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ARTICLE

Synthesis and biological evaluation of novel Δ^2-isoxazoline fused cyclopentane derivatives as potential antimicrobial and anticancer agents

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Santosh Kumar Prajapti[a], Shweta Shrivastava[b], Umesh Bihade[c], Ajay Kumar Gupta[a], V.G.M. Naidu[b], Uttam Chand Banerjee[c], Bathini Nagendra Babu[a,*]

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As a part of our endeavour toward the synthesis of new heterocyclic bioactive agents, three series of Δ^2-isoxazoline fused cyclopentane derivatives (27 compounds) were synthesized and characterized by IR, ^{1}H NMR, ^{13}C NMR and MS analysis. The newly synthesized target compounds were evaluated for their preliminary *in vitro* antimicrobial and anticancer activities. The results indicated that the compounds **4b**, **4h**, **4i**, **5d** and **5g** displayed remarkable anti-microbial activity with respect to their standard drugs Ampicillin, Gentamycin and Amphotericin B. In preliminary MTT cytotoxicity studies, compound **4i** was found to be equipotent to the standard drug Etoposide against MCF-7. The influence of the most active cytotoxic compound **4i**, on the cell cycle distribution was evaluated in the MCF-7 cell line, which displayed a cell cycle arrest at S phase. Moreover, Acridine orange/ethidium bromide staining, annexin V binding assay and mitochondrial membrane potential revealed that the compound **4i** can induce cell apoptosis in MCF-7 cells. Compounds **4b** and **4h** are potential leads as antimicrobials owing to no significant cell toxicity observed in present study. Docking studies revealed that compound **4i** binds to Phe1145, Glh698, Met696, Cys1191, Met1169 and Ile1167 on DNA methyltransferase (DNMT1) protein and inhibition of DNMT1 could be the possible mechanism of action for these compounds.

Introduction

The microbial resistance to the currently available antimicrobial drugs is the major concern area in the treatment of microbial infections. These resistant strains curtail the life span of the drugs.[1] On the other hand, cancer is one of the leading causes of death worldwide. It accounted for 8.2 million deaths (around 17% of all deaths) in 2012 and with an estimated 14 million deaths in 2030.[2] The current scenario highlights the need for the discovery and development of new chemical entity with novel mode of actions.[3] The identification of novel molecule that can be more effective and reliable is still a major challenge for medicinal chemistry researchers.

In recent years, there has been increased attention towards the synthesis of isoxazole/isoxazoline derivatives, since they displayed a significant role in organic and medicinal chemistry. Organic compounds containing isoxazole/isoxazoline motif as a core unit are known to exhibit a wide range of biological activities such as antibacterial,[4,5] antifungal,[6] anticancer,[7] anticonvulsant,[8] anti-inflammatory,[9] antiviral,[10,11] antidepressant,[12] and antithrombic activity.[13] Isoxazole moiety has been found in various drugs such as cloxacillin, dicloxacillin, flucloxacillin which are currently in clinical use as antibiotics.[14,15] In addition, isoxazolines are versatile masked structural entities and allow easy access to amino alcohols,[16,17] hydroxy ketones[18,19] and isoxazolidines,[20,21] which have been played a crucial role in natural product synthesis and several pharmacologically active heterocyclic compounds.[22] On the other hand, synthesis of cyclopentane derivatives has attracted tremendous interest for decades among the researchers due to their potential applications in medicinal chemistry as therapeutic agents. The functionalized cyclopentane derivatives are known to posses various biological activities such as anticancer (by inhibiting Akt phosphorylation[23] and steroid metabolizing enzymes AKR1C1 and AKR1C3[24]), antimicrobial,[25] antimalarial,[25] antiviral,[26] human neurokinin-1 (hNK1) inhibiotors[27] and so on. Also, it is revealed from the literature survey that the isoxazoline fused heterocyclic hybrids as well as different fused nitrogen containing heterocycles, showed a broad range of therapeutic activity (Fig. 1).[28-32] Despite several reports on fused heterocycles, there is continuing demand for development of new methods for the synthesis of novel fused heterocycles due to their plethora of medicinal applications. Thus, based on aforementioned results and taking into account of these structural aspects of

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ecyclopentane and isoxazolines, we anticipate that the hybrid emanating from the incorporation of isoxazoline moiety to cyclopentane scaffold might exhibit clinically useful antimicrobial and anticancer activity. In this communication, we describe the synthesis, antimicrobial and anticancer activity of novel Δ^2-isoxazoline fused cyclopentane derivatives **4a**–**i**, **5a**–**i** and **6a**–**i**. Among these, the most effective cytotoxic compound **4i** was also evaluated for acridine orange/ethidium bromide staining, annexin V binding assay, mitochondrial membrane potential, and cell cycle analysis.

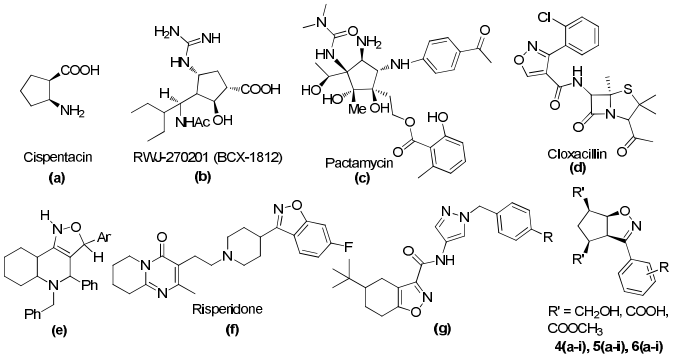
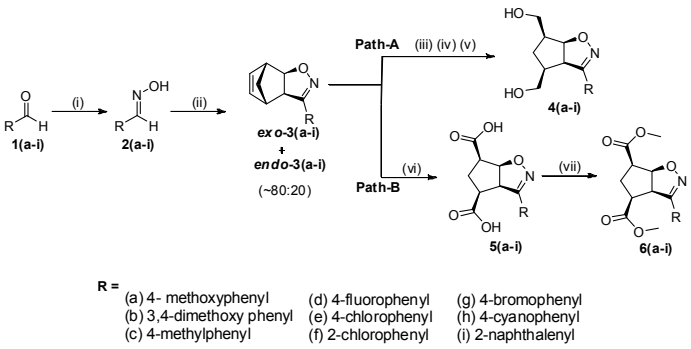


Fig. 1 Structure of some representative cyclopentane, isoxazole and fused isoxazoline derivatives.

Result and discussion

Chemistry

All isoxazoline fused cyclopentane derivatives **4a**–**i**, **5a**–**i**, **and 6a**–**i** were synthesized as racemates by utilizing the [3+2] cycloaddition of nitrile oxide to norbornadiene as depicted in Scheme 1. The aldehydes (**1a**–**i**) were first converted to corresponding aldoximes (**2a**–**i**) *via* reaction with hydroxylamine in presence of sodium hydroxide.[33] The resulted aldoximes (**2a**–**i**) were treated with sodium hypochlorite to generate respective hydroimoyl chloride, followed by dehydrohalogenation with the removal of HCl using 4-dimethylaminopyridine (DMAP) to produce nitrile oxide, which underwent [3+2] cycloaddition with norbornadiene to provide a racemate of *exo* and *endo* isomer of cycloadduct in a ratio of about ~80:20 [7,22]. The mixture of adducts were separated by silica gel column chromatography using 1–5% of EtOAc:Hexane. The structure of cycloadducts were confirmed by ^{1}H NMR and as per the literature precedents.[34,35] In its spectrum, two double doublet (dd) peaks in range of δ ~6.0–6.5 ppm correspond to olefin protons and the chemical shift (δ) at ~5 ppm and ~4 ppm depicted the presence of protons at fused position of cycloadducts **3a**–**i**, which comes as doublet (d) having the same coupling constant value (J = ~8 Hz) confirmed the stereochemistry of *exo* cycloadducts, while double doublet (dd) peak at δ ~5 and δ ~4 with couping constant (J = ~4 Hz, ~9 Hz) was observed in case of *endo* cycloadduct. IR bands in the region of 1585–1620 cm^{-1}, confirmed the presence of C=N bond in cycloadducts. In all cases, *exo* adduct was found to be the major product and these cycloadducts were utilized as key intermediate to carry out further reactions. To obtain isoxazoline fused cyclopentane analogs, *exo* adducts were subjected to oxidative opening of C=C bond. For that purpose, we have utilized two different path for the oxidative cleavage of C=C bond of cycloadducts **3a**–**i** to obtain target compounds **4a**–**i**, **5a**–**i**, and **6a**–**i**. Path-A involves *cis*-dihyroxylation of cycloadducts ***exo*-3a**–**i** in presence of catalytic amount of osmium tetraoxide and *N*-methylmorpholine *N*-oxide[36] followed by oxidative opening using sodium periodate[37] and then reduction[38] of respective di-aldehydes to afford series of diol products of fused isoxazolines **4a**–**i**. IR bands ranging from 3250 to 3400 cm^{-1} indicated the presence of hydroxyl group in the synthesized compounds. The appearance of multiplet signal around δ 3.5–4.0 ppm depicted the presence of CH_2 protons attached to fused bicyclic system of **4a**–**i,** confirmed the oxidative opening of C=C bond in cycloadducts ***exo*-3a**–**i**. In ^{13}C NMR, peak from δ 155–162 ppm indicated the presence C=N of dihydroisoxazole. Path-B involves potassium permanganate for direct opening of C=C bond[39] of ***exo*-3a**–**i** to get series of bi-carboxylic acid substituted isoxazoline fused cyclopentane analogs **5a**–**i**, which on further treatment with catalytic amount of sulfuric acid in methanol[40] yielded methyl ester analog of isoxazoline fused cyclopentane analogs **6a**–**i** in high yields. Disappearance of olefin peak in ^{1}H NMR confirmed the oxidative opening of cycloadducts ***exo*-3a**–**i**. Furthermore, the appearance of peak in the range of δ 171−174 in ^{13}C NMR confirmed the presence of carbonyl carbon in **5a**–**i**. IR bands in range of 2900–3120 and 1690–1720 cm^{-1} indicated O–H and C=O stretching respectively. Compounds **6a**–**i** were confirmed by the characteristic peak of –OCH_3 at range of δ 3.6–3.8 in ^{1}H NMR. The IR spectra showed strong C=O absorption at 1720–1740 cm^{-1}. Furthermore, δ 172–173 in ^{13}C NMR indicated the presence of carbonyl carbon of ester in compounds **6a**–**i**.



Scheme 1 Synthesis of Δ^2-isoxazoline fused cyclopentane derivatives; Reagents and conditions: (i) NH_2OH•HCl, NaOH, EtOH-H_2O (1:1), rt, 4 hr; (ii) Norbornadiene, NaOCl, DMAP, DCM, 0°C to rt, 12 h; (iii) OsO4, NMO, acetone-water (7:3), rt, 24 h; (iv) NaIO4, DCM- aq. NaHCO3 (15:1), 0 °C, 30 min; (v) $NaBH_4$, MeOH, 0 °C, 2 h; (vi) $KMnO_4$, acetone-H_2O (8:2), 0 °C, 3 h (vii) Cat. H_2SO_4, MeOH, 6 hr.

Biological evaluation

***In vitro* antimicrobial activity.** *In vitro* antimicrobial screening of the newly synthesized compounds **4a-i**, **5a-i** and **6a-i** were examined against two Gram-positive bacteria viz; *Bacillus subtilis* and *Bacillus megaterium*, two Gram-negative bacteria viz; *Escherichia coli* and *Pseudomonas Spp.* and one fungal

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strain viz; *Candida albicans*. Cup plate diffusion method[41,42] was used for the determination of the preliminary antibacterial and antifungal activities. Ampicillin, Gentamycin and Amphotericin-B were used as reference drugs. The results illustrated in Table 1 revealed that most of the tested compounds displayed variable inhibitory effects on the growth of the tested Gram positive and Gram negative bacterial strains, and also against antifungal strains. Particularly compounds **4h**

Table 1: *In vitro* antimicrobial activity of compounds **4(a-i), 5(a-i)** and **6(a-i)**.

Compound	Minimum inhibitory concentration (μg/mL)[a]				
	Gram positive		Gram negative		Fungi
	Bs	Bm	Ec	Ps	Ca
4a	-	-	-	-	-
4b	46.8	46.8	**0.92**	**1.2**	**4.6**
4c	-	-	-	-	-
4d	-	-	-	-	-
4e	-	-	-	-	-
4f	74.4	-	-	-	-
4g	48.6	48.6	-	-	-
4h	**21**	**21**	**2.1**	**2.1**	11
4i	**26**	**26**	-	-	-
5a	-	-	-	-	-
5b	-	-	88.4	44.2	81.6
5c	-	-	-	-	-
5d	-	-	78	**7.8**	-
5e	60	30	-	-	-
5f	-	-	-	-	-
5g	**10.5**	**10.5**	**2.2**	22	8.7
5h	-	-	-	-	-
5i	-	-	-	-	-
6a	-	-	-	-	-
6b	-	-	-	-	-
6c	-	-	-	-	-
6d	-	-	-	-	-
6e	-	-	-	-	-
6f	-	-	-	-	-
6g	-	-	-	-	-
6h	-	-	-	-	-
6i	-	-	-	-	-
Ampicillin	**25**	**25**	NT	NT	NT
Gentamycin	NT	NT	**2**	**8**	NT
Amphotericin B	NT	NT	NT	NT	**5**

Bs: *Bacillus subtilis*, Bm: *Bacillus megaterium*, Ec: *Escherichia coli*, Ps: *Pseudomonas Spp.*, Ca: *Candida albicans*.
[a](-): Inactive (MIC > 100 μg/mL).
NT: Not tested.

and **5g** have shown broad spectrum activity. It was observed that, the diol series of Δ^2-isoxazoline substituted with 3,4 dimethoxy phenyl **4b** showed superior activity against *E. coli*. (MIC 0.92 μg/mL), *Pseudomonas Spp.* (MIC 1.2 μg/mL) and *Candida albicans* (MIC 4.6 μg/mL) as compared to their reference drugs, while moderate inhibition was observed against Gram positive bacterial strains. Replacing the diol substitution with bicarboxylic acid **5b** displayed decreased activity against all tested organism. Substitution of Δ^2-isoxazoline diols with *p*-methoxy phenyl **4a**, tolyl **4c**, 4-fluoro phenyl **4d** and 4-chloro phenyl **4e** led to loss in antimicrobial activity. However, the bicarboxylic analog of Δ^2-isoxazoline substituted with 4-fluoro phenyl **5d** was active specifically against Gram negative bacteria, *Pseudomonas Spp.* (MIC 7.8 μg/mL) and *E.coli* (MIC 78 μg/mL), while 4-chloro phenyl substituted compound **5e** showed inhibitory activity only against Gram positive bacterial strains, *Bacillus subtilis* (MIC 60 μg/mL), *Bacillus megaterium* (MIC 30 μg/mL). Diols with 2-chloro phenyl **4f** and 4-bromo phenyl **4g** substitution showed moderate activity against Gram positive bacterial strains. However, the bicarboxylic Δ^2-isoxazolines bearing 4-bromo phenyl **5g** substitution displayed excellent activity against *Bacillus subtilis* (MIC 10.54 μg/mL), *Bacillus megaterium* (MIC 10.54 μg/mL), *E. coli* (MIC 2.2 μg/mL) along with good inhibitory action against *Pseudomonas Spp.* (MIC 22 μg/mL) and *Candida albicans* (MIC 8.78 μg/mL). Compound with 4-cyano phenyl substitution **4h** exhibited significant activity against Gram positive and Gram negative bacterial strain as compared to their reference drugs. The Δ^2-isoxazoline diol **4h** also showed good activity against *Escherichia coli* and *Candida albican*s (MIC 11 μg/mL), whereas its carboxylic acid analog **5h** lost their activity against all tested microbial strains. The compound with naphthalenyl substitution **4i** in Δ^2-isoxazoline diol displayed equipotent activity to their reference drug Ampicillin, specifically against Gram positive bacteria, while activity was lost in case of its bicarboxylic acid analog **5i**. On the other hand, the antimicrobial activity was lost against all tested microbial strains in methyl ester analogs of Δ^2-isoxazoline **6a-i**. It is worth mentioning that the compound Δ^2-isoxazoline diol substituted with planar and bulky group (naphthalenyl) **4i** displayed selectivity towards Gram positive bacterial strain. Similarly, the selectivity for Gram positive bacterial strain was also observed in bicarboxylic analog of Δ^2-isoxazoline tethered electron withdrawing with bulky group (4-chloro phenyl) **5e**. Introduction of two methoxy groups (electron donating group) on phenyl ring **4b** in Δ^2-isoxazoline diol enhanced its selectivity to inhibit Gram negative bacterial and fungal growth, while introducing electron withdrawing group (cyano) at para position of phenyl ring **4h** showed broad spectrum antimicrobial activity. Moreover, bicarboxylic analog of Δ^2-isoxazoline substituted with bulky group **5g** (4-bromo phenyl) showed activity against all tested microbial strains.

***In vitro* anticancer activity.** All newly synthesized compounds were evaluated for their *in vitro* cytotoxic activity against five cancer cell lines HT-29 (colon), HeLa (cervix), MCF-7 (breast), A549 (lung) and PC-3 (prostate) cancer cell lines by employing MTT assay.[43] Etoposide was taken as the reference in this study. Concentration response course analysis was performed to determine drug concentrations required to inhibit the growth of cancer cells by 50% (IC_{50}) after incubation for 48 h. The results of *in vitro* anticancer activity revealed that, some of the synthesized compounds exhibited different levels of anticancer properties (Table 2). From the close analysis of the IC_{50} values, it was observed that, Δ^2-isoxazolines, bearing *p*-methoxy phenyl substituent of diol series **4a** showed inhibitory activity against MCF-7 and A-549; however its bicarboxylic compound **5a** lost cytotoxicity against all cancer cell line and its methyl ester analog **6a** displayed activity specifically against HT-29. Δ^2-Isoxazoline, substituted with 3,4 dimethoxy phenyl, only

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bicarboxylic analog **5b** showed moderate cytotoxicity against HT-29. In case of tolyl substitution, only **4c** was shown to be active against HeLa and PC-3. Enhancement in anticancer activity was observed in Δ^2-isoxazoline diol against HT-29 and A-549 when, fluoro group was introduced at *para* position of phenyl ring **4d**. Complete loss of activity was observed in case of its bi-carboxylic acid derivative **5d**; however its ester analog **6d** exhibited moderate activity against HeLa and MCF-7.

Table 2. *In vitro* anticancer activity of compounds **4a-i, 5a-i** and **6a-i**.

Comp	IC_{50} (μg/mL)[a]				
	HT-29	HeLa	MCF-7	A549	PC-3
4a	-	-	23.7±3.25	36.6±4.98	-
4b[c]	-	-	-	-	-
4c	-	26.5±3.51	-	-	28.0±2.54
4d	17.8±4.21	-	-	31.1±5.62	-
4e	22.9±6.74	-	14.7±2.15	-	-
4f	33.8±7.84	-	-	-	-
4g	37.3±	-	-	-	-
4h[c]	-	-	-	-	-
4i	14.3±5.32	24.5±8.43	**11.5±1.45**	-	48.4±5.32
5a	-	-	-	-	-
5b	40.6±7.12	-	-	-	-
5c	-	-	-	-	-
5d	-	-	-	-	-
5e	-	19.0±4.35	28.5±1.87	30.0±6.74	-
5f	-	-	-	-	-
5g	-	45.6±8.32	-	-	-
5h	30.3±2.54	33.3±5.63	-	-	-
5i	29.7±4.87	-	31.8±2.21	-	-
6a	28.9±3.65	-	-	-	-
6b	-	-	-	-	-
6c	-	-	-	-	-
6d	-	20.1±2.56	39.4±4.38	-	-
6e	-	-	-	-	-
6f	-	-	-	-	-
6g	-	27.4±3.48	-	-	-
6h	-	-	-	-	-
6i	-	-	-	-	-
Etopo-side[b]	4.21±0.53	2.35±0.25	**11.95±2.63**	8.9±1.87	7.2±1.37

[a](-): Non-cytotoxic (IC_{50} > 50 μg/mL).
[b]Etoposide was used as reference drug.
[c]Compound tested against normal mammalian breast epithelium cell line (MCF-10A) [IC_{50} > 100 μg/mL]

Furthermore, compound substituted with chloro at *para* position **4e** showed good inhibitory activity against MCF-7 along with HT-29, while bicarboxylic derivative 5e was active against HeLa, MCF-7 and A-549 and loss of cytotoxicity was observed in case of its ester analog **6e**. Substitution of phenyl with chloro at *ortho* **4f** and bromo at *para* position **4g** of Δ^2-isoxazoline diol displayed cytotoxicity specifically against HT-29. Cytotoxicity was lost in *ortho* chloro phenyl substituted bicarboxylic **5f** and its methyl esters analog **6f** whereas, *p*-bromo substituted carboxylic and ester analog **5g**, **6g** were shown to be active specifically against HeLa. In case of 4-CN phenyl substituted Δ^2-isoxazoline, only acid derivative **5h** was found to be active against HT-29 and HeLa. Replacement of phenyl group with the naphthalenyl ring, the fused isoxazoline diol **4i** displayed cytotoxic activitiy towards HT-29, HeLa, MCF-7 and PC-3. However, its acid derivative **5i** was active only against HT-29 and MCF-7 and complete loss of activity was observed in its ester analog **6i**. Significantly, compound **4i** was found to be most potent having comparable IC_{50} value (11.5 ± 1.45 μg/mL) with that of the reference drug, Etoposide (IC_{50} 11.95±2.63 μg/mL) in MCF-7. These observations may promote a further development of Δ^2-Isoxazoline and may lead to compounds with better pharmacological profile than the standard anticancer drugs.

Notably, the compounds **4b** and **4h**, which showed potent antimicrobial activity, were accompanied with no significant cytotoxicity against cancer as well as normal mammalian cells, which might be useful as potential lead for the development of novel antimicrobial agents. However, the compounds **4i** and **5g** showing remarkable antimicrobial activity cannot be considered as good antimicrobials because of their potential cytotoxicity.

Acridine Orange and Ethidium Bromide (AO/EB) staining. The most active cytotoxic compound **4i** was also investigated for the inhibition of cell proliferation and cytotoxicity to understand whether it is due to apoptotic induction or nonspecific necrosis, morphological features were analyzed through Phase-contrast microscopy and AO/EB staining.[44] The morphological abnormalities were studied under a phase-contrast microscope. Cells treated with 5 and 10 μg/ml of compound **4i** for 48 h showed obvious morphological changes, with chromatin condensation, fragmentation and formation of apoptotic bodies. However, the control group (without test compound) showed normal healthy shape with intact nuclei and without any abnormalities (Fig. 2a). Most of the treated cells in all the test concentrations exhibited similar symptoms of apoptosis but the damage was severe in the cells exposed to highest concentration of 10μg/ml (Fig. 2c). The results of light microscopy were consistent with that of fluorescence microscopy using AO/EB (Fig. 3a–c). The bright condensed chromatin identified by AO staining was a clear indication of early apoptosis leading to margination of chromatin into a horseshoe shaped structure. Cells treated with high concentration 10 μg/ml of compound **4i** exhibited intense nuclear fragmentation followed by the formation of apoptotic bodies.

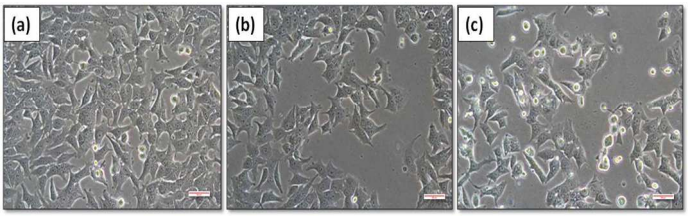

Fig. 2 Morphological changes in MCF-7 cells treated with and without compound **4i** for 48 h. Light microscopy: (a) Control, cells with intact nuclei; (b) Early signs of apoptosis characterized at 5 μg/ml by extremely condensed chromatin;(c) cell membrane blebbing, nuclear fragmentation and chromatin condensation at 10 μg/ml.

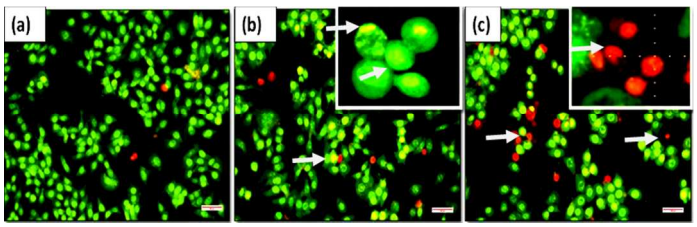

Fig. 3 Morphological changes in MCF-7 cells treated with and without compound **4i** for 48 h. Fluorescence microscopy: (a) Green live cells show normal morphology of control; (b) Early signs of apoptosis characterized at 5 μg/ml by extremely condensed chromatin, which marginated into a horseshoe-shaped structure and cell membrane blebbing, (c) Destructive fragmentation of the nuclei at 10 μg/ml and irregular distribution of chromatin, late apoptotic cells exhibited condensed chromatin and their nuclei, stained red with EB.

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Annexin V binding assay. In the next series of experiment, the apoptotic activity of compound **4i** on human breast cancer (MCF-7) cells was proved using the apoptosis assay based on the annexin V/propidium iodide staining.[45] MCF-7 cells were treated with 5 and 10 µg/ml of compound **4i** for 48 h. As depicted in Fig. 4, the compound **4i** increased the percentage of early apoptosis stage (6.00% to 24.94% and 48.32%, respectively) with the concentration which indicated that compound **4i** induced apoptosis of MCF-7 in a dose dependent manner (Fig. 4).

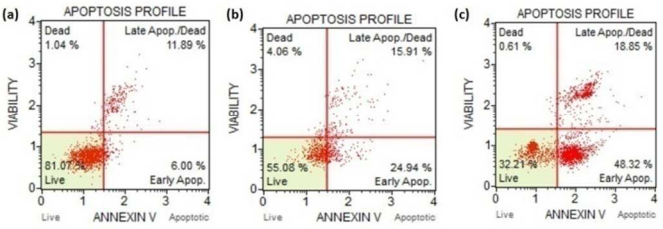

Fig. 4 Annexin V binding assay by Muse™ Cell Analyzer after 48 h of treatment with compound **4i** in MCF-7. The dual parameter dot plots combining annexin V-fluorescein isothiocyanate (FITC) and PI fluorescence show the viable cell population in the lower left quadrant (annexin V-PI-), apoptotic cells in the lower right quadrant (annexin V+ PI-) and the upper right quadrant (annexin V+ PI+), and necrotic cells in the upper left quadrant (annexin V-PI+).

Cell cycle analysis. To gain insight into the mechanisms through which tested compounds reduced the number of adherent cells, flow cytometric analysis[46] was performed after incubation of MCF-7 cells with compound **4i** at selected concentrations for 48 h. Separation of cells in G_0/G_1, S and G_2/M phase was based on fluorescence intensity after staining with propidium iodide. Representative profiles are shown in Fig. 5. In untreated cells, a predominant number of MCF-7 cells were accumulated in G_0/G_1 phase. Compound **4i** induced a marked reduction in the number of cells in the G_0/G_1 phase and an accumulation of cells in the S phase of the cell cycle. MCF-7 cells were treated with 5 & 10 µg/ml of compound **4i** for 48 h, percentage of cell arrest in S phase increased progressively from 12.7, 14.9, and 27.5% respectively (Fig. 5). At 48 h of exposure, the number of cells increased in the S-phase by 2.16 fold, when compared to controls.

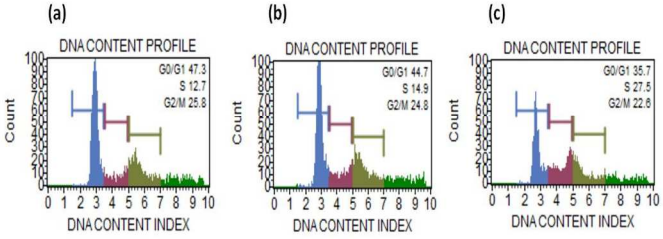

Fig. 5 Effect of compound **4i** on cell cycle progression of MCF-7 cells, (a) Control cells; (b, c) Cells treated with 5 and 10 µg/ml for 48 h followed by analysis of cell cycle distribution using propidium iodide cell staining method. Cell population in each cell cycle phase was numerically depicted. Data represent one of three independent experiments.

Analysis of mitochondrial membrane potential (MMP). In addition, the effect of compound **4i** on mitochondrial membrane potential loss in MCF-7 by was investigated by JC-1 staining followed by fluorescent microscopy.[47] Change in the mitochondrial membrane potential was determined by red verses green fluorescence by JC-1 dye where healthy mitochondria gives out o fluorescence because of J aggregates of JC-1 dyes and dead/apoptotic cells show green fluorescence because of lack of mitochondrial membrane potential (Fig. 6). Changes in the ratio between the measurement at wavelengths of 590 nm (red) and 540 nm (green) fluorescence intensities were in agreement with cytotoxicity potency by inducing significant depolarization of mitochondrial membrane potential in a dose-dependent manner in MCF-7 cells.

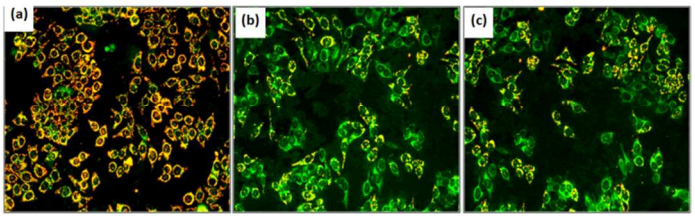

Fig. 6 Determination of mitochondrial membrane potential through JC-1 staining and detection using fluorescent microscopy. Cells were exposed to 0, 5 and 10 µg/ml of compound **4i** for 12 h followed by JC‑1 dye incubation for the final 20 min. After treatment, the mitochondrial membrane potential was found to be interrupted, as evidenced by the migration of JC‑1 dye from the mitochondria into the cytoplasm of treated cells, and the subsequent reduction in the mitochondrial red fluorescence signals.

Molecular docking studies. Biological studies revealed that the molecule induce cell death by arresting cell cycle at S-phase and mitochondrial apoptotic pathway. In order to investigate the possible mechanism, molecular docking was performed for compound **4i** into the binding pocket of the cofactor of human DNA methyltransferase (DNMT1). Coordinates of protein structure were obtained from the Protein Data Bank (PDB ID: 3PTA).[48] Geometry of the molecules was optimized by using the optimized potentials for liquid simulations-2005 (OPLS-2005) force field. Compound **4i** was flexibly docked into the crystallographic structure of the methyltransferase domain of human DNMT1 using Docking Glide Extra Precision (XP), version 6.0[49] (Schrödinger, 2013). For the purpose of docking protocol validation, S-adenosylhomocysteine (SAH) bound to the crystal structure was removed from the binding pocket and docked back into the cofactor binding site. Docking results showed a similar docking pose to the pose of co-crystal ligand with RMSD of 1.11 Å which is in the acceptable range to reproduce the binding mode of SAH. As depicted in Figure 7, the compound interacted with the SAH binding site formed from the Glu1168, Glh1189, Val144, Ile1167, Asn1192, Asp1190, Cys1191, Leu1247, Phe1145, Met1169, Trp1170, Glh698, Met696 and Ala696. The π-π stacking exhibited by adenine moiety with Phe1145 in SAH is compensated by naphthalene ring of compound **4i.** The –OH group of Δ^2-isoxazoline ring displayed hydrogen bonding interaction with Glh698 and Met696. Beside this, docking pose of compound **4i** showed hydrophobic interaction with Met1169, Ile1167, Val1144 and Cys1191. Docking studies of these compounds on DNMT1 protein suggest that DNMT1 inhibition could be the possible mechanism of action for these compounds. The binding pose for molecule **4i** in DNMT1 protein is shown in Fig. 7.

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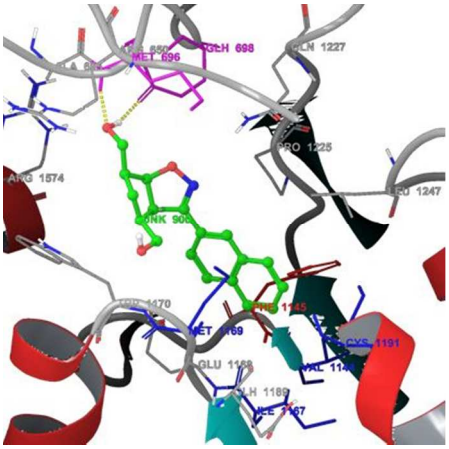

Fig.7 Top view of 3-D image showing interactions of compound **4i** with human DNMT1 protein (PDB ID: 3PTA).

Conclusion

In the present study, a series of Δ^2-isoxazoline fused cyclopentane analogs were synthesized and evaluated for their *in vitro* antimicrobial and anticancer potentials. From the results of the tested compounds, compounds **4b, 4h, 4i, 5d** and **5g** displayed promising antimicrobial activity with reference to standard drugs Ampicillin, Gentamycin and Amphotericin B respectively. Interstingly, the compounds **4b** and **4h** were shown to be non-cytotoxic towards cancer and normal cell lines, demonstrated the potential candidates for antimicrobials. In preliminary MTT cytotoxicity studies, compound **4i** was found to be equipotent to the standard drug Etoposide against MCF-7 cancer cell line. The cell cycle analysis revealed that the compound **4i** arrested the MCF-7 cell cycle at S phase in a dose-dependent manner. Furthermore, Acridine orange/ethidium bromide staining, annexin V binding assay and mitochondrial membrane potential studies evidenced that compound **4i** inhibit the cancer cell growth in MCF-7 through apoptosis. Docking studies of **4i** on DNMT1 protein suggested that DNMT1 inhibition could be the possible mechanism for the cytotoxic activity of these compounds. These preliminary results encourage further investigation on synthesized compounds aiming to the development of new potential bioactive agents.

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Notes and references

[a]Department of Medicinal Chemistry, National Institute of Pharmaceutical Education and Research (NIPER), Balanagar, Hyderabad 500037, India.
[b]Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER), Balanagar, Hyderabad 500037, India.
[c]Department of Pharmaceutical Technology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, S. A. S. Nagar, Punjab 160062, India.

The following supplementary data are available: synthetic procedure and characterization data of synthesized compounds; procedure for *in vitro* biological activity of antimicrobial and anticancer.

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