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PII: S0960-894X(20)30543-6
DOI: https://doi.org/10.1016/j.bmcl.2020.127432
Reference: BMCL 127432

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 27 May 2020
Revised Date: 18 July 2020
Accepted Date: 20 July 2020

Please cite this article as: Sai Mani, G., Anchi, P., Sunkari, S., Donthiboina, K., Godugu, C., Shankaraiah, N., Kamal, A., Synthesis of (*Z*)-3-(arylamino)-1-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)prop-2-en-1-ones as potential cytotoxic agents, *Bioorganic & Medicinal Chemistry Letters* (2020), doi: https://doi.org/10.1016/j.bmcl.2020.127432

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Synthesis of (*Z*)-3-(arylamino)-1-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)prop-2-en-1-ones as potential cytotoxic agents

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
Imidazo[1,5-*a*]pyridines
Cytotoxicity
Cell cycle analysis
Annexin-V
Mitochondrial membrane potential

ABSTRACT

The new derivatives based on (*Z*)-3-(arylamino)-1-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)prop-2-en-1-one scaffold was synthesized and evaluated for their *in vitro* cytotoxic potential against a panel of cancer cell lines, *viz*., A549 (human lung cancer), HCT-116 (human colorectal cancer), B16F10 (murine melanoma cancer), BT-474 (human breast cancer), and MDA-MB-231 (human triple-negative breast cancer). Among them, many of the synthesized compounds exhibited promising cytotoxic potential against the panel of tested cancer cell lines with IC_{50} <30 μM. Based on the preliminary screening results, the structure-activity relationship (SAR) of the compounds was established. Among the synthesized compounds, **15i** displayed a potential anti-proliferative activity against HCT-116 cancer cell line with an IC_{50} value of 1.21±0.14 μM. Flow cytometric analysis revealed that compound **15i** arrested the G0/G1 phase of the cell cycle. Moreover, increased reactive oxygen species (ROS) generation, clonogenic assay, acridine orange staining, DAPI nuclear staining, measurement of mitochondrial membrane potential (ΔΨm), and annexin V-FITC assays revealed that compound **15i** promoted cell death through apoptosis.

Cancer can be considered as one of the hyper-proliferative diseases characterized by the deregulation of cell functioning, which eventually modifies the cell death causing apoptosis. Apoptosis is an important physiological process involved in tissue homeostasis and its inappropriate regulation or disruption leads to numerous diseases including cancer.[1] Various transcription factors are involved in the apoptotic pathway and therefore, anticipated as effective anticancer drug targets.[2] Thus, compounds are designed to induce apoptosis in cancer cells by targeting these specific factors involved in apoptosis.[3,4] In this regard, many drugs are available based on anti-proliferative activity. However, most of the currently available drugs usually act on rapidly dividing metabolically active cells and suffer poor selectivity between normal and cancer cells. Moreover, high toxicity and poor tolerance are the major problems associated with currently available anticancer drugs. Therefore, the discovery and development of new chemical entities which are more selective towards cancerous cells that particularly inhibit key mechanisms involved in the progression and development of tumor.[5] In this regard, various small synthetic molecules are currently being investigated as newer anticancer agents.[6]

Next, nitrogen-containing heterocycles are the most important structural components in many pharmacologically active molecules. According to the U.S.FDA database 59% of the approved drugs contain a nitrogen-bridged fused heterocycles.[7] Among these heterocycles, imidazoles represent a significant class of heterocyclic molecules with a promising biological and pharmaceutical importance. They are found to be common moieties in highly important biomolecules such as histamine, biotin, and in numerous alkaloids that displays a wide spectrum of biological properties. One of the interesting and most widely used heterocyclic motifs from this category is imidazopyridine,[8] a bicyclic N-fused imidazole which was known to exhibit a broad spectrum of biological activities for various targets such as positive inotropic agents,[9] thromboxane synthase inhibitors,[10] antiviral,[11] antibacterial,[12] estrogen production suppressors,[13] anxioselective and hypnoselective agents.[14] Moreover, imidazopyridines display cytotoxic activity through various molecular mechanisms, for instance through the inhibition of vascular endothelial growth factor (VEGF)-receptor KDF (Kinase insert domain receptor) and through the initiation of apoptosis.[15] Recently, we have reported the synthesis of imidazopyridine-linked oxadiazoles (**1**),[16] imidazopyridine oxindoles (**2**),[17] imidazopyridine-linked benzimidazoles (**3**),[18] bisindole-imidazopyridine derivatives[19] as potential cytotoxic agents as shown in **Figure 1**.

Moreover, numerous small molecules containing arylaminoprop-2-en-1-ones are reported to possess anticancer activity as shown in **Figure 1**. In continuation to our earlier efforts, we have designed and synthesized a series of (*Z*)-(diphenyl pyrazolyl)-2-propenones (**5**),[20] (*Z*)-(arylamino)-pyrazolyl/isoxazolyl-2-propenones,[21] benzo[*d*]imidazo[2,1-

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b]th ...ilic arylpropenones[23] as effective apoptosis-inducing and potential cytotoxic agents. Furthermore, these studies also revealed that the inclusion of propen-1-one moiety into the molecules has resulted in compounds with promising cytotoxic activity. For instance, (*Z*)-1-aryl-3-arylamino-2-propen-1-ones (**4**)[24] exhibited effective cytotoxicity towards various tested cancer cell lines with anti-tubulin activity by arresting the G2/M phase of the cell cycle.

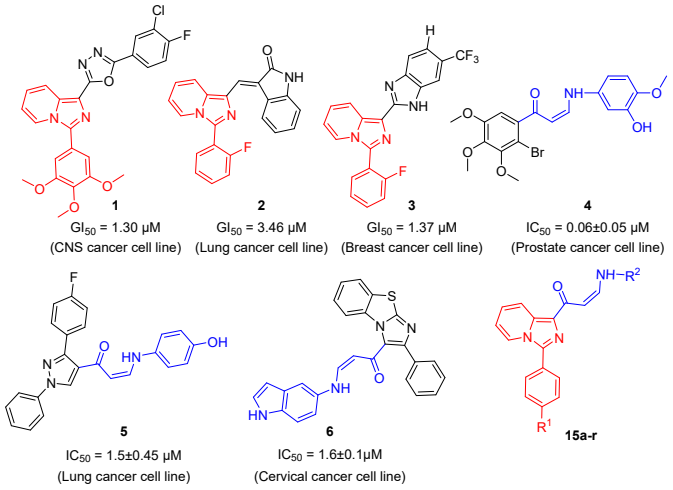


Figure 1. Chemical structures and anticancer activities of imidazopyridines **1**–**3**, aminoprop-2-en-1-one derivatives **4**–**6** and (*Z*)-3-(arylamino)-1-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)prop-2-en-1-ones **15a**–**r**.

The development of small molecules through molecular hybridization from known structural motifs is one of the current trends in drug discovery. It is anticipated that the improved cytotoxicity may be obtained by the structural conjugation of two potent pharmacophoric units. Hence, herein we have designed and synthesized a new series of imidazopyridyl-linked arylaminoprop-2-en-1-ones by replacing oxadiazole group of imidazopyridine-linked oxadiazole derivatives[16] (**1**) with 3-arylamino-2-propen-1-one group of (*Z*)-1-aryl-3-arylamino-2-propen-1-ones[24] as shown in **Figure 2**. Thus, a new series of compounds related to this template with diverse substituents on both biologically active pharmacophoric subunits have been synthesized.

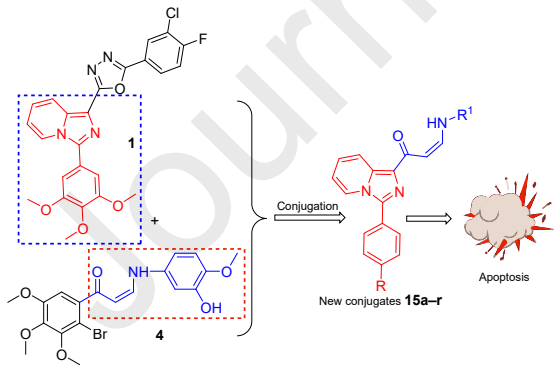
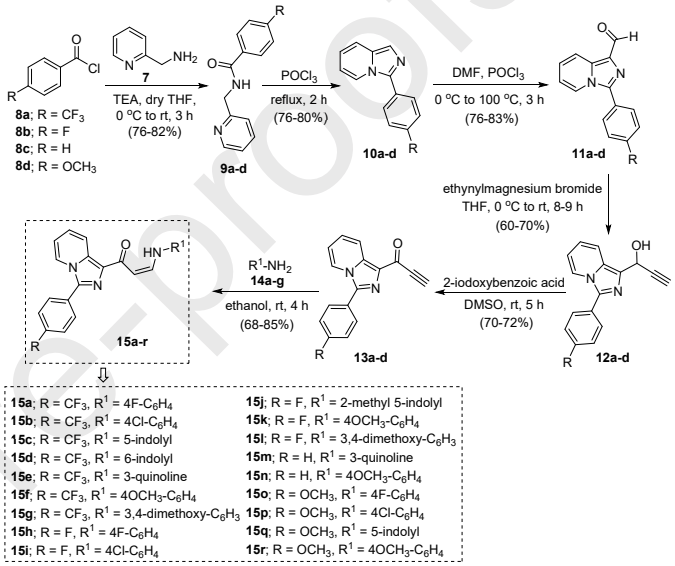


Figure 2. The conjugation of scaffolds **1** and **4** leads to new series of (*Z*)-3-(arylamino)-1-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)prop-2-en-1-ones **15a**–**r**.

The imidazopyridine-linked 3-arylaminoprop-2-en-1-one conjugates **15a**–**r** were synthesized by employing a general synthetic approach as illustrated in **Scheme 1**. The target compounds were synthesized from various substituted 1-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)prop-2-yn-1-ones **13a-d** and different substituted anilines **14a-g**. The key intermediates such as imidazo[1,5-*a*]pyridinyl propynones **13a-d** were synthesized ...amination of 2-pyridyl-methanamine (**7**) on substituted benzoyl chlorides **8a-d** in the presence of triethylamine to deliver *N*1-(2-pyridylmethyl)-substituted benzamides **9a-d** which were cyclized into the corresponding imidazo[1,5-*a*]pyridines **10a-d** by refluxing in $POCl_3$. The imidazo[1,5-*a*]pyridine derivatives **10a-d** were formylated under Vilsmeier-Haack conditions with $POCl_3$ in dry DMF provided 3-(substituted phenyl)imidazo-[1,5-*a*]pyridine-1-carbaldehyde intermediates **11a-d**.[18] Later, these aldehydes were treated with ethynyl magnesium bromide to afford 1-aryl-2-propyn-1-ols **12a-d**. Next, oxidation of **12a-d** with 2-iodoxybenzoic acid (IBX) in the presence of dimethyl sulfoxide (DMSO) gave 1-aryl-2-propyn-1-ones **13a-d**.[20] Finally, condensation of **13a-d** with different aryl amines **14a-g** in ethanol at room temperature provides (*Z*)-3-(arylamino)-1-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)prop-2-en-1-ones **15a-r** in good to excellent yields (68-85%, **Scheme 1**).



Scheme 1. Schematic representation for the synthesis of imidazopyridine-linked 3-arylaminoprop-2-en-1-ones **15a-r**.

All the synthesized compounds **15a-r** were assessed for their *in vitro* cytotoxic potential against a panel of selected cancer cell lines, *viz*., A549 (human lung cancer cell line), HCT-116 (human colorectal cancer cell line), B16F10 (murine melanoma cancer cell line), BT-474 (human breast cancer), and MDA-MB-231 (human triple-negative breast cancer cell line) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[25] The obtained values were compared to the standard drug nocodazole as shown in **Table 1** and expressed in IC_{50} values. Interestingly, the screening results revealed that the compounds **15b**, **15c**, **15f**, **15h**, **15i**, **15k**, **15n**, and **15q** displayed a potential cytotoxic activity (IC_{50} <30 μM) against all the tested cell lines. Based on the acquired cytotoxicity assay results on different cancer cell lines, a structure-activity relationship (SAR) is established. The effect of different groups on the imidazo[1,5-*a*]pyridine ring as well as aryl/heteroaryl amine-linked to prop-2-en-1-one was demonstrated by synthesizing the compounds containing both electron-withdrawing and electron-donating substituents. Compounds containing H, F atoms, and CF_3, OMe groups on the phenyl group linked to imidazo[1,5-*a*]pyridin-1-yl residue showed superior cytotoxic activity. On the other hand, arylamine residue containing compounds such as **15a**, **15b**, **15f**, **15g**, **15h**, **15i**, **15k**, **15l**, **15n**, **15o**, **15p**, and **15r** (Table 1) displayed good anticancer effects against the selected cancer cell lines compared to heteroaryl amines except the compounds containing 5-indolylaryl group (**15c** and **15q**). The activity of the

com tent showed decreased activity against all the tested cancer cell lines. The introduction of the methyl group on 2-position of the indolylaryl group (**15j**) showed decreased cytotoxicity. It was noted that the introduction of the chloro group on the aryl amine-linked to prop-2-en-1-one (**15b**, **15i**, and **15p**) displayed potent cytotoxic activity against all the tested cancer cell lines followed by a methoxy group (**15f**, **15n**, and **15r**). However, the introduction of an additional OMe group on the meta position of aryl amine-linked to prop-2-en-1-one (**15l**) showed a significant reduction in potency. Moreover, compounds bearing the fluoro group on *para*-position of arylamino prop-2-en-1-one display reduced cytotoxicity. Among all the synthesized compounds **15c**, **15i**, and **15q** exhibited potent cytotoxic activity against all the tested cancer cell lines. Based on these observations, structure-activity relationship (SAR) of (*Z*)-3-(arylamino)-1-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)prop-2-en-1-ones was derived and depicted as shown in **Figure 3**.

Table 1. *In vitro* cytotoxicity (IC_{50} in μM) for the compounds **15a**–**r** on different cancer lines[a]

Compound	A549[b]	HCT-116[c]	B16F10[d]	BT-474[e]	MDA-MB-231[f]
15a	>30	>30	>30	>30	>30
15b	18.48±2.74	11.52±0.50	>30	3.82±0.93	3.84±0.72
15c	5.19±0.30	27.14±1.45	18.20±0.96	5.19±0.06	1.18±0.066
15d	>30	>30	>30	>30	>30
15e	>30	>30	>30	>30	>30
15f	21.83±0.14	7.72±1.25	>30	>30	>30
15g	>30	>30	>30	>30	>30
15h	13.18±0.39	>30	>30	16.94±1.64	25.11±1.58
15i	5.09±1.32	1.21±0.14	11.00±1.74	7.03±1.73	4.25±0.37
15j	>30	>30	>30	>30	>30
15k	19.15±4.41	>30	2.56±0.08	15.67±5.25	>30
15l	>30	>30	>30	>30	>30
15m	>30	>30	3.78±0.13	19.70±1.05	12.28±0.49
15n	>30	7.01±0.42	1.79±0.39	12.80±0.33	28.25±5.86
15o	>30	>30	>30	>30	>30
15p	ND	>30	1.24±0.17	37.32±0.89	>30
15q	4.80±1.03	17.4±1.09	5.12±0.33	12.98±0.25	28.69±0.59
15r	ND	>30	2.12±0.22	15.42±0.10	>30
Nocodazole	3.63±0.24	2.82±1.32	3.12±0.77	2.72±1.57	1.83±0.40

[a]50% inhibitory concentration after 48 h of drug treatment and the values are averages of three individual experiments, [b]human lung cancer cell line, [c]human colorectal cancer cell line, [d]murine melanoma cancer cell line, [e]human breast cancer cell line, [f]human triple-negative breast cancer cell line. ND: Not Determined.

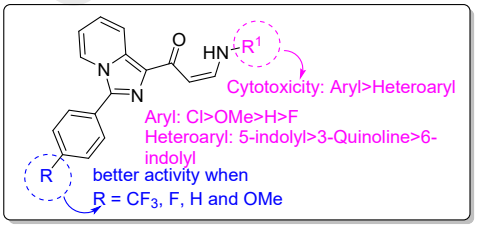


Figure 3. SAR of (*Z*)-3-(arylamino)-1-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)prop-2-en-1-ones.

compound **15i** was selected as a model substrate against HCT-116 cell line to carry further experiments to identify the mechanism of cancer cell growth inhibition

Many cytotoxic compounds displayed their cytotoxicity by arresting at a particular checkpoint of the cell cycle or through the induction of apoptosis or by a collective effect of both cell cycle arrest and apoptosis.[26] *In vitro* screening results revealed that compound **15i** exhibited significant cytotoxic activity against human colon cancer cell line (HCT-116). Hence, it was considered of interest to know whether this cell growth inhibition was due to cell cycle arrest or not. In the present study, HCT-116 cells were treated with the potential conjugate **15i** at a concentration of 0.5, 1.0, 2.5 μM for 48 h. Flow cytometric analysis revealed that compound **15i** arrested G0/G1 phase of the cell cycle in a dose-dependent manner i.e. the ratio of HCT-116 cells increased in G0/G1 phase from 28.2% at control, 31.5% at 0.5 μM, 38.7% at 1 μM and 41.9% at 2.5 μM as shown in **Figure 4**. Therefore, the acquired results concluded that compound **15i** produces cytotoxicity by inducing G0/G1 phase of cell cycle arrest.

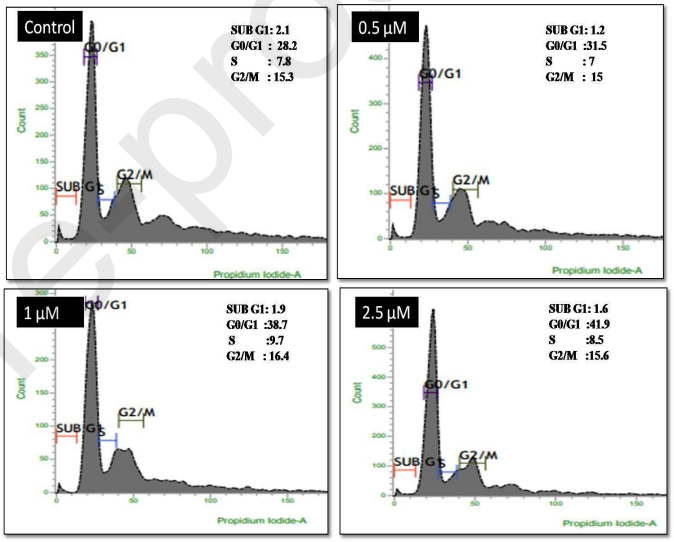


Figure 4. Cell cycle analysis of HCT-116 cells upon treatment with compound **15i** by flow cytometry. DNA histogram depicts various stages of cell cycle i.e., SubG1, G0/G1, S, G2/M. Each phase of treated cell cycle results was evaluated with DMSO control cells, cell cycle analysis. 10,000 events were examined for the results.

The proliferative potential and ability of adhered cells to form colonies from a single cell to multicellular solid tumors can be evaluated using the colony formation assay. Therefore, it is the method to determine the effectiveness of cytotoxic molecules based on cell reproductive death and cell survival. In this assay, the colony-forming ability of HCT-116 cells was measured by treating media with various concentrations (0.25, 0.5, and 1 μM) of compound **15i** over for 10 days to form colonies. Cells were then stained with crystal violet to visualize the colonies. The results concluded that there is a concentration-dependent inhibition of colony formation by **15i** on HCT-116 cells. Hence, this study confirmed that compound **15i** was efficient in inhibiting the colony formation even at a low concentration and maximum colonies disappeared at 1 μM concentration as shown in **Figure 5**.

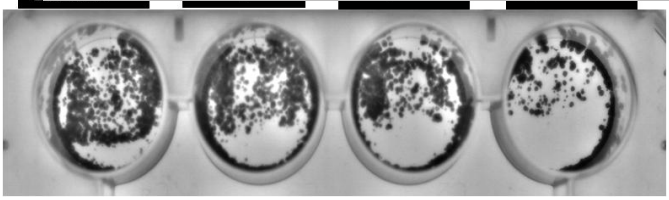


Figure 5. Effect of conjugate **15i** on the colony-forming capability of HCT-116 cells. (i) a) Control cells (HCT-116), b) **15i** (0.25 µM), c) **15i** (0.5 µM), d) **15i** (1 µM), (ii) Bar graph displaying the number of colonies remained following treatment of the cells through increasing concentration of the conjugate **15i**.

To examine whether the treatment with compound **15i** could lead to loss of cell viability and stimulate apoptosis, HCT-116 cells were treated with 0.5, 1, and 2.5 µM concentrations of compound **15i.** Upon 48 h post-treatment, cells were observed through a phase-contrast microscope and representative photographs were taken as shown in **Figure 6**. Results revealed that compound **15i** treated cells show a visible difference in morphology and viability. Based on the results, we concluded that as a concentration of the compound increased, there is a distinct decrease in cell viability with significant morphological changes such as cell shrinkage and detachment of cells from the substratum.

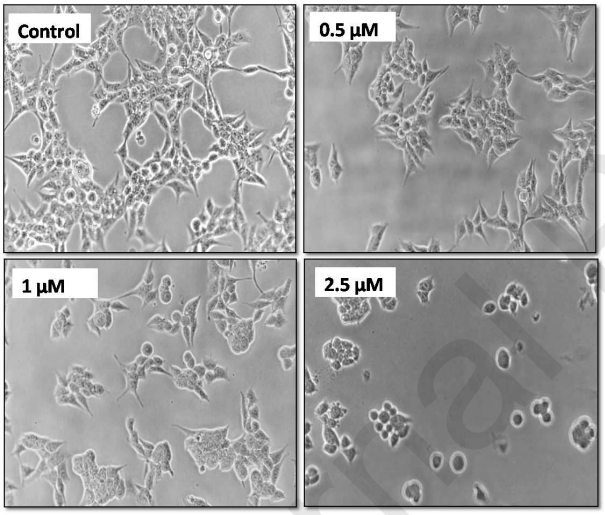


Figure 6. Phase-contrast images of HCT-116 cells treated with 0.5, 1, and 2.5 µM concentration of compound **15i** and observed for the compound induced morphological changes at 200× magnification.

Acridine orange (AO) is a fluorescent cationic dye that allows identifying the apoptotic cells. It can easily traverse through the intact cell membrane and intercalates into double-stranded DNA, releases green colour fluorescence upon excitation at 480-490 nm.[28] HCT-116 cells were incubated with the compound **15i** at 0.5, 1.0, 2.5 µM concentrations for 24 h and stained with acridine orange. After executing this assay, it was observed that the untreated control cells displayed normal homogenous morphology, and cells appeared to be a bright green colouration, indicating healthy and viable cells. However, upon treatment with compound **15i**, we observed irregular distribution of chromatin, membrane blebbing, and also green fluorescence diminished gradually as shown in **Figure 7** in a concentration-dependent manner indicating DNA break down, which is a typical hallmark of apoptosis.

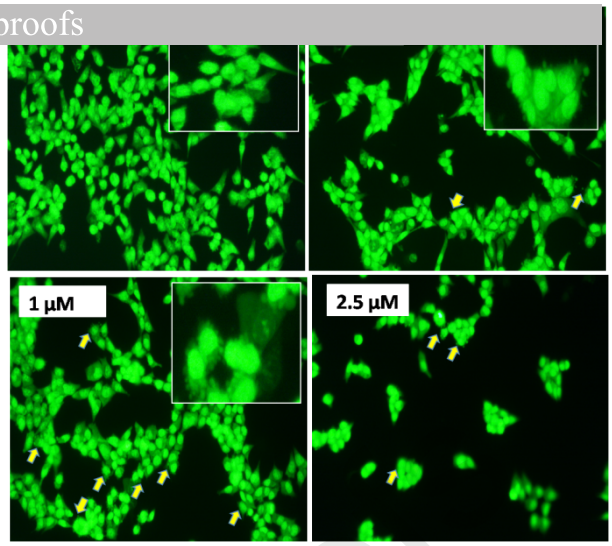


Figure 7. Acridine orange (AO) staining in HCT-116 cell line displaying apoptosis. HCT-116 cells were treated with increased concentrations of compound **15i** and stained with acridine orange. Reduced viable cells and apoptotic features were noticed at 200× magnification.

DAPI is a blue coloured fluorescent dye that stains ds DNA by strongly binding to A-T rich sequence of nucleic acid in a rigid manner. Nuclear modifications that were formed during apoptosis such as condensed nuclei, nuclear-fragmented bodies can be visualized through DAPI staining.[29] To investigate the compound **15i** induced nuclear changes in the HCT-116 cells, the DAPI staining was exploited. HCT-116 cells were treated with various concentrations of **15i** (0.5, 1.0, and 2.5 µM) for 48 h and stained with DAPI. The results from **Figure 8** concluded that **15i** treated HCT-116 cells displayed condensed and micronuclei formation along with the loose cell structure in a dose-dependent manner. Whereas, untreated control cells displayed normal intact nucleus which implies that conjugate **15i** has the potential to induce apoptosis in HCT-116 cells.

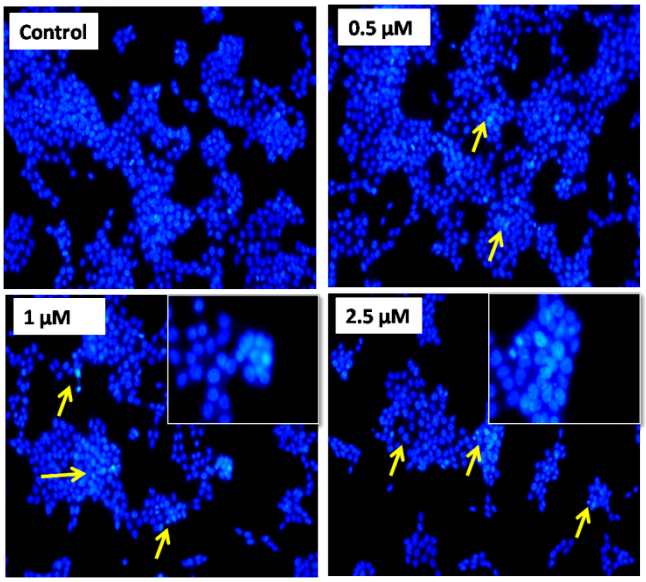


Figure 8. DAPI stain for nuclear morphology in HCT-116 cancer cell lines following 48 h post-treatment with conjugate **15i**. The images were captured with a fluorescence microscope using a DAPI filter at 200× magnification.

An increased level of reactive oxygen species (ROS) causes oxidative damage to mitochondrial permeability transition pore causing depletion of mitochondrial membrane potential and further it leads to the initiation of the intrinsic apoptotic

casc) is
it was considered of interest to identify the effect of **15i** on the ROS generation. Hence, cells were incubated with **15i** for 24 h at 0.5, 1, 2.5 µM concentration, and the intracellular ROS generation was observed by employing 2'-7'-Dichlorodihydrofluorescein diacetate (DCFDA) staining using fluorescent microscopy. DCFDA is a non-fluorescent dye which is altered into a fluorescent green DCF *via* intracellular esterases. The fluorescence intensity of DCF corresponds to the quantity of ROS generated. As shown in **Figure 9**, there is a considerable enhancement of DCF cell population by **15i** as compared to control which indicates that it induces apoptosis in a concentration-dependent manner through intracellular ROS generation in HCT-116 cells. This was also confirmed with the observed fluorescence emission where a considerable increase was observed dose-dependently. Overall, these results conclude that compound **15i** induced apoptosis *via* the mitochondrial pathway.

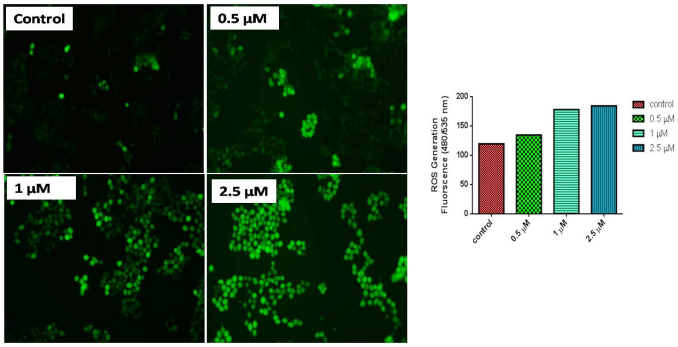


Figure 9. DCFDA images illustrating the effect of compound **15i** on the generation of Reactive Oxygen Species (ROS) in arbitrary units at concentrations 0.5, 1, and 2.5 µM on HCT-116 cancer cell lines. The corresponding graph represents fluorescence emitted at excited and emission wavelengths 480/535 nm in comparison to control at various doses.

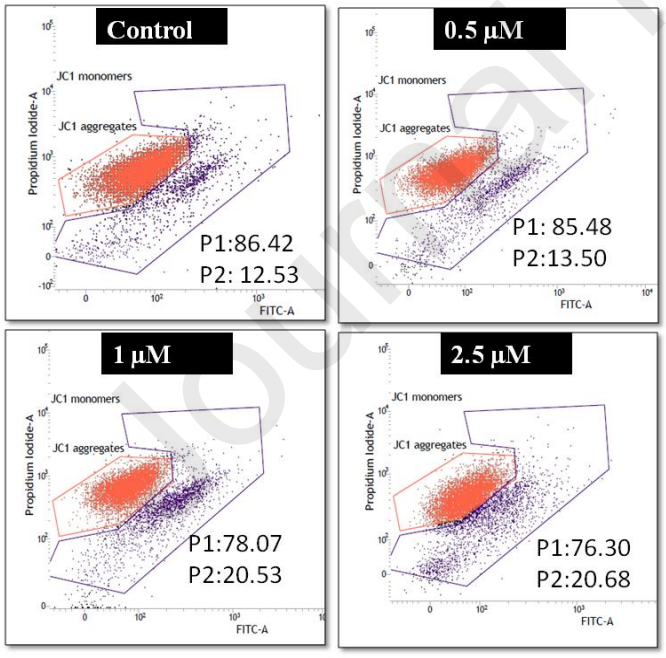


Figure 10. Effect of compound **15i** on mitochondrial membrane potential (ΔΨm) in HCT-116 cancer cells upon treatment with 0.5, 1, and 2.5 µM concentration. Cells were incubated with JC-1 dye and analyzed by flow cytometer (BD FACSVerse™, USA). Values in the figures correspond to the J aggregates and J monomers as P1 and P2 monomers populations respectively.

essential for bio-energetic function and mitochondrial integrity. Alteration of ΔΨm is an indication of early events that occur during apoptosis and chemical-hypoxia induced necrosis.[31] Mitochondrial damage induced by **15i** was estimated by identifying the drop in mitochondrial membrane potential. After 48 h of drug treatment with the compound **15i** at 0.5, 1.0, 2.5 µM concentrations, it was identified that ΔΨm of HCT-116 cells was reduced as shown in **Figure 10**, measured by JC-1 staining. JC-1 is a cationic lipophilic dye employed to stain the mitochondria. JC-1 binds dominantly to J monomers formed during hyperpolarisation in cells which emits fluorescence at 590±17.5 nm. On the other hand, healthy normal cells contain more J aggregates and fewer monomers due to which JC-1 dye could not bind and emits fluorescence at 530±15 nm. Flow cytometric analysis of HCT-116 cells after treatment with compound **15i** revealed that the compound has disturbed the ΔΨm in contrast to control. Based on the acquired results, we have identified an increase of J-monomers (disrupted mitochondrial cells) formed from normal polarised J aggregate. In the present study, the analysis was symbolized as a P1 (normal ΔΨm) and P2 populations (altered ΔΨm).

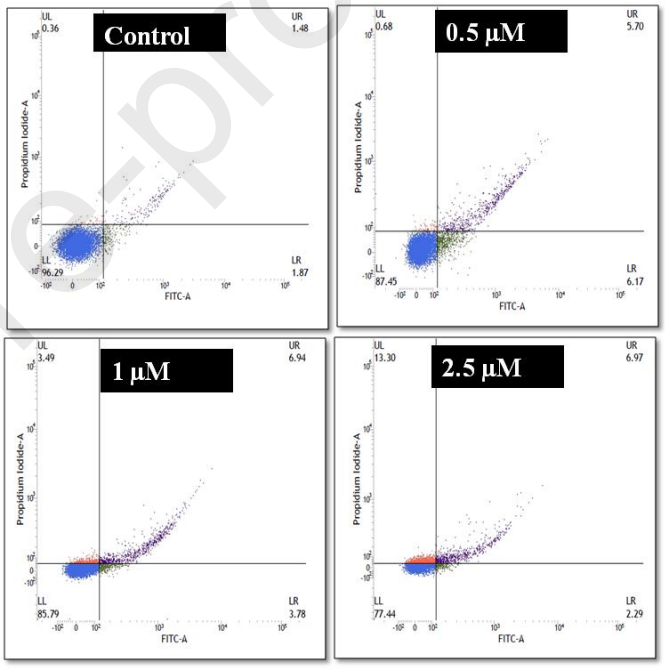


Figure 11. Annexin V-FITC/propidium iodide dual staining assay. HCT-116 cells were treated with compound **15i** and labeled with Annexin V-FITC/PI and analyzed for apoptosis using a flow-cytometer. Cells in the lower left quadrant (Q1-LL: AV^-/PI^-): live cells; lower right quadrant (Q2-LR: AV^-/PI^-): early apoptotic cells; upper right quadrant (Q3-UR: AV^+/PI^+): late apoptotic cells and upper left quadrant (Q4-UL: AV^-/PI^+): necrotic cells.

The apoptotic effect of compound **15i** was further analyzed by annexin V FITC/PI (AV/PI) dual staining assay to observe the incidence of phosphatidylserine externalization and also to identify whether it is due to apoptosis or necrosis.[32] This study assists the detection of live cells (Q1-LL; AV−/PI−), early apoptotic cells (Q2-LR; AV+/PI−), late apoptotic cells (Q3-UR; AV+/PI+) and necrotic cells (Q4-UL; AV−/PI+). In the present study, HCT-116 cells were treated with compound **15i** for 48 h at 0.5, 1, 2.5 µM concentrations to identify the apoptotic effect as shown in **Figure 11**. This assay reveals that compound **15i** has exhibited apoptosis in a dose-dependent manner with an increase in late apoptotic cells i.e. control (1.48%), at 0.5 µM (5.70%), at 1 µM (6.94%) and 2.5 µM (6.97%). Besides, at 1 µM (3.49%)

and Mani exhibited an increase in the necrotic cell population.

In summary, we detailed the design, synthesis, and cytotoxic potential of a series of (*Z*)-3-(phenylamino)-1-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)prop-2-en-1-ones against selected cancer cell lines. The cytotoxic data revealed that compound **15i** displayed an effective cytotoxic potential against the human colorectal cancer cell line (HCT-116). Moreover, detailed biological studies such as cell cycle analysis revealed that **15i** arrest HCT-116 cells in the G0/G1 phase of cell cycle and induced cell death by apoptosis. It was additionally confirmed by subsequent cell viability studies such as clonogenic assay, acridine orange staining, DAPI nuclear staining, generation of ROS, changes in mitochondrial membrane potential, and annexin V–FITC/PI assays. Based on the acquired results, compound **15i** can be considered as a potential lead molecule in the designed chemical library that would become a potential drug for the treatment of colorectal cancer.

Acknowledgments

G. S. M. is thankful to the Department of Pharmaceuticals, the Ministry of Chemicals and Fertilizers Govt. of India, New Delhi for the award of a Research Fellowships. NIPER Research Communication No.: NIPER-H/2020/M021.

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Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Graphical Abstract

Synthesis of (*Z*)-3-(arylamino)-1-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)prop-2-en-1-ones as potential cytotoxic agents

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