Purpose: A series of quinazoline isatine hybrid derivatives were designed and their molecular docking studies were performed to ascertain the inhibition of EGFR by these hybrids.

Methods: Molecular modelling and docking methods were employed to design and synthesize the molecules. The compounds which showed good binding properties were synthesized and characterized. After structural confirmation of these compounds they were evaluated for their antiproliferative activity on OVCAR-3 ovarian cancer cell line.

Results: These compounds were further evaluated for EGFR inhibitory activity and cell migration studies. It was found that the O-05 compound had the most potent inhibitory activity (IC_{50}=2.11 μM for OVCAR-3 and IC_{50}=0.46 μM for EGFR). The O-05 compound was found to be a potential antitumor agent as per its pharmacological activity, molecular docking, and inhibition of OVCAR-3 cells.

Conclusion: The compound O-05 or its structural analogs can be developed into potential lead molecules for the development of potential clinical agents for ovarian cancer.

Key words: antitumor activity, EGFR, isatin, ovarian cancer, quinazolines

Introduction

Epithelial ovarian cancer (EOC) arises either from the mesothelial lining of the ovaries (either from the epithelial surface lining or cortical ovarian cysts formed by invaginations of the surface epithelium) or from the fallopian tube epithelium and contributes up to 90% of ovarian cancers. Earlier studies have reported that overexpression of epidermal growth factor receptor (EGFR) is found in more than 60% of the cases of ovarian cancers [1,2]. Studies that mainly focus on the EGFR inhibition activity have always challenged scientists to find out some new molecules to target EGFR. The EGFR overexpression is reported to range between 5-67% in patients suffering from ovarian cancer [3-6]. Ovarian cancer is a heterogeneous disease with respect to pathology, molecular biology and clinical outcome, suggesting that a single standard treatment is unlikely to benefit all patients. Histologically, most ovarian cancers arise from the distal fallopian tube or ovarian surface and the majority of these EOC are of serous/papillary subtype, followed by endometrioid, mucinous, clear cell and undifferentiated types. These different subtypes - together with other clinical factors including age, performance status, FIGO stage, differentiation, presence of ascites and surgical debulking status - are important prognostic factors [6-10].
It is clear from the published reports that EGFR and its family members have emerged as attractive targets for anticancer therapy. Structurally, the ErbB family members consist of (i) a cysteine-rich, extracellular N-terminal ligand binding domain and a dimerization arm; (ii) a hydrophobic transmembrane domain; and (iii) an intracellular highly conserved cytoplasmic C-terminal tyrosine kinase domain with several phosphorylation sites. Since the intracellular tyrosine kinase domain is highly conserved, the variable extracellular ligand binding domain enables binding to different ligands. Further, the extracellular region of EGFR is subdivided into four domains as I, II, III and IV (Figure 1) [11-15].

This is also proved from the successful stories of anticancer drugs like gefitinib, erlotinib and lapatinib. These drugs have demonstrated the utility of quinazoline derivatives as EGFR inhibitors. Moreover, isatine and its derivatives are also reported for their anticancer activity. So by using hybrid approach, it is worthwhile to synthesize some new hybrid derivatives of isatin and quinazoline. Literature studies have shown that EGFR and N1 atom of the ligand structure can form a bond with an amino group of a receptor [14-17].

To investigate the potential use of hybrid scaffold, new series of quinazoline and isatine derivatives were synthesized as EGFR ATP binding site inhibitors. Quinazoline and its derivatives belong to the nitrogen containing heterocyclic compounds and have attracted the attention of many scientist due to their versatile biological profile. Researchers have already determined many therapeutic activities of quinazoline derivatives, including anticancer [18], antiinflammatory [19], antibacterial [20], analgesic [21], antiviral [22], anticytotoxicity [23], antispass [24], antituberculosis [25], antioxidative [26], antimalarial [27], antihypertensive [28], antiobesity [29], antipsychotic and antidiabetic [30-35]. Moreover, Isatin or 1H-indole-2,3-dione, an indole derivative, is also widely reported for its versatile biological activity including anticancer activity [35-40].

Methods

Molecular docking studies

Docking calculations were carried out for the designed compounds into three dimensional (3D) X-ray structure of EGFR (PDB code: 4I24) [41] using Accelryes Drug Discovery Studio, USA (3.5 version) [42-44]. The ligand structures were drawn in 2D and then they were converted to 3D by using MarvinSketch (ChemAxon). The structures were optimized for the study by generating various conformers using the energy minimization protocol. The crystal structures of EGFR (PDB code:4I24) complex were obtained from the RCSB Protein Data Bank. The ligand structure was imported to the new window with proper names and the program was run to prepare the ligand. Finally, the input ligand was selected (Molecules: All) on protein molecular window and were docked for 4I24. Other parameters for the docking study were kept as default. The Binding Energy and electrostatic Interaction energy was calculated. The docking interactions are summarized in Table 1.

Synthetic study

Synthesis and characterization of compounds (Figure 6)

7-fluoro-quinazolin-4-one (2)

A mixture of 2-amino-4-fluorobenzoic acid (126 g, 0.82 mol) and formamidine acetate (170 g, 1.64 mol) in 2-methoxyethanol (800 mL) was refluxed for 16 hrs, and the resultant solution was concentrated. The crude product obtained was diluted in water. The suspension was washed several time with water, and dried to give pure product (yield 88%). Mp 230-232 °C .1H NMR (500 MHz, DMSO) δ 11.37 (s, 1H), 8.10 (dd, J= 8.5, 6.5 Hz, 1H), 8.14 (s, 1H), 7.46 (dd, J= 10.0, 2.5 Hz, 1H), 7.44 (s, 1H), 7.38(s, 1H), 7.18 (dd, J= 8.5, 6.5 Hz, 1H), 6.84 (dd, J= 10.0, 2.5 Hz, 1H), 7.14 (dd, J= 8.5, 6.5 Hz, 1H), 6.84 (dd, J= 10.0, 2.5 Hz, 1H), 7.14 (dd, J= 8.5, 6.5 Hz, 1H), 6.84 (dd, J= 10.0, 2.5 Hz, 1H), 7.14 (dd, J= 8.5, 6.5 Hz, 1H)
EGFR inhibition by hybrid scaffolds in ovarian cancer

Table 1. Docking analysis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amino acid</th>
<th>Bond</th>
<th>Binding energy</th>
<th>Inhibitory constant (μM)</th>
<th>Intermolecular energy</th>
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</table>

The compounds in bold have good binding energy and good inhibitory constant and were taken for synthetic study and biological evaluation.

7-fluoro-6-nitro-3H-quinazolin-4-one (3)

A portion wise at 0 °C was added to the mixture of fuming nitric acid (105 mL) and concentrated sulfuric acid (100 mL), compound (2) (47.4 g, 0.29 mmol). The resulted mixture was heated at 110 °C for 2 hrs. The solution was cooled to room temperature, then poured onto crush ice mixture (1.5 L) followed by recrystallization by ethanol to give the product 7-fluoro-6-nitro-3H-quinazolin-4-one (yield 53%): M.p. 283-285 °C. 1H NMR (500 MHz, DMSO) δ 12.81 (s, 1H), 8.71 (d, J= 8.5 Hz, 1H), 8.32 (s, H), 7.77 (d, J= 12.0 Hz, 1H).

4-chloro-N-[(4Z)-7-fluoro-6-nitroquinazolin-4(3H)-ylidene] aniline (4a)

A suspension of compound (3) (10.45 g, 50 mmol) in SOCl2 (200 mL) containing 4-5 drops of dimethylformamide (DMF) was refluxed for 4 hrs to give a clear solution. Excess SOCl2 was removed under reduced pressure to obtained the crude product 4-chloro-7-fluoro-6-nitroquinazolin aniline. The obtained product was dissolved in chloroform (200 mL) and the solution of C6H5HN2 (10.5 g, 55 mmol) in ethanol (50 mL) was added. The obtained mixture was stirred at room temperature using mechanical stirrer for 20 min. N-hexane was added to complete the precipitation. The precipitated product was filtered with petroleum ether and dried to give pure product (4a). (yield 95%). 1H NMR (500 MHz, DMSO) δ 11.20 (s, 1H), 9.74 (d, J= 7.5 Hz, 1H), 8.81 (s, 1H), 8.10 (s, 1H), 7.90 (d, J= 12.0 Hz, 1H), 7.80 (dd, J= 7.0, 5 Hz, 1H), 7.40-7.45 (m, 2H).

Figure 2. Structure of T790M EGFR kinase domain co-crystallized with dacomitinib.
(4Z)-4-[(4-chlorophenyl)imino]-6-nitro-3,4-dihydroquinazolin-7-ol (5a)

A solution of 4a (1.0 mmol) in dioxane (5 ml) was refluxed with 50% aqueous NaOH (1 ml) for 2 hrs, cooled, acidified with 10% H2SO4 (10 ml) and extracted with ethyl acetate. The organic layers were combined and dried over anhydrous sodium sulfate. Further, these layers were dried by evaporation under reduced pressure. The crude product was purified by flash column chromatography on silica gel eluting with petroleum ether/Ethyl acetate =2:1 (yield 68%). 1H NMR (500 MHz, DMSO) δ 11.97 (s, 1H), 10.14 (s, 1H), 9.25 (s, 1H), 8.61 (s, 1H), 8.18 (s, 1H), 7.87 (d, J= 8.0 Hz, 1H), 7.37-7.33 (m, 2H), 7.25 (s, 1H).

(4Z)-6-amino-4-[(4-chlorophenyl)imino]-3,4-dihydroquinazolin-7-ol (6a)

SnCl2 (10 mmol) was added at 0°C to a solution of 5a (1.0 mmol) in 10 ml methanol. The resulting mixture was stirred using mechanical stirrer at 0°C for 20-25 min at room temperature for 25 min. The mixture was dried under reduced pressure. The pH of the mixture was adjusted to 6.5-7.5 by adding sodium bicarbonate. The residue was purified by using flash column chromatography on silica gel eluting with chloroform and methanol =10:1 (yield 68%). 1H NMR (500 MHz, DMSO) δ 10.71 (s, 1H), 9.32 (s, 1H), 8.33 (s, 1H), 8.22 (t, J= 8.0 Hz, 1H), 7.85 (d, J= 8.0 Hz, 1H), 7.37 (s, 1H), 7.29 (t, J= 8.0 Hz, 1H).

Synthesis of the final product

(3Z)-3-[(4-{(4-chlorophenyl)amino}-7-hydroxy-
quinazolin-6-yl)imino)-5-fluoro-1,3-dihydro-2H-indol-2-one (O-02); IUPAC: name of compound

To the solution of 6a (1 mmol) a solution of fluoro substituted isatin (1 mmol) in ethanol (25 mL) and a few drops of glacial acetic acid were added. The reaction mixture was refluxed for 6 hrs, and then allowed to cool at room temperature. The resultant product was filtered with water and dried to obtain the pure product 7a (yield 75%). 1H NMR (500 MHz, DMSO) δ 10.31 (s, 1H), 5.35 (s,1H,Ar-OH), 9.30 (s, 1H), 8.31 (s, 1H), 8.20 (t, J= 2.0 z, 1H), 7.85 (d, J= 9.0 Hz, 1H), 7.37 (s, 1H), 8.0 (s, 1H,J=2.0) 7.29 (t, J= 8.0 Hz, 1H), 7.19 (d, J= 9.0 Hz, 1H), 7.00 (s, 1H), 5.24 (s, 2H), FABMS (m/z, 100%): 450 ([M+2], 100%) for C22H13Cl2N5 O2 calculated for C(58.68),H (2.91), N (15.55).

Cell proliferation assay

The cells were put in an 96-well plate overnight in Agar growth medium at 105 cells/well. The next day the medium was changed to medium with 1-2% fetal bovine serum. Serially diluted compound (all synthetic compounds) were added, not exceeding a final 0.1% concentration of DMSO. The plates were then read after 72 hrs of the compound addition using CellTiter - Glo (Promega G7573, Madison, WI). The same procedure was repeated with 84 cells/well. The results are displayed in Table 2.

General procedure for preparation, purification of EGFR, and inhibitory assay

The EGFR inhibitory assay was performed as per the protocol mentioned by Introgen, [Introgen Therapeutics, Inc] and measured compounds against active forms of EGFR (Epidermal growth factor receptor inhibitor). Briefly, 10x concentration stocks of EGFR-WT (PV3872) from Invitrogen and EGFR-T 790M/L858R (40550) (BPS Bioscience, San Diego, CA), 1.13x ATP (AS001A) and Y12 - Sox peptide substrate mix and monitored every 71 sec for 50-120 min at UV absorption wavelength 360/λem485 in a Synergy 4 plate reader from BioTek (Winooski, VT).

Statistics

At the conclusion of each assay, progress curves from each well were examined for linear reaction kinetics and fit statistics (R2, 95% confidence interval, absolute sum of squares). Initial velocity (0 min to +30 min) from each reaction was determined from the slope of a plot of relative fluorescence units vs time (min) and then plotted against inhibitor concentration to estimate apparent IC50 from log[Inhibitor] vs Response, Variable Slope model in GraphPad Prism from GraphPad Software (San Diego, CA). The following reagents were used for the EGFR-WT kinase assay: EGFR kinase (5 nM), ATP (15 μM) and Y12- Sox peptide (5 μM). For
the EGFR-T790M/L858R kinase assay (3 nM), ATP (20-50 μM) and Y12-Sox peptide (5 μM) [44].

**Results**

*Molecular docking*

Sixteen new quinazoline isatine derivatives were designed and structures were docked using Accelyres Drug Discovery Studio 3.5 [41]. The structure of the enzyme T790M EGFR was obtained from Protein data bank (www.pdb.com) (PDB code: 4I24; Figure 2) and was used for docking. The crystal structure was cleaned by deleting the ligand, water and cofactors. This was followed by

![Figure 6. Scheme for synthesis of 3-(4-(4-Substituted phenylamino)-7-hydroxyquinazolin-6-yl)imino)5-Substituted 1,3-dihydro-2H-indol-2-one. Reaction conditions: a: Formanimide acetate, 18 h; b: HNO₃, H₂SO₄, 110°C; c: SOCl₂, reflux, 4 h; d: anilines 50 min at room temperature; e: 50% NaOH, reflux, 3 h; f: SnCl₂2H₂O, 30 min at room temperature; g: sub isatin in EtOH.](image)

![Figure 7. Activity against OVCAR-3 cell line and EGFR inhibition correlation (R² = 0.94).](image)

![Table 3. EGFR inhibitory studies](image)
by adding hydrogen atoms to the protein. Docking calculations were carried out using Accelyres Drug Discovery Studio (3.5 version). The ligand structures were imported to the new window with proper names and program was run to prepare the ligands. Finally, the input ligands were selected (Molecules: All) on protein molecular window and were docked for 4I24. The setup for side-chain flexibility by selection of the all-visible option and the setting for the other selected chain during the docking and other parameters were kept in default. The Binding Energy and Electrostatic Interaction energy was calculated (Docking analysis, Table 1).

Analysis of docking

All the listed conformers were virtually docked at the defined cavity of the receptor; the results of docking and binding energy of every compound with the receptor are listed in Table 1 and in Figures 3-5 using dacomitinib as standard drug.

Synthetic studies

Based on the results of docking the compounds which showed good binding affinity with the receptor were further selected for the synthetic studies. (Figure 6).

Anticancer activity

Anti-proliferative activity

The compounds which showed good binding affinity were synthesized and characterized. After their structural confirmation they were evaluated for their antiproliferative activity against OVCAR-3, OVCAR-8 and OV1065 cell lines. Dacomitinib used standard drug was also tested under similar conditions for comparison. The results were summarized in Table 2.

EGFR inhibitory activity

The compounds that showed good antiproliferative activity against OVCAR-3 cells were further tested for EGFR inhibitory activity. Nearly all the tested compounds showed good EGFR inhibitory activity. Among all the compounds O-05 and O-13 had most potent inhibitory activity with an IC_{50} value of 0.46 and 1.12 μM, respectively. The results are shown in Table 3 and Figure 7.

Inhibition to OVCAR-3 and cell migration studies for O-05

Prior to the study of inhibition of cell migration, we applied CBA-100 with concentration of 0.06, 0.08, 0.1 μM and evaluated its inhibitory activity against OVCAR-3 cell migration. The results are shown in Figure 8.

Discussion

Ligands with good binding score form most stable receptor complex with the receptor. The binding pocket is hydrophobic, thus hydrophobic interaction plays a vital role in affinity to EGFR-TK binding. At least two hydrogen bonds of ligand and the receptor are necessary for small molecules to act as EGFR inhibitors. All the compounds were synthesized in good yield and were confirmed by spectral analysis. As evidenced from the results of antiproliferative activity, the O-02 to O-14 compounds showed good antiproliferative activity on OVCAR-3 cell line displaying IC_{50} values between 2.11 to 11.67 μM. Out of the compounds tested, O-05 showed better inhibitory activity (IC_{50} = 2.11 μM). These results were superior comparing with previously reported studies [10-14]. As for the cell migration studies of the designed compounds IC_{50} was better compared with previously reported results [13,14]. The structural activity relationship studies revealed that the compounds which contain diverse imino groups equally possess different antiproliferative activities, which indicated that the antiproliferative activity of compounds was correlated to the structure of imino group. In addition it was confirmed that electronic withdrawn substituents enhanced the antiproliferative activity. The EGFR activity revealed the relationship between antiproliferative activity and EGFR inhibitory activity. We also studied the correlation between the antiproliferative activity against OV-
CAR-3 cell line and EGFR inhibitory activity and found statistically significant difference ($R^2=0.94$). As evidenced from cell migration studies it was confirmed that the amount of migration was less than the control indicating the potential of O-05 compound for antimitastatic therapy.

**Conclusion**

The synthesized compounds exhibited good antitumor activity against various ovarian cancer cell lines, but strong activity was found only in OVCAR-3 cell line. O-05 compound showed potent activity against OVCAR-3 cell line with the IC$_{50}$ values within an acceptable range. This study revealed that the heterocyclic ring with hydrophobic substitutions is necessary for the fit into the binding with the receptor. The encouraging results of the present study could offer an excellent platform towards discovery of new potent antitumor agents. Moreover, the said compounds may be further tested for their activity against other cancer types.

**Conflict of interests**

The authors declare no conflict of interests.

**References**

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