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Research Article

SYNTHESIS, ANTICANCER ACTIVITY, MOLECULAR DOCKING, AND ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION TOXICITY STUDIES OF NOVEL BENZOTHIAZINES

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ABSTRACT

Objective: A series of benzothiazine compounds were studied for absorption, distribution, metabolism, and excretion (ADME) properties to asses their drug-like properties. Compounds 1-10 with favorable ADME properties were selected for molecular docking studies as PIM1 kinase inhibitors.

Methods: Synthesis of compound 1 and 7 by conventional heating and characterized by various methods. Molecular docking carried out using Glide software; ADME toxicity predicted using QuickPro.

Results: Compound 1 showed a Glide score of −7.622 kcal/mol with good hydrophobic and hydrophilic interactions with PIM1 kinase proteins and appears to be more potent. Structure-activity relationship study was made among the 10 compounds, and a basic template was arrived at. An analysis of the structure - Affinity relationships suggested that the substituent at position three is important in influencing affinity.

Conclusion: Compounds with an alkyl spacer between the carboxyl group and the core benzothiazine ring are required for binding of the compounds with the PIM1 kinase. It was further confirmed by its *in vitro* anticancer activity of compound 1 against K562 cell lines by 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay by exhibiting an IC_{50} value of 36.82 µg/ml.

Keywords: Benzothiazine, Molecular docking, Absorption; distribution; metabolism; and excretion properties, *In vitro* anticancer activity.

INTRODUCTION

Malignant tumors is a global medical burden with almost 12 million new cases coming up every year and more than 7 million deaths overall [1]. In Western countries, cancer death rates remained stable over the past decades and are the most common cause of death between the age of 40 and 79 [2]. The increasing understanding of molecular pathways in cancer diseases has recently shown the way for the development of more specific anticancer agents and also known as targeted therapies [3,4]. Especially, inhibitors of growth factors and their receptor signaling have been investigated extensively and led to the approval of monoclonal antibodies (e.g., bevacizumab, cetuximab, or panitumumab) or small-molecule kinase inhibitors (e.g., erlotinib, sunitinib, or sorafenib) [5]. This latter class of compounds can effectively target intracellular tyrosine kinases [6]. Yet, the specificity of these molecules is typically quite low due to the high homology of tyrosine kinase families [7], and the redundancy of these pathways allows cancer cells to either bypass the inhibited kinase or to develop resistance against a specific inhibitor. Recently, the serine/threonine kinase family has entered the focus of research. Small-molecule inhibitors have been developed and tested in clinical trials for some members of this family, for example, the mammalian target of rapamycin complexes [8-10], polo-like kinases [11,12], or aurora kinases [13-15]. However, the overall efficacy of these inhibitors still remains unsatisfying, with only low response rates and rather marginal improvements concerning overall survival in clinical trials [16-20]. Besides these well-established serine/threonine kinases, members of the PIM kinase family have recently been described to possess oncogenic and survival promoting properties [21,22].

PIM1 represents a constitutively active serine/threonine kinase which is distinct from other classes of kinases by both its molecular structure and its molecular regulation. PIM1 controls oncogenic signaling pathways hypoxia, cell cycle control, or apoptosis resistance and have been shown to be overexpressed in various human cancers and to be associated with metastasis and overall prognosis. Therefore, PIM1 is an interesting novel target for targeted drug therapy. Several classes of PIM1 inhibitors have been described which cover a significant number of different chemical core structures, ranging from indolocarbazoles, bisindolylmaleimides, naphthyridines, pyridazines, and isoxazoles to thiazolidine-2,4-diones, thienopyrimidinones, pyridones, and isoxazoloquinolines [23]. All reported small-molecule PIM1 inhibitors are adenosine triphosphate-competitive binders.

Benzothiazine derivatives have pronounced importance in pharmaceutical chemistry and organic synthesis. Benzothiazines exhibit a wide range of biological properties due to their unique structure; therefore, synthesis of benzothiazines is an area of current interest. In the present study, a bunch of benzothiazine derivatives were explored for their PIM1 inhibitor activity.

EXPERIMENTAL

Synthesis and characterization of compound 1 and 7

1,4-dihydro-2-methoxycarbonylmethyl-3-oxo-2H-1,4-benzothiazine

2-aminobenzenethiol 1 (0.01 mol), maleic anhydride 2 (0.01 mol) in presence of methanol (25 mL), and conc. H_2SO_4 (2 mL) were taken in a 100 mL round bottom flask and subjected to reflux for 2 hrs. The reaction was monitored by thin-layer chromatography (TLC). After completion of the reaction, the reaction mass was cooled and the solid thus obtained was washed with 5% sodium bicarbonate solution and extracted in dichloromethane to afford 3 (yield 82%, m.p. 138-140°C).

Infrared (IR) (KBr, cm^{-1}): 1740, 1690, 1480, 1394 cm^{-1}. ^1H NMR (400 MHz, dimethyl sulfoxide [DMSO]-d_6): δ 2.92 (dd, 1H, CH_2), 2.57 (dd, 1H, CH_2), 3.61 (s, 3H, OCH_3), 3.85 (t, 1H, CH), 6.99-7.32 (m, 4H, ArH), 10.60 (s, 1H, ring N-H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.17 (C=O), 167.05 (C=O), 136.80, 127.77, 127.56, 125.03, 117.05, 122.32 (Ar-C), 51.73 (-OCH_3), 38.87 (-CHCOS), 33.22 (=$COCH_2$).

3-oxo-3,4-dihydro-2H-1,4-benzothiazine-2-carboxylic acid (7)

About 15.2 mL (0.1 mol) of diethyl-malonate and 10.8 mL (0.1 mol) of 2-aminothiophenol were taken in a 50-mL round bottom flask and refluxed for 1.5 hrs. The reaction was monitored by TLC. After the completion of the reaction, the precipitate was filtered, washed with distilled water, and dried. Recrystallization in methanol yielded yellow needle-like crystals (mp = 106°C, 58% yield).

IR (KBr, cm^{-1}): 3136, 1740, 1690, 1480, 1394 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 4.85 (s, 1H, CH), 6.99-7.28 (m, 4H, ArH), 10.90 (s, 1H, ring N-H), 11.1 (s, 1H, COOH). ^{13}C NMR: δ167.1 (C=O), 162.97(C=O), 136.92, 127.71, 127.58, 122.69, 116.10, 122.32 (for aromatic carbon atoms), 40.12 (-CHCOS).

***In vitro* anticancer activity**

3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide [MTT] assay

MTT is a colorimetric assay that measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilized with an organic solvent DMSO (Sigma-Aldrich), and the released, solubilized formazan product was measured at 540 nm. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells [12,24].

The cell culture suspension was washed with 1 ×phosphate buffered saline (PBS) and then added 30 µl of MTT solution to the culture (MTT-5 mg/mL dissolved in PBS). It was then incubated at 37°C for 3 hrs. MTT was removed by washing with 1 ×PBS and 200 bµl of DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cell got lysed and color was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank in an ELISA reader (LISASCAN, Erba) [25-28].

% Viability = (OD of test/OD of control) ×100

Molecular docking

ChemDraw 8.0 is one of the chemical compounds drawing package that allows us to draw chemical structures including organics, organometallics, polymers, and Markush structures. It also includes features such as calculation of molecular properties, e.g., molecular weight, density, molar refractivity, etc., two-dimensional and three-dimensional (3D) structure cleaning and viewing, functionality for naming structures, and prediction of log P. We have drawn 10 synthesized compounds and saved in mol file format and eventually input file for molecular docking studies.

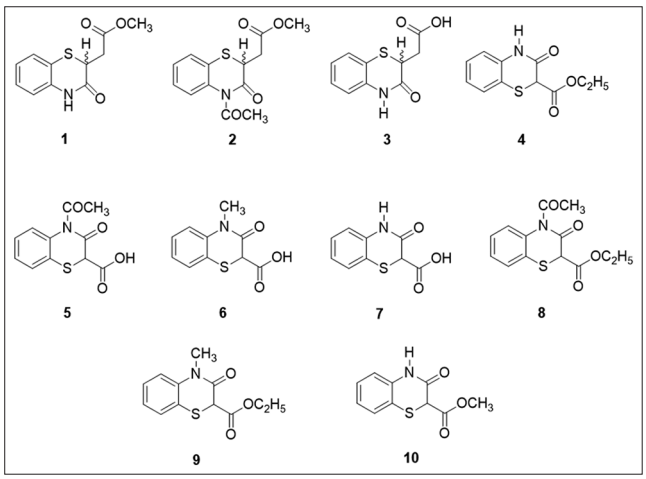
3D NMR structure of PIM1 kinase (PDB ID: 1 YHS) is obtained from the protein data bank from NIH in the USA. Maestro is Schrödinger's powerful, united, multi-platform graphical user interface and was used for our molecular docking studies. QikProp is a quick, accurate, easy-to-use absorption, distribution, metabolism, and excretion (ADME) and is used for our studies [29].

RESULTS AND DISCUSSION

Among the benzothiazine molecules available in our laboratory database compounds 1-10 (Scheme 1) showed favorable ADME characteristics as shown in Table 1.

ADME toxicity properties prediction

We analyzed 44 physically significant descriptors and pharmaceutically relevant properties of a series of benzothiazine compounds, among which were molecular weight, H-bond donors, H-bond acceptors, log P, log P MDCK, log Kp, humoral absorption according to Lipinski's rule of 5 (Table 1). Lipinski's rule of 5 is a rule of thumb to evaluate drug-likeness, or determine if a chemical compound with a certain pharmacological or biological activity has properties that would make it a likely orally active drug in humans. The rule describes molecular properties important for a drug's pharmacokinetics in the human body, including its ADME. These compounds were further evaluated for their drug-like behavior through analysis of pharmacokinetic parameters required for ADME by use of QikProp. For the 10 compounds, the partition coefficient (QPlogPo/w) and water solubility (QPlogS), critical for estimation of absorption and distribution of drugs within the body ranged between 1.139-1.852 and −2.982-1.783. Cell permeability ($QPPCaCO_2$), a key factor governing drug metabolism and its access



Scheme 1: Structure of the compounds 1-10

Table 1: ADME properties of 1-10 as verified by using QikProp (Schrodinger 9.5)

Ligand	QPlogS[a]	$CaCO_2$	Percentage human[b] oral absorption	QPlogKhsa[c]	LogBB[d]	MW[e]	HBD[f]	HBA[g]	QPlog[h] (o/w)	MDCK[i]
1	−2.878	635	86	−0.315	−0.607	237.27	1.00	5.00	1.444	428
2	−2.982	741	89	−0.381	−0.572	279.31	0.00	5.50	1.831	507
3	−2.326	61	66	−0.618	−0.942	223.24	2.00	5.00	1.139	43
4	−2.538	959	91	−0.351	−0.396	237.27	0.00	4.00	1.852	705
5	−2.499	58	68	−0.638	−0.850	251.25	0.00	4.50	1.570	44
6	−1.783	134	74	−0.805	−0.514	223.24	0.00	4.50	1.464	109
7	−2.149	68	70	−0.553	−0.781	209.21	0.00	3.00	1.672	52
8	−2.596	695	88	−0.425	−0.499	279.31	0.00	5.50	1.720	544
9	−2.126	1924	95	−0.603	−0.120	251.30	0.00	5.50	1.564	1505
10	−2.202	757	87	−0.471	−0.412	223.24	0.00	4.00	1.404	545

[a]Predicted aqueous solubility; S in mol/L (acceptable range; −6.5-0.5), [b]Percentage of human oral absorption; (<25% is poor and >80% is high), [c]Prediction of binding to human serum albumin; (acceptable range; −1.0-1.5), [d]Prediction of brain/blood; (acceptable range; −3.0-1.2), [e]Molecular weight (<500 Da), [f]Hydrogen bond donor (<5), [g]Hydrogen bond acceptor (<10), [h]Predicted octanol/water partition co-efficient log p (acceptable range; −2.0-6.5), [i]Predicted apparent MDCK cell permeability in nm/s, ADME: Absorption, distribution, metabolism and excretion

to biological membranes, ranged from 58 to 1924, QPPMDCK ranges from 43 to 1505. Overall, the percentage human oral absorption for the compounds ranged from 66% to 95%. All these pharmacokinetic parameters are within the acceptable range defined for human use, thereby indicating their potential as drug-like molecules.

In the post-genomic era, rational drug design aims to discover small molecules that change the activity of key therapeutic targets which are important for carcinogenesis [1]. Computer-aided design is being used to facilitate the identification of the lead molecule. It reduces the number of compounds to be tested and thereby allows focusing on more promising molecules for lead discovery and optimization. Molecular docking was performed for all the above 10 compounds, and the results are as in Table 2. In general, all the 10 compounds had shown very good interaction with the PIM1 receptor. The Glide score varies from −3.136 to −7.622 kcal/mol, and the Glide energy varies from −20.221 to −48.908 Kcal/mol. An analysis of the structure - affinity relationships suggested that the substituent at position 3 is important in influencing affinity. In fact, compounds with a carboxyl group directly bound to the C3 of the heterocyclic core as in compound **7** are less active toward PIM1, while analogs with an alkyl spacer between the carboxyl group and the core as in compounds 1, 2, and 3 showed good affinity. In compound 7, no hydrogen bond interactions were observed. Only hydrophobic interactions were found between compound 7 with PIM1. The following residues are mainly involved in hydrophobic interactions Val 52, PHE 49, ILE 185, LEU 44, LEU 120, ALA 65, and LEU 174. In the compound 7, when a methylene group was introduced between the carboxyl group and the benzothiazine ring system as in compound 3, the docking score remarkably increases from −5.476 to −6.891 kcal/mol. With compound 3, one hydrogen bond interaction was found between the - NH group of 3 and the –C=O group of ASP 128 with bond length (1.98 Å). Furthermore, the following residues are mainly involved in hydrophobic interactions ILE 185, PHE 49, LEU 44, VAL 52, and VAL 126. Compound on esterification as in compound 1, the docking score further improves to −7.622 kcal/mol.

Upon the examination of docking features between compound 1 and PIM1, it was found one hydrogen bond interaction was present between the −NH group of 1 and the -C=O of ASP 128 with bond length (2.50 Å). The hydrophobic pocket remains the same as that in compound 3 involving interactions with PHE 49, VAL 52, ILE 185, LEU 174, VAL 126, and LEU 44. The docking score of −7.426 kcal/mol was obtained when compound 3 was acylated at NH group. With compound 2 also, it was found only one hydrogen bond interaction was present. The backbone hydrogen atom of the hydrophobic residue of VAL 126 was tightly interacted with oxygen atom of the compound with bond length (1.79 Å). Furthermore, the following residues are mainly involved in hydrophobic interactions PRO 123, ALA 65, VAL 52, LEU 174, and PRO 125. However, without introducing an alkyl spacer between the carboxyl group and the benzothiazine system the docking does not improve with esterification, acylation at NH or alkylation of the nitrogen atom as in compounds 4, 5, 6, 8, 9, and 10. With compounds 4, 6, 9, and 10 no H-bonded interactions were found, only hydrophobic interactions were observed. In compound 5, two hydrogen bond interactions were found. The side chain hydrogen atom of the positively charged residue of ARG 122 strongly interacted with the C=O group of the compound 5 and the backbone hydrogen atom of the hydrophobic residue of VAL 126 nicely interacted with oxygen atom of the compound 5 with bond distances of 1.83 Å and 2.2398 Å, respectively. Furthermore, the following residues are mainly involved in hydrophobic interactions LEU 44, LEU 174, PRO 125, and PRO 123. In compound 8, two hydrogen bond interactions were present with the same residues as that in the compound 5 but with bond distances 1.131 Å and 1.118 Å. The hydrophobic pock*et al*so remains the same. In both the compounds, there is no improvement in the docking score. Among all the compounds, 1 appears to be more potent with respect to the anticancer activity.

Synthesis of the compounds

Since compound 1 has shown very good Glide score and Glide energy, it was synthesized for *in vitro* studies. To have a comparison compound 7 was prepared and tested for its *in vitro* activity. 2-aminobenzenethiol 1 (0.01 mol), maleic anhydride 2 (0.01 mol) in presence of methanol (25 mL), and concentrated H_2SO_4 (2 mL) were refluxed for 2 hrs Scheme 2. The reaction mass was cooled to afford 1.

Diethyl-malonate (0.1 mol) and 2-aminothiophenol (0.1 mol) were refluxed for 1.5 hr to yield 7 (Scheme 3).

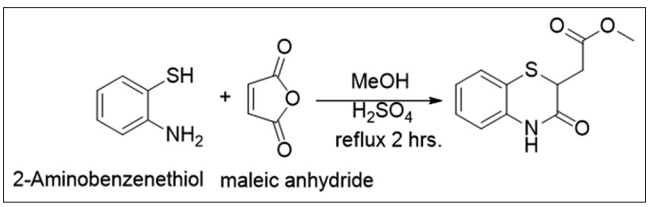
The formation of compound 1 was explained on the basis of IR absorption bands at 1730 and 1690 cm^{-1} due to acid and cyclic carbonyl groups, respectively. In the ^{1}H-NMR spectra, the signal at δ 4.85 for one proton is due to thiazine ring proton. The bunch of signals between 6.99 and 7.28 (m, 4H, ArH) are attributed to aromatic protons, signal at δ 11.1 is due to –COOH and the signal at 10.90 is due to ring N-H protons.

The above assignments were complemented by the ^{13}C NMR signals by exhibiting signals at δ 167.19 and 162.97 due to carbonyl carbons, at δ 136.92, 127.71, 127.58, 122.69, 116.10, and 122.32 due to aromatic carbon atoms and at 40.12 for -CHCOS carbon.

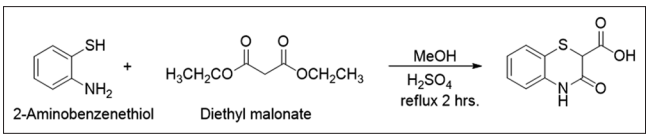
The formation of compound 7 was explained on the basis of absorption bands in IR spectra at 1740 and 1690 cm^{-1} due to ester and cyclic carbonyl groups, respectively. This was also confirmed from the ^{1}H-NMR signal at δ 3.6 due to -OCH_3 group. The pair of doublet of doublets at δ 2.57 and 2.92 each of one proton is due to -CH_2 group. Further, the doublet of doublets at δ 3.85 for one proton is due to thiazine ring proton. The bunch of signals between 6.99 and 7.32 (m, 4H, ArH) are attributed to aromatic protons and the signal at 10.60 is due to ring N-H proton. In the ^{13}C NMR signals at δ 170.17 and 167.05 are due to carbonyl carbons, signals at δ 136.80, 127.77, 127.56, 125.03, 117.05, and 122.32 are due to aromatic carbon atoms, the signal at δ 51.73 for

Table 2: Glide (XP) results of 1-10 usijg of Schrodinger 9.5

Compound name	Glide score (Kcal/mol)	Glide energy (Kcal/mol)	Interacting residues	Distance (Å)
1	−7.622	−48.908	ASP 128	2.5
2	−7.478	−45.769	VAL 126	1.79
3	−6.891	−54.758	ASP 128	1.98
4	−6.006	−43.741	-	-
5	−6.001	−48.591	ARG 122	1.83
			VAL 126	2.2398
6	−5.941	−21.314	-	-
7	−5.476	−35.234	-	-
8	−5.301	−38.521	ARG 122	1.131
			VAL 126	1.118
9	−3.251	−21.31	-	-
10	−3.136	−20.221	-	-



Scheme 2: Synthetic of compound 1 from 2-aminobenzenethiol and maleic anhydride



Scheme 3: Synthetic of compound 7 from 2-aminobenzenethiol and diethylmalonate

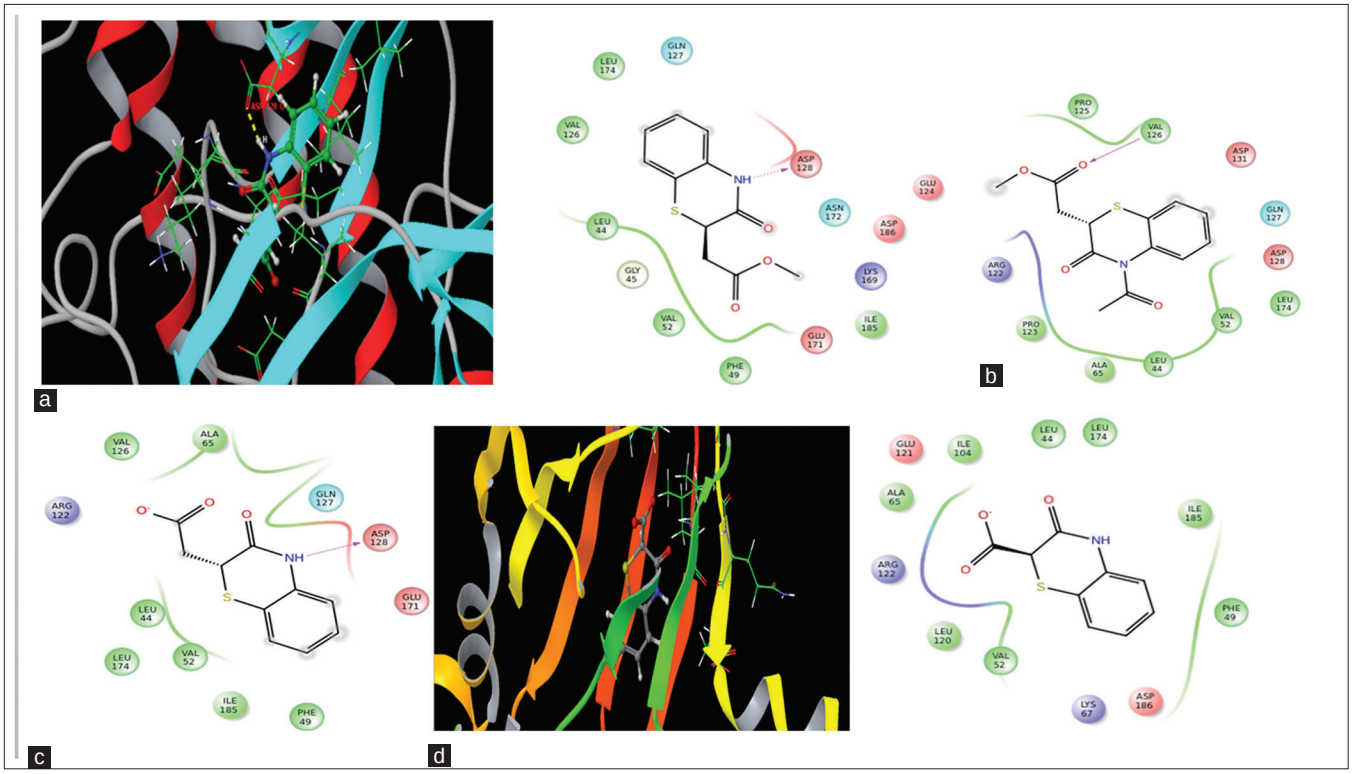


Fig. 1: Binding Interaction of 1, 2, 3 and 7 with target protein PIM1. (a) Three-dimensional (3D) and two-dimensional (2D) structure of target protein PIM1 with 1, (b) 2D structure of target protein PIM1 with 2, (c) 2D structure of target protein PIM1 with 3, (d) 3D and 2D structure of target protein PIM1 with 2

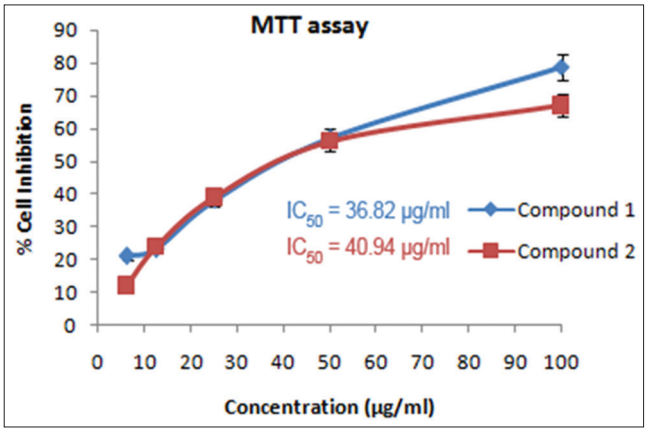


Fig. 2: MTT assay result against K562 cell line Sample size n=3. Values expressed as mean±standard error of the mean

the -OCH_3 carbon atom, 38.87 for -CHCOS carbon and at δ 33.22 -$COCH_2$ methylene carbon.

MTT assay

The effect of 1 and 7 on the proliferation of K562 leukemic cells was verified using MTT assay. K562 cells were treated with 6.25, 12.5, 25, 50 and 100 µg/ml of compounds and were subjected to MTT assay after 48 hrs (Fig. 2). Results showed that cell viability was affected at moderate concentration in case of the benzothiazine compounds 1 and 2. The IC_{50} values of the compounds 1 and 2 were 36.82 µg/ml and 40.94 µg/ml, respectively.

CONCLUSION

A series of benzothiazine compounds were studied for ADME properties to assess their drug-like properties. Compounds 1-10 with favorable ADME properties were selected for molecular docking studies as PIM1 kinase inhibitors. Structure-activity relationship study suggested that compound 1 is more potent when compared with other compounds. It was further confirmed by its *in vitro* anticancer activity of compound 1 against K562 cell lines by MTT assay by exhibiting an IC_{50} value of 36.82 µg/ml. Compound 1 may serve as a basic template for further studies.

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