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Design, synthesis and biological evaluations of 2-aminothiazole scaffold containing amino acid moieties as anti-cancer agents

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Abstract: Due to the emerging mortality rate of colorectal cancer there is a high need for the management and control of this disease. Although several treatment approaches are being developed day by day yet the high incidence rate of colorectal cancer is still not controlled. To ease in the development of treatment therapies for colorectal cancer two derivatives of ethyl 2-aminothiazole 4-carboxylate were designed and synthesized. The compounds Ethyl 2-(2-(1,3-dioxoisoindolin-2-yl)acetamido)thiazole-4-carboxylate (5a) and ethyl 2-(2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanamido)thiazole-4-carboxylate (5b) were characterized and studied for their anti-cancer activities. The *in silico* molecular modeling studies were performed against the target protein beta-catenin which is an important player in the progression of colorectal cancer. The *in silico* ADMET studies were performed to assess the basic physicochemical properties of these compounds. The *in vitro* antiproliferative assay and the enzyme inhibitory assay was performed to validate the role of these compounds in the colorectal cancer. The preliminary cytotoxic assay and the MTT assay of the compounds 5a and 5b against the colorectal cancer cell line HCT 116 showed 60% inhibition of cell proliferation with IC50 of 0.72µM and 1.55µM, respectively. The standard methotrexate showed IC50 of 0.7µM showing potent inhibitory action of these compounds. The *in vitro* validation of the anti-cancer effect of both compounds revealed significant inhibition of beta-catenin concentration at higher doses as compared to control. Both the *in vitro* and *in vivo* assays of compounds showed effective anti-cancer activities and depicts the future potential of these compounds in colorectal cancer.

Keywords: 2-amino-thiazole, molecular docking, *in silico,* colorectal cancer, beta-catenin.

INTRODUCTION

The classes of compounds belonging to heterocyclic group are part of several biologically active agents with widespread use as building blocks in chemistry. 2-aminothiazole, a typical heterocyclic amine is an essential scaffold in the synthesis of many antibiotics, fungicides, anti-oxidants, anti-diabetic (Siddiqui *et al*., 2011), anti-tubercular, anti-viral (Siddiqui *et al*., 2009), anti-inflammatory, anti-convulsant and anti-tumor agents (El-Subbagh *et al*., 1999). Some commonly known pharmaceuticals including talipexole and pramipexole are anti-parkinsonian and dopamine agonists' containing 2-aminothaizole moiety. Tigemonam and Amthamine another 2-aminothiazole containing agents are used as antibacterial and antihistaminic agents, respectively (Zhuravel *et al*., 2005). Another interesting properties of 2-aminothiazole containing agents is that they act as ligands of adenosine receptor antagonist and estrogen receptors (Lin *et al*., 2009).

Many studies have demonstrated cytostatic and cytotoxic activities of 2-aminothiazole against series of cell lines He La L929, HT 29 and T47D as well as against enzymes involved in eicosanoid metabolism. These derivatives also act as inhibitors of *Mycobacterium tuberculosis*, nerve growth factor receptor inhibitor TrkA, inhibitors of MAPK and as vascular adhesion protein-1 inhibitors (VAP-1). The tautomeric equilibriums of 2-aminothiazole and its derivatives have huge importance in biological and industrial sector and has great prominence among scientists (Khalifa, 2018).

Among different cancers, the colorectal cancers (CRCs) undergo a wide range of genetic and epigenetic alterations developing into a group of heterogeneous diseases (Inamura, 2018). It is considered as the third most commonly occurring tumor in oncologic pathology and is expected to overcome the mortality rate of cardiac diseases in the coming years (Siegel *et al*., 2013). It is a prevalent disease in women and in those aged 65-75. The patient suffering from colorectal cancer shows clinical presentation of abdominal pain, chronic bowel movement alterations, nausea, vomiting, involuntary weight loss, anorexia and abdominal distention (Arcos and Tirado, 2009).

Due to the better understanding of carcinogenesis pathways with the help of The Cancer Genome Atlas

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project, progressive development of new potential biomarkers from clinical studies and introduction of novel targeted agents there has been pronounced advancement in the management of CRC in past few years (Ciombor *et al*., 2015). Several biomarkers are known to be involved in the occurrence and progression of colorectal cancer. One of them, beta catenin is an adhesion protein that serves as an intermediate in several transduction pathways including the wnt pathway. It not only have a structural role but is also involved in the activation of several transduction pathways. In our previous work, we identified the potential colorectal cancer targets through system biology approach in which beta-catenin was found to be the candidate biomarker in the progression and development of colorectal tumoriogenesis (Ilyas *et al*., 2020).

The widespread use of 2-aminothiazole derivatives in the management of numerous biological system these derivatives have attracted the attention of several biologists. Our study was also based on the synthesis of this essential scaffold coupled with amino acids, their characterization and study of their important anti-cancer activities. The colorectal cancer targets were identified in our previous study through system biology approach. The differential expression analysis and several bioinformatics tools such as R programming and Bioconductor packages have helped in identifying and studying several important biomarkers in the field of cancer (Altaf *et al*., 2021, Kauffmann *et al*., 2009). About 7 differentially expressed genes were shortlisted, curated and further gene network and protein interaction analysis was performed to understand the role of potential biomarkers in the progression of colorectal cancer (Ilyas *et al*., 2020). The protein target CTNNB1 (beta-catenin) was selected due to its upregulatory role in the development of colorectal cancer. The *in silico* molecular docking studies as well as the *in vitro* cytotoxic and cell inhibition assay was performed to investigate the inhibitory role of these agents in colorectal cancer cell line HCT 116. The cellular role of these agents was also studied against the essential protein beta-catenin involved in the progression of this disease. The study provides important role of these agents in colorectal cancer.

MATERIALS AND METHODS

The melting points of all the compounds were verified with the help of a digital *Gallenkamp* [SANYO] model MPD.BM 3.5 apparatus. Silica gel HF-254 coated plates were used to monitor the reaction completion using thin layer chromatography. The characterization of compounds was done using ^{1}H-NMR (Proton nuclear magnetic resonance) and ^{13}C-NMR (Carbon-13 nuclear magnetic resonance) spectral analysis. *Bruker AV400* spectrophotometer in $CDCl_3$ (Deuterated Chloroform) at 400 *MHz* was used for this purpose and TMS (Tetramethyl silane) was used as an internal standard. Chemical shifts (δ) of all the compounds were measured in ppm (Parts per million). ESI (Electrospray ionization) mass spectra were obtained using Quattro II (Micromass). FT-IR (Fourier transform infrared) spectra were analyzed with the help of *alpha Bruker FT-IR* spectrophotometer (ATR eco ZnSe, V_{max} in cm^{-1}). The elemental analysis was carried out using a LECO-183 CHNS analyzer. The column chromatographic purification was done suing Merck silica gel HF-60 with (4:1 pet.ether:ethyl acetate,) as eluent. The chemicals and reagents used in this study were obtained from Sigma Aldrich Chemical Co., USA.

Standard procedure for the synthesis of ethyl-2-aminothiazole-4-carboxylate

In 53mL of absolute ethanol 0.05 mol ethyl bromopyruvate and 0.10 mol thiourea was refluxed for 24 h. Thin layer chromatography was used to monitor the completion of reaction. The reaction was then allowed to cool at room temperature. The mixture was concentrated in *vacuo* to half the original volume. 2N NaOH was used to alkaline the remaining solution of ethanol by pouring it into water at pH10. The immediate precipitation of light brown solid appeared. For about 10 min the reaction mixture was stirred. Vacuum filtration was used to filter the solid and dried to get final product (Goyal *et al*., 2011, Goyal *et al*., 2019).

2-amino ethyl -1,3-thiazole-4-carboxylate (1)

Light brown crystalline (solid). Yield 70%. M.P. 176 °C. Rf (Retardation factor) 0.19 (pet.ether:ethyl acetate 3:1); IR (ν_{max} cm^{-1}): 3291 (NH_2, str.); 1614 (C=N, str.); 1731 (C=O, ester, str.); 1541 (C=C, str.); ^{1}H-NMR (400 MHz, CD_3OD): 7.5 (s, 1H, H-1); 4.31 (q, *J*=7.2Hz, 2H, H-2); 5.7 (brs, 2H, NH_2); 1.34 (t, *J*=7.1Hz, 3H, H-3); Analytical Calcuation for $C_6H_8O_2N_2S$ C: 41.87; H: 4.69; N: 16.28; found: C: 41.78; H 4.60; N: 16.18.

Synthesis of N, N-phthaloyl protected amino acids

0.03 mol of amino acid (2a, 2b) and 0.03 mol of phthalic anhydride was mixed and heated in an oil bath at 185-190°C for 30 min while stirring the reaction was cooled. In 20 mL of hot methanol the solid material was dissolved and filtered. The products (3a,3b) were allowed to crystallize out slowly IrUU8KOhanol, water mixture (1:1) (Usifoh *et al*., 2001).

2-(1,3-dioxoisoindolin-2-yl)acetic acid (3a)

White crystalline (solid). Yield 89%. m.p 197°C (lit. 197-198 °C). Rf 0.65 (ethyl acetate: pet. ether 3:1).

2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanoic acid (3b)

White crystalline (solid). Yield 90%. m.p 175°C (lit. 178°C). Rf 0.70 (ethyl acetate:pet.ether 3:1).

Synthesis of N,N-phthaloyl amino acids chlorides

5 mmol N,N-Phthaloyl amino acids (3a,3b) in 25 mL dichloromethane, 15 mmol thionyl chloride was added and refluxed for 8h. TLC was used to monitor the reaction competion, excess thionyl chloride was evaporated after

reaction completion. The resulting products (4a,4b) were used without further purification (HASAN *et al*., 2000).

Preparation of amides

To 0.01 mol ethyl-2-aminothiazole-4-carboxylate **(1)** in 50 mL dry toluene, 0.015 mol N,N-phthaloyl amino acids chlorides already dissolved in dry toluene were added drop wise. For 8 h the reaction was heated under reflux. TLC was sued to monitor the reaction. After completion, the solvent was vaporized in *vacuo*. Column chromatography was used to purify the residue obtained using (pet. ether: ethyl acetate, 4:1) as mobile phase (El-Subbagh *et al*., 1999).

Ethyl 2-(2-(1,3-dioxoisoindolin-2-yl)acetamido)thiazole-4-carboxylate (5a)

Brown (solid). Yield 71%. m.p 172 °C. Rf 0.36 (pet.ether: ethyl acetate 3:1); IR (ν_{max} cm^{-1}): 3297 (N-H, str.), 1697 (C=O, amide, str.); 1725 (C=O, ester, str.); 1632,1538 (C=O, phthaloyl amide, str.); ^{1}H-NMR (400 MHz, $CDCl_3$): 9.24 (brs,1H, NH); 7.27 (s, 1H, H-1); 7.91-7.77 (m, 4H, aromatic); 4.51 (s, 2H, H-4); 1.4 (t, *J*=7.2Hz, 3H, H-3); 3.74 (q, *J*=7.1Hz, 2H, H-2); ^{13}C-NMR (400 MHz, $CDCl_3$) δ, ppm: 191.1, 167.4 , 170.3 , 167.2, 139.5, 158.1, 133.4, 129.4, 133.2, 123.2, 130.3, 123.7, 48.4, 113.4, 6.839.7,; Analytical Calculation. for $C_{16}H_{13}O_5N_3S$.(343.35) C: 54.47; N: 12.68; H: 3.61; found: C: 53.71; N: 11.44; H: 3.21. Mass spectrum (ESI) $(M + H)^+$ =344.

Ethyl 2-(2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanamido)thiazole-4-carboxylate (5b)

White crystalline (solid). Yield 87%. m.p 152 °C. Rf 0.28 (pet.ether:ethyl acetate 3:1); IR (ν_{max} cm^{-1}): 3321 (N-H, str.); 1730 (C=O, ester, str.); 1695 (C=O, amide, str.); 1662,1546 (C=O, phthaloyl amide, str.); ^{1}H-NMR (400 MHz, $CDCl_3$): 10.12 (brs,1H, NH); 7.18 (s, 1H, H-1);7.85-7.78 (m, 8H, Ar-H); 5.38 (d, *J*=4.8Hz, 1H, H-4); 4.43 (q, *J*=7.2Hz, 2H, H-2); 1.41 (t, *J*=7.2Hz, 3H, H-3); 3.68 (m, 2H, H-5); ^{13}C-NMR (400 MHz, $CDCl_3$) δ, ppm: 192.2, 167.8 , 170.4 , 138.3, 167.4, 156.5, 137.3, 131.5, 133.7, 132.2, 129.7, 131.2, 130.1, 129.2, 128.6, 123.4,128.3, 123.4, 115.5, 58.3, 35.3, 37.8, 8.4; Anal. Calcd. for $C_{23}H_{19}O_5N_3S$. (433.48) C: 61.45; N: 9.36; H: 4.24: found: C: 60.38; N: 8.52; H: 3.94. Mass spectrum (ESI) $(M + H)^+$ =434.

In silico studies

ADMET studies

An online free web server Swiss ADME predictor was used to study the ADME profile of the synthesized compounds. The tool is used to predict the cheminformaticss, drug like properties and medicinal chemistry of small molecules. The compounds with molecular weight <500 g/mol, hydrogen bond acceptor <10, hydrogen bond donor <5 and number of rotatable bonds <10 are preferred as drug likable compounds. The server also identifies the lipophilic and hydrophilic nature of compounds through Log P and S values. Log P is a degree of lipophilicity of the compounds and is the ratio of concentration of drug substance between two solvents in unionized form. The stronger the lipophilicity of compounds is linked to lower value. The hydrophilicity of the compounds determines the absorption and distribution parameters. The lower water solubility refers to decreased absorption. Log S is the determination of solubility of compound in itself. It is 10 based logarithm of solubility which is calculated in mol/L. The optimum value of Log S for better absorption and distribution lies somewhere between -1 to -4.

The online web server pkCSM was used to test the mutagenic and carcinogenicity of synthesized compounds (Pires *et al*., 2015).

Molecular docking analysis

The molecular docking analysis was performed using software MOE 2010.11 to study the best binding conformation of ligands based on lowest energy scores. Drug Discovery Studio was used to visualize and interpret the results. The active binding sites of target protein was detected by DoG Site Scorer (Volkamer *et al*., 2012). MOE v2010.11 was used to prepare the structure of ligands using the Molecular Builder Module program and were saved as. mdb file. The minimization of energy of ligands was done up to 0.05 Gradient using MMFF94 s force field. The crystallographic structure of Beta catenin (CTNNB1_3SL9, Homo sapiens, Resolution 2.2 Aº) was downloaded from protein data bank. The protein crystal structures mostly contains little or no hydrogen coordinates because of the limited resolution. Therefore, protonation was done using Protonate 3D tools before protein-ligand docking. Energy minimization was then performed with 0.05 Gradient with the help of Amber 99 force field. In order to minimize the energy of protein molecule the energy minimization algorithm of MOE tool was used. The parameters used for minimization of energy were; gradient 0.05, Chiral constraint: Current geometry, Force Field = MMFF94X+Solvation. As the root mean square gradient fall below 0.05 the energy minimization was terminated. GizMOE was used to calculate the initial and final energy in kcal/mol using MMFF94X force field. The minimized structure obtained was then used for Docking as a template. The protein ligand binding was analyzed with the help of MOE docking program in order to analyzer the best conformation and configuration of the ligands having the minimum energy structure. Following parameters were used to carry out the docking analysis, Total Runs: 50, Cycle/Runs : 15, Iteration : 10 000, Potential Energy Grid: ON, Annealing Algorithm : Simulated Annealing.

In vitro studies

In vitro cytotoxic profiling of synthesized compounds

Brine shrimp lethality assay is an important way of identifying the cytotoxic effect of bioactive compounds and is a preliminary assay. At room temperature the brine

shrimp eggs were hatched for 48 hours in simulated sea water in order to get nauplii. The solutions of sample were prepared by dissolving samples at various concentrations of 25 50, 100 and 200 µg/ml in DMSO by serial dilutions. To pre-marked vials the solutions were then added having 10 live nauplii in 5 ml simulated sea water for 24 h. The effectiveness of compounds were assessed by calculating the number of motile nauplii and percent mortality rate (Solis *et al*., 1993). The lethal concentration of the compounds were calculated by the Porbit method (Finney, 1952).

In vitro anticancer activities

The human colorectal cancer HCT 116 cell line ATCC®CCL-247™ [(catalog no: 91091005-1VL) Sigma Aldrich] was used to study the cytotoxic effect of synthesized compounds (Mosmann, 1983). The cell lines were gifted from Department of Biology, Lahore University of Management Sciences (LUMS), Lahore, Pakistan. In T-75 flasks Costar the cells were cultured as monolayer. In 5% CO_2 and 95% air atmosphere supplied incubator at 37ºC subculturing was performed twice a week. In McCoy's 5A medium (Gibco Glasgow) the HCT 116 was cultured. The medium was supplemented with 10% fetal bovine serum FBS (Gibco, Glasgow, UK) and 1% antibiotics penicillin and streptomycin.

The cells were cultures and treated with varying concentration of synthesized compounds at increasing concentration of 25, 50, 100 and 200µg/ml for 24 h. The cells were then treated with 500 µg/ml 3-(4,5-dimethylthiazole-.2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h. To dissolve the solution Dimethyl sulfoxide (DMSO) 100 µl was added. At 570 nm the Absorbance was recorded spectrophotometrically. Graphpad prism was used to calculate the inhibitory rate and graphs were plot against all tested compounds to assess their anticancer activities. Afterwards the IC50 for each compound was calculated.

Estimation of beta-catenin concentration by ELISA

The effect of synthesized compounds on the concentration of beta-catenin was determined to analyze the inhibitory action of synthesized compounds on the target protein beta-catenin. Cells were sub-cultured and counted. In 10 cm cell culture petri dishes $1x10^6$ cells were seeded and were allowed to adhere by incubating overnight at standard tissue culture conditions. The next day, old medium was replaced with fresh medium or test compounds (30 or 100µg/ml) for 24 hours. Cells were then washed with ice-cold PBS and incubated on ice with ice-cold lysis buffer for 30 minutes. Cell scrapers were used to detach the lysed cells and centrifuged at the maximum speed in a cooling centrifuge. Protein concentration was assessed in cell lysate. Control and treated samples (diluted 10 times) along with positive and negative controls were loaded into wells of the 96-well pre-coated ELISA plate in triplicate sets. The procedure was followed as described in the datasheet of the Human CTNNB1 (Catenin, Beta 1) ELISA kit (Catalog no. E-EL-H0666; Elabscience, USA). Change in absorbance reflects the levels of β-catenin in cell lysate.

STATISTICAL ANALYSIS

The statistical analysis was performed by using SPSS ver 25.0 software. One-way ANOVA was done for both the *in vitro* cytotoxicity assay and the percent inhibition of beta-catenin, whereas $p<0.05$ was considered significant.

RESULTS

Chemistry

2-aminothiazole derivatives (5a, 5b) have been synthesized by coupling with amino acids (glycine, phenylalanine). Amino acids undergoes N-protection with phthalic anhydride to give derivatives (3a,3b) that were further converted to respective chlorides (4a,4b) in the presence of thionyl chloride. Finally, condensation of compounds (4a, 4b) with *ethyl-2-aminothiazole-4-carboxylate* (1) afforded amide derivatives (5a, 5b) (Scheme 1). The column chromatography was used to purify the synthesized compounds using (pet. ether: ethyl acetate 4:1) as eluent. All these compounds were characterized with the help of FT-IR, ^{1}H-NMR, ^{13}C-NMR, mass spectrometry & elemental analysis. The appearance of broad singlet for NH proton of amide at δ 9.25 and 10.11 ppm in ^{1}H-NMR spectra followed by the existence of carbonyl stretching vibrations at 1698 and 1695 cm^{-1} in FT-IR spectra confirmed the formation of coupled products (5a and 5b respectively).

ADME and toxicity studies

The Swiss ADME predictor showed that all the compounds have high GI absorption with no BBB permeation, however, the compounds 5a, was the most drug likable molecules with no violations from any drug likeness rules. *Ethyl 2-(2-(1,3-dioxoisoindolin-2-yl)acetamido)thiazole-4-carboxylate (*5a) showed the most drug like properties with no violations of rule, having no CYP inhibition and have high GI absorption. table 1 shows the physicochemical properties of synthesized compounds. The toxicity profile of the compounds obtained from pkCSM are shown in table 2. Both compounds showed to have non-carcinogenic and non-mutagenic properties.

Molecular docking

The binding modes of synthesized ligands were analyzed using the MOE docking program against the target protein CTNNB1. During the docking process the ligands were kept flexible in order to obtain the minimum energy structures. The active binding site of target protein obtained from DoG Site Scorer is presented in fig. 2.

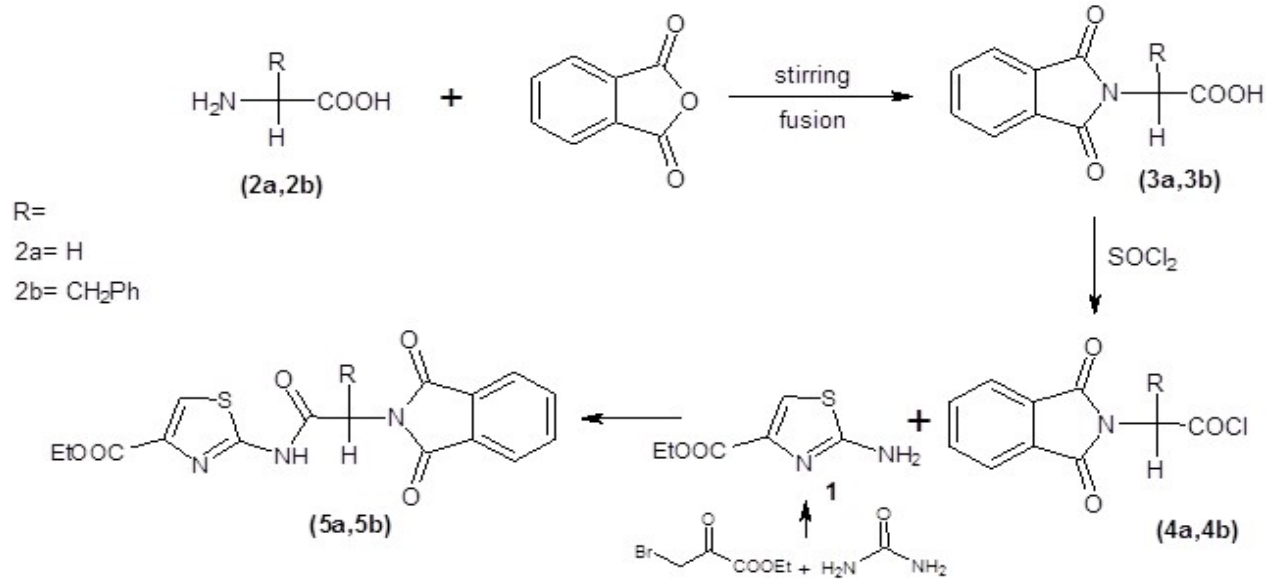

Fig. 1: Scheme 1. Synthesis of ethyl-2-aminothiazole-4-carboxylate derivatives (5a, 5b)

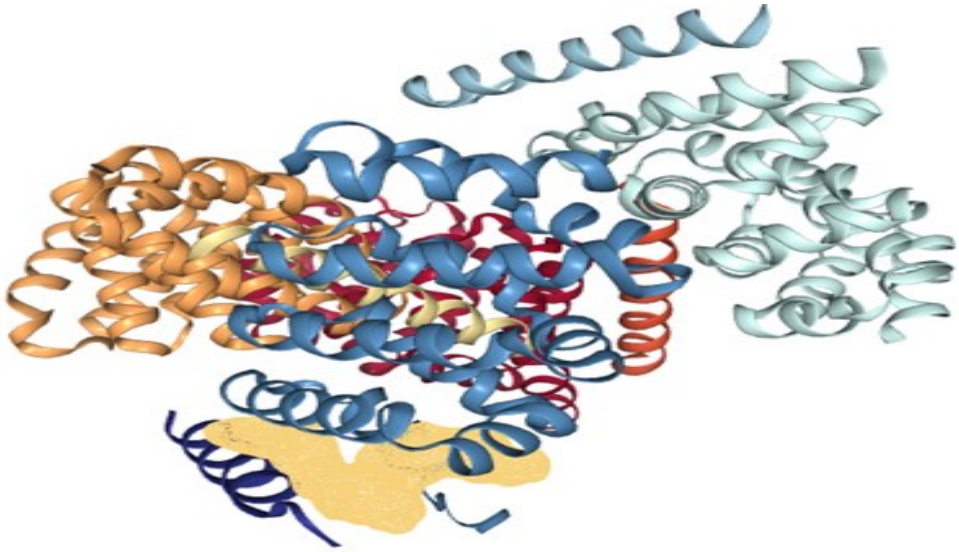

Fig. 2: DoG Site Scorer showing potential binding pocket of 3d protein beta catenin with a drug score of 0.87.

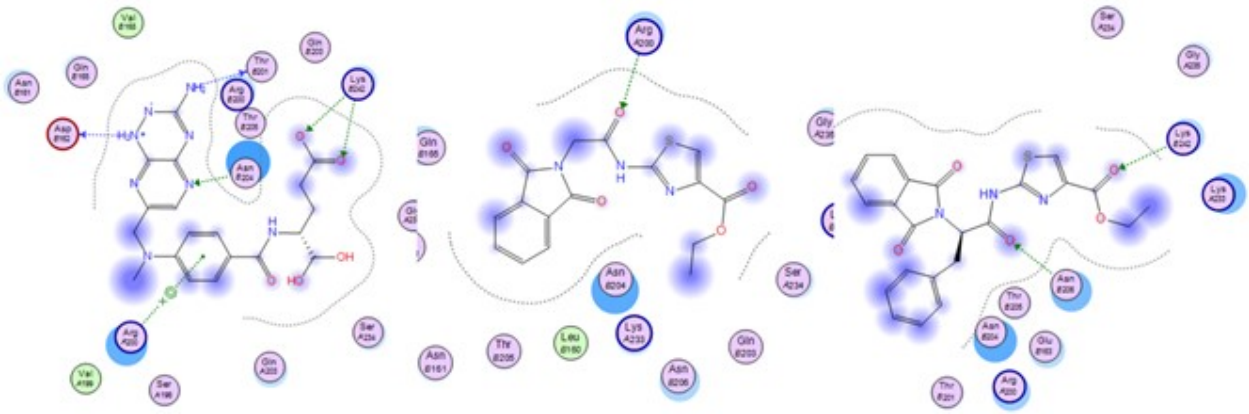

Fig. 3: Residue interactions of standard methotrexate (left) and compounds **5a** and **5b** in the active site.

For the protein-ligand docking analysis of compounds the default parameters were used. The ligands were ranked according to the scoring function generated from GBVI/WSA binding free energy. The ligands with the lowest score were considered the most favorable poses. The best conformation of ligands were screened on the basis of docking score (table 3). All of the compounds showed the lowest binding energy against protein CTNNB1. The compound *Ethyl 2-(2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanamido)thiazole-4-carboxylate (5b)* exhibited the lowest energy score of -5.7201 kcal/mol against CTNNB1 target protein as compared to methotrexate -6.6359 kcal/mol (fig. 3-4). The ligand protein residue interaction visualized by Drug Discovery studio revealed 5a showing amino acid interaction with Arg A200 and the compound 5b showed hydrogen bonding and arene-arene interaction with the amino acid Asn B206 and LysB242 (fig. 3 and 4). The results were further validated through *in vitro* analysis.

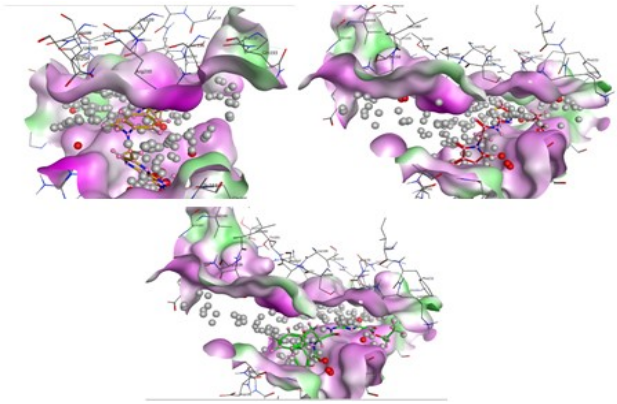

Fig. 4: Comparative binding poses of methotrexate (right) and compounds 5a and 5b inside the active binding site of beta catenin.

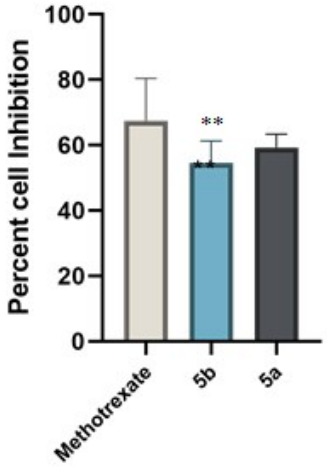

Fig. 5: Cell viability of human HCT 116 cells treated with compounds 5a and 5b for 24h. MTT assay was performed as the viability assay. (**p-value<0.001)

In vitro studies

In vitro preliminary cytotoxic evaluation

The preliminary cytotoxic activities of compounds were determined by brine shrimp lethality assay. Both the tested compounds showed considerable cytotoxic effect. The LC50 of the synthesized compounds are tabulated in table 4.

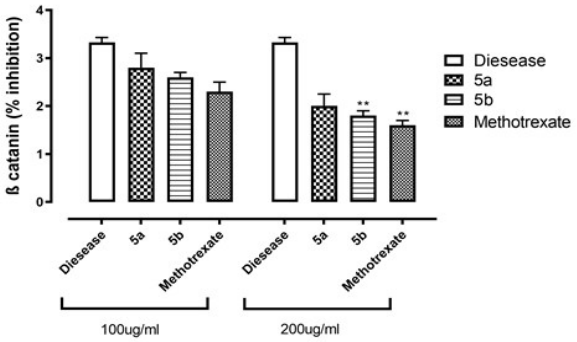

Fig. 6: Effects of tested compounds on HCT 116 cells. The negative dose response graph showed the significant inhibition of cells at 100µg/ml and 200µg/ml with significant reduction of cell concentration at higher dose (**p-value<0.001).

In vitro anticancer evaluation

For the evaluation of the anticancer effectiveness of the synthesized compounds the MTT assay was performed on the human colorectal cancer HCT-116 cell lines. The compounds showed linear increase in the inhibition of cancer cells. Both the compounds showed inhibition of cell proliferation in higher doses. The tested compounds showed 60% inhibition of cancer cells (fig. 5). The compound 5a showed the lowest IC 50 of 0.72µM while the compound 5b showed the highest IC50 of 1.55µM as compared to standard methotrexate having IC50 of 0.7µM. The statistical analysis revealed significant inhibition of cells with $p<0.01$. Both the *in silico* and *in vitro* study exhibited that both the synthesized compounds showed potential activities against colorectal cancer. The molecular docking and inhibitory studies revealed potential inhibitory activities of these compounds against colorectal cancer. The importance of these compounds in cancer inhibition was further validated by *in vitro* enzyme inhibitory assay.

Estimation of beta-catenin concentration

To validate the anti-tumor effects of tested compounds against the target protein beta catenin, the enzyme inhibitory assay was performed. The incubation of colon cancer HCT 116 cell line with the synthesized compounds at concentrations 100µg/ml and 200µg/ml for 72 hours revealed a considerable decrease in the concentration of beta catenin when compared with negative control (fig. 6). The results showed significant effects of these compounds on the concentration of beta catenin along with standard methotrexate with $p<0.001$.

Table 1: Physicochemical properties of synthesized compounds

Compound Code	Molecular weight g/mol	No. of hydrogen bond donors	No. of rotatable bonds	Total polar surface area (Aº)	Log P (iLOGP)	Log S (ESOL)
5a	359.36	359.36	359.36	359.36	359.36	359.36
5b	449.48	449.48	449.48	449.48	449.48	449.48

Table 2: Toxicity profile of synthesized compounds

Compound code	Ames Toxicity	Max tolerated dose (human)	Hepatotoxicity	hERG I inhibitor	hERG II inhibitor
5a	No	0.462	Yes	No	No
5b	No	-0.449	Yes	No	Yes

Table 3: Binding energies and interactions of ligands against target protein CTNNB1

Ligands	CTNNB1		
	Binding free Energy (kcal/mol)	No. of Hydrogen bonds	Interacting residues
5a	-5.6951	1	Arg A200
5b	-5.7948	2	Asn B 206, Lys B 242
Methotrexate	-6.6359	Arene-cation interaction	Lys 242, Arg 200

Table 4: Brine shrimp lethality assay of tested compounds

S. No.	Compounds	% Mortality (µg/ml)				LC 50
		200	100	50	25	
1	5a	100	80	70	50	4.06
2	5b	100	100	80	40	5

STATISTICAL ANALYSIS

The one-way ANOVA of *in vitro* cytotoxic assay revealed significant inhibition of cellular proliferation with $p<0.01$. The compound 5a showed 60% inhibition of cells while 5b showed 57% inhibition having p-value<0.01. the percent inhibition of beta-catenin concentration revealed that higher concentration 200 µg/ml the compound 5b and the standard methotrexate showed maximum inhibition in concentration with **p-value<0.001 (figs. 5 & 6).

DISCUSSION

In this study, two derivatives of 2-aminothiazole were synthesized coupled with amino acids. The compounds were characterized and evaluated for their anti-tumor activities. The *in-silico* physicochemical properties revealed drug-like properties of these compounds and showed non-carcinogenic and non-mutagenic effects. The molecular docking analysis provided lowest binding scores of these compounds with amino acids ArgA200, Asn B 206 and Lys B 242 which are the similar residues as that of standard methotrexate. The binding analysis showed both synthesized compounds bind at the same positions as that of standard methotrexate with almost binding energies close to each other.

The *in vitro* analysis supported the *in silico* data and the cytotoxic assay indicated LC_{50} in the range of 4-5. The cell inhibitory effects of these compounds were confirmed by the MTT assay on the colorectal cancer cell lines HCT116 and the assay revealed IC50 value of 0.72 and 1.55 µM against the compound 5a and 5b, respectively. The standard methotrexate also showed the IC50 of 0.7 µM indicating potent inhibitory effects of these compounds against the colorectal cancer cell line. Previously, Tsai and coworkers synthesized new series of aminothiazole-paeonol derivatives and studied their cytotoxic effects against seven cancer cell lines. The synthesized compounds exhibited the most potent activity against AGS and HT-29 cells (Tsai *et al*., 2016). Their finding suggested the potential activities of 2-aminothiazole derivatives in colorectal cancer. In another study, based on the structural characteristics of crizotinib a series of 2-amino-4-phenylthiazole derivatives were synthesized and screened for their biological activities. One of the compounds showed potential inhibition for HT29 cells having an IC 50 of 2.01µM. the outstanding growth inhibition of CRC cell lines by these derivatives proves their potential role in colorectal cancer (Zhang *et al*., 2018).

One of the key protein acting as an important signaling molecule in the wnt pathway and plays essential role in the tumorigenesis and embryogenesis is β-catenin. It is a multifunctional protein and a structural component of cell-adhesion junctions. The cytoplasmic level of b-

catenin are low in the absence of wnt signaling due to the protein complex that phosphorylate the protein leading to ubiquitin-mediated proteosomal degradation. The levels rise with the activation of wnt signaling causing inactivation of GSK3β. This causes the translocation of β-catenin into the nucleus where it complexes with member of TCF family of transcription factors and activates the expression of target genes. In about ~80% of familial adenomatous polyposis syndrome and sporadic colorectal cancer patients the APC tumor suppressor is mutated. The mutation of APC causes inability of β-catenin degradation leading to nuclear accumulation and transactivation of β-catenin mediated gene transcription. The β-catenin mutations in the phosphorylation sites of GSK3β have been identified in 50% of colorectal cancer patients (Dvory-Sobol *et al*., 2006). The target genes of β-catenin/tcf are c-myc and cyclin D1 which are involved in genetic instability causing tumorigenesis.

Hence, targeting the protein β-catenin or the β-catenin/tcf signaling pathway can serve as an attractive target in the development of a colorectal cancer therapeutic agent. The enzyme linked immunosorbent assay for the protein human beta-catenin was used to identify the inhibitory effects of these compounds on the concentration of protein along with standard methotrexate. Both the compounds along with standard methotrexate showed reduction in the concentration of beta catenin when compared with control (diseased) at 100µg/ml. However, on increasing the concentration of tested compounds at 200µg/ml a significant decline in the concentration of protein was witnessed. Our observation suggests that these compounds are effective inhibitors of cell growth at 100µg/ml. In various types of cancers the deregulation of β-catenin signaling is involved in the development of various malignancies including the colorectal cancer. Due to defective beta-catenin genes or APC the β-catenin/TCF signaling pathway is constitutively active at higher level in various colorectal cancer cells.

This pathway plays essential role in the progression of several cancers suggesting it to be a chief target in the control of cell proliferation or cell death. The assay shows significant effects of these compounds on the concentration of β-catenin. The suppression of beta-catenin was also studied by Lee and coworkers in which they showed down-regulation of β-catenin by capsaicin treatment in APC mutant human colorectal cancer cells. Multiple mechanisms were involved in the capsaicin-mediated down regulation of β-catenin such as suppression of β-catenin gene and greater protein degradation in mutant APC, β-catenin human wild type colorectal cancer cells as well as suppression of TCF4 in APC wild type, β-catenin mutant cancer cells (Lee *et al*., 2012). The study shows potential of these compounds against colorectal cancer, however, further studies are required to process them for their future role in cancer.

CONCLUSION

In this study, two derivatives of 2-amino thiazoles coupled with amino acids that were previously synthesized and characterized were screened and evaluated for their anti-cancer activities against colorectal cancer. The study highlighted the inhibitory effects of these compounds in colorectal cancer providing further opportunities in the development of beta catenin and cancer chemotherapeutic agents. Both the compounds (5a and 5b) showed potent activities with IC50 of 0.7 and 1.55 µM, moreover, the beta-catenin concentrations were highly effected by these compounds at higher doses. Our further studies are based on exploration of enlarge structure activity relationship of these compounds and pre-formulation studies to further investigate their role in cancer.

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