Biochemical Pharmacology, Vol. 55, pp. 1291–1299, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00634-5

Inhibition of Erythrocyte Selenium-Glutathione Peroxidase by Auranofin Analogues and Metabolites

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ABSTRACT. The effect of gold ligation on the inhibition of bovine erythrocyte selenium-glutathione peroxidase (GSH-Px) was examined. The anti-arthritic drug auranofin [2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-*S*)(triethylphosphine) gold(I)] ($Et_3PAuSATg$) and its analogue, Et_3PAuCl, exhibited experimentally equivalent K_i values (11.6 ± 0.8 and 10.8 ± 0.5 μM, respectively), despite the greatly disparate affinities of their ligands for gold(I): 2,3,4,6-tetra-*O*-acetyl-1-thiolato-β-D-glucopyranose ($ATgS^-$) ≫ Cl^-. This similarity reflects ligand exchange reactions that generate the glutathione complex Et_3PAuSG from the excess glutathione (GSH, 1 mM) used in the assay. The K_i values for bis(glutathionato)gold(I) ($Au(SG)_2^-$) and gold(I) thioglucose (AuSTg) were also found to be equal (2.8 ± 0.4 and 2.4 ± 0.5 μM, respectively). This confirms the previous postulate of Chaudiere and Tappel (*J Inorg Biochem* **20:** 313–325, 1984) that $Au(SG)_2^-$ is generated from AuSTg in the presence of excess glutathione. Since auranofin metabolites accumulate in red blood cells, the inhibition of intracellular GSH-Px was examined by using intact erythrocytes. There was greater inhibition of the reaction when the cells were resuspended in isotonic buffer than in whole blood, because serum albumin in the latter competes for the auranofin and decreases the uptake by erythrocytes. After correction for the extent of gold uptake, the K_i values were determined to be the same as those observed for $Au(SG)_2^-$ in the extracellular assay, indicating loss of both the Et_3P and $ATgS^-$ ligands from auranofin. Thus, the inhibition of GSH-Px by gold complexes is dependent on their ligation, and the ultimate gold(I) compound that interacts with erythrocyte GSH-Px in intact red cells, $Au(SG)_2^-$, is radically different from the original auranofin molecule. BIOCHEM PHARMACOL **55**;8:1291–1299, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. auranofin; gold(I) thioglucose; bis(glutathionato)gold(I); inhibition constants; selenium-glutathione peroxidase; erythrocytes; serum albumin

RA‡ afflicts an estimated eight million Americans. Although a cure for RA is still unknown, a small number of disease-modifying antirheumatic drugs have been proven to actually slow down or stop the progress of RA. Among them are the gold-based anti-arthritic drugs (chrysotherapy) that have been used for over 60 years in the treatment of RA. There are three different gold compounds that are approved for use in the United States. The subcutaneously administered drugs Solganol (AuSTg) and Myochrysine (AuSTm) are oligomers (small polymers) with thiolate ligands that bridge between the linear gold(I) ions. The third is a second-generation gold drug, auranofin ($Et_3PAuSATg$), which is administered orally. It is a monomeric linear complex with triethylphosphine (PEt_3) and thiolate ($ATgS^-$) moieties bound to the gold(I) center [1].

After administration of any gold-based anti-arthritic drug, more than 80% of the gold located in the plasma of the bloodstream is associated with serum albumin [2]. When $Et_3PAuSATg$ is administered, slightly more than 50% of the gold accumulates in the erythrocytes [2]. The accumulation by the erythrocytes has been determined to be slightly faster than uptake into the plasma [3]. The efflux of gold(I) out of the erythrocytes occurs only if serum albumin is present in the plasma and is governed by a first-order rate constant of 0.81 ± 0.18 hr^{-1} [4]. The product that forms after gold(I) efflux out of the erythrocytes is an albumin–gold–glutathione complex [4].

GSH-Px (EC 1.11.1.9) catalyzes the reduction of hydrogen peroxide and many organic peroxides to alcohol and water with the use of glutathione as the reducing agent. It is a member of an important class of selenium-containing enzymes as recently reviewed [5]. GSH-Px protects the cell from initiation and propagation of free radical reactions. GSH-Px is found in most tissues, including erythrocytes [6]. The active site for peroxide reduction is a selenocysteine

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‡ *Abbreviations:* λ_{max}, absorbance maximum; δp, ^{31}P NMR chemical shifts; AA, atomic absorption; $ATgS^-$, 2,3,4,6-tetra-*O*-acetyl-1-β-D-thiolato-glucopyranose; $Au(SG)_2^-$, bis(glutathionato)gold(I); AuSTg, gold(I) thioglucose; AuSTm, gold(I) sodium thiomalate; $Et_3PAuSATg$, [2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-*S*)(triethylphosphine)gold(I)]; GSH-Px, selenium-glutathione peroxidase; GSR, glutathione reductase; MeOH, methanol; MOPS, (3-*N*-morpholino)propanesulfonic acid; RA, rheumatoid arthritis; and t-BOOH, *tert*-butylhydroperoxide.

Received 9 June 1997; accepted 6 October 1997.

residue [7], which is a potential gold(I) site. Gold(I) is a soft acid and prefers ligands that are "softer" and can therefore form covalent bonds. Excellent examples of these types of ligands commonly found in the human body are thiolates (i.e. cysteine residues in proteins, free cysteine, and glutathione), and selenolates (found as selenocysteine in some enzymes) [8]. Because selenium is more polarizable then sulfur, and therefore, "softer" in its characteristics, selenium ligands are expected to be better ligands for the "soft" gold atom [8].

A number of studies have been performed on the interaction between AuSTg and glutathione peroxidase isolated from various tissues [9–14]. Tappel and coworkers have determined that AuSTg inhibits the activity of glutathione peroxidase in many tissues [12–14]. Chaudiere and Tappel have shown that AuSTg inhibits glutathione peroxidase activity by binding at the selenocysteine active site [9, 11]. Gold thioglucose was the only gold(I) compound studied in detail. Auranofin is structurally different from AuSTg in that it is a linear monomer with a lipophilic (PEt_3) ligand, which allows for the transport of gold(I) across membranes, yet, the interaction between auranofin and glutathione peroxidase has not been studied extensively. Because significant levels of gold(I) accumulate in the erythrocytes after auranofin administration [2], the effect of $Et_3PAuSATg$ on the activity of erythrocyte glutathione peroxidase is important. The focus of this paper is the determination of inhibition constants of erythrocyte glutathione peroxidase by various gold(I) compounds. The binding of gold compounds to serum albumin affects bioavailability [4], and, therefore, the inhibition of glutathione peroxidase in intact erythrocytes was also investigated in the presence and absence of serum albumin.

MATERIALS AND METHODS

Materials

GSH-Px (from Bovine Erythrocytes, Lot No. 70H9311), GSR (Type III from Bakers yeast, Lot No. 70H82461 and 121H80701), NADPH (Lot No. 031H7010, 031H7075, and 032H7105), Sephadex G-25, and MOPS were purchased from Sigma. BSA (Fraction V: Fatty Acid Ultra Free Lot No. DHD106, DEC106, and EHA107) was purchased from Boehringer Mannheim Biochemicals. AuSTg, GSH, GSSG, t-BOOH, NaCl, KCl, potassium phosphate (mono and dibasic), and NH_4HCO_3) were all purchased from Aldrich. The gold glutathione ($Au(SG)_2^-$) was a gift of Dr. Anne Arendt (nèe Hormann) [15]. The AA standard $KAu(CN)_2$ was purchased from Spex. Auranofin ($Et_3PAuSATg$) and Et_3PAuCl were supplied by SmithKline Beecham). All other chemicals utilized for these studies were reagent grade or better.

AuSTg was dissolved in 5% MeOH (v/v) in either 0.1 M of potassium phosphate, pH 7.2, or 0.1 M of NH_4HCO_3, pH 7.9, while $Au(SG)_2^-$ was dissolved to the appropriate concentration in 0.1 M of potassium phosphate, pH 7.2. $Et_3PAuSATg$ and Et_3PAuCl were dissolved in 100% EtOH and MeOH, respectively, unless otherwise noted. All buffers used for the measurement of gold were prepared in the absence of any sodium.

Enzymatic Assays

GSH-Px activity was assayed spectrophotometrically using a GSR coupled reaction [16]. The assay mixture, unless otherwise noted, consisted of 0.12 mM of NADPH, 1.0 mM of GSH, 0.20 mM of t-BOOH, 4 units/mL of GSR, 0.80 nM of glutathione peroxidase, and 0.10 mM of EDTA in 50 mM of Tris-HCl, pH 7.6, in a final volume of 0.50 mL. All the reaction assays were run at 37°, in a Perkin–Elmer Lambda 6 double beam spectrophotometer, with an RM6 Lauder (Brinkmann Corp.) circulating water bath controller. The decrease in NADP absorbance was followed at 340 nm for at least 2 min for determination of initial rates and other kinetic parameters. All components of the assay mixture except t-BOOH were preincubated at 37° for 2 min prior to initiation with the hydroperoxide.

GSR activity was determined as above, except that the assay mixture consisted of 1.0 mM of GSSG, 1–4 units/mL GSR, 0.12 mM of NADPH, 0.1 mM of EDTA, and 50 mM of Tris–HCl, pH 7.6, at a final volume of 0.50 mL. All reaction components except GSSG were preincubated at 37° for 2 min prior to initiation with GSSG. The spectrophotometric assay was run as described with glutathione peroxidase, except that the amount of GSR used was varied. Various concentrations of $Et_3PAuSATg$ were also added to determine the effect on GSR activity.

Conditions for Gold(I) Inhibition of Glutathione Peroxidase

Under equilibrium conditions the gold(I) compounds were preincubated with glutathione peroxidase and GSH for at least 3 min prior to the addition of any other components of the assay mixture. The components were always added in the following order; 50 mM of Tris-HCl, 0.1 mM of EDTA, gold(I) complex (if added), 1 mM of GSH, 0.80 nM of GSH-Px, 2 units of GSR, 0.12 mM of NADPH, with 0.20 mM of t-BOOH used to initiate the reaction. Controlled reactions, in the absence of gold(I) complexes, were run as a measure of free enzyme activity. All reactions were repeated at least three times.

The activity of glutathione peroxidase was also followed at a limiting concentration of t-BOOH, in the presence of constant GSH, and varying amounts of the gold(I) complexes. The inhibitors were preincubated for 1 min in the presence of GSH and GSH-Px. The components of the reaction assay were as described above, but varying concentrations of t-BOOH (see figure legends) were used to initiated the reaction. The amount of t-BOOH used was always less than NADPH. The t-BOOH concentration was

estimated by the difference between the amount of NADPH before and after the reaction was completed.

Preparation of Erythrocytes

Human erythrocytes (red blood cells) from a single donor (a healthy male, nonsmoker) were collected in 20-mL sterile vacutainers containing sodium heparin. For determination of gold uptake, 1-mL aliquots of the blood were incubated in 1.5-mL Eppendorf tubes in the presence of either 0, 2, 4, 10, 15, 20, or 30 μM of $Et_3PAuSATg$ for 15 min at 21°. The whole blood was centrifuged at 3000 rpm for 10 min at 4° in a Beckmann Microfuge. The plasma layer was removed, measured, and saved for gold determination. The cells were washed two times with ≈300 μL of MOPS/KCl/EDTA buffer (20 mM of MOPS, 150 mM of KCl, and 5 mM of EDTA, pH 7.4), and centrifuged at 3000 rpm for 10 min. The washed erythrocytes were then resuspended in ≈300 μL of MOPS/KCl/EDTA buffer. The resuspended red blood cells were lysed by the addition of 4 mL of H_2O and incubation for 1 h at 21°. Then the lysate was analyzed for glutathione peroxidase activity as described below.

Preparation of Erythrocytes Suspended in Isotonic Saline Buffer

Human erythrocytes (15 mL) collected as described above, were centrifuged at 3500 rpm for 10 min at 4°. The separated cells were washed two times with ≈5.0 mL of 1.0% saline solution and centrifuged at 3500 rpm for 10 min at 4°. The washed erythrocyte pellet was resuspended in ≈5.0 mL of MOPS/KCl/EDTA buffer (described above). Aliquots of 1 mL were removed from the erythrocytes that had been resuspended in isotonic saline buffer and incubated in the presence of either 0, 2, 4, 10, 15, 20, or 30 μM of $Et_3PAuSATg$ for 15 min at 21°. The gold-treated cells were then washed, resuspended, and lysed as described above. The lysate was analyzed for glutathione peroxidase activity as described in the next section.

Assay for Glutathione Peroxidase Activity in Erythrocytes

Conditions for the GSR assay were modified as follows for determining the inhibition of GSH-Px activity in erythrocytes. After intact cells were exposed to $Et_3PAuSATg$ and transferred to fresh medium, they were disrupted and the assay was performed as above (omitting additional $Et_3PAuSATg$). Aliquots of the cells were used to determine the intracellular gold concentrations. Cell lysates prepared from cells not exposed to gold were incubated with $Et_3PAuSATg$ for 2 min in the presence of GSH and assayed as above.

Gold Assays

The concentration of gold was determined by AA spectroscopy on an Instrumentation Laboratories 357 instrument set at 343 nm, a bandwidth of 1 mm, and a current of 5 A. Calibration of the instrument was performed with standard gold solutions that were prepared by dilution of Spex $KAu(CN)_2$ standard (171.4 ppm) to 1.714 ppm and 3.428 ppm in NH_4HCO_3 buffer.

RESULTS

Optimization of the GSH-Px Assay

GSH-Px catalyzes the reduction of organic peroxides by glutathione to form the corresponding alcohol and water, as described by Equation 1.

$$GSH\text{–}Px \qquad (1)$$

$$2\ GSH + ROOH \rightarrow GSSG + ROH + H_2O$$

As described by Paglia and Valentine [16], when this reaction is coupled to GSR (Equation 2), the reaction of glutathione peroxidase can be monitored,

$$GSR \qquad (2)$$

$$GSSG + 2\ NADPH \rightarrow 2\ GSH + 2\ NADP$$

because GSR utilizes the chromophore NADPH, λ_{max} = 340 nm. When GSH, NADPH, and GSR remain in excess of GSH-Px [16], the rate of NADPH disappearance is directly related to the reaction catalyzed by GSH-Px. Chaudiere and Tappel [9] found that adding 10–15 μM of AuSTg to the assay mixture inhibited ≈30–40% of the GSR activity. However, when 4 units/mL of GSR were used in the assay mixture, the activity of GSR, even accounting for the inhibition by the gold(I), was still 30-fold greater than the activity of GSH-Px [9]. Therefore, the effect of $Et_3PAuSATg$ on GSR was also examined as a prelude to the present study. A decrease of ≈20–25% did not occur until 50 μM of $Et_3PAuSATg$ was added. Therefore, if GSR is added at levels of 4 units/mL of assay mixture (≈35 nM), the effect of the gold(I) on the GSR activity is negligible, since the activity of GSR is 30- to 40-fold greater than that of GSH-Px, and conversely, there is no perturbation of the gold species present, since the GSR concentration is much smaller than the lowest gold concentrations (2 μM) used. The absolute reaction rate of the GSH-Px with 1 mM of GSH present in the assay system is 13.6 ± 0.3 μM of NADPH min^{-1}.

Thiomalate, the ligand of the anti-arthritic drug gold thiomalate, is a very potent inhibitor of GSH-Px activity, while thioglucose does not affect the activity of GSH-Px [10]. Since triethylphosphine oxide (Et_3PO) is a product of auranofin metabolism [3, 17], the effect of Et_3PO on the coupled reaction was investigated. When Et_3PO was added within the range of 0 and 100 μM, the GSH-Px activity, 13.6 ± 0.3 μM of NADPH min^{-1}, was unaffected. There-

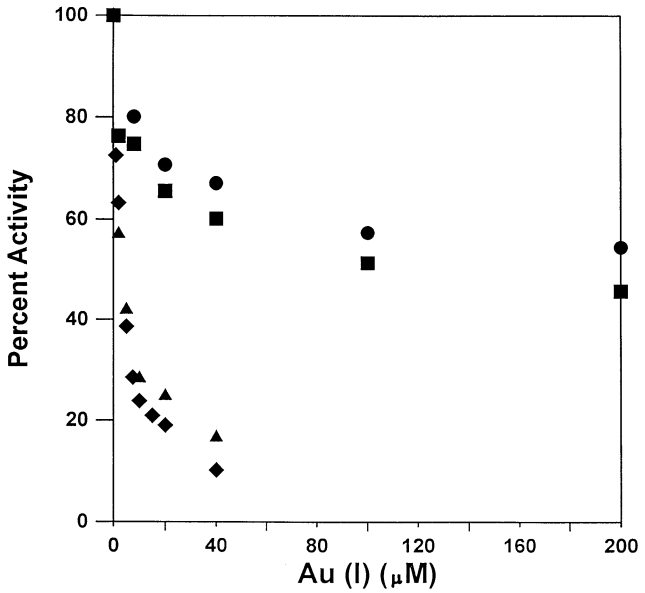

FIG. 1. Inhibition of GSH-Px by gold(I) compounds in the presence of 1.0 mM of GSH at 37°. $Et_3PAuSATg$ (●), Et_3PAuCl (■), AuSTg (▲), and $Au(SG)_2^-$ (◆). The concentrations of all components of the assay mixture are described in Materials and Methods. The amount of inhibition is expressed as a percent of the control activity for glutathione peroxidase. Each data point represents the average of at least three independent measurements. The absolute rate of the GSH-Px reaction in the absence of added inhibitors was 13.6 ± 0.3 μM of NADPH min^{-1}.

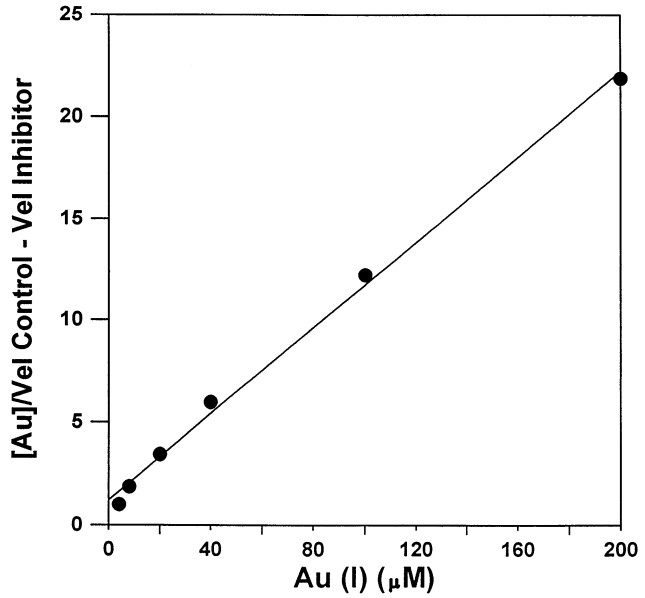

FIG. 2. Determination of K_i for $Et_3PAuSATg$ inhibition of GSH-Px in the presence of 1.0 mM of GSH. All conditions were as described in the legend of Fig. 1. The line was obtained by linear regression of the points (R = 0.9965). The inhibition constant, K_i, for $Et_3PAuSATg$ was obtained from the ratio of intercept/slope. Vel = velocity. Each data point represents the average of at least three independent measurements.

fore, any inhibition of the reaction by auranofin ($Et_3PAuSATg$) or its analogue, Et_3PAuCl, depends on the gold(I) species present.

Inhibition of Glutathione Peroxidase by Et_3PAuX ($X = ATgS^-$, Cl^-)

The effect of $Et_3PAuSATg$ was investigated, since gold(I) species derived from it accumulate in erythrocytes [2]. As can be seen in Fig. 1, the activity of the erythrocyte GSH-Px decreased in the presence of $Et_3PAuSATg$. An inhibition constant, K_i = 11.7 ± 5.1 μM, in the presence of 1 mM of GSH was determined from the linear plot demonstrated in Fig. 2. A single slope was observed over the range of $Et_3PAuSATg$ concentrations utilized in this study. Therefore, it is concluded that gold(I) inhibition occurs via binding at a single site on GSH-Px. A value for K_i (8.2 ± 2.6 μM) was also determined for the reactions run in the presence of 0.25 mM of GSH (Table 1).

A similar study was also employed to analyze the interaction of Et_3PAuCl with erythrocyte glutathione peroxidase. This analogue of auranofin was studied to determine if the substitution of the Cl^- ligand for $ATgS^-$ altered the inhibition constants determined for $Et_3PAuSATg$. The addition of Et_3PAuCl also decreased the activity of GSH-Px (Fig. 1). As for $Et_3PAuSATg$, inhibition via binding at a single site was observed for Et_3PAuCl, with K_i values equal to 10.9 ± 5.5 and 8.1 ± 2.7 μM in the presence of 1.0 and 0.25 mM of GSH, respectively. These values are very close to those determined for $Et_3PAuSATg$ (Table 1).

The type of inhibition (reversible competitive, irreversible competitive, or noncompetitive) occurring between Et_3PAuX and GSH-Px was determined under steady-state conditions. The concentration of GSH was kept at a constant level of 1.0 mM, and the amounts of Et_3PAuX and t-BOOH were varied. The rate of GSH-Px activity as a function of both t-BOOH and $Et_3PAuSATg$ concentration can be seen in Fig. 3. The common intercepts of points approximately at the vertical axis demonstrate that $Et_3PAuSATg$ bound competitively with respect to the peroxide substrate. When the data for both compounds were replotted as described by Dixon [18], the binding constants for $Et_3PAuSATg$ and Et_3PAuCl inhibition were calculated to be 11.6 ± 0.8 and 10.8 ± 0.5 μM, respectively (Fig. 3, inset).

Inhibition of Glutathione Peroxidase by $Au(SG)_2^-$ and AuSTg

The concentration of GSH in erythrocytes has been determined to be between 1 and 10 mM [11], thus suggesting that gold(I) in the cell may bind to this abundant thiol ligand. Therefore, gold glutathione ($Au(SG)_2^-$) was also studied. The extent of inhibition was greater than with either of the triethylphosphine gold(I) complexes (Fig. 1). When the data obtained in the presence of 1 mM of GSH were replotted, a linear relationship was observed, and a

TABLE 1. Comparison of Inhibition constants for various gold(I) complexes

Gold(I) complex	K_i (μM) Erythrocyte GSH-Px 1 mM GSH	K_i (μM) Erythrocyte GSH-Px 0.25 mM GSH	K_i (μM) Liver GSH-Px	Inhibition type
$Et_3PAuSATg$	11.7 ± 5.1* 11.6 ± 0.8†	8.2 ± 2.6		Competitive
Et_3PAuCl	10.9 ± 5.5* 10.8 ± 0.5†	8.1 ± 2.7		Competitive
AuSTg	2.4 ± 0.5	2.1 ± 0.5	2.3‡	Competitive‡
$Au(SG)_2^-$	2.8 ± 0.4			ND§

Values are means ± SD, $N = 3$.
*Fig. 2.
†Fig. 3, Dixon plot.
‡Ref. 9.
§Not determined.

single value for the K_i (2.8 ± 0.4 μM) was calculated (Table 1).

The K_i for AuSTg inhibition of purified GSH-Px from hamster liver was determined previously to be ≈2.3 μM [9, 19]. For a comparison, the inhibition of erythrocyte GSH-Px by AuSTg was studied. Linear relationships yielding single inhibition constants of 2.4 ± 0.5 and 2.1 ± 0.5 μM were observed in the presence of 1.0 and 0.25 mM of GSH, respectively (Table 1). Both values are similar to the K_i values previously determined for purified hamster liver glutathione peroxidase [9] and to the constant for $Au(SG)_2^-$ inhibition of erythrocyte GSH-Px determined here.

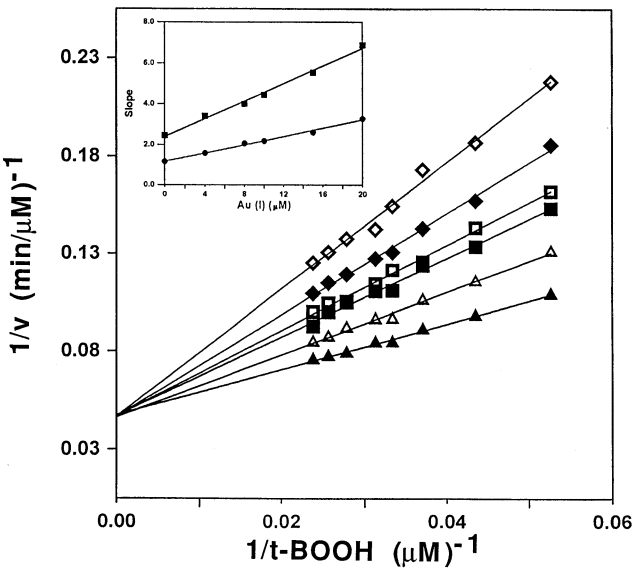

FIG. 3. Double-reciprocal plot of inhibition for GSH-Px by $Et_3PAuSATg$ in the presence of 1 mM of GSH at 37° in Tris–HCl buffer, pH 7.6. All other conditions for the assay mixture are described in Materials and Methods. From bottom to top line, the initial $Et_3PAuSATg$ concentrations were 0, 4, 8, 10, 15, and 20 μM, respectively. Each line was analyzed by linear regression with all values of $R > 0.990$. Inset: The slopes of the lines were plotted as a function of gold(I) concentration. Linear regression analysis yielded an R equal to 0.9933 and 0.9971 for $Et_3PAuSATg$ (●) and Et_3PAuCl (■). The intercept is equivalent to the K_m/V_{max} and the slope is equivalent to the $(K_m/V_{max})/K_i$. Each data point represents the average of at least three independent measurements.

$Et_3PAuSATg$ Dependence of the Glutathione Peroxidase Activity in Erythrocyte Lysate

Erythrocyte (red blood cell) lysate was prepared and used to analyze baseline levels of glutathione peroxidase activity. Since hemoglobin, which also has an absorbance at 340 nm, is present in the lysate, the volume of erythrocytes that could be added was limited by the spectrophotometric sensitivity. The rate of oxidation of NADPH increased linearly with the increasing volume of added cells. The intercept is non-zero, because NADPH is also oxidized by catalase, which is present in erythrocytes [13, 14]. Tappel and coworkers [13, 14] determined that gold(I) does not affect the activity of catalase. Therefore, the inhibition of NADPH oxidation by gold(I) in erythrocytes can be related directly to the inhibition of glutathione peroxidase activity.

The K_i for auranofin inhibition of glutathione peroxidase activity was determined in the lysate. The decrease in glutathione peroxidase activity as a function of added $Et_3PAuSATg$ (Fig. 4) resembled the profile observed earlier with purified enzyme (Fig. 1). From the lysate data, an inhibition constant for the binding of $Et_3PAuSATg$ to glutathione peroxidase was determined to be 8.0 ± 1.6 μM (Table 2). This value is within the experimental error of the inhibition constant determined for $Et_3PAuSATg$ with glutathione peroxidase purified from bovine erythrocytes (Fig. 2; Table 1).

Inhibition of Glutathione Peroxidase in Intact Erythrocytes

To examine the inhibition of GSH-Px after cellular uptake of gold(I), the effect of auranofin on erythrocytes in either whole blood or isotonic buffer was investigated. The intact cells were removed from the native plasma and resuspended in isotonic buffer and exposed to $Et_3PAuSATg$. After an

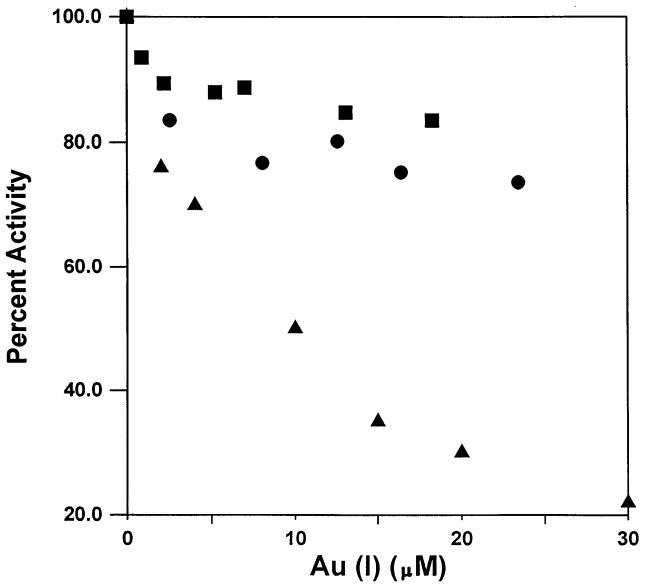

FIG. 4. Effect of gold(I) from $Et_3PAuSATg$ on the activity of GSH-Px in erythrocyte lysate (▲), erythrocytes resuspended in isotonic buffer (●), or whole blood (■). The concentration of all components of the assay mixture are described in Materials and Methods. The final $Et_3PAuSATg$ concentration added to the assay mixture was either 0, 2, 4, 10, 15, 20, or 30 μM. The amount of inhibition is expressed as a percent of the control activity for glutathione peroxidase (13.6 ± 0.3 μM of NADPH min^{-1}). Each data point represents the average of at least three independent studies.

incubation period, the cells were lysed for determination of the gold accumulation and its effect on GSH-Px activity. Typically, 83% of the gold was transported into the cells after 15 min as determined by AA spectroscopy. In all cases, the final gold concentrations determined inside and outside of the erythrocytes accounted for 95–100% of the gold added initially. The activity of glutathione peroxidase in each of the gold-treated cell lysates was determined. As can be seen in Fig. 4, the glutathione peroxidase activity decreased in the presence of increasing gold concentration. When the data were replotted using the lysate gold concentration, the K_i was calculated to be 2.1 ± 1.6 μM (Table 2).

Next, the effect of $Et_3PAuSATg$ on glutathione peroxidase activity was investigated in whole blood. The plasma contains serum albumin, which is a high affinity gold binding site [4, 20, 21], at a concentration of ≈500–600 μM. Therefore, upon addition of $Et_3PAuSATg$ (0–30 μM) to the blood, some gold remains bound to serum albumin and is not transported into the erythrocytes. The distribution of gold(I) between the cells and plasma was ≈57 and ≈43%, respectively. Because the cellular uptake of gold is lower in whole blood, the activity of glutathione peroxidase determined for each of the gold-treated erythrocyte lysates was greater than in isotonic buffer at the same initial gold concentration (Fig. 4). Nevertheless, the inhibition constant for gold(I), 2.0 ± 0.8 μM (Table 2), was the same as that in buffer when the lysate gold concentration was used for the calculation. These values, which are much less than the K_i determined when $Et_3PAuSATg$ was reacted without an intact red blood cell membrane, provide evidence for phosphine displacement to form $Au(SG)_2^-$ and will be addressed in the discussion.

TABLE 2. Inhibition constants for glutathione peroxidase in erythrocytes exposed to $Et_3PAuSATg$

Conditions	K_i (μM)
$Et_3PAuSATg$ + lysed erythrocytes	8.0 ± 1.6
$Et_3PAuSATg$ + erythrocytes in buffer*	2.1 ± 1.6
$Et_3PAuSATg$ + whole blood	2.0 ± 0.8

Values are means ± SD, $N = 3$.
*20 mM of MOPS, 150 mM of KCl, and 5 mM of EDTA, pH 7.4.

DISCUSSION

Tappel and coworkers examined the effect of AuSTg on various GSH-Px enzymes; their studies focused on glutathione peroxidase purified from hamster liver, and in various mammalian tissues [9–14, 19]. AuSTg binds reversibly at the active selenocysteine site of purified hamster liver GSH-Px, with a inhibition constant of 2.3 μM. The mixed bidentate selenolate-thiolate complex of gold that was postulated as an intermediate is consistent with their inhibition studies and the known chemistry of gold [9]. Despite the extensive work by Tappel *et al.* on AuSTg inhibition of GSH-Px, virtually nothing is known about the effect of ligation of the gold by other ligands, except for the case of gold thiomalate, where direct and complete inhibition of GSH-Px by the thiomalate precluded further studies. Unlike AuSTg, the administration of auranofin ($Et_3PAuSATg$) leads to a substantial accumulation of gold(I) metabolites in erythrocytes [22], where there is significant GSH-Px activity. Since gold(I) efflux is an equilibrium phenomenon [4], the results of Intoccia *et al.* [22] suggest that a very high affinity site for gold must be present in the erythrocytes, and that this site can pull the gold away from the high affinity Cys-34 site of serum albumin. Therefore, the present study of erythrocyte GSH-Px with various gold compounds, especially $Et_3PAuSATg$ and complexes of glutathione, the dominant thiol in erythrocytes, was necessary to understand the pharmacological significance of gold inhibition of GSH-Px.

The four compounds investigated included two gold(I) thiolates, AuSTg and $Au(SG)_2^-$, and two phosphine complexes, $Et_3PAuSATg$ and Et_3PAuCl. The K_i values for inhibition of the purified GSH-Px by AuSTg and $Au(SG)_2^-$, despite their different stoichiometries and structures, were experimentally indistinguishable and close to those measured previously [9]. The values for the phosphine complexes were also similar to one another, but larger than those for the simple thiolates. The latter result is especially surprising since chloride is so much weaker than a thiol as a ligand for gold(I).

Sadler and coworkers [23, 24], using NMR spectroscopy,

determined that GSH completely displaces the Cl^- ligand of Et_3PAuCl by ligand exchange as described in Equation 3.

$$Et_3PAuCl + GSH \rightarrow Et_3PAuSG + Cl^- + H^+ \quad (3)$$

Therefore, in the assay system, where 0.25 or 1.0 mM of GSH is present, the species interacting with glutathione peroxidase is Et_3PAuSG, not Et_3PAuCl. Furthermore, equilibrium displacements of the $ATgS^-$ from auranofin ($Et_3PAuSATg$) by protein and low-molecular-weight thiols [17, 20, 21, 23–26] including GSH [23, 24, 27, 28], Equation 4,* have been documented extensively. Although the thiol pK_{SH} values and the NMR

$$Et_3PAuSATg + GSH \rightleftharpoons Et_3PAuSG + HSATg \quad (4)$$

chemical shifts (δ_p) of Et_3PAuSG and $Et_3PAuSATg$ suggest that the ATgSH ligand has a higher affinity than GSH for gold(I) [26], the large excess of GSH (1 mM) over $ATgS^-$ (2–200 μM) shifts the equilibrium to favor the GSH complexes. Thus, the indistinguishable inhibition constants reported here for $Et_3PAuSATg$ and Et_3PAuCl (Table 1) are explained by GSH displacement of the Cl^- and $ATgS^-$ ligands according to Equations 3 and 4.

The inhibition constants determined for both $Et_3PAuSATg$ and Et_3PAuCl in the presence of 0.25 mM GSH were lower than those determined at 1.0 mM (Table 1). This is most likely due to the increased thermodynamic competition between the enzyme and GSH for Et_3PAu^+ at higher GSH levels (Equation 5). The values for K_i are larger than for AuSTg, suggesting that the triethylphosphine ligand common to both Et_3PAuCl and $Et_3PAuSATg$

$$GSH\text{–}PxSeH + Et_3PAuSG \rightleftharpoons GSH\text{–}PxSeAuPEt_3 + GSH \quad (5)$$

weakens the inhibition.

$Au(SG)_2^-$ is an unusually stable dithiolate gold species [29]. Elder *et al.* [29] have observed $Au(SG)_2^-$ formation in the reactions of $Au(CN)_2^-$ with GSH under conditions mimicking red blood cell concentrations. Chaudiere and Tappel [9] postulated that because the GSH concentration was 40-fold greater than the AuSTg concentrations in the enzyme assay, the gold(I) species interacting with glutathione peroxidase may actually be $Au(SG)_2^-$, and not AuSTg (Equations 6 and 7). As demonstrated in Fig. 1, the inhibition patterns of $Au(SG)_2^-$ and AuSTg are similar. The inhibition constant, K_i, for authentic $Au(SG)_2^-$ was experimentally equivalent to the value obtained with AuSTg (Table 1). These results provide distinct experimental confirmation for the hypothesis of Chaudiere and Tappel.

* When 4 mM of GSH and 4 mM of $Et_3PAuSATg$ react in 10% MeOH:90% 100 mM of NH_4HCO_3 buffer, pH 7.9, the ^{31}P NMR spectrum exhibits a single average resonance at 36.3 ppm, intermediate between the resonances of $Et_3PAuSGATg$ (37.0 ppm) and Et_3PAuSG (35.8 ppm). This result indicates ligand rapid exchange and substantial conversion to the glutathione complex. No Et_3PO (61.3 ppm) is observed under these conditions (Isab AA and Shaw CF III, unpublished observations).

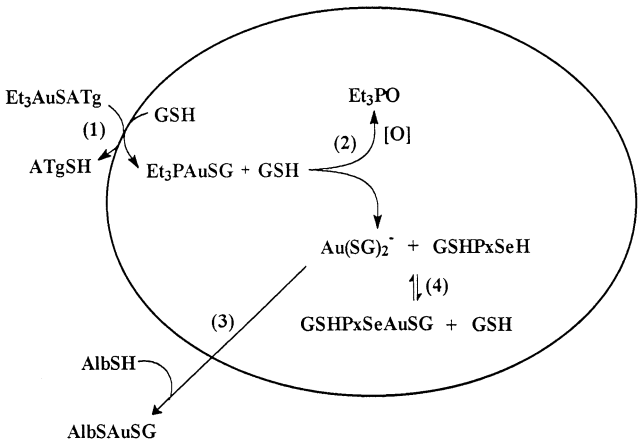

FIG. 5. Reaction of auranofin ($Et_3PAuSATg$) and its metabolites with erythrocyte components. Steps 1–4 have been described independently elsewhere [4, 22–24, 30, 31]. Together they comprise an explanation for the K_i value equal to that of $Au(SG)_2^-$ when inhibition of GSH-Px is measured after exposing intact cells to auranofin.

$$AuSTg + GSH \rightleftharpoons GSAuSTg + H^+ \quad (6)$$

$$GSAuSTg + GSH \rightleftharpoons Au(SG)_2^- + HSTg \quad (7)$$

The inhibition constant for $Et_3PAuSATg$ added to human erythrocyte lysate in the presence of all the cellular components (Table 2) was very similar to the value determined for purified bovine erythrocyte glutathione peroxidase in the presence of 1 mM of GSH (Table 1). The slight difference may be due to small differences between the human and bovine enzymes. To determine the inhibition of glutathione peroxidase in intact erythrocytes, various amounts of $Et_3PAuSATg$ were reacted with either whole blood, or erythrocytes that were resuspended in isotonic buffer in the absence of serum albumin. The inhibition constants determined in whole blood and in buffer, after correcting for the different extent of gold(I) uptake, were indistinguishable from one another (Table 2). Therefore, the presence of extracellular serum albumin does not alter the inhibition constant for gold(I) binding to glutathione peroxidase in the red blood cells, although it reduces the amount of gold(I) entering the cells. However, the inhibition constants when a cell membrane was present differed from those when $Et_3PAuSATg$ was added directly to the lysate. The lower K_i for gold(I) determined when $Et_3PAuSATg$ was added to intact cells (Table 2) was comparable to that for $Au(SG)_2^-$ (Table 1). Therefore, these data indicate that after transport into the erythrocytes the gold(I) from $Et_3PAuSATg$ no longer retains either original ligand. Loss of triethylphosphine is driven by oxidation to Et_3PO, which occurs *in vivo* [22, 30] and in whole blood *ex vivo* [23, 24], but not when $Et_3PAuSATg$ reacts via simple ligand exchange with other thiols [20, 25, 27, 28] as in the cell free assay system.

Figure 5 presents the metabolism of auranofin ($Et_3PAuSATg$) as it interacts with the erythrocyte (red

blood cell). This diagram integrates previous findings [4, 22–24, 30, 31] with the work presented in this study. The Et_3PAu^+ moiety from auranofin is shuttled across erythrocyte and other cell membranes (Fig. 5, step 1), due to the lipophilic triethylphosphine ligand bound to $Et_3PAuSATg$ [30, 31]. Intoccia *et al.* [22] found that greater amounts of gold accumulate in the erythrocytes than in the serum. As previously mentioned, the concentration of GSH in red blood cells can reach levels greater than 1 or 2 mM [11]. Therefore, glutathione is potentially an important ligand in gold metabolism, and Et_3PAuSG has been identified in red blood cells exposed to Et_3PAuCl [23]. It has also been determined, by using triple-labeled $[^3H]Et_3P^{195}Au^{35}SATg$, that upon administration of $Et_3PAuSATg$ to dogs the Au—P bond is broken [22, 30]. The phosphine is oxidized to Et_3PO, which has been found in both the urine and the bloodstream after auranofin administration [22, 30]. Therefore, the triethylphosphine ligand is progressively displaced from gold(I) in the erythrocytes, and the gold(I) complex that remains is $Au(SG)_2^-$ (Fig. 5, step 2). This conclusion derives further support from the work of Shaw *et al.* [4] on gold efflux from erythrocytes. In that study, it was determined that glutathione and albumin are the ligands bound to gold(I) after it effluxes into the plasma (Fig. 5, step 3) [4].

The mechanism represented in Fig. 5 is consistent with and explains the results obtained by utilizing isolated erythrocytes as the source of glutathione peroxidase. As previously demonstrated for other cells [22, 30], the $ATgS^-$ ligand is lost when $Et_3PAuSATg$ is transported into the cytosol [30, 31]. However, this would not affect the inhibition constant, because, as seen in Table 1, when either $Et_3PAuSATg$ or Et_3PAuCl was added to the glutathione peroxidase assay, the K_i values were the same. Yet, the K_i obtained when auranofin was added to intact red blood cells (Table 2) was experimentally indistinguishable from the value obtained for the interaction between authentic $Au(SG)_2^-$ and purified glutathione peroxidase (Table 1). This decline in K_i for auranofin in the presence of an intact membrane supports the postulate that both original ligands of auranofin are displaced by GSH in the cell. The loss of Et_3P, which is not thermodynamically favorable in itself, is driven by its oxidation to Et_3PO in the presence of the erythrocyte membranes (and other oxidants *in vivo*) that are not present in the assay system containing only purified enzyme.

In conclusion, this study demonstrated the following: (1) the ligation of gold altered its ability to inhibit GSH-Px and, by extension, other enzymes; (2) the inhibition constants obtained using $Et_3PAuSATg$ and Et_3PAuCl in the presence of purified enzyme and cell-free extracts were equal due to the generation of Et_3PAuSG by ligand exchange with GSH; (3) the inhibition measured in whole blood yielded the same K_i as $Au(SG)_2^-$; and (4) therefore, the final circulating gold(I) metabolites of auranofin are radically different than the originally administered complex.

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