Synthesis, Antitumor Activity and Docking of 2,3-(Substituted)-1,4-Naphthoquinone Derivatives Containing Nitrogen, Oxygen and Sulfur

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Eleven 2,3-(substituted)-1,4-naphthoquinone derivatives were synthesized in yields ranging from 52-89%. These derivatives were evaluated for their cytotoxic effects on human lungs (H460), triple-negative breast (MDA-MB-231) and ovarian (A2780) cancer cell lines. Compounds 5f and 8 showed IC₅₀ values of 3.048 × 10⁻⁵ mol L⁻¹ and 4.24 × 10⁻⁶ mol L⁻¹ for H460; 5e and 8 showed IC₅₀ values of 2.16 × 10⁻⁵ mol L⁻¹ and 1.60 × 10⁻⁵ mol L⁻¹ for MDA-MB-231, and 5g and 8 showed IC₅₀ values of 2.68 × 10⁻⁶ mol L⁻¹ and 3.89 × 10⁻⁶ mol L⁻¹ for A2780. Additionally, we conducted a docking study with the four most active compounds and the therapeutic targets PI3K and topoisomerase II showing the pharmacophoric conformation of these compounds.

Keywords: 1,4-naphthoquinone, antineoplastic activity, topoisomerase, PI3K, nucleophilic substitution

Introduction

Notwithstanding, the progress observed in cancer treatments in the past decades, epidemiology data, clearly point to urgent new therapeutic approaches to control the disease. Improvements in the quality of life and overall survival rates of cancer patients strongly rely on the development of novel compounds with promising anticancer activity, such as natural or synthetic substances containing the quinone nuclei. The antineoplastic properties and the mechanism of action of quinone derivatives (Figure 1) have been widely studied and it is known that quinone derivatives can inhibit the activity of topoisomerase and telomerase through DNA alkylation or intercalation, inhibiting the heat shock protein HSP90. In particular, the anticancer properties of quinone derivatives seem to be mainly due to the induction of oxidative stress caused by reactive oxygen species, such as superoxide, generated from the reduction of the quinone nucleus by cellular reductases.

These data show that quinone derivatives can have multiple antineoplastic mechanisms, leading our group to postulate that some of them might, as well as, inhibit the activity of...
the phosphatidylinositol 3-kinase (PI3K). This suggestion may open an avenue to use of quinone derivatives as target therapies against PI3K-related pathways, which are frequently regulated in several human cancers, promoting cellular proliferation, differentiation and drug resistant phenotype, among other carcinogenic properties.2

It has been established that many of the biological effects of quinone derivatives depend on their 1,4-naphthoquinone pharmacophore group. Such is the case for antitumor, antiproliferative, antibacterial, anti-inflammatory, antimalarial, antiviral, antifungal and antileishmanial compounds.1 Moreover, the incorporation of nitrogen and sulfur atoms on C2 and C3 of the 1,4-naphthoquinone core has led to the formation of compounds with diverse biological activities, including anticancer activity.3

Thus, considering the importance of the substitution of C2 and C3 of the naphthoquinone core for various biological activities, the literature has provided numerous examples that 2,3-dichloro-1,4-naphthoquinone reacts with nucleophiles to form monosubstituted and disubstituted products and, more commonly, a mixture of both, depending on the softness of the nucleophile used.4,5 However, reactions carried out with electron donor groups such as amines replace just one of the chlorine atoms due to the increasing of the electron density in the naphthoquinone core.6 The second replacement happens when the withdrawing electrons effect is imposed on the naphthoquinone ring or a catalyst, normally containing palladium, is employed in the reaction.2

Herein, we present the 2,3-(substituted)-1,4-naphthoquinone derivatives containing nitrogen, oxygen and sulfur atoms, obtained from 2,3-dichloro-1,4-naphthoquinone 1, 2-methoxy-1,4-naphthoquinone 2 and 1,4-naphthoquinone 3 (Figure 2) and the respective in vitro biological assays for their potential antiproliferative activity. Importantly, the active antineoplastic substances seem to act by multiple cellular pathways, which is an effective strategy to avoid the occurrence of the chemoresistant phenotype frequently observed in tumor cells.

Figure 2. Substrates for synthesis of the desired compounds.

Experimental

General information

All solvents and reagents were commercially purchased and were used without any treatment.

Melting points were determined using Fisatom 430D equipment. Infrared (IR) spectra were recorded on Bomem FTLA2000-102-ABB spectrometer. The 1H nuclear magnetic resonance (NMR) and 13C NMR spectra were obtained on a Varian VNMR spectrometer model 400 (400 MHz) with tetramethylsilane (TMS) as internal standard. NMR analyses of compounds 5a-g, 6a-c and 8 were performed in chloroform deuterated (99.8%) with 1% (v/v) of TMS and stabilized with silver foil (Cambridge Isotope Laboratories, Inc.).

Mass spectra were recorded on ultra-high resolution and accuracy mass spectrometer (model 9.4 T Solarix, Bruker Daltonics), operated in both ionization modes: positive and negative electro spray ionization with Fourier transform ion cyclotron resonance mass spectrometry, ESI(+) and ESI(−)-FT-ICR MS spectra were acquired with resolving power of m/Dm50% ca. 500000, in which Dm50% is the full peak width at half-maximum peak height of m/z 400 and a mass accuracy < 1 ppm. It provides an unambiguous molecular formula assignment for singly charged molecular ions such as [M – H]+ or [M + H]+ and DBE (double bound equivalents) values.

Compounds 5b and 6b were partially soluble in most common deuterated solvents used in NMR and accurate 13C NMR spectra could not be obtained for these compounds.

Synthesis of compounds 5a-g

2-Chloro-3-[(pyridin-2-ylmethyl)amino]naphthoquinone (5a)

A suspension of 2,3-dichloro-1,4-naphthoquinone 1 (0.2270 g, 1.0 mmol), methanol (10 mL), picolylamine 4a (155 µL, 1.5 mmol) and triethylamine (153 µL, 1.1 mmol) was stirred for 2 h at room temperature. The product was filtered, washed with cold methanol to obtain 5a as an orange colored solid (0.2624 g, 88%); melting point (m.p.: 156-157 °C (literature: 126-127 °C)]; 1H NMR (400 MHz, CDCl3) δ 8.65 (d, 1H, J 4.9 Hz, Py-H), 8.15 (dd, 1H, J 7.7 Hz, 1.3, H-8), 8.06 (dd, 1H, J 7.6 Hz, 1.4, H-5), 7.82 (s, 1H, N-H), 7.74-7.69 (m, 2H, H-6,7), 7.63 (td, 1H, J 7.6 Hz, 1.3, Py-H), 7.29 (d, 1H, J 7.9 Hz, Py-H), 7.30-7.22 (m, 1H, Py-H), 5.19 (d, 2H, J 5.0 Hz, CH3); 13C NMR (101 MHz, CDCl3) δ 180.57, 176.78, 155.10, 149.04, 144.39, 136.87, 134.77, 132.65, 132.40, 129.96, 126.77, 126.71, 122.66, 121.72, 48.60; EI-FT-ICRMS (M+): calcd. for C19H14ClN2O2: 299.0587; found: 299.0583 (DBE = 12).
7 h at room temperature. The product was filtered, washed with cold methanol to give 5b as a reddish orange solid (0.2356 g, 89%); m.p.: 215 °C (with decomposition); 1H NMR (400 MHz, CDCl$_3$) δ 8.14 (dd, 1H, J 7.6 Hz, 10.0, H-8), 8.02 (dd, 1H, J 7.7 Hz, 1.0, H-5), 7.73 (td, 1H, J 7.6 Hz, 1.3, H-6 ), 7.63 (td, 1H, J 7.5 Hz, 1.2, H-7), 6.13 (bs, 1H, N-H), 4.00 (dd, 2H, J 13.5 Hz, 6.8, H$_2$-CH$_2$), 3.72 (dd, 1H, J 14.0 Hz, 7.0, HN-CH$_2$), 2.91 (t, 1H, J 6.4 Hz, HN-CH$_2$), 2.12 (dt, 1H, J 13.9 Hz, 7.1, CH$_3$), 1.83 (dt, 1H, J 12.9 Hz, 6.6, CH$_3$); EI-FT-ICRMS (M$^+$) calcd. for C$_{13}$H$_6$ClO$_2$: 265.0744; found: 265.0739 (DBE = 8).

2-Chloro-3-[[2-hydroxyethyl]amino]naphthalene-1,4-dione (5e)

A suspension of 2,3-dichloro-1,4-naphthoquinone 1 (0.2270 g, 1.0 mmol), methanol (10 mL), 2-aminooethanol 4c (121 µL, 2.0 mmol) was stirred for 24 h at room temperature. The product was filtered, washed with cold water to give 5e as an orange solid (0.2356 g, 89%); m.p.: 125 °C (with decomposition; literature: 196 °C); 1H NMR (400 MHz, CDCl$_3$) δ 8.22-8.17 (m, 2H, H-5,8), 8.15-8.10 (m, 2H, H-6,7), 3.59-3.42 (m, 4H, S-CH$_2$), 1.62 (bs, 1H, N-H), 4.00 (dd, 2H, J 13.9 Hz, 6.6, CH$_3$), 1.83 (dt, 1H, J 12.9 Hz, 6.6, CH$_3$); EI-FT-ICRMS (M$^+$) calcd. for C$_{13}$H$_6$ClO$_2$: 265.0744; found: 265.0739 (DBE = 8).

2-Chloro-3-methoxynaphthalene-1,4-dione (5d)

A suspension of 2,3-dichloro-1,4-naphthoquinone 1 (0.2270 g, 1.0 mmol), methanol (10 mL) and triethylamine (84 µL, 0.6 mmol) was stirred for 24 h at room temperature. Then, water was added (15 mL) slowly and stirred for 12 h. The product was filtered, washed with cold water to give 5d as an orange solid (0.1654 g, 66%); m.p.: 125 °C (with decomposition; literature: > 250 °C); 1H NMR (400 MHz, CDCl$_3$) δ 8.07 (dd, 2H, J 5.7 Hz, 3.3, H-5,8), 7.69 (dd, 2H, J 5.7 Hz, 3.3, H-6,7), 3.30 (s, 4H, S-CH$_2$); 13C NMR (101 MHz, CDCl$_3$) δ 178.60, 111.84, 117.42, 113.39, 26.99; EI-FT-ICRMS (M$^+$) calcd. for C$_{13}$H$_6$S$_2$O$_2$: 249.0044; found: 249.0037 (DBE = 8); [M + Na]$^+$: 270.9863; found: 270.9856 (DBE = 8).

2-Chloro-3-[[mercaptomethyl]thio]naphthoquinone (5f)

A suspension of 2,3-dichloro-1,4-naphthoquinone 1 (0.2270 g, 1.0 mmol), 1,2-ethanedithiol 4e (210 µL, 2.5 mmol) was stirred for 1 h at room temperature. Then, water was added (15 mL) slowly and stirred for 12 h. The product was filtered, washed with cold water to give 5f as a purple solid (0.2356 g, 89%); m.p.: 125 °C (with decomposition; literature: 196 °C); 1H NMR (400 MHz, CDCl$_3$) δ 8.22-8.17 (m, 2H, H-5,8), 8.15-8.10 (m, 2H, H-6,7), 3.59-3.42 (m, 4H, S-CH$_2$), 1.62 (bs, 1H, N-H), 4.00 (dd, 2H, J 13.9 Hz, 6.6, CH$_3$), 1.83 (dt, 1H, J 12.9 Hz, 6.6, CH$_3$); EI-FT-ICRMS (M$^+$) calcd. for C$_{13}$H$_6$ClO$_2$: 265.0744; found: 265.0739 (DBE = 8).

2,3-Bis[[2-hydroxyethyl]thio]naphthoquinone (5g)

A suspension of 2,3-dichloro-1,4-naphthoquinone 1 (0.2270 g, 1.0 mmol), acetone (5 mL), 2-mercaptoethanol 4f (210 µL, 2.0 mmol) was stirred for 1 h at room temperature. Then, water was added (10 mL) slowly and stirred for 24 h. The product was filtered, washed with cold water to obtain 5g as an orange solid (0.1614 g, 52%); m.p.: 111-113 °C (literature: 117-118 °C); 1H NMR (400 MHz, CDCl$_3$) δ 8.07 (dd, 2H, J 5.7 Hz, 3.3, H-5,8), 7.72 (dd, 2H, J 5.7 Hz, 3.3, H-6,7), 3.79 (q, 4H, J 4.9 Hz, CH$_2$-OH), 3.47-3.37 (m, 4H, S-CH$_2$), 2.71 (s, 2H, CH$_2$-OH); 13C NMR (101 MHz, CDCl$_3$) δ 178.88, 148.99, 133.81, 132.85, 127.18, 61.94, 38.20; EI-FT-ICRMS (M$^+$) calcd. for C$_{13}$H$_6$S$_2$O$_2$: 309.0255; found: 309.0260 (DBE = 7).

Synthesis of compounds 6a-c

2-[(Pyridin-2-ylmethy]lamino)naphthalene (6a)

A suspension of 2-methoxy-1,4-naphthoquinone 2 (0.0941 g, 0.5 mmol), methanol (10 mL), picolylamine 4a (180 µL, 2.0 mmol) and triethylamine (84 µL, 0.6 mmol) was stirred for 24 h at room temperature. Then, water was slowly added (20 mL) and stirred for 24 h. The product was filtered, washed with cold methanol to obtain 6a as an orange solid (0.1506 g, 58%); m.p.: 146-148 °C (literature: 153-155 °C); 1H NMR (400 MHz, CDCl$_3$) δ 8.63 (dd, 1H, J 4.9 Hz, 0.7, Py-H), 8.12-8.07 (m, 2H, H-5,8), 7.78-7.66 (m, 2H, H-6,7), 7.63 (td, 1H, J 7.5 Hz, 1.2, Py-H), 7.29 (d, 1H, J 8.7 Hz, Py-H), 7.29-7.22 (m, 1H, Py-H), 7.16 (s, 1H, NH),
5.78 (s, 1H, H-4), 4.49 (d, 2H, J=5.2 Hz, NH-CH₃): ¹³C NMR (101 MHz, CDCl₃) δ 183.08, 181.70, 154.51, 149.49, 147.76, 136.91, 134.67, 133.57, 132.04, 130.60, 126.31, 126.17, 122.81, 121.67, 101.74, 47.03; El-FT-ICRMS (M⁺) calcd. for C₁₁H₁₀N₂O₂: 265.0977; found: 265.0971 (DBE = 12); [2M + H]⁺: 529.1876; found: 529.1869 (DBE = 24); [M + Na]⁺: 287.0796; found: 287.0790 (DBE = 12) and [2M + Na]⁺: 551.1695; found: 551.1687 (DBE = 24).

2-[(3-Aminopropyl)amino]naphthoquine (6b)
A suspension of 2-methoxy-1,4-naphthoquinone 2 (0.0941 g, 0.5 mmol), methanol (10 mL), 1,3-diaminopropane 4b (83 µL, 1 mmol) and triethylamine (84 µL, 0.6 mmol) was stirred for 24 h at room temperature. Then, water was slowly added (20 mL) and stirred for 7 h. The product was filtered, washed with cold methanol to obtain 6b as an orange solid (0.1335 g, 58%); m.p.: 124-126 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.10 (dd, 1H, J=7.7 Hz, 1.2, H-8), 8.05 (dd, 1H, J=7.7 Hz, 1.2, H-5), 7.74 (td, 1H, J=7.6 Hz, 1.4, H-6), 7.63 (td, 1H, J=7.6 Hz, 1.3, H-7), 5.91 (s, 1H, NH), 5.76 (s, 1H, H-4), 3.35 (dd, 2H, J=6.6 Hz, CH₂-NH₂), 2.11 (dt, 2H, J=6.9 Hz, NH-CH₃), 1.25 (s, 2H, CH₂); El-FT-ICRMS (M⁺) calcd. for C₁₉H₁₆N₄O₂: 321.1133; found: 231.1118 (DBE = 8).

2-((2-Hydroxyethyl)amino)naphthalene-1,4-dione (6c)
A suspension of 2-methoxy-1,4-naphthoquinone 2 (0.1881 g, 1.0 mmol), methanol (10 mL), 2-aminoethanol 4c (121 µL, 2.0 mmol) was stirred for 32 h at room temperature. The solution was cooled and the product was filtered, washed with cold methanol to obtain 6c as an orange solid (0.1642 g, 76%); m.p.: 156-157 °C (literature: 146-147 °C); ¹H NMR (400 MHz, CDCl₃) δ 8.08 (ddd, 2H, J=18.6 Hz, 7.7, 1.0, H-5,8), 7.73 (td, 1H, J=7.6 Hz, 1.3, H-6), 7.62 (td, 1H, J=7.6 Hz, 1.3, H-7), 6.22 (s, 1H, H-4), 6.19 (s, 1H, H-4), 3.98 (dd, 2H, J=6.6 Hz, NH-CO), 2.11 (dt, 2H, J=6.9 Hz, NH-CH₃), 1.25 (s, 2H, CH₂); El-FT-ICRMS (M⁺) calcd. for C₁₉H₁₄N₂O₃: 313.1082; found: 313.1082 (DBE = 12).

Synthesis of compound 7
2-Methoxynaphthalene-1,4-dione (7)
2-Hidroxy-1,4-naphthoquinone (1.000 g, 5.7 mmol) was dissolved in methanol (50 mL) containing concentrated chloridric acid (0.8 mL). The reaction mixture was maintained under reflux for 4 h. Then, the solution was allowed to cool and the product was filtered and recrystallized from hot water to obtain 7 as a yellow solid (0.7937g, 73%); m.p.: 179-180 °C (literature: 183 °C); ¹³C NMR (400 MHz, CDCl₃) δ 8.19-8.04 (m, 2H, H-5,8), 7.81-7.68 (m, 2H, H-6,7), 6.19 (s, 1H, H-4), 3.92 (s, 3H, OCH₃).

Synthesis of compound 8
2-(((2-Hydroxyethyl)thio)naphthalene-1,4-dione (8)
A solution of 1,4-naphthoquinone 3 (0.0941 g, 0.5 mmol), acetone (2.5 mL) and 2-mercaptopethanol 4f (70 µL, 1.0 mmol) was stirred for 0.5 h at room temperature. The crude mixture was then purified by chromatography (15-25% ethyl acetate:hexane) to obtain product 8 as a yellow solid (0.0449 g, 77%); m.p.: 124-127 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.14-8.04 (m, 2H, H-5,8), 7.97-7.66 (m, 2H, H-6,7), 6.71 (s, 1H, H-4), 3.98 (dd, 2H, J=10.7 Hz, 5.4, CH₂-OH), 3.10 (t, 2H, J=6.1 Hz, S-CH₂), 2.06 (s, 1H, CH₂-OH); ¹³C NMR (101 MHz, CDCl₃) δ 182.04, 181.59, 154.21, 134.42, 133.40, 132.09, 131.84, 127.44, 126.92, 126.59, 59.73, 33.26; ESİ(+)-FT-ICRMS calcd. for C₁₃H₁₀O₅S [M + Na]⁺: 257.0248; found: 257.0244; [2M + Na]⁺: 491.0599; found 491.0596.

In vitro antineoplastic activity evaluation
The in vitro cytotoxicity activity of the synthesized compounds was carried out against three human cancer cell lines of solid tumors namely H460 (non-small cell lung cancer/large cell lung cancer), A2780 (epithelial ovarian carcinoma) and MDA MB-231 (triple negative breast cancer). Cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% of fetal bovine serum (FBS) and antibiotics, at 37 °C in 5% of CO₂. For dimethyl thiazolyl diphenyl tetrazolium bromide (MTT) assay, the cells were plated in 96-well culture dishes at 7.5 × 10³ cell/well, and allowed to recover for 24 h, then, treated for 24 h in a dose-dependent manner with each tested compound at concentrations of 10⁻⁴, 10⁻⁵, 10⁻⁶ and 10⁻⁷ mol L⁻¹. After, was used MTT (5 mg mL⁻¹) to evaluate the cellular metabolic viability. The absorbance at 630 nm was measured on a spectrophotometer (MR-96A, Bioclin).

Control treatments were performed with chemotherapy standard. The results are representatives of three independent experiments, mean and standard-deviation of the absorbances were used to calculate cell metabolic viability, and the IC₅₀ using PrismaGraphPad version 5.1.

Docking
The docking studies were performed in AutoDock Vina software with the ligand and the enzymes PI3Kγ.
Delarmelina et al. (PDB ID: 1E7U) and topoisomerase II (PDB ID: 1QZR). The 3D structures of the compounds were obtained after semi-empirical PM6 optimization. Docking was performed covering an 18 × 10 ×12 Å box size, centered on ligand. After validation of the method with the crystallographic ligands, interaction studies with new compounds were performed.

Results and Discussion

Chemistry

Initially, we carried out monosubstitution of the 2,3-dichloronaphthoquinone 1 with nitrogen nucleophiles (picolylamine 4a, 1,2-diaminopropane 4b and 2-aminoethanol 4c), oxygen nucleophile (methanol 4d), and sulfur nucleophiles (1,2-ethanedithiol 4e and 2-mercaptoethanol 4f) using the various experimental conditions compiled in Table 1.

Table 1. Synthesis of substituted naphthoquinone derivatives from 2,3-dichloro-1,4-naphthoquinone

<table>
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<tr>
<th>Entry No.</th>
<th>Nucleophile</th>
<th>Solvent</th>
<th>Base</th>
<th>time / h</th>
<th>Product</th>
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<td>1</td>
<td>4a</td>
<td>CH₃OH</td>
<td>Et₃N</td>
<td>2</td>
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<tr>
<td>2</td>
<td>4b</td>
<td>CH₃OH</td>
<td>not used</td>
<td>7</td>
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<tr>
<td>3</td>
<td>4c</td>
<td>CH₃OH</td>
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<tr>
<td>4</td>
<td>4d</td>
<td>CH₃OH</td>
<td>Et₃N</td>
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<tr>
<td>5</td>
<td>4e</td>
<td>CH₃OH</td>
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<td>6</td>
<td>4e</td>
<td>(CH₃)₂CO/H₂O</td>
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<td>7</td>
<td>4f</td>
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<td>not used</td>
<td>24</td>
<td><img src="image" alt="5g" /></td>
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</table>

For the synthesis of compound 5a (entry 1, Table 1), triethylamine was used with the nucleophile and methanol as the solvent to facilitate the precipitation of the product, which was obtained with high purity and in an excellent yield. The synthesis of 5b (entry 2, Table 1) followed a similar methodology, except for the absence of triethylamine. Moreover, to the best of our knowledge, the preparation of compound 5b has not been reported before.

In turn, compounds 5c (entry 3, Table 1) was synthesized under milder conditions than that previously published and yet resulted in better yields. Compound 5c was synthesized by Brun et al., reacting 2,3-dichloro-1,4-naphthoquinone, 2-ethanolamine and Et₃N, in diethyl ether solution, obtaining a 46% yields. In the present work, the same product was obtained with a yield of 89% by using methanol as the solvent and excess of nucleophile. The substance 5d (entry 4, Table 1) was prepared before using...
a solution of 2,3-dichloro-1,4-naphthoquinone and sodium methoxide in methanol, where methoxide acted as the nucleophile. In this work, compound 5d was synthesized with a yield of 81% using a solution of 2,3-dichloro-1,4-naphthoquinone, methanol and Et₃N, where methanol was the nucleophile and the solvent, simultaneously.

Preparation of the compound 5f by reacting 1 with 1,2-ethanendithiol 4e (entry 6, Table 1) was particularly challenging since we failed to reproduce the previously described method of nucleophilic substitution in water. Therefore, we enacted several experimental modifications, including different solvents (water, ethanol, methanol and acetone), variable temperatures (reactions were carried out at room temperature (r.t.) and under reflux, different bases such as K₂CO₃ and Et₃N and reaction times ranging from 1-48 h. However, due to the softness and the high nucleophilicity of 4e, the disubstituted cyclized product 5e (entry 5, Table 1) was systematically obtained from an intramolecular nucleophilic substitution (Scheme 1). Heat and the use of the Et₃N base favored generation of the product. The desired compound 5f was obtained only when the reaction was carried out in a mixture of acetone:water at r.t. with excess nucleophile.

In order to obtain the monosubstituted product from 1 and 2-mercaptoethanol 4f, a reaction was carried out under the same optimized conditions as previously described for the formation of 5f. However, the disubstituted product 5g was formed (entry 7, Table 1) from a double intermolecular nucleophilic substitution. No alicyclic product was obtained, which is likely due to the increased hardness and lower nucleophilicity of the hydroxyl group of 2-mercaptoethanol. The monosubstituted product was not obtained by this methodology.

Subsequently, several attempts were made to develop a simple and effective novel methodology that would enable us to obtain the disubstituted compounds from the products 5a and 5f using, initially, nucleophiles 4a and 4e (Table 2). 

In attempt to render the chlorine atom a better leaving group, we added AgNO₃ to the reactional solution, aiming at the complexation of the Ag⁺ ions with the chlorine atom (entries 2-9, Table 2); the same rationale applies for the use of AlCl₃, a Lewis acid with an affinity for oxygen and halogen atoms (entries 10-13, Table 2). However, the two methodologies proved to be unsatisfactory. Next, CeCl₃ was used to impose an electron withdrawing effect on the quinone ring by complexation with the carbonyl group and, thus, increasing the electrophilicity of C₇ and facilitating the nucleophile attack (entry 14, Table 2). Again, an unsatisfactory result was obtained. We next tried to complete the second nucleophilic substitution under Finkelstein conditions using NaI to replace the remaining chlorine by iodine, which is a better leaving group (entries 15 and 16, Table 2). Nonetheless, we did not succeed in obtaining the desired dissubstituted products.

To evaluate the importance of the chlorine at the carbon atom C₇, as well as, the nitrogen substituent at the C₈ in the 5a-c products for antitumor activity, nucleophilic substitution
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reactions at r.t. with 2-methoxy-1,4-naphthoquinone 7 using the 4a picolylamine, 1,2-diaminopropane 4b and 2-aminoethanol 4c nucleophiles, were carried out (Table 3). The compounds 6a17 and 6c18,24 were obtained in good-to-moderate yields by nucleophilic substitution of the methoxyl group in the 2-methoxy-1,4-naphthoquinone 7 by picolylamine and ethanolamine, respectively (Scheme 2). The compound 6b was synthesized following the same method, but using 1,3-diaminopropane as the nucleophile and methanol as the solvent, at room temperature. Moreover, to the best of our knowledge, the preparation of compound 6b has not been reported before.

Scheme 2. Mechanism for nucleophilic substitution on 2-methoxy-1,4-naphthoquinone.

The 2-methoxy-1,4-naphthoquinone 7 was prepared by methylation of 2-hydroxy-1,4-naphthoquinone via heating in methanol and hydrochloric acid, as described in the literature.19

The reaction of 2-methoxy-1,4-naphthoquinone was also tested with the sulfur nucleophiles 4e and 4f; however, distinct from the observation with nitrogen nucleophiles, the reactions with sulfur nucleophiles resulted in a complex mixture of products which were difficultly separated and it was not possible to obtain the desired product. A possible undesired reaction in this system is the oxidative Michael addition.

Because we failed to substitute the methoxyl group in 7 by the sulfur nucleophiles 4e and 4f to obtain the corresponding monosubstituted product containing the sulfur atom in the position 2 of the naphthoquinone core, we then tried to obtain these products by the oxidative Michael addition.

This methodology, which is well recommended, employs cerium(III), copper(II), nickel(II) or gold(III) salts as Lewis acids to promote the Michael addition type in the naphthoquinone, with moderate-to-good results when primary and secondary aliphatic and aromatic amines were used.6,24-27 A comprehensive study of the methods was carried out by Lisboa et al.,25 who obtained better yields when Cu(OAc)2·H2O and acetic acid was used at 60 °C. Interestingly, recent studies have described simplified methods to carry out the referred reaction without the need for a metal, which promotes the oxidative addition of quinone, but using methanol, ethanol, ethanol:water, or even water only as solvents.4,14,28-31

Initially, the oxidative Michael reaction was tested between the 2-mercaptoethanol 4f and the 1,4-naphthoquinone 3, using Cu(OAc)2·H2O as the oxidant and acetic acid or ethanol as solvent, as described in the literature.6 However, in both cases, a complex mixture of products was formed, making them difficult to isolate and purify. Subsequently, the reaction was performed using oxygen as the oxidizing agent and water or ethanol as the solvent. The reaction was performed at room temperature and under reflux, but again the desired product could not be obtained.

Only when acetone was used as the solvent, at r.t., compound 8 was obtained in a 77% yield (Scheme 3). The occurrence of oxidative addition in absence of an external oxidizing agent can be explained by the oxidizing ability of 1,4-naphthoquinone, which can assist in the formation of the desired product, as described by Taylor et al.,32 is worth emphasizing that, to the best of our knowledge, compound 8 has never been before described in the literature.

Table 3. Synthesis of monosubstituted derivatives 6a-c from 2-methoxy-1,4-naphthoquinone 7

<table>
<thead>
<tr>
<th>Entry No.</th>
<th>Nucleophile</th>
<th>Solvent</th>
<th>time / h</th>
<th>Product</th>
<th>Yield / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4a</td>
<td>CH3OH</td>
<td>24</td>
<td>6a</td>
<td>58</td>
</tr>
<tr>
<td>2</td>
<td>4b</td>
<td>CH3OH</td>
<td>7</td>
<td>6b</td>
<td>58</td>
</tr>
<tr>
<td>3</td>
<td>4c</td>
<td>CH3OH</td>
<td>32</td>
<td>6c</td>
<td>76</td>
</tr>
</tbody>
</table>
Synthesis, Antitumor Activity and Docking of 2,3-(Substituted)-1,4-Naphthoquinone Derivatives

The aforementioned optimized oxidative Michael addition was employed with 1,2-ethanediithiol 4e as the nucleophile; however, the respective product was not obtained. We also tested hydrated copper acetate in acetic acid or water; nonetheless, the strategy was proven to be ineffective.

**In vitro antineoplastic activity**

Over the past years, efforts of our research group have been devoted to synthesizing and proving the antineoplastic efficacy of various naphthoquinone derivatives in several human cancer cell lines, such as lung cancer H460 and A549, ovarian cancer A2780, triple negative breast cancer MDA-MB-231 and promyelocytic leukemia HL-60, which are representative cancers with poor prognoses.

In the present study, the *in vitro* cytotoxicity of the synthesized compounds 5a-5g, 6a-6c and 8 was assessed, by a standard colorimetric assay (the MTT method) to estimate IC<sub>50</sub> values, in three human cancer cell lines of solid tumors: H460 (non-small cell lung cancer/large cell lung cancer), A2780 (epithelial ovarian carcinoma) and MDA MB-231 (triple negative breast cancer). For internal experimental controls, the lineages were treated with routinely prescribed chemotherapy agents: etoposide, cisplatin for lung cancer line H460, cisplatin and paclitaxel for epithelial ovarian carcinoma A2780, paclitaxel, and doxorubicin for MDA MB-231 (triple negative breast cancer). All cells were treated continuously for 24 h in a dose-dependent fashion. The results are listed in Table 4.

When analyzing the proliferation of the lung cancer cell line H460, we noted that compound 8 was 10 times more potent than the control drugs at inhibiting H460 proliferation. On the other hand, compounds 5f and 5g showed cytotoxic activities similar to the controls. Thus, the compound containing a sulfur atom at the C<sub>2</sub> position of the naphthoquinone was the most potent drug against the type of lung cancer studied, suggesting that the drug might serve as an alternative, but yet efficient, therapeutic strategy to fight this deadly disease.

For the triple negative breast cancer cell line, MDA MB-231, all tested compounds were less potent than paclitaxel at controlling the cell proliferative activity. Nonetheless, among the tested compounds, substance 8 was the most potent. Of interest, compound 5c, which contains a nitrogen substituent at the C<sub>2</sub> position of the naphthoquinone, was potent against the proliferation of the cancer at the same order of magnitude as compound 8. These observations are of remarkable clinical interest since triple negative breast cancer still challenges the field of medicine due to its low overall survival rates. These low rates are caused, in part, by the relapse of resistant clones, despite the initial satisfactory response to chemotherapy, thus leading to an overall low index of survival.

The *in vitro* screening for ovarian cancer A2780 antiproliferation revealed that compounds 8 and 5g were 10 times more potent than paclitaxel. Other compounds, such as 6a and 6b, exhibited activities similar to paclitaxel, but were more potent than cisplatin against the disease. Overall, and as discussed above, these findings are of major clinical interest since ovarian cancer cells are either refractory or acquire resistant phenotypes, accounting for ovarian cancer second position in the ranking of the most frequent causes of deaths related to gynecological cancers in the world.

Interestingly, we noted that when alkyl groups containing sulfur atom are present at C<sub>2</sub> of the carbon atom of the naphthoquinone core, the resulting compounds are more potent against human cancers than compounds.
containing nitrogen atoms. It is worthwhile pointing out that the most active compounds with substituents at the C2 position of the naphthoquinone core have either –OH or –SH groups in their terminal alkyl chain.

Docking

As previously discussed, PI3K is frequently mutated in human cancers, thus enabling constitutive activation of PI3K-related pathways, as in PI3K/AKT/mTOR pathways. Because the genotype facilitates carcinogenesis and cancer progression,34 targeting PI3K, as well as, its effect or molecules have attracted the attention of cancer research groups in both academia and the pharmaceutical industry. Indeed, there are a few examples in clinical trials now; nonetheless, many have failed to confer sustained disease remission. Therefore, the generation of novel anti-PI3K molecules is urgent. Recently, the elucidation of the crystal structure of PI3Kγ and their complexes with Wortmannim and LY294002 ligands have facilitated the rational design of new inhibitors of the enzyme. Its crystal structure was obtained from the Protein Data Bank (PDB) under code 1E7U (Figure 3). Docking studies were conducted in order to analyze the conformation and binding energy through the AutoDock Vina software.21

Docking studies were also conducted in relation to topoisomerase II, another target of great importance in the study and development of new naphthoquinone substances with potential antineoplastic activity.35,36 The crystal structure of this protein was obtained from the PDB under code 1QZR.

PI3K and topoisomerase II were prepared with their respective ligands, dexrazoxane and Wortmannim, according to the protocol described in the literature.21 Grid boxes with dimensions of 18 × 10 × 12 Å for both receptors and coordinates x = 23.426, y = 62.986 and z = 20.716 (PI3K) and x = 28.166, y = 33.408 and z = 32.263 (topoisomerase II), both centered in the ligand, were constructed to fully cover the active site of the enzymes. Immediately after, the crystallographic ligands were redocked with their receptors in order to validate the efficiency of the docking calculations (Figure 4).

![Figure 3](image3.png)

**Figure 3.** Crystallographic structure representation of PI3K (1E7U) (a) and topoisomerase II (1QZR) (b).

![Figure 4](image4.png)

**Figure 4.** Crystallographic ligands (grey) and redocked ligand. (a): red Wortmannim (1E7U-KWT:PDB) and (b): green dexrazoxane (1QZR-CDX:PDB).

Next, the control drugs Etoposide, a topoisomerase II inhibitor, and Wortmannim, a PI3K inhibitor compound described in the literature,37,38 were superimposed on the binding site of the enzyme, the adenosine triphosphate (ATP) cleft, via the same method. Note that the chemical structure of the ligand was fully optimized using the parametric method 6 (PM6) semi-empirical method.22 As a result, we obtained the superposition of the ligand in the active site of PI3Kγ by calculating the interaction energy ligand/receptor (Table 5). The same procedures of molecular modeling calculations were used for all naphthoquinone derivatives.

The results obtained for the tested compounds (Table 5) indicated interaction energies close to crystallographic reference structures, especially 5a and 6a against PI3Kγ and 6c against topoisomerase II. These results lead us to suppose that the anticancer mechanism of action to these naphthoquinone derivatives occurs through PI3K and topoisomerase II inhibition, as widely reported previously.2

The docking studies are able to identify the best pose of ligands into the binding site. Figure 5 depicts the intermolecular interaction between Wortmannim and 6a against the binding site of PI3K enzyme performing van der Waals interactions with Lys808, Pro810, Trp812, Ile831, Ile879, Glu880, Ile881, Asp950, Phe961, Ile967 amino acids; electrostatic interactions with Met804, Val882, Lys883, Met953, Asp964, Tyr867; and hydrogen bonds with Val882, Lys883, Tyr867, Asp964 amino acids (Figure 6a).

Likewise, the most active compound by docking, 6a, can complex by van der Waals interactions with Ser806, Pro810, Glu880, Lys833, Ile879, Val882, Met953, Phe961, Ile963 amino acids; electrostatic interactions with Met804, Tyr867, Asp964 amino acids. No hydrogen bonds could be observed in these compounds. However, new derivatives with acceptor hydrogen bond can improve the active by an interaction with Tyr867, similar to Wortmannim. In addition, Figure 6c shows the cluster of all ligands in the
binding site. As can be seen, the naphthalene complex is very similar to Wortmannin into the binding site of phosphatidylinositol 3-kinase (PI3K), suggesting this molecular target as a receptor of naphthoquinones.

Similarly, to PI3K enzyme, Figure 6 shows the intermolecular interaction between dexrazone, and 6c. As can be seen, dexrazone performs van der Waals interactions with Tyr28, Tyr144, Leu148 amino acids; electrostatic interactions with Thr27, His28, Asn142 amino acids; and hydrogen bond with Gln365 amino acid, all for both chains of enzyme (a and b) (Figure 6a). Similarly, compound 6c can complex through van der Waals interactions with Thr27, Tyr144, Gln365 amino acids; electrostatics interaction with Gln17, His20, Thr27, Tyr28, Asn142; a hydrogen bond can be observed between 6c and Thr27 (Figure 6b). Finally, Figure 6c shows a cluster formed by dexrazone and naphthoquinones. These results suggest that these compounds can inhibit the topoisomerase II.

In addition, the naphthoquinone derivatives 5c, 5f, and 8 can highlight the electrostatic interaction between the naphthoquinone carbonyl core and amino acids from the Asp964 residue of the PI3K protein (Figure 7).

Another important interaction occurs between –SH and –OH substituents and the C2 naphthoquinone carbon

<table>
<thead>
<tr>
<th>Compound</th>
<th>Interaction Energy / (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wortmannin</td>
<td>–7.7</td>
</tr>
<tr>
<td>Dexrazone</td>
<td>–</td>
</tr>
<tr>
<td>Etoposide</td>
<td>–</td>
</tr>
<tr>
<td>5a</td>
<td>–7.7</td>
</tr>
<tr>
<td>5b</td>
<td>–6.7</td>
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<td>5c</td>
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<td>6c</td>
<td>–6.7</td>
</tr>
<tr>
<td>8</td>
<td>–6.6</td>
</tr>
</tbody>
</table>
with different amino acid residues, such as the electrostatic interaction with the Lys833 to compound 5c and the hydrogen bonds with Asp950 and Lys833 with 5g and 8, respectively. The other interactions of these compounds are van der Waals interactions with different amino acid residues.

The docking of etoposide against the active site of topoisomerase II showed a hydrogen bond between the hydroxyl group and Gly365, electrostatic interactions with the Gly17, Thr27, Tyr28, and van der Waals interactions with Tyr144, as shown in Figure 8.

Naphthoquinone derivatives have in general similar interactions to those presented in the docking etoposide, where the major amino acid residues responsible for this interaction have been Gly365, Gly17, Thr27, Tyr28 and Tyr144 (Figure 9). This similarity likely reflects the interaction energies between naphthoquinone derivatives and etoposide when topoisomerase II is the therapeutic target.

Compound 5c showed two important hydrogen bonds, the amino and hydroxyl groups from the side chain of the substituent (ethanolamine) with the tyrosine amino acid residues glycine 28 and 17. Furthermore, an electrostatic interaction with threonine 27 was observed.
Compounds 5f and 5g showed the same hydrogen bonds with etoposide described for the amino acid residue Gly365. However, the group that was responsible for this interaction in 5f (5g) was the naphthoquinone carbonyl core (the terminal hydroxyl substituent from mercaptoethanol). Besides these interactions, compound 5g showed an electrostatic interaction with the amino acid threonine 27. Compound 8 showed two electrostatic interactions with the amino acid residues Gly17 and Tyr144. van der Waals interactions were also observed with Gly365 and Tyr28.

**Conclusions**

In this work, we have described novel and highly efficient methodologies for preparation of eleven 1,4-naphthoquinone derivatives 5a-g, 6a-c and 8 containing substituents in positions 2 and/or 3 in yields ranging from 38% to 89%. The following compounds showed greater in vitro antitumor activity: 5f and 8 against human lung cancer lines H460; 5c and 8 against triple-negative breast cancer lines MDA-MB-231; 5g and 8 against ovarian cancer lines A2780. The clinical drugs tested in H460, etoposide and cisplatin; and in A2780, cisplatin and paclitaxel, showed higher values of IC_{50} compared with the results obtained for synthesized novel compounds, thus, pointing to the higher antineoplastic potency of the latter. The structure/activity relationship of these compounds showed that bioisosteric substitution for sulfur atoms at the carbon C_{2} in naphthoquinone core, or hydroxyl groups in the substituent chain, are primarily responsible for the biological activity of the compounds. The docking analysis revealed that a possible therapeutic interaction in this interaction in 5f (5g) was the naphthoquinone carbonyl core (the terminal hydroxyl substituent from mercaptoethanol). Besides these interactions, compound 5g showed an electrostatic interaction with the amino acid threonine 27. Compound 8 showed two electrostatic interactions with the amino acid residues Gly17 and Tyr144. van der Waals interactions were also observed with Gly365 and Tyr28.

**Supplementary Information**

Supplementary data (Figures S1-S36) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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