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COMMENTARY

CELLULAR AND MOLECULAR PHARMACOLOGY OF AURANOFIN AND RELATED GOLD COMPLEXES

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Gold complexes have been employed as therapeutic agents in the treatment of rheumatoid arthritis for a number of years. The most commonly used chrysotherapeutic agents have been water soluble, parenterally administered gold(I) thiolates such as gold sodium thiomalate (Myochrysine) and gold sodium thioglucose (Solganol) [1–6]. Subsequently, a number of alkylphosphine gold complexes displayed antiarthritic activity when administered orally to adjuvant arthritic rats. Auranofin (AF§) [(1-thio-β-D-glucopyranose 2,3,4,6-tetraacetato-*S*)-(triethylphosphine)gold(I)] (Fig. 1) was among the most potent and efficacious of the compounds tested [7]. In addition, its activity in animal models was similar to that of parenterally administered gold sodium thiomalate [8]. Since these initial observations, a variety of studies have been reported describing the effects of AF in numerous experimental systems and its clinical efficacy based on data from more than 5000 patients with rheumatoid arthritis [6].

Previous reviews have described the effects of AF on immunologic processes [9–11], on various organs [12], in animals after administration of high doses [12], and in humans with rheumatoid arthritis [2]. AF has also been characterized pharmacokinetically [13–15]. However, little is known about the cellular and molecular mechanisms of action of AF. Nevertheless, during the past several years, substantial progress has been made towards elucidating the molecular actions of AF and other gold complexes. The objectives of this review are to summarize recent progress in the molecular pharmacology of AF, to place these studies in the context of chrysotherapy, and to identify a number of the key remaining questions.

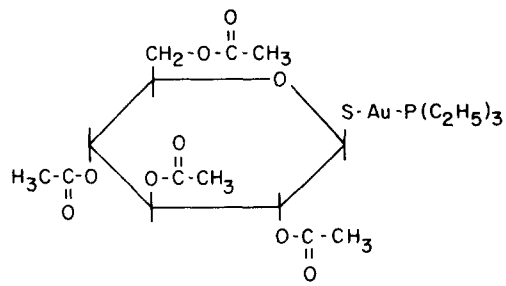

Fig. 1. Structure of auranofin.

Chemistry of AF

Very rarely is it possible to correlate drug actions clearly and directly with the basic chemical properties of the drug. As a rule, there is simply too little information to reduce the complexities of drug interactions with biological systems to the simplicities of basic chemical properties of the drug. However, recent data suggest that correlations between biological effects and chemical characteristics may be surprisingly direct and quite clear for AF. For this reason, a review of the chemistry of AF is appropriate.

Three basic properties of gold complexes are of importance: the oxidation state of the gold, the number and types of ligands coordinated to the gold, and the geometry of the ligands surrounding the gold [16]. These properties will determine the rate and the extent to which the gold will undergo oxidation-reduction and ligand exchange reactions. They will also affect the solubility of the complex and its partition between aqueous and non-aqueous phases.

Gold can exist in several oxidation states, the most common being Au(O), Au(I) and Au(III). In aqueous solutions, Au(III) tends to undergo reduction in the absence of stabilizing ligands. Au(I) generally forms linear, two coordinate complexes in which two ligands are attached on opposite sides of the gold nucleus. Au(III) favors the formation of square planar complexes, in which four ligands are arranged at 90° angles from one another relative to the gold. In order of preference, the ligands that bind to Au(I) are cyanide > thiolphosphines > thiol-esters > nitrogen, chloride ≫

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§ Abbreviations: AF, auranofin; Et_3P, triethylphosphine; TATG, tetraacetylthioglucose; LDH, lactate dehydrogenase; and IC_{50}, (i) concentration of drug required to inhibit 50% enzyme activity, and (ii) drug concentration required to reduce cell survival by 50% on the exponential part of the survival curve.

carboxyl groups. This suggests that thiols such as cysteine or glutathione will be principal targets for gold complexes and that thiol-containing ligands bind more stably than many other ligands [16]. Obviously, the micro environment of the potential ligand, e.g. a sulfhydryl in a protein, may affect reactivities to a significant extent.

AF contains gold in the +1 oxidation state. The gold nucleus is coordinated to triethylphosphine and tetraacetylthioglucose (TATG), ligands that have been reported to stabilize Au(I) in solution. In addition, these ligands make AF lipophilic and, thus, it may partition into cellular membranes easily. One would therefore predict that, in the absence of competing thiol groups or cyanide, AF should be reasonably stable, but that ligand exchange should occur in the presence of competing thiols. The ligands attached to gold in AF can be metabolized. Triethylphosphine can be oxidized, and the resulting triethylphosphine oxide may be toxic to cells. The TATG may be deacetylated and loss of the acetyl groups may result in a more hydrophilic compound [17]. Furthermore, unlike Myochrysine and Solganol, AF cannot polymerize in solution since phosphines are not bridging ligands [16].

Consequently, AF can be considered a relatively stable gold-containing compound that exists as a monomer in solution, is freely soluble in lipid membranes, and is likely to undergo ligand exchange reactions with cellular and extracellular sulfhydryl groups. Ligand exchange reactions may liberate TATG and triethylphosphine which may be metabolized further. Inasmuch as the gold has two potential ligating sites, cross-links between gold and available ligands may be formed in some macromolecules.

Interactions with biologically important molecules

Small molecular weight thiols. AF and other gold-containing complexes have been shown to interact with small molecular weight thiols such as glutathione and cysteine [18]. Inasmuch as glutathione is typically in the highest concentration of the small molecular weight intracellular thiols [19, 20], the most prevalent reactions within the cell are likely to be with glutathione. Low concentrations of low molecular weight cysteine and/or glutathione–gold complexes have been demonstrated in the plasma [21]. More recently, as will be discussed in more detail in a later section, the addition of extracellular glutathione has been shown to inhibit cellular association of AF. Addition of glutathione to the extracellular fluid of cells loaded with gold from AF was shown to enhance cellular efflux of the gold. These data provide additional evidence that glutathione binds to the gold in AF and that it may compete with cellular thiols for gold when present in the extracellular fluid [22].

Interactions with proteins. Although other reactions are possible, the principal reactions with proteins occur with sulfhydryl groups in amino acids such as cysteine. The sulfhydryl group in cysteine is more reactive than the thiolether in methionine [16].

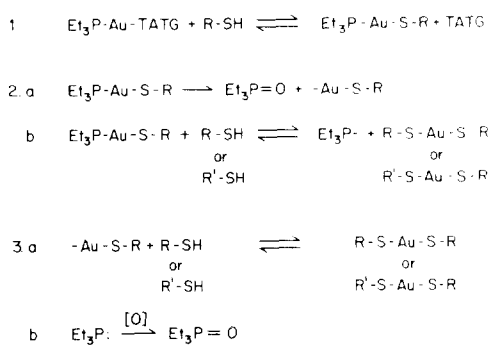


Fig. 2. Generic reaction sequence for auranofin and sulfhydryl-containing proteins. Reaction 1: The tetraacetylthioglucose group (TATG) is displaced by the reactive sulfhydryl group (SH) in the protein (R). Reaction 2a: The triethylphosphine (Et_3P) is oxidized while complexed with the gold protein (Au—S—R), releasing Et_3P=O which is volatile. Reaction 2b: Alternatively, another sulfhydryl group displaces the Et_3P group. The sulfhydryl group may be present in the same protein as in the initial reaction if the protein (R) contains more than one available sulfhydryl group. Alternatively, another protein (R^1) may donate a sulfhydryl. Reaction 3a: If the triethylphosphine is oxidized leaving the gold, the gold in the protein complex (—Au—S—R) has an available coordination site and may interact as above. Reaction 3b: If released via sulfhydryl group displacement, unoxidized triethylphosphine may be oxidized to the volatile triethylphosphine oxide Et_3P=O.

A generic reaction scheme can be proposed for the interactions of AF with proteins. The binding of AF to sulfhydryl-containing proteins proceeds via several steps as described in Fig. 2. In the first step (reaction 1), TATG is displaced by a competing thiol. The rate of this reaction is determined by the reactivity of the leaving group attached to gold (in the case of AF, TATG) and the reactivities and concentrations of competing ligands. Thus, one would predict that the rate of the first reaction would be greater for triethylphosphine gold(I) chloride (SKF 36914) in which the TATG is replaced with Cl, a better leaving group. Support for these concepts derives from studies with albumin and whole cells employing NMR spectroscopy and triply labeled AF [23, *].

The second step in the reaction sequence results in the displacement of the triethylphosphine moiety. The phosphine–gold bond is more stable than the TATG–gold bond and thus the first ligand exchange involves displacement of the TATG. It is unclear whether the triethylphosphine is oxidized prior to or after being displaced (reaction 2a). In either event, the sequence ultimately results in a gold molecule doubly ligated with sulfhydryl groups and triethylphosphine oxide.

Reactions 2b and 3a suggest that the gold in AF may form coordinate bonds with two thiols in the same protein or with a single thiol in each of two proteins. This "cross-linking" reaction is important in several regards. First, once cross-linked, the gold is much less likely to exchange to a new protein or pair of proteins than when linked via a single sulfhydryl group since both sulfhydryls in the bidentate complex must be displaced for the gold to

* D. Ecker, J. Hempel, R. Kirsch and S. T. Crooke, manuscript submitted.

migrate to a new protein or protein pair in the bidentate complex. Second, the formation of intramolecular cross-links may result in significant conformational changes for the affected protein. Conversely, the formation of intermolecular cross-links may radically alter the function of two proteins or such characteristics as membrane fluidity. In reactions in solution, it is likely that intermolecular cross-links will occur only at high protein concentrations. However, in reactions that occur in semiliquid environments, such as membranes, intermolecular cross-links may occur more frequently because of the relatively fixed geometry maintained and the relatively high concentrations of sulfhydryl-containing proteins [24].

Albumin. Albumin is a serum protein that normally contains only one reduced sulfhydryl (cysteine 34) [25]. Because of the relevance of thiols to the pharmacokinetics of AF and the potential to use albumin interactions as a model for protein–AF interactions, a number of studies have been performed. Elegant studies by Shaw and colleagues [23] using both AF and SKF 36914, the chloro analog of AF, have shown that these compounds interact with albumin as would be predicted by the generic model. At high albumin concentrations, intermolecular cross-links are formed. At high chlorotriethylphosphine gold to albumin ratios, secondary interactions between gold and non-sulfhydryl groups in albumin may occur. These secondary interactions do not occur with AF. Similar data have been generated employing radiolabeled AF.*†

Metallothionein. Metallothioneins are evolutionally conserved proteins that are important in heavy metal detoxification [26, 27]. In mammalian cells two metallothioneins have been identified. The genes for metallothionein have been sequenced in several species and shown to be highly conserved [28–30]. Metallothionein contains seven clusters of cysteines, and it is through these clusters that heavy metals are bound. Thus, metallothionein is important not only because of its biological relevance but also since it may serve as a model for proteins that contain multiple sulfhydryl groups.

Several heavy metals bind to and induce increased production of metallothionein including cadmium, copper, zinc, silver, cobalt and mercury [31]. Schmitz *et al.* [32] demonstrated that gold from sodium gold thiomalate could replace zinc or cadmiun from metallothionein. Moreover, when rats were administered gold, the gold coeluted with the metallothionein fraction isolated from liver and kidney [32]. Relatively little is known about the chemical mechanisms of the interactions other than that multiple gold molecules may bind per molecule of metallothionein and the binding appears to occur in the cysteine clusters (for review see Ref. 33).

Enzymes. A large number of enzymes have been reported to be inhibited by various parenterally administered gold complexes [33]. Most of these have been shown to have sulfhydryl groups necessary for activity. However, lysozyme was also shown to be inhibited by sodium gold thiomalate [34]. Furthermore, for most enzymes studied with a variety of gold complexes, the inhibitory concentrations were in the millimolar range. Thus, it is unlikely that such effects are important *in vivo* at therapeutically relevant gold concentrations.

Considerably less is known about the effects of AF on various enzymes. AF was reported to inhibit cathepsin B and cathepsin D in lysates from mouse macrophages with IC_{50} values of 5.4×10^{-4} M and 5.4×10^{-5} M respectively (in the same lysates). No inhibition of β-glucuronidase or LDH was observed [35]. In our laboratory we have studied effects of AF and other gold complexes on purified DNA polymerases of cells and human herpes viruses [36]. AF inhibited DNA polymerase α, β and herpes simplex virus type-1 induced DNA polymerase.

In contrast, no inhibition of DNA polymerase of herpes simplex virus type-2 nor Epstein Barr virus was observed. The inhibition induced by AF and other gold(I) complexes was noncompetitive with regard to DNA and the nucleoside triphosphate substrates, and most likely resulted from interactions with sulfhydryl groups within the DNA polymerase. The K_i for AF and SKF 36914 of DNA polymerase α was 27.8 and 0.9 μM respectively. For gold(III) complexes, the mechanism of inhibition was more complex because of potential interactions with DNA as well as the enzyme.

High concentrations (2×10^{-4} M) of AF have also been shown to inhibit Na^+,K^+-ATPase activity in isolated enterocytes [37]. The authors suggested that this may be a mechanism by which AF induces diarrhea *in vivo*. However, it is not clear that such concentrations are attained in the gastrointestinal tract.

Studies in our laboratory on phospholipases present in mouse macrophages and purified from *Bacillus cereus* suggest that AF may alter the activities of these enzymes. Specifically, the activity of phospholipase C was increased in sonicates of macrophages and purified phospholipase C at concentrations of 0.1 to 0.13 μM AF and 1 to 20 μM AF respectively. These studies suggest that AF may activate phospholipase C at therapeutically relevant concentrations in the macrophage.‡

Interactions with DNA. That gold complexes might interact with DNA was suggested by a number of factors including the similarities with platinum-containing compounds [38]. However, studies employing a variety of techniques including agarose gel electrophoresis, the "snap back" ethidium bromide assay, circular dichroism and assays for cleavage demonstrate no interaction of AF with DNA [36, 37]. This is due to the relative affinities of gold for TATG and phosphine moieties versus the nitrogens in DNA. Inasmuch as gold much prefers the thiol and phosphine ligands in AF to a much greater degree than potential sites on DNA, no ligand exchange takes place. Replacement of the thioglucose with a more active leaving group results in compounds that readily bind DNA. For example, Fig. 3 shows an agarose gel electrophoretic assay of BR322 DNA

* R. M. Snyder and S. T. Crooke, unpublished results.
† D. Ecker, J. Hempel, R. Kirsch, and S. T. Crooke, manuscript submitted.
‡ R. M. Snyder, C. K. Mirabelli, M. Clark and S. T. Crooke, manuscript submitted.

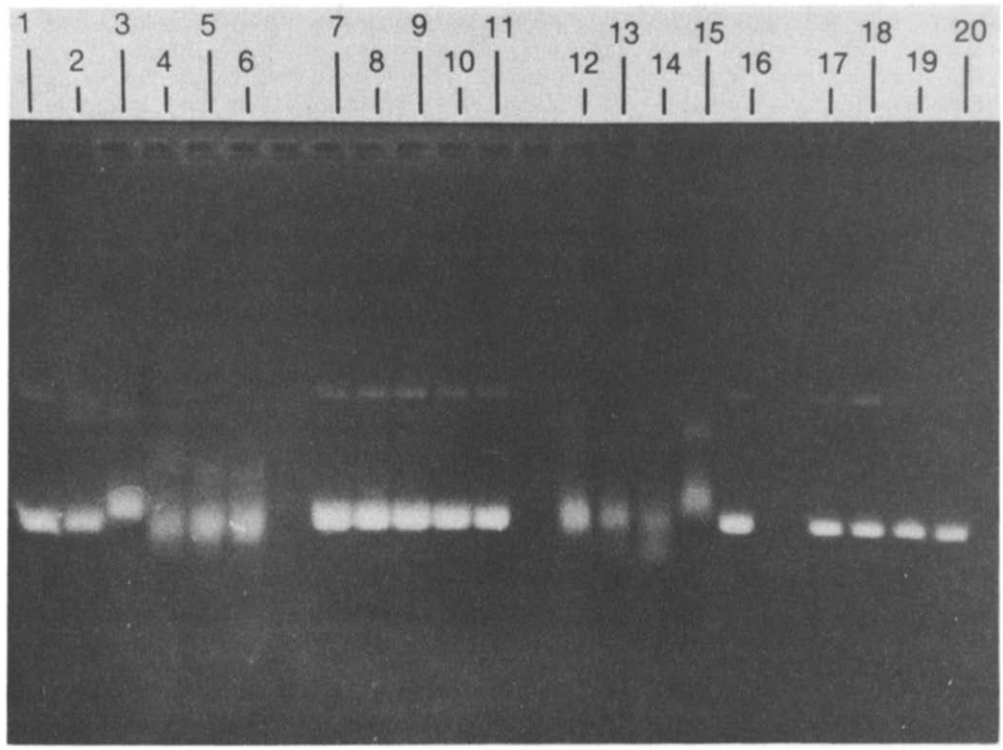
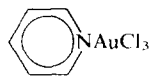


Fig. 3. Conformational changes of pBR322 DNA affected by auranofin, SKF 36914 and $HSGlu(OAc)_4$. Lanes 2–6, 7–11 and 17–19 contain DNA treated with SKF 36914, $HSGlu(OAc)_4$ and auranofin respectively. Lanes 12–16 contain DNA treated with a combination of both SKF 36914 and $HSGlu(OAc)_4$. The drug concentrations are expressed in (drug/nt) ratios as follows: lanes 1 and 20 (0) controls; 2 (0.5); 3 (1.0); 4 (2.0); 5 (4.0); 6 (8.0); 7 (0.5); 8 (1.0); 9 (2.0); 10 (4.0); 11 (8.0); 12–16 each were treated with SKF 36914 at a drug/nt ratio of 8 and $HSGlu(OAc)_4$ at: 12 (0.5); 13 (1); 14 (2); 15 (4); 16 (8); 17 (1); 18 (2); and 19 (8). From Ref. 40.

Table 1. Binding of phosphino gold complexes to pBR322 DNA as measured in the agarose gel electrophoresis assay*

No.	$[CH_3CH_2]_3P—Au—X$ X	DNA binding† 25 mM Tricine‡ pH 7.1	25 mM Sodium borate§ pH 9.5
1	$SGlu(OAc)_4$	neg	neg
4	$SCH(CH_2COOH)COOH$	neg	neg
5	SCH_3	neg	neg
6	CH_3	neg	neg
7	CN	neg	neg
8	$P[CH_2CH_3]_3^+ Cl^-$	neg	neg
3	Cl	neg	pos
9	Br	neg	pos
10	SCN	neg	pos
11	Cl_3	pos	pos
12	Br_3	pos	pos
2	Compound 	pos	pos

* From Ref. 40.

† As determined by agarose gel electrophoresis; neg. no effect on electrophoretic mobility of DNA at drug/nt ratios ≤ 5; pos. effect on electrophoretic mobility of DNA observed at drug/nt ratio ≤ 5.

‡ Incubation of gold complexes with DNA was performed in 25 mM tricine buffer, pH 7.1, and 25 mM $NaNO_3$ at 37° for 5 hr.

§ Incubation of gold complexes with DNA was performed in 25 mM sodium borate, pH 9.5, and 25 mM $NaNO_3$ at 37° for 5 hr.

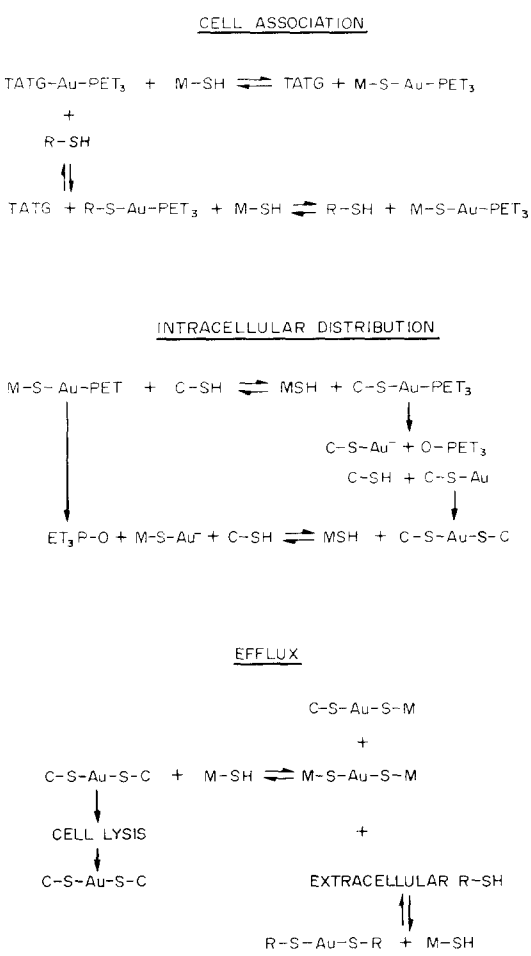


Fig. 4. Model for cell association, intracellular distribution, and efflux of auranofin. From Ref. 22.

treated with AF, triethylphosphine gold(I) chloride (SKF 36914) and tetracetylthioglucose complexes. As can be seen, AF did not bind to DNA, but SKF 36914 in which the TATG is replaced with Cl readily binds. The addition of TATG to SKF 36914 in the presence of DNA inhibited the binding as TATG stabilizes the gold relative to exchange with nitrogens in DNA. Table 1 shows the importance of pH and buffer to interactions of these compounds with DNA and additional structure–activity relationships.

The interactions of gold(III)-containing complexes with DNA differed in several regards from those of gold(I) complexes. The binding of gold complexes to DNA was affected primarily by the ligands attached to the gold. When the gold is associated with two reactive leaving groups, the complexes produce inter- and intrastrand cross-links similar to those produced by cisplatinum [39]. That the effects of gold(III) compounds on DNA are similar to those of platinum(II)-containing compounds is not surprising as both are square planar complexes in which the metal is coordinately ligated to four substituents. Thiol-containing reagents inhibit the binding of gold complexes to DNA. Furthermore, when gold complexes that produce DNA cross-links are allowed to bind to DNA and then are treated with 2-mercaptoethanol, the gold is removed from DNA and single-strand breaks are produced [40, 41]. The DNA breakage produced by gold complexes is independent of oxygen, suggesting that oxygen free radicals are not involved. It is, however, affected by DNA topology. Superhelical DNA is a sensitive substrate, whereas no breakage or linear DNA is observed. Thus, the breaks may result from removal of gold involved in intra- and/or interstrand cross-links that have induced a high degree of torsion in the relatively constrained structure of superhelical DNA [39].

Cellular association, uptake, and distribution

Studies in our laboratory have demonstrated that the gold moiety in AF is taken up, distributed, and eliminated from cells via a sequential thiol exchange process [22]. A model explaining the processes has been proposed and is shown in Fig. 4. This model predicts that the rate-limiting step for cellular uptake is the displacement of tetraacetylthioglucose by membrane-associated thiol groups. Indeed, recent studies (to be published) have shown that the energy of activation (E_a) for cell association correlates with the reactivity of the leaving group. Thus, SKF 36914, triethylphosphine gold chloride, more rapidly associates with cells than AF. Furthermore, several additional steps may involve oxidation of the phosphine. Recent studies have confirmed that inhibition of oxidation of the phosphine does not alter association of gold with cells, but does affect cellular distribution and efflux. The model suggests that gold association, distribution, and efflux will be defined solely by the concentrations and reactivities of thiol-containing compounds in the membrane, cytosol, nucleus and extracellular fluid and is independent of metabolic energy. The model also suggests that intra- and intermolecular cross-links may be produced in proteins by gold(I)-containing compounds. Whether these cross-links occur will be determined by the relative concentrations of various proteins and the number of thiol groups they contain. The production of cross-links depends on the exchange of phosphine which may be dependent on oxidation of the phosphine and, once produced, such cross-links will tend to localize the gold to the site of the cross-link as exchange of both ligands would be required to allow the gold to migrate to new sites [22].

One report has suggested that, when AF is incubated with everted intestinal sacs, AF is deacetylated and absorbed through the intestinal wall with sulfhydryl and phosphine bonds intact [17]. However, the conclusions that this occurred were based on the fact that addition of TATG to the sac contents reformed AF. This would be expected if the compound that crossed the mucosa of the everted sac was Et_3P—Au—. In fact, if the Au—S bond were intact as suggested (Et_3P—Au—TG), extremely high concentrations of TATG would be required to displace the thioglucose and thus regenerate AF.

Cytotoxicity

AF is cytotoxic [39–45] against a variety of cells *in vitro*. The IC_{50} for cytotoxicity for AF is usually in the 1–10 μM range [21, 42, 45]. For example, Fig. 5

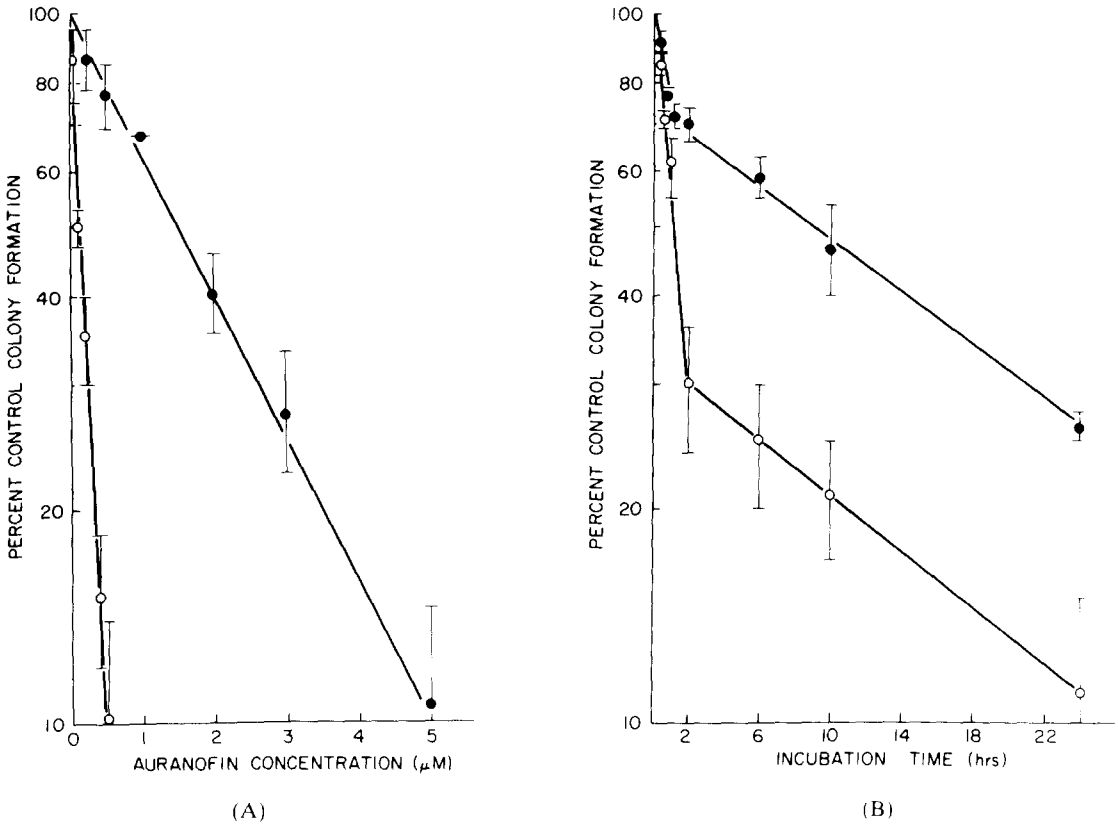


Fig. 5. Cytotoxic activity of auranofin against B16 melanoma cells as measured in the monolayer clonogenic assay: (A) colony formation as a function of auranofin concentration following a 2-hr exposure to drug in the presence (●) and absence (○) of 10% FCS; (B) colony formation as a function of the exposure time of cells to auranofin at 1 (●) and 4 (○) μM. Points, mean of three assays; bars, S.E. From Ref. 42.

shows the effects of AF on B16 melanoma cells *in vitro*. In all cells studied to date, AF induces a monoexponential concentration versus cell survival curve, suggesting comparable sensitivity of all cells in the assay to the drug. Moreover, as observed in using B16 melanoma cells, the cytotoxicity is independent of cell cycle phase. The cytotoxicity of AF and all gold complexes tested to date correlates with the total gold associated with cells. Gold sodium thiomalate, for example, is reported to be minimally cytotoxic. This, we believe, is due to polymerization of this compound resulting in less gold being cell associated *in vitro*. When the cytotoxicities of AF and gold sodium thiomalate are normalized to the total cell associated gold, they are equally potent as cytotoxins. The addition of extracellular thiol-containing compounds such as albumin, fetal calf serum, or glutathione reduces the potency of AF and other gold complexes because they bind the gold, reducing the cell-associated gold [21, 42]. AF and other gold complexes inhibit DNA, RNA and protein syntheses at approximately equivalent concentrations that are in excess of cytotoxic concentrations, and cell death is relatively rapid. These and other data suggest that the cytotoxicity of AF and many other gold complexes may be mediated via effects in the plasma membrane [39].

Although AF is a relatively potent cytotoxic agent *in vitro* against almost all cell types studied, it has a very limited spectrum of activity against tumors *in vivo*. In fact, the only experimental rodent tumor against which AF has been shown to have a significant therapeutic index is P388 leukemia implanted intraperitoneally [46, 47]. Thus, AF does not possess the degree of antitumor activity required for development as an antineoplastic agent, as was suggested previously [45, 46].

In contrast to AF, a number of other gold complexes have been shown to be potent cytotoxins *in vitro* and to have significant *in vivo* antitumor activity [44, 47]. Although potent cytotoxicity *in vitro* does not necessarily predict for *in vivo* antitumor activity, gold compounds lacking *in vitro* cytotoxicity have proven inactive against experimental *in vivo* tumors [47]. Furthermore, *in vitro* cytotoxic potential correlates reasonably well ($r = 0.80$) with the maximal tolerated dose *in vivo*. In the nearly 100 compounds studied, *in vivo* antitumor activity was optimal when gold(I) was ligated with a substituted phosphine and a thio sugar. Modifications of the acetyl groups of the thioglucose in AF resulted in no significant change in *in vitro* or *in vivo* activities [47].

Mechanisms of resistance to cytotoxicity

In our laboratory, several cell lines have been developed that are resistant to AF. One AF resistant

cell line derives from a Chinese hamster ovary cell line resistant to cadmium due to a 6-fold amplification of metallothionein genes [48].

When these cells were exposed to increasing concentrations of AF, subclones were produced that were resistant to 7.5 and 15 μM AF (approximately 15-fold less sensitive than the parent cell line). In this cell line, resistance to AF was clearly mediated via increased production of metallothionein in the presence of AF. This was demonstrated to be due to increased transcription of metallothionein genes [49].

The resistance induced by AF in Chinese hamster cells was much less stable than that induced by cadmium. The metastability of the AF-induced resistance induced by AF was shown to be due to the fact that the half-life of metallothionein induced by AF was significantly shorter than that induced by cadmium. No differences in the stability of the amplified genes, nor the extent of transcription nor stability of the mRNA were observed.* The induction of metallothionein by AF was dependent on gold, and other gold-containing complexes also induced resistance via induction of metallothionein [46]. In contrast, the anticancer agent *cis*-diaminedichloroplatinum II did not induce metallothionein. Nor were cells resistant to either agent cross-resistant to the other.†

Although metallothionein may mediate resistance to AF and related compounds, other mechanisms may also mediate resistance, as we have observed the induction of other proteins in addition to metallothionein.*

Immunologic effects

AF and several related gold complexes were shown to inhibit superoxide production by mouse peritoneal macrophages induced by phorbal myristic acetate [50, 51]. At a concentration of 2 μM, AF inhibited production of interleukin 1 and interleukin 2 by macrophages and inhibited conconavalin A induced mitogenesis in T-lymphocytes [52].

Similarly, macrophages exposed to AF were inhibited from phagocytosis of *Candida albicans*. In this study, differences in potency between AF and gold sodium thiomalate were reported as well. However, it is not clear to what extent the gold sodium thiomalate had undergone polymerization [53].

Conclusions

During the past several years, significant progress in understanding the cellular and molecular mechanisms of action of AF and related gold complexes has been made. Based on results from studies in our laboratory and others, we can conclude that gold is an essential component of the molecule and that the ligands associated with the gold modify the reactivity of the complex, lipophilicity, steric limitations for interactions with macromolecules, and other characteristics. The initial reactions in which AF is involved result from the displacement of TATG which is attached much less stably than the triethylphosphine moiety to the gold. Thus, changes in the ligands such as replacement of the TATG with a more active leaving group, e.g. chloride, result in more reactive compounds.

Although AF and related compounds may react with a number of potential ligands other than thiols, the reactions of physiologic importance are likely to be those involving thiols. Thus, AF and analogs may have effects on a variety of enzymes. The enzymes likely to be affected at lowest AF concentrations are those with sulfhydryl groups that are important to the activity of the enzyme either because of direct involvement in the catalytic site or allosteric effects. Whether the activity of an enzyme is enhanced or inhibited by AF will depend on the accessibility and roles of each of the important sulfhydryls. For example, at relatively low AF concentrations, phospholipase C activity is enhanced. This may be due to interaction with a sulfhydryl group on an inhibitory subunit or modulatory protein associated with the enzyme. Alternatively, it may be due to allosteric effects enhancing the activity of the enzyme.

Cell association, distribution, and efflux of AF are due to a sequential ligand exchange process, a sulfhydryl shuttle. This model is particularly useful in that it explains a variety of phenomena in relatively straightforward chemical terms. Moreover, it is probably a generic mechanism for cell uptake as any agent that can interact with sulfhydryls could undergo such a process. In fact, any compound capable of multiple displacements with any set of ligands could undergo this process. For example, *cis*-diaminedichloroplatinum II might undergo a similar ligand exchange process with various amino groups in cells. Recent studies demonstrate that this may indeed occur [54].

AF as well as other gold complexes are potent cytotoxic agents. Although the mechanism of cytotoxicity of AF is not precisely defined, the data allow us to propose that the cytotoxicity derives from interactions with sulfhydryl groups in specific cellular proteins. A number of lines of evidence suggest that the key lesions may be intra- and intermolecular cross-links in critical membrane-localized proteins. We are currently investigating this possibility. Although gold complexes bind to DNA, DNA is unlikely to be a primary target. For some gold compounds, chromatin and chromatin-associated proteins may be important targets, however.

AF resistance may be induced in cells exposed to AF. In cells we have studied, the resistance is clearly mediated, at least in part, by metallothionein gene activation. AF-induced resistance is much shorter lived than cadmium-induced resistance, and this has been shown to be due to a dramatically shorter half-life of metallothionein induced in the presence of AF and other gold complexes. Cross-resistance to platinum-containing compounds is highly unlikely in cells resistant to gold-containing compounds as the mechanisms of resistance appear to be quite different.

Obviously, many critical questions remain to be answered. Many relate to the cytotoxicity of AF. Are intra- and intermolecular cross-links produced? If so, in what proteins are these lesions most important, and are these proteins in the plasma membrane?

* B. Monia, T. Butt and S. T. Crooke, unpublished observations.

† R. Johnson and C. K. Mirabelli, unpublished observation.

AF induces metallothionein. What are the genetic mechanisms involved? Why is AF-induced metallothionein less stable than that induced in the presence of cadmium or zinc? Are there other mechanisms of resistance to AF and analogs?

AF contains a phosphine moiety. This forms a very stable coordinate bond with the gold. Is the phosphine oxidized before being displaced or after? Perhaps in proteins with a high concentration of sulfhydryl groups such as metallothionein, the formation of the first adduct by displacement of the TATG then favors displacement of the phosphine moiety because of the high density of sulfhydryl groups. In proteins with fewer sulfhydryl groups, perhaps oxidation of the phosphine may be required before displacement. What is the role of the phosphine and phosphine oxide moieties in the cytotoxicity and other activities of AF, and do processes such as lipid peroxidation play a role in oxidizing the phosphine, resulting in a gold molecule capable of cross-linking reactions?

What is the role of cytotoxicity in the antiarthritic activity of AF and analogs? We initiated our studies based on the hypotheses that AF is cytotoxic and that it is relatively more cytotoxic to abnormal cells involved in the initiation or propagation of rheumatoid arthritis, e.g. abnormal macrophages. At present we can conclude that AF is cytotoxic to cells involved in the immune system. We cannot conclude that this is essential to the activity of AF as an antiarthritic. Nor can we draw any conclusions about the relative importance of activities of AF in immunologically active cells versus humoral effects or effects in the afflicted joints.

These and many other questions regarding the biochemical and pharmacological mechanisms of coordinated gold complexes await additional studies.

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