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Synthesis and Preliminary Pharmacological Evaluation of New (±) 1,4-Naphthoquinones Structurally Related to Lapachol

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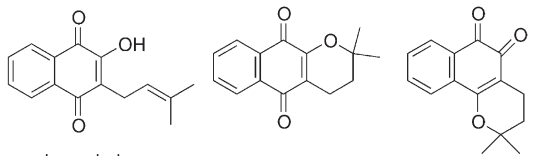
Abstract—Seven new 1,4-naphthoquinones structurally related to lapachol were synthesized from lawsone and oxygenated arylmercurials. These compounds can also be seen as pterocarpan derivatives where the A-ring was substituted by the 1,4-naphthoquinone nucleus. Pharmacological screening provided evidence of significant biological activities, including effects against proliferation of the MCF-7 human breast cancer cell line, against Herpes Simplex Virus type 2 infection, and against snake poison-induced myotoxicity. One derivative displaced flunitrazepam binding and showed benzodiazepine-like activity, suggesting novel neuroactive structural motifs.

Introduction

Naphthoquinones are widespread in nature and play important physiological roles in animals and plants.[1,2] Secondary metabolites bearing in their structure the 1,4- and 1,2-naphthoquinone moieties have been isolated from plants and exhibit interesting biological activities. Lapachol (2-hydroxy-3-prenyl-1,4-naphthoquinone), found in species of *Tabebuia*, was shown to be active against the Walker-256 carcinoma and Yoshida sarcoma.[3,4] Several synthetic derivatives of lapachol have been prepared. Its 2β-*O*-glycoside-tetraacetate derivative, for example, was shown to be active against leukemia,[5] while the tricyclic derivatives furano-1,4-naphthoquinones and pyrano-1,4-naphthoquinones showed, respectively, potent cytotoxicity against cell lines derived from human solid tumors in vitro (A-549, MCF-7, HT-29)[6,7] and antitumor activity, inhibiting the growth of KB cells in culture.[8] Pyrano-1,4-naphthoquinones are also active against Gram-positive bacteria, fungi and mycoplasma.[9]



Among the derivatives of lapachol bearing the 1,2-naphthoquinone nucleus, β-allyl lapachone shows a trypanocidal activity in contaminated blood samples,[10] while the combination of taxol and β-lapachone induced death of cultured cancer cells from ovary, breast, prostate, melanoma, lung, colon and pancreas.[11,12] As part of a program aimed at synthesizing anti-virus and anti-tumor compounds,[13] we report the synthesis and preliminary pharmacological evaluation of new lapachol

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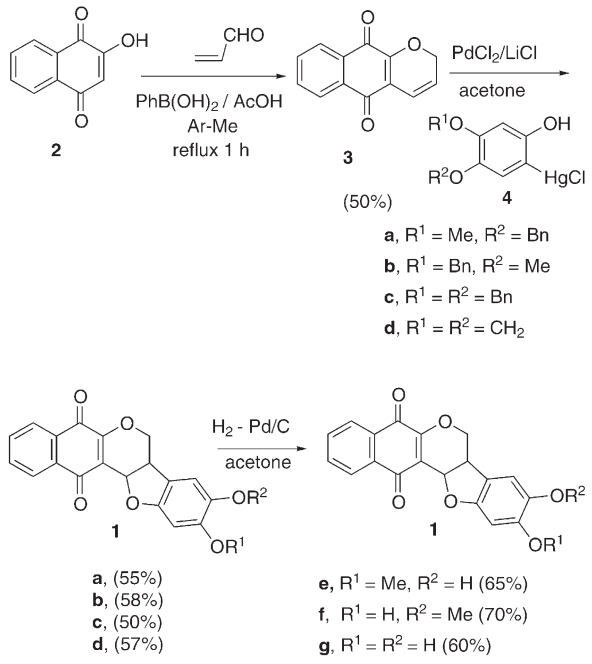
derivatives **1a**–**g** where the conformationally restricted aromatic substituents at C3 were designed to mimic the prenyl side chain of the natural product. These compounds can also be seen as pterocarpan derivatives where the A-ring was substituted by the 1,4-naphthoquinone moiety. The presence of different patterns of oxygenation at the D-ring in compounds **1a**–**g** provides new potential sites for interaction with biological macromolecules.

Results and Discussion

Chemistry

The lapachol derivatives **1a**–**g** were prepared as shown in Scheme 1. Using the protocol described by Snieckus et al. for the synthesis of chromens from phenols,[14] chromen **3** was obtained from commercially available lawsone (**2**), in a one-pot procedure, through the reaction with acrolein in the presence of boronic acid, in 50% yield after chromatographic purification.

The *o*-cloromercuriophenol derivatives **4a**–**d** were prepared from piperonal, vanillin and isovanillin, as previously described by our group.[15] These compounds were allowed to react with chromen **3** in the presence of lithium tetrachloropalladate II and acetone,[16,17] leading to the desired new naphthoquinones **1a**–**d** through a Heck-type coupling reaction, in around 50% yield. These coupling reactions could be accomplished starting from crude **3**, without any loss in the total chemical yield and purity. All naphthoquinones obtained are red solids and could be purified by crystallization. Compounds **1a**–**c** were allowed to react with hydrogen, leading to the phenolic derivatives **1e**,**f** and the catechol derivative **1g**.



Scheme 1.

Cancer cell assays

The antiproliferative activity of derivatives **1a**–**g** was studied using MCF-7 breast cancer cells (Fig. 1). Cells in exponential growth were incubated for 24 h in the absence (control, 100%) or presence of increasing concentrations of the derivatives. The inhibition of cell growth was determined using an in vitro cytotoxicity assay. Stock solutions of the compounds were prepared in dimethylsulfoxide (DMSO) and final solvent concentrations were below the cytotoxic level for MCF-7 cells. The results of three independent experiments indicated that compounds **1e** and **1f** have comparable and significant activities and are the most potent proliferation inhibitors in this series, with mean inhibitory concentrations (IC_{50}) of 5.3 and 7.8 μM, respectively (Table 1). The growth of MCF-7 cells was also inhibited by compounds **1a** and **1d** but less potently (IC_{50} values of 27.7 and 29.0 μM, respectively). The other compounds

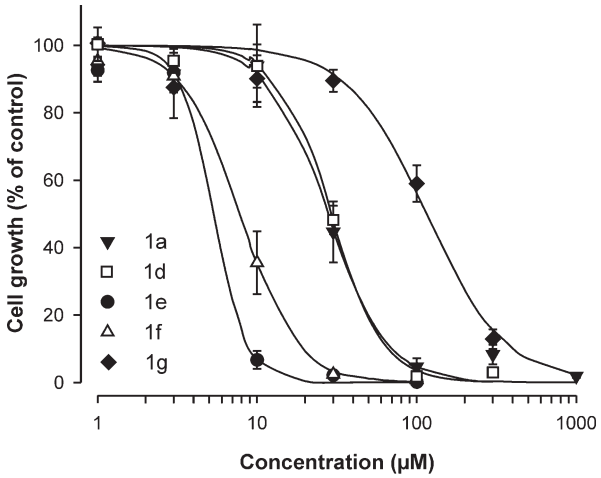


Figure 1. Effect of the derivatives on intact MCF-7 cell proliferation after 24 h of treatment with the compounds. Cells were plated at a density of 1.4×10^5 cells/mL per well using 96-well plates. The cell growth was assessed by the MTT assay and is expressed relative to untreated control cultures (100%). Each point represents the average of at least three independent experiments in which four replicate determinations were made; error bars represent the SEM. The curves were determined by non-linear fitting to the Hill equation. Six to eight concentrations of each compound were used. Some data points (below 1 μM) are not shown, for clarity.

Table 1. Effects of compounds on the growth of MCF-7 cells in culture

Comp.	Cytotoxicity IC_{50} (μM)[a]
1a	27.7±3.3
1b	>100
1c	>100
1d	29.0±1.3
1e	5.3±0.7
1f	7.8±0.6
1g	115.1±24.3

[a]Data represent the mean±SEM of the IC_{50} obtained from at least three independent experiments, each with four replicates. At least five concentrations of each compound were tested to determine the IC_{50} by fitting the Hill equation to the data.

studied in this series were also able to inhibit cell growth in a concentration-dependent manner, but their full effect apparently required higher concentrations that could not be tested due to the limited solubility in DMSO and aqueous solutions.

The *ortho*-quinone β-lapachone was previously shown to kill MCF-7 cells mainly by apoptosis, in a manner independent of cell cycle stage and of the p53/p21 pathway.[18–20] The cytotoxic effect of the quinones has often been ascribed to their redox reactive properties, which may lead to covalent modification of key enzymes, particularly DNA topoisomerases.[21,22] However, the differences in potency among the derivatives shown here may indicate that their antiproliferative effect is not solely due to the reactive quinone group, since their activity was influenced by the pattern of oxygenation in ring D. Furthermore, a recent in vitro study suggested that the 1,4-naphthoquinone α-lapachone inhibits topoisomerase II through a mechanism distinct from that of β-lapachone.[23] Thus, the mechanism of action of the novel series of compounds might be different from that of other quinones.

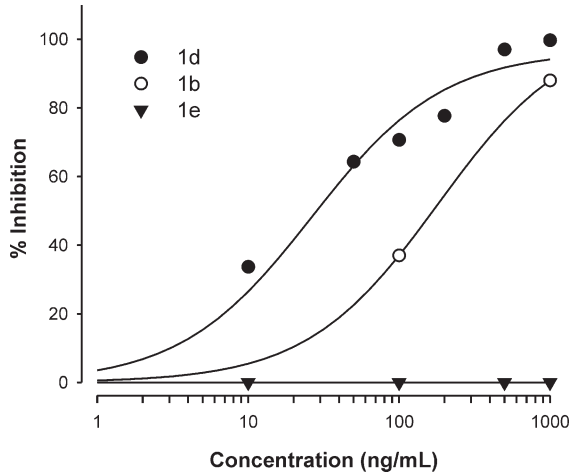


Figure 2. Effect of compounds **1b, 1d,** and **1e** on HSV-2 growth. Vero cells were infected with HSV-2 (strain G) at m.o.i. = 10 p.f.u./cell in the presence of increasing concentrations of the compounds and virus growth was determined by plaque assay at 24 h p.i. Results are expressed as% inhibition relative to virus titers in cells grown in the absence of the compounds. Results obtained in three independent experiments did not differ by more than 6–9% (● is **1d**; ○ is **1b** and ▼ is **1e**).

Viral infection assay

The antiviral activity of derivatives **1a**–**e** was studied using infection with Herpes Simplex Virus type 2 (HSV-2) as a model system. African green monkey (Vero) cells were infected with HSV-2 (strain G) at a multiplicity of infection (m.o.i.) of 10 plaque-forming units (p.f.u.) per cell in the presence or absence of increasing concentrations of the respective compounds. Cell extracts were collected at 24 h post infection (p.i.) and assayed for virus titers by plaque assay, as described.[24–26] Duplicate cultures were mock-infected with phosphate buffered saline instead of virus, exposed to increasing concentrations of compounds **1a**–**e** or DMSO (used as vehicle for compound solubilization) and examined for cytotoxic effects 24 h later. Cytotoxicity was seen in the mock-infected cultures treated with 20–100 μg/mL of DMSO or compounds **1a**–**e**. The results of three independent experiments that did not differ by more than 6–9% indicated that compound **1d** caused a significant concentration-dependent decrease in HSV-2 growth (Fig. 2). The IC_{50} calculated from these data was 25 ng/mL and the IC_{90} was 400 ng/mL. The therapeutic index, expressed as the minimal toxic concentration divided by the minimal concentration inhibiting virus growth was 2000. This value is within the range of most antiviral compounds with clinical effectiveness. HSV-2 growth was also inhibited by compound **1b**, albeit less efficiently (Fig. 2). The approximate IC_{50} for **1b**, obtained from only two drug concentrations, was 180 ng/mL. Virus growth was not inhibited by the other compounds studied in this series.

We interpret these findings to indicate that compound **1d** (and to a lower extent **1b**) has antiviral activity, at least against HSV-2. This is particularly significant since HSV-2 is one of the most widely distributed sexually transmitted infections and its prevalence has recently reached epidemic proportions.[27]

The incidence of resistance to acyclovir (ACV), which is commonly used for the treatment of HSV infections, is increasing rapidly. This is particularly true among immunocompromised individuals in whom resistant strains can cause severe and debilitating disease. Ongoing research is focused on the development of agents that may be effective against ACV-resistant strains,[28–30] most of which are mutated in thymidine kinase (TK).[31] Compound **1d** is particularly promising for the treatment of infections due to ACV-resistant TK mutants because it is unlikely to target the HSV TK. Indeed, β-lapachone is a DNA topoisomerase I inhibitor that induces cell cycle delay at the G_1 to S phase.[11,12] Topoisomerase I was previously implicated in HSV-2 replication.[32] However, additional studies are needed in order to determine the mechanism responsible for HSV-2 growth inhibition by the lapachol derivatives **1d** and **1b** and define the spectrum of their antiviral activity.

Muscle toxicity assay

The activity against muscle damage induced by snake venom was tested in order to identify mixed enzyme-inhibiting properties of the novel naphthoquinones. The crude venom of *Bothrops jararacussu* is myotoxic, mainly due to the presence of proteolytic and phospholipase A_2 activities which interact with tissues inducing hemorrhage and myonecrosis.[33–35] Sarcolemmal damage and myotoxicity induced by *B. jararacussu* crude venom were assessed in vitro by the rate of loss of creatine kinase (CK) from mice isolated extensor digitorum longus (EDL) muscles.[36,37] At 30 μM, six compounds (**1a**, **1b**, **1d**, **1e**, **1f**, and **1g**) antagonized the increase of CK release induced by the venom (Fig. 3A). The compound bearing two hydroxyls in ring D (**1g**) was the most potent and was able to fully inhibit the venom's myotoxic activity with an IC_{50} of 1.3 μM (Fig. 3B). Interestingly, coumestans with the same pattern of

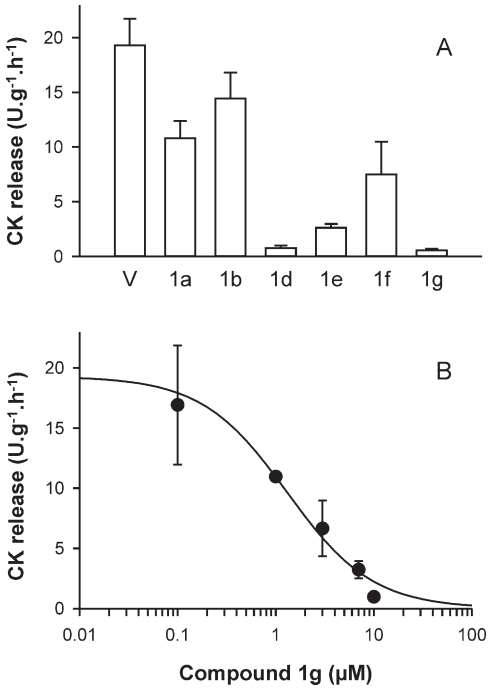


Figure 3. Effect of compounds **1a**, **1b**, **1d**, **1e**, **1f**, and **1g** on CK release induced by *Bothrops jararacussu* venom. (A**)** Bars represent the rate of CK release ($U\,g^{-1}\,h^{-1}$) of mouse EDL muscles exposed during 60 min to *B. jararacussu* venom (*V*; 25 μg/mL) either alone or in the presence of 30 μM of compounds **1a**–**g**. The basal rate of CK release in physiological saline was $0.33\pm0.03\,U\,g^{-1}\,h^{-1}$ ($n=58$). (B) Concentration–effect curve for compound **1g** (0.1–10 μM) plus 25 μg/mL of *B. jararacussu* crude venom. Each point represents the mean ± SEM ($n=3$–7).

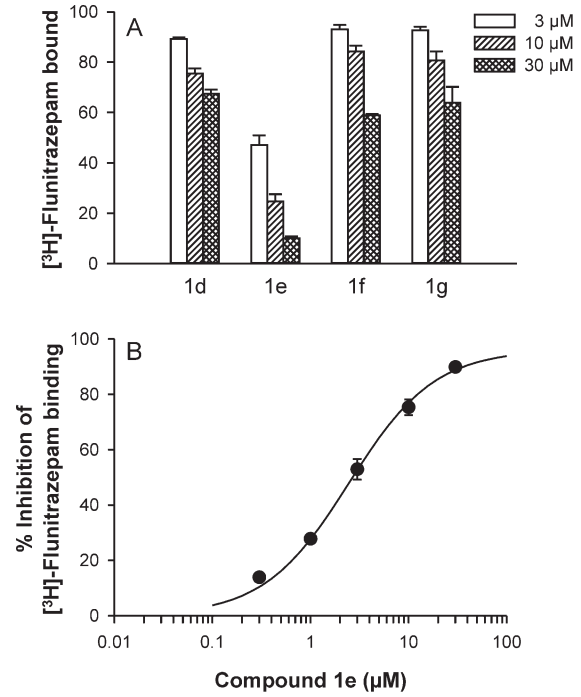


Figure 4. (A) Effect of compounds **1d**–**1g** on 0.2 nM [^{3}H]-flunitrazepam binding to rat brain synaptosomes. (B) Full inhibition curve for compound **1e**, which yielded an IC_{50} of 2 μM. Each point represents the mean ± SEM of 2–4 independent experiments performed in triplicate.

oxygenation in ring D were previously shown to inhibit the cell damage due to various crude venoms or purified protease and phospholipase A_2 toxins, including the natural coumestan wedelolactone[38,39] and some of its derivatives recently described by our group.[15]

Benzodiazepine-like activity assays

In a preliminary pharmacological screening of the novel compounds, their ability to compete with [^{3}H]-flunitrazepam for the binding to benzodiazepine (BZ) receptors in rat brain synaptosomes was assessed. As shown in Fig. 4A, compounds **1d**–**1g** inhibited the binding of [^{3}H]-flunitrazepam in a concentration-dependent manner when tested at 3–30 μM. A complete inhibition curve of the most active compound **1e** (Fig. 4B) yielded an IC_{50} of 2 μM. These results indicate a potential modulatory effect on the γ-aminobutyric acid A ($GABA_A$)/chloride ion channel involved in inhibitory transmission in the central nervous system.[40,41]

The effects of compound **1e** on the $GABA_A$-BZ receptor complex were further investigated using electrophysiological techniques. When applied alone, compound **1e** at 30 μM potentiated the bicuculline-sensitive tonic whole-cell currents due to endogenous GABA in 28 out of 32 rat hippocampal neurons (Fig. 5), but had no effect when currents elicited by GABA were absent or blocked. The augmented current elicited by **1e** was inhibited by the competitive $GABA_A$ antagonist

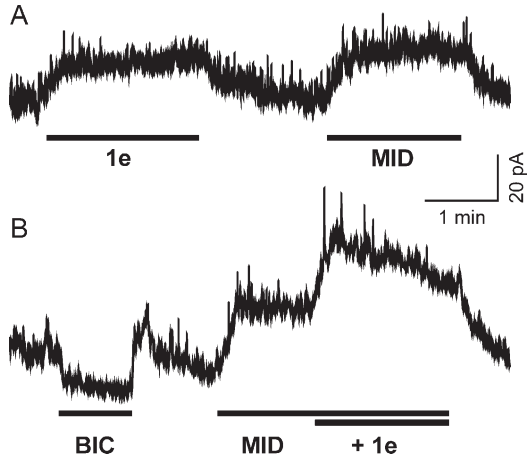


Figure 5. Sample traces showing effects of **1e** in rat hippocampal neurons voltage-clamped at 0 mV. (A) Tonic outward Cl^- currents induced by endogenous GABA were reversibly potentiated by **1e** 30 μM and by midazolam 0.1 μM (MID). (B) Blockade by bicuculline 10 μM (BIC) reveals the magnitude of the tonic GABA-induced current which was potentiated by MID and **1e** in an additive manner. The indicated drugs were applied during the periods corresponding to the thick lines under the traces.

bicuculline 10 μM. The average potentiating effect of **1e** 30 μM was 84 ± 6.7% of that of the full BZ agonist midazolam at 0.1 μM ($n=6$ cells). Addition of **1e** 30 μM to midazolam 0.1 μM caused further current increases ($n=4$). These data suggest that compound **1e** is an agonist at the BZ modulatory site of the $GABA_A$

receptor-channel. Although the potency and the affinity of **1e** seem to be much lower than that of the benzodiazepines, their structures are unrelated, and the investigation of new derivatives will be of significant interest in view of the potential applications of subtype-selective BZ ligands.[41]

Conclusion

In summary, the newly synthesized 1,4-naphthoquinones exhibited a range of significant biological activities, suggesting potential therapeutic value as anticancer and antiviral agents, as well as against the myotoxic effects of crotalid venoms. Compounds **1b** and **1d** were very active against HSV-2 growth, showing IC_{50} values in the submicromolar range. In vivo studies using animal models of viral infections and additional chemical alterations that may enhance their activity and widen their antiviral spectrum are under consideration. Compound **1e** emerged as the most potent inhibitor of breast cancer cell proliferation, but also as a modulator at the BZ binding site, an effect not yet reported for quinones. Considering the known activity of the *ortho*- and *para*-quinones β-lapachone and α-lapachone,[11,12] the naphthoquinone moiety may be essential for the antiproliferative and antiviral effects. However, the substituents in ring D may confer selectivity at the target(s) involved in these effects and may be the main determinants of the activity against cytotoxic snake venom enzymes and of the binding affinity to BZ receptors. The observed differences in potency and selectivity of the compounds may help clarify the structural requirements for binding to their respective molecular targets, and thus deserve further investigation.

Experimental

Chemistry

Melting points were determined with a Thomas-Hoover apparatus and are uncorrected. Column chromatography was performed on silica gel 230–400 mesh (Aldrich). ^{1}H NMR spectra were recorded on a Varian Gemini (200 MHz) instrument using tetramethylsilane (TMS) as standard and $CDCl_3$ as solvent. *J* Values are given in Hz. ^{13}C NMR spectra were obtained at 50 MHz.

2H-Benzo[g]chromene-5,10-dione (3). A solution of lawsone (2) (1,0 g, 5.74 mmol), acrolein (13.2 mL), phenylboronic acid (0.7 g, 5.74 mmol) and glacial HOAc (26.4 mL) in toluene (210.0 mL) was refluxed for 1 h. Optimization of product formation was effected by TLC monitoring. The mixture was cooled, concentrated in vacuo, and the residue was extracted with several portions of EtOAc. The combined extract was washed successively with H_2O, $NaHCO_3$, and brine, dried (Na_2SO_4), the solvent was evaporated in vacuo, and the crude product was purified by flash chromatography on silica gel (EtOAc–hexane 1:9 eluent) to give a red solid (0.6 g, 50%), mp 215–217 °C. ^{1}H NMR ($CDCl_3$) δn 5.1 (dd, J = 3.5, 2.0 Hz, 2H), 5.87 (dt, J = 10.0, 3.5 Hz, 1H), 6.72 (dt, J = 10.0, 2.0 Hz, 1H), 7.48–8.27 (m, 4H). Anal. calcd for $C_{13}H_8O_3$: C, 73.58; H, 3.80. Found: C, 73.56; H, 3.81.

General procedure for the preparation of naphthoquinones (1a–d). Example: naphthoquinone (**1d**). To a mixture of $PdCl_2$ (0.194 g, 1.1 mmol) and LiCl (0.047 g, 1.1 mmol) in acetone (10 mL) was added chromen **3** (0.250 g, 1.1 mmol) in acetone (10 mL). This mixture was stirred for 15 min at 0 °C and then 2-chloromercurio-4,5-methylenedioxyphenol 4 days (0.414 g, 1.1 mmol) in acetone (10 mL) was added to it. The suspension thus obtained was stirred for 12 h at 25 °C. After this time, brine (30 mL) was added to it and the mixture was extracted with acetyl acetate (100 mL), the organic extract dried (Na_2SO_4), and subjected to column chromatography to give the compound **1d** as a red solid (0.218 g, 57%), mp 136–138 °C. ^{1}H NMR ($CDCl_3$) δ 3.44 (m, 1H), 3.76 (t, J = 11.2 Hz, 1H), 4.51 (dd, J = 11.2, 5.1 Hz, 1H), 5.63 (d, J = 6.7 Hz, 1H), 5.91 (d, J = 5.6 Hz, 1H), 5.92 (d, J = 5.6 Hz, 1H), 6.58 (s, 1H), 6.72 (s, 1H), 7.71–7.78 (m, 2H), 8.10–8.25 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 38.47 (C), 67.30 (C), 73.07 (CH), 94.34 (CH), 101.33 (CH_2), 104.36 (CH), 115.86 (C), 118.18 (C), 126.52 (CH), 127.83 (CH), 130.57 (C), 131.85 (C), 133.41 (CH), 134.58 (CH), 142.02 (C), 148.42 (C), 153.74 (C), 157.05 (C), 179.33 (C), 183.32 (C). Anal. calcd for $C_{20}H_{12}O_6$: C, 68.97; H, 3.47. Found: C, 68.95; H, 3.48.

The following naphthoquinones were prepared accordingly.

Naphthoquinone (1a). This compound was obtained by column chromatography as a solid in 55% yield, mp 65–67 °C. ^{1}H NMR ($CDCl_3$) δ 3.43 (m, 1H), 3.73 (t, J = 11.1 Hz), 3.82 (s, 3H), 4.48 (dd, J = 11.3, 4.6 Hz, 1H), 5.61 (d, J = 6.6 Hz, 1H), 5.06 (s, 2H), 6.60 (s, 1H), 6.68 (s, 1H), 7.38–7.60 (m, 5H), 7.73–7.82 (m, 2H), 8.11–8.26 (2H); ^{13}C NMR ($CDCl_3$) δ 38.75 (CH), 56.01 (CH_3), 67.34 (CH_2), 72.76 (CH_2), 72.92 (CH), 96.34 (CH), 112.54 (CH), 114.93 (C), 118.32 (C), 127.50 (CH), 127.85 (CH), 128.37 (CH), 130.64 (CH), 131.09 (CH), 131.93 (CH), 132.53 (CH), 133.36 (CH), 134.58 (CH), 135.48 (CH), 137.15 (C), 142.73 (C), 151.68 (C), 154.06 (C), 157.06 (C), 179.41 (C), 183.38 (C). Anal. calcd for $C_{27}H_{20}O_6$: C, 73.63; H, 4.58. Found: C, 73.64; H, 4.57.

Naphthoquinone (1b). This compound was obtained by column chromatography as a solid in 58% yield, mp 96–98 °C. ^{1}H NMR ($CDCl_3$) δ 3.46 (m, 1H), 3.75 (t, J = 11.7 Hz, 1H), 3.84 (s, 3H), 4.52 (d, J = 11.0, 5.0 Hz, 1H), 5.08 (s, 2H), 6.57 (s, 1H), 6.83 (s, 1H), 7.25–7.58 (m, 5H), 7.65–7.78 (m,2H), 8.08–8.24 (m, 2H); ^{13}C NMR (CDCl3) δ 38.71 (CH3), 57.11 (CH), 67.29 (CH2), 71.00 (CH2), 72.80 (CH), 98.43 (CH), 109.28 (CH), 115.58 (C), 118.31 (C), 126.44 (CH), 127.02 (CH), 127.76 (CH), 128.41 (CH), 130.58 (C), 131.85 (C), 132.46 (CH), 133.34 (CH), 133.53 (CH), 134.49 (CH), 135.43 (CH), 135.43 (CH), 136.54 (C), 144.50 (C), 149.64 (C), 153.16 (C), 157.00 (C), 179.29 (C), 183.29 (C). Anal. calcd for $C_{27}H_{20}O_6$: C, 73.63; H, 4.58. Found: C, 73.65; H, 4.57.

Naphthoquinone (1c). This compound was obtained by column chromatography as a solid in 50% yield, mp 147–149 °C. ^{1}H NMR ($CDCl_3$) δ 3.39 (m, 1H), 3.70 (t, J = 10.8 Hz, 1H), 4.45 (dd, J = 11.2, 5.1 Hz, 1H), 5.05 (s, 2H), 5.07 (s, 2H), 5.57 (d, J = 6.5 Hz, 1H), 6.60 (s, 1H), 6.84 (s, 1H), 7.23–7.74 (m, 10H), 8.06–8.24 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 38.55 (CH), 67.29 (CH_2), 71.18 (CH_2), 72.91 (CH), 73.34 (CH_2), 98.78 (CH), 113.90 (CH), 116.00 (C), 118.29 (C), 126.45 (CH), 126.51 (CH), 127.16 (CH), 127.63 (CH), 128.28 (CH), 130.67 (CH), 130.96 (CH), 131.93 (CH), 132.53 (CH), 133.38 (CH), 134.51 (CH), 135.50 (CH), 136.67 (C), 137.31 (C), 143.38 (C), 151.05 (C), 154.18 (C), 157.07 (C), 179.31 (C), 183.32 (C). Anal. calcd for $C_{33}H_{24}O_6$: C, 76.73; H, 4.68. Found: C, 76.75; H, 4.69.

General procedure for the preparation of naphthoquinones (1e–g). Example: naphthoquinone (**1e**). Naphthoquinone (**1a**) (0.440 g, 0.1 mmol) in acetone was hydrogenated (3 atm) in the presence of Pd/C (10%). After 3 h the catalyst was filtered and the product purified by chromatography on silica to give **1e** in 65% yield, mp 119–121 °C. ^{1}H NMR ($CDCl_3$) δ 3.47 (m, 1H), 3.76 (t, J = 11.0 Hz, 1H), 3.84 (s, 3H), 4.54 (dd, J = 11.0, 5.1 Hz, 1H), 5.61 (d, J = 6.7 Hz, 1H), 6.57 (s, 1H), 6.85 (s, 1H), 7.69–7.83 (m, 2H), 8.09–8.22 (m, 2H). Anal. calcd for $C_{20}H_{14}O_6$: C, 68.57; H, 4.03. Found: C, 68.56; H, 4.01.

The following phenolic derivatives were prepared accordingly.

Naphthoquinone (1f). This compound was obtained by column chromatography as solid in 70% yield, mp 117–120 °C. ^{1}H NMR ($CDCl_3$) δ 3.48 (m, 1H), 3.79 (t, J = 11.1 Hz, 1H), 3.87 (s, 3H), 4.54 (dd, J = 11.1, 4.0 Hz, 1H), 5.62 (d, J = 6.8 Hz, 1H), 6.60 (s, 1H), 6.81 (s, 1H), 7.73–7.83 (m, 2H), 8.12–8.22 (m, 2H). Anal. calcd for $C_{20}H_{14}O_6$: C, 68.57; H, 4.03. Found: C, 68.55; H, 4.00.

Naphthoquinone (1g). This compound was obtained by column chromatography as solid in 60% yield, mp 195–197 °C. ^{1}H NMR ($CDCl_3$) δ 3.56 (m, 1H), 3.77 (t, J = 10.1 Hz, 1H), 4.56 (dd, J = 10.1, 4.8 Hz, 1H), 5.54 (d, J = 6.8 Hz, 1H), 6.36 (s, 1H), 6.88 (s, 1H), 7.28–7.45 (m, 2H), 7.83–8.13 (m, 2H). Anal. calcd for $C_{19}H_{12}O_6$: C, 67.86; H, 3.60. Found: C, 67.87; H, 3.62.

Pharmacology

The effect of the test compounds on MCF-7 cell proliferation was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.[42–44] MCF-7 cells were grown in MEM complete medium containing 1 mM sodium pyruvate (90%), 10% fetal bovine serum and 1% penicillin/streptomycin (all from GibcoBRL) at 37 °C in a humidified atmosphere of 5% CO_2–95% air. Cells were seeded in 96-well flat-bottom plates (Falcon, Becton Dickinson Labware) at 1.4×10^5 cells/mL (100 μL) on day zero. Twenty-four hours later, when the cells were in exponential growth, several concentrations of the test compounds were added. Stock solutions of the compounds in DMSO (stored frozen) were freshly diluted in culture medium and filter-sterilized before addition to the cells. The final concentrations of DMSO had no effect on cellular proliferation. The cells were incubated for 24 h in the absence and presence of the test compounds. After the incubation period, the medium was removed and cells were incubated with 1 mg/mL MTT at 37 °C for 3 h. Reduced MTT was measured at 560 nm using a 96-well plate reader (Victor 1420 Multilabel Counter—Wallac) after solubilization with DMSO and homogenization.

In vitro myotoxicity was evaluated at room temperature as described.[36] Briefly, EDL muscles were blotted, weighed rapidly and then transferred to sample collecting units of 2.5 mL capacity, where they were superfused continuously at a rate of 3 mL/min with physiological saline solution (PSS) equilibrated with 95% O_2–5% CO_2. At 40-min intervals, the solutions perfusing the muscles were collected and replaced with fresh media. The collected samples were stored at 4 °C until their CK activity was determined using a diagnostic kit (Sigma). The rate of CK release from the isolated muscles is expressed as international enzyme units released into the medium per gram of muscle per h of collection ($U\,g^{-1}\,h^{-1}$). The basal release rate refers to the enzyme loss from the muscles into the PSS at the beginning of the experiment, after the preparations had been mounted in the sample collecting units for at least 1 h. The composition of the PSS was (in mM) NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 15, NaH_2PO_4 1, and glucose 11 (pH 7.4 ± 0.02 after equilibration with 95% O_2–5% CO_2). *Bothrops jararacussu* venom (Instituto Vital Brazil, Niterói, RJ, Brazil) alone or associated to the different naphthoquinones was added to the nutrient solution which superfused the isolated muscles.

Specific binding of flunitrazepam was studied in crude synaptosomes prepared from rat brain (without cerebellum and brainstem). Briefly, brains were homogenized in a Potter homogenizer with a motor-driven teflon pestle at 4 °C in 15 volumes of ice-cold 0.32 M buffered sucrose (pH 7.4) per gram of organ. After centrifuging at $1000g_{max}$ for 10 min, the supernatant was centrifuged at $48{,}000g_{av}$ for 20 min to obtain the crude synaptosomes that were resuspended in buffered Krebs solution and stored at −80 °C until use. Synaptosomes (200 μg protein) were incubated at 4 °C for 90 min in a buffered Krebs solution containing 0.2 nM [^{3}H]-flunitrazepam (85 Ci/mmol, New England Nuclear Life Science Products, USA). After incubation, samples were rapidly diluted with 3 mL of ice-cold Tris–HCl buffer and instantaneously filtered on glass fibre filters (GMF 3 type from Filtrak, Germany) under vacuum. Filters were then washed once more with 3 mL buffer, dried, immersed in a scintillation cocktail and the radioactivity retained in the filters was counted with a Packard Tri-Carb 1600 TR liquid scintillation analyser. Nonspecific binding was estimated in the presence of 5 μM unlabeled flunitrazepam.

Hippocampal neurons were isolated from rat fetuses at 18–20 days of gestation, maintained in culture for 14–35

days, and used in whole-cell patch-clamp membrane current recordings as previously described.[45] Compounds were dissolved in the standard bath saline, which consisted of (in mM) NaCl 165, KCl 5, $CaCl_2$ 2, HEPES 5, NaOH 2 (pH 7.35), and solutions were applied to the neurons in fast-switching pulses with a motorized parallel tube array system. Chloride currents induced by endogenous GABA were recorded in the presence of the sodium channel blocker tetrodotoxin (0.3 μM) at a fixed membrane potential of 0 mV, in order to eliminate action potential-dependent activity and spontaneous excitatory synaptic currents.

Data analysis

The concentration–response and binding curves were fitted by either a single-site equation (rectangular hyperbola) or the Hill equation, using nonlinear regression. Data from assays of viral growth, venom-induced CK release and [^{3}H]-flunitrazepam binding were well fitted by a single-site model, whereas cancer cell proliferation data were better fitted by the Hill model with exponents greater than 1.7. Results are expressed as the mean ± SEM.

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