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Manumycin A Is a Potent Inhibitor of Mammalian Thioredoxin Reductase-1 (TrxR-1)

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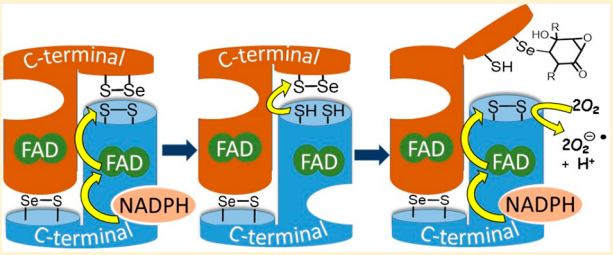
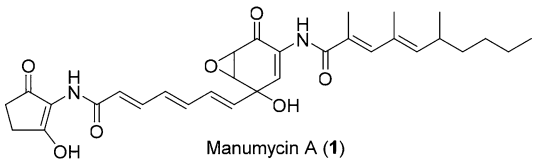
Supporting Information

ABSTRACT: The anticancer effect of manumycin A (Man A) has been attributed to the inhibition of farnesyl transferase (FTase), an enzyme that is responsible for post-translational modification of *Ras* proteins. However, we have discovered that Man A inhibits mammalian cytosolic thioredoxin reductase 1 (TrxR-1) in a time-dependent manner, with an IC_{50} of 272 nM with preincubation and 1586 nM without preincubation. The inhibition of TrxR-1 by Man A is irreversible and is the result of a covalent interaction between Man A and TrxR-1. Evidence presented herein demonstrates that Man A forms a Michael adduct with the selenocysteine residue, which is located in the C-terminal redox center of TrxR-1. Inhibitors of TrxR-1, which act through this mechanism, convert TrxR-1 into a SecTRAP, which utilizes NADPH to reduce oxygen to superoxide radical anion ($O_2^{-\bullet}$).



KEYWORDS: *Thioredoxin reductase, reactive oxygen, oxidative stress, SecTRAP*

Man A (**1**) is a bacterial secondary metabolite that was first isolated from *Streptomyces parvulus* as a result of a random screening program for farnesyl transferase (FTase) inhibitors.[1] FTase catalyzes the post-translational farnesylation of proteins including the *Ras* family of proteins. *Ras* proteins regulate numerous functions, which are related to cell growth, proliferation, and cell signaling. They function by binding to and activating several effector proteins, which regulate critical cellular processes including transcription, translation, cell-cycle progression, and calcium signaling.[2] Upon farnesylation, *Ras* associates with intracellular membranes where it becomes active. FTase inhibitors block farnesylation such that *Ras* remains in the cytosol and does not stimulate its downstream targets. *Ras* is the most frequently mutated oncogene in human cancers with as many as 25% of known human tumors having mutated *Ras*.[3] FTase inhibitors have been discovered and developed for the treatment of cancers[4,5] as well as progeria[6] and parasitic infections.[7−10] Man A inhibits rat brain FTase with a K_i of 1.2 μM[1] and has shown antitumor activity in a variety of cancer cell types[11,12] and tumor models.[13,14]



Manumycin A (**1**)

The tumoricidal activity of manumycin was initially attributed to the inhibition of FTase preventing activation of *Ras*. However, it later became apparent that Man A can stimulate tumor and cell death by pathways that are independent of FTase.[15] Numerous studies report the induction of reactive oxygen species (ROS) or, more specifically, superoxide radical anion ($O_2^{-\bullet}$) in Man A treated cells and tumors.[15−24] However, the mechanism of $O_2^{-\bullet}$ induction has remained undetermined. Herein, we report that manumycin is a potent inhibitor of mammalian thioredoxin reductase-1 (TrxR-1) and inducer of NADPH oxidase activity.

The thioredoxin system is a major regulatory system for the maintenance of redox homeostasis of the cell.[25] It consists of thioredoxin (Trx) and thioredoxin reductase (TrxR). Trx reduces disulfide bridges in protein targets by thiol disulfide exchange. TrxR reduces the oxidized Trx back to its active disulfide form, with the reducing equivalents provided by NADPH. Mammalian TrxR-1 is a homodimer with the monomers aligned antiparallel to each other. NADPH initiates a cascade of redox reactions across three redox active sites: FAD is first reduced by NADPH, next $FADH_2$ reduces an N-terminal disulfide (Cys^{59}-Val-Asn-Val-Gly-Cys^{64} in human TrxR-1), which undergoes thiol disulfide exchange with a C-terminal seleno-sulfide (Cys^{497}-Sec^{498}-Gly in human TrxR-1) on the opposite chain of the dimer. The C-terminal redox center of TrxR-1 undergoes thiol−disulfide exchange with oxidized Trx (Figure 1).

We recently demonstrated that the algal toxin, brevetoxin, is a unique and potent inhibitor of TrxR-1.[26] After making this discovery, we reasoned that other molecules, which are similar

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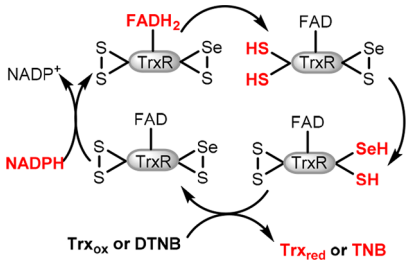

Figure 1. Electron flow in TrxR. Reduced sites shown in red.

in size and functionality, might behave in a similar manner. We examined a series of compounds for their effect on TrxR-1, and while the effect of Man A is not identical to that of brevetoxin, we did discover that it is indeed a potent inhibitor of TrxR-1. Furthermore, through a series of enzyme assays and the use of a selective probe, we have established the mechanism of inhibition of TrxR-1 by Man A. These results should stimulate a reassessment of the tumoricidal activity of Man A in the context of our recent finding.

The effect of Man A on the thioredoxin system was examined using an assay that is based on the reduction of eosin-modified insulin by Trx.[27] In this two-enzyme assay, oxidized Trx is continuously reduced by TrxR-1 with reducing equivalents ultimately provided by NADPH. Man A was incubated (30 min) at concentrations ranging from 0 to 1000 nM with a mixture of prereduced TrxR-1. In this assay, Man A exhibited a dose-dependent inhibition of TrxR-1/Trx activity with an IC_{50} of 572 (±88) nM (Figure 2A).

Mammalian TrxR-1 has a broad substrate specificity and reduces low molecular weight disulfides in addition to Trx. The effect of Man A on the reduction of the disulfide 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) by rat recombinant TrxR-1 was examined at concentrations ranging from 0 to 5000 nM. When prereduced TrxR-1 was incubated with Man A for 1 h prior to the addition of substrate, the reduction of DTNB by TrxR-1 was inhibited with an IC_{50} of 272 (±29) nM. However, when Man A was added simultaneously with DTNB (in other words, without preincubation of TrxR-1 with Man A) TrxR-1 was inhibited with IC_{50} of 1586 (±128) nM (Figure 2B). At a concentration of 5 μM, the inhibition is similar with or without preincubation.

The differences in IC_{50} with and without preincubation suggest that the inhibition of DTNB reduction is time dependent. Figure 2C shows the time course of inhibition of TrxR-1 with 1 μM Man A. Inhibition is observed as early as 2 min of incubation and continues to increase over time.

In an effort to determine if the inhibition of TrxR-1 by Man A is reversible, the DTNB reduction assay was performed as before; however, after 1 h of incubation of prereduced TrxR-1 with 2.45 μM Man A, the mixture was passed through a gel filtration column (MW cutoff of 6000 amu) followed by a 30 min incubation with additional NADPH (Figure 2D). Gel filtration should remove all unbound Man A from the solution, and the subsequent 30 min incubation should allow for reestablishing equilibrium. As shown in Figure 2D, this treatment still resulted in complete inhibition of TrxR-1 demonstrating that the inhibition of TrxR-1 by Man A is irreversible. It is particularly important to note that Man A has no effect on the rate of DTNB reduction when incubated with oxidized TrxR-1. When oxidized TrxR-1 is incubated with Man A for 30 min, followed by gel filtration to remove Man A, TrxR-1 activity is restored completely upon reduction with NADPH. This observation demonstrates that either the N-terminal or C-terminal redox centers, or both, must be reduced for Man A to have an effect on TrxR-1. Furthermore, it suggests that one or both are sites of reactivity with Man A.

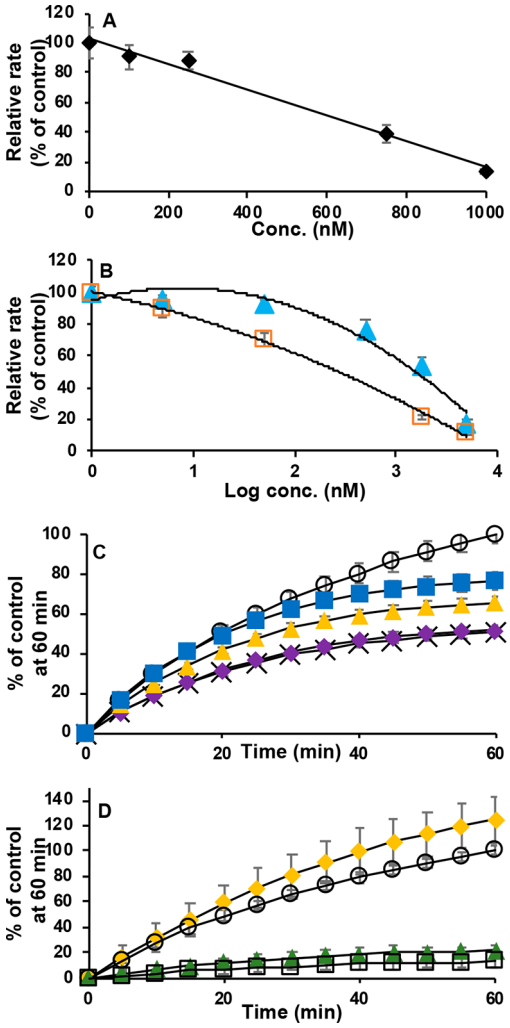

Figure 2. (A) TrxR-1/Trx insulin reduction assay. Percent inhibition by Man A vs concentration. Results are expressed as rate as % of control. (B) Inhibition of DTNB (2 mM) reduction by TrxR-1 (5.6 nM) in the presence of Man A (0−5000 nM). Sixty minute preincubation with Man A (orange □). No preincubation (light blue ▲). Results are expressed as initial rates relative to control (no Man A). (C) Time course inhibition of DTNB reduction by TrxR-1 (5.6 nM) in the presence of Man A (1 μM); control (DMSO only, ○). Incubation times 15 min (X); 10 min (purple ◆); 5 min (yellow ▲); 2 min (blue ■) after Man A addition. (D) Test of irreversibility of inhibition of DTNB reduction by TrxR-1 (5.2 nM) in the presence of Man A (2 μM). TrxR-1 reduced only after removal of Man A by gel filtration (yellow ◆); control (no Man A, ○); Man A not subjected to gel filtration (gray ▲); Man A removed by gel filtration after incubation with reduced TrxR-1 (□).

Numerous electrophiles inhibit mammalian TrxR-1 by alkylating the C-terminal selenocysteine. Many of these electrophiles are α,β-unsaturated carbonyl compounds that irreversibly react with TrxR-1.[25,28−36] Any of the four (mono, di, and tri) unsaturated carbonyl groups of Man A could serve as Michael acceptors and alkylate TrxR-1 at the C-terminal selenocysteine to inhibit the enzyme. Additionally, the epoxide

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may also act as an electrophile. Two Man A derivatives were examined for their ability to inhibit TrxR-1/Trx toward insulin reduction: deoxy-Man A (**2**) and dihydro-Man A (**3**). These results are shown in Figure 3. Neither deoxy-Man A (**2**) nor

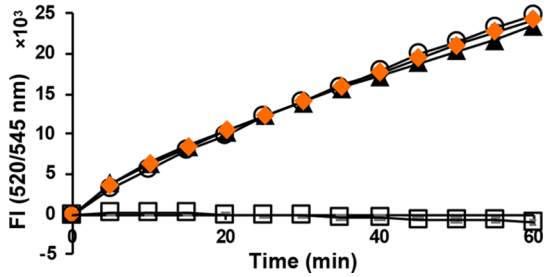
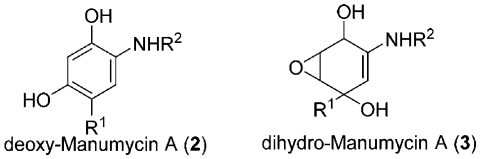


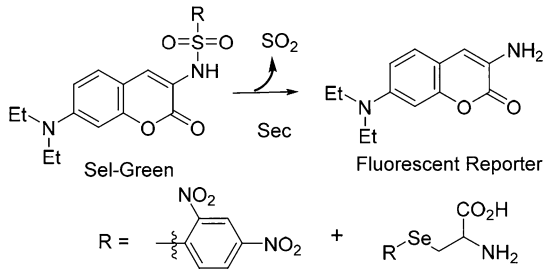
Figure 3. Insulin reduction by TrxR-1/Trx in the presence of 20 μM each of deoxy-Man A (▲); dihydro-Man A (orange ◆); Man A (□); and control (DMSO only, ○).

dihydro-Man A (**3**) inhibits insulin reduction by the TrxR-1/Trx system at concentrations of 20 μM. This demonstrates that while Man A has several electrophilic sites, the site of reactivity with TrxR-1 must be the α,β-unsaturated cyclohexenone.



The selenol selective probe Sel-green[37] was used to evaluate the reactivity of Man A and its derivatives with selenocysteine. The strongly nucleophilic, reduced selenol undergoes nucleophilic aromatic substitution with the probe, which then releases the fluorophore as shown in Scheme 1. When selenocysteine is

Scheme 1. Nucleophilic Substitution Reaction of Sel-green Probe by Reduced Selenocysteine



alkylated, it will not react with the probe, and fluorescence will be suppressed. Figure 4 demonstrates that the release of fluorescent reporter by selenocysteine is completely inhibited in the presence of Man A. Under the same conditions, deoxy-Man A shows only slight inhibition, indicating that Man A reacts readily with selenocysteine, while its derivatives do not.

The chemical modification of the C-terminal selenocysteine of TrxR-1 yields a SecTRAP (selenium compromised thioredoxin reductase-derived apoptotic proteins), which promotes both apoptosis and necrosis via oxidative stress and increased intracellular reactive oxygen species (ROS) production.[38] Both curcumin and juglone modified TrxR-1 have demonstrated strongly induced NADPH oxidase activity producing $O_2^{-\bullet}$ in the presence of oxygen via the N-terminal

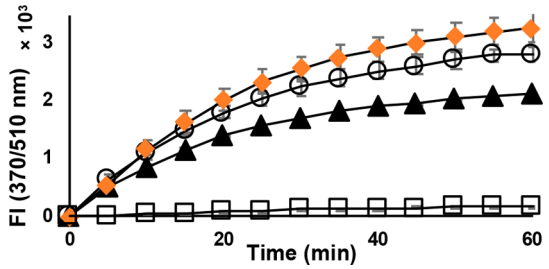


Figure 4. Reaction of reduced L-selenocysteine (20 μM) with Sel-green probe (20 μM) in the presence of Man A and derivatives (20 μM): Control (DMSO only, ○); deoxy-Man A (▲); dihydro-Man A (orange ◆); Man A (□).

(Cys[59]/Cys[64]) redox center.[28,39,40] In addition to increased ROS, Man A treated cells have shown NADPH oxidase activity,[21] decreased TrxR-1 activity,[16] and decreased Trx expression.[20] One reason for activation of NADPH oxidase activity may be due to the alkylation of TrxR-1, producing a SecTRAP. Transfection of cells with SOD and TrxR cDNA or pretreatment with ROS scavengers has been shown to block the adverse effects of Man A.[15,41] The NADPH oxidase activity of TrxR-1 in the absence of a disulfide substrate was examined (Figure 5A). When preincubated with 62.5 μM Man A, the consumption of NADPH by TrxR-1 is increased 10-fold relative to the control.

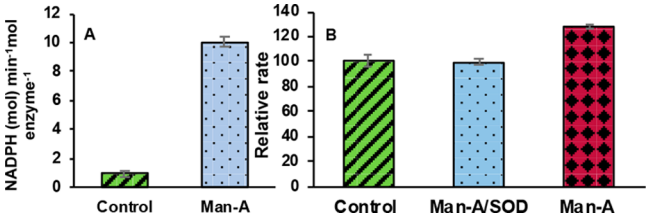


Figure 5. (A) NADPH oxidase activity of TrxR-1 (0.2 μM) induced by Man A (62.5 μM). (B) Reduction of cytochrome C (100 μM) by TrxR-1 (100 nM) in the presence of Man A (62.5 μM); Man A and SOD (62.5 μM and 6 units/well); control (DMSO only).

The reduction of cytochrome C in the presence of Man A treated TrxR-1 was monitored over time with and without the addition of SOD (Figure 5B). Superoxide can reduce cytochrome C. If superoxide is produced by Man A treated TrxR-1, then the addition of SOD should inhibit cytochrome C reduction. This was indeed the case. The rate of cytochrome C reduction was decreased by 28% in the presence of SOD when compared to the Man A treated sample alone.

Finally, Man A has numerous electrophilic sites that may serve as sites of reactivity. Given that living cells contain numerous endogenous nucleophiles, it may be difficult for Man A to reach its target TrxR-1 in living cells. The TrxR-1 activity of human lymphoblast (GM02152) cell lysate in the presence of Man A (1 μM) was analyzed using the insulin reduction assay (Figure 6). The ratio of initial rates for control/Man A treated sample is 2.3:1. There appears to be a longer induction time in the Man A treated sample. However, the manufacturer of the assay kit recommends comparing rates between 15 and 45 min. Here, the relative rate for the control/Man A treated sample is 1.2:1.

In conclusion, we have demonstrated that Man A is an irreversible inhibitor of human TrxR-1. The mechanism of inhibition of TrxR-1 by Man A is very likely by acting as a

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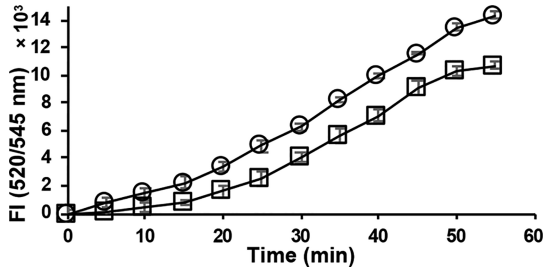

Figure 6. TrxR-1/Trx insulin reduction assay with cell homogenate (GM02152, 15.6 μg) in the presence of Man A (1 μM): control (DMSO only, ○); Man A (□).

Michael acceptor to the nucleophilic Sec residue in the C-terminal redox center of the enzyme. These findings should stimulate a reconsideration of the mechanism for the antitumor activity of Man A.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00489.

Experimental procedures for all enzyme and biochemical assays, cell culture, and harvesting and lysis conditions (PDF)

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Author Contributions

The manuscript was written through contributions of all authors who have given approval to the final version. K.R. designed the research project. A.T. conducted all experiments. K.R. wrote the manuscript. K.R. and A.T. revised the manuscript. A.T. wrote and K.R. revised the Supporting Information.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Man A, manumycin A; Deoxy-Man A, deoxymanumycin A; Dihyro-Man A, dihydromanumycin A; FTase, farnesyl transferase; TrxR1, mammalian cytosolic thioredoxin reductase 1; NADPH, nicotinamide adenine dinucleotide phosphate; Sec-TRAP, selenium compromised thioredoxin reductase apoptotic proteins; Trx, thioredoxin; FAD, flavin adenine dinucleotide; ROS, reactive oxygen species; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); amu, atomic mass unit; SOD, superoxide dismutase

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