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INHIBITION OF MICROSOMAL LIPID PEROXIDATION AND CYTOCHROME P-450-CATALYZED REACTIONS BY NITROFURAN COMPOUNDS

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(5-Nitro-2-furfurylidene)amino compounds bearing triazol-4-yl, benzimidazol-1-yl, pyrazol-1-yl, triazin-4-yl or related groups (a) stimulated superoxide anion radical generated by rat liver microsomes in the presence of NADPH and oxygen; (b) inhibited the NADPH-dependent, iron-catalyzed microsomal lipid peroxidation; (c) prevented the NADPH-dependent destruction of cytochrome P-450; (d) inhibited the NADPH-dependent microsomal aniline 4-hydroxylase activity; (e) failed to inhibit either the cumenyl hydroperoxide-dependent lipid peroxidation or the aniline-4-hydroxylase activity, except for the benzimidazol-1-yl and the substituted triazol-4-yl derivatives, which produced minor inhibitions. Reducing equivalents enhanced the benzimidazol-1-yl derivative inhibition of the cumenyl hydroperoxide-induced lipid peroxidation. The ESR spectrum of the benzimidazol-1-yl derivative, reduced anaerobically by NADPH-supplemented microsomes, showed characteristic spin couplings. Compounds bearing unsaturated nitrogen heterocycles were always more active than those bearing other groups, such as nifurtimox or nitrofurazone. The energy level of the lowest unoccupied molecular orbital was in fair agreement with the capability of nitrofurans for redox-cycling and related actions. It is concluded that nitrofuran inhibition of microsomal lipid peroxidation and cytochrome P-450-catalyzed reactions was mostly due to diversion of reducing equivalents from NADPH to dioxygen. Trapping of free radicals involved in propagating lipid peroxidation might contribute to the overall effect of the benzimidazol-1-yl and substituted triazol-4-yl derivitives.

KEY WORDS: nitrofurans, nifurtimox, lipid peroxidation, aniline-4-hydroxylase, superoxide, LUMO energy.

ABBREVIATIONS: 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-triazole(I), 3,5-bis(methylthio)-4-[NF]-1,2,4-triazole; NF-triazine, 3-thioxo-4-[NF]-1,2,4-triazin-5-one; nitrofurazone, 5-nitro-2-furaldehyde semicarbazone; DMFA, N,N-dimethylformamide; *t*-BuOOH, *tert*-butyl hydroperoxide; CuOOH, cumenyl hydroperoxide; SOD, superoxide dismutase; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; MDA, malondialdehyde; LUMO, lowest unoccupied molecular orbital.

INTRODUCTION

Nitrofuran compounds are frequently used in human medicine as antimicrobial and antiparasitic agents. Among these compounds stands nifurtimox, one of the most

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successful drugs used for the treatment of acute forms of American trypanosomiasis (Chagas' disease).[1,2] Nifurtimox redox-cycling in the presence of oxygen and NADPH-supplemented microsomes, either from *Trypanosoma cruzi* (the agent of Chagas' disease) or the mammalian host, generates reactive oxygen species. These species may explain nifurtimox trypanocidal action on *T. cruzi*, its toxicity for the human host and its mutagenicity in bacteria.[3–14] Furthermore, electron diversion to dioxygen at cytochrome P-450 reductase level contributes to other effects of nifurtimox, such as the inhibition of microsomal lipid peroxidation.[12]

Mester *et al.*[15] synthesized a series of new nifurtimox analogues in which the tetrahydro-4*H*-thiazine-1,1′-dioxide group was replaced by unsaturated five- or six-membered nitrogen heterocycles. Most of these compounds were superior to nifurtimox, as regards (a) *in vitro* activity on *T. cruzi*[15] and *Salmonella typhimurium*;[16] (b) inhibition of glutathione reductase;[17] and (c) production of superoxide anion radical by NADH-supplemented lipoamide dehydrogenase.[18] The ESR spectra of the corresponding radical anions showed hyperfine spin couplings restricted to the nitrofuran moiety, the spin density at the nitro group nitrogen being greater than with nifurtimox, nitrofurazone and nitrofurantoin radicals.[19] Taking into account the foregoing, in the present study we have examined the new nitrofuran derivatives as (a) inducers of superoxide production; (b) inhibitors of lipid peroxidation in rat liver microsomes and (c) inhibitors of the cytochrome P-450-catalyzed aniline 4-hydroxylase reaction. Our results demonstrate the superior activity of the new nitrofuran compounds and confirm the postulated mechanism of nitrofuran inhibition of lipid peroxidation in rat liver microsomes.[12]

MATERIALS AND METHODS

Microsomal preparations

Microsomes were obtained from the livers of 20-h fasted, male Wistar rats, 240–280 g, fed a Purine-like rat chow. Some rats were pre-treated with phenobarbital (40 mg/Kg i.p. in 0.5 ml of 0.15 M NaCl) for 4 days and were used 20 h after the last dose of the inducer. The homogenate and the microsomal fraction were prepared as described.[12] Microsomes were washed twice with 150 mM KCl by centrifugation for 1 h at 105,000 *g*. The pellet was resuspended in 150 mM KCl and either used immediately or stored in liquid nitrogen. Investigation of SOD in the microsomal suspension was negative whereas measurements of catalase activity yielded 9.0 m units/mg protein.

Determination de-superoxide anion radical

Production of $O_2^{\cdot-}$ was determined by the adrenochrome[20] and the cytochrome *c*[21] assays. With the former method, the reaction mixture contained microsomes (0.27 mg protein/ml), 1.0 mM epinephrine, 130 mM KCl, 23 mM KH_2PO_4–Na_2HPO_4 buffer, pH 7.4 and NADPH-generating system (0.55 mM $NADP^+$, 5.5 mM 6GP, 1.2 units/ml G6PD, 5.5 mM $MgCl_2$); total volume was 3.0 ml. Control samples were supplemented with SOD (6.0 units/ml), as indicated in Table I legend. The reaction was started with G6P and the initial rate was measured by the increase of absorption at 480 − 575 nm ($\varepsilon = 2.96\,mM^{-1}cm^{-1}$). With the cytochrome *c* method, the reaction mixture contained microsomes (0.13 mg/ml protein), 130 mM KCl, 23 mM..KH_2PO_4–Na_2HPO_4,

pH 7.4, NADPH-generating system (as above) and acetylated cytochrome *c* as indicated in the Results section; total volume was 3.0 ml The initial reaction rate was measured by the increase of absorption at 550 − 540 nm (ε = 19 $mM^{-1}cm^{-1}$). Absorption measurements were performed with the Aminco DW 2a™ UV/VIS spectrophotometer at 30°.

Assay of lipid peroxidation

For the assay of NADPH-dependent lipid peroxidation, the incubation mixture contained liver microsomes (1.5 mg protein/ml), the NADPH-generating system (as above), 1.7 mM ADP, 0.1 mM $FeCl_3$, 130 mM KCl and 23 mM KH_2PO_4–Na_2HPO_4, pH 7.4. The $FeCl_3$–ADP mixture was prepared separately before beginning the experiments. After thermal equilibration at 37°, the reaction was started by addition of G6PD. Aliquots (1.0 ml) were withdrawn at the times indicated, chilled to 0°, and MDA formation was measured. For the CuOOH-Fe-induced peroxidation, the incubation mixture consisted of 0.11 mM EDTA, 0.1 mM $FeSO_4$, 0.175 mM CuOOH, microsomes, KCl and phosphate buffer as described for the assay of the NADPH-dependent lipid peroxidation. The reaction was started by adding CuOOH. The nitro compounds were added dissolved in DMFA. Controls received the same volume of solvent whose concentration never exceeded 1% (v/v); DMFA failed to affect the rate of lipid peroxidation. The final volume of the incubation mixture was always 3.0 ml. Incubations were performed in a New Brunswick Gyratory shaker, at 60 cycles/min and 37°, under air. Other experimental conditions were as described in the Results section.

Enzyme assays

Aniline 4-hydroxylase activity was measured by determining 4-aminophenol production, which was estimated colorimetrically by the indophenol reaction.[22] For the assay using NADPH as electron donor, the reaction mixture contained microsomes (1.7 mg protein/ml), 1.25 mM aniline, 6.25 mM $MgCl_2$, 2.5 mM 6GP, G6PD (0.5 units/ml), 0.125 mM $NADP^+$, 0.1 M KH_2PO_4–Na_2HPO_4, pH 7.4, preflushed with oxygen; incubation was for 20 min at 37°. For the assay using CuOOH, the reaction mixture contained microsomes (2.0 mg protein/ml), 3.0 mM aniline, 0.25 mM CuOOH and 80 mM Tris–HCl buffer, pH 7.6. Incubation was for 30 min at 37°. NADPH-cytochrome P-450 reductase activity was measured with NADPH as substrate and cytochrome *c* as artificial electron acceptor.[23] The reductase-catalyzed transfer of electrons to cytochrome *c* was measured at 550 − 540 nm. NADH-cytochrome b_5 reductase was estimated by the rate of reduction of potassium ferricyanide,[24] followed at 420 nm. Measurements were performed at 30° using the Aminco DW 2a™ UV/VIS spectrophotometer. Other experimental conditions are described in the Results section. SOD[25] and catalase[26] were measured as described in the corresponding References.

Analytical methods

MDA and protein content of microsomal membranes were determined as described, respectively.[27,28] Nitrofurans did not interfere with the MDA assays. Cytochrome P-450 was estimated as described by Omura and Sato.[29] Cytochrome b_5 was estimated

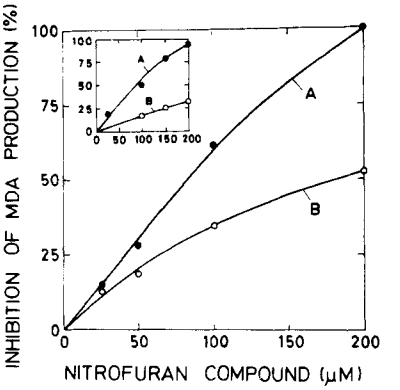

FIGURE 1 Effect of NF-benzimidazole (A) and NF-triazole (B) concentration on the rate of lipid peroxidation by microsomes incubated with the NADPH-generating system. Time of incubation, 1 h; other conditions were as described in the Materials and Methods section. *Inset*: results with phenobarbital-induced microsomes. Microsomal cytochrome P-450 content (nmol/mg protein): 1.78 (phenobarbital-induced microsomes) and 1.11 (control microsomes).

triazol-4-yl derivatives proved to be the most effective; (b) with CuOOH-iron, significant inhibitions were obtained only with NF-triazole(I) and NF-benzimidazole but this inhibition was lesser than those observed with the NADPH-iron system. While the results obtained with NADPH-iron indicate inhibition of lipid peroxidation initiation, those with CuOOH-iron point to inhibition of propagation. However, the latter effect seemed to be a minor one, considering the relatively high nitrofuran concentration used in this experiment.

NF-benzimidazole also inhibited the *t*-BuOOH-initiated lipid peroxidation (Figure 2). Interestingly enough, the addition of NADPH to the *t*-BuOOH system enhanced the effect of NF-benzimidazole since the inhibition of MDA formation increased from 20–21% in the absence of NADPH to 50–52% in its presence (incubation time, 30 and 45 min, respectively; Figure 2). These results indicate that the antioxidant effect of NF-benzimidazole depended on its being a substrate of the microsomal NADPH-cytochrome P-450 reductase and consequently, an antioxidant

TABLE III
Effect of nitrofuran compounds on lipid peroxidation by liver microsomes incubated with the NADPH–Fe or the CuOOH–Fe systems

Nitrofuran compound	MDA equivalents (nmol/mg protein)	
	NADPH–Fe	CuOOH–Fe
None (control)	36 ± 1.3	8.3 ± 0.4
Nitrofurazone	21 ± 0.7 (42)	8.5 ± 1.9 (NS)
NF-triazole (I)	10 ± 2.9 (71)	6.2 ± 0.1 (25)
NF-triazole	10 ± 1.8 (72)	7.9 ± 0.4 (NS)
NF-pyrazole	10 ± 2.1 (72)	7.3 ± 0.1 (NS)
NF-benzimidazole	4.1 ± 0.3 (89)	6.8 ± 0.1 (18)

Microsomes were incubated for 60 min with the reaction mixtures which composition was described in the Materials and Methods section; 200 μM nitrofuran compound. The figures represent the mean ± S.E.M. of the experimental values ($n = 6$). In parenthesis, percentage inhibition of MDA production. In all cases $P < 0.05$, except where indicated NS (not significant).

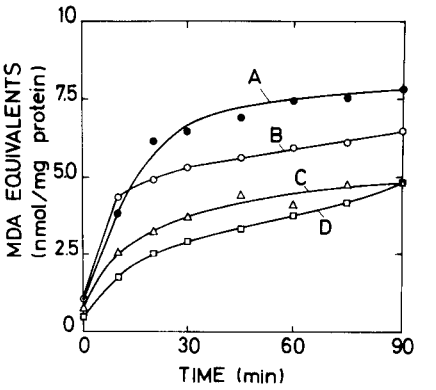

FIGURE 2 Effect of NF-benzimidazole and NADPH on *t*-BuOOH-induced microsomal lipid peroxidation. The reaction mixture consisted of microsomes (1.4 mg protein/ml), 130 mM KCl, 23 mM KH_2PO_4–Na_2HPO_4, pH 7.4, and the following additions: sample A, 2.6 mM *t*-BuOOH and 0.5 mM NADPH; B, 2.6 mM *t*-BuOOH; C, 2.6 mM *t*-BuOOH and 100 μM NF-benzimidazole; D, 2.6 mM *t*-BuOOH, 0.5 mM NADPH and 100 μM NF-benzimidazole. Other conditions were as described in the Materials and Methods section.

effect of the nitroanion radical might contribute towards the inhibition of lipid peroxidation.

Loss of microsomal cytochrome P-450 is associated with NADPH-induced lipid peroxidation[38,39] and, accordingly, nitrofuran inhibition of the latter prevented cytochrome P-450 destruction. Figure 3 shows the inhibitory effects of nifurtimox and NF-benzimidazole on the time-course of cytochrome P-450 destruction and Table IV summarizes the effect of NF-benzimidazole on the variation of cytochrome P-450, cytochrome b_5 and the corresponding reductases after one hour incubation of microsomes with the NADPH-generating system. It is to be seen that (a) lipid peroxidation produced 95% destruction of cytochrome P-450 (experiments A and B); (b) NF-benzimidazole prevented cytochrome P-450 loss to a significant degree (91%; exper-

TABLE IV

Cytochrome P-450, cytochrome b_5, NADPH-cytochrome P-450 reductase and NADH-cytochrome b_5 reductase in microscomes preincubated with nitrofuran compounds and NADPH-generating system.

Sample	NF-benzimidazole	Cytochrome P-450	Cytochrome b_5	P-450 reductase	b_5 reductase
A	None (control)	1.1 ± 0.1[a]	0.62 ± 0.02[a]	163 ± 2[b]	3.1 ± 0.1[c]
B	None (control)	0.1 ± 0.1	0.60 ± 0.01	150 ± 14	3.3 ± 0.4
C	50 μM	0.9 ± 0.2[d]	0.70 ± 0.02	160 ± 6	3.0 ± 0.1

Samples contained microsomes (1.5 mg protein/ml), 23 mM KH_2PO_4 – Na_2HPO_4, pH 7.4, 130 mM KCl and the following additions: A, DMFA; B, DMFA and NADPH-generating systems; C, nitrofuran compound and NADPH-generating system; total volume 6.0 ml. After 60 min incubation at 37°, samples were centrifuged at 105,000 *g* for 30 min, the microsomes were suspended in 0.1 M phosphate buffer, pH 7.4, and cytochrome content and enzyme activities were measured. Other experimental conditions were as described in the Materials and Methods section. Values represent the mean ± S.E.M. of at least 3 measurements.

[a] In nmol/mg protein; [b] in nmol/min/mg protein; [c] in μmole/min/mg protein. [d] Sample B *versus* sample C; $P < 0.05$.

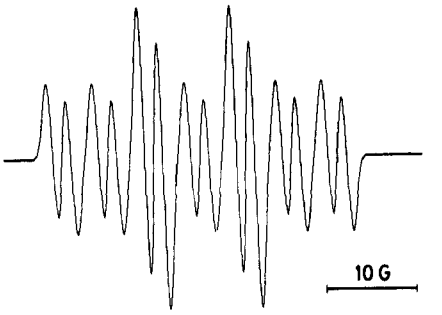

FIGURE 4 Computer simulated ESR spectrum of NF-benzimidazole nitroanion radical. The reaction mixture consisted of 2.0 mM NF-benzimidazole, microsomes (6.0 mg protein/ml) and NADPH generating system in 50 mM Tris–HCl, pH 7.4. Anaerobiosis was obtained as described.[19] Instrumental conditions: 55 mW microwave power; 100 kHz microwave modulation frequency; 0.80 Gpp modulation intensity; 0.20 G modulation amplitude; 1 s time constant; 6 G/min scan rate and 1×10^6 gain. Other conditions were as described in Reference 19 and in the Materials section. Hyperfine spin coupling constant for nitroanion radicals (G): 1 N(NO_2), 10.30; 1 H(4), 2.06; 1 H(3), 0.60; 1 N(CH=N), 5.16.

DISCUSSION

In a previous study, Dubin *et al.*[12] were able to observe that nifurtimox and nitrofurazone inhibited NADPH-iron-induced lipid peroxidation in rat liver microsomes. The nitrofurans strongly stimulated the microsomes NADPH-oxidase activity, thus producing electron diversion which was considered to be the main cause of the inhibition of lipid peroxidation. The results reported here show that nitrofuran compounds bearing unsaturated nitrogen heterocycles were more effective than nifurtimox or nitrofurazone, both as superoxide generators (Table I) and as inhibitors of lipid peroxidation (Table III). Production of the nitro anion radical by these compounds, as illustrated by results with NF-benzimidazole in Figure 4, fits in well with the generally accepted mechanism of oxyradical production by nitrofuran redox-cycling.[1,19] Neither the fatty acid chains of the microsomal lipids nor membrane-bound proteins, such as cytochrome P-450, cytochrome b_5 and the corresponding dehydrogenases, were affected, however, by the products of nitrofuran redox-cycling (Table IV). These negative results support electron diversion to oxygen as a futile pathway for reducing equivalents, otherwise utilized for initiating lipid peroxidation or cytochrome P-450-dependent reactions. In this context, it should be noted that NADPH-cytochrome P-450 reductase is anchored in the endoplasmic reticulum membrane solely by a short hydrophobic segment, the conformation of the enzyme catalytic domain being independent of the hydrophobic membrane-bound domain.[43] The membrane-bound enzyme can interact with molecules (naphthoquinones and nitrofurans) in the aqueous phase and accordingly, production of toxic radicals by the redox-cycling of these molecules would occur far removed from targets such as lipid chains and membrane-bound enzymes. Furthermore, $OH^{\cdot}$ radicals resulting from Fenton chemistry, because of their high reactivity, would not survive for more than a few collisions after their formation.

In a series of related chemical structures, each particular substituent adds a constant contribution to the activity of the parent molecule. The results here described demonstrate the influence of the NF-counterpart on the assayed nitrofurans activity.

Thus, linkage of the NF-moiety to an unsaturated heterocycle produced more active inhibitors of lipid peroxidation (Table III) or the cytochrome P-450-catalyzed reaction (Table V) than linkage to the tetra-4*H*-thiazine-1,1′-dioxide or the hydrazine carboxamide groups (in nifurtimox and nitrofurazone, respectively). Interesting enough, activity correlations such as the one represented by the series NF-triazole(I) > NF-benzimidazole > NF-pyrazole > nifurtimox ⩾ nitrofurazone (Tables I and III) were observed as regards (a) LUMO energy levels for the neutral molecule (Table VI); (b) nitrofuran capability for catalyzing ascorbate oxidation (Table VI); (c) nitrofuran interactions with glutathione reductase[17] and lipoamide dehydrogenase.[18] Therefore, secondary effects resulting from the specific properties of the non-nitrofuran moiety modulated each compound activity. In this connection, the hydrophobicity[41] and the affinity of the substituent group for cytochrome P-450 may be significant.

The relatively high capability of the new nitrofuran derivatives for redox-cycling and oxyradical production (Table I), taken together with their inhibitory action on cytochrome P-450 catalyzed reactions (Table V) and glutathione reductase[17] leads one to assume a greater toxicity of these compounds for the mammalian host that would make them unsuitable as chemotherapeutic agents for Chagas' disease.

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