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2-Phenylaminonaphthoquinones and related compounds: Synthesis, trypanocidal and cytotoxic activities

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ABSTRACT

A series of new 2-aminonaphthoquinones and related compounds were synthesized and evaluated in vitro as trypanocidal and cytotoxic agents. Some tested compounds inhibited epimastigote growth and trypomastigote viability. Several compounds showed similar or higher activity and selectivity as compared with current trypanocidal drug, nifurtimox. Compound **4l** exhibit higher selectivity than nifurtimox against *Trypanosoma cruzi* in comparison with Vero cells. Some of the synthesized quinones were tested against cancer cells and normal fibroblasts, showing that certain chemical modifications on the naphthoquinone moiety induce and excellent increase the selectivity index of the cytotoxicity (**4g** and **10**). The results presented here show that the anti-*T. cruzi* activity of 2-aminonaphthoquinones derivatives can be improved by the replacement of the benzene ring by a pyridine moiety. Interestingly, the presence of a chlorine atom at C-3 and a highly lipophilic alkyl group or aromatic ring are newly observed elements that should lead to the discovery of more selective cytotoxic and trypanocidal compounds.

1. Introduction

Infectious diseases and cancer are responsible for a large number of worldwide mortality. Cancer is one of the top ten leading causes of death. It is estimated that 7.6 million people died of cancer in 2008[1,2] and, if current trends continue, this number will rise to 9 million people by 2015.[3] On the other hand, infectious diseases still constitute a global health problem, causing the death of 8.8 million people in 2008.[1] Several neglected diseases are encountered among those with the highest economic burden. A specific parasitosis, Chagas disease (American trypanosomiasis, caused by *Trypanosoma cruzi*), affects more than 8 million people in Latin America, and causes approximately 10,000 deaths annually, which in the Americas is a higher figure than for malaria, and it is responsible for 89% of deaths from tropical-clustered diseases in the region.[1] In addition, Chagas disease causes over US$1.2 billion/year of productivity loss in seven of the countries where it is endemic.[4] Although both cancer and Chagas disease have different etiologies, they share metabolic and pathophysiological features such as glutathione metabolism, some signal transduction pathways, tissue invasion mechanisms and immune evasion strategies. Thus, the information obtained from a therapeutic target in any one of these cell types can be a useful tool for drug research in the other. As a result, several reports describe the effect of various compounds from natural extracts or new synthetic molecules against both *T. cruzi* and cancer cells.[5–12] On the order hand, a considerable number of natural and synthetic quinones (Fig. 1), have shown interesting biological properties, such as such as antimalarial (calothrixin A and B),[13–16] antibacterial (cribostatin I and streptonigrin),[17,18] antitumor (streptonigrin, calothrixin A and B and griffithazanone A),[19–22] and fungicidal activities.[23] These compounds have some important structural features, such as the 2-aminonaphthoquinone moiety (**1**), or 2-aminoquinoline- and isoquinoline-5,8-diones, or a substituted phenylamino group at C-2 (Fig. 1). It is known that the presence of the nitrogen atom, allows modulation of the

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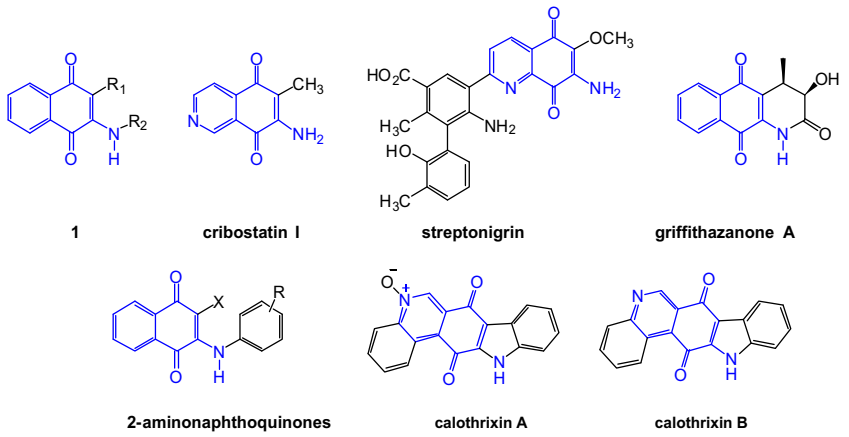


Figure 1. Structures of representative 2-amino-1,4-napthoquinone-type compounds with biological activities.

substituent's effects on the electronic properties of the quinone system, as well as modification of the geometry of the quinone molecules and their reduction intermediates.

In recent years, we have synthesized several series of 2-aminonaphthoquinone-type compounds such as benzocarbazolequinones, indazolylnaphthoquinones, benzoquinolinequinones, and related heterocyclic compounds.[24–28] Most of these compounds showed micromolar IC_{50} values against several series of tumor cell lines and trypanosome cultures.[25,28] One of the aims of this work was to analyze the influence of the enlargement of the heteropolycyclic system on their trypanocidal and cytotoxic effects. Since no data have been reported regarding the influence of the donor-acceptor and lipophilic properties of 2-phenylaminonaphthoquinones on *T. cruzi* cultures and their cytotoxic activity on normal and cancer cells, we synthesized a variety of these compounds to evaluate their trypanocidal activity and cytotoxic properties against different morphological stages of *T. cruzi* and a panel of four cell lines, including non-tumor dermal human fibroblast (DHF), and three human-derived tumor cell lines, namely PC-3 (prostate), MDA-MB231 and MCF-7 (breast).

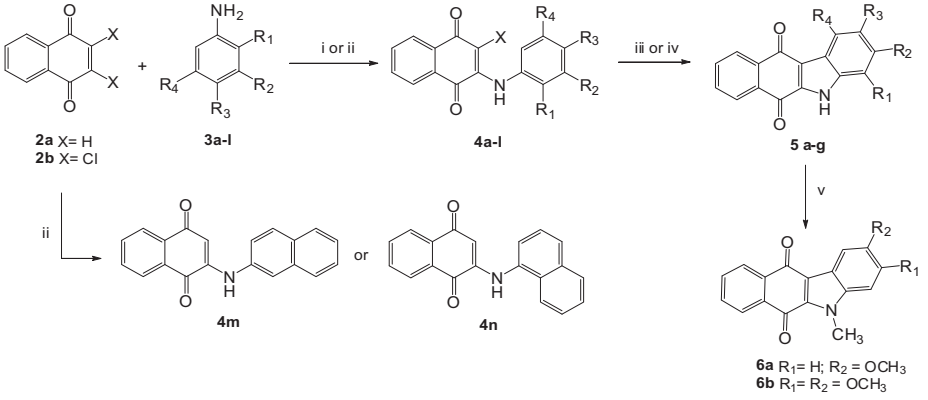
2. Results and discussion

2.1. Chemistry

The synthesis of 2-phenylaminonaphthoquinones (**4a**–**n**) and related 5*H*-benzo[*b*]carbazole-6,11-dione derivatives (**5a**–**d**) followed the general pathway outlined in Scheme 1. The first step was the substitution of the acceptor quinone nucleus (**2a**–**b**) by several aniline derivatives (**3a**–**l**) and 1- or 2-naphthylamine, which were achieved using two different methodologies (*i* or *ii*) reported in the literature.[29–31] Using methodology *ii*, and $CeCl_3{\cdot}7H_2O$ as Lewis acid catalyst, increased yields of the entire series were obtained (Fig. 1 and Table 1). In parallel, to study the importance of the nitrogen atom in the tricyclic system, we decided to obtain several benzocarbazolequinones, because some

Table 1
2-Phenylaminonaphthoquinones prepared by amination of quinones **2a**–**b**

Compound	X	R_1	R_2	R_3	R_4	Yield (%)	
						method i	method ii
4a	H	H	H	H	H	—	89
4b	H	OCH_3	H	H	H	62	67
4c	H	H	OCH_3	H	H	57	100
4d	H	H	H	OCH_3	H	49	78
4e	H	H	OCH_3	OCH_3	H	29	—
4f	H	OCH_3	H	OCH_3	H	65	—
4g	H	OCH_3	H	H	OCH_3	50	81
4h	H	H	H	$O(CH_2)_5CH_3$	H	74	74
4i	H	H	H	OH	H	—	86
4j	H	CH_3	H	CH_3	H	—	99
4k	Cl	OCH_3	H	H	OCH_3	—	83
4l	Cl	H	H	$O(CH_2)_5CH_3$	H	—	92
4m	H	—	—	—	—	—	74
4n	H	—	—	—	—	—	55



Scheme 1. Reagents and conditions: (i) ethanol, rt, 24–72 h; (ii) aniline **3a**–**l** or α- or β-naphthylamine, 0.22 equiv $CeCl_3{\cdot}7H_2O$, ethanol, rt, 12–24 h; (iii) 0.9 equiv $Pd(OAc)_2$, benzoquinone, AcOH, reflux, 12–24 h; (iv) 2–10 mol % $Pd(OAc)_2$, pivalic acid, 140 °C, 12–24 h ; (v) (1) KOH, ethanol, rt, (2) CH_3I, acetone, rt, 6 h (85% of yield for **6a** and 91% for **6b**).

of these and other compounds related to **4a**–**n** exhibit antineoplastic and antiparasitic activity.[25,28] For the biaryl cyclodehydrogenation of **4** leading to benzocarbazolequinones we used the procedure developed by Luo et al.,[31] which is a process catalyzed by palladium(II) in acetic acid (methodology *iii*). The use of pivalic acid as solvent, at a higher temperature, increased the yield in this step (*iv*).[32] To study the effect of hydrogen bonding involving the indole nucleus, we alkylated the indole nitrogen position using classical conditions.[33] The structures of the new members of the series were established on the basis of their spectral properties (IR, ^{1}H NMR, ^{13}C NMR and HRMS) (Table 2).

To obtain analogs of phenylaminonaphthoquinones and analyze the consequences of the isosteric replacement of a benzene ring by pyridine, we synthesized compound **10**, using conditions reported by Yoshida et al.[34] Thus, reaction of quinolequinone (**8**) with aniline **9g** was regioselective, using nickel(II) (Scheme 2). Attempts to cyclize **10** under similar conditions to those used for **5a**–**d** were unsuccessful. Other compounds related to **10** have been synthesized by our group (**11** and **12**)[35] and their trypanocidal effect has not been studied previously. Benzofuranoquinone **15**, was prepared by brominating phenol **13** and subsequent oxidation of dibromophenol **14**.[36] The reaction of bromobenzoquinone **15** with 2,5-dimethoxyaniline, or with an excess of diazomethane afforded arylaminobenzofuranquinone **16** or furoindazolequinone **17**, respectively (Scheme 3).

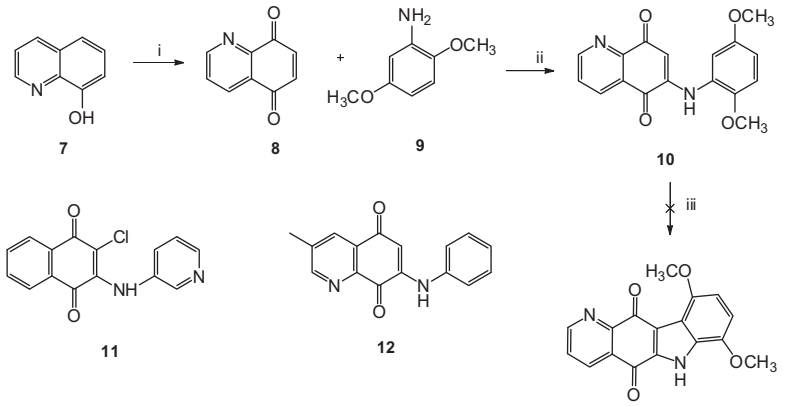
Considering that the synthesis of carbazolediones have some limitations, our group has reported a new strategy to obtain these compounds through conversion of 2-hydroxy-3-phenyl-1,4-naphthoquinones into the tetracyclic system (Scheme 4).[37–39,24] This methodology started from the coupling of isochroman-1,4-dione **18** with nitrobenzaldehydes **19a**,**b** to give 3-benzylideneisochroman-1,4-diones **20a**,**b**. The rearrangement of **20a**,**b** under basic conditions yielded 3-aryl-2-hydroxy-1,4-naphthoquinones **21a**,**b**, which were converted into 5*H*-benzo[*b*]carbazolequinones **5a** and **5h** by reduction with $NaBH_4$ in isopropanol at room temperature.

Table 2
Benzocarbazoles prepared by cyclodehydrogenation of 2-phenylaminonaphthoquinones

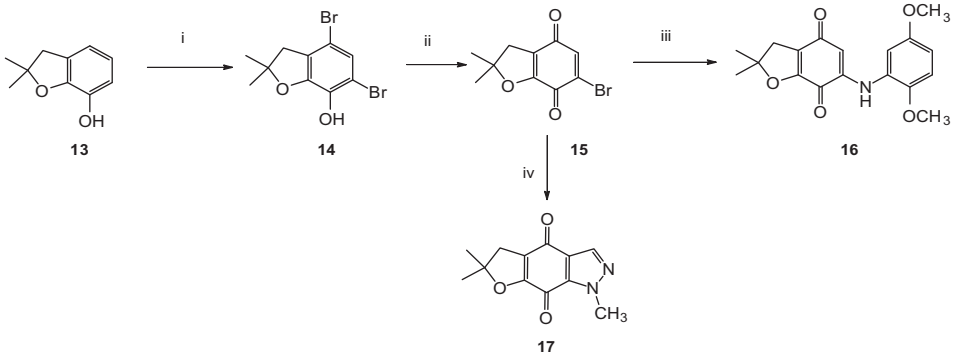
Compound	R_1	R_2	R_3	R_4	Yield (%)	
					method iii	method iv
5a	H	H	H	H	43	50
5b	OCH_3	H	H	H	67	100
5c	H	OCH_3	H	H	21	40
5d	H	H	OCH_3	H	40	80
5e	OCH_3	H	H	OCH_3	40	75
5f	H	H	$O(CH_2)_5CH_3$	H	67	50
5g	H	OCH_3	OCH_3	H	42	—

2.2. Bioactivity: trypanocidal effect

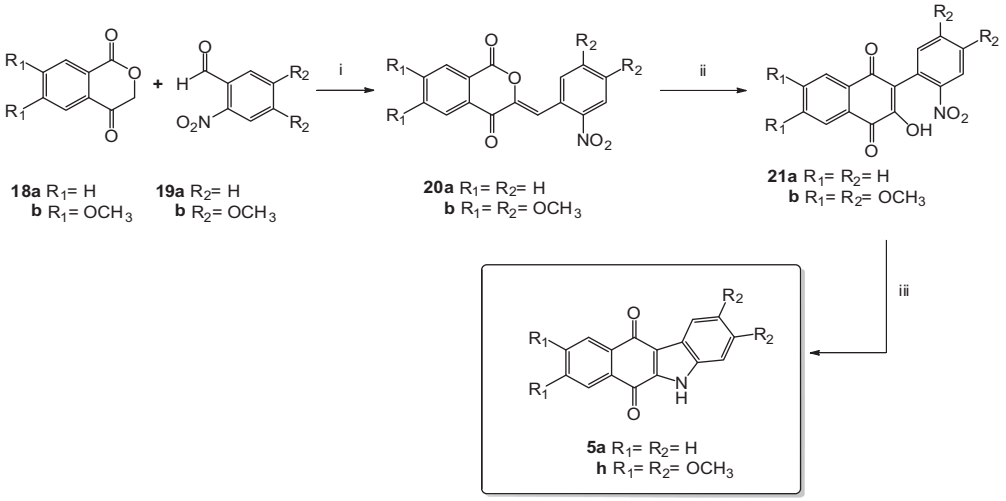
Trypanocidal activity of all the new compounds was tested against *T. cruzi* epimastigotes, and those that exhibited higher activity than nifurtimox, used here as the reference trypanocidal drug, were also assayed against trypomastigotes. The quinones were incorporated into the culture medium at different concentrations. Growth inhibition until day 10 was evaluated in comparison to controls at day 5. To quantify the relative efficacy of the quinones in vitro compared with the standard drug, the IC_{50} values were determined. Table 3 shows the effect of the quinones tested



Scheme 2. Reagents and conditions: (i) IBDA, CH_3CN–H_2O, 15 min, rt; (ii) $NiCl_2$, ethanol, rt, 12 h; (iii) $Pd(OAc)_2$, benzoquinone, AcOH, reflux, 12–24 h.



Scheme 3. Reagents and conditions: (i) Br_2, $CHCl_3$, 0 °C, 1.5 h (83%);[36] (ii) CrO_3, AcOH–H_2O (1:1), rt, 1 h (51%);[36] (iii) 2,5-dimethoxyaniline, ethanol, 0.5 equiv $CeCl_3{\cdot}7H_2O$, rt, 80%; (iv) CH_2N_2, Et_2O, 15 min, 0 °C, 67%.



Scheme 4. Reagents and conditions: (i) NaOAc, AcOH, 60 °C, 6 h; (ii) NaOMe, MeOH, , rt, 12 h; (iii) $NaBH_4$, ^{i}PrOH, rt, 4 h.[37,38,40]

Table 3
Effect of 2-aminophenylnaphthoquinones and related compounds upon culture growth of *T. cruzi* and selectivity index versus Vero cells

Compound	Epimastigote IC_{50} (μM)[a]	% trypomastigote mortality [b]	Vero IC_{50} (μM)[a]	Selectivity index[c]
4a	>25*	–	>10*	–
4b	3.49 ± 0.3	18.78 ± 1.8	>25*	–
4c	>25*	–	>25*	–
4d	>25*	–	>25*	–
4e	~25	–	>25*	–
4f	>25*	–	>25*	–
4g	23.30 ± 4.7	–	>50*	–
4h	1.72 ± 0.3	69.63 ± 4.2	27.04 ± 6.1	16
4i	>25*	–	>25*	–
4j	>25*	–	>25*	–
4k	>100*	–	>100*	–
4l	6.04 ± 0.1	46.30 ± 4.1	195.85 ± 26.9	32
4m	9.02 ± 0.2	86.02 ± 2.6	6.97 ± 0.3	0.77
4n	15.90 ± 1.3	59.67 ± 7.7	>25*	–
5a	>10*	–	>10*	–
5b	20.86 ± 2.0	12.21 ± 3.0	21.73 ± 5.8	0.95
5c	> 25*	–	>25*	–
5d	>50*	–	>50*	–
5e	>100*	–	>100*	–
5f	>100*	–	>100*	–
5g	n.d.	–	n.d.	–
5h	>100*	–	> 100*	–
6a	>25*	–	>25*	–
6b	>100*	–	>100*	–
10	2.45 ± 0.1	32.62 ± 4.6	15.43 ± 4.5	6.3
11	>100*	–	>100*	–
12	5.79 ± 0.4	58.20 ± 8.4	16.16 ± 1.3	2.7
16	12.35 ± 0.5	7.50 ± 2.4	15.54 ± 2.2	1.3
17	2.53 ± 0.6	38.57 ± 9.1	13.40 ± 2.1	5.3
21a	>100*	–	>100*	–
21b	>100*	–	>100*	–
Nifurtimox	21.05 ± 0.1	42.62 ± 3.7	411.13 ± 7.6	20

n.d.: not determined.
* Values of IC_{50} higher than the solubility in the medium.
[a] The results are means of three independent experiments.
[b] Drug concentrations used were the IC_{50} values on epimastigotes.
[c] Selectivity index: expressed as the ratio of IC_{50} in Vero cells to IC_{50} in epimastigotes.

on the growth of Tulahuen strain *T. cruzi* epimastigotes, at their respective IC_{50} concentrations. Some of these compounds showed remarkable trypanocidal effects. Among them, compound **4h** displayed the most potent inhibitory activity (IC_{50} = 1.72 ± 0.3 μM for epimastigotes). However, those compounds with IC_{50} values higher than 20 μM are equipotent with nifurtimox, and thus relatively devoid of pharmacological interest.

To validate our hypothesis regarding the 2-phenylaminonaphthoquinone structural pattern, several chemical modifications were carried out on this moiety, as shown in Figure 2. In order to find out a possible structure–activity relationship, a set of stereoelectronic properties were calculated (Table 4), and used in an attempt to understand this biological activity and to contribute to the design of more effective trypanocidal drugs.

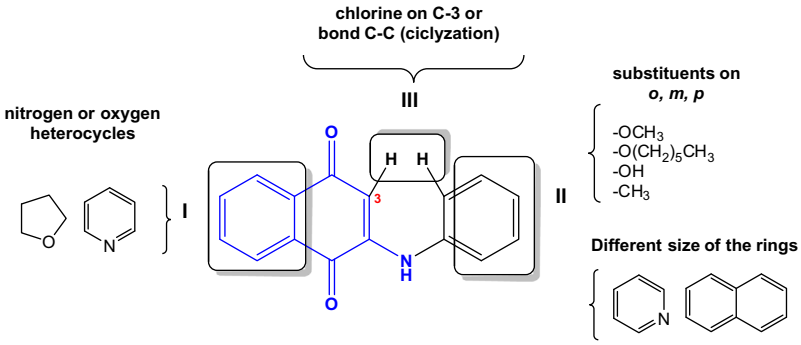


Figure 2. Main chemical modifications of the 2-aminonaphthoquinone framework for trypanocidal and cytotoxicity studies.

2.2.1. Trypanocidal effect on the epimastigote form

Replacement of the benzene by a pyridine ring (modification **I**, Fig. 2), increases the effects on the epimastigote form. For example, compound **10** is almost ten times more potent than its carbocyclic analog **4g**, with IC_{50} values of 2.45 and 23.3 μM, respectively. Furthermore, compound **12** is at least four times more active than **4a**. These results are in agreement with previous results from our group that indicate the importance of nitrogen substitution in the aromatic ring for the activity of related compounds.[25,28] To understand this behavior we examined the HOMO–LUMO energy gaps in these compounds (Table 4). These gaps are quite small for **4g** and **10** on one hand, and **4a** and **12** on the other, but a different distribution of the HOMO and LUMO can be seen in Figure 3, where nitrogen substitution in the aromatic ring (**4g**–**10**) leads to more extensive electron flow in the HOMO. This change is associated with a more planar conformation for **10**, which might also be related to the molecule's access to a specific biological target.

Although the dihydrofuro derivative **16** was less active than **10**, **16** is still more potent than **4g** (12.36 vs 23.30 μM) suggesting that an extended aromatic system is not a necessary feature. Compound **17**, an indazole-furan derivative is equipotent with **10**, highlighting the apparent role of a nitrogen heterocyclic system fused to the quinone ring. An important result of chemical modification **I**, is that these compounds are more active than nifurtimox.

Chemical modification **II** represents the broadest range of substitution patterns in all the compounds tested. From Table 3 it is possible to infer that either increasing volume or lipophilicity of the arylamine moiety (**4h**, **4m**, **4n**) favors the trypanocidal effect, particularly on the epimastigote form of *T. cruzi*. Surprisingly, while methoxy (**4c**–**g**), hydroxy (**4i**) and methyl (**4j**) substitution at the benzene ring lead to inactive derivatives, the 2′-methoxy compound (**4b**) is a remarkable exception (IC_{50} = 3.49 μM) and is seven times more potent than nifurtimox. The 2′-methoxylated derivative is probably more easily reduced because its HOMO–LUMO gap (17.26 eV) is lower than that **4a** (17.61 eV). Compounds **4h, 4m** and **4n** (IC_{50} = 1.72, 9.02 and 15.90 μM, respectively) are more potent than nifurtimox (21.05 μM). These three very lipophilic compounds are presumably penetrating better parasite's plasma membrane by virtue of their high calculated log*P* values (5.55 for **4h**, 4.38 for **4m** and 4.25 for **4n**, Table 4).

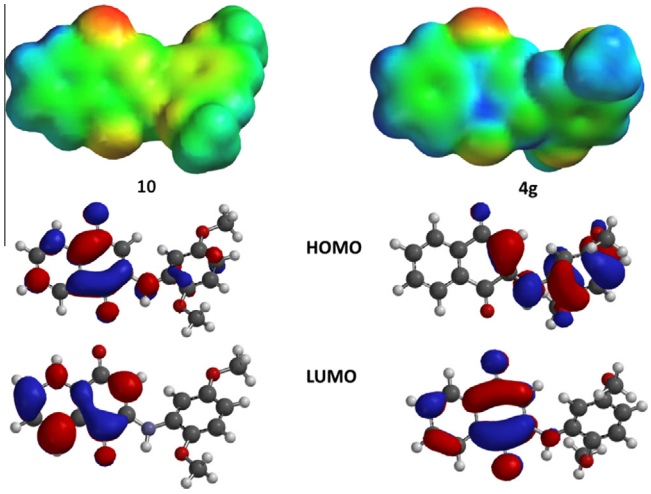


Figure 3. Electrostatic potential and frontier orbitals for **4g** and **10**.

Table 4
Calculated properties for the studied compounds

Compound	Polar surface area (Å^2)	Log*P*	Dipole (D)	HOMO (eV)	LUMO (eV)	HOMO–LUMO Gap
4a	36.64	3.06	1.93	−8.32	9.29	17.61
4b	44.08	2.88	1.45	−8.15	9.11	17.26
4g	52.47	2.99	2.11	−8.09	9.03	17.12
4h	44.90	5.55	3.25	−8.34	9.36	17.70
4k	52.51	3.90	2.60	−7.84	8.57	16.41
4l	44.94	6.46	4.58	−8.35	9.07	17.42
4m	36.96	4.38	1.86	−7.92	8.88	16.80
4n	36.31	4.25	1.99	−7.91	8.91	16.82
5b	54.99	1.90	1.30	−8.13	8.96	17.09
5e	38.90	2.06	1.44	−8.24	9.88	18.82
10	62.16	2.34	2.20	−8.01	8.95	16.96
11	46.20	2.69	4.78	−8.74	9.30	18.04
12	46.43	2.91	0.26	−8.39	9.35	17.74
16	60.39	2.12	1.14	−8.06	8.97	17.03
17	49.07	0.48	2.00	−9.44	9.90	19.34
Nifurtimox	84.64	0.89	9.57	−9.75	10.47	20.22

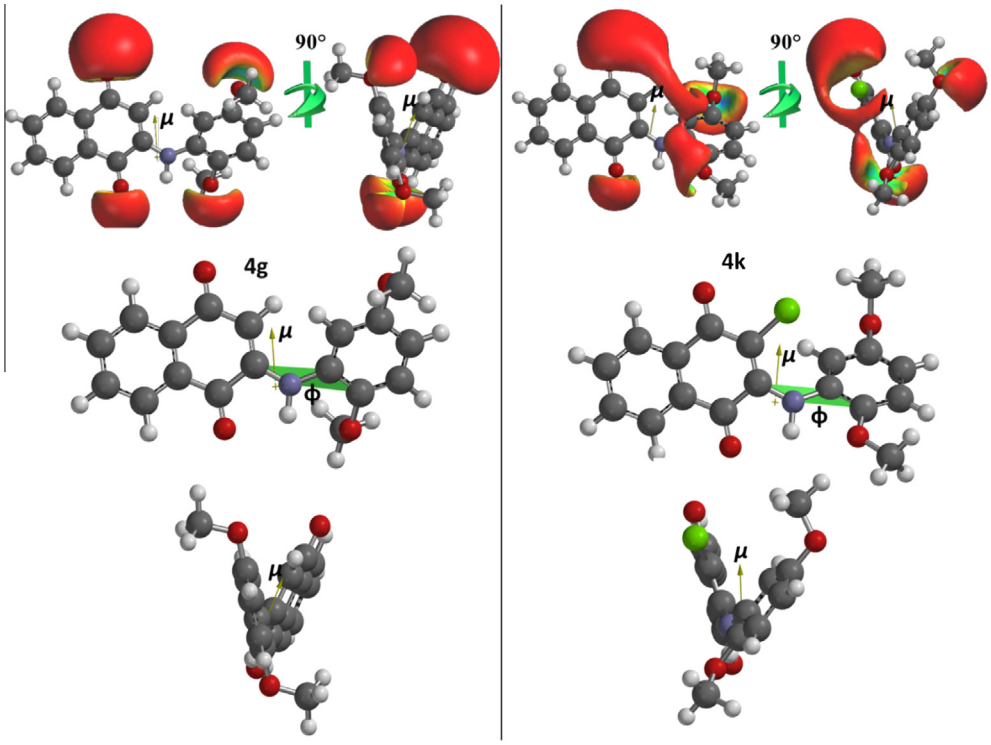


Figure 4. Geometry analysis of **4g** and **4k**.

Regarding modification **III**, replacement of the hydrogen atom at C-3 by chlorine afforded **4k** and **4l**. Both compounds showed decreased trypanocidal effect compared with their unhalogenated analogs **4g** and **4h**. Nevertheless, in **4l** an almost four-fold loss of activity against epimastigotes is compensated by a very significant decrease in its toxicity toward Vero cells and therefore greater selectivity than nifurtimox (see an analysis of this aspect in Section 2.2.3). Fig. 4 shows that substitution of H by Cl at C-3 (**4g** vs **4k**) modifies the overall geometry of the molecule and the electron distribution around the quinone system. In both cases in which comparison is possible, chlorination at C-3 is detrimental for the trypanocidal activity in spite of the concomitant increase in lipophilicity. The very low activity of compound **11** suggests that—in contrast to the introduction of nitrogen atom in the quinone moiety—when the arylamine moiety is heterocyclic, there is no favorable effect on trypanocidal potency, possibly due to the sharply reduced lipophilicity.

Cyclization of the 2-phenylaminonaphthoquinone system to yield the planar, more conjugated benzocarbazole derivatives does not increase and may actually be detrimental to the trypanocidal effect (**5a**–**5f**). Nevertheless, **5b** is still at least as active as nifurtimox, corroborating the finding that an *o*-methoxy group on the phenylamine moiety is a favorable feature. N-methylation of **5e** and **5f** to yield **6a** and **6b** gave no useful results. As shown in Figure 5, for **4g** and **5e** the electronic potential becomes quite uniform in the benzocarbazole compound although the HOMO orbital arrangement is similar in both. The MO energies diminish only slightly on cyclization, but log*P* and polar surface area decrease quite considerably from 2.99 to 2.06 and from 52.47 to 38.90 Å^2 (Table 4), for **4g** and **5e**, respectively.

Finally, the 2-hydroxynaphthoquinones **21a**–**21b** (with a similar structural pattern to lapachol), starting materials for the synthesis of benzocarbazoles **5e**–**f**, were also evaluated for their activity versus *T. cruzi*. Compounds **21a**–**21b** showed very poor effect, suggesting that the 2-phenylaminonaphthoquinone moiety is pivotal for the trypanocidal activity.

2.2.2. Trypanocidal effect on the trypomastigote form

Due to the relevance of the trypomastigote form in the infective cycle of *T. cruzi*, the next step in the search for new candidate drugs against Chagas disease is to evaluate the more active compounds in the epimastigote assays vs trypomastigotes. With this aim, the percentage mortality of trypomastigotes was determined at the IC_{50} values for the compounds that proved to be more active than nifurtimox against epimastigotes (Table 3). The results shown in Table 3 indicate that compounds **4h**, **4l**, **4m**, **4n** and **12** are similarly toxic to both parasite forms (46–87% mortality of trypomastigotes at epimastigote IC_{50}). These are interesting results because they show that these compounds retain the trypanocidal effect at different stages of the parasite's life cycle and sometimes with higher activity against the infective form (**4h** and **4m**). It seems likely that these results are related to the high lipophilicity of **4h** and **4l**–**n**. However, some compounds with calculated log P $\leqslant$ 2.9 (**4b**, **5b**, **10**, **12**, **16** and **17**, Table 4), while being at least as active as nifurtimox against epimastigotes, and with the exception of **12**, are less so against trypomastigotes and no relationship to their lipophilicity is apparent. This reduced potency might be related to a lesser ability of these structures to cross the trypomastigote membrane. This membrane is rich in *trans*-sialidase, and has a high concentration of sialic acid and an increased negative charge.[41] Therefore, uncharged molecules with substituents that present more non-bonding electrons or more than two heteroatoms might experience electronic repulsion and difficulty in entering the membrane.

2.2.3. Selectivity of trypanocidal compounds (Selectivity index, SI)

A potential antichagasic drug must show low toxicity in mammalian host cells and for this reason, the compounds with the strongest trypanocidal effects vs epimastigotes, their cytotoxic effects in Vero cell cultures were determined. It is well known that naphthoquinones generate reactive oxygen species (ROS) and as expected, several of the tested compounds were

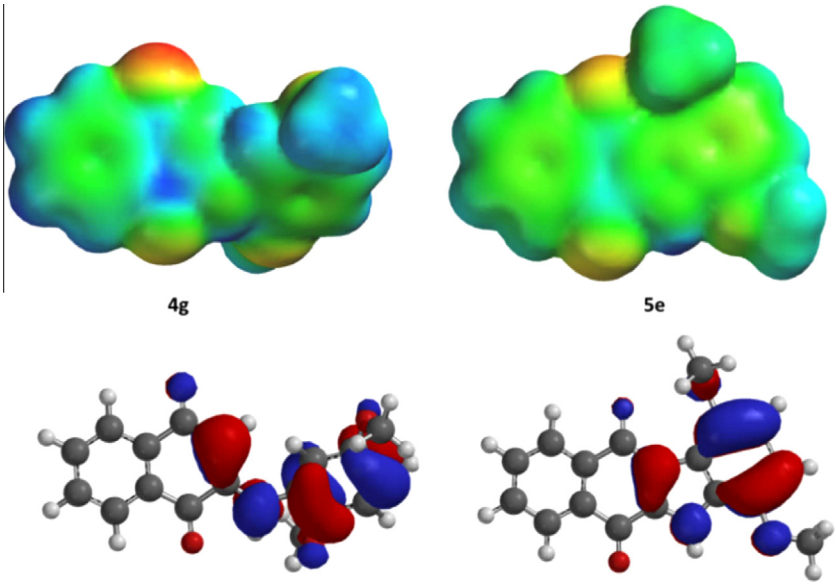


Figure 5. Electronic potential and HOMO of **4g** and **5e**.

quite cytotoxic. Table 3 shows—for the compounds with higher epimastigote toxicity—the IC_{50} values for Vero cell proliferation and their selectivity indices as the ratios of the IC_{50} values in both assays. These results indicate that most of these compounds are less selective than nifurtimox (SI = 20), although the potent **4h** is almost as selective (SI = 16). Quite unexpectedly **4l**, though not the most potent compound of the series, showed rather low toxicity (IC_{50} = 196 μM and SI = 32), possibly due to the substitution of hydrogen by halogen at C-2. Interestingly, **4l** is as potent as nifurtimox against trypomastigotes at its epimastigote IC_{50}. Consequently, among all the compounds tested here, **4l** is not only three times more potent than nifurtimox against both the epimastigote and the trypomastigote forms of *T. cruzi*, but also exhibits greater selectivity in regard of its toxicity toward Vero cells. These two favourable features seem to stem from the simultaneous presence of the halogen atom on the quinone ring and the lipophilic hexyloxy group on the arylamino substituent. In contrast, the remaining compounds displayed in Table 3 inhibited Vero cell proliferation within the expected range, and were therefore practically unselective and therefore lacking of clinical interest.

2.3. Bioactivity: cytotoxicity in human cell lines

A representative subset of the arylaminonaphthoquinones and related compounds described here, were tested for their antiproliferative effects against a hormone-independent and a hormone-responsive breast cancer line (MDA-MB231 and MCF-7), prostate cancer line (PC-3) and a normal human dermal fibroblast line (DHF). In the literature quinone/hydroquinone cytotoxicity is usually attributed to two processes: redox cycling of quinones, resulting in the generation of ROS which can damage biomolecules and inhibition of mitochondrial function and electrophilic arylation of critical cellular nucleophiles. Both mechanisms can result in oxidative stress and cell death.[42,43,29,44]

A conventional colorimetric assay was set up to estimate the IC_{50} values, which represent the concentration of a drug that is required for 50% inhibition in vitro after 72 h of continuous exposure to the test compounds. Four serial dilutions (from 12.5 to 100 μM) for each sample were evaluated in triplicate and doxorubicin was used as the reference drug.

Table 5 shows the IC_{50} values for **4b–g**, **5b**, **5d**, **6a–b** and **10**. In general, activity of these quinones was scattered among cell

Table 5
Antiproliferative activity of selected arylaminonaphthoquinone derivatives and related compounds against tumor cell lines and normal fibroblasts

Compounds	IC_{50} (μM)			
	PC-3	MCF-7	MDA-MB231	DHF
4b	>100	>100	>100	>100
4c	>100	>100	>100	>100
4d	35.4 ± 2.98	>100	>100	65.4 ± 9.48
4e	10.4 ± 1.01	22.2 ± 3.02	10.2 ± 1.51	>100
4f	60.0 ± 8.29	>100	>100	>100
4g	1.03 ± 1.14 (>97)*	0.21 ± 0.31(>480)	87.5 ± 9.34	>100
4h	>100	>100	>100	>100
4l	27.9 ± 4.5	>100	>100	>100
5b	>100	>100	>100	>100
5d	>100	>100	>100	>100
6a	>100	>100	>100	>100
6b	>100	>100	>100	>100
10	0.53 ± 1.01(42)	0.12 ± 1.02(188)	8.8 ± 1.23	22.5 ± 4.87
Doxorubicin	0.54 ± 0.36 (~15)	0.32 ± 0.24(25)	0.31 ± 0.13(25)	7.86 ± 1.34

n.d.: not determined.
* In parentheses, the Selectivity index (SI) expressed as the ratio of IC_{50} in DHF to IC_{50} in cancer cells.

cultures with variable sensitivity. Quinones **4b**–**g**, **5b**, **5d**, **6a**–**b** lack cytotoxic effects on cancer cells and together with those compounds with IC_{50} values higher than 10 μM (**4d**–**f**), are relatively uninteresting. Only compounds **4g** and **10** showed remarkable cytotoxicity, with their most potent inhibitory activity on PC-3 and MCF-7 cells. **4g** was more selective (SI >97 and 480 for PC-3 and MCF-7, respectively) than **10** (SI = 42 and 188 for PC-3 and MCF-7, respectively) and both compounds over more selective that the reference drug. Considering that **4g** and **10** are isosteres, these results are in agreement with the trypanocidal effects showed by these compounds, where once again the replacement of the benzene by a pyridine ring increases the cytotoxic effect but not the selectivity.

A full cytotoxicity analysis for the other naphthoquinones assayed in *T. cruzi* is being carried out to understand the structural requirements for obtaining compounds with selective activity toward cancer cells. However, the present results seem to suggest that coplanarity of the tetracyclic quinone ring (**5a**–**b** and **6a**–**b**) and the substitution pattern on the phenyl amino moiety (**4b**–**f**) are related to reduced cytotoxicity and selectivity.

3. Conclusion

A study of mostly new 2-arylaminonaphthoquinone derivatives (14), related 5*H*-benzo[*b*]carbazole-6,11-diones (8) and a few additional quinone compounds (9), against *T. cruzi* epi- and trypomastigotes, and of some of them against cancer cells and normal fibroblasts, showed that certain chemical modifications on the naphthoquinone moiety increase the trypanocidal and cytotoxic effects. Several of these compounds were more potent than the reference drug nifurtimox versus both stages of the parasite life cycle, and exhibited increased selectivity against *T. cruzi* in comparison with Vero cells, most notably **4l**. Cytotoxicity against prostate and mammary cancer cells in vitro was also quite selective (vs normal fibroblasts) in the case of **4g** compared with the reference drug. A preliminary analysis confirms an earlier conclusion that the replacement of a benzene ring by nitrogen isosteres condensed to the naphthoquinone core is an important feature to increase these activities. However, attempts to reveal general structure-activity relationships using stereoelectronic and lipophilicity properties were unsuccessful. Nevertheless, the presence of a chlorine atom at C-3 and a highly lipophilic alkyl group or aromatic ring are newly observed elements that should lead to the discovery of more selective cytotoxic and trypanocidal compounds.

4. Experimental

4.1. Chemistry

4.1.1. Materials and measurements

Melting points were determined on a Kofler Thermogerate apparatus and are uncorrected. Infrared spectra were recorded on a JASCO FT/IR-400 spectrophotometer. Nuclear magnetic resonance spectra were recorded, unless otherwise specified, on a Bruker AM-400 instrument using deuterochloroform solutions containing tetramethylsilane as internal standard. Mass spectra were obtained on a HP 5988A mass spectrometer. HPLC-MS experiments were performed on an Exactive Plus Orbitrap MS Thermo Scientific. Thin layer chromatography (tlc) was performed using Merck GF-254 type 60 silica gel. Column chromatography was carried out using Merck type 9385 silica gel. Compounds **4a**–**d**, **4f**–**g**, **4i**, **4k**, **4m**, **5a**–**b**, **5d**, **5g** and **6a** were identified by comparing their spectral properties (Melting point, IR, ^{1}H NMR and ^{13}C NMR) to those reported for these compounds in the literature.[24,26,29,33,41–43] The purity of the compounds was determined by tlc and high-resolution mass spectrometry (HRMS).

4.1.2. Synthesis

4.1.2.1. General synthetic procedures for 2-arylaminonaphthoquinones (4a–4n). *Method i*: In a reaction flask a suspension of naphthoquinone **2a** (300 mg, 1.89 mmol) in 10 mL EtOH (abs.) was placed under magnetic stirring at 10 °C until the solid dissolved completely. To this solution was added dropwise a solution of the corresponding amine (0.95 mmol) in 5 mL of EtOH, also at 10 °C. After the addition, the reaction mixture was allowed to reach room temperature and was stirred for another 24–48 h, after which the EtOH was removed in a rotary evaporator. The solid residue was purified by column chromatography on silica gel.

Method ii: In a reaction flask was prepared a 10 mL solution of naphthoquinone **2a** (300 mg, 1.89 mmol) or **2b** (300 mg, 1.32 mmol), the corresponding amine (0.95 mmol and 0.66 mmol respectively) and 0.42 mmol of $CeCl_3{}^*7H_2O$ in 10 mL of EtOH. The reaction mixture was stirred at room temperature for 12–24 h until disappearance of the naphthoquinone, and the EtOH was evaporated. The residue was redissolved in CH_2Cl_2, the organic phase was washed successively with 2 M HCl and saturated NaCl solution, dried with Na_2SO_4 and concentrated to dryness. The crude product was purified by column chromatography on silica gel.

*4.1.2.1.1. 2-(Phenylamino)naphthalene-1,4-dione (**4a**).* Red solid. Yield by method ii (192 mg, 89%), mp: 190–192 °C. (lit 189–190 °C)[33]. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 6.42 (s, 1H), 7.21 (t, J = 7.4 Hz, 1H), 7.27 (d, J = 7.9 Hz, 2H), 7.42 (t, J = 7.9 Hz, 2H), 7.58 (s, 1H), 7.66 (td, J = 1.2, 7.6 Hz, 1H), 7.75 (td, J = 1.2, 7.6 Hz, 1H), 8.11 (m, 2H). ^{13}C NMR ($CDCl_3$) δ ppm: 103.4, 122.6 (2C), 125.6, 126.2, 126.5, 129.7 (2C), 130.4, 132.3, 133.2, 134.9, 137.4, 144.7, 182.1, 183.9. IR (KBr, cm^{-1}): 3317, 1668, 1639, 1596. MS (ESI): 250.0 ($C_{16}H_{11}NO_2$ $[M+H]^+$).

*4.1.2.1.2. 2-(2-Methoxyphenylamino)naphthalene-1,4-dione (**4b**).* Red solid. Yield by method i (165 mg, 62%). Yield by method ii (180 mg, 67%), mp 145–147 °C. (lit 147–148 °C).[33] ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.92 (s, 3H), 6.49 (s, 1H), 7.00 (m, 2H), 7.15 (dt, J = 1.1, 7.9 Hz,1H), 7.42 (d, J = 7.9 Hz, 1H), 7.66 (dt, J = 1.1, 7.9 Hz, 1H), 7.76 (dt, J = 1.2, 7.6 Hz,1H), 7.99 (s, 1H), 8.12 (m, 2H). ^{13}C NMR ($CDCl_3$) δ ppm: ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm: 55.77, 103.61, 111.15, 120.88, 121.07, 125.46, 126.10, 126.53, 126.96, 130.53, 132.28, 133.30, 134.77, 143.97, 151.17, 182.12, 183.96. IR (KBr, cm^{-1}): 3307, 1672, 1631, 1597. MS (ESI): 280.0 ($C_{17}H_{13}NO_3$ $[M+H]^+$).

*4.1.2.1.3. 2-(3-Methoxyphenylamino)naphthalene-1,4-dione (**4c**).* Red solid. Yield by method i (152 mg, 57%). Yield by method ii (266 mg, 100%), mp 157–159 °C (lit 160–163 °C).[33] ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.83 (s, 3H), 6.45 (s, 1H), 6.74 (dd, J = 8.3, 2.0 Hz, 1H), 6.80 (t, J = 2.2 Hz, 1H), 6.86 (dd, J = 7.9, 1.8 Hz, 1H), 7.31 (t, J = 7.6 Hz, 1H), 7.56 (s, 1H), 7.65 (td, J = 7.6, 1.3 Hz, 1H), 7.75 (td, J = 7.6, 1.3 Hz, 1H), 8.10 (dt, J = 7.6, 1.2 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ ppm: 55.4, 103.8, 108.4, 110.9, 114.8, 126.1, 126.5, 130.3, 130.4, 132.3, 133.2, 134.8, 138.6, 144.5, 160.6, 182.0, 183.9. IR (KBr, cm^{-1}): 3227, 1675, 1605, 1594. MS (ESI): 280.0 ($C_{17}H_{13}NO_3$ $[M+H]^+$).

*4.1.2.1.4. 2-(4-Methoxyphenylamino)naphthalene-1,4-dione (**4d**).* Red solid. Yield by method i (130 mg, 49%). Yield by method ii (207 mg, 78%), mp 145–147 °C. (lit. 155–157 °C).[31] ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.83 (s, 3H), 6.22 (s, 1H), 6.94 (d, J = 8.8 Hz, 2H), 7.20 (d, J = 8.8 Hz, 2H), 7.43 (s, 1H), 7.65 (td, J = 1.1, 7.6 Hz, 1H), 7.75 (td, J = 1.1, 7.6 Hz, 1H), 8.07–8.10 (m, 2H). ^{13}C NMR ($CDCl_3$) δ ppm: 55.6, 102.5, 114.9 (2C), 124.8 (2C), 126.1, 126.4, 130.0, 130.4, 132.2, 133.4, 134.9, 145.7, 157.7, 182.2, 183.7. IR (KBr, cm^{-1}): 3226, 1679, 1621, 1604. MS (ESI): 280.0 ($C_{17}H_{13}NO_3$ $[M+H]^+$).

*4.1.2.1.5. 2-(3,4-Dimethoxyphenylamino)naphthalene-1,4-dione (**4e**).* Red solid. Yield by method i (85 mg, 29%), mp 125–127 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.90 (d, J = 5.1 Hz, 6H), 6.28 (s,

1H), 6.78 (d, J = 1.9 Hz, 1H), 6.85 (d, J = 8.8 Hz, 1H), 6.90 (d, J = 8.5 Hz, 1H), 7.44 (s, 1H), 7.66 (t, J = 7.4 Hz, 1H), 7.76 (t, J = 7.4 Hz, 1H), 8.11 (m, 2H). ^{13}C NMR ($CDCl_3$) δ ppm: 56.1, 56.2, 102.8, 107.4, 111.7, 115.6, 126.1, 126.4, 130.3, 130.4, 132.2, 133.4, 134.9, 145.5, 147.3, 149.7, 182.1, 183.7. IR (KBr, cm^{-1}): 3292, 1675, 1596, 1571. MS (ESI): 310.0 ($C_{18}H_{15}NO_4$ $[M+H]^+$). HRMS for ($C_{18}H_{15}NO_4$ [M]). Calcd: 309.1001. Found: 309.2015.

*4.1.2.1.6. 2-(2,4-Dimethoxyphenylamino)naphthalene-1,4-dione (**4f**).* Red solid. Yield by method i (190 mg, 65%), mp 149–151 °C. (lit. 150–153 °C).[45] ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.82 (s, 3H), 3.86 (s, 3H), 6.28 (s, 1H), 6.43–6.59 (m, 2H), 7.29 (d, J = 8.6 Hz, 1H), 7.52–7.83 (m, 3H), 8.10 (d, J = 7.6 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ ppm: 55.6, 55.8, 99.5, 102.6, 104.0, 119.9, 123.0, 126.0, 126.4, 130.5, 132.1, 133.5, 134.7, 144.8, 152.9, 158.2, 182.2, 183.7. IR (KBr, cm^{-1}): 3306, 1693, 1681, 1576. MS (ESI): 310.0 ($C_{18}H_{15}NO_4$ $[M+H]^+$).

*4.1.2.1.7. 2-(2,5-Dimethoxyphenylamino)naphthalene-1,4-dione (**4g**).* Red solid. Yield by method i (146 mg, 50%). Yield by method ii (237 mg, 81%), mp 127–129 °C. (lit 128–129 °C).[29] ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.80 (s, 3H), 3.87 (s, 3H), 6.53 (s, 1H), 6.65 (dd, J = 1.9, 8.9 Hz, 1H), 6.87 (d, J = 8.9 Hz, 2H), 7.02 (d, J = 2.8 Hz, 1H), 7,66 (td, J = 1.1, 7.6 Hz, 1H), 7.75 (td, J = 1.1, 7.5 Hz, 1H), 8.02 (s, 1H) 8.08–8.15 (m, 2H). ^{13}C NMR ($CDCl_3$) δ ppm: 55.9, 56.2, 104.0, 107.9, 109.1, 111.7, 126.1, 126.5, 127.7, 130.5, 132.3, 133.2, 134.8, 143.7, 145.4, 153.8, 182.0, 183.9. IR (KBr, cm^{-1}): 3349, 1673, 1637, 1602. MS (ESI): 310.0 ($C_{18}H_{15}NO_4$ $[M+H]^+$).

*4.1.2.1.8. 2-(4-Hexyloxyphenylamino)naphthalene-1,4-dione (**4h**).* Red solid. Yield by method i (245 mg, 74%). Yield by method ii (245 mg, 74%), mp 97–101 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.83 (t, J = 6.0 Hz, 3H), 1.21–132 (m, 4H), 1.36–1.40 (m, 2H), 1.63–1.77 (m, 2H), 3.86 (t, J = 6.5 Hz, 2H), 6.13 (s, 1H), 6.83 (d, J = 8.6 Hz, 2H), 7.08 (d, J = 8.6 Hz, 2H), 7.38 (s, 1H), 7.54 (t, J = 7.5 Hz, 1H), 7.64 (t, J = 7.5 Hz, 1H) 7.99 (d, J = 7.5 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ ppm: 14.0, 22.6, 25.7, 29.2, 31.6, 68.4, 102.4, 115.4 (2C), 124.8 (2C), 126.1, 126.4, 129.8, 130.4, 132.1, 133.4, 134.8, 145.6, 157.2, 182.1, 183.7. IR (KBr, cm^{-1}): 3215, 1678, 1660, 1615. MS (ESI): 350.0 ($C_{22}H_{23}NO_3$ $[M+H]^+$). HRMS for ($C_{22}H_{23}NO_3$ $[M+H]^+$). Calcd: 349.1678. Found: 350.1726.

*4.1.2.1.9. 2-(4-Hydroxyphenylamino)naphthalene-1,4-dione (**4i**).* Brown solid. Yield by method ii (216 mg, 86%), mp 248–249 °C. (lit. 250–252 °C).[31] ^{1}H NMR (400 MHz, DMSO) δ ppm: 5.88 (s, 1H), 6.83 (d, J = 8.8 Hz, 2H), 7.16 (d, J = 8.8 Hz, 2H), 7.76 (td, J = 1.4, 7.5 Hz 1H), 7.84 (td, J = 1.3, 7.5 Hz, 1H), 7.94 (dd, J = 1.0, 7.6 Hz, 1H), 8.04 (dd, J = 1.0, 7.6 Hz, 1H), 9.06 (s, 1H), 9.57 (s, 1H). ^{13}C NMR (DMSO) δ ppm: 106.0, 121.0 (2C), 130.5, 131.0 (2C), 131.2, 134.2, 135.7, 137.6, 138.1, 140.1, 152.3, 160.6, 186.9, 187.3. IR (KBr, cm^{-1}): 3303, 1671, 1622, 1597. MS (ESI): 265.0 ($C_{16}H_{11}NO_3$ $[M+H]^+$).

*4.1.2.1.10. 2-(2,4-Dimethylphenylamino)naphthalene-1,4-dione (**4j**).* Orange solid. Yield by method ii (260 mg, 99%), mp 155–158 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 2.23 (s, 3H), 2.34 (s, 3H), 5.90 (s, 1H), 7,06 (d, J = 8.0 Hz, 1H), 7.10 (s, 1H), 7.14 (d, J = 8.0 Hz, 1H), 7.27 (s, 1H), 7.65 (td, J = 1.3, 7.5 Hz, 1H) 7.74 (td, J = 1.3, 7.5 Hz, 1H) 8.06–8.14 (m, 2H). ^{13}C NMR ($CDCl_3$) δ ppm: 12.9, 16.2, 98.2, 120.2, 121.4, 121.6, 122.9, 125.8, 127.3, 127.4, 128.0, 128.4, 128.7, 130.1, 132.1, 141.4, 177.5, 178.9. IR (KBr, ν, cm^{-1}): 3283, 1681, 1615, 1595. MS (ESI): 278.0 ($C_{18}H_{15}NO_2$ $[M+H]^+$). HRMS for ($C_{18}H_{15}NO_2$ $[M+H]^+$). Calcd: 277.1103. Found: 278.1156.

*4.1.2.1.11. 2-Chloro-3-(2,5-dimethoxyphenylamino) naphthalene-1,4-dione (**4k**).* Purple solid. Yield by method ii (188 mg, 83%), mp 146–149 °C (lit. 146 °C).[29] ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.77 (s, 3H), 3.81 (s, 3H), 6.57 (d, J = 2.8 Hz, 1H), 6.71 (dd, J = 2.9, 8.9 Hz, 1H), 6.82 (d, J = 8.9 Hz, 1H), 7.61 (s, 1H), 7.68 (t, J = 7.4 Hz, 1H), 7.76 (t, J = 7.5 Hz, 1H), 8.10 (d, J = 7.6 Hz, 1H), 8.19 (d, J = 7.6 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ ppm: 55.9, 56.1, 110.9, 111.3, 111.4, 115.1, 126.9, 127.0, 127.1, 130.0, 132.6, 132.9, 134.9, 141.7, 146.8, 152.9, 177.4, 180.4. IR (KBr, cm^{-1}): 3314, 1676, 1655, 1576. MS (ESI): 345.0 ($C_{18}H_{14}ClNO_4$ $[M+H]^+$).

*4.1.2.1.12. 2-Chloro-3-(4-(hexyloxy)phenylamino) naphthalene-1,4-dione (**4l**).* Purple solid. Yield by method ii (233 mg, 92%), mp 130–133 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.91 (t, J = 6.9 Hz, 3H), 1.28–1.41 (m, 4H), 1.46 (m, 2H), 1.73–1.86 (m, 2H), 3.96 (t, J = 6.6 Hz, 2H), 6.85 (d, J = 8.8 Hz, 2H), 7.03 (d, J = 8.8 Hz, 2H), 7.60–7.70 (m, 2H), 7.74 (td, J = 1.0, 7.6 Hz, 1H), 8.08 (d, J = 7.5 Hz, 1H), 8.16 (d, J = 7.5 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ ppm: 14.0, 22.6, 25.7, 29.2, 31.6, 68.3, 113.4, 126.3, 126.9, 127.0, 127.8, 129.8, 130.1, 132.7, 132.8, 134.7, 135.0, 141.8, 157.4, 177.3, 180.6. IR (KBr, cm^{-1}): 3224, 1677, 1634, 1606. MS (ESI): 385.0 ($C_{22}H_{22}ClNO_3$ $[M+H]^+$). HRMS for ($C_{22}H_{22}ClNO_3$ $[M+H]^+$). Calcd: 383.1288. Found: 384.1334.

*4.1.2.1.13. 2-(Naphthalen-2-ylamino)naphthalene-1,4-dione (**4m**).* Orange solid. Yield by method ii (210 mg, 74%), mp 189–191 °C. (lit 191–192 °C).[46] ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 6.59 (s, 1H); 7.37 (dd, J = 1.8, 8.7 Hz, 1H), 7.47–7.52 (m, 2H), 7.68 (t, J = 7.5 Hz, 1H) 7.71–7.86 (m, 5H), 7.88 (d, J = 8.7 Hz, 1H) 8.14 (t, J = 6.8 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ ppm: 103.8, 119.3, 121.6, 125.9, 126.2, 126.6, 127.1, 127.5, 127.8, 129.8, 130.4, 131.1, 132.4, 133.3, 133.8, 134.9, 144.5, 182.1, 184.0. IR (KBr, cm^{-1}): 3301, 1668, 1627, 1596. MS (ESI): 300.0 ($C_{20}H_{13}NO_2$ $[M+H]^+$).

*4.1.2.1.14. 2-(Naphthalen-1-ylamino)naphthalene-1,4-dione (**4n**).* Red solid. Yield by method ii (156 mg, 55%), mp 156–158 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 6.00 (s, 1H), 7.43–7.61 (m, 4H), 7.66–7.82 (m, 4H), 7.91 (d, J = 3.8 Hz, 2H) 8.09 (d, J = 7.3 Hz, 1H). 8.17 (d, J = 7.3 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ ppm: 103.9, 121.8, 122.4, 125.6, 126.2, 126.5, 126.8, 127.0, 127.4, 128.8 (2C), 130.5, 132.3, 132.9, 133.3, 134.6, 134.9, 146.5, 182.2, 183.8. IR (KBr, cm^{-1}): 3343, 1669, 1640, 1610. MS (ESI): 299.0 ($C_{20}H_{13}NO_2$ $[M+H]^+$).

4.1.2.2. General synthetic procedure for benzocarbazolequinones 5a–5g. *Method iii*: In a 25 mL two-necked flask fitted with a coil condenser and kept under a dry inert atmosphere, 50 mg (0.17 mmol) of the corresponding phenylaminonaphthoquinone, 18.4 mg (0.17 mmol) of 1,4-benzoquinone and $Pd(OAc)_2$ (38 mg, 0.15 mmol) were poured into 10 mL of glacial AcOH and the resulting suspension was refluxed for about 24 h. The hot suspension was filtered and the AcOH was removed in rotary evaporator. The solid residue was purified by recrystallization with CH_2Cl_2 in some cases and in others by column chromatography on silica gel.

Method iv: The corresponding naphthoquinone (0.5 mmol), $Pd(OAc)_2$ (2–10 mol %), K_2CO_3 (10 mol %) and pivalic acid (PivOH, 450 mg, 0.44 mmol) were mixed in a test tube which was immersed in an oil bath at 110–120 °C. The PivOH melted and the mixture was stirred for 14–48 h at this temperature. The solution was allowed to cool to room temperature, diluted with CH_2Cl_2, washed with saturated Na_2CO_3 solution, dried with anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The pure product was purified by column chromatography on silica gel.

*4.1.2.2.1. 5H-Benzo[b]carbazole-6,11-dione (**5a**).* Orange solid. Yield by method iii (21 mg, 43%). Yield by method iv (62 mg, 50%), mp 305–306 °C. (lit. 307–310).[47] ^{1}H NMR (400 MHz, DMSO) δ ppm: 7.36 (t, J = 7.5 Hz, 1H), 7.44 (t, J = 7.6 Hz, 1H), 7.59 (d, J = 8.2 Hz, 1H), 7.82 (m, 2H), 8.09 (t, J = 7.0 Hz, 2H), 8.20 (d, J = 7.9 Hz, 1H), 13.04 (s, 1H). ^{13}C NMR (DMSO) δ ppm: 114.3, 117.8, 122.8, 124.3, 124.4, 126.4, 126.5, 127.4, 133.1, 133.6, 134.5, 134.7, 137.6, 138.7, 178.0, 180.8. IR (KBr, cm^{-1}): 3254, 1663, 1645, 1621.

*4.1.2.2.2. 4-Methoxy-5H-benzo[b]carbazole-6,11-dione (**5b**).* Orange solid. Yield by method iii (32 mg, 67%). Yield by method iv (69 mg, 50%), mp 253–255 °C (lit. >250 °C).[48] ^{1}H NMR (400 MHz,

DMSO) δ ppm: 3.97 (s, 3H), 6.98 (d, J = 7.7 Hz, 1H), 7.28 (t, J = 7.9 Hz, 1H), 7.74–7.88 (m, 3H), 8.09 (ddd, J = 1.6, 5.8, 7.2 Hz, 2H), 13.23 (s, 1H). ^{13}C NMR (DMSO) δ ppm: 56.1, 107.2, 114.7, 118.3, 125.4, 125.9, 126.4, 126.5, 129.6, 133.2, 133.7, 134.4, 134.6, 137.4, 147.9, 177.6, 181.0. IR (KBr, cm^{-1}): 3273, 1654, 1267.

*4.1.2.2.3. 3-Methoxy-5H-benzo[b]carbazole-6,11-dione (**5c**).* Orange solid. Yield by method iii (10 mg, 21%). Yield by method iv (55 mg, 40%), mp 294–296 °C. ^{1}H NMR (400 MHz, DMSO) δ ppm: 3.86 (s, 3H), 7.09 (dd, J = 2.5, 9.0 Hz, 1H), 7.50 (d, J = 9.0 Hz, 1H), 7.62 (d, J = 2.4 Hz, 1H), 7.81 (td, J = 1.4, 7.4 Hz, 1H), 7.86 (td, J = 1.5, 7.4 Hz, 1H), 8.10 (td, J = 1.2, 7.7 Hz, 2H), 13.00 (s, 1H). ^{13}C NMR (DMSO) δ ppm: 55.8, 102.6, 115.5, 117.5, 118.9, 125.3, 126.5 (2C), 133.2, 133.6, 134.0, 134.6, 134.7, 137.5 157.4, 177.7, 180.7. IR (KBr, cm^{-1}): 3202, 1664, 1635, 1263. MS (ESI): 278.0 ($C_{17}H_{11}NO_3$ [M+H]$^+$). HRMS for ($C_{17}H_{11}NO_3$ [M+H]$^+$). Calcd: 277.0739. Found: 278.0793.

*4.1.2.2.4. 2-Methoxy-5H-benzo[b]carbazole-6,11-dione (**5d**).* Red solid. Yield by method iii (19 mg, 40%). Yield by method iv (111 mg, 80%), mp 298–300 °C. (lit. 299–300 °C).[49] ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.94 (s, 3H), 7.06 (dd, J = 2.4, 8.8 Hz, 1H), 7.43 (d, J = 8.8 Hz, 1H), 7.13 (dd, J = 1.2, 7.6 Hz, 1H), 7.71 (m, 2H), 7.76 (d, J = 2.4 Hz, 1H), 8.22 (dd, J = 1.2, 7.6 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ ppm: 55.6, 102.6, 114.1, 119.2, 126.1, 126.5, 132.7, 132.9, 133.4, 133.8, 133.9, 134.7, 136.8, 136.9, 157.4, 170.8, 173.8. IR (KBr, cm^{-1}): 3242, 1667, 1642, 1526. MS (ESI): 278.0 ($C_{17}H_{11}NO_3$ [M+H]$^+$).

*4.1.2.2.5. 1,4-Dimethoxy-5H-benzo[b]carbazole-6,11-dione (**5e**).* Orange solid. Yield by method iii (21 mg, 40%). Yield by method iv (115 mg, 75%), mp 286–288 °C. ^{1}H NMR (400 MHz, DMSO) δ ppm: 3.92 (s, 3H), 4.00 (s, 3H), 6.67 (d, J = 2.0 Hz, 1H), 7.27 (d, J = 2.0 Hz, 1H), 7.84–7.94 (m, 2H), 8.12–8.18 (m, 2H), 13.26 (s, 1H) ^{13}C NMR ($CDCl_3$) δ ppm: 55.9, 56.3, 99.7, 118.0, 125.3, 126.0, 126.4, 126.5, 133.4, 133.6, 134.5, 137.0, 148.5, 158.7, 177.2, 180.9. IR (KBr, cm^{-1}): 3254, 1663, 1645, 1621. MS (ESI): 308.0 ($C_{18}H_{13}NO_4$ [M+H]$^+$). HRMS for ($C_{18}H_{13}NO_4$ [M+H]$^+$). Calcd: 307.0845. Found: 308.0897.

*4.1.2.2.6. 2-Hexyloxy-5H-benzo[b]carbazole-6,11-dione (**5f**).* Orange solid. Yield by method iii (40 mg, 67%). Yield by method iv (87 mg, 50%), mp 186–187 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0,85 (m, 3H), 1.18 (m, 6H), 1.76 (m, 2H), 3.95 (s, 2H), 6.77 (d, J = 8.0 Hz, 1H), 6.96 (d, J = 8.8 Hz, 1H), 7.40 (d, J = 8.8 Hz, 1H), 7.45 (s, 1H), 7.83 (d, J = 8.0 Hz, 1H), 8.12 (d, J = 8.8 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ ppm: 13.5, 24.1, 25.1, 28.6, 29.0, 72.8, 103.7, 109.0, 114.6, 118.0, 125.2, 125.8, 130.4, 151.5, 152.4, 158.2, 176.6, 180.8. IR (KBr, cm^{-1}): 3264, 2955, 2925, 2852, 1664, 1261. HRMS for ($C_{22}H_{21}NO_3$ [M+H]$^+$). Calcd: 347.1521. Found: 348.1571.

*4.1.2.2.7. 2,3-Dimethoxy-5H-benzo[b]carbazole-6,11-dione (**5g**).* Purple solid. Yield by method iii (22 mg, 42%), mp 275–276 °C. (lit. 275–277 °C).[37] ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.92 (s, 3H), 3.93 (s, 3H), 7.04 (s, 1H), 7.62 (s, 1H), 7.83–7.91 (m, 2H), 8.12–8.15 (m, 2H), 12.92 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 56.1 (2C), 95.6, 102.3, 117.9, 126.4 (2C), 133.5, 133.6 (2C), 134.2, 134.4, 134.5, 135.8 149.2, 151.4, 176.6, 181.0. IR (KBr, cm^{-1}): 3248, 1643, 1588.MS (ESI): 308.0 ($C_{18}H_{13}NO_4$ [M+H]$^+$).

4.1.2.3. General synthetic procedure for *N*-methyl-benzocarbazolediones (6a–b). To a solution of benzocarbazoledione **5c** (140 mg, 0.50 mmol) or **5g** (154 mg, 0.50 mmol) in 15 mL of EtOH in a 25 mL flask was added 4.42 mmol of KOH pellets and the mixture was stirred at room temperature until complete dissolution. The EtOH was removed under vacuum and the residue was treated with acetone (15 mL) and methyl iodide (3.54 mmol). The product obtained was purified by column chromatography on silica gel.

*4.1.2.3.1. 3-Methoxy-5-methyl-5H-benzo[b]carbazole-6,11-dione (**6a**).* Orange solid. Yield (124 mg, 85%), mp 246–249 °C. (lit. 250–253 °C).[31] ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.91 (s, 3H), 4.19 (s, 3H), 6.76 (s, 1H), 7.02 (d, J = 8.2 Hz, 1H), 7.68 (br s, 2H); 8.15 (m, 2H), 8.29 (d, J = 8.7 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ ppm: 32.0, 55.6, 92.6, 100.0, 115.7, 118.1, 119.3, 124.7, 126.1, 126.3, 132.8, 133.4, 133.7, 134.0, 134.5, 141.5, 160.2, 178.5, 181.4. IR (KBr, cm^{-1}): 3448, 1655, 1618, 1513. MS (ESI): 292.0 ($C_{18}H_{13}NO_3$ [M+H]$^+$).

*4.1.2.3.2. 2,3-Dimethoxy-5-methyl-5H-benzo[b]carbazole-6,11-dione (**6b**).* Orange solid. Yield (146 mg, 91%), mp 278–280 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.98–4.09 (m, 6H), 4.23 (br s, 3H), 6.79 (d, J = 5.5 Hz, 1H), 7.68 (s, 2H), 7.82 (d, J = 5.5 Hz, 1H), 8.17 (d, J = 6.4 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ ppm: 32.2, 56.2, 56.3, 92.2, 102.9, 117.5, 118.9, 120.7, 126.0, 126.3, 132.8, 133.3, 133.6, 134.0, 135.7, 149.3, 151.5, 177.9, 181.6. IR (KBr, cm^{-1}): 3424, 1649, 1590, 1508. MS (ESI): 322.0 ($C_{19}H_{15}NO_4$ [M+H]$^+$).

4.1.2.4. Synthetic procedure for 7-((2,5-dimethoxyphenyl)amino)quinoline-5,8-dione (10). A solution of **8** (6.38 mmol)[34] in 100 mL EtOH was added to a solution of amine **9** (18.8 mmol) and $NiCl_2$ (3.14 mmol) in 50 mL EtOH. The reaction mixture was stirred at 30 °C in the presence of air for 12 h, and was monitored by thin layer chromatography. After the reaction was completed, the EtOH was removed under reduced pressure leaving a residue to which was added a cold aqueous 1% solution of HCl in order to decompose the Ni complex. The product was extracted repeatedly with $CHCl_3$ and then with water, dried with Na_2SO_4 and the solvent removed. The product obtained was purified by column chromatography on silica gel. Purple solid, yield (1.68 g, 85%), mp 70–80 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.80 (s, 3H), 3.88 (s, 3H), 6.71 (br s, 1H), 6.89 (d, J = 7.2 Hz, 1H), 7.02 (s, 1H), 7.53–7.71 (m, 2H), 7.98 (S, 1H), 8.45 (d, J = 4.7 Hz, 1H), 9.05 (s, 1H). ^{13}C NMR ($CDCl_3$) δ ppm: 55.9, 56.2, 104.9, 108.1, 109.8, 111.9, 126.4, 127.1, 127.4, 134.4, 143.4, 145.5, 148.9, 153.8, 155.2, 181.8, 182.2. IR (KBr, cm^{-1}): 3328, 1673, 1630, 1570. HRMS for ($C_{17}H_{14}N_2O_4$ [M+H]$^+$). Calcd: 310.0954. Found: 311.1075.

4.1.2.5. 6-((2,5-Dimethoxyphenyl)amino)-2,2-dimethyl-2,3-dihydrobenzofuran-4,7-dione (16). According to method ii, the product was prepared in a flask containing 10 mL of ethanol, 100 mg (0.39 mmol) of quinone **15**, 119 mg (0.78 mmol) of 2,5-dimethoxyaniline and 75 mg (0.20 mmol) of $CeCl_3$*$7H_2O$. The mixture was stirred for 2 h to room temperature and then solvent was removed under reduced pressure. The crude product was purified by chromatographic column using a mixture of $CHCl_3$/AcOEt (4/1) as mobile phase. Purple solid, yield (78 mg, 61%), mp: 82–84 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 1.57 (s, 6H), 2.89 (s, 2H), 3.77 (s, 3H), 3.85 (s, 3H), 6.06 (s, 1H), 6.65 (dd, J = 2.9, 8.9 Hz, 1H), 6.65 (dd, J = 2.9, 8.9 Hz, 1H), 6.86 (d, J = 8.9 Hz, 1H), 6.95 (d, J = 2.8 Hz, 1H), 8.16 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 28.33, 39.39, 55.90, 56.24, 93.25, 97.76, 107.83, 109.80, 111.76, 115.05, 127.39, 144.35, 145.53, 153.77, 161.57, 179.13, 179.52. IR (KBr, cm^{-1}) 3308, 1655, 1614, 1576, 1208, 1111. HRMS for ($C_{18}H_{19}NO_5$ [M+H]$^+$). Calcd: 329.1263. Found: 330.1312.

4.1.2.6. 5,6-Dihydro-1,6,6-trimethyl-1H-furo[3,2-f]indazole-4,8-dione (17). In a flask containing 15 mL Et_2O and 200 mg (1.12 mmol) of quinone **15**, cooled at 0 °C, it was added an excess of diazomethane. The reaction was left under magnetic stirring at 0 °C for 15 min. The solvent was the removed under reduced pressure and the reaction mixture was purified by recrystallization in MeOH. Orange solid, yield (174 mg, 67%), mp 205–206 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 2.17 (s, 6H), 2.91 (s, 2H), 4.20 (s, 3H); 7.83 (s, 1H). ^{13}C NMR ($CDCl_3$) δ ppm: 28.3, 30.9, 38.8, 39.3, 93.4 120.4, 120.7, 136.5, 160.5, 173.8 175.3. IR (KBr, cm^{-1}): 3448, 1681, 1645. MS (ESI): 232.0 ($C_{12}H_{12}N_2O_3$ [M+H]$^+$). HRMS for ($C_{12}H_{12}N_2O_3$ [M+H]$^+$). Calcd: 232.0848. Found: 233.0904.

4.2. Biology

4.2.1. Trypanocidal assay

4.2.1.1. Cell viability assay. The effect of drug treatments on *T. cruzi* and Vero cells was evaluated using the MTT assay as a viability test.[49] Briefly, 10 μL of 5 mg/mL tetrazolium dye (MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) plus 0.22 mg/mL phenazine methosulfate (electron carrier) were added to each well of a 96-well plate containing Vero cells or epimastigotes in 100 μL RPMI 1640 without phenol red. Drugs, dissolved in DMSO, were added to culture media at the concentrations shown in figures and tables. DMSO final concentration was less than 0.25% v/v. After incubation for 4 h at 37 °C or 28 °C (for Vero cells or epimastigotes, respectively), the water-insoluble formazan generated was dissolved by addition of 100 μL of 10% w/v SDS in 0.01 M HCl. The plates were further incubated overnight at 37 °C, and optical density (OD) of the wells was determined using a microplate reader (Asys Hitech, Austria) at 570 nm. Under these conditions, the OD is directly proportional to the viable cell number in each well. All experiments were performed at least three times and data are shown as the means and their standard deviations from triplicate cultures.

4.2.1.2. Epimastigote culture growth. Epimastigotes of the Dm28c strain were grown at 28 °C in modified Diamond's medium as described previously.[50] The cultures were initiated with a cell density of 3×10^6 epimastigotes/mL. Cell density was measured by direct counting with a hemocytometer. Compounds dissolved in DMSO were added to the epimastigote suspension, with a final concentration of DMSO below 1%. After 24 h, viability of the epimastigotes was determined by MTT reduction as described above.

4.2.1.3. *T. cruzi* trypomastigotes. Vero cells (ATCC® CCL-81™) were infected with bloodstream trypomastigotes from *T. cruzi*-infected Balb/c mice. Vero cell cultures infected with trypomastigotes were incubated at 37 °C in RPMI medium (5% FBS), in humidified air and 5% CO_2 for 5–7 days. After that time, the culture medium was collected and centrifuged at 3000×*g* for 5 min, and the trypomastigote-containing pellet was resuspended in RPMI supplemented with 5% fetal bovine serum and penicillin–streptomycin at a final density of 10^7 parasites/mL.[51] IC_{50} values were obtained by non-linear regression fitting data to a four-parameter equation model, using GraphPad Prism 5.0 software.

4.2.2. Viability or cytotoxic assay

4.2.2.1. Cell lines. The experimental cell cultures were obtained from the American Type Culture Collection (Rockville, MD, USA). MCF-7 and MDA-MB231 cells (estrogen receptor-positive and -negative human breast cancer cells, respectively), PC-3 cells (prostate cancer cells) and non-tumoral dermal human fibroblasts (DHF) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin and 1 mM glutamine. Cells are seeded into 96-well microtiter plates in 100 μL at a plating density of 3×10^3 cells/well. After 24 h incubation at 37 °C under a humidified 5% carbon dioxide atmosphere to allow cell attachment, the cells were treated with different concentrations of drugs and incubated for 72 h under the same conditions. Stock solutions of compounds were prepared in DMSO and the final concentration of this solvent was kept constant at 0.1%. Control cultures received DMSO alone.

4.2.2.2. In vitro growth inhibition assay. The sulforhodamine B assay was used according to the method of Skehan et al. 1990,[52] with some modifications.[53] Briefly, the cells were set up $2–3 \times 10^3$ cells per well of a 96-well, flat-bottomed 200 μL microplate. Cells were incubated at 37 °C in a humidified 5% CO_2/95% air mixture and treated with the compounds at different concentrations for 72 h. At the end of drug exposure, cells were fixed with 50% trichloroacetic acid at 4 °C (final concentration 10%). After washing with water, cells were stained with 0.4% sulforhodamine B (Sigma–Aldrich, St. Louis, MO), dissolved in 1% acetic acid (50 μL/well) for 30 min, and subsequently washed with 1% acetic acid to remove unbound stain. The protein-bound stain was solubilized with 100 μL of 10 mM unbuffered Tris base, and the cell density was determined using a fluorescence plate reader (wavelength 540 nm). Values shown are the mean ± SD of three independent experiments in triplicate.

4.3. Computational

4.3.1. Ligands

The geometries of all ligands involved in this study were fully optimized using the Hartree–Fock method with the standard basis set, 6-31G*. Electrostatic potential (ESP) charges were derived from the calculations. All calculations were performed using the Gaussian03 suite.[54] Druglikeness properties were predicted for all compounds using from Molsoft LLC and Visual Molecular Dynamics program (VMD).[55]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.07.030.

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