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EFFECTS OF LIGANDS ON GOLD INHIBITION OF SELENIUM GLUTATHIONE PEROXIDASE

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Abstract—Gold inhibits selenium-dependent glutathione peroxidase (GSH Px) *in vitro*. Chrysotherapy has been used for over five decades without complete understanding of its pharmacodynamics. This study shows that gold is potentially an inhibitor of GSH Px *in vivo*. Reported are conditions for assay of GSH-Px activity showing that the inhibition by gold species can be reversed. The study demonstrates the high affinity of gold for the selenohydryl-active site of GSH Px relative to the affinities of other physiological ligands; GSH Px was inhibited to a greater extent by a small molecular-weight fraction isolated from kidneys of gold-treated rats than by the fraction isolated from kidneys of rats not treated with gold. The data provide a new perspective on the action of gold-containing drugs *in vivo*.

Gold-containing drugs are used in the treatment of human rheumatoid arthritis [1] and other inflammatory disorders [2]. Recent findings in this laboratory show that gold interacts with selenium *in vivo* [3] and is a strong inhibitor of selenium-glutathione peroxidase (GSH:H_2O_2 oxidoreductase, EC 1.11.1.9) (GSH Px)† *in vitro* [4]. Purified GSH Px is very sensitive to gold inhibition *in vitro*. High concentrations of gold accumulate in tissues during chrysotherapy [5, 6]; however, direct measurement of *in vivo* inhibition of the enzyme by soluble gold is difficult. Assay of GSH Px activity in tissue homogenates requires dilution of the activity and, therefore, of any inhibitory species of gold that may be present.

The bioinorganic chemistry of Au(I) has been described [7, 8]. Sulfhydryl groups are stable water-soluble ligands for Au(I). The overwhelming abundance of available sulfhydryl ligands relative to selenohydryl ligands *in vivo* makes significant inhibition of GSH Px under physiological conditions questionable. The present study addressed the question of whether *in vivo* inhibition of GSH Px by gold could occur after administration of gold-containing drugs.

Inhibition of GSH Px activity in preparations from a variety of tissues and species by gold drugs was tested. The ability of other ligands to alter GSH Px inhibition by gold drugs was demonstrated. Additionally, a small molecular-weight fraction isolated from kidneys of gold-treated rats inhibited GSH Px.

MATERIALS AND METHODS

Enzyme sources. Purified bovine erythrocyte GSH Px (BvGP) (Sigma, St. Louis, MO) and a 105,000 *g* supernatant fraction of homogenized liver from a rat fed a standard diet (RLS) were used as enzyme sources to test the effect of gold and other ligands on activity of the enzyme. The apparent GSH transferase activity present in RLS was measured by the difference in activity using cumene hydroperoxide and hydrogen peroxide as substrates. The GSH transferase activity was approximately 12% of the total activity. Testing of RLS for GSH transferase by cyanide inhibition indicated an absence of activity. Erythrocytes and polymorphonuclear leukocytes (neutrophils) were isolated from blood of a healthy human adult female. Erythrocytes were separated from whole heparinized blood by centrifugation at 1000 *g* and washed three times with 0.9% NaCl. The erythrocytes were then diluted in 19 vol. of distilled water. Neutrophils were separated from heparinized blood diluted 1:1 with Hanks' balanced salt solution by centrifugation through one-quarter volume of a mixture containing 10% Hypaque (Winthrop Laboratories, New York, NY) and 6.4% Ficoll (Pharmacia Fine Chemicals, Piscataway, NJ) at 350 *g* for 20 min at room temperature. The plasma and monocyte layers were removed, and the neutrophil-containing fraction was treated with 3% Dextran T500 (Pharmacia) to sediment erythrocytes. The supernatant fraction was centrifuged at 300 *g* for 10 min at 4°. The remaining erythrocytes were lysed by adding 20 ml of 0.2% NaCl, mixing for 30 sec, and adding 20 ml of 1.6% NaCl to achieve isotonicity. Neutrophils were washed twice in calcium- and magnesium-free Hanks' balanced salt solution and resuspended at approximately 5×10^7 cells/ml.

Assays. GSH Px activity was measured using a coupled assay [9], which is an appropriate method for measuring gold inhibition of GSH Px [4]. Unless indicated otherwise, the concentration of GSH was

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† Abbreviations: GSH Px, selenium-dependent glutathione peroxidase; BvGP, bovine erythrocyte glutathione peroxidase; RLS, rat liver soluble fraction; and KCF, kidney cytosol fraction.

0.25 mM. Gold thioglucose (GTG) (Sigma) and SKF D-39162, *S*-triethyl phosphine gold 2,3,4,6-tetra-acetyl-1-thioglucopyranoside (Auranofin), were tested as enzyme inhibitors. SKF D-39162 was dissolved in 70% ethanol at 20 mM. Aliquots of this stock solution were added to the reaction mixtures at the concentrations indicated. SKF D-39162 was a gift of the Smith Kline & French Co., Philadelphia, PA. Potential reversers of inhibition, henceforth referred to as ligands, were added to reaction mixtures that contained gold prior to starting the reaction by addition of cumene hydroperoxide. Sodium cyanide was added to the reaction mixture containing gold after the reaction was started.

Ligands. NaCN, KI (both from J. T. Baker, Phillipsburg, NJ), KCl, NaSCN (Mallinckrodt, Paris, KY), KBr (Kodak, Rochester, NY), selenomethionine, selenocystine, cysteine, cystine, β-mercaptoethanol, and egg lysozyme (Sigma) were used as ligands. Human and bovine albumin (fraction V), human transferrin, and histidine monochloride were purchased from Nutritional Biochemicals, Cleveland, OH. Bovine γ-globulins (fraction II) were purchased from Calbiochem, La Jolla, CA.

Concentrated protein solutions, 25–50 mg/ml, were dialyzed overnight against 10 mM Tris–HCl, 0.1 mM EDTA, and 0.25 mM GSH to remove small molecular weight compounds and to reduce sulfhydryl groups. Aliquots of the protein solution were incubated with 5 or 10 nmoles GTG at 37° for 15 min before addition of BvGP and other components of the reaction mixture. The mixture was incubated for a further 5 min at 37° before the reaction was started by addition of cumene hydroperoxide.

Selenocysteine was prepared by reduction of selenocystine. To 1.35 mg selenocystine (Sigma) was added 1.65 ml of a mixture that contained 200 μl of β-mercaptoethanol, 423 μl of 2 M ammonium hydroxide, and 3.45 ml of 1 mg/ml sodium borohydride. The mixture was heated for 5 min at 45° and then dried at 45° under nitrogen. The molar equivalent of cystine (0.98 mg) was treated in the same manner. After drying, the volume of liquid in each tube was brought to 1 ml with 0.01 M HCl–1 mM dithioerythritol to give an 8 mM solution of selenocysteine or cysteine.

Protein-free fractions were obtained from gold-treated or control rat kidney soluble fractions. The kidney soluble fraction was the 100,000 *g* supernatant obtained from 1:1 tissue-distilled water homogenates. Aliquots (1 ml) of the supernatant fractions were loaded into Centrifree filtration devices (Amicon, Danvers, MA) that had been washed three times with distilled water to remove azide. The devices were centrifuged for 30 min at 1500 *g*. The clear filtrates were kept on ice until they were added to the reaction mixtures.

Data analysis. For each combination of ligand and GTG concentrations tested, the fraction of enzyme activity (relative to uninhibited activity) in the presence of both agents was compared to the fraction of enzyme activity in the presence of the ligand and GTG at the appropriate concentration. When the fraction of enzyme activity in the presence of both agents was greater than the product of the fractions of enzyme activity in the presence of either gold or the ligand alone, the ligand was considered antagonistic to the inhibition by gold [10].

The degree to which enzyme activity is restored in the presence of a known concentration of reversing agent is a measure of the ratio of K_r to K_i and ligand to inhibitor ratio. Rearranging the terms in a formula from Webb [10] gives the following formula:

$$K_r = \frac{K_i}{\frac{I_t}{R_t}\left(\frac{1}{i_r} - \frac{1}{i_0}\right)}$$

K_r is the dissociation constant for ligand and inhibitor; K_i is the inhibition constant for GTG (2.3 μM) [4]; I_t is the GTG concentration; R_t is the ligand concentration (this equation is valid only if $R_t > I_t$); i_r is the fraction of enzyme activity inhibited in the presence of R (ligand); and i_0 is the fraction of enzyme activity inhibited by GTG alone.

RESULTS

GSH Px inhibition by gold drugs. The inhibition curves obtained with the impure enzyme sources, RLS, human erythrocytes, and human neutrophils, by GTG were similar to the curves obtained with purified hamster liver [4] and purified BvGP (Fig. 1). The inhibition of BvGP by SKF D-39162 was similar to that by GTG on a nanomole gold basis, but inhibition by SKF D-39162 was less potent for RLS GSH Px activity (Fig. 2). Because the assay used can measure activity of any GSH transferase in RLS, a small amount of this activity may account for some of the differential effects.

Gold-containing tissues as GSH Px inhibitor. To show that there were small molecular weight gold species in the cell cytosol capable of inhibiting GSH Px in gold-injected animals, kidney homogenates from four rats injected with approximately 30 mg of GTG over a 21-day period and containing an average of 520 ppm gold were fractionated to obtain a protein-free fraction from cell cytosol (KCF). Addition of KCF from kidneys of control and GTG-injected animals to GSH Px demonstrated that KCF from control rats was inhibitory, but that KCF from gold-treated rats was a more potent inhibitor (Fig. 3A).

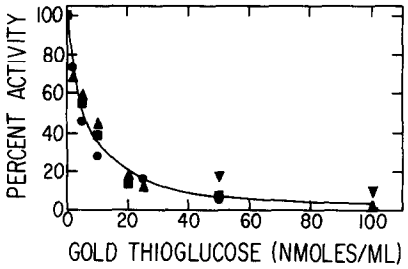

Fig. 1. Inhibition of glutathione peroxidase activities by gold thioglucose. Key: purified bovine erythrocyte glutathione peroxidase (●), rat liver soluble fraction (▲), human erythrocyte lysate (■), and human neutrophil lysate (▼). Points represent means of at least four determinations for rat liver soluble fraction, three determinations for bovine erythrocyte glutathione peroxidase, and single determinations for erythrocyte and neutrophil activities.

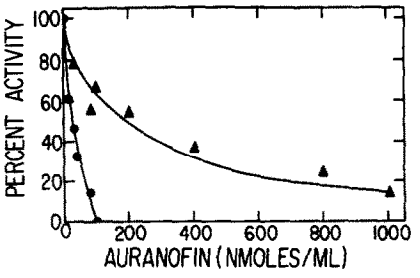

Fig. 2. Inhibition of purified bovine erythrocyte glutathione peroxidase (●) or rat liver soluble fraction (▲) by SKF D-39162. Points represent a single determination.

KCF was incubated with BvGP for 5 min at 37° before assay. The additive inhibitory effect of gold and KCF was demonstrated by adding GTG to the reaction mixture in the presence and absence of KCF (Fig. 3B). The amount of gold required to cause the same degree of inhibition that could be attributed to factors other than KCF was interpolated from Fig. 1. The gold concentration in undiluted KCF prepared from the four separate kidneys corresponded to 12–18 nmoles/ml on the basis of its GSH Px inhibition. The KCF fraction was too small to determine gold by atomic absorption spectroscopy.

Ligands as reversers of inhibition. The affinity of gold for the active site of GSH Px as compared to its affinity for other potential ligands is demonstrated by the ability of these ligands to reverse the inhibition by GTG. Table 1 lists compounds tested for their abilities to increase GSH Px activity in the presence of GTG. The increase in enzyme activity in the presence of the ligand plus GTG over the activity in the presence of GTG alone is described as an antagonism between the ligand and GTG. Cyanide, cysteine, selenocysteine, and several proteins, human and bovine albumin, γ-globulin fraction, and lysozyme were antagonistic to GTG inhibition of BvGP.

Cyanide (10 mM) reversed the inhibition of RLS GSH Px at all gold concentrations when GSH was present at concentrations of 1 mM or higher. At GSH concentrations of 0.5 mM or lower, reversal of enzyme inhibition was incomplete (Fig. 4). Cyanide

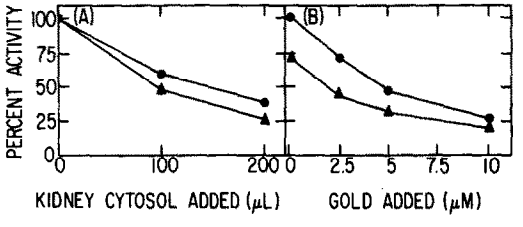

Fig. 3. Inhibition of bovine glutathione peroxidase. (A) Kidney cell cytosol fraction from control (●) or gold thioglucose-injected rats (▲) was added to a 1-ml reaction mixture and preincubated at 37° for 5 min. Points for control cytosol represent two determinations. Points for gold-containing cytosol are means of four separate cytosols tested individually. (B) Additive inhibition by gold thioglucose and kidney cell cytosol fraction. Conditions: added gold thioglucose (●), and gold thioglucose plus 50 μl kidney cytosol fraction from control rats (▲) in a 1-ml reaction mixture. Points represent a single determination.

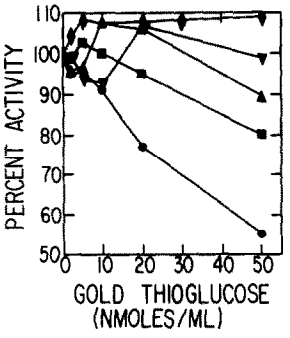

Fig. 4. Inhibition of rat liver soluble glutathione peroxidase by gold thioglucose in the presence of various concentrations of glutathione and 10 mM sodium cyanide. Key: 0.10 mM GSH (●), 0.25 mM GSH (■), 0.50 mM GSH (▲), 1.0 or 5.0 mM GSH (◆), 2.5 mM GSH (▼). Points represent the mean of two determinations.

only partially reversed gold inhibition of purified BvGP and, when present at concentrations above 0.2 mM, the enzyme reaction rate decreased (data not shown). Cysteine and selenocysteine reversed the inhibition of RLS GSH Px.

The kinetic rate of GSH Px is proportional to the available GSH concentration when hydroperoxide concentration is limiting. The ability of GSH to alter gold binding to the enzyme was tested at GSH concentrations from 0.1 to 5.0 mM, and the enzyme activity at each GTG concentration is expressed in Fig. 5 as the percentage of the rate at each GSH concentration in the absence of GTG. Figure 5 shows that, despite activation of the enzyme by increased GSH concentration, the percentage of enzyme activity was constant over the range of 0.1 to 2.5 mM GSH for each GTG concentration. At 5.0 mM GSH, the hydroperoxide concentration used in these determinations was limiting.

The halide salts, azide, and thiocyanate did not increase the activity of GSH Px in the presence of GTG. Selenomethionine was not antagonistic to the inhibition.

The relative affinity of each ligand tested as a reverser of gold inhibition may be quantitatively decribed by its dissociation constant with gold. The K_r for ligands shown to be antagonistic to GTG

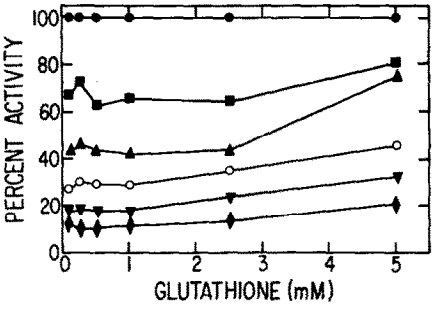

Fig. 5. Gold thioglucose inhibition of rat liver soluble glutathione peroxidase at various GSH concentrations expressed relative to the activity at the same GSH concentration in the absence of gold thioglucose. Key: no GTG (●); 2 (■), 5 (▲), 10 (○), 20 (▼), or 50 (◆) μM GTG. Points represent the mean of two determinations.

Table 1. Ligand reversal of glutathione peroxidase inhibition by gold

Ligand	Concentration	Glutathione peroxidase source	Gold thioglucose (μM)	Effect
	mM			
KCl	10	Bovine	5–50	None
KI	10	Bovine	5–50	None
KBr	20	Bovine	5–50	None
NaSCN	2, 5	Bovine	10	None
NaN_3	10	Bovine	10	None
NaCN	0.01–10	Bovine, rat	2–50	Antagonism*
Glutathione	0.1–5.0	Rat	2–50	None
β-Mercaptoethanol	0.5, 0.1, 0.2	Bovine	10	None
Cysteine (by reduction)	0.08, 0.16	Bovine, rat	10, 20, 50	Antagonism
Cysteine	0.16	Bovine	10	Antagonism
Selenocysteine (by reduction)	0.08, 0.16, 0.4	Bovine, rat	10, 20, 50	Antagonism
Selenomethionine	1	Bovine	5–50	None
Histidine	0.5–10	Bovine	20	Antagonism
	mg/ml			
Bovine albumin	10, 15	Bovine	5, 10	Antagonism
Human albumin	10, 15	Bovine	5, 10	Antagonism
Bovine γ-globulins	10, 15	Bovine	5, 10	Antagonism
Human transferrin	6, 9	Bovine	10	Antagonism
Egg lysozyme	2.5, 5.0, 7.5	Bovine	5, 10, 20	Antagonism

* Antagonism is defined as an increase in glutathione peroxidase activity in the presence of ligand plus gold thioglucose over the activity in the presence of gold thioglucose alone.

Table 2. Calculated dissociation constants of ligands for gold (K_r)

Ligand	K_r* (μM)	K_r† (μM)
Selenocysteine (by reduction)	43 ± 27‡ (9)§	43 (2)
Cysteine (by reduction)	59 ± 6 (3)	50 (2)
Sodium cyanide	106 ± 20 (4)	134 ± 34 (3)
γ-Globulins	187 ± 56 (3)	ND‖
Bovine albumin	197 ± 20 (3)	ND
Human albumin	242 ± 96 (3)	ND
Egg lysozyme	538 ± 161 (3)	ND
Histidine	10^4 (3)	ND
Human transferrin	$>10^5$ (2)	ND

* Calculated using reversal of BvGP inhibition.
† Calculated using reversal of RLS inhibition.
‡ Values are means ± S.D. when more than two assays were averaged.
§ Number of assays.
‖ Not determined.

inhibition of GSH Px *in vitro* (Table 1) were calculated. The ligands are listed in Table 2 in order of strongest to weakest in affinity for gold on the basis of the K_r.

DISCUSSION

GTG inhibited GSH Px activity in a variety of species and tissues, including rat liver, bovine and human erythrocytes, and human neutrophils. The extent of inhibition of comparable amounts of enzyme activity by equal amounts of gold was similar, regardless of the enzyme source or purity. Approximately 50% inhibition occurred at 5 μm GTG. The K_i for GTG inhibition of purified hamster liver enzyme is 2.3 μM [4]. The difference could be due to variations in the GTG preparations, which may contain oxidation products of thioglucose [11].

SKF D-39162 was not as potently inhibitory to either BvGP or RLS GSH Px as was GTG, even though the two compounds contain the same molar amount of gold. Possible reasons for the difference are that SKF D-39162 does not undergo rapid ligand exchange with thiolates, as GTG does with GSH [8], or SKF D-39162 may be too bulky or too hydrophobic to be accommodated in the GSH Px active site. SKF D-39162 is lipid soluble rather than water soluble. The lower inhibitory potency of SKF D-39162 for RLS can also be attributed to the drug binding to hydrophobic sites on the other proteins and lipid components in the enzyme preparation.

Demonstration of a kidney cytosolic small mol-

ecular-weight fraction that is inhibitory to GSH Px is not surprising. Small molecules, including cysteine or selenocysteine and possibly mercaptans in cytosol, can inhibit the enzyme [12]. Inhibition of GSH Px in the cytosolic environment is complex. The inhibition by KCF from gold-treated rat kidney was 25% greater than that by KCF from control kidneys. The additive inhibition of GTG and KCF demonstrates that reversal of inhibition by physiological ligands, as in the defined assays reported here, is not complete. Since the KCF fraction represented a dilution of the original cytosol prepared from the homogenate, the free intracellular gold concentration may have been two or three times higher. The gold concentration reported for kidney soluble fraction from gold-treated rats was as much as 30% of the total tissue concentration [13, 14]. Most of the gold is presumed bound to cytosolic proteins [15], and the KCF was a protein-free fraction.

Analysis of the interaction of gold with cysteine by assay of GSH Px activity was complicated by an inhibitory effect of cysteine on the enzyme in the absence of gold. Cysteine acts as a competitive inhibitor of GSH Px in one step of the reaction mechanism, but it can substitute for GSH in another step [16, 17]. The analysis of the interaction of gold with selenocysteine by assay of GSH Px activity was also complicated. Selenocysteine has some catalytic activity like that of GSH Px. With the selenocysteine prepared with dithioerythritol, there may have been some effect from the dithioerythritol that could not be eliminated. Human and bovine albumins, γ-globulins, histidine, and cyanide also slightly decreased the reaction rate of GSH Px. The presence of lysozyme in the assay mixture increased the GSH Px activity approximately 20%.

Iodide is the softest base among the halides found *in vivo*. Both mono- and tri-iodide are known to complex with gold [18]. Thyroid gland hyperplasia in dogs administered high levels of gold drugs indicates the possibility of *in vivo* binding of iodine by gold [19]. In the present study, however, iodide did not decrease GSH Px inhibition by GTG; therefore, it could not compete with selenium as a gold ligand. Thiocyanate has nucleophilic properties and contains a charged sulfur group but, surprisingly, it did not reverse inhibition by GTG. These data are in agreement with the prediction by Sadler [7] that the affinity of cyanide for gold would be stronger than would the affinity of histidine, chloride, or carboxyl groups. Sadler [7] also suggested that Au(I) would have a stronger affinity for selenium than sulfur ligands.

The ability of proteins to reverse inhibition indicates the presence of gold-binding sites on the proteins. The presence of either numerous low-affinity or unique but high-affinity binding sites on proteins could influence inhibition by binding gold. The association constants reported for gold binding to albumin range from 2×10^2 to $1.5 \times 10^6\ M^{-1}$ [8, 20]. Mason [20] reported that gold binds to albumin at a single site, with an affinity constant of $6.1 \times 10^3\ M^{-1}$. The inverse of the affinity constant would be 164 μM, or approximately the value calculated herein for K_r for both albumin sources tested as reversers of inhibition. Albumin is the main plasma gold-binding protein [20]. Lysozyme was tested as a reverser of inhibition because it contains a free sulfhydryl group, and γ-globulins were tested because they contain a large number of disulfide bonds (20–25/molecule). Transferrin is a glycoprotein that binds ferric iron. Proteins were required in micromolar amounts to exhibit measurable antagonism to GSH Px inhibition by gold, indicating the presence of only specific, high-affinity binding sites. Transferrin was the only protein that did not have some gold-binding capacity. These findings are consistent with the observation that crude GSH Px preparations are as sensitive to GTG inhibition as are the purified enzymes.

The reversal of inhibition by cyanide and the results obtained with various GSH concentrations are difficult to explain. One possibility is that a mixed-ligand coordination complex is formed between cyanide, GSH, and gold as the ratio of GSH to cyanide is increased. This complex might be more stable than the dicyanide–gold complex. Another possibility is that cyanide binds to an oxidized form of GSH Px [21], and the BvGP is more susceptible than the RLS to this action, either because of its purity or an altered property of the active site.

The data show that the selenocysteine active site of GSH Px is among the biological ligands with the highest affinity for gold administered as GTG, and they support the idea that gold will bind selenium *in vivo*. Binding of Au(I) to other ligands, cysteine, GSH, or protein thiols, does not preclude its exchange with the GSH Px active site. Gold in contrast to mercury or silver, exchanges ligands *in vivo* [8]. Therefore, the presence of either protein or thiol-bound gold species provides an environment where significant binding to selenocysteine-containing proteins may occur *in vivo*.

As much as 80% of the selenium in the rat is bound to protein as selenocysteine [22]. Studies have shown that selenide exists in rat liver [23]. Gold binding to selenide may be important in blocking GSH Px synthesis in gold-treated rats [3]. GSH Px inhibition would decrease the antioxidant protection of cells.

The presence of 10 mM cyanide during assay of GSH Px in tissue fractions reverses inhibition by gold. After gold loading, assay of tissues in the presence of cyanide provides a means to eliminate possible residual inhibition by endogenous gold species.

The use of kinetic analysis to predict *in vivo* inhibition of enzymes is not without limitations. For example, secondary inactivation is an important consideration. However, the case for classical, single substrate-utilizing enzymes would be less complex than for GSH Px, which exhibits a ter–uni ping–pong mechanism [24]. Investigation of enzyme reactions with inhibitors and reversers is not new; however, the only investigations done have used thiols to reverse inhibition of sulfhydryl-containing enzymes by *p*-chloromercuribenzoate [10]. In these studies, the ability of thiols to reverse inhibition was assumed to be a criterion for demonstrating sulfhydryl group inactivation.

The idea of using kinetic analysis as an approximation of *in vivo* activity should be of utility to both pharmaceutical and toxiological development and testing. Use of *in vitro* assay of relative affinities of

pharmacons for enzymes could predict volume of distribution, specificity, and major side effects.

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