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Original article

Synthesis and anticancer potential of certain novel 2-oxo-*N*'-(2-oxoindolin-3-ylidene)-2*H*-chromene-3-carbohydrazides

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ABSTRACT

Treatment of ethyl 3-hydrazinyl-3-oxopropanoate (**6**) with indoline-2,3-dione derivatives **7a**–**g** gave ethyl 3-oxo-3-(2-(2-oxoindolin-3-ylidene)hydrazinyl)propanoates **8a**–**g** which were allowed to react with the appropriate salicylaldehyde **9a** and/or **9b** to furnish the chromene-based hydrazones **10a**–**i**. Compounds **10a**–**i** displayed a significant activity against HT-29 colon cancer cell line and a moderate activity against leukemia K562 cell line. Compound **10f** emerged as the most active congener toward HT-29 colon cancer cell line with $IC_{50} = 7.98 \pm 0.05$ μM whereas compound **10c** exhibited the best anti-proliferative activity against leukemia K562 cell line with $IC_{50} = 9.44 \pm 0.02$ μM. Moreover, compound **1e** showed 87.81 ± 7% inhibition of side population (SP) HT-29 colon cancer stem cells.

1. Introduction

Cancer is one of the most dreaded diseases of mankind and it is the principal cause of mortality worldwide. Currently, one in 4 deaths in the United States is due to cancer and more than ten million new cancer cases occur annually [1]. There are many reasons for the difficulty in the control and treatment of cancer including the clonal evaluation of cancer types and the genetic and epigenetic instability of cancer. The recently proposed cancer stem cell (CSC) hypothesis states that a small percentage of tumor cells are responsible for tumor initiation and progression through unlimited self-renewal. Numerous studies have identified CSCs in leukemia [2], breast [3], brain [4], lung [5], colon [6], and other cancers. Side population (SP) cells have been isolated from several solid tumors in a technique based on the exclusion of Hoechst dye 33342 or other dyes, which occurs primarily through the activity of membrane pumps encoded by multidrug-resistance gene 1 (MDR1) and breast cancer-resistance gene 2 (ABCG2). SP cells resist cytotoxic agents and hence often survive longer. Additionally, SP cells isolated from patients or from metastatic cell lines are enriched with colorectal CSCs/progenitor cells or stem/progenitor cells [7]. The tumorigenic potential of SP cells has been detected in various cancer models [8,9]. Accordingly, SP cells are considered a potential target in cancer therapy because they are thought to be a source of CSCs.

Colorectal cancer is the most common malignancy of the gastrointestinal tract [10] and causes 655,000 deaths worldwide every year [11]. Many treatment protocols have been applied to mange colorectal cancer, but a complete cure has not been achieved. This failure to achieve a cure may be due to the presence of colorectal CSCs that are resistant to chemotherapy and/or radiation therapy. Therefore, it is important to use therapies that target stem cells as well as proliferating cells to cure colorectal cancer [12].

Chromene-2-ones are widely distributed in natural products and they display diverse biological activities. Chromene-containing compounds exert their anti-cancer activities *via* inhibition of steroid sulfatase, aromatase, and/or carbonic anhydrase enzymes [13–15]. On the other hand, indoline-2,3-diones exhibit anticancer

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activities by inhibiting tyrosine kinase or caspase-3/7. In 2006, the FDA approved an indoline-2,3-dione derivative, Sunitinib (Sutent)®, for the oral management of renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumor (GIST) [16]. Additionally, we have documented the synthesis of certain novel indoline-2,3-diones that display potent and selective anti-proliferative activity against multidrug-resistant cancer cells [17,18].

An examination of the literature revealed that Soliman et al. obtained salicyaldehyde azine (**3a**) instead of the expected hydrazide **2a** for the reaction of ester **1a** with hydrazine hydrate (Fig. 1) [19]. Recently, malonohydrazide (**4**) along with salicyaldehyde azine (**3a**) were isolated from the reaction of **1a** with hydrazine hydrate and a mechanism for their formation was proposed [20]. In the same vein, Sammour et al. reported that the reaction of ethyl 6-bromo-2-oxo-2*H*-chromene-3-carboxylate (**1b**) with hydrazine hydrate gave compounds **3b** and **4** instead of hydrazide **2b** (Fig. 1) [21]. Additionally, Mohamed et al. [22] published the reaction of ethyl 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylate (**1c**) with hydrazine hydrate to give compounds **3c** and **4** instead of hydrazide **2c** (Fig. 1).

Interestingly, Ma et al. documented that the reaction of ethyl 7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxylate (**1d**) with hydrazine hydrate gave hydrazide **2d** (Fig. 1) [23], which was rigorously confirmed [24]. These variable findings in the reaction of ethyl chromene-3-carboxylates **1a–d** with hydrazine hydrate demonstrate that the type and position of the substituent affect significantly on the pyran-2-one ring stability.

In view of the aforementioned premises, and in a continuation of our interest in the synthesis of hydrazone-based compounds with anticancer activity [17,18,25–29], we aimed to report herein the synthesis and *in vitro* antiproliferative activity of the title compounds **10a–i** toward a panel of cancer cell lines as well as their inhibitory activity on side population cancer stem cells.

2. Results and discussion

2.1. Chemistry

Hydrazides are versatile tools for the construction of several functionalized heterocycles with interesting biological activities [30–36]. Therefore, the synthesis of hydrazide-containing compounds has drawn considerable interest [37,38]. The common route for the synthesis of chromene-based hydrazones is the reaction of the appropriate hydrazide **2** with indoline-2,3-diones. Many reports indicate that hydrazide **2a** is produced from the reaction of ester **1a** with hydrazine hydrate (Fig. 1) and that **2a** can be used as a key starting material in the synthesis of several heterocyclic compounds and hydrazones [39–48]. However, the ring opening of chromene-2-ones under moderate conditions has been reported where the aliphatic pyran-2-one ring in the chromene system is highly reactive. It undergoes ring opening at the lactone acyl center under nucleophilic attack as well as under nucleophilic conjugate addition at the carbon–carbon double bond [49].

These findings encouraged us to establish an alternative route for synthesis of the targeted chromene-based hydrazones **10a–i** starting from ethyl 3-hydrazinyl-3-oxopropanoate (**6**) (Scheme 1) instead of esters **1a**,**b**. Consequently, treatment of **6** with the appropriate indoline-2,3-dione derivative **7a–g** in refluxing ethanol resulted in the formation of ethyl 3-oxo-3-(2-(2-oxoindolin-3-ylidene)hydrazinyl) propanoates **8a–g** (Scheme 1).

Condensation of hydrazones **8a–g** with the appropriate salicyaldehyde **9a** or **9b** in the presence of piperidine yielded 2-oxo-*N*′-(2-oxoindolin-3-ylidene)-2*H*-chromene-3-carbohydrazides **10a–i**. IR spectra of compounds **10a–i** revealed three carbonyl absorption bands around 1700 cm^{-1} as well as two NH absorption bands in the 3450–3100 cm^{-1} region. Additionally, their ^1H NMR spectra showed two D_2O-exchangeable signals in the region δ 9.07–11.3 and δ 11.0–11.95 due to NH of isatin and hydrazone functionalities, respectively.

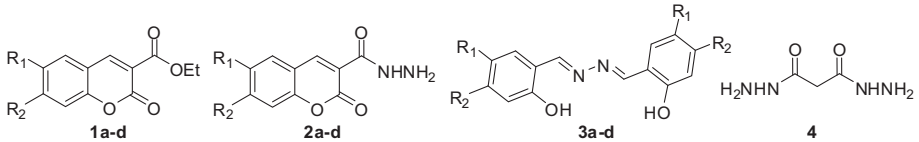
2.2. In vitro antiproliferative activity

In vitro antiproliferative activity of compounds **10a–i** against leukemia K562, breast MDA-MB-468 and colon HT-29 cell lines is displayed in Table 1. Compound **10c** (R/R_1 = H/Cl) exhibited the highest antiproliferative activity toward leukemia K562 cell line with IC_{50} = 9.44 ± 0.02 μM followed by compounds **10d** (IC_{50} = 12.08 ± 0.01 μM) and **10f** (IC_{50} = 12.99 ± 0.1 μM) which contain bromine and methoxy substituents at five position of indoline-2,3-dione moiety, respectively. The antiproliferative activity of the other synthesized congeners is in the following decreasing order: **10a** (IC_{50} = 25.5 ± 08 μM) > **10b** > **10i** > **10g** > **10e** > **10h** (IC_{50} = 36.99 ± 0.09 μM). On the other hand, compound **10h** (IC_{50} = 15.48 ± 0.02 μM) is the most active candidate among all the synthesized compounds **10a–i** against breast MDA-MB-468 cell line while compound **10f** exhibited the weakest antiproliferative activity with IC_{50} = 43.97 ± 0.17 μM.

The best *in vitro* antiproliferative profile of compounds **10a–i** is showed against colon HT-29 cancer cell line where compound **10f** is the most active congener with IC_{50} = 7.98 ± 0.05 μM. Compound **10f** has un-substituted chromene moiety and a substituent, methoxy group, endowed with negative inductive and positive mesomeric effects at the five position of indoline-2,3-dione nucleus. Compounds **1e** (R/R_1 = H/Me), **1g** (R/R_1 = H/NO_2) and **1d** (R/R_1 = H/Br) exhibited a comparable biological activity to that of compound **10f** with IC_{50} = 8.42 ± 0.01, 9 ± 0.01 and 10.05 ± 0.03 μM, respectively. The decreasing order of antiproliferative activity of the rest compounds toward colon HT-29 cancer cell line is as follows: **1h** (IC_{50} = 12.25 ± 0.04 μM) > **1b** > **1i** > **1a** > **1c** (IC_{50} = 27.1 ± 0.46 μM).

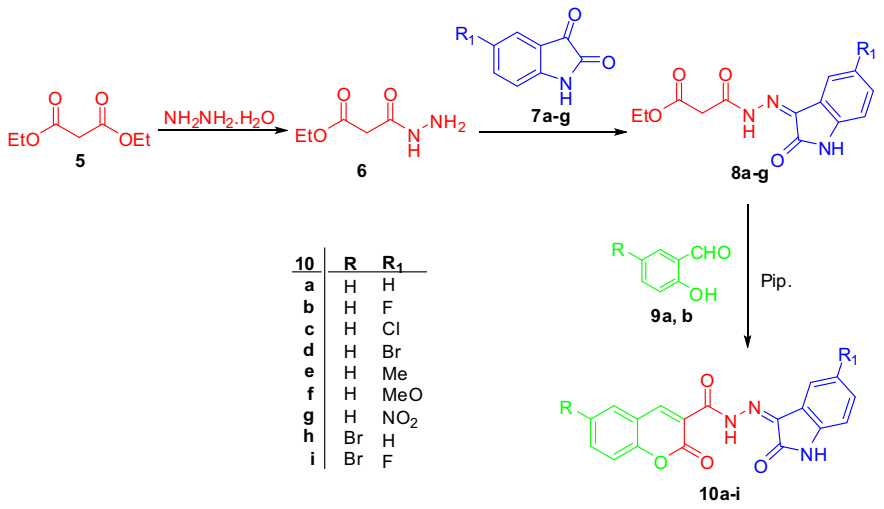
2.3. Side population cancer stem cells inhibitor activity

The inhibitory activity of the target compounds **10a–i** against side population colon HT-29 cancer stem cells was performed at 10 μM (Table 2 and Fig. 2). Compound **1e** (R/R_1 = H/Me) is the most active inhibitor displaying 87.8 ± 7% inhibition as compared with the reference compound, Verapamil, which showed 95 ± 8%



1-3: **a**, R_1 = R_2 = H; **b**, R_1 = Br, R_2 = H; **c**, R_1 = H, R_2 = OH; **d**, R_1 = H, R_2 = $N(Et)_2$

Fig. 1. The structure of compounds **1–3** (**a–d**) and **4**.



10	R	R_1
a	H	H
b	H	F
c	H	Cl
d	H	Br
e	H	Me
f	H	MeO
g	H	NO_2
h	Br	H
i	Br	F

Scheme 1. Synthesis of hydrazones **10a**–**i**.

inhibition. On the other hand, compounds **10f** (R/R_1 = H/MeO) and **10i** (R/R_1 = Br/F) increased the side population, while the rest compounds showed variable inhibitions varied from 78.05 ± 7% for **10h** (Br/R_1 = Br/F) to 46.36 ± 14% for **10a** (R/R_1 = H/H).

3. Conclusion

Synthesis and *in vitro* antiproliferative activity of certain new 2-oxo-*N*′-(2-oxoindolin-5-substituted-3-ylidene)-2*H*-chromene-3-carbohydrazides **10a**–**i** toward leukemia K562, breast MDA-MB-468 and colon HT-29 cell lines are reported. The title compounds **10a**–**i** exhibited good antiproliferative profile against colon HT-29 cell line where compounds **10f** and **10e** emerged as the most active candidates with IC_{50} = 7.98 ± 0.05 and 8.42 ± 0.01 μM, respectively. Additionally, the target compounds **10a**–**i** displayed a moderate inhibitory activity for side population (SP) colon HT-29 cancer stem cells. Compound **10e** showed 87.81 ± 7% inhibition of SP colon HT-29 cancer stem cells where the reference compound, verapamil, exhibited 95 ± 8% inhibition.

4. Experimental

4.1. Chemistry

4.1.1. General

Infrared (IR) Spectra were recorded as KBr disks using the Perkin Elmer FT-IR Spectrum BX apparatus. Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. NMR Spectra were scanned in DMSO-d_6 on a Jeol NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Chemical shifts are expressed in δ-values (ppm) relative to TMS as an internal standard. Coupling constants (*J*) are expressed in Hz. D_2O was added to confirm the exchangeable protons. Mass spectra were measured on an Agilent Triple Quadrupole 6410 QQQ LC/MS equipped with an ESI (electrospray ionization) source.

4.1.2. General procedure for the synthesis of ethyl 3-oxo-3-(2-(2-oxoindolin-3-ylidene)hydrazinyl)propanoates *8a*–*g*

Ethyl 3-hydrazinyl-3-oxopropanoate (**6**) was prepared from diethyl malonate (**5**) and hydrazine hydrate according to the reported method (m.p. 68–69 °C) [50]. A solution of compound **6** (1.46 g, 10 mmol) and the appropriate indoline-2,3-dione **7a**–**g** (10 mmol) in absolute ethanol (30 mL) was refluxed for 1 h and then left to cool. The solid formed was collected by filtration, washed with ethanol to give hydrazones **8a**–**g** which were used for the next step without any further purification.

4.1.3. General procedure for the synthesis of 2-oxo-*N*′-(2-oxoindolin-3-ylidene)-2H-chromene-3-carbohydrazides *10a*–*i*

To a solution of the appropriate hydrazone **8a**–**g** (1 mmol) and salicylaldehyde (**9a**) or 5-bromosalicylaldehyde (**9b**) (1 mmol) in absolute ethanol (25 mL), a catalytic amount of piperidine (0.3 mL) was added. The reaction mixture was refluxed for 1 h. The formed precipitate was filtered off, washed with ethanol, dried and

Table 1
In vitro antiproliferative activity for compounds **10a**–**i** on K562 chronic myelogenous leukemia, MDA-MB-468 breast cancer and HT-29 colon cancer cells.

Compound **10**	R	R_1	IC_{50} (μM)[a]		
			Leukemia K562	Breast MDA-MB-468	Colon HT-29
a	H	H	25.5 ± 08	17.2 ± 0.01	16.7 ± 0.3
b	H	F	25.77 ± 0.07	17.52 ± 0.01	16.25 ± 0.5
c	H	Cl	9.44 ± 0.02	16.45 ± 0.11	27.1 ± 0.46
d	H	Br	12.99 ± 0.13	27.97 ± 0.02	10.05 ± 0.3
e	H	Me	35.01 ± 0.43	18.21 ± 0.04	8.42 ± 0.01
f	H	MeO	12.08 ± 0.01	43.97 ± 0.17	7.98 ± 0.05
g	H	NO_2	27.64 ± 0.48	31.01 ± 0.15	9 ± 0.01
h	Br	H	36.99 ± 0.09	15.48 ± 0.02	12.25 ± 0.4
i	Br	F	27.4 ± 0.22	40.25 ± 0.24	16.58 ± 0.7

[a] IC_{50}: concentration of the compound (μM) producing 50% cell growth inhibition after 48 h of compound exposure, as determined by the WST-1 assay. Each experiment was run at least two times, and the results are presented as average values ±S.D.

Table 2
Side population inhibition activity on HT-29 colon cancer stem cells for compounds **10a**–**i**.

Compound **10**	R	R_1	Side population Inhibition (%) ± SD at 10 μM
a	H	H	46.36 ± 14
b	H	F	68.36 ± 8
c	H	Cl	73.81 ± 7
d	H	Br	73.81 ± 7
e	H	Me	87.81 ± 7
f	H	MeO	In[a]
g	H	NO_2	58.54 ± 7
h	Br	H	78.05 ± 7
i	Br	F	In[a]
Verapamil			95 ± 8

[a] Increase the side population cancer stem cells.

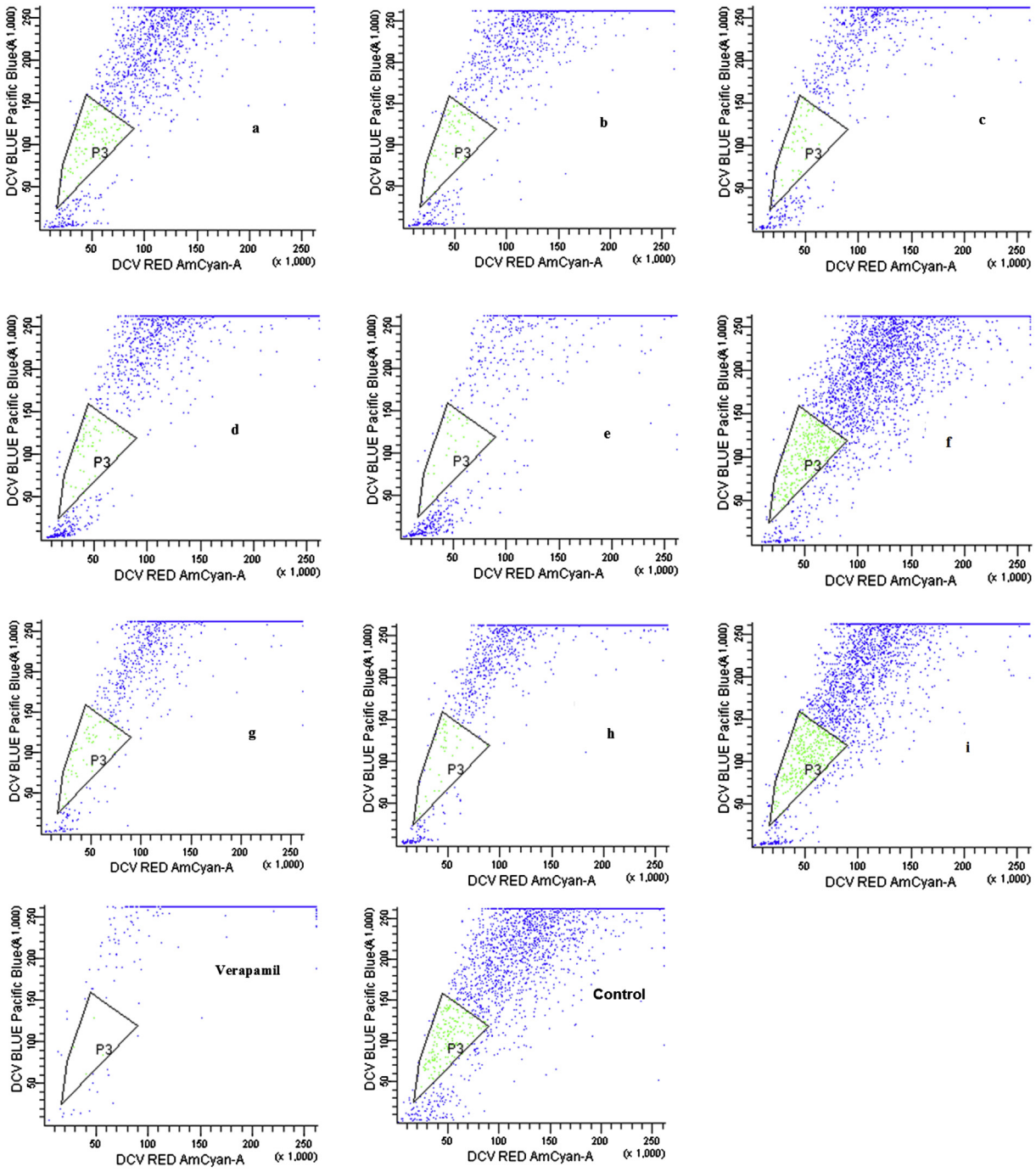


Fig. 2. The histograms of flow cytometric identification of side population cells in HT-29 colon cancer cells for compounds **10a**–**i** and verapamil.

recrystallized from EtOH/DMF to give the title carbohydrazides **10a**–**i**.

*4.1.3.1. 2-Oxo-N′-(2-oxoindolin-3-ylidene)-2H-Chromene-3-carbohydrazide (**10a**).* Yield (69%); mp 318–320 °C; IR (KBr): ν 3450–3164 (2NH), 1705 (3C=O) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.93–9.13 (m, 9H, 8ArHs and 1H of pyran), 11.13 (s, D_2O exch., 1H, NH of isatin), 11.89 (s, D_2O exch., 1H, NH of hydrazide); ^{13}C NMR (DMSO-d_6): δ 116.8, 116.9, 118.9, 119.7, 120.2, 125.6, 127.8, 128.1, 129.6, 130.9, 131.0, 134.5, 136.8, 141.4, 150.1, 154.5, 161.6, 165.8; ESI MS *m/z*: 334.1 $[M + 1]^+$, 356.1 $[M + 23]^+$.

*4.1.3.2. N′-(5-Fluoro-2-oxoindolin-3-ylidene)-2-oxo-2H-chromene-3-carbohydrazide (**10b**).* Yield (62%); mp 310–312 °C; IR (KBr): ν 3450–3197 (2NH), 1700 (3C=O) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.93–9.14 (m, 8H, 7ArHs and 1H of pyran), 11.19 (s, D_2O exch., 1H, NH of isatin), 11.89 (s, D_2O exch., 1H, NH of hydrazide); ESI MS *m/z*: 352.1 $[M + 1]^+$.

*4.1.3.3. N′-(5-Chloro-2-oxoindolin-3-ylidene)-2-oxo-2H-chromene-3-carbohydrazide (**10c**).* Yield (70%); mp > 360 °C; IR (KBr): ν 3450–3196 (2NH), 1734–1654 (3C=O) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.93–9.12 (m, 8H, 7ArHs and 1H of pyran), 11.13 (s, D_2O exch., 1H,

NH of isatin), 11.89 (s, D_2O exch., 1H, NH of hydrazide); ^{13}C NMR (DMSO-d_6): δ 116.8, 117.0, 118.9, 119.9, 125.8, 127.2, 127.9, 129.4, 130.8, 133.5, 134.5, 134.9, 141.4, 145.2, 149.9, 154.5, 161.6, 165.3; ESI MS *m/z*: 368.1 $[M + 1]^+$, 390.1 $[M + 23]^+$.

*4.1.3.4. N′-(5-Bromo-2-oxoindolin-3-ylidene)-2-oxo-2H-chromene-3-carbohydrazide (**10d**).* Yield (70%); mp 302–304 °C; IR (KBr): ν 3442–3100 (2NH), 1701 (3C=O) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.97–8.93 (m, 8H, 7ArHs and 1H of pyran), 11.13 (s, D_2O exch., 1H, NH of isatin), 11.90 (s, D_2O exch., 1H, NH of hydrazide); ESI MS *m/z*: 412.1 $[M]^+$.

*4.1.3.5. N′-(5-Methyl-2-oxoindolin-3-ylidene)-2-oxo-2H-chromene-3-carbohydrazide (**10e**).* Yield (73%); mp 310–312 °C; IR (KBr): ν 3400–3197 (2NH), 1700 (3C=O) cm^{-1}; ^1H NMR (DMSO-d_6): δ 2.48 (s, 3H, CH_3), 6.89–8.92 (m, 8H, 7ArHs and 1H of pyran), 11.13 (s, D_2O exch., 1H, NH of isatin), 11.90 (s, D_2O exch., 1H, NH of hydrazide); ^{13}C NMR (DMSO-d_6): δ 20.5, 116.8, 116.9, 118.9, 119.9, 125.8, 127.2, 127.9, 129.4, 130.9, 134.5, 134.9, 141.4, 145.2, 148.2, 149.9, 154.5, 161.6, 164.8; ESI MS *m/z*: 348.1 $[M + 1]^+$, 370.1 $[M + 23]^+$.

*4.1.3.6. N′-(5-Methoxy-2-oxoindolin-3-ylidene)-2-oxo-2H-chromene-3-carbohydrazide (**10f**).* Yield (60%); mp > 360 °C; IR (KBr): ν 3380–3197 (2NH), 1701 (3C=O) cm^{-1}; ^1H NMR (DMSO-d_6): δ 3.34 (s, 3H, OCH_3), 6.95–8.93 (m, 8H, 7ArHs and 1H of pyran), 11.13 (s, D_2O exch., 1H, NH of isatin), 11.89 (s, D_2O exch., 1H, NH of hydrazide); ESI MS *m/z*: 364.1 $[M + 1]^+$, 386.2 $[M + 23]^+$.

*4.1.3.7. N′-(5-Nitro-2-oxoindolin-3-ylidene)-2-oxo-2H-chromene-3-carbohydrazide (**10g**).* Yield (62%); mp 320–322 °C; IR (KBr): ν 3300–3197 (2NH), 1700 (3C=O) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.95–8.93 (m, 8H, 7ArHs and 1H of pyran), 11.13 (s, D_2O exch., 1H, NH of isatin), 11.95 (s, D_2O exch., 1H, NH of hydrazide); ESI MS *m/z*: 379.2 $[M + 1]^+$, 401.1 $[M + 23]^+$.

*4.1.3.8. 6-Bromo-2-oxo-N′-(2-oxoindolin-3-ylidene)-2H-chromene-3-carbohydrazide (**10h**).* Yield (74%); mp 308–310 °C; IR (KBr): ν 3446–3229 (2NH), 1707 (3C=O) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.94–8.95 (m, 8H, 7ArHs and 1H of pyran), 11.13 (s, D_2O exch., 1H, NH of isatin), 11.90 (s, D_2O exch., 1H, NH of hydrazide); ESI MS *m/z*: 413.9 $[M + 1]^+$, 436.0 $[M + 23]^+$.

*4.1.3.9. 6-Bromo-N′-(5-fluoro-2-oxoindolin-3-ylidene)-2-oxo-2H-chromene-3-carbohydrazide (**10i**).* Yield (65%); mp > 360 °C; IR (KBr): ν 3450–3228 (2NH), 1706 (3C=O) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.82–9.90 (m, 7H, 6ArHs and 1H of pyran), 11.18 (s, D_2O exch., 1H, NH of isatin), 11.93 (s, D_2O exch., 1H, NH of hydrazide); ESI MS *m/z*: 430.0 $[M]^+$, 454.0 $[M + 23]^+$.

4.2. In vitro antiproliferative activity

Antiproliferative activity of the title compounds **10a–i** was evaluated at Stem Cell Therapy Program, King Faisal Specialized Hospital and Research Center, P.O. 3354, Riyadh 11211, Saudi Arabia. *In vitro* antiproliferative activity was measured by the cell growth inhibition assay. The general *in vitro* antitumor evaluation of the test compounds **10a–i** was conducted by use WST-1 reagent for determination of IC_{50} for each compound and the results are given in Table 1. K562 chronic myelogenous leukemia, MDA-MB-468 breast cancer and HT-29 colon cancer cell lines were purchased from the American Type Culture Collection. Cells were maintained in RPMI 1640 (Sigma), supplemented with 10% FBS (Lonza), 100 IU/mL penicillin, 100 mg/mL streptomycin and 2 mmol/L L-glutamine (Sigma). Cells were seeded into 96-well plates at $0.4*10^4$/well and incubated overnight. The medium was replaced with fresh one containing the desired concentrations of the test compounds. After 48 h, 10 μl of the WST-1 reagent were added to each well and the plates were re-incubated for 4 h at 37 °C. The amount of formazan was quantified using ELISA reader at 450 nm.

4.3. Side population cancer stem cells inhibitor activity

The side population cancer stem cells inhibitor activity of the test compounds **10a–i** was evaluated at Stem Cell Therapy Program, King Faisal Specialized Hospital and Research Center, P.O. 3354, Riyadh 11211, Saudi Arabia. Side population staining was performed by using Vybrant® DyeCycle Violet Assay Kit (Molecular Probe) following the manufacturer's recommendations. Briefly, $1*10^6$ of HT-29 colon cancer cells suspended in 1 mL of DMEM medium and 10 μM of Vybrant® DyeCycle Violet were added and incubated for 90 min in 37 °C. Cells were centrifuged and washed by the medium. As a control, cells were treated with 100 μM Verapamil (sigma), which blocks the action of the transporter responsible for dye exclusion. The analyses were performed on a FACS LSRII (BD Biosciences). Debris and cell clusters were excluded during side-scatter and forward-scatter analyses. Results are given in Table 2 and Fig. 2.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.09.060.

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