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Inhibition of lysosomal cysteine proteases by chrysotherapeutic compounds: a possible mechanism for the antiarthritic activity of Au(I)

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Abstract—Although Au(I) complexes have been used to treat rheumatoid arthritis for over 75 years, their mechanism of action is still poorly understood. A family of enzymes responsible for joint destruction in rheumatoid arthritis, the cathepsins, has been discussed as a possible biological target of Au(I). In this study, inhibition of the cathepsins by known Au(I) drugs and related compounds was investigated. The compounds tested inhibited cathepsin activity with IC_{50} values as low as 600 nM. More typical IC_{50} values were in the 50–200 μM range. Although the gold complexes are not extremely potent cathepsin inhibitors, it is likely that this inhibition is biologically relevant given the high concentrations of Au(I) in the serum and joints of patients undergoing chrysotherapy. While it is likely that there are multiple targets of Au(I) in vivo, inhibition of the cathepsins would provide protection against the joint destruction that is a hallmark of rheumatoid arthritis and is one possible mechanism for Au(I) antiarthritic activity.

Throughout history, gold has been peddled as a cure for many different ailments. The earliest putative therapeutic use of gold can be traced to China in 2500 BC.[1] Whether worn as a protective amulet or ingested as a colloidal suspension, the therapeutic effects (not to mention actual gold content) of gold preparations were often dubious at best. In 1890, the discovery that gold cyanide could inhibit the growth of *Mycobacterium tuberculosis* led to a systematic investigation of the therapeutic properties of gold.[2] The successful treatment of rheumatoid arthritis by injected Au(I) compounds was first reported in the 1930s.[3] Surprisingly little progress has been made in chrysotherapy over the years; sodium aurothiomalate (myochrysin) and sodium aurothioglucose (solganol), two of the compounds first reported in 1935, are still in use in the clinic today. In the 1970s, a new compound, triethylphosphine(2,3,4,6-tetra-O-acetyl-β-1-D-thiopyranosato-S)gold(I) (Auranofin, Fig. 1), was introduced.[4] The advantages of this compound include oral administration rather than intramuscular injection and a lower incidence of side effects as compared to the other two compounds.[5] Despite dramatic results in some cases, gold is currently used as a drug of last resort because of intolerable side effects experienced by nearly half of the patients.[6,7]

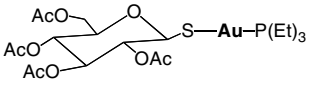

Figure 1. The molecular structure of auranofin.

The pharmacokinetics of chrysotherapeutic compounds has been well studied over the years. Upon ingestion or injection, the Au(I) ligands readily dissociate and exchange with biological thiolates. Ligand exchange occurs most rapidly with halide ligands, followed by thiolate ligands. Phosphine ligands exchange more slowly, but are still cleared from the system much more quickly than the Au(I) itself.[2,8] All of the ligands used in chrysotherapy have much shorter systemic half-lives than gold; the ligands are largely excreted within 24 h, while the half-life of gold in the body is 20 days.[8] During the course of treatment, gold builds up in the serum of patients, reaching concentrations between 5 and 25 μM.[9] In serum, Au(I) binds preferentially to thiolate ligands with low pK_a values, such as cys34 of albumin, which appears to be the main carrier of Au(I) in the body.[1,10] Although Au(I) is distributed throughout the body, it accumulates in the lysosomes and the synovium.[7,11]

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Once the gold has been absorbed into the bloodstream, its mechanism of action is not clearly understood. Many pathways have been proposed, and it is likely that therapeutic activity is derived from a combination of biological effects. Gold may act at the cellular level by inhibiting T cell proliferation and modulating the immune system.[12] Gold has also been shown to induce mito- chondrial membrane permeability[13] and to inhibit osteoclastic bone resorption.[14] At the level of transcription, gold may inhibit the activation of NF-κB, a transcription factor responsible for the production of TNF and other key inflammatory cytokines.[15,16] Gold may also interfere with homeostasis of the copper(I) ion, binding to Cu(I)-responsive transcription factors, and other Cu(I) trafficking proteins.[17]

The ability of Au(I) to undergo facile ligand exchange, especially with biological thiolates with low pK_a values, indicates that gold may inhibit the activity of several different enzymes involved in inflammation and joint destruction.[8,18] Proteolytic enzymes responsible for collagen, bone, and extracellular matrix degradation in rheumatoid arthritis have been investigated as possible targets of Au(I) antiarthritic activity. Au(I) inhibits human neutrophil collagenase, a zinc-dependent protease, by replacing zinc from a zinc-binding site distal to the active site.[19] In addition, Au(I) has been reported to inhibit some serine-dependent proteases through an unknown mechanism.[20]

Of the enzymes involved in joint inflammation and destruction in rheumatoid arthritis, the cathepsin family of lysosomal enzymes is of special interest because, in addition to their ability to cause bone resorption and extracellular matrix degradation, they are cysteine-dependent proteases and contain an activated cysteine in their active sites. The cathepsins are particularly intriguing targets because they are prevalent in the lysosomes and in the inflamed arthritic joints, two sites where Au(I) readily accumulates in the body. Coordination of Au(I) to the active site cysteine should result in inhibition of enzyme activity, which in turn would be expected to provide protection for the arthritic joints. Previous studies of the ability of chrysotherapeutic agents to inhibit the cathepsins have largely employed cell lysates and impure cathepsin preparations.[21–24] Although inhibition of cathepsin activity has been observed, conflicting accounts of the potency, reversibility, and mode of inhibition have been published. A brief report on the interaction of Au(I) with pure cathepsins described inhibition at low micromolar concentrations of Au(I).[25] In this study, we investigated the ability of a series of metal complexes to inhibit purified cathepsins. Au(I) complexes with known therapeutic properties were tested as well as therapeutically inactive Au(I) and Ag(I) complexes.

The ability of gold complexes to inhibit members of the cathepsin family of proteases was investigated.[26,27] As shown in Figure 2 and Table 1, the different gold complexes investigated inhibited the cathepsins to varying degrees. In all cases, $NaAuCl_4$ showed the most potent inhibition, followed by myochrysin and auranofin. In contrast, as shown in Figure 3, the biologically inactive $Au(dppe)_2Cl$ complex[28] shows no inhibition of the cathepsins up to the limit of complex solubility. The observed differences in potency are not due to an inherent difference between the inhibitory properties of Au(I) and Au(III), as the Au(III) is readily reduced to Au(I) by DTT under the conditions of the experiment. Rather, the variance in IC_{50} values observed for the different gold complexes is consistent with the differential labilities of the gold ligands. As mentioned earlier, halide ligands are more labile than thiolates, which in turn exchange more readily than phosphine ligands.[8] Because ligand exchange is required for the gold to bind to the enzyme, it is not surprising that the IC_{50} values for this series of gold complexes follow this trend, with the biologically inactive complex $Au(dppe)_2Cl$ containing bidentate chelating phosphine ligands showing no reactivity at all. Sodium tetrachloroaurate, myochrysin, and auranofin all inhibit the cathepsins. The most potent inhibition is observed when $NaAuCl_4$ is added to hlCatB, with an IC_{50} value of 600 nM.

Table 1. Comparison of IC_{50} and K_i values for the cathepsins with different gold complexes

Enzyme	Inhibitor	IC_{50} (μM)	K_i (μM)
Paramecium tetraurelia cathepsin L	Auranofin	>250	500 ± 100
	Myochrysin	250	240 ± 30
	$NaAuCl_4$	40	56 ± 7
Human liver cathepsin L	Auranofin	>250	>1000
	Myochrysin	90	110 ± 15
	$NaAuCl_4$	60	48 ± 20
Human liver cathepsin B	Auranofin	250	440 ± 250
	Myochrysin	150	180 ± 40
	$NaAuCl_4$	0.6	4.2 ± 3

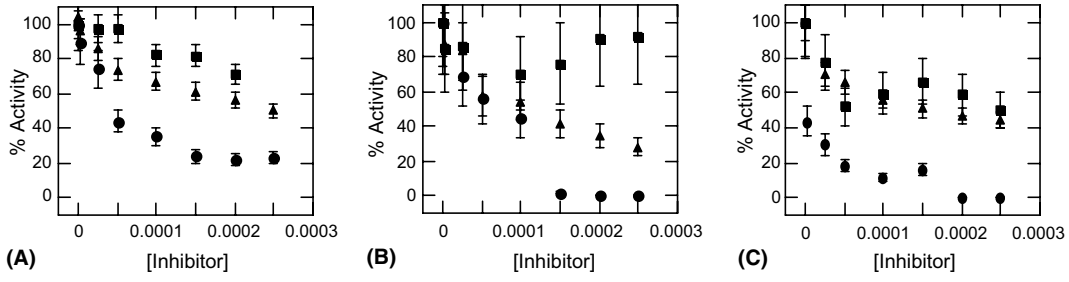

Figure 2. IC_{50} curves for (■) auranofin, (▲) myochrysin, and (●) $NaAuCl_4$ inhibition of (A) pCatL, (B) hlCatL, and (C) hlCatB.

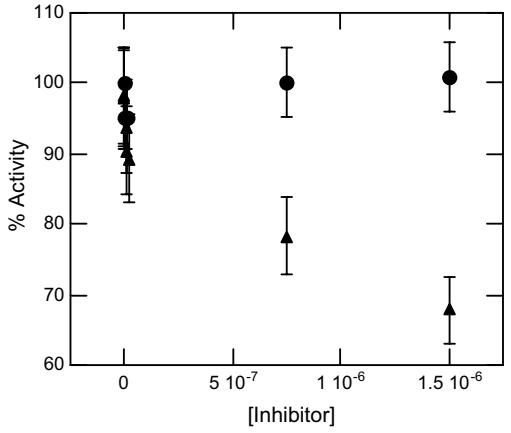

Figure 3. Comparison of IC_{50} curves for (●) $Au(dppe)_2Cl$ and (▲) myochrysin inhibition of pCatL.

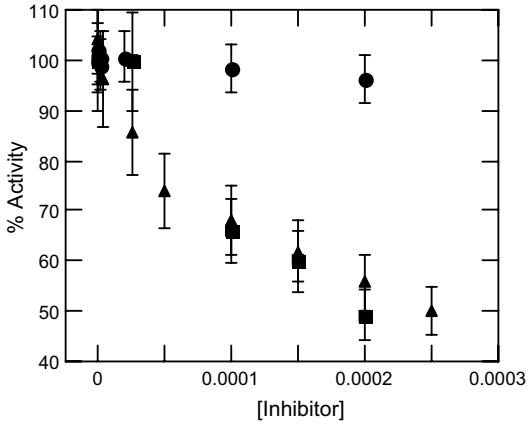

Figure 4. Comparison of IC_{50} curves for (●) thiomalic acid, (■) NaAg(thiomalate), and (▲) myochrysin inhibition of pCatL.

The inhibitory properties of similar metal complexes and the thiomalate ligand alone were also investigated. The results are shown in Figure 4.[30] Sodium silver thiomalate was investigated as an analog of myochrysin.[20] This complex did not inhibit the cathepsins under the standard reaction conditions, but when a buffer containing KNO_3 rather than NaCl was used, inhibition similar to that displayed by myochrysin was observed. Because of the insolubility of silver chloride and the prevalence of the chloride ion in the body, enzyme inhibition by Ag(I) is unlikely to have any biological relevance. Of greater interest is the result that the thiomalate ligand is unable to inhibit the enzyme. This is in line with results of other researchers who have found that it is the gold ion itself, not the ligands, that is necessary for therapeutic activity.[4,29]

The mode of inhibition was investigated, and results are tabulated in Table 2.[31] By varying both substrate and inhibitor concentration, we found that the K_m of the reaction is not dependent on auranofin concentration. Similar results were obtained using myochrysin as the inhibitor. These data are consistent with noncompetitive

Table 2. K_m values for pCatL in the presence of varying concentrations of auranofin

[Auranofin]	K_m values (μM)
300 μM	49 ± 20
30 μM	47 ± 20
3 μM	32 ± 15
300 nM	27 ± 15

inhibition by the gold complexes, which has been observed by other researchers.[24] Furthermore, in noncompetitive inhibition, K_i is equal to IC_{50} for a given substrate, and can be calculated from a plot of the initial rate of reaction as a function of inhibitor concentration (Eq. 1)[32] The K_i values for the interaction of each gold complex with the three cathepsins were determined according to Eq. 1 and are tabulated in Table 2. The K_i values closely mirror the IC_{50} values obtained from the data in Figure 2.

$$v_i = \frac{V_{max}}{\left(1 + \frac{K_m}{[S]}\right)\left(1 + \frac{[I]}{K_i}\right)} \quad (1)$$

Intuitively, one would expect the Au(I) to coordinate to the active site cysteine in the enzyme, abolishing activity. This model is not inconsistent with noncompetitive inhibition, as both Au(I) and peptide substrate could bind in the active site. Proteolytic substrates recognize and bind their substrates through amino acid recognition sites adjacent to the active site. Au(I) coordination to the active site cysteine would not occlude these adjacent binding pockets, allowing both substrate and inhibitor to occupy the active site simultaneously. Work is currently underway in our laboratory to identify the site of the Au(I)–cathepsin interaction.

Contrary to reports in the literature,[24,25] Au(I) cathepsin inhibition was not readily reversible upon addition of excess thiolate. The ability of both thiomalate and glutathione to reverse Au(I) inhibition was investigated. After a 1 h preincubation of myochrysin and cathepsin, no increase in activity was observed upon addition of thiolate. It is possible that the source of enzyme may underlie the observed discrepancy; these studies were done using highly active purified enzyme while previous studies were done on unpurified cell extracts. Perhaps the impure enzymes were not fully activated prior to the previously reported inhibition experiments and that the additional activity observed after addition of glutathione was due to activation of uninhibited enzyme rather than a reversal of the Au(I) inhibition of the enzyme.

Au(I) is a moderately potent, noncompetitive inhibitor of the cathepsin family of lysosomal cysteine proteases in vitro. Since therapeutically relevant complexes inhibit the cathepsins and therapeutically inactive compounds do not, the assay for cathepsin inhibition reported herein may be useful as a model for the discovery of novel complexes with therapeutic potential. Using this approach, promising complexes could be identified through a simple enzyme assay prior to in vivo testing.

The IC_{50} values of the clinically useful gold(I) compounds are in the mid to high micromolar range. Despite their limited potency, the inhibition of the cathepsins by Au(I) is likely to be relevant in vivo based on the amount of Au(I) available in the inflamed joints of rheumatic patients. While inhibition of this family of enzymes responsible for extracellular matrix degradation, bone resorption, and joint destruction could help protect the joints of rheumatoid arthritis patients undergoing chrysotherapy, Au(I) could also inhibit other enzymes with catalytically important cysteine residues. The Au(I) may also play a host of other roles in alleviating the symptoms of rheumatoid arthritis, and a complete picture of the molecular mechanism of Au(I) antiarthritic activity remains to be elucidated.

Acknowledgements

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26. Enzyme activity assays: Standard conditions used to determine the activity of the purified enzymes against *Z*-Phe-Arg-MCA are described below. Substrate stocks were dissolved in DMSO and aliquots taken such that each reaction had the same amount of DMSO, which never exceeded 5% of the total reaction volume. The buffer used for all studies contained 20 mM sodium acetate, pH 5.5, 50 mM sodium chloride, 1.0 mM EDTA, and 0.01% Brij 35, with 400 μM DTT added to the buffer just prior to each reaction. In each reaction, the concentration of enzyme was fixed between 1 and 10 nM and the substrate concentration varied from 5 nM to 100 μM. The increase in fluorescence as the substrate was hydrolyzed was measured every 60 s for 30 min. Fluorescence was converted into concentration of 7-amino-4-methylcoumarin (MCA) produced using a standard curve of MCA fluorescence, and the data was fit to the Michaelis–Menten equation, providing kinetic parameters for each enzyme.
27. Enzyme inhibition assays: Inhibition assays were performed using a fixed enzyme concentration of between 1 and 10 nM and a fixed substrate concentration of 20 μM. Stock solutions and serial dilutions of the metal complexes were made in DMSO for auranofin, myochrysin, and $NaAuCl_4$, in methanol for $Au(dppe)_2Cl$, and in water for NaAg(thiomalate). Enzyme activity was measured in the presence of between 3 nM and 300 μM metal complex, except in the case of $Au(dppe)_2Cl$, which was insoluble at concentrations above 1 μM.
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31. To determine the mode of inhibition, four different reactions were run where the substrate concentration was varied from 20 to 90 μM at inhibitor concentrations of 300 nM, 3, 30, and 300 μM.
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