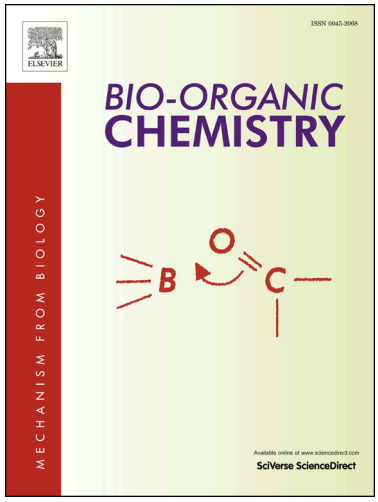

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PII: S0045-2068(16)30068-2
DOI: http://dx.doi.org/10.1016/j.bioorg.2016.09.002
Reference: YBIOO 1948

To appear in: *Bioorganic Chemistry*

Received Date: 10 June 2016
Revised Date: 1 September 2016
Accepted Date: 2 September 2016

Please cite this article as: A.V. Subba Rao, M.V.P.S. Vishnu Vardhan, N.V. Subba Reddy, T. Srinivasa Reddy, S.P. Shaik, C. Bagul, A. Kamal, Synthesis and Biological Evaluation of Imidazopyridinyl-1,3,4-oxadiazole conjugates as Apoptosis Inducers and Topoisomerase IIα Inhibitors, *Bioorganic Chemistry* (2016), doi: http://dx.doi.org/10.1016/j.bioorg.2016.09.002

Synthesis and Biological Evaluation of Imidazopyridinyl-1,3,4-oxadiazole conjugates as Apoptosis Inducers and Topoisomerase IIα Inhibitors

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Abstract

A series of imidazopyridinyl-1,3,4-oxadiazole conjugates were synthesized and investigated for their cytotoxic activity and some compounds showed promising cytotoxic activity. Compound **8q** (NSC: 763639) exhibited notable growth inhibition that satisfies threshold criteria at single dose (10 µM) on all human cancer cell lines. This compound was further evaluated at five dose levels (0.01, 0.1, 1, 10 and 100 µM) to obtain GI_{50} values ranging from 1.30-5.64 µM. Flow cytometric analysis revealed that compound **8q** arrests the A549 cells in sub G1 phase followed by induction of apoptosis which was further confirmed by Annexin-V-FITC, Hoechst nuclear staining, caspase 3 activation, measurement of mitochondrial membrane potential and ROS generation. Topo II mediated DNA relaxation assay results showed that conjugate **8q** could significantly inhibit the activity of Topo II. Moreover, molecular docking studies also indicated binding to the topoisomerase enzyme (PDBID 1ZXN).

Keywords: Imidazopyridine, oxadiazole, cytotoxicity, topoisomerase, molecular docking.

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1. Introduction

Cancer is a group of heterogeneous diseases with dysregulation of cell functioning, which ultimately alter the gene transcription programs in the cells which causing death. It is remained as an unsolvable challenge to both physicians and scientists second leading cause of death in developing as well as developed countries.[1,2] Although chemotherapy is the mainstay of cancer therapy, the use of available chemotherapeutics is often limited mainly due to undesirable side effects and it clearly underscores the need of developing novel chemotherapeutic agents for more effective cancer treatments.[3] The search for such potential anticancer agents has been designed to the discovery of small synthetic molecules with promising anticarcinogenic activity. Apoptosis or programmed cell death is a critical cellular process for the maintenance of tissue homeostasis and normal development of multicellular organisms. Inappropriate regulation of apoptosis signaling pathways may lead to several diseases including cancer.[4] In tumor cells, the pathways that lead to apoptosis are disrupted or impaired due to which cells undergo malignant transformation. Therefore, targeting critical apoptosis regulators with a goal to induce apoptosis in cancer cells has emerged as an attractive strategy in cancer chemotherapy.[5] DNA topoisomerase (topo) is recognized as an important target in anticancer drug discovery.[6-10] Topoisomerases I and II are nuclear enzymes that modulate in maintaining the topological changes of DNA and play a crucial role in the cellular processes such as transcription, replication and chromosome segregation to resolve the topological problems of DNA double helix during the repeated cycles of DNA cleavage and ligation.[11] Human topo II exists in two isoforms namely topo-IIα and topo-IIβ and the level of topo-IIβ is constant throughout the cell cycle, whereas the level of topo-IIα is higher in rapidly proliferating cells.[12] From the recent advances it has been evident that drug molecules that act on topo II enzyme can be divided into topo-II poisons that form a stabilized cleavable covalent ternary complex and cause apoptosis by blocking the relegation and enzyme release step. Whereas, topo-II catalytic inhibitors act on the catalytic steps.[13] ATP sensitive topo-II poisons may specifically target on ATP-bound topo-II and it works as a sliding protein clamp.[14] It has long been accepted that topoisomerases are valuable therapeutic targets with the development of a number of topo II inhibitors, such as etoposide (**A**, **Figure 1**).[15]

Nitrogen heterocycles are the most significant structural components of pharmaceuticals. Analysis of database of U.S. FDA approved drugs reveal that 59% of unique small-molecule drugs contain a nitrogen-bridgehead fused heterocycles.[16] Among synthesized small molecules, imidazopyridine[17] is one of the most important heterocyclic systems from the bicyclic N-fused imidazoles which are known to display a wide range of activities for diverse number of targets. Imidazopyridines are known to display broad spectrum of biological activities such as inhibitors of aromatase estrogen production suppressors,[18] platelet aggregation inhibitors, positive inotropic agents,[19] thromboxane synthetase inhibitors,[20] antibacterial,[21] antiviral,[22] hypnoselective and anxioselective activities.[23] Imidazole ring containing N-fused bicyclic system has possessed marketed drugs, such as zolpidem (hypnotic, **B**) and zolimidine (antiulcer, **C**), possess structural resemblance to these antitopoisomerase II biaryls (**Figure 1**).[24,25] Moreover, 1,3,4-oxadiazoles are pharmalogically important molecules that are known to possess significant biological activities such as antibacterial,[26] anti-inflammatory,[27] anticonvulsant,[28] anticancer,[29] analgesic,[30] antiviral[31] and antifungal properties.[32] Further, 1,3,4-oxadiazole heterocycles are very good bioisosteres of amides and esters, which can contribute substantially in increasing pharmacological activity by participating in hydrogen bonding interactions with the receptors.[33]

As part of our ongoing efforts to discover newer anticancer agents, we previously reported the synthesis of a number of heterocyclics like imidazo[1,5-*a*]pyridine-benzimidazole hybrids,[34] oxindole derived imidazopyridines, (**D, Figure 1**)[35] imidazo[1,5-*a*]pyridine–PBD conjugates[36] and 2-aryl-1,2,4-oxadiazolo-benzimidazoles conjugates.[37] The promising biological activity exhibited by such conjugates prompted us to explore some newer molecules by linking the two pharmacophores such as imidazopyridine and oxadiazole scaffolds with a view to enhance their anticancer activity. In this context, we have designed and synthesized imidazopyridine-oxadiazole conjugates (**E**, **8a–s**, **Figure 1**) that were evaluated for their anticancer potential at the Development Therapeutic Program (DTP) of the National Cancer Institute (NCI), USA, against a panel of sixty cancer cell lines and their detailed biological implication relating to apoptotic cell death apart from their effect on DNA topoisomerase-IIa inhibition.

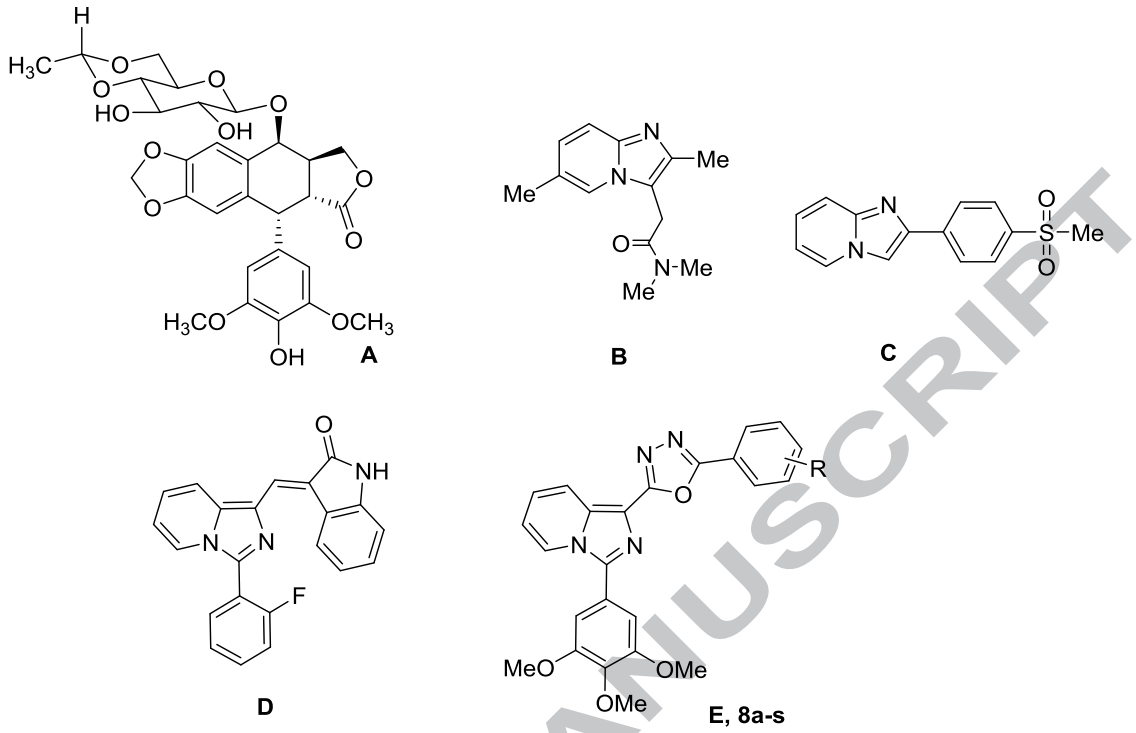
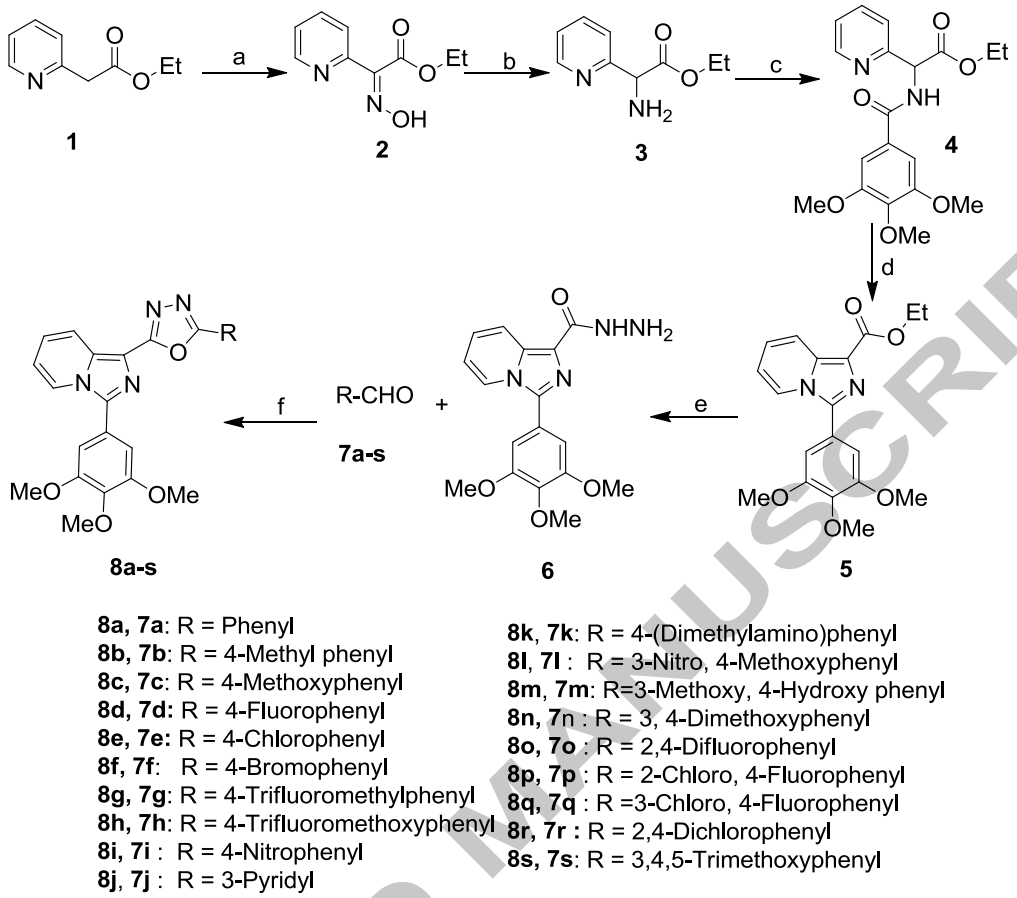


Figure 1: Topoisomerase II inhibitor etoposide (**A**), imidazopyridine derivatives (**B, C, D** and target molecules **E**, **8a-s**).

2. Results and discussions

2.1 Chemistry

The synthesis of imidazopyridine oxadiazole conjugates (**8a-s**) was carried out by using commercially available ethyl 2-(2-pyridyl)acetate (**1**) as the starting material and is illustrated in **Scheme 1**. The reaction of **1** with sodium nitrite in acetic acid yielded **2** with 83% yield which was reduced with Pd/C in methanol to give the amino intermediate (**3**) in 76% yield. Treatment of this amine (**3**) with trimethoxybenzoylchloride and triethylamine in tetrahydrofuran at 0 °C afforded the amide (**4**) with 74% yield and the cylization of this amide was carried out by using phosphorous oxychloride at 120 °C to provide imidazo[1,5-*a*]pyridine intermediate (**5**) with 70% yield.[38] The reaction of **5** with hydrazine hydrate in isopropanol afforded the corresponding hydrazide in 82% yield. The desired oxadiazole conjugates (**8a-s**) were obtained by the reaction of Schiff bases, which were obtained from the hydrazide and aldehydes (**7a-s**), in the presence of iodine and potassium carbonate in dimethyl sulphoxide at 80 °C [39] with range of 63-74% yields.



Scheme : *Reagents and Conditions*: a) aq.$NaNO_2$, AcOH, 3 h, 83%; b) Pd/C, MeOH, 8 h, 76%; c) 3,4,5-trimethoxy benzoyl chloride, TEA, Dry THF, 0 °C to rt, 3 h, 74%; d) $POCl_3$, 120 °C, 3 h, 70%; e) $NH_2NH_2{\cdot}H_2O$, isopropanol, 80 °C, 6 h, 82%; f) I_2, K_2CO_3, DMSO, 80 °C, 4 h, 63-74%.

3.0 Biology

3.1 Cytotoxic activity

All the synthesized oxadiazole linked imidazopyridines (**8a-s**) were submitted to the National Cancer Institute (NCI), Bethesda for evaluation of cytotoxic activity. Initially these were evaluated at a single concentration of 10 µM towards a panel of approximately sixty cancer cell lines derived from nine different cancer types (leukemia, lung, colon, CNS, melanoma, ovarian, renal, prostate and breast). Primary cytotoxic assays were performed according to the NCI protocols.[40-42] These compounds were added at a single concentration and the cell culture was incubated for 48 h and end point determinations were made by using SRB method. The results of

each compound are reported as the percentage of growth (GP) of the treated cells in comparison to the untreated control cells as shown in **Table 1**. Some structure activity relationship could be observed based on the number of cell lines that displayed sensitivity towards each of the newly synthesized compounds. Among all compounds, the compounds **8c**, **8k**, and **8l** having electron releasing groups (Methoxy and N-Methyl) at para- position of aromatic ring have shown better anti-proliferative activity with 63.05, 69.69 and 69.61 % mean growth. The activity was also influenced by the presence of halogen substituents like fluorine, chlorine and bromine at para position. For instance, compounds **8d** (fluoro), **8e** (chloro) and **8f** (bromo) showed moderate growth inhibition. In contrast, compounds having di substitution with fluoro group at *para* and chloro at *meta* position resulted in most active compound **8q** with 33.54 mean growth percent. Based on the results of primary screening, **8q** was selected for the five dose screening to determine its GI50 (the molar concentration required to cause 50% growth inhibition) and the data is shown in **Table 2**.

Table 1: Sensitivity, NSC: code, growth percent, mean growth percent of NCI cancer cell lines treated with synthesized compounds (10 µM)

Compound	NSC code	Mean growth	Range of growth	Most sensitive cell line	Growth % of most sensitive cell line
8a	**NSC:** 761871 / 1	81.62	14.74 to 116.16	OVCAR (Ovarian)	14.74
8b	**NSC:** 761874 / 1	78.69	37.16 to 115.02	RPMI-8226 (Leukemia)	37.16
8c	**NSC:** 761861 / 1	63.05	18.57 to 105.78	UO-31 (Renal)	18.57
8d	**NSC:** 761872/ 1	78.96	45.80 to 113.02	RPMI-8226 (Leukemia)	45.80
8e	**NSC:** 761860 / 1	77.54	34.82 to 126.65	RPMI-8226 (Leukemia)	34.82
8f	**NSC:** 763709 / 1	86.46	38.71 to 121.17	HOP-62(Non-Small Cell Lung Cancer)	38.71
8h	**NSC:** 761124 / 1	72.12	35.10 to 115.79	UO-31 (Renal)	35.10
8j	**NSC:** 761877 / 1	100.09	60.85 to 129.20	RPMI-8226 (Leukemia)	60.85
8k	**NSC:** 761875 / 1	69.69	22.10 to 106.60	RPMI-8226(Leukemia)	22.10
8l	**NSC:** 763622 / 1	69.61	8.84 to 105.54	EKVX(Non-Small Cell Lung Cancer)	8.84
8m	**NSC:** 763623 / 1	91.47	54.08 to 115.18	CCRF-CEM(Leukemia)	54.08
8o	**NSC:** 761878 / 1	97.76	74.67 to 126.97	SR(Leukemia)	74.67
8q	**NSC:** 763639 / 1	33.54	-65.54 to 72.94	HOP-62 (Non-Small Cell Lung Cancer)	33.54
8r	**NSC:** 761829 / 1	84.86	46.01 to 105.50	UO-31 (Renal)	68.52

This compound was dissolved in DMSO and evaluated for its cytotoxic activity at five concentrations at 10 fold dilutions (the highest being 10^{-4} M and others 10^{-5} M, 10^{-6} M, 10^{-7} M and 10^{-8} M). The results showed that the **8q** exhibited remarkable cytotoxic activity against most of the tested tumor cell lines with GI50 values in the range of 1.30-5.46 µM. This compound exhibited a distinctive pattern of sensitivity against some individual cell lines (**Table 2**), as well as a broad spectrum of cytotoxic activity. With regard to the sensitivity against some individual cell lines, it exhibited remarkable growth inhibition against SNB-75 (CNS cancer), HOP-92 (non-small cell lung cancer), A498 (renal), HCT-116 (colon) BT-549 (breast) cancer cell lines with GI50 values 1.30, 1.55, 1.55, 1.60 and 1.67 µM and less sensitive against HCC-2998

(colon) , CAKI-1 (renal) and NCI-H322M (non-small cell lung) cancer cells with GI50 5.64, 5.46, 5.05 μM. This data revealed an obvious sensitivity profile towards CNS cancer subpanel (GI_{50} value ranging from 1.30 to 3.09 μM), least for SNB-75 and maximum for SF-295 cell line. This compound also proved to be sensitive towards all the tested breast cancer cell lines with GI50 less than 3.39 μM.

Table 2: Cytotoxicity of conjugate **8q** against a panel of 60 human cancer cell lines.

Panel/Cell Line	GI_{50} (μM)[a]	Panel/Cell Line	GI_{50}(μM) [a]	Panel/Cell Line	GI_{50} (μM) [a]
Leukemia		***CNS Cancer***		OVCAR-8	1.69
K-562	4.34	SF-268	2.03	NCI/ADR-RES	1.88
RPMI-8226	1.79	SF-295	3.09	SK-OV-3	2.10
		SF-539	2.59	***Renal Cancer***	
Non-Small Cell		SNB-19	1.93	786-0	2.93
Lung Cancer		SNB-75	1.30	A498	1.55
A549/ATCC	1.95	U251	1.86	ACHN	2.29
EKVX	4.19			CAKI-1	5.46
HOP-62	2.19	***Melanoma***		RXF 393	2.62
HOP-92	1.55	LOX IMVI	3.83	SN12C	2.42
NCI-H226	2.38	MALME-3M	3.83	TK-10	2.40
NCI-H23	4.00	M14	3.27	UO-31	2.20
NCI-H322M	5.05	MDA-MB-435	3.09	***Prostate Cancer***	
NCI-H460	1.99	SK-MEL-2	2.27	PC-3	2.11
NCI-H522	1.92	SK-MEL-28	2.11	DU-145	2.56
Colon Cancer		SK-MEL-5	3.03	***Breast Cancer***	
COLO 205	4.68	UACC-257	2.23	MCF7	2.06
HCC-2998	5.64	UACC-62	2.03	MDA-MB-231/ATCC	2.15
HCT-116	1.60	***Ovarian Cancer***			
HCT-15	4.55	IGROV1	3.61	HS 578T	2.51
HT29	2.20	OVCAR-3	2.76	BT-549	1.67
KM12	2.48	OVCAR-4	1.83	T-47D	2.10
SW-620	3.05	OVCAR-5	4.57	MDA-MB-468	3.39

[a] Compound concentration required to decrease cell growth to half that of untreated cells. [b] (**8q**, NSC 763639)

3.2 Cell cycle analysis

Cell cycle analysis plays an important role in studies for anticancer screening, mechanism and cytokinetic research.[43] To gain further insight into the mode of action of **8q** as the most potent compound, we examined its effect on the cell cycle by flow cytometry in A549 cells. Interestingly, a concentration dependent change was observed in the cell cycle pattern (**Figure 2**

and **Table 3**). These results clearly demonstrate that the treatment of cells for 48 h with 2 and 4 µM of compound **8q** causes cell cycle arrest at sub G1 phase from 29.52% to 41.70% as well as subsequent reduction of cell population in G1 phase from 60.72% to 49.30% as seen in **Figure 2** and **Table 3**.

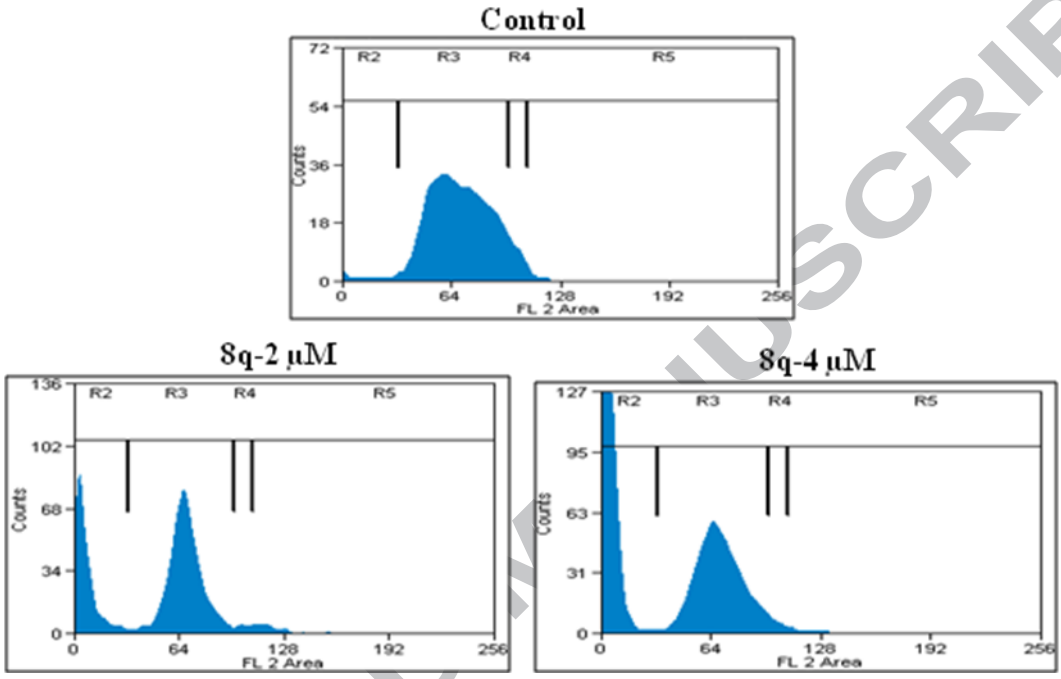


Figure 2: Flow cytometric analysis in A549 lung cancer cell lines after treatment with compound **8q** at 2 and 4 µM concentrations for 48 h.

Table 3: Effect of compound **8q** on cell cycle phase distribution in A549 cells.

Compound	Conc. [µM]	Cell cycle phase [%] Sub G1	G1	S	G2/M
Control		7.05	78.03	7.26	7.39
8q	2	29.52	60.72	2.20	7.56
8q	4	41.70	49.30	3.47	5.53

3.3 Caspase-3 Activation

Activation of caspases plays a vital role for the initiation and execution of the apoptotic process. Among the caspases, caspase-3 is one of the key effector caspases which cleave multiple proteins in cells, leading to apoptotic cell death.[44] It is well known that the cell cycle arrest at Sub G1 phase is shown to induce cellular apoptosis,[45] hence it was considered of interest to examine whether the cytotoxicity of **8q** is by virtue of apoptotic cell death. We examined that activation of caspase-3 in A549 cells treated with compound **8q** at 2 and 4 µM concentration. The results indicate that there is nearly 3 to 4-fold induction in caspase-3 activity relative to the control as shown in **Figure 3**. This indicates that 8q activates caspase activity and has the ability to induce apoptosis in A549 cancer cell lines.

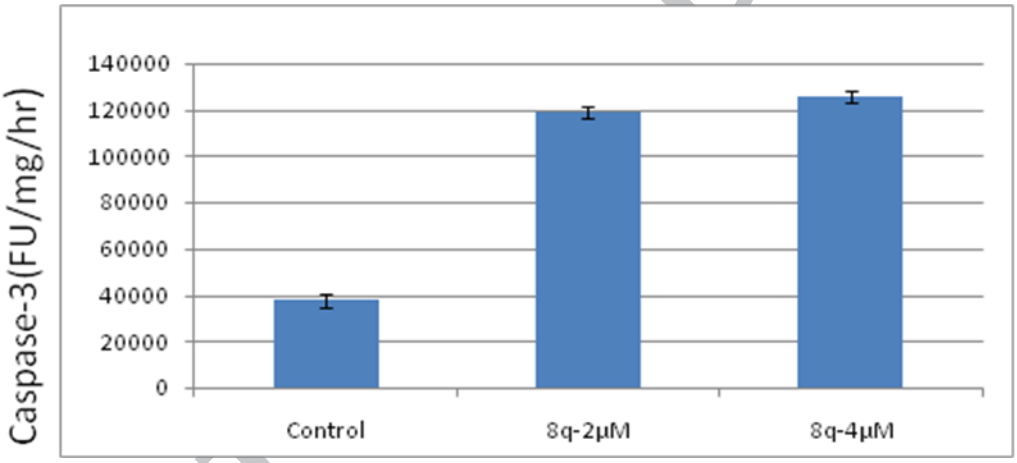


Figure 3: Effect of the compound **8q** on caspase-3 activity. A549 cells were treated with the conjugate **8q** for 48 h with 2 and 4 µM concentrations. Values indicated are the mean ± SD of two different experiments performed in triplicate.

3.4 Effect on DNA (Tunel assay and Hoechst staining)

A terminal transferase dUTP nick end labelling (TUNEL) assay[46] was carried out with a view to understand the effect of **8q** on DNA by treating with A549 cells at 2 µM for 24 h. The **Figure 4a** illustrates that DNA fragmentation (green FITC staining) was observed in the treated cells whereas there is no fragmentation in the untreated cells, thereby suggesting apoptotic cell death. Moreover, Hoechst DNA staining also indicated the formation of apoptotic bodies (blebbing) in

cells treated with conjugate **8q**, which is more prominent than untreated cells, as apparent in **Figure 4b** and the results demonstrated that **8q** have apoptotic-inducing ability.

4a)

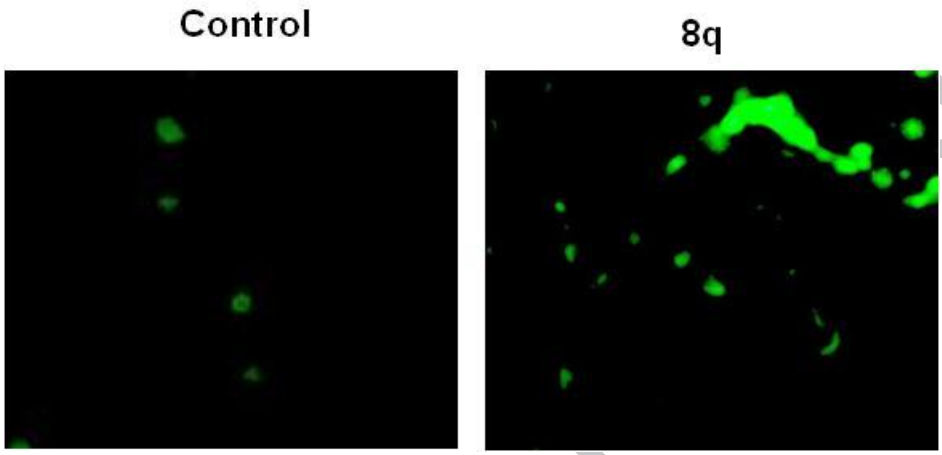


Figure 4a: Effect of compound on DNA fragmentation. A549 cells were treated with conjugate **8q** at 2 µM for 24 h. DNA fragmentation was monitored by primary antibody binding to fragmented DNA, followed by detection with FITC-labeled secondary antibody. Apoptotic cells are thus stained green. Control cells yield only back ground color. Apoptotic nature of the conjugate **8q** was detected by fragmented nucleus in the compound treated cells.

4b)

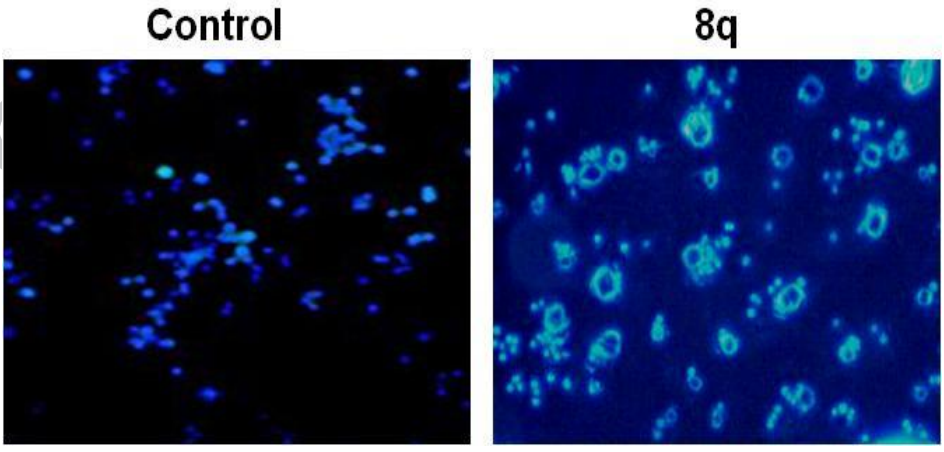


Figure 4b: Effect of conjugate **8q** on apoptosis**.** A549 cells were treated with conjugate **8q** at 2 µM for 24 h. Hoechst 33258 was used to stain the nuclei of both control and conjugate **8q**-

treated cells. As above, the apoptotic nature of the conjugate **8q** is apparent by fragmented nuclei in compound-treated cells.

3.5 Measurement of mitochondrial membrane potential ((ΔΨm)

Mitochondrial membrane potential regulates the mitochondrial permeability and plays an important role in triggering apoptosis. Loss of ΔΨm leads to depolarization of mitochondrial membrane leading to collapse of mitochondrial functions ensuing cell death.[47] In order to further investigate the apoptosis-inducing effect of **8q**, changes in mitochondrial membrane potential was measured by using JC-1 staining. Thus A549 cells were treated with **8q** at 2 µM concentration for 24 h and were analyzed for aggregates to monomers by using JC-1 dye. The percentages of JC-1 aggeregates in control cells was 6.8 % whereas in the treated compound **8q** the percentage increases to 21.5 % thus indicating loss of ΔΨm and provides evidence of apoptosis for **8q** treated cells with low mitochondrial potential as shown in **Figure 5**. These results demonstrate that mitochondrial pathway is probably playing a key role in modulating cell apoptosis caused by **8q**.

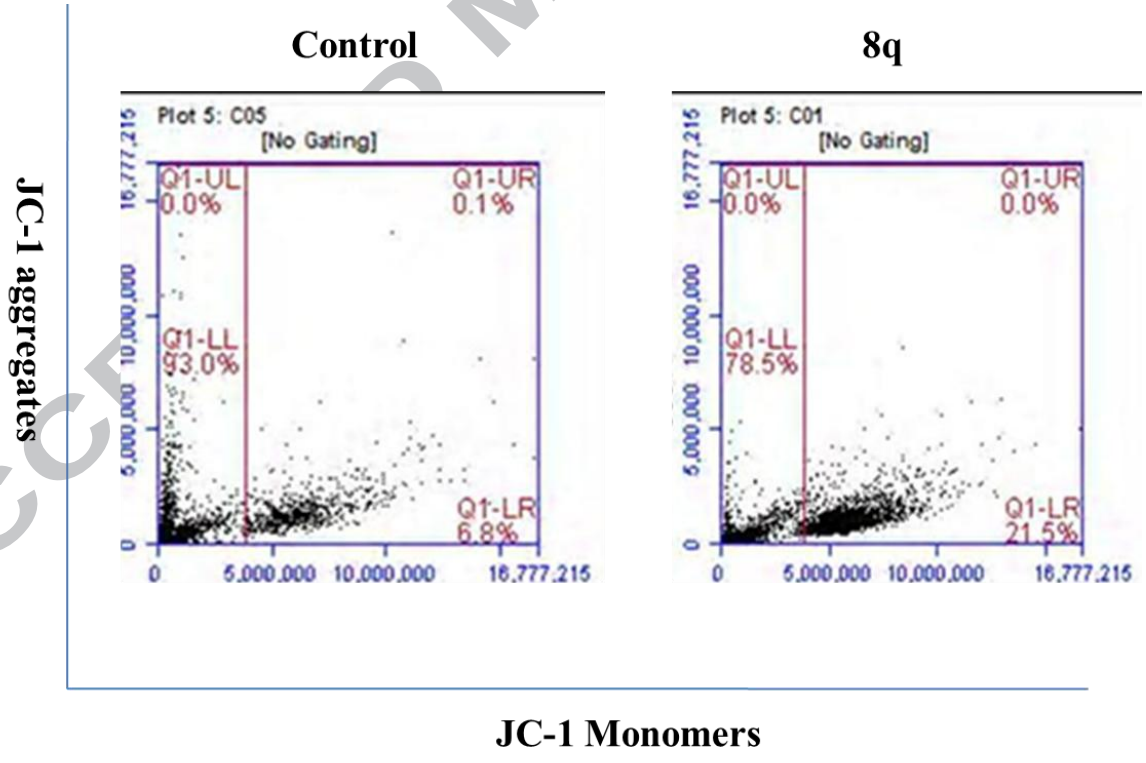


Figure 5: The loss of mitochondrial membrane potential was monitored by JC-1 staining of A549 cells treated with compound **8q** and samples were then subjected to flow cytometry analysis on a FACScan (Becton Dickinson).

3.6 Effect on intracellular ROS generation

Mitochondrial membrane depolarization is associated with mitochondrial production of ROS.[45] Therefore, we investigated whether ROS production has increased after treatment with the test compounds. Apoptosis can be initiated by oxidative stress which may be mediated by the generation of reactive oxygen species (ROS). [48,49] As **8q** is cytotoxic and induces apoptosis in A549 cells, it was considered of interest to understand its effect of **8q** on the generation of ROS. Therefore, cells were incubated with **8q** for 24 h at 2 µM concentration and the intracellular generation of ROS was examined using DCFH-DA dye by fluorescent microscopy. As shown in **Figure 6**, there was a significant increase in DCF positive cell population by **8q** as compared to the control which reveals that it induces apoptosis through intracellular ROS in A549 cells. Altogether, these results indicate that this compound induced apoptosis through the mitochondrial pathway.

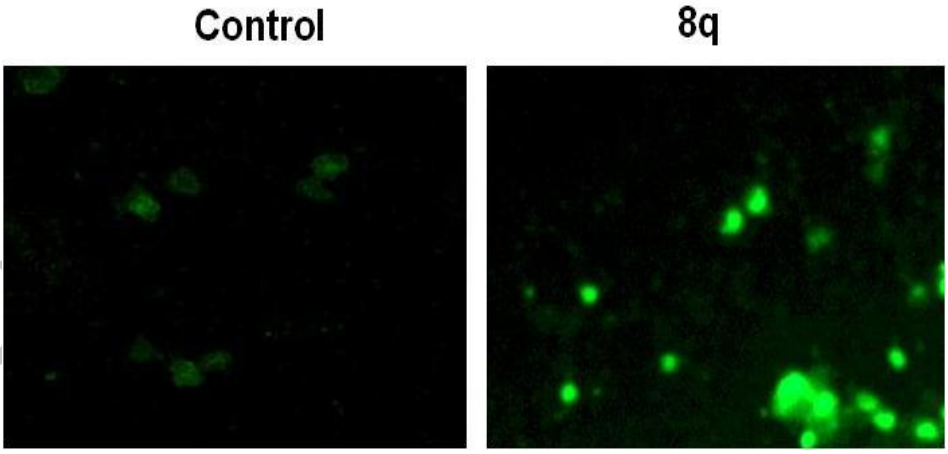


Figure 6: The effect of conjugate **8q** on the ROS production in A549 cells.

3.7 Annexin V-FITC staining

The apoptotic effect of **8q** was further evaluated by Annexin V FITC/PI (AV/PI) dual staining assay[50] to examine the occurrence of phosphatidylserine externalization and also to understand

whether it is due to physiological apoptosis or nonspecific necrosis. Thus A549 cells were treated with **8q** for 48 h at 2 µM concentrations to examine the apoptotic inducing ability. From the results it was observed that **8q** showed 22.9 % of apoptosis, whereas 14.9 % of apoptosis was observed in case of control (untreated cells) as shown in **Figure 7**. Thus this experiment suggested that **8q** induces apoptotic cell death in A549 cells.

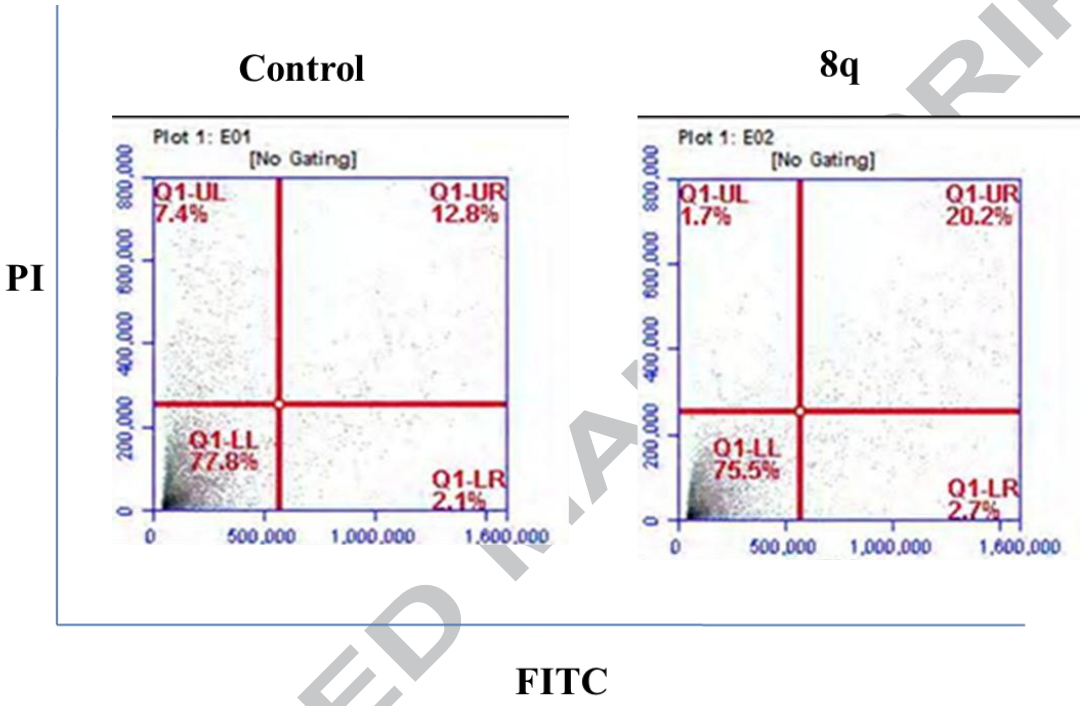


Figure 7: Annexin V-FITC staining. A549 cells were treated with compounds **8q** at 2 µM concentration for 48 h.

3.8 Effect on topoisomerase - IIα protein on 8q

DNA Topoisomerase exists in two isoforms and it is well established that throughout the cell cycle of rapidly proliferating cells topo IIα is relatively expressed higher than topo I.[51] DNA-topoisomerase II is a nuclear enzyme that controls the DNA structure by catalyzing the DNA cleavage and relegating the phosphodiester bonds. In the recent years, DNA topoisomerase II inhibitors have gained importance as clinically useful chemotherapeutical agents. The protocols described are of assays used to assess new chemical entities for their ability to inhibit both forms of DNA topoisomerase [52]. The main factors, which must be considered for assaying enzymatic activity, are temperature, pH, ionic strength and the proper concentrations of the essential

components like substrates and enzymes. Nevertheless, many enzymes, especially those from mammalian sources, possess a pH optimum near the physiological pH of 7.5, and the body temperature of about 37 °C can serve as assay temperature, although because of experimental reasons frequently 25 °C is preferred. But in many cases the particular features of the individual enzyme dictate special assay conditions, which can deviate considerably from recommended conditions. In addition, exact values for the concentrations of assay components such as substrates and enzymes cannot be given, unless general rules depending on the relative degree of saturation can be stated. Enzymes display their highest activity at their respective optimum conditions, deviations from the optimum conditions cause a reduction of the activity, depending on the degree of the deviation. Based on the above information the concentration of the sample is taken more for enzymatic assays to get the efficient results.[53] Topoisomerase II inhibition studies were performed by using topoisomerase II Drug Screening Kit (TG 1009, Topogen, USA),[54] which is suitable for screening the enzyme inhibition by both catalytic inhibitory compounds (CICs) and interfacial poisons (IFPs). CICs inhibit the activity of the enzyme, whereas IFP compounds stimulate the formation of the cleavage complexes and block the relegation which leads to form linear DNA. Catenated DNA in the presence of topo II enzyme incubated with etoposide and conjugate **8q** at concentration of 100 µM (lane E, F in control, **Figure 8**) did not show the formation of linear DNA, but most of the catenated DNA was highly super coiled and remained in the wells documenting the formation of only nicked circular and relaxed DNA, indicates that the conjugate **8q** interference in the catalytic activity of the topo II enzyme and suggesting that the role of **8q** as catalytic inhibitory compound. This assay suggests that conjugate **8q** affect the topoisomerase II enzyme activity.

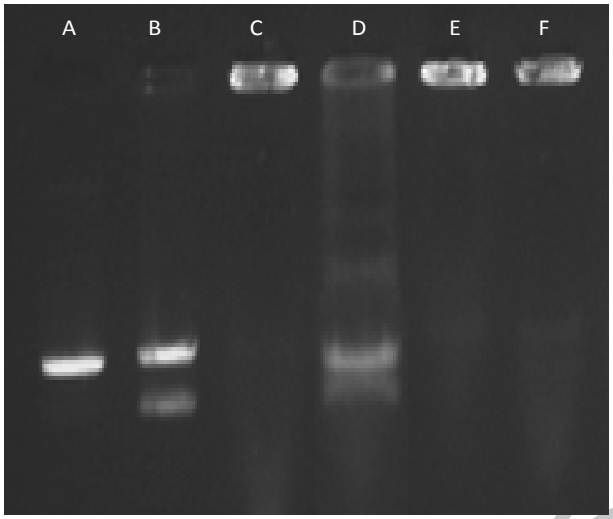


Figure 8: A: Linear DNA; B: Decatenated DNA; C: Catenated DNA; D: Catenated DNA+topo IIα; E: Catenated DNA+topo IIα+etoposide (100 μM); F: Catenated DNA+topo IIα+**8q** (100 μM).

3.9 Molecular Docking studies

The cell cycle analysis and topo-II assay prompted us to do molecular docking studies on topo-II to understand the precise nature of binding and to develop an insight into the atomic level interactions by such molecules. Docking studies were performed on **8q** and topo-II structure was obtained from Protein Data Bank (PDBID 1ZXN).[55] Necessary corrections to the protein were carried out using Protein Preparation Wizard from Schrodinger package. Geometry of the molecule was optimized in Gaussian 09 using PM3 semi-empirical method.[56] Additional docking studies were performed using AutoDock 4.2 docking software.[57] Grids for the docking studies were prepared around ATP binding site using ATP as a center for the grid. For the validation of docking protocol, docking studies on reference ligand (ADP) were performed. Binding pose of reference ligand shows RMSD 0.91 with docking score -8.84. The binding pose (as in **Figure 9**) shows that this molecule binds effectively in the ATP binding pocket with docking score -8.29. The trimethoxyphenyl group is deeply buried in the ATP binding pocket whereas 5-phenyloxadiazole ring is situated towards the surface. Superimposed pose of **8q** with cocrystal ligand shows that trimethoxyphenyl ring was near to the purine ring where nitrogen of purine ring and methoxy group of trimethoxyphenyl ring forming hydrogen bonding interactions

with same amino acid Asn120. Sugar moiety of ADP is superimposed with imidazopyridine ring of **8q**. The trimethoxyphenyl ring shows the hydrophobic interactions with the Glu87, Ile88, Asn91, Ala92, Asn95, Ile118, Asn120, Gly124, Ala167, Thr215, Cys216, Ile217 amino acids, oxygen of the 3-methoxy substituent shows hydrogen binding interactions with the side chain of Asn120 and phenyl ring shows π-π interactions with Phe142. The imidazopyridine ring exhibits hydrophobic interactions with the Ile141, Thr147, Ser148, Ser149, Lys168 amino acids. The 5-phenyloxadiazole ring was involved in the hydrophobic interactions with Asp94, Gln97, Arg98, Asn150, Thr159, Gly161 amino acids. Further Lys157 involves in the cation-π interactions with the phenyl ring and nitrogen of oxadiazole ring forms hydrogen bonding side chain of Asn150. Thus, these molecular docking studies along with the biological assay data suggest that conjugate **8q** is a potential topo-II inhibitor.

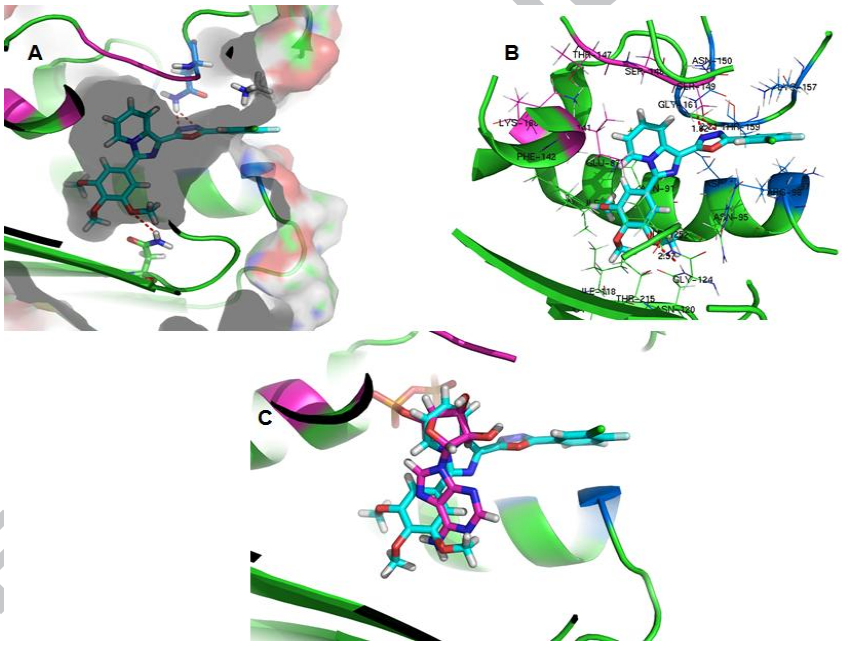


Figure 9: (**A**) Binding pose of **8q** with surface Reference Ligand: (B) Docking pose for **8q** in ATP binding site of Topo-II. Molecule shown in peru color, protein in green color, amino acid forming hydrogen bond in magenta color and amino acid having π interaction are in violate color. (**C**) Superimposed pose of **8q** with cocrystal ligand ADP.

4.0 Conclusion

In conclusion, a series of imidazopyridinyl-1,3,4-oxadiazole conjugates were synthesized and evaluated for their cytotoxic activity against a panel of sixty human tumor cell lines. Among them conjugate **8q** exhibited significant cytotoxic activity with GI_{50} values ranging from 1.30-5.64 µM and arrests the cells at subG1 phase of the cell cycle. The Topo II inhibitory activity of these conjugate was tested by relaxation and cleavage complex in vitro assay. Molecular docking of **8q** in the Topo II binding site reveals the possible binding mode with the target. Interestingly, **8q** induces apoptosis through caspase-3 activation, nuclear fragmentation condensation and disruption of mitochondrial membrane potential as well as increase in the intracellular ROS levels and Annexin-V. Overall, the present study demonstrates that the synthesis of imidazopyridinyl-1,3,4-oxadiazole conjugates as promising anticancer agents with apoptotic-inducing activity and inhibition of the topoisomerase IIα and it could be concluded that the **8q** may be used as a new template for development of chemotherapeutics.

5.0 Experimental Section

5.1. Chemistry

All chemicals and reagents are commercially available and were used directly without further purification. Reactions were monitored by TLC performed on silicagel glass plate containing 60 GF-254, and visualization was achieved by UV light or iodine indicator. Column chromatography was performed with Merck 60–120 mesh silica gel. ^{1}H and ^{13}C NMR spectra were recorded by using Varian and Avance instruments. Chemical shifts are expressed in parts per million (δ in ppm) downfield from internal TMS and coupling constants are expressed in Hz. ^{1}H NMR spectroscopic data are reported in the following order: multiplicity (s, singlet; brs, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet), coupling constants in Hz, number of protons. ESI mass spectra were recorded on a Micro mass Quattro LC using ESI^{+} software with capillary voltage 3.98 kV and an ESI mode positive ion trap detector. Melting points were determined with an Electro thermal melting point apparatus, and are uncorrected.

5.1.1. Ethyl 2-hydroxyimino-2-(2-pyridyl)acetate (2) [38]:

A solution of 5g (0.03 mol) of ethyl (2- pyridyl) acetate (**1**) in 10 mL of glacial acetic acid was cooled in an ice bath and 2.20 g (0.032 mol) of sodium nitrite in 5 ml of water was added over a period of 20 minutes, keeping the temperature between 15 to 25 °C. The mixture was stirred for

an additional 30 minutes, 50 mL of water was added, and the stirring continued for two hours. The crystals were washed with water, washed three times with 5% sodium bicarbonate solution and again with water. Recrystallization from methanol yielded 4.9 g (83%) of white needles. mp: 147−152 °C; ^{1}H NMR (300 MHz, $CDCl_3$): 7.65 (s, 1H), 6.95-6.89 (m, 2H), 6.75 (s, 1H), 3.98 (q, J = 6.7 Hz, 2H), 1.54 (t, J = 6.7 Hz, 3H); MS (ESI, m/z): 195 $(M+H)^+$.

5.1.2. Ethyl 2-amino-2-(2-pyridyl)acetate (3) [38]:

To a solution of ethyl 2-hydroxyimino-2- (2-pyridyl)acetate (**2**) (4 g, 0.02 mol) in 50 mL of methanol was added catalytic amount of 10% palladium on activated carbon. The mixture was stirred overnight under hydrogen, the reaction mixture was filtered, and the filtrate was evaporated. The crude product **3** was used directly in next step without further purification. MS (ESI, m/z): 181 $(M+H)^+$.

5.1.3. Ethyl 2-(pyridin-2-yl)-2-(3,4,5-trimethoxybenzamido)acetate (4) [38]:

To a stirred solution of ethyl 2-amino-2-(pyridin-2-yl)acetate **3** (3.8 g, 0.035 mol) in dry THF, triethylamine (1.1 mmol) and trimethoxybenzoylchloride (4.9 g, 0.035 mol) were added at 0 °C. The reaction mixture was stirred for 3 h and the reaction was monitored by TLC. After completion of reaction, the solution was concentrated in vacuo. The residue was partitioned between water and ethyl acetate, and the aqueous layer was extracted with ethyl acetate. The combined extracts were dried over Na_2SO_4 and concentrated. The crude was further purified by column chromatography using ethyl acetate/hexane (5:5) to obtain the pure product in 74% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.36 (m, 1H), 8.11 (d, J = 7.5 Hz, 1H), 7.82 (d, J = 6.7 Hz, 1H,) 7.58 (m, 1H), 7.16 (dd, J = 9.8, 7.5 Hz, 1H), 6.98 (s, 2H), 5.47 (d, J = 6.5 Hz, 1H,), 4.32 (q, J = 6.9 Hz, 2H), 3.92 (s, 3H), 1.52 (t, J = 6.9 Hz, 3H); MS (ESI, m/z): 375 $[M+H]^+$;

5.1.4. Ethyl 3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridine-1-carboxylate (5):

The reaction mixture of **4** (3g, 8 mmol) and $POCl_3$ was refluxed for 3h. After completion reaction, the reaction mixture was poured in ice-water and quenched with saturated $NaHCO_3$. This water layer was extracted with ethyl acetate and the combined extracts were dried and concentrated under vaccum. The crude obtained was further furified by column chromatography to obtain pure product as yellow solid in 70% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.29 (dd, J = 16.9, 8.2 Hz, 2H), 7.17 – 7.09 (m, 1H), 6.98 (s, 2H), 6.83 – 6.76 (m, 1H), 4.55 (q, J = 13.5, 6.7 Hz, 2H), 3.92 (s, 6H), 3.91 (s, 3H), 1.47 (t, J = 6.7 Hz, 3H); MS (ESI, m/z): 357 $[M+H]^+$. HRMS (ESI, m/z) calcd for $C_{19}H_{21}O_5N_2$: 357.14463 $[M+H]^+$; found: 357.14459.

5.1.5. 3-(3,4,5-Trimethoxyphenyl)imidazo[1,5-*a*]pyridine-1-carbohydrazide (6):

To a solution of compound **5** (2 g, 5.6 mmol) in isopropanol, hydrazine hydrate (0.84 mL, 16.8 mmol) was added and the reaction mixture was heated at 80 °C. Then the reaction mixture was cooled to room temperature and the solvent was removed. The residue was diluted with water and extracted with ethyl acetate. The combined organic extracts were dried over Na_2SO_4 and concentrated under vaccum. The crude obtained was further furified by column chromatography to yield pure product in 82% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.86 (s, 1H), 8.44 (d, *J* = 9.1 Hz, 1H), 8.26 (s, 1H), 7.11- 7.06 (m, 1H), 6.95 (s, 2H), 6.80-6.76 (m, 1H), 3.94 (s, 6H), 3.93 (s, 3H); MS (ESI, m/z): 343 $[M+H]^+$. HRMS (ESI, m/z) calcd for $C_{17}H_{19}O_4N_4$: 343.13976 $[M+H]^+$; found: 343.13971.

5.1.6. General procedure for the synthesis of imidazopyridine-oxadiazoles (8a-s)

A solution of acyl hydrazine **6** (1mmol) and aromatic aldehydes **7a-s** (1 mmol) in *i*-PrOH (10 mL) was refluxed until the reaction was completed (monitored by TLC, 3−5 h), and then the solvent was evaporated under reduced pressure. To the resulting residue dissolved in DMSO (5 mL), potassium carbonate (3 mmol) and iodine (1.2 mmol) were added in sequence. Then the reaction mixture was heated to 80 °C and stirred until the completion of reaction. After being cooled to room temperature, it was treated with 5% $Na_2S_2O_3$ (20 mL), extracted with ethyl acetate the combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated under vaccum to obtain crude products. The crude obtained was purified by column chromatography using ethyl acetate and hexane as eluent to afford pure products (**8a-s**).

5.1.6.1. 2-Phenyl-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8a):

The title compound was obtained from compound (**6)**, and benzaldehyde (**7a**) following the general procedure described above. Yield 72%; yellow solid; mp: 230-233 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.36 (dd, *J* = 15.4, 8.2 Hz, 2H), 8.28 – 8.23 (m, 2H), 7.56 – 7.49 (m, 3H), 7.13 (dd, *J* = 9.0, 6.0 Hz, 1H), 7.04 (s, 2H), 6.77 (t, *J* = 7.5 Hz, 1H), 3.96 (s, 6H), 3.91 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 163.31, 161.27, 153.79, 139.72, 132.41, 131.35, 128.85, 126.94, 124.21, 124.07, 123.49, 122.34, 119.81, 114.73, 105.98, 60.99, 56.41; MS (ESI, m/z): 429 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{24}H_{21}O_4N_4$: 429.17753 $[M+H]^+$; found: 429.17752.

5.1.6.2. 2-p-Tolyl-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8b):

The title compound was obtained from compound (**6)** and 4- methybenzaldehyde (**7b**) following the general procedure described above. Yield: 69%; yellow solid; mp: 210-213 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.39 (d, *J* = 9.2 Hz, 1H), 8.35 (d, *J* = 7.3, Hz, 1H), 8.12 (d, *J* = 8.2 Hz, 2H), 7.34 (d, *J* = 8.0 Hz, 2H), 7.17-7.13 (dd, *J* = 6.4, 2.2 Hz, 1H), 7.04 (s, 2H), 6.83 (t, *J* = 6.4 Hz, 1H), 3.96 (s, 6H), 3.94 (s, 3H), 2.44 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 163.46, 160.98, 153.77, 141.80, 139.64, 139.28, 132.28, 129.59, 126.89, 124.21, 123.41, 122.31, 121.17, 119.80, 116.35, 114.70, 105.96, 60.93, 56.41, 21.59; MS (ESI, m/z): 443 [M+H]+; HRMS (ESI, m/z) calcd for $C_{25}H_{23}N_4O_4$: 443.17107 $[M+H]^+$; found: 443.17102.

5.1.6.3. 2-(4-Methoxyphenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8c):

The title compound was obtained from compound (**6)** and 4- methoxybenzaldehyde (**7c**) following the general procedure described above. Yield: 71%; yellow solid; mp: 240-244 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.37 (dd, *J* = 16.8, 8.2 Hz, 2H), 8.14 (d, *J* = 8.8 Hz, 2H), 7.09 (dd, *J* = 8.9, 6.4 Hz, 1H), 6.99 (d, *J* = 10.1 Hz, 4H), 6.77 (t, *J* = 6.7 Hz, 1H), 3.97 (s, 6H), 3.91 (s, 3H), 3.89 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 163.27, 162.01, 160.80, 153.77, 139.58, 139.27, 132.19, 128.71, 124.26, 123.31, 122.28, 119.85, 116.55, 114.69, 114.30, 105.96, 60.98, 56.41, 55.33; MS (ESI, m/z): 459 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{25}H_{23}N_4O_5$: 459.16607 $[M+H]^+$; found: 459.16595.

5.1.6.4. 2-(4-Fluorophenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4 oxadiazole (8d):

The title compound was obtained from compound (**6)** and 4- fluorobenzaldehyde (**7d**) following the general procedure described above. Yield: 67%; yellow solid; mp: 220-224 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.40 (dd, *J* = 13.4, 8.2 Hz, 2H), 8.27 (dd, *J* = 8.8, 5.3 Hz, 2H), 7.23 (t, *J* = 8.6 Hz, 2H), 7.14 (dd, *J* = 9.0, 6.5 Hz, 1H), 7.03 (s, 2H), 6.81 (t, *J* = 6.8 Hz, 1H), 3.99 (s, 6H), 3.94 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.28, 162.93, 162.59, 153.88, 139.82, 132.52, 129.32, 129.21, 124.24, 123.64, 122.43, 120.39, 119.88, 116.42, 116.13, 114.84, 106.05, 61.06, 56.49; MS (ESI, m/z): 447 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{24}H_{20}O_4N_4F$: 447.14624 $[M+H]^+$; found: 447.14616.

5.1.6.5. 2-(2-Chlorophenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8e):

The title compound was obtained from compound (**6)** and 2- chlorobenzaldehyde (**7e**) following the general procedure described above. Yield: 66%; yellow solid; mp: 196-200 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.40 (dd, *J* = 11.3, 8.2 Hz, 2H), 8.17 (dd, *J* = 7.4, 2.0 Hz, 1H), 7.60 (d, *J* = 7.5 Hz, 1H), 7.51 – 7.43 (m, 2H), 7.22 – 7.13 (m, 1H), 7.05 (s, 2H), 6.86 (t, *J* = 6.3 Hz, 1H), 3.97 (s, 6H), 3.95 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.48, 162.59, 153.58, 139.71, 132.37, 129.68, 129.27, 124.68, 123.11, 122.78, 120.29, 119.77, 116.82, 116.03, 114.94, 106.05, 60.56, 56.67; MS (ESI, m/z): 463 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{24}H_{20}O_4N_4Cl$: 463.11667 $[M+H]^+$; found: 463.11660.

5.1.6.6. 2-(4-Bromophenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8f):

The title compound was obtained from compound (**6)** and 4- bromobenzaldehyde (**7f**) following the general procedure described above. Yield: 74%; yellow solid; mp: 225-229 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.41 (dd, *J* = 15.2, 8.2 Hz, 2H), 8.14 (d, *J* = 8.6 Hz, 2H), 7.69 (d, *J* = 8.6 Hz, 2H), 7.24 – 7.15 (m, 1H), 7.04 (s, 2H), 6.86 (t, *J* = 6.7 Hz, 1H), 3.99 (s, 6H), 3.96 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.61, 162.43, 153.78, 139.61, 132.45, 129.86, 129.32, 124.32, 123.65, 122.26, 120.77, 119.78, 116.81, 116.00, 114.33, 105.89, 60.79, 55.89; MS (ESI, m/z): 507 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{24}H_{20}O_4N_4Br$: 507.06673 $[M+H]^+$; found: 507.06669.

5.1.6.7. 2-(4-(Trifluoromethyl)phenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8g):

The title compound was obtained from compound (**6)** and 4-(trifluoromethyl)benzaldehyde (**7g**) following the general procedure described above. Yield: 69%; yellow solid; mp: 243-247 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.44 (d, *J* = 9.2 Hz, 1H), 8.39 (d, *J* = 8.1 Hz, 2H), 7.81 (d, *J* = 8.3 Hz, 2H), 7.71 (dd, *J* = 5.7, 3.3 Hz, 1H), 7.53 (dd, *J* = 5.7, 3.3 Hz, 1H), 7.04 (s, 2H), 6.86 (t, *J* = 6.8 Hz, 1H), 3.98 (s, 2H), 3.95 (s, 1H); ^{13}C NMR (75 MHz, DMSO): δ 165.39, 162.59, 153.45, 139.87, 132.38, 129.72, 129.16, 124.81, 123.85, 122.71, 120.47, 119.58, 116.69, 116.19, 114.72, 106.09, 61.06, 56.49; MS (ESI, m/z): 497 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{25}H_{20}O_4N_4F_3$: 497.14256 $[M+H]^+$; found: 497.14259.

5.1.6.8. 2-(4-(Trifluoromethoxy)phenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8h):

The title compound was obtained from compound (**6)** and 4-(trifluoromethoxy)benzaldehyde (**7h**) following the general procedure described above. Yield: 70%; yellow solid; mp: 220-225

°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.40 (dd, *J* = 9.7, 8.6 Hz, 2H), 8.33 – 8.26 (m, 2H), 7.39 (d, *J* = 8.1 Hz, 2H), 7.23 – 7.16 (m, 1H),7.05 (s, 2H), 6.90 – 6.84 (m, 1H), 3.98 (s, 6H), 3.96 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 162.21, 161.68, 153.93, 140.03, 139.49, 132.85, 127.25, 126.01, 124.15, 123.94, 122.51, 119.83, 114.93, 106.06, 61.08, 56.48; MS (ESI, m/z): 513 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{25}H_{20}F_3O_5N_4$: 513.13862 $[M+H]^+$; found: 513.13856.

5.1.6.9. 2-(4-Nitrophenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8i):

The title compound was obtained from compound (**6)** and 4- nitrobenzaldehyde (**7i**) following the general procedure described above. Yield: 71%; yellow solid; mp: 245-249 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.48 – 8.37 (m, 6H), 7.23 (dd, *J* = 9.0, 6.5 Hz, 1H), 7.04 (s, 2H), 6.90 (t, *J* = 6.8 Hz, 1H), 3.99 (s, 6H), 3.96 (s, 3H); ^{13}C NMR (75 MHz, DMSO): δ 165.81, 162.57, 153.86, 144. 91, 139.86, 132.52, 129.21, 126.63, 124.24, 123.64, 120.39, 119.32, 116.13, 114.84, 106.11, 65.92, 56.89; MS (ESI, m/z): 474 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{24}H_{20}O_6N_5$: 474.14132 $[M+H]^+$; found: 474.14119.

5.1.6.10. 2-(Pyridin-3-yl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8j):

The title compound was obtained from compound (**6)** and 3- pyridinecarboxaldehyde (**7j**) following the general procedure described above. Yield: 64%; yellow solid; mp: 251-255 °C;

^{1}H NMR (300 MHz, DMSO): δ 9.45 (s, 1H), 8.75 (d, *J* = 4.3 Hz, 1H), 8.51 (d, *J* = 8.1 Hz, 1H), 8.41 (dd, *J* = 17.9, 8.8 Hz, 2H), 7.46 (dd, *J* = 8.1,5.6 Hz, 1H), 7.15 (dd, *J* = 9.6, 6.7 Hz, 1H), 7.00 (s, 2H), 6.87 (t, *J* = 6.98 Hz, 1H), 3.96 (s, 6H), 3.91 (s, 3H); ^{13}C NMR (75 MHz, DMSO): δ 166.81, 163.89, 153.62, 146. 82, 144. 68, 139.46, 136.82, 127. 62, 124.24, 123.64, 120.39, 119.52, 116.37, 114.78, 106.09, 65.84, 56.75; MS (ESI, m/z): 430 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{23}H_{20}N_5O_4$: 430.15110 $[M+H]^+$; found: 430.15103.

5.1.6.11. N,N-Dimethyl-4-(5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazol-2-yl)aniline (8k):

The title compound was obtained from compound (**6)** and 4-(dimethylamino)benzaldehyde (**7k**) following the general procedure described above. Yield: 69%; yellow solid; mp: 135-140 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.38 (dd, *J* = 15.9, 8.1 Hz, 2H), 8.07 (d, *J* = 8.8 Hz, 2H), 7.12 – 7.05 (m, 1H), 7.04 (s, 2H), 6.76 (d, *J* = 8.9 Hz, 3H), 3.99 (s, 6H), 3.93 (s, 3H), 3.10 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.04, 160.18, 153.74, 152.09, 139.39, 139.19, 131.92, 128.34, 124.38,

123.00, 122.18, 119.92, 114.60, 111.47, 111.09, 105.94, 60.97, 56.41, 40.06; MS (ESI, m/z): 472 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{26}H_{26}N_5O_4$: 472.19730 $[M+H]^+$; found: 472.19728.

5.1.6.12. 2-(4-Methoxy-3-nitrophenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8l):

The title compound was obtained from compound (**6)** and 4-methoxy-3-nitrobenzaldehyde (**7l**) following the general procedure described above. Yield: 66%; yellow solid; mp: 248-251 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.40 (t, *J* = 8.4 Hz, 2H), 7.99-7.87 (m, 3H), 7.22 (dd, *J* = 8.7, 6.9 Hz, 1H), 7.04 (s, 2H), 6.89 (t, *J* = 6.9 Hz, 1H), 4.10 (s, 3H), 3.96 (s, 6H), 3.95 (s, 3H); ^{13}C NMR (75 MHz, DMSO): δ 164.31, 161.27, 153.79, 153.33, 143.93, 139.72, 132.41, 131.35, 128.85, 126.94, 124.21, 123.49, 122.34, 119.81, 114.73, 105.94, 60.99, 56.41, 54.98; MS (ESI, m/z): 504 $[M+H]^+$. HRMS (ESI, m/z) calcd for $C_{25}H_{21}N_5O_7Na$: 526.13472 $[M+Na]^+$; found:526.13470.

5.1.6.13. 2-Methoxy-4-(5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazol-2-yl)phenol (8m):

The title compound was obtained from compound (**6)** and 4-hydoxy-3-methoxybenzaldehyde (**7m**) following the general procedure described above. Yield: 63%; yellow solid; mp: ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.38 (dd, *J* = 12.5, 8.2 Hz, 2H), 7.78 (dd, *J* = 8.3, 1.7 Hz, 1H), 7.73 (d, *J* = 1.6 Hz, 1H), 7.19 – 7.12 (m, 1H), 7.05 (d, *J* = 3.5 Hz, 1H), 7.04 (s, 2H), 6.84 (t, *J* = 6.4 Hz, 1H), 4.02 (s, 3H), 3.96 (s, 6H), 3.94 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.54, 162.45, 159.72, 158.64, 156.79, 156.12, 153.53, 150.39, 149.75, 139.64, 132.54, 125.73, 125.62, 120.62, 117.34, 113.91, 109.73, 106.47, 60.98, 56.41, 55.39; MS (ESI, m/z): 475 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{25}H_{23}N_4O_6$: 475.16118 $[M+H]^+$; found: 475.16115.

5.1.6.14. 2-(3,4-dimethoxyphenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8n):

The title compound was obtained from compound (**6)** and 3,4-dimethoxybenzaldehyde (**7n**) following the general procedure described above. Yield: 67%; yellow solid; mp: 248-252 °C; ^{1}H NMR (300 MHz, CDCl3): δ 8.43 (d, *J* = 9.1 Hz, 1H), 8.36 (d, *J* = 7.1 Hz, 1H), 7.81 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.72 (d, *J* = 1.6 Hz, 1H), 7.12 (dd, *J* = 9.0, 6.5 Hz, 1H), 7.03 (s, 2H), 6.96 (d, *J* = 8.4 Hz, 1H), 6.80 (t, *J* = 6.7 Hz, 1H), 4.02 (s, 3H), 3.98 (d, *J* = 3.7 Hz, 9H), 3.93 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 163.32, 160.89, 153.80, 152.13, 151.65, 149.15, 139.59, 139.31, 132.27, 124.20, 123.38, 122.27, 120.52, 119.87, 116.62, 114.73, 111.96, 109.43, 105.96, 61.97, 56.40,

56.20, 55.96; MS (ESI, m/z): 489 $[M+H]^+$. HRMS (ESI, m/z) calcd for $C_{26}H_{25}N_4O_6$: 489.17615 $[M+H]^+$; found: 489.17607.

5.1.6.15. 2-(2,4-difluorophenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8o)

The title compound was obtained from compound (**6)** and 2,4-difluorobenzaldehyde (**7o**) following the general procedure described above. Yield: 67%; yellow solid; mp: 218-220 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.39 (d, *J* = 9.1 Hz, 2H), 8.24 (dd, *J* = 14.9, 8.6 Hz, 1H), 7.28 (s, 1H), 7.19 (dd, *J* = 9.5, 6.6 Hz, 1H), 7.07 (dd, *J* = 13.1, 4.8 Hz, 3H), 6.86 (t, *J* = 6.8 Hz, 1H), 3.96 (d, *J* = 4.9 Hz, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 163.77, 161.47, 153.84, 150.13, 149.75, 139.97, 139.48, 137.34, 137.22, 132.64, 131.21, 131.13, 124.16, 123.72, 122.45, 119.75, 116.05, 114.77, 112.42, 112.22, 106.10, 61.01, 56.47; MS (ESI, m/z): 465 $[M+H]^+$. HRMS (ESI, m/z) calcd for $C_{24}H_{19}N_4O_4F_2$: 465.13658 $[M+H]^+$; found: 465.13636.

5.1.6.16. 2-(2-Chloro-4-fluorophenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8p)

The title compound was obtained from compound (**6)** and 2-chloro-4-fluorobenzaldehyde (**7p**) following the general procedure described above. Yield: 69%; yellow solid; mp: 225-230 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.36 (dd, *J* = 15.4, 8.1 Hz, 2H), 8.14 (dd, *J* = 8.7, 5.9 Hz, 1H), 7.31 (dd, *J* = 8.4, 2.3 Hz, 1H), 7.14 (dd, *J* = 14.1, 5.4 Hz, 2H), 7.01 (s, 2H), 6.82 (t, *J* = 6.5 Hz, 1H), 3.93 (s, 6H), 3.92 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 162.34, 160.82, 158.84, 157.25, 156.73, 156.12, 153.20, 150.42, 149.82, 139.82, 132.62, 125.88, 125.31, 120.74, 117.18, 113.82, 109.75, 106.17, 60.52, 56.37; MS (ESI, m/z): 481 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{24}H_{19}N_4O_4ClF$: 481.10736 $[M+H]^+$; found: 481.10739.

5.1.6.17. 2-(3-Chloro-4-fluorophenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8q)

The title compound was obtained from compound (**6)** and 3-chloro-4-fluorobenzaldehyde (**7q**) following the general procedure described above. Yield: 73%; yellow solid; mp: 220-224 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.42 (dd, *J* = 15.8, 8.1 Hz, Ar-H, 2H), 8.34 (dd, *J* = 6.9, 2.1 Hz, Ar – H, 1H), 8.17 (ddd, *J* = 8.5, 4.5, 2.1 Hz, Ar-H, 1H), 7.33 (dd, *J* = 10.9, 6.2 Hz, Ar-H, 1H), 7.17 (dd, *J* = 9.0, 6.6 Hz, Ar-H, 1H), 7.02 (s, Ar-H, 2H), 6.84 (t, *J* = 6.5 Hz, Ar-H, 1H), 3.99 (s, 2×OMe, 6H), 3.94 (s, 1×OMe, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 161.51 (Ar-C), 160.13 (Ar-C), 159.46 (Ar-C), 158.95 (Ar-C, d, *J* = 234.2 Hz, C-F), 158.43 (Ar-C), 157.92 (Ar-C), 153.57

(Ar-C), 150.13 (Ar-C), 149.75 (Ar-C), 139.78 (Ar-C), 132.50 (Ar-C), 125.26 (Ar-C), 124.14 (Ar-C), 120.96 (2×Ar-C), 117.15 (Ar-C), 113.33 (Ar-C), 109.52 (Ar-C), 106.17 (Ar-C), 60.28 (2×OMe-C), 56.24 (1×OMe-C); MS (ESI, m/z): 481 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{24}H_{19}N_4O_4ClF$: 481.10737 $[M+H]^+$; found: 481.10720.

5.1.6.18. 2-(2,4-dichlorophenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8r)

The title compound was obtained from compound (**6)** and 2,4-dichlorobenzaldehyde (**7r**) following the general procedure described above. Yield: 66%; yellow solid; mp: 220-224 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.39 (ddd, *J* = 14.8, 7.9, 4.1 Hz, 2H), 8.11 (d, *J* = 8.5 Hz, 1H), 7.61 (d, *J* = 2.0 Hz, 1H), 7.42 (dd, *J* = 8.5, 2.1 Hz, 1H), 7.18 (ddd, *J* = 9.2, 6.5, 0.9 Hz, 1H), 7.03 (s, 2H), 6.87 – 6.83 (m, 1H), 3.95 (s, 6H), 3.94 (s, 3H). MS (ESI, m/z): 498 $[M+H]^+$. HRMS (ESI, m/z) calcd for $C_{24}H_{19}N_4O_4Cl_2$: 498.07822 $[M+H]^+$; found: 498.07794.

5.1.6.19. 2-(3,4,5-Trimethoxyphenyl)-5-(3-(3,4,5-trimethoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1,3,4-oxadiazole (8s):

The title compound was obtained from compound (**6)** and 3,4,5-trimethoxybenzaldehyde (**7s**) following the general procedure described above. Yield: 72%; yellow solid; mp: 173-176 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.45 (d, *J* = 9.2 Hz, 1H), 8.38 (d, *J* = 7.2 Hz, 1H), 7.48 (s, 2H), 7.19 (dd, *J* = 8.8, 6.1 Hz, 1H), 7.05 (s, 2H), 6.90 – 6.84 (m, 1H), 4.00 (s, 6H), 3.97 (s, 6H), 3.95 (d, *J* = 1.6 Hz, 6H); ^{13}C NMR (75 MHz,$CDCl_3$): δ 163.31, 161.21, 153.88, 153.55, 139.67, 139.43, 132.49, 130.84, 128.74, 125.09, 123.57, 122.30, 119.97, 119.21, 114.85, 105.99, 104.18, 60.98, 56.44; MS (ESI, m/z): 519 $[M+H]^+$; HRMS (ESI, m/z) calcd for $C_{27}H_{27}N_4O_7$: 518.18734 $[M+H]^+$; found: 519.18715.

6.0 Biology

6.1. In vitro growth inhibition

The screening of anticancer activity was evaluated by the NCI, USA, according to standard procedures (http://dtp.nci.nih.gov/ branches/btb/ivclsp.html).[58]

6.2. Cell cycle analysis

Flow cytometric analysis (FACS) was performed to evaluate the distribution of the cells through the cell cycle phases. A549, lung cancer cells were incubated with compounds **8q** at concentrations of 2 and 4 μM for a period of 48 h. Untreated and treated cells were harvested,

washed with PBS, fixed in ice-cold 70% ethanol and stained with propidium iodide (Sigma Aldrich). Cell cycle was performed by flow cytometry (Becton Dickinson FACS Caliber).[59]

6.3. Caspase 3 activity

Caspase-3 assay was conducted for detection of apoptosis in lung cancer cell line (A549). The commercially available apoptosis detection kit (Sigma-Caspase 3 Assay kit, Colorimetric) was used. A549 cells were treated with the most active compound **8q** at 2 and 4 μM for 48 h. After 48 h of treatment, cells were collected by centrifugation, washed once with PBS, and cell pellets were collected. Collected cell pellets were suspended in lysis buffer and incubated for 15 min. After incubation, cells were centrifuged at 20,000 rpm for 15 min and collected the supernatant. Supernatants were used for measuring caspase 3 activity using an ELISA-based assay, according to the manufacturer's instructions.

6.4. Tunel assay

After incubation with conjugate **8q at** 2μM for 24 h, cells were rinsed three times with phosphate-buffered saline (PBS) and fixed with methanol. Based on the TUNEL protocol (DeadEnd™ Fluorometric TUNEL System) cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate and then rinsed twice with PBS. The DNA nick-labeling reaction was performed using 50 μL TUNEL reaction mixture, including 45 μL enzyme solution and 5 μL nucleotide mix at 37 ºC for 60 min. Then the samples were rinsed three times with PBS and analyzed under a fluorescence microscope.

6.5. Mesurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential (ΔΨm) was determined by using JC-1 dye. Cell-permeable cationic carbocyanine dye JC-1 (Sigma aldrich India, Catlog- CS0390), also known as 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodine, emits green fluorescence (525 nm) in its monomeric form. However, upon transfer to the membrane environment of a functionally active mitochondrion, it exhibits an aggregation dependent orange-red fluorescence (emission at 590 nm). Briefly, cultures were treated with the test drugs for 24 h. After drug treatment the cells were incubated with JC-1 dye for 20 min at 37 °C. After incubation cultures were used for the measuring mitochondrial membrane potiential (ΔΨm), according to the manufacturer's instructions

6.6. ROS Generation

To evaluate intracellular reactive oxygen species (ROS) levels, 2',7'-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes) fluorescent dye was used to clarify this issue. The nonpolar DCFH-DA is converted to the polar derivative DCFH by esterases when it is taken up by the cell. DCFH is nonfluorescent but is rapidly oxidized to the highly fluorescent DCF by intracellular H_2O_2 or nitric oxide. A549 cells were treated with compound **8q** at 2 µM concentration for 24 h. To determine the production of ROS, cells were treated with 2 µM of DCFH2-DA at 37°C for 30 min, and the fluorescence of DCF was measured at 530 nm after excitation at 485 nm (DCFH2-DA, after deacetylation to DCFH2, is oxidized intracellularly to its fluorescent derivative, DCF).[60]

6.7. Flow cytometric evaluation of apoptosis

A549 (1×10^6) were seeded in six-well plates and allowed to grow overnight. The medium was then replaced with complete medium containing 2 µM concentration of compound **8q** for 48 h along with vehicle alone as control. After 48 h of drug 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.[61] 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.

6.8. Topoisomerase II inhibition assay

Topoisomerase II inhibition assay was determined by the ATP dependent decatenation of kDNA. Topoisomerase-II activity was determined using kit (TG 1009, Topogen, USA)[62]. Reactions were carried out in 20 µL and contained 120 mM KCl, 50 mM Tris–HCl, pH 8, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM ATP, 30 mg/ml bovine serum albumin, 200–300 ng of kDNA, and topoisomerase II. The amount of topoisomerase II (5 units) was adjusted in preliminary experiments to decatenate approximately 100% of the kDNA under our assay conditions. The reactions were incubated at 37 ^{0}C for 30 min and terminated by the addition of 2 µL of a stop buffer containing 10% (w/v) SDS and 2 µL 0.5 mg/ml proteinaseK and then incubated for 10 min at 37 ^{0}C. After completion of the reaction catenated and decatenated reaction products were separated by 1% agarose gel electrophoresis stained with 0.5 µg/ml ethidium bromide (EtBr) in

TAE buffer (40 mM tris-acetate, pH 8.0, and 1 mMEDTA). Authentic decatenated kDNA and linear kDNA (Topogen Inc.) were run as controls to identify decatenated kDNA. The gels were run at 100 V for about 1hr and visualized under UV transillumination (BIO RAD gel doc XR+, USA).

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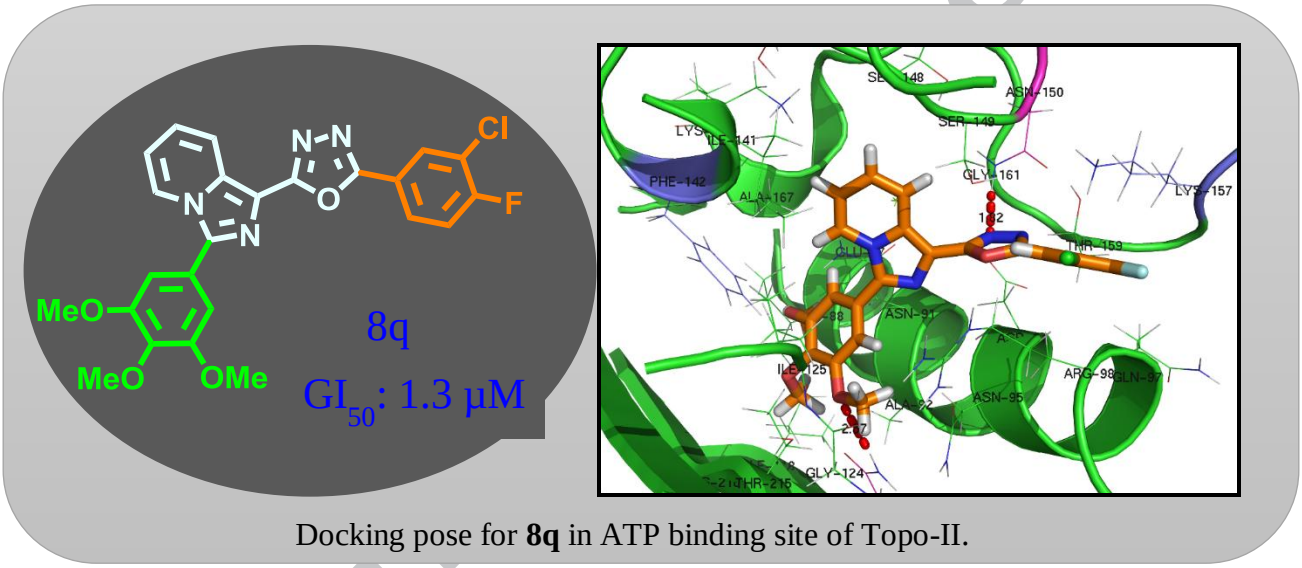
Graphical abstract

A series of imidazopyridinyl-1,3,4-oxadiazole conjugates were synthesized and evaluated for their anticancer potential against a panel of sixty human tumor cell lines. One of the compounds **8q** displayed good antiproliferative activity against A549 cell line by inhibiting topoisomerase IIα and induced apoptosis.



Docking pose for **8q** in ATP binding site of Topo-II.

Highlights

- New imidazopyridine-oxadiazole conjugates (**8a-s**) were designed, synthesized and evaluated their cytotoxic activity against a panel of NCI cancer cell lines.
- Among them, **8q** showed notable activity GI_{50} values ranging from 1.30-5.64 μM.
- This compound **8q** significantly inhibits the Topo IIα
- The molecular docking studies also indicated binding to the topoisomerase enzyme (PDBID 1ZXN).