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ORIGINAL RESEARCH

Synthesis and antitumor studies of novel benzopyrano-1,2,3-selenadiazole and spiro[benzopyrano]-1,3,4-thiadiazoline derivatives

S. I. El-Desoky · F. A. Badria · M. A. Abozeid · E. A. Kandeel · A. H. Abdel-Rahman

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Abstract A convenient and efficient synthetic protocol of new selenadiazole and thiadiazoline derivatives incorporating benzopyranone moiety from readily available starting materials was described. Reaction of different 2,2-dialkyl and 2,2-spirocycloalkyl dihydrobenzopyranones **1a–e** with semicarbazide hydrochloride and thiosemicarbazide afforded the corresponding semicarbazones **2a–e** and thiosemicarbazones **3a–e**, respectively. Furthermore, cyclization of the semicarbazones **2a–e** via oxidation using selenium dioxide gave a novel series of chromenoselenadiazoles **4a–e**. A series of spirobenzopyrano-1,3,4-thiadidazolines **5a–e** were synthesized by refluxing of the thiosemicarbazones **3a–e** in acetic anhydride. The synthesized compounds were tested in vitro against four cancer cell lines namely: MCF-7, VERO, WI-38, and HEPG-2. In vivo studies were also performed using Ehrlich ascites carcinoma for antitumor activity. Interestingly, Compounds **4b** and **5a** showed significant antitumor activities and were capable to improve the hematological parameters as well as increase the mean survival time of the mice bearing tumor.

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Introduction

Substituted benzopyran-4-ones are common among natural products and they have been used to prepare various heterocyclic ring systems. On the other hand, the interesting pharmacological activities of selenium heterocycles are well known (Gleiter and Schehlmann, 1990; Lalezari *et al*., 1974). In addition, selenium is a key component of several major metabolic pathways in human, including thyroid hormone metabolism, antioxidant defense system, and immune function (Chen *et al*., 2009). Also, selenium supplementation could reduce the incidence of various cancer types such as prostate, lung, colon, and liver cancers (Chen *et al*., 2009; El-Bayoumy and Sinha, 2004).

It is well known that a number of heterocyclic compounds containing nitrogen and sulfur heteroatoms exhibited a wide variety of biological activities (Ellis, 1977; Joshi *et al*., 2006; Sharma and Sarita, 1994). Moreover, the diazole system is found in numerous antiparasitic, fungicidal, and anti-inflammatory drugs (Baht *et al*., 2005). Some 1,2,3-selena and 1,3,4-(thia)diazoles were found to possess antitumor activity (Klayman and Gunther, 1972; Atta *et al*., 2010).

In view of remarkable pharmacological efficiency of selenadiazole and thiadiazoline derivatives and in continuation of our studies in the chemistry of chromones (Atta *et al*., 2010; El-Desoky *et al*., 1997; Abdel-Rahman *et al*., 2005; El-Subbagh *et al*., 2000; El-Shafei *et al*., 2009), we report herein the synthesis of 1,2,3-selena and 1,3,4-(thia)diazoles incorporated benzopyranone scaffold to be evaluated pharmacologically as antitumor agents.

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Results and discussion

Chemistry

The naturally occurring 6-hydroxy-2,2-dimethyl-2,3-dihydrobenzopyran-4-one (**1a**), which was previously isolated from *Calea cuneifolia* DC and *Gynura elliplia*, possesses anti-platelet aggregation activity (Lourenco *et al*., 1981; Lin *et al*., 2000). Also, some benzopyranone derivatives were used as I_{Ks}-channel blockers (Gerlach *et al*., 2001). In the present work, the latter interesting biogenetic chromanone as well as a series of other structurally related dialkyl and spirocycloalkylchromanones are used as precursors for the construction of novel benzopyranoselenadiazole and spirobenzopyranothiadiazoline derivatives hoping to obtain promising biologically active compounds.

Refluxing of 2,5-dihydroxyacetophenone with different ketones namely: acetone, butanone, cyclopentanone, cyclohexanone, or cycloheptanone in the presence of catalytic amount of pyrrolidine (Kabbe and Widdig, 1982) gave a quantitative yield of the naturally occurring 2,2-dimethyl-2,3-dihydrobenzopyran-4-one (**1a**) and a series of the structurally related derivatives **1b–e**. The melting points and spectral data of **1a–d** are in agreement with the reported data (Tripathi *et al*., 2009). The reaction of **1a–e** with semicarbazide hydrochloride in the presence of sodium acetate and few drops of glacial acetic acid gave the corresponding semicarbazones **2a–e**. Also, the reaction of **1a–e** with thiosemicarbazide led to the formation of thiosemicarbazone derivatives **3a–e**.

On the other hand, heating of compounds **2a–e** with equimolar amounts of selenium dioxide gave a series of chromenoselenadiazole derivatives **4a–e**, respectively. The construction of chromenoselenadiazoles **4a–e** was achieved via cyclocondensation of semicarbazones **2a–e** using selenium dioxide in acetic acid (Lalezari *et al.*, 1969). Moreover, the reaction of thiosemicarbazone derivatives **3a–d** with acetic anhydride afforded the corresponding spirochroman-4,5′-Δ^2-1,3,4-thiadiazoline derivatives **5a–d** in very good yields (Scheme 1). The construction of 1,3,4-thiadiazoline ring is mostly achieved by heterocyclization of thiosemicarbazones by acylation with acid anhydrides or acid chlorides (Kubota *et al.*, 1980).

All of the synthesized compounds were established on the basis of analytical and spectral data (C.f. Experimental section). The ^{1}H-NMR spectrum of **2b** revealed the aromatic and methylene protons, in addition to protons of NH_2, NH, and OH which were exchangeable with D_2O at δ 6.42, 8.85, and 9.35, respectively. Additionally, the ^{1}H-NMR spectra of **4a–e** revealed the absence of methylene protons of the chroman ring, in addition to the exchangeable protons except hydroxyl group proton.

As a result of the diastereotopic nature of methylene protons related to the chroman ring of thiadiazoline derivatives **5a–d**, they appeared in the ^{1}H-NMR spectra at two different chemical shifts within $\delta = 2.37$–2.65 and 3.28–3.61 ppm with coupling constant 13.20–14.10 Hz. This marked difference between the chemical shifts not only results from the inductive effect exerted by the heteroatoms, but also due to a spatial effect probably exerted by the acetyl groups. The ^{1}H-NMR of **5b** obviously revealed the presence of two isomers I and II (Fig. 1). The percentages of both isomers were calculated according to the integration curves of $-CH_3$ signal in both of them. Depending upon the stereochemistry of isomer II, methyl group attached to C-2 is in the same direction to nitrogen atom attached to C-4, therefore its chemical shift has a higher value (1.35 ppm) than that of the isomer I (1.32 ppm). Moreover, the ^{13}C-NMR spectrum of **5d** confirmed the ring closure by the appearance of a signal at *circa* 75.86 ppm assigned to C-2 in thiadiazoline ring, and signals of carbonyl moieties incorporated in the molecule at $\delta =$ 168.95, 169.61, and 170.21 ppm. The mass spectra of **5b** and **5c** showed the molecular ion peaks at *m/z* 405 (M^+, 21.20 %) and 417 (M^+, 28.60 %), respectively, which are in agreement with their molecular formula.

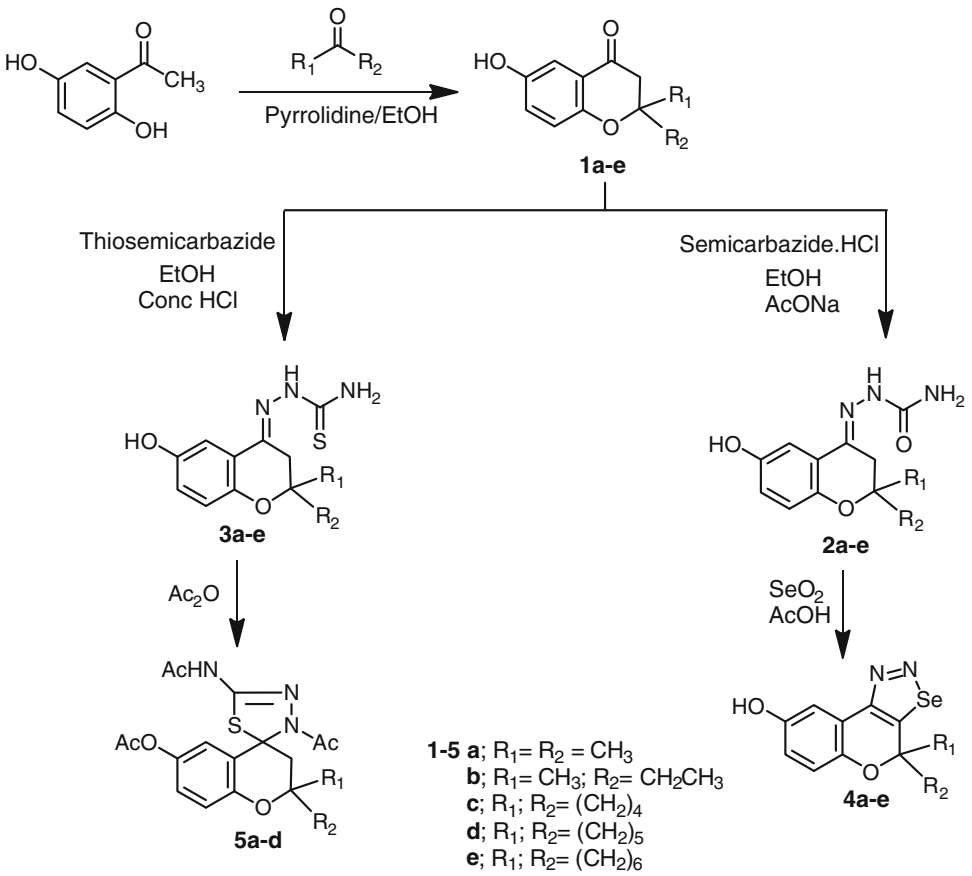
Antitumor studies

Antitumor screening test

The cytotoxic effects of the synthesized compounds against four different cell lines: MCF-7 (cells from breast cancer), VERO (African green monkey kidney cells), WI-38 (fibroblast cells), and HEPG-2 (hepatoma cells) were evaluated using 5-fluorouracil (5-FU) as a reference cytotoxic drugs (Table 1). The results showed that compounds **2b**, **3b**, **4a**, **4b**, **4c**, **5c**, and **5d** were comparable to that of 5-FU (positive control) while compound **5a** is more cytotoxic agent than 5-FU (Table 1).

The result of antitumor screening allows the following assumptions about the structure activity relationships (SARs) of the synthesized compounds: (a) the cytotoxic activity increases by transforming benzopyranones derivatives **1a–c** into their respective semicarbazones **2a–c** and thiosemicarbazones **3a–c** (Dutta *et al*., 2005). (b) Further enhancement of cytotoxic activity was obtained via transformation of semicarbazones derivatives to corresponding selenadiazole derivatives as demonstrated in conversion of **2a** and **2b** to corresponding **4a** and **4b**, respectively. These results are in full agreement with the previous obtained data on the role of synthetic selenadiazole derivatives in induction of caspase- and P53-dependent apoptosis in breast carcinoma cells (Chen *et al*., 2009). (c) The appreciable cytotoxicity of thiadiazoline derivatives, especially **5a**, may be correlated with the presence of thiadiazoline moiety in their molecular structures and this is in agreement with the reported results (Ying *et al*., 2007). (d) As

Scheme 1 Synthesis of chromenoselenadiazoles **4a–e** and spirochroman-4,5′-Δ^2-1,3,4-thiadiazolines **5a–d**



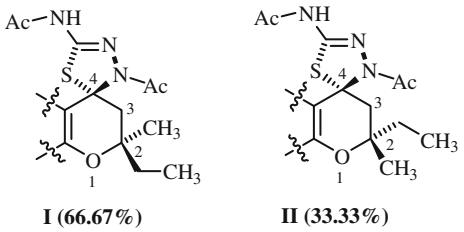


Fig. 1 Two isomers of **5b**

the size of the aliphatic moiety increases, the cytotoxic activity decreases, this may be due to the steric hindrance which takes place by increasing of the side chain size (You *et al.*, 1998).

In vivo study using Ehrlich ascites carcinoma (EAC) assay

The promising results of compounds **4b** and **5a** in the antitumor screening test prompted us to study the effect of the above two compounds on the viability of EAC, in addition to the effect on the hematological status including hemoglobin (Hb), hematocrit (HCT), and white blood cells (WBCs) on EAC-bearing mice. And as a continuation of this work, the effect on the mean survival time (MST) and consequently percentage increase in life span (%ILS) was studied. Adult swiss female albino mice were chosen for in vivo studies.

A comparison was made among five groups of mice ($n = 7$) on the 14th day after incubation. The groups comprised of: (1) normal mice, (2) control (EAC-bearing mice), (3), (4), and (5) EAC-bearing mice treated for the first 9 days with 5-FU, **4b**, and **5a**. The results of in vivo study are shown in Table 2.

We may infer the following information from Table 2: (a) In the fifth day after inoculation of Ehrlich cells in mice, increase in body weight and ascites was observed clearly, and also the mice became slow and inactive. (b) Mice which received compounds **4b**, **5a,** and 5-FU did not show observed manifestation of abdominal ascites. (c) Hematological parameters of EAC-bearing mice on the 14th day showed significant changes when compared with normal mice. (d) Mice treated with compounds **4b** and **5a** showed a dramatic reduction to the total WBC and reciprocally an increase in the hemoglobin content and RBCs even more than those treated with 5-FU. (e) Mice treated with compounds **4b** and **5a** showed a reduction of the tumor size even when compared to 5-FU. (f) Mice treated with

Table 1 In vitro cytotoxicity of tested compounds on different cell lines

Compound	IC_{50} (mM)			
	MCF-7	VERO	WI-38	HEPG-2
1a	0.41	0.42	0.43	0.48
1b	0.15	0.15	0.15	0.15
1c	0.64	0.74	0.51	0.46
1d	0.09	0.09	0.21	0.19
1e	1.51	1.32	1.43	1.45
2a	0.15	0.17	0.14	0.12
2b	0.07	0.05	0.07	0.06
2c	0.11	0.09	0.10	0.10
2d	0.07	0.08	0.16	0.15
2e	1.12	1.13	1.12	1.12
3a	0.14	0.15	0.12	0.14
3b	0.06	0.06	0.06	0.06
3c	0.10	0.09	0.10	0.10
3d	1.34	0.84	1.12	0.82
3e	1.62	1.44	1.51	1.44
4a	0.06	0.05	0.07	0.06
4b	0.06	0.06	0.05	0.05
4c	0.06	0.06	0.07	0.06
4d	0.12	0.10	0.11	0.09
4e	1.49	1.19	1.61	1.15
5a	0.03	0.03	0.03	0.03
5b	1.01	0.63	0.84	0.62
5c	0.05	0.04	0.04	0.05
5d	0.08	0.08	0.08	0.07
5-FU	0.02	0.05	0.02	0.07

IC_{50} inhibition concentration that reduce survival to 50 %, *5-FU* 5-fluorouracil

compounds **4b** and **5a** exhibited an increase in the MST and consequently improvement of the percentage increase in life span (%ILS) over those treated with 5-FU.

The above-mentioned results lead to a conclusion that compounds **4b** and **5a** might be considered as potential anticancer agents. However, further pharmacokinetics, bioavailability, and preclinical studies will be conducted in the near future.

Conclusion

The present study reports an efficient and convenient synthesis of novel series of selenadiazoles **4a–e** and thiadiazolines **5a–e**. The naturally occurring 6-hydroxy-2, 2-dimethyl-2,3-dihydrobenzopyran-4-one (**1a**) as well as a series of other structurally related dialkyl and spiro-cycloalkyl chromanones were used as starting materials. The cytotoxic effects of the synthesized compounds against four different human cell lines such as MCF-7, VERO, WI-38, and HEPG-2 were evaluated. The results revealed that, compounds **2b**, **3b**, **4a**, **4b**, **5c,** and **5d** were similar to that of 5-fluorouracil (IC_{50}: 0.02–0.07 mM). Whereas, compound **5a** (IC_{50} = 0.03 mM) is more cytotoxic than 5-FU. Moreover, in vivo antitumor study using Ehrlich ascites carcinoma (EAC) revealed that there are an improvement in the hematological parameters of tumor-bearing mice, decrease in the tumor volume, and increases in the life span to higher extent compared with 5-FU. Finally, compounds **4b** and **5a** might be used as potential anticancer agents.

Experimental

General

All melting points are uncorrected and were recorded on an open glass capillaries using a Gallenkamp apparatus. The IR spectra (ν/cm^{-1}) (KBr) were recorded on Perkin Elmer Infrared Spectrophotometer Model 157. The ^{1}H-NMR spectra were run on Bruker AC 300 MHz Spectrophotometer. ^{13}C-NMR spectra were recorded on JOEL (at 500 MHz) using TMS as an internal reference and $CDCl_3$ and DMSO-d_6 as solvents, and chemical shift (δ) values are

Table 2 Effect of compounds **4b** and **5a** on hematological parameters, viability of EAC, MST, and %ILS

Groups	Hb (12–16 g/dL)	HCT (35.0–50.0 %)	WBCs (4–11) × 10^3 cm	Viability of Ehrlich cells (10^6/mL)	MST (survival/day)	%ILS
Normal	13.90	53.60	8.40	–	–	–
Control	8.70	35.50	38.60	220.00	9.10	–
5-FU	10.00	42.30	13.80	123.00	15.70	72.53
4b	10.50	43.70	11.50	107.00	15.80	73.63
5a	11.90	45.00	12.80	110.00	18.10	98.90

Normal test without EAC, *Control test* with EAC, *5-FU* 5-fluorouracil, *Hb* hemoglobin, *HCT* hematocrit, *WBCs* white blood cells, *MST* mean survival time, *%ILS* percentage increase in life span

recorded in ppm. The mass spectra (EI) were run at 70 eV with JEOL JMS600 equipment. Elemental analyses (C, H, and N) were carried out at the Microanalytical Center at Cairo University, Egypt. The results were found to be in good agreement with the calculated values. Follow-up of the reactions was made by thin layer chromatography (TLC) on Silica gel pre-coated aluminum sheets (type 60 F254, Merck, Darmstadt, Germany) and the spots were detected by exposure to UV lamp at λ_{254} nm.

Synthesis

*General procedure for the synthesis of (2,2-dialkyl) and (spirocycloalkyl)benzopyranones **1a–e***

Equimolar amounts of 2,5-dihydroxyacetophenone (1.52 g, 10 mmol) and ketone derivatives (10 mmol) namely: acetone, butanone, pentanone, hexanone, or heptanone in ethanol containing a catalytic amount of pyrrolidine (1 mL) was added. The reaction mixture was refluxed for 10–15 h (TLC control). The solvent was evaporated under reduced pressure and dried well. The obtained residual syrup was purified with column chromatography using petroleum ether/ethyl acetate as eluent to give compounds **1a–e**.

*6-Hydroxy-2,2-dimethyl-2,3-dihydrobenzopyran-4-one **(1a)*** Yield (1.48 g, 77 %); yellow needles; m.p. 140–141 °C; [petroleum ether/ethyl acetate 4:1]. Melting point and spectral data were in complete agreement with the published data (Gerlach *et al*., 2001; Tripathi *et al*., 2009).

*2-Ethyl-6-hydroxy-2-methyl-2,3-dihydrobenzopyran-4-one **(1b)*** Yield (1.43 g, 70 %); deep yellow crystals; m.p. 60–61 °C; [petroleum ether/ethyl acetate 4:1]. Melting point and spectral data were in complete agreement with the published data (Tripathi *et al*., 2009).

*6-Hydroxy-2,2-tetramethylene-2,3-dihydrobenzopyran-4-one **(1c)*** Yield (1.48 g, 68 %); pale yellow crystals; m.p. 118–120 °C; [petroleum ether/ethyl acetate 4:1]. Melting point and spectral data were in complete agreement with the published data (Tripathi *et al*., 2009).

*6-Hydroxy-2,2-pentamethylene-2,3-dihydrobenzopyran-4-one **(1d)*** Yield (2.13 g, 92 %); yellow crystals; m.p. 116 °C; [petroleum ether/ethyl acetate 4:1]. Melting point and spectral data were in complete agreement with the published data (Tripathi *et al*., 2009).

*6-Hydroxy-2,2-hexamethylene-2,3-dihydrobenzopyran-4-one **(1e)*** Yield (1.56 g, 64 %); faint orange crystals; m.p. 126 °C; [petroleum ether/ethyl acetate 4:1]; IR (KBr): ν/cm^{-1} = 3425–3300 (OH, broad), 1668 (C=O), 1617 (Ar); ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.20–2.20 (m, 12H, 6 CH_2), 2.72 (s, 2H, H-3), 5.70 (s, broad, 1H, OH, exchangeable with D_2O), 6.86 (d, 1H, H-8, J = 9 Hz), 7.06 (dd, 1H, H-7, J = 3.3, 9 Hz), 7.33 (d, 1H, H-5, J = 3.3 Hz). Anal. Calcd. for $C_{15}H_{18}O_3$ (246.13): C, 73.15; H, 7.37 %. Found: C, 73.32; H, 7.46 %.

*General procedure for the synthesis of 2,2-dialkyl and (spirocycloalkyl) 2,3-dihydrobenzopyran-4-one semicarbazones **2a–e***

A solution of **1a–e** (10 mmol), semicarbazide hydrochloride (2.23 g, 20 mmol), dry sodium acetate (2 g) in dry ethanol (20 mL), and a catalytic amount of glacial acetic acid (1 mL) was refluxed with stirring for 1–5 h (TLC control). The reaction mixture was poured onto crushed ice after cooling. The formed precipitate was filtered off, washed with water, dried, and recrystallized from ethanol to give compounds **2a–e**.

*6-Hydroxy-2,2-dimethyl-2,3-dihydrobenzopyran-4-one semicarbazone **(2a)*** Yield (1.82, 73 %); yellow crystals; m.p. 212–214 °C; Melting point and spectral data were in complete agreement with the published data (Quilico *et al*., 1950).

*2-Ethyl-6-hydroxy-2-methyl-2,3-dihydrobenzopyran-4-one semicarbazone **(2b)*** Yield (1.43 g, 70 %); pale yellow crystals; m.p. 100–101 °C; IR (KBr): ν/cm^{-1} = 3444, 3330, 3316, 3226 (OH, NH, NH_2), 1675 (C=O, amide), 1581 (Ar); ^{1}H-NMR (DMSO-d_6) δ (ppm): 0.80 (t, 3H, $CH_2\underline{CH_3}$, J = 7.5 Hz), 1.17 (s, 3H, CH_3), 1.51 (dq, 1H, $\underline{CH_a}CH_3$), 1.63 (dq, 1H, $\underline{CH_b}CH_3$), 2.64 (s, 2H, H-3), 6.42 (s, 2H, NH_2, exchangeable with D_2O), 6.62 (d, 1H, H-8, J = 9 Hz), 6.67 (dd, 1H, H-7, J = 2.7, 9 Hz), 7.37 (d, 1H, H-5), 8.85 (s, broad, 1H, NH, exchangeable with D_2O), 9.35 (s, broad, 1H, OH, exchangeable with D_2O). Anal. Calcd. for $C_{13}H_{17}N_3O_3$ (263.13): C, 59.30; H, 6.51; N, 15.96 %. Found: C, 59.54; H, 6.45; N, 15.69 %.

*6-Hydroxy-2,2-tetramethylene-2,3-dihydrobenzopyran-4-one semicarbazone **(2c)*** Yield (2.53 g, 92 %); colorless crystals; m.p. 103–104 °C; IR (KBr): ν/cm^{-1} = 3430, 3362, 3284–3217 (OH, NH, NH_2), 1692 (C=O, amide), 1583 (Ar); ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.70–1.90 (m, 8H, 4 CH_2), 2.76 (s, 2H, H-3), 6.41 (s, 2H, NH_2, exchangeable with D_2O), 6.60 (m, 2H, H-7 and H-8), 7.38 (d, 1H, H-5, J = 3 Hz), 8.88 (s, 1H, NH, exchangeable with D_2O), 9.31 (s, 1H, OH, exchangeable with D_2O). Anal. Calcd. for $C_{14}H_{17}N_3O_3$ (275.13): C, 61.08; H, 6.22; N, 15.26 %. Found: C, 61.22; H, 6.41; N, 15.50 %.

*6-Hydroxy-2,2-pentamethylene-2,3-dihydrobenzopyran-4-one semicarbazone (**2d**)* Yield (2.60 g, 90 %); pale yellow crystals; m.p. 188–190 °C; IR (KBr): $\nu/cm^{-1} = 3467$, 3349–3261 (OH, NH, NH_2), 1673 (C=O, amide), 1573 (Ar); ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.20–1.80 (m, 10H, 5 CH_2) 2.65 (s, 2H, H-3), 6.39 (s, 2H, NH_2, exchangeable with D_2O), 6.64–6.67 (m, 2H, H-7 and H-8), 7.36 (d, 1H, H-5, $J = 2.10$ Hz), 8.80 (s, broad, 1H, NH, exchangeable with D_2O), 9.36 (s, 1H, OH, exchangeable with D_2O). Anal. Calcd. for $C_{15}H_{19}N_3O_3$ (289.14): C, 62.27; H, 6.62; N, 14.52 %. Found: C, 62.47; H, 6.81; N, 14.73 %.

*6-Hydroxy-2,2-hexamethylene-2,3-dihydrobenzopyran-4-one semicarbazone (**2e**)* Yield (2.25 g, 74.3 %); pale red crystals; m.p. 118 °C; IR (KBr): $\nu/cm^{-1} = 3465$, 3390–3200 (OH, NH, NH_2), 1675 (amide), 1571 (Ar); ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.36–1.80 (m, 12H, $6CH_2$), 2.71 (s, 2H, H-3), 6.40 (s, 2H, NH_2, exchangeable with D_2O), 6.70–6.76 (m, 2H, H-7 and H-8), 7.20 (d, 1H, H-5, $J = 3$ Hz), 8.47 (s, broad, 1H, NH, exchangeable with D_2O), 9.41 (s, 1H, OH, exchangeable with D_2O). MS (EI, 70 eV) *m/z* (%) = 304 [M^++1] (20.15), 303 [M^+] (98.29), 302 [M^+−1] (7.60), 286 [M^+–NH_3] (49.15), 260 (68.57), 246 (100), 203 (68.35). Anal. Calcd. for $C_{16}H_{21}N_3O_3$ (303.16): C, 63.35; H, 6.98; N, 13.85 %. Found: C, 63.51; H, 6.87; N, 13.71 %.

*General procedure for the synthesis of 2,2-dialkyl and (spirocycloalkyl) 2,3-dihydrobenzopyran-4-one thiosemicarbazones **3a–e***

A mixture of chromanones **1a–e** (10 mmol), thiosemicarbazide (1 g, 11 mmol) in dry ethanol (25 mL), and a catalytic amount of conc. HCl (1 mL) was refluxed with stirring for 3–5 h (TLC control). The reaction mixture was cooled, poured onto crushed ice (100 g) thereafter, while stirring was continued for 1 h. The formed precipitate was filtered off, washed well with cold water, dried, and recrystallized from ethanol to give compounds **3a–e**.

*6-Hydroxy-2,2-dimethyl-2,3-dihydrobenzopyran-4-one thiosemicarbazone (**3a**)* Yield (1.86 g, 70 %); yellow crystals; m.p. 238–239 °C; IR (KBr): $\nu/cm^{-1} = 3426$, 3351, 3270 (OH, NH, NH_2), 1606 (C=N), 1197 (C=S); ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.27 (s, 6H, 2 CH_3), 2.81 (s, 2H, H-3), 6.63 (d, 1H, H-8, $J = 9$ Hz), 6.74 (dd, 1H, H-7, $J = 2.70$, 9 Hz), 7.49 (d, 1H, H-5, $J = 2.70$ Hz), 7.86 (s, 1H, NH_2, exchangeable wtih D_2O), 8.24 (s, 1H, NH_2, exchangeable wtih D_2O), 8.90 (s, 1H, NH, exchangeable wtih D_2O), 10.22 (s, 1H, OH, exchangeable wtih D_2O). Anal. Calcd. for $C_{12}H_{15}N_3O_2S$ (265.09): C, 54.32; H, 5.70; N, 15.84; S, 12.08 %. Found: C, 54.50; H, 5.88; N, 15.57; S, 12.27 %.

*2-Ethyl-6-hydroxyl-3-methyl-2,3-dihydrobenzopyran-4-one thiosemicarbazone (**3b**)* Yield (2.09 g, 75 %); pale brown crystals; m.p. 200–201 °C; IR (KBr): $\nu/cm^{-1} = 3424$, 3363, 3276 (OH, NH, NH_2), 1606 (C=N), 1197 (C=S); ^{1}H-NMR (DMSO-d_6) δ (ppm): 0.90(t, 3H, $\underline{CH_3}CH_2$, $J = 7.80$ Hz), 1.18 (s, 3H, CH_3), 1.54 (dq, 1H, $\underline{CH_a}CH_3$), 1.65 (dq, 1H, $\underline{CH_b}CH_3$), 2.74 (d, 1H, H_a-3, $J_{gem} = 17.10$ Hz), 2.84 (d, 1H, H_b-3, $J_{gem} = 17.10$ Hz), 6.65 (d, 1H, H-8, $J = 8.70$ Hz), 6.70 (dd, 1H, H-7, $J = 3$, 8.70 Hz), 7.48 (d, 1H, H-5, $J = 3$ Hz), 7.85 (s, 1H, NH_2, exchangeable with D_2O), 8.23 (s, 1H, NH_2, exchangeable with D_2O), 8.90 (s, 1H, NH, exchangeable with D_2O), 10.31 (s, 1H, OH, exchangeable with D_2O). MS (EI, 70 eV) *m/z* (%) = 280 [M^++1] (0.08), 279 [M^+] (0.29), 278 [M^+−1] (0.09), 160 (41), 137 (81), 64 (100). Anal. Calcd. for $C_{13}H_{17}N_3O_2S$ (279.10): C, 55.89; H, 6.13; N, 15.04; S, 11.48 %. Found: C, 55.72; H, 6.33; N, 15.18; S, 11.71 %.

*6-Hydroxy-2,2-tetramethylene-2,3-dihydrobenzopyran-4-one thiosemicarbazone (**3c**)* Yield (2.32 g, 80 %); pale yellow crystals; m.p. 233–234 °C; IR (KBr): $\nu/cm^{-1} = 3439$, 3362, 3284 (OH, NH, NH_2), 1607 (C=N), 1195 (C=S); ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.40–1.80 (m, 8H, 4 CH_2), 2.92 (s, 2H, H-3), 6.64 (d, 1H, H-8, $J = 9$ Hz), 6.72 (dd, 1H, H-7, $J = 3$, 9 Hz), 7.49 (d, 1H, H-8, $J = 3$ Hz), 7.86 (s, 1H, NH_2, exchangeable with D_2O), 8.23 (s, 1H, NH_2, exchangeable with D_2O), 8.91 (s, 1H, NH, exchangeable with D_2O), 10.28 (s, 1H, OH, exchangeable with D_2O). Anal. Calcd. for $C_{14}H_{17}N_3O_2S$ (291.10): C, 57.71; H, 5.88; N, 14.42; S, 11.00 %. Found: C, 57.82; H, 5.94; N, 14.34; S, 11.20 %.

*6-Hydroxy-2,2-pentamethylene-2,3-dihydrobenzopyran-4-one thiosemicarbazone (**3d**)* Yield (2.44 g, 80 %); yellow crystals; m.p. 122 °C; IR (KBr): $\nu/cm^{-1} = 3422$, 3298–3200 (OH, NH, NH_2), 1589 (C=N), 1208 (C=S); ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.34–1.97 (m, 10H, $5CH_2$), 2.56 (s, 2H, H-3), 6.45 (s, broad, 3H, NH, NH_2, exchangeable with D_2O), 6.80 (d, 1H, H-8), 7.25 (dd, 1H, H-7), 7.35 (d, 1H, H-5), 8.77(s, broad, 1H, OH, exchangeable with D_2O). Anal. Calcd. for $C_{15}H_{19}N_3O_2S$ (305.12): C, 58.99; H, 6.27; N, 13.76; S, 10.50 %. Found: C, 58.71; H, 6.41; N, 13.90; S, 10.69 %.

*6-Hydroxy-2,2-hexamethylene-2,3-dihydrobenzopyran-4-one thiosemicarbazone (**3e**)* Yield (2.23 g, 70 %); yellow crystals; m.p. 184 °C; IR (KBr): $\nu/cm^{-1} = 3415$, 3320, 3276 (OH, NH, NH_2), 1594 (C=N), 1218 (C=S); ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.20–1.90 (m, 12H, 6 CH_2), 2.83 (s, 2H, H-3), 6.60–6.80 (m, 2H, H-7, H-8), 7.46 (d, 1H, H-5, $J = 2.70$ Hz), 7.84 (s, 1H, NH_2, exchangeable with D_2O), 8.22 (s, 1H, NH_2, exchangeable with D_2O),

8.88 (s, broad, 1H, NH, exchangeable with D_2O), 10.37 (s, 1H, OH, exchangeable with D_2O). Anal. Calcd. for $C_{16}H_{21}N_3O_2S$ (319.14): C, 60.16; H, 6.63; N, 13.16; S, 10.04 %. Found: C, 60.41; H, 6.83; N, 13.37; S, 10.21 %.

*General procedure for the synthesis of 4,4-dialkyl and (spirocycloalkyl)benzopyrano[4,3-d] (1,2,3)selenadiazoles **4a–e***

A solution of compounds **2a–e** (5 mmol) in glacial acetic acid (15 mL) was warmed at 60 °C with stirring, then selenium dioxide (0.55 g, 5 mmol) was added portionwise during a period of 30 min and stirring was continued for further 4–10 h (TLC control). After completion of the reaction, the reaction mixture was filtered to remove the deposited selenium. The filtrate was poured onto crushed ice and the obtained solid was filtered off, washed thoroughly with cold water, sodium carbonate solution, and water. The obtained product after drying was purified with column chromatography using petroleum ether/ethyl acetate as eluent to give compounds **4a–e**.

*8-Hydroxy-4,4-dimethylbenzopyrano[4,3-d](1,2,3)selenadiazole (**4a**)* Yield (1.04 g, 74 %); yellowish brown crystals; m.p. 168–170 °C; [petroleum ether/ethyl acetate 7:3]; IR (KBr): ν/cm^{-1} = 3500–3250 (OH, broad), 1502 (N=N); ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.77 (s, 6H, $2CH_3$), 5.35 (s, broad, 1H, OH, exchangeable with D_2O), 6.83 (dd, 1H, H-7, J = 2.70, 8.70 Hz), 6.87 (d, 1H, H-6, J = 8.70 Hz), 7.79 (d, 1H, H-9, J = 2.70 Hz); ^{13}C-NMR ($CDCl_3$) δ (ppm): 30.39, 80.05, 111.58, 117.46, 118.52, 145.82, 151.09, 153.71, 159.60. Anal. Calcd. for $C_{11}H_{10}N_2O_2Se$ (281.99): C, 46.99; H, 3.58; N, 9.96 %. Found: C, 46.72; H, 3.80; N, 9.76 %.

*4-Ethyl-8-hydroxy-4-methylbenzopyrano[4,3-d](1,2,3)selenadiazole (**4b**)* Yield (1.05 g, 71 %); yellow crystals; m.p. 108 °C; [petroleum ether/ethyl acetate 7:3]; IR (KBr): ν/cm^{-1} = 3400–3200 (OH, broad), 1504 (N=N); ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.03 (t, 3H, $\underline{CH_3}$–CH_2, J = 7.20 Hz), 1.72 (s, 3H, CH_3), 2.02 (q, 2H, $\underline{CH_2}$–CH_3, J = 7.20 Hz), 6.75–6.90 (m, 3H, H-7, H-6, H-9), 7.77 (s, broad, 1H, OH, exchangeable with D_2O). MS (EI, 70 eV) *m/z* (%) = 297 [M^++2] (12.59), 296 [M^++1] (89.96), 295 [M^+] (6.41), 294 [M^+−1] (44.29), 267 [M^+–N_2] (24.72), 253 [M^+–cyclopropane] (100), 188 [M^+–selenadiazirene] (96.17), 173 (78.30), 131 (70.79). Anal. Calcd. for $C_{12}H_{12}N_2O_2Se$ (296.01): C, 48.82; H, 4.10; N, 9.49 %. Found: C, 48.96; H, 4.31; N, 9.63 %.

*8-Hydroxy-4,4-tetramethylene benzopyrano[4,3-d] (1,2,3)selenadiazole (**4c**)* Yield (1.12 g, 73 %); yellow crystals; m.p. 144 °C; [petroleum ether/ethyl acetate 7:3]; IR (KBr): ν/cm^{-1} = 3448 (OH, free), 3500–3200 (OH, broad, bonded), 1503 (N=N); ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.80–2.38 (m, 8H, 4 CH_2), 6.72 (dd, H-7, J = 3 Hz, 8.70 Hz), 6.86 (d, 1H, H-6, J = 8.70 Hz), 7.43 (d, 1H, H-9, J = 3 Hz), 9.34 (s, 1H, OH, exchangeable with D_2O). MS (EI, 70 eV) *m/z* (%) = 309 [M^++2] (6.66), 308 [M^++1] (47.25), 307 [M^+] (4.01), 279 [M^+–N_2] (5.66), 251 (37.62), 199 (100), 115 (54.41). Anal. Calcd. for $C_{13}H_{12}N_2O_2Se$ (308.01): C, 50.83; H, 3.94; N, 9.12 %. Found: 50.91; H, 3.74; N, 9.10 %.

*8-Hydroxy-4,4-pentamethylene benzopyrano[4,3-d](1,2,3)selenadiazole (**4d**)* Yield (1.17 g, 73 %); yellow crystals; m.p. 184 °C; [petroleum ether/ethyl acetate 7:3]; IR (KBr): ν/cm^{-1} = 3378 (OH, free), 3530–3210 (OH, broad, bonded), 1500 (N=N); ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.38–2.20 (m, 10H, 5 CH_2), 6.74 (d, 1H, H-7, J = 7.80 Hz), 6.93 (d, 1H, H-6, J = 8.70 Hz), 7.44 (s, 1H, H-9), 9.34 (s, 1H, OH, exchangeable with D_2O). Anal. Calcd. for $C_{14}H_{14}N_2O_2Se$ (322.02): C, 52.35; H, 4.39; N, 8.72 %. Found: C, 52.10; H, 4.21; N, 8.42 %.

*8-Hydroxy-4,4-hexamethylene benzopyrano[4,3-d](1,2,3)selenadiazole (**4e**)* Yield (1.24 g, 74 %); yellow crystals; m.p. 144 °C; [petroleum ether/ethyl acetate 7:3]; IR (KBr): ν/cm^{-1} = 3600–3200 (OH, broad, bonded), 1500 (N=N); ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.30–2.50 (m, 12H, $6CH_2$), 6.80–7.10 (m, 3H, H-7, H-6, H-9), 7.72 (s, 1H, OH, exchangeable with D_2O). Anal. Calcd. for $C_{15}H_{16}N_2O_2Se$ (336.04): C, 53.74; H, 4.81; N, 8.36 %. Found: C, 53.95; H, 4.60; N, 8.40 %.

*General procedure for the synthesis of spirochroman-4, 5′-Δ^2-1,3,4-thiadiazolines **5a–d***

A mixture of **3a–d** (10 mmol) in freshly distilled acetic anhydride (20 mL) was refluxed on water bath (90 °C) for 5–20 h (TLC control). After completion of the reaction, the reaction mixture was poured onto crushed ice with vigorous stirring. The formed precipitate was filtered off, washed with water, dried, and purified by column chromatography using petroleum ether/ethyl acetate as eluent to give compounds **5a–d**.

*6-Acetoxy-4′-acetyl-2′-acetylamido-2,2-dimethylspirochroman-4,5′-Δ^2-1,3,4 thiadiazoline (**5a**)* Yield (3.08 g, 70 %); pale brown crystals; m.p. 144 °C; [petroleum ether/ethyl acetate 4:6]; IR (KBr): ν/cm^{-1} = 3218 (NH), 1758 (CH_3–COO), 1702, 1680 (CH_3CONH, two groups), 1621 (C=N). ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.34 (s, 3H, CH_3), 1.46 (s, 3H, CH_3), 2.00 (s, 3H, $\underline{CH_3}$–COO–), 2.236 (s, 3H, $\underline{CH_3}$–CON), 2.243 (s, 3H, $\underline{CH_3}$–CON), 2.43 (d, 1H, H_a-3, J_{gem} = 13.80 Hz), 3.42 (d, 1H, H_b-3, J_{gem} = 13.80 Hz), 6.73 (d, 1H, H-8, J = 8.70 Hz), 6.89 (dd, 1H, H-7, J = 3,

8.70 Hz), 7.13 (d, 1H, H-5, $J = 3$ Hz), 9.64 (s, br., 1H, NH, exchangeable with D_2O). Anal. Calcd. for $C_{18}H_{21}N_3O_5S$ (391.12): C, 55.23; H, 5.41; N, 10.73; S, 8.19 %. Found, 55.43; H, 5.21; N, 10.53; S, 8.00 %.

6-Acetoxy-4′-acetyl-2′-acetylamido-2-ethyl-2-methylspirochroman-4,5′-Δ^2-1,3,4-thiadiazoline (**5b**) Yield (3.11 g, 77 %); yellow crystals; [petroleum ether/ethyl acetate 4:6]; m.p. 110 °C; IR (KBr): $\nu/cm^{-1} = 3222$ (NH), 1760 (CH_3COO), 1698, 1680 (CH_3CON, two groups), 1621 (C=N). ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.00 (m, 6H, 2$\underline{CH_3}CH_2$, isomer I, II), 1.32 (s, 3H, CH_3, isomer I), 1.35 (s, 3H, CH_3, isomer II), 1.55 (m, 2H, $\underline{CH_2}CH_3$, isomer II), 1.75 (m, 2H, $\underline{CH_2}CH_3$, isomer I), 1.98 (s, 6H, 2$\underline{CH_3}COO$, isomer I, II), 2.25 (s, 12H, 4$\underline{CH_3}CON$ groups, isomer I, II), 2.40 (d, 1H, H_a-3, $J_{gem} = 13.20$ Hz, isomer I), 2.50 (d, 1H, H_a-3, $J_{gem} = 14.10$ Hz, isomer II), 3.34 (m, 2H, H_b-3, isomer I, II), 6.71 (d, 2H, H-8, isomer I, II), 6.87 (m, 2H, H-7, isomer I, II), 7.12 (d, 2H, H-5, isomer I, II), 9.68 (s, 1H, NH, exchangeable with D_2O, isomer I), 9.85 (s, 1H, NH, exchangeable with D_2O, isomer II). MS (EI, 70 eV) *m/z* (%) = 407 [M^++2] (8.50), 406 [M^++1] (17.80), 405 [M^+] (21.20), 363 [M_1, M^+–CH_2CO], 304 [M_2, M^+–$C_3H_4N_2OS$] (27.10), 293 [M_3, M_1–CH_2CO, CH_2=CH_2] (50.00), 262 [M_4, M_2–CH_2CO] (60.20), 251 [M_3–CH_2CO or CH_3–CH=CH_2] (85.60), 233 [M_5–CH_2CH_2, $H^\bullet$] (100), 208 (43.20), 191 (37.30), 136 (24.60), 100 (20.30). Anal. Calcd. for $C_{19}H_{23}N_3O_5S$ (405.14): C, 56.28; H, 5.72; N, 10.36; S, 7.91 %. Found: C, 56.42; H, 5.93; N, 10.25; S, 7.70 %.

6-Acetoxy-4′-acetyl-2′-acetylamido-2,2-tetramethylenespirochroman-4,5′-Δ^2-1,3,4-thia-diazoline (**5c**) Yield (2.92, 70 %); colorless crystals; m.p. 154–155 °C; [petroleum ether/ethyl acetate 4:6]; IR (KBr): $\nu/cm^{-1} = 3218$ (NH), 1762 (CH_3COO), 1699, 1673 (CH_3CONH, two groups), 1620 (C=N); ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.66–2.03 (m, 8H, 4 CH_2), 2.09 (s, 3H, $\underline{CH_3}COO$), 2.24 (s, 3H, $\underline{CH_3}CON$), 2.27 (s, 3H, $\underline{CH_3}CON$), 2.37 (d, 1H, H_a-3, $J_{gem} = 13.80$ Hz), 3.61(d, 1H, H_b-3, $J_{gem} = 13.80$ Hz), 6.69 (d, 1H, H-8, $J = 9$ Hz), 6.86 (d, 1H, H-7, $J = 2.70$, 9 Hz), 7.13 (d, 1H, H-5, $J = 2.70$ Hz), 10.40 (s, 1H, NH, exchangeable with D_2O). MS (EI, 70 eV) *m/z* (%) = 418 [M^++1] (6.60), 417 [M^+] (28.60), 416 [M^+−1] (19.80), 375 [M_1, M^+–CH_2CO] (52.7), 332 [M_2, M_1–Ac] (16.50), 316 [M_2–O] (26.40), 293 [M_3, M_1–cyclohexene], 251 [M_3–CH_2CO](100), 199(46.20), 115(30.80), 65(50.50). Anal. Calcd. for $C_{20}H_{23}N_3O_5S$ (417.14): C, 57.54; H, 5.55; N, 10.07; S, 7.68 %. Found: C, 57.74; H, 5.36; N, 10.27; S, 7.73 %.

6-Acetoxy-4′-acetyl-2′-acetylamido-2,2-pentamethylenespirochroman-4,5′-Δ^2-1,3,4-thiadiazoline (**5d**) Yield: (4.09 g, 95 %); colorless crystals; m.p. 130 °C; [petroleum ether/ethyl acetate 4:6]; IR (KBr): $\nu/cm^{-1} = 3224$ (NH), 1756 (CH_3COO), 1706, 1650 (CH_3CONH, two groups), 1621 (C=N). ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.20–1.90 (m, 10H, 5CH_2), 2.01 (s, 3H, $\underline{CH_3}COO$), 2.22 (s, 3H, $\underline{CH_3}CON$), 2.23 (s, 3H, $\underline{CH_3}CON$), 2.48 (d, 1H, H_a-3, $J_{gem} = 14.10$ Hz), 3.30 (d, 1H, H_b-3, $J_{gem} = 14.10$ Hz), 6.78 (d, 1H, H-8, $J = 8.70$ Hz), 6.90 (dd, 1H, H-7, $J = 2.70$, 8.70 Hz), 7.09 (d, 1H, H-5, $J = 2.70$ Hz), 9.44 (s, 1H, NH, exchangeable with D_2O); ^{13}C-NMR ($CDCl_3$) δ (ppm): 21.26, 21.50, 22.85, 24.32, 25.45, 32.65, 38.11, 45.30, 74.83, 75.86, 118.43, 120.03, 123.11, 125.38, 144.32, 145.52, 150.34, 168.95, 169.61, 170.21. Anal. Calcd. for $C_{21}H_{25}N_3O_5S$ (431.15): C, 58.45; H, 5.84; N, 9.74; S, 7.43 %. Found: C, 58.25; H, 5.74; N, 9.93, S, 7.33 %.

Antitumor studies

Antitumor screening test

Samples were prepared for assay by dissolving in 50 µL of DMSO and diluting aliquots into sterile culture medium at 0.4 mg/mL. These solutions were subdiluted to 0.02 mg/mL in sterile medium and the two solutions were used as stocks to test samples at 100, 50, 20, 10, 5, 2, and 1 mg/mL in triplicate in the wells of microtiter plates (El-Subbagh *et al.*, 2000).

The compounds were tested for cytotoxic activity against MCF-7 (cells from breast cancer), VERO (African green monkey kidney cells), WI-38 (fibroblast cells), and HEPG-2 (hepatoma cells).

The compounds were assayed in triplicate on monolayers grown in Dulbeccos modified Eagle's medium supplemented with 10 % (v/v) calf serum (HyClone Laboratories, Ogden, UT), 60 mg/mL Penicillin G, and 100 mg/mL streptomycin sulfate maintained at 37 °C in a humidified atmosphere containing about 15 % (v/v) CO_2 in air. All medium components were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise indicated. Cells stocks were maintained at 34 °C in culture flasks filled with medium supplemented with 1 % (v/v) calf serum. Subcultures of cells for screening were grown in the wells of microtiter trays (Falcon Microtest III 96-wells trays, Becton–Dickinson Labware, Lincolin Park, NJ) by suspending cells in medium following trypsin–EDTA treatment, counting the suspension with a hemocytometer, diluting in medium containing 10 % calf serum to 2×10^4 cells per 200 mL culture, aliquoting into each well of a tray, and culturing until confluent.

Microtiter trays with confluent monolayer cultures of cells were inverted, the medium shaken out, and replaced with serial dilutions of sterile compounds in triplicate in 100 µL medium followed by titered virus in 100 µL medium containing 10 % (v/v) calf serum in each well. In each tray, the last row of wells was reserved for controls that were not treated with compounds. The trays were cultured for 96 h.

The trays were inverted onto a pad of paper towels, the remaining cells rinsed carefully with medium, and fixed with 3.7 % (v/v) formaldehyde in saline for at least 20 min. The fixed cells were rinsed with water and examined visually. The cytotoxic activity is identified as confluent, relatively unaltered monolayers of stained cells treated with compounds. Cytotoxicity was estimated as the concentration that caused approximately 50 % loss of the monolayer. 5-fluorouracil was used as a positive control.

In vivo study using EAC assay

Adult Swiss female albino mice (20–25 g) were purchased from a local breeder and were housed in microlon boxes in a controlled environment (temperature 25 °C and 12 h dark/light cycle) with standard laboratory diet and water (El-Shafei *et al*., 2009).

Ascites fluid was withdrawn under aseptic conditions from the peritoneal cavity of a EAC-bearing mouse (purchased from National Cancer Institute, Cairo, Egypt) by needle aspiration, and the EAC cells were suspended in normal saline (0.9 % sodium chloride solution) so that each 0.2 mL contains 2×10^6 viable EAC cells and they were microscopically examined for their viability using trypan blue stain.

Mice were divided into five groups (7 each) as follows: (1) normal mice, (2) control group (EAC only), (3) tumor-bearing mice treated intraperitoneally (i.p.) with 5-fluorouracil (20 mg/kg/day) as a standard anticancer drug dissolved in saline (positive control), (4), and (5) tumor-bearing mice treated intraperitoneally with the tested compounds (50 mg/kg/day) suspended in saline. EAC cells suspension (0.2 mL containing 2×10^6 cells per mouse) was inoculated (i.p.) into the last four groups only. Twenty four hours after inoculation, 100 µL of the treatment as a solution in saline was injected (i.p.) into groups (3, 4, and 5) once a day for nine consecutive days. The control group was treated with the same volume of normal saline solution. The mice were observed for 15 days. The activity was assessed using peritoneal cells count and hematological parameters.

Acute toxicity studies The LD_{50} values of compounds **4b** and **5a** were evaluated for their acute toxicity in mice after administration by i.p. route in the ranges of 213.5–286.1 and 303.4–401.1 mg kg^{-1} for compounds **4b** and **5a**, respectively.

Compounds **4b** and **5a** resulted in acute toxic manifestation but caused no obvious neurotoxic reaction including tremor, twitch, jumping, drowsy, and exhibited a decrease in locomotor activity after the administration of the compounds at doses above 500 mg kg^{-1}. Of the tested compounds, compound **5a** displayed the lowest acute toxicity with LD_{50} value of 348.4 (303.4–401.1) mg kg^{-1} vs. compound **4b** which showed LD_{50} value of 247.1 (213.5–286.1) mg kg^{-1}.

*Effect of compounds **5a** and **4b** on hematological parameters* In order to detect the influence of the tested compounds on the hematological status of EAC-bearing mice, a comparison was made among the groups of mice on the 14th day after inoculation. Blood was withdrawn from each mouse from retro orbital plexus, and the white blood cells (WBC) count, red blood cells (RBC) count, hemoglobin, and HCT were determined.

Determination of viable cells count of EAC After 5 days of treatment, 100 µL samples of EAC cells (from three mice) were taken in each group, and 20-fold dilution in saline was prepared. The cells were stained with trypan blue and the viable cells were counted using a hemocytometer. The mean value of total number of viable cells per group (treated) was compared with those of the control group.

*Effect of compounds **4b** and **5a** on survival time* The mean survival time (MST) of each group, consisting of seven mice was noted. The antitumor efficacy was compared with that of 5-FU, for 9 days. The MST of the treated groups was compared with that of the control group using the following calculation:

Percentage increase in life span over control (%ILS) = [MST of treated group − MST of control group] × 100 − 100; where, MST = mean survival time (days of each mouse in a group)/Total no. of mice (Sur and Ganguly, 1994).

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