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Synthesis and in Vitro Evaluation of Potential Antichagasic Hydroxymethylnitrofurazone (NFOH-121): A New Nitrofurazone Prodrug

Man-Chin Chung,[a] Rafael Victório Carvalho Güido,[a] Tatiane Favarato Martinelli,[b] Marinei Ferreira Gonçalves,[c] Michelle Carneiro Polli,[b] Katia Cirlene Alves Botelho,[b] Eliana Aparecida Varanda,[d] Walter Colli,[c] M. Terêsa M. Miranda[c] and Elizabeth Igne Ferreira[b,*]

[a]*Lapdesf- Laboratório de Pesquisa e Desenvolvimento de Fármacos, Departamento de Fármacos e Medicamentos, Faculdade de Ciências Farmacêuticas, UNESP, Araraquara, Caixa Postal 502, CEP 14.801-902, SP, Brazil*
[b]*Departamento de Farmácia, Faculdade de Ciências Farmacêuticas, USP, Caixa Postal 66083, CEP 05389-970, SP, Brazil*
[c]*Departamento de Bioquímica, Instituto de Química, USP, Caixa Postal 26077, CEP 05513-970, SP, Brazil*
[d]*Departamento de Ciências Biológicas, Faculdade de Ciências Farmacêuticas, UNESP, Araraquara, Caixa Postal 502, CEP 14.801-902, SP, Brazil*

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Abstract—The synthesis of mutual prodrugs of nitrofurazone with primaquine, using specific and nonspecific spacer groups, has been previously attempted seeking selective antichagasic agents. The intermediate reaction product, hydroxymethylnitrofurazone (NFOH-121), was isolated and tested in LLC-MK_2 culture cells infected with trypomastigotes forms of *Trypanosoma cruzi* showing higher trypanocidal activity than nitrofurazone and benznidazol in all stages. The mutagenicity tests showed that the prodrug was less toxic than the parent drug. Degradation assays were carried out in pH 1.2 and 7.4.

Introduction

Chagas' disease is an important social and medical ailment for people living in the Americas.[1–5] It is endemic in 21 countries, with approximately 16–18 million individuals infected with *Trypanosoma cruzi* and about 100 million people at risk of contracting the parasitosis, including those living in northern hemisphere regions that received intense migratory currents from Ibero-American countries. Current therapy is based on nifurtimox and benznidazol, which are mostly effective in the acute phase of the disease and may cause serious adverse side effects.[5] In Brazil, the only drug available in the market is benznidazol (Benz) in spite of the claim that Brazilian *T. cruzi* strains are more resistant to this drug than those from other countries in South America.[6] Thus, new and better drugs are urgently needed to face that serious situation. The driving force for antichagasic development has been selectivity of action, through the knowledge of biochemical targets in the parasite. Some new compounds showed to be promising.[7]

Despite their toxicity, nitroaromatic compounds[7–11] are, generally, very active against *T. cruzi* and have been considered as important leads for molecular modification.

Nitrofurazone (NF), a 5-nitro-2-furfurilidenesemicarbazone, is primarily an antimicrobial agent active against Gram-positive microorganisms and used only in topical infections due to its side effects.[5] Nevertheless, Gonçalves and co-workers[12] found trypanocidal activity in that drug. Its effect could be attributed to a trypanothione reductase inhibition.

Trypanothione reductase of *T. cruzi* has been considered a key enzyme in oxidative metabolism of the parasite[13–17] and nitrofurane derivatives have shown to

*Corresponding author. Tel.: +55-11-3091-3654; fax: +55-11-3815-4418; e-mail: hajudan@usp.br

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produce irreversible inactivation of that enzyme in anaerobic as opposed to aerobic conditions. In the initial step, these compounds, as well as substituted naphthoquinones, are reduced by the enzyme.[15,18] The final products of such reaction are oxidized by molecular oxygen and act as enzymatic inhibitors, blocking the reduction of the physiological substrate, trypanothione dissulfide.

Molecular modification[19,20] has been the most promising approach to introduce new drugs in therapeutics. Prodrug design, or latentiation, is a molecular modification process commonly employed with the purpose of obtaining better drugs, especially at the viewpoint of pharmacokinetics.[21,22] Hydroxymethyl derivatives are prodrugs whose main goal is to obtain more hydrophilic derivatives than the parent compound. In general, NH-acidic drugs such as amides, imides and ureides are potential targets to *N*-hydroxymethylation.[20]

We have worked on the preparation of selective nitrofurazone-primaquine (Chung et al., in preparation) mutual prodrugs. Dipeptides or succinic acid have been used as spacer groups. The dipeptidyl-primaquine compounds were reported as potential antichagasic prodrugs.[23] The hydroxymethylnitrofurazone (NFOH-121) used as intermediate to obtain the succinic acid-containing prodrugs unexpectedly showed to be a very potent antichagasic drug, as tested in vitro. Thus, the mutagenicity and degradation of NFOH-121 at pH 1.2 and 7.4 were also examined.

Materials and Methods

Materials

Nitrofurazone (NF) used was synthesized by Prof. Dr. Leoberto Costa Tavares from FCF-USP (São Paulo, Brazil). Potassium carbonate was from Ecibra and all other reagents were from Merck, analytical grade.

Apparatus

The ^{1}H and ^{13}C NMR spectrometries were carried out in a NMR Bruker Spectrometer AC-200 T, 200 MHz, in Central Analítica of IQ-UNESP, Araraquara, SP. The solvent used was DMSO-d_6. Elemental analyses of carbon, hydrogen and nitrogen were carried out in an Elemental Analyser 240 CHN, Perkin-Elmer, from Central Analítica of IQ-USP, São Paulo. UV–visible analysis was performed in a Beckman DU70 spectrophotometer in FCF-USP, São Paulo. Melting points were determined in a Büchi capilar apparatus and they were not corrected.

Degradation analysis were monitored using a Varian ProStar 330HPLC system coupled to photodiode array detector and a J.T. Baker C-18, 5 μ, 300 Å, 0.46×25.00 cm column. Mobile phases used were: sodium acetate buffer pH 4.6 and acetonitrile (79:21, v/v). The flow rate was 1.0 mL/min and the wavelength was 365 nm.

General synthetic methods

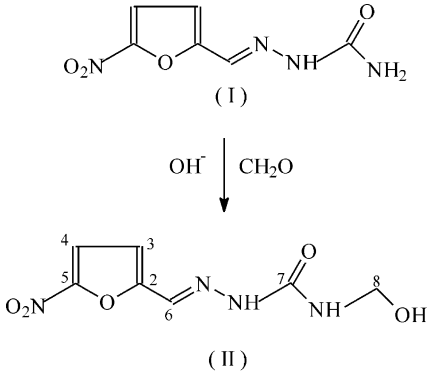
The synthesis of hydroxymethylnitrofurazone (**II**, **NFOH-121**) was carried out in alkaline conditions, using the method described by Yamaoka et al.[24] Scheme 1 outlines the procedure used to synthesize this derivative. In alkaline medium, nitrofurazone (**I**) (5.0 mmol), K_2CO_3 (5.0 mmol), water (50 mL) and 18 mL of formaldehyde were mixed. The reaction was carried out for 49 h at room temperature, being monitored by TLC using silica-gel and the following mobile phase: chloroform/methanol/acetic acid, 85:10:5, v/v/v. The suspension formed was then filtered. The resulting product was washed with methanol and recrystallized from methanol/water. Yield: 56%. Melting point: 150–154 °C. Elemental analysis: calcd: 36.84% C; 3.51% H; 24.56% N; found: 37.20% C; 3.26% H; 24.09% N. ^{1}H NMR (DMSO-d_6) δ 7.81 (s, 1H, CH), 7.77–7.75 (d, 1H, Het-H), 7.68–7.61 (t, 1H, NH), 7.22–7.20 (d, 1H, Het-H), 4.62–4.59 (d, 1H, CH_2); ^{13}C NMR (DMSO-d_6) δ 127.89 (C2), 112.72 (C3), 115.17 (C4), 151.36 (C5), 152.86 (C6), 154.60 (C7), 63.22 (C8).

Solubility determination

The solubility of the hydroxymethyl derivative of nitrofurazone or of the parent compound in 0.1 M hydrochloric acid was determined following Bundgaard and Johansen.[25] Briefly, an excess of each compound was dispersed in 25 mL of the solvent stirred for 24 h at 22° C and filtered. The resulting saturated solution of nitrofurazone or its derivative was diluted with 0.1 M hydrochloric acid 10- and 20-fold, respectively. The absorbances of the solutions were measured at 285 nm. The concentrations of the compounds were calculated from standard curves.

Decomposition of *N*-hydroxymethylnitrofurazone (NFOH-121)

The decomposition of the *N*-hydroxymethyl derivative of nitrofurazone was determined according to Bundgaard and Johansen[25] by trapping the formaldehyde released with semicarbazide hydrochloride in buffer solution, pH 7.4, at 37 °C and detecting the corresponding semicarbazone at 235 nm. Such decomposition at pH 1.2 and 7.4 at 37 °C was also monitored by



Scheme 1. Synthesis of hydroxymethylnitroturazone (II, NFOH-121).

HPLC. Briefly, solution of NFOH-121 (15 μg/mL) in sodium acetate (pH 1.2 and 7.4) was prepared and maintained under stirring at 37 °C. Samples were collected at 0, 15, 30 min and 1, 2, 4, 8, 16 and 24 h (pH 1.2) or at 0, 15, 30 min and 1, 2, 4, 8, 16, 24, 48, 72, 120 and 144 h (pH 7.4) of incubation and were analyzed in triplicate. The results were the mean of the values.

Biological in vitro test

Trypanosomes. Trypomastigotes forms of *Trypanosoma cruzi*, Y strain, were used in the in vitro assays. The organism was maintained in continous culture in Dulbecco's modified Eagle's medium (Dulbecco and Freeman, 1959) supplemented with $NaHCO_3$ (1.2 g/L), penicillin (500,000 U/L) and streptomycin (100 mg/L) (DME medium), containing 10% of heat-inactivated fetal bovine serum at 37 °C.

Cell culture assays. The in vitro testing was performed by seeding 24/2 mL wells tissue culture slides with 2×10^4 Rhesus monkeys renal epithelial cells (*Macaca mullata*), LLC-MK_2 (provided by Adolf Lutz Institute, São Paulo, Brasil) maintained in DME supplemented with 5% of heat-inactivated fetal bovine serum. Slides were incubated at 37 °C in an atmosphere containing 5% CO_2 in air. Confluent cells were used for subcultivation. The cell monolayer was washed with 10 mM phosphate buffer 150 mM NaCl (PBS) and treated with 0.1% of trypsin in PBS containing 1 mM of EDTA. The subcultivation was repeated every 7 days. Drugs were dissolved in DMSO or water and diluted to the desired concentrations with medium. Final solvent concentrations did not exceed 0.1% (v/v). Trypomastigotes (1×10^6/mL) were inoculated 24 h after the cells layers were seeded. Fresh medium with or without drugs in concentrations of 5 and 10 μM was added to the cells every 24 h of the incubation period. The in vitro activity was determined by counting the number of organisms at 6, 7, 8, 12, 14, 15, and 18 days after infection. The organisms were counted separately as trypomastigotes and amastigotes.

Mutagenicity assay. The method of direct incubation in plate according to Maron and Ames[26] was performed. Culture of *S. typhimurium* TA98 strain in the agar minimum glucose medium (AMG)—agar solution, Vogel Bonner E(VB) 50×, and 40% glucose solution—was used. DMSO solutions of NFOH and NF at concentrations of 5, 10, 25, and 100 nmol/mL were used in triplicate. Positive control of 4-nitro-phenylendiamine and 2-anthramine negative control of DMSO run in paralel. The influence of metabolic activation was tested by adding 500 μL of S9 fraction of mouse liver treated with Aroclor 1254, obtained from Moltox, Inc. (Annapolis, MD, USA). The revertant number was manually counted and the mutagenic rate (MR)—ratio between the average of the revertant number per plate for each concentration and the average of the number of revertants of the negative control-determined. The sample was considered to be mutagenic when the number of revertant colonies was at least the double of the negative control ($MI \geq 2.00$).

Results and Discussion

The activity of NFOH-121 was very high in both trypomastigotes and amastigotes, comparatively to that of benznidazol. The activity of 5 μM NFOH in trypomastigotes was quite similar to that found with 10 μM NF (Fig. 1).

With amastigotes, benznidazol showed again slightly lower activity (81.1%) than that observed with the nitrofurane derivatives: 97.2, 99.6, and 100% for 5 and 10 μM NF and 5 μM NFOH-121, respectively (Fig. 2).

Particularly interesting is the fact that while the cell infection by both trypomastigotes and amastigotes was reactivated in the 15th day after benznidazol treatment, there was no recovery of those forms when cells were treated with NFOH-121. Although this observation deserves further studies, it is very suggestive of a higher effectiveness of the prodrug when compared to the classical compound used in therapeutics.

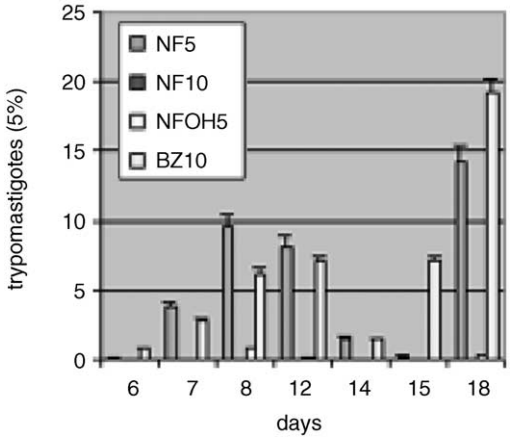


Figure 1. Effect of 5 μM NF (nitrofurazone), 10 μM NF, 5 μM NFOH-121 and 10 μM Benz (benznidazol) on the percentage of trypomastigotes in the 6, 7, 8, 12, 14, and 15th days after cell infection with *T. cruzi* trypomastigotes. Control: 100% trypomastigotes.

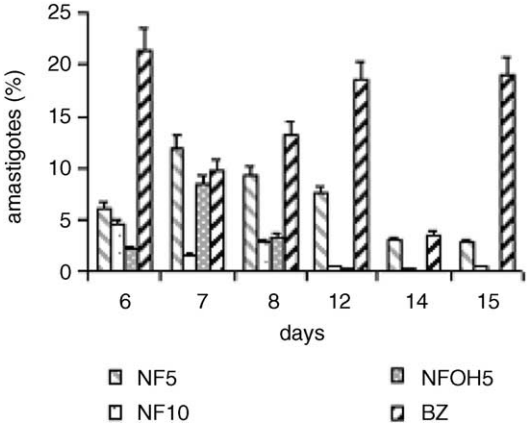


Figure 2. Effect of 5 μM NF, 10 μM NF, 5 μM NFOH and 10 μM Benz on the percentage of amastigotes in the 6, 7, 8, 12, 14, and 15th days after cell infection with *T. cruzi* trypomastigotes. Control: 100% amastigotes.

Those biological results are very encouraging if it is considered that the hydroxymethylated compound is less toxic than nitrofurazone. Indeed, NFOH-121 and nitrofurazone have been compared and, as indicated in Figure 3 and Table 1, the mutagenic activity of the former showed to be about 4 times lower (Fig. 3, Table 1). The decrease in the number of revertants observed at the maximum concentration of nitrofurazone can be explained by its toxicity for the *Salmonella* strain used.

The higher activity of NFOH-121 when compared to 10 μM NF or 10 μM benznidazol may be due to an increased uptake of the hydroxylated compound facilitated by its higher hydrosolubility. In fact, generally the *N*-hydroxymethyl derivatives of amide- or imide-type are more water soluble than the parent compounds. The replacement of a hydrogen bound to a nitrogen atom by a hydroxymethyl group may decrease the intra- or intermolecular hydrogen bonds, leading to a consequent diminution of the melting point and an enhancement in water solubility.[20] We have proved this as the solubility of hydroxymethyl derivative (0.992 mg/mL) was about 1.5-fold that of nitrofurazone (0.657 mg/mL) and its melting point was substantially lower (150–154 °C) than that of nitrofurazone (240–244 °C). This is also confirmed by a decreasing in the logP value from 0.23 (octanol–water[27]) for nitrofurazone to −0.19 (BioByte Demo) for NFOH-121.

The general mechanism for the decomposition of a hydroxymethyl derivative of acidic compounds at pH 1.4 involves a stepwise pathway with an *N*-hydroxymethyl anion as an intermediate that undergoes a rate-determining N-C cleavage, recovering the drug (Scheme 2). This is a spontaneous reaction.[20] Following the method of Bundgaard and Johansen[25] we have determined the decomposition of hydroxymethylfurazone at pH 7.4, 37 °C. Figure 4 shows the spectra of this derivative in the presence and absence of semicarbazide in comparison to that of semicarbazide plus formol, which leads to semicarbazone. A slight shift in the wavelength at the same region of the blank was observed for hydroxymethylnitrofurazone plus semicarbazide, indicating formol release.

We have confirmed the degradation at pH 1.2 and 7.4 by HPLC analysis. In acidic medium, NFOH-121 was fully decomposed into NF in 16 h (Fig. 5). Interestingly, such decomposition initiates after 1 h of incubation, but the release of NF takes one extra hour to occur. As in the meantime byproducts are formed in the incubation medium, we suggest that the conversion of NFOH-121 into NF occurs in two steps through the formation of an intermediate.

The half-time ($t_{1/2}$) of the prodrug at pH 1.2 was 1.5 h, as calculated by the graphic method in semilogarithmic scale. The backward constant rate (K_b) calculated by the equation $K_b = \ln/t^{1/2}$ was 0.46 h^{-1}. This acidic instability must be considered in in vivo assays.

The time-course of the prodrug degradation at pH 7.4 is shown in Figure 6. After 144 h of incubation its initial amount (18 μg) decreases to 10 μg and 6.0 μg of the drug is formed. At this pH we did not observe formation of byproducts in the incubation medium. Calculated half-time ($t_{1/2}$) of the prodrug was 134 h and $K_{b\ NFOH\ 7,4}$ was 0.005 h^{-1}.

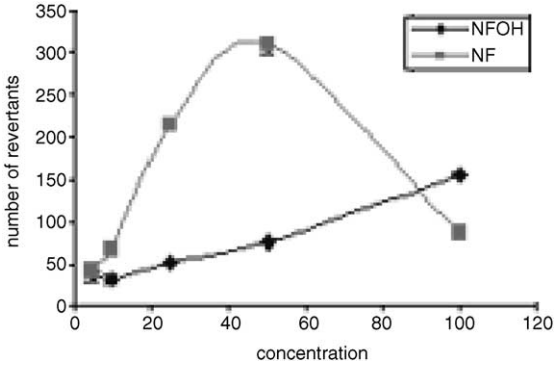
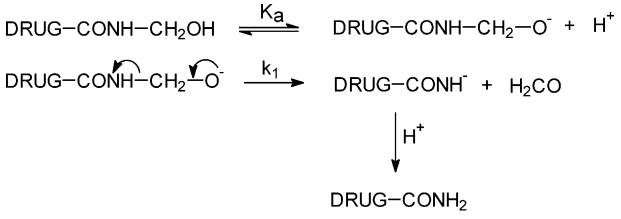


Figure 3. Mutagenic activities of NFOH-121 and NF in *S. typhimurium* TA98 strain.

Table 1. Mutagenicity rates (MR) of NFOH and NF on TA98 *S.typhimurium* strain

Concentration (nmol)	MR per plate	
	NFOH-121	NF
5.00	1.77	1.64
10.00	1.47	2.68[a]
25.00	2.36*	8.60[a]
50.00	3.45*	12.40[a]
100.00	7.09*	Toxicity

[a]Mutagenic activity (MR ≥ 2.00).



Scheme 2. Degradation of a *N*-hydroxymethyl derivative pH 7.4.

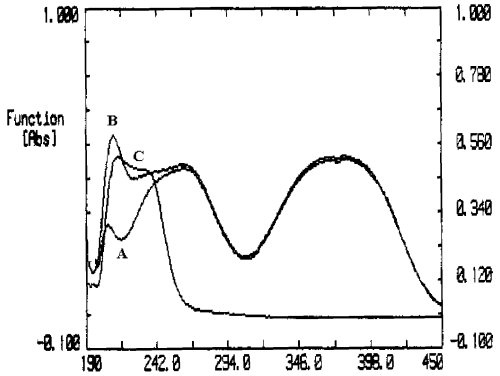


Figure 4. Comparative UV–visible spectra of hydroxymethylnitrofurazone in the presence (A) and in the absence (B) of semicarbazide and of semicarbazone plus formol (C).

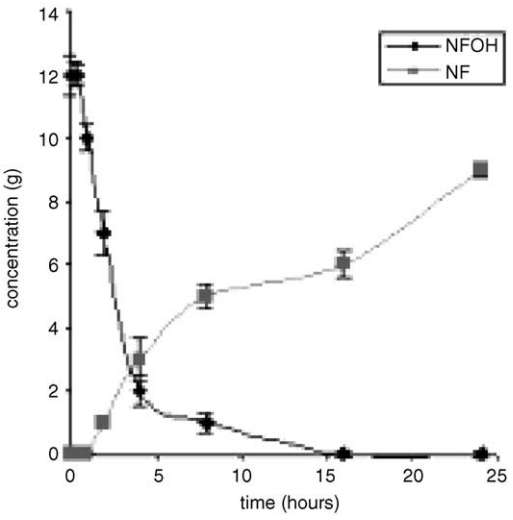


Figure 5. Degradation of NFOH-121 at pH 1.2.

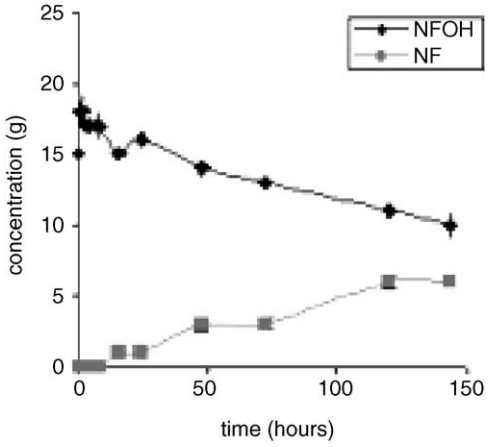


Figure 6. Degradation of NFOH-121 at pH 7.4.

These data led to the conclusion that 50% of NFOH-121 conversion is sufficient to cause a significant diminution in both amastigotes and trypomastigotes percentages in the 6th day of assay. Thus, in such aspect this is more active than NF.

In summary, the prodrug NFOH-121 seems to be a very promising compound for in vivo studies. Notwithstanding, further in vitro studies are needed to establish the intimate mechanism.

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