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Synthesis and studies of the antifungal activity of 2-anilino-/2,3-dianilino-/2-phenoxy- and 2,3-diphenoxy-1,4-naphthoquinones

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Abstract Several synthetic and natural naphthoquinone derivatives have been associated with antifungal activity. *Candida albicans* is a fungus that is known to exist in the normal human flora, but under certain conditions it can cause mild to fatal infections. Its pathogenicity has been associated with fungus conversion from cellular yeast to filamentous form **Y**–**M**. Inhibition of this process by several anilino-, dianilino-, phenoxy-, and diphenoxy-1,4-naphthoquinones was investigated in order to find some correlation between structure, redox properties and biological activity.

Keywords 1,4-Naphthoquinone derivatives · *Candida albicans* · Antifungal compounds · Reduction potential

Introduction

Several synthetic and natural organic compounds containing a quinone moiety in their structure have been associated with different biological activities. In some cases, the biological activity of quinones has been related to their redox properties and their capacity to accept one or two electrons to form the corresponding radical anion ($Q^{\cdot -}$) and hydroquinone radical dianion (Q^{2-}). These intermediate species interact with crucial cellular molecules such as oxygen, DNA and proteins, modifying their biological properties [1–3]. Therefore, studies on the synthesis,

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structure and physicochemical properties of novel quinone compounds with more selective biological activity are basic for future medicinal applications.

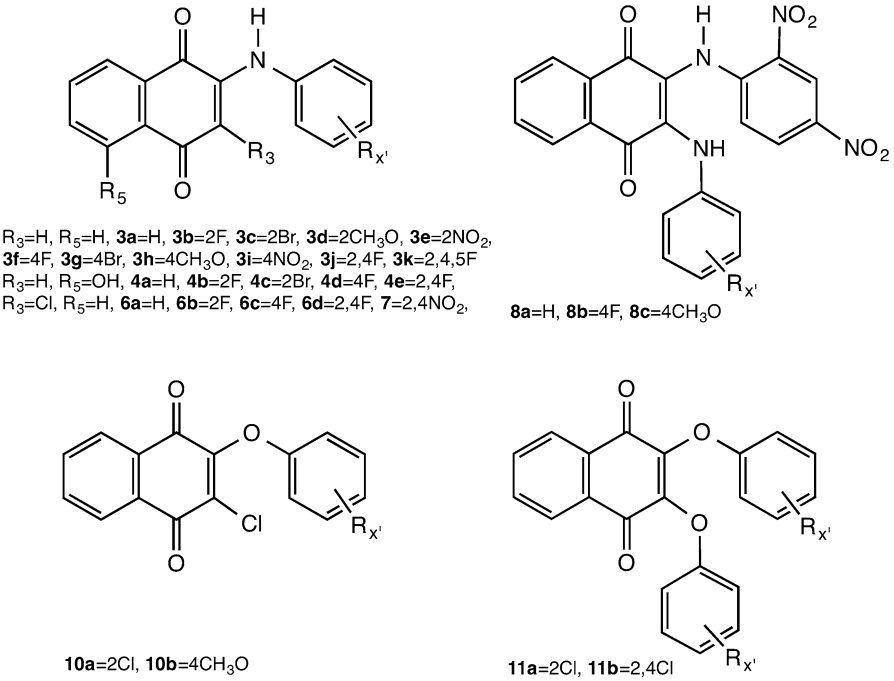
1,4-Naphthoquinones, possessing a substituent like an anilino, phenoxy or arylthio group in the two position, have been the subject of intensive research. Many of them find use in a variety of medicinal and biological applications, such as antituberculars [4], antimalarials [5, 6], antibacterials [7, 8], antitumor agents [9, 10], larvicides, herbicides and fungicides [11–14]. It has been indicated that the presence of a nitrogen, sulphur or oxygen atom allows modulation of the substituent effects on the electronic properties of the quinone system.

Candida albicans is a dimorphic fungus that is known to exist in the normal human flora [15]. However, it could cause mild to fatal opportunistic infections and it is particularly dangerous in people with a compromised immune system such as patients with cancer or AIDS [16, 17]. It is the most common fungal pathogen associated with cancer. In general, the term candidiasis involves a wide range of infections that range from minor vaginitis [18, 19] to systemic and quite life-threatening diseases. There are three major forms of the disease: genital, oral, and invasive or deep candidiasis. In this latter form, the fungus invades internal organs and it is associated with a mortality rate of 40–60 % [20–24].

Its pathogenicity has been associated with fungus conversion from cellular yeast to filamentous form **Y–M** [25]. Patients with severe candidiasis generally have filaments of *C. albicans* penetrating the infected tissue [26, 27]. Filamentous penetration through the gastrointestinal tract is most likely the origin of the majority of deep or invasive candidiasis. In the laboratory, *C. albicans* can be converted from the cellular yeast to the filamentous form **Y–M** under certain conditions. Therefore, this in vitro experiment could provide a convenient methodology to test potential compounds aimed to inhibit this highly pathogenic conversion [28–32]. Since there are only a couple of reports on this type of study, we investigated the activity of several anilino and phenoxy derivatives of 1,4-naphthoquinone (Scheme 1) to inhibit *C. albicans* conversion to **Y–M**.

Results and discussion

In a general method to prepare 2-anilino-1,4-naphthoquinone, a substituted aniline is added, in a 1,4-type manner, to the naphthoquinone structure [33, 34]. However, this methodology is unpractical and requires tedious chromatographic purifications since it gives low yields with aromatic amines containing electron-withdrawing groups [35]. There are some reports on the use of Lewis acids to catalyze this reaction [36, 37]. In recent studies, we reported the preparation of novel 2-fluoroanilino and 2-nitroanilino derivatives of 1,4-naphthoquinones in the presence of a Lewis acid and strong oxidation agent such as $CeCl_3$ or $FeCl_3$ [38–41]. The catalyst facilitates an oxidative coupling between a given aniline and naphthoquinone and, as a consequence, the reaction is cleaner. Several 2-anilino-1,4-naphthoquinones **3a–k** were prepared (Scheme 2) following the procedure previously reported by us [38–41]. An ethanolic solution of 1,4-naphthoquinone and a catalyst ($CeCl_3$ or $FeCl_3$) was prepared and stirred to activate the quinone. Then,



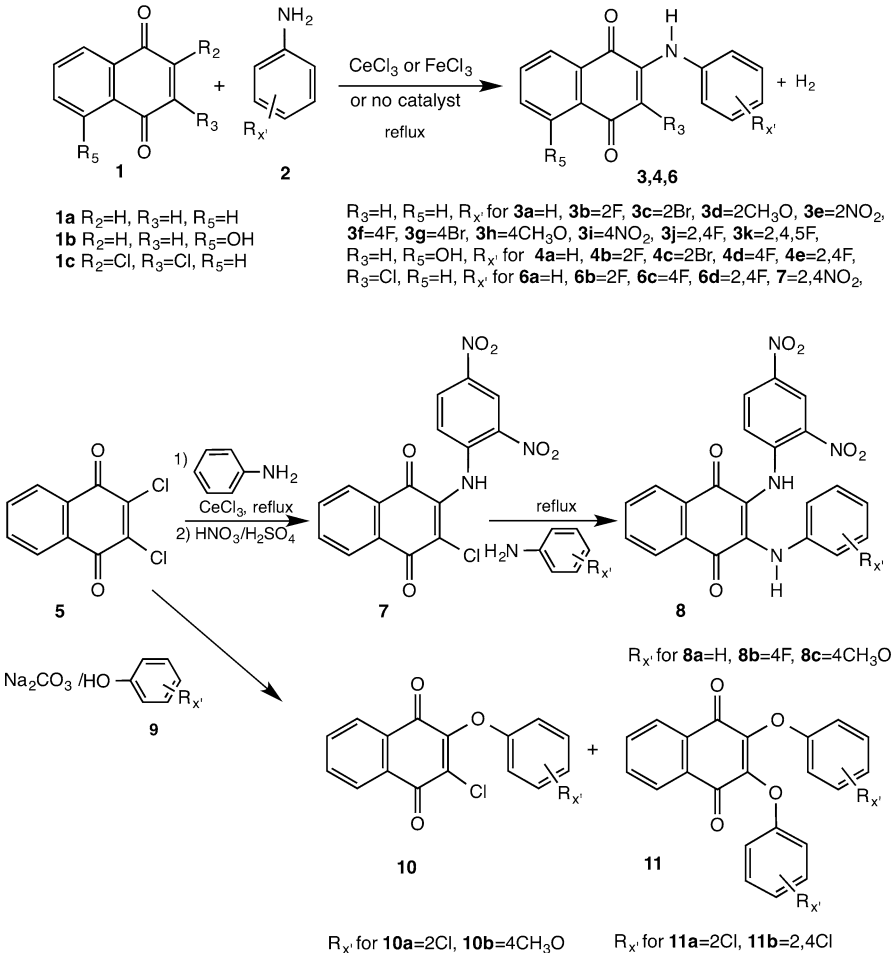
Scheme 1 Structure of 1,4-naphthoquinone derivatives

an ethanolic solution of aniline was added and the reaction mixture was refluxed for several hours. The solid obtained was filtrated, washed with cold ethanol and recrystallized.

A number of 5-hydroxy-1,4-naphthoquinones display different biological properties including antimalarial, antibacterial, and antifungal activity [11]. Therefore, we also synthesized a series of 5-hydroxy-2-(anilino)-1,4-naphthoquinones **4a–e** following the same procedure (Scheme 2) previously described [38, 39]. In this case, the 5-hydroxy substituent also favors the formation of one adduct with only trace amounts of other secondary products.

Another method to prepare 2-anilino-1,4-naphthoquinones consists of the nucleophilic displacement of an already halogenated 1,4-naphthoquinone. We performed the preparation of 2-chloro-3-(anilino)-1,4-naphthoquinones **6a–6d** (Scheme 2) following the procedure reported in the literature [42] with some modifications [43]. 2,3-Dichloro-1,4-naphthoquinone **5** (1 mol) was dissolved in methanol and a large excess of aniline (2–4 mol) was added. The solution was refluxed for several hours (2–10) to obtain a solid crystalline product.

Dianilino derivatives **8a–c** were prepared in two steps (Scheme 2). 2-Anilino-3-chloro-1,4-naphthoquinone **6a** was nitrated with a mixture of concentrated acids (HNO_3 and H_2SO_4) to give the 2-chloro-3-(2,4-dinitroanilino)-1,4-naphthoquinone **7** [44]. To achieve the reaction of this latter quinone (1 mol) with aniline, 4-methoxy or 4-fluoroaniline, an excess of the amine (15 mol) was required with a reflux in methanol for several hours (10–15) [45].



Scheme 2 Synthesis of 1,4-naphthoquinone derivatives

Since the presence of chlorine atoms and phenoxyl groups on the structure of quinone has been demonstrated to improve antifungal activity [11], some chlorophenoxy-naphthoquinone derivatives **10a–b** and **11a–b** (Scheme 2) were prepared following the literature procedure with some modifications [46]. 2,3-Dichloro-1,4-naphthoquinone was added to a mixture of a substituted phenol and sodium carbonate in a minimal amount of DMSO. The reaction mixture was stirred at room temperature (RT). The pure products were separated by column chromatography.

Biological activity

Several naphthoquinone derivatives have shown potent antifungal activity against pathogenic fungi [11]. However, there are no previous reports on their activity to

inhibit morphological transition of *C. albicans* from yeast to mycelium **Y–M** [47]. The minimum concentration of 1,4-naphthoquinone derivatives required to inhibit this **Y–M** conversion was determined by microscopic observation. *C. albicans* cells were incubated in Lee's medium [48] at regular human temperature (37 °C). At the initial time (Fig. 1a), *C. albicans* cells showed an ovoid structure (5–7 μm in diameter). After 30–45 min of incubation (Fig. 1b), blastospores showed the first emission growth, that eventually gave rise to germ tube formation (Fig. 1c). Germ tube growth was characterized by the appearance of septa and a continued elongation (Fig. 1d) with a length 5–6 times the diameter of a blastospore at 3 h of incubation (Fig. 1e).

The 1,4-naphthoquinone derivatives studied **3a–11b** presented different degrees of inhibition activity against **Y–M** conversion (Table 1). A series of 2-anilino compounds **3a–3k** were tested. For some of the compounds, having a substituent in an *ortho* or *para* position (either electron donor or electron acceptor) had a very minor or no effect on the inhibition since the minimum inhibitory concentration (MIC) values were similar to the ones obtained with unsubstituted aniline **3a**. Exceptions are compounds **3b** and **3j** that have a fluorine atom in the *ortho* position and have MIC values of 31.25 and 1.25, respectively.

These results on biological activity are quite interesting since they present some correlation with our recent studies on spectral and electrochemical characterization of 2-(fluoroanilino)-1,4-naphthoquinones [38, 39]. UV–Vis spectral analysis of several derivatives indicated the existence of an intramolecular electronic transfer from the fluoro substituent on aniline to the 1,4-naphthoquinone moiety. In agreement with this donor–acceptor character, the cyclic voltammograms gave two one-electron reduction waves to the corresponding anion ($E^{I}_{1/2}$) and dianion ($E^{II}_{1/2}$) intermediates.

In general, fluoro substituents reduced the electron density of the electroactive naphthoquinone and facilitated the reduction process. In halogenated compounds,

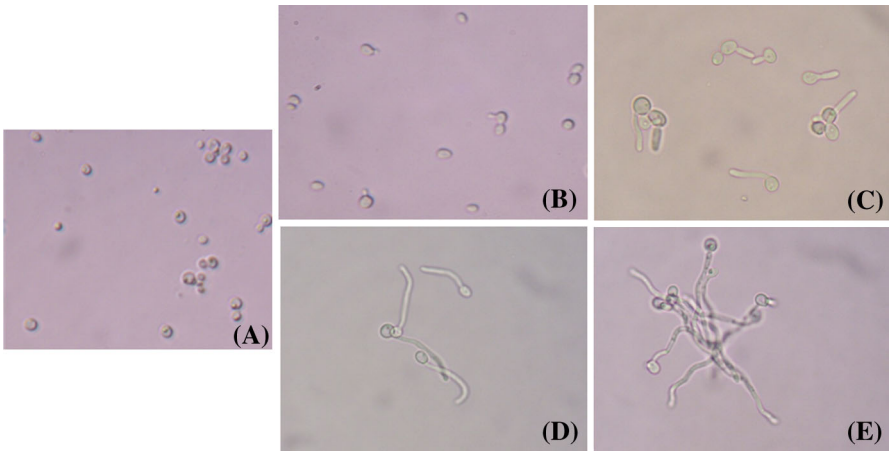


Fig. 1 *Candida albicans* mycelia growth under microscopic observation (magnification ×40). Conditions 37 °C and 200 rpm shaking in Lee's medium. **a** Initial time, **b** 30–45 min, **c** 1 h, **d** 2 h and **e** 3 h of incubation

Table 1 Structures and inhibition of *C. albicans* **Y–M** conversion for 1,4-naphthoquinone derivatives

Compound	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	$R_{6'}$	R_3	R_5	MIC[a] (μg/mL) *C. albicans*[b]
3a	H	H	H	H	H	H	H	500.00
3b	F	H	H	H	H	H	H	31.25
3c	Br	H	H	H	H	H	H	500.00
3d	CH_3O	H	H	H	H	H	H	500.00
3e	NO_2	H	H	H	H	H	H	na[c]
3f	H	H	F	H	H	H	H	250.00
3g	H	H	Br	H	H	H	H	250.00
3h	H	H	CH_3O	H	H	H	H	500.00
3i	H	H	NO_2	H	H	H	H	500.00
3j	F	H	F	H	H	H	H	1.95
3k	F	H	F	F	H	H	H	125.00
4a	H	H	H	H	H	H	OH	31.25
4b	F	H	H	H	H	H	OH	7.80
4c	Br	H	H	H	H	H	OH	250.00
4d	H	H	F	H	H	H	OH	125.00
4e	F	H	F	H	H	H	OH	62.50
6a	H	H	H	H	H	Cl	H	62.50
6b	F	H	H	H	H	Cl	H	15.62
6c	H	H	F	H	H	Cl	H	250.00
6d	F	H	F	H	H	Cl	H	125.00
7	NO_2	H	NO_2	H	H	Cl	H	125.00
8a	H	H	H	H	H	R_3^d	H	31.25
8b	H	H	F	H	H	R_3^d	H	31.25
8c	H	H	CH_3O	H	H	R_3^d	H	62.50
10a	Cl	H	H	H	H	Cl	H	3.90
10b	H	H	CH_3O	H	H	Cl	H	250.00
11a	Cl	H	H	H	H	R_3^e	H	1.95
11b	Cl	H	Cl	H	H	R_3^f	H	0.97

[a] The MIC value was defined as the lowest concentration of the naphthoquinone derivative required to inhibit the conversion of yeast to mycelium **Y–M** of *Candida albicans*

[b] Fungi tested: *Candida albicans* ATCC 26555

[c] na: No activity

[d] R_3: 2,4-dinitroanilino

[e] R_3: 2-chlorophenoxy

[f] R_3: 2,4-dichlorophenoxy

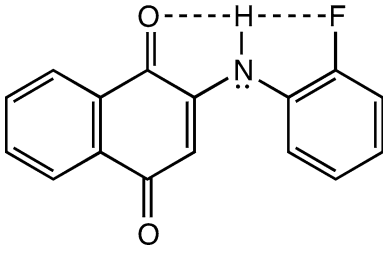
this effect is modulated by simultaneous electron attracting and donor properties of the substituents [49]. Thus, strong electron-withdrawing substituents like fluoro and chloro displaced the redox potentials to less negative values and made the reduction of a substituted quinone easier than that of 1,4-naphthoquinone. Furthermore, a slightly larger effect is observed when an *ortho*-fluoro substituent is present in the

aniline due to an intramolecular bifurcated or three-center hydrogen bond (Scheme 3) [50]. Therefore, the biological activity of 2-(fluoroanilino)- and 2-(2,4-difluoroanilino)-1,4-naphthoquinones could be related to their redox properties (Table 2) and the presence of an intermolecular hydrogen bond. These physicochemical properties enhance their capacity to form intermediate species (radical anion or dianion) that easily interact with different cellular molecules such as oxygen, DNA and proteins.

It has been reported that the presence of a hydroxyl group adjacent to the carbonyl group on 1,4-naphthoquinone affects its biological properties [51]. In general, 5-hydroxy-1,4-naphthoquinone derivatives **4a–d** showed improved activity as inhibitors of **Y–M** transition in comparison with non-hydroxylated analogues **3a–c** and **3f**. As an example, **4b** gave a smaller MIC (7.80 μg/mL) relative to the one observed (31.25 μg/mL) for **3b**. Once again, an intramolecular hydrogen bond between the phenolic hydrogen and the quinone oxygen might explain some of these findings. The introduction of a hydroxyl group at the C5 position of the 1,4-naphthoquinone structure increases its reduction potential by means of an intramolecular hydrogen bond that favors the formation of an anion or dianion [51]. This physicochemical effect might be responsible for the observed enhancement in biological activity.

In order to determine the effect of a chlorine atom in the inhibition of **Y–M** transition, several 2-(anilino)-3-chloro-1,4-naphthoquinone derivatives were prepared **6a–d**. In compound **6a**, the simple substitution of a hydrogen by a chlorine atom on 1,4-naphthoquinone ring, induced the activity to increase (MIC of 62.50 μg/mL) relative to compound **3a** (MIC of 500.00 μg/mL). Furthermore, an increased in the activity by 50 % was observed for *ortho*-fluoro derivative **3b** with an MIC of 31.25 μg/mL upon addition of a chlorine atom to give **6b** with an MIC of 15.62 μg/mL. However, the addition of a chlorine atom on **3j** derivative reversed the biological activity for this compound, increasing the MIC from 1.95 μg/mL with **3j** to 125.00 μg/mL with **6d**. This latter observation indicates that the introduction of an electron-withdrawing chloro substituent at the quinone does not improve biological activity.

In previous reports, 2,3-disubstituted-1,4-naphthoquinone with arylthio and arylamino substituents demonstrated some antifungal activity on diverse pathogenic fungi [11–14]. In this instance, 2-(anilino)-3-(2,4-dinitroanilino)-1,4-naphthoquinone derivatives **8a–c** were prepared and their inhibitory activity on germ tube formation **Y–M** of *C. albicans* was evaluated. The presence of an additional



Scheme 3 Structure of 2-(2-fluoroanilino)-1,4-naphthoquinone showing the effect of fluorine through a bifurcated or three-center hydrogen bond

Table 2 Correlation of electrochemical parameters of 1,4-naphthoquinone derivatives with inhibition of **Y–M** *Candida albicans* transition

Compound	Substituent	Activity MIC[d] (μg/mL)	[c]$E^{II}_{1/2}$
3h	4-CH_3O	500.00	−1809[b]
3a	H	500.00	−1685[b]
3g	4-Br	250.00	−1642[b]
3b	2-F	31.25	−1636[a]
3j	2,4-F	1.95	−1636[a]

[a] Determined by cyclic voltammetry at 100 mV/s [38]

[b] Determined by cyclic voltammetry at 100 mV/s [49]

[c] $E^{II}_{1/2}$ is the second wave reduction potential

[d] The MIC value was defined as the lowest concentration of naphthoquinone derivative required to inhibit the conversion of yeast to mycelium **Y–M** of *Candida albicans*

nitroaniline moiety on the 1,4-naphthoquinone in compound **8a** gave an enhanced activity (MIC of 31.25 μg/mL), relative to 2-chloro-3-(2,4-dinitroanilino)-1,4-naphthoquinone **7** (MIC of 125.00 μg/mL). Nitro groups are known to have strong electron-withdrawing properties due to inductive and resonance effects and favor reduction of NQ [49].

2-Chloro-3-phenoxy-1,4-naphthoquinone derivatives have been used as synthetic intermediates for heterocyclic compounds with promising biological activity [46]. Several phenoxy-1,4-naphthoquinone **10a–b** and 2,3-bis(phenoxy)-1,4-naphthoquinone **11a–b** were prepared. Experimental studies (Table 1) indicated a remarkable activity for 2-chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone **10a**, 2,3-bis(2-chlorophenoxy)-1,4-naphthoquinone **11a** and 2,3-bis(2,4-dichlorophenoxy)-1,4-naphthoquinone **11b** with MIC values of 3.90, 1.95 and 0.97 μg/mL, respectively. Therefore, these compounds were shown to be the most active inhibitors against **Y–M** transition on *C. albicans*. In these latter cases, a chlorophenoxyl substituent must exert a strong electron-withdrawing effect reducing the electron density on the quinone ring, thus favoring reduction to generate intermediate radical anions. A similar effect has been previously reported for chloroanilino substituents [49].

Experimental

Materials and methods

All chemicals were reagent grade and used without further purification. Melting points were measured with a Fisher Johns apparatus and are uncorrected. UV–Vis spectra were obtained on a Shimadzu UV-2401 PC spectrophotometer. IR spectra were recorded on a Nicolet Nexus 470 FT-IR spectrophotometer using KBr pellets, and values are given in cm^{-1}. NMR spectra were obtained on a Varian Mercury

400 MHz model spectrometer using $CHCl_3$ or DMSO as a solvent and TMS as internal standard. Mass spectra were recorded on a Finnigan MAT 8200 spectrometer at 70 eV.

General procedures for the synthesis of 1,4-naphthoquinone derivatives

General procedure for 2-(anilino)-1,4-naphthoquinone ***3a–k*** *and 2-(anilino)-5-hydroxy-1,4-naphthoquinone* ***4a–e*** These compounds were prepared by a method previously reported in the literature by us [38–41]. 1,4-Naphthoquinone or 5-hydroxy-1,4-naphthoquinone (1 mmol) was dissolved in ethanol (10 mL) and an amount (0.1 mmol) of catalyst ($FeCl_3$ or $CeCl_3$) was added. The reaction mixture was stirred for 15 min to allow the reaction between the Lewis base (1,4-naphthoquinone) and catalyst. A solution of the substituted aniline (1 mmol) in ethanol (10 mL) was slowly added and the mixture was refluxed for 4 h. TLC analysis indicated only one product was formed. The resulting solid was filtered, washed with cold ethanol and recrystallized from ethanol. Total characterization of compounds **3a**–**k** has been previously reported [38–41].

2-(Anilino)-5-hydroxy-1,4-naphthoquinone ***4a*** Orange-red solid (73 %), mp: 224 °C; UV–Vis, λ_{max} (log ε): 413 (3.74), 271 (4.29), 205 (4.40); IR (KBr, cm^{-1}): 3278 (N–H), 3052 (C–H), 1632 (C=O), 1578 (C=C), 1515 (N–H), 1273 (C–N), 1238 (C–CO–C), 731, 693 (C–H); ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.53 (s, 1H, O–H), 9.28 (s, 1H, N–H), 7.72 (t, $J = 7.6$ Hz, 1H, H7), 7.44 (t, $J = 7.2$ Hz, 2H, H3′, H5′), 7.42 (d, $J = 7.4$ Hz, 1H, H6), 7.37 (d, $J = 7.8$ Hz, 2H, H2′, H6′), 7.24 (t, $J = 7.2$ Hz, 1H, H4′), 7.21 (d, $J = 7.4$ Hz, 1H, H8), 6.02 (s, 1H, H3); EM (m/z): 265 $[M]^+$ (100 %). The exact mass was 265.0739 amu, the mass observed was 265.0711 amu for $C_{16}H_{11}NO_3$.

2-(2-Fluoroanilino)-5-hydroxy-1,4-naphthoquinone ***4b*** Orange-red solid (100 %), mp: 238 °C; UV–Vis, λ_{max} (log ε): 412 (3.72), 267 (4.19), 204 (4.36); IR (KBr, cm^{-1}): 3300 (N–H), 3053 (C–H), 1628 (C=O), 1596 (C=C), 1504 (N–H), 1373 (C–N), 1264 (C–CO–C), 1174 (C–F), 761 (C–H); ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.46 (s, 1H, O–H), 9.16 (s, 1H, N–H), 7.72 (dt, $J = 7.8$ Hz, $J = 0.8$ Hz, 1H, H7), 7.41 (dd, $J = 7.4$ Hz, $J = 0.9$ Hz, 1H, H6), 7.35 (m, 3H, H3′, H4′, H5′), 7.31 (m, 1H, H6′), 7.25 (dd, $J = 7.4$ Hz, $J = 0.8$ Hz, 1H, H8), 5.47 (s, 1H, H3); ^{19}F NMR: −120.115 ppm; EM (m/z): 283 $[M]^+$ (100 %). The exact mass was 283.0645 amu, the mass observed was 283.0611 amu for $C_{16}H_{10}FNO_3$.

2-(2-Bromoanilino)-5-hydroxy-1,4-naphthoquinone ***4c*** Yellow-orange solid (86 %), mp: 186 °C; UV–Vis, λ_{max} (log ε): 413 (3.72), 267 (4.19), 205 (4.40); IR (KBr, cm^{-1}): 3306 (N–H), 1641 (C=O), 1612, 1586 (C=C), 1465 (N–H), 1250 (C–CO–C), 739 (C–H); 1248 (C–N), 694 (C–Br), ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.46 (bs, 1H, O–H), 9.14 (bs, 1H, N–H), 7.78 (dd, $J = 8.20$ Hz, $J = 1.5$ Hz 1H, H3′), 7.72 (dt, $J = 7.8$ Hz, $J = 1.2$ Hz, 1H, H7), 7.51 (dd, $J = 7.4$ Hz, $J = 1.2$ Hz, 1H, H6), 7.42 (m, 2H, H4′, H5′), 7.30 (dd, $J = 7.4$ Hz, $J = 1.5$ Hz, 1H, H6′), 7.26 (dd, $J = 7.4$ Hz, $J = 1.2$ Hz, 1H, H8), 5.37 (s, 1H, H3); EM (m/z):

343/345 $[M]^+$ (24/24 %). The exact mass was 342.9844 amu, the mass observed was 342.9813 amu for $C_{16}H_{10}BrNO_3$.

2-(4-Fluoroanilino)-5-hydroxy-1,4-naphthoquinone ***4d*** Wine-red solid (87 %), mp: 246 °C; UV–Vis, λ_{max} (log ε): 413 (3.73), 269 (4.26), 225 (4.08), 205 (4.39); IR (KBr, cm^{-1}): 3216 (N–H), 3000 (C–H), 1629 (C=O), 1606, 1585 (C=C), 1509 (N–H), 1327 (C–N), 1312 (C–CO–C), 1228 (C–F), 811 (C–H); ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.45 (s, 1H, O–H), 9.23 (s, 1H, N–H), 7.71 (dt, $J = 7.8$ Hz, $J = 0.8$ Hz, 1H, H7), 7.44 (dd, $J = 7.4$ Hz, $J = 0.9$ Hz 1H, H6), 7.39 (dd, $J = 8.6$ Hz, $J = 5.1$ Hz, 2H, H2′, H6′), 7.26 (dd, $J = 8.20$ Hz, $J = 5.1$ Hz, 2H, H3′, H5′) 7.22 (d, $J = 7.8$ Hz, 1H, H8), 5.90 (s, 1H, H3); ^{19}F NMR: −119.858 ppm; EM (m/z): 283 $[M]^+$ (100 %). The exact mass was 283.0645 amu, the mass observed was 283.0611 amu for $C_{16}H_{10}FNO_3$.

2-(2,4-Difluoroanilino)-5-hydroxy-1,4-naphthoquinone ***4e*** Orange solid (73 %), mp: 236 °C; UV–Vis, λ_{max} (log ε): 412 (3.73), 264 (4.17), 235 (4.09), 205 (4.35); IR (KBr, cm^{-1}): 3344 (N–H), 1635 (C=O), 1600, (C=C), 1488 (N–H), 1251 (C–N), 1137 (C–F), 863, 824 (C–H); ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.44 (bs, 1H, O–H), 9.12 (bs, 1H, N–H), 7.72 (dt, $J = 7.8$ Hz, $J = 0.8$ Hz, 1H, H7), 7.51 (dd, $J = 7.4$ Hz, $J = 0.9$ Hz, 1H, H6), 7.42 (m, 2H, H5′, H6′), 7.25 (dd, $J = 8.2$ Hz, $J = 0.8$ Hz, 1H, H-8), 7.19 (dt, $J = 8.9$ Hz, $J = 3.1$ Hz, 1H, H3′), 5.42 (s, 1H, H3); ^{19}F NMR: −111.565, −115.301 ppm; EM (m/z): 301 $[M]^+$ (100 %). The exact mass was 301.0550 amu, the mass observed was 301.525 amu for $C_{16}H_9F_2NO_3$.

General synthetic procedure for 2-chloro-3-(anilino)-1,4-naphthoquinones ***6a–d*** These compounds were prepared following the literature procedure [42] with some modifications [43]. A mixture of 2,3-dichloro-1,4-naphthoquinone (5 mmol) and several equivalents of the corresponding aniline (aniline and 4-fluoroaniline, 10 mmol; 2-fluoroaniline and 2,4-difluoroaniline, 20 mmol) in 50 mL of methanol was stirred and refluxed for several hours. The reaction progress was monitored by TLC. After the reaction was finished, the mixture was kept in an ice-water bath. The precipitated product was collected by filtration, washed with cold methanol and purified by recrystallization from CH_2Cl_2.

2-Chloro-3-(2-fluoroanilino)-1,4-naphthoquinone ***6b*** Red solid (90 %), mp: 176 °C; UV–Vis, λ_{max} (log ε): 464 (3.64), 274 (4.53), 208 (4.59); IR (KBr, cm^{-1}): 3255 (N–H), 1671 (C=O), 1596, 1567 (C=C), 1503 (N–H), 1332 (C–N), 1292 (C–CO–C), 1264 (C–F), 1141 (C–Cl), 762 (C–H); ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.23 (s, 1H, N–H), 8.01 (dd, $J = 7.6$ Hz, $J = 1.4$ Hz, 2H, H5, H8), 7.86 (td, $J = 7.6$ Hz, $J = 1.4$ Hz, 1H, H6), 7.78 (td, $J = 7.6$ Hz, 1.4 Hz, 1H, H7), 7.33 (td, $J = 9.2$ Hz, $J = 6.6$ Hz, 1H, H3′), 7.25 (m, 2H, H4′, H5′), 7.18 (td, $J = 9.2$ Hz, $J = 7.1$ Hz, 1H, H6′); ^{19}F NMR: −122.81 ppm; EM (m/z): 301/303 $[M]^+$ (100/35 %). The exact mass was 301.0306 amu, the mass observed was 301.0300 amu for $C_{16}H_9ClFNO_2$.

2-Chloro-3-(4-fluoroanilino)-1,4-naphthoquinone ***6c*** Red solid (98 %), mp: 235 °C; UV–Vis, λ_{max} (log ε): 474 (3.69), 275 (4.53), 208 (4.67); IR (KBr,

cm^{-1}): 3259 (N–H), 1674 (C=O), 1599, 1567 (C=C), 1491 (N–H), 1332 (C–N), 1290 (C–CO–C), 1215 (C–F), 1140 (C–Cl), 829 (C–H); ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.35 (s, 1H, N–H), 8.05 (dd, $J = 7.6$ Hz, $J = 1.4$ Hz, 2H, H5, H8), 7.85 (td, $J = 7.6$ Hz, $J = 1.4$ Hz, 1H, H6), 7.75 (td, $J = 7.6$ Hz, $J = 1.4$ Hz, 1H, H7), 7.15 (dd, $J = 8.6$ Hz, $J = 5.0$ Hz, 4H, H2′, H3′, H5′, H6′); ^{19}F NMR: −116.24 ppm; EM (m/z): 301/303 $[M]^+$ (73/30 %). The exact mass was 301.0306 amu, the mass observed was 301.0300 amu for $C_{16}H_9ClFNO_2$.

2-Chloro-3-(2,4-difluoroanilino)-1,4-naphthoquinone ***6d*** Red solid (92 %), mp: 187–188 °C; UV–Vis, λ_{max} (log ε): 459 (3.71), 272 (4.60), 209 (4.76); IR (KBr, cm^{-1}): 3237 (N–H), 1680 (C=O), 1606, 1569 (C=C), 1498 (N–H), 1331 (C–N), 1293 (C–CO–C), 1246 (C–F), 1138 (C–Cl), 846 (C–H); ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.18 (s, 1H, N–H), 8.01 (dd, $J = 7.8$ Hz, $J = 1.5$ Hz, 2H, H5, H8), 7.86 (td, $J = 7.6$ Hz, $J = 1.4$ Hz, 1H, H6), 7.79 (td, $J = 7.6$ Hz, $J = 1.4$ Hz, 1H, H7), 7.38 (dd, $J = 8.9$ Hz, $J = 6.2$ Hz, 1H, H6′), 7.31 (td, $J = 10.6$, $J = 8.4$, 1H, H5′), 7.08 (td, $J = 10.0$ Hz, $J = 2.74$ Hz, 1H, H3′); ^{19}F NMR: −113.05, −117.69 ppm; EM (m/z): 319/321 $[M]^+$ (100/39 %). The exact mass was 319.0212 amu, the mass observed was 319.0210 amu for $C_{16}H_8ClFNO_2$.

General synthetic procedure for 2-chloro-3-(2,4-dinitroanilino)-1,4-naphthoquinone ***7*** This compound was prepared by direct nitration of 2-anilino-3-chloro-1,4-naphthoquinone **6a** according to literature procedure [42] with some modifications [43]. A solution of concentrated acids HNO_3/H_2SO_4 (8/2 mL) was slowly added to **6a** (9 mmol). The resulting mixture was stirred at RT for 4 h. To induced precipitation, a volume of cold water (100 mL) was added. The precipitate was collected by filtration, washed with $NaHCO_3$ solution (5 %), cold water and ethanol.

General synthetic procedure for 2-(anilino)-3-(2,4-dinitroanilino)-1,4-naphthoquinone ***8a–c*** Preparation and characterization of these compounds has been recently reported by us. [45]. A solution of **7** (1 mmol) in 50 mL of ethanol and a large amount of substituted aniline (15 mmol) were placed in a flask. The mixture was refluxed vigorously for several hours (10–15). Cold distilled water was added and the reaction mixture was placed in an ice-bath. The product was separated by filtration and purified by column chromatography (silica gel/$CHCl_3$).

General synthetic procedure for 2-chloro-3-(phenoxy)-1,4-naphthoquinone ***10a–b*** *and 2,3-bis(phenoxy)-1,4-naphthoquinone* ***11a–b*** These compounds were obtained from the reaction between 2,3-dichloro-1,4-naphthoquinone **5** and a given phenol **9** according to the procedure reported in the literature [46] with several modifications [43]. A mixture of substituted phenol (4 mmol) and Na_2CO_3 (4 mmol) was stirred in 15 mL of DMSO at RT for 15 min. An amount of **5** (2 mmol) was slowly added. The reaction was monitored by TLC. When the reaction was finished, 20 mL of cold distilled H_2O was added and the precipitate was filtered and washed with cool methanol. The crude product was purified by silica gel column chromatography (silica gel/$CHCl_3$:Hex, 1:1).

2-Chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone ***10a*** Orange solid (50 %), mp: 162 °C; UV–Vis, λ_{max} (log ε): 335 (3.44), 276 (4.07), 251 (4.12), 240 (4.11), 203 (4.43); IR (KBr, cm^{-1}): 1669 (C=O), 1611 (C=C), 1249, 1229 (C–O), 1054 (C–Cl), 753, 690 (C–H); ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.11 (dd, $J = 7.6$ Hz, $J = 1.5$ Hz, 1H, H5), 7.96 (dd, $J = 7.4$ Hz, $J = 1.5$ Hz, 1H, H8), 7.92 (td, $J = 7.4$ Hz, $J = 1.5$ Hz, 2H, H6), 7.87 (td, $J = 7.4$ Hz, $J = 1.5$ Hz, 2H, H7), 7.57 (dd, $J = 8.0$ Hz, J = 1.5 Hz, H3′), 7.30 (dd, $J = 8.2$, $J = 1.4$ Hz, 1H, H6′), 7.23 (td, $J = 7.9$ Hz, $J = 1.5$ Hz, 1H, H5′), 7.14 (td, $J = 7.6$ Hz, $J = 1.4$ Hz, 1H, H4′); EM (m/z): 318/320/322 $[M]^+$ (14/10/2 %). The exact mass was 317.9850 amu, the mass observed was 317.9810 amu for $C_{16}H_8Cl_2O_3$.

2-Chloro-3-(4-methoxyphenoxy)-1,4-naphthoquinone ***10b*** Purple solid (90 %), mp: 127 °C; UV–Vis, λ_{max} (log ε): 336 (3.31), 277 (4.17), 244 (4.22), 222 (4.23), 206 (4.40); IR (KBr, cm^{-1}): 3076 (=C–H), 2923 (C–H), 1676 (C=O), 1590 (C=C), 1241, 1204 (C–O), 717 (C–Cl), 854 (C–H); ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.19 (dd, $J = 7.6$ Hz, $J = 1.7$ Hz; 1H, H5), 8.03 (dd, $J = 7.4$ Hz, $J = 1.6$ Hz, 1H, H8), 7.78 (td, $J = 7.4$ Hz, $J = 1.5$ Hz, 1H, H6), 7.74 (td, $J = 7.6$ Hz, $J = 1.6$ Hz, 1H, H7), 6.96 (dd, $J = 9.2$ Hz, J = 2.5 Hz, 2H, H2′, H6′), 6.84 (dd, $J = 9.12$, $J = 2.5$ Hz, 2H, H3′, H5′); EM (m/z): 314/316 $[M]^+$ (100/37 %). The exact mass was 314.0346 amu, the mass observed was 314.0312 amu for $C_{17}H_{11}ClO_4$.

2,3-Bis-(2-chlorophenoxy)-1,4-naphthoquinone ***11a*** Yellow solid (48 %), mp: 153 °C; UV–Vis, λ_{max} (log ε): 335 (3.52), 270 (4.10), 250 (4.26), 204 (4.55); IR (KBr, cm^{-1}): 2923 (C–H), 1669 (C=O), 1611 (C=C), 1249, 1229 (C–O), 1054 (C–Cl), 753, 690 (C–H); ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.01 (dd, $J = 5.5$ Hz, $J = 3.3$ Hz, 2H, H5, H8), 7.89 (dd, $J = 5.5$ Hz, $J = 3.3$ Hz, 2H, H6, H7), 7.42 (dd, $J = 8.0$ Hz, $J = 1.0$ Hz, 2H, H3a′, H3b′), 7.33 (dd, $J = 8.0$ Hz, $J = 1.0$ Hz, 2H, H6a′, H6b′), 7.15 (td, $J = 7.6$ Hz, $J = 1.0$ Hz, 2H, H5a′, H5b′), 7.04 (td, $J = 7.6$ Hz, $J = 1.0$ Hz, 2H, H4a′, H4b′); EM (m/z): 410/412/414 $[M]^+$ (4.6/4/1.3 %). The exact mass was 410.0113 amu, the mass observed was 410.0100 amu for $C_{22}H_{12}Cl_2O_4$.

2,3-Bis-(2,4-dichlorophenoxy)-1,4-naphthoquinone ***11b*** Yellow solid (85 %), mp: 153 °C; UV–Vis, λ_{max} (log ε): 334 (3.55), 274 (4.15), 250 (4.34), 205 (4.68); IR (KBr, cm^{-1}): 3087, 3027 (C–H), 1670, 1665 (C=O), 1592 (C=C), 1264, 1234 (C–O), 1102 (C–Cl), 866, 821 (C–H); ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.01 (dd, $J = 5.6$ Hz, $J = 3.3$ Hz, 2H, H5, H8), 7.89 (dd, $J = 5.6$ Hz, $J = 3.3$ Hz, 2H, H6, H7), 7.63 (d, $J = 2.5$ Hz, 2H, H3a′, H3b′), 7.42 (d, $J = 8.9$ Hz, 2H, H6a′, H6b′), 7.27 (dd, $J = 8.8$ Hz, $J = 2.54$ Hz, 2H, H5a′, H5b′); EM (m/z): 478/480/482 $[M]^+$ (13.8/17.8/9.9 %). The exact mass was 477.9333 amu, the mass observed was 477.9305 amu for $C_{22}H_{10}Cl_4O_4$.

Inhibition of ***Y–M*** *C. albicans transition*

Germ tube inhibition assay The 1,4-naphthoquinone derivatives were dissolved in DMSO as 10 mg/mL (stock) and diluted appropriately in sterile Lee's medium [48]

just before use to obtain the required concentrations. Identical amounts of DMSO were added to control the solvent effect. *C. albicans* ATCC strain 26,555 was used in this study. Cells were maintained and subcultivated on Sabouroud dextrose agar at 28 °C. The final pH was adjusted to 5.6. Inhibition assays were performed in liquid cultures using Lee's medium. The composition of medium (per liter) was as follows: 2.5 g anhydrous K_2PO_4H; 0.2 g $MgSO_4{\cdot}7H_2O$; 5 g anhydrous $(NH_4)_2SO_4$; 5 g NaCl; 12.5 g glucose; 0.5 g prolina; 0.001 g biotin. The final pH was adjusted to 6.8. To study the effect of 1,4-naphthoquinone derivatives on the **Y**–**M** transition of *C. albicans*, stationary-phase cells were collected by centrifugation (5000*g* × 10 min) and inoculated into appropriate volume of Lee's medium and the initial concentration was regulated adjusting the optical density at 600 nm between 0.10 and 0.15. A volume of 475 µL of yeast suspension was transferred to 5-mL tubes and incubated at 37 °C and 200 rpm in an orbital incubator (New Brunswick Sci., Co.). 25 µL of a naphthoquinone derivative solution was inoculated into yeast suspension in incubation with decreasing concentrations of derivative (1:2 serial dilutions) after 30–45 min of incubation since this is the time required for germ tube formation (Fig. 1b). Samples were observed in an optical microscope (40× magnification) after 1–3 h. All the experiments were performed in triplicate and twice independently. All tests were run in duplicate.

Growth conditions C. albicans yeast cells were propagated in Lee's medium as follows: an aliquot of 50 mL with 7 µg of cells (dried weight per mL of medium) was dispensed into a 250-mL Erlenmeyer flask. The culture was incubated at 28 °C and 200 rpm in an orbital incubator (New Brunswick Sci., Co.). Cellular growth was monitored by measuring the optical density at 600 nm using a Shimadzu UV-160-A spectrophotometer. The cellular amount was determined using absorbance–cellular dried weight/ml of the medium relation curve. The culture was incubated by 12–15 h to obtain 0.6 absorbance, a value that corresponds to medium point exponential growth. To obtain a saturated culture containing synchronized cells, the yeast cells were collected by centrifugation (3500 rpm, 10 min), washed with sterile distilled water two times and re-suspended in 25 mL of sterile distilled water to a final concentration of 1 mg/mL. The culture was incubated at 28 °C and 200 rpm in an orbital incubator for 1–2 h and then stored for several hours (12–24) at 4 °C (metabolic repose) [47]. In this form, yeast cells must be kept a maximum of 48 h before inhibition germ tube and inhibition growth assays.

Conclusions

Structural analysis of several 1,4-naphthoquinone derivatives revealed that their capacity to inhibit *C. albicans* **Y**–**M** transition is related to the position and nature of the substituent groups and the presence of intramolecular hydrogen bonds on the naphthoquinone structure.

Having a chlorophenoxyl substituent or an intramolecular hydrogen bond due to the presence of a 2-fluoroanilino or 5-hydroxyl substituent on 1,4-naphthoquinone structure enhances its biological activity. These structural features facilitate quinone reduction to give a reactive intermediate that could easily interact with several

biomolecules. These results could lead to the generation of new selective compounds as an alternative treatment for infections caused by fungi or *C. albicans*.

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