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Synthesis and evaluation of 2-heteroaryl and 2,3-diheteroaryl-1,4-naphthoquinones that potently induce apoptosis in cancer cells

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This article describes the preparation of 2-heteroaryl and 2,3-diheteroaryl-1,4-naphthoquinones by an environmentally benign short synthetic route with the goal of finding 1,4-naphthoquinone derivatives that induce apoptosis in cancer cells. We have identified three most active naphthoquinones 10, 12 and 15 that potently induce apoptosis in human cervical carcinoma (HeLa) cells. One of these three compounds perturbed both microtubule and actin filaments.

Introduction

During recent years biological effects of 2-heteroaryl and 2,3-diheteroaryl-1,4-naphthoquinones have led to synthesis of 1,4-naphthoquinone derivatives by nucleophilic substitution and addition reactions in non aqueous medium leading to mixture of products and lower yields.[1-10] This prompted us to devise a new synthetic route for regioselective synthesis of 2-heteroaryl and 2,3-diheteroaryl-1,4-naphthoquinone derivatives in high yields by carrying out nucleophilic substitution reactions "in-H_2O" using micelles as catalyst not reported earlier for the title compounds.

Naphthoquinones have a wide spectrum of anticancer activity.[11] They covalently bind and intercalate into DNA, which leads to DNA breakage and chromosomal aberration.[12,13] Naphthoquinones are also able to modulate receptor tyrosine kinase activity.[14,15] Recently naturally occurring 1,4-naphthoquinone derivatives[16] as well as synthetic 1,4-naphthoquinones[17] have been found to induce apoptosis in HL-60 (human promyelocytic leukemia cells) and HT29 (human colon adenocarcinoma *cell* line).

One of the recent approaches in drug design which includes pharmacophore modelling and three dimensional quantitative structure-activity relationship (3D-QSAR)/ comparative molecular similarity indices analysis (CoMSIA) methods applied to 2,3-disubstituted-1,4-naphthoquinones and heterocyclic quinones possessing nitrogen and sulfur atoms have unraveled the pronounced cytotoxic effects of these compounds.[18,19]

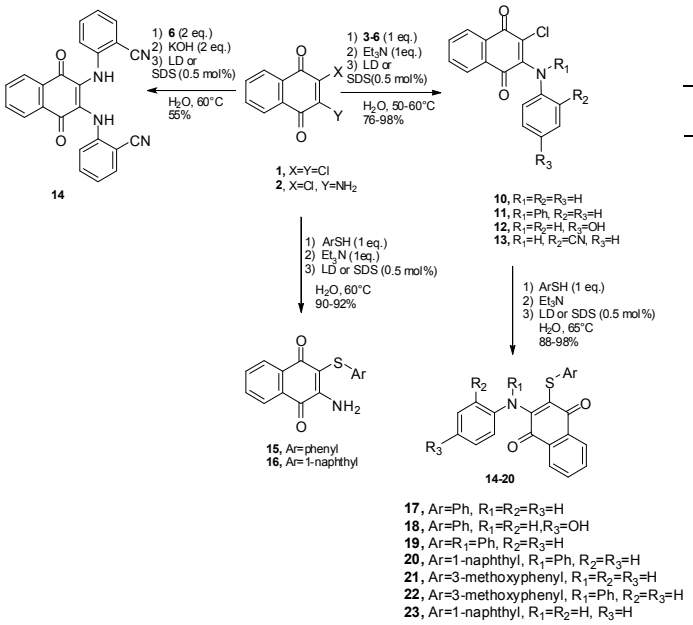
Results and discussion

In the course of a medicinal chemistry program aimed at discovering 1,4-naphthoquinone derivatives containing nitrogen and sulfur atoms endowed with cytotoxic activity, we have synthesized a series of 2-chloro-3-(*sec/tert.*-arylamino)-naphthalene-1,4-diones **10-13**, 2,3-diarylaminonaphthalene-1,4-dione **14,** 2-amino-3-arylsulfanyl-naphthalene-1,4-diones **15, 16** and 2,3-diheteroaryl-naphthalene-1,4-diones **17-23**.

Recently organic reactions performed in water have attracted considerable attention with respect to unique property of water in promoting reactions and enhancing selectivity.[20] The surfactant-type catalyst plays a dual role both as a catalyst to activate the substrate molecules and as a surfactant to increase the concentration of organic reactions to form micelle particles in water which have been extensively used.[21,22]

The synthesis of 1,4-naphthoquinone derivatives **10-23** in water was performed as described in Scheme 1. Nucleophilic substitution reaction of 2,3-dichloro-1,4-naphthoquinoe **1** with primary and secondary aryl amines **3-6** in H_2O using Et_3N as a base and LD or SDS[23,24] as catalyst resulted in the regioselective synthesis of 2-chloro-3(*sec/tert*-arylamino)-naphthalene-1,4-diones **10-13** in 74-99% yield. The reaction of **1** with 2-aminobenzonitrile **6** (2 eq.) under identical conditions afforded **14** regioselectively in moderate yield. 2-Chloro-3-amino-1,4-naphthoquinone **2** on nucleophilic substitution reaction with aryl thiol **7** and **9** (1 eq.) under identical conditions resulted in the formation of **15** and **16** respectively in excellent yields. In order to synthesize 2,3-diheteroaryl-

naphthalene-1,4-diones **17-23**; **10-13** were reacted with aryl thiols **7-9** under identical conditions to afford **17-23** in excellent yields.

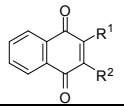


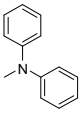
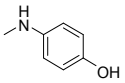
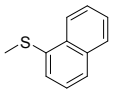
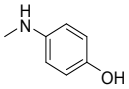
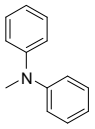
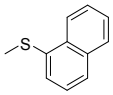
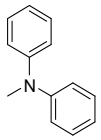
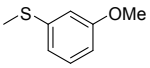
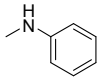
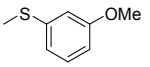
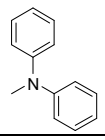
Scheme 1. Synthesis of 1,4-naphthoquinone derivatives **10-23**

The use of surfactants LD or SDS invariably leads to almost identical yields of products **10-23.** It is pertinent to note that use of LD or SDS as a catalyst in presence of Et_3N results in excellent yields of **10-23**. The absence of catalyst results in poor yields of **10-23** (0-25%) in presence of bases (KOH, Et_3N etc.). In the presence of LD or SDS "In H_2O", micelles[25] are formed and are responsible for observed catalytic influence during the course of reactions outlined in Scheme 1. The synthesis of 1,4-naphthoquinone derivatives **10-23** carried out by us in non aqueous solvents is uneconomical and involves longer duration of time for completion of reaction[26,27].

The antiproliferative activities of compounds **10-23** were tested against HeLa cells proliferation at 5 μM. At 5 μM, compounds **10-23** showed different levels of inhibition on HeLa cells proliferation (Table 1). The compounds **10, 12** and **15** were found to inhibit HeLa cells proliferation more than 80% suggesting that these compounds have most potent activity among the synthesized derivatives (Table 1).

Table 1. Mean % inhibition of HeLa cells proliferation by naphthoquinone series of compounds at 5 μM concentration.



Compd	R1	R2	logP[a]	Mean% inhibition
10	-Cl		1.5	88 ± 2
11	-Cl		3.79	46 ± 7
12	-Cl		1.2	96 ± 7
14			2.26	59 ± 6
15		-NH2	0.95	81 ± 17
16		-NH2	1.95	40 ± 4
18			2.75	33 ± 12
19			5.34	35 ± 16
20			6.34	48 ± 4
21			3.01	53 ± 3
22			5.21	51 ± 18

[a] logP was calculated using ChemBioDraw Ultra 10.0

Compounds **10**, **12** and **15** were selected for further studies and were found to inhibit HeLa cells proliferation in a concentration dependent manner. Half maximal inhibitory concentrations (IC_{50}) of compounds **10, 12** and **15** were determined by SRB assay[28,33] and their IC_{50} was found to be 3.9 ± 0.3, 3.2 ± 0.7, and 3.7 ± 1 μM, respectively (Table 2). Under similar conditions, cisplatin, a widely used anticancer agent, inhibits the proliferation of HeLa cells with IC_{50} value of 8 ± 1 μM.[33]

Table 2. IC_{50} values of compounds **10, 12** and **15** in HeLa cells

Compounds	IC_{50} (μM)
10	3.9 ± 0.3
12	3.2 ± 0.7
15	3.7 ± 1
Cisplatin	8±1

Regarding Structure Activity Relationship (SAR) among **10, 11** and **12,** replacement of H by the bulky phenyl substituent leads to considerable decrease in mean % inhibition of HeLa cells proliferation. The introduction of OH group leads to enhancement of mean % inhibition of HeLa cells proliferation leading to identification of **12** as the most active compound of the series. The SAR among **14-22** leads to infer that replacement of H by phenyl and naphthyl leads to decrease in activity. Thus in general the presence of larger number of bulky groups in compounds **10-22** lead to decrease in antiproliferative activity.

The structures of compound **12** and compound **10** are quite similar, so we have chosen compound **12** for further analysis as its cytotoxicity on HeLa cell proliferation was comparatively higher than that of the compound **10**. Apoptosis is considered to be an important mechanism of cancer cell death. Therefore, we performed Annexin V/PI staining to determine the effects of compounds **12** and **15** on apoptosis.[29,30] Compound **12** significantly increased the Annexin V positive cell population as compared to control (Figure 1). The number of Annexin V positive cells was almost double the number of PI positive cells suggesting that cell death occurred through apoptosis (Table 3).

Table 3. % of Annexin V and PI positive cells in the presence of compound **12**.

Compound **12** (μM)	% Cells with Annexin V staining	% Cells with PI staining
0	2	1
3	17	7
6	30	12
9	43	21

Compound **15** also increased the number of Annexin V positive cells (Table 4) but the effect was less pronounced as compared to compound **12.**

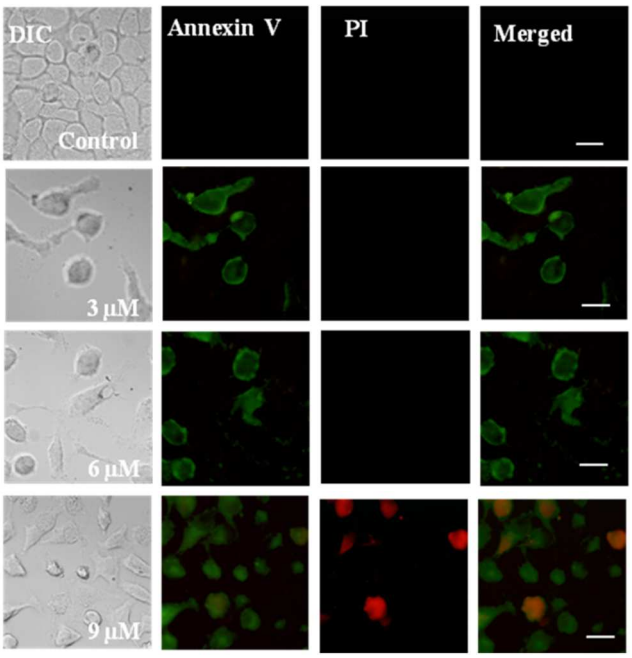


Figure 1. Annexin V and PI staining in the presence of Compound **12** (Scale bar 10 μm).

Table 4. % of Annexin V and PI positive cells in the presence of compound **15**

Compound **15** (μM)	% Cells with Annexin V staining	% Cells with PI staining
0	2.2	1
4	10	6
8	25	9
12	33	12

Thus, Annexin V/PI staining results suggest that compound **12** is most potent apoptosis causing derivative among the synthesized naphthoquinone series of compounds **10-23**.

Upon initiation of apoptosis, caspase 3 is known to be activated and translocated to the nucleus.[31] Thus, the determination of caspase 3 localization can indicate the activation of cell death via apoptosis. Both compound **12** (Figure 2) and compound **15** caused an increase in the nuclear localization of caspase 3 in the HeLa cells (Table 5 and Table 6). However, the effect of compound **15** was lesser as compared to compound **12** (Table 5 and 6).

Table 5. Increase in Caspase 3 localisation in presence of compound **12**.

Compound **12** (μM)	% Cells with caspase 3 staining
0	2 ± 1
3	15 ± 3
6	25 ± 2
9	41 ± 3

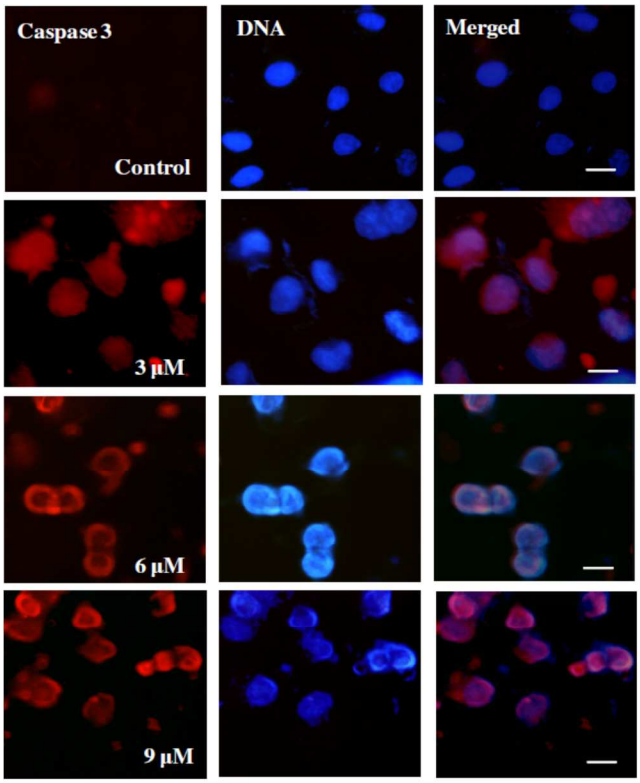
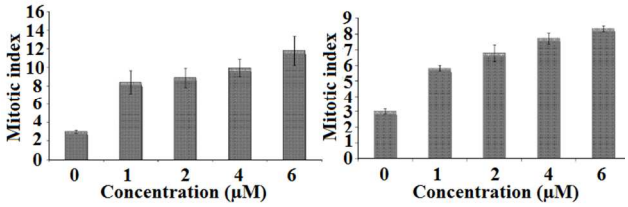


Figure 2. Increased caspase-3 nuclear localization in presence of compound **12** (Scale bar 10 µm).

Table 6. Increase in Caspase 3 localisation in presence of compound **15**.

Compound **15** (µM)	% Cells with caspase 3 staining
0	2 ± 1
4	12 ± 4
8	20 ± 2
12	27 ± 3



Compound **12** mitotic index Compound **15** mitotic index

Figure. 3 Effects of Compound **12** & **15** on the mitotic index in HeLa cells

Further, we investigated the effects of compound **12** and **15** on HeLa cell cycle progression by determining mitotic index[32] (Figure 3) and phosphohistone staining.[30] In control, 3% cells were found to be in mitosis whereas 11.7 ± 0.4 % and 8.3 ± 0.3 % cells were present in mitosis in presence of 6 µM compound **12** and compound **15**, respectively. Compound **12** and **15** treatment also increased the number of phosphohistone positive cells. For example, 3.2 ± 0.3% control HeLa cells displayed phosphohistone staining while 13.3 ± 1% and 12.7 ± 5% cells showed phosphohistone staining in the presence of 9 µM compound **12** and 20 µM compound **15**, respectively.

Furthermore, upon short exposure of compound **12** to HeLa cells, microtubules and actin network got disassembled, while short exposure of compound **15** did not seem to cause any effect on microtubule and actin network. The actin network of 20 µM compound **12** treated HeLa cells was disassembled in a similar fashion as 60 µM Cytochalsin D, a known actin depolymerizing agent. Compound **12** also caused an increase in the cyclin B1 staining[33] (Table 7).

Table 7. Compound **12** treatment increased nuclear localization of cyclin B1.

Concentration (µM)	Cyclin B1 positive cells (%)
0	3.3
3	11.5
6	15.2
9	17.3

The redox potential being the most significant physicochemical property determining the cytotoxicity of naphthoquinone derivatives, the reactive oxygen species (ROS) formed due to quinone redox cycling seem to be relevant to cytotoxicity of 1,4-naphthoquinone derivatives in addition to their hydrophobicities.[10]

Conclusion

In summary, we have identified a potent 1,4-naphthoquinone derivative **12** that selectively induces apoptosis in HeLa cells as well as environmentally benign synthetic route for the synthesis of compound **12** and other derivatives has been developed. We are currently studying the details of selectivity of the potent naphthoquinone derivatives that induce apoptosis in HeLa cells.

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Experimental Section

General

^{1}H and ^{13}C NMR spectra were recorded on Perkin Elmer model R.32 spectrophotometers (300 MHz H, 75 MHz C) in deuterated chloroform ($CDCl_3$) or deuterated dimethylsulfoxide (DMSO-d_6). The data is reported as follows: chemical shifts in ppm (δ), multiplicities are indicated as s-singlet; d-doublet; t-triplet; q-quartet; m-multiplet, br - broad. Coupling constants, J, are reported in Hz. Infrared spectra were recorded on FTIR 8201 PC, Schimadzu spectrophotometers in KBr discs, and the peaks reported in cm^{-1}. All compounds showed satisfactory

elemental analysis for C, H, N and S. Progress of reactions and purity of compounds were monitored by thin layer chromatography (TLC), which was performed on silica gel G and the plates were visualized by visible, UV light and Iodine (I_2). Spectral facilities and elemental micro-analyses were carried out by SAIF division of Central Drug Research Institute, Lucknow, India. Melting points were determined on an electrically heated Townson Mercer melting point apparatus and are uncorrected. Chemicals and reagents were purchased from Aldrich and used as received.

General procedure for the synthesis of 1,4-Naphthoquinone derivatives (10-23):

1 mmol nucleophile (**3-6** or **7-9**) was added to 5 mL aqueous suspension of surfactant (0.5 mole % LD) and Et_3N/ KOH (1 mmol). After stirring for five minutes, quinone **1/ 2/ 10-13** (1 mmol/ 2 mmol) was added and stirred at temperature. (Table 2, supporting information). The products were filtered after neutralization with 5% HCl, if required, followed by column chromatography (hexane/EtOAc) of the product to yield the desired products (**10-23**): (Table 2, supporting information).

2-chloro-3-(phenylamino)naphthalene-1,4-dione (10):[26,27]

Red powder from methanol; yield 99%; mp: 210-211 °C; IR (KBr): 1597 and 1675 (>C=O of quinone), 3243 (N-H) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 8.19 (d, J=8.0 Hz, 1H), 8.12 (d, J=8.0 Hz, 1H), 7.77 (t, J=8.0 Hz, 1H), 7.69 (t, J=8.0, 1H), 7.35 (m, 3H), 7.22 (t, J=7.6, 1H), 7.09 (d, J=7.6, 2H). ^{13}C NMR (100 MHz, $CDCl_3$+DMSO-d_6): δ 115.1, 124.7, 125.9, 127.3, 127.3, 128.7, 130.2, 132.9, 133.3, 135.3, 137.9, 142.1, 177.8, 180.8. Anal. Calcd. for $C_{16}H_{10}ClNO_2$ (284): C, 67.74; H, 3.55; N, 4.94. Found: C, 67.10; H, 3.53; N, 4.91; Beilstein test:[34] Cl positive.

2-chloro-3-(diphenylamino)naphthalene-1,4-dione (11):[26,27]

Yellow fine needle shaped crystals from methanol which on exposure in air changed color yellow to fluorescent light green.; yield 62%; mp: 130-132 °C IR (KBr): 1592 and 1678 (>C=O of quinone) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 8.22 (m, 1H), 8.15 (m, 3H), 8.09 (m, 3H), 7.81 (m, 1H), 7.75 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 127.3, 127.3, 128.7, 131.2, 131.4, 134.3, 134.7, 157.2, 179.9, 180.1; MS: 360 (M^+). Anal. Calcd. For $C_{22}H_{14}ClNO_2$ (360): C, 73.44; H, 3.92; N, 3.89 Found: C, 72.96; H, 3.98; N, 3.92; Beilstein test:[34] Cl positive.

2-chloro-3-(4-hydroxyphenylamino)naphthalene-1,4-dione (12):[26,27]

Violet powder from methanol; yield 96%; mp: 181 °C; IR (KBr): 1593 and 1678 (>C=O of quinone) 3269 (N-H), 3468 (O-H) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 8.19 (d, J=7.6, 1H), 8.12 (d, J=7.6, 1H), 7.90 (s, 1H), 7.75 (t, J=7.6, 1H), 7.69 (t, J=7.6, 1H), 7.35 (d, 2H), 7.05 (d, 2H), 4.75 (s, 1H).^{13}C NMR (100 MHz, $CDCl_3$+Acetone-d_6): δ 112.1, 114.4, 126.2, 126.9, 127.0, 129.3, 129.4, 131.9, 134.3, 139.0, 142.0, 155.51, 179.9, 180.1; Anal. Calcd. for $C_{16}H_{10}ClNO_3$ (300): C, 64.12; H, 3.36; N, 4.67; Found: C, 63.92; H, 3.42; N, 4.72; Beilstein test:[34] Cl positive.

2-(3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylaminobenzonitrile (13):[26,27]

The general procedure was followed for 24 h to give orange crystals on crystallization with EtOAc/hexane; yield 76%; mp: 233-234 °C; IR (KBr): 554, 680, 734, 836, 879, 1002, 1158, 1229, 1274, 1380, 1478, 1539, 1591, 1679, 2364, 3450, cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.08 (d, J=7.6 Hz, 1H), 7.31 (d, J=7.6 Hz, 1H), 7.56-7.82 (m, 4H), 7.67 (s, 1H) 8.19 (dd, J=7.6 Hz, 1.0 Hz, 2H); $^{13}CNMR(CDCl_3)$: 99.6, 113.3, 117.1, 118.3, 119.4, 127.3, 128.8, 130.1, 131.3, 133.9, 134.2, 135.1, 136.6, 151.7, 152.2, 178.3, 179.1; MS: 309 (MH^+); Anal. Calcd. ($C_{17}H_9ClN_2O_2$): C, 66.14; H, 2.94; N, 9.07; Found: C, 65.85; H, 2.70; N, 8.94; Beilstein test:[34] Cl positive.

2,2'-(1,4-dioxo-1,4-dihydronaphthalene-2,3-diyl)bis(azanediyl)dibenzo nitrile (14):[26,27]

Dark red solid after crystallization with EtOAc; yield 55%; mp 258-259 °C; IR (KBr): 505, 617, 645, 716, 761, 793, 851, 912, 1016, 1095, 1135, 1241, 1287, 1332, 1383, 1469, 1509, 1598, 1677, 2222, 2366, 3265, 3416 cm^{-1}; ^1H NMR ($CDCl_3$ +DMSO-d_6): δ 6.72-7.32 (m, 6H), 7.67-7.80 (m, 6H), 8.15 (dd, J=5.5 Hz, 3.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$+DMSO-d_6): δ 99.1, 118.5, 119.0, 119.8, 120.8, 126.9, 131.9, 134.8, 135.4, 137.0, 151.2, 181.8; MS: M^+ (M^++1): 391; Anal. Calcd. ($C_{24}H_{14}N_4O_2$): C, 73.84; H, 3.61; N, 14.35; Found: C, 74.02; H, 3.82; N, 14.60.

2-amino-3-phenylsulfanyl-[1,4]naphthoquinone (15):[26,27]

Red crystals after crystallization with methanol; yield 92%; mp 160-161 °C; IR (KBr): 1598 and 1676 (>C=O of quinone), 3243 and 3462 (NH_2) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 8.08 (m, 2H), 7.65 (m, 2H), 7.38 (m, 2H), 7.26 (s, 3H), 7.09 (bs, 2H); ^{13}C NMR (100 MHz, $CDCl_3$+DMSO-d_6): δ 126.0, 126.9, 127.1, 127.3, 129.5, 131.1, 132.9, 134.2, 135.5, 136.5, 153.9, 179.3, 180.7; Anal. Calcd. for $C_{16}H_{11}NO_2S$ (281): C, 68.31; H, 3.94; N, 4.98; S, 11.40; Found: C, 67.98; H, 3.88; N, 4.93; S, 11.35.

2-amino-3-(naphthalen-1-ylsulfanyl)-[1,4]naphthoquinone (16):[26,27]

Brown powder from methanol; yield 90%; mp 170-171 °C; IR (KBr): 1585 and 1662 (>C=O of quinone), 3270 and 3440 (NH_2) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 8.17 (m, 2H), 7.98 (m, 2H), 7.77 (m, 4H), 7.50 (m, 3H), 7.30 (bs, NH_2); ^{13}C NMR (100 MHz, $CDCl_3$+DMSO-d_6): δ 125.0, 126.0, 126.1, 126.9, 127.1, 127.1, 127.5, 128.2, 128.9, 129.0, 131.2, 131.9, 133.0, 134.0, 134.2, 134.3, 135.5, 153.9, 179.4, 180.7; Anal. Calcd. for $C_{20}H_{13}NO_2S$ (331): C, 72.49; H, 3.95; N, 4.23; S, 9.68; Found: C, 71.92; H, 3.92; N, 4.27; S, 9.60.

2-phenylamino-3-(phenylthio)naphthalene-1,4-dione (17):[26,27]

Brown solid from methanol; yield 99%; mp 143-145 ^{o}C; IR (KBr): 1589 and 1664 (>C=O of quinone), 3265 (N-H) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (d, J=7.6, 1H), 8.13 (d, J=7.6, 1H), 7.93 (bs, 1H), 7.76 (t, J=7.6, 1H), 7.69 (t, J=7.6, 1H), 7.17-7.15 (t, 2H), 7.13-6.98 (m, 4H), 6.75-6.68 (m, 4H); Anal. Calcd. for $C_{22}H_{15}NO_2S$ (357): C, 73.93; H, 4.23; N, 3.92; S, 8.97; Found: C, 74.22; H, 4.21; N, 3.98; S, 9.03.

2-(4-hydroxyphenylamino)-3-(phenylthio)naphthalene-1,4-dione (18):[26,27]

Shining brown solid from methanol; yield 88%; mp 220-221 ^{o}C; IR (KBr): 1546 and 1624 (>C=O of quinone), 3315 (N-H), 3375 (O-H) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (d, J=7.2, 1H), 8.12 (d, J=7.2, 1H), 7.90 (bs, 1H), 7.68-7.76 (m, 3H) 7.31 (m, 1H), 7.03-7.05 (m, 3H), 6.77-6.80 (m, 2H), 6.62 (m, 3H); Anal. Calcd. for $C_{22}H_{15}NO_3S$ (373): C, 70.76; H, 4.05; N, 3.75; S, 8.59; Found: C, 70.02; H, 4.01; N, 3.70; S, 8.63.

2-(diphenylamino)-3-(phenylthio)naphthalene-1,4-dione (19):[26,27]

Red oil; yield 90%; IR (KBr): 1590 and 1678 (>C=O of quinone) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.04-8.08 (m, 4H), 7.94-7.98 (m, 2H), 7.60-7.80 (m, 4H), 7.43-7.49 (m, 3H), 7.02-7.11 (m, 3H), 6.60-6.69 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 127.0, 127.3, 127.6, 127.8, 128.0 (4C), 129.4, 129.5, 130.7, 131.7, 131.8, 134.0, 134.4, 137.5, 178.5, 180.8. Anal. Calcd. for $C_{28}H_{19}NO_2S$ (434): C, 77.57; H, 4.42; N, 3.23; S, 7.40; Found: C, 77.42; H, 4.38; N, 3.18; S, 7.45.

2-(diphenylamino)-3-(naphthalene-1-ylthio)naphthalene-1,4-dione (20):[26,27]

Yellow powder from methanol; yield 98%; mp: 120-121 ^{o}C; IR (KBr): 1590 and 1678(>C=O of quinone) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.04-8.08 (m, 4H), 7.94-7.7.98 (m, 2H), 7.60-7.80 (m, 9H), 7.43-7.49 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$+DMSO-d_6): δ 125.6, 125.8, 125.9, 126.0, 126.6, 127.0, 127.5, 127.7, 128.3, 128.6, 130.0, 130.6, 130.7, 131.3, 131.5, 132.8, 133.0, 133.3, 158.5, 178.8, 181.1; Anal. Calcd. for $C_{32}H_{21}NO_2S$ (484): C, 79.48; H, 4.38; N, 2.90; S, 6.63; Found: C, 78.98; H, 4.34; N, 2.92; S, 6.58.

2-(3-methoxyphenylthio)-3-(phenylamino)naphthalene-1,4-dione (21):[26,27]

Shining brown solid from methanol; yield 99%; mp: 143-145 ^{o}C; IR (KBr): 1587 and 1662 (>C=O of quinone), 2359 and 2925 (OMe), 3310 (N-H) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (d, J=7.6, 1H), 8.13 (d, J=7.6, 1H), 7.96 (bs, 1H), 7.77 (t, J=7.6, 1H), 7.69 (t, J=7.6, 1H)7.19-7.17 (t, 2H, Ar-H), 7.15-7.09 (t, 1H, Ar-H), 6.96-6.92 (t, 1H, Ar-H), 6.73-6.71 (d, 2H, Ar-H), 6.60-6.58 (d, 1H, Ar-H), 6.38-6.39 (d, 1H), 6.22 (s, 1H, Ar-H), 3.63 (s, 3H, OCH_3), Mass (Fab): M^+=387, M^++1=388, M^++2=389; ^{13}CNMR (400 MHz, $CDCl_3$): δ 55.0, 112.0, 112.7, 113.0, 114.1, 120.6, 123.0, 124.6, 126.7, 127.0, 127.7, 129.0, 130.3, 132.7, 133.3, 134.7, 136.3, 142.6, 159.3, 180.7, 181.0 Anal. Calcd. for $C_{23}H_{17}NO_3S$ (387): C, 71.30; H, 4.42; N, 3.62; S, 8.28; Found: C, 71.24; H, 4.39; N, 3.58; S, 8.32.

2-(diphenylamino)-3-(3-methoxyphenylthio)naphthalene-1,4-dione (22):[26,27]

Red oil; yield 95%; IR (KBr): 1588 and 1662 (>C=O of quinone), 2359 and 2925 (OCH_3) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03-8.07 (m, 4H), 7.92-7.96 (m, 2H), 7.59-7.78 (m, 4H), 7.43-7.45 (m, 2H), 7.02-7.11 (m, 3H), 6.62-6.70 (m, 3H), 3.73 (s, 3H, OCH_3). Anal. Calcd. for $C_{29}H_{21}NO_3S$ (464): C, 75.14; H, 4.57; N, 3.02; S, 6.92; Found: C, 75.42; H, 4.58; N, 3.08; S, 7.00.

2-(naphthalene-1-ylthio)-3-(phenylamino)naphthalene-1,4-dione (23):[26,27]

Light brown solid from methanol; mp: 119-120 ^{o}C; yield 96%; IR (KBr): 1590 and 1662 (>C=O of quinone), 3329 (N-H) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.04-8.08 (m, 4H), 7.94-7.98 (m, 2H), 7.68-7.80 (m, 4H), 7.43-7.49 (m, 3H) 7.32-7.26 (m, 2H), 6.62-6.56 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$+DMSO-d_6): δ 123.29, 125.15, 126.03, 126.08, 126.60, 126.75, 127.71, 127.95, 128.82, 130.89, 131.48, 133.00, 133.36, 133.73, 133.87, 135.28, 136.87, 141.93, 143.13, 181.24, 181.60; Anal. Calcd. for $C_{26}H_{17}NO_2S$ (407): C, 76.64; H, 4.21; N, 3.44; S, 7.87; Found: C, 75.92; H, 4.19; N, 3.41; S, 7.91.

Evaluation of anticancer activity:

SRB assay:

Human cervical carcinoma (HeLa) cells were cultured in Minimal Essential medium (Hi Media) containing 10% fetal bovine serum.[35] For cell proliferation assay, 1×10^5 cells/ml were seeded in 96 well plates and for microscopic studies, 0.5×10^5 cells /ml were grown as monolayer on glass cover slips.[35] After 24 hours of seeding, 5 μM of each of the compounds tested was added to the wells and control cells were treated with vehicle (0.1% DMSO). Concentration of DMSO was kept 0.1% in both control and treated cells. Cells were incubated with the compounds for 24 hours and then, sulforhodamine B assay was performed.[28] The half maximal inhibitory concentration of **10**, **12** and **15** compounds on HeLa cell proliferation was determined after one cell cycle using sulforhodamine B assay.[28]

Annexin/ PI and caspase 3 staining:

HeLa cells were incubated with either vehicle or different concentrations of compound **12** (3 μM, 6 μM, 9 μM) and compound **15** (4 μM, 8 μM, 12 μM) for 24 h. Then, cells were stained with FITC labeled Annexin V and propidium iodide.[29,30] The cells were observed with an Eclipse TE 2000U microscope (Nikon, Japan) at 40 X magnification. The images were analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).[29,30] HeLa cells were incubated in the absence or presence of different concentrations of compound **12** (0, 3, 6 and 9 μM) and compound **15** (0, 4, 8 and 12 μM) for 24 hours. Then, cells were fixed with 3.7% (v/v) formaldehyde and caspase 3 staining was performed using

anticaspase 3 antibody (1:200 dilution). The coverslips were washed with BSA/PBS at room temperature and then incubated with secondary antibody (anti-mouse Alexa-568 conjugate) for 1 h at 1:200 dilution. To visualize nuclei, cells were stained with Hoechst 33258 (1µg/mL). Immuno-stained cells were examined using 40 X objective.

Determination of mitotic index:

HeLa cells were treated without or with different concentrations of compound **12** and compound **15** for 24 hours. The mitotic index was determined by staining DNA with Hoechst 33258 (1 µg/mL) for 10 min as described previously.[32] 500 cells were counted for each data set. HeLa cells were incubated without or with 3, 6 and 9 µM compound **12** for 24 hours. Subsequently, cells were fixed with 3.7% (v/v) formaldehyde. The cyclin B1 staining was done by incubating cells with 1:300 dilution of anti-cyclin B1 antibody.[33] To visualize nuclei, cells were stained with Hoechst 33258 (1µg/mL). Immuno-stained cells were examined using a 40X objective.[32]

Microtubule and actin staining:

HeLa cells were incubated without or with either 20 µM of compound **12**, or 20 µM of compound **15** for 2.5 h at 37 °C. After treatment, cells were fixed with 3.7% (v / v) formaldehyde. Microtubules, actin and DNA were stained as described previously.[29] Immuno-stained cells were examined using a 40X objective.

Notes and references

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† Electronic Supplementary Information (ESI) available: [copies of Spectra of final compounds and figures related to biological activity]. See DOI: 10.1039/b000000x/

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23. (a) LD is a common Laundry detergent (Common washing powder used for washing of cloths in home) which contains alkylbenzenesulfonates. It is economically more viable than SDS (Sodium Dodecyl Sulfate), triton X-100 or other costly surfactants. We preferred to use LD as surfactant. Laundry detergent (LD) as surfactant is Ariel, detergent product made in India by Proctor and Gambel home products Ltd. Mumbai 400099 (Tel +912224942113, E-mail: intouch.lm@pg.com) No: 4902430214 933, MRP: Rupees 2.00 packed 03/2009, Net

weight 14 g. The cost of SDS is Rs. 1123.00 for 25 g in the Sigma Aldrich catalog (436143-25G)-2008. (b) In place of Ariel, other laundry detergents (washing powder) can also be used. For details refer to http://enwikipedia.org/wiki/Laundry detergent.

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Graphical Abstract

Synthesis and evaluation of 2-heteroaryl and 2,3-diheteroaryl-1,4-naphthoquinones that potently induce apoptosis in cancer cells

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