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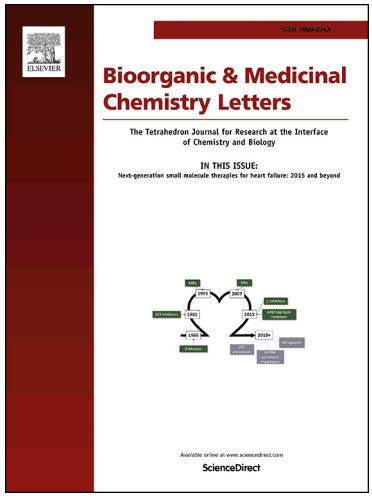

Synthesis and anticancer activity studies of indolylisoxazoline analogues

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Synthesis and anticancer activity studies of indolylisoxazoline analogues

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ABSTRACT

A new library of thirteen indolylisoxazolines **6a-m** has been synthesized by the treatment of indolylchalcones with hydroxylamine hydrochloride. Evaluation of anticancer activity of indolylisoxazolines **6a-m** led to the identification of potent compounds **6c-d**, **6i** and **6l**, with IC_{50} ranging 2.5-5.0 μM against the tested cancer cell lines. Using a number of complementary techniques such as acridine orange/ethidium bromide staining, PARP1 cleavage and DNA strand breaks assay, we show that the compounds **6c** and **6i** induce apoptosis in highly aggressive C4-2 cells. Our data further revealed that **6c** and **6i** inhibited C4-2 cells proliferation without inducing ROS. Finally, we show that compounds **6c** and **6i** also potently inhibit cell migration, indicating these compounds have the potential to serve as effective anti-cancer agents.

Indole scaffold is frequently present in many important synthetic and natural drug molecules including anticancer, anti-oxidants, anti-inflammatory, analgesics and anti-pyretic drugs.[1,2] Greater versatility and biodiversity of indole nucleus makes it highly privileged motif for the target-based drug design and development of anticancer agents. In the last decade, importance of indole motif in the anticancer drug development is reflected by the identification of many indole-based natural and synthetic anticancer agents with distinct mechanism of actions.[3-5] Among the indole-based compounds, indolylazoles containing five- and six-membered heterocycles linked indole have received greater attention due to their unique properties such as stability and hydrophilic nature, which improves aqueous solubility and thus simplify the formulation and *in vivo* uses (Fig 1).[6] 5-(3'- Indolyl) oxazoles isolated from different micro-organisms are reported to display interesting biological activities. For example, Labradorins **1** and **2** were found to be potential inhibitors (GI_{50} = 9.6-9.8 μg/mL) of lung cancer cell line.[7,8] Inspired by naturally occurring indolylazoles, we identified 5-(3-indolyl)-1,3,4-oxadiazoles **2** and indolyl-1,2,4-triazoles **3** as potent anticancer agents.[9] Some of the indolylazoles **3** are reported to show selective cytotoxicity against prostate cancer cell line (IC_{50} = 0.8 μM) and found to inhibits tubulin polymerization.[5] Compound A–289099 **(4)** with oxazoline ring was recognized as an orally active antimitotic agent with most promising anticancer property (IC_{50} = 6.2 nM) against NCI-H460 cells.[10]

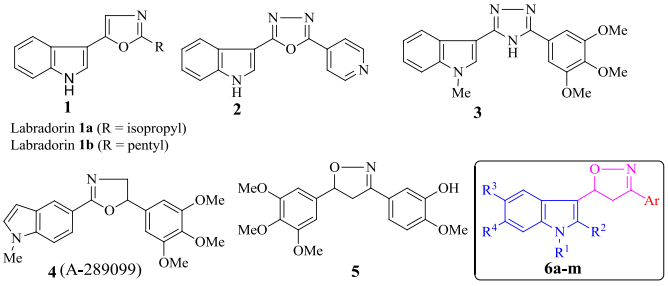

Fig. 1. Representative examples of cytotoxic indolyl(aryl)azoles

In recent years, accumulating evidences have revealed that isoxazolines possess important biological properties such as anticancer, antimicrobial, fungicidal, anticonvulsant, anti-inflammatory.[11] Some of the isoxazolines containing molecules have been found to elicit interesting anticancer activities with improved pharmacokinetics profile. For example, diaryl analogues **5** demonstrated potent cytotoxic activity by blocking most of the cancer cells in G2 phase.[12] Isoxazoline linked dihydro-quinazolinones significantly reduced the growth of cancer cell lines, and disrupted tubulin polymerization.[13]

As a part of our search for novel anticancer agents, in the present study, we designed indole-based heterocycles by linking indole and (hetero) aryl moieties through stable isoxazoline scaffold found in many biologically active molecules and drugs.[14-17] Many anticancer agents with substantial cytotoxicity have been

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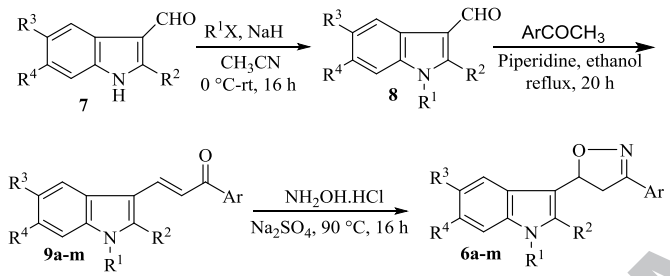
prepared by replacing alkenyl bond of Combrastatin with isoxazoline moiety.[10] With the broad spectrum activities observed for indole derivatives and isoxazolines, we envisioned linking two heterocyclic units in single molecule that could lead to compound with improved efficacy and selectivity.

Indolylisoxazolines **6a-m** were synthesized from the reaction of appropriate indolylchalcones **9a-m** with hydroxylamine hydrochloride in ethanol under refluxing conditions as outlined in Scheme 1. Firstly, substituted indole-3-carboxaldehydes **8** were achieved from Vilsmeier-Haack formylation of commercially available indoles, followed by the *N*-alkylation of **7** with an appropriate alkyl halide in the presence of sodium hydride. This afforded substituted indole-3-carboxaldehydes **8** in excellent yields by incorporating necessary modifications in reported procedures.[18] Next, condensation reaction of aldehyde **8** with diverse acetophenones under basic conditions led to indolyl chalcones **9a-m** in good to excellent yields. Finally, treatment of **9a-m** with hydroxylamine hydrochloride in the presence of a catalytic amount of anhydrous sodium sulfate in ethanol afforded indolylisoxazolines **6a-m** in 60–80% yields.

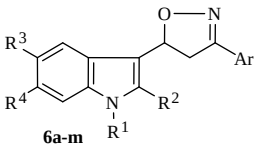


Scheme 1. Synthesis of indolylisoxazolines **6a-m**

The prepared indolylisoxazolines **6a-m** was fully characterized using IR, NMR (^{1}H & ^{13}C) and mass spectral data. In ^{1}H NMR spectra of **6a-m** revealed the appearance of a triplet at $\delta \sim 6.10$ ppm (methyne proton of isoxazoline) in addition to two doublet of doublet at $\delta \sim 3.82$ ppm and 3.65 ppm (methylene protons of isoxazoline). The ^{13}C NMR spectra of **6a-m** exhibited two characteristic signals at $\delta \sim 40$ ppm and ~ 80 ppm assigned to methyne and methylene carbons of isoxazoline ring. The HRMS spectra of isoxazolines **6a-m** showed molecular ion peak in agreement with the calculated mass. Based on HPLC analysis, purity of the synthesized compounds **6a-m** was found to be greater than 97%.

Synthesized indolylisoxazolines **6a-m** were screened for *in vitro* cytotoxicity against six human cancer cell lines: castration-resistant prostate cancer cell lines (22Rv1 & C4-2), PTEN-/- prostate cancer cell line (PC3), human embryonic kidney cell line (HEK293), pancreatic cancer cell line (BxPC3) and triple negative breast cancer cell line (MDA-MB-231) using the MTT assay. The IC_{50} values (in μM) were used to determine the growth inhibition in the presence of indolylisoxazolines **6a–m** against the tested cancer cell lines and Doxorubicin was used as a positive control. In our previous report, 3,4,5-trimethoxyphenyl and 4-pyridyl bearing chalcones were found to be potent against a panel of cancer cells.[18,19] In light of favourable effects observed with these derivatives on antitumor activity, isoxazolines **6a-i** with similar structural motifs were prepared.[18] Compound **6a** without any substituent on indole ring was found to be moderately active against the tested cancer cell lines. Next, introduction of a methoxy group at position-5 of indole ring resulted in analogue **6b** with reduced activity. Interestingly, by shifting the methoxy group from position-5 to position-6 in indole ring led to **6c** (IC_{50} = 3.5-16.5 μM) with substantial improvement in cytotoxicity. Replacement of the methoxy group with a fluorine atom was tolerated (MeO: **6c** IC_{50} = 3.5–16 μM vs F: **6d** IC_{50} = 3.66–5 μM). Insertion of a methyl group at position-2 of indole ring (compound **6e**) displayed moderate to good cytotoxicity. Recently, chlorobenzylindole and pyridyl motifs have been reported to be beneficial for the cytotoxicity.[4] By incorporating these important structural units, we prepared isoxazolines **6f-i** with cytotoxicity in micromolar range. The analogue **6i** with (4-chlorobenzyl)-2-methylindole and pyridyl moieties was found to be the most potent compound with IC_{50} of 2.5 μM against C4-2 and HEK293 cell lines. In subsequent modifications, we prepared derivatives **6l** and **6m** possessing 3,4,5-trimethoxyphenyl fragment which is vital in many anticancer agents, especially in various tubulin binding agents.[18] Compound **6l** with indole and 3,4,5-trimethoxyphenyl moieties displayed significant cytotoxicity ($IC_{50} \sim 4.5$ μM). Incorporation of C5-methoxy in indole ring (compound **6m**) or alteration of any methoxy group in trimethoxyphenyl fragment (compounds **6j** & **6k**) are detrimental for the activity.

Table 1. *In vitro* anticancer activity studies of indolylisoxazolines **6a-m** (IC_{50} μM)



Compd	R^1	R^2	R^3	R^4	Ar	22Rv1	C4-2	HEK293	BxPC-3	MDAMB-231	PC3
6a	H	H	H	H	4-pyridyl	> 100	12.75	> 100	30.5	19.66	> 100
6b	H	H	OMe	H	4-pyridyl	35.5	> 100	> 100	68.5	> 100	> 100
6c	H	H	H	OMe	4-pyridyl	16.32	**3.5**	**4.16**	9.5	7.38	10.45
6d	H	H	H	F	4-pyridyl	**3.66**	8	9.5	9.5	**5**	12.9
6e	H	CH_3	H	H	4-pyridyl	17.33	45.1	> 100	95.5	26.6	21.2
6f	4-ClBz	H	H	H	4-pyridyl	9.6	11.42	10.16	> 100	13.66	25.69
6g	4-ClBz	H	OMe	H	4-pyridyl	10.6	> 100	14.16	> 100	26	55.21
6h	4-ClBz	H	H	OMe	4-pyridyl	17.3	> 100	37.16	> 100	> 100	95.1
6i	4-ClBz	CH3	H	H	4-pyridyl	8.33	**2.5**	**2.5**	13.5	6.66	11.2
6j	H	H	H	H	3,4-$(OCH_2O)C_6H_3$	> 100	15.81	> 100	27	26.6	> 100
6k	H	H	H	H	3,4-$(OCH_3)_2C_6H_3$	16.66	65.5	> 100	> 100	> 100	70.14
6l	H	H	H	H	3,4,5-$(OCH_3)_3C_6H_2$	**4.51**	**5**	**5**	13.5	11.66	15.5
6m	H	H	OMe	H	3,4,5-$(OCH_3)_3C_6H_2$	11.33	71.2	45.4	> 100	26	42.58
Doxorubicin						6.32	3.25	3.9	9.12	6.25	9.58

*The activity data represent mean values of experiments conducted in triplicates at three independent times

Structure–activity relationship studies of the prepared indolylisoxazolines **6a-m** suggested that isoxazolines substituted as 6-methoxyindole, 6-fluoroindole, *N*-chlorobenzyl-2-methylindole and indole at position-5, and at position-3 with pyridyl and trimethoxyphenyl are favourable for the cytotoxicity (Fig. 2)

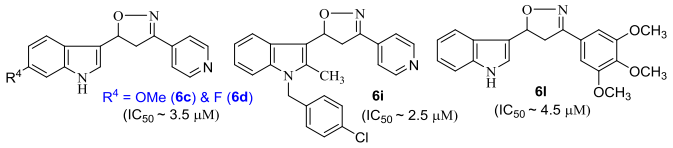


Fig. 2. Identified potent indolylisoxazolines

To investigate the mechanism of cell death induced by these compounds, we conducted various assays. Acridine orange (AO) stains both live and dead cells, whereas ethidium bromide (EB) stains only dead cells. Therefore, AO/EB staining was used to examine whether cell death occur *via* apoptosis or necrosis. Effect of indolylisoxazolines on the morphological changes of C4-2 cells is illustrated in Fig 3A. Incubation of compounds **6i** (IC_{50} = 2.5μM) and **6c** with C4-2 cells (IC_{50} = 3.5 μM) for 48 h resulted in typical nuclear fragmentation (red), whereas no visible changes in cell nucleus and cell integrity was observed for the control cells. The results of AO/EB staining revealed the compounds **6c** and **6i** triggered apoptosis in C4-2 cells.[20]

PARP is a eukaryotic DNA-binding protein that specifically recognizes the single stranded breaks formed during DNA damage. Once PARP detects the single stranded breaks it binds to the DNA, undergoes the structural change and begins the synthesis of a polymeric adenosine diphosphate ribose (poly (ADP – ribose) or PAR) chain which acts as a signal for the other DNA-repairing enzymes.[14] PARP1 cleavage assay (Fig. 3B) shows that indolylisoxazolines **6c** and **6i** induced apoptosis in C4-2 cells. C4-2 cells were treated with compounds **6c** and **6i** and level of cleaved PARP was observed. The results revealing the possible occurrence of DNA damage and apoptosis.[10]

DNA fragmentation is a key feature of apoptosis, a type of programmed cell death. Apoptosis is characterized by the activation of caspase-3 activated DNase (CAD).[20] CAD is normally inhibited by the protein, inhibitor of Caspase Activated DNase (ICAD). During apoptosis, the apoptotic effector caspase, caspase-3, cleaves ICAD and causes CAD to become activated.[21] Thus, we conducted DNA ladder assay[22] to verify that the cell death triggered by compounds **6c** and **6i** was indeed apoptosis. As shown in Fig. 3C, treatment of C4-2 cells with compounds **6c** and **6i** at 10 μM concentration for 48 h, triggered the formation of DNA ladder, which confirms that these compounds induce apoptosis.

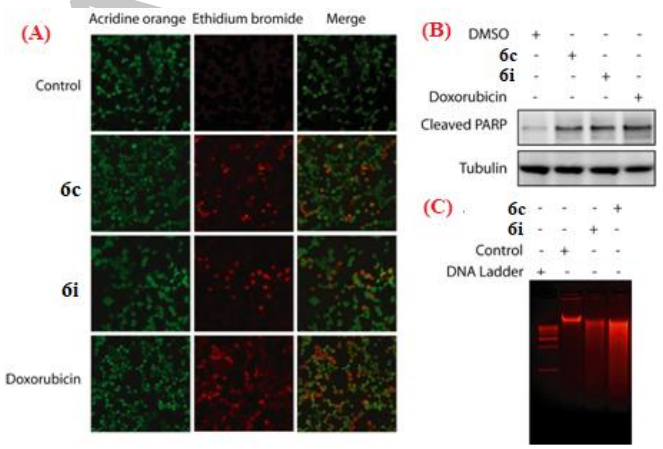


Fig. 3. Apoptosis inducing effects of **6c** and **6i** in C4-2 cells.

Previous studies have shown that compounds bearing indole scaffolds can induce cell death by increasing reactive oxygen species (ROS). ROS can include peroxides, superoxides, hydroxyl radicals and singlet oxygen. ROS are formed as a natural byproduct of the oxygen metabolism. Therefore, we measured whether compounds **6c** and **6i** induce ROS accumulation using DCFDA staining in C4-2 cells. H_2O_2-treated C4-2 cells were used as a positive control. ROS levels were quantified in untreated and C4-2 cells treated with H_2O_2 (10 μM), **6c** (10 μM) or **6i** (10 μM) for 48 h. While H_2O_2-treated cells displayed high DCFDA staining, **6c** and **6i**-treated cells shows similar DCFDA staining, indicating that these compounds do not induce apoptosis *via* ROS upregulation (Fig. 4)

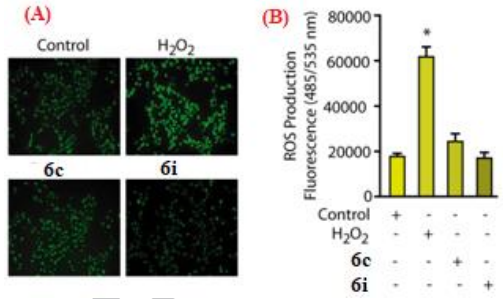


Fig. 4. Quantification of ROS levels induced by **6c** and **6i**

In solid cancers, metastasis is responsible for 90% of mortality. Therefore, we investigated whether these compounds inhibit cell migration. Wound healing assay is a classical and commonly used method for studying cell migration, which mimics wound healing *in vivo*.[23] In this assay, a scratch is introduced in a monolayer of cells and then images are taken at the beginning and at regular intervals to access cell migration rates. After cells were wounded, they were further incubated for 12h, 24h and 48h. The cells were fixed and images were captured, which showed that both compounds **6c** and **6i** potently inhibit C4-2 cell migration (Fig. 5). These results indicate that compounds **6c** and **6i** have the potential to serve as effective anti-cancer agents.

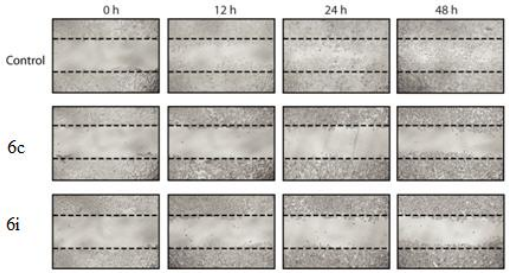


Fig. 5. Inhibition of cell migration on C4-2 cells effected by **6c** and **6i**

In summary, we have prepared indolylisoxazolines and some of them showed high potency in inhibiting the growth of 22Rv1, C4-2, PC3, HEK293, B**x**PC-3, and MDA-MB-231 cancer cell lines at micromolar concentrations. Compounds **6c-d**, **6i** and **6l** were significantly cytotoxic against all six cancer cell lines with selectivity towards C4-2 and HEK293 cells. Importantly, two of the tested compounds **6c** and **6i** also exhibited strong anti-cell motility property in C4-2 cells. The preliminary anticancer activity studies of indolylisoxazolines revealed that CH_3 group at second position of the indole ring, protection of indole nitrogen with chlorobenzyl group and insertion of OMe group at sixth position of indole ring are beneficial for anticancer activity and selectivity. Identified indolylisoxazolines are potent anticancer agents and could be explored for specific biological effects at molecular level.

Acknowledgments

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Supplementary Material

Supplementary data (general experimental procedures and analytical spectra of final compounds) associated with this article can be found, in the online version, at http://dx.doi.org/.

Graphical Abstract

Synthesis and anticancer activity studies of indolylisoxazoline analogues

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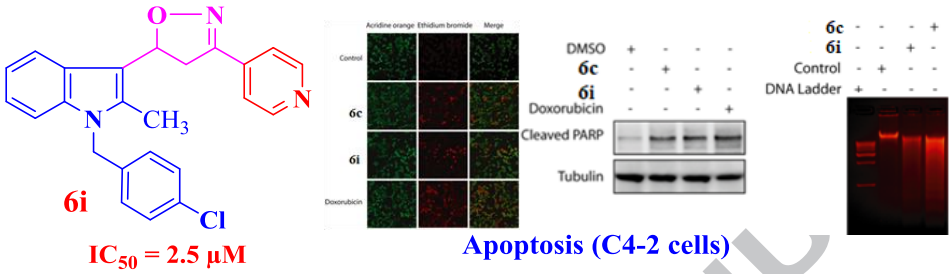

Highlights

- A series of indolylisoxazolines were prepared and assessed for their cytotoxicity.
- Isoxazoline derivatives **6c-d**, **6i** and **6l** displayed potent cytotoxicity (IC_{50} = 2.5-5.0 μM).
- Isoxazolines **6c** and **6i** induced apoptosis and inhibited C4-2 cell migration.