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Novel naphthoquinone derivatives: Synthesis and activity against human African trypanosomiasis

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ABSTRACT

A series of naphthoquinone derivatives has been synthesized and tested for its biological activity against human African trypanosomiasis. The use of reverse micellar medium not only enhanced the conversion rate, but also showed selectivity towards mono-coupled product in aryl chloride–aniline coupling reactions. Two derivatives of naphthoquinone (**9b** and **9c**) exhibited potent activity against *Trypanosoma brucei* in vitro with low cytotoxicity.

The Human African trypanosomiasis (HAT), normally known as sleeping sickness, is a perilous and neglected parasitic disease. It is prevalent in at least 36 sub Saharan Africa countries. Most (96%) cases of HAT are caused by the *Trypanosoma brucei gambiense* protozoa, which is transmitted from infected animals to humans by bites of blood sucking tsetse flies (*Glossina* genus).[1–3] The medications currently used for treatment are unsafe and inefficient; for example, treatment with melarsoprol (a trivalent arsenical derivative) often results in fatal encephalopathy, agranulocytosis, and myocardial damage.[4,5] There is a dearth of effective treatment regimens to fight against HAT. The negligence towards this disease engenders a demand for new research projects to produce drugs which are effective against the disease as well as safe for human use.

During research conducted on the chemical genetics of *Plasmodium falciparum*, potential anti-malaria agents, such as naphthoquinones, were discovered through use of screening techniques such as high through-put screening (Scheme 1).[6]

A process development study of transition metal catalyzed coupling reactions is one of the most important areas for organic scientists because of the applications of these reactions in pharmaceuticals;[7] especially in synthesizing bioactive compounds to fight against various diseases such as parasitic diseases. One such class of drugs is naphthoquinones which has shown anti-malarial, anti-cancer, anti-diabetic, anti-fungal, anti-bacterial and anti-inflammatory activities since 1969,[8–10] until recently, where epoxy-1,4-naphthoquinones have also been used as inhibitors of human leukemia (THP1) cell proliferation.[11] Atovaquone is an example of a naphthoquinone that is in use as an antimalarial agent. It is believed that it interferes with the mitochondrial respiratory chain of the *Plasmodium* sp.[12] Because of their positive antimalarial activities; we tested the naphtaquinones against HAT and performed a structural activity relationship study. In a continuation of our ongoing research focused on novel chemical entities with antimalarial or antitrypanosomal activities we aim on synthesizing various bioactive compounds; examples include derivatives of hydroxypyrid-2-ones,[13] febrifugine,[14] and fexinidazole,[15]. Our previous structure–activity relationship study[13] revealed the following requirements for bioactivity against *T. brucei*: an electron withdrawing moiety, an aromatic ring (good source of electrons), an electronegative halogen and its position in the molecule. We decided to design a molecule which included a halogen at the 2nd position and an electron donating and/or withdrawing substitution on the aromatic rings (X, R^1 and R^2, respectively in Scheme 1). In the proposed molecule, substitution of R^1 and R^2 on various positions aid in our understanding of the effect of substitution on activity against *T. brucei*. Hence, we decided to synthesize a series of substituted naphthoquinone derivatives (Scheme 1).

Mital et al. synthesized various 2-substituted 1,4-naphthoquinone derivatives by using 2-bromonaphthalene as the starting material in a Heck coupling reaction followed by oxidation.[10] Similarly, some scientists used palladium catalyzed Suzuki cross coupling reaction of aryl chlorides and Boronic acids.[16] Tandon et al. have done exceptional work in chemoselective coupling reactions by using water as a solvent.[17–19] However, coupling reaction in water (Tandon process) has limitation of substrate specificity.[18] The catalytic amination of aryl halides, called as Buchwald–Hartwig reaction, has been proved to be an useful method for preparing a variety of arylamines.[20–25]

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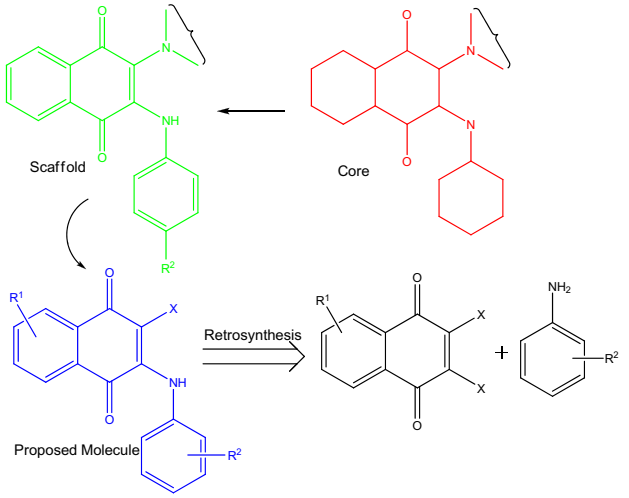


Scheme 1. Retrosynthetic approach for naphthoquinone derivatives. R^1 and R^2 = electron withdrawing or donating functional groups; X = halogen.

The design of novel, eco-friendly and cost effective synthetic processes for use in industry is an important goal. One of the best approaches involves the use of micellar micro-reactors, which possess a controlled balance of amphiphilicity and the ability to affect selectivity towards particular products.[26–32] In this study, we present the catalytic coupling reaction of anilines and aryl halides (Buchwald–Hartwig reaction) for synthesizing substituted naphthoquinones using reverse micellar micro-reactors.

We coupled 2,3-dichloronaphthalene-1,4-dione (RCl) and aniline ($R'NH_2$) using NaOH, L (10%), and $[cinnamylPdCl]_2$ (5%), in surfactant (sodium lauryl sulphate, SLS)–toluene as the reaction media and we not only observed a high % conversion but also enhancement in the selectivity towards **1b** (Table 1, entry 3).[33] The selectivity towards **1b** was very surprising because substitution of the activating group NHR on **1b** was expected to enhance the rate of formation of di-coupled **1a** product.

The above reaction suggest that in the presence of reverse micellar media (60 mM SLS surfactant) gave good yield of coupling reaction between aniline and 2,3-dichloronaphthalene-1,4-dione with reduced quantities of Pd catalyst and L (5 mol % Pd and 10 mol % L). It was also observed that the presence of surfactant micelles enhanced the % conversion and the selectivity towards the mono-coupled **1b** product of the reaction. Gas chromatography technique was employed to determine the exact % conversion to product **1**, and results were further confirmed by using ^1H and ^{13}C NMR spectroscopy.

The application of the process to derivatives of 2,3-dichloronaphthalene-1,4-dione with different substituted anilines is shown in Table 2, in which electron withdrawing and donating groups on aniline and electron withdrawing group (NO_2) on 2,3-dichloronaphthalene-1,4-dione gave good yields. The substitution pattern (2nd, 3rd and 4th position) on aniline does not appear to affect the yield.

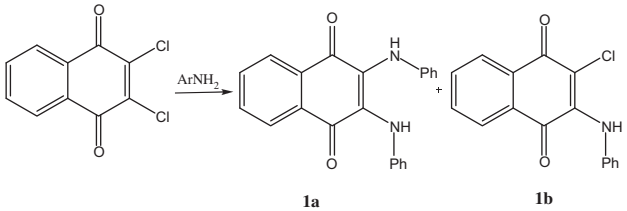
The synthesized compounds were evaluated on the basis of their ability to inhibit cell proliferation of *T. brucei* rhodesiense in culture.[34] The growth inhibitory activity against L-6 rat skeletal muscle myoblast cells was determined to establish a cellular therapeutic index.[35] The relationship between toxicity of naphthoquinone derivatives and substituted functional groups on the structure is still unknown and perplexing. Some naphthoquinone derivatives have shown to possess haemolytic activity and cause nephrotoxicity.[36,37] Hence, before proposing naphthoquinone derivatives as biologically active compounds, along with activity determination, in vitro toxicological studies becomes a prerequisite.

Compounds with mono-substitution (**b** and **c**) showed higher *T. brucei* rhodesiense inhibitory activity compared to di-substituted isomers (**a**). The presence of an electron-withdrawing chlorine group and phenyl amine ring with high electron density at two adjoining carbon atoms in the structure seems to be the main reason for this activity. This also showed the importance of selectivity towards mono-substituted product in coupling reaction. Compounds **1a** and **1b** showed effective *T. brucei* inhibitory activity and low cytotoxicity (Table 2, entry 1). However, compounds **2–4** showed lower inhibition activity compared to **1** but had low cytotoxicity (Table 2, entries 3–8). The decrease in the *T. brucei* inhibitory activity was attributed to the presence of an electron-donating group (Me) on the phenyl amine ring.

On comparing the *T. brucei* inhibitory activity of compounds **2–4** and **5–7** there is no correlation between the activity and the position of electron-donating group on phenyl amine ring (Table 2, entries 3–8 and 9–17).

To study the effect of electron withdrawing groups (NO_2 and CF_3) on the *T. brucei* inhibitory activity, we tested compounds **5–11** (Table 2, entries 9–27). We found that the presence of electrophile NO_2 on naphthalene-1,4-dione ring (with electron donating Me group on phenyl amine ring) in compounds **5–7** increased *T. brucei* inhibitory activity with a slight increase in cytotoxicity compared to compounds **2–4**. Surprisingly, compounds **8a** and **8b** with a CF_3 (electron withdrawing group) at the 2nd position on phenyl amine ring (without the presence of Me group on phenyl amine ring) showed *T. brucei* inhibitory activity with average cytotoxicity (Table 2, entries 18 and 19). Also the presence of NO_2 on naphthalene-1,4-dione ring and CF_3 on phenyl amine ring in compounds

Table 1
Synthesis of 2-chloro-3-(phenylamino)naphthalene-1,4-dione

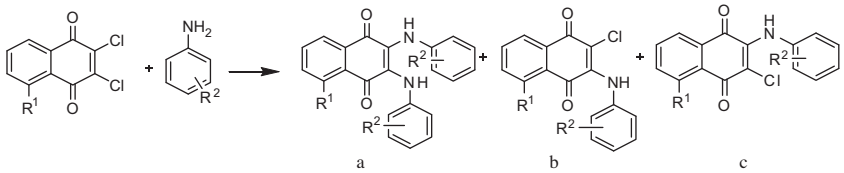


1a **1b**

Entry	Reaction conditions	% Total conversion[a] (% selectivity)	Isolated yield (%)
1	Pd (10 mol %), L (20 mol %), THF (5 mL), 24 h, 70 °C	35, **1a** (90), **1b** (10)	**1a** (27), **1b** (5)
2	Pd (10 mol %), L (20 mol %), Toluene (5 mL), 24 h, 110 °C	2	—
3	Pd (5 mol %), L (10 mol %), Toluene (5 mL), SLS (60 mM), 5 h, 20 °C	62, **1a** (7), **1b** (93)	**1a** (3), **1b** (55)

[a] Determined by gas chromatograph. Each experiment was done three times.

Table 2
Scope of the reaction in micellar micro-reactors and pharmacological properties of synthesized naphthoquinones



Entry	Substrate	% Total conversion[a] (% selectivity)	Isolated yield (%)	IC_{50} versus *T. brucei*[b] (μM)	IC_{50} versus L-6 rat skeletal myoblast cells[b] (μM)
1	**1a**, R^1 = H, R^2 = H	62 (7)	3	0.67 ± 0.1	378 ± 15
2	**1b**, R^1 = H, R^2 = H	62 (93)	55	0.17 ± 0.1	417 ± 20
3	**2a**, R^1 = H, R^2 = 2-Me	65 (10)	5	20 ± 3	>450
4	**2b**, R^1 = H, R^2 = 2-Me	65 (90)	56	18 ± 3	>450
5	**3a**, R^1 = H, R^2 = 4-Me	65 (8)	3	24 ± 3	>450
6	**3b**, R^1 = H, R^2 = 4-Me	65 (92)	55	20 ± 3	>450
7	**4a**, R^1 = H, R^2 = 3-Me	66 (9)	4	21 ± 3	>450
8	**4b**, R^1 = H, R^2 = 3-Me	66 (91)	58	18 ± 3	>450
9	**5a**, R^1 = NO_2, R^2 = 2-Me	58 (8)	3	7 ± 1	186 ± 10
10	**5b**, R^1 = NO_2, R^2 = 2-Me	58 (45)	25	4 ± 1	195 ± 10
11	**5c**, R^1 = NO_2, R^2 = 2-Me	58 (47)	25	3 ± 1	210 ± 10
12	**6a**, R^1 = NO_2, R^2 = 4-Me	60 (9)	4	12 ± 2	172 ± 10
13	**6b**, R^1 = NO_2, R^2 = 4-Me	60 (44)	23	8 ± 1	186 ± 10
14	**6c**, R^1 = NO_2, R^2 = 4-Me	60 (47)	26	6 ± 1	169 ± 10
15	**7a**, R^1 = NO_2, R^2 = 3-Me	60 (7)	3	15 ± 2	195 ± 10
16	**7b**, R^1 = NO_2, R^2 = 3-Me	60 (46)	25	10 ± 2	214 ± 10
17	**7c**, R^1 = NO_2, R^2 = 3-Me	60 (47)	26	11 ± 2	223 ± 10
18	**8a**, R^1 = H, R^2 = 2-CF_3	62 (5)	2	0.45 ± 0.1	292 ± 15
19	**8b**, R^1 = H, R^2 = 2-CF_3	62 (95)	57	0.15 ± 0.1	307 ± 15
20	**9a**, R^1 = NO_2, R^2 = 2-CF_3	60 (9)	4	0.09 ± 0.01	88 ± 5
21	**9b**, R^1 = NO_2, R^2 = 2-CF_3	60 (45)	24	0.07 ± 0.01	75 ± 5
22	**9c**, R^1 = NO_2, R^2 = 2-CF_3	60 (46)	24	0.05 ± 0.01	95 ± 5
23	**10a**, R^1 = H, R^2 = 4-CF_3	61 (11)	4	2 ± 0.2	276 ± 10
24	**10b**, R^1 = H, R^2 = 4-CF_3	61 (89)	52	0.8 ± 0.1	292 ± 10
25	**11a**, R^1 = NO_2, R^2 = 4-CF_3	58 (8)	3	4 ± 0.5	97 ± 5
26	**11b**, R^1 = NO_2, R^2 = 4-CF_3	58 (44)	23	2 ± 0.2	121 ± 10
27	**11c**, R^1 = NO_2, R^2 = 4-CF_3	58 (48)	25	0.8 ± 0.2	115 ± 10

[a] Determined by gas chromatograph.
[b] IC_{50} values are means standard deviations; each experiment was done three times.

9a–c showed highest *T. brucei* inhibitory activity in the series with low cytotoxicity (Table 2, entries 20–22). The reason for this enhancement in *T. brucei* inhibitory activity may be the balance of strong electron withdrawing groups in the structure that is, CF_3 on phenyl amine ring, NO_2 on naphthalene ring and Cl on 1,4-dione ring.

To evaluate the importance of the position of CF_3 group on phenyl amine ring with respect to the inhibition of *T. brucei* cell proliferation, we compared the activities of compounds **9** and **11** (or **8** and **10**) (Table 2, entries 20–27). The decrease in the *T. brucei* inhibitory activities of compounds with CF_3 at the 4th position in phenyl amine ring (compounds **10** and **11**) supported the postulation that the positioning of a strong electron withdrawing groups in the structure of naphthoquinones an important factor for *T. brucei* inhibitory activity.

The exact mechanism of action for this series of naphthoquinones against *T. brucei* is still unknown. However, to get glimpse about the relationship of the structure and the activity we evaluated the % conversion of compounds 2,3-dichloronaphthalene-1,4-dione, **1a**, **9b** and **9c** in the presence of cysteine in ringer solution[38] (Fig. 1). The higher rate of consumption of compounds **9b** and **9c** than 2,3-dichloronaphthalene-1,4-dione and **1a** indicates the comparison of the structures with respect to activity. The presences of a phenyl amine ring (a good source of electron density) with strong electrophile CF_3 group in the structure of **9c**, may cause protonation at C4 carbonyl group to form reactive intermediate which may react with a nucleophile (i.e., cysteine and lysine); similar protonation was observed previously in the case of lawsone

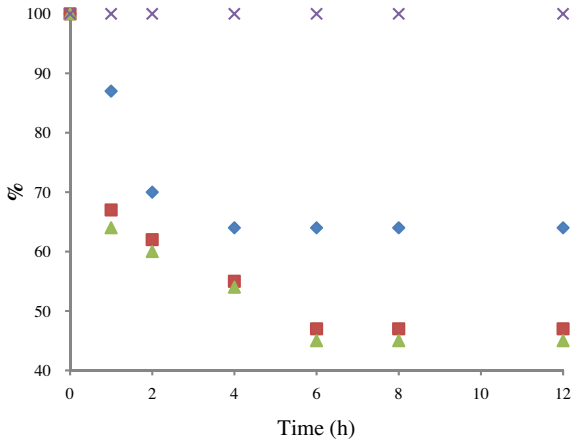


Figure 1. The consumption study of compounds 2,3-dichloronaphthalene-1,4-dione (×), **1a** (◆), **9b** (■) and **9c** (▲) (1 mmol) in the presence of cysteine (5 mmol) in Ringer solution at 37 °C. % Conversion was determined by gas chromatograph.

acetate.[39] The presence of electrophilic groups like Cl, NO_2 and CF_3 (at the 2nd position in phenylamine ring) in the structure may cause an increase in electrophilicity of the ring. These groups may also act as good leaving groups in the binding step of the cysteine thereby, conferring activity of **9c** against *T. brucei*.

It seems that these compounds are affecting against polyamine biosynthesis[40] in the parasite. If activities of compounds **9** and **11**

compared, then it would appear that the presence of CF_3 at 4th position on phenylamine ring may not act as good leaving group as compared to the CF_3 at 2nd position due to less steric hindrance. Hence, the position at which these electrophilic groups reside in the structure effect on the *T. brucei* inhibitory activity.

In conclusion, coupling of substituted RCl and anilines has been achieved using reverse micellar media. It resulted in selectivity towards mono-coupled substituted naphthoquinones. Compounds **9b** and **9c** showed excellent *T. brucei* inhibitory activity with low cytotoxicity. It seems that the electron withdrawing group such as NO_2, CF_3 and Cl and electron density rich groups (phenylamine ring and aromatic ring) at particular positions on the naphthoquinone as the backbone structure are important to *T. brucei* inhibitory activity.

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33. L (93.30 mg, 0.20 mmol, 20 mol %), and $(cinnamylPdCl)_2$ (52 mg, 0.10 mmol, 10 mol %) were added in 2.5 mL solution of SLS (100 mM) in toluene. This mixture was stirred under reflux for 5 min, under nitrogen atmosphere in 50 cm^3 glass round bottom flask with cylindrical shape, 1 cm length magnetic stirrer. The following mixture was then added; 2,3-dichloro-1,4-naphthoquinone (227 mg, 1 mmol, 1 equiv), aniline (0.1 mL, 2 mmol, 2 equiv) and powdered sodium hydroxide (44 mg, 1.1 mmol) in 2.5 mL solution of SLS (100 mM) in toluene. Reaction mixture was stirred for 5 h. A solution of CTAB (100 mM) in toluene (5 mL) was added to the reaction mixture which was agitated for an additional 10 min. The mixture was filtered through a plug of Celite, the filtrate concentrated under reduced pressure, and then purified by flash chromatography on silica gel using hexane:ethyl acetate 9:1 to give 2,3-bis(phenylamino)naphthalene-1,4-dione (**1a**); yield: 10.2 mg (3% yield), 2-chloro-3-(phenylamino)naphthalene-1,4-dione (**1b**) yield: 161.3 mg (57% yield). The experiments were performed in replicates of three. The variation in the results from the reported average values was within ±0.75%.
34. Parasites *T. brucei* rhodesiense were grown in mice to subsaturation density (3×10^8 cells/mL of blood). One milliliter of infected blood was collected with 0.2 vol of citrate glucose anticoagulant and 0.1 M sodium citrate/0.04 M glucose at pH 7.7 and was diluted in 9 mL of HMI-9 medium. Samples were centrifuged at 200 rpm for 5 min at room temperature. The supernatant with trypanosomes was transferred to a culture flask and incubated at 37 °C for 2 h to settle remaining blood cells. Trypanosomes in the supernatant were then cultured in an atmosphere of 5% CO_2 at 37 °C, in HMI-9 medium supplemented with 50 μM streptomycin/penicillin, 10% heat inactivated FBS and 10% Serum Plus to a density of 1×10^6 cells/mL. Cultures were fed daily by adding fresh medium (2.5 mL) after removing an equal volume and routinely diluted 1:10 to 1:20 daily to maintain densities in the range of 10^4 cells/mL. Cell proliferation tests were performed in 96-well tissue culture white polycarbonate flat bottom sterile plate by adding 100 μL of the diluted culture to each well. One microliter of compound diluted in DMSO was added by pin-transfer. Plates were incubated for 48 h at 37 °C then equilibrated at room temperature for 30 min and 50 μL of Cell Titer Glo was added to each well. Plates were shaken on a gyratory shakefor 5 min at room temperature (500 RPM). The plates were then read after 10 min in a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and emission wavelength of 588 nm. Fluorescence development was measured and expressed as percentage of the control. Data were transferred into the graphic program Softmax Pro (Molecular Devices) to calculate IC_{50} values.
35. Series of compounds were tested for toxicity in vitro against L-6 rat skeletal muscle myoblast cells. Toxicity tests were performed in 96-well tissue culture plates with the protein binding dye sulforhodamine B (SRB). Series of compounds were serially diluted and added to empty wells of the 96-well plate. The wells were immediately seeded with the cell line L-6 (rat skeletal muscle myoblasts) at 2×10^3 cells/100 μL, 50 μL per well in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat inactivated FBS. A threefold serial dilution ranging from 90 to 0.13 mg/mL of compounds in test medium was added. Corresponding solvent blanks were run for each test. Plates were incubated at 37 °C for 72 h in a humidified incubator containing 5% CO_2. After 72 h under culture conditions, cells were fixed to the plate by layering 50% trichloroacetic acid (TCA) at 4 °C over the growth medium in each well to make a final TCA concentration of 10%. Cultures were incubated at 4 °C for 1 h, and then washed three times with water and air dried. Wells were stained for 45 min with 0.4% (wt/vol) SRB in 1% acetic acid and washed three times with 1% acetic acid. Cultures were air dried, and bound dye was solubilized with 10 mM Tris base (pH 10.5) for 15 min on a gyratory shaker at room temperature (500 rpm). Spectra MAX Plus microtiter plate reader (Molecular Devices) was used to measure the optical density at 490–530 nm wavelength range.
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38. The consumption study was performed in tissue culture white polycarbonate flat bottom sterile plates by adding 100 μL of the Ringer solution (NaCl (6 g), $CaCl_2$ (0.1 g), KCl (0.079 g), $NaHCO_3$ (0.1 g), EDTA (1 g), H_2O (1 L)). One millimolar of compound diluted in DMSO and 5 mmol of cysteine were added after stirring. Plates were incubated at 37 °C then equilibrated at room temperature for 5 min and performed gas chromatography analysis. We also tried to do this study in the presence of HMI-9 medium; however, due to the presence of various amino acids in that medium, extraction and analysis of % conversion for compounds became very complicated.
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