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

J ELMGREEN, I AHNFELT-RØNNE, AND O H NIELSEN

From the 1Department of Rheumatology TTA, Rigshospitalet, University of Copenhagen; the 2Department of Pharmacology, Leo Pharmaceutical Products; and the 3Department of Medical Gastroenterology C, Herlev Hospital, University of Copenhagen, Denmark

SUMMARY The effect of auranofin on human neutrophil (PMN) 5-lipoxygenase activity and leucotriene B4 (LTB4) chemotaxis was investigated. [1-14]C 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. This cell type may constitute more than 90% of the cellular exudate in synovial fluid and is abundant in the inflamed synovial membrane and at the interface of cartilage with pannus. Local secretion of arachidonic acid metabolites, formed mainly via the 5-lipoxygenase pathway, and of oxygen free radicals, and lysosomal enzymes, especially natural proteases, may be essential for perpetuation of inflammation and for tissue destruction.

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. Accordingly, high concentrations of LTB4 are found in the synovial fluid from patients with active rheumatoid arthritis. LTB4 is a proinflammatory mediator, which activates human PMNs with respect to chemotaxis and aggregation, and, further, it is a complete secretagogue in PMNs, showing a physiological profile similar to that of calcium ionophore A23187. Thus inhibitors of LTB4 synthesis and LTB4 actions may show anti-inflammatory properties.

Auranofin, a new gold compound for oral treatment of rheumatoid arthritis, has been shown to affect many of the activities of PMNs in acute inflammation. 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-hydroxyeicosatetraenoic 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...
healthy volunteers who had taken no drugs, including salicylates, for at least four weeks.

Neutrophils were isolated by a modification of Böyum’s method 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 μmoll) (mol. wt 678.5) (Smith, Kline and French, Solna, Sweden) dissolved in 0-1% ethanol. Nordihydroguaiaretic acid (10 μmol/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 LTB4 (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 [1-14C]arachidonic acid (37 000×103 Bq/5×10⁹ cells, 2.2×10⁹ Bq/mmol) (Amersham International, Buckinghamshire, UK) for five hours at 37°C to obtain steady state labelling of intracellular pools of arachidonic acid. 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 μmol/l. Extracellular fluid containing radiolabelled metabolites was prepared by instantaneous removal of the cells by centrifugation (8000 g, one minute) through dibutyl phthalate:dimethyl phthalate 3:1 (density 1-033 g/ml) before extraction of eicosanoids.

The radioactive metabolites were then separated by thin layer chromatography and measured by autoradiography and laser densitometry as previously described. 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. The concentration of auranofin necessary for 50% inhibition of arachidonic acid catabolism (IC₅₀) via the 5-lipoxygenase pathway was calculated by interpolation from the logarithmic dose-response curves.

**Inhibition of human neutrophils by auranofin**

In a separate series of six experiments [1-14C]arachidonic acid labelled PMNs were incubated with auranofin (31 μmol/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 (2×10⁶/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. An optimal concentration of LTB₄ (10 nmol/l), which earlier had been shown to be a potent chemoattractant, was chosen as activating agent. 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. Chemotaxis was corrected for spontaneous migration towards Gey's solution.

Inhibition of PMN migration by auranofin was expressed as an IC₅₀ 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) 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, LTB₄ 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 metabolites of LTB₄ (20-OH-LTB₄ and 20-COOH-LTB₄), whereas only about 2% was cyclooxygenase products, tentatively defined as 12-hydroxyeicosatetraenoic acid. Release of LTB₄ 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 LTB₄ was depressed markedly with a median IC₅₀ value for LTB₄ and 5-HETE of 17-4 μmol/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 $1.7 \times 10^2$ Bq/ml (1.2–2.3).

The established lipoxygenase inhibitor nordihydroguaiaretic acid (10 μmol/l) included to validate the assay nearly abolished (>95% inhibition) the formation of the two 5-lipoxygenase metabolites, LTB$_4$ and 5-HETE ($p<0.01$).

REVERSIBILITY AND VIABILITY
The 5-lipoxygenase inhibitory effect of auranofin (31 μmol/l) was almost reversible. Thus an inhibition of LTB$_4$ 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 LTB$_4$ at a concentration of 31 μmol/l, the median IC$_{50}$ value being 45 μmol/l.

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

<table>
<thead>
<tr>
<th>Concentration of auranofin (mol/l)</th>
<th>0</th>
<th>10$^{-6}$</th>
<th>10$^{-5}$</th>
<th>10$^{-4}$</th>
<th>10$^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioactivity released</td>
<td>6.7</td>
<td>6.8</td>
<td>6.8</td>
<td>6.5</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>(2.4–9.7)</td>
<td>(2.6–8.9)</td>
<td>(2.2–9.3)</td>
<td>(2.4–9.6)</td>
<td>(2.9–9.3)</td>
</tr>
</tbody>
</table>
Inhibition of human neutrophils by auranofin 137

(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) respect-
ively. 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 investiga-
tions.⁵⁰–⁵² During clinical situations, with a
conventional dose of auranofin of 6 mg/day, the
serum concentration of auranofin is approximately
1 µmol/l,⁵³ and the concentration of gold com-
ounds in synovial fluid is assumed to be in the
range of 1.5–112.0 µmol/l, with a median of
29 µmol/l.⁵⁴ Thus the concentration range selected
for the present investigation was of pharmacological
relevance.

When endogenous arachidonic 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 produc-
tion 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 cytochala-
sin B as challenger for the PMNs.⁵⁵ 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.⁵⁶ 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.⁵⁷ As it has been
reported that the potency of some 5-lipoxygenase
inhibitors—for example, benoxaprofen, is de-
pendent on the cell stimulus, whereas the potency of
others—for example, BW755c, is not,⁵⁸ it was
decided to study the effect of auranofin on a
functional aspect of a physiological stimulus associ-
ated with 5-lipoxygenase activation—namely,
chemotaxis of PMNs in response to LTB₄; the action
by LTB₄ being qualitatively similar to that of
A23187.⁵⁹

The influence of auranofin on neutrophil chemo-
taxi has earlier been studied using the agarose
technique, and it was found that PMNs preincu-
bated with auranofin migrated a shorter distance
than controls when stimulated with serum or bac-
terial factors—that is, cytotoxins with different non-
specific receptors on cell surfaces.⁶¹ The present
study using the highly purified eicosanoid LTB₄,
activating PMNs through specific surface receptors,⁶²,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 aurano-
fin, 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 eico-
sanoids also have the potential for release of tissue
destructive oxygen free radicals.⁶³

The authors are grateful to Helma Furhauge, Hanne Kargaard, and
Bente Nielsen for skilful technical assistance. This study was
supported by grants from handelsgartner Ove William Buhl
Olesen’s and aegterfælle Edith Buhl Olesen’s Foundation, Else and
Mogens Wedell-Wedellsborg’s Foundation, Direktør Jacob Mad-
sen’s and hustru Olga Madsen’s Foundation, and the Danish
Medical Research Council.

References

1 Weissmann G. Activation of neutrophils and the lesions of
2 Palmer D G. Total leucocyte enumeration in pathologic
3 Mohr W, Westerhellweg H, Wessinghage D. Polymorphonu-
clear granulocytes in rheumatic tissue destruction. III. An
electron microscopic study of PMNs at the pannus-cartilage
junction in rheumatoid arthritis. Ann Rheum Dis 1981; 40:
396–9.
4 Klichstein L B, Shapleigh C, Goetzl E J. Lipoxygenase of
arachidonic acid as a source of polymorphonuclear leukocyte
chemotactic factors in synovial fluid and tissue in rheumatoid
5 Henderson B, Higgs G A, Moncada S, Salmon J A. Synthesis
of eicosanoids by tissues of the synovial joint during the
development of chronic erosive synovitis. Agents Actions 1980;
17: 360–2.
6 Ward P, Johnson K J, Till G O. Oxygen radicals, neutrophils,
and acute tissue injury. In: Taylor A E, Matalon S, Ward P.,
eds. Physiology of oxygen radicals. Baltimore: Williams and
7 Weissmann G. Lysosomal mechanisms of tissue injury in
8 Elmgreen J, Nielsen OH, Ahnfelt-Rønne I. Enhanced capacity
for release of leucotriene B₂ by neutrophils in rheumatoid
9 Davidson E M, Rae S A, Smith M J H. Leukotriene B₂, a
mediator of inflammation present in synovial fluid in rheuma-
10 Ford-Hutchinson A W, Bray M A, Doug M V, Shipley M E,
Smith M J H. Leukotriene B₂, a potent chemokinetic and


