Bioorganic Chemistry 87 (2019) 484–494

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Synthesis and biological evaluation of new bisindole-imidazopyridine hybrids as apoptosis inducers

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ARTICLE INFO

Keywords:
Bisindole-imidazopyridine
Heterocyclic
ROS
Cytotoxicity
Apoptosis

ABSTRACT

A series of diindolylmethanes (**5a-t**) were designed, synthesized, and examined for their cytotoxicity against four human cancer cell lines like prostate (DU-145), lung (A549), breast (MCF-7) and cervical cancer (HeLa). These results revealed that among all the hybrids, two (**5k** and **5r**) were identified and exhibited significant cytotoxic effect against A549 cancer cells with IC_{50} values of 1.65 ± 0.3 and 1.80 ± 0.8 μM respectively. To investigate the reasons for the cytotoxic activity, the conventional biological assays were carried out with **5k** and **5r** on the A549 cancer cells. Both hybrids led to the arrest of A549 cell lines at the G2/M phase of the cell cycle and strongly induced apoptosis. Further the apoptotic effects of **5k** and **5r** were confirmed by ROS, annexin-V FITC, and mitochondrial membrane potential. Moreover, structure–activity relationships were elucidated with various substitutions on these hybrids.

1. Introduction

Indoles and their derivatives are well known as an important class of heterocyclic compounds in many distinct areas of biology and medicinal chemistry, probably one of the most important frameworks in drug discovery [1,2]. Bisindole nucleus is present in many natural products isolated from marine sources with broad spectrum of biological activities [3]. Interestingly the bisindoles are active metabolites of indole containing natural products responsible for their anticancer activity. Indole-3-carbinol (I3C, **1**) is an ingredient of cruciferous vegetables known for suppressing the proliferation and induces apoptosis in various cancer cells [4,5]. In many early-phase clinical trial studies indicated that I3C works like a prodrug, which forms active metabolites in the stomach [6,7], which are the acid condensation products of I3C usually referred to as oligomer, that exerts *in vivo* antitumor effects. Among the 20 oligomers namely diindolylmethane (DIM, **2**), is the major oligomer that reduces prostate tumor growth in mice [8]. Various anticancer bisindole derivatives of DIM have been developed by different research groups across the globe. Further investigations showed that diindolylmethane is a strong mitochondrial Hþ-*α*TPase inhibitor and stimulates mitochondrial reactive oxygen species (ROS) production [9]. Thus DIM could be considered an important class of anticancer chemotherapeutic lead that may provide a number of medicinally useful compounds.

On the other hand, nitrogen-bridgehead fused heterocycles containing an imidazole ring are common structural components in several pharmacologically important molecules that display a wide range of activities for diverse number of targets. One of the most widely used heterocyclic systems from this research group is the imidazopyridines [10], which shows a broad spectrum of biological activities such as inhibitors of aromatase, estrogen production suppressors [11], positive inotropic agents [12], platelet aggregation inhibitors and thromboxane synthetase inhibitors [13]. Imidazopyridines exhibit cytotoxic activity through different molecular mechanisms such as vascular endothelial growth factor (VEGF)-receptor KDR (kinase insert domain receptor) inhibition and the induction of apoptosis [14,15]. Recently, we have reported that the synthesis of imidazopyridine-oxindole (**3**) [16] and imidazopyridine-benzimidazole derivatives (**4**) [17,18] as potential

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https://doi.org/10.1016/j.bioorg.2019.03.061
Received 22 June 2018; Received in revised form 19 February 2019; Accepted 21 March 2019
Available online 22 March 2019

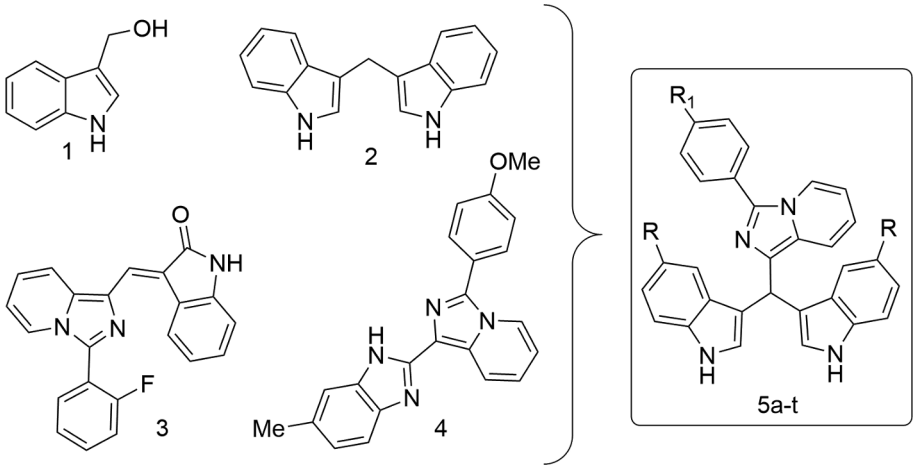


Fig. 1. Lead structure of biologically active antitumor agents. (**1**, Carbinol), (**2**, Dimethylindole), and imidazopyridine containing scaffolds (**3**, **4,** and **5a-t**).

anticancer agents.

As a part of our continuous research to develop potent anticancer agents and considering the diverse chemotherapeutic potential of bisindole and imidazopyridine scaffolds, we have planned to explore the potential beneficial effects of incorporating both the scaffolds in a single structure. Thus, a new hybrid has been designed by combining both these active scaffolds as shown in Fig. 1. The present work describes the synthesis of a series of bisindole-imidazopyridine hybrids (**5a-t**) and their evaluation for cytotoxic activity apart from their effect of apoptotic cell death.

2. Results and discussions

2.1. Chemistry

Synthesis of the bisindole-imidazopyridine hybrids (**5a-t)** was accomplished as described in Scheme 1. The intermediates *N*1-(2-pyridylmethyl)-substituted benzamides (**8a-e)** were prepared by the reaction of different substituted benzoyl chlorides (**7a-e**) and 2-pyridylmethanamine (**6**) in the presence of triethylamine. The cyclization of *N*1-(2-pyridylmethyl)-substituted benzamides (**8a-e)** in $POCl_3$ gave phenyl substituted imidazo[1,5-*a*]pyridines (**9a-e**) in good yields, which were subjected to Vilsimier-formylation with $POCl_3$ in dry DMF to give 3-(substituted phenyl)imidazo[1,5-*a*]pyridine-1-carbaldehyde (**10a-e**). The reaction between (**10a-e**) and substituted indoles (**11a-d**) in ethanol using sulfamic acid afforded thebisindole-imidazopyridine hybrids (**5a-t**) in excellent yields.

2.2. Biology

2.2.1. Cytotoxic activity

All the synthesized bisindole-imidazopyridine hybrids (**5a-t)** were evaluated for their antiproliferative activity against four human tumor cell lines: DU-145 (prostate cancer), A549 (lung cancer), MCF (breast cancer), and HeLa (cervical cancer) employing MTT assay [19]. DIM was used as the reference standard and the results obtained are shown in Table 1. Interestingly but not surprisingly, all the compounds (**5a-t**) showed more pronounced activity against A549 cells compared to other cancer cell lines tested. These conjugates displayed cytotoxic activity comparable to the standard DIM.

Based on the results obtained from the cytotoxicity assay, the most cytotoxic conjugates (**5k** and **5r**) were taken up for the detailed biological studies, such as cell cycle analysis, reactive oxygen species (ROS) generation, apoptosis determination, and measurement of mitochondrial membrane potential.

2.2.2. Cell cycle analysis

Many cytotoxic compounds exert their effect by blocking cell cycle progression or by inducing apoptosis or combined effect of both [20,21]. In order to understand the mode of action of these potential conjugates such as (**5k** and **5r**), we examined their effects on cell cycle progression by flow cytometry analysis on A549 cancer cells. In this study, A549 cells were treated with mentioned conjugates at concentrations of 1.5 and 3 μM for 24 h. The data obtained clearly indicates that DIM, conjugates **5k** and **5r** are altering the cell cycle progression profoundly. The compounds **5k** at 1.5 μM and 3 μM shows 18.11% and 21.43% in G2/M phase. Where in **5r** has shown 18.58% and 23.91% in G2/M phase arrest at 1.5 μM and 3 μM respectively. Whereas DIM showed 24.27% arrest in G2/M at 5 μM concentration. Moreover, percentage distribution of cells in various phases of the cell cycle is indicated by the bar graph (Fig. 2).

2.2.3. Measurement of reactive oxygen species (ROS)

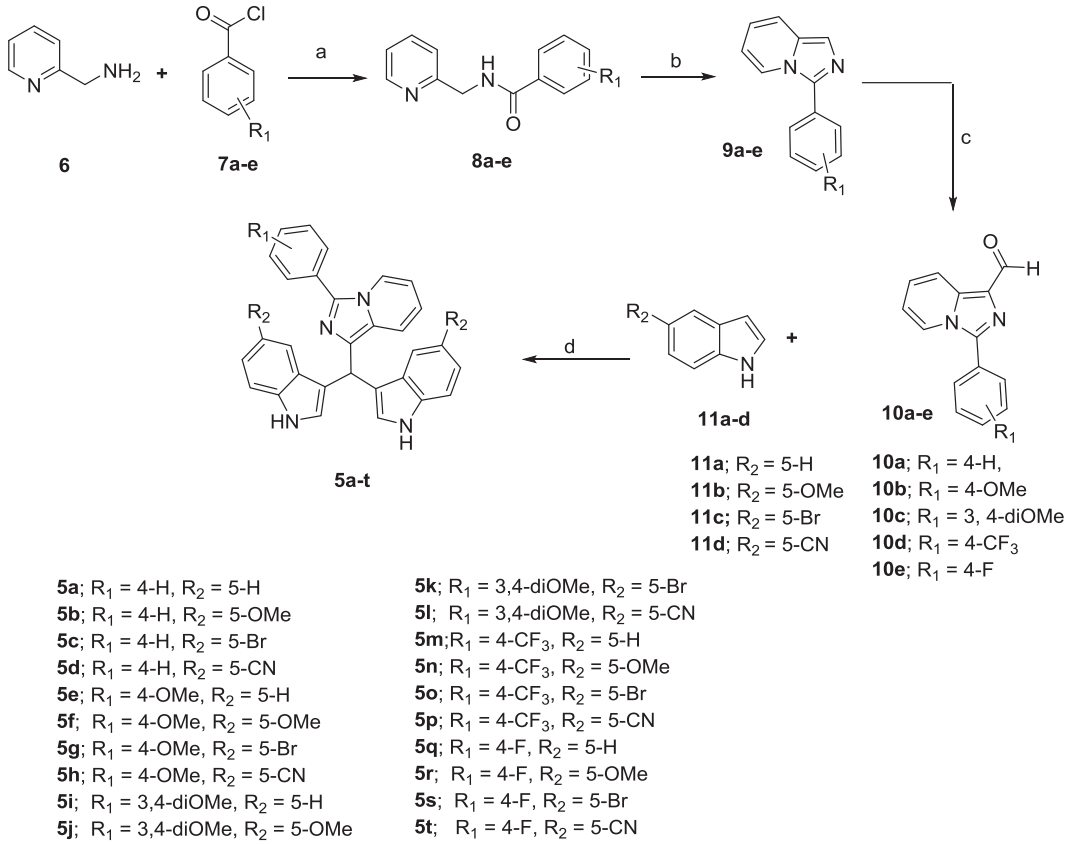
Various categories of chemotherapeutic drugs, either natural or synthetic induce ROS signals, which is an important strategy in drug development studies. Accumulation of ROS in the A549 cells treated with DIM, **5k**, and **5r** for 24 h was estimated by DCFDA. 2′, 7′– dichlorofluorescindiacetate a non-fluorescent substance and is converted to 2′, 7′–dichlorofluorescein (DCF) inside the cells through oxidation by ROS. Upon treatment with **5 k** and **5r** at indicated concentrations increased ROS production was observed in a dose dependent manner (Fig. 3). However, this is not comparable to DIM.

2.2.4. Determination of apoptosis by using annexin V-FITC/PI

Quantification of apoptosis induced by DIM, **5k**, and **5r** was determined by using annexin V-FITC/PI staining. This assay provides the extent of live, early apoptotic, late apoptotic, and necrotic cells as shown in Fig. 4. The percentage of early apoptotic cells in the **5k** and **5r** increased at 1.5 μM (26.13% and 37.93%) to 3 μM (28.05% and 48.77%), whereas DIM has shown 24.56% at 5 μM. Therefore, these results prove that the conjugates **5k** and **5r** induce apoptosis in a dose dependent manner.

2.2.5. Measurement of mitochondria membrane potential (ΔΨm)

Mitochondria membrane potential (ΔΨm) was determined by using JC-1 dye. Intact mitochondrion with energized membrane potential shows red aggregates in presence of JC-1 dye. Potent apoptotic inducers manifest green fluorescence with JC-1 dye by losing the membrane potential as de-energized. Cells treated with DIM, **5k**, and **5r** showed the increased green fluorescence as compared to untreated cells (Fig. 5).



Scheme 1. Synthesis of Imidazo[1,5-*a*]pyridine-bisindole conjugates (**5a-t**). Reagents and Conditions: (a) TEA, dry THF, 0 °C to rt, 3 h; (b) $POCl_3$, reflux, 3 h; (c) DMF, $POCl_3$, 0 °C, 3 h; (d) EtOH, Cat. Sulfamic acid, rt, 8 h.

Table 1
Cytotoxicity of bisindole-imidazopyridine hybrids (**5a-t**) against a panel of human cancer cell lines.

Compound	DU-145[b]	A549[c]	MCF-7[d]	HeLa[e]
5a	22.4 ± 1.8	5.1 ± 1.2	22.2 ± 1.1	16.7 ± 1.8
5b	9.8 ± 0.6	3.7 ± 0.89	9.7 ± 0.06	8.8 ± 0.3
5c	8.9 ± 0.7	5.5 ± 0.04	19.7 ± 1.4	15.2 ± 1.3
5d	12.9 ± 0.2	6.9 ± 0.2	16.8 ± 0.1	11.6 ± 1.3
5e	10.0 ± 0.3	10.5 ± 0.4	12.8 ± 0.5	11.8 ± 0.02
5f	13.0 ± 1.3	10.8 ± 0.6	15.5 ± 0.04	11.4 ± 6.7
5g	11.8 ± 0.14	9.5 ± 0.7	14.7 ± 0.5	11.2 ± 1.4
5h	13.5 ± 0.7	11.7 ± 1.1	16.5 ± 0.9	23.7 ± 1.7
5i	9.4 ± 0.7	11.9 ± 0.5	12.4 ± 1.07	11.7 ± 0.05
5j	11.4 ± 0.6	11.5 ± 0.2	16.8 ± 0.9	15.3 ± 0.3
5k	**4.7 ± 0.8**	**1.65 ± 0.3**	**2.1 ± 0.5**	**6.7 ± 1.5**
5l	9.3 ± 0.7	10.9 ± 0.1	15.7 ± 1.2	10.9 ± 0.07
5m	13.8 ± 0.4	16.6 ± 0.6	18.9 ± 0.8	13.9 ± 0.6
5n	9.5 ± 0.3	3.9 ± 0.2	12.7 ± 0.4	13.0 ± 3.2
5o	10.8 ± 0.8	2.0 ± 0.2	11.6 ± 0.3	12.6 ± 1.5
5p	10.6 ± 0.8	3.2 ± 0.4	11.9 ± 0.5	13.0 ± 0.5
5q	15.1 ± 0.6	11.6 ± 0.9	10.2 ± 1.1	11.1 ± 0.5
5r	**7.9 ± 1.6**	**1.80 ± 0.8**	**2.3 ± 0.2**	**7.2 ± 2.0**
5s	17.8 ± 0.4	12.3 ± 0.2	15.5 ± 4.2	13.2 ± 0.7
5t	37.9 ± 1.2	13.2 ± 1.5	16.3 ± 0.7	17.2 ± 0.01
DIM	7.8 ± 0.12	4.9 ± 0.08	5.3 ± 1.88	10.8 ± 0.18

[a]50% Inhibitory concentration after 48 h of drug treatment.
[b] Human prostate cancer.
[c] Human lung cancer.
[d] Human breast cancer.
[e] Human cervical cancer. Results are represented as mean ± SD of three independent experiments, each performed in triplicates.

2.3. Structure-activity relationship

The structure-activity relationship (SAR) is established based on cytotoxic results against four different cell lines (Table 1). The effect of various groups on the imidazopyridine ring as well as indole ring were exemplified by preparing analogs possessing both electron donating as well as electron withdrawing substituents viz. methoxy, dimethoxy, bromo, cyano, fluoro, and trifluoromethyl groups. It was observed that hybrids with either unsubstituted or substituted imdiazopyridine (**5a-5t**) showed weaker potency against all the cancer cell lines expect few hybrids such as **5b, 5k, 5n, 5o, 5p,** and **5r** which are active against A549 cell line. Methoxy substituent on the imidazopyridine ring (**5e-h**) showed significant loss in potency against all the cell lines, comparably better activity was observed with unsubstituted hybrids (**5a-d**) against A549 cell lines. Introduction of an additional methoxy group on the imidazopyridine ring (**5i-l**) also resulted in no improvement in the potency, except **5k** which dramatically increased the potency in all the cell lines especially in A549 by **5k** with IC_{50} value with 1.65 μM. Moreover, introduction of strong electron withdrawing substituent like trifluoromethyl (**5m-5p**) and fluoro (**5q-5t**) on the imidazopyridine ring also did not improve the potency against all the cancer cell lines except A549 cell line where compounds **5n, 5o, 5p** and **5r** showed significant cytotoxicity with values ranging from 1.8 to 3.9 μM. However, for MCF-7 cell line, fluoro substituent increased the cytotoxicity activities of the hybrids. In addition, we further examined the effect of various groups such as methoxy, bromo and cyano on the indole ring at the 5-position. Unsubstituted indole ring containing hybrids (**5a, 5e, 5i, 5m,** and **5q**) showed decreased activity against all the cell lines evaluated. Electron-releasing methoxy group on the indole ring (**5b, 5f, 5j,**

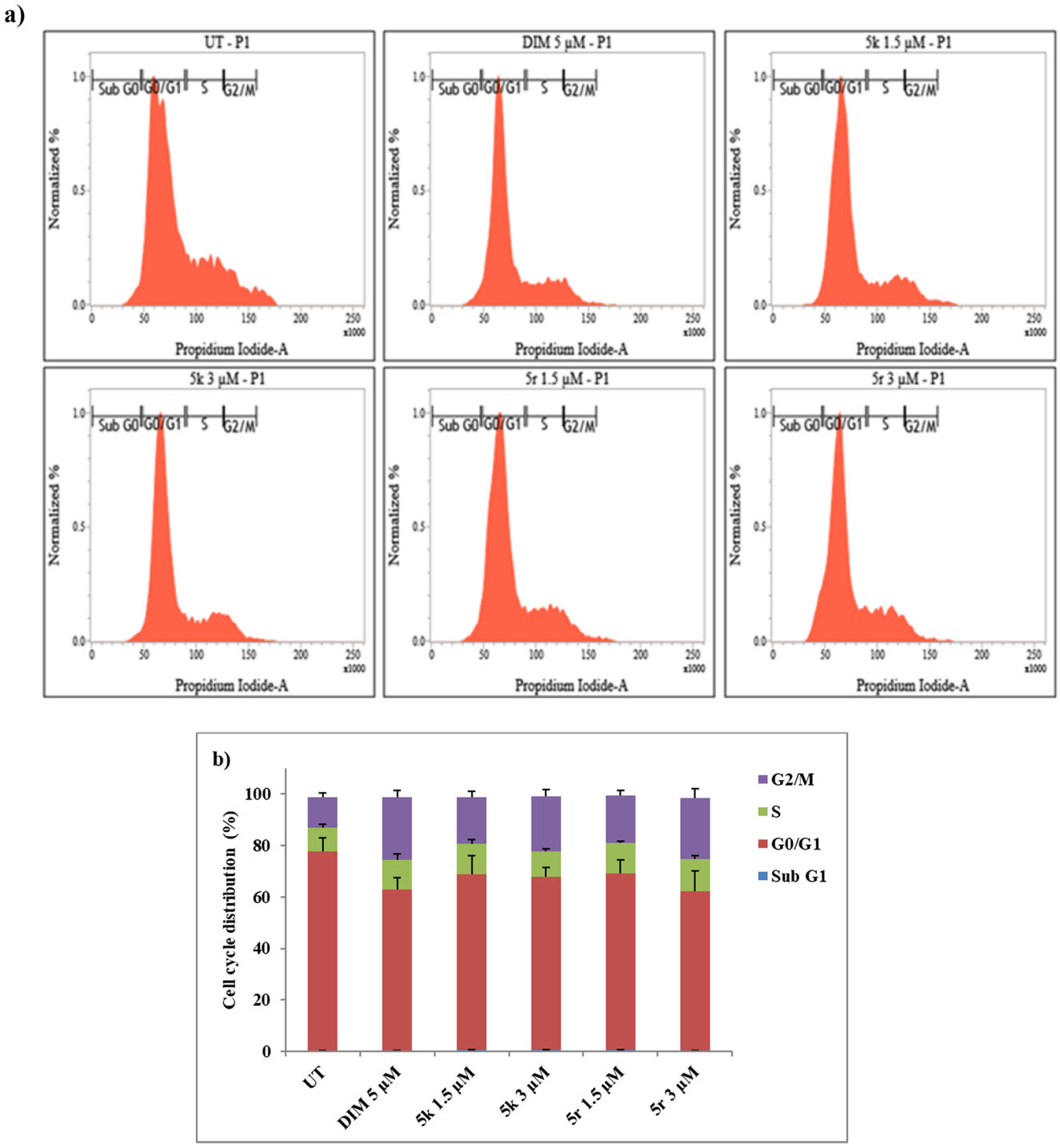


Fig. 2. Cell cycle analysis. A549 cells were treated without or with **DIM**, **5k,** and **5r** at indicated concentrations for 24 h. DNA content in each phase was measured by propidium iodide (PI) staining. Data was represented as histograms (a) and bar graphs (b). Error bars are indicative of mean ± SD of two independent experiments.

5n, and **5r**) substantially altered the potency. Particularly, hybrids **5b, 5n,** and **5r** resulted in a 4 to 6-fold increase in antiproferative activity against A549 cell lines. Among which, 5r is the potent most hybrid with IC50 values ranging from 1.65 to 6.7 µM against all the cell lines. Electron-withdrawing substituents such as bromo and cyano have no substantial effect on the cytotoxicity, where bromo substituted hybrids (**5c, 5g, 5k, 5o,** and **5s**) were comparably more potent than the corresponding cyano substituentuted hybrids (**5d, 5h, 5l, 5p,** and **5t**) in A549 cell lines. Among all the synthesized hybrids, **5k** and **5r** were the potent most compounds against all the cell lines, particularly against A549 cell lines.

3. Conclusion

A series of diindolylmethanes (**5a-t**) were designed, synthesized using sulfhamic acid and evaluated for their cytotoxicity against three human cancer cell lines like prostate (DU-145), lung (A549), breast (MCF-7) and cervical cancer (HeLa) with IC_{50} values ranging from 1.6

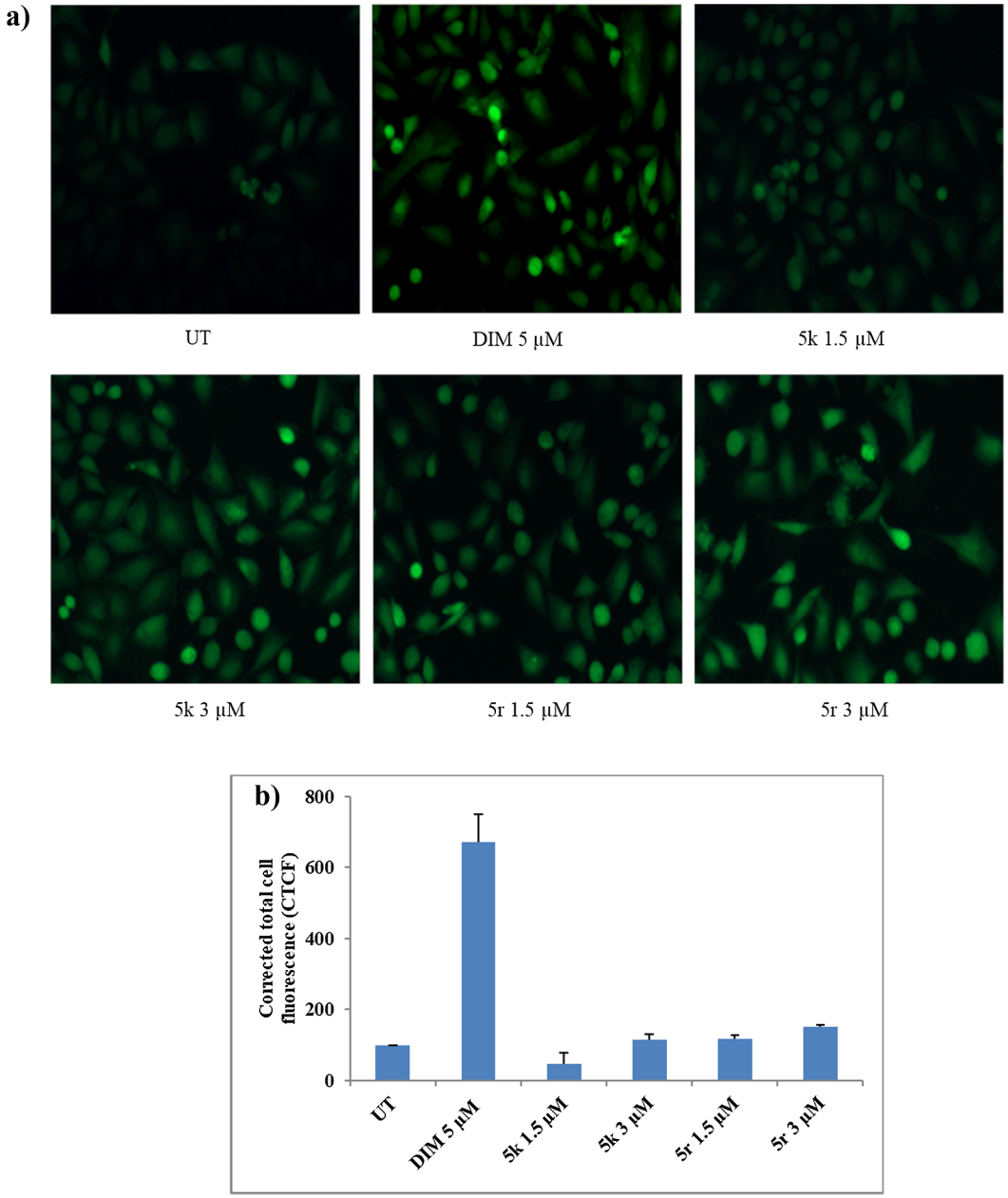


Fig. 3. Determination of ROS generation in A549. Fluorescent images are obtained from the cells treated without or with the DIM**, 5k,** and **5r** for 24 h and then observed the production of ROS by DCFDA (a). Bar graph represents the mean fluorescence intensity of corrected total cell fluorescence (CTCF) (b). Error bars are indicative of mean ± SD of two independent experiments, each performed in triplicates.

to 22.4 µM. Among them, two (**5k** and **5r**) were identified and exhibited significant cytotoxic effect against A549 cancer cells with IC_{50} values of 1.65 ± 0.3 and 1.80 ± 0.8 µM respectively. To investigate the reasons for the cytotoxic activity, the conventional biological assays were carried out with **5k** and **5r** on the A549 cancer cells. Both hybrids led to the arrest of A549 cell lines at the G2/M phase of the cell cycle and strongly induced. Further the apoptotic effects of **5k** and **5r** were confirmed by ROS, annexin-V FITC, and mitochondrial membrane potential. Moreover, structure–activity relationships were elucidated with various substitutions on these hybrids. Detailed biological studies like ROS, annexin-V FITC and mitochondrial membrane potential demonstrate that these hybrids induce apoptotic cell death. These results suggest that such hybrids have the potential to be developed as new template for the potential anticancer agents.

4. Material and methods

4.1. Chemistry

All reagent, starting materials, and solvents were purchased from commercial sources and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded with 300, 400, and 500 MHz spectrometer in $CDCl_3$, DMSO-d_6 solutions with tetramethylsilane (TMS) as an internal standard. High-resolution mass spectra (ESIHRMS) were obtained by using ESI-QTOF mass spectrometer. All reactions were monitored by thin-layer chromatography (TLC) using precoated silica gel 60 F254 Merck and components were visualized by exposure to UV light. Column chromatography was performed using silica gel (60–120 mesh). Melting points were determined on an electro thermal melting point apparatus and are uncorrected.

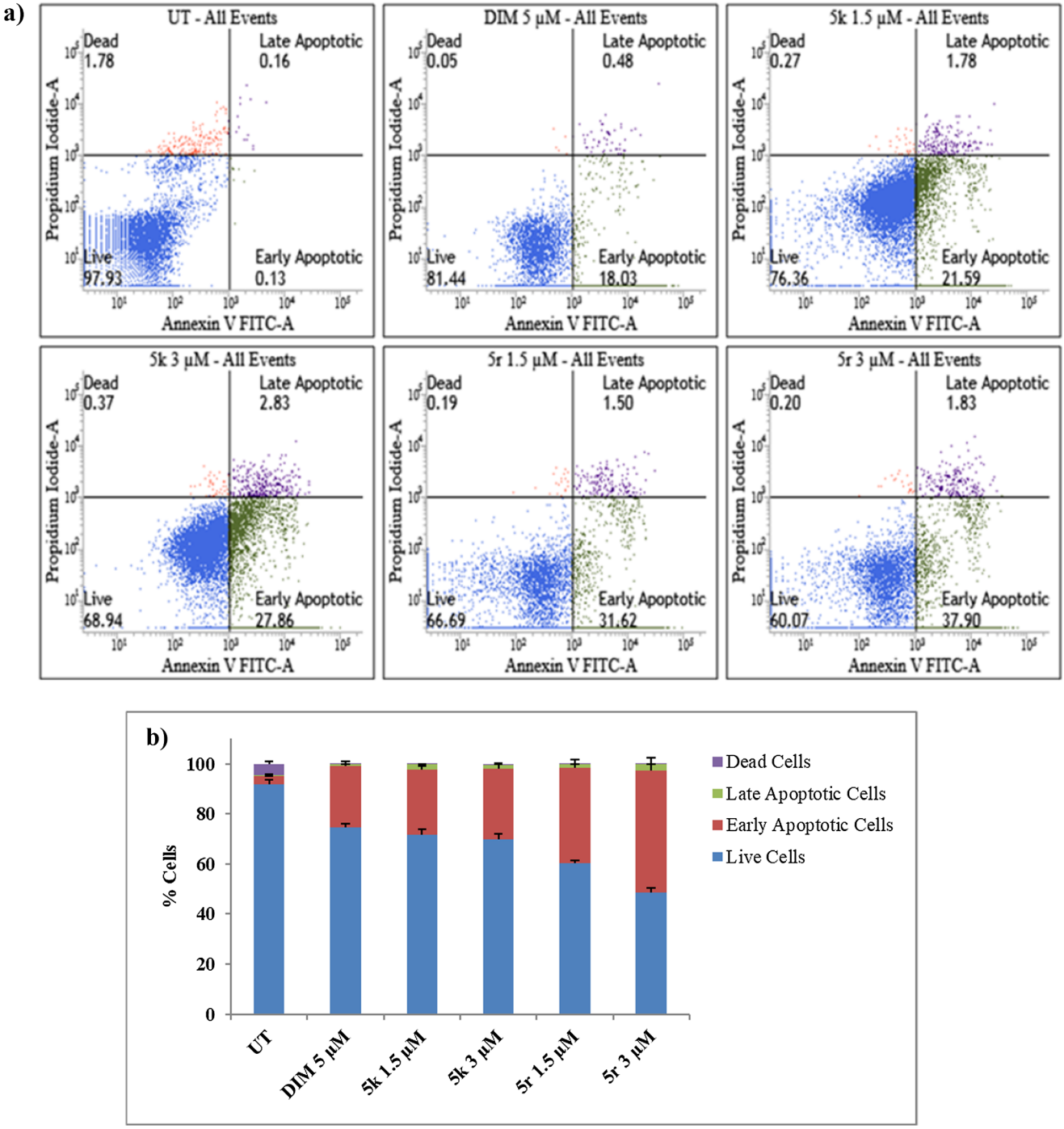


Fig. 4. Compounds induce apoptosis in A549 cells. Cells were treated without or with **DIM**, **5k,** and **5r** for 24 h. Cells were labelled with Annexin V FITC, PI and analyzed by flow cytometry. Percentage of cells (a) in each phase is represented in the bar graph (b). Error bars are indicative of mean ± SD of two independent experiments.

General procedure for the synthesis of *N*1-(2-pyridylmethyl) substituted benzamides (8a-e) Method A: To a stirred solution of 2-pyridylmethanamine 6 (1 mmol) in dry THF was added triethylamine (3.0 mmol) followed by substituted benzoylchlorides (7a-e, 1.1 mmol) at 0 °C. The reaction mixture was stirred for 3 h and the reaction was monitored by TLC. After the completion of the reaction, THF was removed under vacuum to get the crude products. This was further purified by column chromatography (EtOAc-Hexane) to obtain the pure compounds (8a-e).

***N*1-(2-Pyridylmethyl) benzamide (8a):** This compound was prepared according to the method A, employing benzoylchlorides 7a (1 mmol) to obtain the pure product 8a. Yield: 76%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.53 (d, J = 4.7 Hz, 1H), 7.88 (d, J = 6.4 Hz, 2H), 7.67 (t, J = 7.7 Hz, 2H), 7.47–7.39 (m, 3H), 7.32 (d, J = 7.9 Hz, 1H), 7.20 (t, J = 5.0 Hz, 1H), 4.73 (d, J = 4.7 Hz, 2H); MS (ESI): *m/z* 213 $[M + H]^+$.

***N*1-(2-Pyridylmethyl)-4-methoxybenzamide (8b):** This compound was prepared according to the method A, employing 4-methoxy benzoylchloride 7b (1 mmol) to obtain the pure product 8b. Yield: 82%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.56 (d, J = 4.8 Hz, 1H), 7.83 (d, J = 8.7 Hz, 2H), 7.68 (t, J = 7.8 Hz, 1H), 7.47 (brs, 1H), 7.32 (d, J = 7.8 Hz, 1H), 7.21 (t, J = 5.8 Hz, 1H), 6.93 (d, J = 8.7 Hz, 2H), 4.75

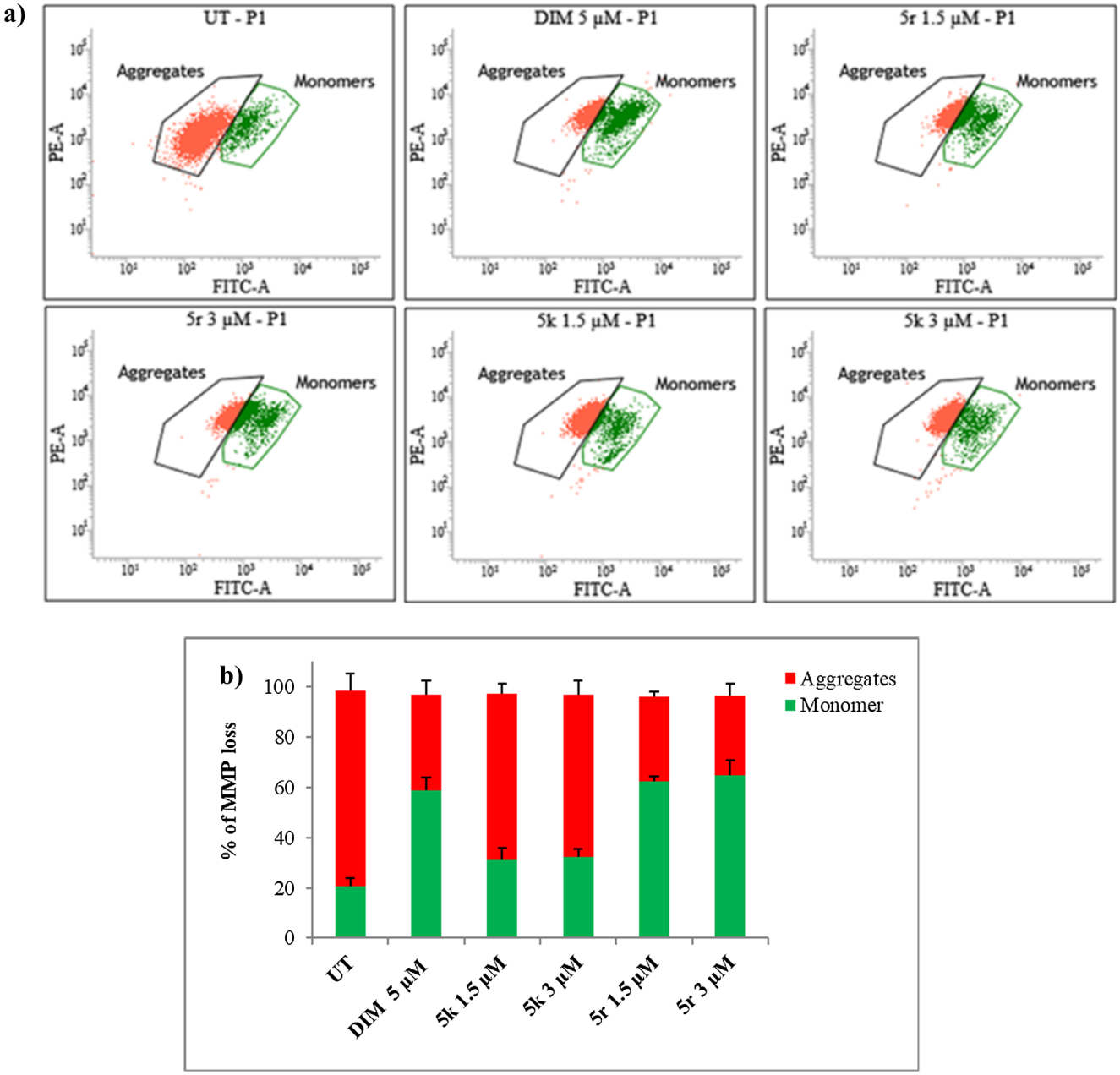


Fig. 5. Compounds induce mitochondrial membrane potential in A549 cells. (a) Cells were treated without or with DIM**, 5k,** and **5r** for 24 h and observed the changes in mitochondrial membrane potential by flow cytometry. (b) Bar graph represents the percentage distribution of J-*a*ggregates and J-monomer cells in all groups. Error bars are indicative of mean ± SD of two independent experiments.

(d, J = 4.8 Hz, 2H), 3.85 (s, 3H); MS (ESI): m/z 243 $[M + H]^+$.

***N*1-(2-Pyridylmethyl)-3,4-dimethoxybenzamide (8c):** Compound was prepared according to the method A, employing 3,4-dimethoxy benzoylchloride 7c (1 mmol) to obtain the pure product 8c Yield 78%, ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.52 (d, J = 4.5 Hz, 1H), 7.70–7.62 (m, 2H), 7.45 (d, J = 2.2 Hz, 1H), 7.39–7.31 (m, 2H), 7.20 (dd, J = 6.0, 5.2 Hz, 1H), 6.82 (d, J = 8.3 Hz, 1H), 4.70 (d, J = 5.2 Hz, 2H), 3.92 (s, 3H), 3.90 (s, 3H); MS (ESI): m/z 273 $[M + H]^+$.

***N*-(Pyridin-2-ylmethyl)-4-(trifluoromethyl)benzamide (8d):** This compound was prepared according to the method A, employing 4-trifluoromethyl benzoylchloride 7c (1 mmol) to obtain the pure product 8c. Yield: 77%; ^{1}H NMR (300 MHz,$CDCl_3$) δ 8.51 (d, J = 5.2 Hz, 1H), 8.15 (brs, 1H), 7.96 (d, J = 7.5 Hz, 2H), 7.63 (d, J = 8.3 Hz, 2H), 7.30 (d, J = 7.5 Hz, 1H), 7.19(q, J = 5.2, 1.5 Hz, 1H), 4.71 (d, J = 4.5 Hz, 2H); MS (ESI): m/z281 $[M + H]^+$.

***N*1-(2-Pyridylmethyl)-4-fluorobenzamide (8e)**: This compound was prepared according to the method A, employing 4-fluoro benzoylchloride Yield 75%, ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.55 (d, J = 4.6 Hz, 1H), 8.00 (brs,1H), 7.72 (d, J = 8.1 Hz, 2H), 7.53 (t, J = 6.2, 6.0 Hz, 1H), 7.40 (d, J = 8.1 Hz, 2H), 7.25 (m, 1H), 7.2 (dd, J = 6.2,5.6 Hz, 1H), 4.71 (d, J = 5.0 Hz, 2H); MS (ESI): m/z 231 $[M + H]^+$.

General procedure for the synthesis of substitutedphenylimidazo[1,5-*a*] pyridine (9a–e) Method B: To *N*1-(2-pyridylmethyl)-substituted benzamides (8a-e) (1 mmol), 4 mL of $POCl_3$ was added and refluxed for 3 h. This was poured into cold water and neutralized with $NaHCO_3$ solution. This water layer was extracted three times with ethylacetate. The combined organic phases were dried over anhydrous Na_2SO_4 and evaporated under vacuum. Thus, the residue obtained was purified by column chromatography using ethylacetate and hexane as solvent system to afford the pure compounds (9a-e).

3-Phenylimidazo[1,5-*a*] pyridine (9a): This compound was

prepared according to the method B. Yield: 78%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.24 (d, J = 7.5 Hz, 1H), 7.76 (d, J = 6.7 Hz, 2H), 7.50–7.36 (m, 5H), 6.67 (dd, J = 9.0, 6.7 Hz, 1H), 6.51 (t, J = 6.7 Hz, 1H); MS (ESI): m/z 195 $[M + H]^+$.

3-(4-Methoxyphenyl)imidazo [1,5-*a*] pyridine (9b): This compound was prepared according to the method B. Yield: 80%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.34 (d, J = 8.7 Hz, 1H), 8.32 (d, J = 6.8 Hz, 1H), 7.71 (d, J = 8.7 Hz, 2H), 7.32–7.17 (m, 1H), 7.07 (d, J = 8.7 Hz, 2H), 6.98 (s, 1H), 6.88 (t, J = 6.8 Hz, 1H), 3.90 (s, 3H); MS (ESI): m/z 225 $[M + H]^+$.

3-(3,4-Dimethoxyphenyl)imidazo [1,5-*a*] pyridine (9c): Yield 76%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.18 (d, J = 7.2 Hz, 1H), 7.47 (s, 1H), 7.40 (d, J = 9.3 Hz, 1H), 7.30 (s, 1H), 7.27 (d, J = 8.3 Hz, 1H), 6.93 (d, J = 7.2 Hz, 1H), 6.64 (dd, J = 7.2, 6.2 Hz, 1H), 6.49 (t, J = 7.2, 6.2 Hz, 1H), 3.90 (s, 3H), 3.87 (s, 3H); MS (ESI): m/z 255 $[M + H]^+$.

3-(4-(Trifluoromethyl)phenyl)imidazo [1,5-*a*] pyridine (9d): This compound was prepared according to the method B. Yield: 78%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.28 (d, J = 7.5 Hz, 1H), 7.91 (d, J = 8.3 Hz, 2H), 7.73 (d, J = 8.3 Hz, 2H), 7.54 (s, 1H), 7.47 (d, J = 9.0 Hz, 1H), 6.73 (q, J = 6.7, 2.2 Hz, 1H), 6.59 (t, J = 6.0 Hz, 1H); MS (ESI): m/z 263 $[M + H]^+$.

3-(4-Fluorophenyl)imidazo [1,5-*a*] pyridine (9e): Yield 85%, ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.16 (d, J = 7.5 Hz, 1H), 7.75 (dd, J = 9.0, 5.2 Hz, 2H), 7.49 (s, 1H), 7.47 (d, J = 9.0 Hz, 1H), 7.26–7.17 (m, 2H), 6.69 (dd, J = 9.0, 6.7 Hz, 1H), 6.53 (t, J = 6.7, 6.0 Hz, 1H); MS (ESI): m/z 213 $[M + H]^+$.

General procedure for the synthesis of 3-(substituted phenyl)-imidazo [1,5-*a*] pyridine-1-carbaldehydes (10a-e) Method C: To an ice-water cooled solution of substituted phenyl imidazo[1,5-*a*]pyridines (9a-c) (1 mmol) in DMF(1.4 mmol) was added drop wise $POCl_3$ (1.4 mmol)with stirring, and then reaction mixture was heated to 100 °C for 2 h. After the completion of the reaction, the reaction mixture was poured into ice water and quenched with ammonium hydroxide solution. The aqueous solution was extracted with ethylacetate and the resultant organic layer was dried over an hydroussodium sulphate and evaporated under vacuum. Thus, the residue obtained was purified by column chromatography using ethylacetate and hexane as solvent system to afford pure compounds (10a-e).

3-Phenylimidazo [1,5-*a*] pyridine-1-carbaldehyde (10a): This compound was prepared according to the method C. Yield:80%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.10 (s, 1H), 8.39 (d, J = 8.3 Hz, 2H), 7.77 (d, J = 6.6 Hz, 2H), 7.58–7.46 (m, 3H), 7.22(dd, J = 9.4, 6.7 Hz, 1H), 6.86 (t, J = 6.7 Hz, 1H); MS (ESI): m/z223 $[M + H]^+$.

3-(4-Methoxyphenyl)imidazo [1,5-*a*] pyridine-1-carbaldehyde (10b): This compound was prepared according to the method C. Yield: 76%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.16 (s, 1H), 8.34(d, J = 8.7 Hz, 2H), 8.31 (d, J = 6.8 Hz, 1H), 7.26–7.23 (dd, J = 8.7 Hz, 2H), 7.07 (d, J = 8.7 Hz, 2H), 6.88 (t, J = 7.8 Hz, 1H),3.90 (s, 3H); MS (ESI): m/z 253 $[M + H]^+$.

3-(3,4-dimethoxyphenyl)imidazo [1,5-*a*] pyridine-1-carbaldehyde (10c): This compound was prepared according to the method C. Yield 82%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.12 (s, 1H), 8.35 (d, J = 5.6 Hz, 2H), 7.30 (s, 1H), 7.25 (s, 1H), 7.21 (t, J = 7.2, 6.4 Hz, 1H), 6.96 (d, J = 8.0 Hz, 1H), 6.84 (t, J = 7.2, 6.2 Hz, 1H), 3.97 (s, 3H), 3.95 (s, 3H); MS (ESI): m/z 283 $[M + H]^+$.

3-(4-(Trifluoromethyl)phenyl)imidazo [1,5-*a*] pyridine-1-carbaldehyde (10d): This compound was prepared according to the method C. Yield: 80%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.16(s, 1H), 8.42–8.38 (m, 2H), 7.95 (d, J = 8.3 Hz, 2H), 7.83 (d, J = 8.3 Hz, 2H), 7.34–7.29 (q, J = 6.0, 3.0 Hz, 1H), 6.97 (t, J = 7.5 Hz, 1H); MS (ESI): m/z 291 $[M + H]^+$.

3-(4-(Fluoro)phenyl)imidazo [1,5-*a*] pyridine-1-carbaldehyde (10e): This compound was prepared according to the method C. Yield: 80%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.17 (s, 1H), 8.37 (dt, J = 9.1, 1.1 Hz, 1H), 8.31 (dt, J = 7.1, 1.0 Hz, 1H), 7.82–7.77 (m, 2H), 7.33–7.24 (m, 3H), 6.96–6.89 (m, 1H); MS (ESI): m/z 241 $[M + H]^+$.

General procedure for the synthesis of compounds (5a-t) Method D: To a solution appropriate aldehyde (10a-e) in ethanol (10 mL), appropriate indole substituents (11a-e) (2.5 mmol) and catalytic amount of sulfamic acid dissolved in H_2O (5 mL) were sequentially added, and then the reaction mixture was refluxed for 8 h. After the completion of reaction, solvent was removed by vacuum to obtain crude product, which was extracted with ethyl acetate. The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated to obtain crude products, which were further purified by column chromatography using ethylacetate and hexane as solvent system to afford pure compounds (5a-t). The purities of isolated products were determined by HPLC column Symmetry [R]-C18, 5 μM, 4.6 nm × 254 nm, 75% AcCN, 25% NH_4OAc, P^H-4.2 adjusted with formic acid.

Di(1*H*-indol-3-yl)methyl)-3-phenylimidazo [1,5-*a*] pyridine (5a):This compound was prepared according to the method D. Yield: 93%; R_f = 0.64 (ethyl acetate/*n*-hexane, 1:1); Melting point: 275–277 °C; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 10.19 (s, 1H), 8.20–8.08 (m, 1H), 7.71 (d, J = 7.3 Hz, 2H), 7.47–7.30 (m, 5H), 7.27 (d, J = 8.1 Hz, 2H), 7.22–7.14 (m, 1H), 6.96 (t, J = 7.7 Hz, 2H), 6.89 (s, 2H), 6.80 (t, J = 7.4 Hz, 2H), 6.47–6.38 (m, 2H), 6.20 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6) δ135.64, 134.90, 134.20, 129.23, 127.74, 127.14, 126.69, 126.43, 125.85, 122.52, 120.07, 119.86, 118.21, 117.68, 117.21, 116.51, 116.41, 111.89, 110.36, 32.64; HRMS (ESI) Calcd for $C_{30}H_{23}N_4[M + H]^+$ 439.1917, found 439.1912. Purity by HPLC UV (254 nm) ESI-MS: 97.30%.

1-(Bis(5-methoxy-1*H*-indol-3-yl)methyl)-3-phenylimidazo [1,5-*a*] pyridine (5b): This compound was prepared according to the method D. Yield: 92%; R_f = 0.42 (ethyl acetate/*n*-hexane, 1:1); Melting point: 280–282; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.59 (s, 2H), 8.39 (d, J = 7.2 Hz, 1H), 7.86–7.81 (m, 2H), 7.71 (d, J = 9.1 Hz, 1H), 7.53 (t, J = 7.3 Hz, 2H), 7.42 (t, J = 7.4 Hz, 1H), 7.22 (s, 1H), 7.20 (s, 1H), 7.18 (d, J = 2.3 Hz, 2H), 7.04 (d, J = 2.4 Hz, 2H), 6.72–6.68 (s, 1H), 6.67 (d, J = 2.4 Hz, 1H), 6.65 (d, J = 2.4 Hz, 1H), 6.62 (td, J = 7.3 1.2 Hz, 1H), 6.17 (s, 1H), 3.61 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 152.47, 135.51, 135.39, 131.50, 130.23, 128.85, 128.01, 127.27, 127.12, 127.01, 123.98, 121.55, 118.31, 117.68, 117.11, 113.15, 111.77, 110.40, 101.56, 55.11, 32.28; HRMS (ESI) Calcd for$C_{32}H_{27}N_4O_2[M + H]^+$ 499.2134, found 499.2202. Purity by HPLC UV (254 nm) ESI-MS: 96.22%.

1-(Bis(5-bromo-1*H*-indol-3-yl)methyl)-3-phenylimidazo [1,5-*a*] pyridine (5c):This compound was prepared according to the method D. Yield: 86%; R_f = 0.74 (ethyl acetate/*n*-hexane, 1:1); Melting point: 273–275; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6)δ 10.77 (s, 2H), 8.26 (d, J = 6.7 Hz, 1H), 8.04–7.95 (m, 1H), 7.78 (d, J = 7.7 Hz, 2H), 7.65 (s, 1H), 7.47 (t, J = 7.7 Hz, 3H), 7.36 (t, J = 7.4 Hz, 1H), 7.26–7.18 (m, 2H), 7.14–7.01 (m, 4H), 6.66–6.48 (m, 2H), 6.14 (s, 1H);'^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6)δ 136.16, 134.35, 133.73, 132.85, 128.71, 127.20, 126.87, 126.56, 126.05, 125.78, 123.30, 121.77, 120.24, 119.75, 116.64, 116.23, 115.43, 111.57, 111.49, 109.51, 31.21; HRMS (ESI) Calcd for $C_{30}H_{21}N_4Br_2[M + H]^+$ 595.0033, found 594.9945. Purity by HPLC UV (254 nm) ESI-MS: 87.07%.

3,3′-((3-Phenylimidazo [1,5-*a*] pyridin-1-yl)methylene)bis(1*H*-indole-5-carbonitrile) (5d):

This compound was prepared according to the method D. Yield: 84%; R_f = 0.25 (ethyl acetate/*n*-hexane, 1:1); Melting point: 278–280; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 11.41 (s, 2H), 8.39 (d, J = 7.1 Hz, 1H), 8.39 (s, 2H), 7.90 (d, J = 9.0 Hz, 1H), 7.84 (d, J = 7.4 Hz, 2H), 7.59–7.54 (m, 4H), 7.53–7.43 (m, 3H), 7.36 (d, J = 8.2 Hz, 2H), 6.80 (t, J = 6.6 Hz, 1H), 6.67 (t, J = 6.6 Hz, 1H), 6.46 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 138.03, 135.86, 128.91, 127.49, 127.42, 127.18, 126.25, 126.09, 125.97, 125.20, 125.14, 123.43, 121.72, 121.57, 120.88, 118.37, 117.93, 117.87, 113.43, 112.78, 100.05, 30.97; HRMS (ESI) Calcd for $C_{32}H_{21}N_6[M + H]^+$ 489.1828, found 489.1874. Purity by HPLC UV (254 nm) ESI-MS: 92.27%.

1-(Di(1*H*-indol-3-yl)methyl)-3-phenylimidazo [1,5-*a*] pyridine (5e): This compound was prepared according to the method D. Yield: 90%; R_f = 0.35 (ethyl acetate/*n*-hexane, 3:2); Melting point: 281–283; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 10.73 (s, 2H), 8.27 (d, J = 7.3 Hz, 1H), 7.73 (d, J = 8.7 Hz, 2H), 7.44 (d, J = 8.1 Hz, 2H), 7.37 (d, J = 7.9 Hz, 2H), 7.22–7.10 (m, 5H), 7.04–6.93 (m, 5H), 6.93–6.84 (m, 1H), 6.49 (s, 1H), 3.91 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6) δ 159.31, 136.56, 135.75, 134.93, 130.02, 129.15, 126.84, 123.45, 122.72, 121.40, 120.88, 119.27, 118.43, 118.22, 117.58, 117.45, 114.40, 112.99, 111.46, 55.31, 33.01; HRMS (ESI) Calcd for $C_{31}H_{25}N_4O$ $[M + H]^+$ 469.2028, found 469.2024. Purity by HPLC UV (254 nm) ESI-MS: 86.27%.

1-(Bis(5-methoxy-1*H*-indol-3-yl)methyl)-3-(4-methoxyphenyl) imidazo [1,5-*a*] pyridine (5f): This compound was prepared according to the method D. Yield: 94%; R_f = 0.26 (ethyl acetate/*n*-hexane, 3:2); Melting point: 253–255; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 9.67 (s, 2H), 8.02 (dd, J = 12.5, 7.9 Hz, 1H), 7.59 (d, J = 8.7 Hz, 2H), 7.15 (d, J = 8.7 Hz, 2H), 7.12–7.05 (m, 1H), 6.93 (d, J = 8.7 Hz, 2H), 6.83 (dd, J = 11.8, 1.9 Hz, 4H), 6.63 (dd, J = 8.7, 2.3 Hz, 2H), 6.39–6.31 (m, 2H), 6.10 (s, 1H), 3.77 (s, 3H), 3.57 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.19, 152.61, 135.60, 135.14, 131.63, 128.97, 127.15, 126.78, 124.10, 122.79, 121.50, 118.40, 117.41, 117.28, 114.43, 113.01, 111.93, 110.54, 101.68, 55.30, 55.25, 32.47; HRMS (ESI) Calcd for $C_{33}H_{29}O_3N_4$[M + H]$^+$ 529.2234, found 529.2232. Purity by HPLC UV (254 nm) ESI-MS: 97.81%.

1-(Bis(5-bromo-1*H*-indol-3-yl)methyl)-3-(4-methoxyphenyl) imidazo [1,5-*a*] pyridine (5 g): This compound was prepared according to the method D. Yield: 87%; R_f = 0.41 (ethyl acetate/*n*-hexane, 3:2); Melting point: 253–255; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 10.44 (s, 1H), 8.15 (d, J = 6.2 Hz, 1H), 7.72 (d, J = 8.8 Hz, 1H), 7.54 (d, J = 1.5 Hz, 1H), 7.48 (d, J = 1.7 Hz, 1H), 7.33 (s, 1H), 7.18 (d, J = 1.8 Hz, 1H), 7.15 (d, J = 1.8 Hz, 1H), 7.12–7.05 (m, 3H), 6.99 (s, 2H), 6.70–6.60 (m, 2H), 6.27 (s, 1H), 3.88 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6) δ 159.44, 143.28, 134.36, 134.00, 128.82, 127.10, 125.84, 124.17, 122.73, 120.29, 118.49, 117.38, 114.47, 113.80, 113.71, 113.48, 112.38, 112.26, 110.48, 54.27, 31.30;HRMS (ESI) Calcd for $C_{31}H_{23}ON_4Br_2$[M + H]$^+$ 625.0195, found 625.0052. Purity by HPLC UV (254 nm) ESI-MS: 96.85%.

3,3′-((3-(4-Methoxyphenyl)imidazo [1,5-*a*] pyridin-1-yl)methylene)bis(1*H*-indole-5-carbonitrile) (5 h): This compound was prepared according to the method D. Yield: 86%; R_f = 0.20 (ethyl acetate/*n*-hexane, 3:2); Melting point: 284–286; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 10.69 (s, 2H), 8.18–8.12 (m, 1H), 7.83 (s, 2H), 7.70 (d, J = 8.6 Hz, 2H), 7.43 (s, 1H), 7.40 (s, 1H), 7.31 (d, J = 1.5 Hz, 1H), 7.29 (d, J = 1.3 Hz, 1H), 7.16–7.10 (m, 1H), 7.09–7.04 (m, 2H), 7.03 (d, J = 8.6 Hz, 2H), 6.55–6.45 (m, 2H), 6.26 (s, 1H), 3.86 (s, 1H);^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6)δ159.35, 138.32, 136.17, 133.24, 129.09, 126.90, 126.33, 125.88, 125.04, 123.47, 122.47, 121.28, 120.82, 118.19, 117.85, 114.23, 112.90, 112.65, 100.42, 55.16, 32.23; HRMS (ESI) Calcd for$C_{33}H_{23}ON_6$[M + H]$^+$ 519.19279, found 519.19295. Purity by HPLC UV (254 nm) ESI-MS: 92.20%.

1-(Di(1*H*-indol-3-yl)methyl)-3-(3,4-dimethoxyphenyl)imidazo [1,5-*a*] pyridine (5i): This compound was prepared according to the method D. Yield: 94%; R_f = 0.35 (ethyl acetate/*n*-hexane, 3:2); Melting point: 203–205; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.75 (s, 2H), 8.36–8.29 (m, 1H), 7.59–7.49 (m, 4H), 7.36–7.28 (m, 4H), 7.15.7.10 (m, 2H), 7.20 (d, J = 8.6 Hz, 2H), 7.00 (t, J = 7.7 Hz, 2H), 6.86 (t, J = 7.7 Hz, 2H), 6.66–6.53 (m, 2H), 6.22 (s, 1H), 3.85–3.79 (m, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 148.93, 148.83, 136.43, 135.71, 134.98, 126.76, 123.38, 122.87, 121.67, 120.79, 120.07, 119.40, 119.29, 118.33, 117.57, 117.52, 115.01, 113.02, 111.89, 111.99, 111.38, 55.65, 55.56, 32.69; HRMS Calcd for $C_{32}H_{27}N_4O_2$[M + H]$^+$ 499. 2134, found 499.2131. Purity by HPLC UV (254 nm) ESI-MS: 98.45%.

1-(Bis(5-methoxy-1*H*-indol-3-yl)methyl)-3-(3,4-dimethoxyphenyl)imidazo [1,5-*a*] pyridine (5j): This compound was prepared according to the method D. Yield: 96%; R_f = 0.16 (ethyl acetate/*n*-hexane, 3:2); Melting point: 255–257; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 9.80 (s, 2H), 8.21–8.11 (m, 1H), 7.34–7.28 (m, 2H), 7.25 (s, 1H), 7.22 (s, 1H), 7.20–7.12 (m, 1H), 7.03–6.98 (m, 1H), 6.95–6.89 (m, 5H), 6.72 (dd, J = 8.7, 2.1 Hz, 2H), 6.47–6.39 (m, 1H), 6.21 (s, 1H), 3.93 (s, 3H), 3.91 (s, 3H), 3.66 (s, 6H);^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6) δ 151.37, 147.65, 147.61, 146.50, 145.41, 134.35, 133.41, 130.45, 125.75, 125.63, 122.68, 121.60, 119.86, 118.59, 117.15, 115.65, 111.28, 110.42, 110.23, 110.15, 109.18, 100.11, 99.99, 54.29, 53.90, 32.26; HRMS(ESI) Calcd for $C_{34}H_{31}O_4N_4$[M + H]$^+$ 559.2345, found 559.2299. Purity by HPLC UV (254 nm) ESI-MS: 97.52%.

1-(Bis(5-bromo-1*H*-indol-3-yl)methyl)-3-(3,4-dimethoxyphenyl)imidazo [1,5-*a*] pyridine (5k): This compound was prepared according to the method D. Yield: 92%; R_f = 0.42 (ethyl acetate/*n*-hexane, 3:2); Melting point: 213–215; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 10.55 (s, 2H), 8.23–8.17 (m, 1H), 7.62–7.57 (m, 2H), 7.35–7.32 (m, 1H), 7.29 (d, J = 8.5 Hz, 2H), 7.23–7.18 (m, 2H), 7.16–7.10 (m, 2H), 7.05–7.01 (m, 1H), 7.00–6.95 (m, 2H), 6.55–6.48 (m, 2H), 6.17 (s, 1H), 3.96–3.91 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6) δ 147.62, 147.01, 133.87, 126.96, 126.50, 123.44, 121.95, 121.23, 120.23, 119.96, 119.53, 118.64, 116.71, 116.25, 115.36, 111.80, 111.55, 110.58, 110.12, 109.64, 110.19, 54.36, 54.28, 31.56; HRMS(ESI) Calcd for $C_{32}H_{25}Br_2N_4O_2$[M + H]$^+$ 655.0.344, found 655.0330. Purity by HPLC UV (254 nm) ESI-MS: 98.42%.

3,3′-((3-(3,4-Dimethoxyphenyl)imidazo [1,5-*a*] pyridin-1-yl) methylene)bis(1*H*-indole-5-carbonitrile) (5 l): This compound was prepared according to the method D. Yield: 90%; R_f = 0.13 (ethyl acetate/*n*-hexane, 3:2); Melting point: 219–221; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 10.91 (s, 2H), 8.19 (m, 1H), 7.91–7.77 (m, 2H), 7.42 (d, J = 7.4 Hz, 1H), 7.29 (s, 1H), 7.18 (d, J = 7.5 Hz, 1H), 7.10 (s, 2H), 7.01 (d, J = 7.2 Hz, 1H), 6.57–6.44 (m, 2H), 6.25 (s, 1H), 3.92 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6) δ 148.14, 147.29, 137.56, 137.50, 135.33, 126.16, 125.39, 124.82, 124.15, 122.65, 120.38, 119.91, 119.87, 119.06, 119.92, 114.24, 111.97, 111.54, 110.68, 110.30, 99.81, 99.71, 54.86, 54.79, 32.15; MS(ESI): *m/z* 548 $[M + H]^+$. Purity by HPLC UV (254 nm) ESI-MS: 91.13%.

1-(Di(1H-indol-3-yl)methyl)-3-(4-(trifluoromethyl)phenyl)imidazo [1,5-*a*] pyridine (5 m): This compound was prepared according to the method D. Yield: 88%; R_f = 0.75 (ethyl acetate/*n*-hexane, 1:1); Melting point: 263–265; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.74 (s, 1H), 8.45 (d, J = 6.6 Hz, 1H), 8.03 (d, J = 7.9 Hz, 2H), 7.85 (d, J = 7.8 Hz, 2H), 7.62 (d, J = 8.9 Hz, 1H), 7.47 (d, J = 7.9 Hz, 1H), 7.32 (d, J = 8.1 Hz, 1H), 7.12 (d, J = 1.8 Hz, 1H), 7.00 (t, J = 7.5 Hz, 1H), 6.85 (t, J = 7.5 Hz, 1H), 6.77–6.66 (m, 1H), 6.25 (s, 1H);^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6) δ 136.21, 135.58, 134.01, 133.48, 128.51, 127.27, 126.42, 125.13, 125.09, 124.46, 123.03, 120.78, 120.53, 120.41, 118.87, 118.52, 117.86, 117.44, 117.05, 113.00, 110.74, 33.47; MS (ESI): *m/z* 507 $[M + H]^+$. Purity by HPLC UV (254 nm) ESI-MS: 98.43%.

1-(Bis(5-methoxy-1*H*-indol-3-yl)methyl)-3-(4-(trifluoromethyl) phenyl)imidazo [1,5-*a*] pyridine (5n): This compound was prepared according to the method D. Yield: 90%; R_f = 0.60 (ethyl acetate/*n*-hexane, 1:1); Melting point: 213–215; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.60 (s, 2H), 8.52 (d, J = 7.3 Hz, 1H), 8.09 (d, J = 8.2 Hz, 2H), 7.86 (d, J = 8.3 Hz, 2H), 7.78 (d, J = 9.1 Hz, 1H), 7.20 (d, J = 8.6 Hz, 2H), 7.18 (d, J = 2.2 Hz, 2H), 7.03 (d, J = 2.4 Hz, 3H), 6.80–6.76 (m, 1H), 6.71 (t, J = 7.3 Hz, 1H), 6.66 (dd, J = 8.6, 2.4 Hz, 2H), 6.20 (s, 1H), 3.61 (s, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 152.57, 136.44, 134.06, 133.93, 131.57, 128.85, 128.08, 127.90, 127.53, 127.04, 126.02, 125.80, 125.75, 124.08, 122.41, 121.89, 118.57, 118.43, 116.95, 113.84, 111.87, 110.50, 101.57, 55.18, 32.34; HRMS (ESI) Calcd for $C_{33}H_{26}F_3N_4O_2$[M + H]$^+$ 567.2008, found 567.2009. Purity by HPLC UV (254 nm) ESI-MS: 92.97%.

1-(Bis(5-bromo-1*H*-indol-3-yl)methyl)-3-(4-(trifluoromethyl) phenyl)imidazo [1,5-*a*] pyridine (5o): This compound was prepared

according to the method D. Yield: 86%; R_f = 0.82 (ethyl acetate/*n*-hexane, 1:1); Melting point: 271–273; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.01 (s, 2H), 8.53 (d, J = 7.3 Hz, 1H), 8.10 (d, J = 8.1 Hz, 2H), 7.91 (d, J = 9.1 Hz, 1H), 7.87 (d, J = 8.3 Hz, 2H), 7.82 (d, J = 1.8 Hz, 2H), 7.32 (d, J = 2.2 Hz, 2H), 7.30 (d, J = 8.6 Hz, 2H), 7.12 (dd, J = 8.6, 1.9 Hz, 1H), 6.86–6.81 (m, 1H), 6.77–6.72 (m, 1H), 6.32 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ135.47, 135.00, 134.10, 133.93, 128.31, 127.99, 127.63, 125.94, 125.74, 125.69, 124.97, 123.13, 122.35, 121.89, 121.74, 120.96, 118.84, 118.15, 116.95, 113.92, 113.35, 110.69, 31.41; HRMS (ESI) Calcd for $C_{31}H_{20}Br_2F_3N_4$[M + H]$^+$ 663.0007, found 663.00088. Purity by HPLC UV (254 nm) ESI-MS: 96.37%.

3,3′-((3-(4-(Trifluoromethyl)phenyl)imidazo[1,5-*a*] pyridin-1-yl)methylene)bis(1*H*-indole-5-carbonitrile) (5p): This compound was prepared according to the method D. Yield: 84%; R_f = 0.50 (ethyl acetate/*n*-hexane, 1:1); Melting point: 277–279; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.42 (s, 2H), 8.53 (d, J = 7.1 Hz, 1H), 8.17 (s, 2H), 8.10 (d, J = 7.7 Hz, 2H), 7.96 (d, J = 8.8 Hz, 2H), 7.87 (d, J = 7.9 Hz, 2H), 7.60–7.45 (m, 3H), 7.37 (d, J = 8.2 Hz, 2H), 6.88 (t, J = 6.6 Hz, 1H), 6.75 (t, J = 6.6 Hz, 1H), 6.49 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 148.73, 148.27, 138.02, 134.90, 134.39, 133.90, 128.86, 128.02, 127.74, 126.23, 126.09, 125.76, 125.72, 125.65, 125.06, 123.47, 122.96, 122.00, 120.87, 119.15, 118.24, 117.98, 114.00, 112.77, 100.08, 30.89; MS (ESI): m/z 557 [M + H]$^+$. Purity by HPLC UV (254 nm) ESI-MS: 98.33%.

1-(Di(1*H*-indol-3-yl)methyl)-3-(4-fluorophenyl)imidazo[1,5-*a*] pyridine (5q): This compound was prepared according to the method D. Yield: 88%; R_f = 0.66 (ethyl acetate/*n*-hexane, 1:1); Melting point: 281–283; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 10.20 (s, 2H), 7.68–7.58 (m, 1H), 7.44–7.32 (m, 3H), 7.44–7.13 (m, 4H), 6.96 (t, J = 7.3 Hz, 2H), 6.89 (d, J = 1.5 Hz, 2H), 6.81 (t, J = 7.6 Hz, 2H), 6.52–6.42 (m, 2H), 6.22 (s, 1H);^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6) δ 159.46, 156.18, 134.94, 133.97, 130.38, 129.35, 129.19, 125.75, 125.16, 123.26, 121.79, 120.43, 120.36, 119.21, 117.51, 116.56, 116.27, 115.80, 114.61, 114.32, 111.16, 109.78, 31.60; HRMS (ESI) calcd for $C_{30}H_{22}N_4F$ [M + H]$^+$457.1823, found 457.1807. Purity by HPLC UV (254 nm) ESI-MS: 97.51%.

1-Bis(5-methoxy-1*H*-indol-3-yl)methyl)-3-(4-fluorophenyl)imidazo[1,5-*a*] pyridine (5r): This compound was prepared according to the method D. Yield: 90%; R_f = 0.50 (ethyl acetate/*n*-hexane, 1:1); Melting point: 305–307; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 10.04 (s, 2H), 8.11–8.04 (m, 1H), 7.75–7.62 (m, 3H), 7.21–7.08 (m, 4H), 6.88 (d, J = 1.9 Hz, 2H), 6.80 (d, J = 2.2 Hz, 2H) 6.61 (dd, J = 8.8, 2.4 Hz, 2H), 6.45–6.40 (m, 2H), 6.08 (s, 1H), 3.56 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6) δ 162.75, 159.47, 151.83, 134.14, 133.94, 130.88, 128.70, 128.59, 126.42, 126.15, 125.59, 125.55, 123.19, 119.91, 117.66, 116.40, 116.04, 114.89, 114.61, 112.01, 110.90, 109.78, 100.36, 54.42, 32.77; HRMS (ESI) calcd for $C_{32}H_{26}FN_4O_2$ [M + H]$^+$517.2040, found 517.2040. Purity by HPLC UV (254 nm) ESI-MS: 96.47%.

1-(Bis(5-bromo-1*H*-indol-3-yl)methyl)-3-(4-fluorophenyl)imidazo[1,5-*a*] pyridine (5 s): This compound was prepared according to the method D. Yield: 86%; R_f = 0.73 (ethyl acetate/*n*-hexane, 1:1); Melting point: 283–285; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.00 (s, 2H), 8.30 (s, 1H), 7.90–7.82 (m, 2H), 7.76 (s, 2H), 7.70 (t, J = 7.0 Hz, 1H), 7.61–7.54 (m, 1H), 7.46–7.37 (m, 2H), 7.33–7.27 (m, 3H), 7.11 (d, J = 8.5 Hz, 2H), 6.82–6.77 (m, 1H), 6.68 (t, J = 6.5 Hz, 1H), 6.30 (s, 1H);‘ ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.53, 158.06, 135.00, 134.85, 131.84, 131.11, 130.90, 128.33, 127.15, 124.96, 123.12, 122.25, 122.19, 121.71, 118.29, 117.79, 117.09, 116.39, 116.18, 113.33, 113.03, 110.70, 30.62; HRMS (ESI) calcd for $C_{30}H_{20}Br_2FN_4$[M + H]$^+$613.0039, found 612.9979. Purity by HPLC UV (254 nm) ESI-MS: 95.29%.

3,3′-((3-(4-Fluorophenyl)imidazo[1,5-*a*] pyridin-1-yl)methylene)bis(1*H*-indole-5-carbonitrile) (5t): This compound was prepared according to the method D. Yield: 85%; R_f = 0.40 (ethyl acetate/*n*-hexane, 1:1); Melting point: 294–296; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 11.15 (s, 2H), 8.23 (d, J = 7.0 Hz, 1H), 7.92 (s, 2H), 7.82 (dd, J = 8.7, 5.4 Hz, 2H), 7.54–7.40 (m, 4H), 7.34–7.20 (m, 5H), 6.68–6.55 (m, 2H), 6.31 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.75, 160.48, 138.34, 135.34, 134.12, 130.06, 129.95, 127.42, 126.77, 126.51, 126.31, 125.36, 123.83, 121.78, 121.21, 118.79, 118.61, 118.11, 116.36, 116.07, 113.82, 113.10, 100.33, 31.35; HRMS (ESI) Calcd for $C_{32}H_{20}N_6F$ [M + H]$^+$ 507.17280, found 507.17187. Purity by HPLC UV (254 nm) ESI-MS: 97.95%.

4.2. Biology

4.2.1. Cytotoxic activity

The cytotoxic activity of the compounds was determined by using MTT assay [22]. Cells were seeded in 100 µL DMEM, supplemented with 10% FBS in each well of 96-well microculture plates and incubated for 24 h at 37 °C in a CO_2 incubator. After incubation, cells were treated with test compounds of desired concentrations for 48 h. After 48 h incubation, 10 µL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/mL) was added to each well and the plates were further incubated for 4 h. Then the supernatant from each well was carefully removed, formazon crystals were dissolved in 100 µL of DMSO and absorbance at 570 nm wavelength was recorded.

4.2.2. Cell cycle analysis

Effect of **5k** and **5r** on DNA content by cell cycle progression was assessed using A549cells. Cells (2×10^5/well) were incubated in a 6-well plate with incomplete media (serum free) for 24 h, to bring all cells in a synchronized state (Margit Rosner, 2013). [23,24] After 24 h, incomplete media was replaced with complete media without or with DIM**, 5k,** and **5r** at indicated concentrations and further incubated for 24 h. The cells were washed thrice with PBS, harvested, fixed in ice cold PBS in 70% ethanol, and stored at −20 °C for 30 min. After fixation, these cells were incubated with RNase A (0.1 mg/mL) at 37 °C for 30 min, stained with PI (50 µg/mL) for 30 min on ice in dark, and then measured for DNA content using BD FACSVerse flow cytometer (Becton Dickinson, USA).

4.2.3. Measurement of reactive oxygen species (ROS)

ROS production from A549 cells treated with DIM, **5k,** and **5r** conjugates was measured by using 2′,7′–dichlorofluorescin diacetate (DCFDA) dye and the method was followed by Reddy, T. Srinivasa, et al. (2015) [25,26]. A549 cells were seeded in a 6-well plate at a density of 2×10^5 cells/well and incubated overnight. After incubation, media was replaced with fresh medium containing DIM, **5k** and **5r** conjugates at indicated concentrations for 24 h. After 24 h incubation, medium was replaced with fresh medium containing DCFDA (20 mM) and incubated in dark for 30 min at room temperature. Cells were washed with PBS twice and fluorescence intensity was measured by fluorescence microscope (Olympus-1X71SIF-3), capture 7 pro software.

4.2.4. Determination of apoptosis by annexin V-FITC/PI stain

Induction of apoptosis was determined by using the Annexin V-FITC apoptosis detection kit (Sigma Aldrich cat. No: APOAF) (Deng, Sanming, et al., 2012) [27]. Briefly, cells were seeded in 6-well plate at a density of 1×10^5 cells/well and allowed to attach overnight, followed by treatment with DIM, **5k,** and **5r** at 1 and 2 µM concentrations for 24 h. After 24 h, media was discarded, and cells were washed twice with PBS. Gently trypsinize and resuspend the cells in 1x binding buffer at a concentration of 1×10^6 cells/mL. Incubate the cell suspension with 5 µL Annexin V- FITC and 10 µL of PI for 10 min at room temperature and protect from light. Analysis was carried out by flow cytometry (FACSVerse, Becton-Dickinson, USA).

4.2.5. Measurement of mitochondria membrane potential ($\Delta\Psi m$)

Mitochondrial membrane potential was measured by according to

the manufacturer's protocol BD^{TM} Mitoscreen kit. Briefly, 2×10^5 cells were seeded in to the 6-well plates and incubated for overnight. Cell culture medium was replaced with fresh medium containing DIM, **5k,** and **5r** at indicated concentrations for 24 h. After incubation, cells were trypsinized and collected into 15 mL tubes and centrifuged at 400*g* for 5 min at room temperature. To the cell pellet 0.5 mL of freshly prepared JC-1 working solution was added and incubated for 15 min at 37 °C in CO_2 incubator. After incubation, cells were washed twice with 1x assay buffer at room temperature. Cells were resuspended in 0.5 mL of 1x assay buffer and analyzed by flow cytometry (Reers, Martin, et al., 1995) [28].

Acknowledgements

The authors thank CSIR-IICT, India and UGC, New Delhi, India for the award of fellowships and the author RS thank CSIR-HRDG (13(8906-A)/2017-pool). Manuscript Communication Number: IICT/Pubs./2018/180

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