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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 7035–7041
Bioorganic & Medicinal Chemistry

Synthesis and potent antitumor activity of new arylamino derivatives of nor-β-lapachone and nor-α-lapachone

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Received 18 May 2007; revised 18 July 2007; accepted 19 July 2007
Available online 22 August 2007

Abstract—Several arylamino derivatives of nor-β-lapachone were synthesized in moderate to high yields and found to show very potent cytotoxicity against six neoplastic cancer cells: SF-295 (central nervous system), HCT-8 (colon), MDAMB-435 (breast), HL-60 (leukaemia), PC-3 (prostate), and B-16 (murine melanoma), with IC_{50} below 1 μg/mL. Their cytotoxicities were compared to doxorubicin and with their synthetic precursors, β-lapachone and nor-β-lapachone. The activity against a normal murine fibroblast L-929 showed that some of the compounds were selective against cancer cells. The absence of hemolytic activity (EC_{50} > 200 μg/mL), performed with erythrocyte suspensions, suggests that the cytotoxicity of the compounds was not related to membrane damage of mouse erythrocytes. For comparison purposes, one isomeric compound based on nor-α-lapachone was also synthesized and showed lower activity than the related *ortho*-derivative. The modified arylamino quinones appear as interesting new lead compounds in anti-cancer drug development.

1. Introduction

Cancer is the second leading cause of death worldwide after cardiovascular diseases. Considerable insight has been gained into the mechanisms by which some chemicals affect cellular growth and how this knowledge has been used the design of new chemotherapeutic drugs,[1,2] providing more selectivity toward cancer cells than to normal cells leading to lower side effects.

Mass screening programs of natural products by the National Cancer Institute have identified the quinone moiety as an important pharmacophoric element for cytotoxic activity.[3] On the basis of biological and structural properties, 1,2- and 1,4-naphthoquinones are considered privileged structures in medicinal chemistry.[4] In fact, quinone moieties are present in many drugs such as anthracyclines, mitomycin, and mitoxantrones, which are used clinically in the therapy of solid cancers.

Quinones play a pivotal role in energy metabolism and in many other key processes mainly in chemotherapy where redox cycling drugs are utilized. Many efficient antineoplastic drugs are quinone derivatives,[2–7] or drugs such as etoposide that can easily be converted to quinones by in vivo oxidation. Apart from antitumor activities, quinones have been studied for molluscicidal[8–10] leishmanicidal,[11] antifungic,[12] and trypanocidal[13,14] activities.

It has been described in the literature that the biological profiles of these molecules are centered on their *ortho*- or *para*-quinonoid moiety.[14,15] This group generally accepts one and/or two electrons to form the corresponding

Keywords: Lapachones; Dihydrofuranonaphthoquinones; Cancer; Cytotoxicity.
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doi:10.1016/j.bmc.2007.07.043

radical anion or dianion species, or protonated species in situ, the driving force of the process being the formation of a fully aromatic system.[4] In such way, the semi-quinone radicals, formed directly by one electron reduction and indirectly after two electron reduction, in the presence of O_2 redox cycling, accelerate the intracellular hypoxic conditions, also increasing the redox state of the cells, by producing reactive oxygen species, such as superoxide anion radical, hydrogen and lipid peroxides, and hydroxyl radicals.[16,17] In addition to their ease of reduction, another main chemical property of quinones which also contributes to their biological activity is the reaction with O-, N- or S-nucleophiles in a Michael-type 1,4-addition.[4] Exposure of the cells to high amounts of quinones may saturate the detoxification system and often leads to a significant depletion of the reduced thiol pool, or thiolated enzymes, by alkylation, causing irreversible changes and cell death.[4] Due to these mechanisms, quinones show cytotoxicity to cancer cells and also to normal cells.[2,4,15]

Since the finding that lapachol (**1**), a natural naphthoquinone, proved to have antitumor activity against carcinoma Walker 256,[18] many other natural and synthetic naphthoquinones were reported as potent antitumoral compounds.[19] Among the natural cytotoxic naphthoquinones, β-lapachone (**2b**) is the most studied in the last years. This quinone can be isolated from plant extracts of *Tabebuia avellanedae*. It has been intensely investigated for clinical use as trypanocidal compound[20,21] in both acute and chronic infections and also against HIV-1 replication.[22] However, the most important applications of this compound are related to its action against several cancer cells. Indeed, it is reported to present a significant anti-neoplastic activity against human cancer cell lines from leukemia,[23] prostate,[24] malignant glioma,[25] hepatoma,[26] colon,[27] breast,[28] ovarian,[29] and pancreatic[30] tumors, at concentrations in the range of 1–10 μM (IC_{50}). Other studies have shown that in combination with taxol, β-lapachone is an effective agent against human ovarian cancer and prostate xenografts in mice.[29] For nor-β-lapachone (**2a**),[31,32] cytotoxicity against KB (human epidermal carcinoma), HeLa (human cervical carcinoma), and HepG2 (human hepatocellular carcinoma) was measured with results in the range of 2–2.5 μM,[31] using MTT colorimetric method.[33]

More recently, trying to search for compounds with lower side effects and with improvement of their biological activity, several heterocyclic derivatives of **2b** (oxyrane **3**,[34] oxazoles **4**,[35] imidazoles **5**,[36] and phenazines **6**[37]) were synthesized (Fig. 1).

The design of new anticancer compounds based on the structure of **2a** and its *para*-isomer **15** (Fig. 1 and Scheme 1) is far less studied than those based on **2b**, which has been extensively studied in recent years including modifications on the quinone and pyran moieties. In this paper, we present our efforts on the preparation of new derivatives of nor-β-lapachone (**2a**) and nor-α-lapachone (**15**), containing amino groups, and the study of their cytotoxic activities against six neoplastic cancer cells: SF-295 (central nervous system), HCT-8 (colon), MDAMB-435 (breast), HL-60 (leukemia), PC-3 (prostate), and B-16 (murine melanoma) and toward one normal cell, the murine fibroblast L-929, all lines originating from the National Institute of Health, Bethesda, Maryland. In this regard, the compounds **9**–**14** and **17** were synthesized, introducing the phenyl group at C-3 position of the dihydrofuran ring of **2a** and **15**, intending to bring to this ring the profile of **14** (Scheme 1), recently published by two of us as a potent trypanocide,[38] and exploring additional biological activities.

2. Results and discussion

2.1. Chemistry

As shown in Scheme 1, the synthesis of **9**–**14** was carried out in one-pot reaction. The first step involved in situ preparation of 3-bromo-nor-β-lapachone (**8**), which was converted into the arylamino derivatives (**9**–**14**) in moderate to high yields (from 50% to 95%, Scheme 1). The synthesis of **17** was carried out from **15**[31,32] through the compound **16,** generated in situ, that reacted with aniline giving the desired product in 70% yield. This compound was synthesized in order to compare its bioactivity with that of the isomeric system.

The structures of the compounds were confirmed by the use of spectroscopic techniques, such as ^{1}H and ^{13}C NMR, infrared, and by high-resolution electron-impact mass spectra, which data clearly confirm the substitution of the bromine atom of the starting materials by arylamino groups in both *ortho*- and *para*-derivatives.

2.2. Biology

The compounds **2a**, **2b**, **9**–**14**, and **17** were tested in vitro against six cancer cells and a normal murine fibroblast L-929, in comparison to doxorubicin, the positive control, by using MTT assay.[39] The concentrations that induce 50% inhibition of cell growth (IC_{50}) in μg/mL are reported in Table 1. Compounds were classified by their activity as highly active ($IC_{50} < 1$ μg/mL), moderately active (1 μg/mL < IC_{50} < 10 μg/mL), or inactive (10 μg/mL > IC_{50}).[40]

The great majority of the compounds are strongly cytotoxic against all cancer cell lines with IC_{50} below 1 μg/mL, except for compounds **9** and **17**. No difference in cytotoxic efficacy was observed on cells treated with β-lapachone (**2b**) and nor-β-lapachone (**2a**). The halogenated compounds **10**, **11**, and **12**, along with the nitroderivative **13**, were the most potent ones. The fluorinated and the nitro compounds did not discriminate between cancer and normal cells. For compounds **11** and **12**, the activity toward cancer cells was two times higher in relation to normal cells. All compounds showed a higher cytotoxicity for breast cancer (MDA-MB-435) cell line when compared to doxorubicin, a fact that supports their anti-cancer activity. In the case of prostate (PC-3) cell line, the results are quite similar.

In general, the presence of electron-withdrawing groups in the arylamino moiety (**10**, **11**, **12**, and **13**) enhanced

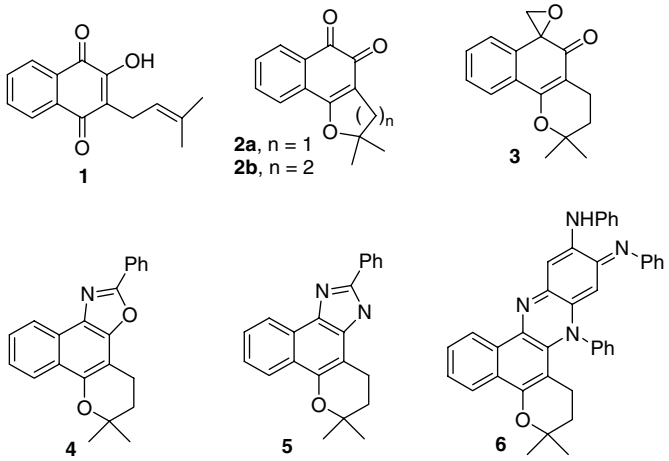
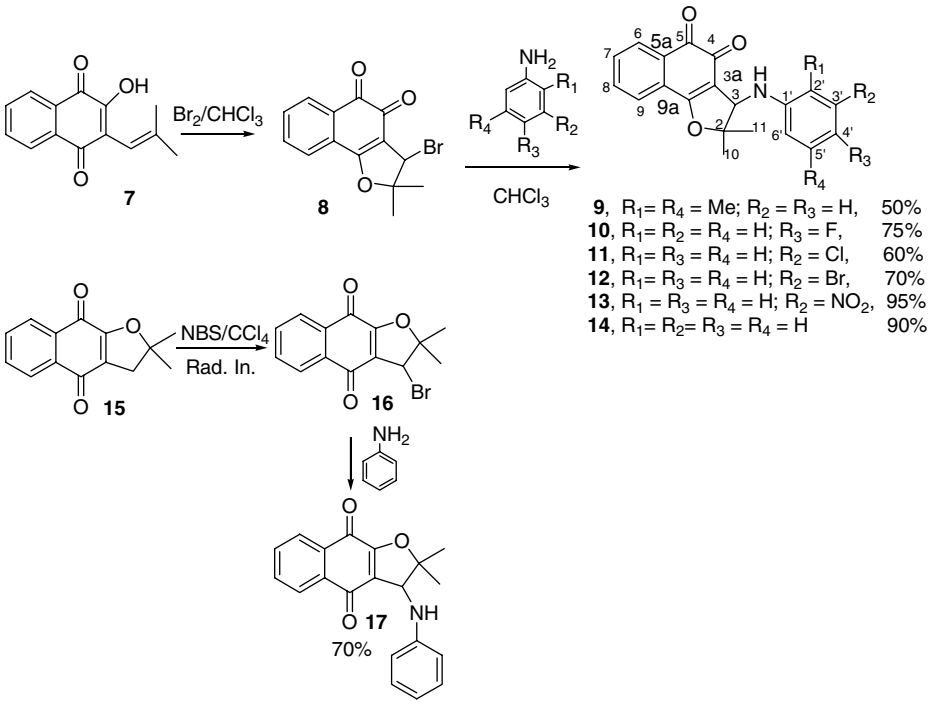


Figure 1. Some important derivatives of **1** and **2**.



Scheme 1. Synthetic route for preparing *ortho*-naphthoquinones **9**–**14** and *para*-naphthoquinone **17**.

the anticancer activity of the substances, while, despite only one example, the electron donating methyl group in compound **9** affects the activity in the opposite way. However, the latter *ortho*-quinones showed higher cytotoxic selectivity for breast cancer (MDA-MB-435) and prostate cancer (PC-3) cell lines when compared to the other cell lines and greater IC_{50} for normal cell line (L929) what can indicate their specificity to cancer cell lines, being a positive fact (Table 1).

Comparing compounds **14** and **17**, it is noticeable that the *ortho*-quinone **14** shows higher activity than its *para*-isomer, as seen before[40] as well as for trypanocidal activity.[14] Despite the higher activity of *ortho*-quinone **17**, when compared to the corresponding *para*-quinones, there are several reports in the literature describing the synthesis and high anti-cancer activity of *para*-naphthoquinones having several appendages on the quinonoid system.

The absence of hemolytic activity ($EC_{50} > 200$ μg/mL) suggests that the cytotoxicity of these compounds is not related to membrane damage of mouse erythrocytes. Experiments concerning the elucidation of the mechanism of anti-cancer action are under way. However, as evidenced for **2b**, a large number of targets and great complexity is expected.[29]

3. Conclusions

The presence of the redox center of the quinones is described in the literature as important to antitumor activity. In this work, we designed and synthesized a

Table 1. Cytotoxic activity expressed by IC_{50}in μg/mL (μmol L^{-1}) of compounds for cancer cell lines[a]

Compounds	HL-60	HCT-8	MDA-MB-435	SF295	B16	PC-3	L-929
9	1.27 (3.66) 1.07–1.51 (3.08–4.35)	1.59 (4.58) 1.03–3.47 (2.96–9.99)	0.27 (0.78) 0.22–0.33 (0.63–0.95)	2.08 (5.99) 1.29–3.34 (3.71–9.61)	3.03 (8.72) 2.51–3.66 (7.22–10.53)	0.32 (0.92) 0.30–0.35 (0.86–1.01)	>5 (>14.39)
10	0.32 (0.95) 0.16–0.66 (0.47–1.96)	0.51 (1.51) 0.42–0.62 (1.24–1.84)	0.12 (0.36) 0.11–0.14 (0.33–0.41)	0.59 (1.75) 0.49–0.71 (1.45–2.10)	0.42 (1.24) 0.32–0.55 (0.95–1.63)	0.31 (0.92) 0.29–0.35 (0.86–1.01)	0.52 (1.54) nd
11	0.39 (1.10) 0.30–0.50 (0.85–1.41)	0.51 (1.44) 0.41–0.63 (1.16–1.78)	0.17 (0.48) 0.15–0.20 (0.42–0.56)	0.36 (1.02) 0.23–0.56 (0.65–1.58)	0.56 (1.58) 0.41–0.78 (1.16–2.20)	0.49 (1.38) 0.41–0.59 (1.16–1.67)	1.16 (3.28) 0.90–1.56 (2.59–4.41)
12	0.50 (1.26) 0.36–0.69 (0.91–1.74)	0.70 (1.76) 0.54–0.92 (1.36–2.31)	0.20 (0.50) 0.15–0.27 (0.38–0.68)	0.93 (2.34) 0.59–1.45 (1.49–3.65)	0.48 (1.21) 0.38–0.60 (0.96–1.51)	0.46 (1.15) 0.38–0.57 (0.95–1.43)	1.02 (2.57) 0.91–1.14 (2.29–2.87)
13	0.33 (0.91) 0.25–0.45 (0.69–1.23)	0.64 (1.76) 0.53–0.78 (1.45–2.14)	0.23 (0.63) 0.18–0.30 (0.49–0.82)	0.50 (1.37) 0.37–0.68 (1.01–1.87)	0.43 (1.18) 0.36–0.50 (0.98–1.37)	0.41 (1.12) 0.34–0.50 (0.93–1.37)	0.42 (1.15) 0.37–0.48 (1.01–1.32)
14	0.66 (2.07) 0.14–3.17 (0.44–9.93)	0.99 (3.10) 0.73–1.34 (2.29–4.20)	0.30 (0.94) 0.26–0.34 (0.81–1.06)	0.89 (2.79) 0.66–1.22 (2.07–3.82)	0.74 (1.47) 0.67–0.82 (2.10–2.57)	0.31 (1.19) 0.36–0.41 (1.13–1.28)	2.72 (8.52) 2.20–3.36 (6.89–10.52)
17	2.99 (9.36) 2.67–3.34 (8.36–10.46)	1.43 (4.48) 1.04–1.92 (3.26–6.01)	0.89 (2.79) 0.59–1.35 (1.85–4.23)	2.52 (7.89) 1.84–3.44 (5.76–10.77)	1.00 (3.13) 0.76–1.32 (2.38–4.13)	1.34 (4.20) 1.14–1.56 (3.57–4.88)	3.08 (9.64) 2.40–3.94 (7.51–12.34)
2b	0.40 (1.65) 0.36–0.43 (1.49–1.78)	0.20 (0.83) 0.18–0.21 (0.74–0.87)	0.06 (0.25) 0.04–0.08 (0.16–0.33)	0.22 (0.91) 0.18–0.27 (0.74–1.11)	nd	nd	nd
2a	0.40 (1.75) nd	0.31 (1.36) 0.27–0.35 (1.18–1.53)	0.07 (0.31) 0.05–0.09 (0.22–0.39)	0.36 (1.58) 0.30–0.43 (1.31–1.88)	nd	nd	nd
Doxorubicin	0.02 (0.04) 0.01–0.02 (0.02–0.04)	0.04 (0.07) 0.03–0.05 (0.05–0.09)	0.47 (0.86) 0.34–0.65 (0.62–1.20)	0.25 (0.42) 0.17–0.36 (0.35–0.46)	0.03 (0.05) 0.02–0.04 (0.04–0.07)	0.24 (0.44) 0.21–0.27 (0.39–0.50)	nd

nd, not determined.

[a] Data are presented as IC_{50} values and 95% confidence intervals obtained by nonlinear regression for all cell lines from three independent experiments. Doxorubicin was used as positive control. Only compounds with IC_{50} values lower than 5 μg/mL at least for one cell line were considered active.

set of new nor-β-lapachone-related compounds with modifications in the dihydrofuran ring moiety and determined their cytotoxicity against six cancer cell lines. The results of the anti-cancer activities of the naphthoquinones **9**–**14** over human tumor cell lines indicate the potential of these compounds for the treatment of cancer. Most of the compounds were cytotoxic against the six cancer cell lines tested with IC_{50} below 1 μg/mL. Despite the fact that the two naphthoquinones nor-β-lapachone (**2a**) and β-lapachone (**2b**) and doxorubicin were more potent or quite similar to the new synthetic compounds, the substituted arylamino quinones appear as interesting new prototype compounds to be explored and open new perspectives to the development of more potent and selective anti-cancer drugs. Regarding the hemolytic assay, none of the compounds was capable to cause hemolysis in mouse erythrocytes, even at the highest concentration (200 μg/mL). Absence of lytic effects suggests that the mechanism of cytotoxicity of the substances is not related with membrane disruption and is probably related to more specific pathways of the cells.

4. Experimental

4.1. Chemical synthesis

4.1.1. General remarks. Melting points were obtained on a Reichert hot stage microscope and are uncorrected. Analytical grade solvents were used. The solvents were previously purified as described in the literature.[41] Column chromatography was performed on silica-gel (Acros Organics 0.035–0.070 mm, pore diameter ca. 6 nm). Infrared spectra were recorded on a Perkin-Elmer FT-IR Spectrometer. Ultraviolet (UV)–visible (VIS) spectra were obtained on a Shimadzu spectrophotometer, with wavelengths expressed in nm and extinction coefficients, ε, in mol cm^{-1}. NMR spectra in $CDCl_3$ solutions were recorded on a Varian Unity Plus 300 instrument. High-resolution electron-impact mass spectra (70 eV) were obtained using a MAT8500 instrument.

4.1.2. Synthetic procedures. Nor-lapachol (**7**) {2-hydroxy-3-(2-methyl-propenyl)-[1,4]-naphthoquinone}, nor-β-lapachone (2,3-dihydro-2,2-dimethylnaphtho[1,2-*b*]furan-4,5-dione) (**2a**), 3-bromo-nor-β-lapachone (3-bromo-2,3-dihydro-2,2-dimethylnaphtho[1,2-*b*]furan-4,5-dione) (**8**), 3-phenylamino-2,2-dimethyl-2,3-dihydronaphtho[1,2-*b*]furan-4,5-dione (**14**), nor-α-lapachone (2,3-dihydro-2,2-dimethyl[2,3-*b*]furan-4,9-dione) (**15**), and 3-bromo-nor-α-lapachone (3-bromo-2,3-dihydro-2,2-dimethyl[2,3-*b*]furan-4,9-dione) (**16**) were prepared from lapachol (**1**) by standard procedures.[31,38,42]

4.1.3. General procedures for preparing 9–13. To a solution of nor-lapachol (**7**) (228.0 mg, 1 mmol) in 25 mL of chloroform, 2 mL of bromine (26 mg, 38 mmol) was added. The bromo intermediate **8** (3-bromo-2,2-dimethyl-2,3-dihydro-naphtho[1,2-*b*]furan-4,5-dione) precipitated immediately as an orange solid. Over this mixture, an excess of the appropriate arylamine was added and, then, the mixture was stirred for 30 min. The crude reaction product was poured into 50 mL of water. The organic phase was separated and washed with 10% HCl (3× 50 mL), dried over sodium sulfate, filtered, and evaporated under reduced pressure to yield a red solid, which was purified by column chromatography in silica-gel, eluted with an increasing polarity gradient mixture of hexane and ethyl acetate (9/1 to 7/3).

4.1.4. 3-(2,5-Dimethylphenylamino)-2,2-dimethyl-2,3-dihydronaphtho[1,2-*b*]furan-4,5-dione (9). The reaction of **7** (228 mg, 1 mmol), 2 mL bromine (26 mg, 38 mmol), and 5 mL of 2,5-dimethylphenylamine (4.86 g, 40 mmol) produced **9** (174 mg, 0.50 mmol, 50% yield) as a brown solid (mp 197–200 °C); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.14 (1 H, ddd, J = 7.3, 1.7, 0.7 Hz), 7.76–7.62 (3H, m), 6.95 (1H, d, J = 7.8 Hz), 6.53 (1H, d, J = 7.1 Hz), 6.34 (1H, s), 4.85 (1H, s), 2.30 (3H, s), 2.07 (3H, s), 1.71 (3H, s), 1.58 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 180.8 (C═O), 175.2 (C═O), 169.3 (C_0), 145.2 (C_0), 136.4 (C_0), 134.5 (CH), 132.4 (CH), 131.1 (C_0), 130.1 (CH), 129.4 (CH), 127.4 (C_0), 124.9 (CH), 119.4 (C_0), 118.3 (CH), 115.2 (C_0), 111.0 (CH), 96.7 (C_0), 61.7 (CH), 27.2 (CH_3), 21.6 (CH_3), 21.5 (CH_3), 16.9 (CH_3); IR ν_{max} (cm^{-1}, film) 3385 (R_2NH), 1648 (C═O), 1615 (C═O); EI-HRMS (*m*/*z*) 347.15210. Calcd for $C_{22}H_{21}NO_3$: 347.15214.

4.1.5. 3-(4-Fluorophenylamino)-2,2-dimethyl-2,3-dihydronaphtho[1,2-*b*]furan-4,5-dione (10). The reaction of **7** (228 mg, 1 mmol), 2 mL of bromine (26 mg, 38 mmol), and 5 mL of 4-fluorophenylamine (5.8 g, 52.2 mmol) produced **10** (253 mg, 0.75 mmol, 75% yield) as a red solid (mp 211–214 °C); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.10 (H_6 or H_9, dd, J = 7.7, 2.2 Hz), 7.74-7.61 (H_6 or H_9; H_7 and H_8, m), 6.89 ($H_{3'}$ and $H_{5'}$, t, J = 8.5 Hz), 6.53 ($H_{2'}$ and $H_{6'}$, dd, J = 9.2, 4.4 Hz), 4.72 (H_3, s), 1.66 (H_{10} or H_{11}, s), 1.58 (H_{11} or H_{10}, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 180.7 (C_5), 175.2 (C_4), 169.5 (C_{9b}), 155.0 ($C_{4'}$, d; J = 234.8 Hz), 143.3 ($C_{1'}$), 134.5 (C_6, or C_7, or C_8 or C_9), 132.4 (C_6, or C_7, or C_8 or C_9), 131.0 (C_{5a}), 129.4 (C_6, or C_7, or C_8 or C_9), 127.2 (C_{9a}), 124.9 (C_6, or C_7, or C_8 or C_9), 115.6 ($C_{3'}$ and $C_{5'}$, d, J = 22.6), 115.0 (C_{3a}), 113.9 ($C_{2'}$ and $C_{6'}$, d, J = 7.7), 96.6 (C_2), 62.3 (C_3), 27.2 (C_{10} or C_{11}), 21.6 (C_{11} or C_{10}); IR ν_{max} (cm^{-1}, film) 3371 (R_2NH); 1611 (C═O), 1639 (C═O); EI-HRMS (*m*/*z*) 337.11140. Calcd for $C_{20}H_{16}O_3FN$: 337.11142.

4.1.6. 3-(4-Chlorophenylamino)-2,2-dimethyl-2,3-dihydronaphtho[1,2-*b*]furan-4,5-dione (11). The reaction of **7** (228 mg, 1 mmol), 2 mL of bromine (26 mg, 38 mmol), and 5 mL of 3-chlorophenylamine (6.0 g, 47.0 mmol) produced **11** (212 mg, 0.6 mmol, 60% yield) as a red solid (mp 219–221 °C); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.11 (1 H, ddd, J = 7.3, 1.2, 0.7 Hz), 7.75–7.62 (3H, m), 7.10 (1H, t, J = 8.1 Hz), 6.72 (1H, ddd, J = 7.8, 1.7, 0.7 Hz), 6.56 (1H, t, J = 2.2 Hz), 6.46 (1H, ddd, J = 8.0, 2.2, 0.7 Hz), 4.78 (1H, s), 1.68 (3H; s), 1.58 (3H; s); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 180.7 (C═O), 175.2 (C═O), 169.5 (C_0), 148.2 (C_0), 134.9 (C_0), 134.5 (CH), 132.5 (CH), 131.0 (C_0), 130.2 (CH), 129.4 (CH), 127.1 (C_0), 125.0 (CH), 118.0 (CH), 114.6 (C_0), 112.7 (C_0), 111.2 (CH), 96.6 (C_0), 61.3 (CH), 27.2 (CH_3),

21.6 (CH_3); IR ν_{max} (cm^{-1}, film) 3365 (R_2NH), 1598 (C=O), 1639 (C=O); EI-HRMS (*m*/*z*) 353.08180. Calcd for $C_{20}H_{16}ClNO_3$: 353.08187.

4.1.7. 3-(4-Bromophenylamino)-2,2-dimethyl-2,3-dihydronaphtho[1,2-*b*]furan-4,5-dione (12). The reaction of **7** (228 mg, 1 mmol), 2 mL of bromine (26 mg, 38 mmol), and 5 mL of 3-bromophenylamine (7.9 g, 45.0 mmol) produced **12** (279 mg, 0.70 mmol, 70% yield) as a red solid (mp 213–215 °C); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.11 (1H, ddd, J = 7.3, 1.2, 0.7 Hz), 7.75–7.62 (3H, m), 7.02 (1H, t, J = 8.0 Hz), 6.85 (1H, ddd, J = 7.8, 1.7, 0.7 Hz), 6.72 (1H, t, J = 2.2 Hz), 6.50 (1H, ddd, J = 8.3, 2.4, 0.9 Hz), 4.78 (1H, s), 1.68 (3H, s), 1.57 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 180.7 (C=O), 175.2 (C=O), 169.7 (C_0), 148.3 (C_0), 134.5 (C_0), 132.5 (CH), 130.1 (C_0), 130.5 (CH), 129.4 (CH), 127.1 (C_0), 125.0 (CH), 123.1 (C_0), 120.8 (CH), 115.6 (CH), 114.6 (C_0), 111.6 (CH), 96.6 (C_0), 61.2 (CH), 27.3 (CH_3), 21.6 (CH_3); IR ν_{max} (cm^{-1}, film) 3362 (R_2NH), 1600 (C=O), 1639 (C=O); EI-HRMS (*m*/*z*) 397.03130. Calcd for $C_{20}H_{16}BrNO_3$: 397.03136.

4.1.8. 2,2-Dimethyl-3-(4-nitro-phenylamino)-2,3-dihydronaphtho[1,2-*b*]furan-4,5-dione (13). The reaction of **7** (228 mg, 1 mmol), 2 mL of bromine (26 mg, 38 mmol), and 5 mL of 3-nitrophenylamine (3.1 g, 22.4 mmol) produced **13** (346 mg, 0.95 mmol, 95% yield) as a red solid (mp 215–217 °C); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.01 (1 H, dd, J = 8.2, 1.0 Hz), 7.76–7.60 (3H, m), 7.49 (1H, dd, J = 8.0, 1.8 Hz), 7.38 (1H, t, J = 2.0 Hz), 7.21 (1H, t, J = 8.0 Hz), 6.85 (1H, dd, J = 8.0, 1.8 Hz), 4.84 (1H, s), 1.73 (3H, s), 1.59 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 180.6 (C=O), 175.2 (C=O), 170.2 (C_0), 148.9 (C_0), 148.0 (C_0), 134.6 (CH), 132.6 (CH), 130.9 (C_0), 129.5 (CH), 129.3 (CH), 127.4 (C_0), 125.1 (CH), 118.5 (CH), 114.3 (C_0), 112.2 (CH), 106.6 (CH), 96.6 (C_0), 61.1 (CH), 27.3 (CH_3), 21.6 (CH_3); IR ν_{max} (cm^{-1}, film) 3360 (R_2NH), 1615 (C=O), 1651 (C=O), 1531–1351 (NO_2); EI-HRMS (*m*/*z*) 364.35150. Calcd for $C_{20}H_{16}N_2O_5$: 364.35152.

4.1.9. 2,2-Dimethyl-3-phenylamino-2,3-dihydro-naphtho[2,3-*b*]furan-4,9-dione (17). Compound **16** (307.14 mg, 1 mmol) was dissolved in 5 mL of aniline (5.1 g, 58 mmol) and the mixture was left under stirring for 30 min, followed by the addition of 50 mL of water. The organic phase was extracted with dichloromethane, washed with HCl 10% (3× 50 mL), dried over sodium sulfate, and filtered. The solvent from the crude was evaporated under reduced pressure and it was purified by column chromatography in silica-gel, using eluents with an increasing polarity gradient mixture of hexane and ethyl acetate (9/1 to 7/3). 2,2-Dimethyl-3-phenylamino-2,3-dihydro-naphtho[2,3-*b*]furan-4,9-dione (**17**) (223.5 mg, 0.7 mmol, 70% yield) was obtained as a brown solid (mp 205–207 °C). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.90 (1H, s), 8.13–8.07 (2H, m), 7.78–7.67 (2H, m), 6.65 (2H, dd, J = 7.5, 0.9 Hz), 7.23 (2H, t, J = 7.2 Hz), 6.79 (1H, tt, J = 7.2, 0.9 Hz), 1.67 (3H, s), 1.57 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 181.7 (C=O), 178.6 (C=O), 159.5 (C_0), 147.3 (C_0), 134.5 (CH), 133.1 (C_0), 133.0 (CH), 131.4 (C_0), 129.3 (CH), 126.2 (CH), 126.0 (CH), 122.5 (C_0), 117.9 (CH), 112.9 (CH), 94.9 (C_0), 61.9 (CH), 27.0 (CH_3), 21.4 (CH_3); IR ν_{max} (cm^{-1}, film) 3383 (R_2NH), 1617 (C=O), 1688 (C=O), EI-HRMS (*m*/*z*) 319.12080. Calcd for $C_{20}H_{17}NO_3$: 319.12084.

4.2. Anticancer assay

4.2.1. Cytotoxicity against cancer cell lines. Compounds (0.01–5 μg/mL) were tested for cytotoxic activity against six cancer cell lines: SF-295 (Central Nervous System), HCT-8 (colon), MDAMB-435 (breast), HL-60 (leukemia), PC-3 (prostate), B-16 (murine melanoma), and one normal cell L-929 (murine fibroblast) (National Cancer Institute, Bethesda, MD). All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% CO_2. Each compound was dissolved with DMSO and diluted with water to obtain a concentration of 1 mg/mL. They were incubated with the cells for 72 h. The negative control received the same amount of DMSO (0.005% in the highest concentration). Doxorubicin (0.1–0.58 μg/mL) was used as a positive control. The cell viability was determined by reduction of the yellow dye 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a blue formazan product as described by Mosmann.[39]

4.2.1.1. Cell membrane disruption. The test was performed in 96-well plates using a 2% mouse erythrocyte suspension in 0.85% NaCl containing 10 mM $CaCl_2$, following the method described by Jimenez et al.[43] The compounds diluted as mentioned above were tested at concentrations ranging from 1.5 to 200 μg/mL. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the liberated hemoglobin was measured spectrophotometrically at 540 nm. DMSO was used as a negative control and Triton X-100 (1%) was used as positive control. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the liberated hemoglobin was measured spectrophotometrically at 540 nm. ED_{50} is the calculated effective dose that induced lysis on 50% that of the Triton X-100.

Acknowledgments

This work was supported by CNPq (National Council of Research of Brazil), into the framework of instituto do Milênio-INOFAR, CNPq/MS/Neoplasias, RENORBIO, along with FINEP, CAPES, BNB (Banco do Nordeste do Brasil), FAPERJ, FAPEAL, UFF, UFRJ, UFC, and UFAL.

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