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ACCELERATED COMMUNICATION

Inhibition of DNA Binding and Transcriptional Activity of a Nuclear Receptor Transcription Factor by Aurothiomalate and Other Metal Ions

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SUMMARY

The antirheumatic gold salt aurothiomalate (AuTM) has cellular actions that are consistent with modulation of gene expression. We have tested the hypothesis that an important mode of action of AuTM is inhibition of binding of certain transcription factors to regulatory elements in DNA. The chemistry of transcription factors containing the zinc finger motif makes them candidates for such an interaction with AuTM. In this regard, the interaction of a steroid hormone receptor, the progesterone receptor (PR), with its DNA response element (PRE) was chosen as a suitable model. Nuclear extracts of T-47D human breast cancer cells rich in PR were incubated with radiolabeled PRE, and binding was determined by gel retardation assay. Preincubation of nuclear extract with AuTM caused a concentration-dependent inhibition of binding of PR to PRE (IC_{50}, approximately 3 μM). Other metal ions inhibited binding at higher concentrations, in a rank order correlating with their binding affinity for thiols. Thiomalic acid had no effect in the absence of gold in this system. To test the effect of AuTM on PR-mediated transcription, we transfected the progestin-inducible expression vector pMSG-CAT into T-47D cells. Transfected cells were incubated in the absence or presence of AuTM and treated with the synthetic progestin ORG2058, to induce chloramphenicol acetyl transferase (CAT) activity. With 10 and 100 μM AuTM, there was inhibition to 67 ± 3% (p = 0.012) and 42 ± 8% (p = 0.008) of CAT specific activity, respectively, compared with controls. These results demonstrate that AuTM can regulate gene expression and that inhibition of binding of a transcription factor to its response element is a likely mechanism. This provides a molecular model for further study of the antirheumatic action of gold salts.

The molecular mode of action of gold salts used in the treatment of rheumatoid arthritis is unknown, despite their important role in therapy for many years (1). AuTM is one of the few agents that has been shown to slow the rate of rheumatoid erosions, and in some patients it can achieve a complete remission (2–4). Unfortunately, the response is highly variable between individuals, with some patients having no apparent benefit and the majority having only a partial improvement. With knowledge of the molecular mode of action of AuTM, it is hoped that its efficacy may be enhanced or that nonresponders might be identified before therapy. It may also be possible to exploit the same mechanism with other, less toxic, drugs.

Many cellular events that occur with gold treatment, including reduced production of specific proteins such as interleukin-1 (5–9), collagenase (10), HLA-DR antigen (11, 12), and complement (12), are consistent with decreased transcription of multiple genes. In contrast, the reported inhibition of a variety of preformed enzymes is probably less relevant to the mode of action of gold salts, because the concentrations required for *in vitro* inhibition are much higher than the peak serum gold concentrations, of 30–40 μM, that are achievable during therapy. Moreover, the actions of very high concentrations of gold on preformed enzymes have not provided a satisfactory explanation for the effects of gold on the inflammatory process (13, 14).

We have hypothesized that gold salts act by inhibiting the binding of transcription factors to their specific DNA response elements in promoter and enhancer regions of genes, thereby modulating transcription rates. In particular, it is proposed that Au^+ may occupy the metal binding site in transcription factors containing the zinc finger motif, analogous to the displacement of Zn^{2+} by Au^+ in metallothioneins (15). In each zinc finger, there is one Zn^{2+} bound to either four cysteines or two cysteines and two histidines, in a tetrahedral coordination

ABBREVIATIONS: AuTM, aurothiomalate; FCS, fetal calf serum; CAT, chloramphenicol acetyl transferase; MMTV-LTR, mouse mammary tumor virus long terminal repeat; PR, progesterone receptor; PRE, progesterone response element; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

structure that is essential for DNA binding and transcriptional regulation (16, 17). Cd^{2+}, which also has affinity for the thiolate anion in cysteine and a preferred tetrahedral coordination structure, can perform the same function as Zn^{2+}, whereas other transition metal ions that have been tested are insufficient for DNA binding when incubated with the zinc-depleted apoprotein (18). Au^{+} has a particularly high affinity for thiolate anions, with which it forms linear complexes with a coordination number of two (13, 19). The structure, and therefore function, of a zinc finger transcription factor might be expected to be perturbed if ion exchange of Zn^{2+} for Au^{+} or addition of Au^{+} into the metal binding site took place.

In this study, PR was used as a model zinc finger transcription factor. Like all members of the nuclear receptor superfamily, PR has two zinc fingers, each with four essential cysteines (16). Upon activation by a progestin, PR binds with high affinity and specificity to a PRE in the promoter region of progestin-responsive genes. We have found that AuTM inhibits the *in vitro* binding of PR to PRE at concentrations low enough to be therapeutically relevant. In addition, AuTM inhibits the transciption of a transfected reporter gene under the control of a progestin-responsive promoter in T-47D breast cancer cells.

Materials and Methods

Cell culture. T-47D cells were supplied by E. G. and G. Mason Research Institute (Worcester, MA) for the National Cancer Institute Breast Cancer Program Cell Culture Bank, and stock cultures were maintained by weekly passage in RPMI 1640 medium (Cytosystems, Sydney, Australia) supplemented with 6 mM glutamine, 10 μg/ml human insulin, and 10% FCS (Commonwealth Serum Laboratories, Melbourne, Australia) and buffered with 14 mM sodium bicarbonate and 20 mM HEPES. Stock cultures were kept antibiotic free and have been shown to be free of *Mycoplasma* contamination. During experiments, the culture medium was changed to 5% FCS, and 20 μg/ml gentamicin was included.

Preparation of nuclear extract. Subconfluent 150-cm^2 flasks of T-47D cells were incubated for 1 hr at 37° with 5 nM ORG2058 (Amersham, Sydney, Australia) and harvested with trypsin/EDTA (0.05% trypsin, 0.02% EDTA, 5 min, 37°). Cells were washed once in culture medium and once in homogenization buffer (15 mM Tris·HCl, pH 7.5, 1 mM EDTA, 2 mM dithiothreitol, 10% glycerol) and a mixture of protease inhibitors, including 1 μg/ml pepstatin A, 47 μg/ml leupeptin, 100 μg/ml bacitracin, and 77 μg/ml aprotinin (Sigma, St Louis, MO), and were kept on ice. Cell pellets were suspended in approximately 50 μl of homogenization buffer/10^7 cells and were homogenized in a Teflon-glass Potter-Elvehjeim homogenizer. Homogenates were centrifuged at 800 × *g* for 20 min, supernatants were discarded, and the nuclear pellet was washed twice in homogenization buffer by resuspension and centrifugation at 800 × *g*. Nuclear pellets were suspended in an equal volume of extraction buffer (20 mM HEPES, pH 7.9, 600 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 2 mM dithiothreitol, 20% glycerol), extracted on ice for 1 hr with repeated resuspension, and centrifuged at 100,000 × *g* at 4° for 60 min. Protein determinations were made by a dye-binding method (Bio-Rad, Sydney, Australia). Supernatants were frozen at −70° until required.

Hormone-binding assay. Aliquots of nuclear extracts were incubated for 16 hr at 4° with 10 nM [^{3}H]ORG2058, in the presence or absence of 1000-fold excess (10 μM) unlabeled ORG2058. Free ORG2058 was adsorbed with dextran-coated charcoal, which was then pelleted by centrifugation. Receptor-bound hormone in the supernatant was determined by liquid scintillation counting.

Gel retardation assay. Aliquots of nuclear extract from ORG2058-treated cells, containing approximately 0.03 pmol of PR, as determined by hormone-binding assay, and 15 μg of nuclear protein, were preincubated for 60 min at 4° with 2 μg of poly(dA-dT)·poly(dA-dT) and 0.5 μg of sheared *Escherichia coli* DNA (Pharmacia, Sydney, Australia), in binding buffer (10 mM HEPES, pH 7.9, 100 mM NaCl, 1 mM dithiothreitol, 0.05 mM EDTA, 2.5 mM $MgCl_2$, 6% glycerol, 2% Ficoll) to a volume of 28 μl. In some experiments, metal ions, thiol agents, or antibodies to PR were included in the preincubation. All metal salts and thiol agents were supplied by Aldrich (Milwaukee, WI) or Sigma, except for D-penicillamine, which was supplied by Fluka (Buchs, Switzerland). Monoclonal antibodies hPRa6 and hPRa7 (20) were the kind gift of Dr. C. Clarke, Cancer Biology Division, Garvan Institute. Subsequently, 0.3 ng, in 2 μl, of a synthetic, PRE-containing, double-stranded, high performance liquid chromatography-purified oligonucleotide, end-labeled with [γ-^{32}P]ATP (Amersham, Sydney, Australia) by T_4 polynucleotide kinase (Bresatec, Adelaide, Australia), was added, and the mixture was incubated for an additional 120 min at 4°. The oligonucleotide containing a PRE (5′-gatcCTTTGGTTACAAACTGTTCTTAAAACGAG-3′:3′-GAAACCAATGTTTGACAAGAATTTTGCTCctag-5′) corresponds to the distal domain (−189 to −162) of the hormone response element of the MMTV-LTR (21), with overhanging *Bam*HI sites. Samples were electrophoresed at room temperature on a 5% polyacrylamide gel (29:1, w/w, ratio of acrylamide to bisacrylamide), in 0.3× TB buffer (27 mM Tris-borate, pH 7.9), at 200 V or 1.5 mA/cm. Gels were dried under vacuum and autoradiographed with Kodak X-OMAT film at −70°, with intensifying screens. Radioactive bands were quantified by Cerenkov counting after excision from the dried gel.

Gene transfection into T-47D cells. Cells were plated into 150-cm^2 flasks (2.5 × 10^6 cells/flask) in RPMI 1640 medium, containing 5% FCS, 3 days before transfection. On the morning of transfection, medium was changed to Dulbecco's modified essential medium (Flow/ICN, Sydney, Australia), pH 7.3–7.4, containing 5% FCS, and cells were transfected, using the calcium phosphate precipitation method (22), with 40 μg/flask of the expression vector pMSG-CAT (Pharmacia). This vector contains the CAT gene under the control of the progestin-inducible MMTV-LTR promoter. Cells were subjected to osmotic shock for 45 sec with 15% glycerol, 4 hr after transfection, and exposure of cells to the DNA continued for 18 hr. Cells were harvested, mixed to reduce variations due to inter-flask differences in transfection efficiences, replated at 2 × 10^5 cells/9.62-cm^2 well (Linbro, McLean, VA), and incubated in 5% CO_2. When the cells had adhered to the plastic (6 hr after plating), AuTM in aqueous solution, or vehicle, was added (20 μl added to 2 ml of culture medium) and cells were incubated for an additional 16 hr and then treated with 10 nM ORG2058. This was followed by an 18-hr incubation, after which time cells were harvested, counted, lysed by freeze thawing in 0.25 M Tris·HCl, pH 7.8, and heated to 65° for 10 min to inactivate endogenous deacetylase. After centrifugation, an aliquot was removed for measurement of CAT activity by a nonchromatographic method (23). CAT activity per well was normalized for total protein per well. Preliminary experiments have shown that T-47D cells remain in exponential growth for at least 5 days in concentrations of AuTM ranging up to 100 μM, without any evidence of cytotoxicity.

Results

Gel retardation assay demonstrates PR binding PRE. The upper band in Fig. 1 was identified as the PR/PRE complex, based on two criteria. Firstly, sequence-specific binding was demonstrated, because it was competed for by an excess of unlabeled PRE oligonucleotide. Secondly, the addition of monoclonal antibodies demonstrated that PR was present in the sequence-specific band. Antibody hPRa6 is directed against the B isoform of PR (20) and, hence, caused a supershift of the AB and BB dimers, leaving the AA dimer as a faint smear. Antibody hPRa7 is directed against both isoforms (20) and caused a supershift of the entire PR/PRE complex. In the autoradiographs shown here, the triplet of AA, AB, and BB

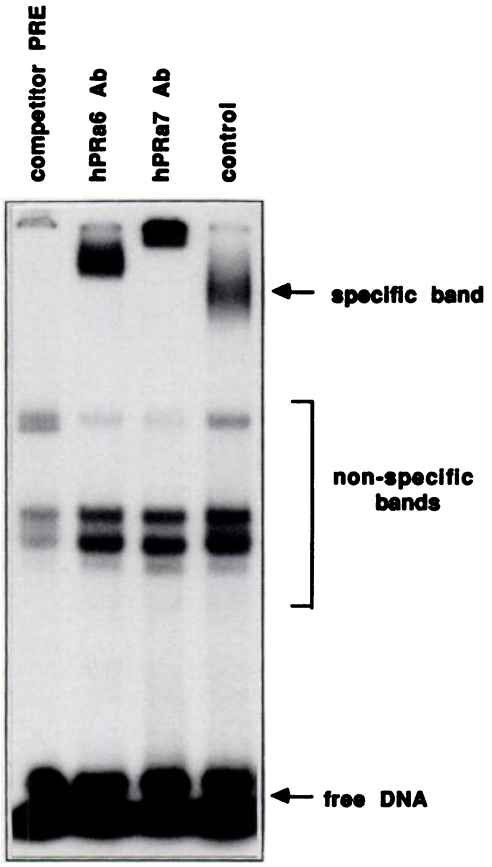

Fig. 1. PR binding to the PRE, measured by gel retardation assay. Nuclear extracts from T-47D cells treated with ORG2058 were incubated with radiolabeled PRE oligonucleotide, and samples were analyzed by nondenaturing 5% polyacrylamide gel electrophoresis. Addition of unlabeled competitor PRE oligonucleotide, in 125-fold excess of labeled PRE, abolished the slow moving band, indicating that it represents sequence-specific binding of a protein to the PRE. Preincubation of the nuclear extracts with monoclonal antibody to PR (hPRa6 or hPRa7) caused a supershift of the specific band, thus demonstrating that it contains PR. In the case of hPRa6, which recognizes only the B isoform of PR, the supershift occurs with the AB and BB dimers of PR, leaving a faint indication of the AA dimer.

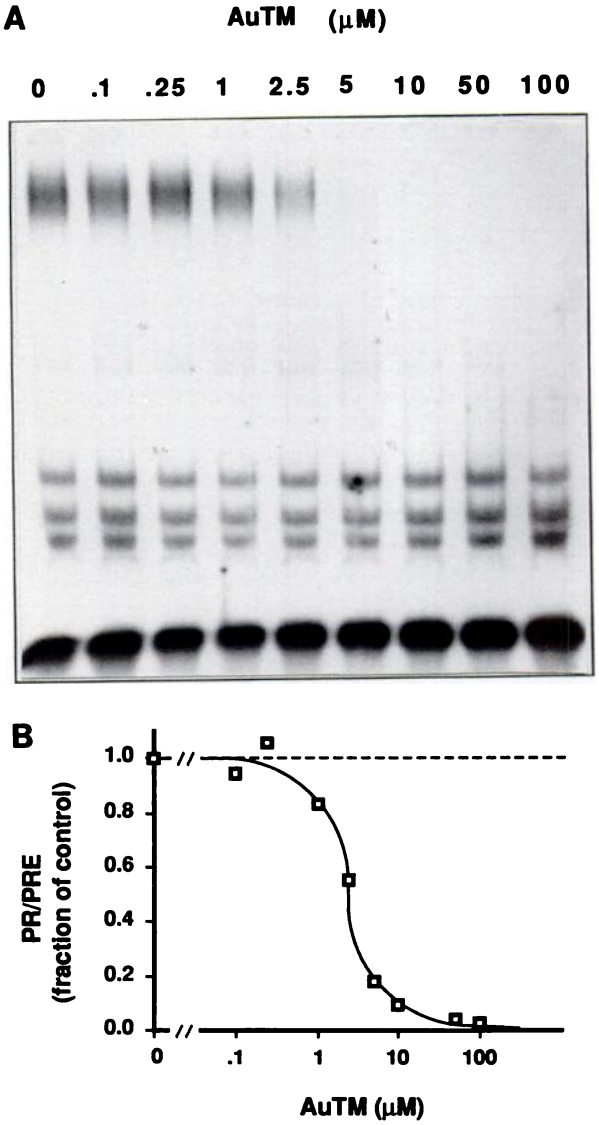

Fig. 2. Effect of AuTM on the binding of PR to PRE. A, Nuclear extracts were preincubated for 60 min with AuTM before incubation with radiolabeled PRE. Samples were analyzed by gel retardation assay, with the final concentrations of AuTM as indicated. B, The slow moving bands representing the PR/PRE complex were excised from the dried gel, quantified by Cerenkov counting, and plotted as a fraction of the control lane. These results are indistinguishable from two other similar experiments.

isoforms was not resolved. The reduction in the intensities of the nonspecific bands with the monoclonal antibodies may be related to the increased total protein added to these lanes. The lack of a supershift indicates that antibody has not bound to the complex.

AuTM inhibits binding of PR to PRE. Preincubation of nuclear extract with different concentrations of AuTM before binding to PRE resulted in a concentration-dependent inhibition of binding, with an IC_{50} of approximately 3 μM AuTM (Fig. 2). Below 1 μM the effect of AuTM was minimal, and above 10 μM the inhibition of binding was almost complete. In contrast to the effects of AuTM on this sequence-specific binding is the observation that the faster moving bands, representing nonspecific protein-DNA binding, were unaffected by AuTM, except at very high concentrations of 100 μM.

Steady state is achieved within a 30-min preincubation of AuTM with PR in the nuclear extract. AuTM is polymeric in aqueous solution and depolymerizes on addition to a protein solution, such that Au^+ distributes to different binding sites (13). Preincubation of nuclear extract was performed in order to allow Au^+ to come to equilibrium with PR and other binding sites before addition of ^{32}P-labeled PRE. A second incubation of 90 min at 4° was then performed to allow complete binding of PR to PRE (21). At preincubation times of 30–150 min, there was no difference in the inhibitory effect of 2.5 μM AuTM on PR binding to PRE, suggesting that the free concentration of Au^+ had reached a steady state, whereas simultaneous addition of AuTM and labeled PRE led to greater inhibition (Fig. 3). In all other experiments, preincubations were for 60 min, to allow ample time in case 30 min is near the shoulder of the equilibration curve.

Thiomalate and other thiols do not affect binding of PR to PRE. Nuclear extract was preincubated with thiomalic acid at 10 and 100 μM. Binding of PR to PRE was unaffected (Fig. 4), indicating that Au^+ is the active moiety in AuTM. Similarly, the thiol agents D-penicillamine, D-cysteine, gluta-

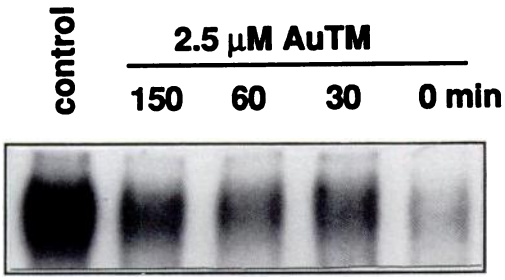

Fig. 3. Effect of preincubation of nuclear extract with AuTM on the binding of PR to PRE. Nuclear extracts were preincubated with AuTM, at a final concentration of 2.5 μM, for 0, 30, 60, and 150 min before addition of radiolabeled PRE. The reaction mix was incubated for an additional 90 min and analyzed by gel retardation. Only the specific PR/PRE band is shown. In the control lane, vehicle (water) was substituted for AuTM.

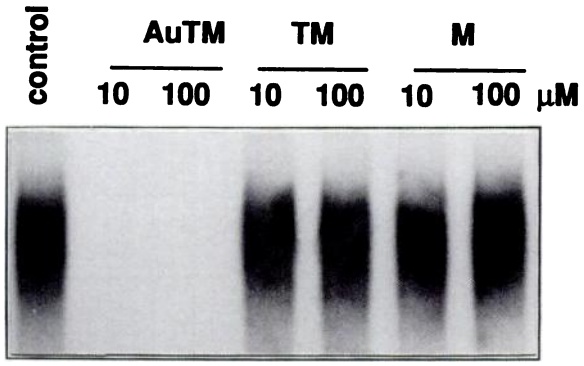

Fig. 4. Effect of thiomalate and malate on the binding of PR to PRE. Nuclear extracts were preincubated for 60 min with water (vehicle, negative control), AuTM (positive control), thiomalic acid (*TM*), or malic acid (*M*), at the indicated concentrations, before addition of radiolabeled PRE. The reaction mix was incubated for an additional 90 min and analyzed by gel retardation.

thione, and dithiothreitol had no effect in this system (data not shown).

Excess Zn^{2+} inhibits the binding of PR to PRE. We performed an experiment in which nuclear extract was preincubated with both Zn^{2+} and AuTM, expecting to find competitive effects. Surprisingly, Zn^{2+} did not enhance the binding of PR to PRE in the presence or absence of AuTM; rather, the addition of Zn^{2+} inhibited binding, and the inhibitory effects were additive when both Zn^{2+} and AuTM were used together (Fig. 5).

Transition metal ions inhibit the binding of PR to PRE at higher concentrations than AuTM, corresponding approximately to their thiol-binding affinities. Nuclear extract was preincubated with AuTM and a selection of transition metal chlorides, at concentrations of 10 and 100 μM, before gel retardation assay (Fig. 6). Consistent with previous experiments, AuTM profoundly inhibited binding of PR to PRE at 10 μM. The next most potent were Cu^{2+}, Zn^{2+}, and Cd^{2+}, consecutively, which had only mild to negligible effects at 10 μM but profound effects at 100 μM. Co^{2+} had a slight effect at 100 μM, whereas Fe^{3+}, Ni^{2+}, Mn^{2+}, Mg^{2+}, and Ca^{2+} had no effect. This rank order, shown in Table 1, correlates approximately with their rank order of affinities for thiols (24, 25). The nonspecific bands were relatively unaffected.

Progestin-induced CAT activity in T-47D cells transfected with pMSG-CAT is reduced by AuTM treatment. The vector pMSG-CAT was transfected into T-47D cells as a measure of PR-mediated transcription. Induction of the transfected cells with the synthetic progestin ORG2058 produced a 25-fold induction of CAT activity (Fig. 7). Prior treatment of cells with 0, 10, and 100 μM AuTM resulted in a marked reduction in the response to progestin. Mean ± standard error (of triplicates) CAT activities were 88 ± 2, 64 ± 3, and 46 ± 5 nmol/min/μg of protein, respectively. Two-tailed paired *t* test showed treatment with 10 μM AuTM ($p = 0.012$) and 100 μM AuTM ($p = 0.008$) to be significantly different from controls. The background CAT activity measured in mock-transfected cells was subtracted, and specific CAT activities, expressed as a percentage of untreated controls, were 67 ± 3% with 10 μM AuTM and 42 ± 8% with 100 μM AuTM.

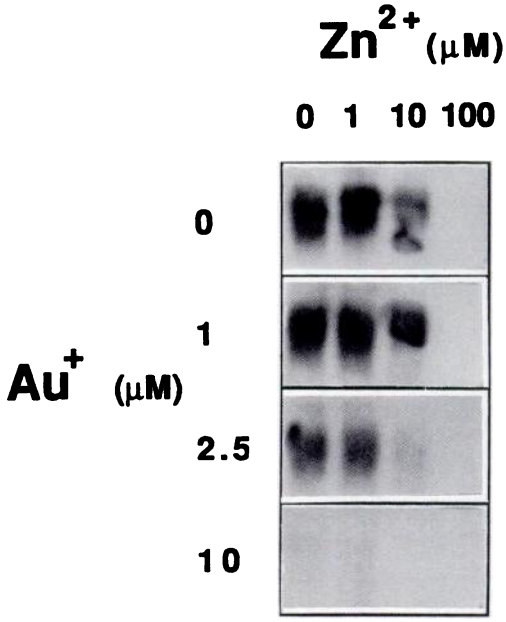

Fig. 5. Effect of Zn^{2+}, with and without AuTM, on the binding of PR to PRE. Nuclear extracts were preincubated for 60 min with $ZnCl_2$, AuTM, or both, at the indicated concentrations, and subjected to gel retardation with radiolabeled PRE. Only the specific PR/PRE bands are shown. Lanes with 0 and 1 μM AuTM are from the same gel and the lanes with 2.5 and 10 μM AuTM are from another gel, which was electrophoresed and autoradiographed simultaneously under identical conditions.

Discussion

The results presented have demonstrated that AuTM can inhibit the *in vitro* binding of PR to a PRE, whereas nonspecific protein-DNA binding is relatively unaltered. Such an effect is consistent with our hypothesis that Au^+ interacts with the zinc finger but does not exclude alternative means of preventing the association of the transcription factor with its specific DNA response element. Gold complexes can bind directly to DNA (26), but it is unlikely that such a mechanism accounts for the observed inhibition of sequence-specific binding to the exclusion of nonspecific binding. Interaction of gold with a third factor, which indirectly affects PR and PRE, is possible. However, the simplest explanation of the results is that Au^+ alters the properties of PR by binding to a critical thiol, or thiols, either within the zinc finger domain, as originally hypothesized, or elsewhere in the protein. This is supported by the known behavior of Au^+, which is invariably bound to cysteine residues when in solution with proteins (13, 19), and by the finding reported here that the effectiveness of other metal ions in

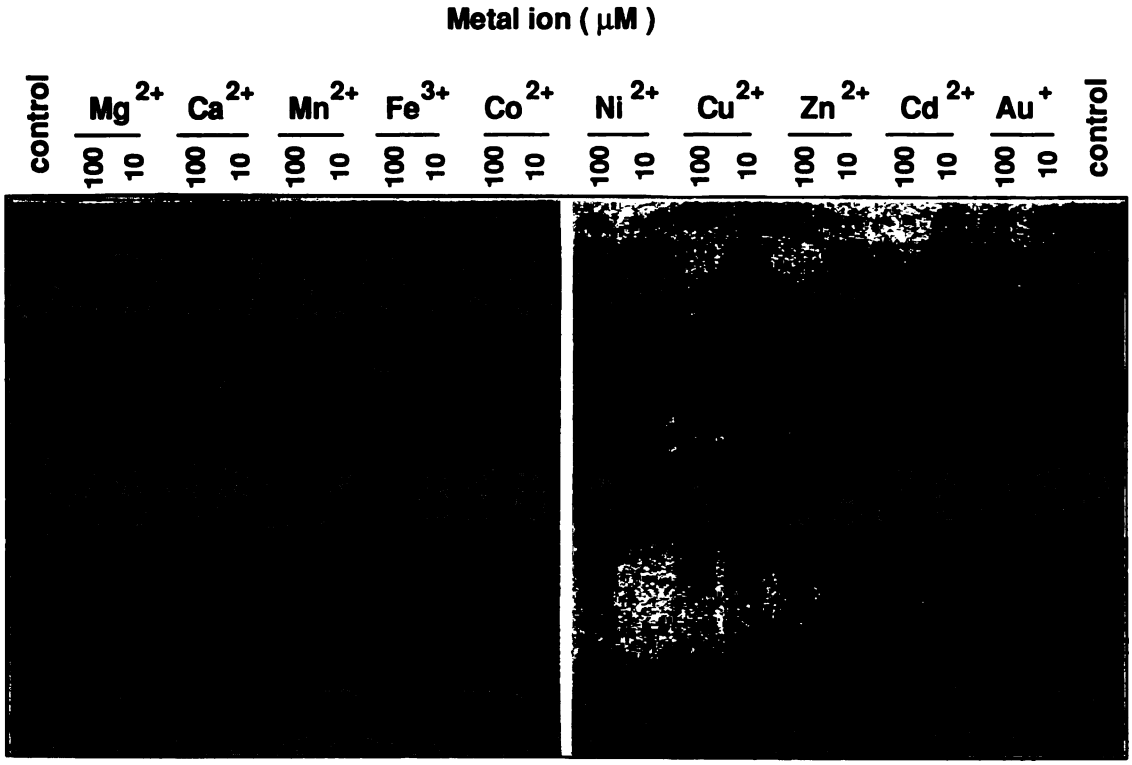

Fig. 6. Relative effect of transition metal ions on the binding of PR to PRE. Nuclear extracts were preincubated for 60 min with the indicated metal ions (all added as chlorides, except for AuTM), at final concentrations of 10 and 100 μM, and subjected to gel retardation. The relative effects of metal ions on the intensity of the PR/PRE band in this experiment are summarized in Table 1.

TABLE 1

Relative inhibitory effect of metal ions on the binding of PR to PRE

Relative effects were determined by ranking the visual intensities of the specific bands in Fig. 6.

	Inhibitory effect			
	Au^{+}	$Cu^{2+} > Zn^{2+} > Cd^{2+}$	Co^{2+}	Fe^{3+}, Ni^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}
10 μM	Profound	Mild	None	None
100 μM	Profound	Profound	Mild	None

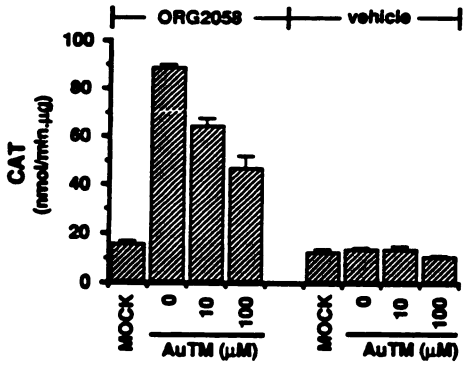

Fig. 7. Effect of AuTM on transcription of a progestin-inducible expression vector, pMSG-CAT, transfected into T-47D breast cancer cells. The vector pMSG-CAT, comprising the progestin-inducible MMTV-LTR promoter linked to the CAT reporter gene, was transiently transfected into T-47D cells by the calcium phosphate precipitation method. Transfected cells were incubated at 37° with 0, 10, or 100 μM AuTM for 16 hr, followed by incubation with either 10 nM ORG2058 or vehicle (ethanol) for an additional 18 hr, and were then harvested. Cells subjected to mock transfection were treated in parallel. CAT activity of harvested cells is expressed as the CAT activity (nmol/min), as determined by a non-chromatographic method, divided by the total protein (μg).

inhibiting the sequence-specific binding correlates with the rank order of the thiol-binding affinity (24, 25) of those metal ions. Indeed, the proposed mechanism for the action of gold is not specific for just one metal ion but is general for species with high affinity for thiols. Consistent with this is evidence supporting the antiinflammatory actions of Cu^{2+} (27) and Zn^{2+} (28, 29).

Because zinc finger proteins require zinc for DNA binding (18, 30, 31), it may seem surprising that additional zinc inhibited binding of functional PR to PRE. A similar observation has been made by Freedman *et al.* (18), who found that zinc and cadmium added to the metal-depleted glucocorticoid receptor DNA-binding domain restored DNA-binding activity, but at higher concentrations did not. The precise mechanism is unknown but presumably involves either addition of more metal ions into the zinc finger structure or binding of metal ions to a functionally sensitive site elsewhere in the protein. It should be noted that a number of metal ions, including Cu^{2+}, Mn^{2+}, Fe^{2+}, and Ni^{2+}, have been found to be ineffective (or, in the case of Co^{2+}, weakly effective) for restoring DNA-binding activity of a zinc-depleted zinc finger protein (18, 32), presumably because they do not conform to a tetrahedral coordination structure. Here we have shown that some metal ions can modulate the DNA-binding activity by acting as inhibitors, irrespective of whether they can participate in a functional finger structure.

The finding that inhibition of binding occurs at an IC_{50} of only 3 μM AuTM is significant, because this is within the range of serum concentrations achieved during therapy of patients with rheumatoid arthritis (13). Unfortunately, little is known

of available concentrations of Au^+ in the cell nucleus. Nevertheless, 3 μM is markedly lower than the K_i values for the various enzymes that are inhibited by AuTM, all of which require inhibitory concentrations above that which can be achieved therapeutically (13).

A long-standing question exists as to whether Au^+, thiomalate, or both is/are the therapeutically active component(s) of AuTM. Most investigators believe that Au^+ is active, although thiomalate may provide some additional benefit, possibly by the same mechanism as other thiol-containing antirheumatics such as D-penicillamine, tiopronin, bucillamine, and captopril (1, 33). It is conceivable that thiol drugs form disulfide bonds with the same "sensitive" cysteines affected by Au^+ and inhibit transcription in the same way, thus explaining the similarities between gold and penicillamine therapy (1). In the experiments reported here, thiol agents, including D-penicillamine, did not inhibit PR binding to PRE. This could be simply because thiol agents do not work in this way or because the experimental conditions lacked appropriate free radical catalysts (34).

The ability of AuTM to inhibit transcription of the PR-dependent pMSG-CAT reporter gene was tested in T-47D breast cancer cells because of their high concentration of PR, rather than any perceived relevance of these cells to rheumatic diseases. The weak inhibition of CAT activity (67 ± 3% of control for 10 μM AuTM in culture medium) in T-47D cells may reflect low sensitivity of this particular cell line to gold. It is known that gold is concentrated in the synovial tissue of patients treated with AuTM (35, 36), indicating that some specificity of action occurs due to increased uptake in certain cell types. Sensitivity of a particular cell type may also depend on the number of intracellular high affinity gold binding sites, such as metallothioneins, that reduce the availability of free gold (37).

We do not suggest that the antirheumatic effects of AuTM are via PR in particular. It is more likely that AuTM affects multiple transcription factors, which in turn regulate the expression of numerous genes. In addition, other unrelated actions of AuTM may also be taking place. The model for the molecular action of antirheumatic gold drugs that is described here is currently being extended to other transcription factors.

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