Design, Synthesis and Biological Evaluation of Morpholinated Isatin-Quinoline Hybrids as Potential Anti-Breast Cancer Agents.

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Research Article

Keywords: Isatin, Quinoline, Morpholine, Molecular hybridization, Breast cancer.

Posted Date: June 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-595432/v1

Abstract

Keeping in view the emerging need of potent and safer anti-breast cancer agents as well as pharmacological attributes of isatin, quinoline and morpholine derivatives, novel hydrazone linked morpholinated isatin-quinoline hybrids has been designed, synthesized and evaluated as anti-breast cancer agents. Synthesized hybrid compounds were preliminary screened against two breast cancer cell lines (MCF-7 and MDA-MB-231). Almost all synthetics showed potent inhibitory potential against hormone positive MCF-7 cells while inactive against hormone negative MDA-MB-231 cells. Potent compounds were further evaluated against L929 (noncancerous skin fibroblast) cell line and found highly selective for MCF-7 cells over L929 cells. Cell cycle analysis confirmed that most potent compound **AS-4** (MCF-7: GI_{50} = 4.36 μM) cause mitotic arrest at G_2/M-phase. Due to higher selectivity toward estrogen receptor alpha (ERα) dependent MCF-7 cells various binding interactions of **AS-4** with ERα are also streamlined, suggesting the capability of **AS-4** in completely blocking ERα. Overall study suggest that, **AS-4** can act as a potential lead for further development of potent and safer anti-breast cancer agents.

1. Introduction

Cancer remains the most difficult disease to treat with second leading cause of deaths around the globe, responsible for approximately 9.6 million deaths in 2018. In fact 1 out of 6 deaths is due to cancer and low as well as middle income nations have 70 % share in it [1]. Rapidly dividing cells of the organs like breast, skin and uterine are more fond to mutations as compared to the cells of other organs of human body thus at high risk to develop cancer. Breast cancer is most commonly occurring cancer type in women and cause significant morbidity and mortality [2]. In 2019, it was responsible for 41760 deaths among women and men in US alone [3]. In India situation is more alarming where BC has 25–32% share in all cancer cases. A survey report issued by Indian Council of Medical Research (ICMR) in 2016 estimated 14.5 million new cancer patients at that time which were estimated to be lifted up to 17.3 million in current 2020 [4].

Estrogen is a primary female sex hormone that play an essential role in the growth and development of mammary glands. Interaction of estrogen with its estrogen receptors (ERα and ERβ) are reported to play an important role in proliferation of mammary cells. All these facts resulted in the emergence of selective estrogen receptor modulators (SERMs) in drug development field. MCF-7 cells which are ERα dependent in nature and are sensitive to SERMs while MDA-MB-231 cells are ERβ dependent in nature in which the expression of estrogen, progesterone and HER2 receptors is absent thus also known as triple negative breast cancer cells [5–8]. US Food and Drug Administration (US-FDA) has already approved triarylethylene derivatives like raloxifene, tamoxifen and toremifene as SERMs with anti-breast cancer profile [9]. Among them, tamoxifen is a first line drug and widely prescribed for the treatment of breast cancer. It responds to approximately 70% of ERα cases while other need adjuvant therapy that generally relapse [10–12]. Additionally, tamoxifen adversely affect the endometrium leading to endometrial cancer while another SERM raloxifene has hot flashes, insomnia, dizziness, and melancholy like side effects [13]. Thus there is a global need to develop novel potent and safer anti-breast cancer agents.

Isatin (2,3-Indolinedione) is a well-known pharmacologically active scaffold with broad range of biological activities such as anticancer, antibacterial, antifungal, antidepressant, anticonvulsant, anti-HIV etc [14–17]. It is widely distributed in plants and marine based natural products including fungal metabolites [18]. Sunitinib III (sutent) is an isatin based drug that has been recently approved for clinical use by US-FDA for the treatment of gastrointestinal stromal tumors and advanced renal cell carcinoma [19]. Another isatin based candidate BIBF1120 II (triple angio-kinase inhibitor) is in phase III clinical trials for the diagnosis of non-small cell lung cancer [20]. Numerous reports (Fig. 1) are available that showcase the anti-breast cancer efficacy of isatin based hybrids including isatin-benzothiazole (**1**), Isatin-chalcone hybrids (**2**), Isatin-benzimidazole (**3**) and Isatin-benzoazine hybrids (**4**) [21–24].

Quinoline (benzopyridine) is a well know biologically active nucleus that has been widely distributed in natural products and represents a family of compounds called quinoline alkaloids. Some of the quinoline alkaloids are well known for their anticancer potential including berberine, camptothecin, chelidonine, chelerythrine, dictamine etc. Inspired from the anticancer potential of quinoline nucleus, synthetic anticancer analogues has been successfully developed by the researchers and are under clinical trial such as bosutinib, levatinib, cbazantinib and tipifarnib [25]. Halogen substituted quinoline compounds especially chloro substituted ones are currently gaining interest particularly due to their anticancer potential [26]. Some chloroquinoline based molecular hybrids (Fig. 1) has been reported as potential anti-breast cancer agents (**5–8**) [27–30].

Morpholine is a unique nucleus with nitrogen and oxygen embedded in it and highly popular in medicinal chemists due to its unique biochemical features. Oxygen atom present in the outer end significantly increase the affinity of morpholine ring with donor-acceptor type interactions with enzymatic receptors. Oxygen atom also decrease the basicity of nitrogen present in the ring via electronegative effect. Due to its unique biochemical properties, researchers are widely exploiting this nucleus for developing anticancer drugs with minimal or no side-effects. Morpholine containing drug named gefitinib has been approved by US-FDA for the treatment of metastatic non-small cell lung cancer while various other morpholine containing compound like GDC-0941, WAY-600, Foretinib, Copanlisib etc are under clinical trials [31]. Various hybrids molecules containing morpholine nucleus (Fig. 1) has been reported as potential anti-breast cancer agents (**3, 8, 9**) [23, 30, 32].

Molecular hybridization is a well-established stratagem in drug development in which two bioactive moieties are combined together with or without any linker to get a single hybrid molecule having properties of both parent moieties with higher potency, reduced toxicity and minimized resistance [18]. CUDC-907 is a well-known example for molecular hybridization which is a hybrid molecule of vorinostat (a potent histone deacetylase inhibitor approved by FDA in 2006 for treatment of T-cell lymphoma) and GDC-0941 (a phosphoinositide 3-kinase inhibitor) which was more effective in both *in vitro* and *in vivo* models with no systemic toxicity and resistance. This hybrid molecule has recently passed the phase I clinical trial for advanced solid tumor and lymphoma treatment and entered in phase II clinical trial. Thus molecular hybridization would be an efficient approach for the development of potent and safer drug candidates with minimum resistance [33].

Hydrazone is a versatile moeity in medicinal chemistry that has been widely employed in architecting broad range of pharmacologically active compounds including antibacterial, antifungal, anticonvulsant, analgesic and anticancer etc. Various isatin based hybrids linked to other biological moieties through hydrazone has been reported with admirable anticancer activity [24]. Due to dominant and favorable profile of hydrazone in anticancer area, it has been selected to be utilized into target hybrid molecules (**10**) to connect Quinoline with morpholinated isatin (Fig. 1).

Considering the alarming heath issue of breast cancer and lack of potent and safer anti-breast cancer agents, present study targets the synthesis of hydrazone linked morpholinated isatin-quinoline hybrids and evaluation against MCF-7 and MDA-MB-231 cell lines along with effect on cell cycle. Compounds with promising activity were evaluated for cytotoxicity against L929 (noncancerous skin fibroblast) cell line. Furthermore, the binding interactions of most potent compound with target ERα were explored via molecular docking studies.

2. Results And Discussion

2.1. Chemistry

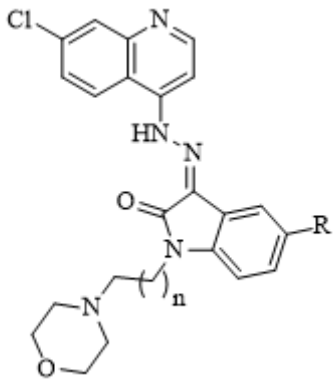
Synthesis of targeted hybrids was conducted via a series of chemical reactions (Scheme 1), initiated from various substituted isatins (**11**). Isatins were reacted with various alkylated morpholines (**12**) with halogen on alkyl end in the presence of K_2CO_3 using DMF as solvent. Reaction was carried out at room temperature and monitored by TLC. After completion, reaction mixture was poured on crushed ice and precipitates so obtained were subjected to column chromatography to get pure 1-(2-morpholinoalkyl)indoline-2,3-diones (**13**). Simultaneously, 4,7-dichloroquinoline (**14**) was treated with hydrazine hydrate in ethanol under reflux, yielded 7-chloro-4-hydarzinylquinoline after washing and crystallization with ethanol itself (**15**) [34]. 1-(2-morpholinoalkyl)indoline-2,3-diones and 7-chloro-4-hydarzinylquinoline were further refluxed together in ethanol with few drops of glacial acetic acid and monitored for progress by TLC. After completion, reaction kept overnight at room temperature for precipitation. Precipitates obtained were further washed and recrystallized with ethanol to get desired morpholinated isatin-quinoline hybrids (**AS-1** to **AS-18**). All chemical reactions were proceeded very smoothly and hybrids were obtained in decent yields. Chemical structures of targeted hybrids were characterized through ^{1}H and ^{13}C NMR along with elemental analysis and were accordance with assumed structures.

2.2. Biological evaluation

Synthesized compounds were evaluated for their cytotoxic activities on two breast cancer cell lines using MTT assay. One was MCF-7, which is ERα dependent and hormone positive cell line while another one was MDA-MB-231, which is ERβ dependent and hormone negative cell line. Preliminary screening of test compounds was performed by using initial concentration of 100 μM each. Compounds showing percentage growth inhibition (Table 1) greater than 60 % were only considered as active and further

evaluated for GI_{50} values (Table 2) using different concentrations of test compounds against sensitive breast cancer cell line along with L929 (noncancerous skin fibroblast) cell line. In preliminary screening all synthesized compounds showed potent growth inhibition potential against MCF-7 cell line while found inactive against MDA-MB-231 at 100 μM concentration that makes the hybrid molecules, selective inhibitors of hormone positive breast cancer. Among the screened hybrids compounds, compounds with two carbon alkyl chain between morpholine and isatin (**AS-1** to **AS-6**) showed more sensitivity toward MCF-7 cells as compare to those with three (**AS-7** to **AS-12**) and four carbon chain (**AS-13** to **AS-18**). Suggesting that two carbon chain length between morpholine and isatin is most suitable for anticancer activity. Furthermore, only hybrids with two carbon chain length between morpholine and isatin (**AS-1** to **AS-6**) were able to show growth inhibition more than 60 % thus explored for GI_{50} values. GI_{50} values generated an exclusive relationship between the activity and electronic environment at 5th position of isatin nucleus. **AS-4** with fluoro substitution (R = F) was most potent among all synthesized compounds with GI_{50} value of 4.36 μM, followed by **AS-3** (GI_{50} = 9.22 μM) with chloro (R = Cl) and **AS-2** (GI_{50} = 18.62 μM) with bromo (R = Br) substitution, which suggest that electronegative halogen groups at isatin are most suitable for the anticancer activity. On the other hand, in **AS-5** and **AS-6** i.e. methyl (R = CH_3) and methoxy (R = OCH_3) substitutions on isatin lowered the activity with GI_{50} values of 24.23 and 22.19 μM that suggest that electropositive substitutions around isatin are unfavorable for the anticancer potential. Unsubstituted isatin in hybrid molecule (**AS-1**) give GI_{50} values of 28.27 μM which is even lower than **AS-5** and **AS-6**. Thus, the overall preference order for R becomes F > Cl > Br > OCH_3 > CH_3 > H and for carbon chain length between isatin and morpholine, it is n = 1 > 2 > 3 that generates a beautiful structure activity relationship. Active compounds when further evaluated against L929 cell line, most of them (**AS-1**, **AS-2**, **AS-5** and **AS-6**) were found inactive with GI_{50} values above 100 μM while **AS-3** and **AS-4** showed GI_{50} values of 78.63 and 52.42 μM respectively. Most potent compound showed selectivity index of 12.03 between MCF-7 and L929 cell line that makes it highly selective for breast cancer cells over normal fibroblast cells.

Table 1 Various morpholinated isatin-Quioline hybrids with their percentage growth inhibition against breast cancer cell lines at 100 µM.



Code	R	n	Percentage growth inhibition (100 μM)	
			MCF-7 Hormone positive breast cancer cell line	MDA-MB-231 Hormone negative breast cancer cell line
AS-1	H	1	65	6
AS-2	Br	1	79	10
AS-3	Cl	1	85	17
AS-4	F	1	93	22
AS-5	CH_3	1	75	13
AS-6	OCH_3	1	71	15
AS-7	H	2	39	-[a]
AS-8	Br	2	48	4
AS-9	Cl	2	53	9
AS-10	F	2	56	17
AS-11	CH_3	2	43	-[a]
AS-12	OCH_3	2	47	-[a]
AS-13	H	3	11	-[a]
AS-14	Br	3	15	-[a]
AS-15	Cl	3	14	8
AS-16	F	3	18	12
AS-17	CH_3	3	11	-[a]
AS-18	OCH_3	3	13	-[a]

[a]No growth Inhibition detected

Table 2 GI_{50} values of active morpholinated isatin-Quioline hybrids against MCF-7 (Hormone positive breast cancer) and L929 (noncancerous skin fibroblast) cell lines along with selectivity index.

Code	GI_{50} (μM)		Selectivity Index (SI)
	MCF-7	L929	
AS-1	28.27	>100	-[c]
AS-2	18.62	>100	-[c]
AS-3	9.22	79.63	8.64
AS-4	4.36	52.42	12.02
AS-5	24.23	>100	-[c]
AS-6	22.19	>100	-[c]
Plumbagin	3.5	-[b]	-[c]
Tamoxefene	50	-[b]	-[c]

[a]SI value > 3 is considered to be highly selective. [b]Not tested. [c]Not calculated

Effect of most potent compound **AS-4** on cell cycle distributions is further evaluated on most sensitive MCF-7 cells. MCF-7 cells were treated with **AS-4** with a concentration of 4.36 μM (GI_{50} value) for 24 hours. Results revealed that **AS-4** cause significant accumulation of MCF-7 cells in G_2/M-phase (50.1 %) at GI_{50} concentration as compare to control (28.04 %) while in reduced accumulation was observed in G_0/G_1-phase (24.1 %) and S-phase (18.06 %) as compare to control which was 36.2 % in G_0/G_1-phase and 28.8 % in S-phase (Fig. 2). Thus, overall results suggest that **AS-4** hinders the proliferation of MCF-7 cells by arresting them at G_2/M-phase that ultimately lead to cell death.

2.3. Molecular docking study

Among the synthesized compounds **AS-4** was emerged as potent inhibitor of MCF-7 breast cancer cells which are hormone positive in nature and ERα dependent. On the other hand, compounds showed insignificant inhibition against MDA-MB-231 which is ERα negative cell line thus it can be concluded that the inhibitory pattern of by **AS-4** may go through the inhibition of ERα. Thus molecular docking studies were performed to get insight into various molecular interactions possibly responsible for the modulation of ERα by **AS-4**. For that purpose, the X-ray crystallographic structure of human estrogen receptor alpha (ERα) in complex with its selective antagonist 4-hydroxytamoxifen (PDB entry: 3ERT; Resolution: 1.9 Å), was employed [REF PDB]. Accuracy of docking protocol was validated by docking co-crystallized ligand 4-hydroxytamoxifen into its binding site. The program was capable to reproduce best fit confirmation of 4-hydroxytamoxifen in chain A with root mean square deviation (RMSD) value of 0.7756, indicating the

reliability of docking protocol. After that **AS-4** was docked into 4-hydroxytamoxifen binding site, and best pose with − 10.1584 score was selected for discussion (Fig. 3).

Overall binding pattern of **AS-4** with its binding site disclose that compound is well settled in the cavity which is stabilized through various electrostatic interactions. Major interactions of **AS-4** with ERα include π-σ, π- π stacked, π-alkyl, salt bridge attractive charge, halogen interaction, C-H bond, π-donor hydrogen bond and conventional hydrogen bond interaction. Chloroquinoline moiety of **AS-4** is well stabilized in the cavity formed by Ala350, Leu387, Met357, Trp383, Leu536 and Leu354 (hydrophobic residues). Chloroquinoline moiety is seemed to be sandwiched between Trp383, Ala350, Leu387 and Leu536 stabilized through π-σ, π- π stacked and π-alkyl type interactions. Long distanced (π-orbital; d = 2.843 Å) π-alkyl interaction of chloro group on quinoline with aromatic residue of Trp383 is also observed. Short distanced conventional hydrogen bond interaction (H-bond acceptor; d = 1.876 Å) is observed between the Asp351 and hydrazone linkage that proves importance of this linkage in strong binding of **AS-4** with active site. Halogen type interaction was observed between flouro group at isatin and Met528. Additional π-donor hydrogen bond interaction was also observed between isatin and Cys530. Alkyl chain between isatin and morpholine seems to interact with Asp351 through C-H bond interaction. A salt bridge attractive charge interaction is also observed between nitrogen group of morpholine and carboxylic oxygen of Asp351. Additional C-H bond interactions are observed between Asp351 and morpholine moiety. Another conventional hydrogen bond (H-bond acceptor; d = 2.692 Å) is observed between the oxygen of morpholine and Met528. Overall study seems to propose that **AS-4** has been adequately decorated with small, rigid and planer groups showcasing ideal scaffold that is able to complete the pharmacophoric need for ERα inhibition.

3. Conclusion

In the present study, hydrazone linked morpholinated isatin-quinoline hybrids has been designed as anti-breast cancer agents and synthesized in good yields that were characterized by using ^{1}H and ^{13}C NMR along with elemental analysis. All compounds were preliminarily screened against one hormone positive (MCF-7) and one hormone negative (MDA-MB-231) breast cancer cell line. Compounds showed good growth inhibition against hormone positive MCF-7 cells while were inactive against hormone negative MDA-MB-231 cells. Potent compounds were further evaluated for cytotoxicity against L929 (noncancerous skin fibroblast) cell line and found highly selective for MCF-7 over L929 cells. Cell cycle analysis confirm that compounds cause mitotic arrest at G_2/M-phase. Since compounds showed potent activity and selectivity toward ERα dependent MCF-7 cells thus various binding interactions of most potent compound **AS-4** (MCF-7: GI_{50} = 4.36 μM) with ERα are also streamlined. Overall study suggest that **AS-4** can act as a hit lead for further development of potential and safer anti-breast cancer agents.

4. Experimental

4.1. Materials and measurements

All chemicals and reagents were procured form Sigma Aldrich, Spectrochem and CDH, India and utilized without any further purification. All yield mentioned are refer to the isolated compounds after purification process. Characterization of synthesized compounds was done via spectroscopic techniques such as ^{1}H and ^{13}C NMR along with elemental analysis. NMR spectra were recorded on Avance III HD 500 MHz Bruker Biospin and JOEL 400 MHz using DMSO-d_6. Chemical shifts in ^{1}H NMR were reported in δ values relative to TMS as internal standard (0.00 ppm). Splitting patter in obtained ^{1}H NMR spectra are reported as s: singlet, d: doublet, m: multiplet, br: broad peak and coupling constants (J) in hertz (Hz).

4.1.1. Procedure for synthesis of 1-(2-morpholinoalkyl)indoline-2,3-diones

Isatin (1 equiv) was mixed with K_2CO_3 (1.5 equiv) and dissolved in DMF. Mixture was allowed to stir for 20 min. After 20 min, 4-(2-Chloroethyl)morpholine hydrochloride was added to the reaction mixture and allowed to stir further at room temperature until reaction was completed (monitored by TLC). On completion, reaction mixture was added in crushed ice and allowed to stand for a while (until the ice completely melts to form water and precipitates completely settled down). Precipitates formed were filtered and dried. Obtained product was further purified using column chromatography using petroleum ether/ethylacetate (9:1) to get 1-(2-morpholinoethyl)indoline-2,3-dione (**16**). Characterization data for 1-(2-morpholinoethyl)indoline-2,3-dione is as follows:

Yield 83%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 7.65–7.60 (1H, m), 7.52–7.50 (1H, m), 7.18 (1H, J = 8, d), 7.08 (1H, J = 8, t), 3.77–3.74 (2H, m), 3.47–3.45 (4H, m), 2.51–2.48 (2H, m), 2.40–2.36 (4H, m). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 184.05, 158.67, 151.31, 138.80, 125.01, 123.70, 117.95, 111.55, 66.72, 55.27, 53.76, 37.47. Anal. Calcd for $C_{14}H_{16}N_2O_3$: C, 64.60; H, 6.20; N, 10.76; Found: C, 64.58; H, 6.21; N, 10.74.

The same procedure described above was utilized in the synthesis of remaining 1-(2-morpholinoalkyl)indoline-2,3-diones.

4.1.2. Procedure for synthesis of 7-chloro-4-hydarzinylquinoline

4,7-dichloroquinoline (1 equiv) was dissolved in absolute ethanol and hydrazine hydrate (15 equiv) added drop wise with stirring. Reaction mixture so obtained was refluxed for 3 hours. After that reaction mixture was cooled down to room temperature and kept overnight for precipitation. Yellow precipitates so obtained were filtered, washed with ethanol (10 mL) twice and further recrystallized using ethanol to get desired 7-chloro-4-hydarzinylquinoline with 80% yield and m.p. 223–225 ºC [34].

4.1.3. Procedure for synthesis of morpholinated isatin-quinoline hybrids (AS-1 to AS-18)

1-(2-morpholinoalkyl)indoline-2,3-dione (1 equiv) and 7-chloro-4hydarzinylquinoline (1 equiv) were dissolved in absolute ethanol and few drops of glacial acetic acid was added to it. Reaction mixture was allowed to reflux until both reactants are not completely consumed (monitored by TLC). On completion, reaction mixture was cooled down to room temperature and kept overnight for precipitation. Precipitates so obtained were filtered, washed with ethanol (10 mL) twice and further recrystallized using ethanol to get desired morpholinated isatin-quinoline hybrids. Characterization data of all synthesized morpholinated isatin-quinoline hybrids is as follows:

(Z)-3-(2-(7-chloroquinolin-4-yl)hydrazono)-1-(2-morpholinoethyl)indolin-2-one (AS-1): Yield 76%, ^{1}H NMR (DMSO-d_6, 500 MHz, δ, TMS = 0): 12.21 (1H, br), 8.53 (1H, J = 10, d), 8.43–8.41 (1H, m), 7.90 (1H, J = 10, d), 7.85 (1H, m), 7.50–7.47 (1H, m), 7.32–7.28 (1H, m), 7.15 (1H, J = 10, d), 7.08–7.04 (2H, m), 3.85–3.82 (2H, m), 3.50–3.48 (4H, m), 2.53–2.50 (2H, m), 2.42 (4H, s). ^{13}C NMR (DMSO-d_6, 125 MHz, δ, TMS = 0): 165.35, 143.48, 142.30, 140.17, 138.64, 136.77, 130.67, 127.45, 125.52, 120.93, 117.96, 109.18, 100.53, 66.71, 55.89, 55.83, 36.99. Anal. Calcd for $C_{23}H_{22}ClN_5O_2$: C, 63.37; H, 5.09; N, 16.07; Found: C, 63.34; H, 5.06; N, 16.05.

(Z)-5-bromo-3-(2-(7-chloroquinolin-4-yl)hydrazono)-1-(2-morpholinoethyl)indolin-2-one (AS-2): Yield 68%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 12.94 (1H, br), 8.82–8.80 (1H, m), 8.06–8.05 (1H, m), 7.86–7.82 (3H, m), 7.73–7.72 (1H, m), 7.62–7.60 (1H, m), 7.33–7.31 (2H, m) 4.29–4.27 (2H, m), 3.80–3.78 (4H, m), 2.08–2.07 (4H, m), 1.94(2H, s). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 154.02, 153.89, 152.67, 152.41, 143.33, 139.53, 138.64, 138.60, 128.61, 126.70, 119.50, 115.74, 113.84, 63.53, 58.58, 55.29, 51.88, 19.31. Anal. Calcd for $C_{23}H_{21}$ $BrClN_5O_2$: C, 53.66; H, 4.11; N, 13.60; Found: C, 53.65; H, 4.08; N, 13.57.

(Z)-5-chloro-3-(2-(7-chloroquinolin-4-yl)hydrazono)-1-(2-morpholinoethyl)indolin-2-one (AS-3): Yield 81%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 13.63 (1H, br), 8.86–8.85 (1H, m), 8.09 (1H, s), 7.95–7.94 (1H, m), 7.81–7.80 (3H, m), 7.52–7.50 (1H, m), 7.34–7.33 (1H, m), 3.76 (2H, s), 3.54 (4H, s), 2.65 (4H, s), 2.38–2.37 (2H, m). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 155.16, 154.18, 151.32, 151.04, 142.83, 138.13, 138.05, 137.83, 127.31, 126.10, 118.91, 115.14, 113.14, 62.93, 58.02, 54.79, 51.17, 18.97. Anal. Calcd for $C_{23}H_{21}Cl_2N_5O_2$: C, 58.73; H, 4.50; N, 14.89; Found: C, 58.69; H, 4.46; N, 14.85.

(Z)-3-(2-(7-chloroquinolin-4-yl)hydrazono)-5-fluoro-1-(2-morpholinoethyl)indolin-2-one (AS-4): Yield 73%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 13.64 (1H, br), 8.85 (1H, s), 8.09 (1H, s), 7.95–7.93 (1H, m), 7.81–7.79 (1H, m), 7.76–7.75 (1H, m), 7.63–7.61 (1H, m), 7.34–7.33 (2H, m), 4.07–4.06 (2H, m), 3.63 (4H, s), 2.57–2.53 (2H, s), 2.50 (4H, m). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 157.13, 155.12, 152.66, 152.14, 143.27, 139.32, 139.13, 138.43, 129.12, 125.16, 119.07, 115.22, 114.23, 62.83, 58.62, 54.89, 51.78, 19.22. Anal. Calcd for $C_{23}H_{21}ClFN_5O_2$: C, 60.86; H, 4.66; N, 15.43; Found: C, 60.83; H, 4.62; N, 15.41.

(Z)-3-(2-(7-chloroquinolin-4-yl)hydrazono)-5-methyl-1-(2-morpholinoethyl)indolin-2-one (AS-5): Yield 83%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 13.65 (1H, br), 8.84–8.83 (1H, m), 8.08 (1H, s), 7.93–7.92 (1H, m), 7.80–7.78 (1H, m), 7.71–7.70 (1H, m), 7.58 (1H, s), 7.29–7.27 (1H, m), 7.20–7.18 (1H, m), 4.12 (2H, s), 3.61 (4H, s), 2.55 (5H, s), 2.38 (4H, s). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 157.22, 155.36, 152.64, 152.48, 143.87, 139.22, 138.79, 138.04, 128.79, 125.11, 119.16, 115.94, 114.29, 62.46, 58.36, 54.97, 51.49, 19.82, 18.23. Anal. Calcd for $C_{24}H_{24}ClN_5O_2$: C, 64.07; H, 5.38; N, 15.57; Found: C, 64.04; H, 5.36; N, 15.55.

(Z)-3-(2-(7-chloroquinolin-4-yl)hydrazono)-5-methoxy-1-(2-morpholinoethyl)indolin-2-one (AS-6): Yield 86%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 13.68 (1H, br), 8.84 (1H, m), 8.09 (1H, s), 7.96–7.92 (1H, m), 7.82–7.76 (2H, m), 7.36 (1H, s), 7.25–7.24 (1H, m), 7.06-7.00 (2H, m), 4.00 (3H, s), 3.77 (2H, s), 3.63 (4H, s), 2.65–2.64 (2H, m), 2.60–2.57 (4H, m). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 157.16, 155.59, 152.49, 152.47, 143.49, 139.34, 138.47, 138.49, 128.99, 125.46, 119.49, 115.74, 114.97, 62.32, 58.49, 54.22, 51.79, 19.58, 21.64. Anal. Calcd for $C_{24}H_{24}ClN_5O_3$: C, 61.87; H, 5.19; N, 15.03; Found: C, 61.85; H, 5.16; N, 15.06.

(Z)-3-(2-(7-chloroquinolin-4-yl)hydrazono)-1-(3-morpholinopropyl)indolin-2-one (AS-7): Yield 76%, ^{1}H NMR (DMSO-d_6, 500 MHz, δ, TMS = 0): 12.22 (1H, br), 8.54 (1H, J = 10, d), 8.44–8.40 (1H, m), 7.91 (1H, J = 10, d), 7.86 (1H, m), 7.49–7.46 (1H, m), 7.31–7.26 (1H, m), 7.12 (1H, J = 10, d), 7.06–7.02 (2H, m), 3.88–3.84 (2H, m), 3.52–3.50 (4H, m), 2.51–2.48 (2H, m), 2.10 (4H, s), 1.95–1.93 (2H, m). ^{13}C NMR (DMSO-d_6, 125 MHz, δ, TMS = 0): 165.33, 143.47, 142.22, 140.37, 138.49, 136.79, 130.32, 126.51, 125.18, 120.95, 116.22, 109.79, 100.48, 66.39, 55.27, 55.66, 36.41, 24.16. Anal. Calcd for $C_{24}H_{24}ClN_5O_2$: C, 64.07; H, 5.38; N, 15.57; Found: C, 64.04; H, 5.34; N, 15.53.

(Z)-5-bromo-3-(2-(7-chloroquinolin-4-yl)hydrazono)-1-(3-morpholinopropyl)indolin-2-one (AS-8): Yield 68%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 12.98 (1H, br), 8.81–8.83 (1H, m), 8.05–8.04 (1H, m), 7.85–7.81 (3H, m), 7.75–7.73 (1H, m), 7.60–7.58 (1H, m), 7.31–7.29 (2H, m) 4.28–4.26 (2H, m), 3.82–3.80 (4H, m), 2.10–2.08 (4H, m), 1.96–1.94 (2H, m), 1.92 (2H, s). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 155.18, 156.32, 153.98, 151.49, 141.22, 139.16, 137.12, 136.27, 128.62, 126.80, 119.92, 115.22, 113.84, 63.32, 58.49, 55.19, 51.17, 24.41, 19.32. Anal. Calcd for $C_{24}H_{23}BrClN_5O_2$: C, 54.51; H, 4.38; N, 13.24; Found: C, 54.47; H, 4.35; N, 13.20.

(Z)-5-chloro-3-(2-(7-chloroquinolin-4-yl)hydrazono)-1-(3-morpholinopropyl)indolin-2-one (AS-9): Yield 81%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 13.62 (1H, br), 8.85–8.84 (1H, m), 8.09 (1H, s), 7.91–7.89 (1H, m), 7.77–7.76 (3H, m), 7.51–7.49 (1H, m), 7.33–7.32 (1H, m), 3.74 (2H, s), 3.56 (4H, s), 2.63 (4H, s), 2.35–2.33 (2H, m), 1.93–1.91 (2H, m). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 154.89, 154.12, 152.22, 151.32, 141.53, 137.93, 137.25, 136.83, 127.51, 126.19, 118.53, 115.59, 113.32, 62.22, 58.49, 53.21, 51.49, 23.16, 18.32. Anal. Calcd for $C_{24}H_{23}Cl_2N_5O_2$: C, 59.51; H, 4.79; N, 14.64; Found: C, 59.47; H, 4.75; N, 14.60.

(Z)-3-(2-(7-chloroquinolin-4-yl)hydrazono)-5-fluoro-1-(3-morpholinopropyl)indolin-2-one (AS-10): Yield 72%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 13.64 (1H, br), 8.87 (1H, s), 8.08 (1H, s), 7.95–7.93 (1H, m), 7.81–7.79 (1H, m), 7.76–7.75 (1H, m), 7.61–7.59 (1H, m), 7.31–7.29 (2H, m), 4.15–4.13 (2H, m), 3.51 (4H, s), 2.55–2.53 (2H, s), 2.50 (4H, m), 1.96–1.94 (2H, m). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 158.22, 155.79, 152.63, 152.05, 143.17, 139.49, 139.02, 138.65, 129.32, 125.27, 118.22, 115.37, 114.21, 62.49, 58.57, 54.34, 51.27, 24.59, 18.36. Anal. Calcd for $C_{24}H_{23}ClFN_5O_2$: C, 61.60; H, 4.95; N, 14.97; Found: C, 61.57; H, 4.92; N, 14.93.

(Z)-3-(2-(7-chloroquinolin-4-yl)hydrazono)-5-methyl-1-(3-morpholinopropyl)indolin-2-one (AS-11): Yield 86%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 13.63 (1H, br), 8.82–8.80 (1H, m), 8.09 (1H, s), 7.94–7.93 (1H, m), 7.81–7.79 (1H, m), 7.73–7.71 (1H, m), 7.57 (1H, s), 7.28–7.26 (1H, m), 7.21–7.19 (1H, m), 4.14 (2H, s), 3.63 (4H, s), 2.54 (5H, s), 2.35 (4H, s), 1.98–1.96 (2H, m). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 156.26, 155.17, 152.18, 152.07, 143.22, 139.36, 138.21, 138.17, 128.51, 125.22, 119.37, 115.51, 114.41, 62.37, 58.16, 54.32, 51.48, 24.46, 19.32, 18.26. Anal. Calcd for $C_{25}H_{26}ClN_5O_2$: C, 64.72; H, 5.65; N, 15.09; Found: C, 64.69; H, 5.61; N, 15.05.

(Z)-3-(2-(7-chloroquinolin-4-yl)hydrazono)-5-methoxy-1-(3-morpholinopropyl)indoli n-2-one (AS-12): Yield 81%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 13.62 (1H, br), 8.82 (1H, m), 8.07 (1H, s), 7.96–7.94 (1H, m), 7.80–7.78 (2H, m), 7.39 (1H, s), 7.23–7.21 (1H, m), 7.12–7.08 (2H, m), 4.12 (3H, s), 3.71 (2H, s), 3.65 (4H, s), 2.64–2.63 (2H, m), 2.59–2.57 (4H, m), 1.95–1.93 (2H, m). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 157.29, 155.78, 152.23, 152.17, 143.32, 140.29, 138.49, 138.13, 128.31, 125.42, 119.65, 116.41, 114.47, 62.79, 58.34, 54.47, 52.75, 24.87, 21.34, 19.35. Anal. Calcd for $C_{25}H_{26}ClN_5O_3$: C, 62.56; H, 5.46; N, 14.59; Found: C, 62.52; H, 5.43; N, 14.57.

(Z)-3-(2-(7-chloroquinolin-4-yl)hydrazono)-1-(4-morpholinobutyl)indolin-2-one (AS-13): Yield 74%, ^{1}H NMR (DMSO-d_6, 500 MHz, δ, TMS = 0): 12.23 (1H, br), 8.51 (1H, J = 10, d), 8.45–8.43 (1H, m), 7.88 (1H, J = 10, d), 7.85 (1H, m), 7.46–7.45 (1H, m), 7.32–7.29 (1H, m), 7.14 (1H, J = 10, d), 7.07–7.03 (2H, m), 3.81–3.79 (2H, m), 3.49–3.47 (4H, m), 2.86–2.83 (2H, m), 2.22 (4H, s), 1.75–1.73 (2H, m), 1.51–1.49 (2H, m). ^{13}C NMR (DMSO-d_6, 125 MHz, δ, TMS = 0): 165.47, 143.32, 142.41, 140.47, 139.28, 135.51, 132.17, 127.32, 126.22, 121.17, 116.26, 109.69, 100.12, 66.51, 55.23, 55.47, 36.12, 25.27, 24.37. Anal. Calcd for $C_{25}H_{26}ClN_5O_2$: C, 64.72; H, 5.65; N, 15.09; Found: C, 64.69; H, 5.63; N, 15.07.

(Z)-5-bromo-3-(2-(7-chloroquinolin-4-yl)hydrazono)-1-(4-morpholinobutyl)indolin-2-one (AS-14): Yield 71%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 12.95 (1H, br), 8.86–8.84 (1H, m), 8.07–8.06 (1H, m), 7.83–7.80 (3H, m), 7.76–7.74 (1H, m), 7.62–7.60 (1H, m), 7.33–7.31 (2H, m) 4.29–4.27 (2H, m), 3.80–3.79 (4H, m), 2.11–2.09 (4H, m), 1.95–1.93 (2H, m), 1.90 (2H, s), 1.55–1.53 (2H, m). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 155.32, 156.27, 153.41, 151.56, 141.37, 139.57, 137.48, 136.42, 128.63, 126.71, 119.64, 115.32, 113.59, 63.41, 58.79, 54.32, 51.48, 25.27, 24.63, 19.47. Anal. Calcd for $C_{25}H_{25}BrClN_5O_2$: C, 55.31; H, 4.64; N, 12.90; Found: C, 55.29; H, 4.63; N, 12.87.

(Z)-5-chloro-3-(2-(7-chloroquinolin-4-yl)hydrazono)-1-(4-morpholinobutyl)indolin-2-one (AS-15): Yield 79%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 13.64 (1H, br), 8.82–8.80 (1H, m), 8.09 (1H, s), 7.92–7.91 (1H, m), 7.76–7.73 (3H, m), 7.52–7.51 (1H, m), 7.34–7.33 (1H, m), 3.73 (2H, s), 3.57 (4H, s), 2.63 (4H, s), 2.35–2.33 (2H, m), 1.94–1.92 (2H, m), 1.53–1.50 (2H, m). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 154.93, 154.22, 152.49, 151.24, 141.47, 137.95, 137.32, 136.48, 127.12, 126.49, 118.27, 115.16, 113.41, 62.32, 58.10, 53.25, 51.47, 25.53, 23.36, 18.32. Anal. Calcd for $C_{25}H_{25}Cl_2N_5O_2$: C, 60.25; H, 5.06; N, 14.05; Found: C, 60.22; H, 4.99; N, 13.98.

(Z)-3-(2-(7-chloroquinolin-4-yl)hydrazono)-5-fluoro-1-(4-morpholinobutyl)indolin-2-one (AS-16): Yield 76%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 13.66 (1H, br), 8.85 (1H, s), 8.09 (1H, s), 7.96–7.94 (1H, m), 7.83–7.81 (1H, m), 7.73–7.71 (1H, m), 7.63–7.61 (1H, m), 7.32–7.30 (2H, m), 4.27–4.25 (2H, m), 3.56 (4H, s), 2.56–2.51 (2H, s), 2.50 (4H, m), 1.95–1.93 (2H, m), 1.49–1.47 (2H, m). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 158.36, 155.47, 152.32, 152.49, 143.34, 139.97, 139.43, 138.46, 129.05, 125.49, 118.34, 115.61, 114.34, 62.32, 58.49, 54.79, 51.18, 25.69, 24.63, 18.01. Anal. Calcd for $C_{25}H_{25}ClFN_5O_2$: C, 62.30; H, 5.23; N, 14.53; Found: C, 62.34; H, 2.19; N, 14.47.

(Z)-3-(2-(7-chloroquinolin-4-yl)hydrazono)-5-methyl-1-(4-morpholinobutyl)indolin-2-one (AS-17): Yield 75%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 13.34 (1H, br), 8.81–8.80 (1H, m), 8.08 (1H, s), 7.95–7.94 (1H, m), 7.81–7.80 (1H, m), 7.72–7.70 (1H, m), 7.58 (1H, s), 7.25–7.23 (1H, m), 7.20–7.18 (1H, m), 4.32 (2H, s), 3.59 (4H, s), 2.67 (5H, s), 2.32 (4H, s), 1.95–1.93 (2H, m), 1.54–1.52 (2H, m). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 155.32, 154.87, 152.11, 152.37, 143.37, 139.47, 138.41, 138.64, 128.48, 125.11, 119.57, 115.48, 114.34, 62.32, 58.49, 54.11, 51.37, 25.57, 24.63, 19.27, 18.41. Anal. Calcd for $C_{26}H_{28}ClN_5O_2$: C, 65.33; H, 5.90; N, 14.65; Found: C, 65.29; H, 5.95; N, 14.61.

(Z)-3-(2-(7-chloroquinolin-4-yl)hydrazono)-5-methoxy-1-(4-morpholinobutyl)indolin-2-one (AS-18): Yield 82%, ^{1}H NMR (DMSO-d_6, 400 MHz, δ, TMS = 0): 13.60 (1H, br), 8.84 (1H, m), 8.09 (1H, s), 7.98–7.96 (1H, m), 7.81–7.79 (2H, m), 7.40 (1H, s), 7.24–7.22 (1H, m), 7.11–7.07 (2H, m), 4.14 (3H, s), 3.73 (2H, s), 3.61 (4H, s), 2.65–2.64 (2H, m), 2.61–2.59 (4H, m), 1.96–1.94 (2H, m), 1.53–1.51 (2H, m). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, TMS = 0): 157.41, 155.38, 152.11, 152.02, 143.37, 140.41, 138.57, 138.49, 128.36, 125.12, 119.31, 116.47, 114.12, 62.37, 58.45, 54.31, 52.23, 25.75, 24.47, 21.21, 19.58. Anal. Calcd for $C_{26}H_{28}ClN_5O_3$: C, 63.22; H, 5.71; N, 14.18; Found: C, 62.19; H, 5.66; N, 14.15.

4.2. Biological evaluation

4.2.1. Cytotoxicity screening

Cell culturing: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin mixture was used to maintain cell lines (MCF-7, MDA-MB-231 and L929: acquired from American Type Culture Collection, ATCC, USA) at 37°C in humidified (containing 5% CO_2) atmosphere.

MTT assay

96-well plates seeded with cells (5×10^3 cells/well) were treated with test compounds (100 μM) for 24 hours under same conditions used in maintaining cells. After 24 hours of incubation, MTT reagent (5 mg/mL) was added to each well of the plate. DMSO (100 μL) was added to solubilize formazan crystals [35, 36]. Absorption changes were noted down using microplate reader at 490 nm. Cell viability was calculated using following equation.

Cell viability = 100 × [Optical density of treated wells – Optical density of blank wells/Optical density of control wells – Optical density of blank wells]

Compounds showing cell viability less than 60 % relative to untreated control cells were subjected to GI_{50} determination by treating cells with different concentrations (1, 10, 25, 50, 100 μM) of test compounds using same procedure described above and interpreting concentration response curve.

4.2.2. Cell cycle analysis

Most potent compound **AS-4** against MCF-7 cell line was subjected to cell cycle phase distribution analysis [37] and was performed using BD Cycletest plus DNA Kit (BD Biosciences) according to manufacturer's instructions. MCF-7 cells (4×10^5 cells/well) were seeded in 6-well plates allowed for attachment. After 24 h, cells were treated with GI_{50} concentration of **AS-4**. After treatment, floating as well as adhered cells were collected in falcon tubes (15 mL) and subjected to centrifugation for 5 minutes. The cell pellet obtained after centrifugation was washed twice with PBS. After this, cell pellet was fixed by using ethanol (70%, 1 mL) and kept at -20°C for 2 hr. After that, cells were washed with PBS again. Then 250 μL of solution A (trypsin buffer) was added to each tube and allowed to stand for 10 minutes at room temperature followed by the addition of 200 μL of solution B (trypsin inhibitor and RNase buffer). After 10 minutes, 200 μL of cold solution C (PI stain solution) was added and incubated for 1 hour in dark on ice. The stained cells were analyzed using BD Accuri software by flow cytometry (BD Accuri C6 Flow Cytometer, BD Biosciences).

4.3. Molecular docking study

Crystal structure of human estrogen receptor alpha (ERα) in complex with its selective antagonist 4-hydroxytamoxifen (PDB entry: 3ERT; Resolution: 1.9 Å) was retrieved from Protein Data Bank [38]. Preparation of structures was done using the drug design platform LeadIT [39]. Co-crystalized ligand 4-hydroxytamoxifen from chain A was used to define the binding site in ERα with the radius of 6.50 Å. Structure of **AS-4** was drawn on ChemDraw Ultra (2013), and its energy was minimized by using MM2 force field in Chem3D Ultra software (Cambridge, USA) [40]. Prepared **AS-4** structure was used as protonated in aqueous solution and docked into prepared binding site of ERα using the FlexX docking module in LeadIT. All FlexX solutions yielded were scored by using a consensus scoring function (CScore) and ranked accordingly. Top best pose with the highest score was selected for further investigation of the interactions [18]. 3D enzyme-hybrid and monomer-hybrid interactions were visualized using Discovery Studio Visualizer [41].

Declarations

Conflict of interest

The authors confirm that this article content has no conflicts of interest.

Acknowledgments

Authors are grateful to the University Grants Commission for providing funds under National Fellowship for Other Backward Classes (NFOBC) to AS, RUSA Component 4.0.The authors are also thankful to Guru Nanak Dev University, Amritsar for providing various facilities to carry out the research work.

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Figures

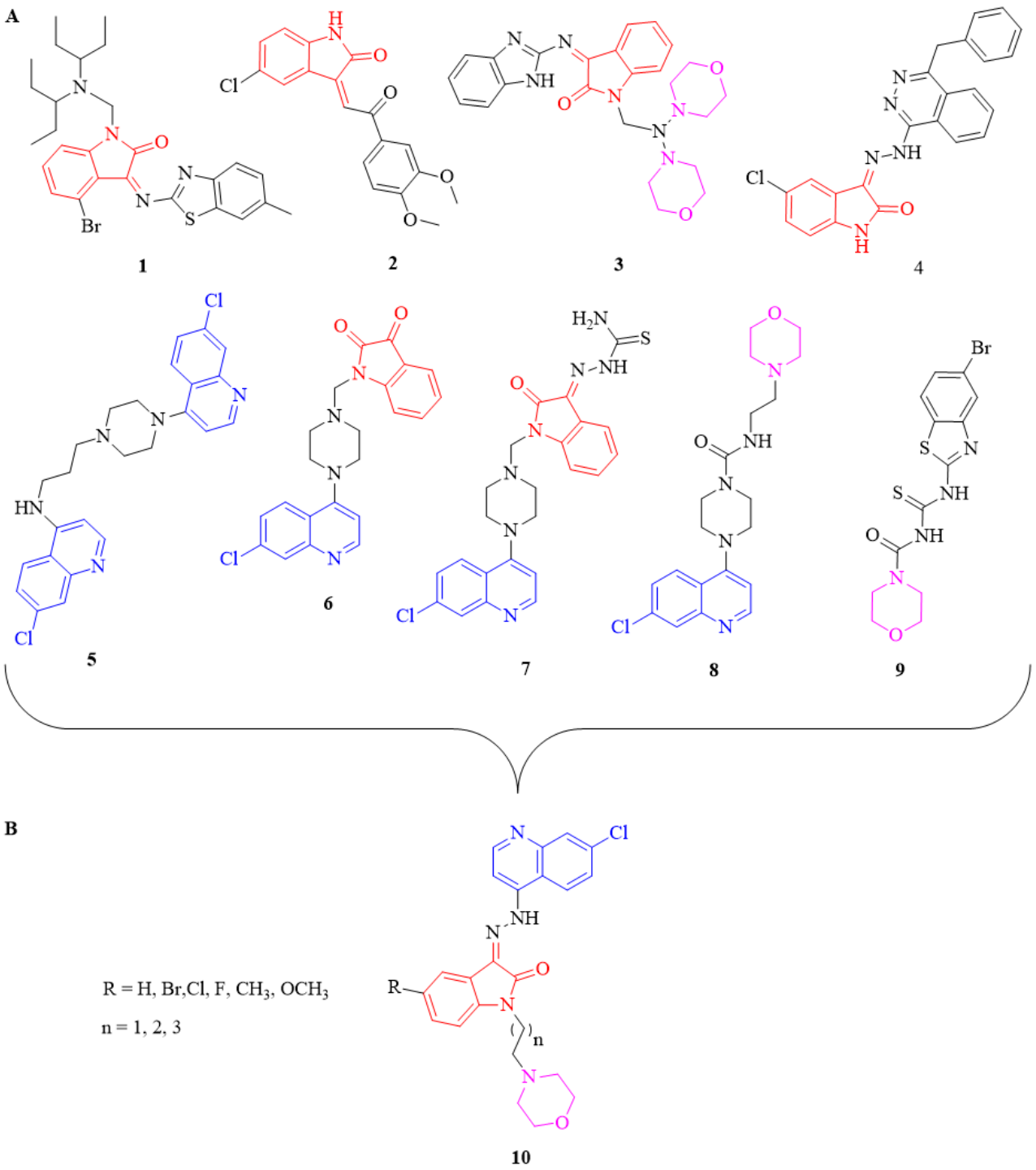

Figure 1

A.Various hybrid molecules containing isatin (1-4, 6, 7), chloroquinoline (5-8) and morpholine (3, 8-9) nucleus having anti-breast cancer activity; B. Designed morpholinated isatin-quinoline hybrids (10).

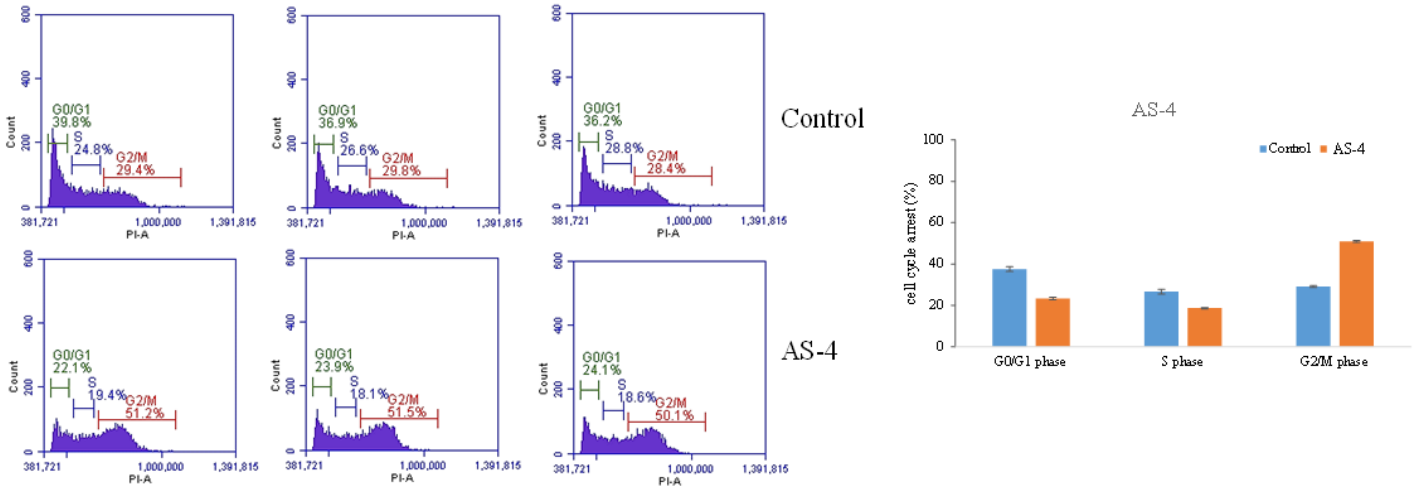

Figure 2

Flow cytometric analysis of compound AS-4 on MCF-7 cells showcasing cell cycle arrest at G2/M phase (Results represents triplicate experiment and provided as mean values).

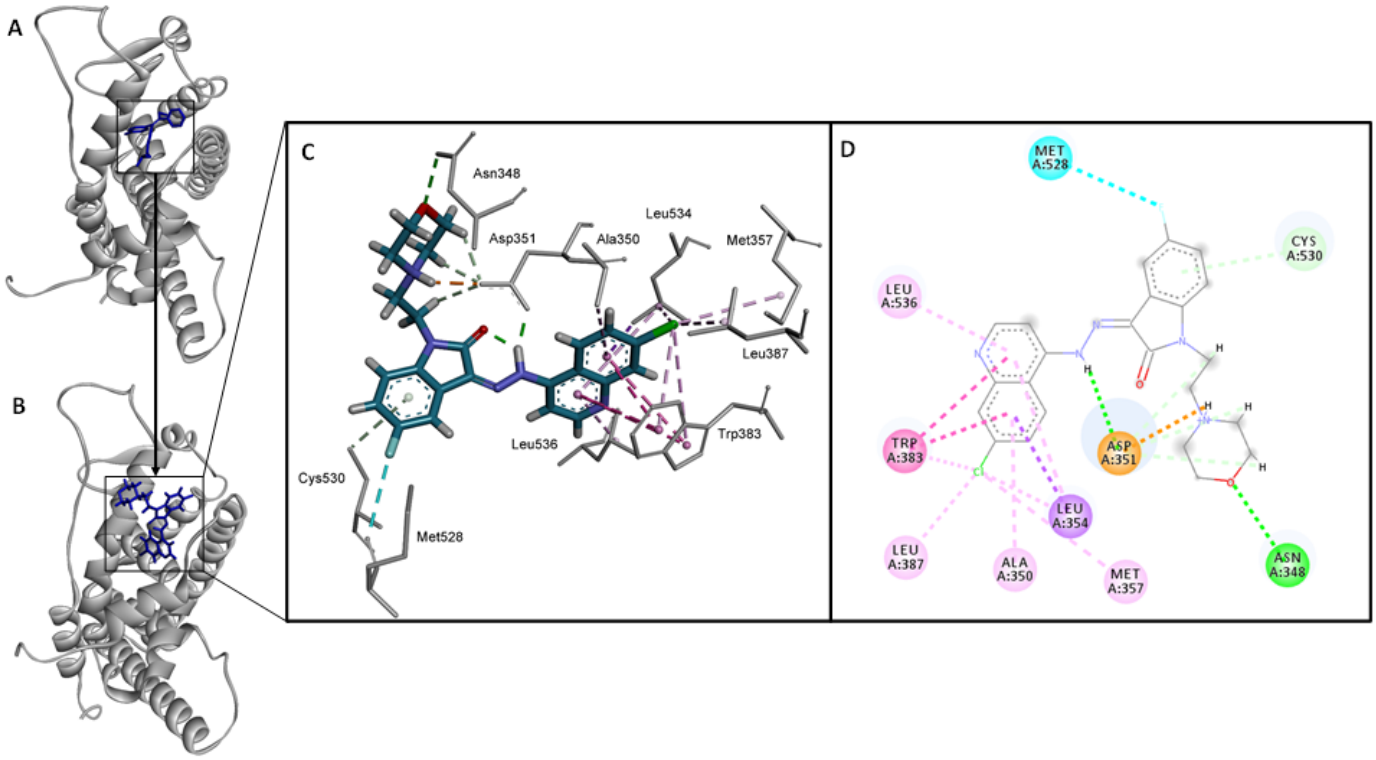

Figure 3

A. Human estrogen receptor alpha (ERα) in complex with its selective antagonist 4-hydroxytamoxifen (PDB entry: 3ERT; Resolution: 1.9 Å); B. AS-4 docked on binding site of ERα; C. 3D view of interactions of AS-4 with residues of binding site of ERα; D. 2D view of interactions of AS-4 with residues of binding site of ERα.

Supplementary Files

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