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Corresponding Author: Dr. John Wataha, PhD, DMD

Corresponding Author's Institution: Medical College of Georgia

First Author: Yo Omata, PhD

Order of Authors: Yo Omata, PhD; Matthew Folan; Melissa Shaw; Regina LW Messer, PhD; Petra E Lockwood; David T Hobbs, PhD; Serge Bouillaguet, PD; Hidehiko Sano, PhD; Jill B Lewis, PhD; John Wataha, PhD, DMD

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Methods: We exposed rat-TrxR1 to auranofin, gold sodium thiomalate, sodium aurothiosulfate, triphenyl phosphine gold chloride, or gold acetate, and measured TrxR activity ex-vivo. We then compared TrxR1 inhibitory levels of gold compounds to those that inhibited mitochondrial activity of THP1 monocytes and OSC2 epithelial cells, estimated by succinate dedhydrogenase activity.

Results: All gold compounds inhibited TrxR1 at concentrations ranging from 5-4000 nM (50% inhibitory concentration). The oxidation state of gold did not correlate with inhibitory potency, but ligand configuration was important. Au(I)-phosphine compounds (triphenyl phosphine gold chloride and auranofin) were the most potent inhibitors of TrxR. All TrxR1 inhibitory concentrations were sublethal to mitochondrial activity in both THP1 and OSC2 cells.

Conclusions: Diverse types of gold compounds may be effective inhibitors of TrxR1 at concentrations that do not suppress cellular mitochondrial function. Inhibition may be optimized to some degree by altering the ligand configuration of the compounds. These results support future study of a variety of Au compounds for therapeutic development as inhibitors of TrxR1.

Sublethal concentrations of diverse gold compounds inhibit mammalian cytosolic thioredoxin reductase (TrxR1)

Yo Omata [a], Matt Folan [b], Melissa Shaw [b], Regina L. Messer [b], Petra E. Lockwood [b], David Hobbs [c], Serge Bouillaguet [d], Hidehiko Sano [a], Jill B. Lewis [b], John C. Wataha [b]

[a]Hokkaido University, Sapporo, Japan

[b]Medical College of Georgia, Augusta, GA

[c]Savannah River National Laboratory, Aiken, SC

[d]University of Geneva, Geneva, Switzerland

Running Title: Diverse gold compounds inhibit TrxR1

Corresponding author:

John C. Wataha DMD, PhD
Medical College of Georgia
Augusta, GA 30912-1160

Phone: (706) 721-2991
Fax: (706) 721-3992
E-mail: watahaj@mail.mcg.edu

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Abstract

Thioredoxin reductase (TrxR) reduces thioredoxin (Trx), thereby contributing to cellular redox balance, facilitating the synthesis of deoxy-ribose sugars for DNA synthesis, and regulating redox-sensitive gene expression. Auranofin is a gold compound that potently inhibits TrxR. This inhibition is one suspected mechanism of auranofin's therapeutic benefit in the treatment of rheumatoid arthritis. The use of other gold compounds to treat cancer or inflammatory disease may rely on their ability to inhibit TrxR. In the current study, we tested the hypothesis that a variety of gold compounds may inhibit TrxR.

Methods: We exposed rat-TrxR1 to auranofin, gold sodium thiomalate, sodium aurothiosulfate, triphenyl phosphine gold chloride, or gold acetate, and measured TrxR activity ex-vivo. We then compared TrxR1 inhibitory levels of gold compounds to those that inhibited mitochondrial activity of THP1 monocytes and OSC2 epithelial cells, estimated by succinate dedhydrogenase activity.

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1. **Introduction**

Thioredoxin reductase (TrxR) is an NADPH-dependent oxido-reductase containing flavin adenine dinucleotide (FAD) and several cysteine thiols that are critical to its catalytic mechanism (Becker et al., 2000; Mustacich et al., 2000; Gromer et al., 2004). Mammalian 'large' TrxR, which differs markedly from the smaller so-called 'bacterial' form, is further distinguished by a selenocysteine residue on an exposed peptide arm near the carboxy terminus (Williams et al., 2000) (Fig. 1). The accessible nature of this selenocysteine residue is thought to afford mammalian TrxR the ability to reduce a diverse repertoire of substrates including hydrogen peroxide, insulin, and the redox-enzyme thioredoxin (Trx) (Gromer et al., 2004). TrxR is a critical regulator of Trx redox balance, both in the cytosol (as TrxR1, Gromer et al., 2005) and in the mitochondria (as Trx2, Rigobello et al., 2004a). Trx plays an essential role in the synthesis of deoxy-ribose sugars for DNA and in maintenance and perhaps regulation of nuclear redox balance critical to transcription factor activity and gene expression (Arnér and Holmgren, 2000). The increasingly apparent central role of Trx in cellular biochemical processes has made TrxR an attractive target to treat cancer, microbial infections, and inflammatory diseases (Novelli et al., 1999; Becker et al., 2000; Rigobello et al., 2004; Nguyen et al., 2005).

Gold compounds have been used for at least 100 years to treat a variety of illnesses, but their modern use has been largely restricted to treating rheumatoid and other arthrides. Because they have serious toxicologically based side effects including proteinuria, diarrhea, bone marrow suppression, and skin rashes (Parish, 1992; Kean et al., 1997; Eisler, 2003), the clinical use of gold compounds has waxed and waned with the development of other compounds. Yet their clinical effectiveness, particularly in refractory rheumatoid arthritis, has given them a persistent therapeutic role worldwide. The two most commonly used gold-based drugs to treat rheumatoid arthritis are auranofin (AF) and gold sodium thiomalate (GSTM). Although mechanisms for both the therapeutic and toxicological effects of gold compounds

remain unclear, the ability of auranofin to suppress TrxR activity at nanomolar concentrations has been proposed as playing a major role in its therapeutic effects (Gromer et al., 2004). GSTM also has been reported to inhibit TrxR activity (Fricker et al., 1998; Rigobello et al., 2004b).

The clinical toxicity of gold-based drugs, coupled with sparse knowledge of their mechanism of action, has curtailed the development of new gold-based drugs or wider use of existing drugs for arthritis or other diseases. Yet, because of the biochemical importance of TrxR, the ability of AF to effectively inhibit TrxR activity strongly suggests that gold compounds might be used to limit the rate of cell division or over-activation of targets of redox-sensitive transcription factors. Central to the potential therapeutic roles of any gold compound is an ability to inhibit TrxR activity at a sublethal concentration. Several types of Au(I) and Au(III) complexes are known to inhibit TrxR1 (Gromer et al., 2004) and TrxR2 (Rigobello et al., 2004a,b).

In the current study, we investigate the hypothesis that diverse types of gold compounds are capable of inhibiting mammalian thioredoxin reductase (TrxR1). Our results suggest that neither gold oxidation state nor ligand configuration exclusively predict TrxR1 inhibitory potency, but that several diverse types of gold compounds are effective inhibitors of TrxR at nanomolar concentrations that do not suppress cellular mitochondrial function. Our results further suggest that other gold compounds might be synthesized to optimize or customize TrxR inhibition.

2. Material and methods

2.1 Gold compound inhibitors

Gold compounds (Table 1) were selected based on known ability of AF and GSTM to inhibit TrxR (Fricker et al., 1996; Simon et al., 2000; Gromer et al., 2004) or proposed use as therapeutic agents for arthritis or cancer (Messori et al., 2000; Che et al., 2003; Messori et al., 2004). Stock solutions of the gold compounds were added to the enzyme mixture to provide an appropriate final concentration range before the enzyme mixture was added to the substrate-

buffer mixture. Concentrations were selected based on literature, and were refined in subsequent experiments to find a range resulting in enzyme inhibition, where possible (Table 1). Where non-water solvents were used to dissolve the stock solutions of the compounds, the final concentrations of the solvents were < 0.1%. In selected cases, experiments were repeated without enzyme to test the direct reduction of DTNB by the gold compounds.

2.2 TrxR1 assay

TrxR1 activity (rat-TrxR, 0.13 U/μL, Sigma-Aldrich) was estimated by an *in vitro* reduction of DTNB (5,5'-dithiobis(2-nitrobenzoic acid), Sigma-Aldrich) to 5'-thionitrobenzoic acid (TNB) using a procedure adapted from those previously published (Holmgren et al., 1995). The conversion of DTNB to TNB (a strongly yellow-colored product) was measured spectrophotometrically at 412 nm. Stock TrxR1 was diluted with enzyme buffer (1 mM EDTA, 10% glycerol, 300 mM NaCl, and 50 mM Tris, pH 7.5) to 0.013 U/μL, and 7.7 μL of this solution was used per reaction to give a final enzyme concentration of 0.10 U/100 μL. DTNB was prepared in ethanol at 63 mM and used at a final concentration of 0.25 mM. A reaction buffer (100 mM K_3PO_4, 10 mM EDTA, 0.2 mg/mL bovine serum albumin, 0.24 mM NADPH in water at pH 7.5) was used to dilute the DTNB and enzyme to 100 μL per reaction. For experiments that used thioredoxin as the substrate, thioredoxin (Trx, human, oxidized, 10-100 μM, Sigma) was added instead of DTNB, and disappearance of NADPH was monitored at 340 nm.

Immediately after addition of the enzyme (n = 4 per condition), the formation of product (DTNB substrate) or disappearance of NADPH (Trx substrate) was monitored for up to 15 min (DTNB) or 60 min (Trx). Levels of product were plotted vs. time with and without various gold compound inhibitors (see below). Blank solutions contained no substrate or enzyme, and controls were run to assure the enzyme and substrate dependence of the conversions.

2.3 Cellular metabolic activity

We assessed the metabolic impact of the gold compounds by measuring succinate

dehydrogenase (SDH) activity to estimate mitochondrial function (Wataha et al., 1992). Because the monocyte is a key mediator of many chronic inflammatory diseases (Auger and Ross, 1992; Bondeson, 1997) and a target of anti-rheumatic compounds, we used the human monocytic THP1 cell line. Because many cancers are epithelial in nature and gold compounds have been proposed as anticancer drugs (Messori et al., 2000; Messori and Marcon, 2004; Rigobello et al., 2004a), we also used the human OSC2 epithelial cell line. THP1 (THP1, ATCC TIB 202) were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 100 μg/mL streptomycin, 100 units/mL penicillin (all from Gibco BRL), and 50 μM β-mercaptoethanol (Sigma-Aldrich). OSC2 (provided courtesy of Dr. Tokio Osaki, Japan) were maintained in 50/50 vol% DMEM/F12 supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 5 μg/mL of hydrocortisone (all from Gibco BRL). Cells were plated in 96-well format (50,000 cells/well in 200 μL for THP1; 12,500 cells/cm^2 in 200 μL for OSC2) for 24 h, then the gold compounds were added from sterile stock solutions to provide a range of concentrations (Table 1). All compounds were soluble in water except AuTPP, which was dissolved in acetone and AF, which was dissolved in ethanol. The final concentrations of these latter solvents in the cell-cultures was 0.5%, which pilot studies showed had no significant effects on mitochondrial activity.

The activity of succinate dehydrogenase (SDH), an enzyme unique to the mitochondria and central to mitochondrial function, was measured (n = 8/condition) using MTT (3-[4,5-dimethylthiazoyl-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma) reduction. This assay was structured to estimate cellular mitochondrial activity (Wataha et al., 1992). Control wells received only vehicle and were used to normalize SDH activity of Au-treated wells.

3. Results

Initial studies established the reliability of the TrxR assay using the DTNB surrogate substrate (Fig. 2). TrxR1 activity was dependent on both the concentration of DTNB and TrxR1,

strongly suggesting that the product detected at 412 nm was the result of TrxR1 activity. As expected, there was no color produced when either enzyme or substrate were withdrawn from the reactants. From these initial studies, we selected an enzyme concentration of 0.10 U and a DTNB concentration of 0.25 mM to measure TrxR1 inhibition in subsequent experiments. To further verify the system, we used the native Trx (oxidized) substrate and 0.10 U of TrxR1, measuring the disappearance of the NADPH co-substrate at 340 nm. NADPH disappeared with its consumption in the reduction of Trx over the same time course (about 15 minutes). TrxR1 activity also was dependent upon Trx concentration. We did not use Trx routinely as a substrate because of its expense and because the DTNB conversion gave a much stronger signal (0.2 OD over 15 min vs. 0.06 OD for Trx, Fig. 2). Furthermore, the precision of the DTNB conversion was better (coefficient of variation of about 10% vs. 20% for the Trx).

Next, we used auranofin (AF) to verify inhibition of TrxR1 (Fig. 3). AF inhibited TrxR activity by 50% at 5-10 nM and by 100% above 15 nM. When Trx (oxidized) was used as a substrate, AF was more potent, inhibiting TrxR completely above 1 nM. When 10 nM AF was used to inhibit DTNB conversion and DTNB concentration was increased by 20-fold, AF inhibition was not reversed, suggesting that inhibition of TrxR1 by AF is noncompetitive with regard to DTNB.

Based on the initial tests of our model (Figs. 2,3), we assessed the ability of five other gold compounds to inhibit TrxR1 activity (Fig. 4). Of these, Au(III) was the most potent, inhibiting TrxR1 activity by 50% at approximately 75 nM. AuTPP and AuTS were less potent than Au(III), and were equal in their ability to inhibit TrxR1 above 100 nM. GSTM and AuOAc were the least potent inhibitors of TrxR1, requiring $\geq$ 500 nM for inhibition. The differential between AF (the most potent inhibitor at 5-10 nM) and AuOAc (the least potent at about 4000 nM was nearly 1000-fold.

In our initial studies of GSTM at concentrations greater than 1000 nM, we observed DTNB conversion of DTNB rather than the expected decrease in DTNB conversion. Concentrations of GSTM above 1000 nM produced a time-dependent increase in reduced DTNB. Further

experiments showed that this reduction occurred even in the absence of the enzyme, and was strongly dependent on the concentration of GSTM (Fig. 5), suggesting a direct chemical reduction of DTNB by GSTM. However, other gold compounds did not exhibit similar behavior, even at concentrations well above those necessary to inhibit TrxR1 activity (Fig. 5, data for compounds other than AF and Au(III) not shown).

Next, we assessed the ability of the gold compounds to inhibit cell mitochondrial function (Fig. 6). In human monocytic cells and epithelial cells, gold compounds did not suppress mitochondrial function at concentrations well above those which inhibited TrxR1 activity. For example, AuTPP significantly inhibited TrxR1 activity above 100 nM, but had little or no effect on mitchondrial activity below 250 nM. For GSTM, concentrations of 500 nM inhibited TrxR1, but concentrations up to 500,000 nM (500 μM) did not suppress mitochondrial function. The response of OSC2 and THP1 cells to the gold compounds were largely equivalent in the doses tested.

4. Discussion

The DTNB model was well-suited to estimate TrxR1 activity (Fig. 2). DTNB reduction was enzyme- and substrate-concentration dependent and behaved similarly to the Trx substrate. Auranofin inhibited the enzyme at concentrations in the range previously reported for DTNB-TrxR (Fig. 3, top; Gromer et al., 2004). Interestingly, AF was more potent (< 1 nM for 50% inhibition) when Trx was the substrate compared to DTNB (5-10 nM). This difference has not been previously reported. The higher sensitivity of Trx reduction may have been caused by its large size (12 kD) relative to DTNB, or the difference in redox potentials of the two substrates. Access of the selenocysteine of TrxR1 to the catalytic selenocysteine site of Trx may have been more easily disrupted by AF than for DTNB.

The current data demonstrate clearly that AF inhibition was not reversible by adding excess DTNB substrate (Fig. 3, bottom), suggesting that AF-TrxR1 interactions are robust and not competitive with regard to DTNB. Irreversibility could have been caused by either stable binding

of AF to the active site(s) of the enzyme or a site distant from the active site that altered the catalytic function of the active sites. The irreversibility of AF inhibition may have important clinical consequences in terms of the persistence of its therapeutic affects and irreversibility of its side effects. These effects are known clinical attributes of AF when it has been used to treat rheumatoid arthritis (Simon et al., 2000; Eisler et al., 2003). Although we did not investigate the reversibility of inhibition of the other gold compounds in the current study, these experiments are logical directions for future study.

Our results showed that diverse types of gold-based compounds inhibit mammalian TrxR1 activity (Fig. 4), and these results are congruent with previous reports on TrxR2 inhibition (Rigobello et al., 2004a,b). In each case, concentrations in the current study required to inhibit TrxR1 were below lethal concentrations in vitro in monocytes (Tiekink et al., 2002; Omata et al., 2005), which is one cell type that has been suggested as a therapeutic target of chrysotherapy in rheumatoid arthritis (Eisler et al., 2003). We further demonstrated that TrxR1 inhibitory levels were below those necessary to inhibit cell mitochondrial activity of two types of human cells (Fig. 6), further supporting the possibility of TrxR1 inhibition without compromise of general cell metabolism.

Gold compounds varied significantly in their potency against TrxR1 activity, but we did not observe a trend suggesting that Au(I) was more potent than Au(III) (Fig. 4). This conclusion is tentative, however, because it is difficult to know the oxidation state of the gold atom once it is placed into the enzyme reaction buffer. In addition, the enzyme itself may change the oxidation state of the gold atom. Other studies suggest that Au(III) from the chloride salt may be rapidly reduced in the cell and may act as Au(I) (Omata et al., 2005). Other metallo-organic Au(III) compounds that have been previously tested were less potent inhibitors of TrxR2 than AF or GSTM (Rigobello et al., 2004a).

Among the Au(I) compounds, the phosphine-coordinated compounds (AuTPP and AF) were more potent than the thiol coordinated compound (GSTM), and this difference suggests that

ligand configuration plays at least some role in the ability of gold compounds to inhibit TrxR1. A similar contrast was observed among the Au(III) compounds; Au(III) was nearly 60-fold more potent than AuOAc. The ability of every compound we tested to inhibit TrxR1 activity at subtoxic levels strongly suggests that other gold compounds might be developed as inhibitors, and that it is the gold center that is the fundamental cause of inhibition in these compounds. Yet, the results also suggest that the activity of the gold center can be tempered by modifying ligand configuration. Factors such as compound size, shape, and the affinity of Au for its ligands also may influence inhibitory potency.

The different potencies of the various gold compounds tested and the ability of ligand configuration to alter potency suggest one mechanism for TrxR1 inhibitory activity. Au(I) and to a lesser extent Au(III) are known binders of selenium (Marcon et al., 2002). Because this selenium center is critical to TrxR1 catalytic activity (Becker et al., 2000; Gromer et al., 2004), the accessibility of selenium on the carboxy terminus of mammalian TrxR1 suggests that selenium is one reasonable target of the large gold ion (Fig. 1). Under this hypothesis, ligands that deferred coordinate binding with Au to Se such as phosphorous (AF, AuTPP) would be more potent inhibitors than those such as sulfur (GSTM) because Au is less likely to relinquish the sulfur-linked ligand to engage selenium. This hypothesis may be tested in future studies by constructing a series of Au-based compounds with ligands of different Au affinity and testing their TrxR1 inhibitory potency. This strategy may help predict which compounds might be inhibitors of small (bacterial) thioredoxin reductase, which has no selenium and a catalytic center based exclusively on the thiol groups of cysteine (Gromer et al., 2004). The ability of both Au(I) and Au(III) compounds to inhibit glutathione peroxidase (another selenoenzyme), but not glutathione reductase (which lacks selenium) supports this hypothesis (Rigobello et al., 2004a). Finally, although selenium may have more affinity for gold than sulfur, the interactions of Au(I) and Au(III) compounds with the nonenzymatic sources of thiol such as gluathione cannot be ignored in trying to understand the cellular redox effects of gold (Dickinson and

Forman, 2002; Filomeni et al., 2002).

We report here for the first time that GSTM can reduce DTNB without TrxR (Fig. 5). We did not observe this direct chemical conversion for other gold compounds (Fig. 5), and conversion by GSTM occurred only at relatively high concentrations (> 1 μM). Nevertheless, direct conversion should be checked in future studies that assess the ability of new gold compounds to inhibit TrxR1.

In summary, we show in the current study that a number of gold-based compounds inhibit thioredoxin reductase at sublethal concentrations, extending the previously reported inhibition of TrxR1 by auranofin and gold sodium thiomalate and reports of inhibition of Trx2 by gold. We report here that TrxR1 inhibition is not necessarily dependent on the initial gold oxidation state, but that ligand configuration does regulate the potency of inhibition. We further show that auranofin inhibits TrxR1 noncompetitively with regard to the DTNB substrate.

Conflict of Interest Statement

No outside financial arrangements or interests exist that would constitute a conflict of interest.

Acknowledgements

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Table 1

Gold Compound	Abbrev.	Ox. State	Source	Stock Solvent	TrxR1 Conc. Range (nM)	SDH Conc. Range (nM)
Auranofin	AF	Au(I)	Alexis Corp	ethanol	0.5-15	NA
Gold sodium thiomalate	GSTM	Au(I)	Sigma-Aldrich	water	500-1000	10000-500000
Triphenylphosphine gold chloride	AuTPP	Au(I)	J & J Materials	acetone	10-100	10-250
Gold chloride, tri-hydrate	Au(III)	Au(III)	Sigma-Aldrich	water	10-500	10000-500000
Sodium aurothiosulfate	AuTS	Au(III)	J & J Materials	water	100-1000	200-5000
Gold acetate	AuOAc	Au(III)	J & J Materials	water	100-20000	10-5000

The cytotoxic effect of AF was not assessed because if has been previously published (Messori et al., 2000; Rigobello et al., 2004a).

Figures and Figure Legends

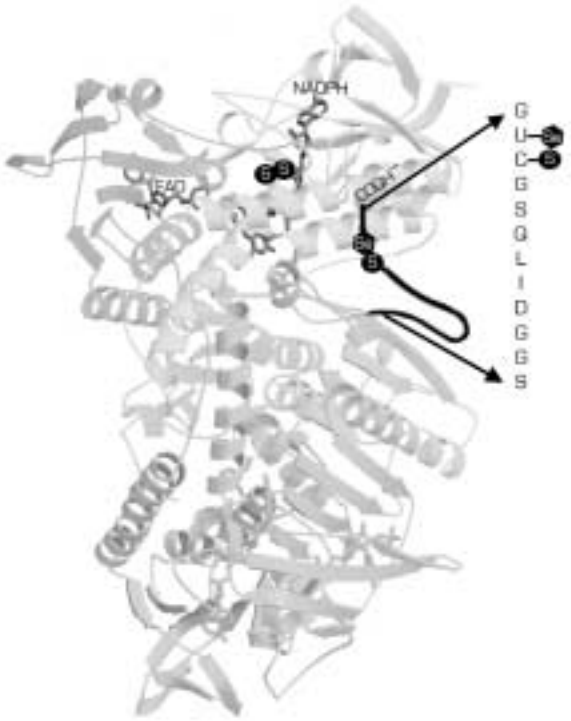

Fig. 1. Ribbon diagram of rat TrxR1 structure. The enzyme has two catalytic centers. The first, associated with FAD and NADPH, is recessed in a cleft of the enzyme. The second is sterically unhindered on an 'arm' near the carboxy terminus. The latter site contains selenocysteine that is essential to catalytic activity (Becker et al., 2000; Gromer et al., 2004).

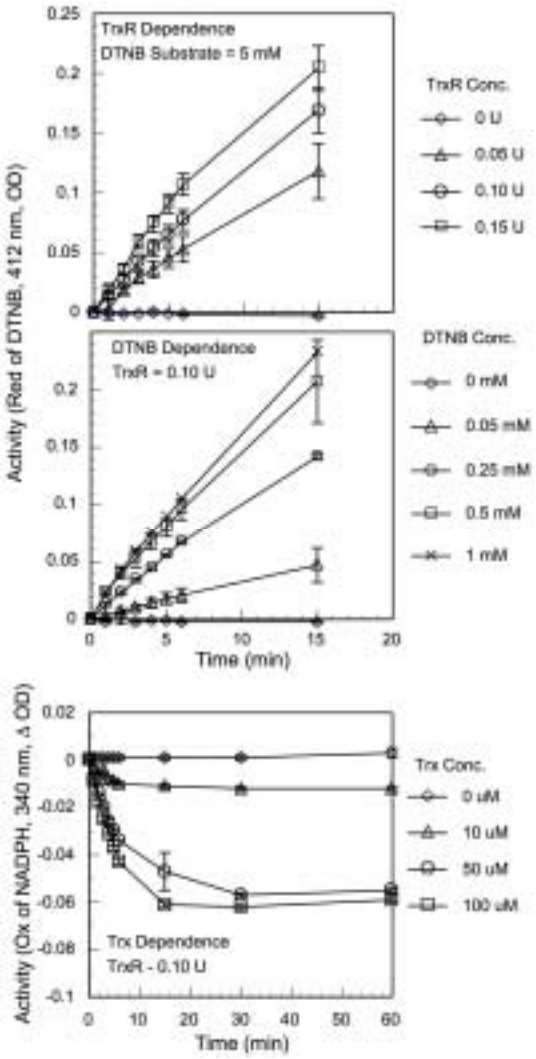

Fig. 2.

Top: Dependence of DTNB reduction by rat-TrxR1 on TrxR1 concentration. TrxR1 concentrations ranged on 0-0.15 U. 5 mM of the surrogate substrate DTNB was used to assess enzyme activity by measuring optical density (OD) at 412 nm as a functioin of time after substrate addition. Error bars represent one standard deviation of the mean (n=3 per condition).

Middle: Dependence of DTNB reduction by rat-TrxR1 on DTNB concentration. DTNB concentrations ranged from 0- 1 mM; TrxR1 concentration was 0.10 U. Activity of the enzyme was estimated by measuring optical dentistry (OD) at 412 nm. Error bars represent one standard deviation of the mean (n=3 per condition).

Bottom: Dependence of NADPH consumption on Trx concentration. Rat-TrxR1 (0.1 U) was exposed to Trx (1-100 μM) as a substrate. Activity of the enzyme was estimated by measuring the OD from the consumption of NADPH at 340 nm. Error bars represent one standard deviation of the mean (n = 2 per condition).

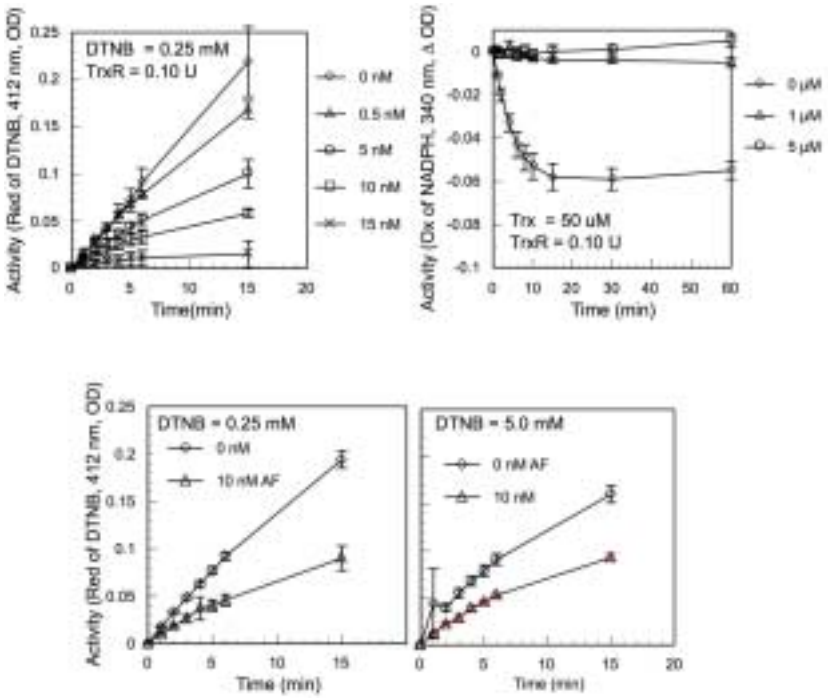

Fig. 3.

Top: AF inhibition of TrxR1 assessed by DTNB reduction (upper left) or Trx reduction (upper right). 0.1 U of TrxR1 was exposed to 0.25 mM DTNB and 0-15 nM of auranofin (AF). Enzyme activity was estimated as described in Fig. 2.

Bottom: Irreversibility of AF inhibition by excess DTNB. 0.1 U TrxR1 was exposed to 0 or 10 nM of AF, then DTNB at 0.25 or 5.0 mM. Enzyme activity was estimated as described in Fig. 2.

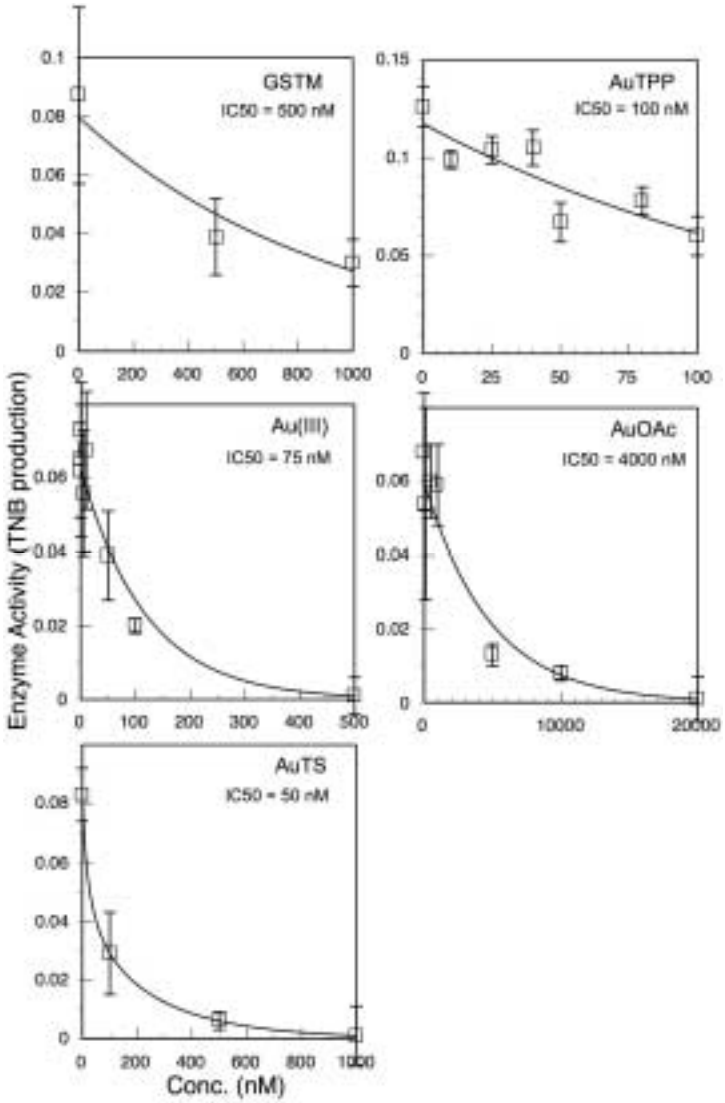

Fig. 4. Inhibition of TrxR1 activity (DTNB method, 0.25 mM) by gold sodium thiomalate (GSTM), gold triphenyl phosphine chloride (AuTPP), gold chloride trihydrate (Au(III)), gold acetate (AuOAc), and sodium aurothiosulfate (AuTS). The enzyme activity was assessed 5 min after addition of DTNB (y axis is OD of TNB production). The approximate 50% inhibitory concentration (IC50, estimated from graphs) is listed for each compound. Error bars represent one standard deviation of the mean (n=3 per condition).

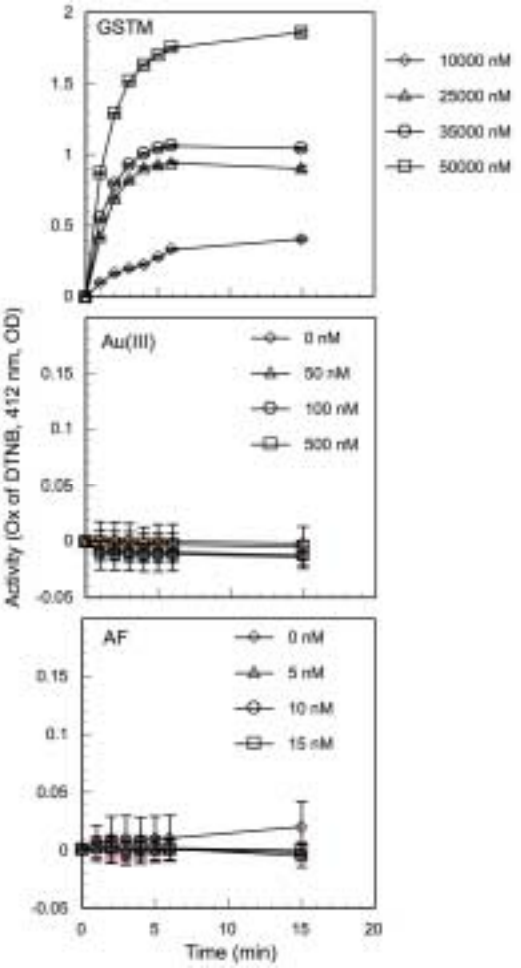

Fig. 5. Direct reduction of DTNB by gold sodium thiomalate (GSTM), gold chloride trihydrate (Au(III)), or auranofin (AF). Reduction was estimated by measuring optical density (OD) at 412 nm without enzyme present. DTNB concentrations were 0.25 nM. Error bars indicate one standard deviation of the mean (n=3 per condition). Note: different scales on y-axes. Experiments for AuTS, AuOAc, and AuTPP are not shown here but were similar in result to AF and Au(III).

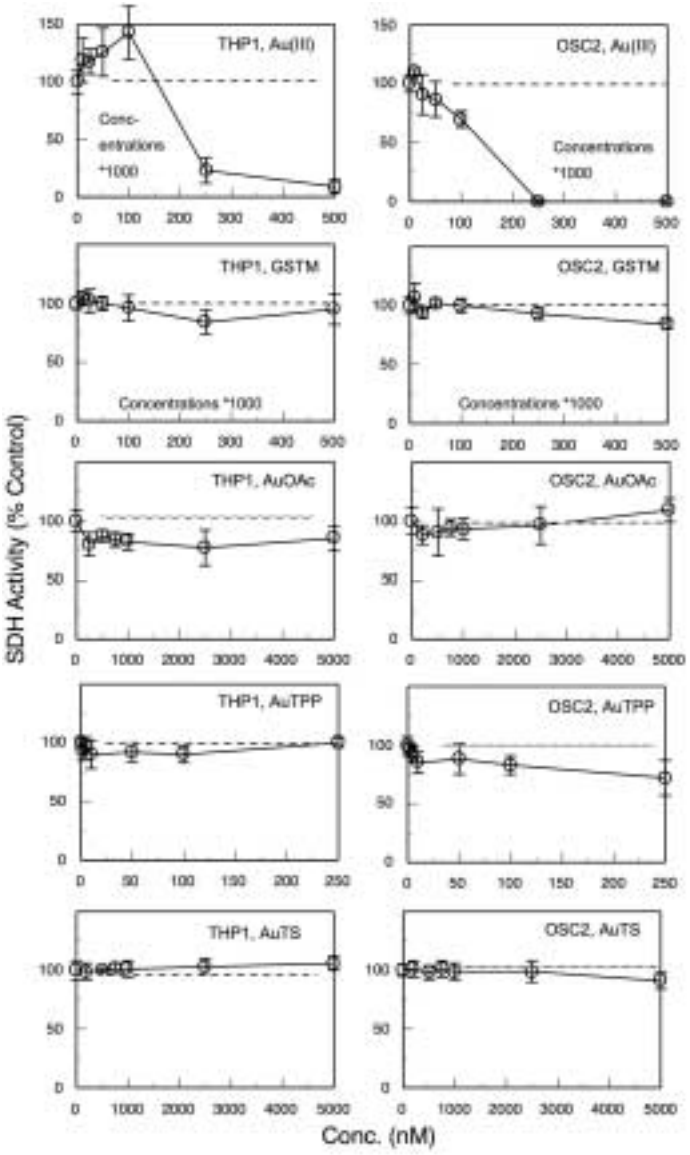

Fig. 6. Inhibition of cellular mitochondrial activity of THP1 monocytic cells or OSC2 epithelial cells by gold compounds gold sodium thiomalate (GSTM), gold triphenyl phosphine chloride (AuTPP), gold chloride trihydrate (Au(IIII)), gold acetate (AuOAc), or sodium aurothiosulfate (AuTS). Cells were exposed to a range of concentrations of the gold compounds (Table 1) for 24, after which mitochondrial succinate dedhydrogenase (SDH) activity was measured. Error bars indicate standard deviations (n = 8 per condition). SDH mitochondrial activity was expressed as a percentage of vehicle controls.

Dear Dr. Acosta:

We have revised our paper in response to the reviewers' comments, and we appreciate the time they took to critique our work. We feel their scrutiny has helped us write a better manuscript. We list below changes we have made so that you and the reviewers can better assess the status of the revised manuscript.

General (improvements made overall)

1. Thioredoxin nomenclature. The reviewers' comments made us realize that we should have specified which TrxR we tested. Thus, we have adjusted the nomenclature to TrxR1 or TrxR2 reserving TrxR for general discussion. We have also adjusted the title to be more accurate.

2. References. In addition to the references requested by the reviewers, we have added several references expanding background on oxidative stress and glutathione. We have also added citations to the figure legends and Table where helpful to the reader.

Reviewer 1:

1. Additional references. We agree that the references cited by the reviewer were extremely relevant and should have been included. We apologize for this oversight. We have added reference to these works in numerous areas of the introduction and discussion (examples-not inclusive. p.1, para 1, ll 10-11 and last sentence; p. 2, para 1, last line; p. 2, para 2, last sentence; p.6, para 1, line 5; p. 9, para 2 .ll 1-3).

2. Delete keyword rheumatoid arthritis. Although we do not see the problem with this keyword; we have deleted it as requested.

Reviewer 2:

1. Discussion of mechanism. We have added a discussion of a paper by Rigobello to add new dimensions to a potential mechanism of Au inhibition of TrxR (p. 10, para 2). We have added references and suggested broader potential effects of Au on glutathione p.10 para 2). Finally, we have added discussion of potential variables that might affect the ability of Au compounds to inhibit TrxR (p 10, para10, last line).

We hope we have adequately addressed the concerns of the reviewers. Please contact us if you have further questions. Thank you for your further consideration.

Fig01RibbonStructure

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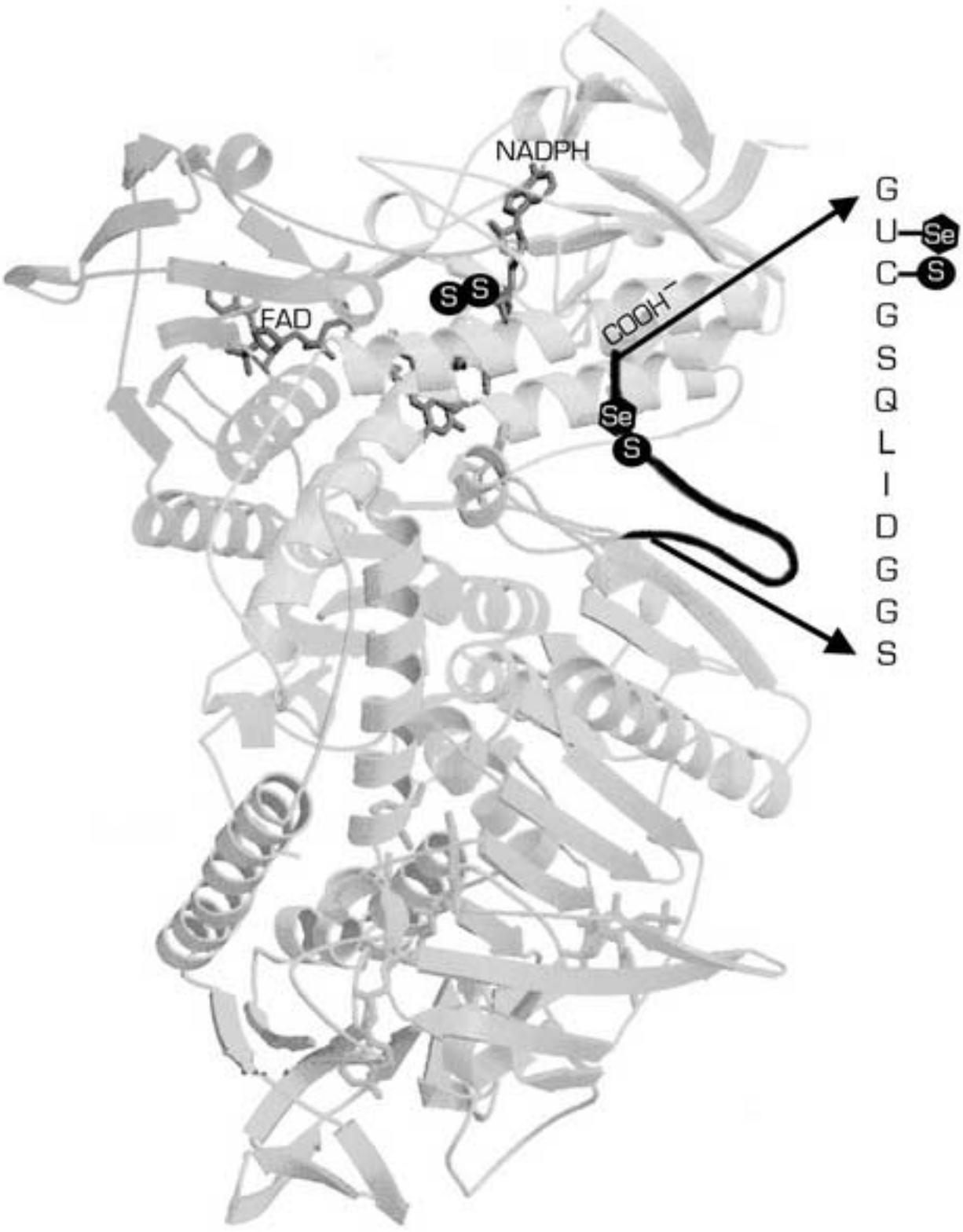

Fig02EnzymeActivity

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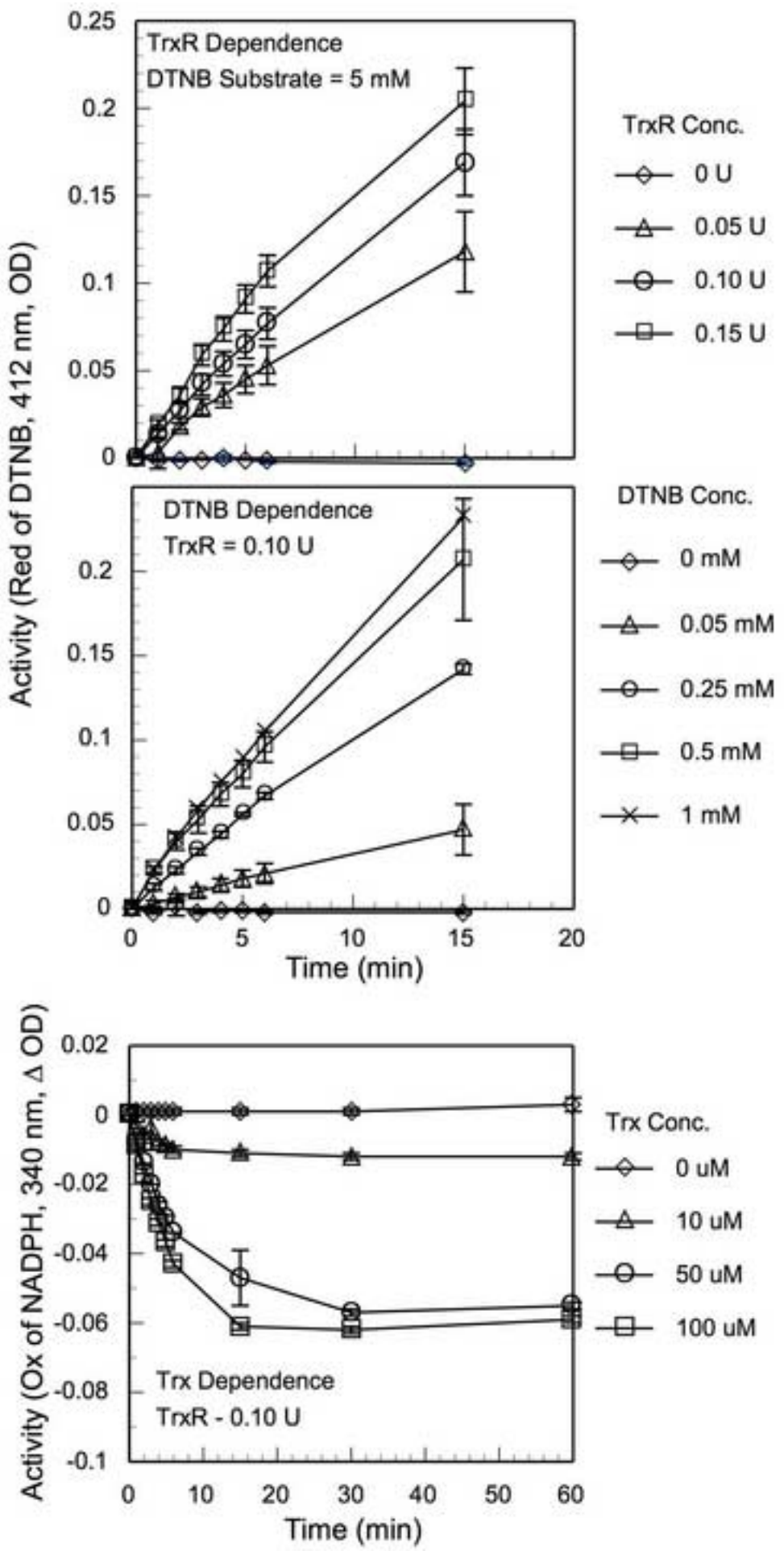

Fig03AuranofinInhib

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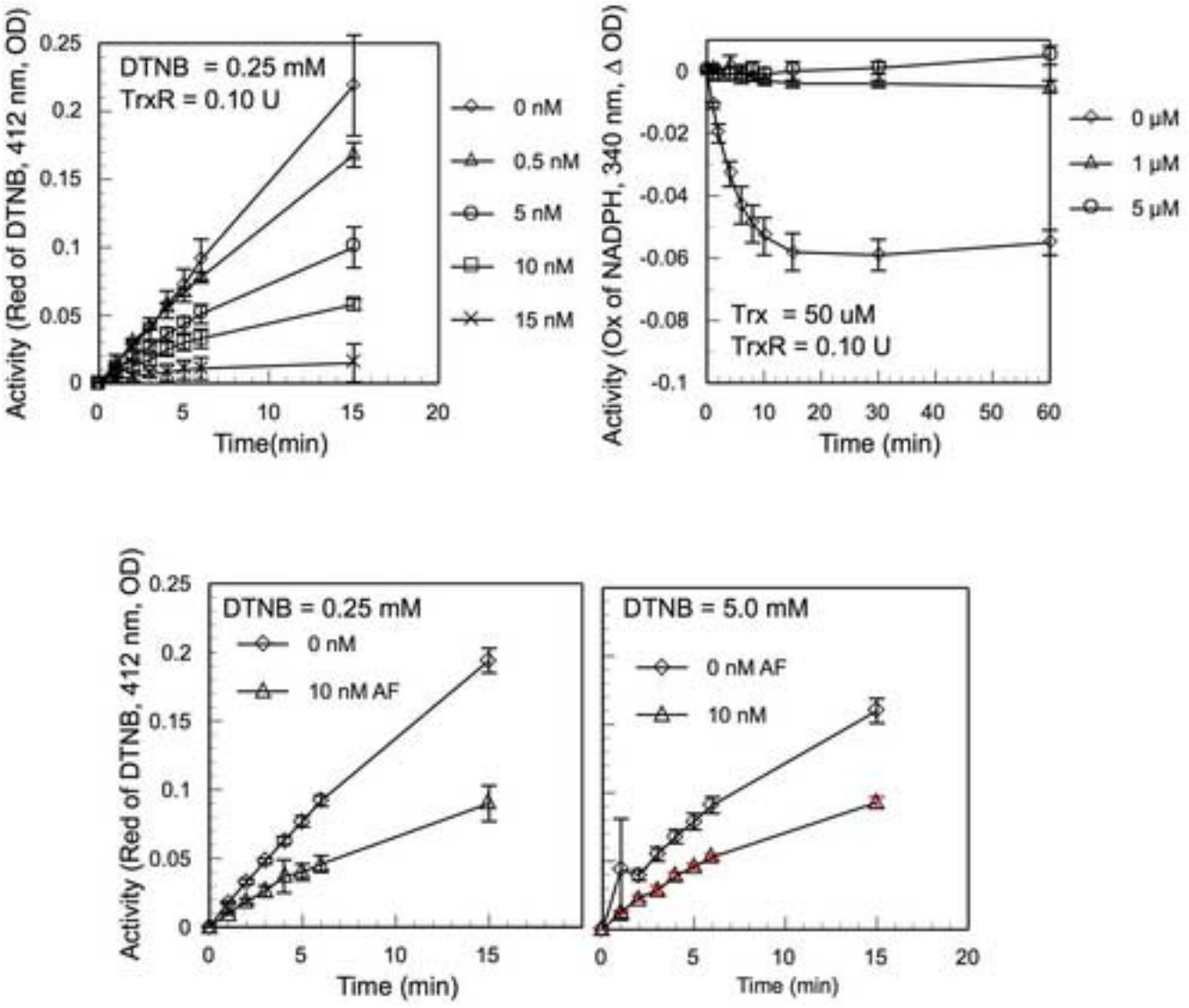

Fig04GoldCmpdInhib

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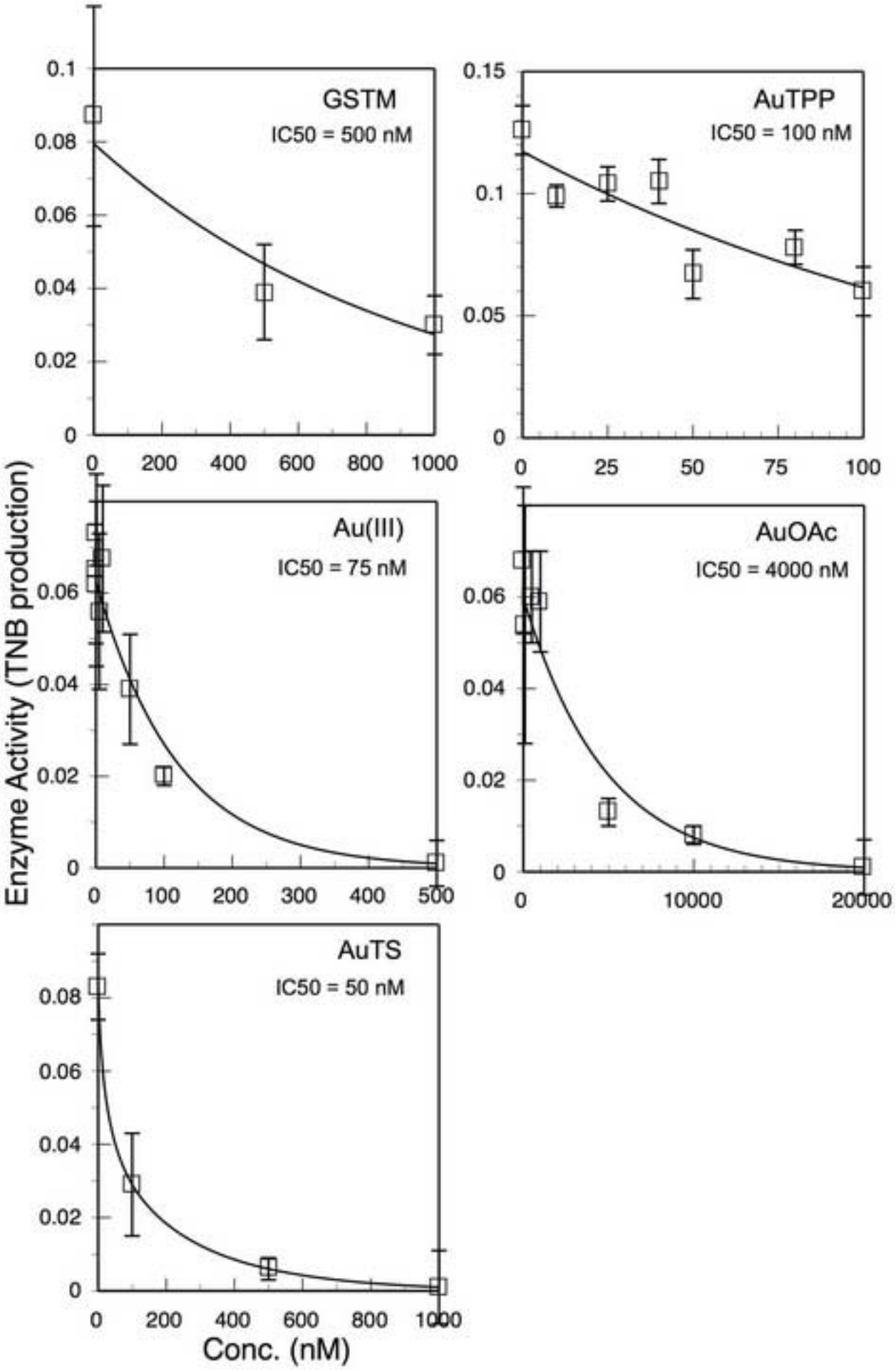

Fig05DirectGSTM

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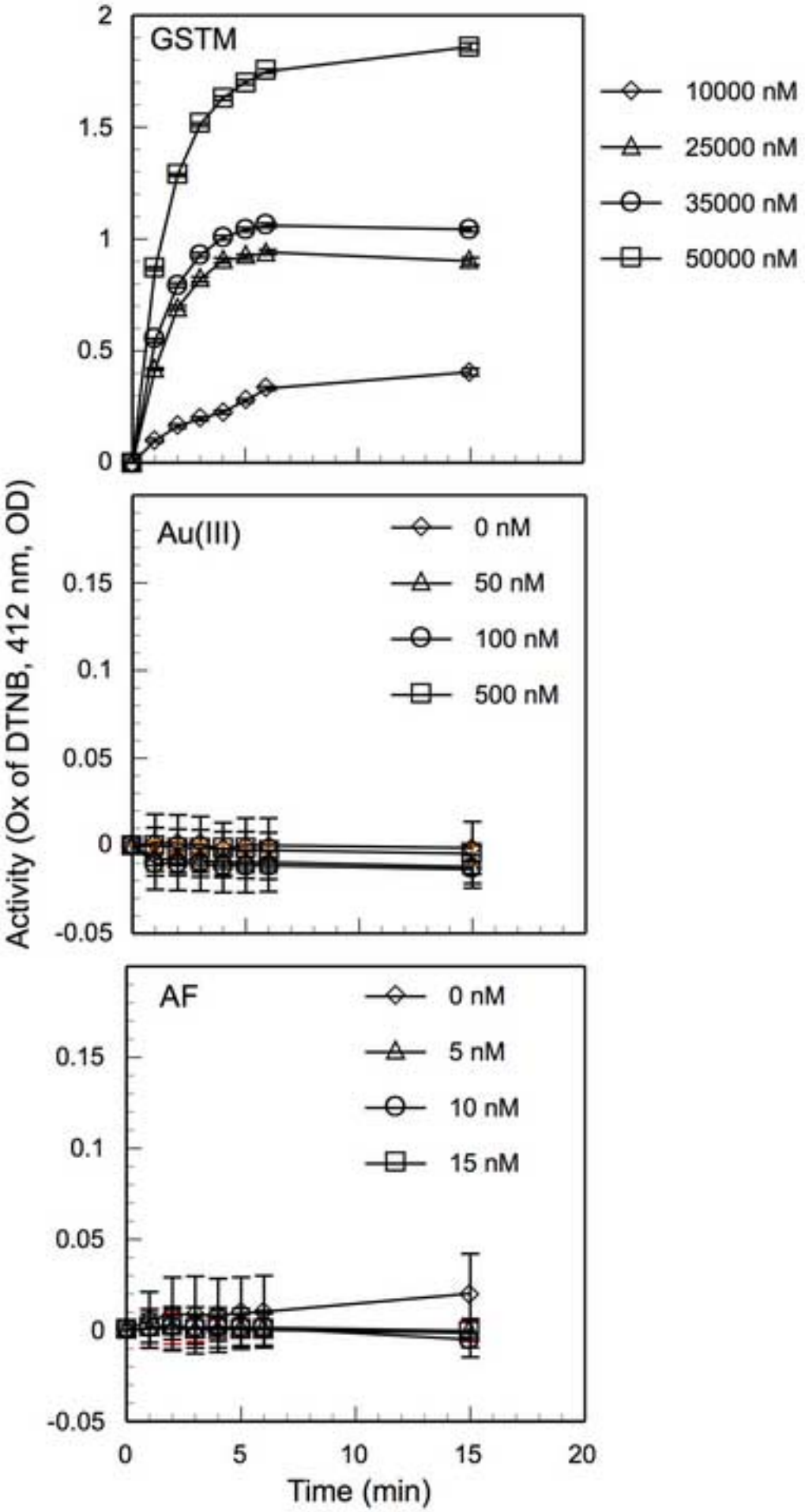

Fig06MetabolicResponse

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