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Design, synthesis, molecular docking and biological activity evaluation of some novel indole derivatives as potent anticancer active agents and apoptosis inducers

Ahmed M. Sh. El-Sharief[a], Yousry A. Ammar[a,*], Amany Belal[b,c,*], Marwa A.M. Sh. El-Sharief[d,e], Yehia A. Mohamed[a], Ahmed B.M. Mehany[f,*], Gameel A.M. Elhag Ali[a], Ahmed Ragab[a,*]

[a] Chemistry Department, Faculty of Science, Al-Azhar University, Nasr City, Cairo 11284, Egypt
[b] Medicinal Chemistry Department, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62514, Egypt
[c] Pharmaceutical Chemistry Department, College of Pharmacy, Taif University, Taif 21974, Saudi Arabia
[d] Applied Organic Chemistry Department, National Research Centre, Cairo, Egypt
[e] Faculty of Science and Arts, Mohail Asser, King Khalid University, Saudi Arabia
[f] Zology Department, Faculty of Science, Al-Azhar University, Nasr City, Cairo 11284, Egypt

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ABSTRACT

Reaction of 5-morphilinosulfonylisatin **(1)** with acetophenones **(2a–e)** afforded 3-hydroxy-3-substituted-2-oxoindoles **3a-e**, when treated with acetic acid the expected 3-phenacylidene-2-oxoindoles **4a-d** and 4-hydroxy-5′-(morpholinosulfonyl) spiro [chromene-2, 3′-indolin]-2′-one **6** were obtained. Isatin derivative **(1)** was stirred with cyano derivatives to produce the arylidines **(7a-c)**, while under reflux condition, it gave pyrrolo[2,3–*b*] indoles **(8, 9)**. Moreover, istain **(1)** reacted with pyrazolo-5-one or 3-substituted phenol in presence of malononitrile to afford spiroxindole derivatives **(10a,b)** and **(11a,b)**. Also, compounds **(10a,b)** and **(11a,b)** were obtained through cyclization of **(7a)** with pyrazolo-5-one or 3-substituted phenol. The obtained compounds were identified by IR, ^{1}H NMR, ^{13}C NMR and elemental analysis. Anticancer activity against three cancer cell lines (HepG-2, HCT-116 and MCF-7) were evaluated using sulforhodamine B assay method. Compounds **4b**, **4c**, **7a**, **7c** and **9** showed broad spectrum anticancer activity on the three tested cell lines with IC_{50} values less than 10 μM. Cell cycle analysis was performed for the most promising derivatives, compounds **4b** and **7c** arrested HepG-2 cells at G2-M phase, while compounds **7a** and **9** accumulated cells at G0-G1 phase, all of them induced apoptosis at priG1 phase in the range of (11.32–19.17%). Additionally compounds **4b**, **7a** and **9** showed more potent activity against EGFR than Lapatinib, their IC_{50} values are from 0.019 to 0.026 μM while IC_{50} of Lapatinib is 0.028 μM. Molecular docking studies were conducted to investigate the binding mode, amino acid interactions and free binding energy of these potent derivatives.

1. Introduction

With the ecological changes and environmental deterioration, cancer has become the second cause of mortality in the world. Development of effective and specific anticancer agents is urgently needed because of severe toxicity and resistance associated with the existing drugs. Anticancer agents usually exert their biological effect through various intracellular targets; therefore, current research mainly focuses on therapeutic targets involved in cell proliferation. However, identification of the exact target for particular class of compounds is one of the challenges [1–4]. These circumstances are driving the attention of researchers to develop new moieties with required characteristics. Minimum side effects and maximum therapeutic effect with least dose, may be regarded as the ideal requirements of cancer chemotherapy. Many studies revealed that isatin is a privileged lead molecule for scheming potential bioactive agents, and their derivatives constitute an important class of heterocyclic compounds and are shown to possess a broad spectrum of bioactivity [5–11]. The indigoids are a group of isatins that have lately emerged as a promising scaffold for anticancer activity. Furthermore, indole substituted heterocycles at the 3-position are considered as an interesting bioactive natural products and pharmaceutical compounds [12,13]. Especially, some isatin

* Corresponding authors at: Medicinal Chemistry Department, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62514, Egypt (A. Belal).
E-mail addresses: yossry@yahoo.com (Y.A. Ammar), abilalmoh1@yahoo.com (A. Belal), abelal_81@yahoo.com (A.B.M. Mehany), ahmed_ragab@azhar.edu.eg (A. Ragab).

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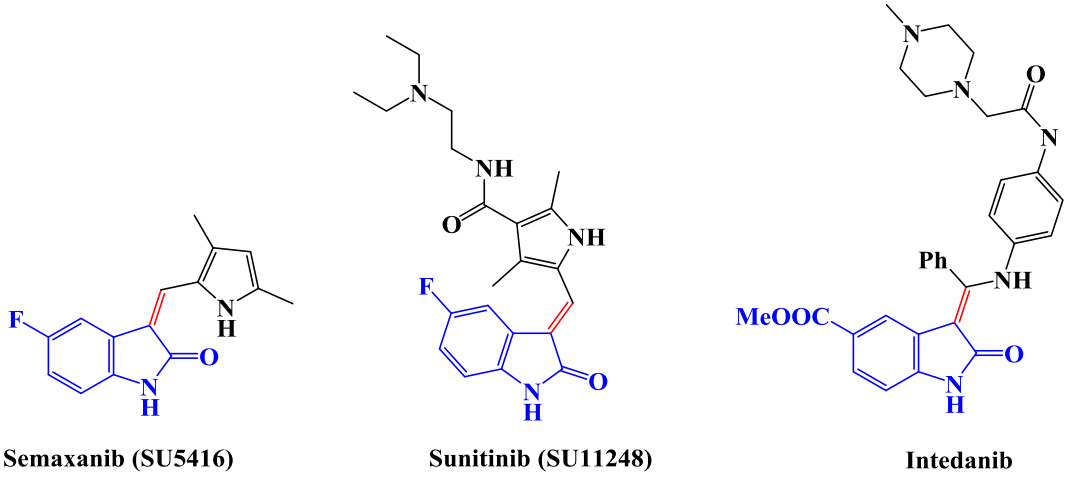


Fig. 1. Some commercial anticancer drugs with indole moiety.

derivatives have been developed as commercial anticancer drugs such as Sunitinib, Intedanib, Semaxanib (Fig. 1) [14,15].

In addition, the analogous of isatin derivatives displayed inhibitory activity against eLF2 kinase activator [16], TNF-α, CDK2 [17] and SARS protease [18]. Furthermore, isatin incorporating a quaternary carbon center at the C_3 position are omnipresent in nature and exploited as building blocks for alkaloid synthesis as well as development of possible therapeutic agents [19]. Spirooxindole compounds were evaluated for in vitro cytotoxicity against a panel of five human cancer cell lines including lung (A-549), CNS (SK-*N*-SH), breast (MCF-7), liver (Hep-2) and prostate (DU-145) by using MTT assay [20,21]. The spiro isatin represents the main scaffold in natural alkaloids such as horsifikine, spirotryprostatine A and B, elacomine, etc. (Fig. 2) and also acts as effective non-peptide inhibitor of the p53-MDM2 interactions [22].

Indeed, sulfonamides constitute an important class of compounds having different pharmacological applications in the treatment of a wide variety of diseases [23–25]. They have also been reported to possess various types of biological properties such as anticancer [26], antibacterial [27], antimicrobial [28], antimalarial [29], antihypertensive [30], antitumor [23]. Their anticancer activity was found to take place through a variety of mechanisms such as disruption of the microtubule assembly, cell cycle arrest at G1 phase, functional suppression of the transcriptional activator NF-Y, angiogenesis and carbonic anhydrase inhibition [24,25]. The most prominent mechanism was the inhibition of carbonic anhydrase isozymes [31,32]. In view of these observations and for continuation of our medicinal program [33–39], that aims at the discovery of novel biologically important heterocyclic compounds, we synthesized a new class of isatin sulfonamide conjugates and spiroisatins, hoping to obtain novel functional molecules with potent antiproliferative effects on tumor cells as the results may provide useful information for design of novel chemotherapeutic drugs. The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases and is upregulated in many cancer cell types, including breast cancer, head and neck squamous cell carcinoma (HNSCC) [40]. In our previous study we have noticed the following: substitution of morpholino isatin at position 3 improved the anticancer activity [41]. In addition, comp. **I** (Fig. 3) showed inhibition of EGFR at nanomolar range. These facts encouraged us to continue our previous work by designing (Fig. 3) and synthesizing the morpholinoisatin scaffold and substitute it at position 3 with different bioactive pharmacophores, fusing it with other bioactive heterocycles and finally designing spiro derivatives of this scaffold hoping to obtain more active anticancer derivatives with potential activity against EGFR TKs.

2. Results and discussion

2.1. Chemistry

Synthesis of some new 2-oxoindole, Pyrrolo[2,3–*b*]indoles and spiroindole derivatives having sulfonamide moiety were obtained through chalone intermediates, these derivatives have a great influence on structure activity relationships [42]. 5-morphilinosulfonylisatin **(1)** was prepared *via* chlorosulfonation of isatin followed by nuceophillic substitution of morpholine according to literature procedure [41]. In the present study, 5-morphilinosulfonylisatin **(1)** was used as starting material to prepare the new derivatives (Schemes 1–3). Reaction of 5-morphilinosulfonylisatin **(1)** with acetophenone derivatives **(2a-e)** in methanol and N,N-diethyl amine (DEA) as a catalyst gave 3-hydroxy-3-substituted 2-oxindoles **(3a-e)**, where the desired product were obtained by addition of acetyl group carbanion to the deficient carbonyl

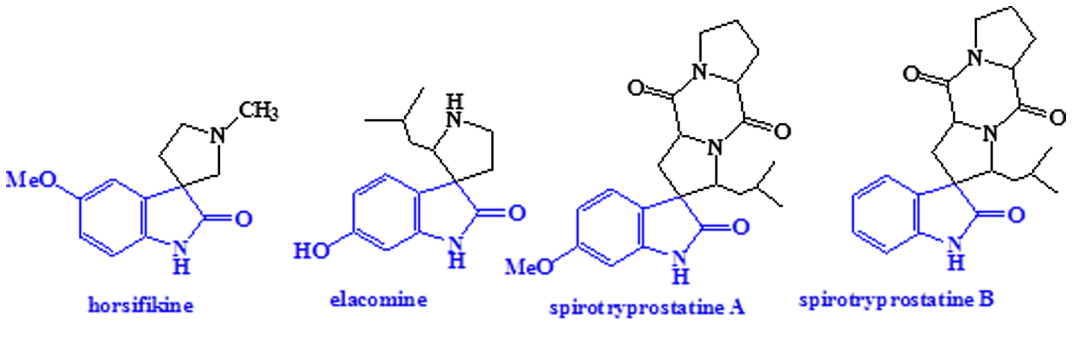


Fig. 2. Alkaloids with spiro indole scaffold.

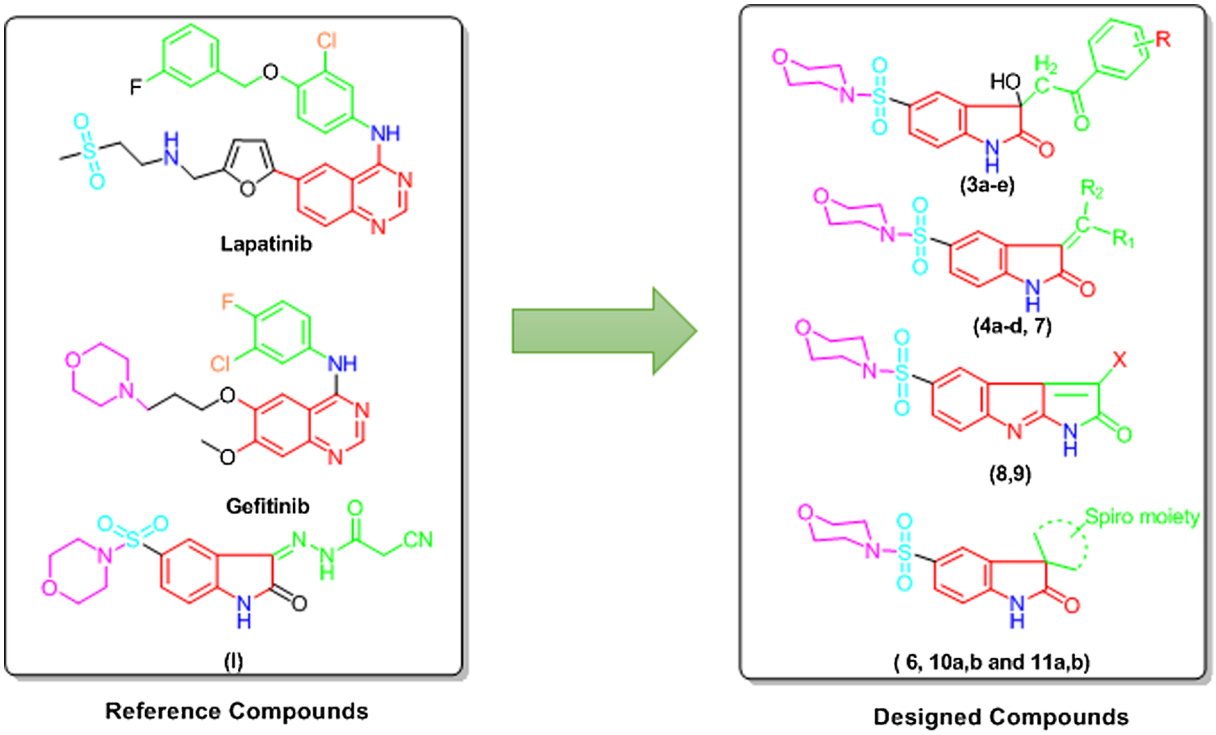
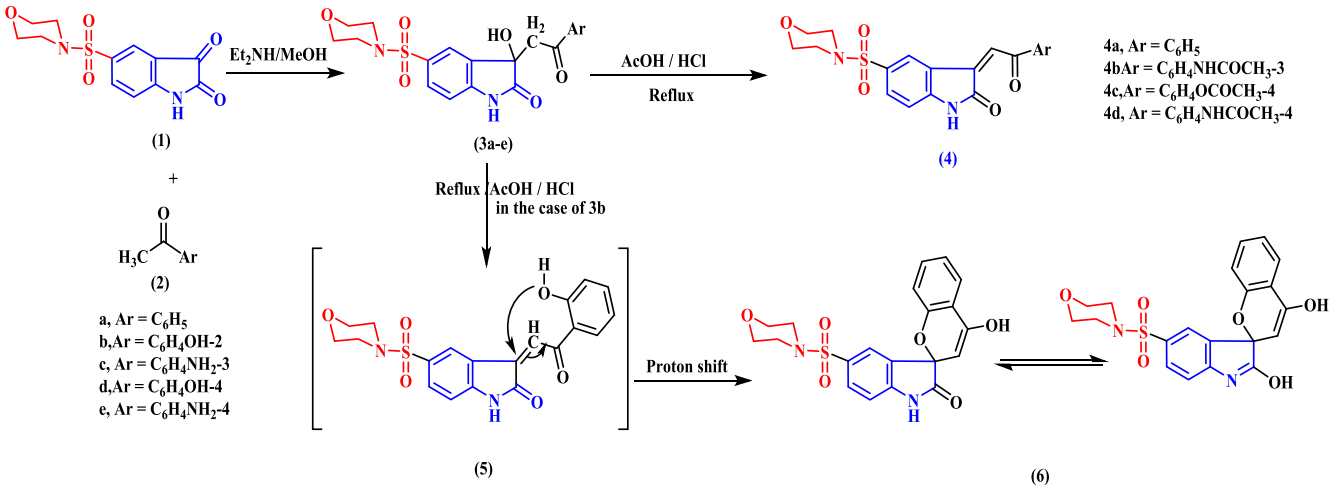


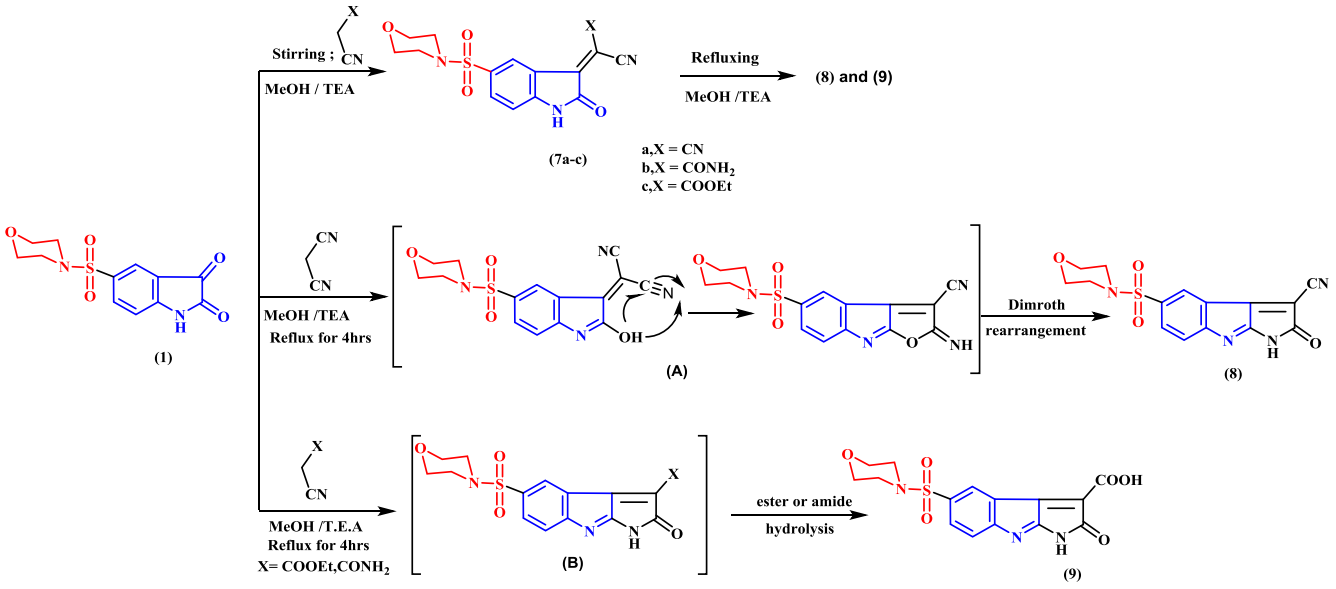
Fig. 3. Rational design of the target Indole derivatives Bearing Sulfonamide Moiety.

function of isatin, the structure of compounds **(3a-e)** were ascertained by spectral data. The IR spectrum of compound **(3b)** showed absorption bands at 3223, 3112 and 1715 cm^{-1} corresponding to OH, NH and carbonyl groups. Its ^{1}H NMR spectrum (DMSO-d_6) showed four triplets at δ 2.67, 2.73, 3.55 and 3.58 ppm corresponding to morpholine protons, two doublet signals at δ 3.7, 4.32 ppm corresponding to $-CH_2$ protons, Furthermore, three singlet signals at δ 6.38, 10.85, 11.22 ppm for 2 OH and NH groups respectively. ^{13}C NMR spectrum of **(3b)** displayed signals for morpholine, CH_2, C3-indolinone carbon at 46.26, 65.61, 47.82, 73.17 in addition to aromatic carbons and two C=O carbons at 178.77, 202.17 ppm. In the same manner, ^{13}C NMR spectrum of **(3d)** displayed signals at δ 46.22, 65.59 related to morpholin, 47.42 for methylene group, 73.3 (C_3-OH), (Ar-C) at 111.82–149.72, (Ar-C-OH) at 165.88 and 177.78, 197.87 for two carbonyl groups (Scheme 1).

3-Phenacylidene-2-indolinone derivatives **(4a-d)** were obtained from **(3a,c-e)** via aldol condensation, spectral data illustrated that both the terminal amino and hydroxy groups underwent acetylation **(3b-e)** (Scheme 1). Structures of **(4a-d)** were confirmed by spectral data and elemental analyses. IR spectrum of **4a** displayed stretching signals for NH and 2CO at 3175, 1715 & 1660 cm^{-1}. The ^{1}HNMR spectrum (DMSO-d_6) of **(4a)** exhibited a lack of $-CH_2$ and $-$OH protons and showed morpholinyl, methine and NH protons at δ 2.77, 3.62, 8.33 and 11.35 ppm. Also, ^{13}C NMR spectrum for this derivative showed presence of morpholinyl, aromatic and methine carbons at δ 46.21 and 65.69, 111.27–149.2 as well as two singlet signals at δ 168.61, 191.81 ppm related to two carbonyl groups. The spectroscopic data agreed with the assigned structure of acetylated derivatives **(4b-d)**. For example, IR spectrum of **(4c)** showed characteristic stretching frequencies for NH, 2C=O, and C=N at 3119 cm^{-1} 1731, 1715 cm^{-1} 1613 cm^{-1}. ^{1}H NMR spectrum (DMSO-d_6) of **4d** showed a singlet signal at δ 2.09 ppm for methyl protons, four triplet signals at δ 2.69, 2.92 and 3.61, 3.68 ppm for morpholinyl protons, multiplet aromatic and methine protons signals at δ 6.65–8.23 in addition to two singlet signals



Scheme 1. Synthesis of 2-oxindole derivatives **(3a-e)**, 3-Phenacylidene-2-indolinone **(4a-d)** and spiro derivatives **(6).**



Scheme 2. Synthesis of arylidine of 2-oxoindole derivatives **(7a-c)** and pyrolo[2,3–*b*]indoles derivatives **8** and **9**.

for 2 NH at δ 10.43 and 11.32 ppm.

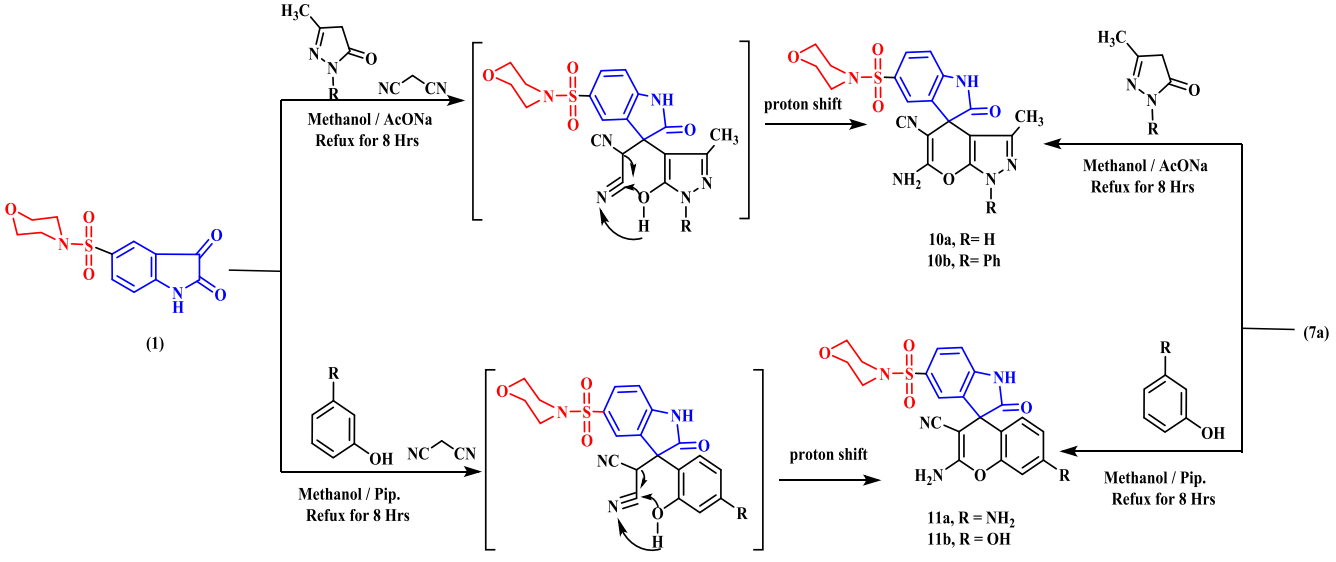
As shown in Scheme 1, compound **(3b)** was heated under reflux in acetic acid containing drops of hydrochloric acid to afford the spiro 2-oxoindole **(6)**; the hydroxyl group in ortho position was attacked by β-carbon of chalcone double bond to afford the intermediate **(5)** which tautomerized to form spiro structure **(6)** (Scheme 1). IR spectrum of **(6)** showed significant stretching frequencies at 3386, 3103, 1718 and 1614 cm^{-1} related to −OH, NH, CO, and C=N, respectively. ^{1}H NMR spectrum (DMSO-d_6) of **(6)** showed two singlet signals at δ 8.81, 9.18 ppm for OH, −NH protons, 2 triplet signals for morpholine protons at δ 2.99 and 3.66 ppm, multiplet aromatic and methine protons at δ 7.03–8.38 ppm. Its ^{13}C NMR spectrum exhibited three singlet signals at δ 46.31, 65.82, 87.17 ppm for morpholine and spiro carbons, signals at δ 118.35–160.19 ppm related to aromatic carbons and a singlet signal at 167.04 due to carbonyl group.

Our work was extended to study the reactivity of isatin derivatives **(1)** towards some active acetonitrile derivatives under different reaction conditions. Thus, when isatin derivatives **(1)** stirred with malononitril, ethyl-cyanoacetate or cyanoacetamide in methanol catalyzed with triethyl amine (TEA) at room temperature afforded the corresponding arylidine of 2-oxoindole derivatives **(7a-c)** (Scheme 2). Whilst, refluxing **(1)** with acetonitrile derivatives under the same reaction conditions afforded pyrrolo[2,3–*b*]indoles derivatives **(8)** and **(9)** (Scheme 2).

The chemical structure of **(7)** was confirmed by spectral and elemental analyses. IR spectrum of **(7a)** showed characteristic bands for NH, (CN) and C=O at the corresponding frequencies 3371, 2236 and 1741 cm^{-1}. ^{1}H NMR spectrum (DMSO-d_6) of **(7a)** exhibited two triplets at δ 2.92, 3.66 ppm assignable to morpholine protons, three singlet signals at δ 7.19, 7.92, 8.18 ppm appear as one singlet and two doublets due to aromatic protons and singlet signal at 11.76 related to −NH of isatin. In addition, its ^{13}C NMR exhibited three signals at δ 46.22, 65.73 and 164.27 ppm due to morpholine moiety and C=O carbons, in addition to signals between 119.66 and 150.23 ppm corresponding to aromatic carbons.

On the other hand, upon treatment of 5-morphilinosulfonylisatin **(1)** with malononitrile in methanol and in the presence of triethylamine as a catalyst under reflux conditions caused cyclization and Dimroth rearrangement to afford the corresponding Pyrrolo[2,3–*b*]indole **(8).**

On the same manner carboxylic acid derivative **(9)** was obtained by



Scheme 3. Synthesis of spiroindole derivatives **10a,b** and **11a,b.**

refluxing 5-morphilinosulfonylisatin **(1)** with ethyl cyanoacetate or cyanoacetamide, however, hydrolysis occurred to give carboxylic acid derivative (**9**) as shown in Scheme 3. Moreover, Pyrrolo[2,3–*b*]indoles **(8)** and **(9)** can also be obtained through refluxing **(7a-c)** in MeOH/TEA mixture. The mechanistic equations for formation of pyrrolo[2,3–*b*]indoles **(8)** and **(9)** are illustrated in Scheme 2. IR spectrum of **(8)** showed an absorption bands at 3396, 2201 and 1761 cm^{-1} due to NH, CN and −C═O functions. ^{1}H NMR spectrum (DMSO-d_6) of **(8)** exhibited two triplets and singlet signals for morpholinyl and NH protons at δ 2.92, 3.67 and 11.75 ppm. In case of amide and ester, the IR spectrum of **(9)** showed lake of (CN), COOEt and $CONH_2$ groups and broad OH group appeared due to presence of carboxyl group.^{1}H NMR spectrum of **(9)** showed two singlet signals at δ 9.51, 11.44 ppm related to NH and OH groups, in addition to characteristic signals for morpholine and aromatic carbons 46.33, 65.76 and 110.58–133.93 ppm respectively.

Multi-component reactions (MCRs) have emerged as a powerful tool for the construction of novel and complex molecular structures due to their advantages over conventional multi-step synthesis [43,44]. Therefore. Heating 5-(morpholinosulfonyl)isatin **(1)**, malononitrile and either 5-methyl-2,4-dihydro-3H-pyrazol-3-one derivatives, or m-aminophenol or resorcinol in a molar ratio as a ternary mixture in absolute methanol containing catalytic amount of a base afforded the expected spiroindoles **(10a,b)** and **(11a,b)**. The chemical structure of **(10a,b)** and **(11a,b)** was in coincidence with spectral and elemental analyses. IR spectrum of **(10a)** as a representative example displayed stretching absorbing bands at 3441, 3339, 3217, 2190, 1712 attributed to NH_2, NH, CN and CO. Its ^{1}H NMR spectrum (DMSO-d_6) showed singlet signal for methyl at δ 1.57 ppm, multiplied signals at δ 7.19–7.7 ppm for 5H aromatic and NH_2 protons, in addition to two singlet signals at δ 11.17, 12.41 ppm indicating the presence of 2NH, ^{13}C NMR spectrum of these compound exhibited two signals for morpholinyl at δ 46.40, 65.68 as well as CH_3 at δ 9.48 and spiro carbon at δ 47.79 ppm, in addition to Ar-C at δ 110.82–155.3 ppm, finally C_6-NH_2 appeared at δ 178.65.

Structures of spiroindoles **(10a,b)** and **(11a,b)** were obtained through refluxing of **(7a)** with either 5-methyl-2,4-dihydro-3H-3-oxo-pyrazole or phenol derivatives under the same conditions. ^{1}H NMR spectrum (DMSO-d_6) of **(11a)** revealed two triplets for morpholinyl moiety at δ 2.79, 3.58 ppm, three singlet signals related to two NH_2 and NH at δ 5.43, 7.21 and 10.93 respectively where two amino groups attached to C_7 and C_2 on chromene moiety.^{13}C NMR spectrum of **(11a)** revealed 46.28, 65.67 (morpholinyl carbons), 50.32 (C-Spiro), 53.67 (C-CN), as well as aromatic carbon at δ 100.62–150.02 ppm and (−C_7-NH_2), (−C═O), (C_2-NH_2) at δ 150.48, 162.26, 180.01 ppm respectively. The postulated mechanism for the reaction of compound **(7a)** with either pyrazole or phenol derivatives is illustrated in Scheme 3.

2.2. Biological evaluation

2.2.1. Antiproliferative activity

The target compounds were evaluated for the cytotoxicity against three cancer cell lines including human hepatocellular carcinoma cell line **(HepG-2)**, colon carcinoma cell line **(HCT-116)** and mammary gland breast cancer cell line **(MCF-7)** using sulforhodamine B colorimetric (SRB) cell proliferation assay, doxorubicin was used as a reference, the obtained IC_{50} values are expressed in μM and represented in Table 1.

The target compounds showed moderate to excellent cytotoxic activity against the three tested cell lines, Compounds **9** and **4c** exhibited the best activity against HepG-2 and HCT-116 with IC_{50} values of (3.63 ± 0.07) and (5.07 ± 0.12) for compound **9** and (4.41 ± 0.12) and (3.55 ± 0.09) μM for compound **4c** which were more active than doxorubicin. Most of the tested compounds have shown significant selectivity towards HepG-2 and HCT-116 more than MCF-7 cell line.

The results indicated that the 3-Phenacylidene-2-indolinone **(4a-d)** derivatives were more active than the corresponding 2-oxoindole **(3a-e)** analogues. This can be attributed to conjugation effect (chalcone system), and substitution in position 4 for arylidine of 3-Phenacylidene-2-indolinone **(4a-d)** causes more activity than position 3 and unsubstituted derivatives (Table 1). As found in Table 1, it is readily observed that there is a significant difference in cytotoxic activity when various alkyl groups are introduced into the phenyl ring of acetophenone derivatives in **3** and **4**. The Presence of hydroxy group in 2-oxoindole derivatives affected its activity, so introduction of another hydroxyl group is required to study structure activity relationship. Introducing second hydroxy group to aryl group of 3-hydroxy-5-(morpholinosulfonyl)-3-(2-oxo-2-arylethyl)indolin-2-one in **3b** and **3d** causes an increase in activity in position 4 than 2 of aryl that occure in 3-substituted indole (10.53 ± 0.4, 12.66 ± 0.51, 17.18 ± 0.72) and (108.52 ± 1.08, 63.63 ± 2.5, 124 ± 4.8) μM respectively on all cell lines and replacement of hydroxy group in position 4 of aryl of (3-hydroxy-5-(morpholinosulfonyl)-3-(2-oxo-2-arylethyl)indolin-2-one) by amino group **3e** enhanced the activity (10.79 ± 0.51, 8.74 ± 0.33, 13.34 ± 0.58) μM.

Spiro structure **6** with chromene-2,3′-indolin, showed moderate anticancer activity, with IC_{50} value less than 35 μM against all the tested cell line (34.39 ± 0.75, 26.67 ± 1.1, 32.63 ± 1.21) μM. Arylidine of 2-oxoindoles derivatives **(7a,c)** showed excellent cytotoxic activity, they showed a broad-spectrum activity against all the tested cell lines. Pyrrolo[2,3–*b*]indoles **(8)** and **(9)** exhibited to be the best active compounds of the series especially dihydropyrrolo[2,3–*b*]indole-3-carboxylic acid **(9)** which is considered the highest cytotoxic compound among all the tested compounds in this study; where (**9**) showed results better than the reference drug on HepG-2, HCT-116 and near to it on MCF-7. Spiroindoles **(10a,b)** and **(11a,b)** showed poor activity against all the tested cell lines. Most of synthetized compounds showed good activity than isatin itself. Finally, the presence of chalcone system **(4a-d)** especially **4b**, **4c** and carboxyl group in Pyrrolo[2,3–*b*]indoles derivatives **(9)** enhanced the antitumor activity and led to results near to or better than the reference drug.

Table 1
Cytotoxic activity of the synthesized compounds against cancerous cell lines.

Sample	IC_{50} (μM) ± S.E.		
	HEPG-2	HCT-116	MCF-7
1	28.54 ± 2.5	59.97 ± 0.09	67.93 ± 0.19
3a	123.77 ± 2.14	149.71 ± 3.1	113.39 ± 4.5
3b	108.52 ± 1.08	63.63 ± 2.5	124 ± 4.8
3c	89.97 ± 1.11	70.69 ± 3.11	80.88 ± 3.01
3d	10.53 ± 0.4	12.66 ± 0.51	17.18 ± 0.72
3e	10.79 ± 0.51	8.74 ± 0.33	13.34 ± 0.58
4a	8.59 ± 0.31	7.99 ± 0.37	11.36 ± 0.44
4b	6.09 ± 0.32	5.32 ± 0.21	9.56 ± 0.47
4c	4.41 ± 0.12	3.55 ± 0.09	6.67 ± 0.36
4d	6.26 ± 0.22	8.24 ± 0.32	14.46 ± 0.62
6	34.39 ± 0.75	26.67 ± 1.1	32.63 ± 1.21
7a	6.08 ± 0.34	5.96 ± 0.22	6.34 ± 0.21
7b	8.62 ± 0.3	8.31 ± 0.25	11.87 ± 0.41
7c	5.19 ± 0.14	7.70 ± 0.34	6.39 ± 0.22
8	4.42 ± 0.09	10.29 ± 0.33	13.11 ± 0.44
9	3.63 ± 0.07	5.07 ± 0.12	6.64 ± 0.25
10a	59.84 ± 2.11	68.19 ± 2.1	118.39 ± 3.9
10b	29.05 ± 1.5	25.31 ± 1.4	33.75 ± 1.8
11a	77.15 ± 2.1	65.74 ± 1.8	85.14 ± 3.1
11b	95.92 ± 2.2	99.93 ± 2.7	123.52 ± 4.8
Isatin	32.31 ± 0.45	21.56 ± 0.37	41.83 ± 0.67
Doxorubicin	5.59 ± 0.55	7.03 ± 0.21	4.89 ± 0.47

Three independent experiments were performed for each concentration.

2.2.2. In-vitro flow cytometric (cell cycle) analysis

Anticancer drugs can motivate cell death through obstruction accompanied by cell-cycle mechanism, while others follow cell death approach through programmed pathway [45]. Measuring cell cycle arrest and induction of apoptosis in different phases to gain evidence

Table 2
Results of cell cycle analysis in HepG2 expressed by (%) of cells in each phase when treated with compounds **4b**, **7a**, **7c** and **9**.

Compound no.	Results			
	%G0-G1	%S	%G2-M	%Pre-G1
4b	51.13	13.65	35.22	11.32
7a	63.01	23.77	13.22	14.41
7c	63.52	14.47	22.01	13.25
9	76.22	8.53	15.25	19.17
HepG-2 cells	58.77	25.01	16.22	2.15

regarding the role of the synthesized compounds in growth inhibition of cancer cells and induction of apoptosis was performed. Thus, we started to investigate the effect of compounds **4b, 7a, 7c** and **9** on cell cycle distribution in HepG-2 cells, they were treated with 5 μM from each compound and the obtained results were represented in Table 2 and Fig. 4.

From the previous results in Table 2 and Fig. 4, the results showed that cells were arrested in G2-M phase by compounds **4b** and **7c** (35.22, 22.01) %, while compounds **7a** and **9** accumulated cells at G0-G1 phase (63.01 and 76.22) % respectively (Table 2). The induced apoptosis by the tested compounds at priG1 phase were in the range from 11.32 to 19.17%.

2.2.3. Annexin V-FITC apoptosis assay

The mechanism of cell death was analyzed by flow cytometry method using Annexin V /propidium iodide (PI) double staining. In these studies, the tested compounds (**4b**, **7a**, **7c** and **9**) and HepG2 cells were incubated for 24 h with 5 μM from each tested compound. The obtained results were presented in Table 3 and Figs. 5 and 6. The percentage of apoptosis induced by compounds (**4b**, **7a**, **7c** and **9**) were (92.12, 92.09, 63.21, 92.57%) respectively. So that, we can say that treatment of HepG-2 cells with compounds **4b**, **7c** and **9** leads to increase in percentage of apoptotic cells from 0.67% for control untreated cells to be 79.01, 41.89 and 63.25 respectively, in early stage while percentage were 64.21% for **7a**. As well as in late stage the percentage of apoptosic cells range from 13.11 to 29.32% compared to control (0%). The results clearly indicate that the tested compounds (**4b**, **7a**, **7c** and **9**) induced apoptosis in HepG-2 cell line.

Table 3
Percent of cell death induced by compounds **4b, 7a, 7c** and **9** on HepG2 cells.

Compound no.	Apoptosis			Necrosis
	Total	Early	late	
4b	93.19	79.01	13.11	1.07
7a	92.37	64.21	27.88	0.28
7c	63.53	41.89	21.32	0.32
9	92.79	63.25	29.32	0.22
HepG-2 cells	0.69	0.67	0.0	0.12

2.2.4. In vitro EGFR inhibition assay

The broad spectrum anticancer active agents **4b, 7a, 7c** and **9** (their IC_{50} values were less than 10 μM on the three tested cell lines) were evaluated for inhibitory activity against EGFR, lapatinib was used as standard EGFR inhibitor. The obtained IC_{50} values are reported and summarized in Table 4, compound **4** showed potent inhibitory activity, nearly 1.5 of lapatinib potency, in addition compounds **7a** and **9** showed to be more potent than the reference drug lapatinib, IC_{50} values were 0.0266, 0.0253 and 0.0283 μM respectively. Replacement of the cyano group in compound **7a** with ethyl ester group **7c** caused a dramatic decrease in its EGFR inhibitory activity, IC_{50} values were 0.0266 and 1.382 μM respectively.

3. Molecular docking studies

In this research work, molecular docking studies were carried out to get better insight into the binding modes, binding free energies and amino acid interactions of the most active derivatives into ATP binding site of EGFR. Compounds **4b**, **7a** and **9** that showed inhibitory activity against EGFR in a nanomolar scale, IC_{50} values are 19, 26 and 25 nmol respectively, were selected to be docked into EGFR binding site and their results were compared with the cocrystallized ligand erlotinib. Verification step was performed after downloading the pdb file: 1 M17, water was removed, hydrogen atoms were added, and binding site was detected, then erlotinib was removed and redocked again into the binding site, its docking score energy was −27.01 kcal/mole and the nitrogen atom of its quinazoline scaffold formed one hydrogen bond with Met769 amino acid. Our promising derivatives **4b**, **7a** and **9** when docked to the same binding site as erlotinib (Figs. 7–9) revealed binding free energy with a minus score, which indicate quick fitting into EGFR binding site, their energies were −20.61, −15.30 and −13.54 kcal/

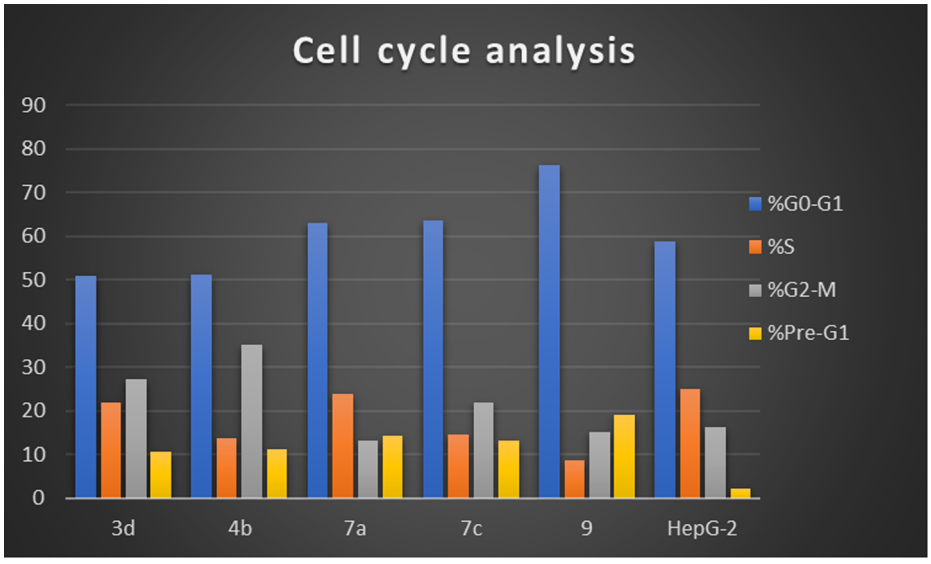


Fig. 4. Cell cycle analysis and apoptosis effect in HepG2 cell treated with compounds **4b**, **7a**, **7c** and **9**.

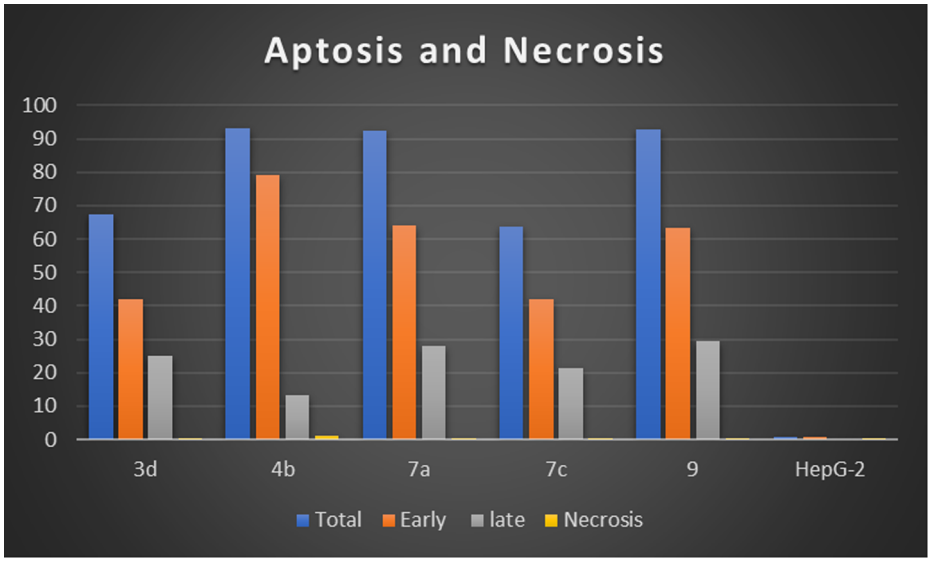


Fig. 5. Percent (%) of cell death induced by compounds **4b**, **7a**, **7c** and **9** on HepG2 cells.

mole respectively. As for amino acid interactions, compound **4b** showed one hydrogen bond with Thr766 through the oxygen atom of the morpholine moiety, in addition carbonyl group and NH group substituents at position 3 formed two additional hydrogen bonds with Cys773 and Asp776 respectively (Fig. 7). Compound **7a** showed the same amino acid interaction as erlotinib (Fig. 8) through the carbonyl group of its indole scaffold with Met769 amino acid. Compound **9** formed three hydrogen bonds (Fig. 9), one of them with Cys773 amino acid through SO_2 group and two hydrogen bonds with Lys721 amino acid through two carbonyl groups present as substituents in the pyrrolidine nucleus which was fused with our indole scaffold. These obtained results confirm the promiscuity of these derivatives to form good binding with EGFR binding site and this may help us understand their good activity against EGFR TKs.

4. Conclusions

Herein, the authors have reported an expeditious strategy utilizing 5-(morpholinosulfonyl) indoline-2,3-dione as a key starting material to construct 2-oxoindoles derivatives, fused pyrrole[2,3–*b*]indoles and spiro indole derivatives. Spiro derivatives showed the least anticancer activity of the synthesized indole derivatives. However, the fused derivative **9** in addition to 3-substituted derivatives **4b**, **7a** and **7c** showed potent anticancer activity on the three tested cell lines. Moreover, compounds **4a**, **7a** and **9** showed to be more potent than lapatinib against EGFR, their molecular docking studies supports their good activity towards these kinases. Cell cycle was arrested at G2/M phase by compounds **4b** and **7c**, at G0-G1 phase by compounds **7a** and **9**. In addition all four promising derivatives showed to induce apoptosis at pri-G1 phase.

5. Experimental

All solvents and reagents were freshly distilled and purified according to standard procedures. All melting points are recorded on digital Gallen Kamp MFB-595 instrument and are uncorrected. The IR spectra (KBr) (cm^{-1}) were measured on a Shimadzu 440 spectrophotometer. ^{1}H NMR spectra (δ, ppm) were obtained in deutrated dimethyl sulfoxide (DMSO-d_6) and ^{13}C NMR at 125 MHz, spectra were obtained on a Varian Gemini 500 (500 MHz) spectrometer, using TMS as an internal standard; chemical shifts are reported as δ ppm units. The data were presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, app = apparent), coupling constant(s) in Hertz (Hz), and integration. Mass spectra were recorded on Thermo Scientific ISQLT mass spectrometer at the Regional Center for Mycology and Biotechnology, Al-Azhar University. Elemental analyses were carried out at Micro Analytical Unit, Cairo University.

5.1. Chemistry

5.1.1. Synthesis of 3-hydroxy-5-(morpholinosulfonyl)-3-(2-oxo-2-arylethyl)indolin-2-one (3)

A mixture of 5-(morpholinosulfonyl)isatin **(1)** (0.01 mol) and acetophenone derivatives **(2a-e)** (0.01 mol) was dissolved in methanol (10 mL) containing (3.0 mL) of diethyl amine in a 100 mL conical flask and the mixture was stirred at room temp., until the solid product so formed (the progress of the reaction was monitored by TLC). The product formed was collected by filtration and crystallized from the proper solvent.

5.1.2. 3-Hydroxy-5-(morpholinosulfonyl)-3-(2-oxo-2-phenylethyl)indolin-2-one (3a)

Yield (81%) as yellow crystals from ethanol; mp: 260–262 °C; IR: ν/cm^{-1}:3219 (br OH), 3109 (NH-isatin), 3052 (CH-Ar), 2961, 2909, 2851 (CH-aliph.), 1715 (C=O-isatin), 1680 (C=O-acetyl); ^{1}H NMR: δ/ppm 2.66, 2.73 (2t, 4H, J = 3.0 Hz, N-2CH_2), 3.49, 3.55 (2t, 4H, J = 3.25 Hz, O-2CH_2), 3.64 (d, 1H, J = 17.5 Hz), 4.32 (d, 1H, J = 18.0 Hz), 6.35(s, 1H, OH, exchangeable with D_2O), 7.06 (d, 1H, J = 8.0 Hz, Ar-H), 7.49–7.52 (m, 2H, Ar-H), 7.59 (d, 1H, J = 8.5 Hz, Ar-H), 7.64–7.68 (m, 2H, Ar-H), 7.89–7.91 (m, 2H, Ar-H), 10.83 (s, 1H, NH; exchangeable with D_2O); ^{13}C NMR: 46.24 (N-2CH_2), 47.12 (CH_2), 65.6 (O-2CH_2), 73.21 (C-OH), 109.05, 118.35, 119.95, 120.05, 122.94, 127.21, 128.46, 129.21, 136.31, 138.72, 141.88, 147.8 (C-OH), 177.04, 197.98 (C=O); MS: (Mwt.: 416): m/z, 416 [M^+, (19.68%)], 77 (100%); Anal. Calcd for $C_{20}H_{20}N_2O_6S$ (416.10): C, 57.68; H, 4.84; N, 6.73; Found: C, 57.52; H, 4.95; N, 6.76%.

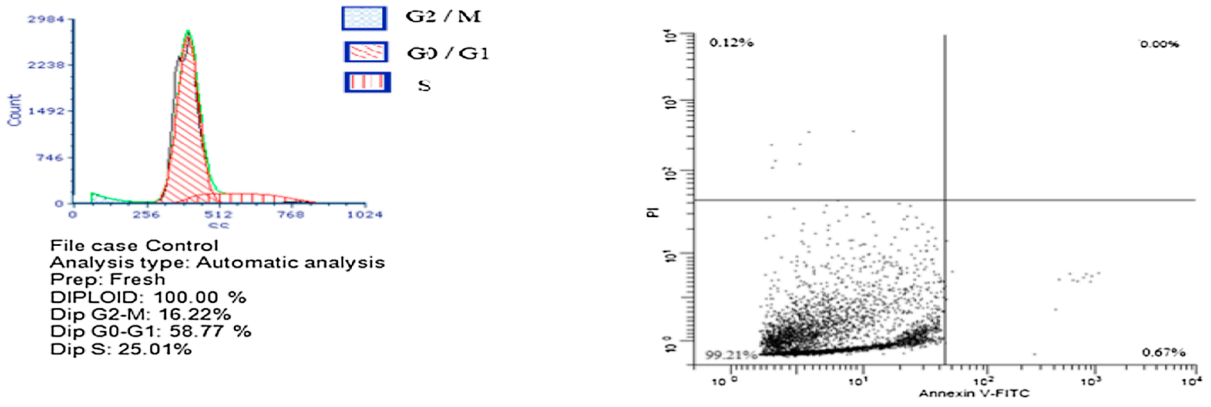
5.1.3. 3-Hydroxy-3-(2-(2-hydroxyphenyl)-2-oxoethyl)-5-(morpholinosulfonyl) indolin-2-one (3b)

Yield (65%) as yellow crystals from ethanol; mp: 289–291 °C; IR: ν/cm^{-1}: 3223 (br OH), 3112 (NH-isatin), 3050 (CH-Ar), 2965, 2892, 2851 (CH-aliph.), 1715 (br C=O-isatin, acetyl); ^{1}H NMR: δ/ppm 2.67, 2.73 (2t, 4H, J = 2.75 Hz, N-2CH_2), 3.55, 3.58 (2t, 4H, J = 3.25 Hz, O-2CH_2), 3.7 (d, 1H, J = 17.5 Hz), 4.32 (d, 1H, J = 18.0 Hz), 6.38 (s, 1H, OH, exchangeable with D_2O), 6.91–6.94 (m, 2H, Ar-H), 7.07 (d, 1H,

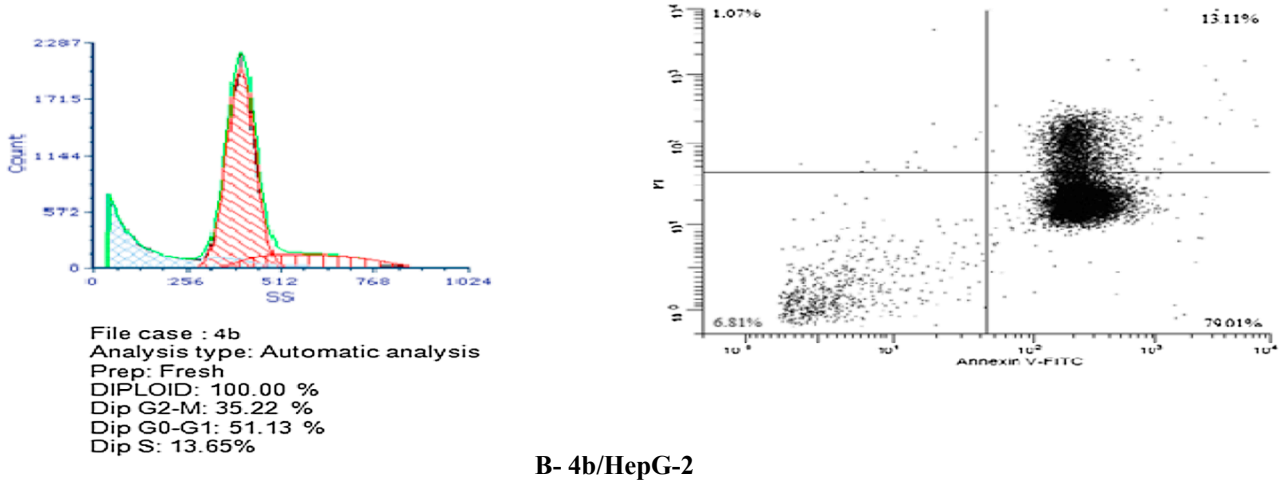
J = 8.5 Hz, Ar-H), 7.48 (s, 1H, Ar-H), 7.59–7.65 (m, 2H, Ar-H), 7.76 (d, 1H, J = 8.0 Hz, Ar-H), 10.85, 11.22 (2s, 2H, NH, OH; exchangeable with D_2O); ^{13}C NMR: 46.26 (N-2CH_2), 47.82 (CH_2), 65.61 (O-2CH_2), 73.17 (C-OH), 110.21, 118.01, 119.71, 121.63, 126.73, 126.98, 130.48, 131.08, 133.00, 136.44, 147.61, 160.27 (C-OH), 178.77, 202.17 (C=O); Anal. Calcd for $C_{20}H_{20}N_2O_7S$ (432.45): C, 55.55; H, 4.66; N, 6.48; Found: C, 55.19; H, 4.53; N, 6.67%.

*5.1.4. 3-(2-(3-Aminophenyl)-2-oxoethyl)-3-hydroxy-5-(morpholinosulfonyl) indolin-2-one (**3c**)*

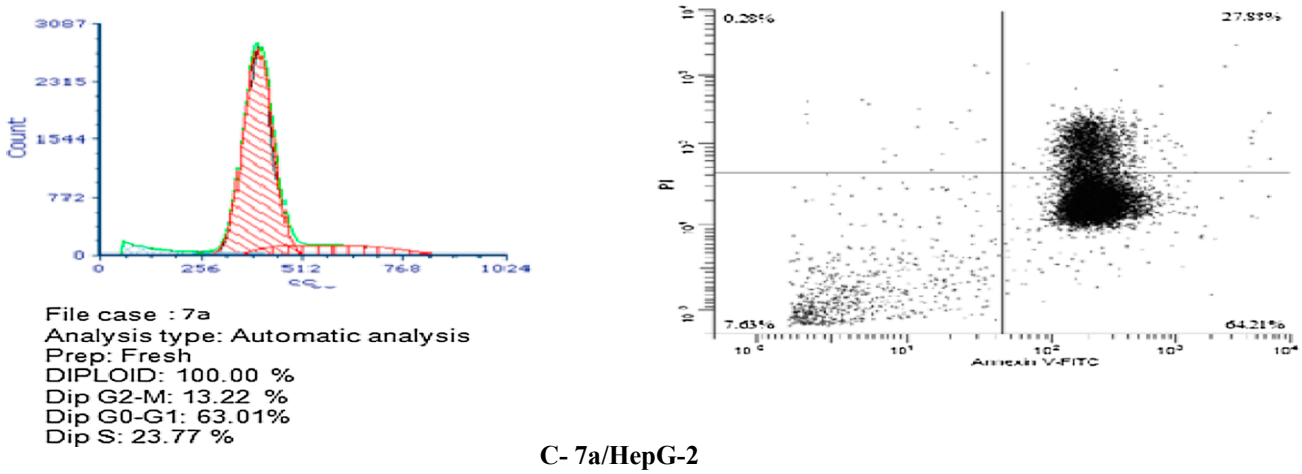
Yield (71%) as yellowish orange powder from ethanol; mp: 186–188 °C; IR: ν/cm^{-1}: 3446, 3218, 3127 (NH-isatin), 3052 (CH-Ar), 2975, 2855 (CH-aliph.), 1715, 1679 (br C=O-isatin, acetyl); ^1H NMR: δ/ppm 2.85 (t, 4H, N-2CH_2), 3.59 (t, 4H, O-2CH_2), 3.67(d, 1H, J = 16.4 Hz), 4.18 (d, 1H, J = 17.2 Hz), 5.33 (s, 2H, NH_2, exchangeable with D_2O), 6.36 (s, 1H, OH, exchangeable with D_2O), 6.79–6.81 (m, 1H, Ar-H), 7.07 (d, 1H, J = 8.0 Hz, Ar-H), 7.11 (d, 1H, J = 8.4 Hz, Ar-H), 7.15 (d, 1H, J = 8.0 Hz, Ar-H), 7.6 (d, 1H, J = 8.8 Hz, Ar-H), 7.67 (s, 1H, Ar-H), 8.1 (s, 1H, Ar-H), 10.81 (s, 1H, NH; exchangeable with D_2O); MS: (Mwt.: 431): m/z, 431 [M^+, (17.8%)], 169 (100%); Anal. Calcd for $C_{20}H_{21}N_3O_6S$ (431.46): C, 55.68; H, 4.91; N, 9.74; Found: C, 55.34; H, 4.87; N, 9.96%.



A-Control HepG-2

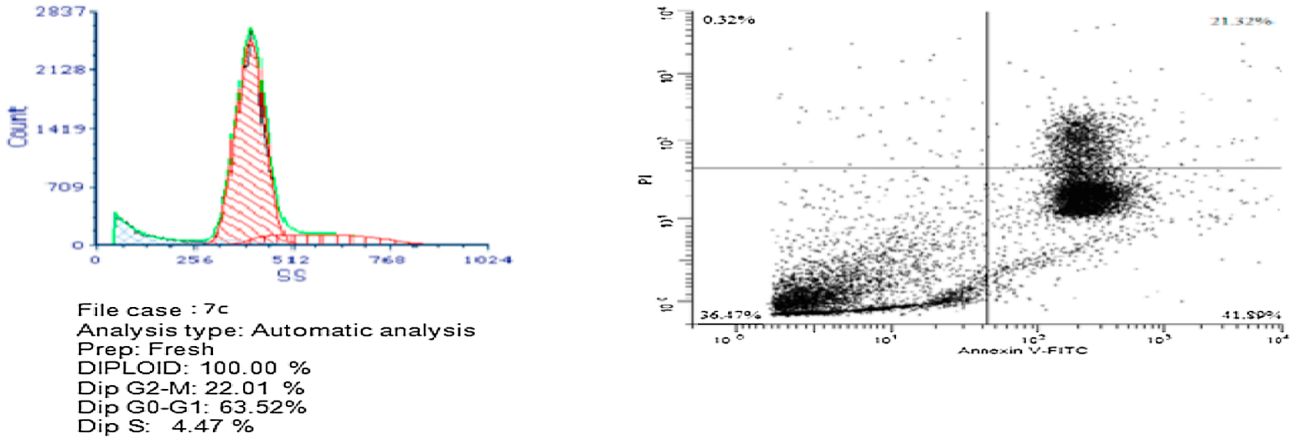


B- 4b/HepG-2

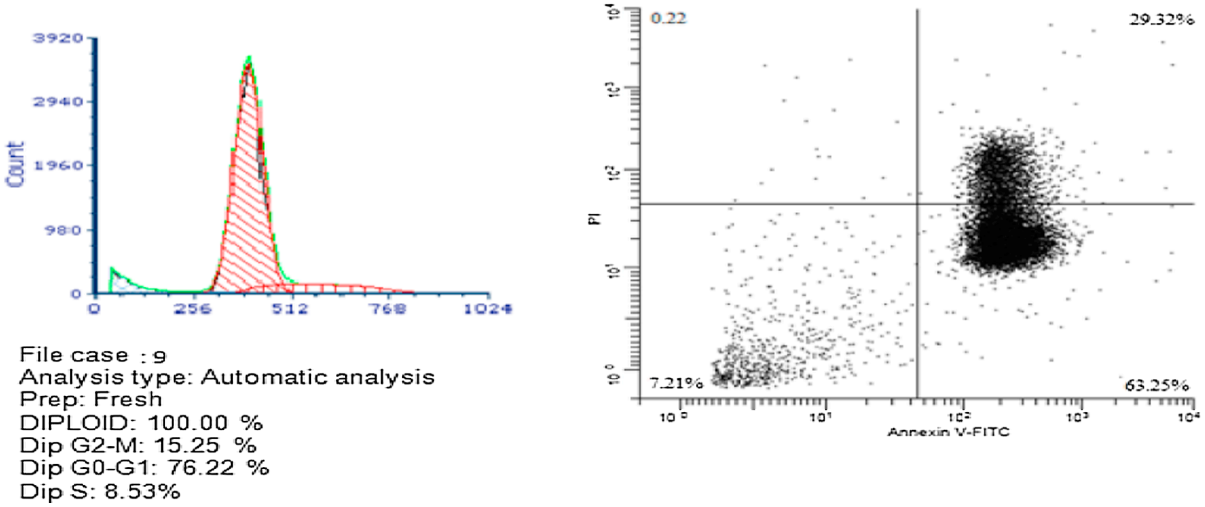


C- 7a/HepG-2

Fig. 6. Cell cycle analysis: A-control HepG-2, B- compound **4b**, C- compound **7**a, D- compound **7c** and compound **9** by flow cytometry using PI staining method.



D- 7c/HepG-2



E- 9/HepG-2

Fig. 6. (*continued*)

Table 4
IC_{50} values of the tested compounds (**4b, 7a, 7c, 9** and lapatinib) against EGFR.

Comp. No.	EGFR IC_{50} (μM)
4b	0.0191
7a	0.0266
7c	1.382
9	0.0253
Lapatinib	0.0283

IC_{50} values are the mean of two independent experiments.

*5.1.5. 3-Hydroxy-3-(2-(4-hydroxyphenyl)-2-oxoethyl)-5-(morpholinosulfonyl) indolin-2-one (**3d**)*

Yield (74.6%) as yellow crystals from ethanol; mp: 208–210 °C; IR: ν/cm^{-1}: 3438, 3331 (2OH), 3224 (NH-isatin), 3063 (CH-Ar), 2975, 2895, 2858 (CH-aliph.), 1714, 1666 (C=O); ^{1}H NMR: δ/ppm 2.62, 2.71 (2t, 4H, N-2CH_2), 3.55, 3.58 (2t, 4H, O-2CH_2), 3.66 (d, 1H, J = 18.0 Hz), 4.20 (d, 1H, J = 17.5 Hz), 6.31 (s, 1H, OH), 6.82 (m, 2H, Ar-H), 7.04 (d, 1H, J = 9.0, Ar-H), 7.56–7.57 (m, 1H, Ar-H), 7.59–7.60 (m, 1H, Ar-H), 7.63 (s, 1H, Ar-H), 7.79 (d, 1H, J = 8.5 Hz, Ar-H), 8.10, 10.79 (2s, 2H, NH and OH; exchangeable with D_2O); ^{13}C NMR: 46.22 (N-2CH_2), 47.42 (CH_2), 65.59 (O-2CH_2), 73.3 (C-OH), 111.82, 113.63, 115.69, 119.81, 120.14, 131.11, 133.16, 133.72, 138.32, 149.72, 162.85, 165.88 (Ar-C-OH), 177.78, 197.87 (C=O); Anal. Calcd for $C_{20}H_{20}N_2O_7S$ (432.45): C, 55.55; H, 4.66; N, 6.48; Found: C, 55.19; H, 4.53; N, 6.97%.

*5.1.6. 3-(2-(4-aminophenyl)-2-oxoethyl)-3-hydroxy-5-(morpholinosulfonyl)indolin-2-one (**3e**)*

Yield (76.5%) as yellowish orange powder from ethanol; mp:dec. 170–172 °C; IR: ν/cm^{-1}: 3460 (OH), 3348, 3262, 3122 (NH_2, NH-isatin), 3048 (CH-Ar), 2983, 2912, 2848 (CH-aliph.), 1764, 1717 (br C=O-isatin, acetyl); ^{1}H NMR: δ/ppm 2.89 (t, 4H, J = 4.4 Hz, N-2CH_2), 3.59 (t, 4H, J = 3.2 Hz, O-2CH_2), 3.65 (d, 1H, J = 20.8 Hz), 4.03 (d, H, J = 20.0 Hz), 6.01 (s, 2H, NH_2, exchangeable with D_2O), 6.27 (s, 1H, OH, exchangeable with D_2O), 6.51–6.57 (m, 2H, Ar-H), 7.16 (d, 1H, J = 8.0 Hz, Ar-H), 7.65–7.7 (m, 2H, Ar-H), 7.92 (d, 1H, J = 8.4 Hz, Ar-H), 8.09 (s, 1H, Ar-H), 10.72 (s, 1H, NH; exchangeable with D_2O); Anal. Calcd for $C_{20}H_{21}N_3O_6S$ (431.46): C, 55.68; H, 4.91; N, 9.74; Found: C, 55.34; H, 4.87; N, 9.96%.

*5.1.7. Synthesis of 5-(morpholinosulfonyl)-3-(2-oxo-2-arylethylidene) indolin-2-one (**4a-d**)*

A solution of 3-hydroxy-5-(morpholinosulfonyl)-3-(2-oxo-2-arylethyl)indolin-2-one **(3)** (0.001 mol) in 15 mL of acetic acid with 0.5 mL of concentrated hydrochloric acid were heated at 95 °C on steam bath for 2 h, the mixture allowed to stand at room temp. The solid products so formed after poured on crushed ice was collected by

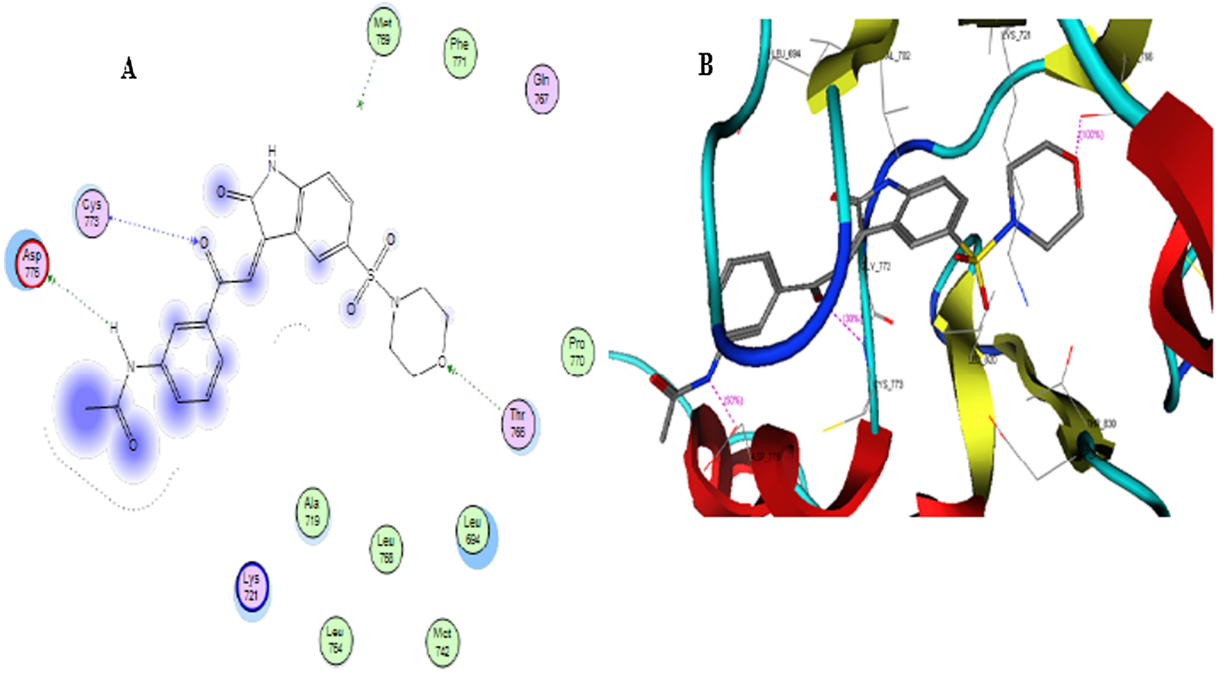


Fig. 7. 2D (A) and 3D (B) binding mode of compound **4b** inside EGFR binding site.

filtration and crystallized from the proper solvent.

*5.1.8. 5-(Morpholinosulfonyl)-3-(2-oxo-2-phenylethylidene) indolin-2-one (**4a**)*

Yield (76.3%) as yellow crystals from ethanol; mp: 214–216 °C; IR: ν/cm^{-1}: 3175 (NH-isatin), 3100 (CH-Ar), 2978, 2859 (CH-aliph.), 1715, 1660 (C═O); ^{1}H NMR: δ/ppm 2.77 (t, 4H, N-2CH$_2$), 3.62 (t, 4H, O-2CH$_2$), 7.13 (d, 1H, J = 8.0 Hz, Ar-H), 7.61–7.64 (m, 2H,Ar-H), 7.71–7.74 (m, 2H, Ar-H), 7.85 (s, 1H,Ar-H), 8.19–8.11 (m, 2H, Ar-H), 8.33 (1s, 1H, –CH methine), 11.35 (1s, 1H, NH; exchangeable with D$_2$O); ^{13}C NMR: 46.21 (N-2CH$_2$), 65.69 (O-2CH$_2$), 111.27, 120.76, 126.36, 127.74, 128.91, 129.25,129.72, 133.1, 134.82, 135.22, 137.18, 149.2, 168.61, 191.82 (2C═O); Anal. Calcd for C$_{20}$H$_{18}$N$_2$O$_5$S (398.34): C, 60.29; H, 4.55; N, 7.03; Found: 60.51; H, 4.11; N, 6.96%.

*5.1.9. N-(3-(2-(5-(Morpholinosulfonyl)-2-oxoindolin-3-ylidene)acetyl) phenyl)acetamide (**4b**)*

Yield (88.6%) as yellow crystals from ethanol; mp: dec.210–212 °C; IR: ν/cm^{-1}: 3341, 3317 (−NH, −NH-isatin), 3087 (CH-Ar), 2975, 2918, 2858 (CH-aliph.), 1726, 1666 (C═O); ^{1}H NMR: δ/ppm 2.08 (s, 3H, CH$_3$), 2.77, 2.92 (2t, 4H, J = 3.6 Hz, N-2CH$_2$), 3.62, 3.67 (2t, 4H, J = 4.1 Hz, O-2CH$_2$), 7.11–7.14 (m, 1H, Ar-H), 7.24–7.25 (m, 1H, Ar-H), 7.52–7.56 (m, 1H, Ar-H), 7.72 (d, 1H, J = 8.8 Hz, Ar-H), 7.75–7.76 (m, 1H, Ar-H), 7.81 (s, 1H, Ar-H), 7.95–7.97 (m, 1H, Ar-H), 8.31 (1s, 1H, −CH methine), 10.24, 11.37 (2S, 2H, 2NH; exchangeable with D$_2$O); MS: (Mwt.: 455): m/z, 455 [M$^+$, (5.2%)], 360 (100%); Anal. Calcd for C$_{22}$H$_{21}$N$_3$O$_6$S (455.12): C, 58.01; H, 4.65; N, 9.23; Found: 57.84; H, 4.53; N, 9.52%.

*5.1.10. 4-(2-(5-(Morpholinosulfonyl)-2-oxoindolin-3-ylidene)acetyl) phenyl acetate (**4c**)*

Yield (71.3%) as yellow crystals from ethanol; mp: 208–210 °C; IR:

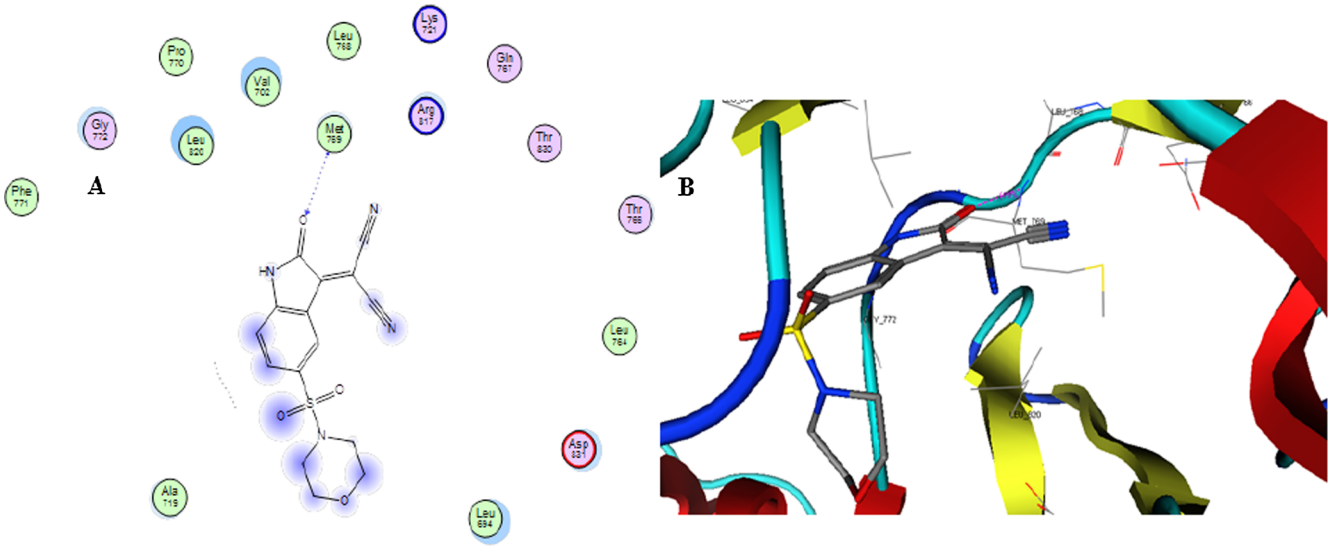


Fig. 8. 2D (A) and 3D (B) binding mode of compound **7a** inside EGFR binding site.

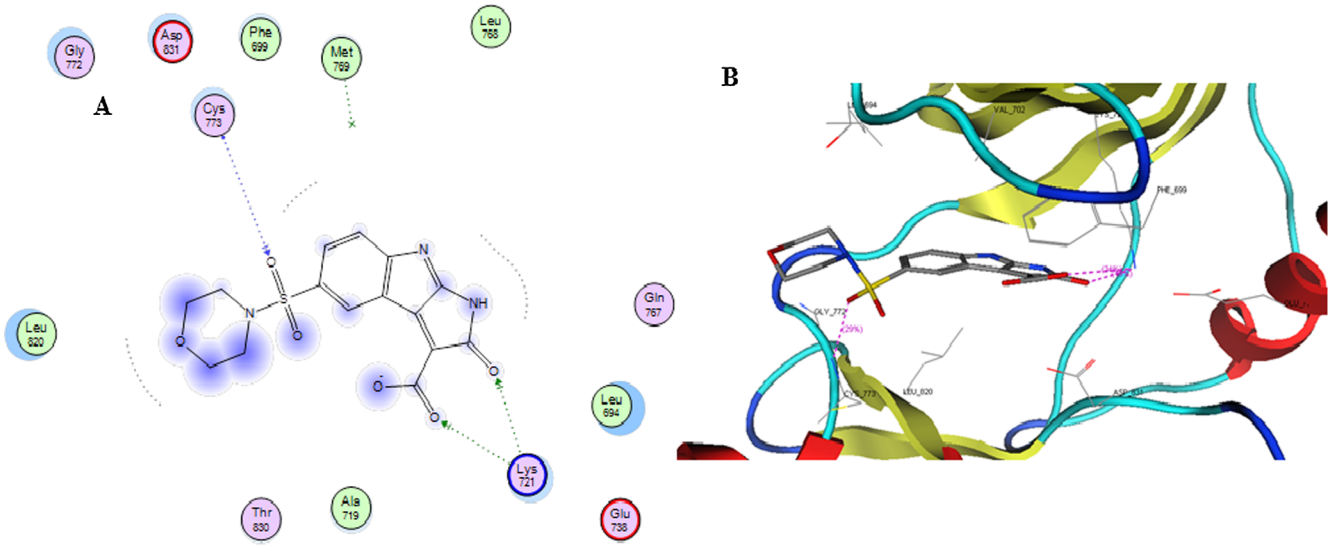


Fig. 9. 2D (A) and 3D (B) binding mode of compound **9** inside EGFR binding site.

ν/cm^{-1}: 3119 (NH-isatin), 3082 (CH-Ar), 2980,2902, 2862 (CH-aliph.), 1731, 1715 (C=O); ^{1}H NMR: δ/ppm 1.92 (s, 3H, CH_3), 2.97 (t, 4H, N-2CH_2), 3.66 (t, 4H, O-2CH_2), 7.69 (s, 1H, Ar-H), 7.76–7.77 (m, 2H, Ar-H), 7.84 (d, 1H, J = 8.5 Hz, Ar-H), 8.04 (d, 1H, J = 8.5 Hz, Ar-H), 8.25 (d, 1H, J = 8.5 Hz, Ar-H), 8.31 (d, 1H, J = 8.5 Hz, Ar-H), 8.58 (s, 1H, −CH methine), 11.49 (S, H, NH; exchangeable with D_2O); Anal. Calcd for $C_{22}H_{20}N_2O_7S$ (456.47): C, 57.89; H, 4.42; N, 6.14; Found: 57.83; H, 4.45; N, 6.31%.

*5.1.11. N-(4-(2-(5-(Morpholinosulfonyl)-2-oxoindolin-3-ylidene)acetyl) phenyl)acetamide (**4d**)*

Yield (68.1%) as yellow crystals from ethanol; mp: 237–239 °C; IR: ν/cm^{-1}: 3361, 3221 (NH,NH-isatin), 3055 (CH-Ar), 2975, 2920, 2858 (CH-aliph.), 1717, 1654 (C=O); ^{1}H NMR: δ/ppm 2.09 (s, 3H, CH_3), 2.69, 2.92 (2t, 4H, J = 3.5 Hz, N-2CH_2), 3.61,3.68 (2t, 4H, J = 4.4 Hz, O-2CH_2), 6.65 (d, 1H, J = 8.4 Hz, Ar-H), 7.13 (d, 1H, J = 8.4 Hz, Ar-H), 7.66–7.71 (m, 1H, Ar-H), 7.72 (s, 1H, Ar-H), 7.78 (d, 1H, J = 7.6 Hz, Ar-H), 7.79–7.82 (m, 1H, Ar-H), 8.09 (d, 1H, J = 8.8 Hz, Ar-H), 8.23 (1s, 1H, –CH methine), 10.43, 11.32 (2s, 2H, 2NH; exchangeable with D_2O); Anal. Calcd for $C_{22}H_{21}N_3O_6S$ (455.12): C, 58.01; H, 4.65; N, 9.23; Found: 57.84; H, 4.53; N, 9.52%.

*5.1.12. 4-Hydroxy-5′-(morpholinosulfonyl)spiro[chromene-2,3′-indolin]-2′-one(**6**)*

Yield (71.3%) as yellow crystals from ethanol; mp: 208–210 °C; IR: ν/cm^{-1}: 3386 (OH), 3103 (NH-isatin), 3059 (CH-Ar), 2977, 2860 (CH-aliph.), 1718 (br C=O); ^{1}H NMR: δ/ppm 2.99 (2t, 4H, N-2CH_2), 3.66 (2t, 4H, O-2CH_2), 7.03 (d, 1H, J = 8.5 Hz, Ar-H), 7.05 (s, 1H, Ar-H), 7.4 (s, 1H, –CH chromene), 8.07–8.09 (m, 2H,Ar-H), 8.19 (d, 1H, J = 8.0 Hz, Ar-H), 8.36–8.38 (m, 2H, Ar-H), 8.81, 9.18 (2s, 2H, NH and OH; exchangeable with D_2O); ^{13}C NMR: 46.31 (N-2CH_2), 65.82 (O-2CH_2), 87.17, 118.35, 119.95, 120.05, 122.31, 122.94, 127.21, 128.16, 129.53, 130.31, 133.91, 138.72, 147.80, 159.75, 160.19, 167.04 (C=O); MS: (Mwt.: 414): m/z, 414 [M$^+$, (54.57%)], 138 (100%); Anal. Calcd for $C_{20}H_{18}N_2O_6S$ (414.43): C, 57.96; H, 4.38; N, 6.67; Found: 57.43; H, 4.57; N, 7.01%.

*5.1.13. Synthesis of new arylidine derivatives from isatin sulfonamide (**7a-c**)*

A suspension of compound **(1)** (0.001 mol) and nitrile derivatives (malononitril, ethyl cyanoacetate, cyanoacetamide) (0.001 mol), in MeOH (10 mL) with catalytic amount of triethyl amine (3drops) were stirred at r.t., completion of the reaction was monitored by TLC. The obtained powders were filtered off, washed with ethanol and re-crystallized with ethanol: DMF: (2:1) mixtures.

*5.1.14. 2-(5-(Morpholinosulfonyl)-2-oxoindolin-3-ylidene) malononitrile (**7a**)*

Yield (91.3%) as red powder; mp 310–312 °C; IR: ν/cm^{-1}: 3371 (NH), 3094 (CH-Ar), 2986, 2920, 2871 (CH-aliph.), 2236 (CN), 1741 (C=O); ^{1}H NMR: δ/ppm 2.92 (t, 4H, J = 4.5 Hz, N-2CH_2), 3.66 (t, 4H, J = 4.5 Hz,O-2CH_2), 7.19 (d, 1H,J = 8.5 Hz, Ar-H_4), 7.92 (d, 1H, J = 8.5 Hz, Ar-H), 8.18 (s, 1H, Ar-H), 11.76 (S, H, NH; exchangeable with D_2O); ^{13}C NMR: 46.22 (N-2CH_2), 65.73 (O-2CH_2), 85.26, 112.34, 112.66 (2CN), 119.66, 125.13, 128.97, 136.84, 150.03, 150.23, 164.27(C=O);MS: (Mwt.: 344): m/z, 344 [M$^+$, (27.05%)], 153 (100%); Anal. Calcd for $C_{15}H_{12}N_4O_4S$ (344.35): C, 52.32; H, 3.51; N, 16.27; Found: 52.12; H, 3.36; N, 16.62%.

*5.1.15. (E)-2-cyano-2-(5-(morpholinosulfonyl)-2-oxoindolin-3-ylidene) acetamide (**7b**)*

Yield (84%) as orange powder; mp: 266–268 °C; IR: ν/cm^{-1}: 3391, 3309, 3189 (NH_2,NH), 3083 (CH-Ar), 2981, 2904, 2860 (CH-aliph.), 2216 (CN), 1729, 1690 (2C=O); ^{1}H NMR: δ/ppm 2.86 (t, 4H, J = 4.5 Hz, N-2CH_2), 3.65 (t, 4H, J = 4.5 Hz, O-2CH_2), 7.15 (d, 1H, J = 9.2 Hz, Ar-H), 7.79 (d, 1H, J = 8.75 Hz, Ar-H), 7.94 (s, 1H, Ar-H), 8.53, 8.69, 11.55 (3s, 3H, NH_2, NH; exchangeable with D_2O); ^{13}C NMR: 46.24, 46.36 (N-2CH_2), 65.26, 65.79 (O-2CH_2), 111.79, 111.90, 114.83, 119.73, 125.38, 128.30, 134.16, 138.66, 148.31, 161.96, 165.68 (2C=O); Anal. Calcd for $C_{15}H_{14}N_4O_5S$ (362.36): C, 49.72; H, 3.89; N, 15.46; Found: 49.61; H, 3.76; N, 15.54%.

*5.1.16. (E)-ethyl 2-cyano-2-(5-(morpholinosulfonyl)-2-oxoindolin-3-ylidene)acetate (**7c**)*

Yield (73.8%) as yellowish orange powder; mp 255–256 °C; IR: ν/cm^{-1}: 3198 (NH), 3091 (CH-Ar), 2976, 2914, 2859 (CH-aliph.), 2233 (CN), 1730, 1697 (2C=O); ^{1}H NMR: δ/ppm 1.37 (t, 3H, J = 7.25 Hz, CH3), 2.92 (t, 4H, J = 4.0 Hz, N-2CH_2), 3.66 (t, 4H, J = 4.25 Hz, O-2CH_2), 4.41 (q, 2H, J = 7.0 Hz, CH_2), 7.12 (d, 1H, J = 8.5 Hz, Ar-H), 7.77 (d, 1H, J = 8.5 Hz, Ar-H), 7.83 (s, 1H, Ar-H), 11.61 (s, H, NH; exchangeable with D_2O); ^{13}C NMR: 14.1 (CH_3), 46.24, 46.36 (N-2CH_2), 65.75, 65.80 (O-2CH_2), 66.82 (CH_2), 99.31, 110.61, 111.96, 121.94, 127.50, 129.62, 133.28, 133.96, 135.20, 148.64, 169.43, 170.3 (2C=O); Anal. Calcd for $C_{17}H_{17}N_3O_6S$ (391.40): C, 52.17; H, 4.38; N, 10.74; Found: 52.26; H, 4.47; N, 10.65%.

*5.1.17. Synthesis of 5-(Morpholinosulfonyl)-2-oxo-2,8-dihydropyrrolo [2,3–b] indolederivatives (**8, 9**)*

5.1.17.1. Method (A). An equimolar amount of 5-(morpholinosulfonyl) isatin **(1)** (0.001 mol), and active nitrile (malononitril, ethyl cyanoacetate, cyanoacetamide) (0.001 mol), were dissolved in absolute methanol (10 mL) catalyzed with triethylamine were heated under reflux for 4 h until the solid product precipitate on hot then poured in crushed ice and finally recrystallized from proper solvent.

5.1.17.2. Method (B). A solution of **(7a-c)** was heated under reflux for 2–4 h in Methanol catalyzed with triethyl-amine then the reaction was monitored by TLC until complete reaction occurs, finally poured in cold water and recrystallized from proper solvent.

*5.1.18. 5-(Morpholinosulfonyl)-2-oxo-2,8-dihydropyrrolo[2,3–b]indole-3-carbonitrile (**8**)*

Yield (82.5%) as red powder from DMF; mp: dec. 240–242 °C; IR: ν/cm^{-1}: 3396 (NH), 3071 (CH-Ar), 2997, 2973, 2859 (CH-aliph.), 2201 (CN), 1761 (C═O); ^{1}H NMR: δ/ppm 2.92 (t, 4H, J = 4.4 Hz, N-2CH_2), 3.67 (t, 4H, J = 4.4 Hz,O-2CH_2), 7.20 (d, 1H, J = 8.4 Hz, Ar-H_7), 7.93 (d, 1H, J = 8.0 Hz, Ar-H_8), 8.18 (s, 1H, Ar-H_4) 11.75 (s, H, NH; exchangeable with D_2O); ^{13}C NMR: 46.11 (N-2CH_2), 65.76 (O-2CH_2),112.66, 119.42, 125.13, 128.34, 130.42, 131.82, 132.82, 136.84, 142.49, 150.63, 169.48 (C═O);MS: (Mwt.: 344): *m/z*, 344 [M^+, (6.7%)], 92 (100%); Anal. Calcd for $C_{15}H_{12}N_4O_4S$ (344.35): C, 52.32; H, 3.51; N, 16.27; Found: 52.12; H, 3.36; N, 16.62%.

*5.1.19. 5-(Morpholinosulfonyl)-2-oxo-2,8-dihydropyrrolo[2,3–b]indole-3-carboxylic acid (**9**)*

Yield (82.5%) as red powder from dioxane; mp: 315–317 °C; IR: ν/cm^{-1}: 3561 (br OH), 3201 (NH), 3071 (CH-Ar), 2968, 2916, 2859 (CH-aliph.), 1700 (C═O); ^{1}H NMR: δ/ppm 2.91 (t, 4H, J = 4.0 Hz, N-2CH_2), 3.64 (t, 4H, J = 4.0 Hz, O-2CH_2), 7.1 (d, 1H, J = 8.8 Hz, Ar-H), 7.73 (d, 1H, Ar-?H), 7.75 (s, 1H, Ar-H), 9.51, 11.44 (2s, 2H, OH, NH; exchangeable with D_2O); ^{13}C NMR: 46.33 (N-2CH_2), 65.76 (O-2CH_2), 110.58, 121.90, 127.46, 129.58, 133.26, 133.93, 148.60, 169.40 (2C═O);MS: (Mwt.: 363): *m/z*, 363 [M^+, (10.46%)], 102 (100%); Anal. Calcd for $C_{15}H_{13}N_3O_6S$ (363.34): C, 49.59; H, 3.61; N, 11.57; Found: 49.89; H, 3.36; N, 11.29%.

*5.1.20. Synthesis of 5-(morpholinosulfonyl)-1′H-spiro[indoline-3,4′-pyrano[2,3–c]pyrazol]-2-one derivatives (**10a-b**) and 2-amino-7-alkyl-5′-(morpholinosulfonyl)-2′-oxospiro [chromene-4, 3′-indoline]-3-carbonitrile (**11a,b**)*

5.1.20.1. Method (A). A mixture of 5-(morpholinosulfonyl)isatin **(1),** malononitrile and either 2,4-dihydro-3H-pyrazol-3-one derivatives or phenol derivatives were dissolved in absolute methanol (30 mL), catalyzed with fused sodium acetate or piperidine were heated under reflux until complete reaction obtained (progress with TLC), then poured in crushed ice and finally recrystallized from proper solvent.

5.1.20.2. Method (B). A mixture of **(7a)** was heated with either 2,4-dihydro-3H-pyrazol-3-one derivatives or phenol derivatives in Methanol (30 mL) catalyzed with fused sodium acetate or piperidine for 8 h then the reaction was monitored by TLC until complete reaction occurs, finally poured in ice and recrystallized from proper solvent.

*5.1.21. 6′-Amino-3′-methyl-5-(morpholinosulfonyl)-2-oxo-1′H-spiro [indoline-3,4′-pyrano[2,3–c]pyrazole]-5′-carbonitrile (**10a**)*

Yield (80.1%) as yellow powder from ethanol; mp: 258–259 °C; IR: ν/cm^{-1}: 3441, 3339, 3217 (NH_2,NH), 3101 (CH-Ar), 2971, 2984, 2843 (CH-aliph.), 2190 (CN), 1712 (C═O) 1645 (C═N); ^{1}H NMR: δ/ppm 1.57 (s, 3H, CH_3), 2.80 (t, 4H, J = 5.0 Hz, N-2CH_2), 3.60 (t, 4H, J = 4.5 Hz, O-2CH_2), 7.19 (d, 1H, J = 8.5 Hz, Ar-H), 7.35 (s, 2H, NH_2, exchangeable with D_2O), 7.39 (s, 1H, Ar-H), 7.7 (d, 1H, J = 8.0 Hz, Ar-H), 11.17, 12.41 (2s, 2H, 2NH; exchangeable with D_2O); ^{13}C NMR: 9.48 (CH_3), 46.40 (N-2CH_2), 47.79(C-Spiro), 54.48(C-CN), 65.68 (O-2CH_2), 94.82, 110.82, 118.85, 124.24, 128.55, 130.36, 133.88, 135.2, 146.70, 155.3, 163.16 (C═O) 178.65 (C-NH_2); Anal. Calcd for $C_{19}H_{18}N_6O_5S$ (442.45): C, 51.58; H, 4.10; N, 18.99; Found: 51.32; H, 4.16; N, 19.19%.

*5.1.22. 6′-Amino-3′-methyl-5-(morpholinosulfonyl)-2-oxo-1′-phenyl-1′H-spiro [indoline-3, 4′-pyrano [2,3–c] pyrazole]-5′-carbonitrile (**10b**)*

Yield (71.25%) as yellow powder from ethanol; mp: 170–172 °C; IR: ν/cm^{-1}: 3441, 3346, 3222 (NH_2, NH), 3079 (CH-Ar), 2969, 2921, 2859 (CH-aliph.), 2200 (CN), 1736 (C═O) 1613 (C═N); ^{1}H NMR: δ/ppm 1.58 (s, 3H, CH_3), 2.95 (t, 4H, J = 4.75 Hz, N-2CH_2), 3.66 (t, 4H, J = 5.75 Hz, O-2CH_2), 7.22 (s, 2H, NH_2, exchangeable with D_2O), 7.42 (d, 1H, J = 7.5 Hz, Ar-H), 7.31–7.45 (m, 1H, Ar-H), 7.46–7.53 (m, 1H, Ar-H), 7.59 (d, 1H, J = 8.5 Hz, Ar-H), 7.63–7.68 (m, 1H, Ar-H), 7.73 (d, 1H, J = 8.5 Hz, Ar-H),7.82 (d, 1H, J = 7.25 Hz, Ar-H), 8.49 (s, 1H, Ar-H), 11.43 (s, H, NH; exchangeable with D_2O); MS: (Mwt.: 518): *m/z*, 518 [M^+, (18.64%)], 385 (100%); Anal. Calcd for $C_{25}H_{22}N_6O_5S$ (518.55): C, 57.91; H, 4.28; N, 16.21; Found: 58.18; H, 4.21; N, 15.91%.

*5.1.23. 2,7-diamino-5′-(morpholinosulfonyl)-2′-oxospiro[chromene-4,3′-indoline]-3-carbonitrile (**11a**)*

Yield (75.58%) as white powder from dioxane; mp: 220–221 °C; IR: ν/cm^{-1}: 3446,3367,3323 (NH_2, NH-isatin), 3089 (CH-Ar), 2975, 2920, 2864 (CH-aliph.), 2187 (CN), 1726 (C═O) 1612 (C═N); ^{1}H NMR: δ/ppm 2.79 (t, 4H, J = 3.6 Hz, N-2CH_2), 3.58 (t, 4H, J = 4.4 Hz, O-2CH_2), 5.43 (s, 2H, NH_2; exchangeable with D_2O), 6.14 (d, 1H, J = 8.4 Hz, Ar-H), 6.23 (s, 1H, Ar-H), 6.24–6.26 (m, 1H, Ar-H), 7.15 (d, 1H, J = 8.0 Hz, Ar-H), 7.21 (s, 2H, NH_2 exchangeable with D_2O), 7.25–7.26 (m, 1H, Ar-H), 7.63–7.66 (m, 1H, Ar-H), 10.93 (s, H, NH; exchangeable with D_2O); ^{13}C NMR: 46.28 (N-2CH_2), 50.31 (C-spiro), 53.67 (C-CN), 65.67 (O-2CH_2), 100.60, 106.19, 110.76, 111.82, 119.05, 124.40, 127.11, 130.11, 135.80, 147.29, 150.02, 150.48, 162.26 (C═O), 180.01 (C-NH_2); Anal. Calcd for $C_{21}H_{19}N_5O_5S$ (453.47): C, 55.62; H, 4.22; N, 15.44; Found: 55.93; H, 4.41; N, 15.24%.

*5.1.24. 2-Amino-7-hydroxy-5′-(morpholinosulfonyl)-2′-oxospiro [chromene-4,3′-indoline]-3-carbonitrile (**11b**)*

Yield (79.89%) as white powder from dioxane; mp: 267–269 °C; IR: ν/cm^{-1}, 3421, 3328, 3210 (OH, NH_2, NH-isatin), 3079 (CH-Ar), 2974, 2934 (CH-aliph.), 2181 (CN), 1724 (C═O) 1616 (C═N); ^{1}H NMR: δ/ppm 2.79 (t, 4H, J = 4.4 Hz, N-2CH_2), 3.58 (t, 4H, J = 4.4 Hz, O-2CH_2), 6.34 (d, 1H, J = 9.2 Hz, Ar-H), 6.46–6.48 (m, 2H, Ar-H), 7.18 (d, 1H, J = 8.0 Hz, Ar-H), 7.3 (s, 2H, NH_2 exchangeable with D_2O), 7.34 (s, 1H, Ar-H), 7.68 (d, 1H, J = 10.0 Hz, Ar-H), 9.94, 11.00 (2s, 2H, OH, NH; exchangeable with D_2O); ^{13}C NMR: 46.29 (N-2CH_2), 50.37 (C-spiro), 53.62 (C-CN), 65.67 (O-2CH_2), 103.26, 110.25, 110.91, 113.43, 118.82, 124.53, 127.72, 128.50, 130.29, 135.49, 147.27, 149.99, 158.69, 162.08 (C═O), 179.68 (C-NH_2); MS: (Mwt.: 454): *m/z*, 454 [M^+, (31.05%)], 98 (100%); Anal. Calcd for $C_{21}H_{18}N_4O_6S$ (454.46): C, 55.50; H, 3.99; N, 12.33; Found: 55.84; H, 4.06; N, 12.04%.

5.2. Biological evaluation

5.2.1. Antiproliferative activity

In vitro cytotoxicity of all synthetized compounds were evaluated by using colorimetric assay method (SRB) against three human tumor cell lines including Mammary gland breast cancer cell line (MCF-7), human hepatocellular carcinoma cell line (HepG-2), colon carcinoma cell line (HCT116), they were obtained from VACSERA- Cell Culture Unit, Cairo, Egypt. For comparison, doxorubicin was used as a standard reference drug. RPMI-1640 medium, SRB (SulphoRhodamine-B), DMSO (Dimethyl sulfoxide) and doxorubicin were purchased from (sigma co., St. Louis, USA). Fetal bovine serum was obtained from (GIBCO, UK). The cells were cultured in RPMI-1640 medium with 10% fetal bovine

serum. Antibiotics (penicillin 100 units/mL and streptomycin 100 μg/mL) were added at 37 °C in a 5% CO_2 incubator. The cells were seeded in a 96-well plate at a density of 1.0×10^4 cells/well at 37 °C for 48 h under 5% CO_2. After incubation, the cells were treated with different concentrations of the tested compounds and incubated for 24 h. Then the medium was discarded. Fixation was carried out by 10% trichloroacetic acid (TCA) 150 μL/well for 1 h at 4 °C, then wash by water 3 times (TCA reduce SRB protein binding). Wells were stained by SRB 70 μL/well for 10 min at room temperature with 0.4% 70 μL/well (keep in dark place). Then washed with acetic acid 1% to remove unbound dye (end point: colorless drainage). The plates were subjected to air drying for 24 h. The dye were solubilized with 50 μL/well of 10 mMtris base (PH 7.4) for 5 min on a shaker at 1600 rpm. The optical density (OD) of each well was measured at 570 nm with an ELISA microplate reader (EXL 800 USA). The inhibitory concentration at 50% (IC_{50}) was determined from the exponential curve of viability versus concentration. The viability was calculated, as (A570 of treated samples/A570 of untreated sample) × 100 and The IC_{50} values were calculated using the Microsoft Excel. The data were recorded and analyzed to estimate the effects of the tested compounds on cell viability and growth; IC_{50} values for the tested compounds are reported in Table 1, three independent experiments for each concentration were performed. as previously reported [42].

5.2.2. Cell cycle analysis

5.2.2.1. Cell cycle analysis. The selected compounds were treated with (0.1% DMSO) vehicle or 5 μM of **4b, 7a, 7c** and **9** compounds for 24 h. over HepG2 cells that seeded in a 6-well plate at concentration of 1×10^5cells per well, then incubated for 24 h. After that cells were harvested and fixed for 12 h using ice-cold, 70% ethanol at 4 °C. Removal of ethanol and washing cells with cold PBS was done. Then incubated for 30 min at 37 °C in 0.5 mL of PBS containing 1 mg/mL Rnase. The cells were stained for 30 min with propidium iodide in the dark. Then flow cytometer was used to detect DNA contents [46].

5.2.2.2. Annexin-V assay. A solution from **4b, 7a, 7c** and **9** (0.1% DMSO) or 5 μM were treated over HepG2 cells that seeded in a 6-well plate (1×10^5 cells/well), incubated for 24 h, The cells were then harvested, washed using PBS and stained for 15 min at room temperature in the dark using annexin V-FITC and PI in binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$ at pH 7.4), then analyzed by the flow cytometer [47].

5.2.3. EGFR inhibition assay

EGFR inhibition activity was evaluated for the new representative active compounds **4b, 7a, 7c** and **9** using HepG2 cancer cells using cloud clone SEA757Hu 96 Kit, following the same instructions from the manufacturer protocol. The fraction of the EGFR was measured in the presence of the tested compounds using the equation: E (%) = E max/(1 + [I]/ID_{50}), E max is the activity in the absence of the inhibitor, [I] is the inhibitor concentration and ID_{50} is the inhibitor concentration when E (%) = 0.5 E max, then the dose–response curve was plotted, values represent a mean of two independent experiments as previous work [42].

5.3. Molecular docking

1M17 was downloaded from pdb [48], it has erlotinib as the co-crystallized ligand with ATP binding site of EGFR TKs, refined, energy minimized, the binding site was detected using alpha site finder in MOE software (Molecular Operating Environment 10.2008 provided with chemical computing group, Canada.), then saved as Moe file to be ready for docking process. Verification process was performed by redocking of erlotinib into EGFR binding site using default set in MOE software. The selected compounds were drawn as 2D structures by chemdraw, then submitted to MOE software, hydrogenated and energy minimized and saved as mdb file for docking into the same binding site of erlotinib.

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