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PAPER

Investigation of triazole linked indole and oxindole glycoconjugates as potential anticancer agents: Novel Akt/PKB signaling pathway inhibitors†‡

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In continuation of our venture towards the synthesis of novel bioactive agents, the two sets of triazole linked glycoconjugates were synthesized respectively from indole/oxindole (29 compounds) and were further characterized by IR (infrared spectroscopy), ^{1}H NMR (nuclear magnetic resonance), ^{13}C NMR and mass spectral analysis. The newly synthesized target compounds were evaluated for their preliminary *in vitro* anticancer activity against DU145 (prostate cancer), HeLa (cervical cancer), A549 (lung cancer) and MCF-7 (breast cancer) cell lines. In the sulforhodamine B (SRB) assay, the results indicated that the compounds **5f** (indole derivative) and ***E*-9b** (oxindole derivative) displayed remarkable cytotoxic activity against the DU145 cells. Moreover, the colony formation assay (soft agar assay) revealed that the compounds **5f** and ***E*-9b** can inhibit the growth and proliferation of DU145 cells. The impact of the most active cytotoxic compounds **5f** and ***E*-9b** on the cell cycle distribution was assessed in DU145 cells, which displayed a cell cycle arrest at the sub-G1 phase. Next, the compounds **5f** and ***E*-9b** were tested for caspase activation in the DU145 cells, and the results specified that the these compounds have capability to induce apoptosis in cells through an intrinsic pathway leading to subsequent cell death. Further studies also confirmed that the compounds **5f** and ***E*-9b** act against protein kinase B (Akt/PKB) pathway to inhibit proliferation of cancer cells. Thus, the compounds **5f** and ***E*-9b** could be the novel potential anticancer leads as the normal cell line NIH/3T3 (fibroblast) studies showed no significant cytotoxicity.

Introduction

Nowadays, cancer has gradually become the leading cause of mortality worldwide, seriously endangering the health and life of humans for a long period. It has accounted for 8.5 million deaths (around 17% of all deaths) in 2012, and estimated to cause 14 million deaths in 2030.[1] Despite the huge efforts to implement novel chemotherapeutic strategies for the successful treatment of different types of cancer, this disease still remains as one of the major concerns globally.[2] Hence, there is an urgent need to search for some newer and safer anticancer agents that have broader spectrum of cytotoxicity towards tumor cells.

The protein kinase B (Akt/PKB) pathway is an important new target for molecular therapeutics, as it functions as a key nodal point for transducing extracellular (growth factor and insulin) and intracellular (receptor tyrosine kinases, Ras and Src) oncogenic signals.[3,4] Obstruction of Akt signaling results in apoptosis and growth inhibition of tumor cells with an increased amount of Akt. The observed reliance of certain tumors on Akt signaling for survival and growth has broad implications for cancer therapy, offering the prospective for targeted tumor cell killing.[5] In the last few years, through combinatorial chemistry, high-throughput, virtual screening, and traditional medicinal chemistry, a number of inhibitors of the Akt pathway have been identified.[6]

The 3-substituted indole and oxindole scaffolds possess various biological activities and continues to be a main structural unit of many natural and pharmacologically active compounds (Figure 1).[7-9] Recently, new compounds bearing 3-substituted indole and oxindole moieties designed to target Akt pathway have been described.[10,11]

Molecular hybridization which covalently combines two or more drug pharmacophores into a single molecule is an effective tool to design more active novel chemical entities.[12-14] Besides, the hybrids may also minimize the redundant side effects and allow synergic action.[15,16] On the other hand, the copper-catalyzed azide–alkyne cycloaddition (CuAAC) with carbohydrate based azide substrates is now usually applied in drug discovery and has proven exceptionally pliable both in the preparation of glycoconjugate small molecule inhibitors against a range of enzymes and to affix carbohydrates to biomolecules.[17] Glycopyranosyl have advantages of being stable and are inert towards a wide range of reaction conditions and most importantly, they are readily synthesized anomerically pure.[18]

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In this context, we desire to exploit azidosugars as the diversity surrogates to couple with the alkyne-modified 3-substituted indoles and oxindoles. These 3-substituted indole and oxindole derivatives may have the advantages of structural diversity, improved solubility and most important tunable anticancer activity.

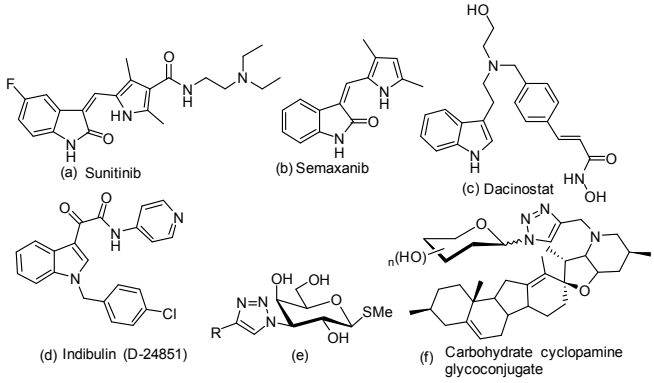


Fig. 1: Structure of some representative bioactive compounds containing indole, oxindole, triazole and glycosyl triazole moieties

Results and discussion

Chemistry

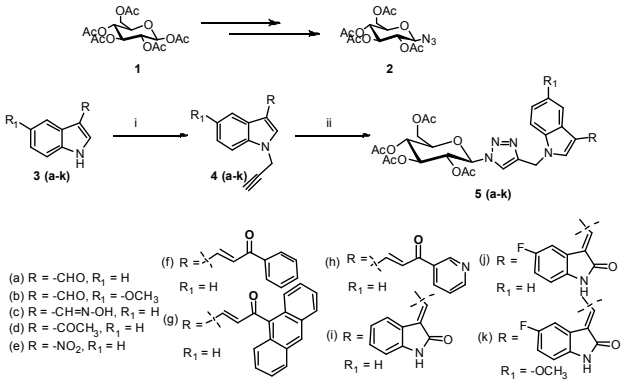
The synthesized glycoconjugate compounds of the present study comprises three core structural elements: (i) a sugar moiety (β-D-glucopyranoside), (ii) a 1,2,3-triazole ring, and (iii) a substituted indole/oxindole. The target glycoconjugates **5(a-k)**, ***E*-9(a-j)** and ***Z*-9(a-j)** were synthesized in two sets of molecules using substituted indole and oxindole as starting materials.

In the first series (Scheme 1), substituted indoles **3(a-k)** were subjected to *N*-propargylation in the presence of a base to give respective alkynes **4(a-k)**. These alkynes were further reacted with β-D-glucopyranosyl azide (**2**) *via* copper(I)- catalyzed Huisgen 1,3-dipolar cycloaddition to furnish triazole linked indole glycoconjugates **5(a-k)**.

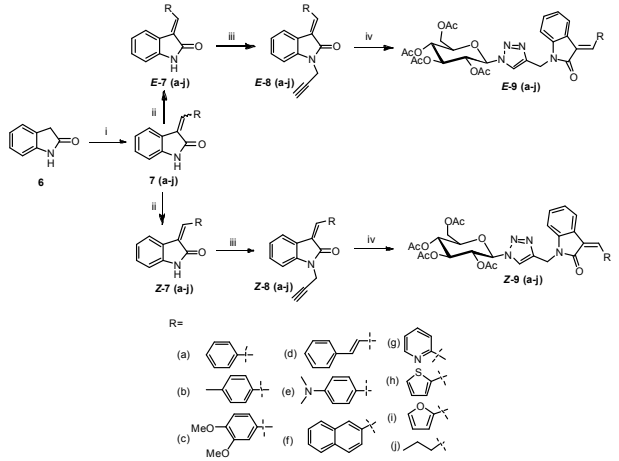
For the preparation of azide precursor, required for the synthesis of proposed glycoconjugates, we have utilized commercially available 1,2,3,4,6-penta-*O*-acetyl-D-glucopyranose **(1)** as the starting material. The β-D-glucopyranosyl azide **(2)** was synthesized *via* bimolecular displacement of the halide substituent of glycosyl halide precursor with an azide nucleophile (Scheme 1).[19]

In the second series (Scheme 2), the well-known Knoevenagel condensation was employed on oxindole by using different aromatic, heteroaromatic and aliphatic aldehydes to afford 3-alkenyl oxindoles **7(a-j)** in a mixture of *E*/*Z* isomeric forms.[20,21] These *E*/*Z* isomers were separated by using the conventional column chromatography and were characterized with the help of ^{1}H NMR. The chemical shift values of the separated isomers **[*E*-7(a-j)** and ***Z*-7(a-j)]** were carefully compared with previous literature.[22-28] The compound ***E*-7g** was obtained in selective *E* form as reported in the literature,[26] whereas the compound ***Z*-7c** was formed as the *Z* isomer with negligible amount of the *E* isomer.[24] In the next step, these isomerically pure 3-alkenyl oxindoles were next subjected to *N*-propargylation for the synthesis of respective alkynes ***E*-8(a-j)** and ***Z*-8(a-j)**. These terminal alkynes bearing substrates ***E*-8(a-j)** and ***Z*-8(a-j)** were further subjected to [3+2] cycloaddition with β-D-glucopyranosyl azide (**2**) to afford triazole linked oxindole glycoconjugates ***E*-9(a-j)** and ***Z*-9(a-j)**.

Herein, the triazole linker serves as a biocompatible, non-labile covalent spacer between the acetylated glucose and the substituted indole/oxindole.



Scheme 1: Synthesis of 3-substituted indole triazole linked glycoconjugates; Reagents and conditions: (i) propargyl bromide, K_2CO_3, acetonitrile, reflux, 6 h; (ii) D-glucopyranosylazide, CuI, *t*-butanol/water (1:1), 40 °C, 48 h.



Scheme 2: Synthesis of 3-substituted oxindole triazole linked glycoconjugates; Reagents and conditions: (i) aldehyde, piperidine, ethanol, reflux, 3-4 h; (ii) isomers separated by using column chromatography; (iii) propargyl bromide, K_2CO_3, acetonitrile, reflux, 6 h; (iv) D-glucopyranosyl azide, CuI, *t*-butanol/water (1:1), 40 °C, 48 h.

Biological evaluation

***In vitro* anticancer activity.** All the newly synthesized compounds were evaluated for their *in vitro* cytotoxic activity against DU145, HeLa, A549 and MCF-7 cancer cell lines by employing the sulforhodamine B (SRB) assay.[29] Doxorubicin 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 revealed that the synthesized target compounds exhibited different levels of anticancer properties (Table 1, Table 2). From the close examination

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of the IC_{50} values (Table 1), it was observed that the indole glycoconjugate substituted with –CHO functional moiety at position 3 (**5a)** showed inhibitory activity against DU145, HeLa and MCF-7, while no activity was observed in the case of A549 cells. The compound substituted with -CHO and -OMe at positions 3 and 5 of indole respectively showed decrease in the activity for DU145 and A549 cell lines, whereas complete loss of inhibition was seen against HeLa and MCF-7 cell lines **(5b)**. The glycoconjugate substituted with oxime **(5c)** showed inhibitory activity especially against DU145 and MCF-7 cells. On the other hand, the indole glycoconjugate substituted with the acetyl group **(5d)** displayed modest activity specifically against DU145 cell line. The compound with nitro substitution at position 3 of the indole displayed moderate activity towards DU145, A459, and MCF-7 cell lines **(5e)**. Interestingly, the cross aldol product substituted with the phenyl ring **(5f)** showed enhancement in the anticancer activity towards all tested cancer cell lines. However, the bulky group substituted cross aldol product of glycoconjugate **(5g)** led to the decreased anticancer activity, while the pyridine substituted analogue **(5h)** demonstrated improvement in the activity for DU145, A549 and MCF-7 cell lines. Using oxindole, an attempt was made to synthesize the Knoevenagel product **(5i)** of the glycoconjugate **5a**, which displayed inhibition specifically against the MCF-7 cell line. While, the glycoconjugate substituted with 5-fluoro oxindole at position 3 of indole **(5j)** showed modest inhibition towards DU145 and A549 cell lines. The indole compound (**5k**) substituted with 5-fluoro oxindole at the 3rd and methoxy at the 5th position was found to be inactive towards all tested cancer cell lines.

Table 1. *In vitro* anticancer activity of triazole linked indole glycoconjugates derivatives (IC_{50} μM)

C	DU145	A549	HeLa	MCF-7	NIH/3T3
5a	58.7± 8.2	NA	44.5±4.3	25.2±2.1	NA
5b	136.1±5.3	106.3±8.3	NA	NA	NA
5c	88.4±10.2	NA	NA	51±3.7	26.2±0.2
5d	75.2±13.6	NA	NA	NA	NA
5e	60.9±9.4	93.2±6.7	NA	NA	20.5±2.1
5f	14.4±0.5	16.3±2.3	33.0±4.8	23.9±3.5	NA
5g	58.1±12.6	46.8±5.0	165.2±7.9	44.0±0.3	NA
5h	29±2.8	39.8±1.6	NA	22.3±2.3	NA
5i	NA	NA	NA	22.2±1.9	NA
5j	87.4±5.3	36.9±6.5	NA	40.4±1.9	62.5±2.6
5k	NA	NA	NA	NA	NA
3f	NA	NA	NA	NA	NA
1	NA	NA	NA	NA	NA
DX	5.4±0.03	7.1±0.01	7.2±0.1	6.1±0.9	10.1±0.03

C: Compound; NA: Not active (IC_{50} > 200 μM); DX: Doxorubicin

In the second set of the glycoconjugates (Table 2), it was observed that the phenyl substituted *E*- and *Z*- isomers of oxindole glycoconjugates **(*E*-9a** and ***Z*-9a)** showed almost similar activities against all tested cancer cell lines. Enhancement in the inhibitory activity was observed in case of tolyl substituted *E*- and *Z*- isomers **(*E*-9b** and ***Z*-9b)** selectively towards DU145 cell line, whereas complete loss of activity was observed against the MCF-7 cells. The compound substituted with 3,4-dimethoxy phenyl moiety **(*Z*-9c)** displayed good inhibition against all tested cancer cell lines. *E*-isomer of cinnamyl substituted analogue **(*E*-9d)** was found to be active only against DU145 and MCF-7, while its Z-isomer **(*Z*-9d)** showed inhibition against all tested cancer cells. Both *E*- and *Z*-isomers of oxindole glycoconjugate with the *N*,*N* dimethyl phenyl substitution **(*E*-9e** and ***Z*-9e)** showed excellent activity towards all cancer cell lines. The *E* isomer of naphthalenyl substituted analogue **(*E*-9f)** showed moderate activity against DU145, A549, and MCF-7, whereas its *Z*-form **(*Z*-9f)** lost its activity against DU145 and HeLa cell lines. The *E*-isomer of pyridinyl substituted compound **(*E*-9g)** displayed modest anticancer activity. The thiophene substituted *E*-isomer of oxindole glycoconjugate **(*E*-9h)** showed moderate to good inhibitory activity against all cancer cell lines. However, complete loss of activity was seen against the HeLa in case of *Z*-isomer **(*Z*-9h)**. Furthermore, the *E*-isomer of furyl substituted analogue **(*E*-9i)** exhibited good inhibition against all cancer cell lines, whereas activity of its *Z*-isomer was found to be insignificant **(*Z*-9i)**. In case of the oxindole glycoconjugates substituted with aliphatic chain, the *E*-isomer **(*E*-9j)** showed promising results while its *Z*-isomer **(*Z*-9j)** led to decrease in the anticancer activity. All compounds were tested against a non-cancerous cell line NIH/3T3 (fibroblast). The compounds ***Z*-9b** and ***Z*-9i** were found to be cytotoxic against NIH/3T3 cells, while the compounds **5f** and ***E*-9b** displayed promising results in case of non-cancerous cell as compared to their reference drug. The compound **5f** was found to be inactive against NIH/3T3 cells, whereas the compound ***E*-9b** showed IC_{50} against the same cell line at 73.3 μM.

Table 2. *In vitro* anticancer activity of triazole linked oxindole glycoconjugates derivatives (IC_{50} μM)

C	DU145	A549	HeLa	MCF-7	NIH/3T3
***E*-9a**	22.8±10.1	20.6±5.3	37.1±3.5	30.2±1.7	NA
***Z*-9a**	24.1±3.6	17.9±2.5	33.3±4.8	24.3±5.2	22.8±2.0
***E*-9b**	9.3±0.5	16.0±0.4	85.6±8.5	NA	73.3±3.6
***Z*-9b**	9.4±0.8	16.5±1.6	NA	NA	9.0±1.2
***Z*-9c**	24.9±7.2	23.3±0.1	63.4±0.5	23.5±0.6	NA
***E*-9d**	17.1±2.0	NA	NA	53.2±8.8	12.3±5.2
***Z*-9d**	19.5±3.9	58.4±29.3	98.1±5.6	34.1±5.8	25.9±6.3
***E*-9e**	11.2±0.9	19.4±3.5	26.3±3.8	25.2±2.2	12.7±3.2
***Z*-9e**	10.1±0.03	17.7±2.0	25±5.4	29.8±2.4	17±0.09
***E*-9f**	43.7±2.3	30.9±6.3	NA	32.7±1.6	28.4±1.2
***Z*-9f**	NA	62.2±7.1	NA	46.5±6.7	80±6.9
***E*-9g**	48.3±2.0	26.1±2.8	51.7±0.6	32.1±7.5	NA
***E*-9h**	23.4±0.7	20.8±1.4	36.9±2.2	28.1±1.8	48.1±3.5
***Z*-9h**	22.8±0.2	16.1±1.9	NA	36.1±10.8	14.6±2.5
***E*-9i**	10±0.3	14.5±0.05	29.9±2.1	29.1±2.4	31.8±1.6
***Z*-9i**	16.9±1.1	41.1±10.8	NA	38.2±9.4	13.3±1.4
***E*-9j**	19.7±3.9	24.1±1.8	NA	22.6±5.8	NA
***Z*-9j**	26.6±5.3	40.4±13.1	46.3±4.3	25±2.4	NA
***E*-7b**	NA	NA	NA	NA	NA
DX	5.4±0.03	7.1±0.01	7.2±0.1	6.1±0.9	10.1±0.03

C: Compound; NA: Not active (IC_{50} > 100 μM); DX: Doxorubicin

Additionally, the building blocks of target compounds i.e. acetylated glucose **(1)**, 3-substituted oxindole **(*E*-7b)** and 3-substituted indole **(3f)**, 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 in case of triazole linked indole and oxindole glycoconjugates rather than the individual units of the target compounds.

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Analysis of the SRB assay results suggests DU145 cells are more sensitive towards both the series of compounds. The impact of modification of the "R" group in indole and oxindole derivatives is interesting in the light of the results of the SAR study, which suggests that substituted phenyl moiety at 3rd position is optimal for anticancer activity. On the other hand indole and oxindole derivatives bearing bulky substitutions at this position demonstrated decrease in the activity. Further derivatization mostly resulted in a significant loss of activity. Among all compounds synthesized, compounds **5f** and ***E*-9b** showed the most significant cytotoxicity to cancer cell lines and were comparatively safer against NIH/3T3 cells. These preliminary results encourage further investigation on the synthesized compounds aiming to the development of novel potential anticancer agents.

Change in morphology. Out of all the compounds in the series synthesized, compounds **5f** and ***E*-9b** were found to have promising anti-cancer activity, especially against DU145 (prostate cancer) cell line. As these compounds were cytotoxic towards DU145 cells with IC_{50} values of 9.3 µM **(*E*-9b)** and 14.4 µM **(5f)**, we further tested their ability to affect the morphology of the cells.[30] The cells were treated with the compounds **5f** and ***E*-9b** respectively in increasing concentrations. After 48 h of incubation, it was observed that the cells began to lose their characteristic morphology (Figure 2A and 2B). Thus, it was confirmed that the compounds have an effect on the morphology of cells.

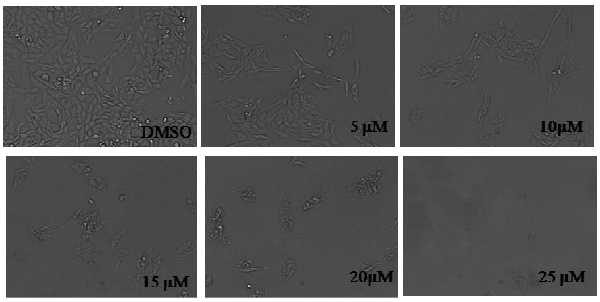


Fig. 2A: DU145 cells were treated with **5f** at the given concentrations and DMSO taken as a control. After 48 h of incubation, the morphology of the cells changes with increasing concentration of compound.

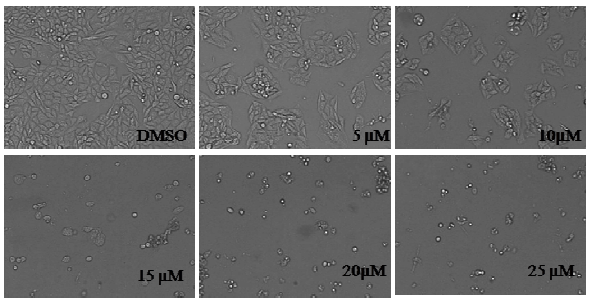
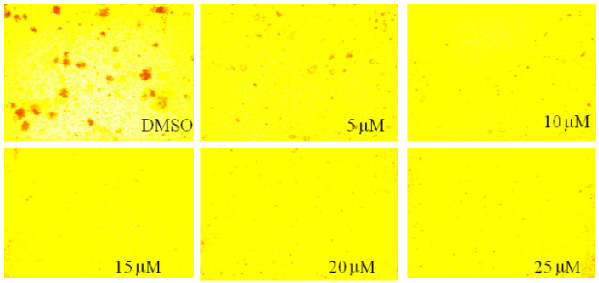


Fig. 2B: DU145 cells were treated with ***E*-9b** at the given concentrations and DMSO taken as a control. After 48 h of incubation, the morphology of the cells changes with increasing concentration of compound.

Colony Formation (Soft Agar) Assay. Prostate cancer cells have an ability of continuous proliferation. To test whether the compounds affect this property of the cells, a colony formation assay (soft agar assay) which mimics an *in vivo* environment was conducted.[30] From the assay, it was clearly demonstrated that the compounds **5f** and ***E*-9b** inhibit the growth of cells in a dose dependent manner.

It was clearly observed that the compounds inhibit anchorage-independent growth of the cells, when the cells were treated with the compounds in increasing concentrations. Thus, the long term effects of the compounds **5f** and ***E*-9b** were confirmed on DU145 (prostate cancer cells). The inhibition of growth and proliferation



was observed at 5 µM in case of cells treated with compound **5f** (Figure 3A), while the inhibition was seen at 10 µM and higher concentrations in cells treated with compound ***E*-9b** (Figure 3B).

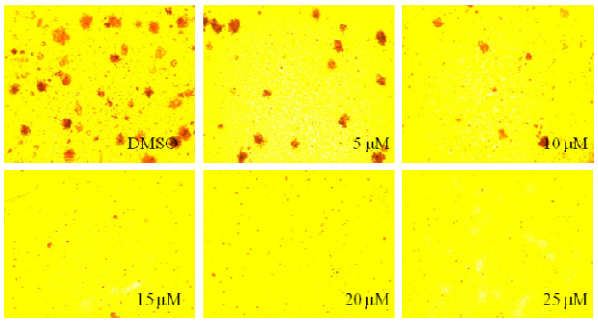


Fig. 3A: Effect of **5f** on the inhibition of colony formation and growth of cells when the cells were treated with **5f**. DMSO was taken as a control. Representative images of the colony-forming assay are shown here. The ability of the cells to proliferate and form colonies decreases with increasing concentration of **5f**.

Fig. 3B: Long term effect of ***E*-9b** on the colony-forming ability of DU145 cells. DU145 cells were treated with indicated concentration of ***E*-9b** and were allowed to grow for 9 days to form colonies. Representative images of the colony-forming assay are shown here. Number of colonies and their size formed by DU145 in soft agar is decreased on exposure to ***E*-9b**. DMSO was taken as a control.

Cell cycle analysis. Many of the cytotoxic compounds exert their growth inhibitory effect by arresting the cell cycle at a specific checkpoint.[31] *In vitro* screening results revealed that the compounds **5f** and ***E*-9b** displayed significant activity against DU145 cells.

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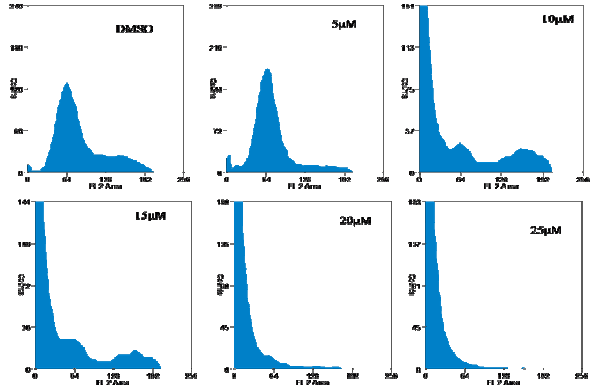

Fig. 4A: Cell cycle analysis of cells treated with **5f**. The cells were stained with propidium iodide and subjected to flow cytometry (FACS). DMSO was taken as a control. It can be observed from the images that the cells accumulate in sub-G1 phase with increasing concentrations as compared to DMSO, with significant increase at 10 μM concentration.

Table 3: DU145 cells were treated with **5f** at varying concentrations (5 μM, 10 μM, 15 μM, 20 μM and 25 μM). The cells were harvested and analyzed *via* flow cytometry. Data represents the cell population (%) in each cell cycle phase.

5f	Sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
DMSO	2.30	61.28	8.62	15.81
5 μM	4.53	75.04	4.72	8.22
10 μM	43.68	21.19	4.41	16.16
15 μM	54.02	21.46	3.82	11.91
20 μM	82.38	12.91	1.34	1.87
25 μM	84.06	11.96	0.80	1.01

Therefore, we herein examined the effect of the compounds **5f** and ***E*-9b** on DU145 cell cycle using flow cytometric analysis. DU145 cells were treated with various concentrations of compounds **5f** and ***E*-9b**, stained with propidium iodide.

Flow cytometry (FACS) was conducted to analyze the cells. From the analysis (Table 3), it was observed that the cells were accumulated in the sub-G1 phase of the cell cycle, with significant increase in cell population at 10 μM concentration in the case of **5f** (Figure 4A).

From the analysis of cells treated with compound ***E*-9b** (Table 4), it was observed that the cells accumulated in sub-G1 phase in a significant manner at 20 μM concentration (Figure 4B). Thus, it was clearly demonstrated that the compounds **5f** and ***E*-9b** cause cell cycle arrest, consequently inhibiting growth and eventual proliferation of cells

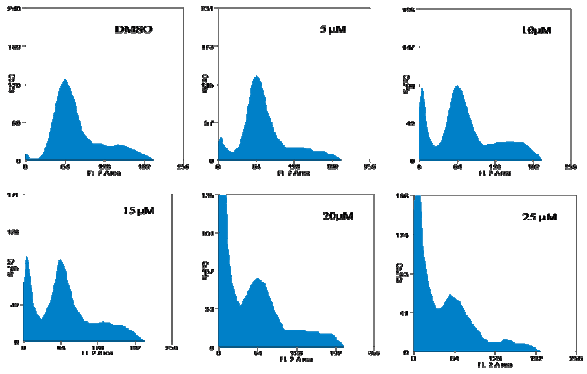

Fig. 4B Cell cycle analysis of cells treated with ***E*-9b**. The cells were stained with propidium iodide and subjected to flow cytometry (FACS). DMSO was taken as a control. It can be observed from the images that the cells accumulate in sub-G1 phase with increasing concentrations as compared to DMSO.

Table 4: DU145 cells were treated with ***E*-9b** at varying concentrations (5 μM, 10 μM, 15 μM, 20 μM and 25 μM). The cells were harvested and analyzed *via* flow cytometry. Data represents the cell population (%) in each cell cycle phase.

E-9b	Sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
DMSO	2.30	61.28	8.62	15.81
5 μM	7.12	59.15	6.85	12.62
10 μM	14.40	48.07	7.56	16.73
15 μM	17.20	46.65	7.64	14.89
20 μM	34.34	40.46	5.72	10.89
25 μM	37.51	41.35	4.86	9.05

Determination of activation of caspase-3, caspase-9 and caspase-8. Cell cycle arrest and inhibition of cell growth mostly leads to the cellular apoptosis. Therefore, in order to investigate whether the compounds induce apoptosis in DU145 cells, caspase activation in cells was studied. Caspases are crucial mediators of programmed cell death (apoptosis). Among them, the caspase-3 is a frequently activated death protease; catalyzing the specific cleavage of many key cellular proteins during apoptosis.[32] It has been previously proven that the activation of caspase-3 occurs in a sequential manner. Caspase-8 and caspase-9 are known to be involved in the activation of caspase-3. Both Caspase-8 and caspase-9 are considered as the initiator caspases.[33,34] To measure the activation of caspases, fluorescence substrates were used. Ac-DEVD AMC was used as the substrate for caspase-3, while Ac-DEVD AFC and Ac-VETD-AMC were used to determine activation of caspase-9 and caspase-8 respectively.

From the assay it was observed that the caspase-8 (Figure 5) and caspase-9 (Figure 6) in cells were activated in response to the treatment with compounds **5f** and ***E*-9b** in a dose dependent manner. As expected, the caspase-3 was also seen to be activated in cells treated with compounds **5f** and ***E*-9b** (Figure 7). The activation of caspase-9 is implicated in the induction of apoptosis *via* mitochondrial mediated intrinsic pathway. Thus, it was evidently confirmed that the compounds **5f** and ***E*-9b** have the ability to induce apoptosis in cells *via* an intrinsic pathway leading to activation of caspase-3 and eventual cell death.

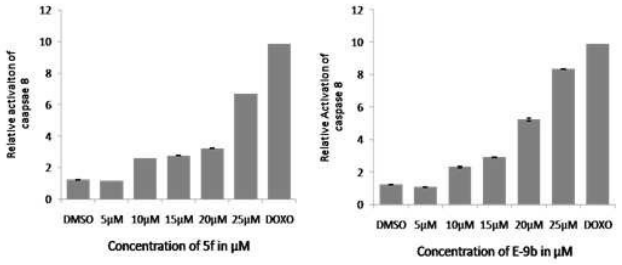

Fig. 5: Activation of caspase-8 was also observed in cells treated with compounds ***E*-9b** and **5f**. After incubation period of 48 h, it was seen that both the compounds cause activation of caspase-8. Doxorubicin (10 μM) was used as a control for activation of caspases. All experiments were carried out in triplicates and mean values are presented here.

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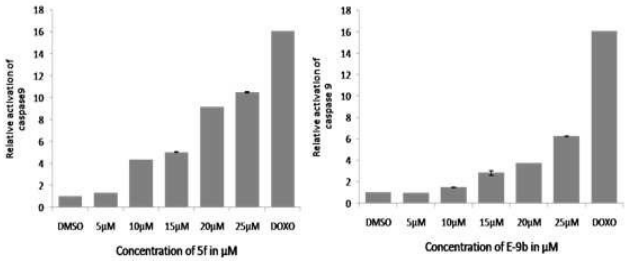

Fig. 6: Relative activation of caspase-9 in cells treated with compounds ***E*-9b** and **5f** respectively. Upon 48 h of incubation it was seen that activation of caspase-9 occurs with increasing concentrations of the compounds. Doxorubicin (10 µM) was used as a control for activation of caspases. All experiments were carried out in triplicates and mean values are presented here.

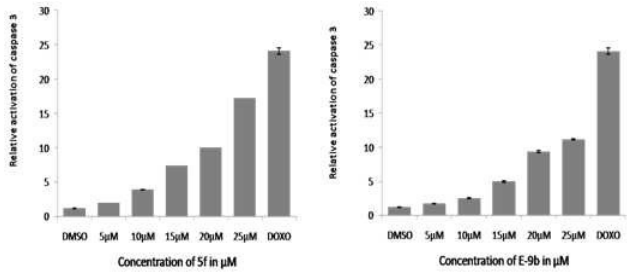

Fig. 7: DU145 cells were treated with increasing concentrations of ***E*-9b** and **5f** respectively. Upon 48 h of incubation it was seen that activation of caspase-3 occurs with increasing concentrations of the compounds. Doxorubicin (10 µM) was used as a control for activation of caspases. All experiments were carried out in triplicates and mean values are presented here.

Akt/PKB and p27 protein expression analysis *via* western blot. As discussed earlier, the compounds **5f** and ***E*-9b** were found to have an anti-proliferative effect on DU145 cells. The colony formation assay suggests that the compounds have the ability to inhibit anchorage-independent cell growth. Primarily, anchorage-independent cell growth occurs by the activation of Akt/PKB pathway.[35-38] This prompted us to conduct a study to determine the effects of compounds **5f** and ***E*-9b** on activation and inactivation of proteins involved in cancer cell proliferation. Thus, the effects of the compounds on the activation of Akt by phosphorylation were measured by using western blotting technique. From the study, a significant decrease in the phosphorylation of Akt in DU145 cells was observed upon the exposure of compounds **5f** and ***E*-9b** (Figure 8). These results clearly suggest that the Akt pathway could be the probable target for inhibition of growth in cancer cell. It has been previously reported that the increased phosphorylation of Akt (pAkt) in tumor samples from patients has been associated with disease progression and increased proliferation.[32] However, compounds **5f** and ***E*-9b** might be targeting Akt directly or other signalling pathways indirectly. Therefore, a precise molecular mechanism for the inhibition of pro-proliferative pathway by the compounds **5f** and ***E*-9b** still remains to be determined.

From the earlier results, it was confirmed that the compounds **5f** and ***E*-9b** have the ability to affect the cell cycle progression in DU145 cells. From the flow cytometric analysis, it was found that both the compounds **5f** and ***E*-9b** cause cell cycle arrest at sub-G1 phase of the cell cycle. Thus, to further verify, we have measured the expression of cell cycle inhibitor p27Kip1 (p27). p27 is known to bind and inhibit the cyclin E- or cyclin A-associated cyclin-dependent kinases (CDKs) 2 and other CDKs, and negatively regulate G1–G2 cell cycle progression.[34] From the analysis, it was observed that the expression of p27 increased with increasing concentrations of the compounds **5f** and ***E*-9b** (Figure 8). This markedly demonstrates that the compounds **5f** and ***E*-9b** act against the cell cycle progression through stabilization of p27Kip1 in the cancer cells. Thus, it was further confirmed that the compounds induce cell cycle arrest and cause eventual apoptosis. We have also analyzed the relative expression of phosphorylated Akt (pAkt) and p27. The expression of pAkt was compared with expression of Akt, whereas p27 Kip1 expression was compared against constitutive β-Actin expression (Figure 9).

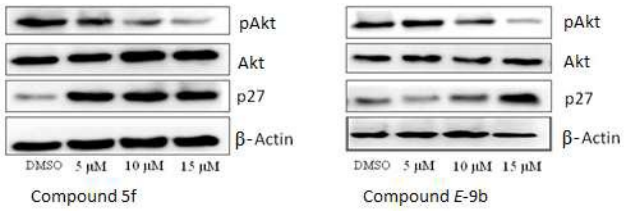

Fig. 8: Upon treatment of cells with **5f** and ***E*-9b**, the protein expression levels of pAkt decreased as compared to Akt. The expression of p27Kip1 protein increased with increasing concentrations of compounds **5f** and ***E*-9b** respectively. β-Actin was considered as a control.

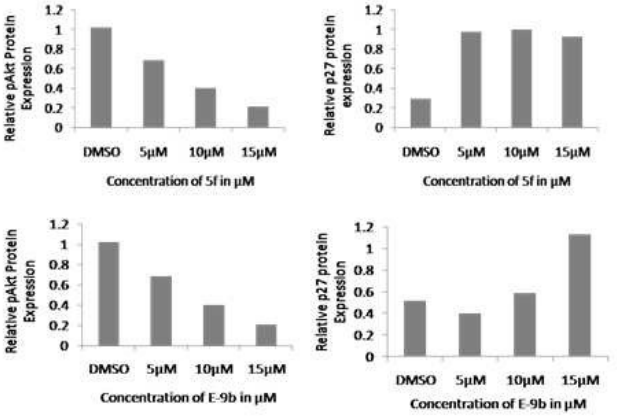

Fig. 9: Relative quantitative analysis of p27 Kip 1 protein expression as compared to constitutive expression of β-Actin protein and pAkt expression in comparison to the Akt expression for cells treated with **5f** and ***E*-9b** respectively. Expression of proteins in cells treated with DMSO was taken as control.

Conclusion

In the current study, two different sets of triazole linked indole and oxindole glycoconjugates were synthesized, and were further evaluated for their *in vitro* anticancer potentials. An initial screening was performed against DU145, HeLa, A549 and MCF-7 cancer cell lines. In preliminary SRB assay, the compound **5f** from indole series and the compound ***E*-9b** from oxindole series were found to be most active against the DU145 (prostate cancer) cell line. Remarkably, the compounds **5f** and ***E*-9b** were found to be non-cytotoxic towards normal cell line NIH/3T3. Next, it was seen that the compounds **5f** and ***E*-9b** have an effect on the morphology of

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DU145 cells. Furthermore, the colony formation assay (soft agar assay) confirmed that compounds **5f** and ***E*-9b** inhibit growth and proliferation of DU145 cells. The cell cycle analysis revealed that the compounds **5f** and ***E*-9b** target the sub-G1 phase of DU145 cell cycle in a dose-dependent manner. Caspase activation studies confirmed that compounds **5f** and ***E*-9b** have the ability to induce apoptosis in cells *via* mitochondrial mediated intrinsic pathway. Further studies also confirmed that compounds **5f** and ***E*-9b** target pro-proliferative pathway (Akt/PKB) thereby inducing growth arrest in cancer cells. These preliminary results inspire further assessment on the synthesized compounds directing to the development of novel potential anticancer agents.

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