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Reversible inhibition of human thioredoxin reductase activity by cytotoxic alkyl 2-imidazolyl disulfide analogues

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Abstract. The thioredoxin/thioredoxin reductase system is important for several aspects of the regulation of cellular proliferation by both intracellular and extracellular mechanisms. The effects of *n*-butyl 2-imidazolyl disulfide (III-2), 1-methylpropyl 2-imidazolyl disulfide (IV-2), and *n*-decyl 2-imidazolyl disulfide (VII-2) on purified human placental thioredoxin reductase activity were examined. The analogues were competitive inhibitors with DTNB for reduction by thioredoxin reductase, with K_i values for III-2, IV-2, and VII-2 being 3.3, 13.0, and 8.6 μ*M*, respectively. The inhibition was noncompetitive with reduced nicotinamide adenine dinucleotide phosphate (NADPH). None of the analogues was a suicide substrate inhibitor of the flavoenzyme. III-2 and VII-2 were metabolized by thioredoxin reductase at about half the rate of DTNB, whereas IV-2 was not detectably metabolized. The second order rate constants for the reactions of III-2 and IV-2 with reduced GSH were 931 and 91 M^{-1} s^{-1}, respectively. The lower reactivity of IV-2 with reduced GSH and the lack of the analogue's metabolism by thioredoxin reductase may be due to the more sterically hindered structure of this analogue. The 50% inhibitory concentrations (IC_{50} values) for the inhibition of serum-dependent cellular proliferation of Swiss 3T3 murine fibroblasts by III-2, IV-2, and VII-2 were 2.0, 3.5, and 4.0 μ*M*, respectively. IV-2 was considerably more potent as an inhibitor of the thioredoxin-dependent cellular proliferation of Swiss 3T3 fibroblasts, showing an IC_{50} value of 60 n*M*. Thus, inhibition of cellular proliferation by alkyl 2-imidazolyl disulfide analogues may involve interaction with thioredoxin, thioredoxin reductase, or an alternative target that is redox-regulated by thioredoxin.

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Abbreviations: *DMEM* Dulbecco's modified Eagle's medium, *DTNB* 5,5′-dithiobis-(2-nitrobenzoic acid), *DTT* dithiothreitol, *FBS* fetal bovine serum, *GSH* glutathione, *Trx* thioredoxin, *TR* thioredoxin reductase

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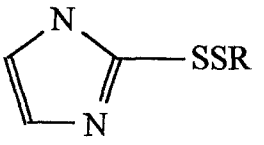
Introduction

The ability to maintain and regulate the intracellular thiol redox environment is mediated in mammalian cells by redox systems such as the GSH/GSH reductase and the thioredoxin/thioredoxin reductase (Trx/TR) redox systems [1]. The Trx/TR redox system has been suggested to be involved in numerous intracellular functions. These include facilitation of protein folding through a protein disulfide isomerase-like mechanism [2], reaction with oxygen radicals [3, 4], activation of the glucocorticoid and interferon-α receptors [5], and the redox regulation of transcription factor activity, for example, AP-1 through Ref-1 [6, 7] and NF-κB [8]. Trx also acts as a cofactor for ribonucleotide reductase, an enzyme involved in the first unique step in DNA synthesis [9]. Trx has extracellular functions and can stimulate cellular proliferation and DNA synthesis in both normal and tumor cells [3, 10].

TR (EC 1.6.4.5) is a flavoenzyme that reduces the active-site cystine residue of oxidized Trx using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Two half-reactions comprise the actual sequence of catalytic events. The first half-reaction involves the reduction of flavine adenine dinucleotide (FAD) by NADPH and electron transfer to the active-site cystine residue of TR. The second half-reaction is the reduction of bound oxidized Trx by TR. Rat liver TR [11] and human placental TR [12] have been isolated as homodimers comprising of 58- and 60 kDa subunits, respectively. Human TR was characterized to be a relatively thermostable enzyme with tightly bound FAD prosthetic groups [12].

We report herein an investigation into agents with the potential to inhibit Trx-stimulated cellular proliferation either by reacting directly with Trx or by inhibiting the

activity of TR. Three disulfide analogues previously examined for cytotoxicity and nonprotein-thiol depletion [13] were considered to be excellent candidates for this type of growth inhibition. This study involved an in vitro analysis of inhibition of human placental TR by cytotoxic alkyl imidazolyl disulfide analogues, a kinetics study between these agents and the nonprotein thiol GSH, and an evaluation of the effect of the analogues on serum- and Trx-stimulated cellular proliferation.



Analogue	R
III-2	$CH_3(CH_2)_2CH_2$-
IV-2	$CH_3CH_2CH(CH_3)$-
VII-2	$CH_3(CH_2)_8CH_2$-

Fig. 1. Alkyl 2-imidazolyl disulfide analogues

Materials and methods

Chemicals

The analogues *n*-butyl 2-imidazolyl disulfide (III-2), 1-methylpropyl 2-imidazolyl disulfide (IV-2), and *n*-decyl 2-imidazolyl disulfide (VII-2) were synthesized as previously reported and recrystallized prior to use [13].

TR purification

Human TR was purified from placenta as described previously [12]. Briefly, one fresh placenta (University Medical Center, University of Arizona) was homogenized in 400 ml of 50 m*M* TRIS-HCl and 1 m*M* ethylenediaminetetracetic acid (EDTA, pH 7.6; solution A), and then centrifuged at 30,000 *g* for 30 min and the supernatant was acid-precipitated. The supernatant was adjusted to pH 7.4 with 1 *M* NH_4OH and applied to a diethylaminoethyl (DEAE)-cellulose column (6×20 cm), and bound protein was eluted with a 400-ml linear 0 to 500-m*M* NaCl gradient in solution A. Fractions containing TR activity were applied to a 2′–5′ adenosine diphosphate (ADP)-agarose (Sigma Chemical Co., St. Louis, Mo.) column (1.5×14 cm). TR activity was eluted with 500 m*M* KCl in solution A and applied to a Blue Sepharose CL-6B (Pharmacia LKB, Piscataway, N.J.) column (1.5×13 cm). TR activity was eluted with a 180-ml linear 0 to 300-m*M* NaCl gradient in solution A and applied to a Mono Q HR 5/5 (Pharmacia LKB, Piscataway, N.J.) column. Activity was eluted with a 60-ml linear 0 to 400-m*M* NaCl gradient in solution A and re-chromatographed twice more on Mono Q as described above to homogeneity. Protein levels were determined by the Bradford dye-binding assay (Bio-Rad Protein Assay Kit, Irvine, Calif.).

TR assay

The assay used to monitor TR activity involved the NADPH-dependent reduction of the disulfide bond in DTNB by TR [11]. Briefly, 300 µl of 100 m*M* potassium phosphate buffer (pH 7.0), 1 m*M* EDTA and 0.2 mg BSA/ml was mixed with 3 µl of 20 m*M* NADPH, 1 µl of 100 m*M* DTNB in dimethylsulfoxide (DMSO), and 0.5–3.0 µl of drug analogues from a 10-m*M* stock solution in DMSO, which at 2% final concentrations alone had no effect on TR activity. Aliquots (0.5–5 µg) of TR were added to the assay mixture, and the change in optical density at 412 nm was monitored over 2 min. The NADPH oxidation assay was performed exactly as described for the DTNB reduction assay, with the only difference being that the rate was monitored by the change in absorbance at 340 nm over time. The kinetic constants (K_M) were determined from Lineweaver-Burke double-reciprocal plots. The K_i values were calculated from the equation

$$K_p = K_M(1 + i/K_i),$$

where K_p represents the new K_M value determined in the presence of a specific concentration of inhibitor (i) in the DTNB reduction assay. Specific activity values were determined from the assay rates as previously described [11]. Ultrafiltrate products were collected after centrifugation through Centricon-10 concentrators with a molecular-weight cut-off of 10 kDa (Amicon, Beverly, Mass.).

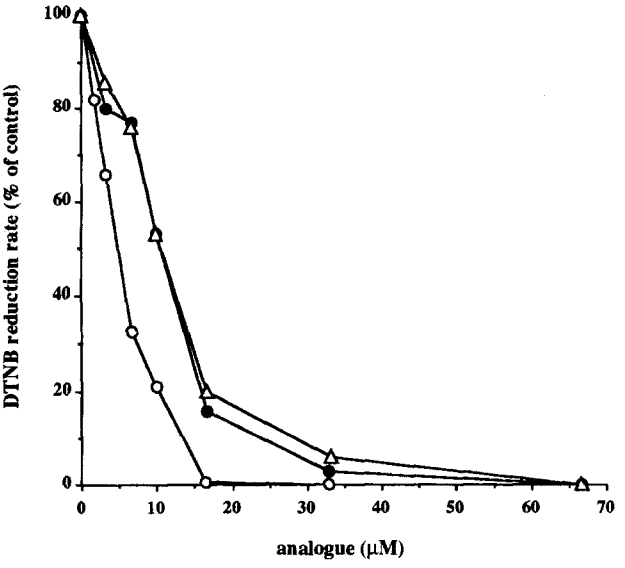


Fig. 2. Inhibition of human TR by alkyl 2-imidazolyl disulfide analogues. TR activity was determined in the DTNB reduction assay as described in Materials and methods. Shown are the dose-response curves for III-2 IV-2 and VII-2 (*open* and *closed circles* and *open triangles*, respectively)

Reactions with GSH

All reactions were followed by monitoring the absorbance at 252 nm, (λ_{max} of 2-mercaptoimidazole) using a Hewlett-Packard 8450A spectrophotometer with a thermostated cuvette holder. In a typical experiment, GSH ($0.8–1.8\times10^{-4}$ *M*) was added to 0.2 *M* phosphate buffer (pH 7.4) in a 1-ml cuvette at 5.0° ±0.1° C. A known quantity of an alkyl 2-imidazolyl disulfide analogue was added to give a final concentration of $0.99–2.43\times10^{-5}$ *M*. The reaction progress was monitored by following the increase in absorbance at 252 nm. At least three trials were recorded for each concentration. For pseudo-first-order kinetics studies, the rate constants were calculated from the appropriate logarithmic relationship by computer assisted linear least-squares analysis [19]. The plot $\ln(A_f - A)$ versus time was found to be linear, indicating a first-order relationship. The pseudo-first-order rate constants (k_1) were found to be directly proportional to the GSH concentration, confirming that the rate of reaction was also of the first order in GSH. The second-order rate constants were therefore obtained by $k_s = k_1/[GSH]$. For stoichiometric measurements, varied concentrations of an

Table 1. K_i values of analogues with human TR[a]

Analogue	K_i (μ*M*)
III-2	3.3
IV-2	13.0
VII-2	8.6

[a] Measured by the DTNB reduction assay

analogue were added to a cuvette containing 2.0×10^{-5} *M* GSH in phosphate buffer (pH 7.4) until no change in final absorbance was observed. $[GSH]_f$ versus $[disulfide]_o$ was plotted to determine their ratio in the reaction (data not shown).

Cell growth

Swiss 3T3 murine fibroblast cells were grown in DMEM containing 10% FBS. Cells in log-phase growth were harvested using 0.5% trypsin and 0.5 m*M* EDTA and plated at 5×10^3 cells/1.6-cm culture well. For growth-inhibition studies, cells were allowed to attach for 4 h and the analogues were added to the media at the indicated concentrations. Control cultures contained the highest final concentration of DMSO (0.1%) without any analogue. Cell numbers were measured 4 days later using an automated cell counter (Coulter, Hialea, Fla.) and all sample cultures were performed in triplicate. For growth stimulation with Trx, Swiss murine 3T3 fibroblast cells ($5-10\times10^4$) were allowed to attach in 35-mm culture dishes with 2 ml of DMEM containing 10% FBS overnight, washed with DMEM, and grown in DMEM containing 0.5% FBS for 24 h. All Trx samples were reduced with a 3-fold excess of DTT prior to their addition to the cultures for a maximal final concentration in the culture of 3.0 μ*M* DTT. Simultaneously 1.0 μ*M* reduced Trx and 0.05–1.0 μ*M* IV-2 were added to each culture, which was then allowed to incubate for 24 h. Control cultures contained 3.0 μ*M* DTT alone or 1 μ*M* Trx reduced with 3.0 μ*M* DTT. Cell numbers were determined using a hemacytometer following detachment of the cells with 0.025% trypsin.

Results

We used purified human placental TR to study the inhibition of this flavoenzyme by alkyl 2-imidazolyl disulfides (Fig. 1). These analogues were found to inhibit the reduction of DTNB by TR, with IC_{50} values being 4 μ*M* for III-2 and 11 μ*M* for both IV-2 and VII-2 (Fig. 2). Kinetics studies showed the analogues to be competitive inhibitors of TR activity with DTNB (Table 1) and noncompetitive inhibitors with NADPH (data not shown).

Metabolism of the analogues by TR was investigated by directly measuring the rate of NADPH oxidation. III-2 and VII-2 were good substrates for reduction by TR, with rates being 1 nmol NADPH oxidized min^{-1} mg^{-1} at 16 μ*M* III-2 and 0.83 nmol NADPH oxidized min^{-1} mg^{-1} at 16 μ*M* VII-2, which were 49% and 37% of the maximum velocity (V_{max}) for DTNB reduction, respectively (Fig. 3). In contrast, no metabolism of IV-2 was detected over a 10-min period (<0.02 nmol NADPH oxidized min^{-1} mg^{-1}).

Preincubation of the analogues with TR and NADPH for 30 min in the absence of DTNB did not result in any change in the rate of DTNB reduction when DTNB was added to the assay mixture (Table 2). Since it was possible that the TR-reduced products of III-2 and VII-2 might reduce DTNB directly in the assay, we examined the ability of a <10-kDa ultrafiltrate collected from a 30-min incubation of sample containing TR, NADPH, and either III-2 or VII-2 to reduce DTNB. The filtrate was not capable of reducing added DTNB as monitored by the change in absorbance at 412 nm (data not shown).

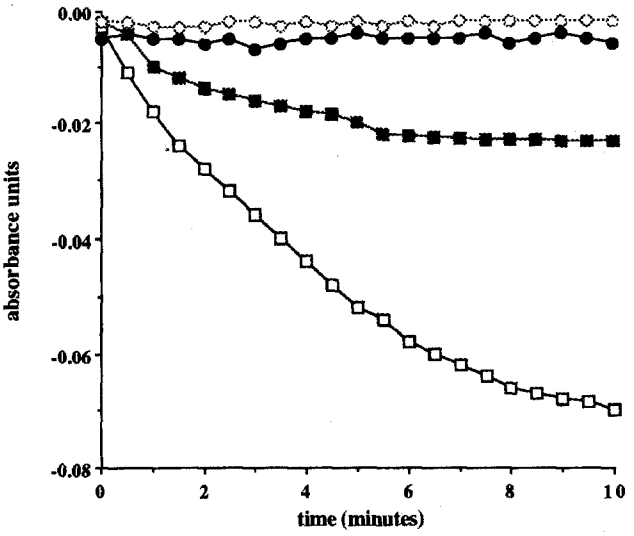


Fig. 3. Metabolism of 2-imidazolyl disulfide analogues by TR. TR was incubated with NADPH alone (*closed circles*) and with either III-2 (*open squares*), VII-2 (*closed squares*), or IV-2 (*open circles*), and the change in absorbance at 340 nm was recorded over a period of 10 min

Table 2. Effect of incubation of analogues with TR on rates of DTNB reduction[a]

Analogue	DTNB reduction (nmol min^{-1} mg^{-1})	
	0 min	30 min
–	2.24	2.20
III-2	1.22	1.14
IV-2	1.77	1.73
VII-2	1.94	1.88

[a] Measured by incubating the analogues at 3.3 μ*M* with TR and NADPH for the indicated periods, followed by the addition of DTNB and monitoring of the change in absorbance at 412 nm

The reaction between the disulfide analogues and GSH, a simple nonprotein thiol, were found to be of the first order with respect to the disulfide and with respect to GSH, indicating overall second-order kinetics. The second-order rate constants between III-2 or IV-2 with GSH (pH 7.4, 5° C) were 931 ± 119 and 91 ± 3 M^{-1} s^{-1}, respectively, and the stoichiometry in both cases was found to be 1:1. Analysis of the reaction mechanism by altering pH and temperature and isolation of the products of the reaction indicated that the reaction proceeds by an S_N2 reaction to produce the mixed disulfide between GSH and the alkyl portion of the disulfide (data not shown; Eq. 1):

$$GSH + RSSR' \rightarrow GSSR + R'SH \quad R = alkyl. \qquad (1)$$

The effect of the analogues on cellular proliferation was determined using Swiss 3T3 murine fibroblasts (Fig. 4A). The 50% growth-inhibitory (IC_{50}) values of III-2, IV-2, and VII-2 in the presence of 10% FBS were determined to be 2.0, 3.5, and 4.0 μ*M*, respectively. In contrast, when cells

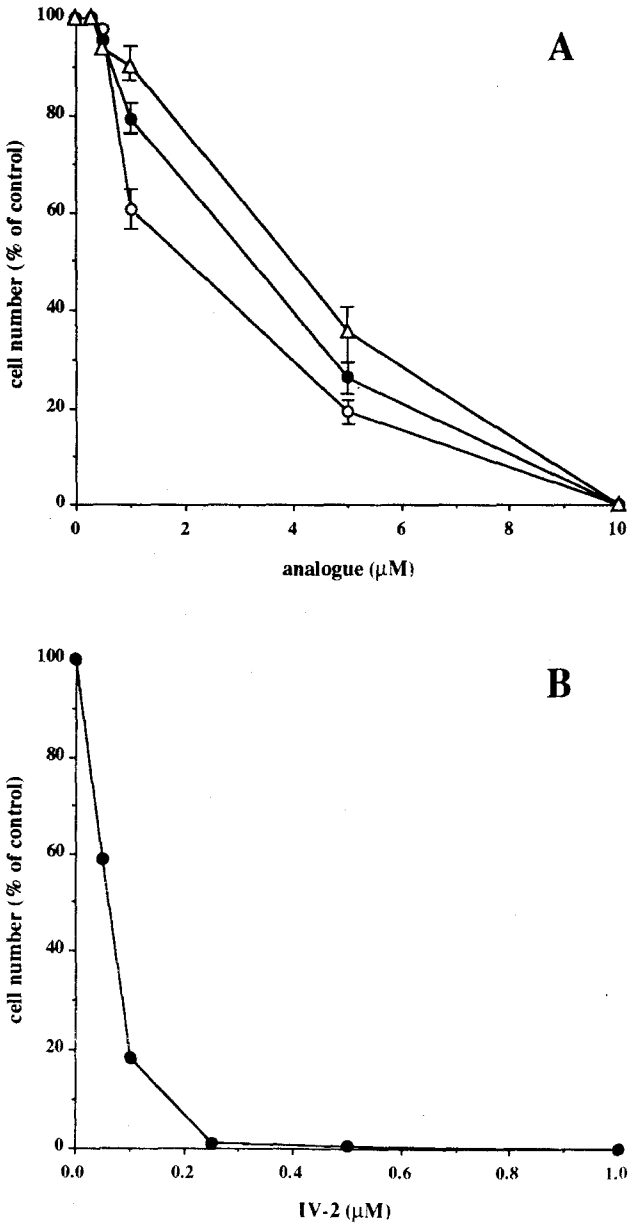


Fig. 4 A, B. Effect of the analogues on cellular proliferation. **A** Murine fibroblasts were grown in the presence of a range of concentrations of III-2, IV-2, and VII-2 (*open* and *closed circles* and *open triangles*, respectively) and 10% FBS. After 4 days of growth, cell numbers were determined as described in Materials and methods. **B** Murine fibroblasts were grown in the presence of 10 μ*M* Trx reduced with 30 μ*M* DTT and a range of concentrations of IV-2. Control cultures contained 10 μ*M* Trx with 30 μ*M* DTT alone. After 24 h of growth, cell numbers were determined as described in Materials and methods

were serum-deprived (0.5% FBS for 24 h) and stimulated with recombinant human Trx, the IC_{50} value for inhibition of Trx-stimulated proliferation by IV-2 was 60 n*M* (Fig. 4B).

Discussion

In this study we examined the properties of a series of alkyl 2-imidazolyl disulfide analogues as inhibitors of purified human placental TR. The analogues were found to be relatively potent inhibitors of DTNB reduction by TR, with IC_{50} values ranging from 4 to 11 μ*M*. Kinetics studies showed that all the analogues were competitive inhibitors of DTNB reduction by TR and noncompetitive inhibitors with NADPH. Analogues III-2 and VII-2 were themselves substrates for metabolism by TR at rates approximately half of those seen with DTNB. In contrast, IV-2 showed no detectable metabolism by TR. These data correlate well with the kinetics study of the reaction between the disulfides and the simple cellular thiol GSH. Since the slower reaction rate between IV-2 and GSH was most likely due to steric hindrance imposed by the bulkier alkyl group, the inability of IV-2 to be metabolized by TR may also be due to steric hindrance. Furthermore, previous studies have shown marked differences in the ability of the analogues to deplete GSH levels in EMT6 cells in culture, with III-2 depleting GSH to 30% of control levels within 30 min whereas IV-2 required 90 minutes to deplete GSH to the same levels [13].

Other reported inhibitors of TR are azelaic acid, which is a competitive inhibitor [14], and the mechanism-based (suicide) substrate inhibitors, antitumor anthracyclines [15], antitumor nitrosoureas [16], and 13-*cis*-retinoic acid [17]. The alkyl 2-imidazolyl disulfide analogues did not show the characteristic time dependence of mechanism-based inhibition of TR. Inhibition of the reduction of DTNB occurred with the analogues acting as competitive substrates (analogues III-2 and VII-2) or binding competitively with DTNB, but without their being reduced themselves (analogue IV-2). Although the analogues can form covalent bonds with GSH, they do not appear to form stable disulfide bonds with the active site cysteines of TR, since this would be expected to lead to irreversible and/or time-dependent inhibition of the flavoenzyme.

We have previously shown that a recombinant form of human Trx, the natural substrate for TR, stimulates cellular proliferation of serum-deprived Swiss 3T3 murine fibroblasts [10]. Furthermore, site-directed mutagenesis of the active-site cysteines in Trx indicated that the stimulation was dependent upon a redox-active form of Trx [10]. Since it has been suggested that an extracellular TR is present on the surface of cultured human keratinocytes [18], it is possible that extracellular Trx may be kept reduced, and biologically active, by a cell-surface extracellular TR. The effect of the analogues on cellular proliferation indicated that the concentration effective for inhibiting cell growth was in the same range as that which inhibited TR activity in the DTNB assay. The analogue IV-2 was shown to be a more potent inhibitor of Trx-dependent than of serum-dependent cellular proliferation at a concentration lower than that required to inhibit TR. We have not determined if there are differences in the growth characteristics of fibroblasts grown in 10% FBS alone versus Trx with 0.5% FBS. Since IV-2 neither is metabolized in vitro by TR nor interacts with cellular thiols, it is possible that this analogue is relatively stable during growth studies. Studies have shown that these disulfide analogues can react in a similar fashion with Trx as with GSH (manuscript in preparation). Our results suggest that the site of action of IV-2 leading to inhibition of cellular proliferation could be either with

extracellular Trx, a surface-bound TR-like activity, or an alternative target that is redox-regulated by Trx.

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