Synthesis and biological evaluation of novel isoxazolines linked via piperazine to 2- benzoisothiazoles as potent apoptotic agents

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Abstract

Synthesis of 3-(4-((3-Phenyl-4,5-dihydroisoxazol-5-yl)methyl)piperazin-1-yl) benzoisothiazole derivatives (**5a-i**), which constitute a new class of isoxazolines, has been accomplished in regio-selective manner. These derivatives have been prepared by employing the reaction between substituted aldoximes (**4a-i**) and 3-(4-Allylpiperazin-1-yl) benzoisothiazole in presence of chloramine-T which afforded in good yields. These compounds were screened for cytotoxic activity on tumor cells. Four among the nine synthesized compounds were found to exhibit potent cytotoxic and antineoplastic activities in comparison to tumour necrosis factor-related apoptosis inducing ligand (TRAIL) protein in mammalian cancer cells. The rest of the derivatives showed moderate activity.

Key words: Benzoisothiazoles, Piperazine, Isoxazole and Cytotoxic activity.

1. Introduction

Increasing cases of neoplastic diseases like cancer is associated with high mortality rate worldwide, accounting for almost 8.2 million deaths [1]. This has stimulated an unprecedented level of research directed towards the discovery of new structure leads that might be of use in designing novel chemotherapeutic drugs. Despite breakthroughs in anticancer therapies, chemotherapy still remains a reliable approach for cancer therapy. A typical attribute of neoplastic cells is their ability to evade apoptosis consequently due to accumulation of mutations that block apoptosis pathways [2]. Many studies have indicated the induction of apoptosis upon efficacious cancer treatment [3]. Whilst chemotherapeutic drugs are known to induce

autophagy, cell cycle arrest and cell senescence [4, 5], it is imperative that there is induction of apoptosis and permanent removal of neoplastic cells for effective cancer chemotherapy. Recently, benzoisothiazole, Isoxazole and piperazine rings have been shown to exhibit varied biological activities including anticancer properties [6–11].

5-((4-(Benzo[d]isothiazol-3-yl)piperazin-1-yl)methyl)-3-phenyl-4,5-dihydro isoxazole is a class of heterocyclic compounds having benzoisothiazole, piperazine and isoxazoline rings in the same system.

Derivatives of 2-Benzo/benzoisothiazolimino-5-arylidene-4-thiazolidinones [12] act as balanced inhibitors of cyclooxygenase/lipoxygenase enzymes. Amidino benzoisothiazole derivatives [13] showed remarkable analgesic activity and antiphlogistic activity. 3-benzoisothiazolyl derivatives [14] exhibit potent affinity for the 5-Hydroxytryptamine-2A (5-HT_{2A}) receptor combined with moderate to low 5-HT_{1A} and D2 affinities. 2-(Benzylideneamino)-benzo[d]isothiazol-3-ones [15] were found to be possessing good antimicrobial activity with the parent compound, 2-amino-benzo[d]isothiazol-3-one being the most effective.

Introduction of the methyl sulfonamide group at the 7-position of the Benzo[d]isothiazole-1,1-dioxides [16] provided an excellent boost in potency (IC₅₀ = 0.005 μ M) but showed poor replicon inhibition Effective Concentration (EC₅₀) = 4.924 μ M). 3-Aminobenzo[d]isothiazole derivatives [17] show a local anaesthetic activity in the rat sciatic nerve model. Benzoisothiazole derivatives [18] linked to N,N-Dimethylformimidamide group was found to significantly inhibit the 5-HT-induced Ca²⁺ increases making them potent 5-HT₆ receptor antagonists.

Isoxazolines are heterocycles that belong to the azole family which have gained much importance in the field of medicinal chemistry as anticancer agents [8–9]. 3,5-Diaryl isoxazoline /isoxazole linked to 2, 3-dihydroquinazolinone [19] exhibits significant anticancer activity against 18 human cancer cell lines with growth inhibition (GI₅₀) values less than 1 mM. Isoxazole linked to pyrrolobenzodiazepine [20] showed promising anticancer activity with GI₅₀ values in the range of <0.1–3.6 μ M. Isoxazole analogues of retinoids and its derivatives [21] have shown good antiproliferative activity and a capability to induce differentiation of tumor cells. Isoxazolo[4,5-d]pyrimidine derivatives [22] significantly inhibited the humoral immune response in vivo to sheep erythrocytes at a dose of 100 mg. Methylenebis-isoxazolo[4,5-b]azepines bearing furan and thiophene rings [23] were shown to be most toxic towards cancer cell lines in comparison with that of standard drug Cisplatin.

5-(3-Alkylquinolin-2-yl)-3-aryl isoxazole derivatives [24] showed excellent potent cytotoxicity against human cancer cell lines (A549, COLO 205, MDA-MB 231 and PC-3) having IC₅₀ values of <12 μ M. Isoxazole derivatives are also known to show good antiproliferative against Hela cells [25]. Derivatives of isoxazole containing 6-hydroxycoumarin [26] showed exceptional cytotoxic activity with IC₅₀ of 8.2 and 13.6 μ M against PC-3 cancer cell line. Isoxazole derivatives [27] showed

potent antibacterial activity against all the tested microorganisms at a concentration of 100 µg/mL similar to that of the standard antibiotic ciprofloxacin. Ether-linked bis(isoxazoline) derivatives [28] were tested for antimicrobial activity by disc diffusion and micro dilution methods, showing moderate to potent inhibition. Piperazine-based compounds [29] were tested for high-throughput screening (HTS) approaches and exhibited submicromolar inhibition of *Mycobacterium tuberculosis* InhA (MtInhA) enzyme activity. Sulfonyl(piperazin-1-yl)benzo[d]isoxazole analogues [30] were screened for *in vitro* anti-tubercular activity against *Mycobacterium tuberculosis* (MTB) H₃₇Rv strain. The titled compounds exhibited Minimum inhibitory concentration (MIC) between 3.125 and >50 mg/mL. Piperazine based derivatives as a new class of S1 ligands. 4-benzyl-1-(3-iodobenzylsulfonyl)piperidine [31] sigma ligand was identified with 96-fold σ_1/σ_2 selectivity ratio ($K_i\sigma_1 = 0.96 \pm 0.05$ nM and $K_i\sigma_2 = 91.8 \pm 8.1$ nM). 1-(4-Substitutedbenzoyl)-4-(4-chlorobenzhydryl)piperazine derivatives [32] showed significant cell growth inhibitory activity on the selected cancer cell lines, especially had lower GI₅₀ values when compared to 5-fluorouracil (5-FU). Arylpiperazine containing 2-fluorophenyl derivatives [33] exhibited strong cytotoxic activities against LNCaP cells (IC₅₀ < 5 µM).

In this study, based on the survey of recent literature studies on benzo[d]isothiazole, isoxazole and piperazine and in our effort to discover potent anticancer agents, we synthesized benzo[d]isothiazole derivatives containing piperazine and isoxazoline rings and screened them for their antineoplastic efficacy using the human breast cancer cell line MDA-MB-231 and the Ehrlich ascites tumor (EAT), murine mammary carcinoma model. As a positive control, we have used the soluble form of recombinant TNF-related apoptosis-inducing ligand (rTRAIL) which has been shown to induce apoptosis selectively in cancer cells like MDA MB 231 [34].

2. Results and discussion

2.1. Chemistry

Synthesis of novel heterocyclic ring systems by using nitron-alkene cycloaddition reaction has gained prominence over the last few decades [35]. In synthetic perspective, nitron-alkene cycloaddition is used to connect multiple pharmacore moieties with a single step [36]. As a result, the cyclo adducts emanated due to nitron-alkene cycloaddition reaction serves an excellent precursors in organic as well as in medicinal chemistry [37].

Based on the above facts, it would thus be interesting to synthesize molecules possessing benzisothiazole, piperazine and isoxazoline rings in the same system and evaluate their biological properties. So an attempt to design, synthesize and evaluation of *in vitro* cytotoxic activity of novel isoxazolines linked via piperazine to 2- benzoisothiazoles is made. Therefore, we describe herein utilization of nitron-alkene cyclo addition as a synthetic method for the preparation of

isoxazolines linked via piperazine to 2- benzoisothiazoles.

Thus the compounds (3-(4-((3-Phenyl-4,5-dihydroisoxazol-5-yl)methyl)piperazin-1-yl)benzo[d]isothiazole) **5(a-i)** were synthesized from starting material Benzo[d]isothiazol-3(2H)-one (**1**), which was treated with phosphoryl chloride (POCl₃) in dichloromethane to get an intermediate 3-chlorobenzoisothiazole which were intercepted *in situ* by piperazine to afford compound 3-(Piperazin-1-yl)benzo[d]isothiazole (**2**). The compound **2** was subjected to allylation with excess of allyl bromide in the presence of tri ethylamine in Dimethyl Formamide (DMF) to get compound 3-(4-Allylpiperazin-1-yl)benzoisothiazole (**3**). With the compound **3** in hand, we turned our attention to find suitable dipole precursors and in turn substituted aldoximes (**4a-i**) are found to be excellent precursors. These aldoximes were prepared from the corresponding aldehydes from the procedure outlined in already reported method [38]. The obtained substituted aldoximes were subjected to nitron-alkene cycloaddition reaction by treatment with 3-(4-Allylpiperazin-1-yl)benzoisothiazole (**3**) and chloramine-T in ethanol at reflux condition for 1 hour. The oximes will undergo oxidative dehydrogenation to yield nitrile oxides. These nitriloxides further react with olefinic group of compound **3** and undergo cycloaddition to get desired 3-(4-((3-Phenyl-4,5-dihydroisoxazol-5-yl)methyl)piperazin-1-yl)benzo[d]isothiazole derivatives (**5a-i**), which contain Benzo[d]isothiazole, piperazine and isoxazoline rings. The numbering of the skeleton is shown in Fig. 1.

3. Materials and methods

3.1. Cell lines and culture media

MDA-MB-231, human triple negative breast cancer cells were procured from National Centre for Cell Science, Pune, India and cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS), (Gibco, USA) and 100 mg/mL penicillin streptomycin (Invitrogen). The Ehrlich ascites tumor cells/mouse mammary carcinoma cells were maintained in Swiss albino mice (6-8 weeks old) obtained from the animal house facility, Department of Studies in Zoology, University of Mysore, Karnataka, India. The animal experiments were approved by the institutional animal care and use committee, University of Mysore, Karnataka, India. The culture medium used throughout these experiments was DMEM. Recombinant human soluble TRAIL was purified from pGEX-2TK plasmid containing TRAIL-GST fusion construct, a kind gift from Dr. Stanley Lipkowitz, National Cancer Institute, Bethesda. Other chemicals and reagents used were of analytical grade.

3.2. Test compound preparation

All the test compounds were dissolved in Dimethyl sulfoxide (DMSO) (0.1%) solvent and filter sterilized using a 0.22µm syringe filter for the *in vitro* cytotoxicity studies. In control samples, equimolar concentration of DMSO was added as vehicle to rule out its cytotoxic effect. As a standard anti-cancer, apoptosis inducing drug, soluble recombinant rTRAIL (25 pM) was used.

3.3. MTT assay to study the effect of compounds on proliferation and the metabolic activity of cancer cells

Human Triple negative breast cancer, MDA-MB-231 cells (1×10^4) were seeded into 96 well tissue culture plate (Nunc MicroWell™) in complete DMEM and incubated overnight at 37°C, 5% CO₂. Media was replaced with fresh media containing different concentrations (2, 5, 10, 20, 40, 80, 100 and 200 µM/ml) of the compounds (**5a**, **5b**, **5c**, **5d**, **5e**, **5f**, **5g**, **5h** and **5i**) in triplicates with appropriate vehicle (0.1% DMSO) treated controls and media blanks. After 16hrs of treatment, 10µl MTT (5mg/ml) was added to each well and incubated for 4 hours at 37°C, 5% CO₂. The resultant purple formazan crystals were solubilized in 100µl of DMSO. The optical density of the coloured solution was measured at a test wavelength of 570nm and a reference wavelength of 630 nm. (Varioskan™ Flash Multimode Reader, Thermo Scientific). The percentage cytotoxicity and IC₅₀ values were calculated with respect to control wells.

3.4. In vivo cytotoxicity of compounds assessed by Trypan blue dye exclusion assay

Mouse mammary carcinoma, EAT cells growing in exponential phase, were seeded in 6 well culture plates at 3×10^4 cells per well in 2ml complete DMEM medium. After 16h, the media was replaced with complete medium containing 50µM of compounds (**5a**, **5b**, **5c**, **5d**, **5e**, **5f**, **5g**, **5h** and **5i**) to the respective wells. Positive control cells were treated with rTRAIL (25 pM) and the negative control cells were vehicle (0.1% DMSO) treated and incubated for 16 h at 37°C with 5% CO₂ in humidified incubator. The semi adherent cells were harvested by centrifugation at 2500 x g for 5 min. The cell pellet was then re-suspended in 100 µl of PBS; 10 µl of the cell suspension was stained with an equal volume of 0.4% trypan blue in 980 µl of PBS and incubated for 2 min at 37°C. The total number of viable cells was estimated by counting using a Neubauer Chamber.

3.5. Apoptosis detection by morphological changes in cells by nuclear staining

Analysis of apoptosis by morphological changes in EAT cells upon treatment with benzoisothiazole derivatives by Acridine orange and Ethidium bromide (AO/EtBr) dual staining was performed. AO is a cell permeable dye that stains both live and dead cells, whereas EtBr only stains nucleus of cells that have lost membrane integrity. Necrotic cells also stain orange, but resemble viable cells.

Briefly, 5×10^4 cells per well were seeded into 6 well plate in complete DMEM media and treated with compounds (**5a**, **5b**, **5c**, **5d**, **5e**, **5f**, **5g**, **5h** and **5i**) (50 μ M), positive control cells were treated with rTRAIL (25 pM) and the negative control cells were vehicle (0.1% DMSO) treated and incubated for 16h. The cells were harvested and washed with PBS. 10 μ l of 1mg/ml AO and EtBr mixture was added to the cell pellet in PBS and 20 μ l of cells were placed on a microscopic slide with a coverslip and immediately analyzed under a fluorescence microscope 40X objective (Zeiss AxioVert). Nuclei were visualized; apoptotic cells in five random fields were counted and photographed.

3.6. Statistics

All experiments were performed in triplicates. Wherever appropriate, the data are expressed as the mean $\pm$ SD and means were compared using one-way analysis of variance. Statistical significance of differences between control, rTRAIL and compound treated cells were determined by Dunnet's test. For all tests, $P < 0.05$ was considered statistically significant.

4. Results

The ^1H NMR spectrum of compound (**5a**) 3-(4-((3-(4-Methoxyphenyl)-4,5-dihydroisoxazol-5-yl)methyl)piperazin-1-yl)benzo [d] isothiazole. (**5a**) ($\text{R} = \text{OCH}_3$), exhibited a doublet at δ 8.07 ($J = 9$ Hz) due to the C_1 and C_5 - protons. A multiplet was observed in the region between δ 7.64 - 7.54 due to C_{17} , C_{19} and C_{20} protons. Triplet was observed at δ 7.46 - 7.41 ($J = 6$ Hz) due to presence of C_{18} - proton. A doublet in the region δ 7.02 ($J = 9$ Hz) due to presence of C_2 and C_4 - protons. A multiplet was observed at δ 4.93 - 4.83 due to C_9 - proton. A singlet in the region δ 3.8 due to presence of C_{22} - proton. A multiplet was observed at the range between δ 3.56 - 3.45 due to C_{12} , C_{14} and C_8 - protons. A multiplet was observed at the range between δ 3.22 - 3.14 due to C_8 - proton. A multiplet was observed at the range between δ 2.78 - 2.63 due to the presence of C_{10} , C_{11} , and C_{13} - protons. The mass spectrum (LCMS) of the compound (**5a**) showed a $[\text{M} + 1]$ peak at 409.1. The ^1H , ^{13}C NMR and Mass spectral (LCMS) data of all compounds are in complete agreement with assigned structure and details are included in the experimental section.

4.1. Cytotoxicity of compounds on MDA-MB-231 cells

The dose response and IC_{50} values of all the nine compounds were determined using the breast cancer cell line MDA-MB-231. These cells were treated with increasing concentrations (2, 5, 10, 20, 40, 80, 100 and 200 $\mu\text{M/ml}$) of each compound for 16 hours. The results revealed that all the test compounds induced cytotoxicity in the tumor cell line, while compounds **5c**, **5g** and **5e** exhibited high

cytotoxicity with IC₅₀ values of 30, 75 and 95 μM/ml respectively. Compounds **5b**, **5d** and **5a** showed intermediate activity and compounds **5f**, **5i** and **5h** were weakly cytotoxic.

4.2. Benzoisothiazole derivatives induced time dependent cell death

The cytotoxic effects of the benzoisothiazole derivatives was determined *in vitro* using EAT cells. The number of viable cells present in the treated cell suspension was counted by differentiating the live cells from the dead ones. A time dependent decrease in viable cells was observed in treated cells. The percentage viability in cells treated with compounds **5c**, **5f**, **5b** and **5e** showed higher cytotoxicity of 53%, 50%, 45.6% and 44.6% compared to the others, while the rTRAIL treated positive control showed 43% cell death in comparison with the vehicle (0.1% DMSO) treated negative control cells as shown in fig 3.

4.3. Benzoisothiazole derivatives induce apoptosis in EAT cells

In order to ascertain the compound induced cytotoxicity was due to apoptosis, EAT cells were subjected to morphological examination by staining the treated and control cells with AO/EtBr. Cells with intact nuclei and cytoplasm fluoresced green. But positive control and compound treated cells showed early apoptotic morphology with membrane blebbing, condensed nuclei and appeared bright green. The late apoptotic cells fluoresced orange-red with condensed half moon shaped or fragmented nuclei. From the data it is clear that out of the nine benzoisothiazole derivatives, **5c**, **5g**, **5b** and **5e** induced apoptosis as early as 16h by 67.3%, 61.3%, 59% and 50% respectively whereas the positive and negative control samples showed 63.6% and 5% apoptosis respectively. From the data shown in Fig. 4A and B, it is clear that 50 μM of these compounds could induce apoptosis in EAT cells which corroborate with the previous results.

4.4. Discussion

Antineoplastic chemotherapeutics drugs kill tumor cells by inducing apoptosis either by intrinsic or extrinsic pathways. Increase in apoptosis induction in cancer cells is due to the sensitivity of the cancer cells towards the chemotherapeutic drug [39]. Thus novel bioactive drugs like ours need to be tested for drug sensitivity with respect to induction of apoptosis.

In *in vitro* conditions, cytotoxic compounds impede with cellular attachment, resulting in morphological changes, contrarily affecting their proliferation rate, or causing cell death [40]. Hence, the effect of compounds on cell proliferation was studied using the MTT assay which is a well-characterized colorimetric assay, based

on the enzymatic reduction of the tetrazolium salt MTT in living, metabolically active cells, but not in dead cells. We determined the median inhibitory concentration (IC₅₀) of the three highly cytotoxic compounds. Since all the eight compounds were cytotoxic in general, they were subjected to further evaluation of their cytotoxic activity on a murine mammary carcinoma (EAT), with the objective of determining the mode of cell death along with a known apoptosis inducing ligand, TRAIL.

Methods like Hoechst staining, PI staining, annexin staining, acidic denaturation, and thermal denaturation, *in situ* nick translation assays have all been developed to study apoptosis induction by monitoring cytological-morphological changes, but have their own shortcomings in processing steps [41]. While MTT assay, a metabolic profiling assay is also generally used as a drug sensitivity assay, but this assay cannot differentiate between apoptotic and necrotic cells. Hence, a qualitative and quantitative assay like the AO/EtBr dual staining is a well suited method to assess apoptotic cell death [42].

In our present study, four out of nine compounds showed potent pro-apoptotic activity in the EAT tumor model, which was assessed by both trypan blue dye exclusion assay and AO/EtBr dual staining, which was on par or better than the rTRAIL induced apoptosis. In terms of the identification of potent and selective activity in MDA MB 231 breast cancer cell lines, the compounds **5c**, **5g** and **5e** exhibited high cytotoxicity. Compounds **5b**, **5d** and **5a** showed intermediate activity and compounds **5f**, **5i** and **5h** were weakly cytotoxic. From the experimental results it is evident that compound **5f** and **5i** show negligible cytotoxic activity and compounds **5c**, **5b**, **5e** and **5g** are cytotoxic and induce apoptosis and this may be due to the substitution of benzyloxy group at the para position as in **5b**, which shows good apoptotic activity and by inclusion of methoxy group at meta position with benzyloxy group as in **5c**, results in increased rate of apoptosis compared to **5b**. When the benzyloxy group is replaced to meta position (**5e**), we observed a decrease in the activity compared to **5b** and **5c**. But in the absence of benzyloxy groups compounds (**5a**, **5f**, **5d** and **5h**) lose their apoptosis inducing ability and the presence of fluorine group (**5i**) at para position decreases the activity drastically.

5. Experimental section.

The melting points were determined by open capillary method using electric melting point apparatus and are uncorrected. The IR spectra were recorded on Agilent Fourier transform infrared (FT-IR) Spectrophotometer. ¹H NMR spectra were recorded on Bruker 400 MHz spectrometer. ¹³C NMR spectra were recorded on Bruker 100 MHz spectrometer by using DMSO-d₆ as a solvent and TMS as an internal standard. The chemical shifts are expressed in δ ppm. The LC-MS spectra were recorded using Agilent-Single Quartz. The purity of the compounds was checked by TLC.

5.1. General procedure for the preparation of compounds **5a-i**

An equimolar mixture of 3-(4-Allylpiperazin-1-yl)benzoisothiazole (1 g, 3.86 mmoles) and aldoximes (**4a-i**)(3.86 mmoles) in ethanol (15 ml) was stirred for 10 minutes at room temperature. To the above reaction mass, chloramine-T (3.86 mmoles) was charged slowly over a period of 10 min. Then the reaction mixture was heated to 60 to 65 °C and maintained for 2hour. The reaction progress was monitored by TLC. Upon completion of the reaction (disappearance of starting material on TLC), Reaction mass was cooled to room temperature and distilled to a minimum volume and dilute with ethyl acetate (5 mL). The above solution was washed with 2% aqueous sodium hydroxide solution (25 mL x 2) followed by with 5% aqueous sodium hydroxide solutions (25 mL x 2). The organic layer was again washed with water (25 mL x 2). The organic layer was concentrated to dryness to afford a crude derivative. The crude derivative was diluted with methanol (40 mL) to precipitate a solid. The solid was filtered off and wash with 10 ml methanol. The solid was dried and further recrystallized from 1:4 ethylacetate and heptane to afford a pure derivative as an off white solid.

5.1.1. 3-(4-((3-(4-Methoxyphenyl)-4,5-dihydroisoxazol-5-yl)methyl)piperazine-1-yl)benzo[d]isothiazole (**5a**).

White powder, yield (75%), m.p.: 109–111 °C. ¹H NMR (400 MHz, DMSO-d₆) δ: 8.07–8.04 (d, J = 9 Hz, 2H), 7.64–7.54 (m, 3H), 7.46–7.41 (t, J = 6 Hz, 1H), 7.02–6.99 (d, J = 9Hz, 2H), 4.93 - 4.83 (m, 1H), 3.80 (s, 3H), 3.56 - 3.45 (m, 5H), 3.22–3.14 (m, 1H), 2.78–2.63 (m, 6H) ppm. ¹³C NMR (100 MHz, DMSO-d₆) δ: 163.48, 163.53, 156.05, 151.95, 128.08, 127.81, 127.32, 124.36, 124.12, 121.95, 121.01, 114.18, 78.80, 61.33, 55.25, 54.86, 52.98, 49.65, 38.68 ppm. LC MS (m/z): [M+H] 409.1. Anal. calcd. for C₂₂H₂₄N₄O₂S (408.16).

5.1.2. 3-(4-((3-(4-(Benzyloxy)phenyl)-4,5-dihydroisoxazol-5-yl)methyl)piperazin-1-yl)benzo[d]isothiazole (**5b**).

Off white solid. yield (78%), m.p.: 130-134°C; ¹H NMR (400 MHz, DMSO) δ: 8.07 - 8.04 (d, 2H, J = 9Hz); 7.64 - 7.53 (m, 3H); 7.47 - 7.31 (m, 6H); 7.10 - 7.07 (d, 2H, J = 9Hz); 5.16 (s, 2H); 4.93 - 4.83 (m, 1H); 3.53 - 3.45 (m, 5H); 3.21 - 3.05 (m, 1H); 2.77 - 2.58 (m, 6H). ¹³C NMR (100 MHz, DMSO) δ: 163.48, 159.59, 156.03, 151.96, 136.72, 128.42, 128.08, 127.87, 127.81, 127.66, 127.32, 124.36, 124.13, 122.17, 121.01, 115.05, 78.82, 69.28, 61.32, 52.98, 49.65, 38.69. LC MS (m/z): 485 (M+H). Anal. calcd. for C₂₈H₂₈N₄O₂S (484.19).

5.1.3. 3-(4-((3-(4-(Benzyloxy)-3-methoxyphenyl)-4,5-dihydroisoxazol-5-yl) methyl)piperazin-1-yl)benzo[d]isothiazole (**5c**).

Off white solid. yield (73%), m.p.: 146-148°C; ¹H NMR (400 MHz, DMSO) δ: 8.07 - 8.04 (d, 2H, J = 9Hz); 7.59 - 7.53 (t, 1H, J = 9Hz); 7.46 - 7.29 (m, 7H); 7.17 - 7.08 (m, 2H); 5.14 (s, 2H); 4.94 - 4.84 (m, 1H); 3.81 (s, 3H); 3.57 - 3.43 (m, 5H); 3.22 - 3.14 (m, 1H); 2.79 - 2.67 (m, 4H); 2.65 - 2.57 (m, 2H). ¹³C NMR (100 MHz,

DMSO) δ : 163.48, 156.27, 151.95, 149.35, 149.06, 136.76, 128.40, 127.88, 127.81, 127.77, 127.32, 124.36, 124.13, 122.40, 121.02, 120.04, 113.14, 109.32, 78.91, 69.80, 61.34, 55.56, 52.99, 49.64, 38.68. LC MS(m/z): 515.1(M+H). Anal. calcd. for $C_{29}H_{30}N_4O_3S$ (514.2).

5.1.4. 3-(4-((3-(3,4-Dimethoxyphenyl)-4,5-dihydroisoxazol-5-yl)methyl)piperazin-1-yl)benzo[d]isothiazole (5d).

Off white solid. yield (67%), m.p.: 152-154°C; 1H NMR (400 MHz, DMSO) δ : 8.07 - 8.04 (d, 2H, J = 3Hz); 7.59 - 7.54 (t, 1H, J = 9Hz); 7.46 - 7.41 (t, 1H, J = 9Hz); 7.27 (s, 1H); 7.19 - 7.16 (m, 1H); 7.03 - 7.00 (d, 1H, J = 9Hz); 4.94 - 4.76 (m, 1H); 3.80 (s, 6H); 3.64 - 3.44 (m, 5H); 3.23 - 3.14 (m, 1H); 2.79 - 2.68 (m, 4H); 2.65 - 2.58(m, 2H). ^{13}C NMR (100 MHz, DMSO) δ : 163.48, 156.29, 151.95, 150.41, 148.72, 127.81, 127.32, 124.36, 124.12, 122.05, 121.01, 120.15, 111.45, 109.03, 78.89, 61.35, 55.54, 55.47, 52.99, 49.64, 38.68. LC MS(m/z): 438.9(M+H). Anal. calcd. for $C_{23}H_{26}N_4O_3S$ (438.17).

5.1.5. 3-(4-((3-(3-(Benzyloxy)-4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl) methyl)piperazin-1-yl)benzo[d]isothiazole (5e).

Off white solid. yield (83%), m.p.: 138 - 140°C; 1H NMR (400 MHz, DMSO) δ : 8.07 - 8.04 (d, 2H, J = 9Hz); 7.59 - 7.54 (t, 1H, J = 9Hz); 7.47 - 7.30 (m, 7H); 7.21 - 7.18 (m, 1H); 7.06 - 7.03 (d, 1H, J = 9Hz); 5.13 (s, 2H); 4.93 - 4.83 (m, 1H); 3.81 (s, 3H); 3.52 - 3.43 (m, 5H); 3.21 - 3.13 (m, 1H); 2.77 - 2.70 (m, 4H); 2.68 - 2.58 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ : 163.48, 156.21, 151.96, 150.77, 147.70, 136.90, 128.38, 127.83, 127.75, 127.32, 124.36, 124.13, 122.00, 121.02, 120.50, 111.78, 110.96, 78.89, 69.92, 61.32, 55.64, 53.00, 49.64, 38.69. EI MS(m/z): 515.1 (M+H). Anal. calcd. for $C_{29}H_{30}N_4O_3S$ (514.2).

5.1.6. 3-(4-((3-(4-Phenyl)-4,5-dihydroisoxazol-5-yl)methyl)piperazin-1-yl)benzo[d]isothiazole (5f).

Off white solid. yield (79%), m.p.: 118-126°C; 1H NMR (400 MHz, DMSO) δ : 8.08 - 8.05(d, 2H, J = 9Hz); 7.71 - 7.68(m, 2H); 7.59-7.41(m, 5H); 4.98 - 4.88 (m, 1H); 3.57 - 3.52 (t, 1H, J = 9Hz); 3.48 - 3.44 (m, 4H); 3.26 - 3.18 (m, 1H); 2.80 - 2.60 (m, 6H); ^{13}C NMR (100 MHz, DMSO) δ : 163.48, 156.58, 151.96, 129.93, 129.50, 128.75, 127.81, 127.32, 126.51, 124.36, 124.12, 121.01, 79.16, 61.28, 52.98, 49.65, 38.39. LC MS (m/z): 379 (M+H). Anal. calcd. for $C_{21}H_{22}N_4OS$ (378.15).

5.1.7. 3-(4-((3-(4-Chlorophenyl)-4,5-dihydroisoxazol-5-yl)methyl)piperazin-1-yl)benzo[d]isothiazole (5g).

Off white solid. yield (82%), m.p.: 132-134°C; 1H NMR (400 MHz, DMSO) δ : 8.07 - 8.04 (d, 2H, J = 9Hz); 7.72 - 7.69 (t, 2H, J = 6Hz); 7.59 - 7.51 (m, 3H); 7.46 - 7.41 (t, 1H, J = 6Hz); 5.0 - 4.90 (m, 1H); 3.57 - 3.43 (m, 5H); 3.25 - 3.17 (m, 1H); 2.82 - 2.57 (m, 6H). ^{13}C NMR (100 MHz, DMSO) δ : 163.47, 155.86, 151.96, 134.49, 128.84, 128.40, 128.26, 127.81, 127.32, 124.36, 124.12, 121.01, 79.52, 61.22, 52.97, 49.65, 38.24. LC MS (m/z): 413.0(M+H). Anal. calcd. for

C₂₁H₂₁ClN₄OS (412.11).

5.1.8. *3-(4-((3-(3,4,5-Trimethoxyphenyl)-4,5-dihydroisoxazol-5-yl)methyl)piperazin-1-yl)benzo[d]isothiazole (5h).*

Off white solid. yield (63%), m.p.: 128-130°C; ¹H NMR (400 MHz, DMSO) δ: 8.08 - 8.05 (d, 2H, J = 9Hz); 7.59 - 7.54 (t, 1H, J = 6Hz); 7.47 - 7.41 (t, 1H, J = 9Hz); 6.97 (s, 2H); 4.93 - 4.92 (m, 1H) 3.83 (s, 6H); 3.70(s, 3H); 3.58 - 3.45 (m, 5H); 3.27 - 3.18 (m, 1H); 2.79 - 2.63 (m, 6H). ¹³C NMR (100 MHz, DMSO) δ: 163.48, 156.59, 152.96, 151.95, 139.05, 127.82, 127.31, 124.96, 124.37, 124.12, 121.01, 104.07, 79.20, 61.33, 60.09, 56.00, 52.99, 49.62, 38.65. EI MS(m/z): 469.0(M+H). Anal. calcd. for C₂₄H₂₈N₄O₄S (468.18).

5.1.9. *3-(4-((3-(4-Fluorophenyl)-4,5-dihydroisoxazol-5-yl)methyl)piperazin-1-yl)benzo[d]isothiazole (5i).*

Off white solid. yield (77%), m.p.: 106-118°C; ¹H NMR (400 MHz, DMSO) δ: 8.08 - 8.05 (d, 2H, J = 9Hz); 7.78 - 7.70 (m, 2H); 7.59 - 7.54 (m, 1H); 7.46 - 7.41 (t, 1H, J = 6Hz); 7.35 - 7.26 (m, 2H); 4.97 - 4.88 (m, 1H); 3.57 - 3.34 (m, 5H); 3.26 - 3.18(m, 1H); 2.80 - 2.62 (m, 6H). ¹³C NMR (100 MHz, DMSO) δ: 164.58, 163.47, 161.30, 155.75, 151.96, 129.25, 128.87, 128.76, 127.80, 127.32, 126.14, 126.10, 125.58, 124.35, 124.12, 121.01, 115.94, 115.65, 79.30, 61.24, 52.97, 49.64, 38.49. EI MS(m/z): 397(M+H). Anal. calcd. for C₂₁H₂₁FN₄OS (396.14).

6. Conclusion

In conclusion we have synthesized novel 5-((4-(Benzo[d]isothiazol-3-yl)piperazin-1-yl)methyl)-3-phenyl-4,5-dihydroisoxazole derivatives. These derivatives were synthesized in moderate to good yields. They were characterized by ¹H, ¹³C NMR and Mass spectroscopy. Our results demonstrate a promising evidence in treatment of cancer using these benzoisothiazole derivatives and further studies on pro-apoptotic, anti-angiogenic, anti-metastatic signal transduction pathways need to be ascertained to know the primary mode of action.

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Table and Figure Legends

Scheme 1. General synthetic route

Table 1. List of is compounds

Fig. 1. Numbering of the compound (5a)

Fig. 2. Cytotoxicity of compounds on MDA-MB-231 cells as assessed by MTT assay: Cells (3×10^4) were treated with test compounds at different concentrations (2, 5, 10, 20, 40, 80, 100 and 200 $\mu\text{M}/\text{ml}$) for 16 h, incubated with MTT solution for 4 h and the formazan crystals were dissolved in DMSO and read at 570nm. Data are presented as the mean $\pm$ SEM of three independent experiments; *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$.

Fig. 3. Cytotoxicity of EAT cells as assessed by Trypan blue dye exclusion assay: Cells (3×10^4) were treated with benzoisothiazole derivatives (50 μM) along with respective positive and negative controls for 45 mins, 1.5h, 3h and 6h intervals. The cells were harvested, washed with PBS and counted after adding trypan blue dye. The viable cells were counted and the percentage viability was calculated. Data are presented as the mean $\pm$ SEM of three independent experiments; ** $P < 0.01$.

Fig. 4. (A and B): Treatment of EAT cells with benzoisothiazole derivatives induced apoptosis, as assessed by nuclear staining and morphological changes using AO/EtBr dual staining. EAT cells 5×10^4 were seeded in 6-well plates, treated with compounds (50 μM) or rTRAIL (25 pM) for positive control and vehicle (0.1% DMSO) treated negative control. The cells were incubated for 16 hours and then harvested, stained, photographed and counted. Data are presented as the mean $\pm$ SEM of three independent experiments; ** $P < 0.01$.

Table 1. List of is compounds

Compounds	5a	5b	5c	5d	5e	5f	5g	5h	5i
R	H	H	OCH ₃	OCH ₃	OCH ₂ Ph	H	H	OCH ₃	H
R ₁	OCH ₃	OCH ₂ Ph	OCH ₂ Ph	OCH ₃	OCH ₃	H	Cl	OCH ₃	F
R ₂	H	H	H	H	H	H	H	OCH ₃	H

Scheme 1

Figure1

Figure2

Figure3

Figure4

A

B