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An evaluation of the anti-inflammatory activity and mechanism of action of three novel auranofin derivatives

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SUMMARY

Gold compounds have been used for the treatment of rheumatoid arthritis since the mid 20th century as a disease modifying anti-rheumatic drug. Auranofin, an oral anti-rheumatic drug, has been used for many years in the treatment of rheumatoid arthritis (RA). Although the drug has been successful in treating the symptoms of RA, many patients discontinue its use due to severe toxicity over long periods of continued treatment. Since the introduction of auranofin in 1985 there has been no new clinically approved gold drug. Drug discovery research is directing focus on overcoming these toxicity problems. Much of the problems related to the toxicity related to auranofin are due to its lipophilicity. As a result, three compounds (Asa-fin, Mpta-fin and Pta-fin) with varying substituents were synthesised and hence the lipophilic- hydrophilic balance was modulated. All compounds including auranofin were tested against normal cells to determine its toxicity as well as its anti-inflammatory activity.

Three novel auranofin derivatives were compared to auranofin with regards to lipophilicity, toxicity and anti-inflammatory properties

The lipophilicity of the three compounds were compared to auranofin using the octanol-water partition coefficient method. All the novel compounds showed variable lipophilicity compared to auranofin, with Pta-fin and Mpta-fin being more hydrophilic than auranofin.

The cytotoxicity of these novel gold compounds Asa-fin, Mpta-fin and Pta-fin were compared to auranofin using primary porcine hepatocytes and chicken embryo fibroblasts cultures. A metabolic assay based on the reactivity of 3-[4,5-dimethylyhiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) with viable cells was done to measure the effect of the drugs on the growth of cultures. All three novel compounds proved less toxicity at comparable concentrations in primary porcine hepatocytes and in fibroblast proliferation, Asa-fin and Mpta-fin proved less toxic than Auranofin.

The Anti-inflammatory activity of the experimental compounds was determined by testing the effects of the experimental compounds on human lymphocyte proliferation. The MTT assay was used to measure the effect of the drugs on the growth of the cell cultures. All three compounds inhibited the proliferation of human lymphocytes with Pta-fin having the least effect.

The effect of these drugs was also evaluated on the reactive oxidant production by chemiluminescence and flow cytometry on resting, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) and Phorbol Myristate Acetate (PMA) stimulated human neutrophils. Oxidant production by neutrophils was measured after a 45-minute incubation period with luminol enhanced chemiluminescence. Treatment of neutrophils with auranofin and the three compounds showed that auranofin, Asa-fin and Mpta-fin had a biphasic activity on hydrogen peroxide production with higher concentrations decreasing hydrogen peroxide production, possibly leading to the anti-inflammatory action of these drugs. With Pta-fin no decrease in hydrogen peroxide was observed. Using flow cytometry three dyes specific to different reactive oxygen species were used. 2', 7'-Dichloroflourescein diacetate (DCFH) is specific for detecting nitric oxide, Dihydrorhodamine 123 (DHR) is specific for detecting hydrogen peroxide and Hydroethidine (HE) is specific for detecting superoxide. Oxidant production was measured after a 30 minute incubation period with the relative dyes on a flow cytometer. Auranofin and Asa-fin decreased hydrogen peroxide and superoxide production. None of the drugs had an effect on nitric oxide production.

The expression of the β_2-integrin adhesion molecule, CR3, on resting and PMA stimulated neutrophils treated with the experimental compounds was measured by flow cytometry. CR3 expression by neutrophils was measured after 10 minute incubation in the dark with CD11b FITC monoclonal antibody. Treatment of neutrophils with auranofin and the three experimental compounds showed a decrease in CR3 expression on resting and stimulated neutrophils, however the effect was more marked in stimulated neutrophils.

The Anti-inflammatory activity of the experimental compounds was determined by testing the effects of the experimental compounds on cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX 2) in resting and lipopolysaccharide (LPS) stimulated human monocytes. COX 1 and 2 production was measured by flow cytometry. Treatment of monocytes with the experimental compounds showed a decrease in COX 2 production in stimulated monocytes but an increase in COX 2 production in resting monocytes. No effect on COX 1 production was observed with the experimental compounds.

Prostaglandin E_2 (PGE_2) was measured with a Prostaglandin E_2 Enzymeimmunoassay (ELISA) kit on human macrophages. Auranofin, Asa-fin, Mpta-fin and Pta-fin inhibited the production of PGE_2. Auranofin and Asa-fin inhibited the PGE_2 directly proportional to the drug concentration.

The effect of these drugs was also evaluated on various inflammatory cytokines using an inflammatory cytokine kit and measured on a flow cytometer. The cytometric bead array (CBA) human inflammation kit was used to quantitatively measure interlukin-8 (IL-8), interlukin-1β (IL-1β), interlukin-6 (IL-6), interlukin-10 (IL-10), tumour necrosis factor alpha (TNFα) and interlukin-12p70 (IL-12p70). Auranofin and Asa-fin decreased IL 10, TNFα, and IL1β in stimulated cells. No effect was observed on IL 8, IL-12p70 and IL 6. With Mpta-fin and Pta-fin, no significant effect was observed in the cytokines tested.

Drug toxicity was evaluated in mice using all four compounds in BALB/c inbred mice. Aspartate transaminase (AST), gamma glutamine transferase (GGT), urea and creatine levels were measured in the test mice. The group receiving the highest dose of Asa-fin showed the greatest elevation of AST . The lowest dose of the auranofin treatment group showed the greatest elevation in GGT, however this increase was not seen in the subsequent higher dosing groups. None of the treatment groups indicated an increase in urea levels. Mpta-fin and Pta-fin showed no increase in the liver enzymes or in urea and creatine.

The results of this work are indicative that novel gold compounds could play a promising role in anti arthritic applications. Asa-fin exhibited similar anti-inflammatory activity to auranofin but *in vivo* toxicity was high. Mpta-fin showed slightly inferior anti-inflammatory activity to auranofin but *in vivo* toxicity profiles were much more promising. Pta-fin showed the least anti-inflammatory activity of the three novel compounds tested with a similar *in vivo* toxicity profile as Mpta-fin.

CHAPTER 1
INTRODUCTION

1. INTRODUCTION

1.1. General

Rheumatoid arthritis (RA) is probably the most common treatable disability in the Western world, affecting approximately 1% of the population. It is by no means a benign, self-limiting disease; rather, it is a source of considerable morbidity and mortality (Pincus and Callahan 1993). Rheumatoid arthritis is characterized by synovial hyperplasia and inflammatory cell infiltration (Harris 1990). Local proliferation and monoclonal expansion of synovial fibroblast-like cells (synoviocytes) contributes to the hyperplasia of synovium that exhibits tumor-like proliferation and invasion to juxta-articular bone and cartilage that results in joint destruction(Qu, Garcia et al. 1994; Imamura, Aono et al. 1998).

Polymorphonuclear leucocytes (PMNL) from patients with RA exhibit an enhanced capacity for metabolism of endogenous arachadonic acid (AA) with increased release of leukotriene B_4 (LTB$_4$) during activation *in vitro* (Elmgreen, Nielsen et al. 1987). Accordingly, high levels of LTB$_4$ are found in the synovial fluid of patients with active RA. LTB$_4$ is a pro-inflammatory mediator, which activates human PNML's with respect to chemokinesis and aggregation, and is a complete secreatagogue in PNML's. According to Arend and Dayer (1990), Prostaglandin E_2, which is found abundantly in the synoviocytes, is induced by monocyte-derived cytokines such as interleukin 1β in the inflamed rheumatoid synovium. Prostaglandin E_2 plays a main role in the angiogenesis of synovium through vascular endothelial growth factor expression (Ben-Av, Crofford et al. 1995), synovial inflammation and the joint erosion of rheumatoid arthritis (Robinson, Tashjian et al. 1975).

At present the drugs used to treat RA range from non-steroidal anti-inflammatory drugs (NSAIDS) to more potent disease-modifying antirheumatic drugs (DMARDS). The lack of a potent, reliable treatment for early RA is a major problem in modern medicine. According to the older treatment strategy of the "treatment pyramid", treatment of newly diagnosed RA was to be started with NSAIDS alone, and if this drug failed to alleviate the symptoms, more potent DMARDS were to be tried (Bondeson 1997). Most of these treatments however cause severe side effects such as stomach problems, heartburn, ulcers and bleeding in the case of NSAIDS and

cataracts, high blood pressure, sleep problems, muscle loss, bruising, thinning of the bones (osteoporosis), weight gain and susceptibility to infections in the case of DMARDS (Wagner, Khanna et al. 2004). There is therefore a need to develop effective anti-inflammatory drugs with less adverse effects.

1.1.1 Antirheumatic gold compounds

Gold compounds, which are disease modifying anti-rheumatoid drugs (DMARD), have been empirically applied to the management of rheumatoid arthritis, but their mechanism of action is obscure. It has been proposed that they possess immunosuppressive activity by inhibiting:

monocyte functions (Lipsky and Ziff 1977; Scheinberg, Santos et al. 1982)

T cell proliferation (Hashimoto, Whitehurst et al. 1994)

T cell function (Hashimoto, Whitehurst et al. 1992)

B cell activation,(Allen 1986; Hirohata 1996)

chemotaxis (Elmgreen, Ahnfelt-Ronne et al. 1989)

phagocytosis (Dimartino and Walz 1977; Davis, Johnston et al. 1983)

superoxide production (Hurst, Gorjatschko et al. 1989)

The hydrophilic gold complexes sodium aurothiomalate and aurothioglucose are administered as intramuscular injections. The inherent problems and difficulty of administering a drug by intramuscular injection caused a drive for the development of an oral gold anti-arthritic compound with an improved toxicological and pharmacological profile (Gottlieb 1982). The main reason for an improved gold compound was that it could be administered orally in low doses, giving stable serum levels of active compound resulting in an improved therapeutic response and reduced toxicity. Auranofin is a mixed gold compound, with a thiol and a triethylphosphine group as ligands, and differs from the earlier antirheumatic gold complexes by being more lipophilic so it could be taken orally (Bondeson 1997). Studies have indicated that auranofin is superior to placebo and marginally less effective but also less toxic than aurothiomalate (Campion, Lebsack et al. 1996). The orally administered gold-based anti-arthritic agent, auranofin, emerged as a clinically useful therapeutic drug for the treatment of rheumatoid arthritis in the late 1970s (Kean, Hart et al. 1997). Although the exact mechanism by which auranofin produces its therapeutic effects are

unclear, auranofin has been documented to inhibit many aspects of immune cell function, including antibody dependent complement lyses, release of lysosomal enzymes, antibody-dependent cellular cytotoxicity, superoxide production, lymphocyte responsiveness, chemotaxis and phagocytosis (reviewed by (Kean, Hart et al. 1997)).

Auranofin is unique since its physical, chemical, pharmacologic, and pharmacokinetic properties differ from other (injectable) gold compounds (Figure 1.1) (Walz, DiMartino et al. 1979; Lewis, Cottney et al. 1980).
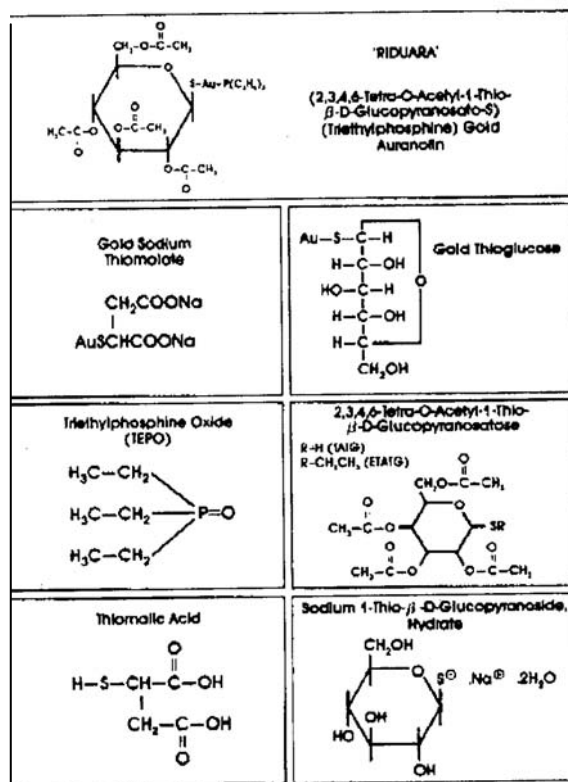
Figure 1.1 Chemical structures of gold compounds (oral and injectable) and their ligands, (Walz, DiMartino et al. 1983).

Evidence suggests that the anti-arthritic action of auranofin may be based upon its immunosuppressive effects, but it has numerous side effects. The most common of these side effects include lower gastrointestinal tract disorders which may include gastrointestinal disorders such as loose stools, abdominal pain and nausea (Bernhard 1982). Haematological side effects such as general leucopoenia (Lewis and Walz

1982), lymphopenia (Hunt and Holt 1981) and neutropenia (Hunt and Holt 1981; Calin, Saunders et al. 1982) have also been reported.

These side effects are related to auranofin's mechanism of action as a DMARD.

Gold compounds have thus aroused interest not only for their clinical properties but also because of their immunopharmacological effects. The major limiting factor has been the high prevalence of side effects reported with gold compounds and in particular the development of haematological reactions and nephrotoxicity. The development of any gold compound with therapeutic benefit similar or superior to current treatments, but with a safer toxicity profile, may be of considerable benefit to both physicians and patients. From studies (Campion, Lebsack et al. 1996), it is thus evident that injectable hydrophilic antirheumatic agents require a greater time period to exert its effects and are more toxic than the lipophilic compound auranofin, which enters cells without difficulty. The toxicity of auranofin however is attributed mainly to its lipophilicity (Campion, Lebsack et al. 1996).

1.1.2 Novel gold compounds

To improve the bioavailability of 2,3,4,6-tetra-O-acetyl-1-thio-B-D-glucopyranosato(triethylphosphine) gold(I) (auranofin) and decrease its side effects, three auranofin derivatives with varying degrees of lipophilicity have been designed and synthesised by Zolisa Sam, under the supervision of Professor Roodt at the University of the Free State. These compounds are 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato(triethylarsine)gold(I) (Asa-fin), 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato(1-methyl-1,3,5-triaza-7-phosphatricyclo[3.3.1.1^{3,7}]decanium-P) gold(I)(Mpta-fin) and 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato(1,3,5-triaza-7-phosphatricyclo[3.3.1.1^{3,7}]decane) gold(I) (Pta-fin). The structures of these compounds can be seen in Figure 1.2 (page 9).

Pta-fin: Pta-fin has an adementyl group. Two nitrogen atoms are exposed per molecule, which makes this compound more polar and thus more soluble in an aqueous medium. The adementyl group however is a very bulky structure and causes steric hindrances which make Pta-fin the most hydrophilic but also the bulkiest compound. This in turn could affect its entrance into a cell. Its effect on the electrons affects its potency.

Mpta-fin: The side group of Mpta-fin is similar to Pta-fin, but is less polar (less hydrophillic) than Pta-fin because only one nitrogen atom is exposed per molecule. The methyl group (CH$_3$) attached to the second nitrogen atom enhances the lipophilicity of this compound. This arrangement makes Mpta-fin a more lipophilic compound than Pta-fin but less lipophilic than Auranofin or Asa-fin

Asa-fin: Asa-fin has a structure very similar to auranofin except that on the side group, the phosphorus atom of auranofin is substituted with an arsenic atom. Both phosphorus and arsenic have vacant d-orbitals which can accept electrons from co-ordinated metal atoms. For this reason substituted phosphines and arsines function as π-electron acceptors as well as σ-electron donors. Such π binding is important for the substitution of low oxide states ie. phenyls.
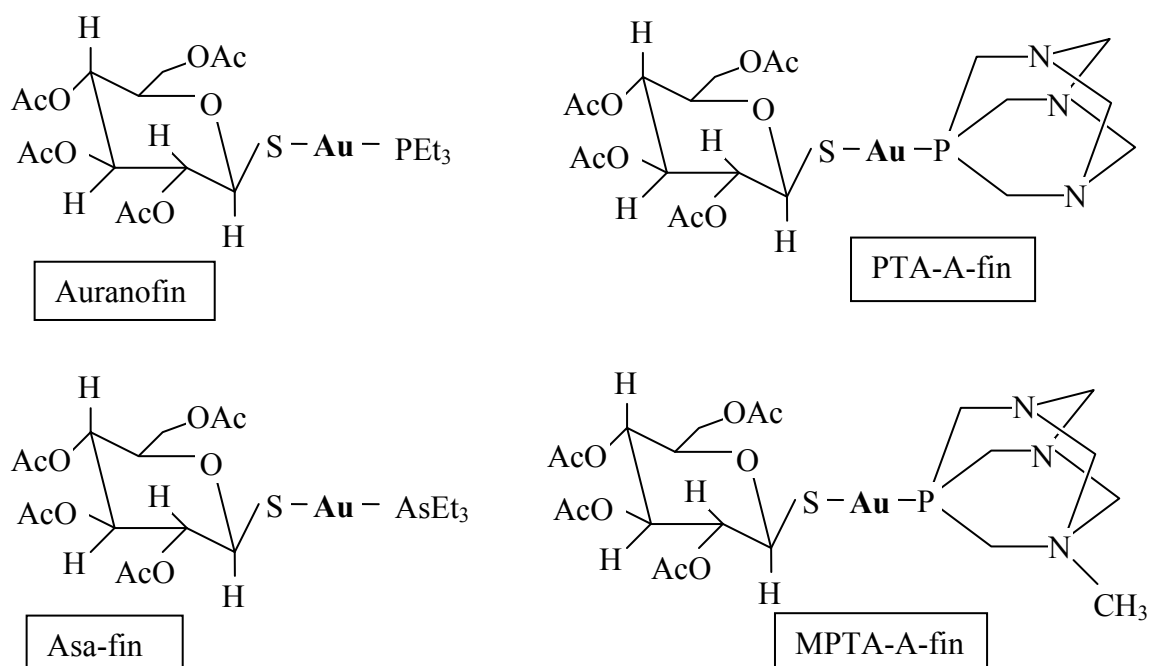
Figure 1.2. The structures of auranofin and its derivatives provided by Zolisa Sam from the University of the Free State

In this study, the lipophilicity, toxicity and anti-inflammatory effects of the experimental drugs were compared to auranofin.

1.2. AIM:

The aim of this study was to test the hypothesis that the three derivatives of auranofin, Asa-fin, Mpta-fin and Pta-fin, are less toxic and more effective than auranofin as anti-inflammatory drugs.

1.3. STUDY OBJECTIVES:

1.3.1 Primary stage

The objectives of this study were to evaluate;

the lipophilicity of the three experimental compounds.

the effects of the experimental compounds on the proliferation of human lymphocytes, porcine hepatocytes and primary chicken embryo fibroblasts

the effects of the experimental compounds on CR3 expression by stimulated human neutrophils.

the effects of the experimental compounds on COX 2 expression by stimulated human monocytes.

the effects of the experimental compounds on superoxide production by human neutrophils.

the effects of the experimental compounds on the production of reactive oxygen species (ROS) by human neutrophils.

the effects of the experimental compounds on prostaglandin E_2 production by human monocyte derived macrophages.

the effects of the experimental compounds on human inflammatory cytokine production

1.3.2 Secondary stage

To determine the acute toxicity of the experimental compounds in mice.

CHAPTER 2
LIPOPHILICITY

2. OCTANOL: WATER PARTITION COEFFICIENT

2.1 Introduction

Lipophilicity of the compounds determines the degree of protein binding and cellular uptake of these compounds. There is a correlation between the lipophilicity of a compound and the degree of selectivity and cytotoxicity potency of a compound (Berners-Price, Bowen et al. 1999). The core properties required to estimate absorption, distribution and transport in biological systems are solubility, lipophilicity, stability and an acid-base structure (Hansch and Leo 1995; Kerns 2001). The degree of lipophilicity will also influence tissue distribution. These properties are used directly or through structure activity relationships to help design active compounds and to determine membrane permeation and toxicity. Lipophilicity is a complex property more often than not determined by the octanol-water partition coefficient.

The exact meaning of hydrophobocity and lipophilicity is vague and often confused with their consequences although the concept is widely used in relation to the sorption of organic chemicals from water (Poole, Gunatilleka et al. 2000) (Dorsey and Khaledi 1993). The tendency of non-polar compounds to prefer a non-aqueous environment over an aqueous environment refers to the hydrophobic effect. The driving force of this process is created by the preference of water to reform an ordered structure. Lipophilicity is an extension of the hydrophobic effect which includes the favorable distribution of a solute between water and organic solvent (cell culture media). Hydrophobocity is therefore not synonymous with lipophilicity but a component of it. The hydrophobic effect is thought to be one of the driving forces for passive transport of drugs through biological membranes (Kerns 2001; Lipinski, Lombardo et al. 2001). Non specific toxicity is expected to correlate with a compound's propensity to accumulate in cell membranes and thus its lipophilicity (Rand 1995).

The octanol-water partition coefficient is one of the most commonly reported physiochemical properties of drugs and industrial chemicals and thus is the scale most commonly used in establishing quantitative-structure activity relationships (QSARs)

(Hansch and Leo 1995) . Lipophilicity/hydrophilicity was determined using the standard octanol/water method of Rideout, Calogeropoulou et al. (1989).

2.2. Aim

In this study the lipophilicity/hydrophilicity of auranofin compared to the novel gold compounds Asa-fin, Mpta-fin and Pta-fin was evaluated using the octanol-water partition coefficient.

2.3. Experimental procedures

A volume of water-saturated octanol and a volume of octanol-saturated water was prepared by shaking equal volumes of octanol and water for 15min. The layers were allowed to separate overnight in a separation funnel. The two fractions were collected separately being careful not to allow cross contamination of one solvent layer onto the other. Hereafter the octanol is referred to as deionised water-saturated octanol and water is referred to as octanol-saturated deionised water. 100μl DMSO was added to the compounds to enhance its solubility. 60μM stock solutions in octanol for each drug (10ml) were made up. Using the 60μM solutions, 40μM and 20μM solutions were prepared by further dilution with octanol, so that the final volume was 5ml. Each of these solutions were added to a 1ml quartz cuvette and analysed separately by UV-visible spectroscopy to give an absorbance maximum at a wavelength of 230 nm. This absorbance corresponded to a C_i value (initial concentration) for each solution. The solutions from the cuvette were emptied and returned to the corresponding 60μM, 40μM and 20μM solutions. 5ml of water was added to each of these solutions so that the final volume was 10ml. Each of the solutions was transferred independently from the 10ml volumetric flasks to separating funnels and shaken vigorously for 5min. The two phases were allowed to settle over a period of 30 min and centrifuged at 150g for 8 min. The aqueous extract was separated being careful to ensure no contamination from the octanol extract and this was then added to a 1ml quartz cuvette for analysis by UV-visible spectroscopy to give an absorbance maximum at a wavelength identified from the C_i determination. This absorbance corresponded to a C_w value for each solution. The concentration of each complex remaining in the octanol layer was determined, C_o, by C_i-C_w. The partition coefficient (PC) was given by C_o/C_w.

PC= C_o/C_w. Results were expressed as the mean log values of the final octanol/water partition coefficient value.

2.4 Results

No statistical analysis of the data could be performed and a descriptive study resulted. Negative Pc values indicate low lipophilicity (more hydrophilicity) while positive values show compounds with high lipophilicity (Figure 2.1, page 15). Auranofin and Asa-fin are more lipophilic compounds as seen from the high positive Pc value. Mpta-fin and Pta-fin appear to be more hydrophilic compounds as seen from the negative Pc value.
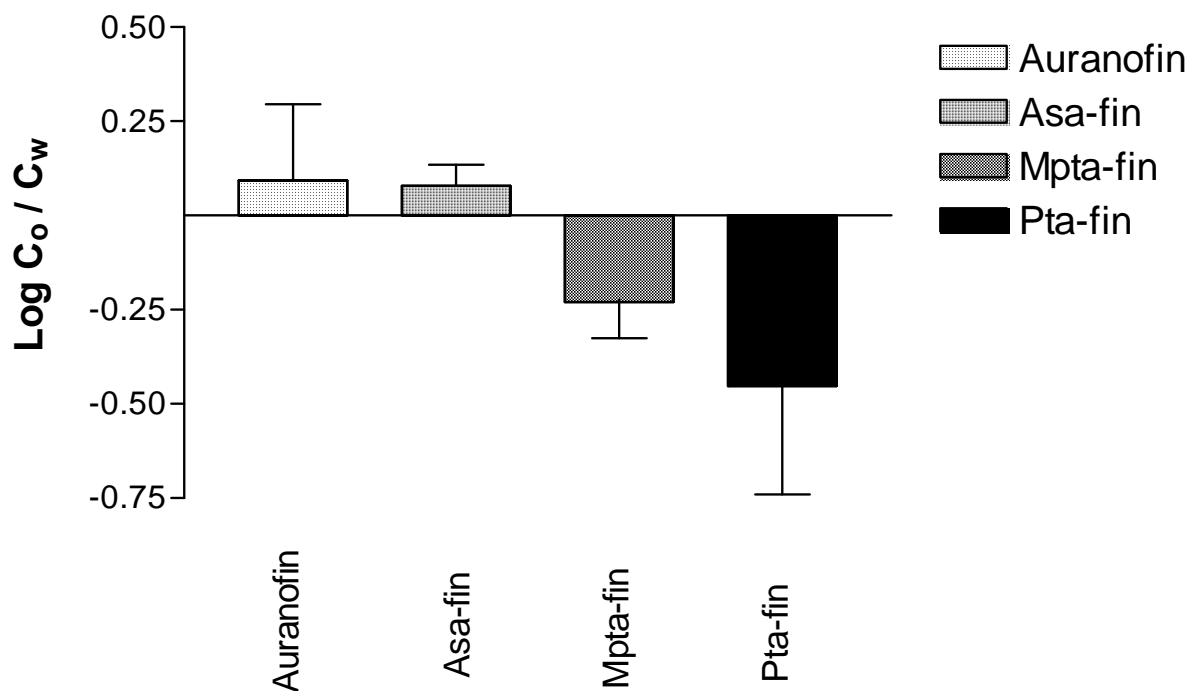
Figure 2.1: The lipophilicity, Log Co/Cw (Log Pc), of auranofin and the three experimental compounds. The concentration of the compounds were 20 μM. Negative Pc values indicate low lipophilicity (more hydrophilicity) while positive values show compounds with high lipophilicity. Auranofin and Asa-fin are lipophilic compounds (as seen from the high positive value) whereas Mpta-fin and Pta-fin are hydrophilic compounds (as seen from the negative value).

2.5. Discussion

From literature and previous studies, it is thus evident that injectable hydrophilic antirheumatic agents require a greater time period to exert their effects and are less toxic than the lipophilic compound, auranofin, which enters cells without difficulty (Walz, DiMartino et al. 1983). The toxicity of Auranofin however is attributed mainly to its lipophilicity. Auranofin and Asa-fin are more lipophilic than Mpta-fin and Pta-fin. This allows Auranofin and Asa-fin to enter cells with more ease, thereby increasing their anti-inflammatory ability but also allowing them to be more toxic (Walz, DiMartino et al. 1983). Mpta-fin and Pta-fin appear to be more hydrophilic compounds, which would mean that the serum concentration of the drug would be high and tissue concentration low. This has an advantage of limiting the side effects experienced that is generally accompanied by gold compounds but has the disadvantage of not having the efficacy of the more lipophilic drugs. One of the most reliable methods in medicinal chemistry to improve *in vitro* activity is to incorporate properly positioned lipophilic groups (Hambley 1997). An example would be the addition of a single methyl group that could occupy a rector 'pocket' and improves binding by approximately 0.7 kcal/mol. In the formation of the novel gold compounds, these principles of altering lipophilicity by adding methyl groups have been experimented with, so toxicity could be limited. As mentioned initially, the usage of the octanol-water partition coefficient (log*P*) method by Hansch and Leo (1995) to determine lipohilicity is widely used in QSAR approaches, but the measurement of log*P* is still not a high-throughput process.

CHAPTER 3
CYTOTOXICITY

3. CYTOTOXICITY

3.1. Introduction

Rheumatoid arthritis is a disease of chronic immunologically-mediated inflammation. The disease is characterised by excessive antibody production which may be the result of B-lymphocyte hyperactivity driven by unrestrained T-helper cells (Barret and Lewis 1986). It has been demonstrated that the generation of hydrogen peroxide is implicated in the induction of apoptosis in human lymphocytes (Yamanaka, Hakoda et al. 1993). Lymphocytes are also involved in the production of proinflammatory cytokines that has been associated with RA (Miossec and van den Berg 1997). The importance of lymphocytes in the pathogenesis of RA is also supported by clinical improvement seen in RA patients after the depletion of recalculating lymphocytes (Karsh, Klippel et al. 1981). Measuring the effect of the experimental compounds on the proliferation of human lymphocytes will thus indicate a possible mechanism in decreasing inflammation.

Cytotoxicity assays are performed to establish sensitivity of cancer cell lines and normal cell cultures to the experimental compounds. A predetermined concentration of cells is exposed to different concentrations of the experimental drug in a 96 well tissue culture plate and incubated for a period of time. Incubation period can range from three to seven days. The results obtained in this experiment enable the researcher to calculate the concentration of the experimental compound that inhibits 50% of growth (IC_{50}). The viable cells in each well of the tissue culture plate is determined by the MTT method (Mosmann 1983). MTT (3-[4; 5 dimethylthiozol-2-yl]-2,5 diphenyl tertazolium bromide) is a pale yellow substrate for assaying cell survival or proliferation. The tetrazolium ring is cleaved in active metabolizing cells by the mitochondrial dehydrogenase enzyme to form dark purple formazan crystals, when incubated with viable cells and is directly proportional to the amount of viable metabolically active cells. The MTT assay is a versatile and quantitative measure and is a convenient method to determine cell survival after treatment with experimental drugs (Mosmann 1983). The MTT assay will be carried out by a method described by Mosmann (1983) with slight modifications.

Chicken fibroblasts were used because it's a primary culture and indicates toxicity of a compound (Glennas, Hunziker et al. 1986). Chicken fibroblasts were isolated from chicken embryos, which are more easily accessible than mouse embryos (Freshney 2000). The primary chicken embryo fibroblasts was isolated by a method described by Freshney (2000).

Hepatocytes were primary cell cultures isolated from porcine livers. This experiment measured toxicity of hepatocytes to the drugs tested. The inhibition of cell growth is also limited to actively growing cells. A decrease in the proliferation of hepatocytes indicates an increase in toxicity.

3.2. Aim

The aim of this study is to evaluate the toxicity of auranofin compared to the three novel auranofin derivatives Asa-fin, Mpta-fin and Pta-fin for primary human lymphocytes, chicken embryo fibroblasts and porcine hepatocytes.

3.3. Cytotoxic assay

3.3.1 Preparation of cells for experimental procedures

Lymphocyte preparation

Venous blood was collected from a healthy volunteer and heparinised (5 units heparin/ml blood). 30ml of heparinised blood was layered onto 15ml Histopaque 1077 (Sigma-Aldrich, Steinheim, Germany) and centrifuged for 25 min at 1800 rpm (650g) (Beckman TJ 6 centrifuge) at room temperature. The top plasma layer was carefully removed and discarded and the lymphocyte / monocyte layer was transferred to 50ml sterile tubes and filled with sterile RPMI-1640 medium without bovine fetal calf serum. After centrifugation for 15 min at 1000 rpm (220g) (Beckman TJ 6 centrifuge), the supernatant fluid was discarded and filled again with RPMI 1640 medium without fetal bovine serum. The latter was centrifuged again for 10 min at 1000rpm (220g) (Beckman TJ 6 centrifuge) and the supernatant discarded. Sterile, ice cold 0.84% ammonium chloride (NH_4CL) was added to the pellet to lyse any remaining red blood cells and the tubes centrifuge for 10 min at 1000 rpm (220g) (Beckman TJ 6 centrifuge). The supernatant was discarded and RPMI medium was added to the

pellet. The resuspended cells were again centrifuged for 10 min at 1000 rpm (220g) (Beckman TJ 6 centrifuge) and the supernatant discarded. The resulting pellet was resuspended in 1ml sterile RPMI + (medium supplemented with 10% bovine fetal calf serum). The cell suspension was diluted to 2×10^6 cells per ml in sterile RPMI+ for use in experimental procedures. Stimulated lymphocytes used in the cytotoxicity tests contained PHA (Phyto-haemaglutenin, Remel, USA). Experimental plates were incubated for 4 days.

Primary chicken fibroblasts

Chicken eggs were incubated at 38.5ºC in a humid atmosphere for 6 days. Eggs were kept in an egg incubator which turned them over once in 24 hours.

The following procedures were carried out under sterile conditions: The egg was placed with its blunt end facing up in a small beaker and swabbed with 70% ethanol. The top of the shell was cracked and the shell peeled off to the edge of the air sac using sterile forceps. The forceps were resterilised to peel off the white shell membrane revealing the chorioallantoic membrane (CAM) below, with its blood vessels. The CAM was pierced with sterile curved forceps and the embryo lifted out by grasping it gently under the head. Embryos were transferred to a sterile Petri dish and decapitated to kill them instantly. All the fat and necrotic material was removed and the embryos transferred to a new sterile Petri dish. Embryos were chopped finely with crossed scalpels into small pieces and transferred into sterile 50ml centrifuge tubes. The tissue was washed with RPMI+ medium (RPMI 1640 supplemented with 1% glutamine, penicillin and streptomycin at 100μg/ml and 10% heat inactivated fetal calf serum) and the pieces allowed to settle. The supernatant was removed and this step repeated two more times. The tissue was transferred into a sterile 100ml glass bottle with a screw cap (Schott bottle) and most of the residual fluid removed. 45ml of PBS (filter sterilised) + 5ml 2.5% trypsin was added and stirred with a magnetic stirrer at about 100rpm for 30min at 37°C. Pieces were allowed to settle and the supernatant (with disaggregated cells in suspension) was poured into a sterile 50ml centrifuge tube and placed on ice. The previous two steps were repeated until disaggregation was complete (3-4 hours). The disaggregated cells in suspension were decanted into new sterile tubes (leaving the debris behind) and then centrifuged at 1000rpm (200g). The supernatant was discarded and the cell pellet resuspended in RPMI + medium. The resulting cell suspension was diluted to 1×10^6 cells per ml and 200 μl of this suspension was added into cell culture flasks containing 15 ml RPMI+. The medium was changed at regular intervals (2-4 days) as the pH decreased (growth medium turned yellow). Experimental plates were incubated for 4 days before cell survival was determined.

Primary porcine hepatocytes

Primary porcine hepatocytes were provided by the Liver Research Unit, University of Pretoria, Faculty of Health Sciences. A suspension of hepatocyte cells were diluted to 1×10^6 cells per ml and used in experimental procedures. The medium was changed at regular intervals (2-4 days) as the pH decreased (when growth medium turned yellow). Experimental plates were incubated for 5-7 days before cell survival was determined.

Isolation of neutrophils from whole blood

Venous blood was collected from healthy volunteers into evacuated potassium EDTA tubes. The freshly collected blood was poured into 50ml graduated plastic centrifuge tubes to a volume of 30 ml. 20 millilitres of Histopaque-1077 (Sigma Diagnostics, St Louis, MO, USA) was under-layered at the bottom of the tube using a sterile pipette. The tubes were centrifuged at 520 g for 30 minutes at 20ºC after which the portion of the upper layer containing the plasma and platelets was removed and discarded. The lymphocyte/monocyte layer was carefully removed together with the bulk of the Ficoll-Hypaque layer. The neutrophil layer is directly above and in contact with the red blood cell concentrate at the Ficoll-Hypaque boundary. The layer was gently removed with minimal inclusion of red cells and diluted to at least six times the volume with cold 0.83% ammonium chloride solution, allowed to stand on ice for 10 minutes during which time the red blood cells lysed, before centrifuging at 480 g for 10 minutes at 10ºC to sediment the granulocytes. If there was still evidence of red blood cells in the pellet the ammonium chloride haemolysis step was repeated. The pellet was washed with Hanks Balanced Salt Solution (HBSS) (Highveld Biological, Midrand, SA.) containing calcium, magnesium, 10% FCS and HEPES buffer, pH 7.4, centrifuged at 480g for 10 minutes at 10ºC and resuspended in cold HBSS to approximately 1/10th of the original volume of blood.

The cells were manually counted (50µl cell suspension added to 450µl counting solution and allowed to stand for at least two minutes before counting in a Neubauer cell counting chamber at 400 x magnification) and the cell suspension further diluted to a concentration of approximately 1×10^7 cells per ml in cold, sterile Hanks Buffered saline solution (HBSS) for the experiment.

3.3.2 Experimental procedures

All procedures were carried out under sterile conditions. Round-bottomed sterile 96-well tissue culture plates were used for all cytotoxicity assays. In preparation of fibroblasts and hepatocyte culture plates, 80 µl of the relevant culture medium and 100 µl of the applicable cell suspension were added to all of the 96 wells. The plates were then incubated at 37ºC in a CO_2 incubator (95%) for one hour before adding 20 µl of the appropriate drug treatment concentration to each of the experimental wells. To the untreated control wells, only 20µl of RPMI+ was added. The final volume in each well was 200 µl.

In preparation of the lymphocyte culture cell plates, 60 µl of RPMI+ culture medium and 100 µl of the lymphocyte cell suspension was added to all of the 96 wells. The plates were then incubated at 37ºC in a CO_2 incubator (95%) for one hour. After the one hour incubation period, 20 µl of the appropriate drug concentration was added to each of the experimental wells. 20 µl of RPMI+ was added to the untreated control cells whereas 20 µl of PHA (2.5µg/ml) was added to the experimental cells.

All experimental plates containing cells were incubated in a CO_2 incubator (95%) in a closed container containing water and incubated at the relevant time periods for the different cells mentioned above. At the termination of the incubation period the viable cells were detected using the MTT staining method. This method is a calorimetric assay using a tetrazolium salt to determine mammalian cell survival and proliferation after treatment with experimental compounds. The assay detects metabolically active cells (Mosmann 1983). 20µl of the MTT stain solution was added to the wells of the different experimental plates and re-incubated for 3-4 hours. At the termination of the incubation period, the plates were centrifuged at 2000rpm (800g) (Beckman TJ 6 centrifuge). Supernatants were removed without disturbing the pellet and washed by adding 150µl PBS and centrifuged for 10 min at 2000rpm (800g) (Beckman TJ 6 centrifuge) where after the supernatants were removed without disturbing the pellet and plates were left for an hour to dry. 100µl DMSO was added to each well to solubulise the formazan crystals. Culture plates containing the DMSO/formazan solution were incubated at room temperature for 1 hour on a shaker and measured spectrophotometrically at a wavelength of 570 nm and a reference wavelength of 630 nm to detect cellular growth and survival after drug treatment. Percentage survival

was calculated and this value was then be used to determine the IC_{50} value. IC_{50} is the concentration (μM) of the experimental compound inducing a 50% decrease in cell growth. These values were determined using the Graphpad Prism software program.

3.4. Expression and statistical analysis of results

Results are expressed as the mean % of untreated controls ± SEM (standard error measurement). ANOVA (analysis of variance) was used to analyse the data. P values were used to compare experimental values with untreated control values. IC50 (inhibitory concentration 50%) values were calculated to express the concentration of the drug that inhibited 50% of the growth of the relevant cells.

3.5 Results

3.5.1 Lymphocyte proliferation assay

All three experimental compounds Asa-fin, Mpta-fin and Pta-fin as well as auranofin, decreased the proliferation of resting and stimulated human lymphocytes in a dose related fashion (Figures 3.1 and 3.2, pages 26 and 27).

Within the concentration ranges of 1.5-100 μM, auranofin significantly decreased ($p<0.001$) the proliferation of resting lymphocytes.

With the treatment of Asa-fin, lymphocyte viability was decreased with significance ($p<0.001$) within the Asa-fin concentration range of 3.1 μM and 100 μM. Asa-fin concentrations below 3.1 μM showed no significant reduction in lymphocyte viability. Mpta-fin exhibited slightly less activity than Asa-fin. Lymphocyte viability was decreased with significance ($p<0.001$) within the concentration range of 6.25 μM and 100μM. All other concentrations tested below 6.25 μM showed no significant decrease in lymphocyte proliferation.

Pta-fin treatment decreased resting lymphocyte proliferation with significance ($p<0.01$) at 25 μM and with higher significance ($p<0.001$) at the concentrations of 50 μM and 100 μM. No significant effects were noted in the concentration range below 25 μM.

Lymphocyte proliferation in PHA- stimulated human lymphocytes was decreased with marked significance ($p<0.001$) upon the treatment of lymphocytes with auranofin from the concentration range of 0.7 µM to 100 µM. At the concentration of 0.4 µM auranofin inhibited the proliferation of stimulated lymphocytes with a significance of $p<0.01$. (Figure 3.2, page 27)

When the stimulated lymphocytes were treated with Asa-fin, proliferation of the lymphocytes was decreased significantly ($p<0.001$) within the concentration range of 1.5 µM to 100 µM. No significant decreases were noted in the proliferation of stimulated lymphocytes treated with concentrations lower than 1.5 µM.

Treatment of the stimulated lymphocytes with Mpta-fin resulted in a clear significant ($p<0.001$) decrease in the proliferation of lymphocytes within the Mpta-fin concentration range of 3.1 µM and 100 µM while no significant decreases in lymphocyte proliferation were noted at Mpta-fin concentrations lower than 3.1 µM.

Treatment of the PHA stimulated lymphocytes with Pta-fin showed significant ($p<0.001$) decreases in the proliferation of human lymphocytes within the concentration range of 12.5 µM to 100 µM. Stimulated lymphocytes treated with concentrations lower than 12.5 µM showed no significant decrease in their proliferation.

IC50 values (Table 3.1, page 32) of auranofin, Asa-fin, Mpta-fin and Pta-fin were found to be 0.964 µM, 2.513 µM, 3.914µM and 12.078µM respectively for resting lymphocytes and 0.976µM, 2.650µM, 3.262µM and 10.493 µM for stimulated lymphocytes. According to these results it can be seen that Asa-fin mimics the activity of auranofin the closest from the three derivatives and Pta-fin the least.
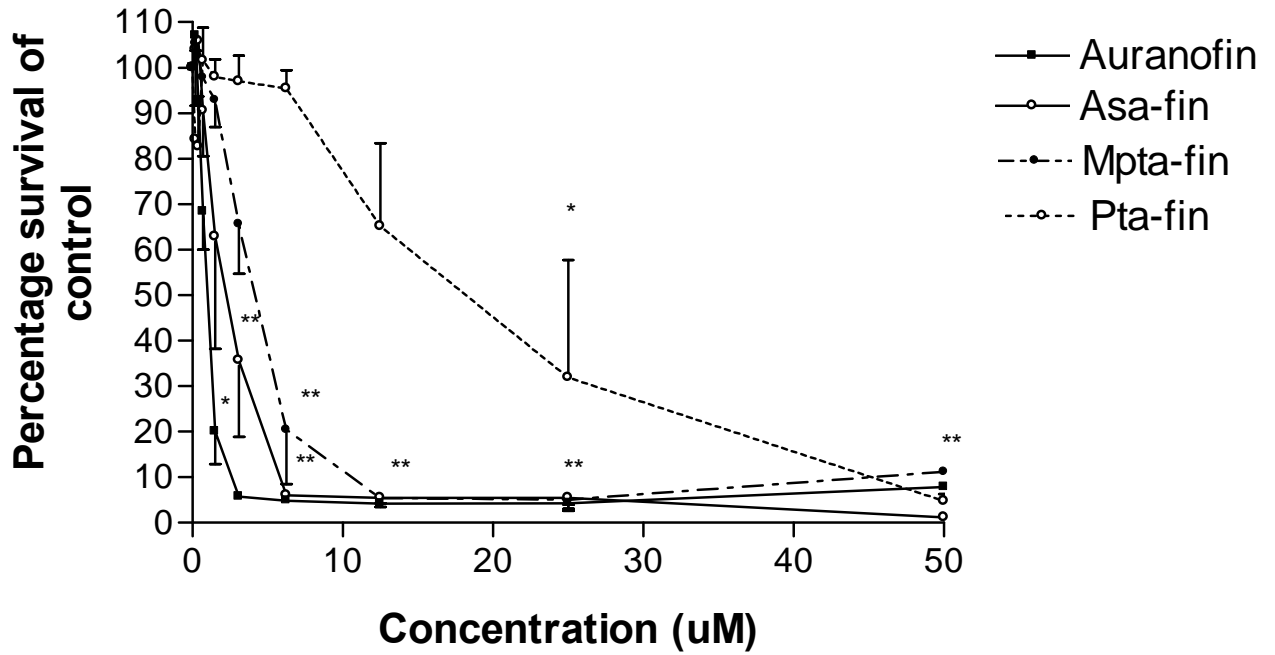
Figure 3.1: The cytotoxicity of Asa-fin, Mpta-fin and Pta-fin compared to auranofin on resting lymphocytes. Significance (p value) was calculated between untreated control values and the relevant treatment groups. Concentrations higher than 50 μM were not shown as no further significant reduction was noted. n=3.

** *p<0.01**

** **p<0.001**
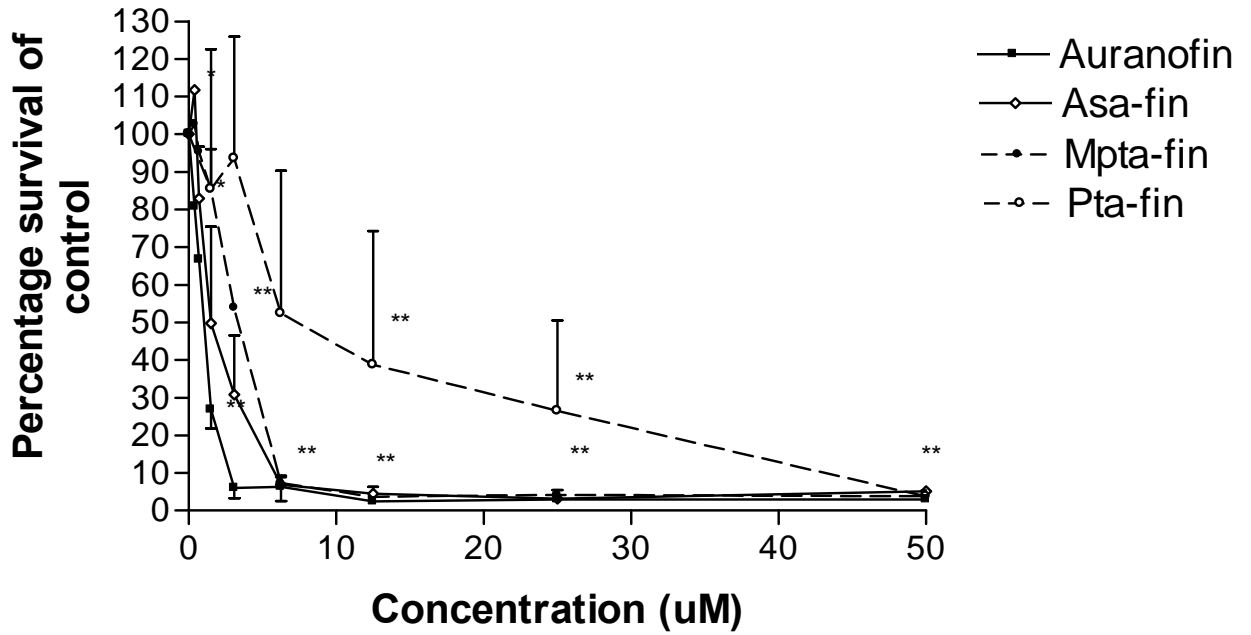
Figure 3.2: The cytotoxicity of Asa-fin, Mpta-fin and Pta-fin compared to auranofin on PHA stimulated lymphocytes. Significance (p value) was calculated between untreated control values and the relevant treatment groups. Concentrations higher than 50 μM were not shown as no further significant reduction was noted. n=3.

** *p<0.01**

** **p<0.001**

3.5.2. Primary chicken embryo fibroblasts

Evaluating the cytotoxic effect of the novel gold compounds compared to auranofin on primary chicken embryo fibroblasts cultures showed that the gold compounds were highly selective and inhibited in a dose response manner of inhibiting fibroblast proliferation (Figure 3.3, page 29).

Upon treatment with auranofin, there was a significant ($p<0.001$) decrease in the proliferation of the primary chicken embryo fibroblasts wihtin the concentration range of 6.125 μM and 50 μM. Concentrations below 6.125 μM auranofin did not significantly inhibit fibroblast proliferation.

The proliferation of fibroblasts occurred at higher concentrations with the novel gold compounds. Asa-fin significantly ($p<0.001$) inhibited fibroblast proliferation within the concentration range of 12.5 μM to 50 μM. Mpta-fin inhibited fibroblast proliferation significantly ($p<0.01$) at 25 μM and highly significantly at 50 μM ($p<0.001$). No significant differences in fibroblast proliferation were seen at concentrations below 25 μM with Mpta-fin. Pta-fin inhibited fibroblast proliferation with significance ($p<0.05$) at 3.1μM and 12.5 μM, with significance at 6.25 μM and with high significance ($p<0.001$) at 25μM and 50 μM.

The IC50 values (Table 3.1, page 32) of auranofin, Asa-fin, Mpta-fin and Pta-fin were found to be 0.784 μM, 3.23 μM, 1.66 μM and 2.614 μM respectively for chicken embryo fibroblasts. According to these results it can be seen that Asa-fin shows the least toxicity followed by Pta-fin and then Mpta-fin. Auranofin exhibits the highest toxicity among the compounds tested.

Figure 3.3: The cytotoxicity of Asa-fin, Mpta-fin and Pta-fin compared to auranofin on primary chicken embryo fibroblasts. Significance (p value) was calculated between untreated control values and the relevant treatment groups. Concentrations higher than 25 μM were not shown as no further significant reduction was noted. n=3.

** *p<0.05**

** **p<0.01**

***p<0.001

3.5.3 Primary porcine hepatocytes

Results similar to the primary chicken embryo fibroblasts were obtained with the primary porcine hepatocytes and the cells again showed a dose related inhibition to the tested compounds (Figure 3.4, page 31).

Hepatocyte cell growth was inhibited significantly ($p < 0.01$) when treated with concentrations of auranofin between 0.4 μM and 1.5 μM. When treated with auranofin concentrations in the range of 3.1 μM to 50 μM, the inhibition of hepatocyte cell growth was highly significant ($p < 0.001$).

Treatment of the hepatocytes with Asa-fin showed that the hepatocyte growth was decreased significantly ($p < 0.001$) when treated with concentrations between 6.25 μM to 50 μM. No significant inhibition of hepatocyte growth was noted with concentrations lower than 6.25 μM Asa-fin.

Mpta-fin inhibited hepatocyte growth significantly ($p < 0.001$) within the concentration range of 3.1 μM to 50 μM, while no significant inhibition of hepatocyte growth was noted with concentrations of Mpta-fin lower than 3.1 μM.

Hepatocyte cell growth was inhibited with significance ($p < 0.01$) at the Pta-fin concentration of 3.1 μM. Highly significant ($p < 0.001$) decreases in hepatocyte growth was noted within the Pta-fin concentration range of 6.25 μM and 100 μM. Concentrations of Pta-fin lower than 3.1 μM showed no significant inhibition of hepatocyte growth.

The IC50 values (Table 3.1, page 32) of auranofin, Asa-fin, Mpta-fin and Pta-fin were found to be 0.716 μM, 4.692 μM, 1.826 μM and 2.697 μM respectively for primary porcine hepatocyte cultures. According to these results it can be seen that Asa-fin shows the least toxicity followed by Pta-fin and then Mpta-fin. Auranofin exhibits the highest toxicity among the compounds tested.

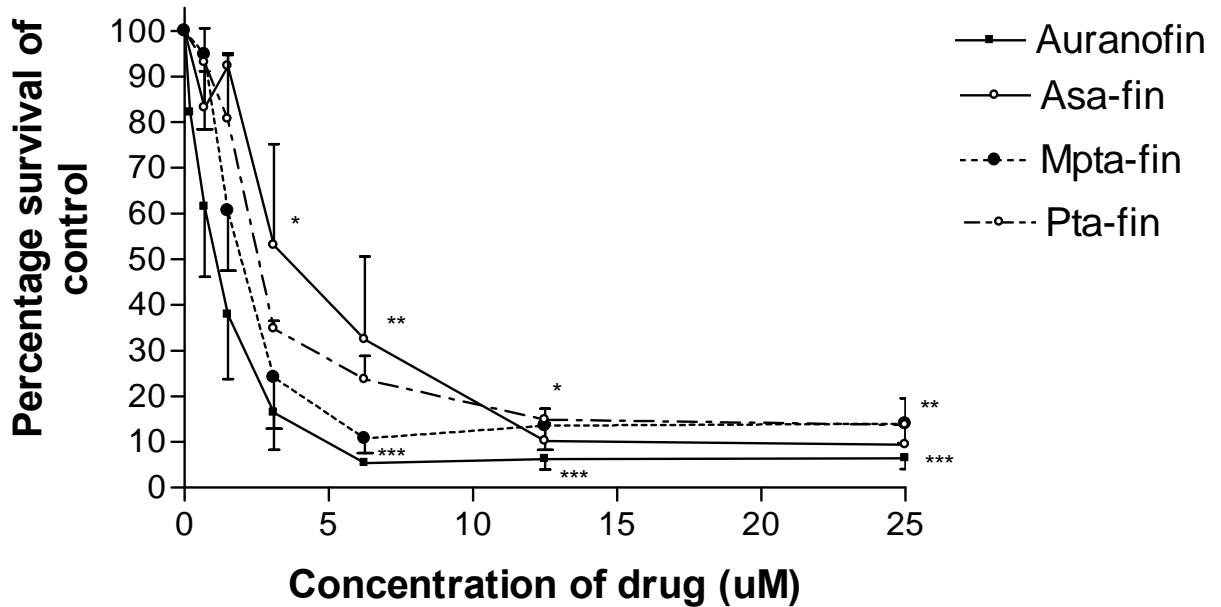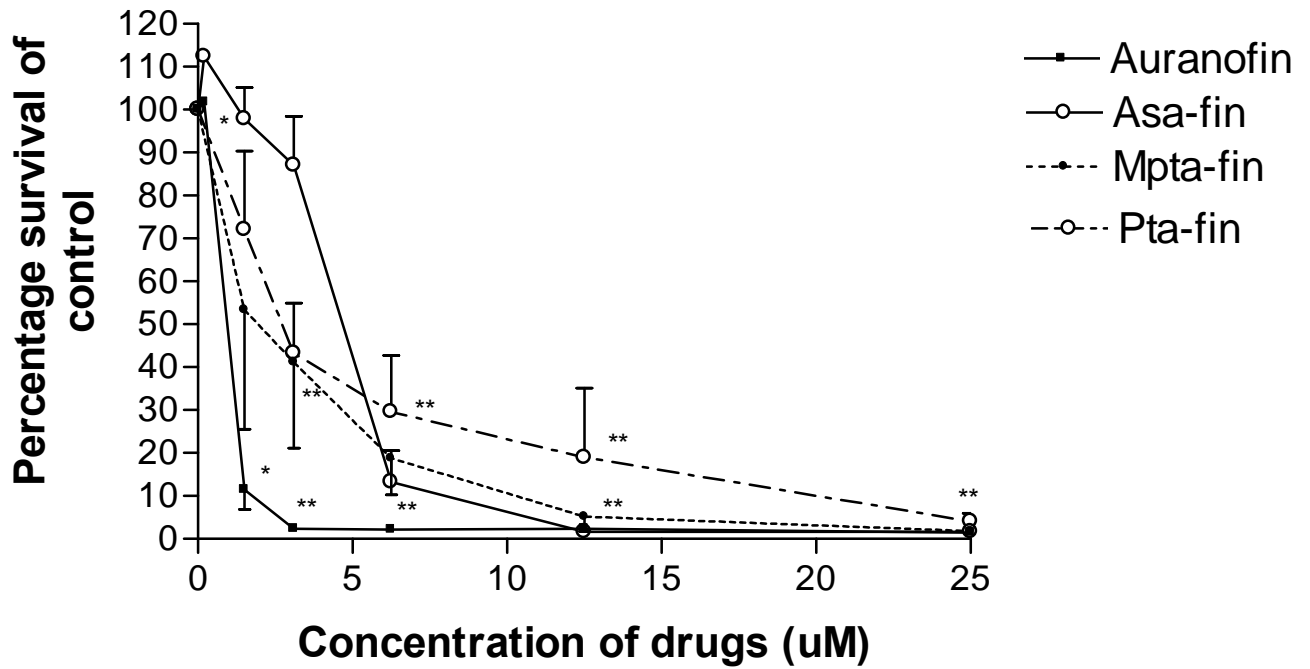**Figure 3.4: The cytotoxicity of Asa-fin, Mpta-fin and Pta-fin compared to auranofin on primary porcine hepatocytes. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=3.**

*p<0.01

**p<0.001

Table 3.1. IC 50 values of auranofin and its derivatives on various cell cultures.

Compounds	IC 50 (μM)			
	Fibroblasts	Lymphocytes (Stimulated)	Lymphocytes (Resting)	Hepatocytes
Auranofin	0.784	0.976	0.964	0.716
Asa-fin	3.23	2.650	2.513	4.692
Mpta-fin	1.663	3.262	3.941	1.826
Pta-fin	2.614	10.493	12.078	2.697

3.6. Discussion

The pathogenic mechanisms driving the synovial inflammation in RA are incompletely understood. It is postulated that the activation of synovial T lymphocytes by antigen-presenting cells is the primary event in the initiation of synovial inflammation. Thus one of the first experiments to be carried out was to determine the effect Auranofin and its various derivatives on the proliferation of resting and PHA stimulated lymphocytes. The *in vitro* effects of auranofin and the novel gold compounds on lymphocyte proliferation appear to be concentration dependent. *In vitro* human lymphocyte proliferation was affected only slightly by auranofin 0.4 μM in stimulated lymphocytes but markedly to almost completely inhibited by higher concentrations of 1.5 μM to 100 μM in resting lymphocytes and 0.7 μM to 100 μM in stimulated lymphocytes. The novel gold compounds also inhibited the proliferation of human lymphocytes in resting and PHA stimulated cells. Asa-fin showed similar activity to auranofin inhibiting proliferation of resting and stimulated lymphocytes from 1.5 μM, whereas Mpta-fin and Pta-fin showed a slightly inferior inhibition to resting and stimulated lymphocytes when compared to auranofin. These effects of inhibiting lymphocyte proliferation in resting and PHA stimulated cells was also noted by (Finkelstein, Roisman et al. 1982; Russell, Davis et al. 1982).

At low concentrations (0.25 to 0.50μg gold /ml), auranofin has been shown to cause a marked *in vitro* stimulation of human natural killer cell activity against RAJI, K-562 and HAE-60 target cells (Russell, Davis et al. 1982). At mostly higher concentrations (0.1 to 10 μg gold/ml), auranofin suppressed both T- and B- lymphocyte proliferation responses *in vitro* via stimulation of suppressor cell activity (Koboyashi, Sato et al. 1982). Possible mechanisms through which the novel gold compounds inhibit the proliferation of resting and stimulated lymphocytes still need to be determined.

The novel gold compounds inhibited the growth of primary chicken embryo fibroblasts and porcine hepatocytes at lower concentrations than auranofin. From the data shown it can be seen that from the novel gold compounds tested, Asa-fin, which is structurally similar to Auranofin, produced similar effects. The drug that showed the least inhibitory effect on resting and stimulated lymphocytes was Pta-fin. The water-soluble derivative, Pta-fin, had IC50 values of 10.493μM and 12.078μM compared to 0.976μM and 0.964μM of auranofin on resting and stimulated lymphocytes (Table 3.1, page 32). When compared to primary porcine hepatocytes treated with auranofin and the derivatives (Figure 3.4, page 31), Asa-fin was the least toxic. Asa-fin had the lowest IC50 values for fibroblasts and hepatocytes of 3.23 μM and 4.692 μM respectively (Table 3.1, page 32).

Because it decreases lymphocyte proliferation at much lower concentrations than the other compounds, it might suggest this could contribute to its anti-inflammatory activity. Pta-fin showed the second least toxicity from the novel compounds but probably the least anti-inflammatory activity due to its effect on lymphocytes. Mpta-fin showed an intermediately activity between Asa-fin and Pta-fin. Ideally we would want drugs to be least toxic to hepatocytes because they are responsible for eliminating the drug from the body. If their functions were compromised due to the presence of a drug at physiological concentration, this would be detrimental to the body. The most promising anti-inflammatory novel compound with the least toxicity to hepatocytes was Asa-fin. The results thus indicate that the novel compounds could possibly play a promising role as anti-rheumatic agents.

CHAPTER 4
EXPRESSION OF CR3

4. EXPRESSION OF CR3

4.1 Introduction

On activation, neutrophils and monocytes express complement receptor 3 (CR3), a β_2 integrin that is expressed exclusively by haemopoietic cells and which is an adhesion molecule which plays a role in cell adhesion, daipedesis and phagocytosis. The expression of CR3 is one of the first measurable responses on the surface of the cells that occurs after stimulation of neutrophils and can be used as a marker of cell response in inflammatory reactions (Harlan 1993). The expression of the β_2-integrin adhesion molecule, CR3, on resting and stimulated neutrophils treated with the experimental compounds will be measured and compared to the untreated control cells. A fluorescent dye labelled monoclonal antibody against CD11b. The CR3 receptors consist of two polypeptide chains: an α chain of 165kDa and a β chain of 97kDa. The α chain is the CD11b cell surface marker molecule whereas the β chain is the CD18 cell surface marker. The dye binds to the CR3 expressed on the plasma membrane, which can be quantitated by standard flow cytometric analysis. The intensity of the fluorescence correlates with the amount of the CR3 expressed on the outer neutrophil membrane. CR3 expression testing is a standard immuno-pharmacological bioassay that is used to study immuno-modulating/suppressing activities of drugs on neutrophil activation. These receptors can be expressed very rapidly, apparently due to expression of preclustered internal reserves (Petty, Francis et al. 1987).

Normally CR3 binds to fibrinogen or ICAM-1 and E-Selectin, the latter two are present on the surface of activated endothelial cells of the vascular system, as an initial stage of diapedesis of the neutrophils. The inflammatory process depends on the migration of large numbers of neutrophils from the vascular system to the affected area where they release oxidative reactants and cytokines that cause the inflammation (Kishimoto, Baldwin et al. 1999). To determine the CR3 expression, the method of Ueda, Rieu et al. (1994) with slight modifications was used.

In this study the cellular expression of CR3 by neutrophils was quantitated by a flow cytometric method based on the method originally reported by Rabinovitch and June (1990) and adapted. Phycoerythrin (PE) conjugated monoclonal antibodies against the

CD11b cell surface marker molecules were added to both resting and stimulated neutrophils. Analysis of the cellular fluorescence intensity was done by flow cytometry to provide a quantitative measure of the total CR3 expressed on the surface of the neutrophils. An Epics XL-MLC flow cytometer (Beckman Coulter, Fullerton, CA) equipped with an air-cooled argon laser operating at 488nm was used in this study. The gated region in the scattergram represents the neutrophils and only the fluorescence within this gated region was analysed. The fluorescent peak channel position in the histogram was used to quantitate the expressed CR3.

4.2. Aim

The aim of this experiment was to determine the anti-inflammatory activity of auranofin compared to the three novel auranofin derivatives Asa-fin, Mpta-fin and Pta-fin by evaluating the expression of CR3 on PMA stimulated human neutrophils.

4.3. Experimental procedures

Whole blood was collected from healthy individuals in heparin tubes (Becton Dickinson, (San Jose, CA. USA). 400 μl of whole blood was placed in 5 ml polypropylene tubes and the tubes were filled with 0.83% NH_4CL solution containing 0.1% EDTA, vortexed briefly and left on ice for 10 minutes to lyse the red blood cells. Tubes were centrifuged for 10 minutes at 500g at room temperature and the supernatant discarded. Tubes were filled with RPMI+ and centrifuged for 10 minutes at 500g at room temperature and the supernatant discarded. 300μl aliquots of RPMI 1640 medium containing 10% FCS were added to each tube and pre-incubated in a water bath at 37ºC for 15 minutes. 100μl of dilutions of the relevant drug was added to the 15 ml tubes. The test compounds were replaced by RPMI+ in the two control tubes; otherwise all additions were identical to the test compounds. After the incubation, 100 μl of freshly prepared phorbol-12-myristate-13-acetate (PMA) (1000ηg/ml) (Sigma Diagnostics, St Louis, MO, USA) was added to the tubes earmarked as stimulated. Resting tubes received RPMI+. The tubes were now further incubated for 30 minutes in the 37ºC water bath and were thereafter quickly transferred to an ice bath and 100μl aliquots removed and added to corresponding clean counting tubes to which 5μl of PE conjugated anti-CD11b monoclonal antibody (Beckman Coulter, Fullerton, CA) had been added. Non-specific binding was

excluded by an inclusion of a relevant isotypic control. Isotypic background controls were provided by the addition of 100μl aliquots from the resting or stimulated control tubes to 5μl PE conjugated anti-mouse IgG. The tubes were incubated for 10 minutes in the dark and then diluted to 600μl with Isoflow® (Beckman Coulter, Fullerton, CA). The suspensions were analysed within an hour on a Epics XL-MLC flow cytometer (Beckman Coulter) equipped with an air cooled Argon ion laser. Time per analysis was set to 100 seconds. The neutrophil population in the scattergram was gated and this region analysed for fluorescent intensity. Quantitation of the CR3 was directly related to the fluorescent signal measured using the median peak position.

4.4. Expression and statistical analysis of results

Results are expressed as the mean % of untreated controls ± SEM (standard error measurement). ANOVA (analysis of variance) was used to analyse data. P values were used to compare experimental values with untreated control values.

4.5. Results

Evaluating the anti-inflammatory effect of the novel gold compounds compared to auranofin on resting and stimulated human neutrophils showed that the gold compounds auranofin, Asa-fin and Mpta-fin showed a dose response manner of inhibiting the expression of CR3 (Figure 4.1- Figure4.3, pages 39- 41).

Upon treatment with auranofin, there was a highly significant ($p < 0.001$) decrease in the expression of CR3 in resting human neutrophils within the concentration range of 1.5 μM and 100 μM. Concentrations below 1.5 μM auranofin did not significantly inhibit the expression of CR3 in resting neutrophils. In PMA stimulated human neutrophils, auranofin decreased the expression of CR3 significantly ($p < 0.05$) at the concentration of 0.2 μM, very significantly ($p < 0.01$) at the concentration of 0.4 μM and highly significantly ($p < 0.001$) within the concentration range of 0.7-100 μM (Figure 4.1, page 39).

With the treatment of Asa-fin, CR3 expression in resting human neutrophils was highly significantly inhibited ($p < 0.001$) within the concentration range of 3.1 μM and 100 μM. Asa-fin concentrations below 3.1 μM showed no significant reduction in the expression of CR3 in resting cells. In PMA stimulated human neutrophils, the

expression of CR3 was inhibited highly significantly (p<0.001) within the concentration range of 1.5 µM and 100 µM. Concentrations below 1.5 µM showed no significant reduction in the CR3 expressed in stimulated human neutrophils (Figure 4.2, page 40).

Mpta-fin exhibited slightly better activity than Asa-fin in resting neutrophils. CR3 expression was decreased with significance (p<0.001) within the concentration range of 1.5 µM and 100 µM. All other concentrations tested below 1.5 µM showed no significant decrease in CR3 expression. Mpta-fin however showed slightly less activity in PMA stimulated human neutrophils by inhibiting the expression of CR3 significantly (p<0.001) in stimulated cells within the concentration range of 3.1 µM to 100 µM. Concentrations below 3.1 µM in stimulated neutrophils showed no significant inhibition of the CR3 expressed.

Treatment of resting neutrophils with Pta-fin exhibited the least activity in the inhibition of the CR3 expressed in resting neutrophils. Pta-fin significantly (p<0.01) inhibited the expression of CR3 in resting neutrophils at the concentration of 25 µM and with high significance at within the concentration range of 50 µM -100 µM. In PMA stimulated human neutrophils, Pta-fin inhibited the expression of CR3 significantly (p<0.001) within the concentration range of 12.5 µM to 100 µM. Concentrations below 25 µM in resting cells and 12.5 µM in PMA stimulated cells exhibited no significant inhibition on the CR3 expressed.
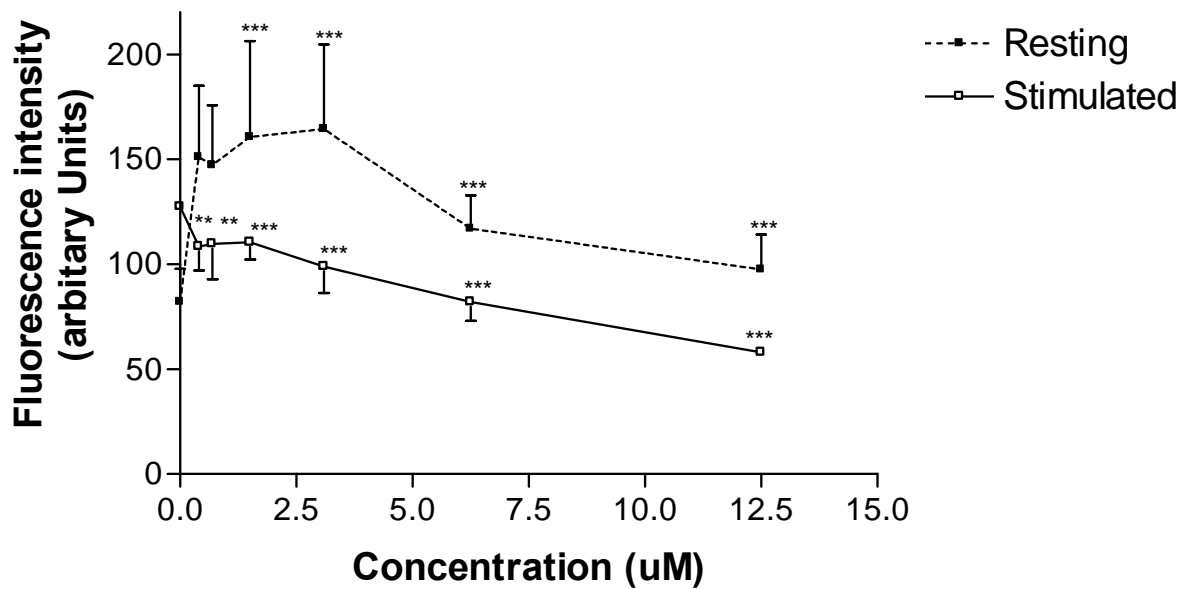
Figure 4.1: The effects of auranofin on the expression of CR3 on human neutrophils. Neutrophils were prepared, incubated in the presence of auranofin (0.3-12,5μM) for 15min. The expression of CR3 was measured on a flow cytometer. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=3.

   *p<0.05
  **p<0.01
***p<0.001
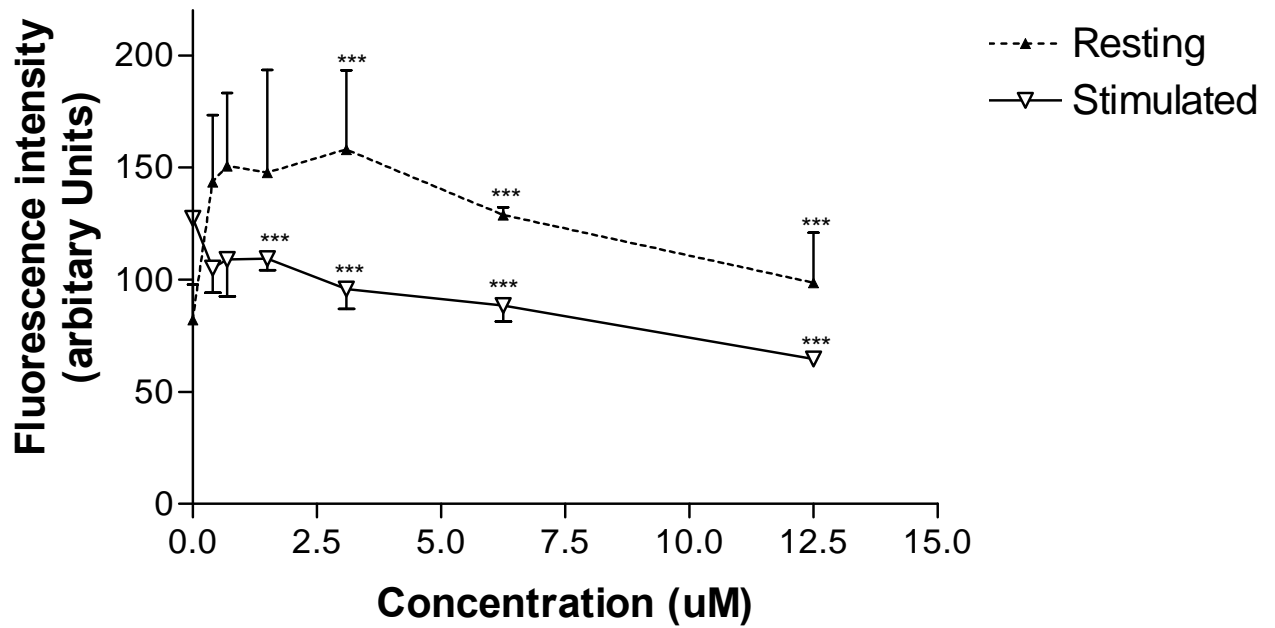
Figure 4.2: The effects of Asa-fin on the expression of CR3 on human neutrophils. Neutrophils were prepared, incubated in the presence of Asa-fin (0.3-12,5μM) for 15min. The expression of CR3 was measured on a flow cytometer. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=3.

*p<0.05

**p<0.01

***p<0.001
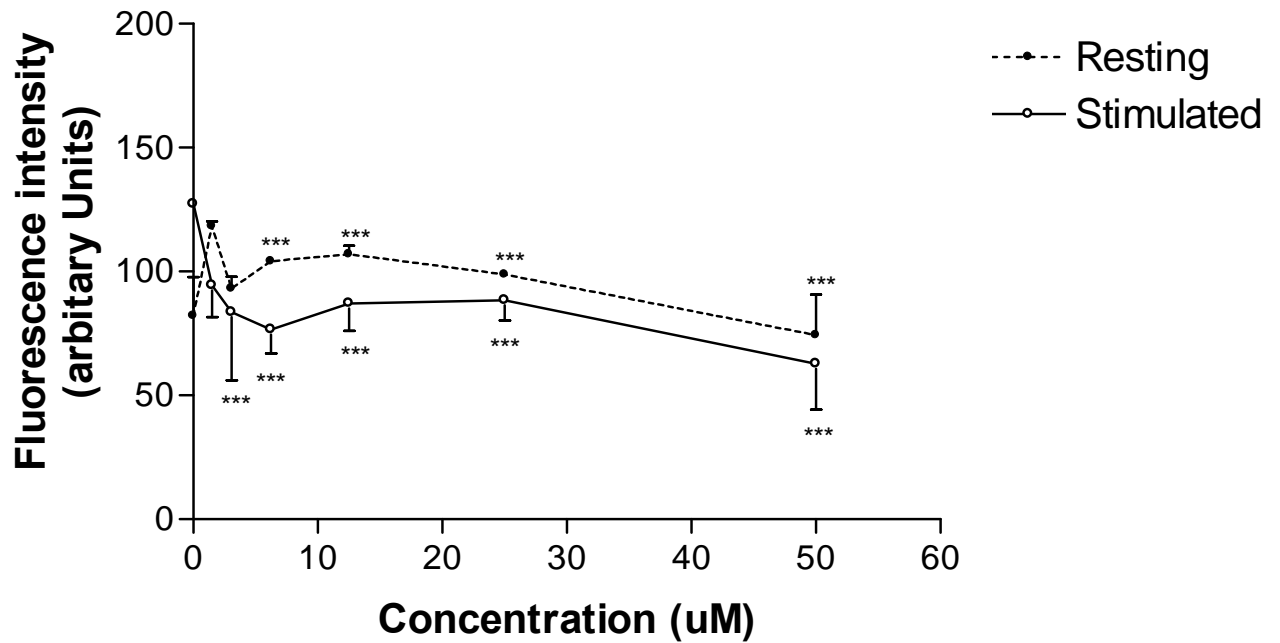
Figure 4.3: The effects of Mpta-fin on the expression of CR3 on human neutrophils. Neutrophils were prepared, incubated in the presence of Mpta-fin (1.5-50μM) for 15min. The expression of CR3 was measured on a flow cytometer. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=3.

   *p<0.05

  **p<0.01

***p<0.001
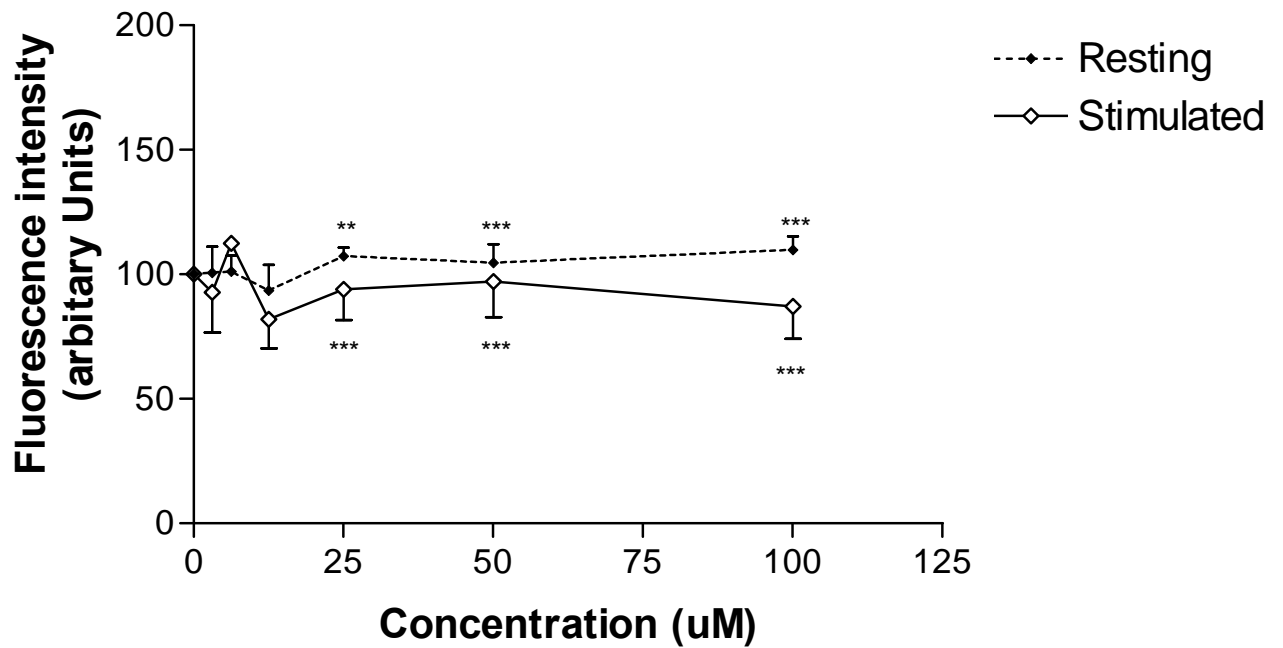
Figure 4.4: The effects of Pta-fin on the expression of CR3 on human neutrophils. Neutrophils were prepared, incubated in the presence of Pta-fin (3.1-100μM) for 15min. The expression of CR3 was measured on a flow cytometer. Concentrations below 25 μM in resting cells and 12.5 μM in PMA stimulated cells exhibited no significant inhibition on the CR3 expressed. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=3.

*p<0.05

**p<0.01

***p<0.001

4.6. Discussion

In this study it was found that Auranofin, Asa-fin and Mpta-fin inhibited the expression of CR3 by activated and resting neutrophils. This indicates a possible mechanism by which the compounds could act as possible anti inflammatory drugs. An overexpression of CR3 is associated with the production of many cytokines, proteolytic enzymes, reactive oxygen and nitrogen intermediates that can cause tissue injury and is commonly found in autoimmune diseases such as RA (Weiss 1989). CR3 is an attractive target for anti-inflammatory drugs. The novel gold compounds also inhibited the expression of CR3 in resting and PMA stimulated human neutrophils. Asa-fin showed the closest activity to auranofin from the three experimental compounds tested in inhibiting the expression of CR3. Asa-fin inhibited the expression of CR3 from 3.1 µM in resting cells and from 1.5 µM in stimulated cells, whereas Mpta-fin inhibited the expression of CR3 from 6.25 µM in resting cells and 3.1 µM in PMA stimulated neutrophils. Pta-fin exhibited the least activity in inhibiting the expression of CR3 with effects noted from 25 µM in resting cells and 12.5 µM in PMA stimulated cells.

Auranofin, Asa-fin and Mpta-fin caused a decrease in CR3 expression of neutrophils at physiological concentrations (1.5- 5.1µM) in whole blood as reported by (Gottlieb 1982) *in vitro* (Figure 4.1- 4.3, pages 39- 41). Pta-fin however failed to inhibit the expression of CR3 at physiological concentrations (Figure 4.4, page 42). The most promising novel compound with CR3 inhibitory effects was Asa-fin. It was also interesting to note that the gold compounds (at low doses) initially caused an increase in the expression of CR3 in resting neutrophils before decreasing the CR3 expression at higher doses. Inhibition of the expression of the CR3 in PMA stimulated neutrophils were not accompanied by an initial increase. These results might suggest better anti-inflammatory activity of auranofin and the novel gold compounds in individuals experiencing inflammation.

CHAPTER 5
COX-1 AND COX-2 ACTIVITY

5. CYCLOOXYGENASE-1 (COX-1) AND CYCLOOXYGENASE-2 (COX-2) EXPRESSION

5.1 Introduction

Cyclooxygenases are pivotal enzymes in cellular prostaglandin biosynthetic pathways and catalyze reactions in which arachadonic acid is converted to the endoperoxidase intermediate, prostaglandin H_2. They have also recently become targets of intense research interest in therapeutic non-steroidal anti-inflammatory drug (NSAID) development. There are two enzymatically active isoforms of the cyclooxygenase enzyme, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX 2) (Fletcher, Kujubu et al. 1992). Both isoforms have similar biological functions but only COX-2 appears to be significantly up regulated during pain and inflammatory disorders (Hinz and Brune 2002). They are located on the luminal surface of the endoplasmic reticulum and on the inner and outer membranes of the nuclear envelope (Spencer, Woods et al. 1998).

COX-1 is expressed in nearly all mammalian tissues and is responsible for the basal prostaglandin synthesis required for cell homeostasis (Smith, Garavito et al. 1996). COX-2 expression is not detected in most tissues under physiological conditions but is inducible by various stimuli (Fletcher, Kujubu et al. 1992; Herschman 1996; Smith, Garavito et al. 1996) . COX- 2 expression is rapidly upregulated during the course of inflammation, following cellular stress, and in response to growth factors, tumor promoters, bacterial endotoxins and inflammatory cytokines (Smith, Garavito et al. 1996; Maloney, Kutchera et al. 1998). Guastadisegni, Nicolini et al. (2002) and Mitchell, Akarasereenont et al. (1994) reported that COX-2 is overtly induced upon stimulation by LPS. COX-2 can be induced in a number of cell types, including fibroblasts, endothelial cells, monocytes and ovarian follicles (Smith, Garavito et al. 1996; Ruitenburg and Waters 2002).
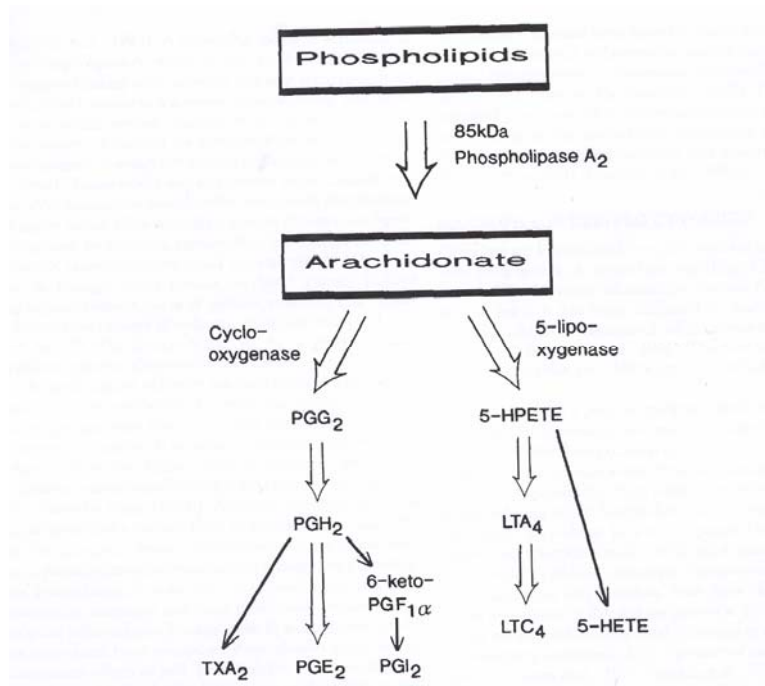
Figure 5.1 Eicosanoid formation in macrophages through the cyclooxygenase and lipoxygenase pathways: PGG$_2$-prostaglandin G$_2$, PGH$_2$- Prostaglandin H$_2$, PGI$_2$- Prostaglandin I$_2$, HPETE- hydroxyl-proxy-eicosateraenoic acid, HETE-hydroxyl-eicosateraenoic acid, LTA4- leucotriene A$_4$, LTC$_4$- leucotrieneC$_4$, TXA$_2$- thromboxane A$_2$ (Bondeson, 1997)

Arachidonic acid metabolites formed by PMNL (polymorphonuclear leukocytes) are predominantly the leukotrienes, formed by the action of 5-lipooxygenase, with leukotriene B$_4$ (LTB$_4$) being the main metabolite (Serhan 1994; Alonso, Bayon et al. 1998). It has been shown that prostaglandin E$_2$ (PGE$_2$) and thromboxane A$_2$ (TxA$_2$) are also synthesized by PMNLs via an inducible cyclooxygenase-2 (COX-2) in response to numerous known PMNL stimuli (Pouliot, Gilbert et al. 1998). The stimulant determines the rate of upregulation of COX-2 in PMNL and it differs greatly from that of the monocytes and macrophages that may have implications in the

control of cell damage by PMNL in diseases like rheumatoid arthritis, sepsis and acute respiratory syndrome (Rocca and Fitzgerald 2002). Similarly inflammatory cells such as neutrophils release platelet activating factor (PAF), which is a very powerful and omnipotent bioactive lipid. It potentiates the inflammatory response of PMNL and eosinophils, possibly through activation of specific G protein type receptors (Prescott 1999).

5.2. Aim

The aim of this study was to evaluate the activity of auranofin compared to the three novel auranofin derivatives Asa-fin, Mpta-fin and Pta-fin on the expression of COX-1 and COX-2 in resting and LPS (lipopolysaccharide) stimulated human monocytes.

5.3. Experimental procedures

Endotoxin activation of monocytes in whole blood

Whole blood was collected from healthy individuals in heparin tubes (Becton Dickinson, San Jose, CA. USA) and 1ml of fresh human whole blood was aliquoted into 15 ml polypropylene tubes. 100µl of dilutions of the relevant drug (different concentrations) was added to the whole blood in the 15 ml tubes. Control tubes received 100µl of RPMI+. Lipopolysaccharide (LPS), (Sigma-Aldrich, Steinheim, Germany) 1.0 µg/ml was added to whole blood as a stimulus. An unstimulated sample (without LPS) was used as a negative control. The samples were incubated for 4 hours in a 37ºC incubator with 7% CO_2.

Intracellular immunoflourescent staining of endotoxin- activated whole blood monocytes

After the incubation with the LPS, 100µl aliquots of whole blood were transferred to 5 ml polypropylene tubes. Samples were treated with 2 ml of lysing solution (as supplied by Becton Dickinson, San Jose, CA. USA) for 10 min in the dark at room temperature and then centrifuged for 5 min at 500g at room temperature. The supernatant was removed and discarded. Samples were permeabilised by incubating them in the presence of 0.5 ml of lysing solution containing 0.2% saponin (Sigma-Aldrich, Steinheim, Germany) for 10 min in the dark at room temperature and then washed by adding 2 ml of phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% sodium azide (NaN3) and centrifuged for 5 min at

500g. The supernatant was removed and discarded. Samples were stained intracellularly by adding 20 µl of Anti-COX-1/Anti-COX-2 antibody solution (Becton Dickinson, San Jose, CA. USA) and incubated for 30 min in the dark at room temperature. Sample tubes were then washed with 2 ml of PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide (NaN3) and centrifuged for 5 min at 500g at room temperature. The supernatants were removed and discarded. Finally samples were resuspended to a final volume of 400 µl in 1% paraformaldehyde (PFA, in PBS, Electron Microscopy Sciences, Fort Washington, PA, 10% EM grade) and stored at 4ºC until ready to be analysed on a flow cytometer (analysis was done within 24 hours).

5.4. Expression and statistical analysis of results

Results are expressed as the mean % of untreated controls ± SEM (standard error measurement). Student's paired t-test was used to analyse data. P values were used to compare experimental values with untreated control values.

5.5. Results

5.5.1 Cyclooxygenase-1 (COX-1) expression

Evaluating the COX-1 inhibiting effect of the novel gold compounds compared to auranofin on resting and LPS stimulated human monocytes showed that, as was the case with auranofin, the novel gold compounds Asa-fin, Mpta-fin and Pta-fin had no effect on the expression of COX-1 by resting and LPS stimulated human monocytes (Figure 5.2- Figure 5.5, pages 49-52).
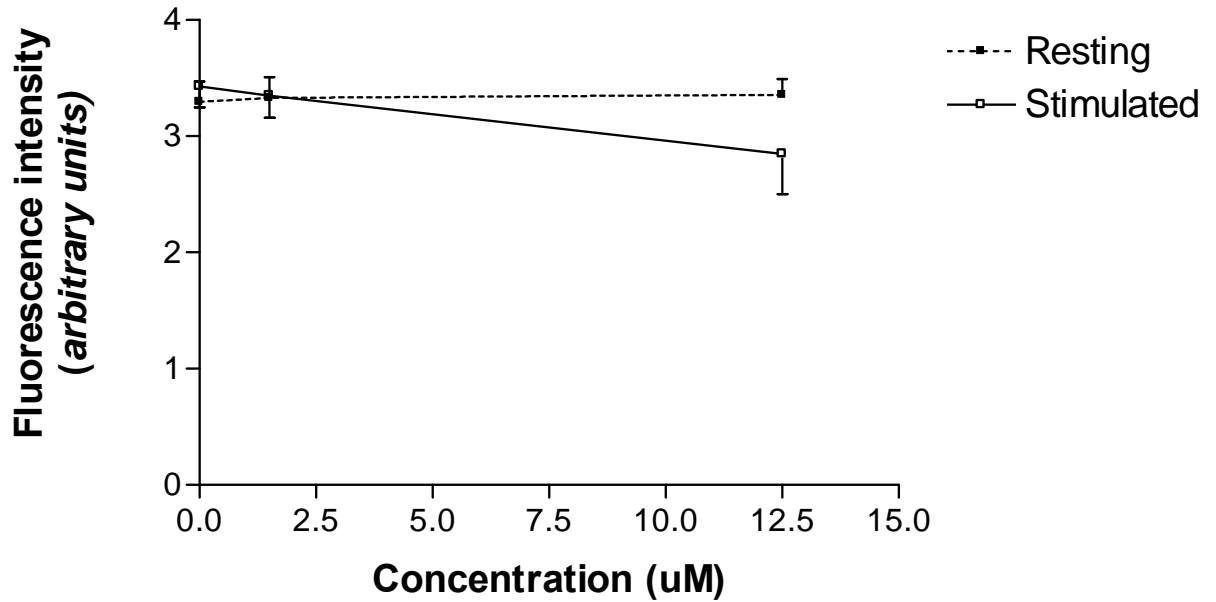
Figure 5.2: The effect of Auranofin on the expression of COX-1 by resting and LPS stimulated human monocytes. Monocytes were incubated in the presence of Auranofin (1.5 and 12.5 µM) for 30 minutes prior to the addition of LPS (1 µg/ml). After the addition of LPS, monocytes were incubated for a further 4 hours. Expression of COX-1 was determined on a flow cytometer using an Anti-Cox1 FITC/ Anti Cox-2 PE antibody. The results are representative of two different experiments using blood from two different donors. n=2.
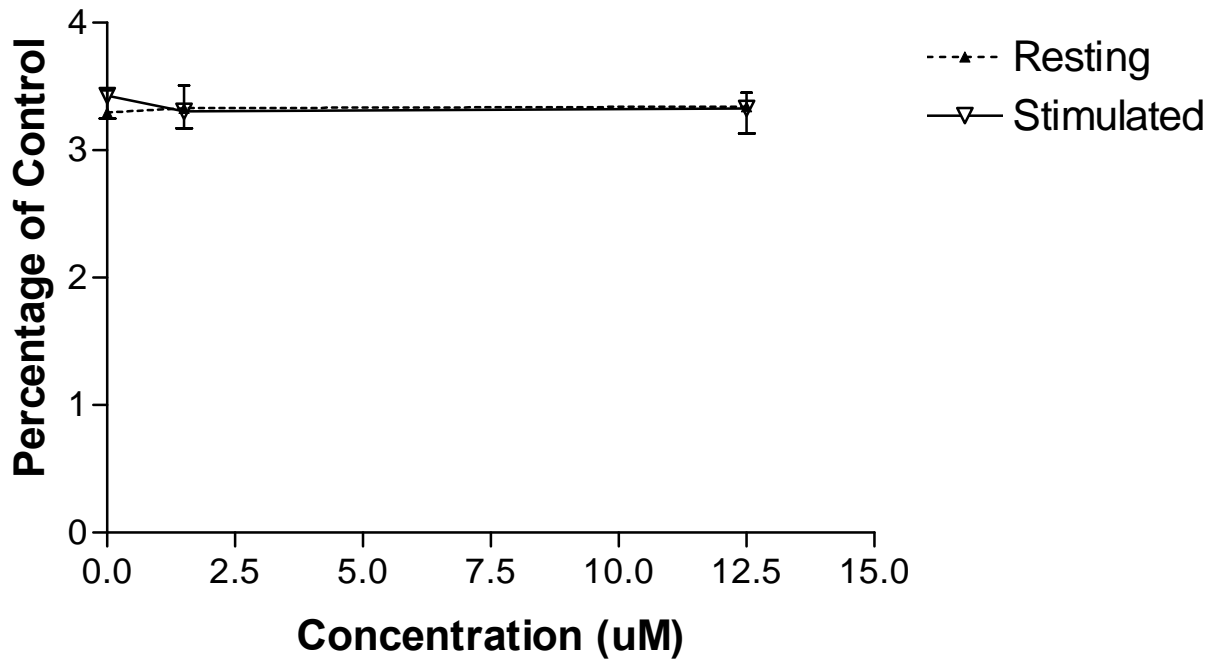
Figure 5.3: The effect of Asa-fin on the expression of COX-1 by resting and LPS stimulated human monocytes. Monocytes were incubated in the presence of Asa-fin (1.5 and 12.5 μM) for 30 minutes prior to the addition of LPS (1 μg/ml). After the addition of LPS, monocytes were incubated for a further 4 hours. Expression of COX-1 was determined on a flow cytometer using an Anti-Cox1 FITC/ Anti Cox-2 PE antibody. The results are representative of two different experiments using blood from two different donors. n=2.
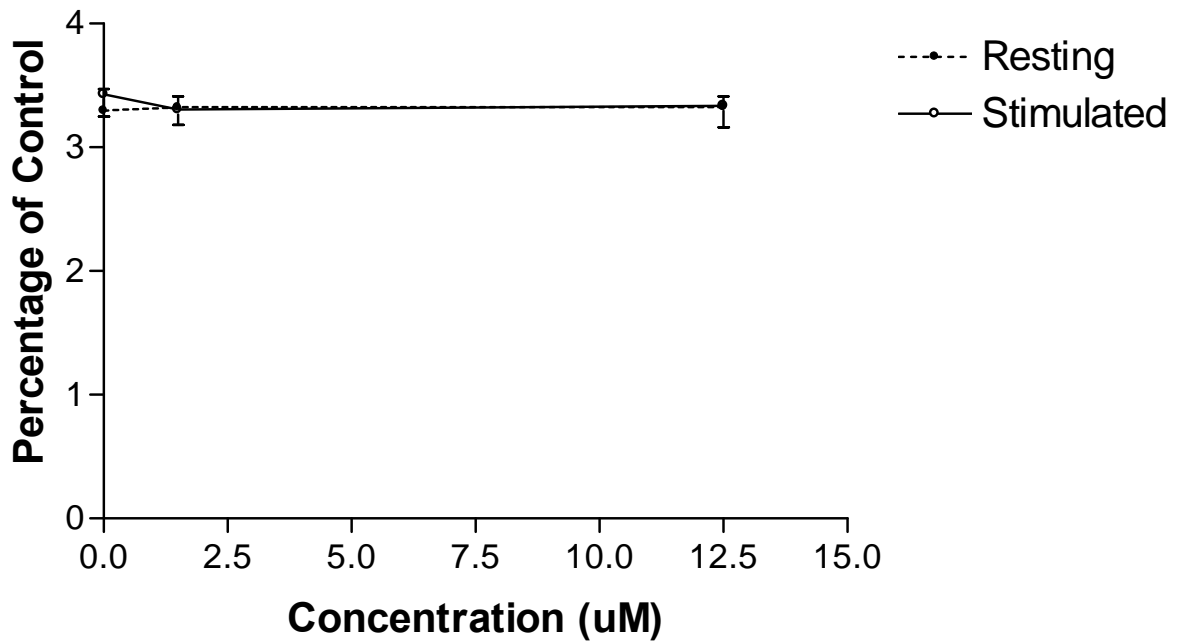
Figure 5.4: The effect of Mpta-fin on the expression of COX-1 by resting and LPS stimulated human monocytes. Monocytes were incubated in the presence of Mpta-fin (1.5 and 12.5 μM) for 30 minutes prior to the addition of LPS (1 μg/ml). After the addition of LPS, monocytes were incubated for a further 4 hours. Expression of COX-1 was determined on a flow cytometer using an Anti-Cox1 FITC/ Anti Cox-2 PE antibody. The results are representative of two different experiments using blood from two different donors. n=2.
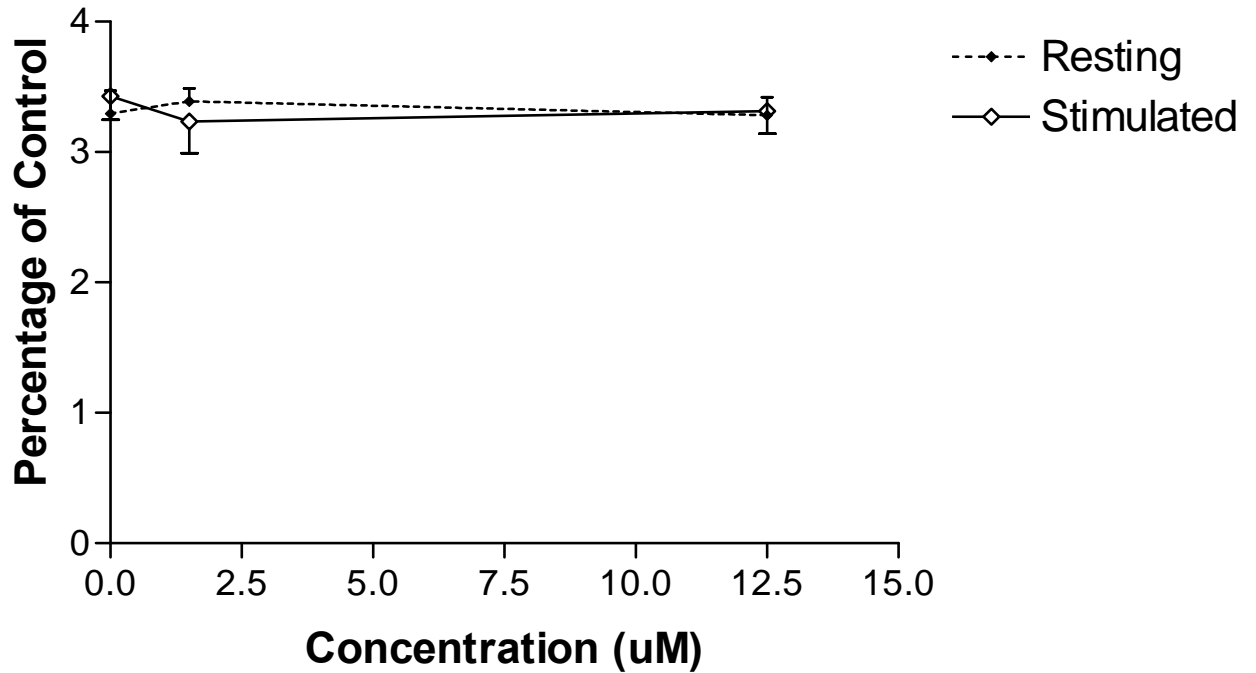
Figure 5.5: The effect of Pta-fin on the expression of COX-1 by resting and LPS stimulated human monocytes. Monocytes were incubated in the presence of Pta-fin (1.5 and 12.5 µM) for 30 minutes prior to the addition of LPS (1 µg/ml). After the addition of LPS, monocytes were incubated for a further 4 hours. Expression of COX-1 was determined on a flow cytometer using an Anti-Cox1 FITC/ Anti Cox-2 PE antibody. The results are representative of two different experiments using blood from two different donors. n=2.

5.5.2 Cyclooxygenase-2 (COX-2) expression

LPS stimulated monocytes that were treated with auranofin 1.5 µM showed a significant (p<0.01) decrease in the COX-2 expressed. At a concentration of 12.5 µM, auranofin caused a decrease in the expression of COX-2 in LPS stimulated monocytes which was highly significant (p<0.001). However in the resting cell suspensions, auranofin caused a significant (p<0.01) increase in the expression of COX-2 after treatment with 12.5 µM auranofin (Figure 5.6, page 54). There were no significant effects on COX-2 expressed in resting monocytes at a concentration of 1.5 µM auranofin.

 Asa-fin significantly decreased the expression of COX-2 in the LPS stimulated monocytes at 1.5 µM (p<0.01) and at 12.5 µM (p<0.05) however there were no significant effects in the expression of COX-2 in the resting monocytes (Figure 5.7, page 55).

For stimulated monocytes treated with Mpta-fin 1.5 µM and 12.5 µM there was a significant (p<0.01) decrease in the expression of COX-2. In resting human monocytes no significant effects in the expression of COX-2 were noted (Figure 5.8).

The novel gold compound, Pta-fin significantly inhibited the expression of COX-2 in LPS stimulated human monocytes at concentrations of 1.5µM (p<0.05) and 12.5 µM (p<0.01) but triggered the expression of COX-2 on resting human monocytes at the concentration 12.5 µM (p<0.05) (Figure 5.9, page 57).
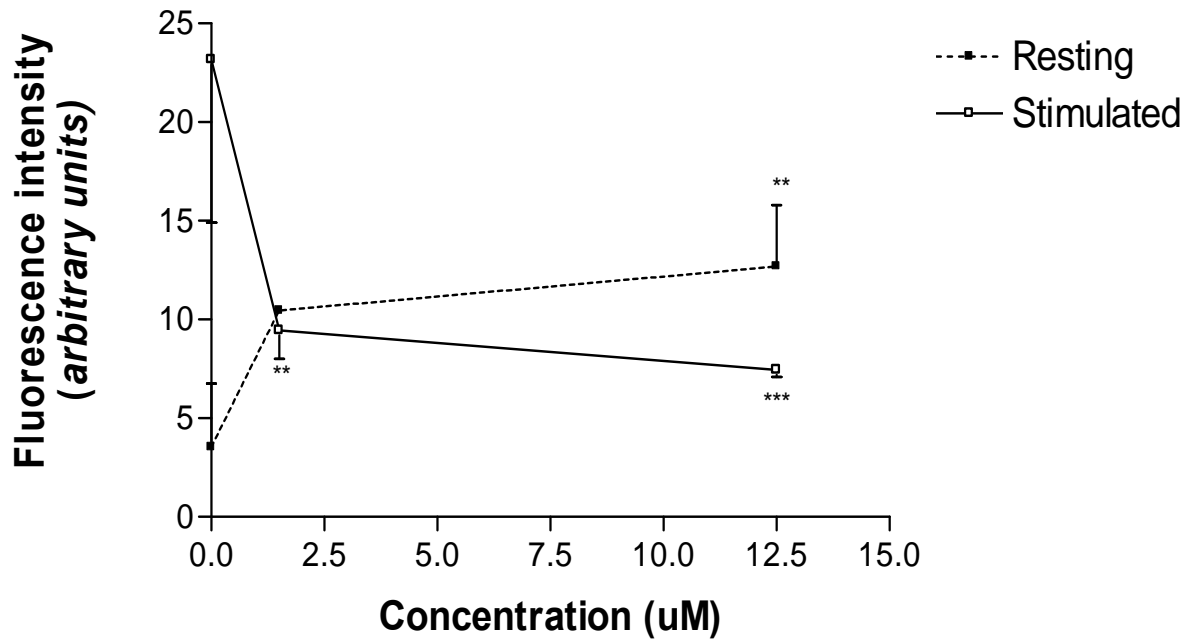
Figure 5.6: The effect of Auranofin on the expression of COX-2 by resting and LPS stimulated human monocytes. Monocytes were incubated in the presence of Auranofin (1.5 and 12.5 µM) for 30 minutes prior to the addition of LPS (1 µg/ml). After the addition of LPS, monocytes were incubated for a further 4 hours. Expression of COX-2 was determined on a flow cytometer using an Anti-Cox1 FITC/ Anti Cox-2 PE antibody. The results are representative of two different experiments using blood from two different donors. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=2.

*p<0.05

**p<0.01

***p<0.001
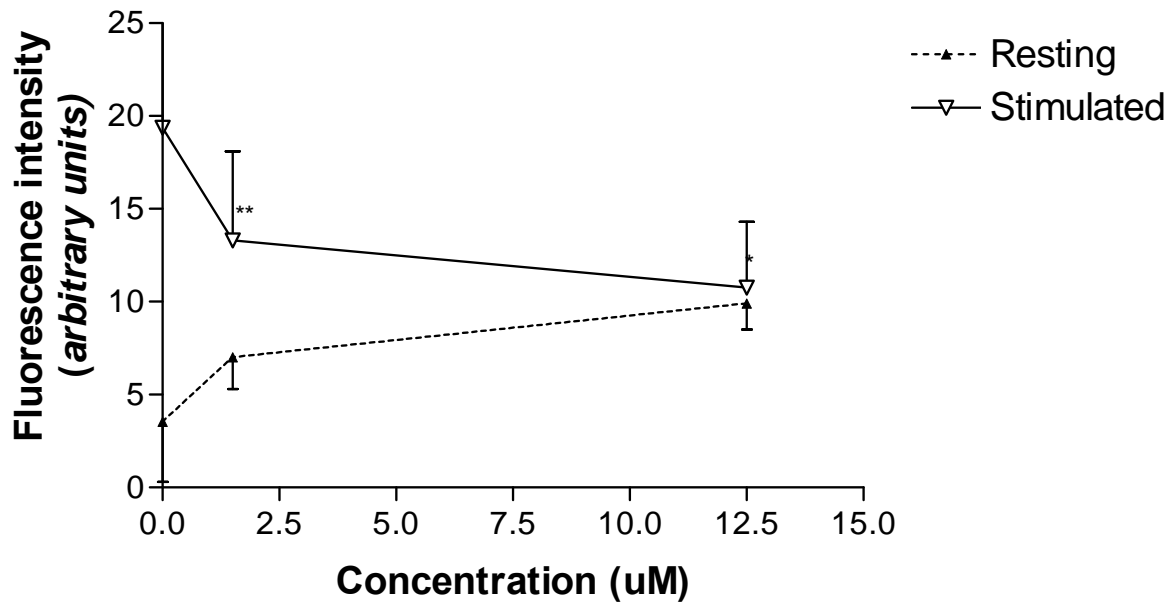
Figure 5.7: The effect of Asa-fin on the expression of COX-2 by resting and LPS stimulated human monocytes. Monocytes were incubated in the presence of Asa-fin (1.5 and 12.5 μM) for 30 minutes prior to the addition of LPS (1 μg/ml). After the addition of LPS, monocytes were incubated for a further 4 hours. Expression of COX-2 was determined on a flow cytometer using an Anti-Cox1 FITC/ Anti Cox-2 PE antibody. The results are representative of two different experiments using blood from two different donors. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=2.

   *$p < 0.05$

  **$p < 0.01$

***$p < 0.001$
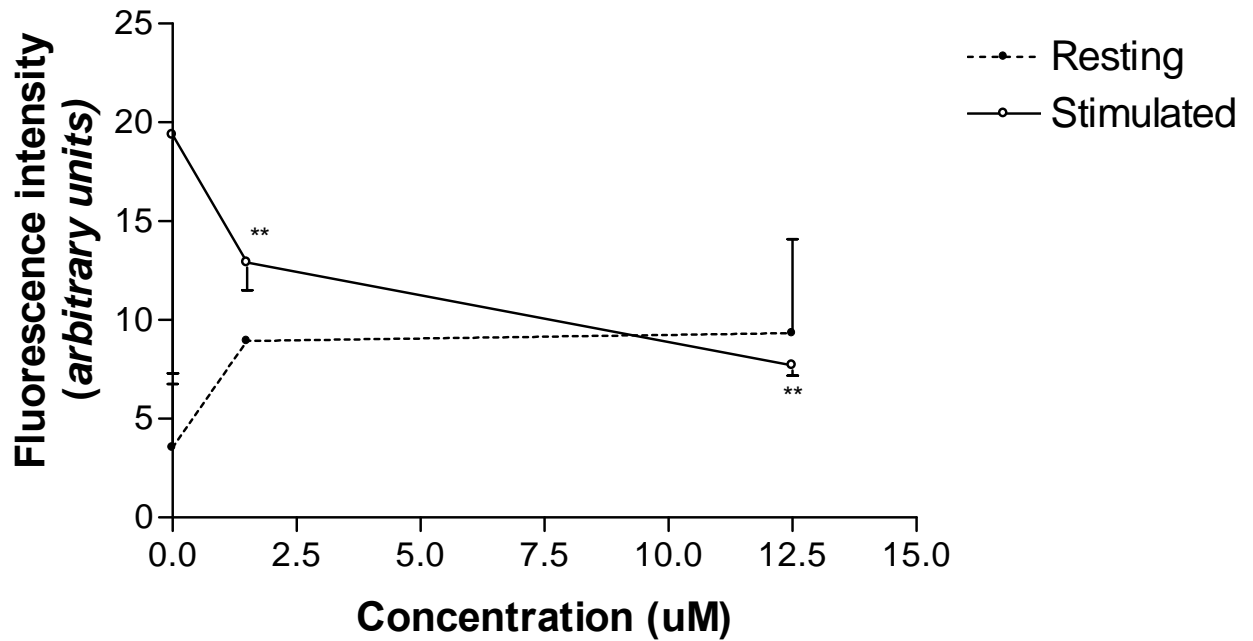
Figure 5.8: The effect of Mpta-fin on the expression of COX-2 by resting and LPS stimulated human monocytes. Monocytes were incubated in the presence of Mpta-fin (1.5 and 12.5 µM) for 30 minutes prior to the addition of LPS (1 µg/ml). After the addition of LPS, monocytes were incubated for a further 4 hours. Expression of COX-2 was determined on a flow cytometer using an Anti-Cox1 FITC/ Anti Cox-2 PE antibody. The results are representative of two different experiments using blood from two different donors. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=2.

 *p<0.05

 **p<0.01

***p<0.001
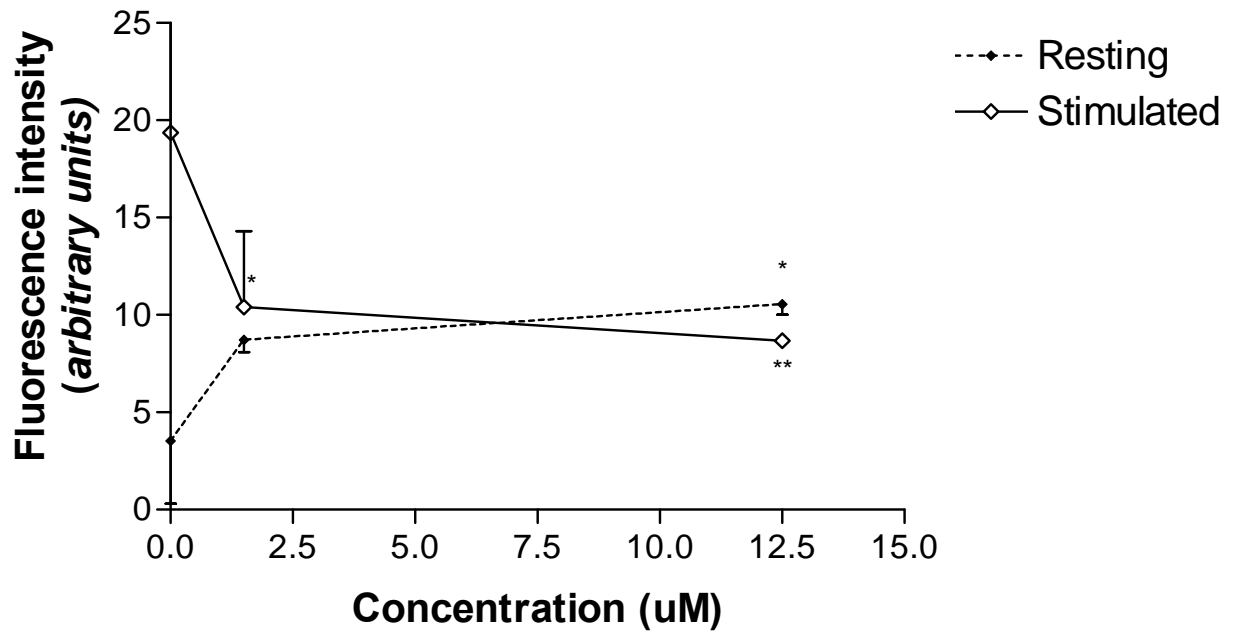
Figure 5.9: The effect of Pta-fin on the expression of COX-2 by resting and LPS stimulated human monocytes. Monocytes were incubated in the presence of Pta-fin (1.5 and 12.5 µM) for 30 minutes prior to the addition of LPS (1 µg/ml). After the addition of LPS, monocytes were incubated for a further 4 hours. Expression of COX-2 was determined on a flow cytometer using an Anti-Cox1 FITC/ Anti Cox-2 PE antibody. The results are representative of two different experiments using blood from two different donors. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=2.

  *p<0.05

  **p<0.01

***p<0.001

5.6. Discussion

In this study it was found that Auranofin, Asa-fin and Mpta-fin inhibited the expression of COX-2 in LPS stimulated human monocytes. This indicates yet another possible mechanism by which the compounds could act as anti inflammatory drugs.

COX-1 is the constitutive enzyme involved in the production of prostaglandins that protect the stomach and kidney from damage. Auranofin and the novel gold compounds did not show any effects on COX-1 expression in resting and LPS stimulated human monocytes. COX-2 is inducible by inflammatory stimuli such as cytokines and produces prostaglandins that contribute to pain and swelling of inflammation (Otto and Smith 1995; Vane and Botting 1998). Pain associated with inflammatory disorders is usually treated with non-steroidal anti-inflammatory drugs (NSAIDs), which produce their therapeutic effect by inhibiting COX enzymes.

In this experiment the effects of Auranofin and the novel derivatives on COX-1 and COX-2 protein expression was determined using flow cytometry. According to literature (Yamada, Niki et al. 1997; Yamada, Sano et al. 1999) auranofin inhibits the expression of COX-2 and the production of PGE_2. Auranofin and the novel compounds decreased the expression of COX-2 at concentrations as low as 1.5 µM (Figure 5.6- Figure 5.9, pages 54-57) but had no effect on the expression of COX-1. It could thus be concluded that the mechanism by which auranofin and the novel compounds decrease the production of PGE_2 is by decreasing the expression of COX-2. It is reported that auranofin inhibited the expression on interleukin 1β (IL-1β) and tumor necrosis factor α (TNF α) mRNA in mouse peritoneal macrophages stimulated by zymosan, LPS or by various intact bacteria (Bondeson 1997). Thus the mechanism by which auranofin and the novel compounds suppress the expression of COX-2 remains to be elucidated. The concentration of Auranofin in serum is estimated to be between 1.5 µM and 5.1 µM (Gottlieb 1982) in patients with rheumatoid arthritis and the tissue concentration of Auranofin has been shown to be relatively low as compared with that of gold sodium thiomalate (Finkelstein, Roisman et al. 1980; Blodgett 1983). It appears that at therapeutic concentrations auranofin, Asa-fin, Mpta-fin and Pta-fin decrease the expression of COX-2 in LPS stimulated monocytes and thus the production of PGE_2, but auranofin and Pta-fin showed an increase in COX-2 expression in resting cells.

This could lead to the postulation that the novel compounds and auranofin exert their activity better in patients who have ongoing inflammation or RA. Kean, Hart et al. (1997) however reported that the effects of intact auranofin are not completely identical to its *in vivo* effects because it dissociates in the plasma membrane of the digestive tract. Thus the *in vitro* results give us an idication of the effectiveness of the novel compounds but the true effect can only be determined *in vivo*.

CHAPTER 6
CHEMILUMINESCENCE AND FLOURESCENCE ASSAY
OF NEUTROPHIL RESPIRATORY BURST

6. CHEMILUMINESCENCE AND FLOURESCENCE ASSAYS OF NEUTROPHIL RESPIRATORY BURST

6.1 Introduction

Polymorphonuclear leukocytes (PMNL)

PMNL are important mediators of innate immunity and play an important part in the eradication of host bacterial and fungal infections (Malech and Gallin 1987; Bainton, Miller et al. 1997). They destroy microbial organisms by producing reactive oxygen species (ROS) (Casimir and Teahan 1994). Although ROS plays an important role in eradicating pathogens there are also several other important mediators such as chemokines, cytokines and chemotactic factors (including C5a and platelet-activating factor, PAF). PMNL are known to transfer molecular oxygen to the superoxide radical anion via a membrane enzyme system and coupled oxidation (Babior 1978). A further respiratory burst results in the formation of highly reactive oxidants such as hydrogen peroxide, the hydroxyl radical and singlet oxygen. The generation of ROS in the respiratory burst is mediated by the multicomponent membrane linked enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system (Dahlgren and Karlsson 1999). The NADPH oxidase system is dissociated and thus inactive in dormant neutrophils. Some components are membrane bound, while others are stored in the cytosol. When activated, the cytosolic components translocate to the plasma membrane to assemble the active oxidase (Stvrtinova, Jakubovsky et al. 1995; Abdalla 2001).

 The primary product of this oxidase system is one-electron reduced oxygen. i.e. superoxide anion. Superoxide also causes the damage of lipids, proteins and DNA (Lee-Ruff 1977). Elevated ROS levels trigger several transcription factors that mediate the secretion of many inflammatory cytokines (Barnes and Karin 1997). Much of the superoxide formed is dismuted by superoxide dismutase (SOD) to produce oxygen and hydrogen peroxide (H_2O_2), which is converted to hypochlorous acid and chloramines inside the phagosome (Babior 1984).

The antimicrobial activity of the neutrophil requires the delivery of the antimicrobial substance to the target cell. This can prevent colonisation of the host cell tissues or eradicate the target cell. Prior to adherence to the target micro-organism the phagocyte consumes oxygen and forms reactive oxygen intermediates upon stimulation with activation of the NADPH oxidase, a process called the respiratory burst. The highly reactive molecules produced by the NADPH oxidase (super oxide, hydrogen peroxide and nitric oxide) may also cause severe tissue damage and induce inflammatory responses (Abdalla 2001). Super oxide generated by the NADPH oxidase serves as the starting material for the production of a variety of ROS including oxidised halogens, free radicals and singlet oxygen. The oxidase can thus be receptor mediated or receptor independent mechanisms. In intact cells, it appears the activated oxidase undergoes a continuous process of activation and deactivation (Cohen and Lippard 2001).

The PMNL are the most populous of the circulating leukocytes but also the shortest-lived cells in circulation with a half life of 10 hours (Bainton 1980). They are produced and mature in the marrow and are released into circulation. Upon stimulation the PMNL migrate by diapedesis (a sequence of attachment to the luminal vascular endothelium followed by a rapid spreading and flattening of the cells onto the endothelium followed by squeezing through the endothelium into the interstitial space) into the tissue where the stimulus originated. They are produced in the bone marrow in response to acute stress irrespective of where the stimulus originates, whether from infection, trauma, noxious stimuli, emotional stress or infarction.

PMNL form the first line of defence against any acute microorganism's assault and are the first leukocytes to migrate into a lesion or region of infection. This migration is a well-coordinated chain of events involving chemotactic molecules (IL-8, PAF, LTB_4), adhesion molecules (β_2-integrins, ICAM-1, ICAM-2, the L-, P-, & E-selectins), the vascular endothelium and several cytokines (IL-1, IL-8, TNF-α, GM-CSF). Many different receptors and molecules are involved, some with multiple functions e.g. L-selectin and CR3 on the PMNL and P- and E-selectins on the vascular endothelium (Witko-Sarsat, Rieu et al. 2000). PMNL binding of endothelial P-selectin increases β_2-integrin mediated adhesion (via increased CR3 molecule expression) and also stimulates production of reactive oxygen species (ROS)

(Ruchaud-Sparagano, Walker et al. 2000). Almost all the chemotactic molecules, phagocytic stimuli, activated complement molecules (C5a) and several cytokines (TNF-α and GM-CSF) stimulate the expression of high levels of CR3 adhesion molecules by PMNL (Stewart and Hogg 1996).

Chemiluminescence assay

Chemiluminescence is the non-thermal release of light from specific molecules that are easily elevated into a higher energy state by chemical reactions, of which the oxidation reactions are the most common (Allen 1986). Analysis of many reactive compounds and biological reactions can be achieved by means of chemiluminescent assays, which is more sensitive (up to 1000x) and specific than spectrophotometric methodologies. Chemiluminescence can replace the use of radioisotopes in several common assays, and is faster, safer and less demanding on facilities and equipment. Elevated ROS levels trigger several transcription factors that mediate the secretion of many inflammatory cytokines and factors (Barnes and Karin 1997). Most ROS are only weakly or not at all luminescent, making the direct measurement of these compounds difficult. A convenient easy method that allows for kinetic analysis of oxidants is to add a labile photo-emitter such as luminol or lucigenin to the reaction mixture (Thorpe and Kricka 1986). Luminescence reagents are relatively stable and non-toxic. In biological assays, reasonable specificity can be achieved (Allen 1986). For this assay the method of Anderson, Goolam Mahomed et al. (1998) was used with slight modifications.

Flow cytometry assay

It may be misleading to depend on a single test to evaluate neutrophil function because various responses can be elicited by a single stimulus, and distinct transduction pathways may independently regulate these processes. Flow cytometry measures fluorescence and light scatter signals of suspended cells through a laser source. Gating techniques allow the analysis of structural and functional parameters in a large population of intact single cells, excluding those that are aggregated or lysed (Duque and Ward 1987; Carulli 1996). Bass and Parce et al. (1983) developed a method to measure the formation of oxidant products by flow cytometry using the dye 2', 7'-Dichloroflourescein diacetate (DCFH). In this method neutrophils are loaded with the DCFH which is transported across the plasma membrane. The polar non-

fluorescent DCFH are concentrated in the cytoplasm when the acetate groups are removed by esterases. During the oxidative burst, DCFH is converted to DCF through oxidation, mainly by hydrogen peroxide. The dye Dihydrorhodamine 123 (DHR) is used in a very similar way resulting in a red fluorescence (Emmendorffer, Hecht et al. 1990; Vowells, Sekhsaria et al. 1995). Strong non-physiologic stimuli such as PMA and calcium ionophores cause a sustained respiratory burst mirrored by high levels of fluorescence in this assay.

The use of flow cytometry to measure the production of hydrogen peroxide offers an easy semi-quantitative technique that is applicable to a few thousand unpurified cells. In contrast to chemiluminescence, the flow cytometric assay can be performed on whole blood samples and the neutrophil responses can be determined using gating techniques (Richardson, Ayliffe et al. 1998). Walrand, Valeix et al. (2003) have indicated that the dye 2', 7'-Dichloroflourescein diacetate (DCFH) is specific for detecting nitric oxide (NO), whereas the dye Dihydrorhodamine 123 (DHR) is specific for detecting hydrogen peroxide (H_2O_2) and the dye Hydroethidine (HE) is specific for detecting superoxide (SO). For this assay the method of Walrand, Valeix et al. (2003) and Ueda, Rieu et al. (1994) was used with slight modifications.

6.2.1 Chemiluminescence
6.2.1.1 Aim
The aim of the experiments in this section of the study was to evaluate the effects of the novel gold compounds Asa-fin, Mpta-fin and Pta-fin, compared to auranofin, on neutrophil oxidant production measured by chemiluminescence.

6.2.1.2 Experimental procedures
Chemiluminescence assays of neutrophil generated oxidants
PMNL were isolated and counted according to the method described in 3.3.1. Three millilitres of a neutrophil suspension at 1x 10^7 cells/ml was transferred to a clean test tube and diluted with an equal volume of 0.5 μg/ml luminol solution (Sigma Diagnostics, St Louis, MO, USA). This cell suspension was incubated on ice for half an hour.

The test was carried out in clear disposable plastic luminescence tubes. A blank, a negative control and a positive control were included in each experimental set. 600μl of the Hanks Balanced Salt Solution (HBSS) without phenol red but containing 10% FCS and HEPES buffer (used to adjust the pH to 7.4) was added to each tube in the test set and 200μl of the luminol cell suspension. These cuvettes were equilibrated at ambient temperature (22ºC) for 25 minutes before being loaded into a preheated (37ºC) BioOrbit 1251 Chemiluminometer carousel where they were incubated for 5 minutes.

The novel gold compounds were made up in HBSS of which 100μl were added to the cell suspensions before incubation at 37ºC. At the end of the 20-minute incubation period, a 100μl aliquot of the appropriate concentration of test compound per cuvette was added, monitoring was initiated and 60 seconds later the cells were stimulated by the addition of 100μl of 10μM FMLP introduced by automated dispenser and mixed. The release of reactive oxidants was seen as an increase in the chemiluminescence. Stimulation was monitored for 3 minutes.

The background tube contained all reagents but no cells, in the negative control the stimulant was replaced by 100μl HBSS and in the positive control the test compound was replaced by 100μl of HBSS.

6.2.1.3. Expression of results

Crude data obtained from chemiluminescence are illustrated graphically as representative graphs (Figure 6.1-6.4, pages 67-70). Only the peak stimulative values from chemiluminescence were used to determine hydrogen peroxide generation from neutrophils. Graphs are representative of four different experiments.

6.2.1.4 Results

The effect of auranofin, Asa-fin, Mpta-fin and Pta-fin on the respiratory burst of FMLP stimulated human neutrophils yielded results illustrated by representative graphs (Figure 6.1 – Figure 6.4, pages 67-70). The representative graphs clearly indicate that auranofin, Asa-fin and Mpta-fin have a biphasic activity on hydrogen peroxide release. Concentrations of 0.5μM and lower have a stimulatory effect on hydrogen peroxide production and concentrations of 0.5 μM and greater inhibit the

production of hydrogen peroxide from FMLP stimulated human neutrophils. Pta-fin did not influence the release of hydrogen peroxide at any of the concentrations tested.
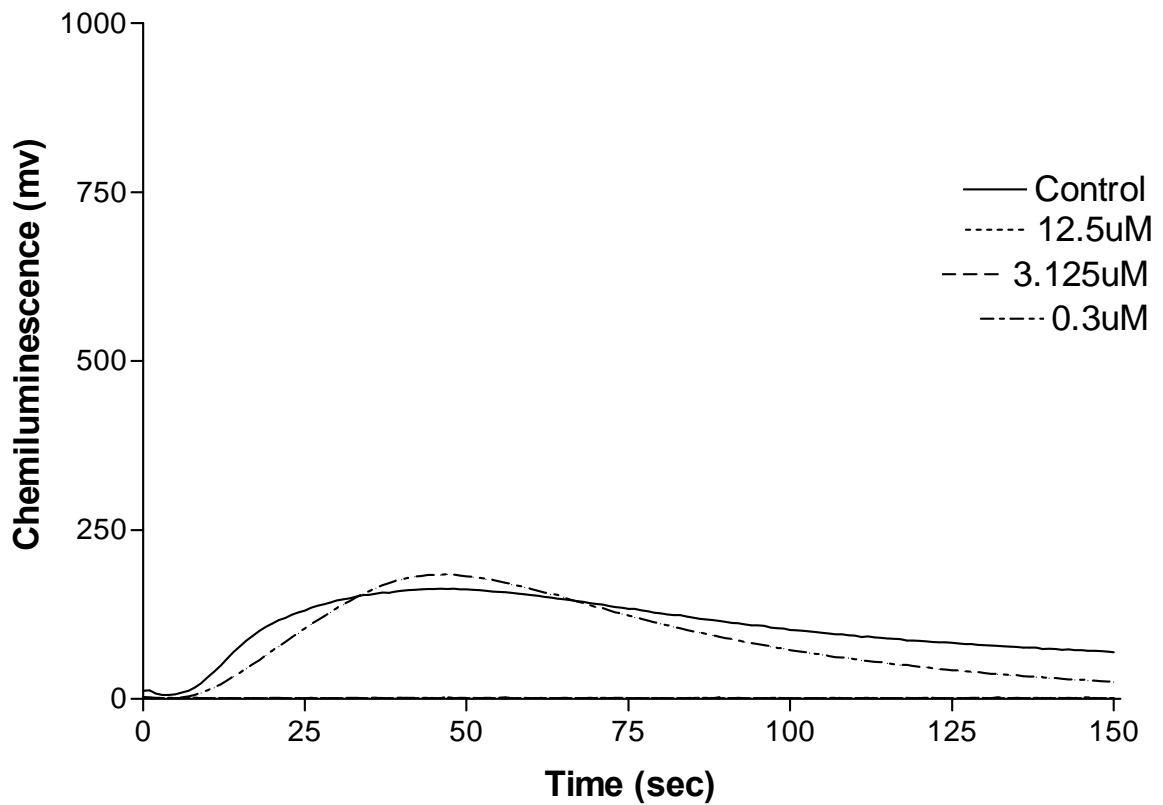
Figure 6.1: The effects of auranofin on luminol enhanced chemiluminescence response by FMLP stimulated human neutrophils. Neutrophils were treated with auranofin (0.3-12,5μM) for 45min. Results indicated are from a single representative experiment.
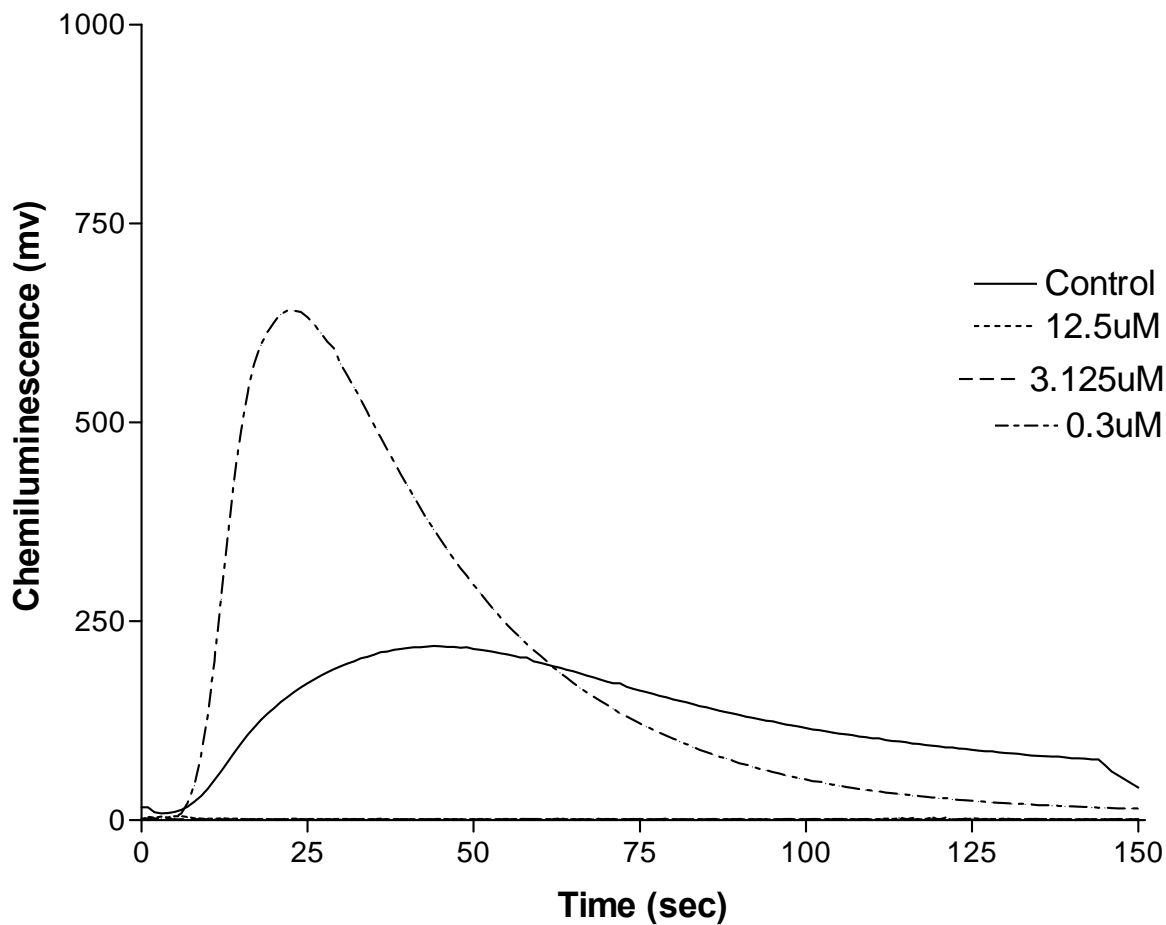
Figure 6.2: The effects of Asa-fin on luminol enhanced chemiluminescence response by FMLP stimulated human neutrophils. Neutrophils were treated with Asa-fin (0.3-12,5µM) for 45min. Results indicated are from a single representative experiment.
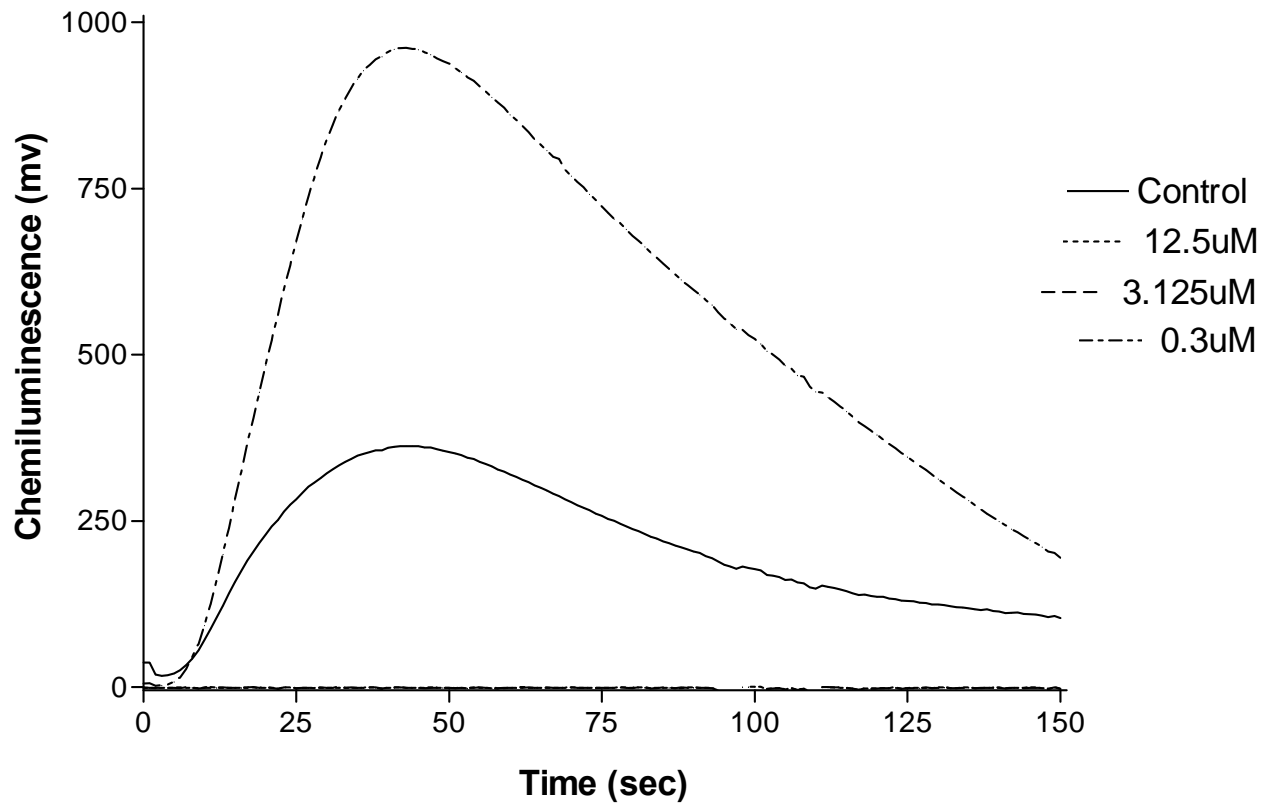
Figure 6.3: The effects of Mpta-fin on luminol enhanced chemiluminescence response by FMLP stimulated human neutrophils. Neutrophils were treated with Mpta-fin (0.3-12,5μM) for 45min. Results indicated are from a single representative experiment.
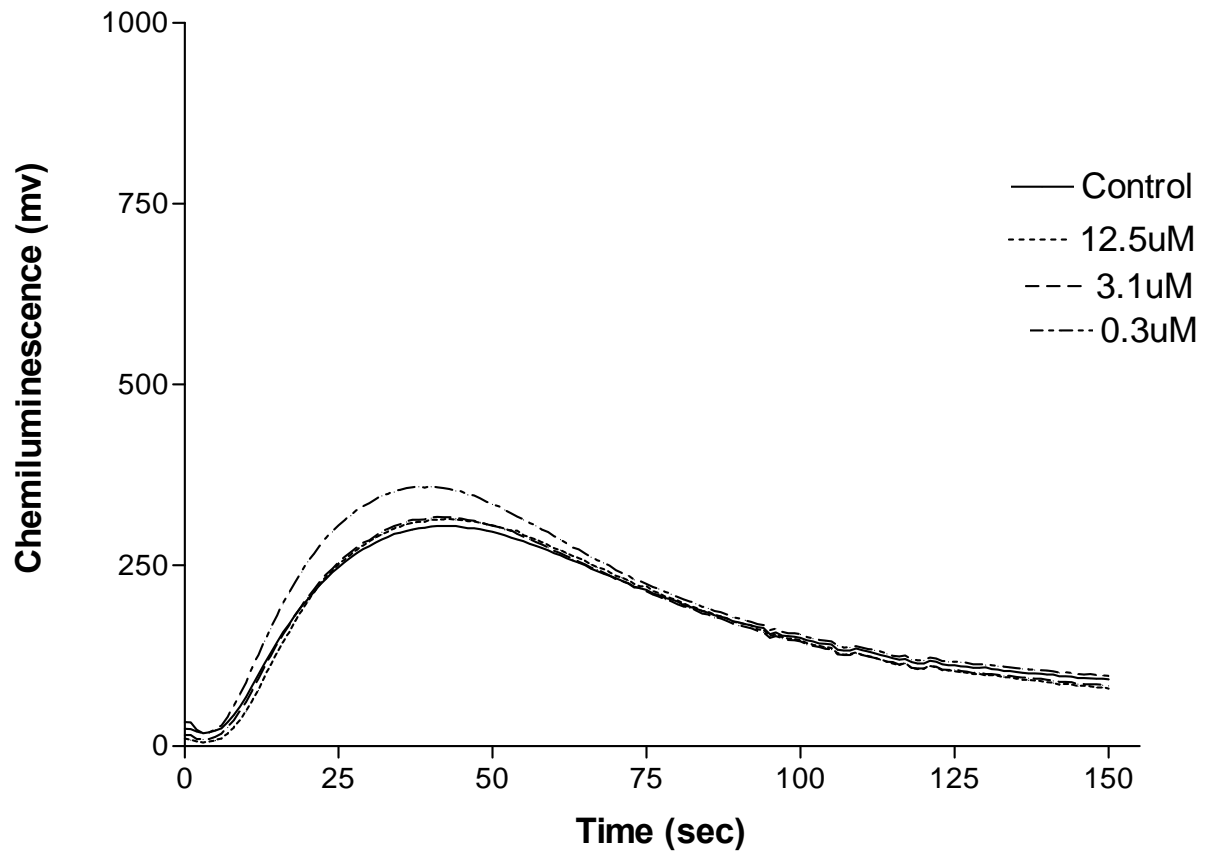
Figure 6.4: The effects of Pta-fin on luminol enhanced chemiluminescence response by FMLP stimulated human neutrophils. Neutrophils were treated with Pta-fin (0.3-12,5μM) for 45min. Results indicated are from a single representative experiment.

6.2.2 Flow cytometry

6.2.2.1 Aim

The aim of the experiment was to evaluate the effects of the novel gold compounds Asa-fin, Mpta-fin and Pta-fin, compared to auranofin on neutrophil oxidant production (superoxide, hydrogen peroxide and nitric oxide) measured by flow cytometry.

6.2.2.2. Experimental procedures

Whole blood was collected from healthy individuals in heparin tubes. 500 µl of whole blood was placed in 5 ml polypropylene tubes. Tubes were filled with 0.83% NH_4CL solution containing 0.1% EDTA, vortexed briefly and left on ice for 10 min to lyse the red blood cells. Tubes were centrifuged for 10 min at 500g at room temperature and the supernatant removed. Tubes were filled with RPMI+ and centrifuged for 10 min at 500g at room temperature and the supernatant removed and discarded. 80 µl RPMI+ and 20 µl of the relevant fluorescent probe (HE-Hydroethidine, DHR-2'7' dichloroflourescein diacetate or DCFH-Dihydrorhodamine 123) (Sigma Diagnostics, St Louis, MO, USA) was added to each tube and incubated for fifteen minutes on a shaker at room temperature. 50µl of dilutions of the relevant drug was added to the 15 ml tubes. Control tubes received 50µl of RPMI+. The cell suspensions were then incubated for 15 min in a 37º water bath and 50 µl PMA (1000ηg/ml) was added to the tubes earmarked as stimulated. Resting tubes received RPMI+ only. Tubes were then further incubated for 10 min in a 37ºC water bath. After incubation the tubes were placed on ice to stop the reaction. 100 µl of the cells from the corresponding tubes together with 500 µl sheath fluid were added to flow cytometry tubes and stored at 4ºC until ready to be analysed on a flow cytometer (analysis was done within 5 hours).

6.2.2.3. Expression and statistical analysis of results

Results are expressed as the mean % of untreated controls ± SEM (standard error measurement). ANOVA (analysis of variance) was used to analyse data. P values were used to compare experimental values with untreated control values.

6.2.2.4. Results

Intracellular super oxide production using hydroethidine (HE)

Auranofin and Asa-fin significantly ($p<0.05$) inhibited the intracellular super oxide production by PMA stimulated human neutrophils at a concentration of 12.5 µM. Low concentrations (0.7 µM and 3.5 µM) of Auranofin and Asa-fin exhibited no significant activity in inhibiting or stimulating superoxide produced by PMA stimulated human neutrophils (Figure 6.5, page 73). Mpta-fin and Pta-fin exhibited no significant effect on intracellular super oxide produced by PMA stimulated human neutrophils throughout the concentration ranges tested (Figure 6.5, page73).

Intracellular hydrogen peroxide (H_2O_2) production using dihydrorhodamine 123 (DHR)

High concentrations (12.5 µM) of Auranofin and Asa-fin significantly inhibited the intracellular H_2O_2 produced from PMA stimulated human neutrophils whereas lower concentrations (0.7 µM and 3.5 µM) had no significant effect on the intracellular H_2O_2 produced by PMA stimulated human neutrophils (Figure 6.6, page74). Mpta-fin and Pta- fin exhibited no significant effect on the internal H_2O_2 produced by PMA stimulated human neutrophils (Figure 6.6, page 74).

Intracellular nitric oxide (NOS) production using 2'7' dichloroflourescein diacetate

Throughout the concentration ranges tested (0.7 µM -12.5 µM), auranofin, Asa-fin, Mpta-fin and Pta-fin had no significant influence on the intracellular NO produced by PMA stimulated human neutrophils (Figure 6.7, page 75). The effect of auranofin, Asa-fin, Mpta-fin and Pta-fin on the respiratory burst of PMA stimulated human neutrophils yielded results illustrated by representative graphs (Figure 6.5 − Figure 6.7, pages 73-75).

The percentage of control values indicating the effect of auranofin, Asa-fin, Mpta-fin and Pta-fin at the concentration of 12.5 µM in the presence of either HE, DHR or DCFH are represented in Table 6.1 (page 76). The results indicate that both auranofin and Asa-fin significantly decreased the superoxide and hydrogen peroxide produced by PMA stimulated human neutrophils. Mpta-fin and Pta-fin had no significant effect on the different ROS produced intracellularly by PMA stimulated human neutrophils.
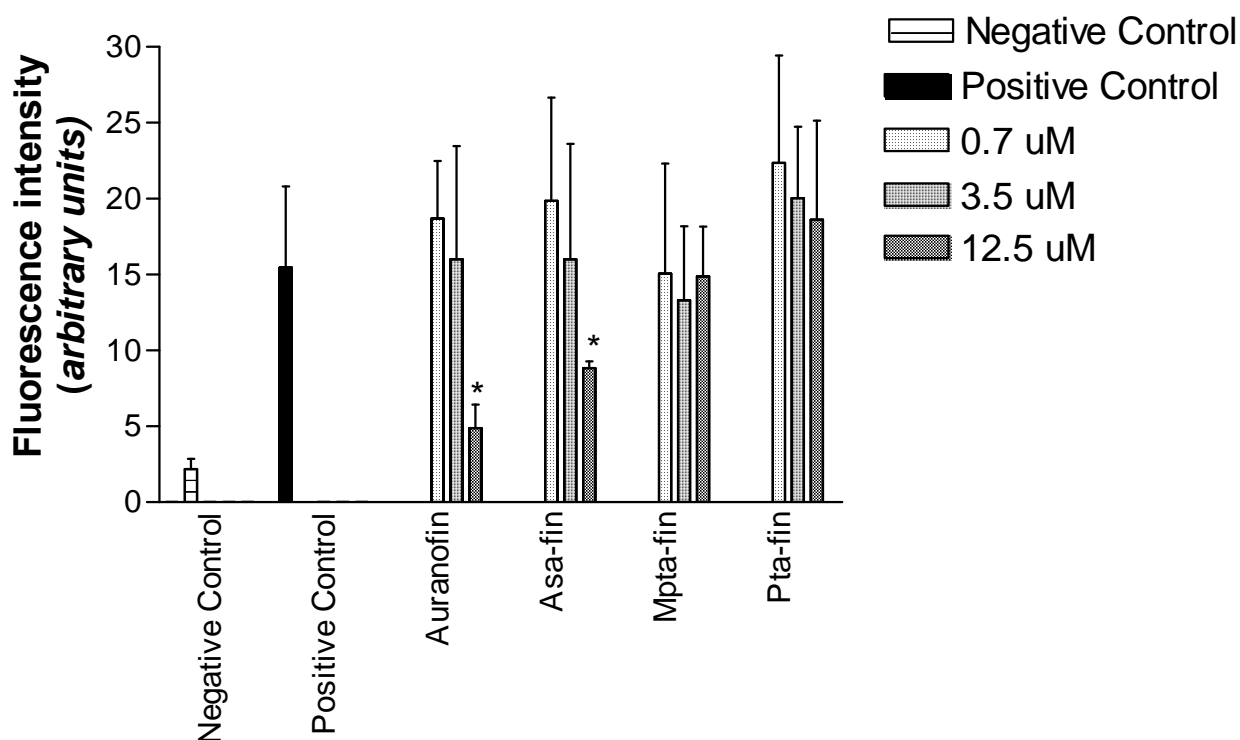
Figure 6.5: Fluorescence intensity of hydroethidine probes indicating the effect of the experimental compounds on superoxide production intracellularly by stimulated human neutrophils. Neutrophils were stimulated with 100 ng/ml PMA in the presence of various concentrations of auranofin, Asa-fin, Mpta-fin or Pta-fin. The results are representative of three different experiments using neutrophils isolated from three different donors. The negative control represents neutrophils treated with no stimulant and no drug compound. The positive control represents neutrophils treated with 100 ng/ml PMA but no drug compound. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=3.

*p<0.05
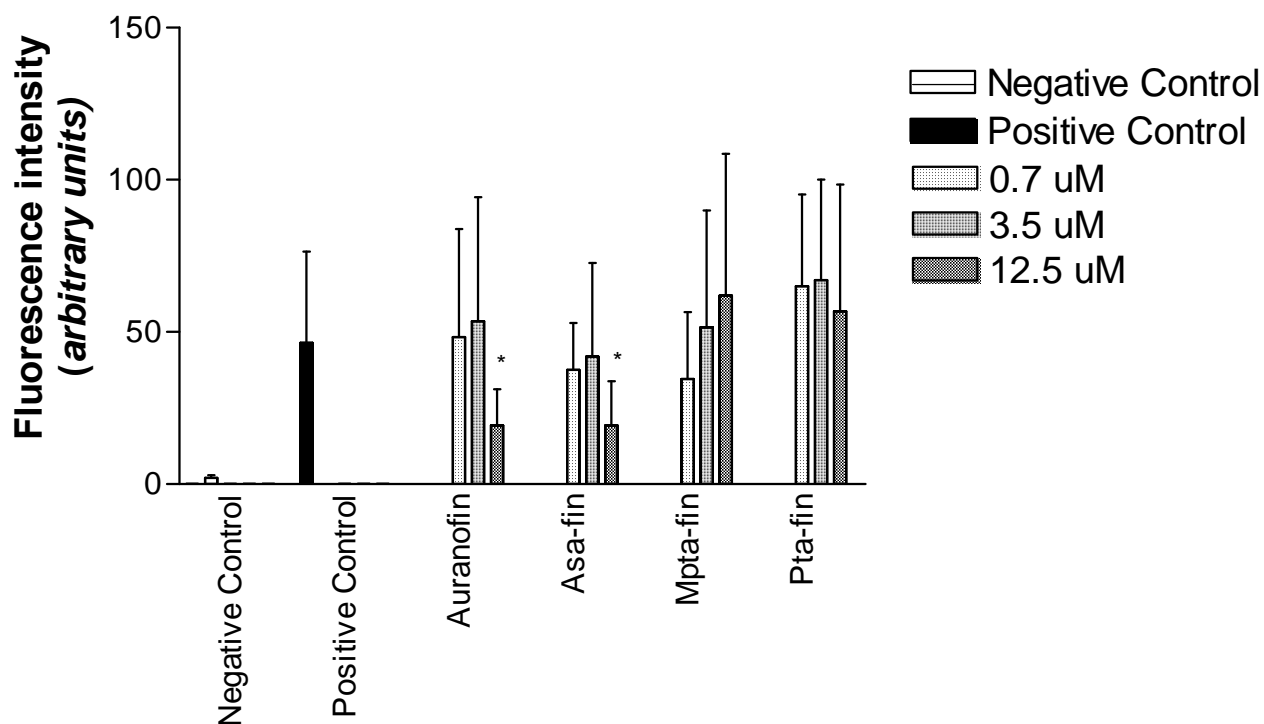
Figure 6.6: Fluorescence intensity of dihydrorhodamine 123 indicating the effect of the experimental compounds on hydrogen peroxide intracellularly by stimulated human neutrophils. Neutrophils were stimulated with 100 ng/ml PMA in the presence of various concentrations of Auranofin, Asa-fin, Mpta-fin or Pta-fin. The results are representative of three different experiments using neutrophils isolated from three different donors. The negative control represents neutrophils treated with no stimulant and no drug compound. The positive control represents neutrophils treated with 100 ng/ml PMA but no drug compound. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=3.

*p<0.05
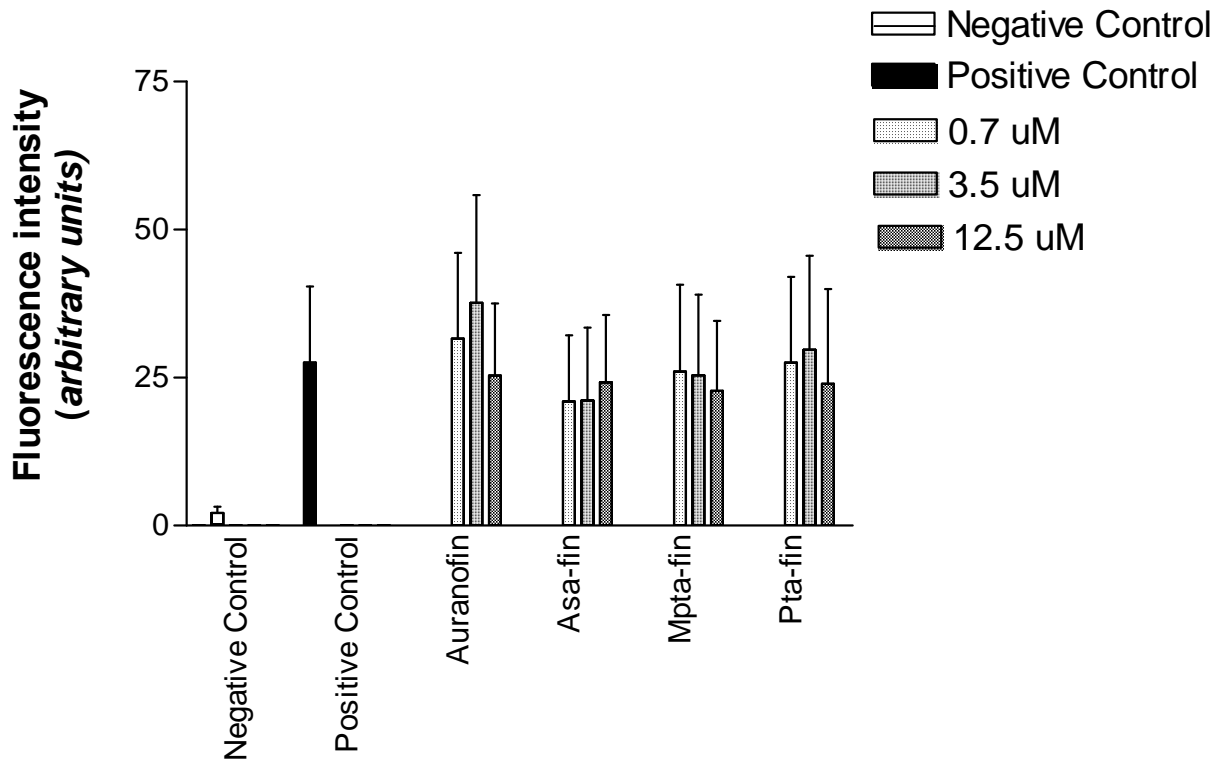
Figure 6.7: Fluorescence intensity of 2'7' dichloroflourescein diacetate indicating the effect of the experimental compounds on nitric oxide production intracellularly by stimulated human neutrophils. Neutrophils were stimulated with 100 ng/ml PMA in the presence of various concentrations of Auranofin, Asa-fin, Mpta-fin or Pta-fin. The negative control represents neutrophils treated with no stimulant and no drug compound. The positive control represents neutrophils treated with 100 ng/ml PMA but no drug compound. Results are representative of three different experiments using neutrophils isolated from three different donors. n=3.

Table 6.1: The effect of auranofin and the novel gold compounds Asa-fin, Mpta-fin and Pta-fin at a concentration of 12.5 μM on the intracellular release of various reactive oxygen species measured by a flow cytometric technique.

Reactive Oxygen Species (ROS)	Auranofin	Asa-fin	Mpta-fin	Pta-fin
Hydrogen Peroxide	41.55*	41.48*	133.20	122.22
Super Oxide	31.6*	45.74*	96.39	120.44
Nitric Oxide	91.95	87.89	82.65	86.98

Results are expressed as the percentage of treatment compound to untreated control. n=3.

*p<0.05

6.3. Discussion

Stimulation of neutrophil membranes generates superoxide by transferring electrons from NADPH inside the cell across the membrane and coupling these to oxygen to produce superoxide, which is a highly-reactive free-radical. Superoxide can form hydrogen peroxide spontaneously or be catalysed by the enzyme super oxide dismutase (SOD). Hydrogen peroxide released by phagocytes can be toxic to a number of targets *in vitro* and is one of the main toxic products released during the respiratory burst, thus perpetuating the inflammatory process (Tsan 1980). Heimburger, Lerner et al. (1998) reported that 1.5 µM auranofin hampered IL-1 induced hyper adhesiveness of human umbilical vein endothelial cells. IL-1 induces the expression of adhesion molecules on endothelial cells specific to neutrophils. Targeting these molecules could lead to an anti-inflammatory action. Mpta-fin and Pta-fin exhibited no effect on the intracellular ROS produced throughout the concentration range tested. Mpta-fin and Pta-fin may have an inhibitory effect at higher concentrations but those concentrations may not be attainable in an *in vivo* system.

To correlate the chemiluminescence data which measures extracellular ROS, different cell permeable probes, viz. HE, DHR and DCFH were used to determine intracellular ROS. DHR reacts directly with H_2O_2, DCFH is oxidised by reactive nitrogen species (RNS) and HE is most responsive to super oxide (Walrand, Valeix et al. 2003). According to literature, auranofin exerts a biphasic effect on superoxide production (Hurst, Gorjatschko et al. 1989; Liu, Akahoshi et al. 2000). It was shown that low concentrations (≤0.5 µM) of auranofin had an enhancing effect on superoxide production, whereas higher concentrations (>0.5 µM-10 µM) produced suppression.

Chemiluminescence results obtained from experiments carried out on human neutrophils, confirm previous studies that auranofin showed a biphasic activity. This biphasic activity was also noted with two of the three novel gold compounds, i.e. Asa-fin and Mpta-fin. At concentrations of 0.5 µM and lower, auranofin increased hydrogen peroxide production. Concentrations above 0.5 µM decreased hydrogen peroxide production (Figure 6.1, page 67). Similarly it was also noted that results obtained from Asa-fin and Mpta-fin followed a similar trend as auranofin whereas

concentrations lower than 0.5 µM of Asa-fin and Mpta-fin increased the luminescence signal indicating an increase in the production of hydrogen peroxide and concentrations of 0.5 µM and higher decreased the production of hydrogen peroxide (Figure 6.2 and Figure 6.3, pages 68 and 69). It is possible to speculate that the drugs may interfere with cellular interactions of neutrophils or may potentiate anti-inflammatory mechanisms. Pta-fin did not show any inhibitory effect on the production of hydrogen peroxide throughout the concentration range tested.

Flow cytometry results indicate that auranofin and Asa-fin inhibited intracellular super oxide and H_2O_2 production in the cell at 12.5 µM (Figure 6.5 and Figure 6.6, pages 73 and 74). All four compounds had no significant effect on the intracellular NO production (Figure 6.7, page 75). Although Yamashita, Niki et al. (1997) observed that auranofin inhibited NO production in a dose dependent manner from 0.3 µM in RAW 264.7 cells that were stimulated with LPS. The mechanism by which auranofin exhibited this activity was due to the inhibition of the expression of inducible NO synthase (NOS) protein. The ability of auranofin to inhibit the production of SO and H_2O_2 correlates with previous studies mentioned in the previous chapter. It has been reported by Minta, Williams et al. (1983) that auranofin inhibits superoxide production in human PMNL cells by the modulation of ligand-receptor coupling and nicotinamide-adenine dinucleotide phosphate oxidase activity.

In literature, the effects of auranofin on the functioning of phagocytic leucocytes have been investigated using a variety of techniques. The chemotactic responses of both monocytes and PMNLs were inhibited by auranofin in a dose dependent manner (Scheinberg, Finkelstein et al. 1979). The effect of gold compounds on the respiratory burst and superoxide production was also investigated. Auranofin, and to a much lesser extent gold thiols, was shown to suppress the respiratory burst as evidenced by a reduction in chemiluminescence (Davis, Miller et al. 1982). In more specific assay systems it has been shown that the generation of superoxide radicals were also inhibited in a dose dependent response in both PMNL and monocytes. This was again more potent with auranofin than with the gold thiols (Davis, Johnston et al. 1983). Miyachi, Yoshioka et al. (1987) showed that auranofin decreased the production of superoxide, hydrogen peroxide and the hydroxyl radical. It is interesting to note that auranofin and the novel compounds Asa-fin and Mpta-fin did not scavenge hydrogen

peroxide and are thus not anti-oxidants, but prevented the ROS from being formed. This has been supported by the Liu, Akahoshi et al. (2000) who reported that treatment of neutrophils with auranofin at a concentration of $5\mu M$ for 24 hours caused neutrophils to lose their capacity to generate superoxide in response to FMLP stimulation.

It is possible to speculate that auranofin and its derivatives may interfere with the cellular interactions of surviving neutrophils or may potentiate anti-inflammatory mechanisms by inhibiting adhesion molecules expressed through decreased ROS production. Through the variation of the method in detecting different ROS, it can be concluded that auranofin influences a variety of cellular functions and in this regard may well provide some important information on the mechanism by which the novel gold compounds inhibit the inflammatory process in rheumatoid arthritis. This activity of preventing ROS from being formed may indicate a different anti-inflammatory mechanism for auranofin and the novel gold compounds Asa-fin and Mpta-fin.

CHAPTER 7
PROSTAGLANDIN E2 (PGE$_2$) INHIBITION

7. PROSTAGLANDIN E$_2$ (PGE$_2$) INHIBITION

7.1 Introduction

In the pathway of prostaglandin synthesis, cyclooxygenase is the key enzyme that catalyzes the conversion of arachidonic acid to prostaglandin E$_2$ (PGE$_2$) via phospholipase enzymes (Figure 5.1, page 46). It is generally accepted that prostaglandin E$_2$ at the inflammatory sites are formed as a result of the production of cyclooxygenase-2. Another constitutive isoform, cyclooxygenase-1, is relevant to the production of prostaglandins that regulate normal cellular processes such as vascular homeostasis regulation, gastric mucosal protection and renal integrity maintenance (Smith, Meade et al. 1994; Vane, Bakhle et al. 1998). PGE$_2$ plays a major role in the angiogenesis of synovium by increasing the expression of vascular endothelial growth factor (Ben-Av, Crofford et al. 1995), synovial inflammation and the joint erosion in rheumatoid arthritis sufferers (Robinson, Tashjian et al. 1975). Cyclooxygenase-2 protein is elevated in the synovial tissue of patients with rheumatoid arthritis (Sano, Hla et al. 1992; Crofford, Wilder et al. 1994; Siegle, Klein et al. 1998), resulting in an increase in PGE$_2$ production (Levi and Shaw-Smith 1994). This thus indicates that a decrease in PGE$_2$ production would decrease inflammation.

Non-steroidal anti-inflammatory drugs (NSAID`s) decrease prostaglandin E2 production mainly by inhibiting cyclooxygenase activity (Vane 1971). The adverse effects of non-steroidal anti-inflammatory drugs are thus suggested to be due to the inhibition of cyclooxygenase-1 (Vane, Bakhle et al. 1998). Gold compounds, which are disease modifying anti-rheumatoid drugs, are used in the management of rheumatoid arthritis. With regards to its effect on arachidonic acid metabolism, previous studies have shown stimulatory effects (Peters-Golden and Shelly 1988); (Klaushofer, Hoffman et al. 1989; Bondeson and Sundler 1993), whereas others have observed inhibitory effects *;* (Yamada, Niki et al. 1997; Yamashita, Niki et al. 1997). To culture macrophages, a method of (Yamashita, Ichinowatari et al. 1999) was used with slight modifications.

7.2. Aim

The aim of the experiment was to evaluate the effects of the novel gold compounds Asa-fin, Mpta-fin and Pta-fin, compared to auranofin on prostaglandin E_2 synthesis.

7.3. Experimental procedures

Culturing of human macrophages

100 μM of whole blood was collected from healthy volunteers in blood bags containing heparin (used for the isolation of lymphocytes). 40 ml of whole blood was collected from the same individual in 8 ml tubes with no additives and allowed to clot for 10 min (used for autologous serum). After clotting, the tubes were centrifuged for 15 min at 1500rpm (460g) (Beckman TJ 6 centrifuge). The supernatant was collected. This was referred to as autologous serum. From the unclotted blood, the lymphocytes were isolated as explained in 3.3.1. RPMI 1640 used to culture the cells were then supplemented with 10 % autologous serum. 100 μl media supplemented with autologous serum was placed in each well in a 96 well plate and incubated at 37ºC in an atmosphere of 5% CO_2 for an hour.

The cell suspension was diluted to 5×10^5 cells (macrophages) per ml in sterile RPMI+ for the experiment and 100 μl of the cell suspension was added to each well. Cells were then incubated at 37ºC in an atmosphere of 5% CO_2 for 2 hours to allow monocytes to attach to the wells. After the incubation period, the non-adherent cells were removed by removing the supernatant with a pipette. Each well was washed twice with RPMI supplemented with autologous serum by adding 50 μl media, slightly shaking the plate and then removing the media with a pipette. 150 μl media supplemented with autologous serum was added to the adherent cells and allowed to incubate at 37ºC in an atmosphere of 5% CO_2 for 7 days. Media was replaced every 3 days. After the 7 day incubation period, the media from the wells were removed and 20 μl of the relevant concentration of drug and 160 μl of media supplemented with autologous serum was added to each well and incubated at 37ºC in an atmosphere of 5% CO_2 for 3 hours. The cultured monocyte derived macrophages were stimulated by adding 20 μl of a 1μg/ml (LPS) solution to the wells earmarked for stimulation. Control tubes received the supplemented media only. The plate was then incubated at 37ºC in an atmosphere of 5% CO_2 for 24 hours.

Measurement of PGE$_2$

PGE$_2$ was measured with a Prostaglandin E$_2$ Enzyme-Linked ImmunoSorbent Assay (ELISA) kit from Amersham Biosciences (Buckinghamshire, England). The test was carried out according to the manufacturer's instructions.

7.4. Expression and statistical analysis of results

Results are expressed as the mean % of untreated control systems ± SEM (standard error measurement). ANOVA (analysis of variance) was used to analyse data. P values were used to compare experimental values with untreated control values.

7.5. Results

The experimental compounds Asa-fin, Mpta-fin and Pta-fin as well as auranofin decreased the production of PGE2 in the stimulated human monocytes in a dose related manner (Figure 7.1, page 84).

There was a highly significant (p<0.001) decrease in the production of PGE$_2$ in LPS stimulated human monocytes treated with auranofin in a dose related manner. The decrease in the production of PGE$_2$ was noted throughout the tested concentration range of 1.5 µM – 25 µM auranofin.

Treatment of the cells with the novel gold compounds, Asa-fin, Mpta-fin and Pta-fin, significantly decreased (p<0.001) PGE$_2$ production in LPS stimulated human monocytes within the concentration range of 1.5 µM – 25 µM compared to the untreated control systems.
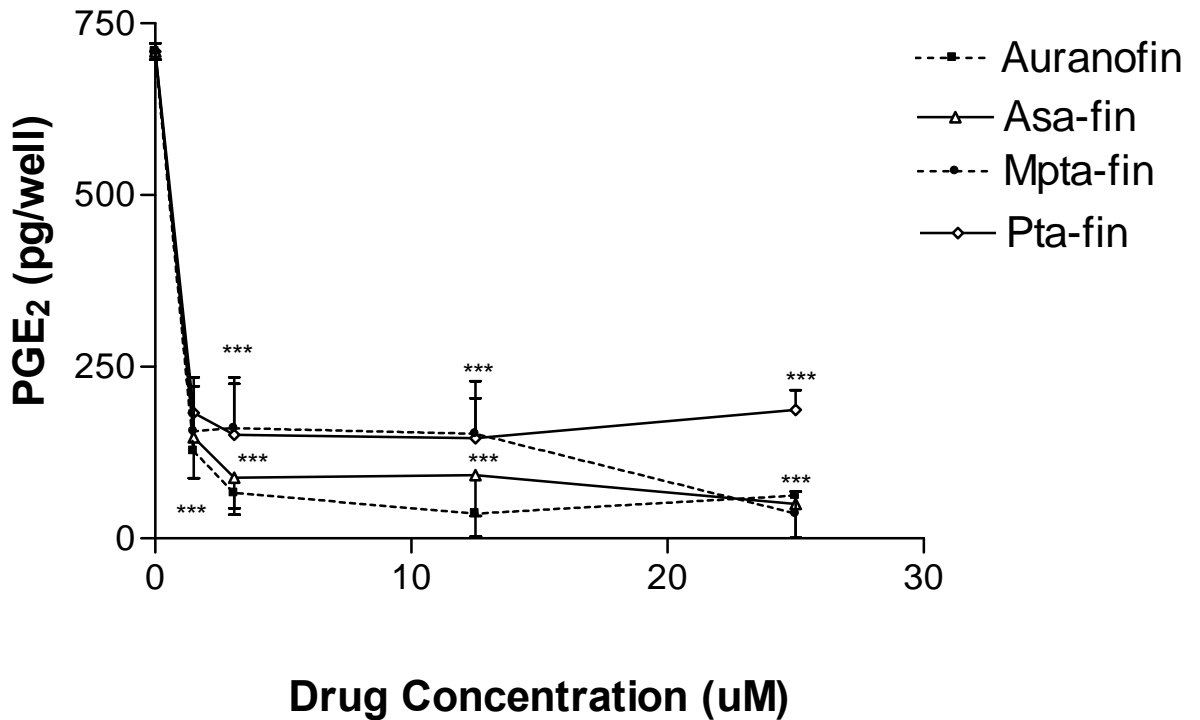
Figure 7.1: The effects of Auranofin, Asa-fin, Mpta-fin and Pta-fin on stimulated human macrophages. Monocytes were incubated for seven days and then exposed to the experimental compounds indicated for three hours before stimulating with LPS and incubating for twenty-four hours. PGE_2 production was detected by ELISA. The results are representative of three different experiments using macrophages isolated from three different donors. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=3.

***p<0.001

7.6. Discussion

In cultures of rat peritoneal macrophages, it was previously reported that Auranofin, at 1-10 μM, inhibited prostaglandin E_2 production induced by a protein kinase C activator, 12-O-tetradecanoylphorbol 13-acetate (TPA) as well as inhibiting the induction of cyclooxygenase-2 (COX 2) protein by lowering the level of COX-2 mRNA (Yamashita, Niki et al. 1997). PGE_2 is produced by macrophages stimulated by lipopolysaccharide (LPS) through the activation of COX-1 and COX-2 pathways. A quantitative analysis of PGE_2 levels in macrophages was a good marker of testing the anti-inflammatory activity of the novel compounds. Gold compounds were known to inhibit prostaglandin synthesis and this has been suggested previously as a possible mechanism of action of auranofin in rheumatoid arthritis (Gottlieb 1977; Bonta, Parnham et al. 1980; Lamprecht, Finkelstein et al. 1981). Lamprecht, Finkelstein et al. (1981) demonstrated that auranofin inhibits prostaglandin activity and not the synthesis thereof. In contrast, (Yamada, Sano et al. 1999) showed that the inhibitory effect of auranofin on PGE_2 synthesis was detected at concentrations of 0.1 μM and higher.

Auranofin, Asa-fin and Mpta-fin decreased the amount of Prostaglandin E_2 produced. Auranofin and the novel gold compounds Asa-fin, Mpta-fin and Pta-fin decreased PGE_2 production at concentrations of 1.5μM (Figure 7.1, page 84). The statistical difference in the PGE_2 concentrations obtained after treatment with auranofin and the novel gold compounds at 3.1 μM ($p < 0.05$) indicate that, although all of the compounds decreased the PGE_2 production, auranofin was superior to the rest of the experimental compounds. Auranofin at the concentration of 3.1 μM differed significantly ($p < 0.05$) in decreasing the amount of PGE_2 produced compared to Pta-fin. These results provide yet another possible mechanism by which auranofin and the novel gold compounds could exert their anti-inflammatory activity.

CHAPTER 8
HUMAN INFLAMMATORY CYTOKINE ASSAY

8. HUMAN INFLAMMATORY CYTOKINE ASSAY: (CYTOMETRIC BEAD ARRAY) (CBA)

8.1 Introduction

Flow cytometry is an analysis tool that allows for the discrimination of different particles on the bases of size and color. The CBA system uses the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. The CBA capture bead suspension allows for the detection of multiple analytes in a small volume sample.

The CBA human inflammation kit was used to quantitatively measure interleukin-8 (IL-8), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor alpha (TNF-α) and interleukin-12p70 (IL-12p70) protein levels in a single sample. In patients with RA, macrophage and fibroblast-like cells in the synovium are highly activated based on their morphology (Kingsley, Pitzalis et al. 1990) and cytokine profile (Firestein and Zvaifler 1990). Products of these cells, such as IL-1, TNF-α, IL-6 and granulocyte macrophage stimulating factor (GM-CSF) are abundant in synovial fluid and tissues (Firestein and Zvaifler 1990). It has been shown *in vivo*, that DMARDS lower levels of acute phase reactants (Sanz and Alboukrak, 1991). Recent strategies of drug development have focussed on neutralising cytokines that are present in inflamed joints. These cytokines reportedly mediate joint inflammation and destruction in RA, and include TNF-α, IL-8 and IL-6 (Keystone 2001).

Bacterial lipopolysaccharide (LPS) and IL-1β induce COX-2 upregulation in macrophages whereas the Th 2 associated cytokines (IL-4, IL-10 and IL-13) suppress this induction (Berg, Zhang et al. 2001). The protein mediators include amongst others the cytokines, chemokines, growth factors, interferons, adhesion molecules and tumour necrosis factors. Cytokines are expressed by myeloid and lymphoid cells and are ligands for receptors on other cells that are involved in the active immune response. The result is often a cytokine cascade where one cytokine initiates the expression of another that again stimulates a third cytokine to be expressed. An example is the LPS induced TNF-α that causes the sequential cascade of IL-1 and IL-6 (Beutler and Cerami 1987), which are both pro-inflammatory cytokines. Cytokines

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are generally stimulants of effects that take effect at gene expression level such as cell proliferation, differentiation, maturation or activation, whereas other cytokines inhibit these same effects. Cytokine production is regulated by inducing stimuli at gene transcription level with transient production and short radii of action.

The interleukins are a subset of 18 cytokines that are produced mainly by leukocytes and that act primarily on other leukocytes. Their functions are diverse, but mostly stimulatory for inflammatory processes (IL-2, IL-12, TNF-α, IL-6) or cell maturation. Some interleukins are anti-inflammatory (IL-1β, IL-4, IL-10, and IL-13) (Smith 1993; Colotta et al. 1994; Mosmann 1994; Levesque and Haynes 1997; Ryan 1997) and usually work in coherently with other interleukins or cytokines.

Stimulated macrophages release pro-inflammatory cytokines and lipid inflammation mediators as well as express MHC class II molecules, all of which can condition dendritic cells or enable presentation of antigen directly (MacMicking et al. 1997).

8.2. Aim

The aim of the experiment was to compare the effects of the novel gold compounds Asa-fin, Mpta-fin and Pta-fin, to auranofin, on the expression of IL-8, IL-1β, IL-6, IL-10, TNFα and IL-12p70.

8.3. Experimental procedures

Lymphocytes were isolated according to 3.3.1 (page 19) and treated with the experimental compounds according to the relevant concentrations for 36 hours in a 37ºC, CO_2 (95%) incubator. After the 36 hour incubation with the experimental compounds, lymphocytes were centrifuged at 810g for 10 minutes. The supernatants were collected and kept in a -70ºC freezer until analysed on a BD FACS array flow cytometer.

Measurement of inflammatory cytokines

Inflammatory cytokines were measured with a CBA human inflammation kit from Becton Dickinson, (San Jose, CA. USA). The test was carried out according to the manufacturer's instructions and analysed on a BD FACS array flow cytometer.

8.4. Expression and statistical analysis of results

Results are expressed as the means ± SEM (standard error of the mean). ANOVA (analysis of variance) was used to analyse data. P values were used to compare experimental values with untreated control values.

8.5. Results

Evaluating the effect of the novel gold compounds compared to auranofin on various inflammatory cytokines showed that the gold compounds auranofin, Asa-fin and Mpta-fin had no significant effect on the production of the cytokines IL-12p70, IL-6 and IL-8 in resting and PHA stimulated cells (Table 8.1 and 8.2, pages 90 and 91). Upon treatment with 0.7 µM auranofin, PHA stimulated cells produced a significantly ($p < 0.01$) lower level of IL-10. Asa-fin at 0.7 µM also decreased the production of IL-10 significantly ($p < 0.05$) in PHA stimulated cells. Auranofin at 0.2 µM and Asa-fin significantly increased the production of TNF-α in PHA stimulated cells with a significance of $p < 0.01$ and $p < 0.05$ respectively. PHA stimulated lymphocytes treated with Asa-fin 0.7 µM produced significantly ($p < 0.01$) more IL-1β. Mpta-fin and Pta-fin had no significant effect on the production of IL-10, TNF-α and IL-1β in resting and PHA stimulated cells (Table 8.1 and 8.2).

Table 8.1: The effect of auranofin and the novel gold compounds Asa-fin, Mpta-fin and Pta-fin on inflammatory cytokines in resting lymphocytes. n=3.

Compound	Concentration	IL-12p70 pg/ml	TNF-α pg/ml	IL-10 pg/ml	IL-6 pg/ml	IL-1β pg/ml	IL-8 pg/ml
		Means ± SEM	Means ± SEM	Means ± SEM	Means ± SEM	Means ± SEM	Means ± SEM
Control		242±45	158±45	169±55	974±585	340±67	46224±16160
Auranofin	0.7 μM	209±67	152±34	101±35	1913 ±1096	283±90	832629 ±406049
Auranofin	0.2 μM	224±22	2059 ±1760	101±16	14212 ±9653	1204 ±948	253850 ±27428
Asa-fin	0.7 μM	511±219	292±60	205±93	3955 ±1764	443±175	240128 ±55926
Asa-fin	0.2 μM	420±29	222±20	140±18	2884 ±998	263±28	93934±21687
Mpta-fin	0.7 μM	239±2	206±12	138±9	1569 ±860	231±29	53085±4523
Pta-fin	0.2 μM	343±19	235±20	180±586	1595 ±350	1936±15	62017±22164

Results are expressed as the means± standard error.

Table 8.2: The effect of auranofin and the novel gold compounds Asa-fin, Mpta-fin and Pta-fin on inflammatory cytokines in PHA stimulated lymphocytes.

Compound	Concentration	IL-12p70 pg/ml	TNF-α pg/ml	IL-10 pg/ml	IL-6 pg/ml	IL-1β pg/ml	IL-8 pg/ml
		Means ± SEM	Means ± SEM	Means ± SEM	Means ± SEM	Means ± SEM	Means ± SEM
Control		179±16	3228±65	1957 ±485	71338 ±19383	2003±274	121873 ±500661
Auranofin	0.7 µM	306±47	20037 ±8049	170±11**	66527 ±28434	10246 ±3526	1402724 ±632327
Auranofin	0.2 µM	382 ±102	39198 ±10151*	936±285	86857 ±10594	24968 ±5913	323936 ±29804
Asa-fin	0.7 µM	326±29	43997 ±20263**	330±162*	67939 ±29512	42054 ±20038**	246125 ±94868
Asa-fin	0.2 µM	347±63	6420 ±1563	1638 ±341	92943 ±4509	2940±630	292633 ±18240
Mpta-fin	0.7 µM	388±87	5964 ±282	1635±11	81358 ±13447	1936±104	263917 ±21179
Pta-fin	0.2 µM	332±36	3647 ±618	1433 ±586	66943 ±10672	266.3±300	236972 ±28160

Results are expressed as the means± standard error. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=3.

*p<0.05

**p<0.01

8.6. Discussion

Several cytokines are produced by PMNL, which appear to play an autocrine regulatory role on PMNLs. The main cytokines expressed by neutrophils are IL-8, a powerful PMNL chemotactic agent and inducer of degranulation, and TNF-α, an adhesion promoting and ROS inducing agent associated with inflammation (Cassatella 1999). Elevated ROS levels are in turn suspected triggers of several transcription factors such as AP1 and NF-κB (nuclear factor kappa B) (Byun, Jeon et al. 2002; Li and Stark 2002) that mediate the secretion of many inflammatory cytokines (Barnes and Karin 1997). Anti-inflammatory cytokines (IL-4, IL-10 and IL-13) appear to inhibit the ROS released by neutrophils. This cytokine expression and control might open new avenues for drug targeting in chronic inflammatory conditions (Witko-Sarsat, Rieu et al. 2000). Gold compounds are reported to inhibit the binding of NF-κB to DNA (Yang, Merin et al. 1995) and NF-κB stimulates the production of interferon-β, TNF-α, IL-2, IL-6 and IL-8. Bondeson and Sundler (1993) have also reported that auranofin inhibits the induction of IL-1β and TNF-α mRNA in mouse macrophages. IL-8 is a neutrophil chemokine and up-regulator of membrane adhesion receptors in neutrophils (Baggiolini, Walz et al. 1989) and the IL-8 gene was reported to co-operatively regulate both NF-κB and transcription factors (Kunsh and Rosen 1993).

It has been shown in this study that auranofin at 0.7μM decreased IL-10 but increased TNF-α production in stimulated cells. Asa-fin at 0.7 μM increased IL-1β and TNF-α production but decreased IL-10 production in stimulated cells. Mpta-fin and Pta-fin did not show any activity against the cytokines at the tested concentrations. Stern, Wataha et al. (2005) reported that monocytes stimulated with LPS and treated with auranofin did not positively or negatively modulate TNF-α and IL-10 secretion but enhanced the IL-6 and IL-8 secretion in LPS induced monocytes

There are many reports on the production or release of IL-1β and TNF-α but many of the results are contradictory. Danis, Kulesz et al. (1989) showed that auranofin had a biphasic effect on LPS induced IL-1β production. Schmidt and Abdulla (1988) showed that auranofin inhibited LPS induced IL-1β production. Evans and Zuckerman (1989) indicated that auranofin (0.15 μM-1.5 μM) inhibited LPS induced

formation of TNF-α in mouse macrophages and human monocytes. This was confirmed by Danis, Franic et al. (1991) who showed that in human monocytes, auranofin inhibited LPS induced IL-1α, IL-1β and TNF-α. Collectively, the data suggest that the responses to gold compounds are not simply immunosuppressive or immunostimulative, but involve complex responses that depend on the type of drug tested, the time of exposure and the concentration of the stimulant used. Further tests may be warranted with different cells, stimulants and drug concentrations to determine conclusively the effect of the novel gold compounds on the production of inflammatory cytokines.

CHAPTER 9
TOXICITY PROFILES IN MICE

9. TOXICITY PROFILES IN MICE

9.1. Introduction

In order to assess the safety of the new treatment before it is administered, tested or made commercially available, animal toxicity testing is necessary according to the regulations of regulatory bodies such as the Medicines Control Council (MCC).

The toxicity of Auranofin has already been determined in Carworth Farm Swiss mice, Charles River rats (Saunders 1983), and dogs (Payne and Arena 1978). Payne and Arena (1978) noted dose related changes in rats, including weight loss, increased salivation, soft faeces, anaemia, and leucocytosis. The haematological abnormalities were believed to be secondary to auranofin- induced gastrointestinal lesions, rather than direct effects on bone marrow. The acute toxicity of auranofin evaluated in mice and rats showed median lethal oral doses of 310 and 265mg/kg respectively. Dogs administered with auranofin 1.8-18 mg/kg/day for 3 months exhibited dose related emesis, diarrhoea, and decreased food consumption and body weight (BW). In high dose groups, decreases in total serum protein and albumin, hyperplasia of thyroid tissues, a sub clinical anaemia with normal bone marrow response was noted (Payne and Arena 1978).

9.2. Aim

The purpose of the toxicity testing was to evaluate and compare the toxicity profiles of auranofin to the novel gold compounds Asa-fin, Mpta-fin and Pta-fin in Balb/C inbred mice.

9.3. Experimental procedures

Experimental animals

Inbred Balb/C mice of 6-8 weeks were used. Animals were housed in groups of three to four in standard mouse cages in rooms with controlled environmental conditions. The animals were fed pellets (EPOL) and water *ad libitum*.

Dosage, route of administration and sample size

Auranofin, Asa-fin, Mpta-fin, and Pta-fin were dissolved in 0.5% gum tragacanth solution and administered at doses of 1ml/100g BW by gavage. The dosages were prepared immediately prior to each oral gavage.

Duration of study:

Each group of mice were gavaged each day for 5 days.

For phase 1, the water/tragacanth solution and the first and second dosages of 18 and 23mg/kg BW respectively were administered each day from day 1 to 5.

Phase 1:

Phase 1 was done over 5 days. 100 mice (10 groups/10 mice per group) were used during the first phase. When no adverse effects were observed after 5 days, phase 2 was followed.

Phase 2:

Phase 2 was carried out over 5 days. A maximum of 100 mice (10 groups/10mice per group) were used during the second phase. In phase 2, the tragacanth solution and the third and fourth dosages i.e. 28mg/kg BW and 33mg/kg BW of drug were administered each day from day 6 to 10. The dosage volume indicates that 1ml of solution was administered for every 100g body weight.

The duration of the study was thus 10 days. Any animal that showed signs of pain and distress were euthanised immediately with CO_2 overdose. If 2 animals in a group showed these signs, the whole group would be euthanised and the procedure repeated with the next (lower) dose level. Directly after termination of the mice, a member of the UPBRC drew maximum blood from them, via cardiac puncture and blood analysis was done.

Evaluation of pain and distress:

The specific side effects observed with auranofin are loose stools. With dosages of >20 mg auranofin /kg BW for 2 weeks or more, an increased muscle tone was observed and animals became easily irritated when handled (Glennas 1985). Animals were monitored for any signs of discomfort or stress. Toxicity would be indicated if adverse effects were observed on the experimental animals or with elevated liver enzymes. Blood was drawn and the clotted blood analysed immediately. The Department of Clinical Pathology at the Faculty of Veterinary Science analyzed

standard liver enzymes and kidney function. The liver enzymes tested were: aspartate transaminase (AST) and gamma glutamine transferase (GGT). Kidney function tests included the determination of urea and creatinine.

9.4. Expression and statistical analysis of results

Results are expressed as the mean % of untreated controls ± SEM. ANOVA was used to analyse data. P values were used to compare experimental values with untreated control values.

9.5. Results

After the administration of the treatment group with a dosage of 18 mg/kg and 23 mg/kg in the auranofin, Asa-fin, Mpta-fin and Pta-fin groups, no abnormalities were detected and the mice were terminated at the end of the 5 days. With the analysis of the data it was noted that the group treated with 18 mg/kg auranofin did not have any significant increase in the AST levels but the GGT levels were significantly ($p<0.001$) increased. This increase was not noted in the GGT levels of the group treated with 23 mg/kg auranofin. The second phase of the study was thus carried out with drug dosages of 28 mg/kg and 33 mg/kg. It was noted that one mouse died in the group treated with 33 mg/kg Asa-fin and there was a noted decrease in activity in the animals in this group. No abnormalities were detected in the group gavaged with 28 mg/kg Asa-fin. After the termination of the animals at the end of the 5 days, the analysis of the blood revealed a significant ($p<0.001$) increase in the AST levels of the group treated with the 33 mg/kg Asa-fin. There were no other statistically significant changes in the AST, GGT, urea and creatinine levels according to the pathology reports of the blood.
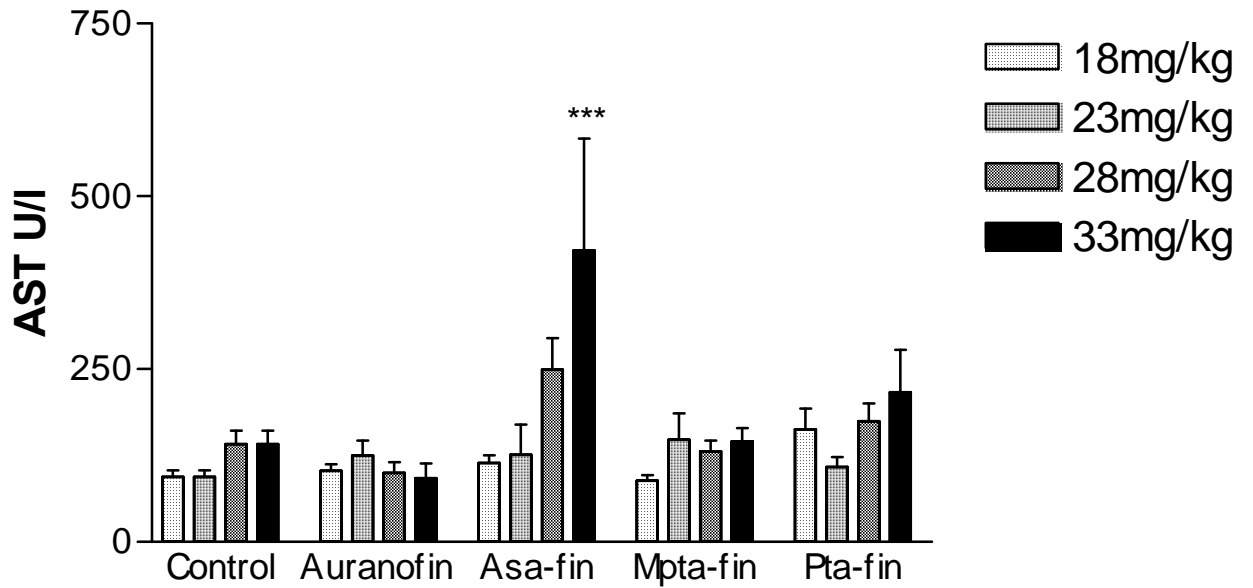
Figure 9.1: The effects of Auranofin, Asa-fin and Mpta-fin on AST levels in Balb/c mice. Mice were gavaged with different dosages of the various drugs for five days. Animals were sacrificed on day five via cardiac puncture and the blood was analysed for changes in the liver enzyme AST. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=10.

***p<0.001
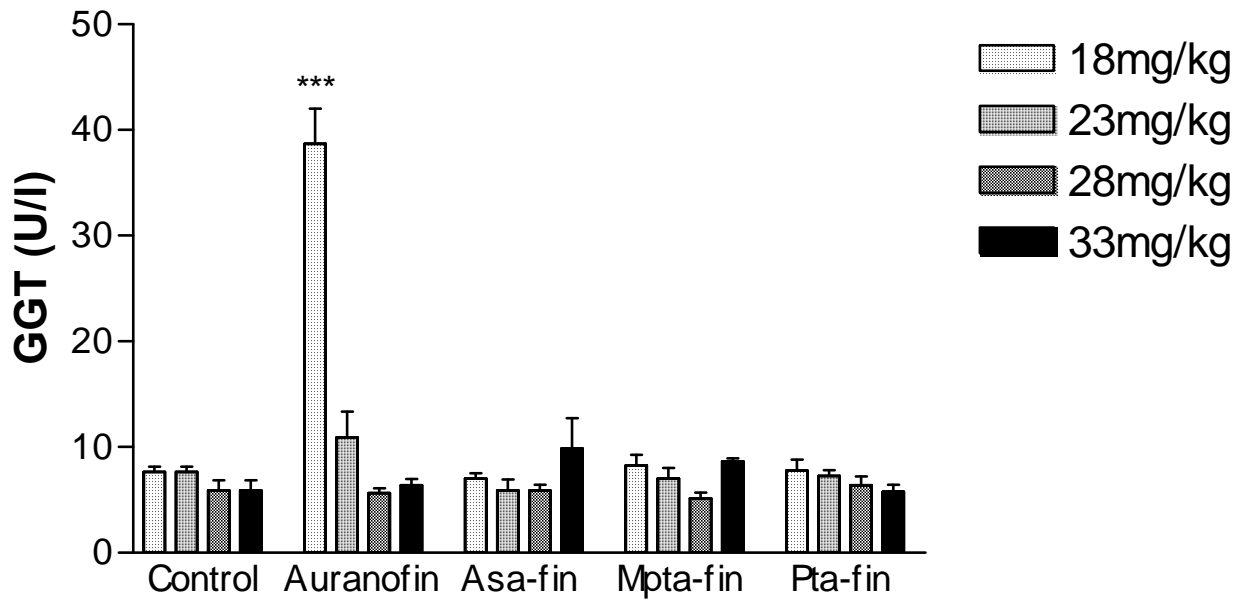
Figure 9.2: The effects of Auranofin, Asa-fin and Mpta-fin on GGT levels in Balb/c mice. Mice were gavaged with different dosages of the various drugs for five days. Animals were sacrificed on day five via cardiac puncture and the blood was analysed for changes in the liver enzyme GGT. Significance (p value) was calculated between untreated control values and the relevant treatment groups. n=10.

***p<0.001
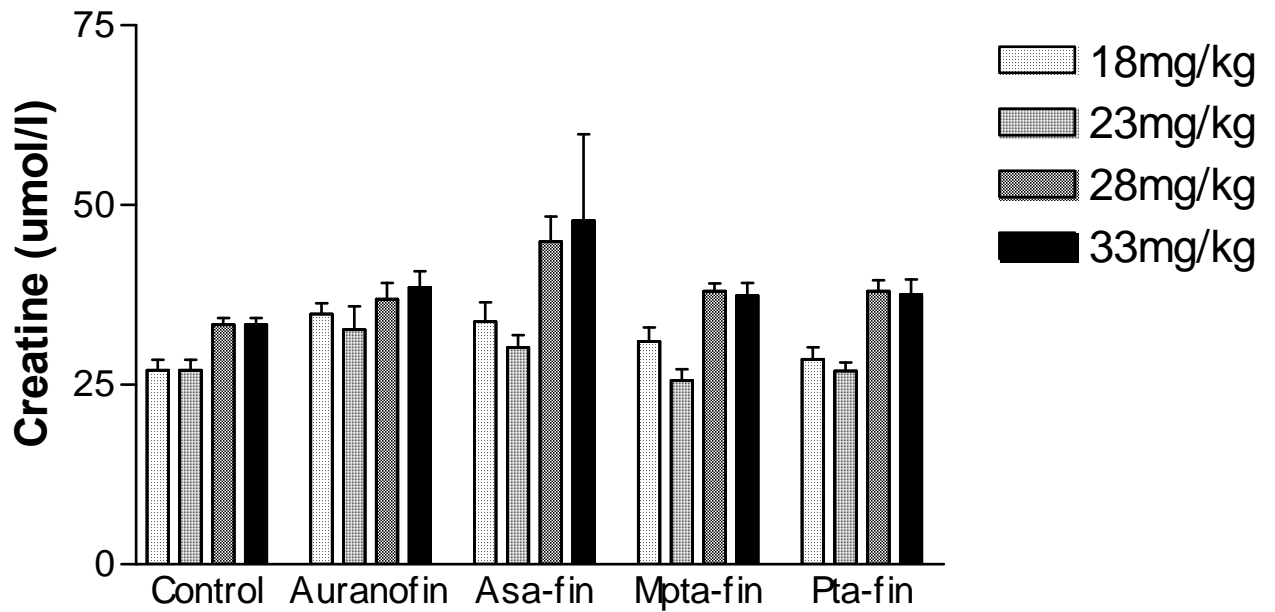
Figure 9.3: The effects of Auranofin, Asa-fin and Mpta-fin on creatinine levels in Balb/c mice. Mice were gavaged with different dosages of the various drugs for five days. Animals were sacrificed on day five via cardiac puncture and the blood was analysed for changes in the creatinine levels. n=10.
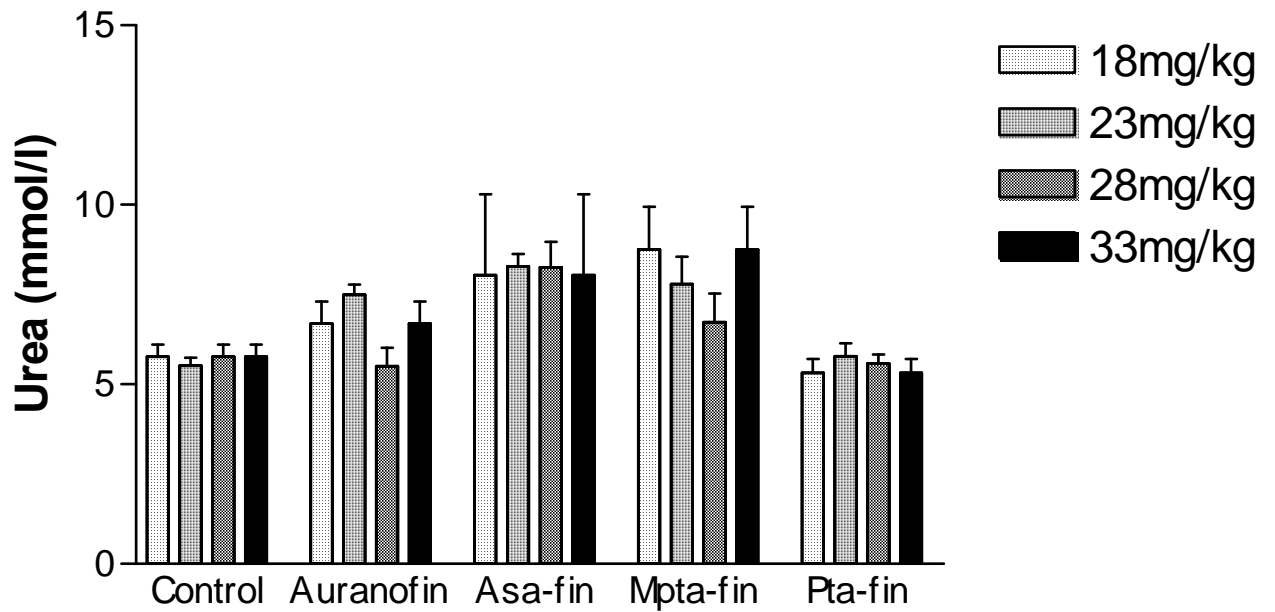
Figure 9.4: The effects of Auranofin, Asa-fin and Mpta-fin on urea levels in Balb/c mice. Mice were gavaged with different dosages of the various drugs for five days. Animals were sacrificed on day five via cardiac puncture and the blood was analysed for changes in the urea levels. n=10.

9.6. Discussion

To evaluate the toxicity of the novel gold compounds 4 different dosages of the compounds were compared, the maximum tolerated dose being 33mg/kg BW for 5 days. Liver enzymes and kidney metabolites were used as parameters of toxicity. The liver enzymes tested were: AST and GGT. Kidney function tests included the determination of urea and creatinine.

Many articles provide *in vitro* and *in vivo* data of auranofin, which is the reference standard of the novel gold compounds. Being novel, there is no literature available for *in vivo* effects of Asa-fin, Mpta-fin and Pta-fin. The *in vitro* results show the effects of the novel compounds as well as auranofin in a variety of cells so as to determine dosages that would not be toxic *in vivo*. Postulating from the *in vitro* data, the gold containing compounds, i.e. Asa-fin, Mpta-fin and Pta-fin, might be less toxic than auranofin. The usual number of mice used per treatment in similar projects reported in the literature is 6-10. This appears to be the minimum number to indicate differences in pathological parameters. During this study, the only group where adverse effects were seen were in the 33mg/kg Asa-fin group. Two mice in this group showed decreased activity. One of those two mice had a hunched appearance and died on day 3 of the second phase of the study. No adverse effects were observed in the mice that received the other drugs even at dosages of 33 mg/kg. Payne and Arena (1978) reported toxicity of Auranofin at 310 mg/kg in rats and 265mg/kg in mice. Hertz, Chevrier et al. (1982) noted no toxicity in rats dosed with 32 mg/kg auranofin and two deaths in rats that were gavaged with 64 mg/kg auranofin.

This is the first documented study that makes use of liver enzymes to quantify the toxicity of auranofin. The AST levels in the groups receiving 33mg/kg Asa-fin was increased significantly compared to the control (Figure 9.1, page 98). The levels of GGT however remained normal with a slight increase (but not significant) of GGT in the group receiving 33 mg/kg Asa-fin (Figure 9.2, page 99). The group that received 18 mg/kg auranofin however shows a significant increase in the GGT levels (Figure 9.2, page 99), but no significant increase in any of the other parameters tested. This increase in the GGT levels were not observed in the groups receiving the higher doses of auranofin viz: 23 mg/kg, 28 mg/kg and 33 mg/kg. There were also no increases in

the other parameters tested or any difference in the animal's behaviour in the group receiving the 18 mg/kg dose of auranofin.

From this study it must be concluded that the gold compounds Mpta-fin and Pta-fin showed similar acute toxicity profiles as auranofin *in vivo*. The compound Asa-fin however showed similar toxicity to auranofin at the lower doses (18, 23 and 28 mg/kg) but proved to be more toxic than auranofin at the highest dose tested (33 mg/kg). These results indicate that the novel gold compounds Mpta-fin are as toxic as auranofin *in vitro* whereas Asa-fin is more toxic than auranofin *in vivo*.

CHAPTER 10
CONCLUSION

10. CONCLUSION

The market for new drugs for rheumatoid arthritis is highly competitive, with considerable research activity and entrants. Market entry, whether into clinical research or commercialisation, is driven by novelty and perceptions of benefit against risk, among other factors.

Improving the molecule of auranofin to develop a more effective and less toxic anti-rheumatic drug would require the molecule to have a greater penetration into affected areas and a greater anti-inflammatory activity at cellular level. Certain heterocyclic carbene complexes of gold(I) have recently been found to induce mitochondrial membrane permeability which is crucial for anti-tumour activity. Nitrogen-rich heterocycles, such as imidazoles, triazoles and tetrazoles are important functionalities in medicinal chemistry. The latter of these compounds are especially versatile ligands that can be used in various coordination modes to metals and to gold(I) in particular. With this in mind three novel gold(I) tetrazolyl- and tetrazolidine-complexes were prepared and characterised. These carbene complexes are obtained by alkylation of tetrazolato gold(I) precursor compounds, which are in turn obtained by two methodologies. Firstly by direct lithiation of tetrazole and transmetallation, and secondly from cyclisation of gold(I) azide with a substituted isocyanide. In the latter regard *tert*-butylisocyanide was found to be highly reactive towards the metal azide. Furthermore, it was reported that when a TMEDA-THF solution was used in lithiation of tetrazoles, the resultant gold complexes undergo homoleptic rearrangements in solution.

The effectiveness of auranofin as an anti-rheumatic drug has widely been researched and is thoroughly documented. However several limitations exist with the use of auranofin as an anti-rheumatic drug due to a number of troublesome and sometimes serious side effects. The most common of these side effects include lower gastrointestinal tract disorders (Bernhard 1982). Haematological side effects such as general leucopoenia (Lewis and Walz 1982), lymphopenia (Hunt and Holt 1981) and neutropenia (Hunt and Holt 1981; Calin, Saunders et al. 1982) have also been reported.

It is of interest to assess the *in vitro* and *in vivo* toxicity and anti-inflammatory activity of the novel anti-rheumatic compounds in serum and tissues in comparison to auranofin.

This research work focused on three potential anti-rheumatic gold compounds, Asa-fin, Mpta-fin and Pta-fin with regards to the *in vitro* and *in vivo* toxicity, anti-inflammatory activity and lipophilicity. From literature and previous studies, it is thus evident that injectable hydrophilic antirheumatic agents require a greater time period to exert their effects and are less toxic than the lipophilic compound, Auranofin, which enters cells without difficulty. The toxicity of Auranofin however is attributed mainly to its lipophilicity. Auranofin and Asa-fin are more lipophilic than Mpta-fin and Pta-fin. This allows Auranofin and Asa-fin to enter cells with more ease, thereby increasing their anti-inflammatory ability but also allowing them to be more toxic. Mpta-fin and Pta-fin are more hydrophilic, which would mean that the serum gold concentration of the drug would possibly be high and tissue concentrations low. This has an advantage of limiting the side effects experienced which generally accompanies treatment with gold compounds but has the disadvantage of not having the efficacy of the more lipophilic drugs.

The beneficial and adverse effects of current gold compounds are clinically well known. This study may lead to a better understanding of the exact action of gold compounds in the treatment of rheumatoid arthritis which may play a pivotal role in the management of this disease.

CHAPTER 11
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11. REFERENCES

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