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DUAL TARGETING OF VEGFR-2 AND C-MET KINASES VIA THE DESIGN AND SYNTHESIS OF SUBSTITUTED BENZYLIDENE-6-(5-CHLOROPYRIMIDIN-2-YL)-9H-PURINE-2,6-DIAMINE DERIVATIVES AS ANGIOGENESIS INHIBITORS

S.A. More[1], N. S. Sakle[2] and S.N. Mokale[1,✉]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Y. B. Chavan College of Pharmacy, Aurangabad-431001, India

[2]Department of Pharmacology, Faculty of Pharmacy, Y. B. Chavan College of Pharmacy, Aurangabad-431001, India

✉Corresponding Author: santoshmokale@rediffmail.com

ABSTRACT

We have designed and synthesized a unique library of benzylidene-6-(5-chloropyrimidin-2-yl)-9H-purine-2,6-diamine derivatives as angiogenesis inhibitors. The designed scaffolds were subjected to docking and ADME prediction studies so as to guage the particular interaction. Further anti-proliferative activity was allotted by employing the SRB method as a target for colorectal cancer on HT-29 and COLO-205 cell lines. The SM-6 derivative showed good anticancer activity and was subjected to in-vitro enzyme inhibition activity using a flow cytometer to test the enzyme inhibition potential. It also induced apoptosis and cell cycle arrest at the G0/G1 phase on HT-29 cells supported by DAPI staining and propidium iodide (PI) staining followed by flow cytometry analyses. These compounds exhibited slight inhibitory effects against VEGFR and c-Met kinases, so their active skeletons warrant further study and will have a positive effect on the event of small anticancer inhibitors of dual-target VEGFR/c-Met kinase.

Keywords: Synthesis, Anti-Proliferative Activity, Cell Cycle Analysis, Apoptosis Assay, VEGFR-2 Inhibitory Assay, Molecular Docking, In-silico ADME Study, MM/GBSA.

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INTRODUCTION

Tumors can be a multi-stage, complex process with life-threatening consequences for people's health and lives.[1] For tumor spread, growth, and survival (RTK) a variety of signal transduction pathways including receptor tyrosine kinases are required for cell differentiation, proliferation, angiogenesis, and apoptosis.[2] In response to ligands, RTK primarily activates transcription factors that mediate target organic phenomenon. RTK signaling pathways are complicated including a wide range of metabolic events and molecular mediators in complex signaling networks.[3] Vascular endothelial protein receptor-2 (VEGFR-2) is taken into account because of the main effector of VEGF/ VEGFR signal transduction in promoting tumor angiogenesis.[4,5] The phosphorylation of VEGFR-2 activates the Raf-1/MAPK/ERK signaling pathway, which results in angiogenesis and improves vascular permeability and tumor migration.[6] Therefore, inhibition of the VEGFR-2 signaling pathway is taken into account in the concert of the foremost important pathway within the development of tumor chemotherapy.[7,8] Mesenchymal epithelial transfer factor tyrosine kinase (c-MET) may be a crucial member of the receptor tyrosine kinases (RTK) family.[9] c-MET is activated by extracellular binding of its ligand, hepatocyte growth factor/ scatter factor (HGF/SF).[10] The aberrant expression of c-MET/ HGF signaling arises from c-MET mutations or overexpression or genomic amplification, which may promote proliferation, migration, invasion, and tumor angiogenesis.[11] The role of c-MET and VEGFR-2 have a synergistic role within the angiogenesis of human cancer. The utilization of dual targets i.e., c-MET and VEGFR-2 inhibitor may act as a necessary element within the development of targeted therapy.[12,13] c-MET is up-regulated in response to VEGFR pathway inhibition and so plays a vital role in tumor angiogenesis and progression.[14] However, the matter of drug resistance frequently arises within the research of single-target drugs and combination drugs. It is found that multi-target drugs may

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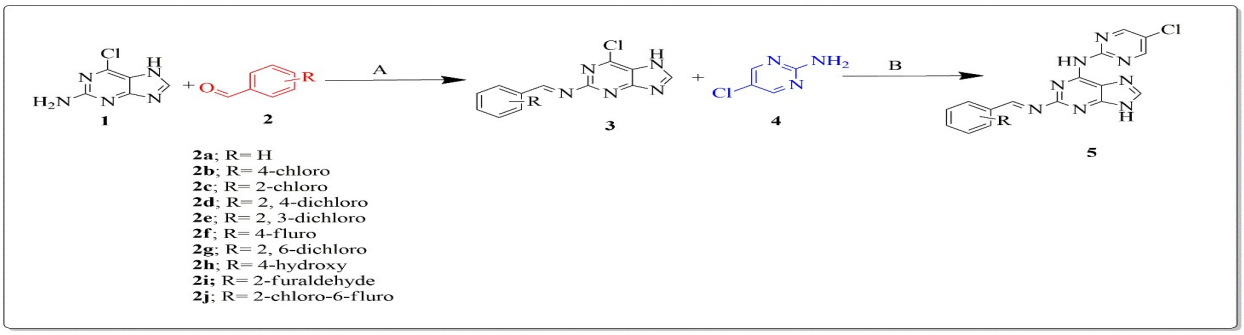
overcome drug resistance and achieve higher efficacy than single-target drugs, which makes the molecules of multi-target drugs widely studied.

EXPERIMENTAL

General procedure for the synthesis of substituted benzylidene-6-chloro-7H-purin-2-amine: The 2-chloro-6,7-dimethoxyquinazolin-4-amine and substituted aldehyde (1:1 Mol) was dissolved in ethanol (10 ml) followed by addition of glacial acetic acid (2 drops). The reaction mixture was allowed to reflux for 24 hours. After completion of the reaction, as indicated by TLC, the reaction mixture was diluted with 100 ml of ice-cold water. The intermediate was filtered and the obtained product was recrystallized using ethanol as a solvent to get fine crystals. General procedure for the synthesis of substituted benzylidene-N6-(5-chloropyrimidin-2-yl)-9H-purine-2, 6-diamine: Once the fine crystals were obtained, the intermediate was reacted to 2-amino-5-cloropyrimidine (1:1mol) by using DMF and anhydrous Potassium Carbonate (3 equivalents).[13] After completion of the reaction, the mixture was diluted with 100 ml of ice-cold water. The final product was filtered and recrystallized by using ethanol as a solvent to get fine crystals (Scheme-1).



Scheme-1: Reagents and Conditions: (1) 2-amino-6-Chloropurine, (2) Benzaldehyde, (a) Glacial Acetic Acid, EtOH, reflux; (3) benzylidene-6-chloro-7H-purin-2-amine (4) 2-amino-5-chloropyrimidine, (b) K2CO3, DMF, reflux and (5) benzylidene-(5-chloropyrimidin-2-yl)-9H-purine-2, 6-diamine

Biological Evaluation

In Vitro Anti-Proliferative Activity

The HT-29 and COLO-205 colorectal cancer cell lines were chosen because docking scores for VEGFR and c-MET were the highest, and overexpression of these two receptors is common in colorectal cancer. SRB test was used to assess the antiproliferative properties of the synthesized compounds against HT-29 and COLO-205 cells. Cells were injected into 96-well microtiter plates in 90L at 5000 cells per well for the current screening experiment. Following cell inoculation, the microtiter plates were incubated for 24 hours at 37°C, 5% CO_2, 95% air, and 100% relative humidity before adding experimental medicines. To prepare a stock of 10-2 concentrations, experimental medicines were solubilized in a suitable solvent. Cells were fixed in situ by the gentle addition of 50 µL of cold 30 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4°C, thereafter SRB assays were performed. The absorbance was read on an ELISA plate reader at a wavelength of 540 nm with a 690 nm reference wavelength.

Enzyme Inhibition Assay

In vitro inhibition kinase assay was carried out by Averin Biotech Pvt. Ltd, Hyderabad. The general procedures were as followed: Culture cells in a 6-well plate at a density of 0.5 x 106 cells/2 ml and incubate in a CO_2 incubator overnight at 37°C for 24 hours. After the incubation period, remove spent media and add IC50 concentrations of compounds and incubate the cell for 72hrs. At the end of the treatment, remove the medium from all the wells into 12 x 75 mm polystyrene tubes and wash with 500 µl PBS (remember to save the PBS in the same tubes). Remove the PBS and add 250 µl of trypsin-EDTA solution and incubate at 37°C for 3-4 minutes. Pour the culture medium back into their respective wells and harvest the cells directly into 12 x 75 mm polystyrene tubes. Centrifuge the tubes for five minutes at 300 x g at 25°C. Carefully decant the supernatant. Wash with PBS twice. Decant the PBS completely. Stain the cells with 20ul of VEGFR-2 and HGF conjugated with PE and incubate at 37 °C for 30s min protected from light. Gently re-suspend cells in 400µl pre-warmed DPBS and analyzed by flow cytometry using the 496 nm laser for excitation and detection at 578nm (FL2).

Molecular Docking, in-silico Physicochemical Properties, and ADME Prediction

Schrodinger Maestro 12.3 was used to do the Molecular Docking. The X-ray co-crystal structure of kinase was downloaded from the RCS Protein Data Bank and constructed using Protein Preparation Wizard, with constraints imposed and default settings utilized in the Schrodinger suite's Maestro graphical user interface. The compounds were exposed to Qikprop, Schrodinger 12.3 molecular properties prediction in order to analyze and predict both physicochemical and pharmacokinetic relevant properties in order to assess the overall quality of developed derivatives as a therapeutic candidate. In order to calculate the ligand binding free energies of the complex system, the prime plug-in was used. MM/MGSB (Molecular mechanics, The Generalized Born Model, and solvent accessibility) was performed to calculate the ligand binding free energies and ligand strain energies for the docked lead compound with c-MET (PDB code: 3LQ8) and VEGFR (PDB code: 4ASD) (Table 4). Polar solvation energies, non-polar solvation energies, and potential energies are the three basic components of binding free energy. Prime MM-GBSA is a plug-in that incorporates the advanced OPLS-2005 force field, the SGB solvation model for polar solvation (GBSA), non-polar solvation (GNP), and molecular mechanics energies (EMM), as well as non-polar accessible surface area and Vander Waals interactions.

RESULTS AND DISCUSSION

***In Vitro* Anti-Proliferative Activity**

The designed synthesized target compounds were evaluated for cytotoxicity against two cell lines viz. HT-29 and COLO-205 (human Colo-rectal cancer; NCI, USA) by Sulforhodamine B (SRB) cell proliferation assay. The results of inhibition were expressed as GI50 i.e. concentration of drug causing 50% inhibition of cell growth values which are summarized in Table-1. The cytotoxicity study clearly indicates that four target compounds showed good cytotoxicity against the HT-29 and COLO-205 cancer cell lines. Among that SM-2 (GI50= 13.28 μM), SM-6 (GI50= 10.75 μM) and SM-9 (GI50= 12.71 μM) showed good activity against COLO-205; whereas, SM-2 (GI50= 13.51 μM), SM-6 (GI50= 10.64 μM) and SM-9 (GI50= 15.82 μM) showed good inhibition activity against HT-29 cell lines.

Table-1: Antiproliferative Screening of the Synthesized Compounds on HT-29 and COLO-205 Cell Lines

Compounds	GI_{50} (μM)	
	HT-29	COLO-205
SM-1	120.06	57.2
SM-2	13.51	13.28
SM-3	145.89	35.43
SM-4	154.62	30.38
SM-5	175.25	125.27
SM-6	10.64	10.75
SM-7	15.74	36.06
SM-8	17.64	12.42
SM-9	15.82	12.71
SM-10	18.94	18.24
Doxorubicin	<10	<10

VEGFR-2 Inhibition Study

As VEGF pathway inhibition may trigger the upregulation of MET expression which may stimulate tumor invasion, a VEGFR-2 inhibition assay was performed. Moreover, the MET signaling pathway is considered as a mechanism of resistance to vascular endothelial growth factors receptor therapy. The most active synthesized compound, SM-6 was subjected to VEGFR-2 inhibitory activity. Doxorubicin was used as positive control which showed the inhibition of 67.45% (COLO-05) and 66.95% (HT-29), whereas synthesized SM-6 have shown 66.76% (COLO-205) and 65.39 (HT-29) inhibition (Table-2).

***In-silico* Screening of Designed Derivatives**

Molecular Docking

The significant inhibitory activity of the synthesized compounds was investigated through molecular docking inside the active site of the VEGFR-2 crystal structure (PDB: 4ASD) and c-MET (PDB: 3LQ8).

Thus, in order to understand the interaction between the designed compounds and kinase, molecular docking was performed using Schrodinger Maestro 12.3 software. The docking study was carried out using Cabozantib as a standard drug in order to find out a better score and interactions. The results are shown in Table-3.

Table-2: Table showing the % of Inhibition of VEGFR-2 Marker in Untreated, Std. and SM-6 Treated COLO-205 and HT-29 Cells

Test/ Marker	VEGFR-2 % of Inhibition	
	COLO-205	HT-29
Cell Control	0.54	0.28
Doxorubicin	67.45	66.95
SM-6	66.76	65.39

Table-3: Docking Scores of the Synthesized Compounds against Selected Receptors with PDB IDs

Compound	VEGFR (PDB ID: 4ASD)	c-MET (PDB ID: 3LQ8)
SM-1	-8.807	-7.174
SM-2	-8.995	-8.793
SM-3	-8.035	-8.229
SM-4	-7.089	-8.551
SM-5	-8.766	-6.671
SM-6	-9.821	-9.940
SM-7	-7.770	-7.159
SM-8	-8.766	-6.790
SM-9	-8.605	-8.119
SM-10	-7.770	-6.921
Cabozantinib	-10.547	-8.473

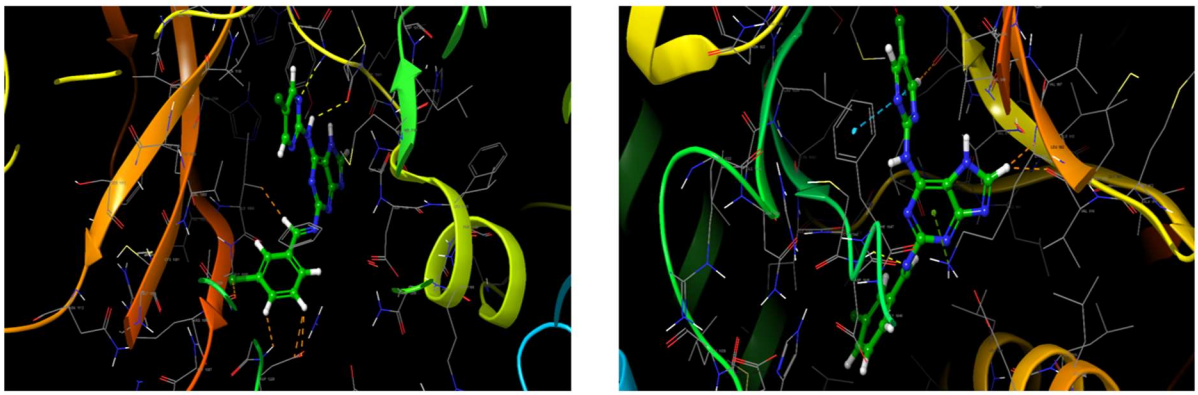


Fig-1: 3D Interaction Diagram of A) SM-6 C-MET (PDB ID: 3LQ8) B) SM-6 VEGFR (PDB ID: 4ASD)

In-Silico Physicochemical Properties and ADME Prediction

In-silico studies were performed for the theoretical prediction of the physicochemical properties of the synthesized compounds. The important parameters were calculated by using Qikprop Schrodinger Maestro 12.3. %ABS was calculated by using the formula: 109-0.345*TPSA (Table-4 and 5).

Table-4: *In-Silico* Physicochemical Properties of the Most Promising Compounds

Compounds	M.W	LogP	QPlogPo/w	n-OH	n-OH-NH	Lipinski's Rule of Five	TPSA	%ABS
SM-1	440.84	3.4	3.061	7.00	2	1	113.768	92.1917
SM-2	419.66	4.8	3.330	7.00	2	0	95.378	92.1945
SM-3	419.66	4.8	3.401	7.00	2	0	95.334	92.2397
SM-4	419.66	4.8	3.373	7.00	2	0	96.275	92.2493
SM-5	403.20	4.4	3.154	7.00	2	0	95.333	92.3145

SM-6	385.21	4.3	2.053	7.00	2	0	104.168	96.1886
SM-7	340.39	2.3	2.894	7.00	2	0	94.724	92.2397
SM-8	368.76	3.9	3.145	7.500	2	0	94.732	94.4065
SM-9	366.76	3.9	1.959	7.500	2	0	117.266	98.8839
SM-10	385.21	4.34	3.146	7.00	2	0	94.758	92.2383

Table-5: *In-silico* ADME Prediction of the Synthesized Compounds

Compound	Number of Metabolites	Predicted ADME		
		Human Oral Absorption (%)	Blood Brain Barrier (BBB)	Plasma Protein binding (PPB %)
SM-1	5	100	0	92.929
SM-2	2	100	0	94.38
SM-3	2	100	0	92.902
SM-4	2	100	0	93.139
SM-5	2	100	0	96.805
SM-6	2	100	0	98.073
SM-7	2	100	0	93.151
SM-8	2	100	0	95.849
SM-9	2	100	0	97.713
SM-10	2	100	0	93.668

CONCLUSION

The above result proves and indicates that SM-6 could serve as an important gateway for the design and development of new multi-target potent inhibitors.

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