Vol. 204, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1994 Pages 224-229

# CHINIFUR, A SELECTIVE INHIBITOR AND "SUBVERSIVE SUBSTRATE" FOR *TRYPANOSOMA CONGOLENSE* TRYPANOTHIONE REDUCTASE

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Received August 11, 1994

**Summary** Nitrofurans with aromatic and heterocyclic substituents inhibit *Trypanosoma congolense* trypanothione reductase (TR) and yeast glutathione reductase (GR), acting as uncompetitive inhibitors vs. NADPH and noncompetitive or uncompetitive inhibitors vs. disulfide substrate. Many of these compounds inhibited trypanothione reductase more effeciently than glutathione reductase. Chinifur (2-{5'-nitro(furo-2'-yl)-ethene-1-yl}-4(N,N-diethylamino)-1-methyl-but-1-yl-aminocarbonyl-4-quinoline) was the most selective inhibitor of, and free radical-generating substrate for, trypanothione reductase ($K_i$= 4.5 μm, TN = 3 $s^{-1}$, $TN/K_m$ = 3.2 x $10^4$ $M^{-1}$ $s^{-1}$), only weakly inhibiting glutathione reductase ($K_i$ = 100 μm). These findings point to the importance of hydrophobic interactions in the design of redox active heteroaromatic compounds acting as selective inhibitors of, and "subversive substrates" for, trypanothione reductase. 

Trypanothione reductase catalyzes the NADPH-dependent reduction of trypanothione, $N^1$,$N^8$-bis(glutathionyl) spermidine, $T(S)_2$, a glutathione analog unique to trypanosomatid parasites (1-3), and serves an important role in their antioxidant defense. Selective inhibitors of trypanothione reductase could be useful as trypanocidal drugs (1-5). Among inhibitors of trypanothione reductase, the use of nitrofurans and quinones seems especially attractive, since trypanothione reductase can reduce these compounds via a one-electron mechanism, thus initiating their redox cycling (4-6). Since these compounds also inhibit other structurally and functionally related antioxidant enzymes, including glutathione reductase (7-9), the design of inhibitors which will selectively interact with trypanothione reductase is desirable. Recent crystallographic studies of trypanothione reductase (10-12) provide some basis for the design of

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Abbreviations: T(S)2, oxidized trypanothione, GSSG, oxidized glutathione; TR, trypanothione reductase; GR, glutathione reductase; TN, turnover number; $TN/K_m$, bimolecular rate constant.

0006-291X/94 $5.00

selective inhibitors which bind in the $T(S)_2$-binding domain, e.g., inhibitors which bear a positive charge and/or a flexible side chain. Indeed, hydrazonoamine-substituted nitrofurans and quinones have been shown to inhibit trypanothione reductase more strongly than glutathione reductase (5). However, the search for novel and selective inhibitors and "subversive" substrates of trypanothione reductase, especially among previously described redox-active compounds, remains desirable.

In this paper we report that chinifur, 2-{5'-nitro(furo-2'-yl)-ethene-1-yl}-4(N,N-diethylamino)-1-methyl-but-1-yl-aminocarbonyl-4-quinoline (IIc, Fig. 1), acts as a selective inhibitor of, and substrate for, *Trypanosoma congolense* trypanothione reductase, but only weakly interacts with yeast glutathione reductase. Parallel studies with a series of nitrofurans and quinones reveal some factors required for the selective inhibition of trypanothione reductase.

## MATERIALS AND METHODS

The overexpressed trypanothione reductase from *T. congolense* was isolated as previously described (13). Glutathione reductase from yeast was purchased from Sigma. The concentration of these enzymes was determined spectrophotometrically using $\varepsilon_{464}$ = 11 $mM^{-1}$ $cm^{-1}$(13). NADPH, cytochrome c, superoxide dismutase, duroquinone, 9,10-phenanthrene quinone, rhein and nitrofurantoin were purchased from Sigma or Aldrich. Trypanothione was purchased from Bachem Bioscience (Switzerland), and glutathione was purchased from Reanal (Hungary).

Nitrofurans Ia-e (Fig. 1) were synthesized by condensation of 5-nitrofuran-2-aldehyde with hydrazides of 4-nitrobenzoic acid (Ia), benzimidazole-2-yl-carbamic acid (Ic), oxalic acid (Ie) and 5(6)-sulphonylhydrazine of benzimidazole-2-yl-carbamic acid methyl ester (Id) or 2,4-dinitrophenylhydrazine (Ib) in acetic acid according to adapted procedures (14). All compounds were characterized by melting point, thin-layer chromatography and $^1H$ NMR Spectroscopy. Nitrofurans IIa-c, synthesized as described (15), were the generous gift of Dr. N.M. Sukhova (Institute of Organic Synthesis, Riga, Lativa). Javanicin was the generous gift of Dr. A.G. Medentsev (Institute of Biochemistry and Physiology of Microorganisms, Pushchino, Russia).

Steady-state rates of NADPH oxidation were determined spectrophotometrically at 340 mm ($\varepsilon_{340,NADPH}$ = 6.2 $mM^{-1}$ $cm^{-1}$) in a Hitachi 557 spectrophotometer. Assays were performed in 0.05 M Hepes, pH 7.5, containing 1 mM EDTA, at 25±0.1°C. Typically, 10-50 μM NADPH, and 20-150 μM trypanothione or 30-300 μM glutathione was used. The nitroreductase activity of trypanothione reductase was monitored by following the reduction of 50 μM cytochrome c ($\varepsilon_{550}$ = 20 $mM^{-1}$ $cm^{-1}$) in the presence of 30 μM NADPH and 5-7 concentrations of nitrofuran. For the determination of the turnover number, TN, and the bimolecular rate constants for the reaction, $TN/K_m$, the steady-state rates were graphically analyzed by Lineweaver-Burk analysis. At saturating concentrations of NADPH and disulfide substrates, the TN of trypanothione reductase and glutathione reductase are 120 $s^{-1}$ and 180$s^{-1}$.

## RESULTS

In accord with previous data (6,8,13), the reactions of trypanothione reductase and glutathione reductase with disulfide substrates follow a 'ping-pong' kinetic mechanism. The TN and other kinetic parameters of both enzymes catalyzing disulfide reductions are similar to previously reported values. All of the nitrofurans investigated (Fig. 1) inhibited trypanothione reductase, acting as uncompetitive inhibitors vs. NADPH. Using saturating concentrations of

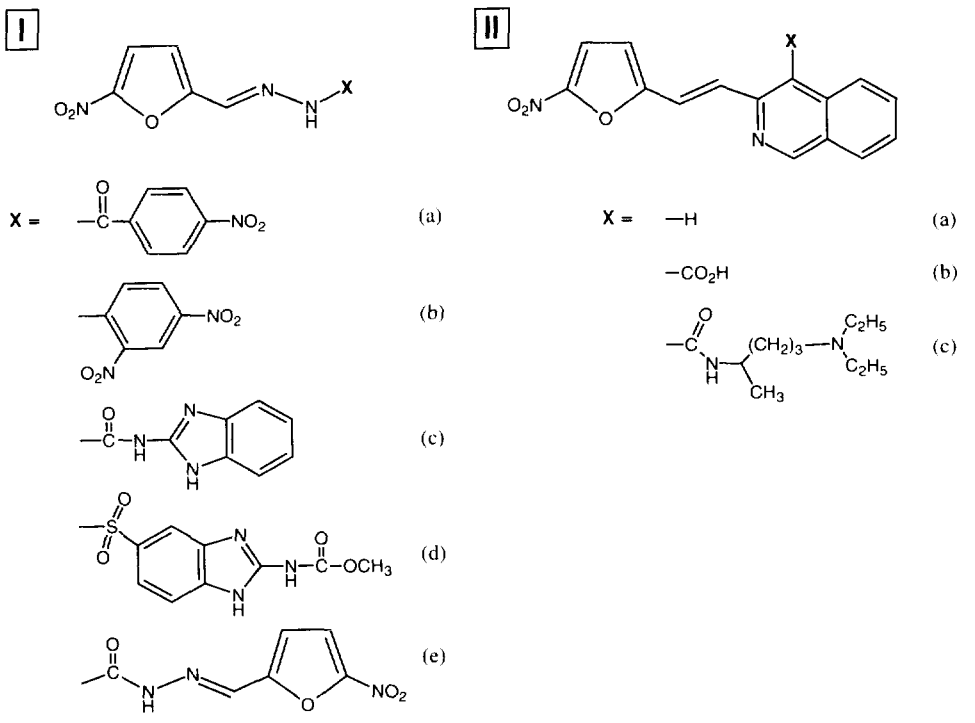


Figure 1. The structural formula of nontrivial nitrofurans.

NADPH and variable concentrations of $T(S)_2$, nitrofurans were either noncompetitive or uncompetitive inhibitors vs. $T(S)_2$. The values of $K_i$ vs. $T(S)_2$, calculated according to Dixon plots, are presented in Table 1. For comparison, the Table also contains the $K_i$ values of nitrofurans vs. GSSG in the glutathione reductase-catalyzed reaction, determined previously (8) or in the present work. The $K_i$ values of several quinones that have been shown to act as inhibitors of glutathione reductase (9) are also presented.

Trypanothione reductase also catalyzed the nitrofuran-dependent reduction of cytochrome c (4,5). This reaction was inhibited 50-80% by 30 μg/ml superoxide dismutase. This indicates that trypanothione reductase reduces nitrofurans to their corresponding anion radicals which can reduce cytochrome c, either directly or via the $O_2/O_2^{\cdot-}$ redox pair (4,5). The following kinetic parameters were determined for the indicated substrates: TN = 3 $s^{-1}$, $TN/K_m$ = 9.2 x $10^4$ $M^{-1}$ $s^{-1}$ (IIc), TN = 0.4 $s^{-1}$, $TN/K_m$ = 1.3 x $10^4$ $M^{-1}$ $s^{-1}$ (Ia), TN = 2 $s^{-1}$, $TN/K_m$ = 6 x $10^3$ $M^{-1}$ $s^{-1}$ (Ie), TN = 0.16 $s^{-1}$, $TN/K_m$ = 1.2 x $10^4$ $M^{-1}$ $s^{-1}$ (Ib), and TN = 2 $s^{-1}$, $TN/K_m$ = 1.6 x $10^3$ $M^{-1}$ s-1 (Id). Other nitrofurans, including nitrofurantoin, exhibited $TN/K_m$ of 170 - 350 $M^{-1}$ $s^{-1}$.

## DISCUSSION

Among the compounds investigated, nitrofurans containing aromatic and heterocyclic substituents generally inhibited trypanothione reductase more strongly than glutathione reductase

TABLE I

The inhibition constants ($K_i$) of nitrofurans and quinones vs. disulfide substrate in the trypanothione reductase and glutathione reductases-catalyzed reactions

| Compound | $K_i$ (μM)[a] | |
|---|---|---|
| | Trypanothione Reductase | Glutathione Reductase |
| Nitrofurantoin | 40 (noncomp.) | 100 (uncomp.)[b] |
| Ia | 55 (uncomp.) | >300 (uncom.) |
| Ib | 75 (uncomp.) | >300 (uncomp.) |
| Ic | 50 (noncomp.) | 160 (uncomp.) |
| Id | 55 (noncomp.) | 220 (uncomp.) |
| Ie | 35 (noncomp.) | 85 (uncomp.) |
| IIa | 22 (uncomp.) | 8 (uncomp.)[b] |
| IIb | 150 (uncomp.) | 5 (uncomp.)[b] |
| IIc | 4.5 (noncomp.)[c] | 100 (uncomp.)[b] |
| 9,10-Phenanthrene quinone | 2.5 (uncomp.)[c] | 2 (uncomp.)[b] |
| Duroquinone | 200 (uncomp.) | 200(uncomp.)[b] |
| Rhein (4,5-dihydroxy-9,10-anthraquinone-2-carbonic acid) | 80 (uncomp.) | 2.6 (uncomp.) |
| Javanicin (2-methoxymethylene-3-methyl-5,8-dihydroxy-7-methoxy-1,4-naphthoquinone) | 50 (uncomp.) | 70 (uncomp.)[b] |

[a]NADPH = 40μM.
[b]Determined previously (8,9).
[c]Nonlinear Dixon plots indicating incomplete inhibition (7) were observed. $K_i$ was estimated from the double-reciprocal plots of inhibition degree vs. inhibitor concentration (7). The maximal degree of inhibition was close to 85%.

(Table). The selectivity of inhibition of glutathione reductase by nitrofuran IIb and rhein, and their weak inhibition of trypanothione reductase, may be explained by the presence of the anionic carboxyl groups in these inhibitors, since the glutathione binding site of glutathione reductase is positively charged (11,12). Other quinones, including dihydroxy-substituted javanicin, exhibit modest specificity of inhibition.

The TN and bimolecular rate constants exhibited by chinifur and other nitrofuran substrates are very similar to the previously reported values of these kinetic constants for the one-electron reduction of ten quinones catalyzed by the *Trypanosoma congolense* trypanothione reductase (6). Chinifur (IIc) was a 4.5μM inhibitor of trypanothione reductase that only weakly inhibited glutathione reductase, suggesting that the structure of chinifur meets the criteria proposed for the efficient binding in the $T(S)_2$-binding domain of trypanothione reductase, i.e., the presence of basic substituents or positive charge, and a flexible side chain (11,12). Among described inhibitors of trypanothione reductase, only nonreducible analogs of $T(S)_2$ (16) and

cationic tricyclic antidepressants (17) act as competitive inhibitors vs. $T(S)_2$. Like the compounds described here, hydrazonoamine-substituted nitrofurans and quinones act as noncompetitive inhibitors vs. $T(S)_2$ (5). The noncompetitive nature of the inhibition by chinifur is maintained in the presence of 2 M $(NH_4)_2SO_4$, i.e., under conditions that resemble those used in the X-ray structural analysis. A possible explanation for the noncompetitive nature of the inhibition by chinifur is that the inhibitor binds both at the trypanothione binding site and to a site similar to the interface cavity of glutathione reductase (Phe-78,78', His-75,75') (18). The binding of quinoidal or aromatic compounds at the interface site of glutathione reductase (18) is probably responsible for the uncompetitive nature of the inhibition of the compounds versus glutathione (7,8). Similarily, the enhancement of the rate and extent of one-electron reduction of quinones by trypanothione reductase by increasing concentrations of the product, $NADP^+$, suggests that quinones bind to and are reduced at, a site which may be functionally equivalent to the interface site of glutathione reductase (6). The data presented here indicates that chinifur, previously known for its bacteriacidal activity (15), may be useful as a potential trypanocidal agent as well.

## ACKNOWLEDGMENTS

The research described in this publication was made possible in part by Grant N LA7000 from the International Science Foundation to Institute of Biochemistry (Vilnius) and NIH GM33449.

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