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INHIBITION OF HUMAN NEUTROPHIL LEUKOTRIENE B_4 SYNTHESIS BY COMBINATION AURANOFIN AND EICOSAPENTAENOIC ACID

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Abstract—It has been demonstrated that both auranofin and eicosapentaenoic acid (EPA) have anti-inflammatory properties and both inhibit neutrophil leukotriene B_4 (LTB_4) synthesis. In the present study, we examined interactions between auranofin and EPA with regard to inhibition of human neutrophil LTB_4 synthesis. Auranofin inhibited A23187-stimulated LTB_4 synthesis, but the dose required for inhibition of LTB_4 was greater than that required for inhibition of other 5-lipoxygenase metabolites; namely, the all-*trans* isomers of LTB_4 and 5-hydroxyeicosatetraenoic acid. These results were explained after a comparison of the rates of synthesis of these 5-lipoxygenase metabolites in the presence and absence of added arachidonic acid which led to the conclusion that leukotriene A hydrolase, the enzyme catalysing the formation of LTB_4, was saturated with substrate and rate-limiting for LTB_4 synthesis during A23187 stimulation. In combination, auranofin and EPA had a simple additive effect on inhibition of the 5-lipoxygenase pathway. Favorable drug/EPA combinations have the potential to provide a beneficial anti-inflammatory effect with lower levels of each component than are required when used individually.

The use of chrysotherapy in the treatment of rheumatoid arthritis is well established although, in various clinical trials which examined the use of auranofin, approximately one-third of patients dropped out due to lack of efficacy [1]. While many cellular and biochemical actions of auranofin have been demonstrated, the pharmacology of its anti-rheumatic effect is not established. Auranofin inhibits neutrophil chemotaxis and the respiratory burst at certain concentrations, but it probably has a bimodal effect with stimulation at lower concentrations (reviewed in Ref. 1). Similarly, it has been reported that auranofin causes dose-dependent stimulation and inhibition of neutrophil leukotriene B_4 (LTB_4‡) [2] and monocyte IL-1 [3] production.

Dietary fish oil is not commonly used as a therapeutic agent in rheumatoid arthritis despite several reports of placebo-controlled studies in which it provided symptomatic benefits in patients [4–9]. Since large doses of fish oil (10–20 g per day) were used in these reported studies for only a modest improvement in symptoms, it is not surprising that it has not been used as an anti-rheumatic agent. In common with auranofin, dietary fish oil or EPA, a 20-carbon n-3 fatty acid present in fish oil, also inhibits neutrophil LTB_4 synthesis [6, 10] and monocyte IL-1 synthesis [9, 11]. Thus, the potential exists for additive interactions between auranofin and fish oil (or EPA) for inhibition of LTB_4 and IL-1, both of which have been implicated as important mediators of inflammation [12].

In this study, we examined the effect of combinations of auranofin and EPA on human neutrophil LTB_4 synthesis. In order to interpret the results, this study also required an examination of the kinetics of various reactions involved in LTB_4 synthesis.

MATERIALS AND METHODS

Neutrophil preparation. Venous blood samples (20 mL) from healthy volunteers were added to tubes containing 4.5% EDTA, pH 7.4 (4 mL) and 6% dextran (4 mL) in water. Erythrocytes were allowed to sediment at 37° after which neutrophils were separated on a discontinuous Percoll gradient [13]. The cells were washed twice in Ca^{2+}, Mg^{2+}-free DPBS and finally resuspended in complete DPBS at 1×10^6 cells/mL.

Neutrophil stimulation. Stock solutions of auranofin (kindly provided by Smith Kline & French Australia, Ltd) were prepared in methanol. For each experiment, fresh 1:10 dilutions were prepared in phosphate buffered saline and 10 μL of the appropriate solutions were added to 1 mL of cell suspension. AA or EPA stock solutions were prepared in methanol. For each experiment, fresh 1:20 dilutions were prepared and 10 μL was added to 1 mL of cell suspension. The final concentration of AA or EPA was 5 μM. Cells were preincubated with fatty acid or auranofin for 10 min at 37° after which they were stimulated by the addition of A23187. After a further incubation for 5 min at 37°, the reaction mixture was acidified with 100 mM citric acid (0.25 mL). 15-HETE was added as an internal

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‡ Abbreviations: LTB_4, leukotriene B_4; IL-1, interleukin-1; EPA, eicosapentaenoic acid; DPBS, Dulbecco's phosphate-buffered saline; AA, arachidonic acid; 5-HETE, 5-hydroxyeicosatetraenoic; LTA, leukotriene A.

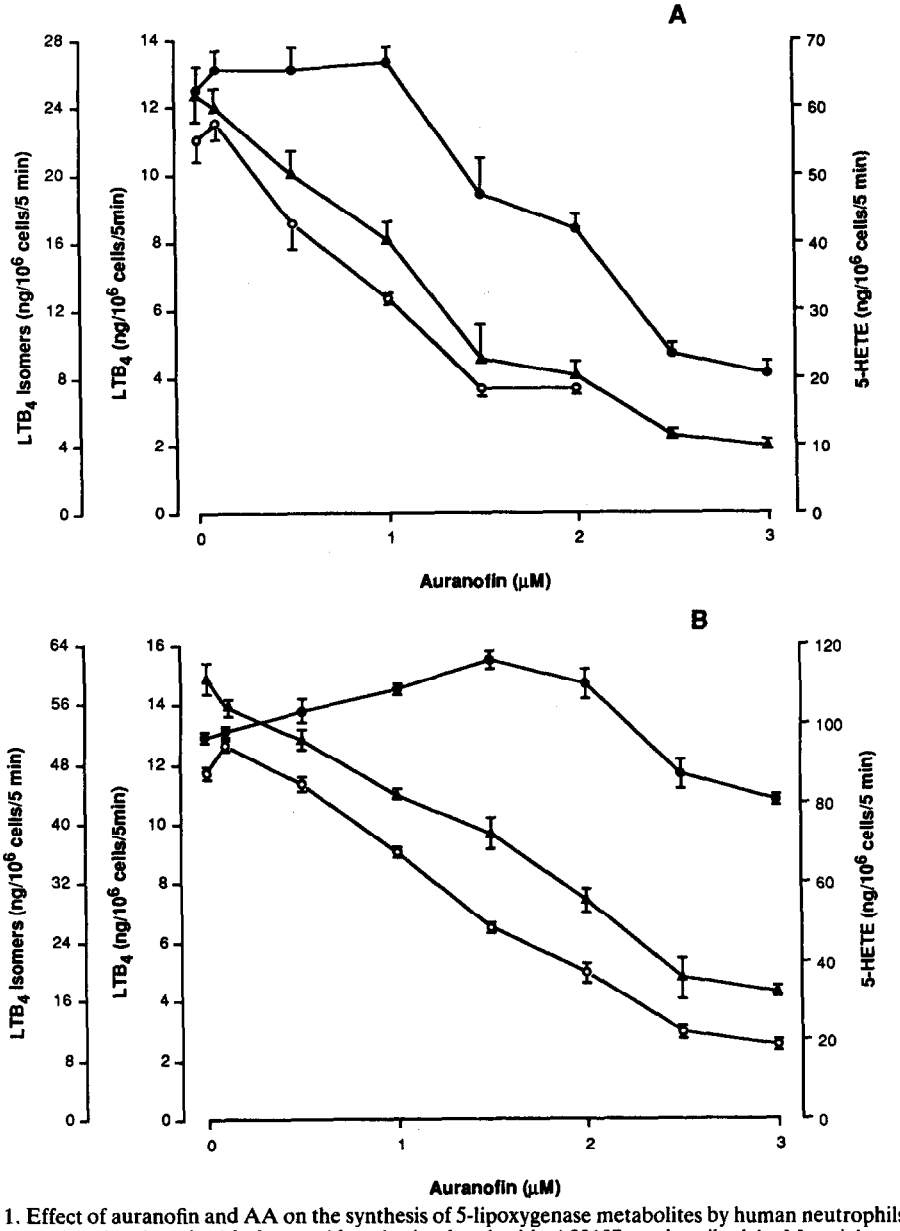

Fig. 1. Effect of auranofin and AA on the synthesis of 5-lipoxygenase metabolites by human neutrophils. Cells were preincubated with fatty acid and stimulated with A23187 as described in Materials and Methods. Values represent the mean ± SEM of triplicate incubations. (A) A23187; (B) A23187 + AA. Note the differences in scale for LTB_4 isomers and 5-HETE between (A) and (B). (●) LTB_4; (○) LTB_4 isomers; (▲) 5-HETE.

standard. After extraction with chloroform/methanol (7:3, v/v), the solvent was evaporated and the residue was dissolved in methanol for HPLC analysis.

Measurement of 5-lipoxygenase metabolites. The 5-lipoxygenase metabolites, LTB_4, the all-*trans* isomers of LTB_4 and 5-HETE, were separated and quantified by reverse-phase HPLC and UV detection as described previously [14]. The total 5-lipoxygenase products were computed by adding the LTB_4, LTB_4 isomers and 5-HETE values for each sample. Although the ω-oxidation of newly formed LTB_4 has been described, the effect is dependent on cell concentration [15]. We observed no disappearance of LTB_4 at incubation times up to 40 min at 1×10^6 cells/mL, which was the cell concentration used for the present study (results now shown). It is noteworthy that LTB_4 levels decreased rapidly after 1 min incubation time at a cell concentration of 1×10^7 cells/mL (results not shown).

RESULTS

At the lower concentrations used, auranofin increased A23187-stimulated LTB_4 synthesis when

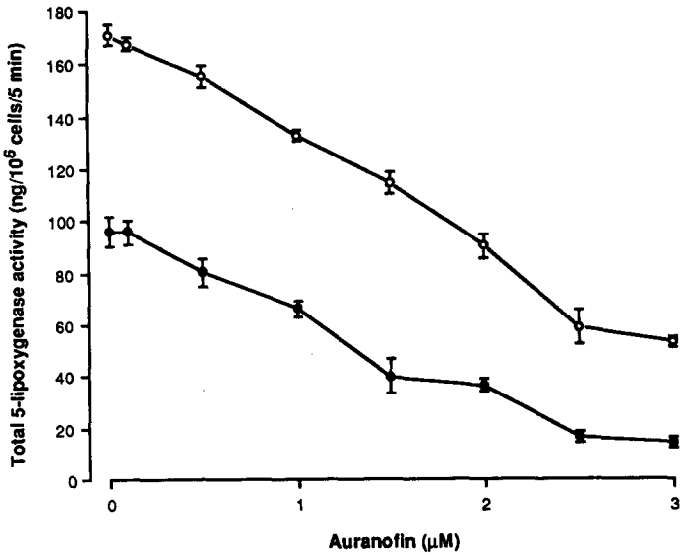

Fig. 2. Effect of auranofin on the synthesis of the total 5-lipoxygenase products by human neutrophils. The total 5-lipoxygenase products were computed as described in Materials and Methods and the values (mean ± SEM) are computed from the data represented in Fig. 1. (●) A23187; (○) A23187 + AA (5 μM).

Table 1. Effect of AA on A23187-stimulated synthesis of 5-lipoxygenase metabolites

	LTB_4	LTB_4 isomers	5-HETE
	(ng/10^6 cells/5 min)		
− AA	12.5 ± 0.7	22.0 ± 1.2	61.1 ± 3.8
+ AA (5 μM)	12.9 ± 0.1	46.8 ± 0.8	111.4 ± 3.8

Results represent the means ± SEM of triplicate determinations.

exogenous AA was present (Fig. 1). Auranofin inhibited LTB_4 synthesis at 1.5–3 μM in the absence, and in the presence of exogenous AA (Fig. 1). In contrast, the synthesis of LTB_4 isomers and 5-HETE was inhibited by a lower concentration of auranofin (0.5 μM) (Fig. 1). It is apparent that auranofin inhibited the synthesis of the total 5-lipoxygenase products (calculated as described in Materials and Methods) in a dose-dependent manner at all concentrations ≥ 0.5 μM and that the dose–response was not altered by the addition of exogenous AA (Fig. 2). In the absence of auranofin, the effect of addition of AA to the A23187-stimulated neutrophils can be examined. Exogenous AA increased the synthesis of 5-HETE and the all-*trans* isomers of LTB_4, but did not alter the synthesis of LTB_4 (Fig. 1 and Table 1).

The addition of EPA to the neutrophils decreased the synthesis of LTB_4 from 16.2 to 6.7, and the total 5-lipoxygenase products from 126 to 54 ng/10^6 cells/5 min (Fig. 3). Auranofin inhibited LTB_4 synthesis at concentrations of 2–3 μM. By contrast, auranofin inhibited the total 5-lipoxygenase products at all concentrations ≥0.5 μM in the absence, or the presence of EPA. At each concentration of auranofin, the percentage inhibition of the total 5-lipoxygenase products by EPA was similar with a mean (±SD) inhibition of 63% (±8) (Fig. 3, inset).

DISCUSSION

Several studies have reported that dietary fish oil supplements have a modest beneficial effect on symptoms in rheumatoid arthritis patients [4–9]. In these previous studies, the existing medication regimen was not changed and thus fish oil was taken against the background of a varied range of anti-inflammatory medication. The controlled use of fish oil as one component in combination chemotherapy has not been considered. Combination chemotherapy with gold and either sulfasalazine, hydroxychloroquine, penicillamine, chlorambucil or levamisole has been examined and while increased efficacy was observed, there was also an increased incidence of toxicity and withdrawals were frequent [16]. The combination of gold and fish oil has not been examined for clinical efficacy, but a favourable interaction with regard to inhibition of key inflammatory mediators would provide a biochemical basis for such a clinical study.

Although both auranofin and fish oil can alter neutrophil LTB_4 synthesis, the effect of combinations of these two agents cannot be predicted. Various effects of auranofin on LTB_4 synthesis have been reported. For example, it has been reported that auranofin stimulates LTB_4 synthesis at low concentrations and inhibits synthesis at higher concentrations [2]. This bimodal effect was evident in the current study when exogenous AA was present in addition to the primary agonist, A23187. In

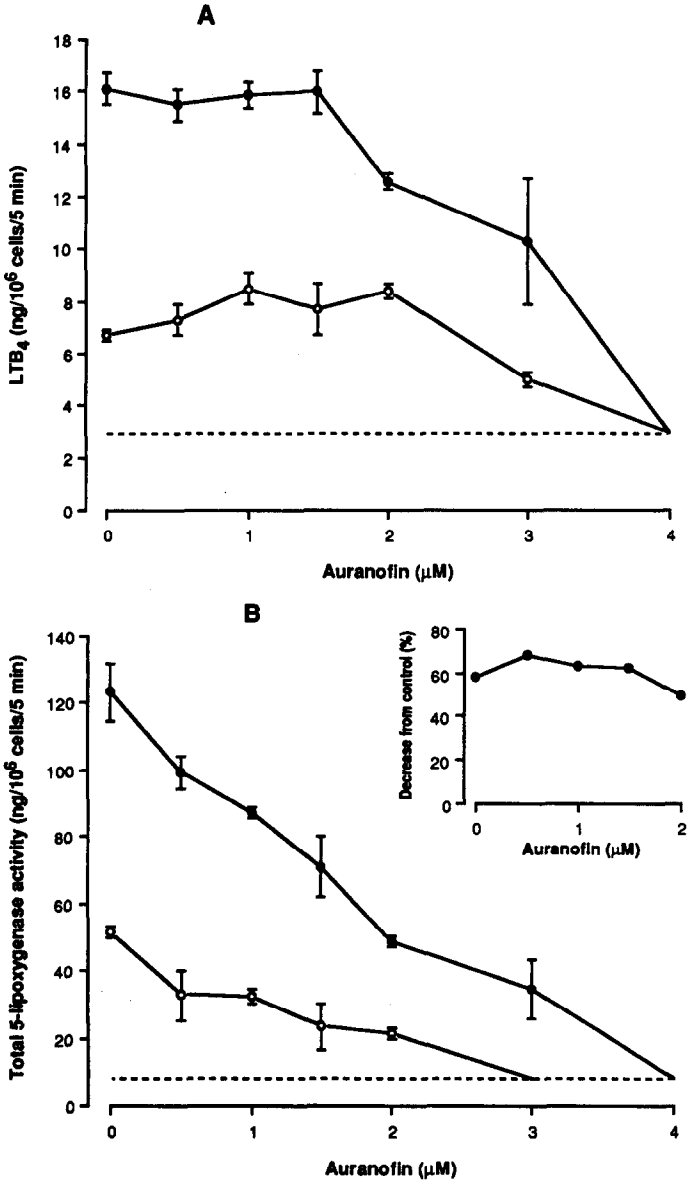

Fig. 3. Effect of auranofin and EPA on the synthesis of (A) LTB_4 and (B) the total 5-lipoxygenase products by human neutrophils. Cells were preincubated with fatty acid and stimulated with A23187 as described in Materials and Methods. Values represent the mean ± SEM of triplicate incubations. The dashed line indicates the minimum detectable amount. The inset shows the decrease (%) in the total 5-lipoxygenase products due to the addition of EPA. (●) A23187; (○) A23187 + EPA (5 μM).

agreement with previous observations [2], the stimulation of LTB_4 synthesis by low auranofin concentrations was associated with an inhibition of 5-HETE and the all-*trans* isomers of LTB_4. As suggested previously [2], this pattern of alterations suggests that auranofin has two actions on this pathway, namely (a) inhibition of an enzymic reaction before the formation of 5-HETE and LTA_4 (the all-*trans* isomers of LTB_4 being non-enzymatic degradation products of LTA_4); and (b) stimulation of LTA hydrolase, the enzyme responsible for conversion of LTA_4 to LTB_4. However, when the effects of auranofin on the total flux of AA into the 5-lipoxygenase pathway are examined by computing the total 5-lipoxygenase products, it is apparent that inhibition is the predominant effect of auranofin on this pathway.

Some of these apparently disparate effects of auranofin on LTB_4 and the other 5-lipoxygenase metabolites may be explained by consideration of the relative kinetics of reactions leading to these metabolites. Stimulation of neutrophils by A23187 leads to increased intracellular Ca^{2+}, stimulation of AA release and translocation of 5-lipoxygenase from

cytosol to membrane which is a necessary process for its activation [17, 18]. LTA hydrolase is a cytosolic enzyme which does not require activation for expression of activity [17]. In the present study, the addition of exogenous AA to A23187-stimulated cells increased the production of LTB_4 isomers and 5-HETE, but did not increase the production of LTB_4. This pattern of response suggests that, when neutrophils are stimulated with A23187, LTA hydrolase is saturated with substrate and is rate-limiting for LTB_4 synthesis. Thus, the effect of auranofin on LTB_4 synthesis will vary, depending on whether LTA hydrolase is saturated with substrate and rate-limiting for LTB_4 synthesis. The conditions used in the present study, i.e. stimulation with A23187 ± AA, are conditions which are commonly used. With the addition of AA as well as A23187, LTA hydrolase may remain saturated in the presence of significant inhibition of 5-lipoxygenase activity. Such a phenomenon may explain the demonstration of LTA hydrolase stimulation by auranofin in the presence, but not the absence of exogenous AA.

Whether the *in vivo* dose–response curve of auranofin concentration versus LTB_4 synthesis is similar to that described herein, is dependent on the conditions of neutrophil stimulation *in vivo*. It is not known whether the stimuli *in vivo* are sufficiently potent activators of 5-lipoxygenase activity to lead to substrate saturation of LTA hydrolase. However, the analysis of the total 5-lipoxygenase products as well as LTB_4 allows an analysis of the effects of auranofin in both situations. If LTA hydrolase is rate-limiting *in vivo*, the LTB_4 response ito auranofin in this study will reflect those conditions. If LTA hydrolase is not rate-limiting *in vivo*, the response of the total 5-lipoxygenase products to auranofin in this study will reflect the response of LTB_4 under such conditions. It is most probable that auranofin inhibits the 5-lipoxygenase metabolites via inhibition of 5-lipoxygenase activity rather than inhibition of AA release [19].

The inhibition of LTB_4 synthesis by EPA may result from dual actions of EPA; firstly, the decrease in membrane phospholipid AA content [14, 20] resulting from dietary EPA, and secondly, inhibition of LTA hydrolase by LTA_5, a 5-lipoxygenase metabolite of EPA [21]. Thus, it could not be predicted whether auranofin and EPA would have nil, or an additive, or a synergistic interaction. The results of the present study indicate that auranofin and EPA have a simple additive interaction for inhibition of both LTB_4 and the total 5-lipoxygenase products.

Previous clinical trials which investigated the symptomatic effects of dietary fish oil in rheumatoid arthritis patients have not considered the effects of the concurrent anti-rheumatic drug usage [4–9]. In none of the trials was the basal medication altered. However, the combination of dietary fish oil with drugs known to have a positive interactive biochemical effect with regard to inhibition of inflammatory mediators, has the potential to increase the anti-inflammatory effect of fish oil and decrease anti-rheumatic drug use. These considerations could form the basis of a second generation of clinical trials investigating the use of dietary fish oil by rheumatoid arthritis patients.

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