Available online at www.sciencedirect.com
ScienceDirect
European Journal of Medicinal Chemistry 44 (2009) 1086–1092
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Original article

2,3-Disubstituted-1,4-naphthoquinones, 12*H*-benzo[*b*]phenothiazine-6,11-diones and related compounds: Synthesis and Biological evaluation as potential antiproliferative and antifungal agents

Vishnu K. Tandon [a,*], Hardesh K. Maurya [a], Ashutosh Tripathi [a], G.B. ShivaKeshava [b], Praveen K. Shukla [b], Pallavi Srivastava [c], Dulal Panda [c,**]

[a] *Department of Chemistry, University of Lucknow, Lucknow 226007, India*
[b] *Division of Fermentation Technology, Central Drug Research Institute, Lucknow 226001, India*
[c] *School of Biosciences and Bioengineering, Indian Institute of Technology, Bombay, Powai, Mumbai 400076, India*

Received 7 January 2008; received in revised form 14 June 2008; accepted 20 June 2008
Available online 2 July 2008

Abstract

A series of 2-chloro-3-arylsulfanyl-[1,4]naphthoquinones (**2**), 2,3-bis-arylsulfanyl-[1,4]naphthoquinones (**3**) and 12*H*-benzo[*b*]phenothiazine-6,11-diones and their analogs **6–8** were synthesized and evaluated for their antiproliferative activity against human cervical cancer (HeLa) cells. Compounds **3a** and **3b** were found to possess most potent antiproliferative and cell killing ability. Compounds **1–8** were also evaluated for antifungal activities. The structure–activity relationship of these compounds was studied and the results show that compound **2a** ($MIC_{50} = 1.56$ μg/mL) exhibited in vitro potent antifungal activity compared to the clinically useful antifungal drug Fluconazole ($MIC_{50} = 2.0$ μg/mL) against *Sporothrix. schenckii*. Compound **2a** ($MIC_{50} = 1.56$ μg/mL) also exhibited same antifungal activity compared to clinically useful drug Amphotericin-B ($MIC_{50} = 1.56$ μg/mL) against *Trichophyton. mentagraphytes*.

Keywords: Antiproliferative; 1,4-Naphthoquinone derivatives; Cytotoxic, human cervical cancer (HeLa) cells; Antifungal

1. Introduction

In recent years increasing recognition of biological activities of natural and synthetic 1,4-naphthoquinone derivatives has stimulated enormous interest in this class of compounds [1–7]. The clinical importance of this class of compounds has led to the development of new agents in which retaining the core 1,4-naphthoquinone moiety could exhibit variety of biological effects including cytotoxic [8–11], antiviral [12], molluscidal [13], anti-inflammatory, antiplatelet, antiallergic [14,15], antimalarial [16], antileishmanial [17], antibacterial, antifungal [18–27] and antiproliferative activities [28].

The recent pharmacophore modelling approach 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 1,4-naphthoquinones **I** (Fig. 1) in human promyelocytic leukemia HL-60 cell line have explained the pronounced cytotoxic activity of these derivatives [1]. The spirohydantoin derivatives **II** (Fig. 1) possessing two nitrogen atoms in the same ring and sulfur atom in the dihydrothieno[2,3-*b*]naphtha-4,9-dione system were found to possess potential cytoxic activity [10].

The profound biological activity exhibited by naphtho[2,3-*b*][1,4]-thiazine-5,10-diones **III** (Fig. 1) by incorporation of additional nitrogen or sulfur atoms in six-membered heterocyclic ring retaining the quinone chromophore has led to development of lead molecule **III** [6].

* Corresponding author. Tel.: +91 9415066847; fax: +91 522 26848.
** Corresponding author.
E-mail address: vishnutandon@yahoo.co.in (V.K. Tandon).

0223-5234/$ - see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2008.06.025

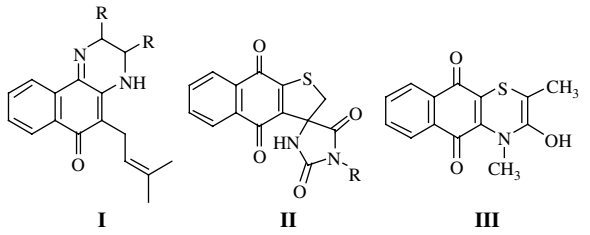


Fig. 1. Lead antiproliferative [10] and antifungal agents [6].

In the course of a medicinal chemistry program aimed at discovering 1,4-naphthoquinone derivatives containing sulfur and nitrogen atoms endowed with cytotoxic activity, we have synthesized a series of 2-chloro-3-arylsulfanyl-[1,4]naphthoquinones **2**, 2,3-bis-arylsulfanyl-[1,4]naphthoquinones **3**, 12*H*-benzo[*b*]phenothiazine-6,11-diones (**6**,**7**) and 7*H*-14-thia-7-aza-benzo[*a*]naphthacene-8,13-dione (**8**).

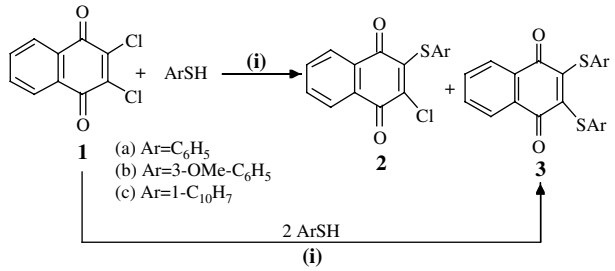
2. Results and discussion

2.1. Chemistry

The precursors, 2-chloro-3-phenylsulfanyl-[1,4]naphthoquinone (**2**) required for the synthesis of **6**–**8**, have been synthesized according to Scheme 1. The reaction of 2,3-dichloro-1,4-naphthoquinone (**1**) with aryl thiols afforded a mixture of 2-chloro-3-arylsulfanyl-[1,4]naphthoquinones (63–80%) (**2**) and 2,3-bis-arylsulfanyl-[1,4]naphthoquinones (20–37%) (**3**). This reaction involves nucleophilic displacement of Cl atom in 2,3-dichloro-1,4-naphthoquinone (**1**) with sulfur nucleophile resulting in the formation of **2**. Nucleophilic displacement reaction of **2** with arylthiols (1:2) resulted exclusively in the formation of 2,3-bis-arylsulfanyl-[1,4]naphthoquinones (**3**) as exhibited in Scheme 1.

The synthesis of 12*H*-benzo[*b*]phenothiazine-6,11-diones (**6**,**7**) and their analog 7*H*-14-thia-7-aza-benzo[*a*]naphthacene-8,13-dione (**8**) was carried out according to Scheme 2.

However, the nucleophilic displacement reaction of **2** with NaN_3 in DMF–H_2O (10:1) afforded 12*H*-benzo[*b*]phenothiazine-6,11-diones and their analogs **6**–**8** as the only isolated products as exhibited in Scheme 2. The mechanism of formation of **6**–**8** from **2** involves nucleophilic displacement of Cl by N_3 followed by elimination of N_2 and formation of nitrene **5**. Intramolecular cyclization further leads to formation of **6**–**8**. In these reactions regioselective intramolecular C–H bond



Scheme 1. Reagents and conditions: (i) EtOH, room temp.

insertion reaction of nitrene **5** with benzene moiety occurred to form cyclized products **6**–**8**. The regioselective intramolecular C–H bond insertion reaction of nitrene with aromatic compounds has earlier been reported by Kim et al. [29]. All synthesized compounds were characterized by ^{1}H NMR and IR spectral data and elemental analytical data.

2.2. Antiproliferative activities

The cells undergo many morphological and biochemical changes during apoptotic cell death. Annexin V is known to bind to the exposed phosphotidylserine residue in the early stage of apoptosis [33]. Propidium Iodide (PI) binds to DNA; however, it enters into the cells only after the disruption of the plasma membrane [34]. FITC-conjugated Annexin V and PI were used to stain the early apoptotic cells and the late apoptotic/necrotic cells, respectively.

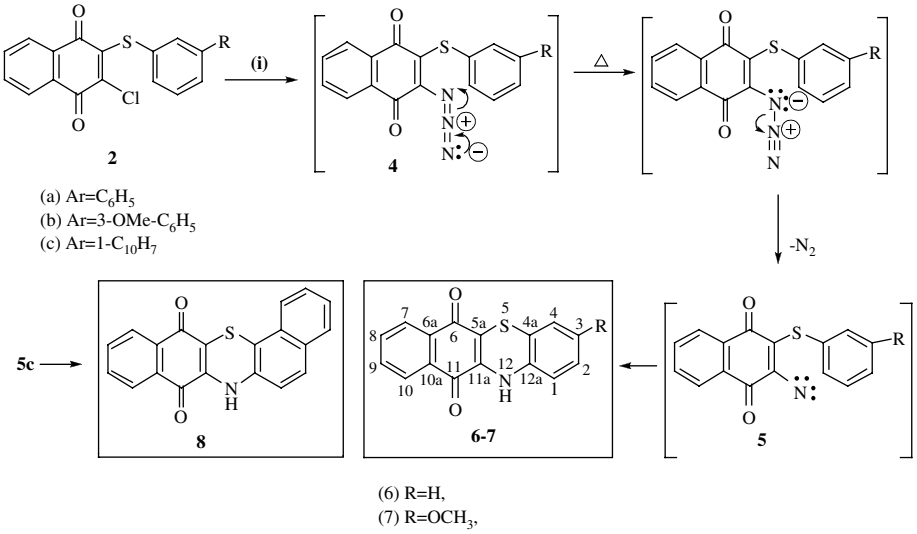
The effects of compounds **1**–**8** on the proliferation of cervical cancer (HeLa) cells were determined by incubating the HeLa cells with different concentrations (1, 5 and 20 μM) of compounds **1**–**8** for 24 h. The inhibitory effects of these compounds on the proliferation of HeLa cells are provided in Table 1. Some of the compounds inhibited proliferation strongly. Compounds **3a** and **3b** were found to be the most active compounds showing 100% inhibition of HeLa cell proliferation at 20 μM concentration, while Noscapine [38] showed 50% inhibition of HeLa cell proliferation at 34 μM concentration. In the presence of **3a** and **3b**, HeLa cells were found to be stained with both of the dyes indicating that these agents induce apoptotic death in HeLa cell (Fig. 2). Further, the cells showed characteristic of apoptosis, i.e. cell rounding, swelling and blebbing in the presence of these compounds.

To find out the mechanism of cell death, we analyzed the effects of **3a** and **3b** on the organizations of cellular microtubules and chromosomes of the HeLa cells by fluorescence microscopy. These compounds did not affect the cellular microtubules and chromosomes of the HeLa cells (data not shown). Further, **3a** and **3b** did not block cell cycle progression at mitosis. For example, percentage of cells in mitosis were found to be 3.4 ± 0.8, 3.5 ± 1.1, 3.8 ± 0.9 and 4.2 ± 1.2 in the absence and presence of 5, 10 and 20 μM of **3b** compound.

The study of structure–activity relationship of **1**–**8** revealed that 2,3-bis-arylsulfanyl substituted-[1,4]naphthoquinones **3a** and **3b** showed pronounced antiproliferative activity against human cervical cancer (HeLa) cells referred in Table 1, than the cyclic analogs **6**–**8**. The introduction of methoxy group in aromatic ring enhances antiproliferative activity at concentration 1 and 5 μM whereas replacement of phenyl by naphthyl group caused decrease in antiproliferative activity. The fact that the acyclic analogs are more potent than the corresponding cyclic analogs has also been reported by Miguel del Corral et. al. [39].

2.3. Antifungal activity

Comparison of antifungal activity of compounds **1**–**8** referred in Table 2 with that of antifungal drug Miconazole



Scheme 2. Reagents and conditions: (i) NaN_3, DMF, H_2O, Δ.

showed that compound **2a** had better activity against *Candida. albicans* and *Cryptococcus neoformans* and had same antifungal profile against *Aspergillus fumigatus* while compound **2b** had same activity against *C. albicans*. On comparison of antifungal activity with that of antifungal drug Nystatin, compound **2a** had better activity against *Sporothrix schenckii* and significant activity against *C. albicans*. Compound **2c** also exhibited better activity against *S. schenckii* when compared with Nystatin. Compound **2a** exhibited better activity than clinically prevalent antifungal drug Fluconazole against *S. schenckii*. Compound **2a** also exhibited similar antifungal activity when compared with Amphotericin-B against *Trichophyton mentagraphytes*.

Table 1
Inhibition of cell proliferation by compounds **1**–**8**

Compound	Conc. (μM)	Inhibition (%)	SD (±)
2a	1	15	2.6
2a	5	29	3.4
2a	20	73	5.8
2b	1	39	2.7
2b	5	48	3.5
2b	20	74	7.6
2c	1	0	0
2c	5	22	5.5
2c	20	69	4.4
3a	1	51	4.6
3a	5	60	3.7
3a	20	100	0
3b	1	64	3.8
3b	5	76	4.2
3b	20	100	0
3c	1	22	3.8
3c	5	30	2.4
3c	20	46	3.5
6	1	5.5	1.2
6	5	6.5	2.2
6	20	42	4.5
7	1	8.5	2.5
7	5	20	3.7
7	20	77	5.6
8	1	12	2.7
8	5	19	4.8
8	20	42	6.4
1	1	28	4.2
1	5	43	3.6
1	20	49	4.8
Noscapine [38]	34	50	3.7

Compounds **1**–**8** inhibit HeLa cell proliferation in a concentration dependent manner. Inhibition of proliferation was determined after one cell cycle using Sulforhodamine B.

Structure–activity relationship in **1**–**8** revealed that presence of chlorine atom in 1,4-naphthoquinone nucleus seemed essential for potent antifungal activity as revealed in Table 2 in compounds **2**(**a**–**c**). Replacement of chlorine atom by SAR in **3**(**a**–**c**) as well as their cyclic analogs **6**–**8** did not result in antifungal activity. Replacement of one chlorine atom by phenyl group in **1** resulted in profound antifungal activity leading to formation of **2a** compared to replacement by 3-methoxy phenyl and 1-naphthyl substituent leading to formation of **2b** and **2c** respectively.

3. Conclusion

In conclusion, we have synthesized a series of 2-chloro-3-arylsulfanyl-[1,4]naphthoquinones (**2**), 2,3-bis-arylsulfanyl-[1,4]naphthoquinones (**3**) and 12*H*-benzo[*b*]phenothiazine-6,11-diones and their analogs (**6**–**8**). The promising compounds **3a** and **3b** have exhibited potent antiproliferative activity compared to the other derivatives of the series against cervical cancer (HeLa) cells. Compound **7** also exhibited significant antiproliferative activity but lesser compared to **3a** and **3b**. Our results suggest that both **3a** and **3b** have potent antiproliferative and cell killing ability. Amongst the most promising antifungal compounds **2a** showed better antifungal activity than clinically prevalent antifungal drug Fluconazole against *S. schenckii* and same antifungal activity when compared with amphotericin-B against *T. mentagraphytes*. Further work is in progress for drug development of compounds **2a**, **3a** and **3b**.

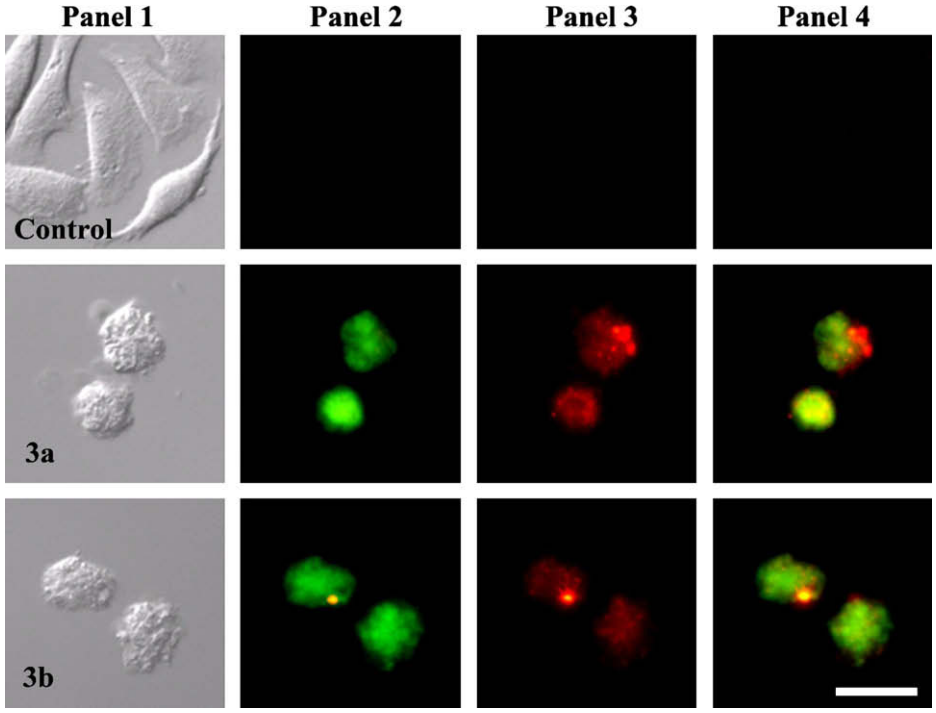


Fig. 2. Compounds **3a** and **3b** induce apoptosis in HeLa cells: HeLa cells were incubated with 5 μM **3a** or **3b** for 24 h and stained with Annexin V and Propidium Iodide (PI) using Annexin V apoptosis detection kit. Panel 1 shows cell morphology using differential interference contrast (DIC) microscopy. Panel 2 shows Annexin V staining, panel 3 shows PI staining and panel 4 is a merged image of panel 2 and panel 3. Bar 20 μm.

4. Experimental

4.1. Materials and methods

The reagents and the solvents used in this study were of analytical grade and were used without further purification. The melting points were determined on an electrically heated Townson Mercer melting point apparatus and are uncorrected. IR spectra were recorded on FTIR 8201 PC, Schimadzu Spectrophotometers on KBr discs. Nuclear magnetic resonance (NMR) spectra were recorded on Perkin–Elmer model R.32 spectrometer using TMS as an internal reference. All compounds showed satisfactory elemental analysis for C, H and N. Progress of reactions and purity of compounds were monitored by thin layer chromatography (TLC), which was performed on silica gel G and compounds were detected with iodine vapors, where required. Spectral analyses and elemental microanalyses were carried out by SAIF division of Central Drug Research Institute, Lucknow, India. Most reagents were purchased from Lancaster, Sigma–Aldrich and Merck.

Table 2
Structures and in vitro antifungal activity for compounds **1–8** (MIC: μg/mL)

Compounds	MIC (μg/mL)					
	C. albicans	*C. neoformans*	*S. schenckii*	*T. mentagraphytes*	*A. fumigatus*	*Candida parapsilosis*
2a	6.25	6.25	1.56	1.56	12.5	6.25
2b	25	25	3.12	3.12	25	25
2c	50	50	6.25	6.25	50	50
3a	>50	>50	50	>50	>50	>50
3b	>50	>50	>50	50	>50	>50
3c	>50	>50	>50	>50	>50	>50
6	>50	>50	50	>50	>50	>50
7	>50	>50	>50	50	>50	>50
8	>50	>50	50	>50	>50	>50
1	0.78	3.12	0.78	1.56	1.56	1.56
Miconazole	25	12.5	[a]	<0.78	12.5	[a]
Nystatin	7.8	3.5	13.2	[a]	[a]	[a]
Fluconazole	1.0	1.0	2.0	0.5	2.0	2.0
Amphotericin-B	0.39	0.78	[a]	1.56	[a]	[a]

[a] Activity not reported.

4.2. General procedure for the synthesis of 2-chloro-3-arylsulfanyl-[1,4]naphthoquinones (**2**) and 2,3-bis-arylsulfanyl-[1,4]naphthoquinones (**3**)

A mixture of 2,3-dichloro-1,4-naphthoquinone (**1**) (10 mmol) and aryl thiols (12 mmol) in abs. EtOH (50 mL) was stirred vigorously for 3–8 h at 40 °C. The resulting solution was concentrated in vacuo and the residue comprising of **2** and **3** was subjected to column chromatography on silica gel using EtOAc/hexane (1:25) and the product was crystallized with suitable solvent to give **2** in 63–80% yield. Analogous reaction of 2,3-dichloro-1,4-naphthoquinone (**1**) (10 mmol) and aryl thiols (20 mmol) in abs. EtOH (50 mL) gave the product **3** in 90–95% yield after crystallization with suitable solvent.

4.2.1. 2-Chloro-3-phenylsulfanyl-[1,4]naphthoquinones (**2a**)

$R_f = 0.4$; orange needles after crystallization with EtOAc/hexane; 80% yield; mp 124 °C; IR (KBr): 1590 and 1666 (>C=O of quinone) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.27 (s, 3H, Ar-H), 7.40 (m, 2H, Ar-H), 7.65 (m, 2H, C_6–H and C_7–H), 8.08 (m, 2H, C_5–H and C_8–H). Anal. Calcd. for $C_{16}H_9ClO_2S$ (300.76): C, 63.90; H, 3.02; S, 10.66. Found: C, 63.68; H, 2.98; S, 10.58; Beilstein test: [35] Cl positive.

4.2.2. 2-Chloro-3-(3-methoxy)phenyl sulfanyl-[1,4]naphthoquinones (**2b**)

$R_f = 0.5$; orange needles after crystallization with EtOAc/hexane; 63% yield; mp 108 °C; IR (KBr): 1589 and 1665 (>C=O of quinone) cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.79 (s, 3H, OCH_3), 6.85–7.20 (m, 4H, Ar-H), 7.73 (m, 2H, C_6–H and C_7–H), 8.15 (m, 2H, C_5–H and C_8–H). Anal. Calcd. for $C_{17}H_{11}ClO_3S$ (330.79): C, 61.73; H, 3.35; S, 9.69. Found: C, 61.92; H, 3.52; S, 9.80; Beilstein test: [35] Cl positive.

4.2.3. 2-Chloro-3-(naphthalen-1-ylthio)naphthalene-1,4-dione (**2c**)

$R_f = 0.5$; orange crystals after crystallization with EtOAc/hexane; 65% yield; mp 180 °C; IR (KBr): 1586 and 1662 (>C=O of quinone) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.50 (m, 3H, Ar-H), 7.77 (m, 4H, Ar-H), 7.98 (m, 2H, C_6–H and C_7–H), 8.17 (m, 2H, C_5–H and C_8–H). Anal. Calcd. for $C_{20}H_{11}ClO_2S$ (350.82): C, 68.47; H, 3.16; S, 9.14. Found: C, 68.50; H, 3.14; S, 9.10; Beilstein test: [35] Cl positive.

4.2.4. 2,3-Bis(phenylthio)naphthalene-1,4-dione(**3a**)

$R_f = 0.3$; dark orange crystals after crystallization with EtOAc/hexane; 95% yield; mp 150 °C; IR (KBr): 1591and 1660 (>C=O of quinone) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.34 (m, 10H, Ar-H), 7.68 (m, 2H, C_6–H and C_7–H), 7.99 (m, 2H, C_5–H and C_8–H). Anal. Calcd. for $C_{22}H_{14}O_2S_2$ (374.48): C, 70.56; H, 3.77; S, 17.13. Found: C, 70.52; H, 3.74; S, 17.09.

4.2.5. 2,3-Bis(3-methoxyphenylthio)naphthalene-1,4-dione (**3b**)

$R_f = 0.3$; dark orange crystals after crystallization with EtOAc/hexane; 93% yield; mp 80 °C; IR (KBr): 1589 and 1662 (>C=O of quinone) cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.77 (s, 6H, 2 × OCH_3), 6.79–7.26 (m, 8H, Ar-H), 7.68 (m, 2H, C_6–H and C_7–H), 8.00 (m, 2H, C_5–H and C_8–H). Anal. Calcd. for $C_{24}H_{18}O_4S_2$ (434.53): C, 66.34; H, 4.18; S, 14.76. Found: C, 66.56; H, 4.32; S, 14.92.

4.2.6. 2,3-Bis(naphthalen-1-ylthio)naphthalene-1,4-dione (**3c**)

$R_f = 0.3$; dark orange crystals after crystallization with EtOAc/hexane; 90% yield; mp 190 °C; IR (KBr): 1591and 1665 (>C=O of quinone) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.44 (m, 6H, Ar-H), 7.69 (m, 4H), 7.80 (m, 2H, C_6–H and C_7–H), 7.98 (m, 2H, C_5–H and C_8–H), 8.09 (m, 4H, Ar-H). Anal. Calcd. for $C_{30}H_{18}O_2S_2$ (474.59): C, 75.92; H, 3.82; S, 13.51. Found: C, 75.89; H, 3.78; S, 13.48.

4.3. General procedure for the synthesis of 12H-benzo[b]phenothiazine-6,11-diones and 7H-14-thia-7-aza-benzo[a]naphthacene-8,13-dione (**6–8**)

Sodium azide (30 mmol) was added to a stirred solution of 2-chloro-3-arylsulfanyl-[1,4]naphthoquinones (**2**) in DMF (15 mL) and H_2O (1.5 mL). The reaction mixture was stirred for 4 h at 100 °C. The resulting solution was poured onto crushed ice (50 g) and the solid precipitate was filtered, dried and subjected to column chromatography on silica gel using EtOAc/hexane (1:15) and the product was crystallized with suitable solvent to give **6–9** in 67–85% yield.

4.3.1. 6H-Benzo[b]phenothiazine-6,11(12H)-dione (**6**)

Red crystals after crystallization with EtOAc/hexane; 85% yield; mp 110 °C; IR (KBr): 1592 and 1665 (>C=O of quinone), 3305 (N–H) cm^{-1}; ^1H NMR ($CDCl_3$): δ 6.00 (bh, 1H, NH), 7.32 (m, 2H, Ar-H), 7.49 (m, 2H, Ar-H), 7.71 (m, 2H, C_6–H and C_7–H), 8.15 (m, 2H, C_5–H and C_8–H). Anal. Calcd. for $C_{16}H_9NO_2S$ (279.31): C, 68.80; H, 3.25; N, 5.01; S, 11.48. Found: C, 68.78; H, 3.22; N, 4.97; S, 11.51.

4.3.2. 3-Methoxy-6H-benzo[b]phenothiazine-6,11(12H)-dione (**7**)

Red crystals after crystallization with EtOAc/hexane; 79% yield; mp 145–147 °C; IR (KBr): 1594 and 1662 (>C=O of quinone), 3310 (N–H) cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.79 (s, 3H, OCH_3), 6.00 (bh, 1H, NH), 6.77 (d, 1H, $J = 6.5$ Hz, C_1–H), 7.08 (m, 1H, C_3–H), 7.38 (m, 1H, C_2–H), 7.63 (m, 2H, C_6–H and C_7–H), 8.09 (m, 2H, C_5–H and C_8–H). Anal. Calcd. for $C_{17}H_{11}NO_3S$ (309.34): C, 66.01; H, 3.58; N, 4.53; S, 10.37. Found: C, 59.98; H, 3.60; N, 4.50; S, 10.40.

4.3.3. 7H-14-Thia-7-aza-benzo[a]naphthacene-8,13-dione (**8**)

Red crystals after crystallization with EtOAc/hexane; 78% yield; mp 151 °C; IR (KBr): 1592 and 1665 (>C=O of quinone), 3304 (N–H) cm^{-1}; ^1H NMR ($CDCl_3$): δ 6.12 (br s, 1H, NH), 7.40 (m, 4H, naphthyl), 7.70 (m, 2H, naphthyl-H), 7.78 (m, 2H, C_6–H and C_7–H), 8.19 (m, 2H, C_5–H and C_8–H). Anal. Calcd. for $C_{20}H_{11}NO_2S$ (329.37): C, 72.93; H,

3.37; N, 4.25; S, 9.74. Found: C, 73.18; H, 3.54; N, 4.34; S, 9.92.

4.4. In vitro antiproliferative assay

The evaluation of antiproliferative activities of compounds **1–8** against human cervical cancer (HeLa) cells was accomplished by cell culture, immuno-fluorescence microscopy and Annexin/Propidium Iodide staining as shown below.

4.4.1. Cell culture

Human cervical cancer (HeLa) cells were grown in Eagle's minimal essential medium (Himedia) with 10% (v/v) fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. All compounds were dissolved in DMSO and the final concentration of DMSO was less than 0.1%. Cells were seeded at 1×10^5 cells/mL in 96-well tissue culture plates. After 24 h, medium was replaced by fresh medium containing DMSO as vesicle (control) or compounds **1–8** and was incubated for one cell cycle (24 h). The cells were stained with 0.4% Sulforhodamine B, and the concentration of the protein-bound dye was detected by measuring the absorbance at 560 nm [30].

4.4.2. Immuno-fluorescence microscopy

The cells were grown on coverslips in a 24-well plate without or with different concentrations of the compounds **1–8** for 24 h. Microtubules and chromosomes were visualized by fluorescence microscopy as described previously [31,32]. Mouse monoclonal anti-α-tubulin antibody and FITC-conjugated anti-mouse IgG antibody were used to stain tubulin and DAPI was used to stain chromosomes. Images were taken with a Nikon eclipse 2000U fluorescence microscope.

4.4.3. Annexin/Propidium Iodide staining

HeLa cells were grown on coverslips at a density of 5×10^4 cells/mL in 24-well plates as described earlier. Cells were incubated with 5 μM concentration of compound **3a** and **3b** for 24 h and stained with Annexin V and Propidium Iodide using Annexin V apoptosis detection kit (Santa Cruz Biotechnology, Inc.). Cells were observed with a Nikon Eclipse TE 2000U microscope. The images were analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

4.5. In vitro antifungal activity evaluation by MIC assay

Compounds **1–8** were evaluated for their in vitro antifungal activity against *C. albicans*, *C. neoformans*, *S. schenckii*, *T. mentagraphytes*, *A. fumigatus* and *Candida parapsilosis* (ATCC 22019) at the Division of Fermentation Technology of Central Drug Research Institute, Lucknow, India. In this process minimum inhibitory concentration of compounds **1–8** was tested according to standard micro broth dilution as per NCCLS [36,37] protocol. Briefly, testing was performed in flat bottom 96-well tissue culture plates (CELLSTAR® Greiner bio-one GmbH, Germany) in RPMI 1640 medium buffered with MOPS (3-[*N*-Morpholino]propane sulfonic acid) (Sigma chem. Co. MO, USA) for fungal strains. The concentration range of tested compounds was 50–0.36 μg/mL for standard compounds. Initial inoculums of fungal strain were maintained at $1–5 \times 10^3$ cells/mL. These plates were incubated in a moist chamber at 35 °C and absorbance at 492 nm was recorded on Versa Max micro plate reader (Molecular devices, Sunnyvale, USA) after 48 h for *C. albicans* and *C. parapsilosis*, 72 h for *A. fumigatus*, *S. schenckii* and *C. neoformans* and 96 h for *T. mentagraphytes* while bacterial strains were incubated for 24 h. MIC was determined as 90% inhibition of growth with respect to control was observed by using SOFTmax Pro 4.3 Software (Molecular Devices, Sunnyvale, USA).

Acknowledgements

We thank Director, SAIF of Central Drug Research Institute, Lucknow, India for spectral, microanalyses data reported in the manuscript. H.K.M. thanks U.G.C., New Delhi, India for award of J.R.F/S.R.F and D.P. thanks Swarnajayanti Fellowships.

References

[1] Pérez-Sacau E, Diaz-Peřate RG, Estévez-Braun A, Ravelo AG, García-Castellano JM, Pardo L, Campillo M. J. Med. Chem. 2007;50:696.
[2] Shimbashi A, Nishiyama S. Tetrahedron Lett. 2007;48:1545.
[3] Tangmouo JG, Meli AL, Komguem J, Kuete V, Ngounou FN, Lontsi D, Veronique PB, Choudhry MI, Sondengam BL. Tetrahedron Lett. 2006; 47:3067.
[4] Ganapaty S, Thomas PS, Karagianis G, Waterman PG, Brun R. Phytochemistry 2006;67:1950.
[5] Kim TJ, Yun YP. Vasc. Pharmacol. 2007;46:43.
[6] Tandon VK, Maurya HK, Yadav DB, Tripathi A, Kumar M, Shukla PK. Bioorg. Med. Chem. Lett. 2006;16:5883.
[7] Kongkathip N, Luangkamin S, Kongkathip B, Sangma C, Grigg R, Kongsaeree P, Prabpai S, Pradidphol N, Piyaviriyagul S, Siripong P. J. Med. Chem. 2004;47:4427.
[8] Liu KC, Li J, Sakya S. Mini-Rev. Med. Chem. 2004;4:1105.
[9] Miguel del Corral JM, Castro MA, Oliveira AB, Gualberto SA, Cuevas C, Feliciano AS. Bioorg. Med. Chem. 2006;14:7231.
[10] Gomez-Monterrey I, Santelli G, Campiglia P, Califano D, Falasconi F, Pisano C, Vesci L, Lama T, Grieco P, Novellino E. J. Med. Chem. 2005;48:1152.
[11] Schulz WG, Skibo EB. J. Med. Chem. 2000;43:629.
[12] Pérez-Sacau E, Estévez-Braun A, Ravelo AG, Ferro EA, Tokuda H, Mukainaka T, Nishino H. Bioorg. Med. Chem. 2003;11:483.
[13] Silva TMS, Camara CS, Barbosa TP, Soares AZ, da Cunha LC, Pinto AC, Vargas MD. Bioorg. Med. Chem. 2005;13:193.
[14] Lien JC, Kuo SC. Chem. Pharm. Bull. 1996;44:1181.
[15] Lien JC, Huang LJ, Wang JP, Teng CM, Lee KH, Kuo SC. Bioorg. Med. Chem. Lett. 1997;5:2111.
[16] Biot C, Bauer H, Schirmer RH, Davioud-Charret E. J. Med. Chem. 2004; 47:5972.
[17] Mantyla A, Garnier T, Rautio J, Nevalainen T, Vepsalainen J, Koskinen A, Croft SI, Jarvinen T. J. Med. Chem. 2004;47:188.
[18] Tandon VK, Yadav DB, Maurya HK, Chaturvedi AK, Shukla PK. Bioorg. Med. Chem. 2006;14:6120.
[19] Tandon VK, Yadav DB, Singh RV, Chaturvedi AK, Shukla PK. Bioorg. Med. Chem. Lett. 2005;15:5324.
[20] Tandon VK, Yadav DB, Singh RV, Vaish M, Chaturvedi AK, Shukla PK. Bioorg. Med. Chem. Lett. 2005;15:3463.
[21] Tandon VK, Chhor RB, Singh RV, Rai S, Yadav DB. Bioorg. Med. Chem. Lett. 2004;14:1079.

[22] Tandon VK, Singh RV, Yadav DB. Bioorg. Med. Chem. Lett. 2004;14: 2901.
[23] Ryu CK, Choi KU, Shim JY, You HJ, Choi IH, Chae MJ. Bioorg. Med. Chem. 2003;11:4003.
[24] Ryu CK, Han JY, Jung OJ, Lee SK, Lee JY, Jeong SH. Bioorg. Med. Chem. Lett. 2005;15:679.
[25] Ryu CK, Kim HJ. Arch. Pharmacol. Res. 1994;17:139.
[26] Ryu CK, Sun YJ, Shim JY, You HJ, Choi KU, Lee H. Arch. Pharmacol. Res. 2002;25:784.
[27] Errante G, Motta GL, Lagano C, Wittebolle V, Sarciror M-E, Barret R. Eur. J. Med. Chem. 2006;41:773.
[28] Shchekotikhin AE, Buyanov VN, Preobrazhenskaya MN. Bioorg. Med. Chem. 2004;12:3923.
[29] Kim Y-S, Lee H-J, Suh M-E, Schallmeyer D, Lee C-O. Bioorg. Med. Chem. 2003;11:1709.
[30] Skehan P, Storeng R, Scudiero D, Monks A, MeMohan J, Vistica D, Warren JT, Bokesh H, Kenney S, Boyd MR. J. Natl. Cancer Inst. 1990;82:1107.
[31] Gupta K, Bishop J, Peck A, Brown J, Wilson L, Panda D. Biochemistry 2004;43:6645.
[32] Panda D, Rathinasamy K, Santra MK, Wilson L. Proc. Natl. Acad. Sci. U.S.A. 2005;102:9878.
[33] Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. J. Immunol. Methods 1995;184:39.
[34] O'Brien MC, Bolton WE. Cytometry 1995;19:243.
[35] Becker H. Organicum (Practical Handbook of Organic Chemistry). MA, USA: Addison-Wesley; 1973. p. 611.
[36] National Committee for Clinical Laboratory Standard. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast, Approved Standard. Document M27-A. Wayne, PA, USA: National Committee for Clinical Laboratory Standards; 1997.
[37] National Committee for Clinical Laboratory Standard. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Conidium Forming Filamentous Fungi: Proposed Standard. Document M38-P. Wayne, PA, USA: National Committee for Clinical Laboratory Standards; 1998.
[38] Zhou J, Gupta K, Aggarwal S, Aneja R, Chandra R, Panda D, Joshi HC. Mol. Pharmacol. 2003;63:799.
[39] Miguel del Corral JM, Castro MA, Gordaliza M, Martin ML, Gamito AM, Cuevas C, Feliciano AS. Bioorg. Med. Chem. 2006;14: 2816.