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Original article

Synthesis and anticancer activity of oxindole derived imidazo[1,5-*a*]pyrazines

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ABSTRACT

A series of oxindole derivatives of imidazo[1,5-*a*]pyrazines were prepared and confirmed by ^{1}H NMR, mass and HRMS data. These compounds were evaluated for their anticancer activity against a panel of 52 human tumor cell lines derived from nine different cancer types: leukemia, lung, colon, CNS, melanoma, ovarian, renal, prostate and breast. Among them compound **7l** showed significant anticancer activity with GI_{50} values ranging from 1.54 to 13.0 μM. Cell cycle arrest was observed in G0/G1 phase upon treatment of A549 cells with 6.5 μM (IC_{50}) concentration of compound **7l** and induced apoptosis. This was confirmed by Annexin V-FITC as well as DNA fragmentation analysis and interestingly this compound (**7l**) did not affect the normal cells.

1. Introduction

One of the major diseases causing death across the world is cancer. Nitrogen-bridgehead fused heterocycles containing an imidazole ring are a common structural moiety in many pharmacologically important molecules that display a wide range of activities for diverse number of targets. One of the most widely used heterocyclic system from this group is imidazopyridine [1]. Imidazopyridines show a spectrum of biological activities like inhibitors of aromatase estrogen production suppressors [2], positive inotropic agents [3], platelet aggregation inhibitors, thromboxane synthetase inhibitors [4], antiviral [5,6], antibacterial [7], hypnoselective, and anxioselective activities [8]. Imidazopyridines exhibit different type of molecular mechanisms in cancer chemotherapy. Recently, Wu and coworkers reported 3,7-diarylimidazopyridines as inhibitors of the vascular endothelial growth factor (VEGF)-receptor KDR [9]. VEGF is a regulator of vascular permeability and an inducer of endothelial cell proliferation, migration, and survival. Activation of the VEGF pathway is a fundamental regulation of angiogenesis, the formation of new capillaries from established blood vessels. In molecular mechanisms, the mitogenic signal of VEGF is mediated through the receptor tyrosine kinase KDR (VEGFR-2) [10]. Substituted 2-(*N*-trifluoroacetylamino)imidazopyridines are known to arrest cell cycle at G2/M phase and induce apoptosis in SK-LU-1 human cancer cell line [11]. Oxindoles are important pharmacophores that are known to enhance anticancer activity of some core molecules. Similarly, substituted *E*-3-(3-Indolylmethylene)-1,3-dihydroindol-2-ones are also reported as anticancer agents and induce apoptosis [12–17]. Therefore in the present study oxindole-derived imidazo[1,5-*a*]pyrazines have been synthesized and evaluated for their anticancer activity. Further the mode of action of one of the potent compound (**7l**) has been investigated.

2. Results and discussion

2.1. Chemistry

Synthesis of the oxindole-derived imidazo[1,5-*a*]pyrazine (**7a-r**) derivatives was accomplished as described in Scheme 1. The intermediates *N*1-(2-pyridylmethyl)-substituted benzamides (**3a-h**) were prepared by the reaction of different substituted benzoylchlorides (**2a-h**) and 2-pyridylmethanamine (**1**) in the presence of triethylamine. The cyclization of *N*1-(2-pyridylmethyl)-substituted benzamides (**3a-h**) in phosphorous oxychloride gave the intermediates substituted phenyl imidazo[1,5-*a*]pyridines (**4a-h**) in good yield. Formylation of substituted phenyl imidazo [1,5-*a*]pyridines (**4a-h**) in DMF and phosphorous oxychloride

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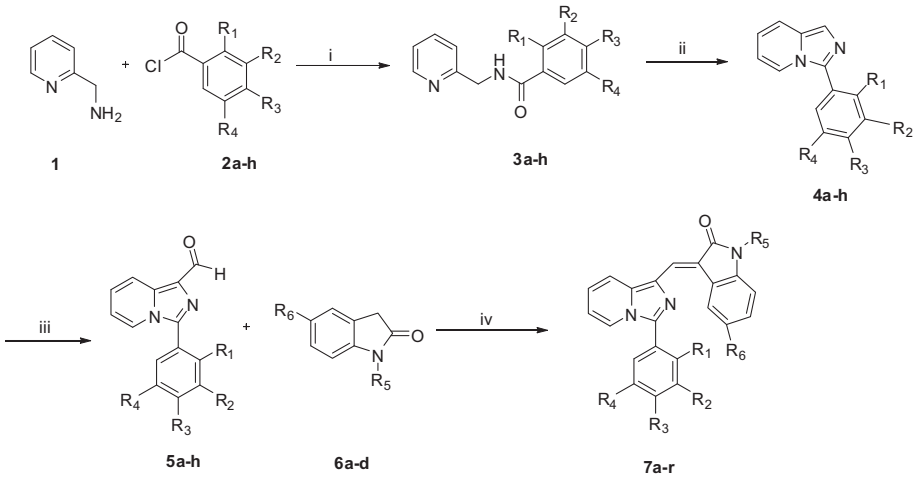


7a = R_1 = H, R_2 = H, R_3 = H, R_4 = H, R_5 = H, R_6 = H
7b = R_1 = H, R_2 = H, R_3 = H, R_4 = H, R_5 = CH_3, R_6 = H
7c = R_1 = H, R_2 = H, R_3 = OCH_3, R_4 = H, R_5 = H, R_6 = H
7d = R_1 = H, R_2 = H, R_3 = OCH_3, R_4 = H, R_5 = CH_3, R_6 = H
7e = R_1 = H, R_2 = H, R_3 = OCH_3 R_4 = H, R_5 = H, R_6 = F
7f = R_1 = H, R_2 = OCH_3, R_3 = OCH_3, R_4 = H, R_5 = H, R_6 = H
7g = R_1 = H, R_2 = OCH_3, R_3 = OCH_3, R_4 = H, R_5 = CH_3, R_6 = H
7h = R_1 = H, R_2 = OCH_3, R_3 = OCH_3, R_4 = OCH_3, R_5 = CH_3, R_6 = H
7i = R_1 = H, R_2 = H, R_3 = CH_3, R_4 = H, R_5 = H, R_6 = H
7j = R_1 = H, R_2 = H, R_3 = CH_3, R_4 = H, R_5 =H, R_6 = F
7k = R_1 = H, R_2 = H, R_3 = CH_3, R_4 = H, R_5 = CH_3, R_6 = F
7l = R_1 = F, R_2 = H, R_3 = H, R_4 = H, R_5 = H, R_6 = H
7m = R_1 = F, R_2 = H, R_3 = H, R_4 = H, R_5 = CH_3, R_6 = H
7n = R_1 = F, R_2 = H, R_3 = H, R_4 = H, R_5 = H, R_6 = F
7o = R_1 = F, R_2 = H, R_3 = H, R_4 = H, R_5 = CH_3, R_6 = F
7p = R_1 = H, R_2 = F, R_3 = H, R_4 = H, R_5 = CH_3, R_6 = H
7q = R_1 = H, R_2 = H, R_3 = F, R_4 = H, R_5 = H, R_6 = H
7r = R_1 = H, R_2 = H, R_3 = F, R_4 = H, R_5 = H, R_6 = F

Scheme 1. Reagents and conditions: (i) TEA, dry THF, 0 °C to rt, 3 h; (ii) $POCl_3$, reflux, 2 h; (iii) DMF, $POCl_3$; (iv) EtOH, piperidine, reflux, 3 h.

gave 3-(substituted phenyl)imidazo[1,5-*a*]pyridine-1-carbaldehyde (**5a-h**) [18].

The reaction between 3-(substituted phenyl)imidazo[1,5-*a*] pyridine-1-carbaldehyde (**5a-h**) and oxindole (**6a-d**) in methanol in the presence of piperidine gave the final oxindole-derived imidazo [1,5-a]pyrazines (**7a-r**) in excellent yield. The structures of these compounds were established on the basis of their spectroscopic data. The ESIMS (mass spectra) of the compounds showed $[M + H]^+$ peaks corresponding to their molecular formula. The purity of new compounds synthesized was verified by ^1H NMR and high resolution mass spectra.

2.2. Anticancer activity

All synthesized new oxindole-derived imidazo[1,5-*a*]pyrazines (**7a-r**) were evaluated by the National Cancer Institute, Bethesda for their anticancer activity at single concentration of 10^{-5} M toward a panel of approximately sixty cancer cell lines. The human tumor cell lines were derived from nine different cancer types: leukemia, melanoma, lung, colon, CNS, ovarian, renal, prostate and breast cancers. Primary anticancer assays were performed according to the US NCI protocol [19–21]. The compounds were added at a single concentration and the cell culture was incubated for 48 h. End point determinations were made with a protein binding dye, sulforhodamine B (SRB). The results for each compound are reported as the percent growth of treated cells when compared to untreated control cells (shown in Table 1). Range of growth% shows the lowest and the highest growth % found among different cancer cell lines.

Table 1
Anticancer screening data in concentration 10^{-5} M.

Compound	Meangrowth %	Range of growth %	Most sensitive cell line	Growth % of the most sensitive cell line
7a	87.51	−1.63 to 123.93	MBA-MB-468 (Breast)	−1.63
7b	85.21	52.68 to 129.58	T-47D (Breast)	52.68
7c	72.54	17.53 to 120.98	ACHN (Renal)	17.53
7d	77.31	−7.02 to 119.01	SR (Leukemia)	−7.03
7e	101.96	48.75 to 126.47	MCF-7 (Breast)	48.75
7f	82.66	21.84 to 117.12	MCF-7 (Breast)	21.84
7g	103.43	70.51 to 138.54	NCI-H522 (Non-small cell lung)	70.51
7h	98.17	42.32 to 126.81	SR (Leukemia)	42.32
7i	78.55	−16.33 to 133.23	DU-145 (Prostate)	−16.33
7j	74.57	−23.07 to 110.36	DU-145 (Prostate)	−23.07
7k	99.39	60.48 to 128.17	NCI-H522 (Non-small cell lung)	60.48
7l	18.49	−68.25 to 86.08	SNB-75 (CNS Cancer)	−68.25
7m	84.72	27.49 to 115.62	DU-145 (Prostate)	27.49
7n	80.90	−7.63 to 130.24	DU-145 (Prostate)	−7.63
7o	99.95	69.77 to 146.09	COLO-205 (Colon)	69.77
7p	70.98	7.47 to 101.45	IGROV1 (Ovarian)	7.47
7q	39.31	39.31 to 118.39	MDA-MB-468 (Breast)	39.31
7r	97.53	−13.90 to 131.85	A498 (Renal)	−13.90

Interestingly, among all the compounds one compound (**7l**) was found active and selected for five dose. In five dose the compound (**7l**) was dissolved in DMSO and evaluated for anticancer activity using five concentrations at 10-fold dilutions, the highest being 10^{-4} M and others 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M. The results of five dose data have been showed in Table 2. Compound (**7l**) is extremely potent with GI_{50} values ranging from 1.54 to 13.0 μM. This compound is active at <2 μM against HCT-116 (*colon cancer*), SNB-75 (*CNS cancer*), OVACAR-3, OVACAR-4, OVACAR-5 (*ovarian cancers*) and MDA-MB- 468 (*breast cancer*) cell lines. It is interesting to note that the mean graph midpoint values of compound **7l** are $\log_{10}$ GI_{50} (−5.34), $\log_{10}$ TGI (−4.82) and $\log_{10}$ LC_{50} (−4.41). These results specify that compound **7l** possess remarkable anticancer activity. This compound was considered for further studies like cell cycle analysis and understanding the mechanism of action.

Specific killing of cancer cells without affecting normal cell growth is a key safety feature of cancer chemotherapy. Therefore compound (**7l**) was evaluated for possible cytotoxicity to normal cells, for example, HEK-293. Compound (**7l**), did not significantly affect the growth of HEK-293, suggesting that the molecule selectively inhibits the growth of cancer cells.

2.3. Cell cycle analysis and apoptotic changes

The tumor suppressor gene p53 is a multifunctional protein responsible for maintaining genomic integrity and its mutation is known to cause tumors in humans [22]. In response to DNA damage, aberrant growth signals, or chemotherapeutic drugs, p53 activates signaling for DNA repair initially, and in failure of DNA repair, induces apoptosis and/or cell cycle arrest [23–25]. Data from MTT assay showed that compound (**7l**) induced significant inhibition of lung cancer cells. It was of interest to understand whether this inhibition of cell growth was on account of cell cycle arrest. Hence, we studied the cell cycle distribution of this compound by treating the cells with propidium iodide-labeling and followed fluorescence-activated cell sorting analysis. Results indicated that compound **7l** (6.5 μM) showed cell cycle arrest at G0/G1 Phase in 24 h and 48 h as compared with untreated control as shown in Fig.1.

Manual field quantification of percent apoptotic cells based on cytoplasmic condensation, presence of apoptotic body, nuclear fragmentation and relative fluorescence in cells treated with test compounds revealed that compound **7l** in A549 cells resulted in significant increase in percentage of apoptotic cell (Fig. 2).

This potent compound **7l** (IC_{50} values of 6.5 μM, against A549 cells) was also investigated for the inhibition of cell growth to understand whether it is due to physiological apoptosis or non-specific necrosis. The ability of compound **7l** to induce apoptosis was measured by Annexin V-FITC and propidium iodide (PI) labeling of A549 cells, after treatment with 6.5 μM for 24 h and 48 h. It was observed that this compound (**7l**) showed significant apoptosis against A549 cells as illustrated in Fig. 3.

Table 2
In vitro cytotoxicity of the compound **7l** on 52 human cancer cell lines.

Panel/Cell Line	GI_{50} (μM)	Panel/Cell Line	GI_{50} (μM)
Leukemia		*Melanoma*	
		MALME-3M	3.07
K-562	6.35	M 14	5.65
SR	11.0	SK-MEL-28	3.71
		LOX IMV	4.15
		SK-MEL-2	6.81
		SK-MEL-5	2.80
Non-small cell lung		UACC-257	5.20
A549/ATCC	3.46	UACC-62	6.32
EKVX	10.6	MDA-MB-435	3.95
HOP-62	2.37	*Ovarian*	
NCI-H226	4.78	IGROV1	2.04
NCI-H23	4.55	OVCAR-3	1.90
NCI-H322M	4.16	OVCAR-4	1.81
NCI-H460	2.17	OVCAR-5	1.80
NCI-H522	2.18	OVCAR-8	2.49
		NCI/ADR-RES	3.36
Colon		SK-OV-3	3.46
COLO 205	4.08	*Renal Cancer*	
HCT-116	1.97	786-0	3.44
HCT-15	13.0	A498	2.81
HT29	3.50	ACHN	3.30
KM12	3.49	RXF 393	3.41
SW-620	3.97	SN12C	4.32
		TK-10	4.05
CNS Cancer		UO-31	2.12
SF-268	4.65		
SF-295	3.65	*Breast Cancer*	
SF-539	3.10	MCF7	2.10
SNB-19	4.70	MDA-MB-231/ATCC	2.27
SNB-75	1.79	BT-549	3.36
U251	2.57	T-47D	2.36
		MDA-MB-468	1.54
Prostate Cancer			
PC-3	6.81		
DU-145	3.26		

2.4. DNA fragmentation

The ability of **7l** for the induction of intranucleosomal DNA fragments at 24 and 48 h of exposure to A549 cells have been demonstrated by agarose gel electrophoresis technique. DNA laddering is significantly observed in the A549 cells exposed to 6.5 uM of **7l**. Our results show that **7l** harbor DNA fragmentation in A549 cells, evident by Fig. 4, which is associated with the last events of drug induced apoptosis.

3. Conclusion

In conclusion, we synthesized a series of oxindole-derived imidazo[1,5-*a*]pyrazines derivatives and evaluated this for anticancer activity. One of the compound **7l** showed significant cytotoxicity against a panel of 52 human cancer cell lines. The FACS analysis showed that the compound **7l** arrests the cell cycle in G0/G1 phase in A549 cells and ultimately leading to cell death. This was further confirmed by Annexin V-FITC, DNA fragmentation analysis and Hoechst staining. Moreover, compound **7l** did not affect the normal cells (HEK-293). Based on these observations, compound (**7l**) could be considered as important lead compound for potential application in anticancer chemotherapy.

4. Experimental

4.1. Chemistry

All chemicals and reagents were obtained from Aldrich (Sigma–Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthew Company, Ward Hill, MA, USA) and were used without purification. Reactions were monitored by TLC, performed on silica gel glass plates containing 60 F-254, and visualization on TLC was achieved by UV light or iodine indicator. Column chromatography was performed with Merck 60–120 mesh silica gel. ^{1}H NMR spectra were recorded on Gemini Varian VXR-unity (400 and 500 MHz) or Bruker UXNMR/XWIN-NMR (300 MHz) instruments. Chemical shifts (δ) are reported in ppm downfield from internal TMS standard. ESI spectra were recorded on Micro mass, Quattro LC using ESI^+ software with capillary voltage 3.98 kV and ESI mode positive ion trap detector. High-resolution mass spectra (HRMS) were recorded on QSTAR XL Hybrid MS/MS mass spectrometer. Melting

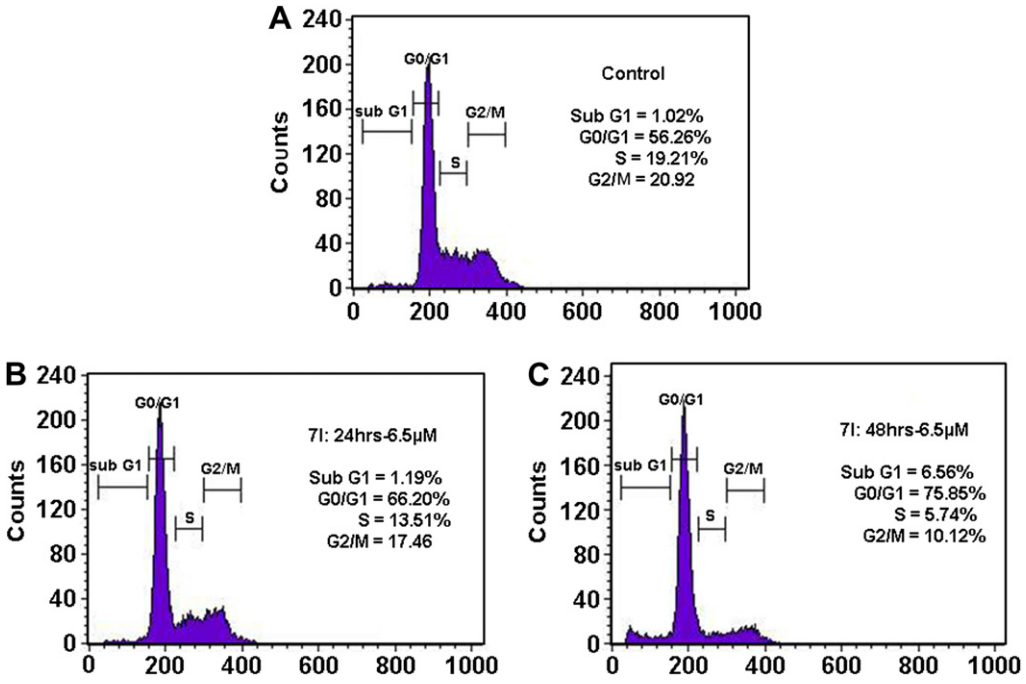


Fig. 1. Cell cycle analysis of 7l in A549 cells, A: Control cells, B: Cells treated with 7l for 24 h in 6.5 μM concentration, C: Cells treated with 7l for 48 h in 6.5 μM concentration.

points were determined with an Electrothermal melting point apparatus, and are uncorrected.

*4.1.1. General procedure for the synthesis of N1-(2-pyridylmethyl)-substituted benzamides (**3a-h**)*

To a stirred solution of 2-pyridylmethanamine (**1**) (1 mmol) in dry THF was added triethylamine (1.1 mmol) followed by substituted benzoylchlorides (**2a-h**) at 0 °C. The reaction mixture was stirred for 3 h and the reaction was monitored by TLC. After completion of reaction, THF was removed under vacuum to get the crude products. This was further purified by column chromatography (EtOAc-Hexane) to get the pure compounds (**3a-h**).

*4.1.1.1. N1-(2-Pyridylmethyl)benzamide (**3a**).* Yield 76%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.53 (d,1H, J = 4.7 Hz), 7.88 (2H, d, J = 6.4 Hz), 7.67 (2H, t, J = 7.7, 6.2 Hz), 7.47–7.39 (3H, m), 7.32 (1H, d, J = 7.9 Hz), 7.20 (1H, t, J = 5.0, 5.0 Hz), 4.73 (2H, d, J = 4.7 Hz); MS (ESI): *m/z* 213 $[M + H]^+$.

*4.1.1.2. N1-(2-Pyridylmethyl)-4-methoxybenzamide (**3b**).* Yield 80%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.56 (d,1H, J = 4.8 Hz), 7.83 (2H, d, J = 8.7 Hz), 7.68 (1H, t, J = 7.8, 5.8 Hz), 7.47 (1H, brs), 7.32 (1H, d, J = 7.8 Hz), 7.21 (1H, t, J = 5.8, 4.8 Hz), 6.93 (2H, d, J = 8.7 Hz), 4.75 (2H, d, J = 4.8 Hz), 3.85 (3H, s); MS (ESI): *m/z* 243 $[M + H]^+$.

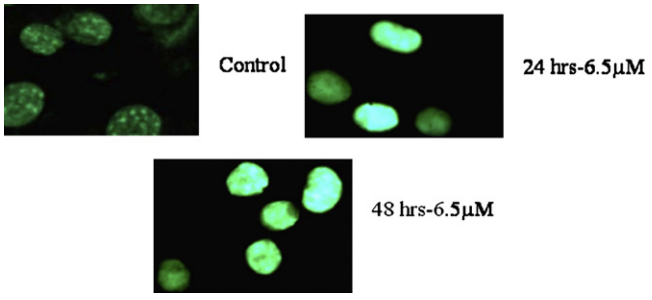


Fig. 2. Hoechst staining of 7l in A549 cells.

*4.1.1.3. N1-(2-Pyridylmethyl)-3,4-dimethoxybenzamide (**3c**).* Yield 78%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.52 (d,1H, J = 4.5 Hz), 7.70–7.62 (2H, m), 7.45 (1H, d, J = 2.2 Hz), 7.39–7.31 (2H, m), 7.20 (1H, dd, J = 6.0, 5.2 Hz), 6.82 (1H, d, J = 8.3 Hz), 4.70 (2H, d, J = 5.2 Hz), 3.92 (3H, s), 3.90 (3H, s); MS (ESI): *m/z* 273 $[M + H]^+$.

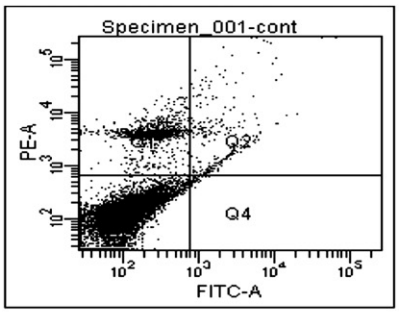
*4.1.1.4. N1-(2-Pyridylmethyl)-3,4,5-trimethoxybenzamide (**3d**).* Yield 76%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.50 (d,1H, J = 4.5 Hz), 7.93–7.90 (1H, brs), 7.67 (1H, t, J = 8.3, 7.5 Hz), 7.34 (1H, d, J = 7.5 Hz), 7.19 (1H, dd, J = 6.7, 5.2 Hz), 7.05 (2H, s), 4.68 (2H, d, J = 5.2 Hz), 3.84 (6H, s), 3.82 (3H, s); MS (ESI): *m/z* 303 $[M + H]^+$.

*4.1.1.5. N1-(2-Pyridylmethyl)-4-methylbenzamide (**3e**).* Yield 74%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.35 (d,1H, J = 4.5 Hz), 8.33 (1H, s), 7.64 (2H, d, J = 8.3 Hz), 7.32 (2H, d, J = 8.3 Hz), 7.20 (1H, dd, J = 6.7, 6.0 Hz), 6.83 (1H, t, J = 8.3, 6.7 Hz), 4.70 (2H, d, J = 5.0 Hz), 2.46 (3H, s); MS (ESI): *m/z* 227 $[M + H]^+$.

*4.1.1.6. N1-(2-Pyridylmethyl)-2-fluorobenzamide (**3f**).* Yield 82%, ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.55 (d,1H, J = 4.8 Hz), 8.12 (1H, t, J = 7.2, 5.6 Hz), 8.06–7.98 (1H, brs), 7.66 (1H, t, J = 8.0, 5.6 Hz), 7.47–7.41 (1H, m), 7.32 (1H, d, J = 8.0 Hz), 7.25 (1H, d, J = 8.0 Hz), 7.19 (1H, dd, J = 7.2, 4.8 Hz), 7.11 (1H, dd, J = 8.0, 4.0 Hz), 4.77 (2H, d, J = 4.8 Hz); MS (ESI): *m/z* 231 $[M + H]^+$.

*4.1.1.7. N1-(2-Pyridylmethyl)-3-fluorobenzamide (**3g**).* Yield 71%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.53 (d,1H, J = 4.5 Hz), 7.80 (1H, brs), 7.70–7.57 (3H, m), 7.38 (1H, dd, J = 8.3, 5.2 Hz), 7.32 (1H, d, J = 7.5 Hz), 7.22–7.14 (1H, m), 4.70 (2H, d, J = 5.2 Hz); MS (ESI): *m/z* 231 $[M + H]^+$.

*4.1.1.8. N1-(2-Pyridylmethyl)-4-fluorobenzamide (**3h**).* Yield 75%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.55 (d,1H, J = 4.6 Hz), 8.00 (1H, brs), 7.72 (2H, d, J = 8.1 Hz), 7.53 (1H, t, J = 6.2, 6.0 Hz), 7.40 (2H, d, J = 8.1 Hz), 7.25 (1H, m), 7.2 (1H, dd, J = 6.2,5.6 Hz), 4.71 (2H, d, J = 5.0 Hz); MS (ESI): *m/z* 231 $[M + H]^+$.



A: Control

Tube: cont

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
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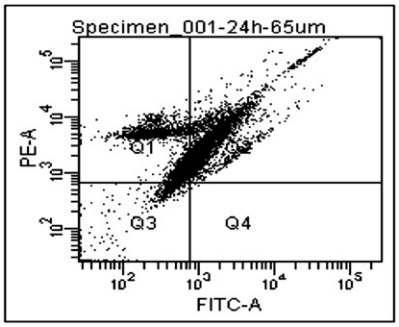
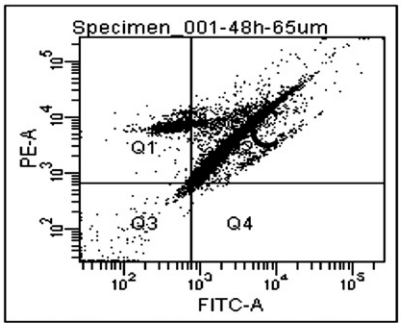
Q2	264	2.6	2.6
Q3	8,570	85.7	85.7
Q4	52	0.5	0.5



B: 24hrs-6.5μM (7l)

Tube: 24h-65um

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Q1	3,122	31.2	31.2
Q2	6,152	61.5	61.5
Q3	719	7.2	7.2
Q4	7	0.1	0.1



C: 48hrs-6.5μM (7l)

Tube: 48h-65um

Population	#Events	%Parent	%Total
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Q2	8,735	87.4	87.4
Q3	343	3.4	3.4
Q4	90	0.9	0.9

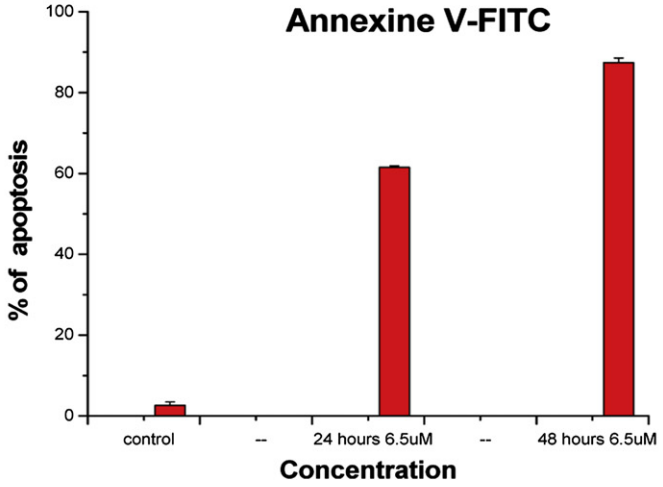

Fig. 3. Annexine V-FITC, (A) Control ells of A549 (B) A549 cells treated with 7l for 24 hrs-6.5 μM($1 \times IC_{50}$) (C) A549 cells treated with 7l for 48 hrs-6.5 μM($1 \times IC_{50}$).

*4.1.2. General procedure for the synthesis of substituted phenyl imidazo [1,5-a]pyridine (**4a-h**)*

To *N*1-(2-pyridylmethyl)-substituted benzamides (**3a-h**), added 4 mL of $POCl_3$ 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 was dried over anhydrous Na_2SO_4 and evaporated under vacuum. The residue, thus obtained was purified by column chromatography using ethylacetate and hexane as solvent system.

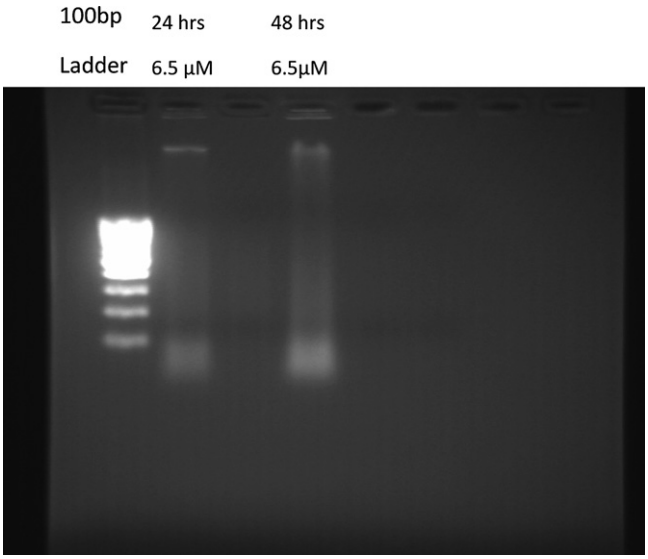

Fig. 4. DNA laddering of 7l on A549 cells.

*4.1.2.1. 3-Phenylimidazo [1,5-a]pyridine (**4a**).* Yield 78%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.24 (1H, d, J = 7.5 Hz), 7.76 (2H, d, J = 6.7 Hz), 7.50–7.36 (5H, m), 6.67 (1H, dd, J = 9.0,6.7 Hz), 6.51 (1H, t, J = 6.7, 6.0 Hz); MS (ESI): m/z 195 $[M + H]^+$.

*4.1.2.2. 3-(4-Methoxyphenyl)imidazo [1,5-a]pyridine (**4b**).* Yield 80%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.34 (1H, d, J = 8.7 Hz), 8.32 (1H, d, J = 6.8 Hz), 7.71 (2H, d, J = 8.7 Hz), 7.32–7.17 (1H, m), 7.07 (2H, d, J = 8.7 Hz), 6.98 (1H, s), 6.88 (1H, t, J = 6.8 Hz), 3.90 (3H, s); MS (ESI): m/z 225 $[M + H]^+$.

*4.1.2.3. 3-(3,4-Dimethoxyphenyl)imidazo [1,5-a]pyridine (**4c**).* Yield 76%, ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.18 (1H, d, J = 7.2 Hz), 7.47 (1H, s), 7.40 (1H, d, J = 9.3 Hz), 7.30 (1H, s), 7.27 (1H, d, J = 8.3 Hz), 6.93 (1H, d, J = 7.2 Hz), 6.64 (1H, dd, J = 7.2, 6.2 Hz), 6.49 (1H, t, J = 7.2, 6.2 Hz), 3.90 (3H, s), 3.87 (3H, s); MS (ESI): m/z 255 $[M + H]^+$.

*4.1.2.4. 3-(3,4,5-Trimethoxyphenyl)imidazo [1,5-a]pyridine (**4d**).* Yield 74%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.39 (1H, d, J = 7.5 Hz), 8.31 (1H, d, J = 9.0 Hz), 7.22 (1H, dd, J = 9.0, 5.2 Hz), 7.08 (1H, s), 6.95 (2H, s), 6.87 (1H, t, J = 6.7, 6.0 Hz), 3.94 (6H, s), 3.90 (3H, s); MS (ESI): m/z 285 $[M + H]^+$.

*4.1.2.5. 3-(4-Methylphenyl)imidazo [1,5-a]pyridine (**4e**).* Yield 82%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.06 (1H, d, J = 7.3 Hz), 7.51 (2H, d, J = 8.1 Hz), 7.32 (1H, s), 7.29 (1H, d, J = 9.0 Hz), 7.14 (2H, d, J = 7.9 Hz), 6.49 (1H, dd, J = 6.4, 6.2 Hz), 6.33 (1H, t, J = 7.3, 7.1 Hz), 2.27 (3H, s); MS (ESI): m/z 209 $[M + H]^+$

*4.1.2.6. 3-(2-Fluorophenyl)imidazo [1,5-a]pyridine (**4f**).* Yield 79%, ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.79–7.72 (2H, m), 7.56 (1H, s), 7.49–7.41 (2H, m), 7.30 (1H, t, J = 7.2 Hz), 7.21 (1H, dd, J = 9.7, 8.9 Hz), 6.74 (1H, dd, J = 6.4, 5.6 Hz), 6.56 (1H, t, J = 7.2, 6.4 Hz); MS (ESI): m/z 213 $[M + H]^+$.

*4.1.2.7. 3-(3-Fluorophenyl)imidazo [1,5-a]pyridine (**4g**).* Yield 72%, ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.24 (1H, d, J = 6.7 Hz), 7.57 (1H, d, J = 7.5 Hz), 7.51–7.41 (4H, m), 7.07 (1H, t, J = 9.8, 8.3 Hz), 6.70 (1H, dd, J = 9.0, 6.7 Hz), 6.54 (1H, t, J = 6.7, 6.0 Hz); MS (ESI): m/z 213 $[M + H]^+$.

*4.1.2.8. 3-(4-Fluorophenyl)imidazo [1,5-a]pyridine (**4h**).* Yield 85%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.16 (1H, d, J = 7.5 Hz), 7.75 (2H, dd, J = 9.0, 5.2 Hz), 7.49 (1H, s), 7.47 (1H, d, J = 9.0 Hz), 7.26–7.17 (2H, m), 6.69 (1H, dd, J = 9.0, 6.7 Hz), 6.53 (1H, t, J = 6.7, 6.0 Hz); MS (ESI): m/z 213 $[M + H]^+$.

*4.1.3. General procedure for the synthesis of 3-(substituted phenyl) imidazo [1,5-a]pyridine-1-carbaldehyde (**5a-h**)*

To an ice-water cooled solution of substituted phenyl imidazo [1,5-*a*]pyridines (**4a-h**) (1 mmol) in DMF (1.4 mmol) was added dropwise and with stirring phosphorus oxychloride (1.4 mmol). A strongly exothermic reaction occurred. After 2 h heating on oil-bath, the mixture was poured into ice water made basic with concentrated ammonium hydroxide and extracted trice with ethylacetate. The organic layer was dried over anhydrous sodium sulfate and evaporated under vacuum. The residue, thus obtained was purified by column chromatography using ethylacetate and hexane as solvent system.

*4.1.3.1. 3-Phenylimidazo [1,5-a]pyridine-1-carbaldehyde (**5a**).* Yield 80%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.10 (1H, s), 8.39 (2H, d, J = 8.3 Hz), 7.77 (2H, d, J = 6.6 Hz), 7.58–7.46 (3H, m), 7.22 (1H, dd, J = 9.4,6.7 Hz), 6.86 (1H, t, J = 6.7 Hz); MS (ESI): m/z 223 $[M + H]^+$.

*4.1.3.2. 3-(4-Methoxyphenyl)imidazo [1,5-a]pyridine-1-carbaldehyde (**5b**).* Yield 76%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.16 (1H, s), 8.34 (2H, d, J = 8.7 Hz), 8.31 (1H, d, J = 6.8 Hz), 7.26–7.23 (2H, dd, J = 8.7 Hz), 7.07 (2H, d, J = 8.7 Hz), 6.88 (1H, t, J = 7.8, 6.8 Hz), 3.90 (3H, s); MS (ESI): m/z 253 $[M + H]^+$.

*4.1.3.3. 3-(3,4-Dimethoxyphenyl)imidazo [1,5-a]pyridine-1-carbaldehyde (**5c**).* Yield 82%, ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.12 (1H, s), 8.35 (2H, d, J = 5.6 Hz), 7.30 (1H, s), 7.25 (1H, s), 7.21 (1H, t, J = 7.2, 6.4 Hz), 6.96 (1H, d, J = 8.0 Hz), 6.84 (1H, t, J = 7.2, 6.2 Hz), 3.97 (3H, s), 3.95 (3H, s); MS (ESI): m/z 283 $[M + H]^{+\cdot}$

*4.1.3.4. 3-(3,4,5-Trimethoxyphenyl)imidazo [1,5-a]pyridine-1-carbaldehyde (**5d**).* Yield 72%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.09 (1H, s), 8.36 (1H, d, J = 7.5 Hz), 8.31 (1H, d, J = 9.0 Hz), 7.22 (1H, dd, J = 6.7, 5.2 Hz), 6.95 (2H, s), 6.87 (1H, t, J = 6.7, 6.0 Hz), 3.94 (6H, s), 3.90 (3H, s); MS (ESI): m/z 313 $[M + H]^+$.

*4.1.3.5. 3-(4-Methylphenyl)imidazo [1,5-a]pyridine-1-carbaldehyde (**5e**).* Yield 75%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.11 (1H, s), 8.32 (2H, d, J = 7.5 Hz), 7.64 (2H, d, J = 8.3 Hz), 7.33 (2H, s, d, J = 7.5 Hz), 7.20 (1H, dd, J = 9.0, 6.7 Hz), 6.84 (1H, t, J = 6.7, 6.0 Hz), 2.46 (3H, s); MS (ESI): m/z 237 $[M + H]^+$.

*4.1.3.6. 3-(2-Fluorophenyl)imidazo [1,5-a]pyridine-1-carbaldehyde (**5f**).* Yield 83%, ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.13 (1H, s), 8.37 (1H, d, J = 8.8 Hz), 7.93 (1H, t, J = 5.6, 5.2 Hz), 7.77 (1H, t, J = 7.3, 7.1 Hz), 7.53 (1H, q, J = 7.1, 6.7 Hz), 7.36 (1H, d, J = 7.1 Hz), 7.30–7.24 (2H, m), 6.90 (1H, t, J = 6.7, 6.6 Hz); MS (ESI): m/z 241 $[M + H]^+$.

*4.1.3.7. 3-(3-Fluorophenyl)imidazo [1,5-a]pyridine-1-carbaldehyde (**5g**).* Yield 78%, ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.12 (1H, s), 8.37 (2H, d, J = 8.3 Hz), 7.60–7.50 (3H, m), 7.28–7.18 (2H, m), 6.90 (1H, t, J = 7.5, 6.0 Hz); MS (ESI): m/z 241 $[M + H]^+$.

*4.1.3.8. 3-(4-Fluorophenyl)imidazo [1,5-a]pyridine-1-carbaldehyde (**5h**).* Yield 70%, ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.11 (1H, s), 8.39 (1H, d, J = 9.0 Hz), 8.30 (1H, d, J = 6.7 Hz), 7.78 (2H, t, J = 8.3, 5.2 Hz), 7.29–7.18 (3H, m), 6.87 (1H, t, J = 6.7 Hz); MS (ESI): m/z 241 $[M + H]^+$.

*4.1.4. General procedure for the synthesis of compounds **7a-r***

The appropriate aldehyde (**5a-h**) (1 mmol) was dissolved in methanol (50 mL) and treated with appropriate oxindole (**6a-d**) (1 mmol) and piperidine (1 mL). the reaction mixture was refluxed 3 h and the precipitate formed on cooling was collected by filtration. Compounds were purified by column chromatography by using chloroform and methanol as solvent system.

*4.1.4.1. 3-[(E)-1-(3-Phenylimidazo [1,5-a]pyridin-1-yl)methylidene]-2-indolinone (**7a**).* Yield 70%, mp: 258–262 °C; ^{1}H NMR (300 MHz, DMSO): δ 10.43 (1H, s), 9.42 (1H, d, *J* = 7.7 Hz), 8.62 (1H, d, *J* = 7.1 Hz), 8.21 (1H, d, *J* = 9.0 Hz), 7.98 (2H, d, *J* = 7.1 Hz), 7.85 (1H, m), 7.74–7.55 (3H, m), 7.30 (1H, dd, *J* = 6.7,6.6 Hz), 7.17 (1H, t, *J* = 7.7, 7.5 Hz), 7.05–6.96 (2H, m), 6.84 (1H, d, *J* = 7.5 Hz); ^{13}C NMR (150 MHz, DMSO-d6): δ 169.95, 141.46, 132.92, 129.80, 129.54, 129.31, 128.17, 128.06, 127.16, 125.96, 125.87, 124.62, 123.78, 123.05, 120.99, 120.81, 120.17, 117.61, 115.55, 108.94; MS (ESI): *m/z* 338 [M + H]$^+$; HRMS calcd for $C_{22}H_{16}N_3O$ [M + H]$^+$ 338.1288, found 338.1288.

*4.1.4.2. 1-Methyl-3-[(E)-1-(3-phenylimidazo [1,5-a]pyridin-1-yl) methylidene]-2-indolinone (**7b**).* Yield 65%, mp: 246–251 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.54 (1H, d, *J* = 7.8 Hz), 8.37 (1H, d, *J* = 6.8 Hz), 8.04 (1H, s), 7.97 (1H, d, *J* = 8.7 Hz), 7.93 (1H, d, *J* = 6.8 Hz), 7.61 (2H, t, *J* = 7.8, 6.8 Hz), 7.54 (1H, d, *J* = 7.8 Hz), 7.28–7.25 (2H, m), 7.12–7.07 (2H, m), 6.83 (1H, d, *J* = 7.8 Hz), 6.80 (1H, t, *J* = 6.8 Hz), 3.34 (3H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ 140.19, 136.09, 134.57, 129.48, 129.42, 129.13, 129.07, 128.15, 128.06, 127.94, 126.52, 123.96, 122.99, 122.82, 122.62, 121.86, 120.55, 118.17, 114.71, 107.05, 26.15; MS (ESI): *m/z* 352 [M + H]$^+$; HRMS calcd for $C_{23}H_{18}N_3O$ [M + H]$^+$ 352.1449, found 352.1434.

*4.1.4.3. 3-(E)-1-[3-(4-Methoxyphenyl)imidazo [1,5-a]pyridin-1-yl] methylidene-2-indolinone (**7c**).* Yield 80%, mp: 286–291 °C; ^{1}H NMR (500 MHz, DMSO): δ 10.23 (1H, s), 9.35 (1H, d, *J* = 7.2 Hz), 8.49 (1H, d, *J* = 7.2 Hz), 8.05 (1H, d, *J* = 9.3 Hz), 7.87 (2H, d, *J* = 8.2 Hz), 7.80 (1H, s), 7.23–7.05 (4H, m), 6.97–6.86 (2H, m), 6.81 (1H, d, *J* = 8.2 Hz), 3.90 (3H, s); MS (ESI): *m/z* 368 [M + H]$^+$; HRMS calcd for $C_{23}H_{18}N_3O_2$ [M + H]$^+$ 368.1399, found 368.1383.

*4.1.4.4. 3-(E)-1-[3-(4-Methoxyphenyl)imidazo [1,5-a]pyridin-1-yl] methylidene-1-methyl-2-indolinone (**7d**).* Yield 76%, mp: 257–262 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.53 (1H, d, *J* = 6.8 Hz), 8.29 (1H, d, *J* = 7.8 Hz), 8.02 (1H, s), 7.93 (1H, d, *J* = 8.7 Hz), 7.83 (2H, d, *J* = 7.8 Hz), 7.33–7.21 (1H, m), 7.20–7.00 (4H, m), 6.83 (1H, d, *J* = 7.8 Hz), 6.76 (1H, t, *J* = 6.8 Hz), 3.92 (3H, s), 3.34 (3H, s); MS (ESI): *m/z* 382 [M + H]$^+$; HRMS calcd for $C_{24}H_{20}N_3O_2$ [M + H]$^+$ 382.1555, found 382.1541.

*4.1.4.5. 5-Fluoro-3-(E)-1-[3-(4-methoxyphenyl)imidazo [1,5-a]pyridin-1-yl]methylidene-2-indolinone (**7e**).* Yield 82%, mp: 317–321 °C; ^{1}H NMR (500 MHz, DMSO): δ 10.03 (1H, s), 8.98 (1H, d, *J* = 8.8 Hz), 8.20 (1H, d, *J* = 6.8 Hz), 7.83 (1H, d, *J* = 9.7 Hz), 7.53 (3H, t, *J* = 8.8, 7.8 Hz), 6.94 (1H, t, *J* = 8.8, 6.8 Hz), 6.84 (2H, d, *J* = 7.8 Hz), 6.67–6.57 (2H, m), 6.44 (1H, dd, *J* = 8.8, 7.8 Hz), 3.92 (3H, s); ^{13}C NMR (150 MHz, DMSO-d6): δ 169.92, 160.23, 155.84, 140.31, 137.53, 136.41, 129.54, 126.69, 124.91, 124.27, 123.87, 121.05, 117.52, 115.49, 114.65, 113.95, 113.63, 113.05, 112.69, 109.06, 55.36; MS (ESI): *m/z* 386 [M + H]$^+$; HRMS calcd for $C_{23}H_{17}FN_3O_2$ [M + H]$^+$ 386.1304, found 386.1288.

*4.1.4.6. 3-(E)-1-[3-(3,4-Dimethoxyphenyl)imidazo [1,5-a]pyridin-1-yl]methylidene-2-indolinone(**7f**).* Yield 68%, mp: 258–263 °C; ^{1}H NMR (300 MHz, DMSO): δ 10.44 (1H, s), 9.47 (1H, d, *J* = 7.5 Hz), 8.66 (1H, d, *J* = 6.9 Hz), 8.18 (1H, d, *J* = 9.2 Hz), 7.85 (1H, s), 7.57 (2H, m), 7.35–7.16 (3H, m), 7.05–6.95 (2H, m), 6.87 (1H, d, *J* = 7.5 Hz), 3.92 (3H, s), 3.90 (3H, s); ^{13}C NMR (150 MHz, DMSO-d6): δ 170.01, 149.87, 149.07, 141.45, 140.06, 136.02, 128.09, 126.89, 126.16, 124.41, 123.97, 123.18, 122.95, 121.38, 120.68, 120.47, 119.98, 117.57, 115.46, 112.01, 111.46, 108.96, 55.67, 55.50; MS (ESI): *m/z* 398 [M + H]$^+$; HRMS calcd for $C_{24}H_{20}N_3O_3$ [M + H]$^+$ 398.1504, found 398.1495.

*4.1.4.7. 3-(E)-1-[3-(3,4-Dimethoxyphenyl)imidazo [1,5-a]pyridin-1-yl]methylidene-1-methyl-2-indolinone (**7g**).* Yield 74%, mp: 218–223 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.59 (1H, d, *J* = 7.3 Hz), 8.37 (1H, d, *J* = 7.1 Hz), 8.03 (1H, s), 7.97 (1H, d, *J* = 9.2 Hz), 7.54 (1H, d, *J* = 6.2 Hz), 7.44 (1H, dd, *J* = 8.3, 8.1 Hz), 7.29–7.23 (1H, m), 7.10–7.01 (3H, m), 6.86–6.75 (2H, m), 4.01 (3H, s), 3.99 (3H, s), 3.34 (3H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ 150.08, 149.76, 149.16, 141.36, 140.02, 136.03, 128.06, 127.85, 126.48, 124.04, 122.85, 122.73, 122.33, 122.15, 121.63, 120.38, 120.00, 118.20, 114.68, 111.77, 111.09, 107.11, 56.00, 55.68, 26.12; MS (ESI): *m/z* 412 [M + H]$^+$; HRMS calcd for $C_{25}H_{22}N_3O_3$ [M + H]$^+$ 412.1661, found 412.1643.

*4.1.4.8. 3-(E)-1-[3-(3,4,5-Trimethoxyphenyl)imidazo [1,5-a]pyridin-1-yl]methylidene-2-indolinone (**7h**).* Yield 65%, mp: 223–228 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.56 (1H, d, *J* = 7.1 Hz), 8.42 (1H, d, *J* = 7.1 Hz), 8.03 (1H, s), 7.99 (1H, d, *J* = 9.0 Hz), 7.31–7.23 (1H, m), 7.15 (2H, s), 7.12–7.00 (2H, m), 6.86–6.80 (2H, m), 3.98 (6H, s), 3.97 (3H, s), 3.34 (3H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ 158.10, 156.20, 153.72, 143.01, 139.96, 136.03, 128.09, 127.97, 126.49, 124.77, 123.86, 122.91, 122.76, 122.61, 121.57, 120.52, 118.18, 114.79, 107.11, 105.42, 60.94, 56.22, 26.09; MS (ESI): *m/z* 442 [M + H]$^+$; HRMS calcd for $C_{26}H_{24}N_3O_4$ [M + H]$^+$ 442.1767, found 442.1749.

*4.1.4.9. 3-(E)-1-[3-(4-Methylphenyl)imidazo [1,5-a]pyridin-1-yl] methylidene-2-indolinone (**7i**).* Yield 71%, mp: 309–315 °C; ^{1}H NMR (300 MHz, DMSO): δ 9.38 (1H, s), 8.39 (1H, d, *J* = 7.5 Hz), 7.56 (1H, d, *J* = 7.1 Hz), 7.28 (1H, s), 7.16 (1H, d, *J* = 9.0 Hz), 6.86 (2H, d, *J* = 7.9 Hz), 6.47 (2H, d, *J* = 8.1 Hz), 6.25 (1H, dd, *J* = 6.6,6.4 Hz), 6.14 (1H, t, *J* = 7.5, 6.7 Hz), 6.03–5.92 (2H, m), 5.81 (1H, d, *J* = 7.5 Hz), 1.92 (3H, s); ^{13}C NMR (150 MHz, DMSO-d6): δ 169.91, 141.44, 140.01, 139.25, 135.98, 129.80, 128.07, 127.94, 127.02, 126.15, 126.07, 124.45, 123.74, 123.04, 122.88, 120.72, 120.09, 117.57, 115.40, 108.88, 20.96; MS (ESI): *m/z* 352 [M + H]$^+$; HRMS calcd for $C_{23}H_{18}N_3O$ [M + H]$^+$ 352.1449, found 352.1442.

*4.1.4.10. 5-Fluoro-3-(E)-1-[3-(4-methylphenyl)imidazo [1,5-a]pyridin-1-yl]methylidene-2-indolinone (**7j**).* Yield 67%, mp: 330–335 °C; ^{1}H NMR (300 MHz, DMSO): δ 9.57 (1H, s), 8.51 (1H, d, *J* = 7.8 Hz), 7.78 (1H, d, *J* = 6.9 Hz), 7.38 (1H, d, *J* = 8.6 Hz), 7.13–6.96 (3H, m), 6.62 (2H, d, *J* = 7.9 Hz), 6.46 (1H, t, *J* = 8.8, 6.9 Hz), 6.27–6.07 (2H, m), 6.02–5.90 (1H, m), 2.03 (3H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ 149.81, 141.39, 139.92, 139.19, 135.78, 129.60, 127.95, 127.81, 127.00, 126.11, 126.00, 124.32, 123.64, 122.97, 122.82, 120.70, 121.10, 117.32, 115.10, 107.13, 27.12, 20.90; MS (ESI): *m/z* 370 [M + H]$^+$; HRMS calcd for $C_{23}H_{17}FN_3O$ [M + H]$^+$ 370.1350, found 370.1367.

*4.1.4.11. 5-Fluoro-1-methyl-3-(E)-1-[3-(4-methylphenyl)imidazo [1,5-a]pyridin-1-yl]methylidene-2-indolinone (**7k**).* Yield 83%, mp: 293–297 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.56 (1H, d, *J* = 7.0 Hz), 7.92 (1H, d, *J* = 7.1 Hz), 7.54 (1H, d, *J* = 8.3 Hz), 7.33–7.01 (3H, m), 6.82 (2H, d, *J* = 8.0 Hz), 6.71–6.52 (1H, t, *J* = 7.8,6.8 Hz), 6.23–6.20 (2H, m), 6.12–6.00 (1H, m), 3.32 (3H, s), 1.98 (3H, s); MS (ESI): *m/z* 384 [M + H]$^+$; HRMS calcd for $C_{24}H_{19}FN_3O$ [M + H]$^+$ 384.1512, found 384.1499.

*4.1.4.12. 3-(E)-1-[3-(2-Fluorophenyl)imidazo [1,5-a]pyridin-1-yl]methylidene-2-indolinone (**7l**).* Yield 78%, mp: 288–293 °C; ^{1}H NMR

(300 MHz, DMSO): δ 9.43 (1H, s), 8.31 (1H, d, J = 7.5 Hz), 7.23 (2H, d, J = 9.6 Hz), 6.94–6.86 (2H, m), 6.71 (1H, q, J = 7.3, 6.4 Hz), 6.54 (2H, q, J = 7.9, 6.7 Hz), 6.32 (1H, dd, J = 6.7,6.4 Hz), 6.15 (1H, t, J = 8.3, 7.7 Hz), 6.03 (1H, t, J = 7.1, 6.9 Hz), 5.92 (1H, t, J = 7.7, 6.9 Hz), 5.84 (1H, d, J = 7.7 Hz); ^{13}C NMR (150 MHz, DMSO-d6): δ 169.90, 158.30, 143.03, 136.02, 135.68, 132.27, 131.71, 128.26, 126.13, 125.44, 124.68, 124.10, 122.90, 122.73, 120.76, 120.58, 117.44, 116.86, 117.74, 116.48, 115.37, 108.92; MS (ESI): *m/z* 356 $[M + H]^+$; HRMS calcd for $C_{22}H_{15}FN_3O$ $[M + H]^+$ 356.1199, found 356.1208.

*4.1.4.13. 3-(E)-1-[3-(2-Fluorophenyl)imidazo [1,5-a]pyridin-1-yl]methylidene-1-methyl-2-indolinone (**7m**).* Yield 72%, mp: 260–265 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.37 (1H, d, J = 7.2 Hz), 7.52 (2H, d, J = 8.2 Hz), 7.20–6.90 (2H, m), 6.85 (1H, m), 6.58 (2H, q, J = 6.8, 6.2 Hz), 6.42 (1H, m), 6.21 (1H, t, J = 6.3, 6.0 Hz), 6.09 (1H, t, J = 7.1, 6.0 Hz), 6.01 (1H, t, J = 7.2, 6.0 Hz), 5.89 (1H, d, J = 6.3 Hz), 3.52 (3H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ 161.61, 158.29, 143.03, 136.02, 135.74, 132.16, 131.69, 131.59, 128.03, 126.50, 125.06, 123.89, 123.55, 123.46, 123.21, 122.76, 121.89, 117.79, 116.47, 116.18, 114.48, 107.09, 26.14; MS (ESI): *m/z* 370 $[M + H]^+$; HRMS calcd for $C_{23}H_{17}FN_3O$ $[M + H]^+$ 370.1355, found 370.1371.

*4.1.4.14. 5-Fluoro-3-(E)-1-[3-(2-fluorophenyl)imidazo [1,5-a]pyridin-1-yl]methylidene-2-indolinone (**7n**).* Yield 65%, mp: 341–346 °C; ^{1}H NMR (300 MHz, DMSO): δ 9.62 (1H, s), 8.42 (1H, d, J = 7.9 Hz), 7.44 (2H, d, J = 8.6 Hz), 7.12–7.03 (2H, m), 6.99–6.81 (1H, m), 6.78–6.61 (2H, m), 6.54 (1H, dd, J = 6.9,6.4 Hz), 6.24 (1H, t, J = 7.1, 6.4 Hz), 6.15 (1H, t, J = 9.2, 8.8 Hz), 6.01–5.93 (1H, m); MS (ESI): *m/z* 374 $[M + H]^+$; HRMS calcd for $C_{22}H_{14}F_2N_3O$ $[M + H]^+$ 374.1099, found 374.1113.

*4.1.4.15. 5-Fluoro-3-(E)-1-[3-(2-fluorophenyl)imidazo [1,5-a]pyridin-1-yl]methylidene-1-methyl-2-indolinone (**7o**).* Yield 60%, mp: 297–302 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.62 (1H, d, J = 8.0 Hz), 7.52 (2H, d, J = 7.9 Hz), 7.11–7.08 (2H, m), 7.03–6.94 (1H, m), 6.87–6.72 (2H, m), 6.64 (1H, dd, J = 6.8,6.5 Hz), 6.32 (1H, t, J = 7.2, 6.8 Hz), 6.23 (1H, m), 6.11–5.98 (1H, m), 3.54 (3H, s); MS (ESI): *m/z* 388 $[M + H]^+$; HRMS calcd for $C_{23}H_{16}F_2N_3O$ $[M + H]^+$ 388.1261, found 388.1249.

*4.1.4.16. 3-(E)-1-[3-(3-Fluorophenyl)imidazo [1,5-a]pyridin-1-yl] methylidene-1-methyl-2-indolinone (**7p**).* Yield 72%, mp: 228–232 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.43 (1H, d, J = 7.5 Hz), 8.39 (1H, d, J = 7.1 Hz), 8.01–7.95 (2H, m), 7.72–7.51 (3H, m), 7.29–7.19 (2H, m), 7.10 (2H, t, J = 8.6, 6.7 Hz), 6.84–6.80 (2H, m), 3.34 (3H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ 161.41, 143.06, 136.19, 130.90, 130.78, 128.49, 128.17, 126.47, 123.72, 123.59, 123.56, 123.20, 122.70, 122.46, 120.98, 118.29, 116.55, 116.26, 115.31, 115.09, 115.00, 107.19, 26.17; MS (ESI): *m/z* 370 $[M + H]^+$; HRMS calcd for $C_{23}H_{17}FN_3O$ $[M + H]^+$ 370.1355, found 370.1366.

*4.1.4.17. 3-(E)-1-[3-(4-Fluorophenyl)imidazo [1,5-a]pyridin-1-yl] methylidene-2-indolinone (**7q**).* Yield 68%, mp: 290–295 °C; ^{1}H NMR (300 MHz, DMSO): δ 10.12 (1H, s), 9.30 (1H, d, J = 7.9 Hz), 8.44 (1H, d, J = 6.6 Hz), 8.05–7.77 (4H, m), 7.34 (2H, t, J = 8.3 Hz), 7.13 (2H, q, J = 8.3, 8.1 Hz), 6.98–6.78 (3H, m); MS (ESI): *m/z* 356 $[M + H]^+$; HRMS calcd for $C_{22}H_{15}FN_3O$ $[M + H]^+$ 356.1193, found 356.1209.

*4.1.4.18. 5-Fluoro-3-(E)-1-[3-(4-fluorophenyl)imidazo [1,5-a]pyridin-1-yl]methylidene-2-indolinone (**7r**).* Yield 81%, mp: 335–340 °C; ^{1}H NMR (300 MHz, DMSO): δ 10.14 (1H, s), 9.47 (1H, d, J = 7.2 Hz), 8.52 (1H, d, J = 6.2 Hz), 8.22–8.02 (3H, m), 7.67 (2H, t, J = 8.1 Hz), 7.32 (2H, q, J = 8.2, 7.9 Hz), 7.02–6.94 (3H, m); MS (ESI): *m/z* 374 $[M + H]^+$; HRMS calcd for $C_{22}H_{14}F_2N_3O$ $[M + H]^+$ 374.1099, found 374.1097.

4.2. Cell culture

The antiproliferative activities of the compounds were determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction assay [26]. 1×10^4 cells/well were seeded in 100 mL DMEM (Dulbecco's Modified Eagle's Medium) 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. Compounds, diluted to the desired concentrations in culture medium. After 24 h of incubation, media was removed and to each well 10 μl of MTT (5 mg/mL) was added and the plates were further incubated for 4 h. Supernatant from each well was carefully removed, formazon crystals were dissolved in 100 μL of DMSO and absorbance at 540 nm of wavelength was recorded.

4.3. In vitro growth percentage

The screening of anticancer compounds in National Cancer Institute is a two step process. In first step the evaluation of all compounds against the 60 cell lines at a single concentration of 10^{-5} M. Compounds which exhibit significant growth inhibition are selected for second step. In second step compounds are evaluated against the 60 cell panel at five concentration levels by the NCI according to standard procedures (http://dtp.nci.nih.gov/branches/btb/ivclsp.html).

4.4. Cytotoxicity assay in HEK-293

Cytotoxic effects of the compounds were determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction assay [26]. 1×10^4 cells/well 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. Compound (**7l**), diluted to the desired concentrations in culture medium. After 48 h of incubation, media were removed and to each well 10 μl of MTT (5 mg/mL) was added and the plates were further incubated for 4 h. Supernatant from each well was carefully removed, formazon crystals were dissolved in 100 μl of DMSO and absorbance at 540 nm wavelength was recorded.

4.5. Cell cycle analysis

To determine the effect of compound on the stages of cell cycle, A549 cells (1×10^6) were seeded in six-well plates and treated with compound **7l** at a final concentration of 6.5 μM for 24 h and 48 h. After 24 h and 48 h treatments, both floating and trypsinized adherent cells were collected and fixed with 70% ethanol. After fixation cells were washed with PBS and stained with 50 μg/mL propidium iodide in hypotonic lysis buffer (0.1% sodium citrate, 0.1% Triton X-100) containing DNase-free RNase-A for 20 min. Stained cells were analyzed using fluorescence-activated cell sorter caliber (Becton Dickinson) [26].

4.6. Morphological analysis for apoptosis with Hoechst staining

Cells were seeded at a density of 10,000 cells over 18-mm coverslips and incubated for 24 h. Then, the medium was replaced, and cells were treated with 6.5 μM 24 h and 48 h. Cells treated with vehicle (0.001% DMSO) were included as controls for all experiments. After overnight treatment, Hoechst 33342 (Sigma–Aldrich) were added to medium at a concentration of 0.5 μg/mL containing 4% paraformaldehyde. After incubation for 30 min 37 °c, cells from each dish were captured from randomly selected fields under fluorescent microscope (Leica, Germany) to qualitatively determine the proportion of viable and apoptotic

cells based on their relative fluorescence and nuclear fragmentation [27].

4.7. Flow cytometric evaluation of apoptosis

A549 cells (1×10^6) were seeded in six-well plates and allowed to grow overnight. The medium was then replaced with complete medium containing 6.5 μM concentration of compound, **7l** for 24 h and 48 h with vehicle alone (0.001% DMSO) as control. After 24 h and 48 h treatment, cells from the supernatant and adherent monolayer cells were harvested by trypsinization, washed with PBS at 3000 rpm. Then the cells (1×10^6) were stained with Annexin V-FITC and propidium iodide using the Annexin-V-PI apoptosis detection kit (Invitrogen). Flow cytometry was performed using a FACScan (Becton Dickinson) equipped with a single 488-nm argon laser as described earlier [28]. Annexin V-FITC was analyzed using excitation and emission settings of 488 nm and 535 nm (FL-1 channel); PI, 488 nm and 610 nm (FL-2 channel). Debris and clumps were gated out using forward and orthogonal light scatter. The experiment was repeated three times.

4.8. DNA laddering assay

Cells were seeded (1×10^6) in six well plates. After incubation for 24 h and 48 h cells were treated with compound **7l** (6.5 μM). After 24 h and 48 h cells were collected and centrifuge 2500 rpm for 5 min at 4 °C. Pellet was collected and washed with Phosphate buffered saline (PBS). And added 100 μl of Lysis buffer, centrifuged at 3000 rpm for 5 min at 4 °c and collected supernant. And add 10 μl of 10% SDS and 10 μl of (50 mg/mL) RNase-A and incubated for 2 h at 56 °c. After that 10 μl (25 mg/mL) Proteinase K and added 65 μl of 10 M Ammonium acetate and 500 μl of ice cold ethanol and mixed well. And this sample was incubated in −80° for 1 h. After incubatation samples were centrifuged at 12000 rpm for 20 min at 4 °c and washed with 80% ethanol air dried for 10 min at room temperature. Dissloved the pellet in 50 μl of TE buffer. After that, DNA laddering was determined by 2% agarose gel electrophoresis. in 50 μl of TE Buffer.

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