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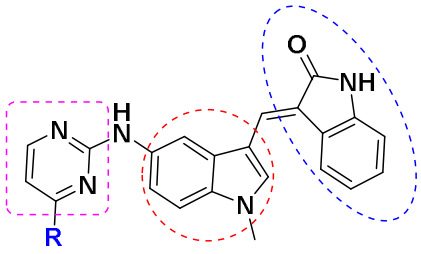

8(a-j)

R = Aryl, Heteroaryl

⟹

*Anticancer
*Morphology study
*Apoptosis
*Cell cycle arrest
*Annexin V binding

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ABSTRACT

In our endeavour towards the development of effective cytotoxic agents, a series of oxindole linked indolyl-pyrimidine derivatives were synthesized and characterized by IR, ^{1}H NMR, ^{13}C NMR and Mass spectral analysis. All the newly synthesized target compounds were assessed against PA-1 (ovarian), U-87MG (glioblastoma), LnCaP (prostate), and MCF-7 (Breast) cancer cell lines for their cytotoxic potential, with majority of them showing inhibitory activity at low micro-molar concentrations. Significantly, compound **8e** was found to be most potent amongst all the tested compounds with an IC_{50} value of (2.43±0.29 μM) on PA-1 cells. The influence of the most active cytotoxic compound **8e** on the cell cycle distribution was assessed on the PA-1 cell line, exhibiting a cell cycle arrest at the G2/M phase. Moreover, acridine orange/ethidium bromide staining and annexin V binding assay confirmed that compound **8e** can induce cell apoptosis in PA-1 cells. These preliminary results persuade further investigation on the synthesized compounds aiming to the development of potential cytotoxic agents.

In recent decades, cancer has achieved an unambiguous distinction for being one of the leading causes of death reported worldwide. About 8.2 million people lost their fight to this dreadful disease in 2012 and the figure has been feared to touch the 14 million mark by 2030.[1] The present scenario highlights the need for the discovery and development of novel drugs with new modes of action.[2] The recognition of new molecule that can be more effectual and reliable is still a major challenge for a medicinal chemist. Hence, this has led several research groups endeavouring for highly potent, effective and economical molecules as anticancer agents, which undoubtedly, is need of the hour. In the last one decade pyrimidine, indole and oxindole derivatives have surfaced up as a diverse class of heterocyclic compounds, reported with a multitude of activities, such as, anticancer, anti-inflammatory, anti-malarial, antifungal, antiviral, anti-HIV, anti-microbial, anti-tubercular, anti-adrenergic, anti-leishmanial, anti-psychotics and lipid lowering agents.[3] Numerous drugs are available in the market containing either of these privileged scaffolds such as, imatinib, sunitinib, dabrafenib, vinca alkaloids displaying potent anticancer activity.[3c,4] It is evidenced from the literatures that scores of pyrimidine, indole and oxindole inhibits the growth of various types of cancer namely, breast, bladder, lung, colon etc. These motifs have been extensively accounted for their ability to target multiple proteins, required at various stages of cancer progression.[4,5]

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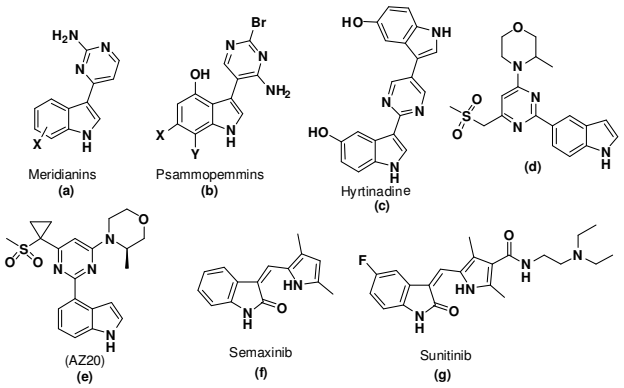


Figure 1. Structure of some representative indolyl-pyrimidine and oxindole derivatives as potential anticancer agents.

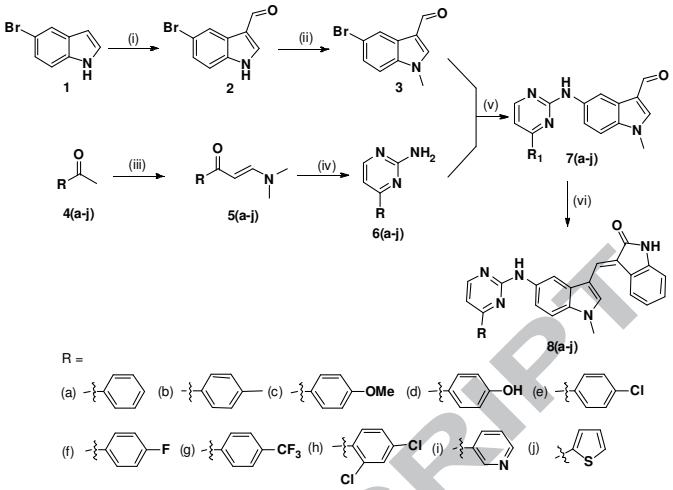
Indole linked pyrimidines are probably one of the most important heterocyclic scaffold present in nature bestowed with significant cytotoxic activity. Extensive database exploration has led to the findings of various naturally occurring indolyl-pyrimidines[3a,3b] such as meridianin, psammopemmins, hyrtinadine and aplicyanin with anticancer potentials (Figure 1).[6,7] Owing to the importance of indole linked pyrimidines in the field of cancer research, various researchers are keenly involved in the development of novel indolyl-pyrimidine derivatives as promising cytotoxic agents (Figure 1).[8] However, till now only one indole linked pyrimidine containing molecule Cediranib

(Recentin), a very potent inhibitor of VEGFR tyrosine kinase, was under phase III clinical trial by AstraZeneca.[9a]

Despite of the great advances in cancer therapy, there has been tremendous growth in the number and types of new anticancer agents containing indolyl-pyrimidine pharmacophore. Since, interesting biological activities are shown by indolyl-pyrimidine derivatives, there has been a considerable curiosity in the synthesis of new hybrid molecules containing indolyl-pyrimidine alongwith oxindole unit, as oxindole containing molecules have shown potent cytotoxic activity.[9b] Thus, our objective is to synthesize indolyl-pyrimidine derivatives with an array of substitutions at it, which may lead us towards an enhancement in cytotoxic activity. In order to develop pertinent leads for prospective antineoplastics, we have introduced appropriate substitutions at C-3 and C-5 positions of indole with oxindole and aminopyrimidines, respectively as well as aromatic substitutions at C-4 position of the aminopyrimidine ring. Furthermore, these compounds have been evaluated for their cytotoxicity. The most active cytotoxic compound was further tested *via* acridine orange staining, annexin V binding assay and cell cycle analysis.

The synthetic protocol to achieve target molecule involved six steps (Scheme 1), which instigates from commercially accessible 5-bromoindole **1** to obtain 5-bromoindole-3-carbaldehyde **2** in quantitative yield, by utilizing Vilsmeier reagent i.e., chloroiminium salt for the formylation at electron rich third position of indole under Vilsmeier-Haack reaction parameters.[10] This was followed by *N*-methylation to acquire 5-bromo-1-methyl-1*H*-indole-3-carbaldehyde **3** in high yield, using reported procedure.[11] On the other hand, different substituted aromatic and heteroaromatic enaminones **5(a-j)** were synthesised by employing respective acetophenones **4(a-j)** and refluxing it with DMF-DMA to obtain desired products in quantitative yields.[12] During the course of product formation, methanol was also formed as a by-product which was distilled off at regular intervals, as it interfered with the proceedings of reaction. The synthesized enaminone intermediates **5(a-j)** were then subjected to reflux with guanidine nitrate in *n*-butanol under basic condition to afford respective aminopyrimidines **6(a-j)** in high yields.[13] Presence of electron withdrawing groups on the ring afforded high yields, as compared to electron donating groups in both the aforementioned reactions. The two privileged moieties **3** and **6(a-j)** were subsequently linked in the penultimate step involving Ullmann type C-N bond coupling reaction to afford compounds **7(a-j)**.[14] In this reaction, CuI was utilized to carry out the coupling under strict anhydrous atmosphere, as the presence of moisture hindered the progress of reaction. As aminopyrimidines are very weak nucleophiles, a strong electron donating agent such as DMEDA was added under basic condition of Cs_2CO_3, to carry out the merging of both the motifs in to a single chemical entity. Finally, the desired product **8(a-j)** was achieved by Knoevenagel condensation[15a,b] of coupling product **7(a-j)** and oxindole under basic condition, to acquire target compounds. The stereochemistry of the final product was confirmed by ^{1}H NMR and the previous literature reports.[15c-g]

The synthesized oxindole linked indolyl-pyrimidine derivatives **8(a-j)** were evaluated for their *in vitro* cytotoxicity against cancer cell lines such as breast cancer (MCF-7), prostate cancer (LnCaP), ovarian teratocarcinoma (PA-1), brain glioblastoma (U-87 MG) and human normal prostate epithelial (RWPE-1) cell lines by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[16] 5-Fluorouracil (5-FU) and Sunitinib were taken as the reference in this study.



Scheme 1: Synthetic scheme for the synthesis of oxindole linked indolyl-pyrimidine derivatives: (i) $POCl_3$, DMF, 0-75 °C; (ii) CH_3I, NaOH, ACN, rt, 3 h; (iii) DMF-DMA, xylene, reflux, 48 h; (iv) guanidine nitrate, NaOH, *n*-butanol, reflux, 12 h; (v) CuI, DMEDA, Cs_2CO_3, 1,4-dioxane, reflux, 20 h; (vi) oxindole, piperidine, EtOH, reflux, 6 h.

The IC_{50} (μM) values (concentration required to achieve 50% inhibition of the tumor growth) of the tested compounds are listed in Table 1. Preliminary cytotoxicity screening of the compounds **8(a-j)** was carried out at 100 μM concentration to determine cell death. The IC_{50} values were not determined for the compound displaying poor activity at this concentration. It is evident from the preliminary results that some of the compounds have shown significant cytotoxicity on human cancer cell lines such as PA-1, U-87 MG and LnCaP. From the close analysis of IC_{50} values (Table 1), it was observed that, oxindole linked indolylpymidine with phenyl substitution **8a** displayed substantial inhibitory activity against all tested cancer cell lines while the phenyl substituted with electron donating groups such as –Me **8b** and –OMe **8c** at *para* position led to loss in cytotoxicity against all cancer cell lines. Enhancement in cytotoxicity was observed against all cancer cell lines, when the phenyl ring is introduced with free hydroxyl group at *para* position of the phenyl ring **8d**. Notably, it showed enhanced inhibitory activity against U-87MG and LnCaP cells as compared to the reference drugs. Interestingly, the compound substituted with a chloro group at *para* position of phenyl ring **8e** has led to further improvements in cytotoxic activity and has displayed enhanced activity against all tested cancer cell lines as compared to the reference drug. Moreover, cytotoxicity was decreased while substituting the phenyl ring with halogen groups such as *p*-F **8f** and *p*-CF_3 **8g** on phenyl ring, although compound **8f** showed promising results against U-87MG cells as compared to their reference drugs. Furthermore, the indolyl-pyrimidine substituted with 2,4 dichlorophenyl **8h** was shown to be specifically active against U-87MG cancer cells. Replacement of phenyl group with the heterocyclic substituents such as pyridine **8i** and thiophene **8j**, displayed improved cytotoxicity towards all the tested cancer cell lines. Fascinatingly, compound **8j** displayed excellent cytotoxic activity against all tested cancer cells, while compound **8i** showed good activity against U-87MG and LnCaP cells. Significantly, compound **8e** was found to be most potent among all the tested compounds with an IC_{50} value (2.43±0.29 μM) on PA-1 cells, while majority of the compounds showed excellent cytotoxic activity against prostate cancers cells.

Table 1. *In vitro* cytotoxic activity of oxindole linked indolyl-pyrimidine derivatives.

Comp.	[a]IC_{50} (µM)				
	PA-1	U-87MG	LnCaP	MCF-7	RWPE-1
8a	17.27±1.07	14.13±0.54	13.42±0.9	42.88±2.20	95.63±1.89
8b	>100	>100	>100	>100	nd
8c	>100	>100	>100	>100	nd
8d	9.27±0.99	13.15±0.32	10.8±0.41	39.63±0.42	58.42±2.97
8e	2.43±0.29	5.82±0.69	6.59±0.66	23.51±1.72	41.05±5.37
8f	31.75±0.91	16.42±0.91	16.34±0.66	48.10±2.04	168.45±3.78
8g	63.94±0.83	25.97±0.8	>100	65.2±12.63	nd
8h	>100	36.79±0.44	>100	>100	nd
8i	18.1±1.82	14.84±0.77	14.15±0.67	46.59±1.74	69.83±2.98
8j	7.94±0.38	7.18±1.54	10.35±0.31	30.75±2.27	64.89±6.52
F[b]	8.51±0.31	15.48±3.50	47.18±2.32	32.27±4.33	nd
S[c]	7.97±1.33	59.75±3.06	12.61±0.99	23.76±0.42	23.0±1.23

[a] 50% inhibitory concentration and mean ± SD of three individual experiments performed in triplicate; [b] 5-Fluorouracil was included as reference standard; [c]Sunitinib; [nd]not determined.

To check the safety and selectivity of the synthesized compounds towards normal cells, we have further performed MTT assay on normal prostate epithelium cells (RWPE-1) for compounds which showed IC_{50} less than 20 µM on LnCaP (prostate cancer) cells. It was quite interesting to observe that the tested compounds displayed more cytotoxicity towards LnCaP cancer cells as compared to RWPE-1 cells (Figure 2). From Table 1 it is clear that compound **8f** was highly selective toward LnCaP cell line (IC_{50} was almost 10.3 fold higher in RWPE-1 cells), when compared to the normal prostate epithelial cells. The highest cytotoxicity of compound **8e** was observed on PA-1 cancer cell line with IC_{50} of 2.43±0.29 µM; hence this compound was selected for further mechanistic analysis on PA-1 cell line. These preliminary results of biological screening of the tested compounds could offer an encouraging support in this field that may lead to the discovery of novel cytotoxic agent.

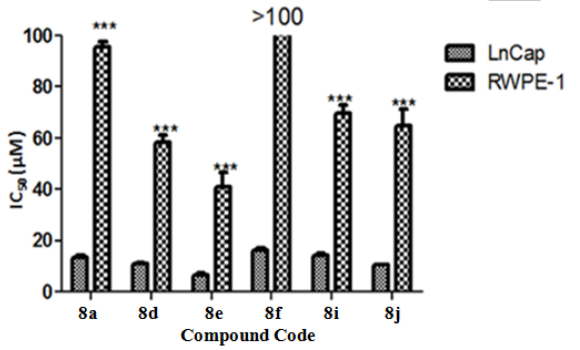


Figure 2. Specificity of compounds **8a**, **8d**, **8e**, **8f**, **8i** and **8j** towards normal prostate epithelial cells (RWPE-1) compared to cancer cells.

The induction of apoptotic bodies formation (consist of damaged organelles and DNA) by anticancer agents, has always been a preferred choice in developing anti-cancer therapeutics. To observe whether the treatment with the compounds could lead to loss of cell viability and induction of apoptosis, PA-1 cells were treated with the most active compound **8e**. Phase contrast microscopy of compound **8e** treated PA-1 cells showed cell shrinkage, cell wall deformation and resulted in reduced number of viable cells, whereas these distinctive morphological features were absent in control as shown in Figure 3.

Acridine orange/ethidium bromide (AO/EB) fluorescent staining assay was performed to differentiate the live, apoptotic and necrotic cells.[17,18] AO permeates the intact cell membrane and stains the nuclei green, whereas EB can stain only cells which has lost membrane integrity and stain the nucleus red. It can be inferred from Figure 4 that the control cells showed normal morphology and appeared green in colour. Fluorescence microscopic images of cells treated with 2.5 µM of compound **8e** clearly showed the morphological changes such as cell shrinkage, membrane blebbing, chromatin condensation and apoptotic body formation, suggesting that the compound **8e** induced cell death in PA-1 cancer cells *via* apoptosis (Figure 4).

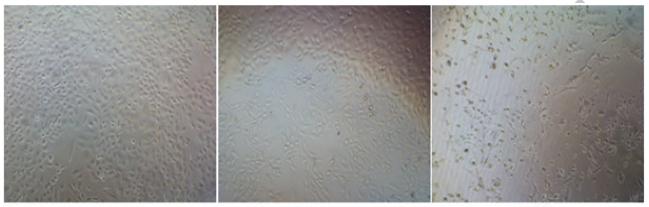


Figure 3. Morphological changes in PA-1 cells. Light microscopy: (a) not treated with compound **8e** was used as control for 24 h; (b) treatment with compound **8e** (1 µM) for 24 h; (c) treatment with compound **8e** (2.5 µM) for 24 h.

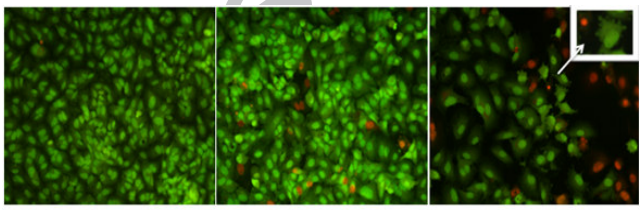


Figure 4. AO/EB staining of compound **8e** in PA-1 cells. Fluorescence microscopy (a) Not treated with compound **8e** was used as control for 24 h; (b) treatment with compound **8e** (1 µM) for 24 h; (c) treatment with compound **8e** (2.5 µM) for 24 h.

The apoptosis inducing effect of compound **8e** on PA-1 cancer cells was further investigated using annexin V-FITC/propidium iodide staining assay.[19] PA-1 cells were treated with two concentrations of 1 and 2.5 µM of compound **8e** for 24 h and further stained with Annexin V-FITC and propidium iodide. As shown in Figure 5, compound **8e** increased the percentage of early apoptotic cells (from 1.65% (ctrl) to 16.68% (1 µM), 25.18% (2.5 µM) respectively) which indicate that **8e** induced apoptosis of PA-1 cancer cells in a dose dependent manner.

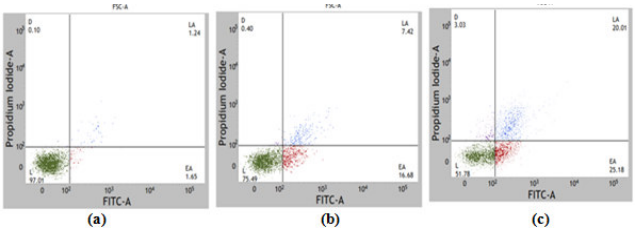


Figure 5. Flow cytometry analysis of apoptotic cells after Annexin V-FITC/propidium iodide (PI) staining (representative result of three independent experiments); L: Live, EA: Early Apoptotic, LA: Late Apoptotic, D: Dead. (a) PA-1 cells without treatment; (b) PA-1 cells treated with 1 µM of **8e** for 24 h; (c) PA-1 cells treated with 2.5 µM of **8e** for 24 h.

Many of the cytotoxic compounds exert their growth inhibitory effect by arresting the cell cycle at a specific checkpoint.[20] *In vitro* screening results revealed that compound **8e** showed significant activity against PA-1 cells. Therefore, we examined the effect of compound **8e** on cell cycle using flow cytometry. PA-1 cells were treated with various concentrations of compound **8e** for 24 h, stained with propidium iodide and samples were analysed by using flow cytometry. The data obtained from the study indicated that majority of control cells (without treatment)

were in G1 phase (73.23%). Treatment with compound **8e** resulted in dose dependent accumulation of cells in G2/M phase. For instance 17.55% and 31.13% of cells were in G2/M phase after treatment with 1 and 2.5 µM concentrations (Figure 6). These results clearly indicate the G2/M phase cell cycle arrest.

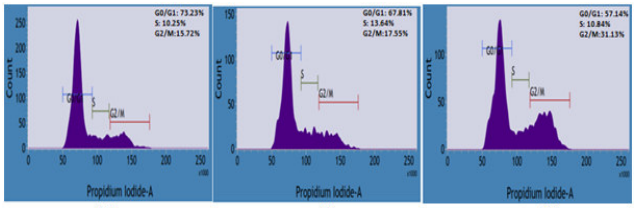


Figure 6. Effect of compound **8e** on cell cycle progression of PA-1 cells, (a) control cells, (b) Cells treated with 1 µM for 24 h, (c) Cells treated with 2.5 µM for 24 h. The analysis of cell cycle distribution was performed by using propidium iodide staining method.

In present study, a panel of oxindole linked indolyl-pyrimidine derivatives were synthesized and evaluated for their *in vitro* cytotoxic potential against four cancer cell lines. The compounds showing promising results (**8a**, **8d**, **8e**, **8f**, **8i**, **8j**) were also evaluated for the cytotoxicity on normal prostate epithelial cell line (RWPE-1). Interestingly, the tested compounds showed more selectivity toward LnCaP cancer cell as compared to the normal prostate epithelial cells. Notably, compound **8e** was found to be most potent amongst all the tested compounds, having IC_{50} value (2.43±0.29 µM) on PA-1 cells, while most of the tested compounds showed enhanced cytotoxicity against prostate cancers cells compared to the reference drugs. The cell cycle analysis results revealed that compound **8e** arrested the PA-1 cell cycle at G2/M phase in dose dependent manner. Furthermore, the apoptotic potential of the most active cytotoxic compound **8e** was assessed by acridine orange/ethidium bromide (AO-EB) staining and annexin binding assay on PA-1 cells. These preliminary results encourage further investigation on synthesized compounds aiming to the development of new potential cytotoxic agents.

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21. Typical reaction procedure, experimental data for all the compounds and general methods are provided in the supporting information.