–130) and 83 µm/45 min (52–124) respectively. Spontaneous migration towards Gey's solution was 27 µm/45 min (16–38).

Discussion

The clinical effect of auranofin in rheumatoid arthritis has been demonstrated in several investigations.^{20–22} During clinical situations, with a conventional dose of auranofin of 6 mg/day, the serum concentration of auranofin is approximately 1 µmol/l,²³ and the concentration of gold compounds in synovial fluid is assumed to be in the range of 1.5–112.0 µmol/l, with a median of 29 µmol/l.²⁴ Thus the concentration range selected for the present investigation was of pharmacological relevance.

When endogenous arachidonate metabolism was investigated in purified human PMNs it was shown that auranofin was a potent inhibitor of the 5-lipoxygenase activity. The IC₅₀ values for production of the two main eicosanoids, 5-HETE and LTB₄, were identical—17–18 µmol/l—which is well within the therapeutic range of auranofin during conventional treatment. These values are a little higher than the 5-lipoxygenase inhibition described by Parente *et al* using f-Met-Leu-Phe and cytochalasin B as challenger for the PMNs.²⁵ They found a significant inhibition of approximately 50% (the IC₅₀ value was not calculated) at 5.8 µmol/l of auranofin. Methodological problems may account for this minor discrepancy.

Calcium ionophore A23187 was chosen for these arachidonic acid studies as 5-lipoxygenase shows an absolute requirement for calcium,²⁶ and A23187 is assumed to produce the maximal synthesis of leucotrienes in response to calcium influx. The sensitivity of the present 5-lipoxygenase assay did not allow measurement of the markedly lower synthesis of 5-lipoxygenase products from PMNs challenged with the physiological stimuli—for example, immune complexes.²⁷ As it has been reported that the potency of some 5-lipoxygenase inhibitors—for example, benoxaprofen, is dependent on the cell stimulus, whereas the potency of others—for example, BW755c, is not²⁸ it was decided to study the effect of auranofin on a functional aspect of a physiological stimulus associated with 5-lipoxygenase activation—namely, chemotaxis of PMNs in response to LTB₄; the action by LTB₄ being qualitatively similar to that of A23187.²⁸

The influence of auranofin on neutrophil chemo-

taxis has earlier been studied using the agarose technique, and it was found that PMNs preincubated with auranofin migrated a shorter distance than controls when stimulated with serum or bacterial factors—that is, cytotoxins with different non-specific receptors on cell surfaces.¹⁴ The present study using the highly purified eicosanoid LTB₄, activating PMNs through specific surface receptors,²⁹ gave an IC₅₀ value of 45 µmol/l with auranofin, again a value within the therapeutic range of auranofin concentrations.

In summary, the present results suggest that locally recruited PMNs may be inhibited by auranofin, leading to a minimised production of tissue destructive and proinflammatory mediators, such as LTB₄ and 5-HETE. Furthermore, auranofin may reduce the migration of PMNs from circulating blood to the affected joints. Thus the inflammation may be further reduced by auranofin because PMNs apart from producing inflammatory active eicosanoids also have the potential for release of tissue destructive oxygen free radicals.³⁰

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