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PAPER

Umbelliferone-oxindole hybrids as novel apoptosis inducing agents†

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In furtherance of our endeavour towards the synthesis of novel bioactive agents, a panel of (*E*)-3-((7-hydroxy-4-methyl-2-oxo-2*H*-chromen-8-yl)methylene)indolin-2-one derivatives were synthesized using diverse 5-substituted oxindoles and 7-hydroxy-4-methyl-2-oxo-2*H*-chromene-8-carbaldehyde (**3a**). These synthesized analogues were further evaluated for their *in vitro* cytotoxic activity against MDA-MB-231 (breast), MCF-7 (breast), DU145 (prostate), PC-3 (prostate) and A549 (lung) cancer cell lines by employing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Among all the synthesized compounds, compounds **7q** (IC_{50} = 9.52 and 9.9 µM) and **7r** (IC_{50} = 15.3 and 11.7 µM) showed the most significant cytotoxicity towards DU145 and PC-3 cancer cell lines respectively. The compounds **7q** and **7r** were found to be comparatively safe towards human normal prostate epithelial (RWPE-1) cells. The apoptosis inducing effect of compounds **7q** and **7r** on PC-3 and DU145 cancer cells was further investigated using annexin V-FITC/propidium iodide staining assay, which confirmed the increase in the percentage of early apoptotic cells. Moreover, the cell cycle analysis revealed cell cycle arrest particularly at G0/G1 phase in PC-3 and DU145 cells. In addition to that, the treatment with compounds **7q** and **7r** led to collapse of the mitochondrial membrane potential (DΨm) and increased levels of reactive oxygen species (ROS) in PC-3 and DU145 cells. Western blotting was performed to examine the appearance of active forms of cytochrome c and cleaved PARP (Poly ADP ribose polymerase), indicator proteins of apoptosis in PC-3 cells; the study confirmed the triggering of mitochondrial mediated apoptotic pathway upon exposure of compounds **7q** and **7r**.

Introduction

Cancer is alone turning into one of the most devastating health problems worldwide as the number of deaths due to it is more than those caused by AIDS, malaria, and tuberculosis combined.[1] According to survey if new treatments are not found by 2030, there will be 27 million people with cancer and 17 million deaths annually.[2] Although there have been untired efforts and screening of myriads of compounds for anti-cancer activities, the ambiguity about the cause of origin of cancer, limitations in its detection at early stage, its direct relation with process of cell division, metastatic nature of cancer cells and lack of drug penetration to cancer tissue are some of the characteristics of this disease which prove as stumbling block in the successful treatment of cancer.[3]

The 3-substituted oxindole forms possess various biological activities and continue to be a main structural unit of many natural and pharmacologically active compounds (Figure 1).[4] For example, sunitinib and indirubin are recognized anticancer agents containing 3-alkenyl-oxindole as basic scaffold.

Umbelliferone, also known as 7-hydroxycoumarin, hydrangine, skimmetine, and β-umbelliferone, is a common natural product of the coumarin family (Figure 1).[5] Umbelliferone primarily occurs in many familiar plants from the Apiaceae (Umbelliferae) family such as carrot, coriander and garden angelica. According to recent studies, umbelliferone was found to exhibit significant anticancer effects *via* the induction of apoptosis, cell cycle arrest and DNA fragmentation in HepG2 cancer cells.[6] Scopoletin, a naturally occurring umbelliferone derivative, can induce cell cycle arrest and increase apoptosis in human prostate tumor cells and human leukemia cell line *via* activation of caspase-3.[7]

Most of the present anticancer drugs generally act on metabolically active or rapidly proliferating cells, and suffer from poor selectivity between normal and cancerous cells. The high toxicity and poor tolerance of the current anticancer drugs generates the need to identify new molecules with potent antitumor activity, low toxicity and minimum side effects.[8]

Molecular hybridization is an effective tool to design more active and novel chemical entities by covalently combining two or more drug pharmacophores into a single molecule.[9] Recently, it has been observed that chromone-pyrimidine, chromone-indolinone, chromonepyrazole, indole-pyrimidine, indole-indolinone, pyrazine–

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coumarins and indole-pyrazole conjugates demonstrated profound growth inhibitory activity against different cancer cells.[10,11]

In this context, we desire to exploit 7-hydroxy-4-methyl-2-oxo-2*H*-chromene-8-carbaldehyde as the diversity surrogate to condense with various 5-substituted oxindoles. These umbelliferone-oxindole derivatives may have the advantages of tunable anticancer activity, low toxicity and structural diversity. Furthermore, the chemical processes and reactions implicated are simple and concise, which make it practicable for the bulk production of important oxindoles derivatives.

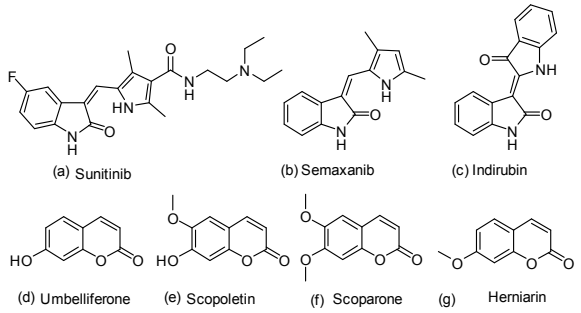


Figure 1: Structure of some representative bioactive oxindole (a, b and c) and umbelliferone (d, e, f and g) derivatives.

Results and discussion

Chemistry

The synthesized 3-umbelliferone substituted oxindoles compounds of the present study comprise two core structural elements: (i) a 5-substituted oxindole and (ii) a 4-methyl umbelliferone moiety.

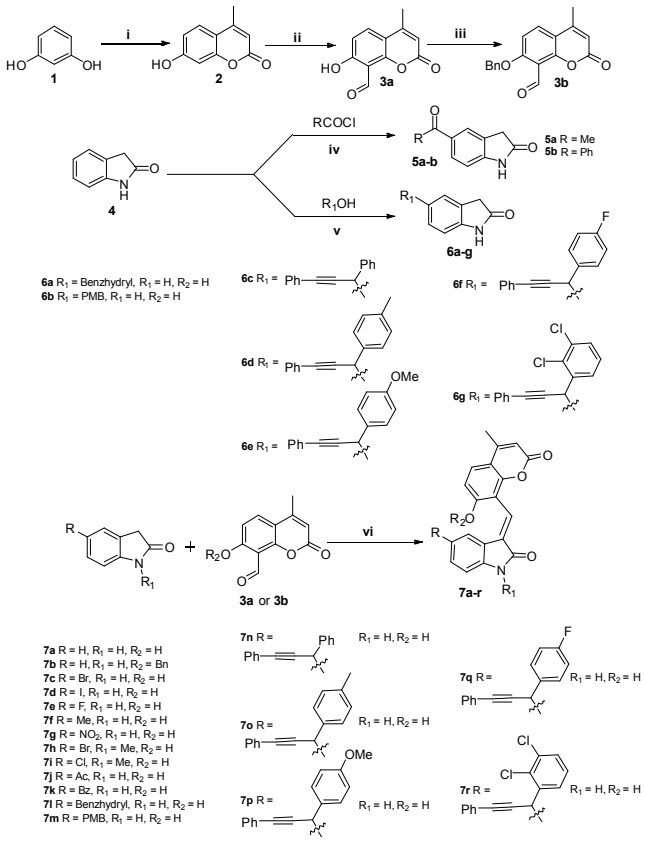
The 4-methyl umbelliferone scaffold required for the syntheses of target compounds was synthesized using resorcinol as the starting material (Scheme 1). Resorcinol (**1**) upon treatment with ethyl acetoacetate and sulphuric acid gave 4-methyl umbelliferone (**2**).[12] Next, the Duff reaction was performed on 4-methyl umbelliferone using urotropine to give the required 7-hydroxy-4-methyl-2-oxo-2*H*-chromene-8-carbaldehyde (**3a**).[13]

Knoevenagel condensation was employed using oxindole (**4**) and aldehyde (**3**) to give target compound **7a** in good yield. Compound **7a** was formed as a single isomer with *E* configuration, confirmed by ^{1}H NMR studies.[14] Next, the reaction was tried using *O*-benzyl protected aldehyde (**3b**) and the compound **7b** was also formed as a single isomer. Encouraged by such interesting observations, different 5-halo substituted oxindole were subjected to Knoevenagel condensation with aldehyde (**3a**) to give corresponding target compounds (**7c-e**) in good to excellent yields. Reactions using 5-methyl and 5-NO_2 substituted oxindoles furnished compounds (**7f-g**) in good yields. *N*-methyl protected 5-halo substituted oxindole substrates upon Knoevenagel condensation with aldehyde (**3a**) gave higher yields (**7h-i**).

To extend the substrate range, diversified 5-substituted oxindoles were synthesized from oxindole. Oxindole (**4**) upon treatment with acid chlorides such as acetyl chloride and benzoyl chloride gave 5-acylated oxindoles (**5a-b**).[15] Reactions with oxindole and bulky alcohols in the presence of Lewis acid gave 5-alkylated oxindoles (**6a-g**).

These 5-substituted oxindoles (**5a-b**) and (**6a-g**) were then subjected to Knoevenagel condensation with aldehyde (**3a**) to give corresponding final compounds (**7j-r**).

It is to be noted that all the synthesized target compounds were obtained specifically in thermodynamically stable *E* configuration form. The configuration can be easily identified by ^{1}H NMR analysis. The deshielding of C4-proton of oxindole could be due to the presence of nearby umbelliferone ring in the *E* isomer form.



Scheme 1: Synthetic scheme for the synthesis of umbelliferone linked oxindole derivatives: (i) ethyl acetoacetate, H_2SO_4, 10 °C, 4 h; (ii) urotropine, sulfuric acid, acetic acid, 120 °C, 4 h; (iii) benzyl bromide, potassium carbonate, acetonitrile, reflux, 6 h; (iv) $AlCl_3$, DMF, reflux, 6 h; (v) *p*TSA, nitromethane, 6 h; (vi) piperidine, ethanol, reflux, 4 h.

Biological evaluation

***In vitro* anticancer activity.** All the newly synthesized compounds were evaluated for their *in vitro* cytotoxic activity against MDA-MB-231, MCF-7, DU145, PC-3 and A549 cancer cell lines by employing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[16] Sunitinib was used as the reference for this study. The concentration response course analysis was performed to determine drug concentrations required to inhibit the growth of cancer cells by 50% (IC_{50}) after incubation for 48 h. The results from the *in vitro* anticancer studies exposed that the synthesized target compounds exhibited different levels of anticancer properties (Table 1). From the close examination of the IC_{50} values, it was observed that the Knoevenagel product (**7a**) of oxindole and aldehyde (**3**) was active only against DU145 cell lines, while the *O*-benzylated product (**7b**) was found to be inactive. Compound bearing 5-bromo (**7c**) substitution showed no activity against all the tested cell lines, whereas compound with 5-iodo (**7d**) substitution

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displayed remarkable activity against A549 cells along with moderate activity towards DU145 and PC-3 cell lines. Target compound with 5-fluro substitution (**7e**) was specifically active against A549, whereas compound with 5-methyl (**7f**) substitution was inactive towards all the tested cell lines. The compound with 5-nitro (**7g**) substitution was also moderately active against A549 cells. Compound bearing 5-bromo *N*-methyl (**7h**) substitutions showed good activity selectively against PC-3 cells, on the other side compound with 5-chloro *N*-methyl (**7i**) substitutions displayed moderate activity against DU145, PC-3 and A549 cell lines. Compound containing 5-acetyl (**7j**) substitution showed moderate activity selectively against PC-3 cells, on the other hand compounds with 5-benzoyl moiety (**7k**) and 5-benzhydryl group (**7l**) were found active only against A549 cells; however the compound bearing 5-*p*-methoxy benzyl (PMB) group (**7m**) displayed good activity against PC-3 and A549 cells and was also moderately active against DU145.

Target compound holding bulky diphenyl propargyl substitution at 5th position of oxindole (**7n**) showed good activity against MCF-7 cells and moderate activity against PC-3, DU145 and A549 cell lines. The compound having *p*-methyl substitution on diphenyl propargyl moiety (**7o**) was found to be moderately active against all the tested cell lines. With *p*-methoxy substitution on diphenyl propargyl moiety (**7p**), the compound was active selectively against prostate cancer cell lines PC-3 and DU145. Interestingly, when diphenyl propargyl moiety was substituted with halogens such as fluoro (**7q**) and chloro (**7r**), the anticancer activity of the compounds was remarkably increased against all the tested cancer cell lines.

Table 1. *In vitro* anticancer activity of umbelliferone-oxindole derivatives (IC_{50} μM)

C	MDA-MB-231	MCF-7	DU145	PC-3	A549	RWPE-1
7a	>50	>50	19.3±0.6	>50	>50	ND
7b	>50	>50	>50	>50	>50	ND
7c	>50	>50	>50	>50	ND	ND
7d	>50	ND	19.8±0.3	25.9±0.2	9.0±1.4	ND
7e	>50	>50	>50	ND	9.7±2.5	ND
7f	>50	>50	>50	>50	>50	ND
7g	>50	ND	>50	ND	38.3±0.5	ND
7h	>50	>50	>50	13.6±0.8	>50	ND
7i	>50	>50	35.4±0.1	22.0±1.6	28.0±0.8	ND
7j	>50	>50	>50	35.0±2.1	>50	ND
7k	>50	>50	>50	>50	49.9±2.0	ND
7l	>50	ND	>50	ND	15.4±0.0	ND
7m	>50	>50	31.6±0.2	19.3±2.3	14.5±1.5	ND
7n	>50	11.6±1.4	32.6±0.4	25.5±2.2	31.3±0.3	ND
7o	34.2±0.3	26.0±2.1	24.1±3.2	17.5±0.5	17.4±0.2	ND
7p	>50	>50	31.7±0.7	16.5±0.3	>50	ND
7q	15.2±0.4	12.4±1.1	9.52±0.3	9.9±0.89	16.4±0.1	23.5±0.39
7r	12.9±0.5	7.4±0.28	15.3±0.2	11.3±0.3	15.3±0.2	25.3±0.58
1	>50	>50	>50	>50	>50	ND
3a	>50	>50	>50	>50	>50	ND
S	7.4±0.5	23.8±0.4	16.3±0.5	12.6±1.0	14.4±0.3	25.2±1.4

C: Compound;; ND: Not determined; S: Sunitinib

The most active compounds **7q** and **7r** were also tested on RWPE-1 cells and were found to be safe compared to the prostate cancer cells.

Additionally, the building blocks of target compounds i.e. oxindole (**1**), 4-methyl umbelliferone aldehyde (**3**), were screened for their anticancer potential and were found to be inactive against all tested cell lines. These results suggest that the enhancement of anticancer activity occurs when the umbelliferone moiety is linked to 5-substituted oxindole rather than the individual units of the target compounds.

Analysis of the MTT assay results suggests PC-3 and DU145 cells are more sensitive towards the synthesized target compounds. The impact of modification of the "R" group at 5th position of oxindole derivatives is interesting in the light of the results of the SAR study, which suggests that bulky diphenyl propargyl moiety at 5th position of oxindole is optimal for anticancer activity. Previous literature reports demonstrated that strong electron withdrawing substitutions such as chloro and fluoro on the aromatic ring increases the lipophilicity of molecules and are responsible for enhanced cytotoxicity.[17,18] Interestingly, electron withdrawing substitutions on diphenyl propargyl moiety at 5th position of oxindole showed better results than electron donating substitutions. Conversely, simple substitutions at this position demonstrated decrease in the activity. Among all compounds synthesized, compounds **7q** and **7r** showed the most significant cytotoxicity towards all the tested cancer cell lines. These primary results persuade further investigation on the synthesized compounds aiming to the development of novel potential anticancer agents.

Acridine orange-Ethidium bromide (AO-EB) staining. Acridine orange/ethidium bromide (AO/EB) fluorescent staining assay was performed to distinguish the live, apoptotic and necrotic cells.[19] AO permeates the intact cell membrane and stains the nuclei green, while EB can stain the cells that have lost their membrane integrity and tinge the nucleus red. From figures 2A and 2B, it can be seen that the control cells display normal healthy morphology with intact nuclear architecture and appeared green in colour.

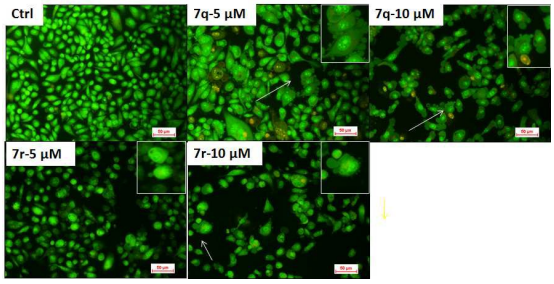

Figure 2A: Morphological changes observed by Acridine orange/Ethidium bromide staining in PC-3 cells. Apoptotic features such as membrane blebbing, chromatin condensation and apoptotic body formations were clearly observed in cells treated with 5 and 10 μM of compounds **7q** and **7r**.

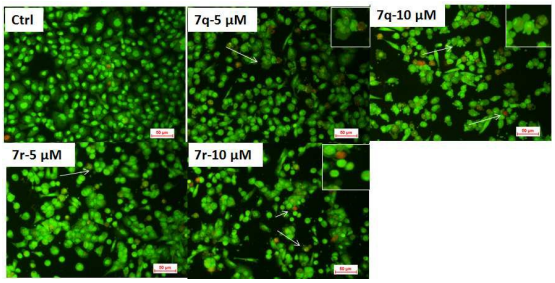

Figure 2B: Morphological changes observed by Acridine orange/Ethidium bromide staining in DU145 cells. Apoptotic features such as membrane blebbing, chromatin condensation and apoptotic body formations were clearly observed in cells treated with 5 and 10 μM of compounds **7q** and **7r**.

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Fluorescence microscopic images of **7q** and **7r** treated PC-3 and DU145 cells have clearly demonstrated morphological changes which are the characteristic features of apoptotic cells such as cell shrinkage, membrane blebbing, chromatin condensation and apoptotic body formation. This confirms that the compounds **7q** and **7r** induced cell death in PC-3 and DU145 cancer cells.

DAPI nucleic acid staining. DAPI (4',6-diamidino-2-phenylindole) is a fluorescent dye that stains nucleus by binding strongly to DNA and thus identifies the nuclear damage or chromatin condensation. The DAPI stains nucleus of the apoptotic cells as bright colored due to the condensed nucleus which is a distinguishing apoptotic characteristic. DAPI staining technique was carried out according to earlier reported method.[20] It can be inferred from the figures 3A and 3B that the nuclear structure of untreated cells was intact, whereas compounds **7q** and **7r** treated cells exhibited horse shaped, fragmented and pyknotic (shrunken) nuclei.

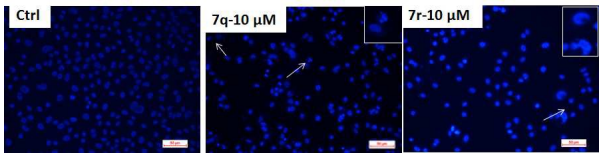

Figure 3A: Assessment of Nuclear morphological changes by DAPI staining in PC-3 cells after 48 h. **7q** and **7r** treated PC-3 cells have displayed nuclear apoptotic characteristics such as nuclear fragmentation and shrunken nuclei.

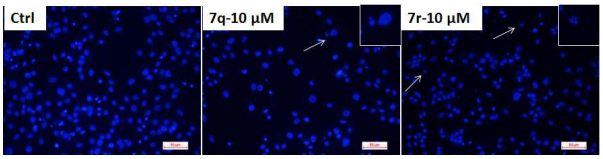

Figure 3B: Assessment of Nuclear morphological changes by DAPI staining in DU145 cells after 48 h. **7q** and **7r** treated DU145 cells have displayed nuclear apoptotic characteristics such as nuclear fragmentation and shrunken nuclei.

Cell cycle analysis. Many of the cytotoxic compounds exert their growth inhibitory effect by arresting the cell cycle at a specific phase of a cell cycle. *In vitro* screening results revealed that the compounds **7q** and **7r** showed significant activity against PC-3 and DU145 cells. Thus, we herein examined the effect of the compounds **7q** and **7r** on cell cycle using propidium iodide staining method (Figure 4A and 4B).[21,22]

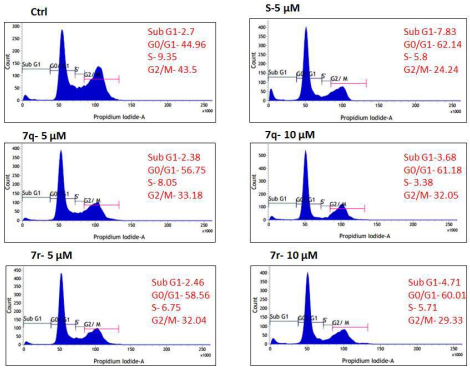

Figure 4A: Effect of compounds on cell cycle distribution as analyzed by flow cytometry using propidium iodide staining method in PC-3 cells. PC-3 cells were treated with designated conc. of **7q**, **7r** and Sunitinib (**S**) for 24 h. The population of cells in each cell cycle phase was numerically depicted. Data shown here is representative of three independent experiments.

PC-3 and DU145 cells were treated with designated concentrations of compounds **7q** and **7r** independently for 24 h. They were stained with propidium iodide and samples were further analysed by flow cytometry. Treatment with the compounds (**7q** and **7r**) at 5 and 10 µM concentrations in PC-3 cells displayed rise in G0/G1 population from 44.96% (control) to 61.18% and 60.01% respectively in a dose dependent manner. Similarly, **7q** and **7r** treated DU145 cells exhibited increase in G0/G1 population of cells from 64.23% (control) to 70.04% and 72.43% respectively in a dose dependent manner. Notably, reference compound Sunitinib (**S**) has demonstrated increase in G0/G1 population from 44.96% (control) to 62.14% and 64.23% (control) to 77.1% in PC-3 and DU145 cell lines at 5 µM respectively. Significant increase in Sub-G1 population in DU145 cells also indicates exertion of apoptosis by the indicated compounds. Overall, the results suggest clear induction of G0/G1 cell cycle arrest by the test compounds.

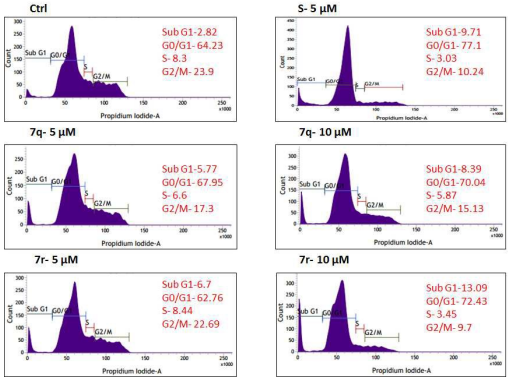

Figure 4B: Effect of compounds on cell cycle distribution as analyzed by flow cytometry using propidium iodide staining method in DU145 cells. DU145 cells were treated with designated conc. of **7q**, **7r** and Sunitinib (**S**) for 24 h. The population of cells in each cell cycle phase was numerically depicted. Data shown here is representative of three independent experiments.

Annexin V binding assay. The apoptosis inducing effect of compounds **7q** and **7r** on PC-3 and DU145 cancer cells was further investigated using annexin V-FITC/propidium iodide staining assay.[23] PC-3 and DU145 cells were treated with 5 and 10 µM of compounds **7q** and **7r** independently for 24 h and stained with Annexin V-FITC and propidium iodide, and samples were analysed by flow cytometry.

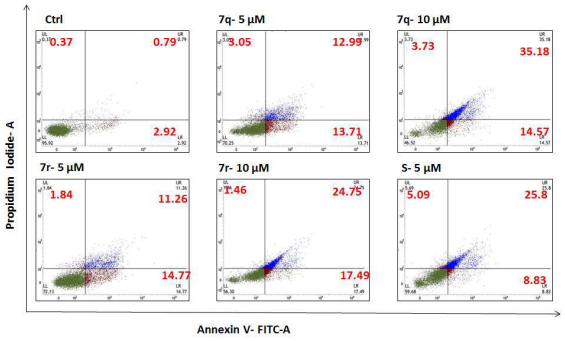

Figure 5A: Annexin V- FITC /PI binding assay in PC-3 cells. PC-3 cells were treated with designated conc. of **7q**, **7r** and Sunitinib (**S**) for 24 h.

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As depicted in figure 5A, **7q** and **7r** treated PC-3 cells demonstrated rise in the total percentage of apoptotic (early and late apoptotic cells- Annexin V +ve cells) and dead cells from 4.08% (untreated) to 53.48% and 43.7% respectively in a dose dependent manner. Similarly, as shown in figure 5B, DU145 cells treated with indicated concentrations of **7q** and **7r** displayed increase in the total apoptotic dead population from 4.38% (untreated) to 54.63% and 46.41% respectively in a concentration dependent manner. Reference compound Sunitinib (**S**) has also displayed rise in apoptotic, dead population from 4.08% (control) to 39.72% and 4.38% (control) to 58.81% in PC-3 and DU145 cells respectively.

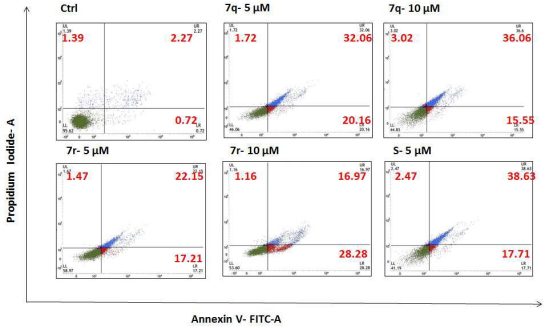

Figure 5B: Annexin V- FITC /PI binding assay in DU145 cells. DU145 cells were treated with designated conc. of **7q**, **7r** and Sunitinib (**S**) for 24 h.

Determination of intracellular ROS generation: The reactive oxygen species generation is one of well characterised mechanisms of many anticancer drugs. Hence, in next array of experiments, we assessed the generation of intracellular reactive oxygen species by compounds **7q** and **7r** in PC-3 and DU145 cells using DCFDA staining method.[24]

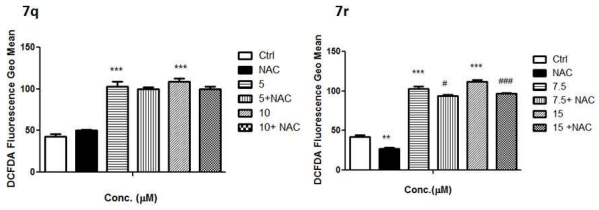

Figure 6A: The effect of compounds **7q** and **7r** on intracellular ROS generation. a) PC-3 cells were treated with various conc. of **7q** for 3 h, with or without 3mM NAC pretreatment for 1 h. b) PC-3 cells were treated with various conc. of **7r** for 3 h, with and without 3 mM NAC pretreatment for 1 h. ROS generation was measured using DCFDA staining by flow cytometry and geomean DCFDA fluorescence are represented in histogram. Values were represented as Mean ±SEM (n=3). Statistical analysis was performed with one way Annova followed by Dunnet's test. *P<0.05 **P<0.01,***P<0.001 , when compared to ctrl. NAC treated groups are compared with their respective compound treated controls. # P<0.05,## P<0.01,### P< 0.001.

Treatment with compounds **7q** and **7r** for 3 h resulted in significant increase in DCFDA fluorescence in a dose dependent manner, signifying ROS accumulating property of compounds (Figure 6A and 6B). Treatment with *N*-acetyl cysteine (NAC) prior to compound treatment has decreased DCFDA fluorescence intensity; this indicated that compounds induced cytotoxicity by ROS generation.

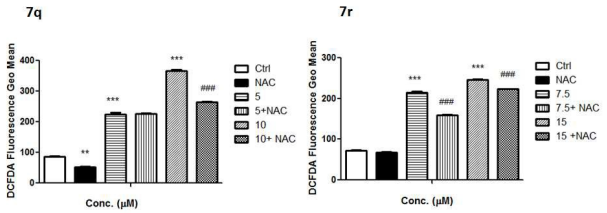

Figure 6B: The effect of compounds **7q** and **7r** on intracellular ROS generation. a) DU145 cells were treated with various conc. of **7q** for 3 h, with or without 3mM NAC pretreatment for 1 h. b) DU145 cells were treated with various conc. of **7r** for 3 h, with and without 3 mM NAC pretreatment for 1 h. ROS generation was measured using DCFDA staining by flow cytometry and geomean DCFDA fluorescence were represented in histogram. Values are represented as Mean ±SEM (n=3). Statistical analysis was performed with one way Annova followed by Dunnet's test. *P<0.05 **P<0.01, ***P<0.001 , when compared to ctrl. NAC treated groups are compared with their respective compound treated controls. # P<0.05,## P<0.01,### P< 0.001.

Assessment of mitochondrial membrane potential (DΨm): Increase in intracellular ROS can cause oxidative stress and lead to the loss of mitochondrial membrane potential. Thus, the effect of compounds **7q** and **7r** on mitochondrial membrane potential (DΨm) was determined by staining with lipophilic cationic JC-1 dye.[25] Healthy polarised mitochondria stains red due to potential dependent formation of J-aggregates, while depolarised mitochondria in apoptotic cells stains green because of J-monomers. Dose dependent increase in green to red fluorescent population ratio from 0.55 to 1.31 and 0.9 was observed with treatment of compounds **7q** and **7r** respectively in PC-3 cells. DU145 cells exposed to the indicated concentrations of compounds **7q** and **7r** also showed enhancement in green to red fluorescence ratio from 0.16 to 5.63 and 0.45 respectively (Figure 7).

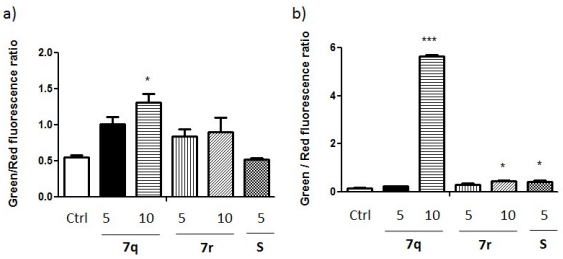

Figure 7: Detection of mitochondrial membrane potential (DΨm) by JC-1 staining. a) Quantitative representation of population of cells with green to red fluorescence ratio in PC-3 cells. b) Quantitative representation of population of cells with green to red fluorescence ratio in DU145 cells. Cells were treated with designated conc. of **7q**, **7r** and Sunitinib (**S**) for 24 h and incubated with 2 µM JC-1 probe and further analyzed by flow cytometer (FACS verse, Becton Dickinson, US).

The compounds **7q** and **7r** displayed prominent effect on green to red fluorescence ratio than the reference compound Sunitinib (**S**). Thus, the results clearly indicate loss of mitochondrial membrane potential by the compounds (**7q** and **7r**) and suggest the involvement of mitochondria dependent apoptotic pathway in their mechanism of action.

Western blotting analysis PARP (Poly ADP ribose polymerase) is 116 kda protein that gets cleaved into 89 kda protein fragment during caspases activation and this cleavage is prominent hallmark of the apoptosis induction. Intrinsic or mitochondria mediated apoptotic pathway can be evidenced with loss of mitochondrial membrane potential usually preceded by the release of cytochrome c into cytosol. Thus, to investigate the changes in protein expression

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levels of proteins cytochrome (cytosolic) and cleaved PARP in PC-3, western blotting analysis was performed (Figure 8).[26] Treatment with **7q** has clearly exhibited 1.88 fold increase in cytosolic levels of cytochrome c at 10 μM, while exposure to **7r** has shown marked increase (8.65 fold) in the protein levels of cleaved PARP. These protein changes were compared with standard drug Sunitinib (**S**). Increase in cleaved PARP level with compound **7r** (6.54 fold at 5 μM) was more pronounced than with Sunitinib treatment (4.53 fold at 5 μM).

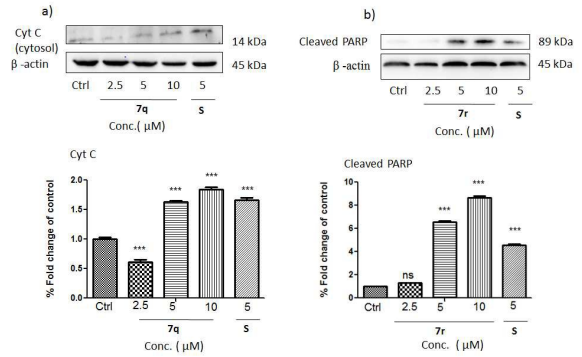

Figure 8: Western blotting analysis of apoptotic protein expression. a) PC-3 cells were treated with 2.5, 5, 10 μM conc. of **7q** and standard drug sunitinib 5 μM for 48 h. b) PC-3 cells were treated with 2.5, 5, 10 μM conc. of **7r** and standard drug Sunitinib (**S**) 5 μM for 48 h. Changes in expression levels of Cyt C and Cleaved PARP are represented. Data shown here is representative of one of the three independent experiments. Representative histograms showing quantitative results for relative levels of Cyt-C of **7q** treated PC-3 cells and cleaved PARP of **7r** treated PC-3 cells. Values are the means ± SEM (n = 3).

Conclusion

In the current study, a series of (*E*)-3-((7-hydroxy-4-methyl-2-oxo-2*H*-chromen-8-yl)methylene)indolin-2-one derivatives were synthesized, and were further evaluated for their *in vitro* anticancer potentials. An initial screening was performed against MDA-MB-231, MCF-7, DU145, PC-3 and A549 cancer cell lines. In MTT assay, the compounds **7q** and **7r** were found to be the most active against the PC-3 and DU145 (prostate cancer) cell lines. Most importantly, the compounds **7q** and **7r** were found to be comparatively safe towards normal cell line RWPE-1. The detailed studies like AO/EB staining, DAPI nuclear staining and Annexin V binding assay suggested that compounds **7q** and **7r** induced apoptosis in PC-3 and DU145 cells. The cell cycle analysis confirmed that the compounds **7q** and **7r** target the G0/G1 phase of PC-3 and DU145 cell cycle in a dose-dependent manner. Moreover, the compounds **7q** and **7r** treatment resulted in collapse of mitochondrial membrane potential and elevated intracellular ROS levels in PC-3 and DU145 cells. Investigation of expression levels of apoptotic proteins cytochrome c and cleaved PARP in PC-3 cells revealed that compounds **7q** and **7r** trigger mitochondrial mediated intrinsic pathway thereby inducing apoptosis in cancer cells. Overall, the current studies demonstrated that dual pharmacophores strategy can be useful in the synthesis of promising chemical entities for the development of cancer therapeutics.

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