Research Article

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Synthesis and antiproliferative evaluation of some 1,4-naphthoquinone derivatives against human cervical cancer cells

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Abstract: In the course of biological properties of quinone derivatives, the N(H)-, S- and S,S-substituted-1,4-naphthoquinones were synthesized by reactions of 2,3-dichloro-1,4-naphthoquinone with different amines (2-morpholinoaniline, tert-butyl 4-aminobenzoate, 4-tert-butylbenzylamine, N-(3-aminopropyl)-2-pipecoline, 2-amino-5,6-dimethylbenzothiazole, N,N'-diphenyl-p-phenylenediamine) and thiolat (sodium 2-methyl-2-propanethiolate). All new products were characterized by MS-ESI, UV-Vis, FT-IR, ^1H NMR, ^{13}C NMR. The antiproliferative activities of these compounds on human cervical cancer (HeLa) cells were evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Although all derivatives inhibited cell growth, the most active compound was 2-(tert-butylthio)-3-chloronaphthalene-1,4-dione **5** ($\text{IC}_{50}=10.16 \mu\text{M}$) against the HeLa cells.

Keywords: Quinones; Amines; Thiols; Antiproliferative activity; HeLa cell.

1 Introduction

Naturally occurring and synthetic naphthoquinones have found wide uses in pharmacological fields with

their antiseptic [1], antifungal, antibacterial [2], anti-inflammatory [3,4], cytotoxic [5-7], anti-tumor [3,8-10], molluscicidal [11], anticancer [12-14], antimicrobial [3,15], antimalarial [16,17], antithrombotic [3], etc. activities. Thus, many naphthoquinone derivatives have been synthesized [7,8,18-21] and some of them have been patented [4,22]. However, there is still a continuous effort to find functionalized naphthoquinones and related compounds. Also, the incorporation of amino, thio, halo or alkyl/aryl group into quinones could be used to improve their antifungal, antibacterial, cytotoxic etc. activities [7,23-25].

Recently, quinones have gained importance for their uses in chemotherapy (anticancer drugs), such as mitomycin C, doxorubicin, daunorubicin, streptonigrin, idarubicin, mitoxantrone, epirubicin etc [12,21,26-29]. Generally, the anticancer properties of the compounds having the quinone structure seem to be due to the induction of oxidative stress [7,30]. Among quinones, 1,4-naphthoquinones such as alkannin, shikonin, 2-hydroxynaphthoquinone, 1,2-pyranonaphthoquinone derivatives have been offered as potential drug candidates for cancer treatment [12,31,32].

Several studies highlight the antiproliferative/anticancer activity of 1,4-naphthoquinone compounds on prostate, breast, lung, colon, brain and pancreas cancer cells [12-14,29,32-34]. For example, some 1,4-naphthoquinone derivatives showed improved antiproliferative activity in comparison to the lead molecule in prostate (DU-145), breast (MDA-MB-231) and colon (HT-29) cancer cell lines [13]. Also, the literature mentions that some compounds similar to those synthesized in this study, for example 2-chloro-3-(N-alkylamino)-1,4-naphthoquinone derivatives exhibit antiproliferative activities on colon (COLO205), brain (U87MG) and pancreas (MIAPaCa2) cancer cells [29]. Besides, the antiproliferative activity of 2-(4-morpholinophenylamino)-3-chloronaphthalene-1,4-dione has been found to be comparable to that of

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the proteasome inhibitor PI-083 [20]. This compound is similar to synthesized compound **3a** in this study.

It has been previously known that some mono-, bis-, tris- and tetrakis-(thio/amino)-quinone derivatives synthesized in our laboratory [35-41] have antifungal, antibacterial, antioxidant and cytotoxic/anticancer activities [38,39]. The N-substituted naphthoquinone compound showed the powerful cytotoxic activity at a concentration of 20 μ M against A549 (lung), MCF-7 (breast), DU145 (prostate), and HT-29 (colon) cancer cell lines [39]. Thus, due to the promising anticancer activities of quinone compounds, we report in this work the synthesis, characterization and antiproliferative activity of N(H)-, S- and S, S-substituted-1,4-naphthoquinones.

2 Experimental

2.1 Chemistry

Melting points were determined using Buchi B-540 equipment. Infrared spectra (IR) were recorded on Thermo Scientific Nicolet 6700 instrument. Mass spectra (MS) were taken from Thermo Finnigan LCQ Advantage MAX, operated in positive and negative ion mode (+ESI and -ESI). ^1H NMR, ^{13}C NMR spectrums were obtained using a Varian Unity Inova (500 MHz) spectrometer by using TMS as the internal standard and deuterated chloroform as solvent. UV-Vis spectra were obtained by using a Lambda 35 UV/Vis Spectrometer (Perkin Elmer) in CHCl_3 . Column chromatography on silica was performed (Merck Kieselgel 60, 70-230 mesh). Unless otherwise stated reagents were purchased from Sigma Aldrich, USA.

2.2 Experimental Design of the Compounds

2-(2-Morpholinophenylamino)-3-chloronaphthalene-1,4-dione (3a). A solution of 1.2 g **1** (5.28 mmol) and 0.94 g 2-morpholinoaniline **2a** (5.28 mmol) in 30 mL ethanol was heated under a reflux condenser without base. The reaction medium was monitored by thin layer chromatography and the mixture was then diluted with water and extracted with chloroform. The CHCl_3 extract was dried with anhydrous Na_2SO_4 and concentrated in under pressure to give a crude product, which was chromatographed with silica gel column to give 0.78 g (40%) **3a**. R_f (CH_2Cl_2): 0.23; Black solid; mp 175-176°C; IR (KBr, cm^{-1}): 3300, 2960, 2852, 1674, 1647, 1604, 1569; UV λ_{max} (CHCl_3) nm (log ϵ): 241 (3.63), 284 (3.93), 521 (3.17); ^1H

NMR (500 MHz, CDCl_3): δ 8.13 (H, dd, CH_{naph} , $J = 7.8$ Hz, 1.5 Hz), 8.02 (H, dd, CH_{naph} , $J = 7.6$ Hz, 1.2 Hz), 7.87 (H, s, NH), 7.70 (H, td, CH_{naph} , $J = 7.6$ Hz, 1.3 Hz), 7.61 (H, td, CH_{naph} , $J = 7.6$ Hz, 1.4 Hz), 7.09 (H, td, CH_{arom} , $J = 7.7$ Hz, 1.6 Hz), 7.04-6.98 (2H, m, CH_{arom}), 6.85 (H, dd, CH_{arom} , $J = 7.8$ Hz, 1.5 Hz), 3.71 (4H, t, 2 $\text{O}-\text{CH}_2$, $J = 4.4$ Hz), 2.89 (4H, t, 2N- CH_2 , $J = 4.4$ Hz); ^{13}C NMR (125 MHz, CDCl_3): δ 179.5, 176.0 (C=O), 143.6, 140.7, 133.9, 131.9, 131.7, 130.8, 129.2, 126.2, 126.1, 125.9, 124.4, 122.8, 118.3, 114.2 (C_{naph} , CH_{naph} , CH_{arom} , C_{arom}), 66.3, 66.2 ($-\text{H}_2\text{C}-\text{O}-$), 50.6, 50.7 ($-\text{N}-\text{H}_2\text{C}-$); MS (+ESI): $m/z = 369.0$ [$\text{M}+\text{H}$] $^+$, MS/MS (+ESI): $m/z = 333.1$ [$\text{M}-\text{Cl}$] $^+$.

Tert-butyl-4-(2-chloro-1,4-dihydro-1,4-dioxonaphthalen-3-ylamino)benzoate (3b). A solution of 1.5 g **1** (6.60 mmol) and 1.28 g tert-butyl 4-aminobenzoate **2b** (6.60 mmol) in 30 mL ethanol was heated under reflux condition without base. The reaction medium was monitored by thin layer chromatography and the mixture was then diluted with water and extracted with chloroform. The CHCl_3 extract was dried with anhydrous Na_2SO_4 and concentrated in under pressure to give a crude product, which was chromatographed with silica gel column to give 1.42 g (56%) **3b**. R_f (CHCl_3): 0.3; Red solid; mp 104-105°C; IR (KBr, cm^{-1}): 3185, 2977, 2931, 1705, 1676, 1651, 1595, 1570; UV λ_{max} (CHCl_3) nm (log ϵ): 240 (4.37), 290 (4.72), 472 (3.83); ^1H NMR (500 MHz, CDCl_3): δ 8.12 (1H, dd, CH_{naph} , $J = 7.8$ Hz, 1.0 Hz), 8.05 (1H, dd, CH_{naph} , $J = 7.6$ Hz, 1.2 Hz), 7.64 (1H, td, CH_{naph} , $J = 7.6$ Hz, 1.3 Hz), 7.71 (1H, td, CH_{naph} , $J = 7.5$ Hz, 1.5 Hz), 7.63 (H, s, NH), 7.90 (2H, dd, CH_{arom} , $J = 8.3$ Hz, 2.1 Hz), 6.97 (2H, d, CH_{arom} , $J = 8.8$ Hz), 1.53 (9H, 3 CH_3); ^{13}C NMR (125 MHz, CDCl_3): δ 179.4, 176.4 (C=O $_{\text{naph}}$), 164.1 ($-\text{O}-\text{C}=\text{O}-$), 140.1, 140.0, 134.1, 132.2, 131.4, 128.9, 128.9, 128.8, 127.5, 126.2, 126.1, 121.7, 121.6, 116.2 (CH_{naph} , C_{naph} , C_{arom} , CH_{arom}), 80.1 (C_{tert}), 27.2, 27.2 (Me); MS (+ESI): $m/z = 384.0$ [$\text{M}+\text{H}$] $^+$, MS (-ESI): $m/z = 382.1$ [$\text{M}-\text{H}$] $^-$, MS/MS (-ESI): $m/z = 326.2$ [$\text{M}-(\text{CCH}_3)_3$] $^-$.

2-(4-Tert-butylbenzylamino)-3-chloronaphthalene-1,4-dione (3c). A solution of 1.0 g **1** (4.4 mmol) and 0.72 g 4-tert-butylbenzylamine **2c** (4.4 mmol) in ethanol at room temperature was stirred without base. The reaction medium was monitored by thin layer chromatography and the mixture was then diluted with water and extracted with chloroform. The CHCl_3 extract was dried with anhydrous Na_2SO_4 and concentrated in under pressure to give a crude product, which was chromatographed with silica gel column to give 0.74 g (47%) **3c**. R_f (CHCl_3): 0.6; Red solid; mp 101-102°C; IR (KBr, cm^{-1}): 3340, 2963, 2904, 2868, 1679, 1646, 1602, 1573; UV λ_{max} (CHCl_3) nm (log ϵ): 241 (3.12), 278 (4.34), 455 (2.38); ^1H NMR (500 MHz, CDCl_3): δ 8.09 (1H, dd, CH_{naph} , $J = 7.8$ Hz, 1.5 Hz), 7.97 (1H, dd, CH_{naph} , $J = 7.8$ Hz, 1.5 Hz), 7.66 (1H, td, CH_{naph} , $J = 7.4$ Hz, 1.1 Hz), 7.56 (1H, td, CH_{naph} , $J = 7.4$ Hz,

1.3 Hz), 7.34 (2H, dd, 2CH_{arom} , $J = 6.4$ Hz, 2.0 Hz), 7.20 (2H, d, 2CH_{arom} , $J = 8.3$ Hz), 6.11 (s, H, NH), 4.95 (d, 2H, $\text{CH}_{2\text{benzyl}}$, $J = 5.9$ Hz), 1.26 (s, 9H, 3CH_3); ^{13}C NMR (125 MHz, CDCl_3): δ 179.5, 175.9 (C=O), 150.2, 143.1, 133.9, 133.8, 131.7, 131.5, 128.9, 128.7, 126.5, 126.0, 125.9, 125.8, 125.6, 125.0 (CH_{naph} , C_{naph} , CH_{arom} , C_{arom}), 47.8 ($\text{CH}_{2\text{benzyl}}$), 33.6 (C_{tert}), 30.3 (3CH_3); MS (+ESI): $m/z = 354.1$ [$\text{M}+\text{H}$] $^+$.

2-(3-(2-Methylpiperidin-1-yl)propylamino)-3-chloronaphthalene-1,4-dione (3d). A solution of 1.0 g **1** (4.4 mmol) and 0.63 g N-(3-aminopropyl)-2-pipecoline **2d** (4.4 mmol) in ethanol at room temperature was stirred without base. The reaction medium was monitored by thin layer chromatography and the mixture was then diluted with water and extracted with chloroform. The CHCl_3 extract was dried with anhydrous Na_2SO_4 and concentrated in under pressure to give a crude product, which was chromatographed with silica gel column to give 0.62 g (40%) **3d**. R_f (EtOH): 0.35; Red viscous oil; IR (KBr, cm^{-1}): 3343, 2933, 2856, 2794, 1678, 1634, 1601, 1570; UV λ_{max} (CHCl_3) nm (log ϵ): 241 (4.19), 276 (4.38), 467 (3.53); ^1H NMR (500 MHz, CDCl_3): δ 8.07 (dd, H, CH_{naph} , $J = 7.8$ Hz, 1.0 Hz), 7.96 (dd, H, CH_{naph} , $J = 7.8$ Hz, 1.0 Hz), 7.63 (td, H, CH_{naph} , $J = 7.6$ Hz, 1.1 Hz), 7.53 (td, H, CH_{naph} , $J = 7.6$ Hz, 1.3 Hz), 7.34-7.48 (sb, H, NH), 3.80-4.10 (m, 2H, CH_2), 2.80-3.0 (m, broad, 2H, CH_2), 1.0-2.5 (m, 14H, $\text{CH}_{2\text{ring}}$, CH_2 , CH_3); ^{13}C NMR (125 MHz, CDCl_3): δ 179.7, 178.0 (C=O), 143.9, 133.7, 131.8, 131.2, 129.1, 125.7, 125.7 (C_{naph} , C-H $_{\text{naph}}$), 55.9, 51.1, 43.8, 32.5, 28.7, 25.2, 24.0, 22.3; 17.3 (CH_3); MS (+ESI): $m/z = 346.9$ [$\text{M}+\text{H}$] $^+$.

2-(5,6-Dimethylbenzo[d]thiazol-2-ylamino)-3-chloronaphthalene-1,4-dione (3e). A solution of 2.0 g **1** (8.8 mmol) and 2-amino 5,6-dimethylbenzothiazole 1.56 g **2e** (8.8 mmol) in 30 mL ethanol was stirred at reflux temperature without base. The reaction medium was monitored by thin layer chromatography and the mixture was then diluted with water and extracted with chloroform. The CHCl_3 extract was dried with anhydrous Na_2SO_4 and concentrated in under pressure to give a crude product, which was chromatographed with silica gel column to give 0.66 g (20%) **3e**. R_f (CHCl_3): 0.1; Dark orange solid; mp 182-183°C; IR (KBr, cm^{-1}): 3106, 3020, 2962, 2922, 2848, 1679, 1669, 1603, 1576, 1457; UV λ_{max} (CHCl_3) nm (log ϵ): 241 (4.63), 286 (4.73), 467 (3.84); ^1H NMR (500 MHz, CDCl_3): δ 8.14 (H, dd, CH_{naph} , $J = 7.3$ Hz, 1.5 Hz), 8.07 (H, dd, CH_{naph} , $J = 7.1$ Hz, 1.2 Hz), 7.73 (H, td, CH_{naph} , $J = 7.6$ Hz, 1.5 Hz), 7.67 (H, td, CH_{naph} , $J = 7.6$ Hz, 1.3 Hz), 7.51 (s, H, CH_{arom}), 7.42 (s, H, CH_{arom}), 2.29 (s, 6H, 2CH_3); ^{13}C NMR (125 MHz, CDCl_3): δ 178.5, 176.6 (C=O), 157.0, 146.8, 140.8, 134.8, 133.9, 133.1, 132.7, 131.0, 129.1, 126.3, 126.1, 123.0, 120.8, 120.4, 19.1 (CH_3), 19.0 (CH_3); MS (+ESI): $m/z = 369.2$ [$\text{M}+\text{H}$] $^+$, MS2 (+ESI): $m/z = 333.4$ [$\text{M}-\text{Cl}$] $^+$.

2-(N-phenyl-N-(4-(phenylamino)phenyl)amino)-3-chloronaphthalene-1,4-dione (3f). A solution of 2.0 g **1** (8.8 mmol) and 2.29 g N,N'-diphenyl-p-phenylenediamine **2f** (8.8 mmol) in 30 mL DMF was stirred at about 120°C. The reaction medium was monitored by thin layer chromatography and the mixture was then diluted with water and extracted with chloroform. The CHCl_3 extract was dried with anhydrous Na_2SO_4 and concentrated in under pressure to give a crude product, which was chromatographed with silica gel column to give 0.79 g (20%) **3f**. R_f (CH_2Cl_2): 0.7; IR (KBr, cm^{-1}): 3320, 3028, 2955, 2918, 2850, 1673, 1595, 1570, 1513, 1496; UV λ_{max} (CHCl_3) nm (log ϵ): 242 (4.78), 277 (4.71), 490 (3.98); ^1H NMR (500 MHz, CDCl_3): δ 8.13 (H, dd, CH_{naph} , $J = 7.6$ Hz, 1.2 Hz), 8.06 (H, dd, CH_{naph} , $J = 7.6$ Hz, 1.2 Hz), 8.02 (s, H, NH), 7.70 (H, td, CH_{naph} , $J = 7.7$ Hz, 1.5 Hz), 7.62 (H, td, CH_{naph} , $J = 7.5$ Hz, 1.5 Hz), 7.29 (t, 4H, CH_{arom} , $J = 7.8$ Hz), 7.10-7.18 (m, 6H, CH_{arom}), 7.02 (d, 4H, CH_{arom} , $J = 6.8$ Hz); ^{13}C NMR (125 MHz, CDCl_3): δ 179.6, 176.5 (C=O), 140.6, 136.5, 134.0, 132.0, 131.6, 128.9, 128.5, 128.4, 127.4, 126.2, 126.0, 124.7, 123.3, 116.8, 114.0.

2-(Tert-butylthio)-3-chloronaphthalene-1,4-dione (5) and 2,3-bis(tert-butylthio)naphthalene-1,4-dione (6). A solution of 0.5 g **1** (2.2 mmol) and 0.24 g sodium 2-methyl-2-propanethiolate **4** (2.2 mmol) in CH_2Cl_2 at room temperature was stirred without base. The reaction medium was monitored by thin layer chromatography and the mixture was then diluted with water and extracted with chloroform. The CHCl_3 extract was dried with anhydrous Na_2SO_4 and concentrated in under pressure to give a crude product, which was chromatographed with silica gel column to give the pure products **5** and **6**:

2-(Tert-butylthio)-3-chloronaphthalene-1,4-dione (5). Yield: 0.09 g (15%); R_f (CHCl_3): 0.6; Orange solid; mp 131-132°C; IR (KBr, cm^{-1}): 1681, 1663; UV λ_{max} (CHCl_3) nm (log ϵ): 250 (4.26), 282 (4.32), 340 (3.65), 444 (3.20); ^1H NMR (500 MHz, CDCl_3): δ 8.1 (q, 2H, CH_{naph} , $J = 5.4$ Hz), 7.70 (p, 2H, CH_{naph} , $J = 4.4$ Hz), 1.40 (s, 9H, 3CH_3); ^{13}C NMR (125 MHz, CDCl_3): δ 179.2, 175.7 (C=O), 150.8 (C-S), 145.5, 133.4, 133.0, 131.2, 130.2, 126.8, 126.4 (C_{naph} and C-H $_{\text{naph}}$), 52.0 (C_{tert}), 31.4 (s, 9H, 3CH_3); MS (+ESI): $m/z = 280.1$ [M] $^+$.

2,3-Bis(tert-butylthio)naphthalene-1,4-dione (6). Known compound [35,42]. mp 103-105°C; (103-105°C [35, 42]). MS (+ESI): m/z [$\text{M}+\text{H}$] $^+$ = 334.5 (m/z [M] $^+$ = 334.7 [35]).

2.3 Cell Culture and Antiproliferative Activity Assay

An antibiotic-antimycotic mixture [penicillin (100 U/mL), streptomycin (100 $\mu\text{g/mL}$), and amphotericin B (0.25 $\mu\text{g/L}$)

mL)] was purchased from Invitrogen, USA. Other reagents were obtained from Sigma Aldrich, USA.

All processes involving cell cultures were carried out in a biological safety cabinet (Class II laminar flow, Bilsen), and cells were grown in a CO₂ incubator (Heraeus D-6450). An inverted microscope (Olympus CK2) was used for cell counting when required.

HeLa cells (10⁵ cells/mL) were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and antibiotic-antimycotic mixture at 37°C in an atmosphere with 5% of carbon dioxide. Synthesized naphthoquinone derivatives and starting compound, 2,3-dichloro-1,4-naphthoquinone **1** were dissolved in EMEM and added to growth medium under aseptic conditions.

The antiproliferative effects of the compounds were examined by using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) test with minor modifications [43]. The assay based on the reduction of MTT to a colored formazan end-product by the action of mitochondrial dehydrogenase in living cells [44]. The cells (1×10⁵ cells/mL) were cultured in 96 well-plates. One day later (after reaching confluence), different concentrations (0.5 μM - 75 μM) of each compound were applied to the cells. At the end of 48 hours of incubation, medium was discarded, and the cells were washed with phosphate buffered saline (PBS). Each well was then loaded with 10 μL MTT stock solution (5 mg/mL) and 90 μL PBS, and the plates were further incubated for 4 hours. Aqueous phase was removed and 200 μL DMSO was added to each well to solubilize the water-insoluble purple formazan crystal. Cell viability was assessed by the measurement of the absorbance at 540 nm in a microplate reader (Eon Microplate Spectrophotometer, Bio-Tek Instruments, Inc. Highland Park, USA). Following formula was used for the calculation:

$$\text{Cell viability (\%)} = (A_{\text{sample}} / A_{\text{control}}) \times 100$$

where A_{sample} is the absorbance of the sample detected for the cells treated with test material and A_{control} is the absorbance of control (untreated cells). The IC₅₀ value (dose of the compound inhibiting 50% viability of HeLa cells) was calculated from a plot between cell viability and concentration of the compound. Each test was performed in triplicate.

2.4 Statistical Analysis

Statistical comparisons for the antiproliferative activity test were made using one-way analysis of variance (ANOVA) module of GraphPad Prism 5. Difference in mean values were considered significant when $P < 0.05$.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and discussion

We initially attempted the synthesis of N(H)-substituted-1,4-naphthoquinones **3a-f** by the reaction of **1** with different amines (2-morpholinoaniline **2a**, tert-Butyl 4-aminobenzoate **2b**, 4-tert-Butylbenzylamine **2c**, N-(3-Aminopropyl)-2-pipecoline **2d**, 2-Amino-5,6-dimethylbenzothiazole **2e**, N,N'-Diphenyl-p-phenylenediamine **2f**) in the absence of a base at room / reflux temperature (Scheme 1). This was then followed by **1** reacted with sodium 2-methyl-2-propanethiolate **4** to obtain S- and S,S- substituted compounds (**5** and **6**, respectively) at room temperature using CH₂Cl₂ as solvent.

¹H and ¹³C NMR spectrums were carried out to obtain characterization of all the synthesized compounds (**3a-f**, **5** and **6**). In the ¹H NMR spectrum of **3a-f**, the signals for protons H_a and H_d of the naphthoquinone ring (Figure 1) in the range δ 7.96-8.14 ppm firstly split into doublets ($J^3 = 7.1-7.8$ Hz) because of adjacent proton and then split into doublets of doublets ($J^4 = 1.0-1.5$ Hz). Similarly, doublets of triplets could be detected for H_b and H_c in the range δ 7.53-7.73 ppm with the corresponding coupling constants ($J^3 = 7.4-7.7$ Hz and $J^4 = 1.1-1.5$ Hz), because of ortho (adjacent) and meta position.

Analyzing the ¹H NMR spectroscopic data of compound **3a** it is obvious that the same expected coupling constants of the protons in ring position of -O-CH₂ and -N-CH₂- are in agreement with the observed triplet peaks (δ 3.71 ppm, $J^3 = 4.4$ Hz and δ 2.89 ppm, $J^3 = 4.4$ Hz, respectively). Its ¹³C NMR spectrum showed signals for the (-H₂C-O-) and (-H₂C-N-) carbons at about δ 66 and 50 ppm and two signals for the carbonyl (C=O) carbons (δ 179.5, 176.0 ppm) as expected. Also, the mass spectrum of **3a** exhibited at m/z 369.0 ([M+H]⁺, 100%) as protonated molecular peak, which is agreement with the molecular formula C₂₀H₁₇ClN₂O₃; 368.81 g.mol⁻¹. Also, this peak selected to fragment in MS/MS (MS2) mode to product m/z = 333.1 [M-Cl]⁺ fragment ion peak.

The fact that the compound **3b** has two types of carbonyl groups can be easily deduced from the peaks at δ 179.4, 176.4 ppm (C=O_{naph}) and δ 164.1 ppm (C=O_{ester}) in

Reagent and conditions: (i) ethanol, reflux (**3a-b**, **3e**); ethanol, room temp. (**3c-d**); DMF, 120 °C (**3f**).
 (ii) CH₂Cl₂, room temp.

Scheme 1: The synthesis of N(H)-, S- and S,S-substituted-1,4-naphthoquinones.

Figure 1: Characterization of quinonoid protons H_{a-d} of the N(H)-substituted 1,4-naphthoquinones (**3a-f**).

the ¹³C NMR spectrum. Also, the chemical shift of δ 80.1 ppm indicates that compound **3b** has tert-butyl carbon [(CH₃)₃C] connected to an oxygen atom and in the negative ion mode, the ESI full mass spectrum of **3b** showed the expected deprotonated peak at m/z 382.1 as [M-H]⁻ and the MS/MS fragmentation spectrum of this peak gave one intense peak appearing at m/z 326.2 as [M-(CCH₃)₃]⁻. Additionally, in the positive ion mode, compound **3b** showed m/z [M+H]⁺ = 384.0 as protonated molecular ion peak (calculated for **3b**: C₂₁H₁₈ClNO₄: 383.82 g.mol⁻¹), supporting its proposed identity.

Reaction of **1** with 4-tert-butylbenzylamine **2c** in ethanol at room temperature led to a red solid compound **3c**. Mass spectrometry data allowed the determination of its protonated molecular ion peak, m/z 354.1 [M+H]⁺, as expected (calculated for **3c**, 353.84 g.mol⁻¹). The doublet at δ 4.95 ppm and the broad singlet at δ 6.11 ppm clearly show the presence of NH-CH₂- and NH protons in the compound **3c**, respectively and ¹³C NMR spectrum showed that both the naphthoquinone's carbonyl (δ 179.5, 175.9 ppm) and NH-CH₂- carbon (47.8 ppm) of **3c**, together.

While compound **3d** revealed proton signals at δ 1.0-4.10 ppm (aliphatic protons and cyclic protons) in their expected positions in the ¹H NMR spectrum, compound **3e** revealed the singlets at δ 7.51 and 7.42 ppm due to the benzothiazole structure in the ¹H NMR spectrum. When the reaction of **1** was carried out with an equivalent of the sodium 2-methyl-2-propanethiolate **4** in dichloromethane without any catalyst at room temperature, mono- and bis-thiosubstituted-1,4-naphthoquinone derivatives (**5** and **6**) were obtained respectively. The mono-thiosubstituted

Figure 2: Antiproliferative effect of the samples on HeLa cells. [3a: $P < 0.0001$, $R^2 = 0.943$; 3b: $P < 0.0001$, $R^2 = 0.922$; 3c: $P < 0.0001$, $R^2 = 0.945$; 3d: $P < 0.0001$, $R^2 = 0.964$; 3e: $P < 0.0001$, $R^2 = 0.956$; 3f: $P < 0.0001$, $R^2 = 0.945$; 5: $P < 0.0001$, $R^2 = 0.911$; 6: $P < 0.0001$, $R^2 = 0.934$. Starting compound 1: $P < 0.0001$, $R^2 = 0.982$. Data are mean $\pm$ SD of percent changes compared with untreated controls ($n=9$)].

product 5 showed the signal of naphthoquinone protons at δ 8.1, 7.70 ppm and of methyl protons at δ 1.40 ppm in the ^1H NMR spectra and the most characteristic two quinonic carbonyl signals (at δ 179.2 and 175.7 ppm) in the ^{13}C NMR spectra.

For all compounds IR spectra were recorded. For all compounds the characteristic stretching vibrations of quinones ($\text{C}=\text{O}$ bonds) appeared in the expected range $1655\text{--}1680\text{ cm}^{-1}$ and absorption bands at about between $3100\text{--}3350\text{ cm}^{-1}$ due to NH groups.

The UV-visible absorption spectra of compounds 3a-e were carried out between 190-600 nm in chloroform at room temperature. 3a-e exhibited a maximum absorbance in the 276-290 nm region ($\pi \rightarrow \pi^*$) and a broad low absorbance in the visible region at 455-521 nm, which could be assigned to $n \rightarrow \pi^*$ transitions. In addition, the S-substituted compound 5 showed four prominent bands at 250, 282, 340, and 444 nm.

3.1 Antiproliferative Activity

The antiproliferative effect of each compound on the HeLa cells was investigated by MTT test and final results were given as mean percentages of control $\pm$ SD. The IC_{50} values were predicted from linear regression analyses (Figure 2). The results indicated that all tested compounds have antiproliferative activity. The highest cytotoxic activity has been determined for compound 5 ($\text{IC}_{50} = 10.16\text{ }\mu\text{M}$) and the antiproliferative capacity of the compounds was found to be in the following order: **5 > 3d > 3f > 3e > 3b > 6 > 3a > 3c**. Among all synthesized compounds, only the 3d, 3f and 5 possessed higher cytotoxic activity than their starting compound 1. Distinct activities of these new products depend on their different compositions. Furthermore, it may be suggested that these factors are directly to or may even be prominent contributors of antiproliferative effects.

Synthetic amino- and thiolated naphthoquinones have been investigated for their cytotoxic activities for many years [12] and as the amino derivatization has been found to enhance biological activity in most cases,

Table 1: The half maximal inhibitory concentration (IC_{50}) of the compounds.

Compound	IC_{50} value
1	17.91 μ M
3a	47.86 μ M
3b	22.54 μ M
3c	52.97 μ M
3d	12.82 μ M
3e	21.53 μ M
3f	16.71 μ M
5	10.16 μ M
6	37.33 μ M

several aminoquinones have been synthesized. The compounds **3a-f** obtained in this study share a common core structure with those synthesized by Pal et al. [29] except the R side chains (Figure 1). Some derivatives of 2-chloro-3-(n-alkylamino)-1,4-naphthoquinones (n-alkyl: methyl, ethyl, propyl and butyl) were found to possess antiproliferative effect on COLO205 (human colorectal adenocarcinoma) and MIAPaCa2 (human pancreatic carcinoma) cell lines whereas no activity was detected on 487MG (human primary glioblastoma) cell line [29]. Thus, in their study, it has been shown that length of the carbon chain in R group has an effect on the activity. The optimal structure of the alkyl group for the activity on COLO205 (IC_{50} = 92.2 μ M) and MIAPaCa2 (IC_{50} = 12.8 μ M) was found as $-C_3H_7$ and as $-C_2H_5$, respectively. The antiproliferative activity of all amino derivatives tested here on HeLa cells is higher than that of alkyl derivatives on COLO205 cells, although they have more complex structures on their side chains. However, only the antiproliferative effect of NH-substituted naphthoquinone derivative **3d** (IC_{50} = 12.82 μ M) on HeLa cells seemed to compete with the effect of L1 (IC_{50} = 11.5 μ M) and L2 (IC_{50} = 12.8 μ M) on MIAPaCa2 cells [29] as their IC_{50} doses were very close (Table 1).

As the reports related to antiproliferative effects of similar compounds are not common in the literature, we also searched the activity of starting compound **1** on HeLa cells, to understand the effect of derivatization on the activity. Previously the IC_{50} dose of 1,4-naphthoquinone on HeLa cells was found as 7.8 μ M [33]. This result could not be interpreted as the incubation time was 72 hours whereas it was 48 hours in this study. Although this compound seems to be effective on HeLa cells as well as on the other cancer cell lines, **3d** seems to be capable to compete with it. Moreover, 2,3-disubstituted-1,4-

naphthoquinone derivatives obtained in this study may exhibit cell-specific or enhanced cytotoxic activities and other biological activities.

On the other hand, as a thiolated naphthoquinone derivative, compound **5** has the highest inhibitory activity among the all compounds tested here. The IC_{50} value of this compound having relatively simple side chain is 10.16 μ M. This result indicated that mono-thiosubstitution enhanced the cytotoxicity. Thus, this compound may be a good candidate for further studies, such as understanding the effects on other cancer cell lines, revealing the action mechanism, and *in vivo* tests.

4 Conclusion

In this paper, we have described the synthesis and structural characterization of some N(H)-, S- and S,S-substituted-1,4-naphthoquinones. The new compounds were characterized by UV-Vis, 1H and ^{13}C NMR, MS (ESI), FT-IR, and their antiproliferative effects were investigated against HeLa cells. Our results indicated that the new naphthoquinone compounds, especially **5** and **3d** might be suggested as potent inhibitors of HeLa cells. But, further work are needed to check their other activities related to anticancer effect, cell specificity, action mechanism and *in vivo* efficacy. Derivatization strategies carried out here might be helpful to get new N(H)-, S- and S,S-substituted-1,4-naphthoquinones with better antiproliferative activity. When the antiproliferative effects of these compounds were determined against human cervical cancer, it was shown that all compounds have antiproliferative properties. In the light of the findings of this study, these new naphthoquinone compounds might be regarded as potential anticancer drugs which could be used in the health and pharmaceutical areas.

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