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Synthesis, antiprotozoal and cytotoxic activities of new *N*-(3,4-dimethyl-5-isoxazolyl)-1,2-naphthoquinone-4-amino derivatives

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Abstract

Three derivatives of *N*-(3,4-dimethyl-5-isoxazolyl)-1,2-naphthoquinone-4-amino (**1**), a compound which exhibits significant activity against *Trypanosoma cruzi* and *Plasmodium falciparum* but with cytotoxicity toward murine L-6 cells, were synthesized with the aim of ameliorating its cytotoxicity. The in vitro antiprotozoal and cytotoxic activities of the synthesized compounds were evaluated against *T. cruzi, Trypanosoma brucei rhodesiense, P. falciparum* and murine L-6 cells. The hydroxymethyl (**2**) and the oxime (**3**) derivatives were active against *T. cruzi*, with IC_{50} values in a range comparable to those of **1** (IC_{50}: 0.65 μg/ml) and benznidazole (IC_{50}: 0.56 μg/ml) while the carboxymethyloxime (**4**) was inactive. Compounds **2** and **3** were cytotoxic toward L-6 cells, with IC_{50} values identical to that of **1** (IC_{50}: 0.50 μg/ml). The results did not support the suggestion that **2** and **3** may be used as prodrugs of **1**.

Keywords: Antiprotozoal agents; Quinones; Trypanocidal activity; Cytotoxicity

1. Introduction

The epidemiological importance of American trypanosomiasis (Chagas' disease) in human pathology is widely known. The prognosis of this disease is serious and the choice of effective medicines is limited; consequently, more research is absolutely necessary to discover and develop new trypanocidal drugs.

Recent studies from our laboratory [1] have shown that *N*-(3,4-dimethyl-5-isoxazolyl)-1,2-naphthoquinone-4-amino (**1**) exhibited significant in vitro antiprotozoal activity against *Trypanosoma cruzi*, the causative agent of Chagas' disease; and *Plasmodium falciparum*, which is accompanied by cytotoxicity toward murine L-6 cells.

Taking into account that one way to ameliorate the cytotoxicity of a compound is to prepare prodrugs [2], it seemed of interest to prepare some derivatives as candidate prodrugs of **1** and to compare their biological and cytotoxic activities with those of **1** and known standard compounds. The results from this study may shed light on whether these principles could be subsequently used in the design of compounds for in vivo evaluation. Thus, in the present work, three new derivatives of **1** (compounds **2–4**, Scheme 1) were synthesized and tested for antiprotozoal and cytotoxic activities against *T. cruzi, Trypanosoma brucei rhodesiense, P. falciparum* and murine L-6 cells, respectively.

2. Experimental procedures

Melting points (m.p., uncorrected) were determined on a Büchi 510. Yields of solids refer to crude products. IR (KBr), mass (at 70 EV), and ^{1}H NMR spectra (chemical shifts in δ, ppm downfield from TMS) were recorded on Nicolet 5-SXC FTIR, Finningan 3300, and Bruker WP 80 SY spectrometers, respectively. Microanalyses (C, H and N) were performed by UMYMFOR, Buenos Aires, Argentina and the values were ±0.4 of theoretical ones. *N*-(3,4-dimethyl-5-isoxazolyl)-4-amino-1,2-naphthoquinone (**1**) and 2-(3,4-dimethyl-5-isoxazolylamino)-*N*-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (**5**) were synthesized by a previously reported procedure [3].

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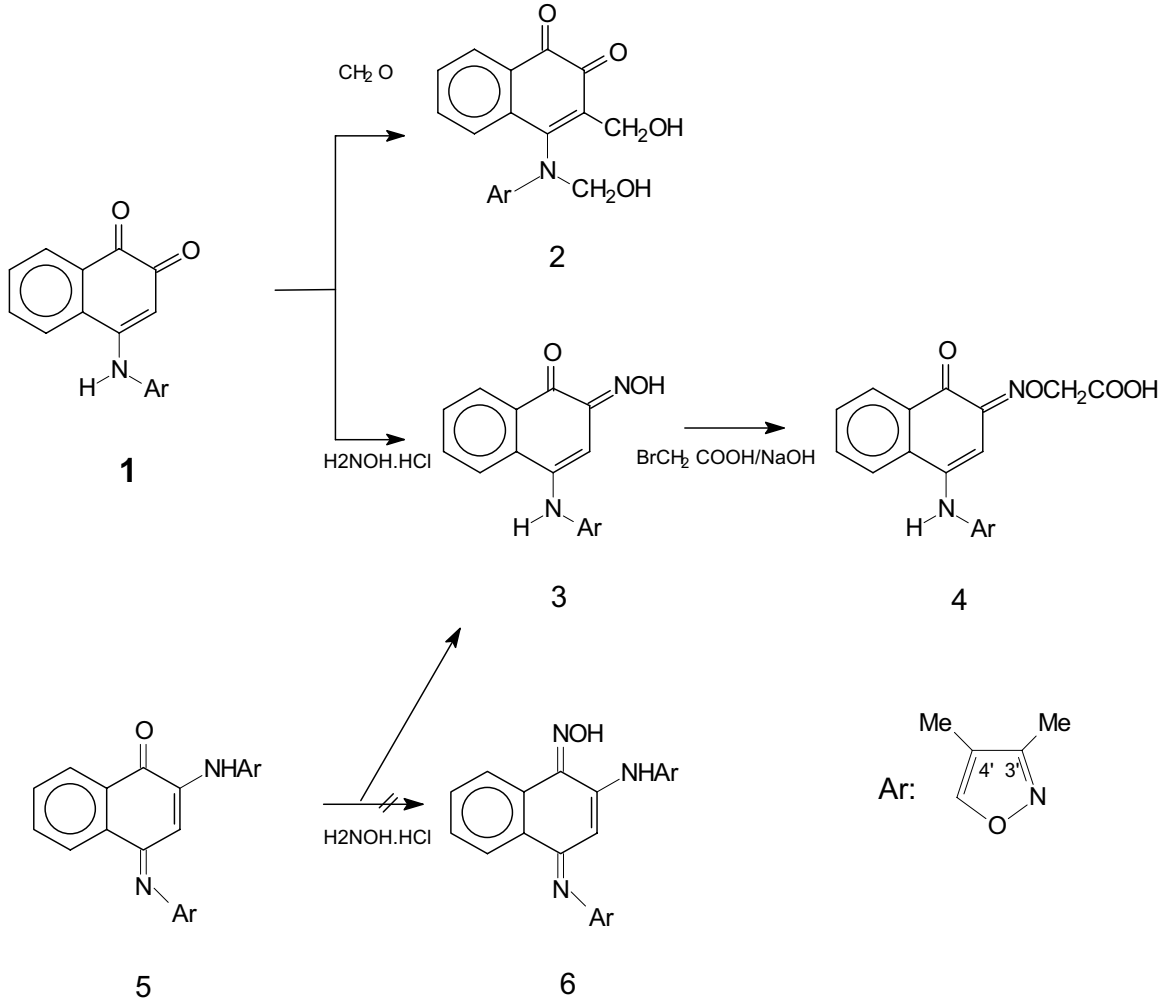

Scheme 1. Reagents and conditions for the synthesis of **2–4**.

2.1. 3-Hydroxymethyl-N-*hydroxymethyl*-N-*(3,4-dimethyl-5-isoxazolyl)-4-amino-1,2-naphthoquinone (**2**)*

A suspension of **1** (0.299 g, 1.1 mmol), 20 ml of formalin (40% formaldehyde in water), 0.015 g (0.11 mmol) of K_2CO_3, 30 ml of ethanol, and 10 ml of water were stirred at room temperature for 96 h. After that, the ethanol was removed under reduced pressure and then 30 ml of water were added. After standing at 5 °C overnight, the resulting precipitate was collected by filtration, washed with water, and dried to give 0.310 g of **2** (86% yield). The crude product was recrystallized from ethanol. M.p.: 225–226 °C (dec.). δ_H (DMSO-d_6): 1.04 (3H, s, Me-4′); 1.89 (3H, s, Me-3′); 3.65 (2H, dd, N–CH_2OH; δ_C: 62.84); 4.73 (2H, dd, 3-CH_2OH; δ_C: 56.28); 6.04 (1H, t, OH); 7.70–8.10 (1H, m, OH); 7.79 (1H, m, H-5); 7.88 (1H, m, H-6); 7.79 (1H, m, H-7); 7.97 (1H, m, H-8) ($J_{5,6}$: 7.45; $J_{5,7}$: 2.64; $J_{5,8}$: 0.54; $J_{6,7}$: 8.42; $J_{6,8}$: 3.24; $J_{7,8}$: 7.72; rms: 0.02). *m/z* (%): 328 (M^+, 3); 102 (23); 68 (15); 42 (47); 31 (100). ν_{max}/cm^{-1}: 3329/3281 (OH); 1676,1628 (CO). Elemental analysis calc. (%) for $C_{17}H_{16}N_2O_5$: C, 62.19; H, 4.91; N, 8.53; found: C, 62.20; H, 5.10; N, 8.67.

2.2. N-*(3,4-dimethyl-5-isoxazolyl)-4-amino-1,2-naphthoquinone-2-one oxime (**3**)*

To a solution of **1** (0.362 g, 1 mmol) in ethanol (80 ml), 1.39 g (20 mmol) of HCl.NH_2OH in water (10 ml) was added, and the mixture was heated under reflux for 4 h. After that, the ethanol was removed under reduced pressure and 50 ml of cold water were added. The resulting precipitate was filtered off, washed with water, and dried to give 0.263 g of **3** (93% yield). The crude product was recrystallized from benzene. M.p.: 211–212 °C (dec.). δ_H (DMSO-d_6): 1.84 (3H, s, Me-3′); 2.16 (3H, s, Me-4′); 7.16 (1H, s, H-3); 7.61 (1H, m, H-7); 7.72 (1H, m, H-6); 8.12 (1H, m, H-8); 8.20 (1H, m, H-5) ($J_{5,6}$: 7.54; $J_{5,7}$: 1.38; $J_{5,8}$: 0.84; $J_{6,7}$: 7.12; $J_{6,8}$: 1.67; $J_{7,8}$: 7.88; rms: 0.12); 8.93 (1H, br s, NH); 12.65 (1H, br s, NOH). *m/z* (%): 283 (M^+, 48); 266 (M-OH, 3); 96 (52); 68 (100). ν_{max}/cm^{-1}: 3325 (NH); 3187 (N–OH); 1644 (CO); 971 (=N–OH). Elemental analysis calc. (%) for $C_{15}H_{13}N_3O_3$: C, 63.60; H, 4.63; N, 14.83; found: C, 63.83: H, 4.85; N, 14.93.

2.3. N-*(3,4-dimethyl-5-isoxazolyl)-4-amino-1,2-naphtoquinone-2-[*O *(carboxymethyl)]-oxime (**4**)*

A mixture of 0.278 g (2 mmol) of bromoacetic acid and 1 g of crushed ice was alkalinized with aqueous NaOH (40%) and added to a solution of **3** (0.283 g, 1 mmol) in ethanol (50 ml). The mixture was stirred at room temperature for 4 d, then was cooled and acidified with aqueous HCl (37%). The resulting precipitate was filtered off, washed with water, and dried to give 0.259 g of **4** (76% yield), which was purified by recrystallization from methanol–water. M.p.: 197–198 °C (dec.). δ_H (DMSO-d_6): 1.85 (3H, s, Me-3′); 2.17 (3H, s, Me-4′); 4.88 (2H, s, CH_2); 7.03 (1H, s, H-3); 7.66 (1H, m, H-7); 7.74 (1H, m, H-6); 8.12 (1H, m, H-8); 8.15 (1H, m, H-5) ($J_{5,6}$: 7.17; $J_{5,7}$: 1.35; $J_{5,8}$: 0.17; $J_{6,7}$: 7.61; $J_{6,8}$: 1.84; $J_{7,8}$: 7.27; rms: 0.07); 9.20 (s, 1H, NH). *m/z* (%): 341 (M^+, 9); 267 (8); 225 (100); 102 (32); 96 (34); 68 (66); 42 (35). ν_{max}/cm^{-1}: 3324 (NH); 2915–2500 (COOH); 1727,1656 (CO). Elemental analysis calc. (%) for $C_{17}H_{15}N_3O_5$: C, 59.82; found: C, 59.96 and HRMS *m/z* calc. for $C_{17}H_{15}N_3O_5$: 341.1012 $[M]^+$; found: 341.0994.

3. Biological screenings

3.1. T. cruzi

Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well/100 μl in RPMI 1640 medium with 10% fetal bovine serum and L-glutamine (2 mM). After 24 h, 5000 trypomastigotes of *T. cruzi* (Tulahuen strain C2C4 containing the β-galactosidase (Lac Z gene)) were added in 100 μl per well with serial drug dilutions. The plates were incubated at 37 °C in 5% CO_2 for 4 d. After 96 h, the substrate (CPRG/Nonidet) was added to the wells. The color reaction that developed during the following 2–4 h was read photometrically at 540 nm. Optical density values were expressed as percentage of the control, and IC_{50} values were calculated from the sigmoidal inhibition curve.

3.2. T. b. rhodesiense

Minimum essential medium (MEM, 50 μl) supplemented according to Baltz et al. [4] with 2-mercaptoethanol and 15% heat-inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions were added to the wells followed by a trypanosome suspension (*T. b. rhodesiense* STIB 900, 50 μl). After incubation for 72 h at 37 °C under a 5% CO_2 atmosphere, Alamar Blue (10 μl) was added to each well and incubation continued for a further 2–4 h. The plate was then read with a Millipore Cytofluor 2300 using an excitation wavelength of 530 nm and an emission wavelength of 590 nm [5]. Fluorescence development was expressed as percentage of the control, and the IC_{50} values determined.

3.3. P. falciparum

Antiplasmodial activity was determined using the *P. falciparum* strains K1 and NF54. A modification of the [^{3}H]-hypoxanthine incorporation assay [6] was used. Briefly, infected human red blood cells were exposed to serial drug dilutions in microtiter plates for 72 h. Viability was assessed by measuring the incorporation of [^{3}H]-hypoxanthine during the final 24 h of incubation by liquid scintillation counting. Counts were expressed as percentage of the control and presented as sigmoidal inhibition curves. IC_{50} values were calculated by linear interpolation selecting values above and below the 50% mark according to Hills et al. [7].

3.4. Cytotoxicity toward L-6 cells

The determination of the cytotoxicity was performed with L-6 rat skeletal myoblast cells according to a previously reported procedure [1]. Briefly, L-6 cells were seeded in 96-well microtiter plates at a density of 10^5/ml in MEM supplemented with 10% heat-inactivated fetal bovine serum. A threefold serial dilution ranging from 90 to 0.123 μg/ml in test medium was added. The plates were incubated as described for the antitrypanosomal assay. After 70 h, Alamar Blue (10 μl) was added to each well and incubation continued for a further 2–4 h. The plate was then processed in the same way as described for *T. b. rhodesiense*.

4. Results

4.1. Chemistry

Reaction of **1** with formaldehyde in basic medium yielded only the bis-hydroxymethyl derivative **2** (Scheme 1). Treatment of **1** with hydroxylamine hydrochloride at reflux yielded the mono-oxime **3** as the only product. Subsequent reaction of **3** with bromoacetic acid in basic medium yielded **4** (Scheme 1). Structures **2–4** were verified by analytical and spectroscopic data obtained by ^{1}H NMR (including the checking of the assignments by spectral simulation with the LAOCOON PC program [8]), IR and MS. In addition, and in order to assign unequivocally the two CH_2OH groups of **2**, the ^{1}H NMR analysis was assisted by means of spin–spin decoupling experiments. Thus, the non-irradiated spectrum showed two double doublets centered at δ_H 3.65 (*N*–C*H*$_2$OH) and δ_H 4.73 (3-C*H*$_2$OH) which were assigned according to Shoolery's effective shielding constants [9]. Irradiation of the NC*H*$_2$ protons led to the collapse of the OH triplet at δ_H 6.04 into an enhanced singlet. Similarly, irradiation of the δ_H 6.04 signal caused enhancement and simplification of the double doublet at δ_H 3.65, showing that the O*H* at δ_H 6.04 is attached to the *N*–*CH*$_2$ group. Then, the other OH proton that falls within the aromatic signals is bonded to the 3-*CH*$_2$ moiety.

It is remarkable that the ^{1}H NMR spectrum of **3** exhibited only one =N–O*H* proton (at δ_H 12.65, the typical range of oximinoketones) [10] pointing out that **3** was configurationally pure as also indicated by TLC results. In addition, the IR spectrum of **3** showed only one C=O absorption at 1644 cm^{-1}

Table 1
In vitro antiprotozoal and cytotoxic activities of **2–4** against *T. cruzi, T. b. rhodesiense, P. falciparum* and murine L-6 cells [a]

Compound	*T. cruzi*	*T. b. rhodesiense*	*P. falciparum* (K1)	(NF54)	Cytotoxicity L-6 cells
Standard	0.56 [b]	0.0061 [c]	0.067[d]	0.003[d]	–
Standard	–	–	0.0015[e]	0.0028[e]	–
1	0.65	0.47	0.11	0.16	0.5
2	0.83	1.14	0.64	0.8	0.5
3	0.68	1.24	2.49	2.46	0.5
4	11.89	32.16	3.41	5.41	17
5	12.85	0.92	0.12	0.19	51

Standards:
[a] The figures reported are IC_{50} values in μg/ml. IC_{50}: the concentration required to give 50% inhibition.
[b] Benznidazole.
[c] Melarsoprol.
[d] Chloroquine.
[e] Artemisinin.

which was attributed to a 1-CO group since in isoxazolyl-naphthoquinones [11,12] the 1-CO was found at ca. 1647 cm^{-1}, while the 2-CO was situated at ca. 1680 cm^{-1}. This assignment was also supported by mechanistic considerations as the bis-isoxazolyl analog of **1** (compound **5**, Scheme 1) under the same reaction conditions, did not yield the oxime **6**, but **3** was obtained as a pure isomer (TLC and ^1H NMR evidence). The formation of **3** from **5** can be rationalized on the basis that **5** in acidic and neutral media undergo irreversible hydrolysis to **1** following the typical mechanism for imines [3].

4.2. Biological and cytotoxic activities

The results of the biological evaluation of **2–4** against *T. cruzi, T. b. rhodesiense, P. falciparum* and murine L-6 cells are presented in Table 1, which also contains data for the compounds **1** and **5** for comparison.

Compounds **2** and **3** exhibited good inhibitory activity against *T. cruzi*, with IC_{50} values in a range comparable to those of **1** (IC_{50}: 0.65 μg/ml) and benznidazole (0.56 μg/ml). In contrast, **3** was inactive with an IC_{50} value similar to that of **5**.

Activity of **2** and **3** against *T. b. rhodesiense* was less pronounced than **1**, while **4** was found to be inactive. These results provide additional evidence that this family of compounds is not active against this parasite [1] as none of the tested compounds appeared more effective than the standard drug melarsoprol (IC_{50}: 0.0061 μg/ml).

In terms of their antimalarial activity, **2–4** were found to be inactive against *P. falciparum* strains K1 (resistant to chloroquine and pyrimethamine) and NF54 (sensitive to all known drugs). Their IC_{50} values were in a range (0.80–5.4 μg/ml) outside the threshold set for activity (0.5 μg/ml).

Compounds **2** and **3** displayed identical cytoxicity to that of **1** (IC_{50}: 0.50 μg/ml), indicating that their good antiparasitic activities could also lack specificity. In contrast, **4** was less cytotoxic than **1**, with an IC_{50} value ca. three times lower than that of **5** (IC_{50}: 51 μg/ml) which showed no cytotoxicity for the L-6 cells [1].

5. Discussion

The validity of the suggestion that **2–4** may be prodrugs of **1** to ameliorate its cytotoxicity was considered by evaluating the data of Table 1. A comparison of the bioactivities of **1** and **2** indicates that the two additional CH_2OH groups, despite their steric hindrance, cause little change in the anti-*T. cruzi* and cytotoxic activities. On the other hand, if **3** is a prodrug of **1**, then the rate of release of the 2-CO group may be reflected by different cytotoxicities as observed for other ketones [2]. Examination of the data (Table 1) reveals identical cytotoxicity for **1** and **3**, suggesting that **3** is active per se; hence, it could not be a prodrug of **1**. Compound **4**, which is not cytotoxic, may be a prodrug inactive in vitro but becoming active after in vivo administration. However, to be useful, the $=NOCH_2CO_2H$ group must enable selective in vivo release of **1** (or **3**) in trypanosomes [13,14].

The chemical modifications studied here indicated that the main structural requirement for the anti-*T. cruzi* and cytotoxic activities of **1** is the presence of the 2-keto moiety or a group sterically or electronically similar (=NOH). The deactivation of the 1,2-naphthoquinone ring through the substitution of the 2-CO group by a carboxymethyloxime (**4**) or an aminoisoxazolyl (**5**) moiety reduces or abolishes both the anti-*T. cruzi* and cytotoxic activity, probably since they modified the redox properties of the naphthoquinone ring or they interfere to some extent with the naphthoquinone ability to bind to DNA [14,15].

In summary, the results reveal good inhibitory activity in vitro of **2** and **3** against *T. cruzi*, which encourages further investigations in order to circumvent the cytotoxic properties of these potential trypanocidal agents.

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