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CONTROL OF OXIDATIVE DAMAGE IN RHEUMATOID ARTHRITIS BY GOLD(I)-THIOLATE DRUGS

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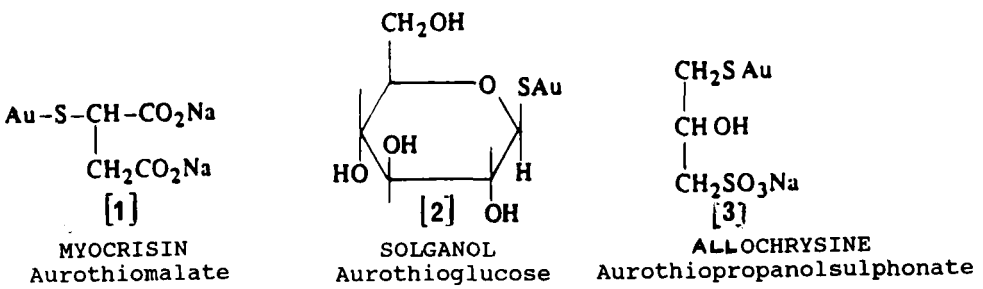
The roles of anti-arthritic gold(I)-thiolate drugs such as disodium aurothiomalate ('Myocrisin') in the modulation or promotion of oxygen radical-mediated oxidative damage *in vivo* are reviewed. In particular, the precise molecular mechanisms by which these novel second-line agents exert their therapeutic effects are discussed in terms of (i) the direct and indirect control of enzymes involved in the generation or scavenging of reactive oxygen speices (ROS) such as superoxide ion, hydrogen peroxide and hydroxyl radical, (ii) the protection of proteins and relevant enzyme systems against attack by ROS and (iii) their direct involvement in the production (at appropriate 'target' sites) or scavenging of ROS *in vivo*. In addition, the role of the orally-effective gold(I)-phosphine complex auranofin in the control of oxidative damage in rheumatoid arthritis is also discussed.

KEY WORDS: Gold, free radicals, rheumatoid arthritis.

INTRODUCTION

Gold(I)-thiolate drugs have been used successfully for many years in the treatment of rheumatoid synovitis, frequently inducing suppression of the disease rather than simple alleviating its symptoms. The usual dosage is equivalent to 30–50 mg of metallic gold per week, the whole course of treatment usually involving a total of 2 g of gold. However, the structural nature of 1:1 gold(I)-thiolate complexes has previously been very poorly defined and it is only within the last few years that attempts to investigate their chemistry have been conducted.[1-5]

The complexes are often formulated[1-3] as simple monomers, [Au(SR)], but it is now



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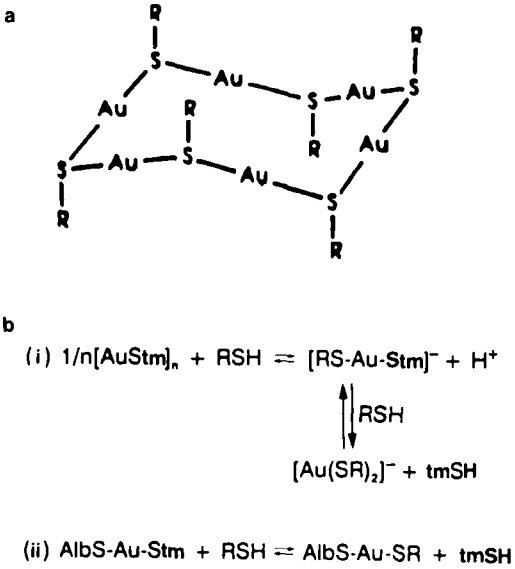


FIGURE 1 (a) Polymeric ring structure for 1:1 gold(I)-thiolate complexes. (b) Scheme for the relase of thiomalate (tmS ^{1}) from gold(I) *in vivo* by (i) direct attack of an endogenous, non-protein-bound thiol (RSH, e.g. cysteine or glutathione) on the oligomeric 1:1 gold(I)-thiomalate complex (1/n[AuStm]$_n$) and (ii) interaction of non-protein-bound thiol with gold(I)-thiomalate bound to the cysteine-34 residue of human serum albumin (AlbS-Au-Stm). [RS-Au-Stm]$^-$ represents a monomeric, mixed-ligand bis-thiolato gold(I) complex containing coordinated thiomalate and [Au(SR)$_2$]$^-$ a monomeric, bis-endogenous thiolato gold(I) complex.

clear that these species are comprised of polymeric rings with gold(I) achieving linear two-coordination via bridging thiolate sulphur ligands[2,5] i.e. [Au(SR)]$_n$ where n is $\sim$ 6 (Figure 1a).

However, in aqueous solution these oligomeric species are able to exist in up to three different structural forms, the exact structural composition being highly dependent upon parameters such as pH, ionic strength and temperature, as has been demonstrated for disodium aurothiomalate and monosodium aurothiopropanolsulphonate.[5,6] This structural heterogeneity is of much significance in studies of the biological chemistry of gold drugs and is likely to have important consequences regarding the molecular pharmacology, biodistribution and mechanisms of action of these novel therapeutic agents. In addition, many commercial preparations contain a slight molar excess of thiolate over gold(I).[2]

Gold(I) is a soft Lewis acid with a high affinity for ligands which contain the thiol group and a low affinity for nitrogen and oxygen donor ligands. Thus it is likely that gold(I) distributes itself primarily amongst protein and non-protein thiol groups *in vivo* and studies of the molecular pharmacology of gold drugs (following their parenteral administration) have revealed that this is indeed the case.[7,8]

For example, in human plasma where the principal source of thiol group is albumin, the gold is predominantly albumin-bound, but smaller amounts are bound to immunoglobulins and aproximately 5% of the total circulating gold is low-molecular-mass or 'free reactive' gold.[9] Despite their low concentration, low-molecular-mass gold species such as the monomeric bis (L-cysteinato)gold(I) complex

($[Au(SCys)_2]^-$)[10] are likely to be of much importance in various transport processes and the attainment of chemical equilibrium of gold amongst numerous protein binding sites. Indeed, a dominating factor in the biological chemistry of gold is the ability of gold(I) to undergo facile thiolate exchange and release reactions, as depicted in Figure 1(b).

1:1 gold(I)-thiolate complexes are presumed to have an intracellular site of action since the gold is eventually localised in intracellular organelles.[11,12] Although neither polymeric aurothiomalate nor protein-bound gold(I) are likely to undergo cellular uptake except by phagocytosis or pinocytosis, it is possible that specific monomeric, bis-thiolato metabolite complexes may be taken up by certain 'target' cells. Moreover, the structural heterogeneity of polymeric 1:1 gold(I)-thiolate complexes is of much biological importance in view of their ability to be phagocytosed as high-molecular-mass materials by cells. It should also be noted that the biodistribution of these therapeutic agents is complicated by the observation that cyanide inhaled in tobacco smoke enhances the uptake of gold into erythrocytes.[13,14] Cyanide ion readily breaks down the polymeric structure of aurothiomalate to form a series of monomeric, two-coordinate gold(I) complexes ($[tmS\text{-}Au\text{-}CN]^-$, $[Au(CN)_2]^-$ and $[Au(Stm)_2]^-$) via ligand-scrambling reations.[15] The aurocyanide complex ($[Au(CN)_2]^-$) is rapidly taken up by red blood cells.

It is generally thought that the delayed, favourable response obtained following the parenteral administration of 1:1 gold(I)-thiolate drugs to rheumatoid patients results from the ability of gold(I) to block some critical thiol group, such as that in immunoglobulin G.[16,17] Indeed, many of the alternative possible modes of action of these gold drugs which include the inhibition of lysosomal enzymes,[18-20] prostaglandin biosynthesis,[21,22] endothelial cell proliferation,[23] thiol-dependent proteases such as cathepsins,[24] ADP or collagen-induced platelet aggregation,[25] human neutrophil collagenase,[26] monocyte migration,[27] and mast cell histamine release,[28] interference with complement activation,[29,30] prevention of the myeloperoxidase-induced inactivaiton of α-1-proteinase inhibitor,[31] restoration of T-cell and T suppressor cell function,[32] alterations in protein interactions,[33-36] and non-specific anti-inflammatory activity[37] may also involve the blockage of sulphydryl group reactivity. These mechanisms of action are also possible mechanisms of action of an endogenous thiol-blocking reagent, postulated to be a ternary copper(II)-histidinate-cystinate complex.[38,39] Consideration of the complex molecular distribution of copper(II) ions in human plasma, which invqlves caeuruloplasmin, albumin (ca. 5% of the total concentration) and low-molecular-mass endogenous ligands, gives rise to the deduction that very small quantities of non-macromolecule-bound copper(II) ions are present by virtue of the nature of the relevant equilibrium expressions. However, circulating human plasma cannot be considered as a medium which involves genuine thermodynamic equilibria and recent attempts to detect low-molecular-mass copper(II) complexes in human biological fluids have indicated that such complexes may be artifactual.[103]

Figure 2 illustrates a possible mechanism for the modulation of the complex thiol-disulphide equilibria *in vivo* by polymeric 1:1 gold(I)-thiolates such as disodium aurothiomalate.

The excess production of superoxide ($O_2^{\cdot-}$), hydroxyl ($^{\cdot}OH$), alkoxyl ($RO^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radical species is an important mechanism of 'oxidative stress' which can give rise to considerable cell and tissue damage *in vivo*.[40] Indeed, there is currently a large amount of experimental evidence available implicating the involvement of these chemically-reactive oxygen radical species in the pathogenesis of inflammatory

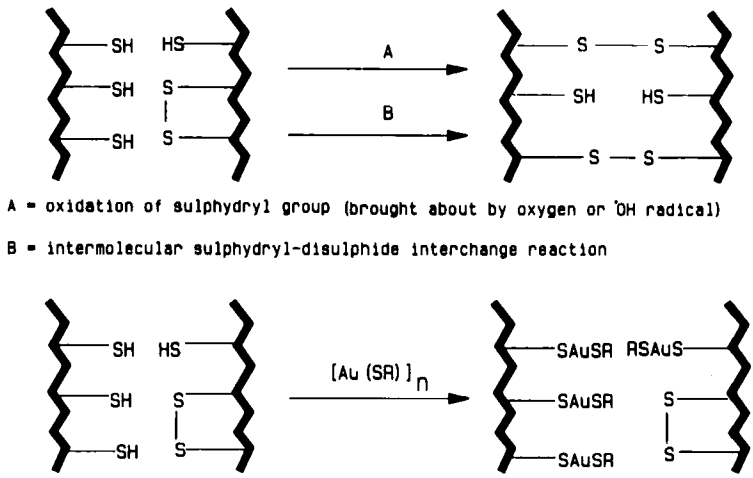


FIGURE 2 Possible mechanism for the regulation of the compelx thiol-disulphide equilibria *in vivo* by gold(I)-thiolate drugs. Pathway A represents oxidation of a protein or membrane sulphydryl group brought about by dioxygen, O_2^-, H_2O_2 or OH radical whilst pathway B represents an intermolecular sulphydryl-disulphide interchange reaction.

joint diseases.[41-45] Hence, it is clear that investigations of the mode of action of gold drugs in current use and also of the design of new gold(I) complexes as therapeutic agents requires the careful consideration of the complex mechanisms by which reactive oxygen species (ROS) exert their damaging effects. Although inhibiition of the generation of ROS by aurothiomalate and the orally-active monomeric, ternary gold(I)-tetra-acetyl thioglucose-triethylphospine complex (2,3,4,6-tetra-O-acetyl-l-thio-β-D-glucopyranosato-S) (triethylphosphine) gold(I): auranofin [4]) has been suggested as a plausible mechanism of action for anti-arthritic gold(I) drugs,[46] there have previously been no studies of the precise molecular mechanisms by which these therapeutic agents extert these effects in one or more stages of the pathogenic process. We postulate that gold(I)-thiolate drugs regulate these processes by two broadly-defined mechanisms: (1) suppression of the deleterious production and action of ROS in biological fluids or on cell membranes, and (2) enhancement of the biologically site-specific generation of ROS within relevant 'target' cells (e.g. endothelial cells), inhibiting their proliferation. We also discuss relevant aspects of the biological chemistry of auranofin, which is now in clinical use for the treatment of rheumatoid arthritis as a second-line therapeutic agent.

Direct and Indirect Control of Enzyme Systems involved in the Production or Transformation of ROS

The possible suppression of hypoxic reperfusion injury by polymeric 1:1 gold(I)-thiolate complexes may provide further evidence for the importance of *in vivo* thiol blockade as a mechanism for their therapeutic efficacy. Hypoxic reperfusion injury, a consequence of a number of pathophysiological and biochemical phenomena

present within the inflamed rheumatoid joint, is a process that is partly mediated by the generation of ROS such as the hydroxyl radical ($^{\cdot}OH$).[43-45] This process gives rise to cell and tissue damage following the re-introduction of dioxygen to the synovium during physiologically-relevant periods of excercise. Xanthine dehydrogenase is a cytosolic enzyme located in capillary endothelium which contains two molybdenum atoms, two flavin adenine dinucleotide (FAD) groups and two each of two different 2Fe–2S clusters. Under normal physiological conditions this enzyme transferes electrons to NAD^+ as it oxidises hypoxanthine or xanthine to uric acid.[47,48] However, the dehydrogenase enzyme is transformed to an oxidase form by Ca^{2+} ion-dependent limited proteolysis or, more likely, by oxidation of essential thiolate (RS^-)[48] functional groups subsequent to the disruption of tissue. Hence, a major consequence of the isolation and purification of the enzyme from biological sources is that it appears as an oxidase, generating $O_2^{\cdot}$ and hydrogen peroxide (H_2O_2) during the conversion of hypoxanthine or xanthine to uric acid. The roles of ROS in postischaemic tissue injury have been extensively reviewed by McCord,[49] who has suggested that conversion of the dehydrogenase form of the enzyme to the oxidase form occurs in ischaemic tissue. In hypoxic tissue, depletion of ATP occurs leading to an accumulation of hypoxanthine, which is oxidised by xanthine oxidase on reperfusion of the tissue with the concomitant adverse production of $O_2^{\cdot-}$ and H_2O_2, leading to tissue damage. Although this hypothesis is supported by much experimental evidence,[50-53] it has recently been challenged by several scientists.[104]

Since oxidation of essential thiolate groups has been implicated as a major pathway for the conversion of xanthine dehydrogenase to its oxidase form,[48] it is possible that suppression of hypoxic reperfusion injury in the inflamed rheumatoid joint may be achieved by exploiting the thiol-blocking activity of 1:1 gold(I)-thiolate complexes.

However, it should also be noted that a specific, highly thermodynamically-favourable blockade of these thiolate groups may result in a suppression of the enzyme's dehydrogenase activity, allowing it to act as an oxidase. Moreover, attack of gold(I) at the iron/sulphur active centres of the enzyme could also lead to its conversion to the oxidase form. Although disodium aurothiomalate undergoes facile ligand-exchange reactions with sulphide ion,[5] it is only poorly reactive with 'labile' sulphide at the active centres of both 2Fe–2S and 8Fe–8S ferredoxins.[54]

Table I shows the influence of various concentrations of disodium aurothiomalate on the relative proportions of xanthine dehydrogenase and xanthine oxidase in rat liver homogenates. From these data, it is clear that at low concentrations, aurothiomalate inhibits the conversion of xanthine dehydrogenase to its oxidase form, whereas at high concentrations (ca.10^{-4} mol dm^{-3}) the conversion is accelerated. The total enzymatic activity (xanthine dehydrogenase plus oxidase) was not inhibitable by aurothiomalate in the concentration range 10^{-8}–10^{-4} mol. dm^{-3}. Interestingly, *p*-hydroxymercuribenzoate, a powerful thiol-blocking reagent has been found to enhance the conversion of the dehydrogenase form of the enzyme to the oxidase form.[48] However, it is important to note that despite the similarities in the chemistry of mercury(II) and isoelectronic gold(I), there are important biologically-relevant differences in the thermodynamics and kinetics of their interactions with thiols. Firstly, the equilibrium constants for the binding of Hg^{2+} or CH_3Hg^+ to endogenous thiols are ca.10^{20} and 10^{15} respectively,[64] whilst the interactions of 1:1 gold(I) thiolates with a second thiol (for example, equation (i) in Figure 1 (b)) have equilibrium constants of ca.10^5 i.e. 1:1 gold(I) thiolates have relatively weak binding constants for their

TABLE 1

a) Influence of increasing concentrations of disodium aurothiomalate (expressed as total gold concentration) on the activity of xanthine dehydrogenase expressed as a percentage of total enzymatic activity (xanthine dehydrogenase plus xanthine oxidase activities). All values are the means of two separate determinations. b) Effect of increasing concentrations of disodium aurothiomalate on the total enzymatic activity of xanthine oxidoreductase (xanthine dehydrogenase plus xanthine oxidase) in rat liver homogenates. Values are expressed in terms of 10^{-3} Units of enzyme/100 mg tissue. Liver homogenates from male Wistar rates were prepared and both xanthine oxidase/xanthine dehydrogenase activities were measured in cytosolic fractions using a spectrophotometric assay based on following the formation of uric acid from its absorbance at 292 nm according to Johnson and Rajagopalan.[116] The assay was performed using molecular oxygen as an electron acceptor (oxidase activity only), and also including 6.7×10^{-4}. dm^{3} NAD^+ as an electron acceptor (dehydrogenase plus oxidase activity). The activity measured was tested for inhibition using 10^{-6} mol. dm^{-3} allopurinol. The effect of aurothiomalate on the % dehydrogenase or total enzymatic activity was investigated by including it in the assay medium or following a preincubation period with the homogenate.

Incubation time (min.)	(a)						
				[Au]/mol. dm^{-3}			
		0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
	0	73	68	69	67	69	15
	45	43	69	67	69	53	27
	(b)						
				[Au]/mol. dm^{-3}			
		10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
	Control	45	47	49	46	44	
	Test	45	50	47	46	41	

reactions with a second equivalent of thiol.[2] Secondly, gold(I) thiolate complexes undergo thiolate-exchange reactions at rapid rates,[65] i.e. they are kinetically labile, whilst the corresponding mercury(II)-thiolate complexes are kinetically inert.

In view of the recent proposal that reaction of disodium aurothiomalate with endothelial cell sulphydryl-containing components is likely to be involved in the mechanism of inhibition of endothelial cell proliferation,[23] it is possible that such inhibition is mediated by the gold(I) blockage of critical thiol(ate) or sulphide ligands present in xanthine dehydrogenase, giving rise to its conversion to the oxidase form of the enzyme with a consequent rise in intracellular O_2^- concentration which in turn leads to endothelial cell dysfunction or death. Effective inhibition of endothelial cell proliferation by gold(I)-thiolate complexes in this manner may be a key pathway in the suppression of inflammatory synovitis by lowering the equantity of small blood vessels available for mononuclear cell infiltration and synovial tissue proliferation.

Intriguingly, Minta and Williams[55] found that aurothiomalte inhibited the enzymatic activity of a subcellular preparation of polymorphonuclear leucocyte (PMNL) NAD(P)H oxidase, the inhibition being more potent than that observed with d-penicillamine, auranofin or its potential metabolite triethylphosphine gold(I) chloride (Et_3PAuCl). Both disodium thiomalate and dithiothreitol inhibited the enzymatic activity of an isolated sample of NAD(P)H oxidase, implicating the involvement of thiol-disulphide interchange reactions in the mechanism of inhibition. In addition, the gold(I)-inhibition of the cellular function of various phagocytes, including chemotaxis of neutrophils,[56] O_2^- production[57] or ROS generation[58] has also been studied. However, Hurst *et al.*[62] found that both gold and d-penicillamine directly or indirectly stimulated O_2^- production by mononuclear phagocytes, and observed a biological difference between responding and non-responding patients treated with either therapeutic agent. They also noted that the capacity of mononuclear phagocytes to generate O_2^- is not directly correlated with disease activity.

Recently, aurothiomalate, aurothioglucose and auranofin have been shown to inhibit the activity of the selenocysteine-containing enzyme glutathione peroxidase[59] (GP_x) which catalyses the reduction of hydroperoxides (equation 1).

$$2\,GSH + H_2O_2 \xrightarrow{GP_x} GSSG + 2H_2O \quad (1)$$

This inhibition involves the formation of a ternary glutathionato-gold(I)-selenocysteine glutathione peroxidase ternary complex (illustrated for aurothioglucose in equation (2)), satisfying the requirement of gold(I) for linear two-coordination. However, there is currently no evidence avilable to suggest that this inhibition actually occurs *in vivo*.

$$GP_x\text{-SeH} + AuStg + GSH \longrightarrow GP_xSe\text{-Au-SG} + tgSH^{\cdot} + H^+ \quad (2)$$

The suppression of GP_x activity in this manner could lead to an increased bioavailable pool of intracellular H_2O_2 which may play a role in biologically 'site-specific' oxidative damage via the Fenton reaction (equation (3)) or the iron-catalysed Haber-Weiss reaction (equation (4)).

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + {}^{\cdot}OH \quad OH^- \quad (3)$$

$$O_2^- + H_2O_2 \xrightarrow[\text{catalyst}]{\text{Fe-complex}} O_2 + {}^{\cdot}OH + OH^- \quad (4)$$

Therapy with gold(I)-thiolate complexes may also lead to the mobilisation of both zinc and copper ions from the cysteine-rich protein metallothionein in patients with inflammatory joint disorders,[60] either by direct displacement or chelation with the thiol ligand as a metabolite. The induction of superoxide dismutase (SOD) activity (equation (5)) may be a very important mechanism by which bioavailable copper ions are able to exert their anti-inflammatory activity.[105]

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (5)$$

The liberated copper ions may also have the ability to participate in beneficial biologically site-specific functions such as the promotion of ROS formation or the induction of H_2O_2 production via the SOD-mimetic activity of one or more of its complexes with endogenous thiols or amino acids, although it is important to appreciate that there is currently no evidence to suggest the presence of bioavailable low-molecular-mass copper complexes in body fluids obtained from patients with rheumatoid arthritis.[103] Notable also are the copper-requiring enzyme lysyl oxidase, which may be involved in the synthesis of connective tissue, and the ability of aqueous Cu(II) ions to inhibit the depolymerisation of hyaluronate.[61]

It is also important to consider the interference of gold(I)-thiolate complexes or their 'free' thiol metabolites with leucocyte myeloperoxidase (MPO) activity. In rheumatoid arthritis the assault of activated leucocytes against articular cartilage gives rise to the leakage of the cell's granular enzymes into extracellular fluid. Of these enzymes, MPO is particularly deleterious since its generates the powerful oxidant hypochlorite ion (OCl^-) from H_2O_2 and chloride ion. Muijsers *et al.*[63] studied the influence of various thiol-containing drugs (including *d*-penicillamine) and aurothioglucose on the activity of a purified MPO preparation. They found that all of the compounds investigated effectively scavenged OCl^-, and that the MPO-H_2O_2-chloride system transformed *d*-penicillamine to its correponding oxidation products, penicillamine disulphide and penicillamine sulphinic acid. Moreover, *d*-penicillamine

converted MPO to its compound III adduct, in which O_2^- ion is bound to the haem group of the enzyme. These studies have implications regarding the potential anti-arthritic activity of the free thiol ligand, released from gold(I) via thiolate-exchange reactions which readily occur *in vivo* (Figure 1 (b)). Indeed, Wasil *et al.*[106] have demonstrated that thiomalate effectively protects α_1-antitrypsin against inactivation by $HOCl/OCl^-$.

Protection of Proteins and Enzyme Systems against Oxidative Damage

It is well known that plasma thiol levels of patients suffering from connective tissue disorders are strikingly lower than those of healthy individuals.[66,67] Although these reduced thiol levels are generally correlated with lowered albumin concentrations resulting from a so-called 'negative acute phase response', it is also possible that the lower concentrations of thiol groups in rheumatoid plasma indicate a disturbance in both the thermodynamic and kinetic aspects of the redox chemistry of endogenous thiols. Hence, the powerful thiol-blocking properties of gold(I)-thiolate complexes and the ability of the unbound thiol(ate) ligand (e.g. thiomalate) to participate in thiol-disulphide interchange reactions serve as potential assets in alleviating this deleterious redox imbalance. Interestingly, it has also been reported that in rheumatoid patients the thiol level is actually increased in the more reduced haemolysate, contrasting with the decreased levels observed in the more oxidised plasma.[68,69] However, these studies remain somewhat speculative since they have been performed using a diversity of thiol assay systems whose results are not unequivocal. Both plasma and haemolysate thiol levels have been reported to return to a value closer to normal during anti-arthritic drug therapy,[70,71] but there is a poor correlation between them, indicating that they may not be closely related. However, reduced glutathione (GSH) levels are controlled by the activity of certain enzymes, some of which occur on the cell's outer membrane. GSH is the main source of low-molecular-mass thiol groups in intracellular fluid (2–3 mmol dm^{-3} in human erythrocytes) and is known to play a major role in the protection of cellular systems from attack by ROS. Only very small quantities of GSH (ca.1–2 μmol. dm^{-3}) are present in human extracellular fluids.[107]

A failure to maintain the required concentrations of reduced thiol in extracellular fluids may be a direct reflection of the adverse production of ROS in these media. Thiols react extremely rapidly with $^{\cdot}OH$ radical (equation (6)), generating thiyl radicals ($RS^{\cdot}$). Moreover, in the presence of dioxygen both thiylperoxy ($RSOO^{\cdot}$) and sulphenyl ($RSO^{\cdot}$) radical species are also formed.[108,113,114]

$$RSH + {}^{\cdot}OH \longrightarrow RS^{\cdot} + H_2O \qquad (6)$$

Protein thiyl radicals produced in this manner have the ability to interact with thiol groups on adjacent proteins to produce macroglobulins or aggregated proteins such as that of immunoglobulin G.[109] These 'unnatural' aggregated macromolecules can stimulate the immune response, leading to the direction of antibodies against them.

Total thiol concentrations in blood plasma (ca. $4–6 \times 10^{-4}$ mol. dm^{-3}) are much higher than the concentrations of circulating gold ($5–25 \times 10^{-6}$ mol. dm^{-3}) during chrysotherapy. However, the observation that the gold distribution between plasma proteins is the same irrespective of the gold concentration for each patient[72,73] indicates that there are dominat equilibria *in vivo* which determine the protein thiol groups with which gold(I)-thiolates interact. Studies of the interaction of various thiols with

polymeric aurothiomalate have revealed that the gold(I) complex readily binds further thiol,[74] a process which involves large changes in the electronic distribution within the ligand attributable to sulphur coordination. These large changes in ligand electron density (which presumably include the sulphur atom) have important biological consequences with regard to the ability of monomeric bis-thiolato gold(I) complexes to function as reducing agents.[2] Indeed, the bis-thiolato gold(I) complex ($[Au(Stm)_2]^-$) which is present in aqueous solutions of commercially-available (solid) disodium aurothiomalate and the autoclaved aqueous solutions of aurothiomalate (Myocrisin) which are used clinically (formed from the reaction of 'excess' thiomalate with the polymeric gold(I) complex) appears to have the ability to act as a reducing agent towards disulphides.[5] This phenomenon is also of relevance to other bis-thiolato gold(I) species which are formed *in vivo*.

In addition to the scavenging of MPO-derived HOCl/OCl^-, the reducing properties of released or 'free' thiolate ligands such as thiomalate are also of pharmacological and immunochemical importance since their reduction of disulphide crosslinks present in macroglobulins may be an important aspect of the mechanism of action of 1:1 gold(I) thiolate drugs.

A possible further example of the thiol-blocking mode of action of gold(I)-thiolate complexes is the suppression of the H_2O_2-mediated oxidative inactivation of the enzyme glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH). Baker *et al.*[75] have postulated that intrachondrocyte oxidant damage is attributable to the oxidation of the sensitive thiol group present at the active centre of G-3-PDH. Similarly, Hyslop *et al.*[76] have investigated the mechanism of H_2O_2-mediated cell injury and found that the glycolytic and mitochondrial pathways of ADP phosphorylation were important intracellular targets.

Interestingly, the corresponding copper complexes of the free thiolate ligands or their corresponding disulphides may also have potential as thiol-blocking agents which could inhibit the free radical-dependent aggregation of proteins in plasma or knee-joint synovial fluid.

Direct Involvement of 1:1 Gold(I)-Thiolate Drugs and their Metabolites in the Production or Scavenging of ROS in vivo

One of the most important features of an inflammatory response is the release of O_2^- and H_2O_2 by inflammatory cells (e.g. PMNL's) into extracellular media. Although there are only small amounts of SOD present in human extracellular fluids, dismutation of O_2^- by this enzyme (equation (5)) during the respiratory burst leads to the production of H_2O_2. Hence, extracellular gold(I)-thiolato species are susceptible to attack by O_2^-, H_2O_2 and ultimately $^{\cdot}OH$ radical. However, this is only likely to occur if the gold(I)-thiolate complexes are present at concentrations sufficient to compete with endogenous species that also have ROS-scavenging ability.

It is also conceivable that one or more of these powerful oxidants has the ability to remove and consume thiolate ligands even from polymeric 1:1 gold(I)-thiolate complexes generating a highly reactive gold(I) ion. Furthermore, intracellular ROS, which can be generated by cells switching from anaerobic to aerobic metabolism, or through the exposure of aerobic cells to a hypoxic environment, have the ability to attack so-called aurosomes in which the gold is present as gold(I) coordinated to two sulphur donor ligands, as indeed it is in polymeric aurothiomalate.[110,111] Figure 3 shows an example of the electron microscopic localisation of gold in a synovial

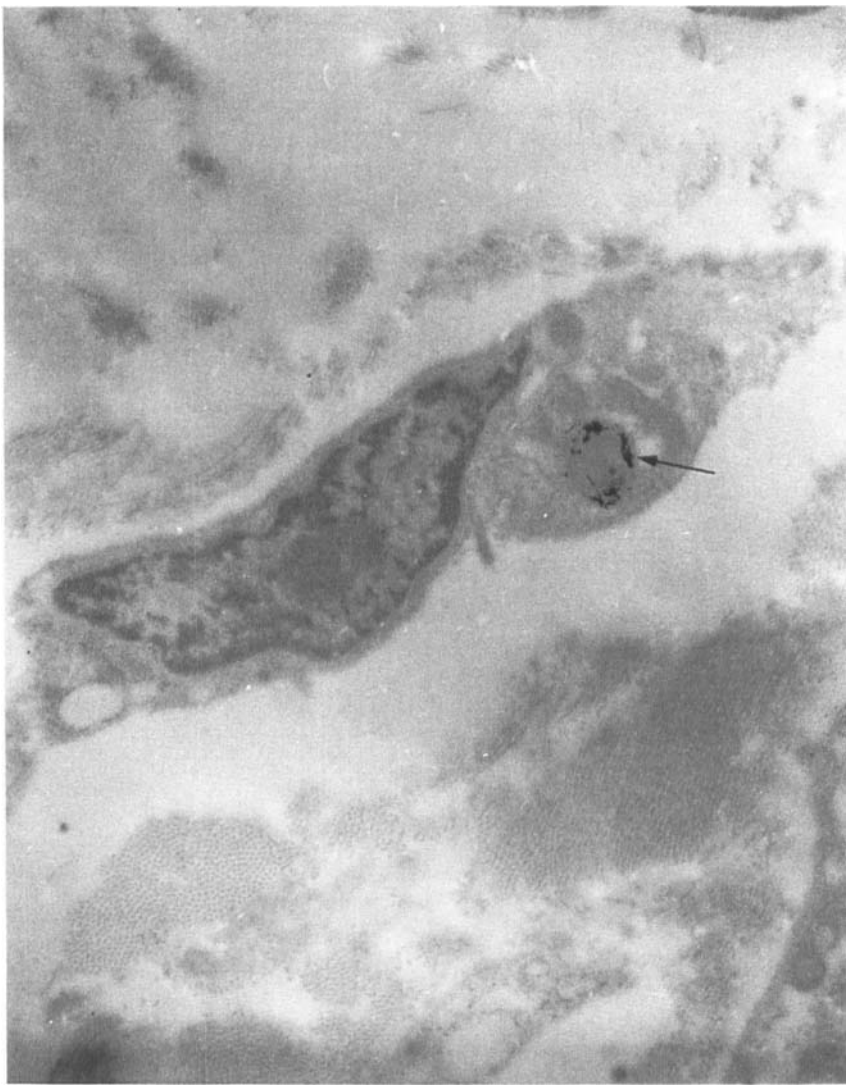


FIGURE 3 Electron micrograph showing the location of gold (arrow) in a synovial macrophage of a patient receiving chrysotherapy with 'Myocrisin'. This unstained section was magnified X 18,600. The identity of the gold was confirmed by electron probe X-ray microanalysis.

macrophage obtained from a rheumatoid patient undergoing therapy with 'Myocrisin'.

Figure 4 shows changes in the electronic absorption spectrum of a phosphate buffered solution of disodium aurothiomalate with time following incubation with H_2O_2. The final spectrum obtained has an absorption band located at 540 nm, characteristic of colloidal gold (gold(O)). The gold(O) is presumably generated by initial H_2O_2 oxidation of coordinated thiomalate followed by disproportionation of the resulting highly-reactive, unstabilised gold(I) ion (equation (7) and (8)).

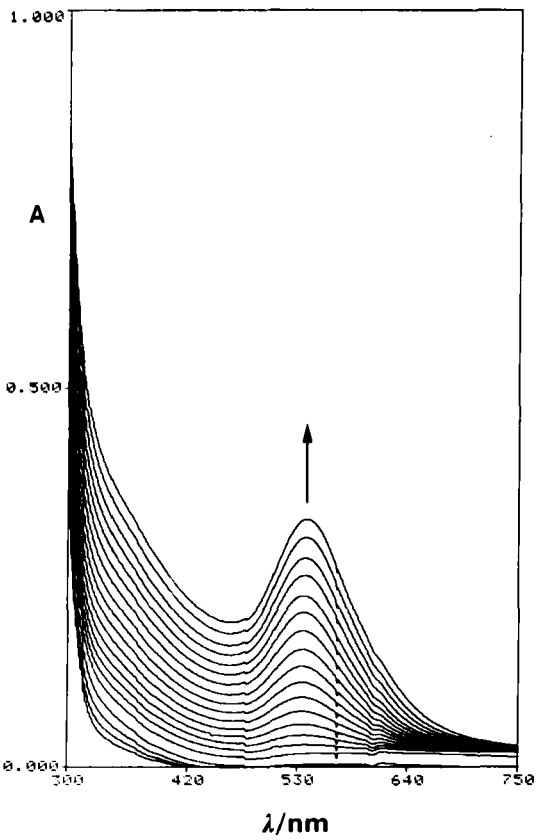


FIGURE 4 Time dependent spectral changes observed following treatment of an aqueous solution of a commercial sample of disodium aurothiomalate with H_2O_2. Eleectronic absorbtion spectra were recorded at 60 min. intervals. The absorbtion maximum produced at 540 nm is attributable to colloidal gold (Au(O)). Reaction mixtures contained, in a final volume of 3.00 ml, 0.10 mol dm^{-3} phosphate buffer pH 7.40, 1.5×10^{-3} mol dm^{-3} gold(I) and 0.10 mol dm^{-3} H_2O_2.

$$[AuStm]_n + H_2O_2 \longrightarrow \text{'Au(I)'} + \text{oxidised thiomalate product} \quad (7)$$

$$3\ \text{'Au(I)'} \longrightarrow 2Au(O) + Au(III) \quad (8)$$

Although the mechanism of this reaction is at present unclear, we have observed induction periods of ca. 30 min. before the reaction begins, suggesting the involvement of a free radical chain reaction. Initiation of this reaction appears to be due to the transfer of an electron from the excess thiomalate impurity to H_2O_2 to initially form both $tmS^{\cdot}$ and $^{\cdot}OH$ radical species. The induction period is presumably attributable to the presence of free radical scavengers in the system (for example, commercial samples of aurothiomalate contain approximately 1/3 molar equivalent of glycerol relative to gold).

Similarly, reaction of the powerful oxidant hypochlorous acid (HOCl) derived from the myeloperoxidase-H_2O_2-chloride system with commercial samples of aurothiomalate yields a pink-coloured gold sol.[77]

The production of Au(O) colloid at the sites of inflammation has much significance in relation to the mechanism of action of anti-arthritic gold(I)-thiolate drugs. For example, colloidal gold is rapidly taken up by macrophages.[112] Moreover, it is possible that in the presence of an endogenous Au(I)-stabilising ligand (e.g. thiols such as cysteine or thiocyanate ion (SCN^-)) colloidal gold is able to promote $^{\cdot}OH$ radical production via a pseudo-Fenton system (equation (9)). Indeed, it has recently been reported that gold metal stimulates the production of small but significant quantities of OH radical when immersed in aqueous H_2O_2 solutions.[78] Small quantities of colloidal gold may be present in injectable 'Myocrisin' clinical vials.

$$Au(O) + H_2O_2 \longrightarrow Au(I) + {}^{\cdot}OH + OH^- \quad (9)$$

The oxidation of metallic gold by atmospheric oxygen in the presence of an Au(I)-stabilising ligand such as cyanide ion (CN^-) is well known and forms the basis of a methodology for the extraction of gold from its ores. In the presence of atmospheric oxygen, gold metal is soluble in a wide range of amino acid solutions.[79] As expected, the sulphur donor ligands cysteine and *d*-penicillamine are the most effective in dissolving Au(O). Using colloidal gold, however, more concentrated solutions (ca. 10^{-4} mol. dm^{-3}) are obtained. L-cysteine readily effects the dissolution of Au(O) colloid into aqueous solution in the presence of O_2. Indeed, the electrode potential (E_{0} 1/2) for the Au(O)/Au(I) couple in the presence of cysteinate ion ($CysS^-$) is 0.144 V,[80] indicating that this process (equation (10)) is thermodynamically favourable. H_2O_2 enhances the dissolution of Au(O) in amino acid solutions by facilitating its oxidation.[79] The occurence of this reaction in appropriate biological microenvironments containing both H_2O_2 and a suitable Au(I)-stabilising ligand may give rise to $^{\cdot}OH$ radical production.

$$Au(O) + 2CysS^- \longrightarrow [Au^{I}(SCys)_2] + e^- \quad (10)$$

It is intriguing to consider that the facile dissolution of gold(O) sols by cyanide ion in the presence of dioxygen[81] may play an important role in the anti-inflammatory activity of gold(I)-thiolate drugs. Cyanide is produced by PMNL's and possibly by monocytes during phagocytosis,[82] and hence $^{\cdot}OH$ radical produced in the immediate vicinity of these potential target cells may form the basis of a possible biological 'site-specific' mechanism for chrysotherapy. In addition, aurocyanide ion ($[Au^{I}(CN)_2]^-$) which is also produced from the reaction of cyanide ion with gold(O) sols could be rapidly taken up by these cells and may affect their function. This hypothesis is supported by the observation that $[Au(CN)_2]^-$ is a powerful inhibitor of chemiluminescence derived from PMNL's and exogenous CN^- greatly potentiates the inhibitory action of aurothiomalate.[15] Moreover, $[Au(CN)_2]^-$ is a potent inhibitor of several enzyme systems, including alcohol dehydrogenase.[83]

It is also significant that polymeric 1:1 gold(I)-thioglucose disproportionates to form metallic gold and thioglucose disulphide (the latter hydrolysing slowly to the sulphinic acid derivative of thioglucose).[84] Occurrence of this reaction *in vivo* may lead to the deposition of Au(O) at a number of specific 'target' sites.

Figure 5 is a schematic representation of the mechanisms by which anti-arthritic gold(I)-thiolate complexes directly promote ROS production within certain target (e.g. endothelial) cells, leading to inactivation of ADP phosphorylation pathways, membrane lipid peroxidation and subsequent cell death. Included is a reaction system involving the direct attack of the thiyl radical ($RS^{\cdot}$ generated by the reaction of

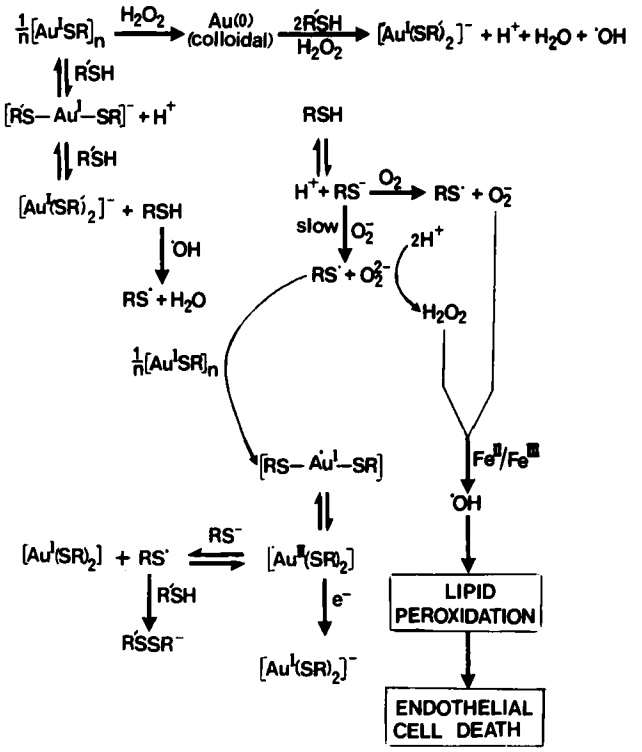


FIGURE 5 Schematic representation of the proposed mechanisms of 'OH radical, O_2^- or H_2O_2 production by anti- arthritic gold(I)-thiolate complexes *in vivo*.

dioxygen, O_2^- or 'OH radical with the parent thiol) at a gold(I) centre in a 1:1 gold(I)-thiolate complex resulting in the formation of an intermediate gold(II)-thiolate complex which is subsequently reduced by further thiol, generating additional thiyl radical. A similar mechanism has been proposed for the raction of benzenethiol with linear, two-coordinate ternary methyl-gold(I)-alkylphosphine complexes which involves the displacement of methyl radicals following RS' radical attack at the metal centre.[85] Interestingly, the SOD-mimetic activity of the multinuclear, mixed oxidation state copper-penicillamine complex ($[Cu_8^{I}Cu_6^{II}(\text{d-pen.})_{12}Cl]^{5-}$ has a mechanism which may involve a copper(I)-stabilised thiyl radical as an intermediate.[86] In addition to their interaction with dioxygen to form $RSO_2^{\cdot}$ and RSO' radical species, thiyl radicals can react with further thiol to form the well-characterised radical ion dimer, $RSSR^-$. Although the toxicological properties of such radicals is currently unclear, it is possible that unlike 'OH radical they may have the ability to diffuse away from their site of formation to attack specific biomolecules. For example, it has been suggested that oxysulphur radicals are sufficiently oxidising to inactivate the elastase-inhibitory activity of α_1-antiproteinase.[115]

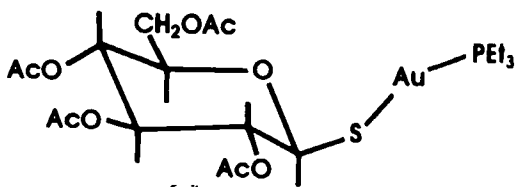
It is also important to note that 'free' thiol metabolites of gold(I)-thiolate drugs are very powerful scavengers of 'OH radical (equation (6)) and this may be of some importance in the protection of extracellular or membrane-bound biomolecules against free radical attack. However, since the accumulation of sufficient concentra-

tions of these thiol metabolites at sites of oxidative damage primarily determines their ability to effectively scavenge 'OH radical *in vivo*, such protection may be limited or even insignificant. Hence, an important role for the gold(I) moiety in the therapeutic action of 1:1 gold(I)-thiolate complexes may involve its transport and delivery of reactive oxygen radical-scavenging thiols to appropriate 'target' sites at wich ROS-mediated oxidant injury occurs.

Interestingly, disodium thiomalate has been found to suppress lipid peroxidation in the microsomal fractions of liver and kidney, whereas aurothiomalate appears to have a slight inducing effect which can be modulated by metallothionein.[87]

Role of Auranofin in the Control of Oxidative Damage

The structure of auranofin is quite distinct from that of 1:1 gold(I)-thiolates in that it is monomeric with gold(I) almost linearly coordianted by phosphorus from triethylphospine (PEt_3) and sulphur from tetra-acetyl-β-D-thioglucose (TATG), [4].



[4]

The thiol ligand adopts the chair conformation both in the solid state and in solution.[88,89] Auranofin is highly lipophilic with poor solubility in water, a factor which probably determines its oral absorption. In acidic solutions[90] or in contact with hamster or rat gut wall,[91] the gold(I)-bound TATG ligand in auranofin is converted to the de-acetylated (and hence more water soluble) species. However, this product, 1-thio-β-D-glucopyranosato-S-(triethylphosphine) gold(I) was found to pass through hamster or rat intestinal wall in an everted gut sac experiment.[91] The degradation of auranofin in acidic methanol solution occurs more rapidly, yielding TATG, triethylphosphine gold(I) chloride (Et_3PAuCl) and a bis-gold(I) sulphonium compound, [(TATG) $(AuPEt_3)_2$].[92]

Despite the incompletely elucidated metabolic fate of auranofin in the stomach, it is apparent that gold(I) complexes containing the intact thiolate-Au(I)-phosphine bonding system are absorbed subsequent to its oral administration.

Although the biological chemistry of gold(I)-phosphine complexes is markedly different from that of polymeric 1:1 gold(I)-thiolates, it is probable that end-products derived from auranofin metabolism are structurally similar to these species. However, the hydrophobic nature of auranofin and its likely Et_3PAuCl metabolite enables it to be delivered to intracellular sites that are inaccessible to gold(I)-thiolates. Furthermore, the Et_3P ligand, on release form gold(I), may have the ability to effect biologically-important reductions. The complex biological chemistry of auranofin has been amply illustrated by ^{31}P NMR studies of its distribution in whole human blood.[2,65] NMR spectra of red cells were found to contain two signals, each attributable to gold(I)-bound phosphine with the second ligands probably thiolate sulphurs (one of which may be due to intact drug). Corresponding spectra of the plasma, however, gave a signal attributable to triethylphosphine oxide (Et_3PO, derived from the oxida-

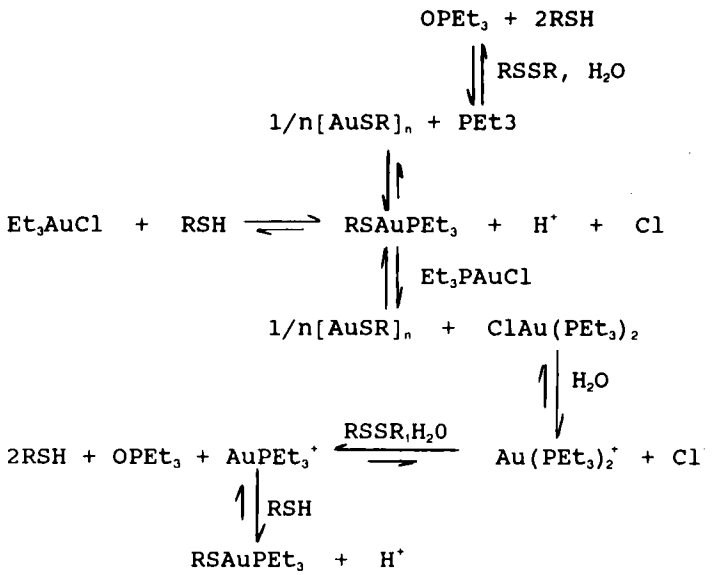


FIGURE 6 Reaction scheme for the control of thiol-disulphide equilibria by triethylphosphine gold(I) chloride (Et_3PAuCl) *in vivo*.

tion of released PEt_3) as well as a broad gold(I)-bound phosphine signal which is possibly due to a protein thiolate-bound $AuPEt_3$ moiety. Both these NMR investigations and the radiolabel studies of Intoccia *et al.*[93] have suggested that plasma may play an important role in the release of the TATG ligand, which in turn may be able to effect the dissociation of interprotein disulphide bonds in extracellular macroglobulins.

The formation of thiolate-substituted auranofin complexes is also of some relevance to the disociation of agregated proteins since both the cysteinato- and glutathionato-Au(I)-triethylphosphine complexes are somewhat unstable and can decompose to the corresponding polymeric 1:1 gold(I)-thiolate complex with concomitant release of reducing PEt_3 [65,94] (equation (11)).

$$Et_3PAuSCys \longrightarrow 1/n[AuSCys]_n + PEt_3 \qquad (11)$$

Reduction of interprotein disulphide crosslinks by released PEt_3 (equation (12)) may also feature as an important aspect of the therapeutic action of gold(I)-phosphine complexes.

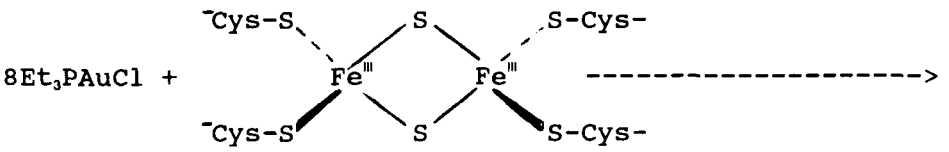
$$PEt_3 + RSSR + H_2O \longrightarrow 2RSH + OPEt_3 \qquad (12)$$

The hydrophobic Et_3PAuCl complex may be produced in the chloride-rich, low pH conditions of the human stomach. It has a powerful ability to block endogenous thiol groups to form $RSAuPEt_3$ adducts. These complexes can further react with excess Et_3PAuCl to form the 3-coordinate bis(phosphine) complex $(Et_3P)_2AuCl$, which is ionised in aqueous solution. Although this species is only poorly reactive towards thiol groups, it reacts with disulphides by virtue of the lability of one of its reducing PEt_3 ligands.[95] There reaction systems are illustrated in Figure 6. Interestingly, both

Et_3PAuCl and $(Et_3P)_2AuCl$ are active against rheumatoid arthritis when administered orally.[96,97]

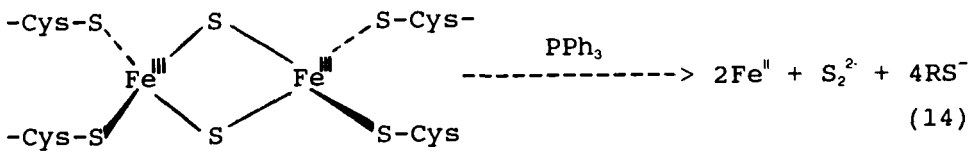
In view of the postulated beneficial, biologically 'site-specific' conversion of xanthine dehydrogenase to its oxidase from within appropriate 'target' cells, it is important to note that as well as having a very high affinity for thiol groups, Et_3PAuCl readily disrupts active iron/sulphide clusters such as those present in ferredoxins.

It reacts with the 2Fe,2S cluster in *Sp. Platensis* ferredoxin or the two 4Fe,4S clusters in *C. Pasteurianum* ferredoxin to yield bis(triethylphosphine) gold(I) sulphide ($(Et_3PAu)_2S$) and $Et_3PAuSCys$-adducts[54] (equation (13)).



$$2(Et_3PAu)_2S + 4(Et_3PAuSCys\text{-}) + 2Fe^{III} \qquad (13)$$

Moreover, release of PEt_3 from auranofin within cellular systems also has important implications for the conversion of xanthine dehydrogenase to its oxidase form *in vivo* since it has been reported that triphenylphosphine (PPh_3) also reacts with iron/sulphide clusters or persulphide species,[98] as depicted in equations (14) and (15).



(14)

$$S_2^{2-} + 4RS^- + 2PPh_3 + 3O_2 \longrightarrow 2Ph_3P{=}S + 2RSSR + 3O_2^{\cdot-} \qquad (15)$$

$$6H^+ \rightarrow 3H_2O_2$$

Interestingly, H_2O_2 is a by-product in this reaction system. Auranofin itself is only poorly reactive towards Fe/S clusters in iron-sulphur proteins.[54]

Notable also is the Et_3PAuCl-induced autoxidation of oxymyoglobin (MbO_2) and oxyhaemoglobin (HbO_2) which appears to involve the release of $O_2^{\cdot-}$. Although the mechanisms of MbO_2 and HbO_2 autoxidation are not fully understood, Wallace *et al.*[100] have described three types of pathway leading to the production of O_2, $O_2^{\cdot-}$ or H_2O_2. The Et_3PAuCl induction of free radical reactions involving dioxygen in this manner could also have important biological consequences.

Minta and Williams[55] explored the influence of both auranofin and Et_3PAuCl on the $O_2^{\cdot-}$ generating system of PMNL's. They found that $O_2^{\cdot-}$ production by PMNL's activated *in vitro* with phorbol-12-myristate-13-acetate (PMA) or N-formyl-methionyl-leucyl-phenylalanine (FMLP) was inhibited by auranofin or Et_3PAuCl in a dose-dependent manner. However, they also found that these therapeutic agents

had no effect on O_2^- production by the hypoxanthine/xanthine oxidase system, indicating that interaction of these gold complexes with essential thiol groups, Fe/S clusters or the sulphido ligand in the Mo=S system of xanthine oxidoreductase is limited to the dehydrogenase form of the enzyme. Both auranofin and Et_3PAuCl inhibited the specific binding of radiolabelled FMLP to PMNL membrane receptors, and did not appear to directly scavenge O_2^-.

Additionally, Corey *et al.*[101] found that both auranofin and a related complex ($Et_3PAuSCH_3$) were effective in quenching singlet oxygen ($O_2^1\Delta g$), an agent capable of causing the peroxidation of polyunsaturated fatty acids in cell membranes. The high atomic number of gold and its heavy-atom promoted spin-orbit coupling are thought to be responsible for the ability of these gold(I) complexes to quench $O_2^1\Delta$ g. Moreover, so-called 'chemical' quenching of $O_2^1\Delta$ g by Au(I) involving charge-transfer or alternatively, oxidative addition to form an unstable Au(III)-peroxy adduct which subsequently decomposes to Au(I) and triplet ('native') O_2, are also possibilities.

CONCLUSIONS

Although the thiolate ligands in 1:1 gold(I)-thiolate drugs may exert a therapeutic effect on release from gold(I) *in vivo*, it is clear that the gold(I) metal centre is an essential pre-requisite for the ability of these novel therapeutic agents to suppress active rheumatoid arthritis. As well as the postulated direct involvement of gold(O) in the biologically site-specific promotion of ROS formation, we also note that the ability of gold(I) to transport electron-donating thiolate ligands (plus Et_3P in the case of auranofin) to certain intra- or extracellular 'target' sites is likely to be an important mechanism in its control of oxygen radical-mediated oxidative damage. In view of this hypothesis, it is important to note that the oral administration of disodium thiomalate failed to act as a suitable alternative to the parenteral administration of its corresponding gold(I) complex, disodium aurothiomalate.[102]

Due to an inappropriate pharmacodisposition of certain anti-arthritic drugs and/or a poor blood perfusion rate, it is somewhat uncertain as to whether various NSAID's actually reach their site of action. Hence, there is a major requirement for the targeting of these therapeutic agents to their sites of action with minimal deleterious side-effects. The direction and control of both the availability and delivery rate of reducing equivalents by gold(I) may serve to aid the rheumatologist's quest for success at the therapeutic level.

Acknowledgements

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