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NITROFURAN INHIBITION OF YEAST AND RAT TISSUE GLUTATHIONE REDUCTASES

STRUCTURE–ACTIVITY RELATIONSHIPS

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Abstract—Nitrofuran derivatives bearing unsaturated five- or six-membered nitrogen heterocycles or related substituents were more effective inhibitors of yeast and rat tissue glutathione reductases than those bearing other groups, such as nifurtimox, nitrofurazone and 5-nitro-2-furoic acid. The inhibitory action proved independent of electron withdrawal from the reduced enzyme, as a consequence of redox-cycling of the nitro group. Uncompetitive kinetics was obtained with nitrofurantoin and nifurtimox. Most of the assayed nitrofurans inhibited the yeast enzyme Coenzyme A glutathione disulfide reductase activity, though less than oxidized glutathione reduction. The transhydrogenase activity was not inhibited to a significant degree. Benznidazole (a 2-nitroimidazole derivative), 2-nitroimidazole, 5-nitroindole and chloramphenicol did not inhibit glutathione reductase. Under the same experimental conditions, liver glutathione peroxidase was not affected by the nitro compounds.

GR† (EC 1.6.4.2) is a ubiquitous FAD-containing enzyme that catalyses the reduction of GSSG at the expense of NADPH [1]. It occurs in two stable forms, E and EH_2. E is characterized by a disulfide between Cys-58 and Cys-63. The two-electron-reduced form, EH_2, contains Cys-58 and Cys-63 as a dithiol. The imidazole of His-467′ is near to all sulfurs taking part in the disulfide bridge exchange and is, therefore, certainly crucial for catalysis [2]. Associated to GP (EC 1.11.1.9), GR may be regarded as an indirect inhibitor of lipid peroxidation [3].

GR preparations from different sources are inhibited by nitro compounds, such as nitrofurantoin, nitrofurazone [4, 5], 5-nitro-2-furfuraldehyde oxime [5], trinitrobenzenesulfonate [6] and nifurtimox (a nitrofuran used for the treatment of Chagas' disease) [7, 8]. To determine the nature of the chemical groups involved in the action of the inhibitors, a number of nitro compounds were studied, including several recently synthesized trypanocides [9], which had not been tested as GR inhibitors.

Figure 1 illustrates the structures of the latter compounds. Complementary experiments were performed on GP.

MATERIALS AND METHODS

Materials. GR from Bakers yeast, Type IV, NADPH-oxidase free, 100–220 I.U./mg protein, was purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. Cytosol GR and GP preparations were obtained from Wistar rats as described in Refs. 7 and 8, the 105,000 *g* supernatant fraction being used without further treatment. Liver mitochondria were obtained by differential centrifugation (11,000–800 *g*) of the liver homogenate in 250 mM sucrose, 1.0 mM EDTA, 5.0 mM Tris–HCl, pH 7.4. The mitochondria were suspended in the same solution plus 1% (w/v) Triton X-100.

Unless stated otherwise, the nitrofurans were synthesized by Mester *et al.* [9] and supplied by Dr. R. Claramunt, Departamento de Química Orgánica, Facultad de Ciencias, U.N.E.D., 28040, Madrid, Spain. Nitrofurantoin, nitrofurazone, 5-nitro-2-furoic acid, 2-nitroimidazole, 5-nitroindole, chloramphenicol, GSH, GSSG, Triton X-100, *thio*-$NADP^+$, CoASSG and DMFA were purchased from the Sigma Chemical Co. Other reagents were as described in Refs. 7 and 8.

Assays. GR and GP activities were measured as in Ref. 8, at 30°. GR reaction mixture contained

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† Abbreviations and chemical names: GR, glutathione reductase; GP, glutathione peroxidase; GSSG and GSH, oxidized and reduced glutathione, respectively; CoASSG, Coenzyme A glutathione disulfide; NF, (5-nitro-2-furfurylidene)amino; nifurtimox, 3-methyl-4-[NF]-tetrahydro-4*H*-thiazine-1,1′-dioxide; NF-triazole, 4-[NF]-1,2,4-triazole; NF-pyrazole, 1-[NF]-pyrazole; NF-benzimidazole, 1-[NF]-benzimidazole; NF-indazole, 1-[NF]-indazole; NF-imidazole, 1-[NF]-imidazole; NF-indole, 1-[NF]-indole; NF-triazole(I), 3,5-bis(methylthio)-4-[NF]-1,2,4-triazole; NF-triazole(II), 1-methyl-3-methylthio-4-[NF]-1,2,4-triazole-5-thione; NF-triazine, 3-thioxo-4-[NF]-6-methyl-1,2,4-triazin-5-one; nitrofurantoin, 1[[(5-nitro-2-furanyl)methylene]-amino]-2,4-imidazolidinedione; nitrofurazone, 5-nitro-2-furaldehyde semicarbazone; benznidazole, *N*-benzyl-2-nitro-1-imidazole-acetamide; DMFA, dimethylformamide; *thio*-$NADP^+$, thionicotinamide adenine dinucleotide phosphate; $I_{0.5\max}$, half-maximal inhibitory concentration; and $I_{\max}$, enzyme inhibition (%) at infinite inhibitor concentration.

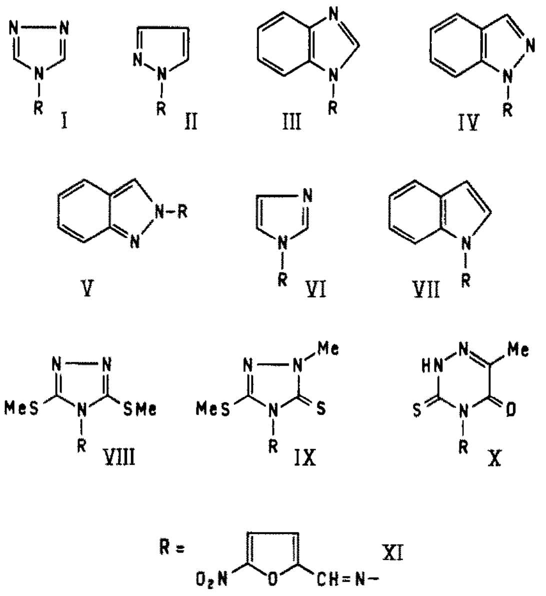

Fig. 1. Structures of the most potent inhibitors of GR. (I) 4*R*-1,2,4-triazole; (II) 1*R*-pyrazole; (III) 1*R*-benzimidazole; (IV) 1*R*-indazole; (V) 2*R*-indazole; (VI) 1*R*-imidazole; (VII) 1*R*-indole; (VIII) 3,5-bis(methylthio)-4*R*-1,2,4-triazole; (IX) 1-methyl-3-methylthio-4*R*-1,2,4-triazole-5-thione; (X) 3-thioxo-4*R*-6-methyl-1,2,4-triazin-5-one; (XI) (5-nitro-2-furfurylidene)amino (*R* or NF in the text). Each compound consists of moieties A (structures I–X) and B (structure XI = R).

50 mM KH_2PO_4–K_2HPO_4, pH 7.4, 1.0 mM GSSG, 0.1 mM NADPH and GR preparation. GP reaction mixture contained phosphate buffer (as above), 0.5 mM GSH, 0.1 mM NADPH, 0.3 to 0.6 I.U./ml yeast GR, 2.6 mM *tert*-butyl hydroperoxide and GP preparation; the final volume was always 3.0 ml. When assaying the effect of pH on GR inhibition, at pH 6 and below 20 mM acetate was included as a buffering substance, the pH values being measured with a glass electrode in the reaction mixture. NADPH oxidation was measured at 340 nm using a 550S Perkin Elmer spectrophotometer. GR transhydrogenase activity was measured by the initial rate of reduction of *thio*-$NADP^+$ with NADPH as the electron donor [10], at 30° and 395 nm, in 0.06 M sodium phosphate, pH 7.6, containing yeast GR (6.0 μg/ml), 25 μM NADPH, 100 μM *thio*-$NADP^+$ and 1.0 mM EDTA. Velocity was calculated as micromoles *thio*-NADPH formed per minute per milligram of enzyme protein, using a molar absorptivity of 1.13×10^4 M^{-1} cm^{-1}. CoASSG reductase activity was measured using a reaction mixture that contained 0.1 M KH_2PO_4, pH 5.5, 25 μM CoASSG, 0.1 mM NADPH, 1.0 mM EDTA and yeast GR (0.8 μg/ml) [1]. $O_2^{\overline{\cdot}}$ generation was measured using the adrenochrome method, as in Ref. 11. Other conditions were as described in Results.

The nitro compounds were added dissolved in DMFA, and controls received the same volume of solvent whose concentration was always below 1% (v/v); DMFA did not affect either GR or GP activity.

The values presented are the average of two or more independent measurements; the experimental values deviated from the mean by less than 5%. Calculations were performed with an Apple II Europlus PC.

RESULTS

Nitrofuran inhibition of yeast GR. Figure 2 shows typical concentration–effect curves. Significant inhibitions were observed in the micromolar concentration range, but in no case was total inhibition observed. The curves were hyperbolic, and double-reciprocal plots of GR inhibition against nitro compound concentration yielded straight lines (the inset in Fig. 2 illustrates typical results.) The latter plots allowed calculation of $I_{0.5\max}$ and $I_{\max}$ values, which are presented in Table 1. It is to be seen that the inhibitory activity was affected markedly by the nature of the substituent attached to the NF group. In this regard, inhibitors could be grouped as follows: (a) the triazine, bis(methylthio)triazole and benzimidazole derivatives, which were the most effective; (b) the pyrazole, triazole, imidazole and imidazolidine (nitrofurantoin) derivatives, with lower activity and (c) nitrofurazone, nifurtimox and 5-nitro-2-furoic acid, which were the least effective. Group (a) were at least 10-fold more active than group (c) members. In the absence of GSSG, none of the compounds assayed stimulated NADPH oxidation (data omitted).

GR kinetics involves loops corresponding to one "ping pong" and one sequential mechanism respectively [12]. Uncompetitive inhibition [13] was obtained with nitrofurantoin (Figs. 3 and 4) and nifurtimox (Figs. 5 and 6). The double-reciprocal plots in the presence of different constant amounts of nitrofurantoin (or nifurtimox) were straight lines parallel to those obtained in the absence of inhibitor.

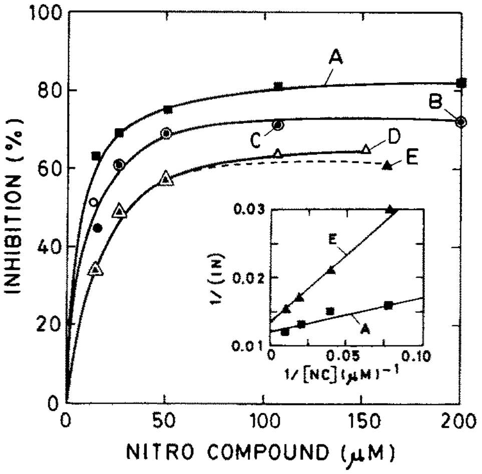

Fig. 2. Effect of increasing concentrations of NF-compounds on yeast GR. The reaction mixture contained GR (0.01–0.03 units/ml) and 5–15 μl of NF-compound solution (final concentration indicated on the abscissa); other conditions were as described in Materials and Methods. A (■), NF-triazine; B (○), NF-benzimidazole; C (●), NF-pyrazole; D (△), NF-imidazole; E (▲), NF-triazole. *Inset*: double-reciprocal plot of percentage inhibition values (IN) versus nitro compound (NC) concentration. Slopes and intercepts were calculated using a linear regression programme; in all cases, $r > 0.98$. A (■) and E (▲) as above.

Table 1. Effects of 5-nitrofuran derivatives on yeast GR

Nitro compound	$I_{0.5\max}$ (μM)
NF-triazine	4.3
NF-triazole(I)	4.4
NF-benzimidazole	7.3
NF-pyrazole	11
NF-triazole	14
NF-imidazole	16
Nitrofurantoin	19
Nitrofurazone	55
Nifurtimox	68
5-Nitro-2-furoic acid	72

Yeast GR, 0.01 to 0.04 I.U./ml; other experimental conditions were as described in Materials and Methods and in the legend of Fig. 2. $I_{\max}$(%): 5-nitro-2-furoic acid, 58; NF-triazole(I), 66; NF-triazole and nitrofurazone, 70; other compounds, 76–83.

Moreover, replots of [NADPH]/v or [GSSG]/v versus [inhibitor] yielded straight lines with slopes depending on [NADPH] or [GSSG]. These lines intersect above the x-axis, at a point corresponding to the K_i values for nitrofurantoin (20 μM) and nifurtimox (78 μM), which agree fairly well with the $I_{0.5\max}$ values of these compounds (Table 1).

Table 2 summarizes experiments on the inhibition of yeast GR with CoASSG as alternative substrate (CoASSG reductase activity) [1, 14]. With CoASSG, the assayed nitro compounds inhibited the enzyme activity less than with GSSG. In contrast to these inhibitions, GR transhydrogenase activity was scarcely inhibited, if at all (experimental data omitted). The results in Fig. 7 with nifurtimox and NF-imidazole indicate pH dependence for these compounds. Thus, the inhibition values at pH 5.5 were 30 and 52%, respectively, whereas at pH 7.7, the corresponding values were 47 and 61%. It remains to be established whether the pH effect was the sole cause of the different activities of the nitro compounds listed in Table 2 with CoASSG and GSSG.

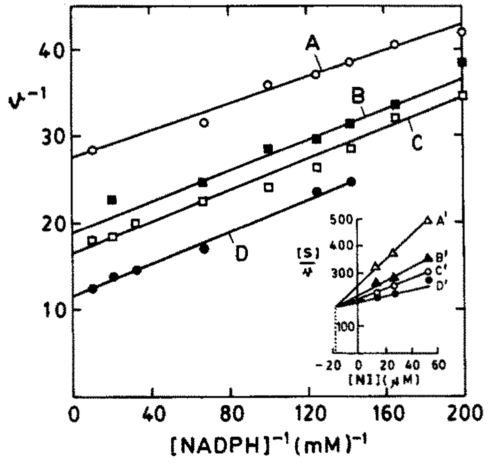

Fig. 3. Effect of different constant concentrations of nitrofurantoin (NI) on yeast GR activity (v) at constant GSSG and increasing NADPH concentrations. Double-reciprocal plot. GR, 75 ng protein/ml (220 U/mg protein); 1.0 mM GSSG: NADPH (mM) as indicated on the abscissa; nitrofurantoin (μM): A, 53; B, 26; C, 13; D, 0 (control). Other experimental conditions were as described in Materials and Methods. $v = \Delta A_{340}$/min. *Inset*: plot of $[S]/v$ against [nitrofurantoin (NI)] (μM); S = [NADPH] (μM): A′ (△), 15; B′ (▲), 10; C′ (○), 8.0; D′ (●), 7.0. Slopes and intercepts were calculated as in the legend of Fig. 2; in all cases $r > 0.98$. The GR preparation K_m values were 5.1 μM (NADPH) and 53 μM (GSSG).

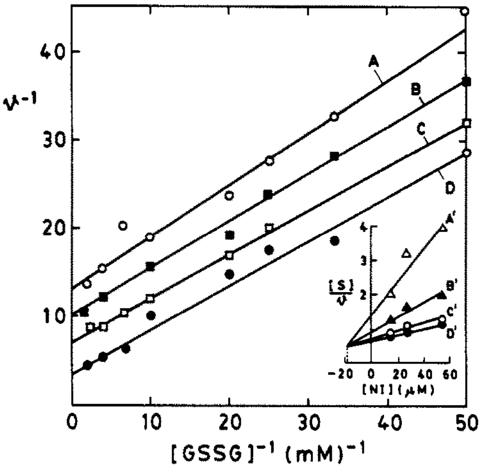

Fig. 4. Effect of different constant concentrations of nitrofurantoin (NI) on yeast GR activity (v) at constant NADPH and increasing GSSG concentrations. GR, 150 ng protein/ml; 0.1 mM NADPH; GSSG (mM) as indicated on the abscissa. Other experimental conditions were as described in Materials and Methods and in the legend of Fig. 3. *Inset*: plot of $[S]/v$ against [nitrofurantoin (NI)] (μM); S = [GSSG] (mM): A′ (△), 0.25; B′ (▲), 0.10; C′ (○), 0.05; D′ (●), 0.04.

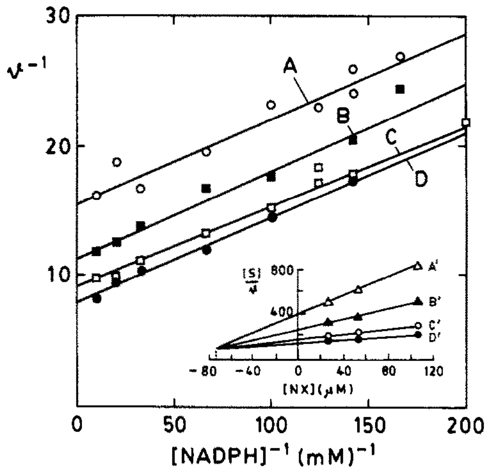

Fig. 5. Effect of different constant concentrations of nifurtimox (NX) on yeast GR activity (v) at constant GSSG and increasing NADPH concentrations. GR, 90 ng protein/ml. Nifurtimox (μM): A, 106; B, 53; C, 26; D, 0. Other experimental conditions were as described in Materials and Methods and in the legend of Fig. 3. *Inset*: plot of $[S]/v$ against [nifurtimox] (μM); S = [NADPH] (μM): A′ (△), 50; B′ (▲), 30; C′ (○), 15; D′ (●), 10.

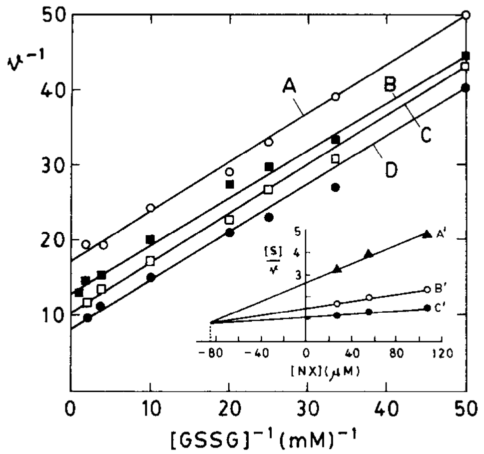

Fig. 6. Effect of different constant concentrations of nifurtimox (NX) on yeast GR activity (v) at constant NADPH and increasing GSSG concentrations. GR, 90 ng protein/ml. Experimental conditions were as described in Materials and Methods and in the legend of Fig. 4. *Inset*: plot of $[S]/v$ against [nifurtimox] (μM); S = [GSSG] (mM): A′ (▲), 0.25; B′ (○), 0.10; C′ (●), 0.05.

Nitrofuran inhibition of rat tissue GR. Figure 8 and Table 3 show the results obtained with liver GRs. Allowance being made for some minor differences which may not be significant, the inhibition pattern was the same for both cytosol and mitochondrial preparations, that is to say, strong inhibition by compounds bearing unsaturated nitrogen heterocycles, against weaker effects of the other compounds. NF-indazole, NF-triazole(II), NF-indole and NF-benzimidazole wee assayed on the cytosol GR and proved to be most effective inhibitors. Heart and kidney GRs were also inhibited by nitro compounds (Fig. 8 and Table 3). The corresponding $I_{0.5\max}$ values were in close agreement with those obtained with the liver enzyme.

Liver, heart and kidney preparations failed to oxidize NADPH in the absence of GSSG and the presence of most of the nitrofurans. However, with the liver extract and NF-triazole, NF-triazole(I) and nifurtimox (all within the 50–200 μM concentration range), NADPH-oxidase activity was observed (data

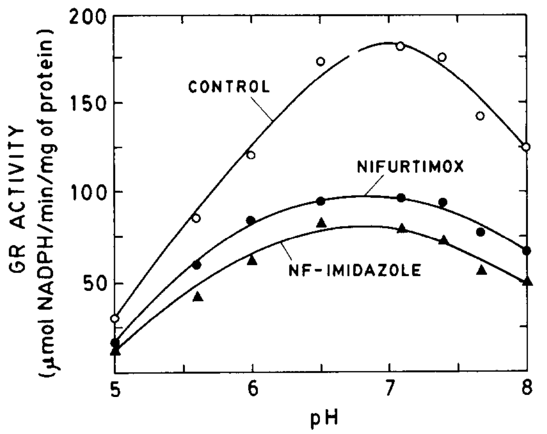

Fig. 7. Effect of pH on yeast GR inhibition by nifurtimox and NF-imidazole. The experimental conditions were as described in Materials and Methods. K: (○) control sample; (●) 53 μM nifurtimox; and (▲) 26 μM NF-imidazole.

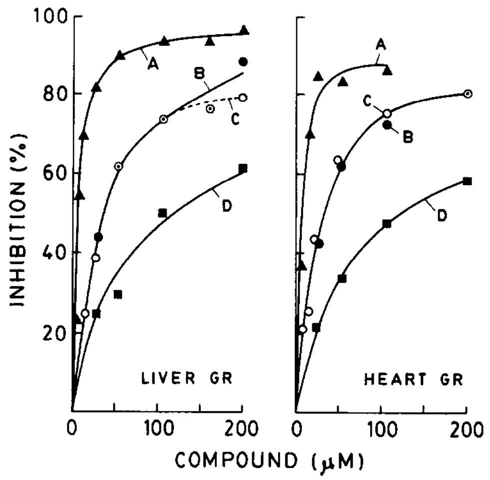

Fig. 8. Inhibition of liver and heart GRs by nitrofurantoin (A), nifurtimox (B), nitrofurazone (C) and 5-nitro-2-furoic acid (D). Liver or heart GR fractions, 0.2 to 0.3 or 0.3 to 0.6 mg protein/ml respectively; other experimental conditions were as described in Materials and Methods and in the legend of Fig. 2.

Table 2. Effects of nitrofuran compounds on CoASSG reductase activity of yeast GR

Nitro compound (106 μM)	Inhibition (%) of CoASSG reduction rate	Inhibition (%) of GSSG reduction rate
NF-imidazole	58	64
NF-triazole(I)	38	66
NF-benzimidazole	36	72
NF-triazole	27	61
Nitrofurantoin	19	60
Nifurtimox	19	48
Nitrofurazone	14	43
NF-triazine	—	82
NF-pyrazole	—	82

Yeast GR (125 I.U./mg protein) 0.8 μg/ml (CoASSG Expt.); other experimental conditions were as described in Materials and Methods and the legend of Table 1. CoASSG reduction rate by the control sample, 11.3 μmol/min/mg protein.

Table 3. Effects of 5-nitrofuran derivatives on liver, heart and kidney GRs

	$I_{0.5\max}$ (μM)			
	Liver GR			
Nitro compound	Cytosolic	Mitochondrial	Heart cytosolic GR	Kidney cytosolic GR
NF-indazole	0.9	—	—	—
NF-triazole(II)	1.3	—	0.5	—
NF-indole	1.5	—	0.7	—
NF-benzimidazole	1.5	—	1.4	0.6
NF-triazole(I)	1.9	3.7	0.9	0.6
NF-pyrazole	2.1	4.1	1.7	1.6
NF-triazine	2.4	2.3	2.8	1.6
NF-imidazole	3.4	3.2	2.5	2.3
Nitrofurantoin	4.1	5.6	5.2	4.9
NF-triazole	8.4		4.6	2.4
Nifurtimox	31	47	32	16
Nitrofurazone	39	25	20	25
5-Nitro-2-furoic acid	65	75	75	78

Liver GR preparation (cytosolic or mitochondrial), 0.3 to 0.8 mg protein/ml; GR specific activity (nmol NADPH/min/mg protein): 66 (cytosolic) and 24 (mitochondrial). Heart GR fraction, 0.3 to 0.6 mg protein/ml; specific activity 12.5 (as above). Kidney GR fraction, 0.12 mg protein/ml; specific activity, 136 (as above). Other experimental conditions were as described in Materials and Methods. $I_{\max}$ (%) for liver cytosolic GR: 5-nitro-2-furoic acid, 78; other compounds, 90–100. Same, for mitochondrial GR: 5-nitro-2-furoic acid, 83; nitrofurazone, 86; NF-benzimidazole, 74; NF-triazine, 88; other compounds, 90–100. Same, for heart cytosolic GR: NF-triazole(II), 88; nitrofurazone, 78; 5-nitro-2-furoic acid, 80; other compounds, 90–100. Same, for kidney cytosolic GR: NF-benzimidazole, 89; NF-triazole, 88; nifurtimox, 82; other compounds, 90–100.

not shown). These activities, which at the highest nitrofuran concentration remained below 10% of the rate of NADPH-oxidation by GSSG, recall similar effects of trinitrobenzenesulfonate [6]. The nitrofuran-induced NADPH-oxidase activity values were accounted for, when calculating GR inhibition. Investigation of O_2^{-} generation was negative, irrespective of the nitro compound assayed (experimental data omitted).

The nitro compounds listed in Table 4 inhibited liver cytosolic GSH-peroxidase less than 6% (experimental data omitted). However, in the absence of GR supplement (yeast GR), the inhibition values followed closely those of liver GR (Table 4), since this latter inhibition prevented GSSG re-reduction (GSH regeneration). Heart and kidney cytosolic GP and liver mitochondrial GP were not inhibited by the nitro compounds, assayed in experimental conditions as described in Table 4 (experimental data omitted).

Table 4. Effects of nitro compounds on liver GSH-peroxidase system (GR–GP)

Nitro compound (μM)	Inhibition (%) of enzyme activity	
	GR	GR–GP
NF-benzimidazole (106)	86	100
NF-triazole(I) (106)	82	100
NF-triazine (106)	91	93
NF-pirazole (106)	92	91
NF-imidazole (106)	89	85
Nitrofurantoin (106)	91	83
NF-triazole (106)	82	70
Nifurtimox (106)	72	79
Nitrofurazone (106)	72	66
5-Nitro-2-furoic acid (106)	49	45
2-Nitroimidazole (200)	0	0
Benznidazole (200)	8.1	2.9
Chloramphenicol (200)	4.5	0
5-Nitroindole (200)	4.5	0

GR and GR–GP, 0.30 or 0.05–0.10 mg liver protein/ml respectively; enzyme activity (nmol NADPH/min/mg protein): 66.2 (GR) and 15.4 (GR–GP); other conditions were as described in Materials and Methods. For the GR–GP system assay the reaction mixture contained 50 mM phosphate buffer, pH 7.4, 0.5 mM GSH, 0.1 mM NADPH, 2.6 mM *tert*-butyl hydroperoxide and liver protein as above.

2-Nitroimidazole, benznidazole, chloramphenicol (a *p*-nitrophenyl derivative) and 5-nitroindole failed to inhibit yeast and rat GRs, at least to a significant degree. Negative results were also obtained as regards GP (experimental data omitted).

DISCUSSION

Among the nitro compounds assayed, only the 5-nitrofuran derivatives were effective inhibitors of GR, thus indicating the relevance of that structure for the inhibitory action. In a series of related chemicals, each particular substituent adds a constant contribution to the activity of the parent molecule [15] and, accordingly, the reported data reflect the influence of the NF counterpart on the activity of the whole molecule. In this connection, it should be noted that linkage of the NF moiety to an unsaturated heterocycle (substituted or not) produced more active inhibitors than linkage to the 4*H*-thiazine (in nifurtimox), hydrazine carboxamide (in nitrofurazone) or carboxyl groups (5-nitro-2-furoic acid). Many descriptors [15] can be chosen to express the influence of a chemical structure for structure–activity relationships, but adequate knowledge of such descriptors is not available for the most active GR nitrofuran inhibitors. Some preliminary information may be gleaned, however, from the fact that the large substituents, such as those in NF-triazoles(I) and (II), NF-indazole and NF-benzimidazole, proved to be more effective than the triazolyl or imidazolyl groups. The results in Figs. 3–6, as well as the relative nitrofuran inactivity on the transhydrogenase reaction (Table 2), fail to support inhibitor competition for substrate binding sites. Moreover, no evidence could be obtained for GR-catalyzed redox-cycling of the nitro group, as expected in the case of nitrofuran interaction with the enzyme flavin site [16]. These negative results rule out diversion of the NADPH-generated electrons towards the nitro compound [17], as the main cause of GR inhibition. In GR active site, the imidazole of His-467′ is crucial for catalysis [2, 18] and, accordingly, unsaturated nitrogen heterocycles could interfere with that imidazole function.

Our observations support the hypothesis [8] that through GR inhibition (followed by GSH-depletion), nitrofurans potentiate the cytotoxic effects of the products of their own redox-cycling. The assayed compounds may well prove candidates for the treatment of both experimental and clinical Chagas' disease [9], unless they are shown to affect mammalian enzymes to a significant degree. In this context, GR inhibition is relevant since the GR–GP system prevents oxidative stress in tissues [7, 8]. The results reported here with liver, heart and kidney GR preparations confirm that potential risk and introduce a note of caution as regards the clinical use of the new nitrofurans, since they inhibit GR more than nifurtimox.

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