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# Mechanisms of Inhibition of the Thioredoxin Growth Factor System by Antitumor 2-Imidazolyl Disulfides

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**ABSTRACT.** The interactions of a series of 2-imidazolyl disulfide antitumor compounds with the thioredoxin reductase(TR)/thioredoxin (hTrx) redox system have been studied. Disulfides **III-2** (*n*-butyl 2-mercaptoimidazolyl disulfide) and **VI-2** (ethyl 2-mercaptoimidazolyl disulfide) were substrates for reduction by TR with $K_m$ values of 43 and 48 μM. Disulfides **IV-2** (1-methylpropyl 2-mercaptoimidazolyl disulfide) and **DLK-36** (benzyl 2-mercaptoimidazolyl disulfide) were competitive inhibitors of the reduction of hTrx by TR with $K_i$ values of 31 μM. None of the disulfides were substrates for reduction by human glutathione reductase. The disulfides caused reversible thioalkylation of hTrx at the redox catalytic site as shown by the fact that there was no thioalkylation of a mutant hTrx where both the catalytic site $Cys^{32}$ and $Cys^{35}$ residues were replaced by Ser. In addition, the disulfides caused a slower irreversible inactivation of hTrx as a substrate for reduction by TR, with half-lives for **III-2** of 30 min, for **IV-2** of 4 hr, and for **IX-2** (*t*-butyl 2-mercaptoimidazolyl disulfide) of 24 hr. This irreversible inactivation of hTrx occurred at concentrations of the disulfides an order of magnitude below those that inhibited TR, and involved the $Cys^{73}$ of hTrx, which is outside the conserved redox catalytic site, as shown by the resistance to inactivation of a mutant hTrx where $Cys^{73}$ was replaced by Ser. Electrophoretic and mass spectral analyses of the products of the reaction between the disulfides and hTrx show that modification of 1–3 Cys residues of the protein occurred in a concentration-dependent fashion. The disulfides inhibited the hTrx-dependent proliferation of MCF-7 breast cancer cells with $IC_{50}$ values for **III-2** and **IV-2** of 0.2 and 1.2 μM, respectively. The results show that although the catalytic sites of TR and hTrx are reversibly inhibited by the 2-imidazolyl disulfides, it is the irreversible thioalkylation of $Cys^{73}$ of hTrx by the disulfides that most probably accounts for the inhibition of thioredoxin-dependent cell growth by the disulfides. BIOCHEM PHARMACOL **55**;7:987–994, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** disulfides; human thioredoxin; thioredoxin reductase; growth factor

hTrx[¶] is a ubiquitous small redox protein that undergoes NADPH-dependent reduction of the conserved active site $Cys^{32}$ and $Cys^{35}$ residues by the flavoenzyme TR [1–3]. hTrx has three other Cys residues, at least one of which, $Cys^{73}$, may be important for biological activity because it stablizes an hTrx homodimer, which is not a substrate for reduction by TR [4]. In the cell, hTrx provides reducing equivalents to intracellular enzymes such as ribonucleotide reductase [1], and through thiol-disulfide exchange hTrx can modulate the activity of proteins [2, 5] including the DNA binding of some of the transcription factors [6–9]. hTrx also acts as a redox-active growth factor [10] by a helper mechanism that sensitizes the cells to growth factors secreted by the cells themselves [11]. hTrx is secreted from cells by a leaderless secretory pathway [12]. Mutant hTrxs, in which the catalytic sites $Cys^{32}$ and $Cys^{35}$ are converted to serines (Ser), either singly or together, are redox inactive and do not stimulate cell growth [10]. hTrx has been found to be over-produced by a number of human primary cancers compared with normal tissue [13–15] and may be acting as an autocrine growth factor for some human cancer cells. We have shown recently that transfection of human cancer cells with a dominant-negative redox inactive mutant hTrx inhibits their anchorage-independent growth *in vitro* and blocks their ability to form tumors *in vivo* [16]. Thus, the TR/hTrx system offers an attractive target for the development of drugs to selectively inhibit cancer cell growth [11].

As part of our studies to identify novel inhibitors of the TR/hTrx system, we examined a series of unsymmetrical 2-imidazolyl disulfides [17], some of which had been found previously to alter the cellular redox state *in vitro* [18]. The 2-imidazolyl disulfides inhibit the growth of cancer cells *in vitro* [18, 19] and show *in vivo* antitumor activity against human tumor xenografts in *scid* mice [19]. The present work reports an examination of the interaction of these 2-imi-

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¶ *Abbreviations:* hTrx, human thioredoxin; TR, thioredoxin reductase; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; DMEM, Dulbecco's Modified Eagle's Medium; 2-ME, 2-mercaptoethanol; and FBS, fetal bovine serum.

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**TABLE 1. Structure of the 2-imidazolyl disulfides and their effects on human TR activity**

| Compound | R | Type | $K_m$ (μM) | $K_i$ (μM) |
|---|---|---|---|---|
| **VI-2** | $-CH_2CH_3$ | Substrate | 48.1 | |
| **III-2** | $-(CH_2)_3CH_3$ | Substrate | 43.1 | |
| **IV-2** | $-CH(CH_3)CH_2CH_3$ | Inhibitor | | 30.8 |
| **IX-2** | $-C(CH_3)_3$ | Non-reactive | | |
| **DLK-36** | $-CH_2C_6H_5$ | Inhibitor | | 30.9 |

NADPH oxidation by TR was measured spectrophotometrically as described in Materials and Methods with either the disulfide as the electron acceptor (substrate) or with hTrx and insulin as the final electron acceptor and the disulfide as inhibitors. The reactions were initiated by the addition of NADPH. $K_m$ and $K_i$ values were calculated from Lineweaver–Burk plots of the data.

dazolyl disulfides with the TR/hTrx system and their effects on hTrx-dependent cell growth. A mechanism for the inhibition of the biological activity of hTrx by the disulfides is proposed that involves thioalkylation of a critical cysteine residue that lies outside the conserved redox catalytic site.

## MATERIALS AND METHODS

### *Preparation of Thioredoxins*

Recombinant hTrx, $Cys^{32} \rightarrow Ser/Cys^{35} \rightarrow Ser$ mutant hTrx (C32S/C35S) and $Cys^{73} \rightarrow Ser$ (C73S) mutant hTrx were prepared and purified as previously described [4, 10]. Twenty-five micromolar stock solutions of the hTrxs in 5 mM of DTT were stored at −20°. Before use, the DTT was removed by passing the hTrx solution through a PD-10 desalting column (Pharmacia). The hTrx solution was kept at 4° and used fresh within 2 hr or hTrx was allowed to oxidize by keeping it at room temperature for at least 3 weeks [17].

### *Drugs*

Unsymmetrical 2-imidazolyl disulfides (see Table 1) were synthesized as described previously [20] and recrystallized prior to use. They were: *n*-butyl 2-mercaptoimidazolyl disulfide (**III-2**); 1-methylpropyl 2-mercaptoimidazolyl disulfide (**IV-2**); ethyl 2-mercaptoimidazolyl disulfide (**VI-2**); *t*-butyl 2-mercaptoimidazolyl disulfide (**IX-2**); and benzyl 2-mercaptoimidazolyl disulfide (**DLK-36**). Stock solutions of the disulfides were prepared at 10 mM in ethanol and diluted in aqueous medium just prior to use. NEM, diamide, and DTT were obtained from Sigma. All other chemicals used were of reagent grade.

### *Cell Growth Studies*

MCF-7 human breast cancer cells were obtained from the American Tissue Type Collection. Cultures were maintained in DMEM containing 10% FBS at 37° and 6% $CO_2$, and passaged at 75% confluence using 0.025% trypsin. The effect of the disulfides on cell proliferation was measured as previously described [11]. Briefly, $10^5$ cells were plated in a 35-mm culture dish in the above medium and, after attachment for 24 hr, their growth was arrested by changing to DMEM with 0.5% FBS for 48 hr. Then the medium was replaced with DMEM containing 10% FBS or 1 μM of hTrx, with or without the disulfides. The cell number was measured 2 days later following detachment with 0.025% trypsin using a hemocytometer. All incubations were conducted in triplicate, and results are expressed as the concentration of disulfide that inhibited cell proliferation by 50% ($IC_{50}$).

### *Inhibition of TR/hTrx by Disulfides*

Human placental TR (sp. act. 33.3 μmol NADPH reduced/min/mg at room temperature) was purified as previously described [21]. Human glutathione reductase (sp. act. 141.2 μmol NADPH reduced/min/mg at room temperature) was purified from aged human red blood cells [22].

The effects of the 2-imidazolyl disulfides on the reduction of hTrx and C73S by TR was measured spectrophotometrically by following the reduction of NADPH at 339 nm [21]. The $K_m$ of disulfides that were substrates for TR was measured using an incubation mixture that contained 2.8 μg/mL of TR, 100 μM of HEPES buffer (pH 7.6), 5 mM of EDTA, and at least six concentrations of disulfide between 10 and 400 μM with the reaction initiated by the addition of 140 μM of NADPH. For disulfides that were inhibitors of TR, the $K_i$ was measured with at least three concentrations of disulfide using an incubation mixture that contained hTrx at 60–240 nM and 1 mg/mL of bovine insulin as the final electron acceptor. In some studies, the disulfides at 3.5 μM were preincubated for up to 24 hr at room temperature in air with 0.7 μM of hTrx or C73S in 100 μM of HEPES buffer (pH 7.6), and 5 mM of EDTA, before measuring the reduction of the hTrx or C73S by TR with 1 mg/mL of bovine insulin as the final electron acceptor. The ability of the disulfides to act as substrates for glutathione reductase was measured by following the reduction of NADPH using an incubation mixture containing 0.15 μg/mL of glutathione reductase, 140 μM of NADPH, 100 μM of HEPES buffer (pH 7.6), 5 mM of EDTA, and the disulfides at 500 μM. The ability of the disulfides to act as inhibitors of glutathione reductase was measured using 50 μM of oxidized glutathione as the final electron acceptor. In both cases, the reaction was initiated by the addition of NADPH.

### *Kinetics of the Reaction between Disulfides and Thioredoxin*

hTrx or C32S/C35S (2 μM) in 0.1 M of sodium phosphate buffer (pH 7.4) was reacted with the disulfides at a concentration of 200 μM at room temperature. The reaction was followed spectrophotometrically as the increase in absorbance at 252 nm due to release of 2-mercaptoimidazole. Initial reaction rates were measured in triplicate.

### *Electrophoresis*

Fresh hTrx, C73S or C32S/C35S, or the same hTrxs treated for 12 hr with 10 mM of diamide, 5 mM of NEM, 10% $H_2O_2$, disulfides **III-2** or **IV-2** (50 and 100 μM), **IX-2** (500 and 1000 μM), or 10 mM of DTT were subjected to electrophoresis on a 24 × 48 cm 16.5% polyacrylamine gel (pH 8.4), containing 0.3% SDS and protein detected by silver staining as previously described [4].

### *Mass Spectrometry*

All studies were performed using an ESI-TOF mass spectrometer constructed at the University of Manitoba [23]. Samples were dissolved in 5 mM of ammonium acetate (pH 6.5) at a final concentration of $10^{-5}$ M and were infused continuously into the ion source at a flow rate of 0.4 μL/min using a Harvard model 11 syringe pump (Harvard Apparatus). The declustering voltage, which controls the kinetic energy of the ions in the instrument interface, was set to 100 V, and the capillary temperature to 110°. Data were acquired in the positive mode, and calibration was performed using the multiply charged ions produced by a separate injection of substance P dissolved in a mixture of water:methanol (1:1) with 1% acetic acid. The samples (200 μL) were assembled in "waterbugs" [24] and dialysed with 8000 MWCO membrane (Spectra/Por 7) against 10 mM of $NH_4OAc$ (pH 6.5) with six changes over 48 hr. Prior to mass analysis, the samples were diluted to a final concentration of 10 μM in 5 mM of $NH_4OAc$ (pH 6.5) (non-denaturing conditions) or in $H_2O$:MeOH (1:1), 1% acetic acid (denaturing conditions). Reduction of the drug was achieved by the addition of 10 μL of 2-ME (100 mM in water) to 30 μL of protein solution (in 10 mM of $NH_4OAc$, pH 6.5). After 24 hr at 37°, the mixtures were dialysed once using a 5000 MWCO filter (Millipore), diluted with water to $10^{-5}$ M protein concentration, and infused into the mass spectrometer.

## RESULTS

### *Cell Growth Inhibition*

The effects of the disulfides on the growth of human MCF-7 breast cancer cells were studied (Fig. 1). The $IC_{50}$ values of **III-2** and **IV-2** with 10% FBS were 35.0 and 3.2 μM, and in the presence of 1 μM of hTrx the $IC_{50}$ values were 0.2 and 1.2 μM, respectively. We have found that C73S stimulates MCF-7 cell growth to the same extent as hTrx [4]; however, disulfides **III-2** and **IV-2** at 1 μM did not inhibit the cell growth caused by 1 μM of C73S (results not shown).

### *Interaction between Disulfides and TR*

Disulfides **III-2** and **VI-2** were substrates for reduction by TR (Table 1). Disulfides **IV-2** and **DLK-36** were not substrates, but were competitive inhibitors of the reduction of insulin by TR and hTrx (Table 1). None of the disulfides were substrates for reduction by human glutathione reductase nor did they inhibit the reduction of oxidized glutathione by glutathione reductase (results not shown).

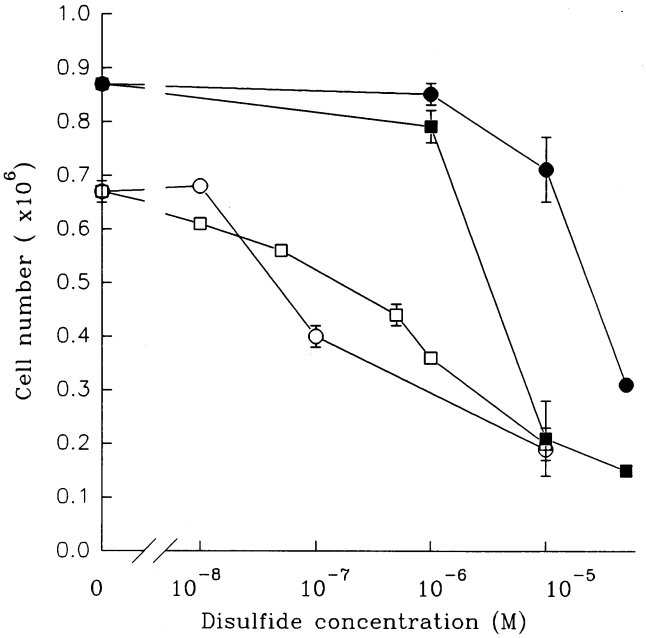


**FIG. 1. Inhibition of hTrx and serum-dependent growth of MCF-7 human breast cancer cells by imidazolyl disulfides** III-2 **(●, ○) and** IV-2 **(■, □). The cells were growth arrested in medium with 0.5% serum for 48 hr at which time there were 0.2 × $10^6$ cells. DMEM with 1 μM of hTrx (open symbols) or 10% serum (closed symbols) was added, and the increase in cell number over a 48-hr period was measured in the presence of various concentrations of the disulfides. Values are means ± SEM, N = 6.**

### *Interaction between Disulfides and Thioredoxin*

Because we had found that the disulfides inhibited hTrx-dependent cell growth at concentrations below those required to inhibit TR we looked for a direct effect of the disulfides on hTrx. The initial reaction between the disulfides and hTrx was measured spectrophotometrically (Fig. 2, A and B). The sharp isosbestic point obtained suggests a direct displacement reaction by the disulfides resulting in the release of 2-mercaptoimidazole (observed at 252 nm) and thioalkylation of hTrx producing the mixed disulfide between 1-methylpropylthiol and hTrx. The initial rate for reaction between hTrx and **III-2** was $7.3 \times 10^{-7}$ M · $sec^{-1}$; for **IV-2**, $2.8 \times 10^{-7}$ M · $sec^{-1}$; for **IX-2**, $3.0 \times 10^{-9}$ M · $sec^{-1}$; and for **DLK-36**, $1.3 \times 10^{-6}$ M · $sec^{-1}$. This is the order of reactivity that would be predicted from the steric and electronic nature of the disulfides' thioalkylating groups. No reaction was observed between the disulfides and either oxidized hTrx (Fig. 2C) or C32S/C35S active site mutant hTrx (not shown), suggesting that the initial reaction between the disulfides and hTrx occurs at the conserved active site Cys residues.

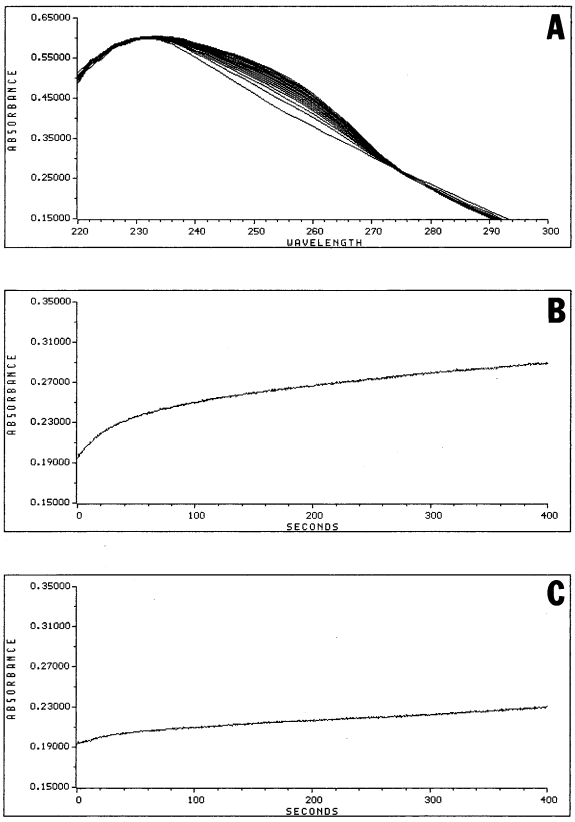


**FIG. 2. (A) Sequential spectrophotometric scans at 2-sec intervals during the reaction between reduced hTrx (1 μM) and** IV-2 **(100 μM) at pH 7.4 and 25°. (B) Time–course of the reaction between** IV-2 **and reduced hTrx measured at 252 nm. (C) Time–course of the reaction between** IV-2 **and oxidized hTrx measured at 252 nm.**

The interaction between the disulfides and hTrx was also examined by gel electrophoresis. Under non-reducing conditions, fresh hTrx gives at least four bands (Fig. 3, I, Lane A), a result that we have shown previously is an artifact of the electrophoresis conditions due to intramolecular disulfide bond formation [4]. Treatment of hTrx with **III-2** and **IV-2** caused conversion to smaller molecular weight bands and the appearance of a new band at 23.3 kDa, which we have shown previously [4] corresponds to the hTrx homodimer (Fig. 3, I, Lanes B–E). These changes were reversed upon treatment with the thiol reducing agent DTT (Fig. 3, II, Lane B compared with Lane A). Treatment of hTrx with the thiol alkylating agent NEM gave a single band at 11.4 kDa (Fig. 3, I, Lane I) that was not reversed upon reduction with DTT or 2-ME (data not shown). Treatment of hTrx with diamide (Fig. 3, I, Lane H), a thiol oxidizing agent, gave bands at 10 and 23.3 kDa. We attribute the bands at 10 kDa to oxidized species of the hTrx monomer, while that at 23.3 results from oxidation to the hTrx homodimer. In contrast, hTrx treated with either **III-2** or **IV-2** at 100 μM followed by 10 mM of diamide did not exhibit increased dimerization and was the same as hTrx treated with disulfide alone (Fig. 3, III, Lanes A and B). These results suggest that **III-2** and **IV-2** interact with a site on hTrx that inhibits dimerization. Based on our previous studies, this is most likely to be $Cys^{73}$, which is essential for stabilization of the hTrx homodimer [4]. Treatment of the Cys73S mutant hTrx with diamide or **IX-2** did not result in homodimerization (results not shown). The relatively unreactive disulfide, **IX-2**, was found to cause predominantly dimerization of hTrx (Fig. 3, I, Lanes F and G), although it was necessary to expose the hTrx to higher concentrations than for the other disulfides to produce the effect.

Mass spectral analyses of hTrx removed from 10 mM DTT and treated with **III-2**, **IV-2**, and **IX-2** have provided data that demonstrate that a thiol/disulfides exchange reaction occurs, producing the thioalkylated hTrx analogue. hTrx removed from DTT was found to have a mass of 11,602 ± 1.3 Da, which corresponds to the calculated value for hTrx (11,607 Da fully reduced) having two disulfide bridges as might be expected for the protein in

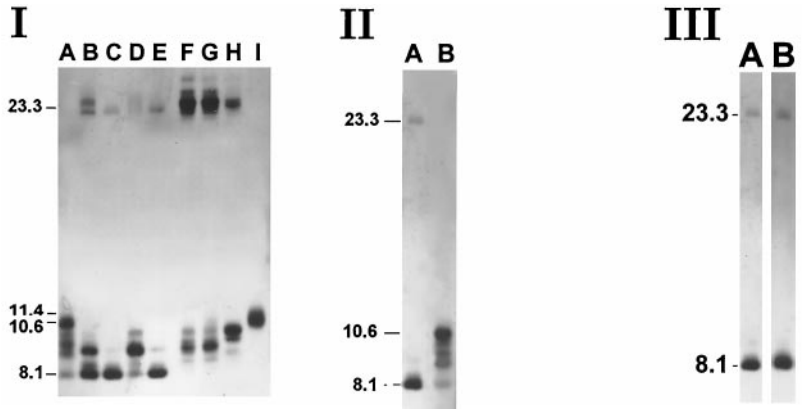


**FIG. 3. (I) SDS–PAGE of reduced recombinant hTrx (Lane A) and hTrx treated with (Lane B) 50 μM of** III-2**; (Lane C) 100 μM of** III-2**; (Lane D) 50 μM of** IV-2**; (Lane E) 100 μM of** IV-2**; (Lane F) 500 μM of** IX-2**; (Lane G) 1000 μM of** IX-2**; (Lane H) 10 mM of diamide; and (Lane I) 10 mM of NEM. Values on the left show the position of molecular weight markers (kDa). (II) SDS–PAGE of hTrx treated with 100 μM of** III-2 **(Lane A) followed by 10 mM of DTT (Lane B). Values on the left show the position of molecular weight markers (kDa). (III) SDS–PAGE of hTrx treated with 100 μM of** III-2 **(Lane A) followed by 10 mM of diamide (Lane B). Values on the left side show the position of molecular weight markers (kDa).**

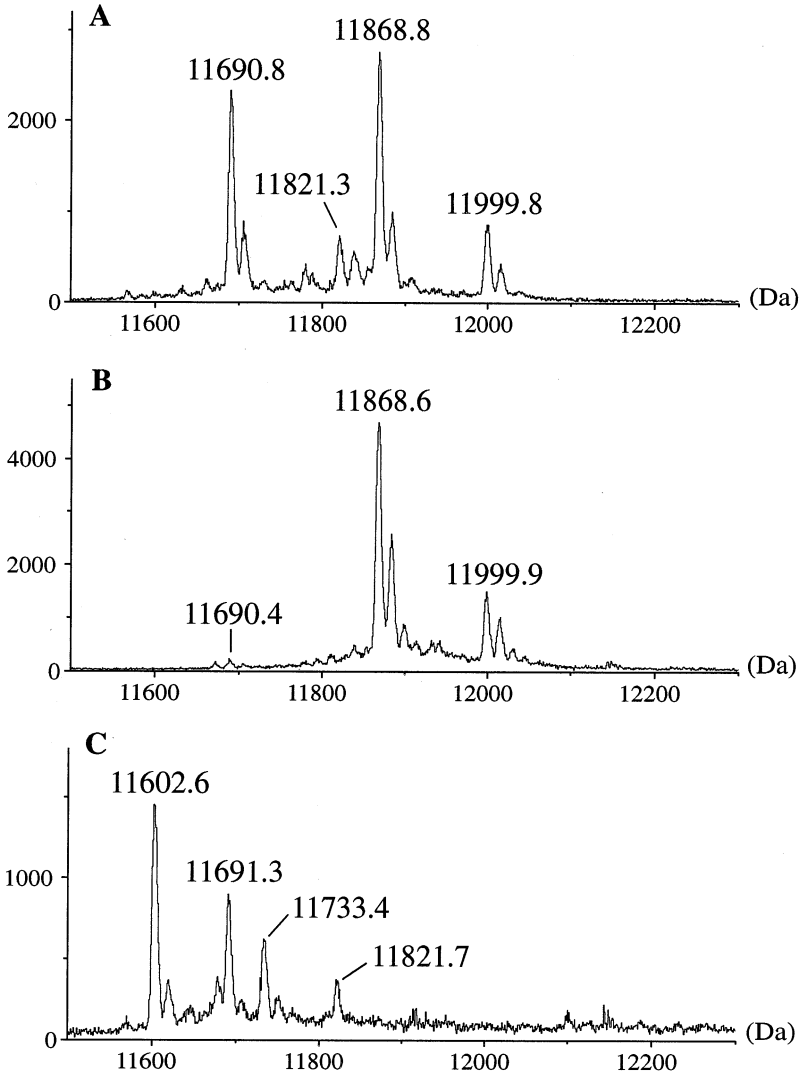


**FIG. 4. Mass spectra of hTrx (20 μM) exposed to (A) III-2 (50 μM); (B) III-2 (100 μM); and (C) of sample 4B exposed to 100 mM of 2-ME for 24 hr. Unlabelled peaks to the right of the major ones (+16 mass units) may be oxidized protein.**

non-reducing conditions. The spectrum of reduced hTrx (25 μM) treated with 50 μM of **III-2** (Fig. 4A) illustrated that two major protein species were present, one with a mass of 11,690.8 Da and another with a mass of 11,868.8 Da. These correspond to a protein molecule modified with one thioalkyl residue [$-S(CH_2)_3CH_3$] and three thioalkyl residues. These modified proteins correspond to the major electrophoretic bands at 9.3 and 8.1 kDa in Fig. 3, I, Lane B. Treatment with 100 μM of **III-2** produced only one major species with a mass of 11,868.6 Da (Fig. 4B), although a very small amount of protein was present with a mass of 11,690.4 Da. These results again correspond to the electrophoresis data (Fig. 3, I, Lane C), which illustrate a major band at 8.1 kDa and a very faint band at 9.3 kDa. If a small proportion of the recombinant protein retains an N-terminal methionine, we would expect to see extra peaks in all the spectra, corresponding to the major peak plus 131 Da. In Fig. 4A, the peaks at 11,821.3 and 11,999.8 Da could be the +M protein with one and three modified residues, respectively. In Fig. 4B, the singly modified +M protein peak would be too small to identify, but the peak at 11,999.9 could be the +M protein with three modified residues. Treatment of the modified hTrx with 100 mM 2-ME for 24 hr at 37° resulted in one major species of mass 11,602 with smaller amounts of 11,691 Da (one modification), and the +M protein at 11,733.4 (11,602 + 131) and 11,821.7 (11,602 + 131 + 89) (Fig. 4C). These results illustrate that reducing conditions removed the thioalkyl residues and again support our proposal that the modifications are the result of thioalkylation of the Cys residues of hTrx. The data also suggest that two of the adducts are reduced more easily than the third, possibly the modified Cys 73. Studies are ongoing using mutant Trx to identify which of the Cys residues are modified.

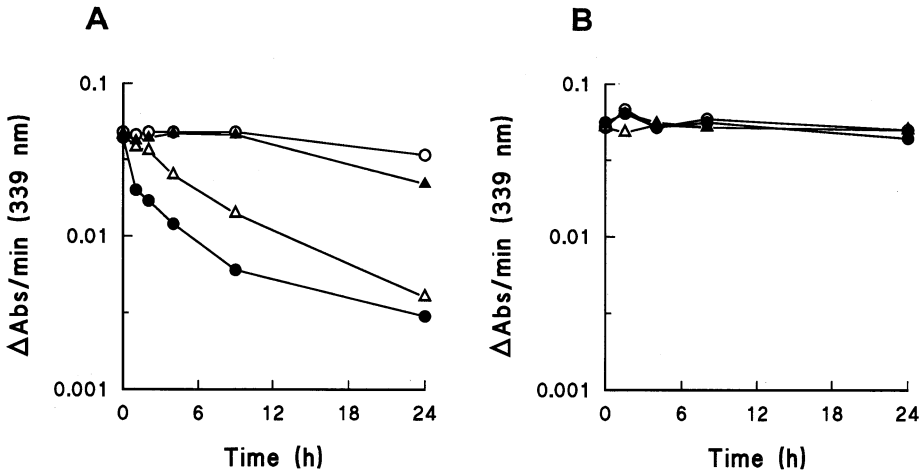


**FIG. 5. Effects of preincubation of hTrx and TR with the disulfides on activity. Key: (○) control; (●) III-2; (△) IV-2; and (▲) IX-2. (A) hTrx (0.7 μM) was incubated with 3.4 μM of disulfide in 100 μM of HEPES buffer (pH 7.4), 5 mM of EDTA at room temperature for up to 24 hr before adding TR, 2.8 μg/mL; insulin, 1 mg/mL; and NADPH, 140 μM. (B) Preincubation of 3.5 μM of disulfide with TR, 2.8 μg/mL, under the same buffer conditions for up to 24 hr before the addition of hTrx, 0.7 μM; insulin, 1 mg/mL; and NADPH, 140 μM. All trials were done in triplicate.**

### *Inactivation of Thioredoxin by the Disulfides*

Preincubation of the disulfides with hTrx resulted in a loss of the ability of hTrx to be reduced by TR with half-lives for the loss by **III-2** of 30 min, **IV-2** of 4 hr, and **IX-2** of 24 hr (Fig. 5). Dialyzing the hTrx against buffer for 24 hr after reaction with the disulfides failed to reverse the inhibition (results not shown). C73S showed no loss of the ability to be a substrate for TR by exposure to any of the disulfides for 24 hr (results not shown). Electrophoretic analyses of hTrx following a 24-hr exposure to **III-2** and **IV-2** showed no evidence of increased homodimer formation over that produced during the 2-hr exposure (results not shown). The results suggest that the disulfides cause irreversible thiolation of hTrx, probably at $Cys^{73}$, resulting in a loss of the ability of hTrx to be reduced by TR. Evidence that the effect is specific for hTrx, and not a general inactivation of proteins by the disulfides, is the absence of inhibition by preincubation of the disulfides with TR (Fig. 5) or glutathione reductase (results not shown).

## DISCUSSION

The 2-imidazolyl disulfides interact with both TR and hTrx. It is postulated that the unbranched alkyl moieties of the disulfides **III-2** and **VI-2**, both of which are substrates for TR, facilitate thiol/disulfide exchange with a Cys residue at the catalytic site of TR and the liberation of 2-mercaptoimidazole (Fig. 6A). There is a subsequent thiol/disulfide exchange to give an oxidized catalytic site followed by reduction by NADPH to regenerate the reduced enzyme. Branching of the alkyl substituent of **IV-2** and the benzyl group of **DLK 36** prevents these compounds from readily undergoing the second thiol/disulfide exchange so that these agents are weak competitive inhibitors of the TR/hTrx system. Extensive branching, as with the *t*-butyl analog, **IX-2**, decreased the reactivity of the disulfide towards the catalytic site Cys residues of TR and prevented **IX-2** from acting as either a substrate or an inhibitor.

There is a rapid reaction of the disulfides with hTrx and liberation of 2-mercaptoimidazole, most likely at the conserved active site $Cys^{32}$ of hTrx, which has been shown to have a lower $pK_a$ than $Cys^{35}$ and is thought to exist as the thiolate anion [25]. Evidence that the catalytic site is the site of reaction is that the C32S/C35S mutant hTrx does not undergo this reaction. The thioalkylated derivative or the oxidized hTrx following a second thiol-disulfide exchange remains a substrate for reduction by TR (Fig. 6B). A slower and irreversible reaction between the disulfides and hTrx results in the inactivation of hTrx as a substrate for TR (Fig. 6C) and ultimately the inhibition of the TR/hTrx system. The inhibition is not seen when C73S is substituted for hTrx in the reaction, suggesting that the reaction occurs at $Cys^{73}$. C73S is as effective as hTrx as a substrate for reduction by TR [4]. Further evidence that thiolation occurs at $Cys^{73}$ is that the disulfides block homodimerization of hTrx caused by diamide, which we have shown previously [4] to involve $Cys^{73}$ homodisulfide bond formation. Thus, the results suggest that while the hydroxyl group of $Ser^{73}$ allows reduction of the conserved redox active site of C73S by TR, thioalkylation of the $Cys^{73}$ in hTrx inhibits this activity. The basis for this inhibition is not clear, but it presumably involves a steric block of the interaction of $Cys^{73}$ with TR. TR has been shown recently to have an unusual selenocysteine group at its C-terminal end, which appears to be essential for catalytic activity [26]. Possibly, an interaction occurs between this selenocysteine and $Cys^{73}$ of hTrx.

The selectivity of the 2-imidazolyl disulfides as inhibitors of thioredoxin compared with other thiol containing pro-

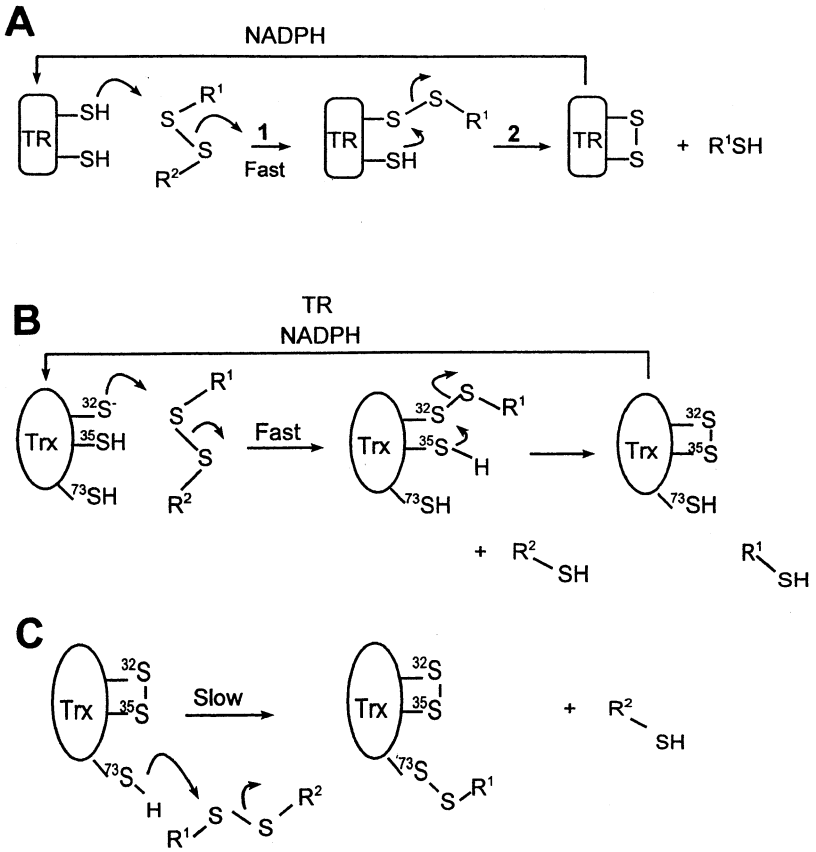


**FIG. 6. Proposed scheme for the reaction of the 2-imidazolyl disulfides ($R^1SSR^2$) with TR and hTrx. (A) Rapid reaction (1) of the disulfide with the catalytic site cysteines of TR liberates 2-mercaptoimidazole ($R^2$-SH). With** III-2 **and** VI-2**, subsequent thiol-disulfide exchange can give an oxidized catalytic site that can be reduced in the presence of NADPH to regenerate TR. Reaction (2) with disulfides** IV-2 **and** DLK 36 **to form the oxidized TR occurs only slowly, resulting in inhibition of the enzyme. (B) Rapid reaction of the disulfide with the conserved catalytic site cysteines of hTrx and subsequent thiol-disulfide exchange to give an oxidized active site. The thiolated or oxidized catalytic site can be reduced by TR in the presence of NADPH to regenerate reduced hTrx. (C) Slower reaction of the disulfide with the non-catalytic site $Cys^{73}$ of hTrx, which is also dependent on the nature of the thiolating substituent, giving a form of hTrx that cannot be reduced by TR.**

teins in the cell remains to be established. We have shown that the disulfides are irreversible inhibitors of thioredoxin but not of thioredoxin reductase or glutathione reductase, presumably because thiolation of the catalytic site cysteine of these enzymes is a reversible process, while the thiolated Cys73 residue of thioredoxin is outside the catalytic site. The thioredoxin system is widely distributed in tissue, and whether the disulfides will be selective in their effects on cancer cells compared with normal cells remains to be fully investigated. The disulfides have shown *in vivo* antitumor activity against human tumor xenografts in *scid* mice [19], although whether this activity is a consequence of the inhibition of thioredoxin and the selectivity of the growth inhibition compared with other fast-growing tissue such as bone marrow and the intestinal mucosa remains to be established.

In summary, our studies have shown that 2-imidazolyl disulfides can interact with both TR and hTrx. The disulfides can be substrates or inhibitors of TR, depending upon the structure of the thiolating substituents. The disulfides also react with a conserved catalytic site cysteine residue of hTrx, and the thiolated or oxidized catalytic site can be reduced by TR. The disulfide also causes a slower thiolation of $Cys^{73}$ of hTrx outside the conserved catalytic site. This $Cys^{73}$-thiolated hTrx is not a substrate for reduction by TR. The reaction with $Cys^{73}$ of hTrx appears to be responsible for the inhibition of the cell growth stimulating activity of hTrx by the 2-imidazolyl disulfides.

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