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Brief Article

3-Arylamino and 3-Alkoxy-nor-β-lapachone Derivatives: Synthesis and Cytotoxicity against Cancer Cell Lines

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Several 3-arylamino and 3-alkoxy-nor-β-lapachone derivatives were synthesized in moderate to high yields and found to be highly potent against cancer cells SF295 (central nervous system), HCT8 (colon), MDA-MB435 (melanoma), and HL60 (leukemia), with IC_{50} below 2 μM. The arylamino para-nitro and the 2,4-dimethoxy substituted naphthoquinones showed the best cytoxicity profile, while the ortho-nitro and the 2,4-dimethoxy substituted ones were more selective than doxorubicin and similar to the precursor lapachones, thus emerging as promising new lead compounds in anticancer drug development.

Introduction

Quinones have been the subject of much interest because of their various biological activities.[1] Reports concerning the biological evaluation of new naturally occurring naphthoquinones (NQs[a]) and semisynthetic analogues containing naphthalenic type structures are constantly increasing.[2] Nearly 300 NQs of related structural types have been isolated from plants, bacteria, and fungi. These naturally occurring compounds have long been used in folk medicine, and more recent studies have proved the therapeutic value of natural and semisynthetic NQs.[3] In the past few decades, a large number of natural NQs have been studied extensively because of their antitumor activity.[4] Many clinically important antitumor drugs containing quinone nuclei, such as anthracyclines, mitoxantrones and saintopin, show excellent anticancer activity.[5] Among the cytotoxic naphthoquinones, β-lapachone **1** has been the most extensively studied in recent years.[6] The semisynthetic nor-β-lapachone **2** and its amino derivatives have been the subject of several studies related to Chagas's disease[7] and as a cytotoxic agent against several cancer cell lines.[8]

To discover cytotoxic naphthoquinones, in the past few years we have synthesized and evaluated the pharmacological activity of naphthodihydrofuranquinones obtained from nor-lapachol **3**, for instance, heterocyclic[9] and arylamino derivatives of nor-β-lapachone **2**,[8] (Scheme 1). We have proved that the insertion of groups in the C-3 position of the dihydrofuran ring intensifies the pharmacological activity of nor-β-lapachone **2** on evaluated cancer cell lines (Scheme 1),[8] mainly upon insertion of an electron-poor arylamino ring modified, for instance, by nitro, fluorine, and bromine groups, with IC_{50} below 1.76 μM.[8]

Following this strategy, we synthesized the first series of substances, new arylaminonaphthoquinones (Scheme 2), where the arylamino ring was substituted by at least one of the following groups: nitro, chlorine, or bromine, together with disubstituted arylaminoquinones, using the methoxy group (resonance electron donating group), halides, and nitro (electron withdrawing group) in different chemical environments. The trypanocidal activity against *Trypanosoma cruzi* (*T. cruzi*) of a few of them, **5**−**8**, has already been reported.[7]

The second series of compounds was planned on the basis of bioisosteric replacement, i.e., NQs-NH-aryl by NQs-O-alkyl. The arylamino and alkoxy-nor-β-lapachone derivatives, described here for the first time, were easily obtained. The methodology employed enabled us to prepare a variety of related analogues with good to excellent yield (Scheme 2).

Chemistry

All the arylamino-nor-β-lapachone derivatives were synthesized as described in the literature.[8] Synthesis was carried out in a one-pot reaction. The first step involved the in situ preparation of 3-bromo-nor-β-lapachone **4** from nor-lapachol **3**,[10] which was converted into the arylamino derivatives **5**−**16** (Scheme 2) in moderate to high yields (from 65% to 95%). The syntheses of **5**−**9** have been previously described.[7,11]

A similar route was used to synthesize the alkoxy-nor-β-lapachone derivatives. Initially, nor-lapachol **3** was prepared using the classical methodology described by Fieser,[12] followed by cyclization in the presence of molecular bromine. Freshly obtained 3-bromo-nor-β-lapachone **4** was subjected to nucleophilic substitution with the respective alcohols. The

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[a] Abbreviations: NQs, naphthoquinones; *T. cruzi*, *Trypanosoma cruzi*; DOXO, doxorubicin; PBMC, peripheral blood mononuclear cells; DMSO, dimethyl sulfoxide.

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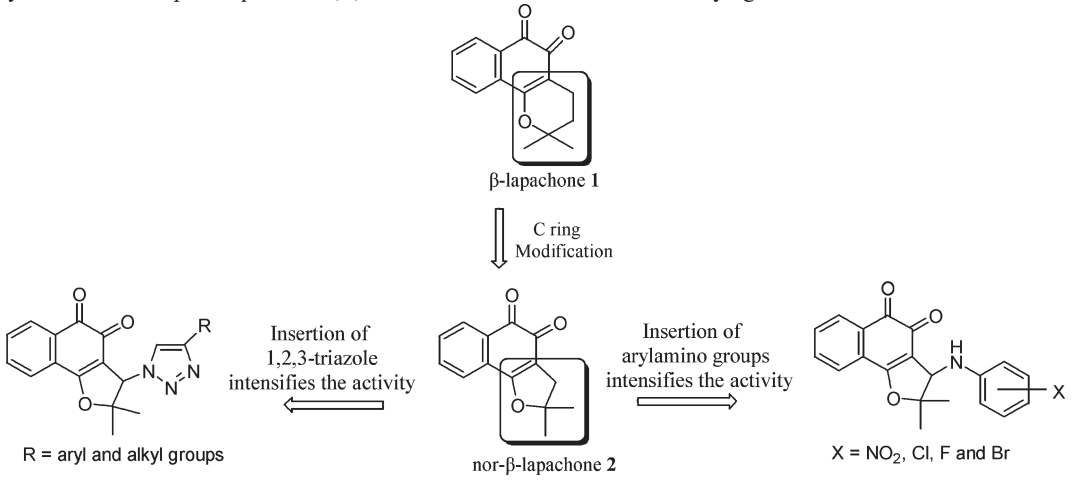
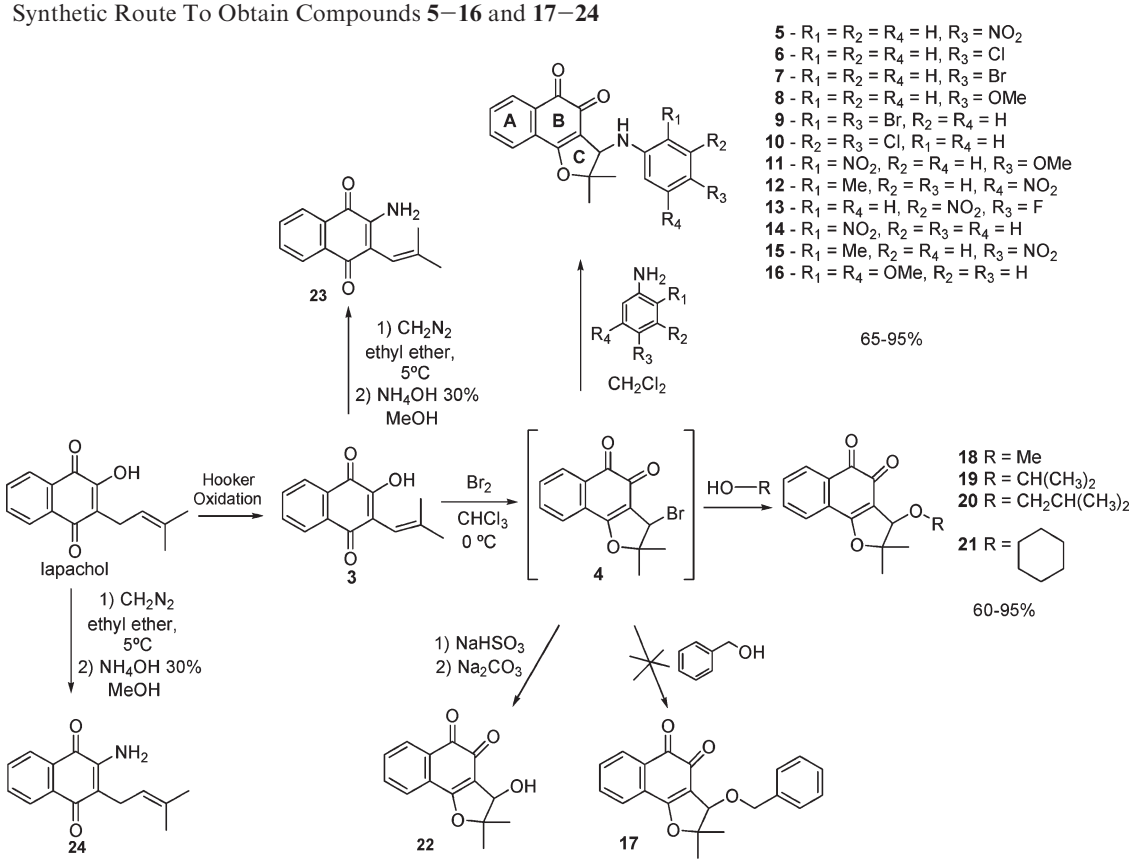
Scheme 1. Arylamino and Naphthoquinone 1,2,3-Triazole Derivatives with Activity against Cancer[8,9]

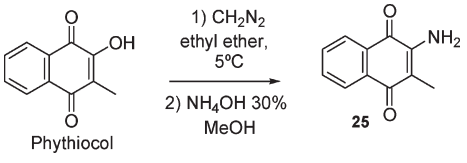


Scheme 2. Synthetic Route To Obtain Compounds **5**−**16** and **17**−**24**



alcohols were chosen according to how easily they were removed at the end of the reaction. In the case of alcohols with high boiling point, such as benzyl alcohol, the isolation of the product was not possible. The compound obtained was unstable when heated, and its distillation and recrystallization were not feasible. Attempts to isolate the product by silica gel column chromatography led to quinone **22**. Because **18** degrades after a few days, it was not pharmacologically evaluated. Substances **24** and **25**, which were subjected to antitumor studies, were obtained from lapachol and phythiocol, respectively, as described in the literature[13] (Schemes 2 and 3). Substance **22** was obtained as described in the literature[10] and used for antitumor screening.

Scheme 3. Synthetic Route To Obtain Compound **25**



The structures of the compounds were confirmed by techniques such as ^{1}H and ^{13}C NMR, IR, high-resolution (electrospray ionization) mass spectrometry, elemental analysis, and literature data.[14] The structures of **11**, **12**, **18**, and **20** were further confirmed by X-ray crystallography.

Table 1. Cytotoxic Activity Expressed by IC_{50} (μM) (95% CI) of Compounds for Cancer Cell Lines, Obtained by Nonlinear Regression for All Cell Lines from Three Independent Experiments[a]

	IC_{50} (μM) (CI 95%)				
compd	HL60	MDA-MB435	HCT8	SF295	PBMC
5	0.96(0.79− 1.15)	0.19 (0.13−0.30)	0.76 (0.54−0.87)	0.82 (0.38−1.70)	5.02 (1.70−5.54)
6	1.24 (1.07−1.44)	0.31 (0.28−0.33)	1.13 (0.70−1.46)	1.32 (1.15−1.55)	3.90 (2.74−5.53)
7	3.76 (3.21−4.41)	0.45 (0.37−0.57)	1.48 (1.15−1.58)	1.83 (1.53−2.20)	3.16 (1.18−7.35)
8	1.97 (1.20−2.08)	0.48 (0.42−0.51)	1.74 (1.60−1.94)	1.11 (0.88−1.45)	4.06 (1.57−5.52)
9	0.67 (0.54−0.81)	0.67 (0.62−0.69)	1.06 (0.88−1.27)	1.82 (1.57−2.13)	2.45 (2.30−2.59)
10	0.54 (0.49−0.64)	1.28 (1.05−1.57)	3.42 (2.52−4.66)	4.89 (4.30−5.56)	2.49 (2.26−2.75)
11	1.34 (1.14−1.57)	0.78 (0.73−0.81)	1.57 (0.48−5.12)	1.77 (1.29−2.56)	3.06 (2.71−3.44)
12	> 13.21	2.85 (2.35−3.51)	> 13.21	> 13.21	> 13.21
13	0.28 (0.23−0.36)	0.39 (0.36−0.41)	1.56 (1.30−1.85)	1.04 (0.88−1.25)	2.11 (1.90−2.35)
14	1.18 (0.82−1.70)	0.74 (0.63−0.85)	1.59 (1.39−1.81)	1.78 (1.61−1.97)	12.02 (10.31−13.99)
15	1.87 (1.66−2.11)	0.52 (0.47−0.58)	1.32 (1.13−1.53)	1.85 (1.66−2.06)	10.65 (8.82−12.87)
16	0.28 (0.26−0.31)	0.39 (0.21−0.42)	0.15 (0.02−0.23)	0.39 (0.26−0.44)	> 13.17
19	2.51 (2.20−2.89)	0.59 (0.2−16.20)	2.61 (2.02−3.38)	4.33 (1.81−10.30)	5.93 (5.20−6.81)
20	1.66 (1.46−1.89)	0.49 (0.03−5.52)	4.39 (3.99−4.82)	3.39 (2.69−4.22)	5.19 (4.49−5.99)
21	1.04 (0.82−1.34)	0.27 (0.21−0.30)	2.02 (1.71−2.35)	2.29 (1.19−4.35)	4.65 (4.04−5.36)
22	4.62 (nd)	1.26 (nd)	4.74 (nd)	4.62 (nd)	nd
23	> 22.0	> 22.0	> 22.0	> 22.0	> 22.0
24	> 20.72	> 20.72	> 20.72	> 20.72	> 20.72
25	> 26.71	> 26.71	> 26.71	> 26.71	> 26.71
doxorubicin	0.03 (0.02−0.04)	0.88 (0.62−1.21)	0.06 (0.04−0.08)	0.41 (0.29−0.44)	0.42 (0.18−0.69)
β-lapachone (**1**)	1.65 (1.49 − 1.78)	0.25 (0.16 − 0.33)	0.83 (0.74 − 0.87)	0.91 (0.74 −1.11)	> 20.6
nor-β-lapachone(**2**)	1.75 (nd)	0.31 (0.22− 0.39)	1.36 (1.18 − 1.53)	1.58 (1.31 − 1.88)	> 21.9

[a] Alamar blue assay was performed with human peripheral blood mononuclear cells (PBMC) after 72 h of drug exposure. nd, not determined.

Results and Discussion

All the arylamino and alkoxy-nor-β-lapachone derivatives **5**−**16**, **19**−**21**, and the substances **22**−**25** were tested in vitro against four cancer cell lines to compare them to doxorubicin (DOXO), the positive control, based on an MTT assay (Table 1).[15] To investigate the selectivity of compounds toward a normal proliferating cell, the Alamar blue assay was performed with peripheral blood mononucluear cells (PBMC) after 72 h of drug exposure. The compounds were classified according to their activity as highly active ($IC_{50} < 2\ \mu M$), moderately active ($2\ \mu M < IC_{50} < 10\ \mu M$), or inactive ($IC_{50} > 10\ \mu M$).[16]

The majority of the arylamino-nor-β-lapachone derivatives present potent activity against all cancer cell lines, but **12**, which showed only reasonable activity against MDA-MB435 ($IC_{50} = 2.85\ \mu M$) and is inactive toward HL60, HCT8, and SF295. The two most active compounds with IC_{50} below $1\ \mu M$ against all cancer cells are **5** and **16**, with even better activities than the standard doxorubicin (toward MDA-MB435) and the precursor **2** and superior to analogue **1** (Table 1).

The most sensitive cell toward quinones is MDA-MB435, as recently observed;[8] for instance, **5**−**9**, **11**, **13**−**21** are more potent than doxorubicin (Table 1), despite being less active than analogues **1** ($IC_{50} = 0.25\ \mu M$) and **2** ($IC_{50} = 0.31\ \mu M$), with the exception of **5** ($IC_{50} = 0.19\ \mu M$) and **21** ($IC_{50} = 0.27\ \mu M$). Compound **13** is more active toward HL60 ($IC_{50} = 0.28\ \mu M$), and **16** [HCT8 ($IC_{50} = 0.15\ \mu M$), HL60 ($IC_{50} = 0.28\ \mu M$)] and **10** [HL60 ($IC_{50} = 0.54\ \mu M$)] also show a different toxicity profile.

Doxorubicin is by far the most active ($IC_{50} = 0.03\ \mu M$) toward HL60 cancer cell line; however, **5**, **6**, **9**−**11**, **13**, **14**, **16**, and **21** are superior in activity when compared to nor-β-lapachone **2** ($IC_{50} = 1.75\ \mu M$) and β-lapachone **1** ($IC_{50} = 1.65\ \mu M$). Compounds **13** and **16** present similar and significant activity ($IC_{50} = 0.28\ \mu M$).

Concerning HCT8 and SF295 cancer cell lines, doxorubicin is again the most active ($IC_{50} = 0.06$ and $0.41\ \mu M$, respectively), with two compounds of the series, **16** ($IC_{50} = 0.15\ \mu M$ and $0.39\ \mu M$, respectively) and **5** ($IC_{50} = 0.76\ \mu M$ and $0.82\ \mu M$, respectively) (Table 1), being more potent than nor-β-lapachone **2** ($IC_{50} = 1.36$ and $1.58\ \mu M$) and β-lapachone **1** ($IC_{50} = 0.83$ and $0.91\ \mu M$).

Despite its mild activity, **12** displays important selectivity against the cancer cell line MDA-MB435 ($IC_{50} = 2.85\ \mu M$).

As for the hydroxyquinone **22** and alkoxy derived quinones **19**−**21**, the results indicated that the bioisosteric replacement did not enhance the cytotoxicity of the compounds but these substances present an apparent selectivity for MDA-MB435 cell line.

For the normal cell PBMC, the IC_{50} values were generally higher than those presented for cancer cell lines (Table 1, column 6). Table 2 lists the selectivity index, represented by the ratio of cytotoxicities between normal cells and different lines of cancer cells.

For **5**, **6**, **14**−**16**, and **19**−**21**, the values of the selectivity index (PMBC vs MDA-MB-435) were significant (> 10).

In terms of cytotoxicity, **5** and **16** showed the best profile and **14** and **16** the highest selectivity, with a much better selectivity index than the one for DOXO and similar to that of the lapachones. Compounds **5**, **14**, and **16** can be considered interesting prototypes for further studies.

The 2-amino-1,4-naphthoquinones **23**−**25** were included as representatives of inactive quinones.

The absence of hemolytic activity ($EC_{50} > 50\ \mu g/mL$), tested with erythrocyte suspensions, suggests that the cytotoxicity of the compounds was not related to membrane damage of mouse erythrocytes.

Conclusions

In this paper, we listed a series of important substances with potent antitumor activity. In general, all the compounds were highly active ($IC_{50} < 2\ \mu M$) and several substances were more active than β-lapachone **1**, an antiprostate cancer prototype currently in phase II studies. Compound **16** presented the best antitumoral profile and selectivity index, even better than doxorubicin, the positive control. Thus, this derivative presents a promising profile for further experimental investigations.

Table 2. Selectivity Index [Ratio between the Cytoxicities, Expressed as IC_{50} (μM), against PBMC and Referenced Cancer Cell Line]

compd	PBMC vs HL 60	PBMC vs MDA MB435	PBMC vs HCT 8	PBMC vs SF 295
5	5.23	26.42	6.60	6.12
6	3.14	12.58	3.45	2.95
7	0.84	7.00	2.13	1.73
8	2.06	8.46	2.33	3.66
9	3.66	3.66	2.31	1.35
10	4.61	1.94	0.79	0.51
11	2.28	3.92	1.95	1.73
12	~1.00	~4.62	~1.00	~1.00
13	7.53	5.41	1.35	2.03
14	10.18	20.48	8.07	5.76
15	5.69	16.20	7.55	6.74
16	47.03	33.77	87.80	33.77
19	2.36	10.05	2.27	1.37
20	3.13	10.59	1.18	1.53
21	4.47	17.22	2.30	2.03
DOXO	14	0.48	7	1.02

Experimental Section

Chemistry. The purity of the compounds was tested by combustion analysis, and it is ≥95%. For details see Supporting Information.

Synthetic Procedures. Lapachol (2-hydroxy-3-(3′-methyl-2-butenyl)-1,4-naphthoquinone) was extracted from the hardwood *Tabebuia* sp. (*Tecoma*) and purified by a series of recrystallizations. Nor-lapachol **3** (2-hydroxy-3-(2-methylpropenyl)[1,4]naphthoquinone) was obtained from lapachol by Hooker oxidation.[12]

The syntheses of **10** and **19** are described below as representatives of the 3-arylamino and 3-alkoxy-nor-β-lapachone series. Detailed syntheses of all other compounds are to be found in the Supporting Information.

General Procedures for Preparing 3-Arylamino-nor-β-lapachones. An amount of 2 mL of bromine (6 g, 38 mmol) was added to a solution of nor-lapachol **3** (228 mg, 1 mmol) in 25 mL of chloroform. The bromo intermediate **4** (3-bromo-2,2-dimethyl-2,3-dihydronaphtho[1,2-*b*]furan-4,5-dione) precipitated immediately as an orange solid. After removal of the bromine, an excess of the appropriate arylamine was added to this mixture and stirred overnight, after which the crude 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 solid, which was purified by column chromatography in silica gel and eluted with an increasing polarity gradient mixture of hexane and ethyl acetate (9/1 to 7/3).

3-(3,4-Dichlorophenylamino)-2,2-dimethyl-2,3-dihydronaphtho-[1,2-*b*]furan-4,5-dione 10. The reaction of nor-lapachol **3** (228 mg, 1 mmol), 2 mL of bromine (6 g, 38 mmol), and 3,4-dichlorobenzenamine (320 mg, 2 mmol) produced **10** (193 mg, 0.5 mmol, 50% yield, mp 218−219 °C). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.10 (1H, dd, J = 6.7, 1.3 Hz), 7.75−7.62 (3H, m), 7.19 (1H, d, J = 8.8 Hz), 6.66 (1H, d, J = 2.7 Hz), 6.42 (1H, dd, J = 8.8, 2.7 Hz), 4.74 (1H, d, J = 6.7 Hz), 4,04 (NH, d, J = 6.7 Hz), 1.67 (3H, s), 1.56 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 180.9, 175.5, 170.0, 147.0, 134.6, 132.8, 132.7, 131.1, 130.7, 129.6, 127.1, 125.1, 120.7, 114.5, 114.2, 112.6, 95.5, 61.4, 27.4, 21.7; EI-HRMS (m/z) $[M + Na]^+$ 410.0321. Calcd for $[C_{20}H_{15}Cl_2NO_3Na]^+$: 410.0326.

General Procedures for Preparing 3-Alkoxy-nor-β-lapachones. An amount of 2 mL of bromine (6 g, 38 mmol) was added to a solution of nor-lapachol **3** (228 mg, 1 mmol) in 25 mL of chloroform. The bromo intermediate **4** (3-bromo-2,2-dimethyl-2,3-dihydronaphtho[1,2-*b*]furan-4,5-dione) precipitated immediately as an orange solid. After removal of the bromine, an excess of the respective alcohol was added to this mixture and stirred (the reaction was monitored by TLC), after which the alcohol was evaporated to yield a solid and the product was recrystallized in appropriate solvent.

3-Isopropoxy-2,2-dimethyl-2,3-dihydronaphtho[1,2-*b*]furan-4,5-dione 19. The reaction of nor-lapachol **3** (228 mg, 1 mmol), 2 mL of bromine (6 g, 38 mmol), and an excess of propan-2-ol produced **19** as an orange solid (170 mg, 0.6 mmol, 60% yield, mp 114−115 °C). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.10 (1H, dd, J = 6.8, 1.3 Hz), 7.70−7.57 (3H, m), 4.62 (1H, s), 6.53 (1H, q, J = 6.1 Hz), 1.61 (3H, s), 1.48 (3H, s), 1.22 (6H, q, J = 6.1 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 181.1, 175.5, 169.9, 134.4, 132.2, 131.2, 129.2, 127.6, 124.8, 116.7, 95.4, 80.7, 72.9, 26.6, 22.4, 22.2, 21.1; EI-HRMS (m/z) $[M + Na]^+$ 309.1108. Calcd. for $[C_{17}H_{18}O_4Na]^+$: 309.1102.

Biology. Cytotoxicity against Cancer Cell Lines. Compounds (0.009−5 μg/mL) were tested for cytotoxic activity against four cancer cell lines: SF-295 (glioblastoma), HCT-8 (colon), MDAMB-435 (melanoma), and HL60 (leukemia) (National Cancer Institute, Bethesda, MD). For details see Supporting Information.

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.[17] For details see Supporting Information.

Inhibition of PBMC Proliferation. Alamar Blue Assay. To investigate the selectivity of compounds toward a normal proliferating cell, the Alamar blue assay was performed with peripheral blood mononuclear cells (PBMC) after 72 h of drug exposure. For details see Supporting Information.

X-ray Crystallographic Analysis. Crystallographic data for **11**, **12**, **18**, and **20** have been deposited with the Cambridge Crystallographic Data Center as Supplementary Publication Nos. CCDC 730149, 730151, 730147, and 730148, respectively. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CH21EZ, U.K. (fax, +44 1223 336 033; e-mail, deposit@ccdc.cam.ac.uk). Figure 1 (in Supporting Information) shows an ORTEP-3 diagram of each molecule.

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Supporting Information Available: Synthesis details of all compounds (except **19** and **10**); X-ray diffraction data, including a file of crystallographic information in Microsoft Word format, for **11**, **12**, **18**, and **20**; HRMS data; elemental analysis data; biology assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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