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Design and synthesis of anti-breast cancer agents from 4-piperazinylquinoline: A hybrid pharmacophore approach

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ABSTRACT

A novel class of 4-piperazinylquinoline derivatives based on the isatin scaffold were designed by molecular hybridization approach and synthesized for biological evaluation. Subsequently, the compounds were examined for their cytotoxic effects on two human breast tumor cell lines, MDA-MB468 and MCF7, and two non-cancer breast epithelial cell lines, 184B5 and MCF10A. Although all compounds examined were quite effective on the breast cancer cell lines examined, the compound 4-bromo-1-[4-(7-chloro-quinolin-4-yl)-piperazin-1-ylmethyl]-1*H*-indole-2,3-dione (**5b**) and N^1-[4-(7-trifluoromethyl-quinolin-4-yl)]-piperazin-1-ylmethyl-4-chloro-1*H*-indole-2,3-dione-3-thiosemicarbazone (**8a**) emerged as the most active among this series. It appeared that both **5b** and **8a** caused apoptosis to MCF7 cancer cells, but not MCF10A non-cancer cells. Thus, 4-piperazinylquinoline linked isatin analog can serve as the prototype molecule for further development of a new class of anti-breast cancer agents.

1. Introduction

The growing incidence of drug resistance to cancer chemotherapeutic agent represents a serious medical problem.[1] Therefore, there is an urgent need to develop new classes of chemotherapeutic agent to treat cancer.[2] A combinational chemotherapeutic drug with different mechanisms of action is one of the methods that are being adopted to treat cancer.[3–6] Besides the exploitation of new targets, there is another approach combining two or more pharmacophores into a single molecule. Therefore, a single molecule containing more than one pharmacophore, each with different mode of action, could be beneficial for the treatment of cancer.[7–9] These 'merged' pharmacophore may be addressing the active site of different targets and offer the possibility to overcome drug resistance. In addition, this approach can also reduce unwanted side effects.[3,4,6,7] The success of this hybridization approach has already been applied in developing novel antibacterial and antimalarial agents to overcome drug resistance.[9–13]

Recently, we have demonstrated that 10 μM chloroquine (Fig. 1, CQ) significantly increases cancer cell killing effects when used in combination with radiation or Akt inhibitors.[3,4,14] Importantly, the CQ-mediated enhancement of cell killing by Akt inhibitors is cancer-specific.[3,4] In continuation of our efforts to develop effective anticancer drugs, we synthesized several CQ-analogs (Fig. 1, **I–III**), and examined their cytotoxic effects on MDA-MB468 and MCF7 breast cancer cell lines. We found that some of these compounds are very effective.[15] The lysosomotropic CQ is accumulated in the lysosomes, raises intra-lysosomal pH, and interferes with autophagosome degradation in the lysosomes. This unique property of CQ and its analogs may be important for the enhancement of cell killing by cancer therapeutic agents in a variety of different tumors.[16] Consistent with data published in the literatures and our results suggested that the 4-aminoquinoline ring system possesses potent anticancer activity.[3,4,15,16]

Published data showed that isatin (1*H*-Indole-2,3-dione) is one of the most effective, newly emerging class of heterocyclic molecules that possesses many interesting and beneficial properties. Importantly, isatin is well tolerated by humans.[17] Several laboratories reported that the isatin scaffold clearly shows anticancer activity against various human tumor cell lines (Fig. 2. I).[8,17–19] We have also found that certain isatin-benzothiazole analogs (Fig. 2. I, II) have anti-breast cancer activity.[8]

A pharmacophore of thiosemicarbazones is known to have anticancer and antiviral activity.[20–22] For example, Methisazone (*N*-methylisatin-β-thiosemicarbazone, Fig. 3) was effective as prophylaxis against smallpox and vaccinia viruses, and 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (Fig. 3, **I**) is

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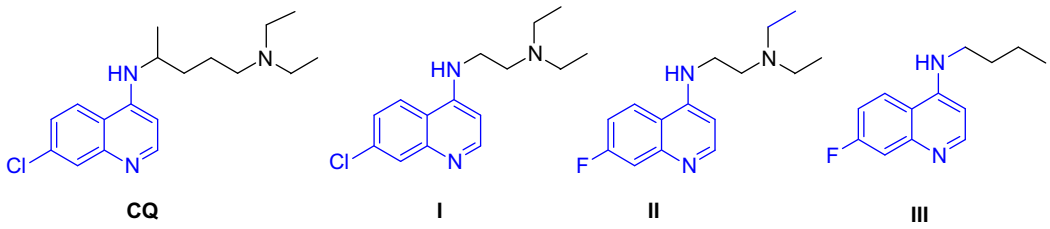


Figure 1. Chemical structures of 4-aminoquinolines reported for anticancer activity.

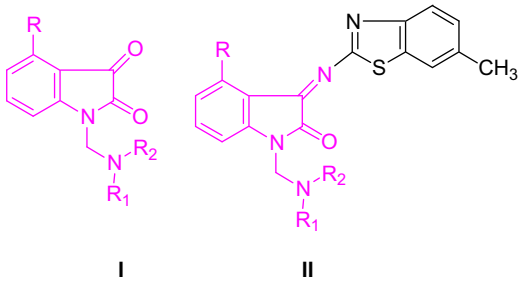


Figure 2. Isatin analogs reported for anti-breast cancer activity from this laboratory.

currently being evaluated by clinical trials against several malignancies including leukemia.[21,22] Hall et al.[23] recently have developed a set of isatin-β-thiosemicarbazones (Fig. 3, **II**) through bioinformatics screening, and found selective activity on the multidrug resistant KB-3-1 and KB-VI cells.

The present work is an extension of our ongoing efforts towards developing effective anticancer agents by a hybrid pharmacophore approach using the 4-piperazinylquinoline scaffold. There are accumulating lines of evidence that hybridization of two or more different bioactive molecules with complementary pharmacophoric functions or with different mechanisms of action often renders synergistic effects.[8–13] Encouraged by these previous reports, we designed three different set of compounds (Fig. 4, Schemes 1 and 2) by hybridizing two or more different pharmacophores in the aim of prospecting their anti-breast cancer potentiality. We then synthesized hybrid compounds by linking the main structural unit of the 4-piperazinylquinoline ring system with the isatin ring system by a Mannich base reaction, and examined their cytotoxic effects on two human breast tumor and two matching non-cancer cell lines.

2. Results and discussion

2.1. Chemistry

The compounds **7a–e** and **8a–8e** described in this study were synthesized by two different synthetic methods as outlined in Schemes 1 and 2. The intermediate compounds 7-substituted-4-piperazin-1-yl-quinoline (**3–4**) were prepared by aromatic nucleophilic substitution on 7-substituted-4-chloro-quinoline with excess of piperazine and triethylamine in neat condition (without solvent). Here, we performed aromatic nucleophilic substitution reaction in excess of amine to avoid the use of phenol as a solvent, which is toxic and prone to polymerization. Upon preparation of the required precursor of secondary amine (**3–4**), we proceeded with the Mannich reaction. The Mannich base analogs **5a–e**, **6a–e**, **7a–e** and **8a–e** were prepared by condensing the active hydrogen atom of istain with formaldehyde and secondary amino function of amino component. A Mannich reaction can be performed via one pot multi-component protocol requiring isatin, an aldehyde component and an amine, or via a preformed iminium ion. Accordingly, molar equivalent amount of paraformaldehyde and an amino component (compound **3** or **4**) were dissolved in ethanol and iminium ion formed in situ was then reacted with isatin and substituted isatin (Scheme 2, **9a–e**) in ethanol reflux for 4 h to furnish the desired *N*-Mannich isatin derivatives (Scheme 1, **5a–e**, **6a–e**; Scheme 2, **7a–e** and **8a–e**). In Scheme 2, the isatin-3-thiosemicarbazones derivatives **9a–e** were synthesized by condensation of isatin with thiosemicarbazide in ethanol reflux for 2 h. In Scheme 1, the final product **7a–e** and **8a–e** were obtained by condensation of *N*-Mannich isatin derivatives with thiosemicarbazide in ethanol reflux for 3 h. The compounds reported in this manuscript have been thoroughly characterized by elemental analysis and spectral data.

2.2. In vitro cytotoxicity

All the compounds synthesized were evaluated for their cytotoxicity on two breast cancer cell lines, MDA-MB468 (a PTEN defective, presumably p53 positive, EGFR positive breast adenocarcinoma cell line) and MCF7 (p53+/-, invasive ductal breast carcinoma) and two non-cancer breast epithelial cell lines, 184B5 and MCF10A. Each compound stored at 20 mM was diluted from 100 μM to 0.0064 μM by fivefold serial dilutions. Cells were treated with each compound for 48 h, followed by measuring cell growth rates by SRB-based spectrophotometry as described previously.[3,9,24,25] The reading of SRB staining is known to accurately reflect the levels of total cellular macromolecules/cell growth/proliferation.[24] The GI_{50} concentration for each compound was

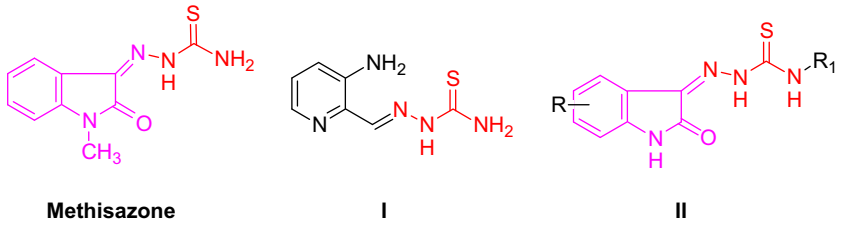


Figure 3. Chemical structures of biologically active thiosemicarbazones.

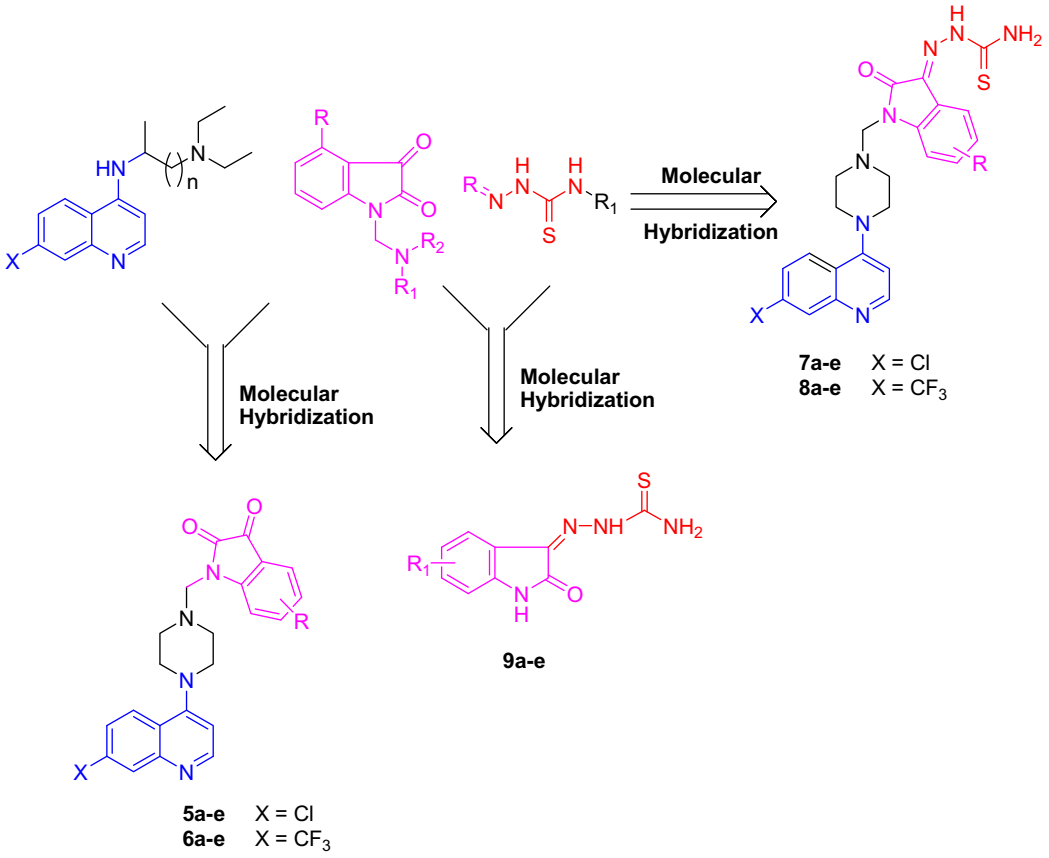
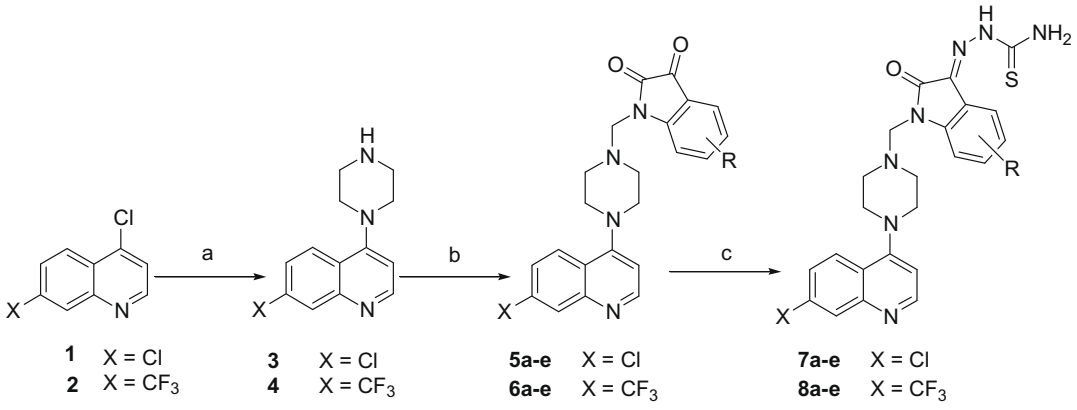


Figure 4. The design of hybrid compounds.

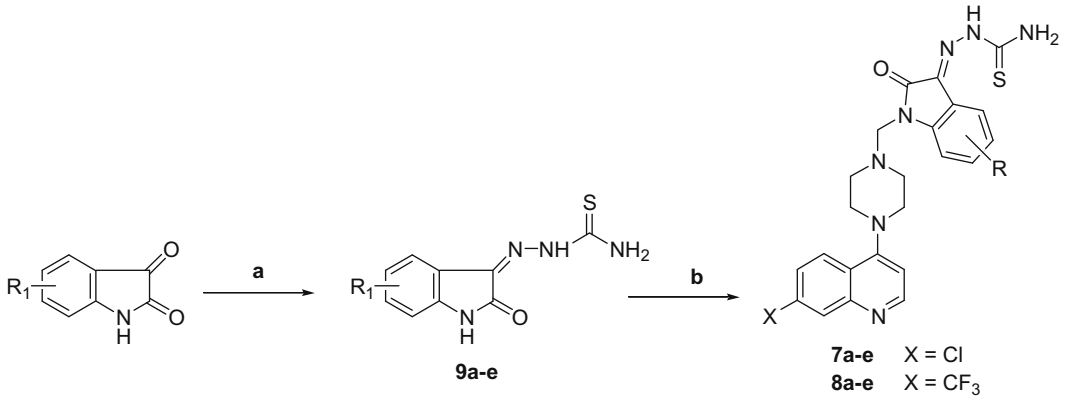


Scheme 1. Synthesis of 4-piperazinylquinoline analogs by Mannich base reaction. Reagents and conditions: (a) piperazine, 80 °C for 1 h and then 130 °C for 7 h; (b) 37% formaldehyde solution, isatin and substituted isatin, ethanol reflux, 4 h; (c) thiosemicarbazide, ethanol reflux, 3 h.

calculated with reference to a control sample, which represents the concentration that results in a 50% decrease in cell growth after 48 h incubation in the presence of the drug. For each compound, 50% growth inhibition (GI_{50}) was calculated from Sigmoidal dose–response curves that were generated with data obtained from two independent experiments carried out each in triplicate and presented in Table 1. The data for CQ and cisplatin were included as references. The resultant data showed that all the compounds had significant cytotoxic effects on the two breast cancer cell lines examined.

Among the twenty-five compounds examined, eight compounds showed GI_{50} ranged 10.34–20.60 μM, fifteen compounds at 21.15–40.36 μM, and two compounds above 52.2, but not more than 57.6 μM on MDA-MB468 cell line. In the MCF7 cells, the GI_{50} values of nine compounds were in the range of 11.44–17.61 μM, eleven compounds 21.56–39.10 μM, the remaining five compounds above 46.63, but not more than 66.78 μM. The difference in the GI_{50} values may be attributable to factors such as the nature of substitutions at 4-piperazinylquinoline ring system and halogen substitution on the 4th and 6th positions of isatin ring system, as well as the genetic and biochemical background of the cell lines.

Compounds derived from 7-chloro substitution on 4-piperazinylquinoline ring system hybridized with 4-bromo (**5b**) or 6-bromo (**5d**) substituted isatin ring system showed an increased



Scheme 2. Synthesis of 4-piperazinylquinoline analogs by Mannich base reaction. Reagents and conditions: (a) thiosemicarbazide, ethanol reflux, 2 h; (b) 37% formaldehyde solution, compound **3** or **4**, ethanol reflux, 4 h.

Table 1
Cytotoxicity of 4-piperazinylquinoline derivatives on human breast cancer cells

Compd No.[a]	X	R	GI_{50}[b,c] (μM)			
			MDA-MB 468	MCF7	184B5	MCF10A
5a	Cl	4-Cl	37.79 ± 1.81	39.11 ± 1.21	77.71 ± 2.23	25.64 ± 0.35
5b	Cl	4-Br	15.88 ± 0.15	15.12 ± 0.34	49.02 ± 1.68	65.62 ± 1.68
5c	Cl	6-Cl	36.62 ± 1.51	26.09 ± 0.89	47.46 ± 1.75	67.78 ± 1.79
5d	Cl	6-Br	35.75 ± 1.41	17.61 ± 0.95	33.22 ± 1.47	17.95 ± 0.21
5e	Cl	H	37.93 ± 1.71	48.29 ± 1.68	71.68 ± 1.97	45.94 ± 2.01
6a	CF_3	4-Cl	28.41 ± 1.21	16.93 ± 0.68	47.43 ± 1.23	24.03 ± 1.02
6b	CF_3	4-Br	52.20 ± 1.98	66.78 ± 1.92	49.67 ± 1.54	37.91 ± 1.32
6c	CF_3	6-Cl	26.06 ± 1.02	15.77 ± 0.45	25.25 ± 0.68	32.77 ± 1.41
6d	CF_3	6-Br	24.82 ± 1.21	28.06 ± 0.78	21.82 ± 0.58	24.45 ± 0.56
6e	CF_3	H	15.27 ± 0.56	33.99 ± 0.96	43.91 ± 1.32	70.02 ± 1.94
7a	Cl	4-Cl	57.61 ± 1.91	57.53 ± 1.29	20.70 ± 0.68	19.78 ± 0.31
7b	Cl	4-Br	40.36 ± 1.71	46.63 ± 2.01	46.36 ± 1.34	29.38 ± 0.49
7c	Cl	6-Cl	26.48 ± 1.02	22.52 ± 0.36	41.33 ± 1.65	64.61 ± 1.97
7d	Cl	6-Br	11.22 ± 0.23	11.44 ± 0.25	13.14 ± 0.45	22.09 ± 0.32
7e	Cl	H	20.60 ± 0.65	23.03 ± 0.85	28.82 ± 0.89	27.72 ± 0.34
8a	CF_3	4-Cl	23.04 ± 0.86	21.56 ± 0.69	87.84 ± 1.85	51.73 ± 1.02
8b	CF_3	4-Br	30.42 ± 0.98	50.63 ± 1.24	26.41 ± 0.75	30.55 ± 0.89
8c	CF_3	6-Cl	11.03 ± 0.65	24.57 ± 0.54	20.13 ± 0.35	38.80 ± 0.77
8d	CF_3	6-Br	39.59 ± 1.23	35.45 ± 0.98	21.36 ± 0.52	26.95 ± 0.63
8e	CF_3	H	10.34 ± 0.23	13.91 ± 0.87	20.17 ± 0.42	22.57 ± 0.79
9a	—	4-Cl	24.04 ± 0.75	30.15 ± 1.02	25.93 ± 0.86	28.69 ± 0.89
9b	—	4-Br	21.15 ± 1.23	14.78 ± 0.83	22.56 ± 0.67	21.36 ± 0.56
9c	—	6-Cl	17.66 ± 0.89	17.23 ± 0.97	40.82 ± 1.58	48.54 ± 1.68
9d	—	6-Br	18.63 ± 0.95	17.88 ± 0.85	34.85 ± 1.23	31.99 ± 1.25
9e	—	H	35.45 ± 1.56	36.93 ± 1.34	60.42 ± 1.85	88.78 ± 1.95
Chloroquine			28.58 ± 1.25	38.44 ± 1.20	76.13 ± 1.13	81.26 ± 1.45
Cisplatin			31.02 ± 0.45	25.77 ± 0.38	25.54 ± 0.35	51.51 ± 0.65

[a] For chemical structures of these compounds, see Figure 4 and Schemes 1 and 2.
[b] GI_{50} values were calculated from Sigmoidal dose–response curves (variable slope), which were generated with GraphPad Prism V. 4.02 (GraphPad Software Inc.).
[c] Values are the mean of triplicates of at least two independent experiments.

cytotoxic effects on MDA-MB468 and MCF7 cells in comparison to isatin ring system having 4-chloro (**5a**) or 6-chloro (**5c**) substitution (Table 1). Furthermore, compounds derived from 7-chloro substitution on 4-piperazinylquinoline ring system hybridized with 4-chloro (**5a**), 4-bromo (**5b**), 6-chloro (**5c**) and 6-bromo (**5d**) substituted isatin ring system showed an increased cytotoxic activity on MDA-MB468 and MCF7 cells in comparison to unsubstituted isatin compound **5e**. This result suggests that hydrophobic substitution (chloro and bromo) on isatin ring system favorable for cytotoxic activity. However, the compounds derived from bioisoteric replacement of chloro group with 7-trifluoromethyl substitution on 4-piperazinylquinoline ring system hybridized with 4-bromo (**6b**) or 6-bromo (**6d**) substituted isatin ring system showed less cytotoxicity on two breast cancer cell lines examined, as compared to isatin ring system having 4-chloro (**6a**) or 6-chloro (**6c**) substitution. This result clearly indicates that a large steric bulk (CF_3) substitution on the 7th position of 4-piperazinylquinoline ring system is not favorable for the enhancement of cytotoxicity on breast cancer cells.

Similarly, compounds derived from 7-chloro substitution on 4-piperazinylquinoline ring system hybridized with 4-bromo (**7b**) or 6-bromo (**7d**) substituted isatin-3-thiosemicarbazone ring system showed an increase in cytotoxic effects on MDA-MB468 and MCF7 cells, compared to the isatin ring system having 4-chloro (**7a**) or 6-chloro (**7c**) substitution. Moreover, the compounds derived from 7-trifluoromethyl substitution on 4-piperazinylquinoline ring system hybridized with 4-bromo (**8b**) and 6-bromo (**8d**) substituted isatin-3-thiosemicarbazone ring system showed lower cytotoxic activity on MDA-MB468 and MCF7 cells than the isatin ring system having 4-chloro (**8a**) or 6-chloro (**8c**) substitution.

Compounds derived from thiosemicarbazone pharmacophore with the isatin ring system with chloro (**9a**, **9c**) or bromo (**9c**, **9d**)

substitution on the 4th or 6th position showed good cytotoxic activity in comparison to unsubstituted compound (**9e**) on two breast cancer cell lines examined. This result also suggests that the essential role of hydrophobic substituent on the isatin ring system for cytotoxicity.

In general, the pharmacophore molecules that were generated by the hybridization of the 4-piperazinylquinoline ring system with isatin-3-thiosemicarbazones were more active than those molecule generated from combining the 4-piperazinylquinoline ring system and an isatin compound. For example, compounds **7d**, **7e**, **8a**, **8b** and **8e** were more active than compounds **5d**, **5e**, **6a**, **6b** and **6e** on the breast cancer cells examined. The GI_{50} values of compound **7c** were 26.48 and 22.52 μM on MDA-MB468 and MCF7, respectively. These values were compared with those obtained with **5c** (i.e., a 4-piperazinylquinoline ring system hybridized with isatin compound), which were 36.62 and 26.09 μM on MDA-MB468 and MCF7 cells, respectively. This data suggests that hybridization of the 4-piperazinylquinoline system with isatin-3-thiosemicarbazones leads to enhanced cytotoxicity on human breast cancer cells, and further validates the concept that anticancer activity can be enhanced by hybridization of two or more of different pharmacophore functional groups.

To gain further insights into the potential of this new series of compounds as an anticancer therapeutic agent, we evaluated 4-piperazinylquinoline-isatin analogs (**5a**–**e** and **6a**–**e**), 4-piperazinylquinoline-isatin-thiosemicarbazone analogs (**7a**–**e** and **8a**–**e**), and isatin-thiosemicarbazone analogs (**9a**–**e**) for cell killing effects on the non-cancer 184B5 and MCF10A breast epithelial cell lines (Table 1). Most of these analogs killed cancer cells more effectively than non-cancer cells. However some of these compound **6b**–**e**, **7a**, **7c**, **7e**, **8c**–**e**, and **9a**–**b** show undistinguished cytotoxicity on immortalized non-cancer cells. The compound **5a**–**e**, **6a**, **6e**, **7b**, **7d**, **8a**, **8b**, **9c** and **9d** showed lesser cytotoxicity on 184B5 and MCF10A cells in comparison to cancer cells. Among these compound **5b**, **8a** and **9c** showed two to four fold lesser cytotoxicity on immortalized non-cancer cells in comparison to breast cancer cells.

The most active compound among the 4-piperazinylquinoline-isatin series was **5b**, which exhibited GI_{50} values of 15.88 and 15.12 μM on MDA-MB468, and MCF7 cells, respectively. This compound showed in vitro cytotoxicity approximately two fold greater than cisplatin. However, **5b** can be significantly more advantageous over cisplatin, since **5b** preferentially inhibited cancer cell growth. For example, GI_{50} values for non-cancer cells were 49.02 (185B5) and 65.62 μM (MCF10A). Thus, the growth inhibition value of **5b** on cancer cells was 3.2-fold (i.e., 49.02 μM/15.5 μM) to 4.2-fold (i.e., 65.62 μM/15.5 μM) higher than non-cancer cells (Table 1). Similarly, compound **8a** was emerged as the most active analog from 4-piperazinylquinoline-isatin-thiosemicarbazone series. The compound **8a** was the most effective as its GI_{50} values were 23.04 and 21.56 μM on MDA-MB468, and MCF7 cells, respectively (Table 1). This data demonstrates that **8a** is slightly more potent than cisplatin. The growth inhibition by **8a** on non-cancer cells was also quite high (87.84 and 51.73 μM for 184B5 and MCF10A cell lines, respectively). Nevertheless, the cytotoxic effect of **8b** on cancer cells was approximately threefold greater than non-cancer cell lines (Table 1). The cell killing effect of these compounds (**5b** and **8a**) on 184B5and MCF10A, an immortalized 'normal' breast cell line was particularly low. It clearly suggests that these compounds have selective cytotoxicidal activity against human breast cancer cells examined.

To gain a better understanding about the mechanisms of action of these compounds, the most cytotoxic compound **5b** and **8a** were further assayed for their effects on cell cycle (by flow cytometry). MCF7 and MCF10A cells were treated with compound **5b** and **8a** at two different concentrations (20 and 40 μM) for 24 h. In MCF7 cells, DMSO treated control group 19% and 13% cells were observed in S and G2/M phase, respectively (Fig. 5a). In MCF7 cells treated with, 20 and 40 μM compound **5b**, 63 and 64% cells were in G1/G0, 18 and 14% cells were in S phase and 14 and 12% cells were in G2/M phase, respectively (Fig. 5b and c). At these concentrations (20 and 40 μM), compound **5b** induced dose-dependent apoptotic cell death in MCF7 cells, and the percentage of apoptotic cells were 6 and 10% by 24 h, respectively. In the compound **8a** treatment group, 64 and 65% cells were in G1/G0, 17 and 16% cells were in S phase, 12 and 11% cells were in G2/M phase, respectively (Fig. 5d and e). The compound **8a** also induced apoptosis in MCF7 cells at these concentrations. The substantial apoptotic cell deaths were observed at higher concentration of these compounds (data not shown). Furthermore, MCF10A cells treated with compounds **5b** or **8a** exhibited a decrease in cells in S phase (Fig. 5g–j) and no significant changes was observed, compared to the control (Fig. 5f). Together compounds **5b** and **8a** showed effective cytotoxicidal activity on MCF7 breast cancer cells.

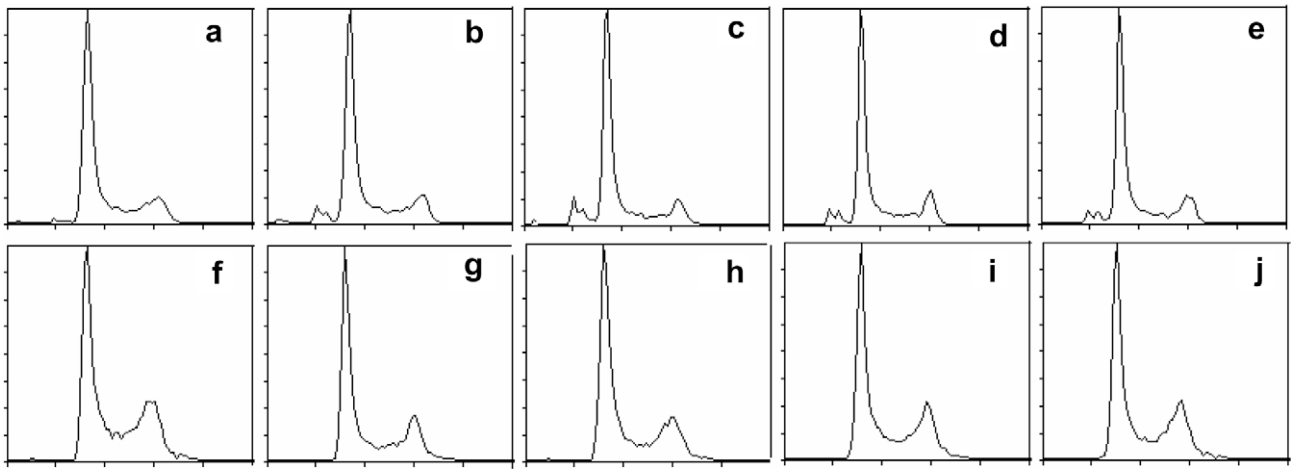


Figure 5. The cytometric profile of the compounds **5b** and **8a**. MCF7 (panels a–e) and MCF10A (panels f–j) cells (2.0×10^6) collected at 24 h post-compound treatment, fixed in 70% ethanol, stained with 100 μg/mL propidium iodide, and cell cycle progression was measured by cytometry (Beckmann Coulter Cytomics FC500). Panels a and f, are negative controls (DMSO treated); samples were treated with **5b**: b (20 μM), c (40 μM), g (20 μM), and h (40 μM). The following samples were treated with **8a**: d (20 μM), e (40 μM), i (20 μM), and j (40 μM).

3. Conclusion

We here describe hybrid pharmacophore design and synthesis of a new series of 4-piperazinylquinoline derivatives. All the compounds derived from 4-piperazinylquinoline exhibited promising anticancer activity against human breast cancer cells in vitro. In particular, the compound 4-bromo-1-[4-(7-chloro-quinolin-4-yl)-piperazin-1-ylmethyl]-1*H*-indole-2,3-dione (**5b**) and N^1-[4-(7-trifluoromethyl-quinolin-4-yl)]-piperazin-1-ylmethyl-4-chloro-1*H*-indole-2,3-dione-3-thiosemicarbazone (**8a**) emerged as the most active compounds of the series. Data from flow cytometry studies suggests that this class of compounds induce the cell death by apoptosis. Furthermore, our data suggest that generating hybrid compounds containing pharmacophore containing 4-piperazinyl-quinoline-isatin derivatives are a promising new approach of developing an effective anticancer agent.

4. Materials and methods

4.1. Experimental

Melting points (mp) were taken in open capillaries on the Complab melting point apparatus. Elemental analysis was performed on a Perkin–Elmer 2400 C, H, N analyzer and values were within the acceptable limits of the calculated values. The ^{1}H spectra were recorded on a DPX-500 MHz Bruker FT-NMR spectrometer using $CDCl_3$ and DMSO-d_6 as solvent. The chemical shifts were reported as parts per million (δ ppm) tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained on a JEOL-SX-102 instrument using fast atom bombardment (FAB positive). The progress of the reaction was monitored on readymade silica-gel plates (Merck) using chloroform/methanol (9:1) as solvent. Iodine was used as a developing agent or by spraying with the Dragendorff's reagent. Chromatographic purification was performed over a silica-gel (100–200 mesh). All chemicals and reagents obtained from Aldrich (USA) were used without further purification.

4.2. Synthesis of 7-substituted-4-piperazin-1-yl-quinoline

A mixture of 7-substituted-4-chloro-quinoline (10.10 mmol), piperazine (2.61 g, 30.30 mmol) and triethylamine (1.4 mL, 10.10 mmol) were heated slowly to 80 °C over 1 h while stirring. The temperature was then increased to 130 °C for 7 h where it was kept for while stirring continuously. The reaction mixture was cooled to room temperature and taken up in dichloromethane. The organic layer was washed with 5% aq $NaHCO_3$, followed by washing with water and then with brine. The organic layer was dried over anhydrous Na_2SO_4 and solvent was removed under reduced pressure, and the residue was then precipitated by addition of mixture of solvent hexane/chloroform (8:2).

4.2.1. 7-Chloro-4-piperazin-1-yl-quinoline (3)

White solid; yield 69%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.31 (br s, 1H, N*H*), 3.15 (s, 4H, N(CH_2CH$_2$)$_2$NAr), 3.18 (s, 4H, N(CH$_2$CH_2)$_2$NAr), 6.80–6.81 (d, *J* = 5.0 Hz, 1H, Ar-*H*), 7.46–7.47 (d, *J* = 5.0 Hz, 1H, Ar-*H*), 7.92–7.94 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 8.01 (s, 1H, Ar-*H*), 8.68–8.69 (d, *J* = 5.0 Hz, 1H, Ar-*H*); ^{13}C NMR (500 MHz, $CDCl_3$): δ 46.10 (2C), 53.58 (2C), 108.97, 121.97, 125.24, 126.09, 128.91, 134.84, 150.22, 151.99, 157.38; ES-MS *m*/*z* 248 [M+H]$^+$. Anal. Calcd for $C_{13}H_{14}ClN_3$: C, 63.03; H, 5.70; N, 16.96. Found: C, 62.99; H, 5.65; N, 16.97.

4.2.2. 4-Piperazin-1-yl-7-trifluoromethyl-quinoline (4)

Pale yellow white solid; yield 68%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.78 (br s, 1H, N*H*), 3.18 (s, 4H, N(CH_2CH$_2$)$_2$NAr), 3.24 (s, 4H, N(CH$_2$CH_2)$_2$NAr), 7.07–7.08 (d, *J* = 5.0 Hz, 1H, Ar-*H*), 7.63–7.65 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 8.13–8.14 (d, *J* = 5.0 Hz, 1H, Ar-*H*), 8.34 (s, 1H, Ar-*H*), 8.81–8.82 (d, *J* = 5.0 Hz, 1H, Ar-*H*); ^{13}C NMR (500 MHz, $CDCl_3$): δ 52.15 (2C), 53.48 (2C), 110.64, 120.79, 125.14, 125.22, 127.76, 130.70, 130.96, 148.73, 152.20, 157.19; ES-MS *m*/*z* 282 [M+H]$^+$. Anal. Calcd for $C_{14}H_{14}F_3N_3$: C, 59.78; H, 5.02; N, 14.94. Found: C, 59.80; H, 4.99; N, 14.92.

4.3. General synthetic procedure for compounds 5a–e and 6a–e

To a solution of isatin or substituted isatin (3.35 mmol) in 5 mL of 99.9% ethanol was added to a mixture of compound (**3** or **4**) (3.35 mmol) and aqueous formaldehyde 37% (3.95 mmol) also dissolved in 10 mL of 99.9% ethanol. The reaction mixture was stirred for 4 h at room temperature, refrigerated for 48 h to form crystals. The crystalline products were separated by filtration, washed and vacuum dried. Recrystallization from ethanol rendered desired products in pure form.

4.3.1. 4-Chloro-1-[4-(7-chloro-quinolin-4-yl)-piperazin-1-ylmethyl]-1*H*-indole-2,3-dione (5a)

Red crystals, yield 68%; mp 118–120 °C; IR (KBr, cm^{-1}): 1725, 1625; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.96 (s, 4H, N(CH_2CH$_2$)$_2$NAr), 3.25 (s, 4H, N(CH$_2$CH_2)$_2$NAr), 4.64 (s, 2H, NCH_2N), 6.84–6.85 (d, *J* = 5.0 Hz, 1H, Ar-*H*), 7.04–7.06 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 7.14–7.15 (d, *J* = 5.0 Hz, 1H, Ar-*H*), 7.44–7.45 (d, *J* = 5.0 Hz, 1H, Ar-*H*), 7.54–7.57 (m, 1H, Ar-*H*), 7.92–7.94 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 8.06 (s, 1H, Ar-*H*), 8.73–8.74 (d, *J* = 5.0 Hz, 1H, Ar-*H*); ^{13}C NMR (500 MHz, $CDCl_3$): δ 50.23 (2C), 52.11 (2C), 62.09, 110.01, 111.14, 115.21, 121.87, 124.59, 126.24, 126.56, 128.49, 131.28, 134.03, 138.90, 150.07, 152.65, 153.34, 156.73, 158.94, 180.49; ES-MS *m*/*z* 441 [M+H]$^+$. Anal. Calcd for $C_{22}H_{18}Cl_2N_4O_2$: C, 59.88; H, 4.11; N, 12.70. Found: C, 59.85; H, 4.09; N, 12.68.

4.3.2. 4-Bromo-1-[4-(7-chloro-quinolin-4-yl)-piperazin-1-ylmethyl]-1*H*-indole-2,3-dione (5b)

Reddish orange crystals, yield 64%; mp 133–134 °C; IR (KBr, cm^{-1}): 1722, 1621; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.96 (s, 4H, N(CH_2CH$_2$)$_2$NAr), 3.25 (s, 4H, N(CH$_2$CH_2)$_2$NAr), 4.64 (s, 2H, NCH_2N), 6.83–6.84 (d, *J* = 5.0 Hz, 1H, Ar-*H*), 7.10–7.11 (d, *J* = 5.0 Hz, 1H, Ar-*H*), 7.32–7.34 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 7.43–7.47 (m, 2H, Ar-*H*), 7.91–7.93 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 8.05 (s, 1H, Ar-*H*), 8.73–8.74 (d, *J* = 5.0 Hz, 1H, Ar-*H*); ^{13}C NMR (500 MHz, $CDCl_3$): δ 50.23 (2C), 52.11 (2C), 62.00, 109.99, 11.56, 116.80, 117.15, 119.64, 121.86, 126.23, 127.72, 128.48, 134.03, 138.84, 150.06, 152.63, 153.67, 156.72, 158.89, 181.01; ES-MS *m*/*z* 487 [M+H]$^+$. Anal. Calcd for $C_{22}H_{18}BrClN_4O_2$: C, 54.40; H, 3.73; N, 11.53. Found: C, 54.39; H, 3.75; N, 11.50.

4.3.3. 6-Chloro-1-[4-(7-chloro-quinolin-4-yl)-piperazin-1-ylmethyl]-1*H*-indole-2,3-dione (5c)

Red crystals, yield 60%; mp 122–123 °C; IR (KBr, cm^{-1}): 1727, 1628; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.90 (s, 4H, N(CH_2CH$_2$)$_2$NAr), 3.27 (s, 4H, N(CH$_2$CH_2)$_2$NAr), 4.62 (s, 2H, NCH_2N), 6.85–6.86 (d, *J* = 5.0 Hz, 1H, Ar-*H*), 7.15 (s, 1H, Ar-*H*), 7.17–7.19 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 7.44–7.46 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 7.59–7.60 (d, *J* = 5.0 Hz, 1H, Ar-*H*), 7.92–7.94 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 8.05 (s, 1H, Ar-*H*), 8.73–8.74 (d, *J* = 5.0 Hz, 1H, Ar-*H*); ES-MS *m*/*z* 441 [M+H]$^+$. Anal. Calcd for $C_{22}H_{18}Cl_2N_4O_2$: C, 59.88; H, 4.11; N, 12.70. Found: C, 59.90; H, 4.13; N, 12.68.

4.3.4. 6-Bromo-1-[4-(7-chloro-quinolin-4-yl)-piperazin-1-ylmethyl]-1*H*-indole-2,3-dione (5d)

Orange crystals, yield 60%; mp 142–143 °C; IR (KBr, cm^{-1}): 1732, 1632; ^1H NMR (500 MHz, $CDCl_3$): δ 2.96 (s, 4H, $N(CH_2CH_2)_2NAr$), 3.27 (s, 4H, $N(CH_2CH_2)_2NAr$), 4.60 (s, 2H, NCH_2N), 6.85–6.86 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.37 (s, 1H, Ar-*H*), 7.43–7.45 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.47–7.48 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.53–7.54 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.92–7.94 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.10 (s, 1H, Ar-*H*), 8.67–8.68 (d, J = 5.0 Hz, 1H, Ar-*H*); ES-MS m/z 487 $[M+H]^+$. Anal. Calcd for $C_{22}H_{18}BrClN_4O_2$: C, 54.40; H, 3.73; N, 11.53. Found: C, 54.38; H, 3.75; N, 11.55.

4.3.5. 1-[4-(7-Chloro-quinolin-4-yl)-piperazin-1-ylmethyl]-1*H*-indole-2,3-dione (5e)

Reddish orange crystals, yield 60%; mp 202–204 °C; IR (KBr, cm^{-1}): 1738, 1619; ^1H NMR (500 MHz, $CDCl_3$): δ 2.92 (s, 4H, $N(CH_2CH_2)_2NAr$), 3.21 (s, 4H, $N(CH_2CH_2)_2NAr$), 4.60 (s, 2H, NCH_2N), 6.82–6.83 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.14–7.18 (m, 2H, Ar-*H*), 7.41–7.43 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.63–7.66 (m, 2H, Ar-*H*), 7.90–7.92 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.03 (s, 1H, Ar-*H*), 8.70–8.71 (d, J = 5.0 Hz, 1H, Ar-*H*); ES-MS m/z 407 $[M+H]^+$. Anal. Calcd for $C_{22}H_{19}ClN_4O_2$: C, 64.94; H, 4.71; N, 13.77. Found: C, 64.96; H, 4.70; N, 13.74.

4.3.6. 4-Chloro-1-[4-(7-trifluoromethyl-quinolin-4-yl)-piperazin-1-ylmethyl]-1*H*-indole-2,3-dione (6a)

Red crystals, yield 58%; mp 144–146 °C; IR (KBr, cm^{-1}): 1725, 1625; ^1H NMR (500 MHz, $CDCl_3$): δ 2.97 (s, 4H, $N(CH_2CH_2)_2NAr$), 3.28 (s, 4H, $N(CH_2CH_2)_2NAr$), 4.66 (s, 2H, NCH_2N), 6.87–6.88 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.05–7.07 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.13–7.15 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.54–7.55 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.65–7.67 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.10–8.12 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.36 (s, 1H, Ar-*H*), 8.84–8.85 (d, J = 5.0 Hz, 1H, Ar-*H*); ES-MS m/z 475 $[M+H]^+$. Anal. Calcd for $C_{23}H_{18}ClF_3N_4O_2$: C, 58.17; H, 3.82; N, 11.80. Found: C, 58.14; H, 3.80; N, 11.76.

4.3.7. 4-Bromo-1-[4-(7-trifluoromethyl-quinolin-4-yl)-piperazin-1-ylmethyl]-1*H*-indole-2,3-dione (6b)

Reddish orange crystals, yield 62%; mp 152–154 °C; IR (KBr, cm^{-1}): 1737, 1615; ^1H NMR (500 MHz, $CDCl_3$): δ 2.92 (s, 4H, $N(CH_2CH_2)_2NAr$), 3.28 (s, 4H, $N(CH_2CH_2)_2NAr$), 4.64 (s, 2H, NCH_2N), 6.94–6.95 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.10–7.11 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.33–7.35 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.45–7.49 (m, 1H, Ar-*H*), 7.66–7.67 (d, J = 5.0 Hz, 1H, Ar-*H*), 8.10–8.12 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.37 (s, 1H, Ar-*H*), 8.82–8.83 (d, J = 5.0 Hz, 1H, Ar-*H*); ^{13}C NMR (500 MHz, $CDCl_3$): δ 49.61 (2C), 51.41 (2C), 61.43, 110.37, 110.73, 116.02, 119.27, 120.08, 122.60, 125.49, 126.75, 127.19, 129.12, 129.38, 138.07, 147.90, 152.04, 152.95, 155.86, 158.02, 180.26; ES-MS m/z 519 $[M+H]^+$. Anal. Calcd for $C_{23}H_{18}BrF_3N_4O_2$: C, 53.19; H, 3.49; N, 10.79. Found: C, 53.23; H, 3.52; N, 10.85.

4.3.8. 6-Chloro-1-[4-(7-trifluoromethyl-quinolin-4-yl)-piperazin-1-ylmethyl]-1*H*-indole-2,3-dione (6c)

Red crystals, yield 58%; mp 162–164 °C; IR (KBr, cm^{-1}): 1735, 1633; ^1H NMR (500 MHz, $CDCl_3$): δ 2.97 (s, 4H, $N(CH_2CH_2)_2NAr$), 3.31 (s, 4H, $N(CH_2CH_2)_2NAr$), 4.64 (s, 2H, NCH_2N), 6.95–6.96 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.15 (s, 1H, Ar-*H*), 7.18–7.19 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.62–7.63 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.66–7.68 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.11–8.13 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.37 (s, 1H, Ar-*H*), 8.83–8.84 (d, J = 5.0 Hz, 1H, Ar-*H*); ^{13}C NMR (500 MHz, $CDCl_3$): δ 49.63 (2C), 51.55 (2C), 61.50, 110.83, 112.15, 116.56, 120.39, 122.88, 123.08, 124.71, 125.05, 126.01, 126.83, 128.98, 129.83, 142.13, 147.94, 152.49, 152.57, 156.03, 181.74; ES-MS m/z 475 $[M+H]^+$. Anal. Calcd for $C_{23}H_{18}ClF_3N_4O_2$: C, 58.17; H, 3.82; N, 11.80. Found: C, 58.21; H, 3.79; N, 11.86.

4.3.9. 6-Bromo-1-[4-(7-trifluoromethyl-quinolin-4-yl)-piperazin-1-ylmethyl]-1*H*-indole-2,3-dione (6d)

Orange crystals, yield 72%; mp 168–170 °C; IR (KBr, cm^{-1}): 1729, 1628; ^1H NMR (500 MHz, $CDCl_3$): δ 2.97 (s, 4H, $N(CH_2CH_2)_2NAr$), 3.32 (s, 4H, $N(CH_2CH_2)_2NAr$), 4.62 (s, 2H, NCH_2N), 6.95–6.96 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.36 (s, 1H, Ar-*H*), 7.38–7.40 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.54–7.55 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.67–7.68 (d, J = 5.0 Hz, 1H, Ar-*H*), 8.11–8.13 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.37 (s, 1H, Ar-*H*), 8.83–8.84 (d, J = 5.0 Hz, 1H, Ar-*H*); ES-MS m/z 521 $[M+H]^+$. Anal. Calcd for $C_{23}H_{18}BrF_3N_4O_2$: C, 53.19; H, 3.49; N, 10.79. Found: C, 53.15; H, 3.45; N, 10.74.

4.3.10. 1-[4-(7-Trifluoromethyl-quinolin-4-yl)-piperazin-1-ylmethyl]-1*H*-indole-2,3-dione (6e)

Reddish orange crystals, yield 65%; mp 166–168 °C; IR (KBr, cm^{-1}): 1727, 1630; ^1H NMR (500 MHz, $CDCl_3$): δ 2.98 (s, 4H, $N(CH_2CH_2)_2NAr$), 3.28 (s, 4H, $N(CH_2CH_2)_2NAr$), 4.65 (s, 2H, NCH_2N), 6.93–6.94 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.12–7.20 (m, 2H, Ar-*H*), 7.64–7.67 (m, 3H, Ar-*H*), 8.10–812 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.35 (s, 1H, Ar-*H*), 8.82–8.83 (d, J = 5.0 Hz, 1H, Ar-*H*); ES-MS m/z 441 $[M+H]^+$. Anal. Calcd for $C_{23}H_{19}F_3N_4O_2$: C, 62.72; H, 4.35; N, 12.72. Found: C, 62.76; H, 4.34; N, 12.74.

4.4. General synthetic procedure for compounds 7a–e and 8a–e (Scheme 1, Method A)

Equimolar quantities of appropriate compound **5a–e** and **6a–e** (0.95 mmol) and thiosemicarbazide (0.95 mmol) were dissolved in warm 99.9% ethanol. The mixture was then continuously stirred at 45 °C for 3 h. After standing for overnight at room temperature, a crystalline product was formed. The product was then separated by filtration, vacuum dried and recrystallized from mixture of solvent hexane/dichloromethane (8:2).

4.5. General synthetic procedure for compounds 7a–e and 8a–e (Scheme 2, Method B)

To a solution of substituted isatin-3-thiosemicarbazone derivative (2.36 mmol) in 5 mL of 99.9% ethanol was added a mixture of compound **3** or **4** (2.36 mmol) and aqueous formaldehyde 37% W/V (2.95 mmol) also dissolved in 10 mL ethanol (99.9%). The reaction mixture was stirred at 45 °C for 4 h. The crystalline product was separated by filtration, vacuum dried and recrystallized from mixture of solvent hexane/chloroform (8:2).

4.5.1. N^1-[4-(7-Chloro-quinolin-4-yl)-piperazin-1-yl]methyl-4-chloro-1*H*-indole-2,3-dione-3-thiosemicarbazone (7a)

Yellowish orange crystals, yield 72%; mp 210–212 °C; IR (KBr, cm^{-1}): 3687, 1620; ^1H NMR (500 MHz, DMSO-d_6 + $CDCl_3$): δ 2.88 (s, 4H, $N(CH_2CH_2)_2NAr$), 3.42 (s, 4H, $N(CH_2CH_2)_2NAr$), 4.70 (s, 2H, NCH_2N), 6.86 (s, 1H, Ar-*H*), 7.05–7.07 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.12–7.14 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.19 (s, 1H, Ar-*H*), 7.37–7.38 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.44 (s, 1H, Ar-*H*), 7.54 (s, 1H, Ar-*H*), 8.43 (s, 1H, Ar-*H*), 8.74 (br s, 2H, NH_2), 12.84 (br s, 1H, NH); ES-MS m/z 514 $[M+H]^+$; $C_{23}H_{21}Cl_2N_7OS$: C, 53.70; H, 4.11; N, 19.06; found: C, 53.72; H, 4.14; N, 19.09.

4.5.2. N^1-[4-(7-Chloro-quinolin-4-yl)-piperazin-1-yl]methyl-4-bromo-1*H*-indole-2,3-dione-3-thiosemicarbazone (7b)

Orange crystals, yield 70%; mp 215–217 °C; IR (KBr, cm^{-1}): 13690, 1618; ^1H NMR (500 MHz, DMSO-d_6 + $CDCl_3$): δ 2.78 (s, 4H, $N(CH_2CH_2)_2NAr$), 2.98 (s, 4H, $N(CH_2CH_2)_2NAr$), 4.68 (s, 2H, NCH_2N), 6.87 (s, 1H, Ar-*H*), 7.11–7.13 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.54–7.58 (m, 2H, Ar-*H*), 7.88–7.90 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.91–7.93 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.00–8.02 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.63 (s, 1H, Ar-*H*), 8.88 (br s, 2H, NH_2), 12.75 (br s, 1H,

NH); ES-MS m/z 590 $[M+H]^+$; $C_{23}H_{21}BrClN_7OS$: C, 49.43; H, 3.79; N, 17.54; found: C, 49.46; H, 3.82; N, 17.56.

4.5.3. N^1-[4-(7-Chloro-quinolin-4-yl)-piperazin-1-yl]methyl-6-chloro-1*H*-indole-2,3-dione-3-thiosemicarbazone (7c)

Red crystals, yield 68%; mp 230–232 °C; IR (KBr, cm^{-1}): 3688, 1618; ^1H NMR (500 MHz, DMSO-d_6 + $CDCl_3$): δ 2.95 (s, 4H, N(CH_2CH_2)$_2$NAr), 3.30 (s, 4H, N(CH_2CH_2)$_2$NAr), 4.65 (s, 2H, NCH_2N), 6.96–6.97 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.10–7.12 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.20 (s, 1H, Ar-*H*), 7.52–7.54 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.60–7.62 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.65–7.67 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.11–8.12 (d, J = 5.0 Hz, 1H, Ar-*H*), 8.70–8.71 (d, J = 5.0 Hz, 1H, Ar-*H*), 9.20 (br s, 2H, NH_2), 12.30 (br s, 1H, NH); ES-MS m/z 515 $[M+H]^+$; $C_{23}H_{21}Cl_2N_7OS$: C, 53.70; H, 4.11; N, 19.06; found: C, 53.74; H, 4.14; N, 19.09.

4.5.4. N^1-[4-(7-Chloro-quinolin-4-yl)-piperazin-1-yl]methyl-6-bromo-1*H*-indole-2,3-dione-3-thiosemicarbazone (7d)

Red crystals, yield 72%; mp 215–217 °C; IR (KBr, cm^{-1}): 3688, 1620; ^1H NMR (500 MHz, DMSO-d_6 + $CDCl_3$): δ 2.90 (s, 4H, N(CH_2CH_2)$_2$NAr), 3.27 (s, 4H, N(CH_2CH_2)$_2$NAr), 4.66 (s, 2H, NCH_2N), 6.91–6.92 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.27–7.29 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.42–7.44 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.47 (s, 1H, Ar-*H*), 7.63–7.65 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.95–7.97 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.52–8.54 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.64 (s, 1H, Ar-*H*), 9.04 (br s, 2H, NH_2), 12.36 (br s, 1H, NH); ^{13}C NMR (500 MHz, $CDCl_3$): δ 50.33 (2C), 52.10 (2C), 61.55, 109.85, 114.53, 119.25, 120.95, 122.57, 124.39, 125.63, 126.29, 126.30, 126.79, 128.08, 130.64, 145.34, 148.46, 153.02, 156.90, 162.28, 179.17; ES-MS m/z 590 $[M+H]^+$; $C_{23}H_{21}BrClN_7OS$: C, 49.43; H, 3.79; N, 17.54; found: C, 49.40; H, 3.75; N, 17.50.

4.5.5. N^1-[4-(7-Chloro-quinolin-4-yl)-piperazin-1-yl]methyl-1*H*-indole-2,3-dione-3-thiosemicarbazone (7e)

Orange crystals, yield 78%; mp 215–216 °C; IR (KBr, cm^{-1}): 3686, 1625; ^1H NMR (500 MHz, DMSO-d_6 + $CDCl_3$): δ 2.99 (s, 4H, N(CH_2CH_2)$_2$NAr), 3.19 (s, 4H, N(CH_2CH_2)$_2$NAr), 4.60 (s, 2H, NCH_2N), 6.98–6.99 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.13–7.14 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.18–7.20 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.35–7.36 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.43–7.44 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.53–7.54 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.98–8.00 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.68–8.69 (d, J = 5.0 Hz, 1H, Ar-*H*), 8.76 (s, 1H, Ar-*H*), 9.11 (br s, 2H, NH_2), 12.48 (br s, 1H, NH); ^{13}C NMR (500 MHz, $CDCl_3$): δ 50.48 (2C), 52.12 (2C), 61.47, 109.94, 111.57, 119.85, 121.10, 121.79, 123.46, 126.26, 126.63, 128.38, 131.51, 131.576, 134.05, 144.29, 149.96, 152.55, 156.74, 162.37, 179.17; ES-MS m/z 481 $[M+H]^+$; $C_{23}H_{22}ClN_7OS$: C, 57.55; H, 4.62; N, 20.43; found: C, 57.53; H, 4.60; N, 20.39.

4.5.6. N^1-[4-(7-Trifluoromethyl-quinolin-4-yl)]-piperazin-1-ylmethyl-4-chloro-1*H*-indole-2,3-dione-3-thiosemicarbazone (8a)

Red crystals, yield 62%; mp 228–230 °C; IR (KBr, cm^{-1}): 3689, 1655; ^1H NMR (500 MHz, DMSO-d_6 + $CDCl_3$): δ 2.94 (s, 4H, N(CH_2CH_2)$_2$NAr), 3.24 (s, 4H, N(CH_2CH_2)$_2$NAr), 4.72 (s, 2H, NCH_2N), 7.13 (s, 1H, Ar-*H*), 7.20–7.21 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.39 (s, 1H, Ar-*H*), 7.45–7.46 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.76–7.78 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.93 (s, 1H, Ar-*H*), 8.20–8.21 (d, J = 5.0 Hz, 1H, Ar-*H*), 8.26 (s, 1H, Ar-*H*), 8.81 (br s, 2H, NH_2), 12.78 (br s, 1H, NH); ES-MS m/z 548 $[M+H]^+$; $C_{24}H_{21}ClF_3N_7OS$: C, 52.60; H, 3.86; N, 17.89; found: C, 52.65; H, 3.82; N, 17.93.

4.5.7. N^1-[4-(7-Trifluoromethyl-quinolin-4-yl)]-piperazin-1-ylmethyl-4-bromo-1*H*-indole-2,3-dione-3-thiosemicarbazone (8b)

Orange crystals, yield 64%; mp 216–218 °C; IR (KBr, cm^{-1}): 3690, 1645; ^1H NMR (500 MHz, DMSO-d_6 + $CDCl_3$): δ 2.94 (s, 4H, N(CH_2CH_2)$_2$NAr), 3.20 (s, 4H, N(CH_2CH_2)$_2$NAr), 4.72 (s, 2H, NCH_2N), 6.95 (s, 1H, Ar-*H*), 7.12–7.14 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.27 (s, 1H, Ar-*H*), 7.37–7.42 (m, 2H, Ar-*H*), 7.73–7.77 (m, 1H, Ar-*H*), 8.20–8.21 (d, J = 5.0 Hz, 1H, Ar-*H*), 8.80 (s, 1H, Ar-*H*), 9.38 (br s, 2H, NH_2), 12.78 (br s, 1H, NH); ES-MS m/z 594 $[M+H]^+$; $C_{24}H_{21}BrF_3N_7OS$: C, 48.66; H, 3.57; N, 16.55; found: C, 48.68; H, 3.60; N, 16.59.

4.5.8. N^1-[4-(7-Trifluoromethyl-quinolin-4-yl)]-piperazin-1-ylmethyl-6-chloro-1*H*-indole-2,3-dione-3-thiosemicarbazone (8c)

Orange red crystals, yield 70%; mp 208–210 °C; IR (KBr, cm^{-1}): 3685, 1639; ^1H NMR (500 MHz, DMSO-d_6 + $CDCl_3$): δ 2.91 (s, 4H, N(CH_2CH_2)$_2$NAr), 3.19 (s, 4H, N(CH_2CH_2)$_2$NAr), 4.60 (s, 2H, NCH_2N), 6.96–6.97 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.08–7.10 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.25 (s, 1H, Ar-*H*), 7.54–7.56 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.60–7.62 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.65–7.67 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.11–8.12 (d, J = 5.0 Hz, 1H, Ar-*H*), 8.72–8.73 (d, J = 5.0 Hz, 1H, Ar-*H*), 8.96 (br s, 2H, NH_2), 12.39 (br s, 1H, NH); ES-MS m/z 548 $[M+H]^+$; $C_{24}H_{21}ClF_3N_7OS$: C, 52.60; H, 3.86; N, 17.89; found: C, 52.55; H, 3.83; N, 17.84.

4.5.9. N^1-[4-(7-Trifluoromethyl-quinolin-4-yl)]-piperazin-1-ylmethyl-6-bromo-1*H*-indole-2,3-dione-3-thiosemicarbazone (8d)

Red crystals, yield 61%; mp 218–220 °C; IR (KBr, cm^{-1}): 2689, 1635; ^1H NMR (500 MHz, DMSO-d_6 + $CDCl_3$): δ 2.80 (s, 4H, N(CH_2CH_2)$_2$NAr), 3.30 (s, 4H, N(CH_2CH_2)$_2$NAr), 4.35 (s, 2H, NCH_2N), 7.08 (s, 1H, Ar-*H*), 7.12–7.13 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.30–7.31 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.39–7.41 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.57–7.59 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.75–7.77 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.20–8.21 (d, J = 5.0 Hz, 1H, Ar-*H*), 8.20–8.21 (d, J = 5.0 Hz, 1H, Ar-*H*), 9.15 (br s, 2H, NH_2), 12.38 (br s, 1H, NH); ^{13}C NMR (500 MHz, $CDCl_3$): δ 50.31 (2C), 52.08 (2C), 61.51,111.35, 114.28, 114.55, 119.25, 120.95, 122.93, 124.39, 125.22, 125.63, 126.30, 126.60, 127.33, 130.63, 145.33, 148.46, 153.02, 156.55, 162.28, 179.18; ES-MS m/z 593 $[M+H]^+$; $C_{24}H_{21}BrF_3N_7OS$: C, 48.66; H, 3.57; N, 16.55; found: C, 48.68; H, 3.59; N, 16.58.

4.5.10. N^1-[4-(7-Trifluoromethyl-quinolin-4-yl)]-piperazin-1-ylmethyl-1*H*-indole-2,3-dione-3-thiosemicarbazone (8e)

Orange crystals, yield 63%; mp 211–212 °C; IR (KBr, cm^{-1}): 3688, 1642; ^1H NMR (500 MHz, DMSO-d_6 + $CDCl_3$): δ 2.90 (s, 4H, N(CH_2CH_2)$_2$NAr), 3.20 (s, 4H, N(CH_2CH_2)$_2$NAr), 4.62 (s, 2H, NCH_2N), 6.97–6.98 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.11–7.12 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.20–7.22 (d, J = 10.0 Hz, 1H, Ar-*H*), 7.33–7.34 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.40–7.41 (d, J = 5.0 Hz, 1H, Ar-*H*), 7.97–7.99 (d, J = 10.0 Hz, 1H, Ar-*H*), 8.67–8.68 (d, J = 5.0 Hz, 1H, Ar-*H*), 8.79 (s, 1H, Ar-*H*), 9.01 (br s, 2H, NH_2), 12.35 (br s, 1H, NH); ^{13}C NMR (500 MHz, $CDCl_3$): δ 50.44 (2C), 52.10 (2C), 61.40, 109.91, 111.59, 118.55, 119.75, 121.00, 121.62, 123.35, 126.46, 126.63, 128.38, 131.51, 131.576, 134.05, 144.29, 149.96, 152.55, 156.74, 162.37, 179.17; ES-MS m/z 515 $[M+H]^+$; $C_{24}H_{22}F_3N_7OS$: C, 56.13; H, 4.32; N, 19.09; found: C, 56.11; H, 4.28; N, 19.13.

4.6. General synthetic procedure for compounds (9a–e)

Equimolar quantities of isatin or substituted isatin (0.95 mmol) and thiosemicarbazide (0.95 mmol) were dissolved in warm 99.9% ethanol. The mixture was then continuously stirred at 45 °C for 2 h. After standing for overnight at room temperature, a crystalline

product was formed. The product was then separated by filtration, vacuum dried and recrystallized from mixture of solvent hexane/dichlormethane (8:2).

4.6.1. 4-Chloro-1*H*-indole-2,3-dione-3-thiosemicarbazone (9a)

Yellow crystals, yield 72%; mp 160–162 °C; IR (KBr, cm^{-1}): 3689, 1665; ^1H NMR (500 MHz, DMSO-d_6): δ 6.79 (s, 1H, Ar-*H*), 7.01 (s, 1H, Ar-*H*), 7.21–7.23 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 9.10 (br s, 2H, NH_2), 11.28 (br s, 1H, NH), 12.76 (br s, 1H, NH); ES-MS *m*/*z* 255 $[M+H]^+$; $C_9H_7ClN_4OS$: C, 42.44; H, 2.77; N, 22.00; found: C, 42.42; H, 2.79; N, 22.07.

4.6.2. 4-Bromo-1*H*-indole-2,3-dione-3-thiosemicarbazone (9b)

Yellow crystals, yield 74%; mp 170–172 °C; IR (KBr, cm^{-1}): 3680, 1641; ^1H NMR (500 MHz, DMSO-d_6): δ 6.86–6.88 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 7.13–7.15 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 7.57 (s, 1H, Ar-*H*), 9.14 (br s, 2H, NH_2), 11.26 (br s, 1H, NH), 12.75 (br s, 1H, NH); ES-MS *m*/*z* 300 $[M+H]^+$; $C_9H_7BrN_4OS$: C, 36.13; H, 2.36; N, 18.73; found: C, 36.15; H, 2.32; N, 18.76.

4.6.3. 6-Chloro-1*H*-indole-2,3-dione-3-thiosemicarbazone (9c)

Yellow crystals, yield 75%; mp 156–158 °C; IR (KBr, cm^{-1}): 3689, 1638; ^1H NMR (500 MHz, DMSO-d_6): δ 6.96–6.98 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 7.56–7.58 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 8.30 (s, 1H, Ar-*H*), 8.90 (br s, 2H, NH_2), 11.13 (br s, 1H, NH), 12.45 (br s, 1H, NH); ES-MS *m*/*z* 255 $[M+H]^+$; $C_9H_7ClN_4OS$: C, 42.44; H, 2.77; N, 22.00; found: C, 42.42; H, 2.73; N, 22.05.

4.6.4. 6-Bromo-1*H*-indole-2,3-dione-3-thiosemicarbazone (9d)

Yellow crystals, yield 76%; mp 180–181 °C; IR (KBr, cm^{-1}): 3682, 1635; ^1H NMR (500 MHz, DMSO-d_6): δ 7.13–7.14 (d, *J* = 5.0 Hz, 1H, Ar-*H*), 7.49–7.50 (d, *J* = 5.0 Hz, 1H, Ar-*H*), 8.26 (s, 1H, Ar-*H*), 8.88 (br s, 2H, NH_2), 11.02 (br s, 1H, NH), 12.52 (br s, 1H, NH); ^{13}C NMR (500 MHz, $CDCl_3$): δ 114.28, 119.85, 122.93, 124.21, 125.63, 131.51, 144.00, 162.94, 179.18; ES-MS *m*/*z* 301 $[M+H]^+$; $C_9H_7BrN_4OS$: C, 36.13; H, 2.36; N, 18.73; found: C, 36.10; H, 2.32; N, 18.75.

4.6.5. 1*H*-Indole-2,3-dione-3-thiosemicarbazone (9e)

Red crystals, yield 78%; mp 151–153 °C; IR (KBr, cm^{-1}): 3679, 1655; ^1H NMR (500 MHz, DMSO-d_6): δ 6.87 (s, 1H, Ar-*H*), 6.98–7.00 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 7.24–7.26 (d, *J* = 10.0 Hz, 1H, Ar-*H*), 7.67 (s, 1H, Ar-*H*), 8.87 (br s, 2H, NH_2), 10.95 (br s, 1H, NH), 12.48 ((br s, 1H, NH); ^{13}C NMR (500 MHz, $CDCl_3$): δ 108.81, 117.83, 118.77, 120.10, 128.86, 129.83, 140.23, 160.53, 176.85; ES-MS *m*/*z* 221 $[M+H]^+$; $C_9H_8N_4OS$: C, 49.08; H, 3.66; N, 25.44; found: C, 49.04; H, 3.64; N, 25.48.

4.7. Cell lines

The human MDA-MB468, MDA-MB231 and MCF-7 breast cancer cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan UT) and 2 mM L-glutamine. 184B5 and MCF10A immortalized breast cells were maintained in mammary epithelial basal medium supplemented with an MEGM mammary epithelial singlequot kit (Cambrex). Cells were grown at 37 °C with 5% CO_2, 95% air under the humidified conditions.

4.8. Reagents

Chloroquine diphosphate and cisplatin were purchased from Sigma–Aldrich Canada Ltd (Oakaville, ON, Canada). All the compounds were dissolved in 10–20 mM dimethyl sulfoxide (DMSO) and stored at −20 °C until use. The stock solution was diluted in culture medium (0.1–100 μM) immediately before use. The final concentration of DMSO in the SRB-based cytotoxicity assays did not exceed 0.1%. To rule out that the DMSO concentration used may affect cell cytotoxicity, culture medium containing equivalent concentration of DMSO was used as a negative control in all experiments. In all studies, the concentration of DMSO used did not notably show any cytotoxicity.

4.9. SRB assay

Cytotoxic effects were determined by a sulforhodamine B (SRB)-based protocol.[3,8,24,25] For a typical screening experiment, 5000–10,000 cells were inoculated into100 μL medium per well of a 96-well microtiter plate as described previously.[24,25] Briefly, after the inoculation, the microtiter plate was incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h, prior to addition of experimental drugs. Some of the sample wells were fixed with 25 μL of 50% trichloroacetic acid (TCA) as a control of the cell population for each cell line at the time of drug addition (Tz). An aliquot of the frozen stock was thawed and diluted to the desired final maximum test-concentration with complete medium. Two to ten fold serial dilutions were made to provide a total of seven drug concentrations (and a control [C]). Following addition of drugs, the culture plate was incubated for additional 48 h. Cells were fixed in situ by slowly adding 25 μL of cold 50% (w/v) TCA (final concentration, 10% TCA), and were then incubated for 60 min at 4 °C. The supernatant was discarded, and the plate was washed five times with tap water, followed by air-dry. 50 μL of SRB solution at 0.4% (w/v) in 1% acetic acid was added to each well, and the plate was incubated for >30 min at room temperature. Unbound SRB was removed by five washes with tap water, followed by air-drying. The cells 'stained' with SRB were solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515–564 nm. The relative growth rate (%) was calculated for each of the compound concentrations according to the following formula:

$$(Ti - Tz)/(C - Tz) \times 100$$

In the formula, time zero (Tz), control growth (C), and OD for different concentration of tested compounds (Ti). The GI_{50} for each compound was obtained from a non-linear Sigmoidal dose–response (variable slope) curve which is fitted by GraphPad Prism v.4.03 software. Values were calculated for each of these parameters if the level of activity was reached. However, if the effect was not reached or was exceeded, the value for that parameter was expressed as greater or less than the maximum or minimum concentration tested.[24,25]

4.10. Flow cytometry

Cells (2.0×10^6) were harvested by centrifugation at 1000 rpm on a bench-top centrifuge for 5 min, followed by fixation with ice-cold ethanol (70%) for 30 min to overnight at −20 °C.[4,8] The ethanol was then removed by centrifugation, and cells were resuspended in 1X PBS solution, followed by centrifuge. The cell pellet was than stained with PI master mix (100 μg/mL RNase A, 100 μg/mL PI, 0.3% Nonidet P-40 and 0.1% sodium citrate in distilled water) for 30 min at 37 °C. DNA content was measured using a Beckmann Coulter Cytomics FC500 (Beckman Coulter, Fullerton, CA), and the proportion of cells in G0/G1, S, and G2/M phases of cell cycle was calculated on the basis of DNA distribution histograms using CXP software.

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