Anti-cancer activity of novel dibenzo[b,f]azepine tethered isoxazoline derivatives

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**Background:** Dibenzoazepine (DB) derivatives are important and valuable compounds in medicinal chemistry. The synthesis and chemotherapeutic properties of naturally occurring DBs and different heterocyclic moiety tethered DBs are reported. Herein, we report the DB-fused hybrid structure that containing isoxazolines (DBIs) and their anti-cancer activity, which could throw light on the structural and functional features of new molecules.

**Results and Conclusion:** The synthesis and characterization of novel ring DB tethered isoxazoline derivatives (DBIs) were carried out. After the detailed structural characterization using 2D-NMR experiments, the compounds were identified as 5-substituted isoxazolines. The effect of newly synthesized DBIs against the invasion of murine osteosarcoma (LM8G7) cells was studied. Among the tested molecules, compound 4g (5-[(3-(4-chlorophenyl)-4,5-dihydroisoxazol-5-yl-methyl]-5H-dibenzo[b,f]azepine), was found to inhibit the invasion of LM8G7 cells strongly, when compared to other structurally related compounds. Cumulatively, the compound 4g inhibited the invasion MDA-MB-231 cells completely at 10 μM. In addition to anti-invasion property the compound 4g also inhibited the migration of LM8G7 and human ovarian cancer cells (OVSAHO) dose-dependently. Compound 4g inhibited the proliferation of LM8G7, OVSAHO, human breast cancer cells (MCF-7) and human melphalan-resistant multiple myeloma (RPMI8226-LR5) cells that are comparable to cisplatin and suramin.

**Keywords:** Dibenzoazepine, Cycloaddition, Isoxazolines, Anticancer agents, ADMET

**Background**

Tricyclic compounds like phenothiazine, acridones, phe-noxazines, benzoazepines and dibenzoazepines are very important class of anticancer agents. Earlier studies have demonstrated that, altering the polarity (incorporation of hydrophilic or hydrophobic groups) at N-position play a vital role to augment the biological activity (Figure 1) [1,2]. Dibenzoazepine (DB) is a diversified core moiety which exhibit antiviral, antiepileptic, anticonvulsant, antimicrobial, antimalarial and anticancer activities [3-5]. 5H-dibenzo[b,f]azepine-5-carboxamide (carbamazepine) is one of the synthesized effective anticonvulsant drugs, which is the most frequently prescribed first-line drug for the treatment of epilepsy [6].

DB-based small molecules are reported to show antioxidant activity and also sirtuin-2 inhibitory activity [7,8]. Azepine moiety containing tricyclic and naturally occurring pyrrolo[2,1-c][1,4]benzodiazepines originated from Streptomyces species, are used as antitumor and antibiotics [9,10]. These compounds exhibit cytotoxic activity by covalent bonding between the C11-position of tricyclic azepine moiety and the N2 of the guanine residues of the minor groove of DNA [11,12]. Synthesis and anticancer activity of tricyclic azepine moiety tethered with piperazine and 1,2,3-triazole moiety are well reported [13]. Several chemical modifications of dibenzoazepine (DB) conjugated to known active moieties of heterocycles to improve the anti-cancer activity has been attempted. However they have not considered for clinical studies due to the problems relating to side effects.

Nucleosides are the building blocks of DNA and become a key molecule in the field of medicinal chemistry for the discovery of new natural nucleoside derivatives.

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So, extensive modifications have been performed on both the heterocyclic base as well as on the sugar moiety. Mainly, the replacement of the ribose ring with an isoxazolidine nucleus has emerged as an interesting class of dideoxynucleoside analogues [14-16]. These analogues undergo phosphorylation by cellular kinases and metabolized by enzymatic systems instead of natural nucleosides, inserted in the DNA growing chain, and finally acting as chain terminators [17,18].

In continuation of our effort to synthesise novel DBs and isoxazoli(di)ne derivatives [19-21] herein we report the synthesis of DB-fused hybrid structure that containing isoxazolines (DBIs) and their anti-cancer activity, which could throw light on the structural and functional features of new molecules.

Results and Discussion
The dipolarophile 2 was prepared by the reaction of commercially available iminostilbene 1 with allylbromide [22]. The N-allyl pendant arrangement of the intermediate 2 showed a major role in the formation of product. It means that, product will be obtained on the basis of either allyl pendant bent towards the tricyclic ring or disposed outside the ring. The X-ray crystallographic studies revealed that the pendant is disposed outside from the tricyclic aromatic ring [22].

The oxime 3 was prepared by the reaction of corresponding aldehyde with hydroxylamine in the presence of sodiumbicarbonate in EtOH/H2O at 60°C [19]. With two scaffolds in hand, the dibenzoazepine derivatives were synthesized in a single step operation via successive 1,3-dipolar cycloaddition reaction. A solution of oxime 3 (2 eq) in dichloromethane was added to a mixture of N-allyl tricyclic amine 2 (1 eq), sodium hypochlorite (4% aqueous solution, 5 eq) and Et3N (0.05 eq) as shown in Scheme 1.

Progress of the reaction was monitored using TLC. After completion of the reaction, the compounds 4(a-h) were purified using column chromatography. The structures of the final products as well as intermediate were confirmed by NMR, mass and elemental analysis.

The proton assignment and regioisomer of the title compound was studied using 1H, 13C NMR and 2D NMR experiments (HMQC, HMBC, and COSY) (Tables 1 and 2). The chemical shift of geminal protons (H4a, H4e) and methine proton (H5) of isoxazoline ring were observed at 3.18, 3.36 and 4.91 ppm (13C; C-4; 3.18 and C-5; 78.17 ppm) respectively. The calculated chemical shifts for geminal protons 4-substituted isoxazoline was assigned at ~5.0 ppm due to the neighboring O-atom effect [23] (Figure 2). Further, HMBC experiment also gave the significant information for the regioselectivity. The geminal protons H4a, H4e showing the cross peak with C-3 (−C = N-) of isoxazoline ring. Similarly, C-5 signal gave cross peak with geminal protons H4a, H4e but not with C-3 (−C = N-) (Table 1). The above data strongly suggested that, the obtained product is 5-substituted isoxazoline.

Biology
DBIs inhibited the invasion of murine osteosarcoma and human breast cancer cells
Invasion and metastasis are the life-threatening aspects of cancer cells [24]. Most of the cancers unmask their
invasive property, and thereby progressing to frank malignancy from pre-existing carcinoma in situ, or from disorders of epithelial proliferation. Hence, we initially studied the effects of DBIs on the invasion of highly metastatic murine osteosarcoma (LM8G7) cells using Matrigel-coated porous membranes. The LM8G7 cells were highly invasive in the assay. The efficacy of the compounds against the invasion of LM8G7 was summarized. Among the tested DBIs, 4g at 1 and 5 μM concentration inhibited the invasion of LM8G7 cells by 67 and 85%, respectively. The results indicated that the compound 4g exhibited dose-dependent anti-invasive property against the LM8G7 cells, when compared to the other structurally similar derivatives (Figure 3). Suramin inhibited the invasion of LM8G7 cells by 73 and 94% at 1 and 5 μM, respectively (data not shown).

Furthermore, we selected the compound 4g and evaluated its effect on the invasion of human breast cancer cells (MDA-MB-231) in vitro. The results of the study revealed that the compound 4g completely suppressed the invasion of MDA-MB-231 cells in a dose-dependent manner (Figure 4).

Compound 4g inhibited the migration of tumor cells
Metastatic tumor cells can migrate from one place to another in the body. So, the effect of compound 4g against the migration of LM8G7 cells was studied. Boydon Chamber assay was performed to examine its effect on migration. Compound 4g inhibited the migration of LM8G7 cells dose dependently (Figure 5) at 0.5 and 1 μM by 47 and 78% respectively. Furthermore, the compound 4g also inhibited the migration of human ovarian (OVSAHO) cells by 40.2 and 84.9% at 0.5 and 1.0 μM, respectively (data not shown).

Compound 4g inhibited the proliferation of tumor cells
We monitored the effects of compound 4g on the proliferation of LM8G7 or OVSAHO cells using real-time cell electronic sensing systemTM (RT-CES). The effects of compound 4g on the proliferation of LM8G7 or OVSAHO cells were monitored dynamically for every
10 min. Compound 4g inhibited the proliferation of LM8G7 and OVSAHO cells dose-dependently with an IC\textsubscript{50} value of 15 \(\mu\)M and 24 \(\mu\)M, respectively, proving its anti-proliferative effect on tumor cells (Figure 6 and 7).

We next investigated the anti-proliferative effect of compound 4g on human melphalan-resistant multiple myeloma (RPMI8226/LR5) cells using MTT assay. It is observed that the compound 4g exerts anti-proliferative
affects on RPMI8226/LR5 at the tested concentration (Figure 8). Furthermore, we investigated the anti-proliferative effect of the compound 4g on human breast cancer (MCF-7) cells. As shown in Figure 9a, it was observed that the compound 4g exerts anti-proliferative effects on MCF-7 cells at various tested concentrations when compared to the control group. Interestingly, we observed that compound 4g had little or no anti-proliferative effect on MCF-10A cells, indicating that it is not substantially cytotoxic to normal cells (Figure 9b). On the other hand, compound 4g moderately inhibited the proliferation of mouse endothelial cells (UV2) with an IC₅₀ value of 62 μM (Table 3). These results show that compound 4g suppressed the proliferation of endothelial cells, but the concentration of compound 4g required to suppress cell proliferation is high. The...
inhibitory activity of compound 4g is comparable to that of reference molecules such as cisplatin and suramin.

**Compound 4g can induce apoptosis in tumor cells**

We evaluated the effect of compound 4g to induce apoptosis in tumor cells to detect early stage of apoptosis. The results indicate that the compound 4g can induce significant apoptosis in a time dependent manner in MCF-7 cells (Figure 10).

**Absorption–distribution–metabolism–excretion–toxicity (ADMET) properties of DBIs**

*In silico* ADMET properties for all the newly synthesized compounds were obtained using Discovery Studio programme (Accelrys Inc., USA). All the DBIs are in accordance with the parameters of the Lipinski’s Rule of Five [25]. The absorption (PSA2D) parameter range was 23 to 66 and also the distribution (AlogP) parameters range lies between 4.6 to 5.9 (Table 4). The ADMET-human intestinal absorption model predicts that these compounds could well absorb in the body. Probably, these compounds are highly penetrable to the blood brain barriers (BBB) after oral administration. Also, the recursive partitioning/classification trees method predicts that the compound can inhibit the CYP2D6 enzyme weakly. These pharmacokinetic parameters well within the acceptable range defined for human use, thereby indicating their potential as drug-like or drug seed molecules.

**Conclusions**

In conclusion, we herein report the incorporation of isoxazoline ring tethered to dibenzo[b,f]azepine for the first time. After the detailed structural characterization using 2D-NMR experiments, the products were confirmed as 5-substituted isoxazolines. Among the tested compounds, compound 4g was found to inhibit the invasion of LM8G7 cells, when compared to other structurally related DBIs. Also, the compound 4g inhibited the invasion MDA-MB-231 cells completely at 10 μM. Evident to invasion, the compound 4g also inhibited the migration of LM8G7 and OVSAHO cells dose dependently. As a result, inhibitory activity of compound
4g on proliferation of LM8G7, OVSAHO, MCF-7 and RPMI8226/LR5 cells and was comparable to that of cisplatin and suramin.

Methods

Chemical synthesis and reagents

Melting points were determined in capillaries on a Tottoli apparatus and are uncorrected. The NMR experiments (1H, 13C, HMBC, HMQC) were carried out at 500 (125) MHz and the reported chemical shifts (δ) are given in ppm and the coupling constants (J) in Hertz (Hz). Multiplicities of NMR signals are designed as s (singlet), d (doublet), m (multiplet, for unresolved lines). Mass spectra were recorded on a Trio 1000 Thermo Quest spectrometer in the electron impact mode or a Platform Micromass spectrometer in the electro spray mode. TLC was performed on silica gel Alugram SilG/UV254 (Macherey-Nagel). The murine osteosarcoma cell line LM8G7, a highly metastatic murine osteosarcoma cell line with the potential to form tumor nodules in the liver, was cloned from LM8G5 cells as described [26,27] and cultured in DMEM supplemented with 10% FBS, streptomycin (100 μg/ml), penicillin (100 units/ml), 100X non-essential amino acids, β-mercaptoethanol (50 μM), 100X sodiumpyruvate, and L-glutamine (2 mM) at 37°C in a humidified 5% CO2 atmosphere. Human ovarian cancer cells (OVSAHO) were procured from ATCC and cultured in RPMI media supplemented with 10% FBS, L-glutamine (2 mM), and NaHCO3 (10%). The cells were grown to 80% confluency before passage, and experiments were restricted to passages 5–20. The human multiple myeloma (MM) cell line RPMI-8226-LR-5 cells were cultured in RPMI 1640 medium.

Table 3 Inhibition of the proliferation of tumor and endothelial cells by compound 4g

| Compound | Anti-proliferative activity (IC50 in μM) |
|----------|----------------------------------------|
|          | LM8G7 | OVSAHO | UV1 |
| 4g       | 15 ± 0.6* | 24 ± 3.6** | 62 ± 1.9 |
| Cisplatin | 30 ± 24 | 15 ± 0.6 | 12 ± 0.4 |
| Suramin  | 12 ± 2.8 | 14 ± 2.4 | 34 ± 1.4 |

*p 0.05 versus control, **p 0.01 versus control, Mann-Whitney U test.
containing 1x antibiotic-antimycotic with 10% FBS. RPMI-8226-LR-5 and MCF-7 cells were cultured in RPMI 1640 medium with 10% FBS supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. MCF-10A cells were cultured in MEGM® mammary epithelial cell complete medium obtained from Lonza, USA [28].

### Table 4 ADMET-properties of the sugar mimetic isoxazoline molecules by use of Discovery Studio 2.5 version

| Compounds | BBB | Solubility | Hepatotoxicity | CYP2D6 | PPB level | ADMET_AlogP98 | ADMET_PSA_2D |
|-----------|-----|------------|----------------|--------|-----------|---------------|---------------|
| 4a        | 0.42| -6.73      | 0.96           | 0.90   | 2         | 5.28          | 66.42         |
| 4b        | 0.42| -6.70      | 0.94           | 0.84   | 2         | 5.28          | 66.42         |
| 4c        | 0.69| -6.49      | 0.80           | 0.83   | 2         | 5.33          | 50.39         |
| 4d        | 0.99| -6.64      | 0.93           | 0.92   | 2         | 5.37          | 32.53         |
| 4e        | 0.73| -6.13      | 0.92           | 0.92   | 2         | 4.66          | 34.86         |
| 4f        | 1.13| -6.76      | 0.95           | 0.96   | 2         | 5.38          | 23.60         |
| 4g        | 1.34| -7.36      | 0.91           | 0.85   | 2         | 5.98          | 23.60         |
| 4h        | 1.26| -7.20      | 0.96           | 0.75   | 2         | 5.79          | 23.60         |

5-\{-3-(2-nitrophenyl)-4, 5-dihydroisoxazol-5-yl-methyl\}-5 H-dibenzo[b,f]azepine 4b

The product is a thick liquid. Yield: 0.224g (65.7 %). 1H NMR (δ ppm, CDCl₃, 500 MHz): δ 3.24 (dd, 1 H, H₄e, J = 14.1, 7.2 Hz); 3.37 (dd, 1 H, H₆e, J = 14.1, 5.0 Hz); 3.45 (dd, 1 H, H₆f, J = 12.8, 6.8 Hz); 4.33 (dd, 1 H, H₆a, J = 12.8, 4.6 Hz); 4.77 (m, 1 H, H₄); 6.70 (d, 2 H, CH, J = 2.1 Hz); 7.4-8.26 (m, 12 H, Ar-H). IR KBr (cm⁻¹): 1315, 1642, 3040, 3072. Anal. Calcd for C₂₉H₂₀N₃O₃: C, 72.24; H, 4.86; N, 10.57. Found : C, 72.45; H, 4.86; N, 10.48.

5-\{-3-(3-nitrophenyl)-4, 5-dihydroisoxazol-5-yl-methyl\}-5 H-dibenzo[b,f]azepine 4c

The product is a thick liquid. Yield: 0.260 g (68.6%). 1H NMR (δ ppm, CDCl₃, 500 MHz): δ 4.4 (d, 2 H, J = 14 Hz), 5.12 (dd, 2 H), 5.28 (dd, 2 H), 5.8 (m, 1 H), 6.76 (s, 2 H), 7.08 (d, 2 H, CH, J = 5 Hz), 6.98-7.12 (q, 4 H), 7.2-7.3 (t, 2 H). IR KBr (cm⁻¹): 1315, 1642, 3040, 3072. Anal. Calcd for C₂₇H₂₆N₂O₄: C, 73.2; H, 5.92; N, 10.41. Found : C, 73.2; H, 5.86; N, 10.41.

5-\{-3-(3,4,5-trimethoxyphenyl)-4, 5-dihydroisoxazol-5-yl-methyl\}5 H-dibenzo[b,f]azepine 4a

The product is a thick liquid. Yield: 0.224g (65.7 %). 1H NMR (δ ppm, CDCl₃, 500 MHz): δ 3.24 (dd, 1 H, H₄e, J = 14.1, 7.2 Hz); 3.37 (dd, 1 H, H₆e, J = 14.1, 5.0 Hz); 3.45 (dd, 1 H, H₆f, J = 12.8, 6.8 Hz); 4.33 (dd, 1 H, H₆a, J = 12.8, 4.6 Hz); 4.77 (m, 1 H, H₄); 6.70 (d, 2 H, CH, J = 2.1 Hz); 7.4-8.26 (m, 12 H, Ar-H). 13C NMR (δ ppm, CDCl₃, 125 MHz): δ 33.8 (C-4), 53.8 (C-6), 56.2 (CH), 130 (CH). MS (ESI + ion): m/z = 443.5 [M + H]⁺. Anal. Calcd for C₂₉H₂₀N₃O₃: C, 72.24; H, 4.86; N, 10.57. Found : C, 72.45; H, 4.86; N, 10.41.

5-\{-3-(4-methoxyphenyl)-4, 5-dihydroisoxazol-5-yl-methyl\}5 H-dibenzo[b,f]azepine 4d

The product is a thick liquid. Yield: 0.224g (65.7 %). 1H NMR (δ ppm, CDCl₃, 500 MHz): δ 3.24 (dd, 1 H, H₄e, J = 14.1, 7.2 Hz); 3.37 (dd, 1 H, H₆e, J = 14.1, 5.0 Hz); 3.45 (dd, 1 H, H₆f, J = 12.8, 6.8 Hz); 4.33 (dd, 1 H, H₆a, J = 12.8, 4.6 Hz); 4.77 (m, 1 H, H₄); 6.70 (d, 2 H, CH, J = 2.1 Hz); 7.4-8.26 (m, 12 H, Ar-H). 13C NMR (δ ppm, CDCl₃, 125 MHz): δ 33.8 (C-4), 53.8 (C-6), 56.2 (CH). MS (ESI + ion): m/z = 443.5 [M + H]⁺. Anal. Calcd for C₂₉H₂₀N₃O₃: C, 72.24; H, 4.86; N, 10.57. Found : C, 72.45; H, 4.86; N, 10.41.
76.2 (C-5), 126.8 (CH), 128–136.0 (Ar-C), 158.1 (C-C=N). MS (ESI + ion): m/z = 387.0 [M + H]+. Anal. calcd for C24 H19 N2ClO: C, 77.82; H, 5.17; N, 7.48. Found : C, 77.9; H, 5.21; N, 7.48.

5-[3-(phenyl)-4,5-dihydroisoxazol-5-yl-methyl]-5 H-dibenzo[b,f] azepine 4f The product is yellow solid. Yield: 0.23 g (72.3 %). 1H NMR (δ ppm, CDCl3, 500 MHz): δ 3.11 (dd, 1 H, H4a, J = -12.0, 5.7 Hz); 4.31 (dd, 1 H, H6e, J = -12.5, 4.1 Hz); 4.84 (m, 1 H, H5); 6.68 (d, 2 H, CH, J = 2.5 Hz); 6.52-8.22 (m, 12 H, Ar-H). 13C NMR (δ ppm, CDCl3, 125 MHz): δ 29.9 (C-4), 36.10 (C-4), 51.8 (C-6), 54.0 (C-5), 112.8 (Ar-C), 125.0 (Ar-C), 128.8-165.9 (Ar-C), 158.80 (C-C=N). MS (ESI + ion): m/z = 353.1 [M + H]+. Anal. calcd for C24 H19 N2FO: C, 77.9; H, 5.21; N, 7.48. Found : C, 77.9; H, 5.21; N, 7.48.

5-[2-(2,6-difluorophenyl)-4,5-dihydroisoxazol-5-yl-methyl]-5 H-dibenzo[b,f] azepine 4h The product is thick liquid. Yield: 0.209 g (69.2 %). 1H NMR (δ ppm, CDCl3, 500 MHz): δ 3.28 (dd, 1 H, H4a, J = -12.5, 4.1 Hz); 4.30 (dd, 1 H, H6e, J = -12.5, 4.1 Hz); 4.84 (m, 1 H, H5); 6.70 (d, 2 H, CH, J = 2.5 Hz); 6.8-7.62 (m, 11 H, Ar-H). 13C NMR (δ ppm, CDCl3, 125 MHz): δ 34.70 (C-4), 51.80 (C-6), 76.61 (C-5), 123.75 (CH), 128.8-165.9 (Ar-C), 158.80 (C-C=N). MS (ESI + ion): m/z = 389.14 [M + H]+. Anal. calcd for C24 H19 N2FO: C, 77.82; H, 5.17; N, 7.48. Found : C, 77.9; H, 5.21; N, 7.48.

Invasion assay
The in vitro invasion assay was performed using bio-coat Matrigel invasion assay system (BD Biosciences, San Jose, CA, USA), according to the manufacturer’s instructions. MDA-MB-231 cells (2 × 10⁵ cells) or LM8G7 cells (2.5 × 10⁵) were suspended in serum-free RPMI 1640 medium or DMEM, respectively and seeded into the Matrigel transwell chambers consisting of polycarbonate membranes with 8-μm pores [29]. After incubation with 1 or 10 μM concentrations of DBIs for 24 h, the upper surfaces of the transwell chambers were wiped with cotton swabs and the invading cells were fixed and stained with crystal violet solution. The invading cell numbers were counted in five randomly selected microscope fields. The % inhibition of the invaded cells was calculated.

In vitro migration assay
The efficacy of LM8G7 cell migration was assessed using the BD BioCoatTM chamber (8 μm PET pores) without Matrigel (BD Biosciences, 8 μm pore size, insert size: 6.4 mm) in vitro [29]. Briefly, single cell suspensions of LM8G7 cells (2.5 × 10⁴) were prepared by detaching and resuspending the cells in DMEM containing 0.1 % BSA. Before the cells were added, the chambers were hydrated for 2 h in an incubator at 37°C. The lower chambers were filled with DMEM containing 5 % FBS. To the upper chamber, LM8G7 or OVSAHO cells and compound 4g in serum-free DMEM was added. After incubation for 24 h, cells that had passed through the BD BioCoatTM chamber and remained attached to the opposite surface of the membrane are stained with Diff-Quick solution and counted in five random microscopic fields per filter. The % inhibition of the migration of LM8G7 or OVSAHO cells by compound 4g was calculated.

Real-time cell proliferation assay
The cell proliferation assay was done using the RT-CES™ system (ACEA Biosciences, San Diego, CA). Briefly, LM8G7 cells (5x10³ cells) were added to ACEA 96X microtiter plates (e-plate™) in 100 μl of medium [30]. In an experiment, cell monolayer was made and the various concentrations of compound 4g (2 to 75 μM), in 150 μl of DMEM were individually added. The effect of the compounds on the proliferation of LM8G7 or OVSAHO cells was continuously monitored up to 48 hours for every 10 min. The proliferation was monitored for a period of 70 h and expressed as a cell
index (quantitative measurement of cell proliferation) as per the manufacturer’s instructions. A cell index was plotted against time (for duplicate experiments). The IC_{50} values were calculated from concentration-response curves by a non-linear regression analysis using the GraphPad Prism (GraphPad Prism Software Inc., San Diego).

**MTT assay**

The anti-proliferative effect of the compound 4g on MCF-7, melphalan-resistant multiple myeloma (RPMI8226/LRS) MCF-10A or UV\(^2\) cells was determined by MTT dye uptake method as described previously [29]. Briefly, the cells were incubated in triplicate in a 96-well plate in the presence or absence of indicated concentration of compound 4g in a final volume of 20 \(\mu\)l for different time intervals at 37°C. Thereafter, 20 \(\mu\)l MTT solution (5 mg/ml in PBS) was added to each well. After a 2 h incubation at 37°C, 0.1 ml lysis buffer (20 % SDS, 50 % dimethylformamide) was added; incubation was continued overnight at 37°C; and then the optical density (OD) at 570 nm was measured by Tecan plate reader.

**Annexin V Assay**

One of the early indicators of apoptosis is the rapid translocation of the membrane phospholipid phosphatidylserine from the cell’s cytoplasmic interface to the extracellular surface and its accumulation there, producing a loss of membrane symmetry that can be detected using annexin V. Briefly, 1 x 10\(^6\) MCF-7 cells were pre-treated with compound 4g (30 \(\mu\)M) for various time points and then subjected to annexin V staining. Cells were washed, stained with FITC-conjugated anti-annexin V antibody, and then analyzed with a flow cytometer (BD FACS Calibur, BD Biosciences, US) [28].

**Authors’ contributions**

MPS and Basappa lead the medicinal chemistry efforts for the project and assisted in writing the manuscript. Basappa and MS conceived the project, established the in vitro assays, and participated in the experimental design and wrote the paper. SN, AC and DSP are responsible for experimental design, supervised MPS and Basappa. All authors read and wrote the paper. SN, AC and DSP assisted in writing the manuscript. Basappa and MS conceived the project, designed the experiments, supervised MPS and Basappa. All authors read and wrote the paper.

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