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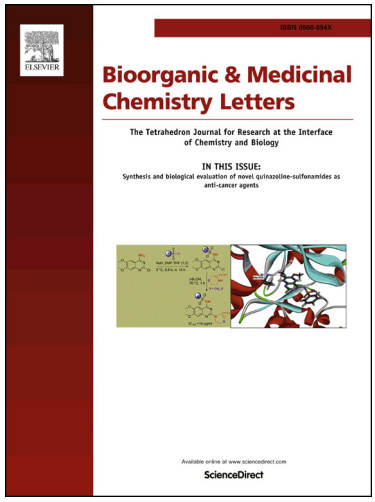

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Design and synthesis of 1,2,3-triazolo-phenanthrene hybrids as cytotoxic agents

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ABSTRACT

A series of new 1,2,3-triazolo-phenanthrene hybrids has been synthesized by employing Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. These compounds were evaluated for their *in vitro* cytotoxic potential against various human cancer cell lines *viz.* lung (A549), prostate (PC-3 and DU145), gastric (HGC-27), cervical (HeLa), triple negative breast cancer (MDA-MB-231, MDA-MB-453) and breast (BT-549, 4T1) cells. Among the tested compounds, **7d** displayed highest cytotoxicity against DU145 cells with IC_{50} value of 1.5±0.09 μM. Further, the cell cycle analysis shown that it blocks G0/G1 phase of the cell cycle in a dose dependent manner. In order to determine the effect of compound on cell viability, phase contrast microscopy, AO/EB, DAPI, DCFDA and JC-1 staining studies were performed. These studies clearly indicated that the compound **7d** inhibited the cell proliferation of DU145 cells. Relative viscosity measurements and molecular docking studies indicated that these compounds bind to DNA by intercalation.

Heterocyclic scaffolds capable of DNA targeting are the most promising agents in the anticancer drug discovery process.[1] Intercalation is one of the major modes of drug-DNA interaction that is exhibited by various potent natural and synthetic antitumor agents such as doxorubicin, acridines, anthraquinones, distamycins, and phenanthrene derivatives.[2] Combilexins are a class of DNA binding agents that exert their mode of action *via* both minor groove binding and intercalation. They are capable of strong binding with DNA than any other class of DNA binders due to their dual mode of binding.[3] Phenanthroindolizidine alkaloids were originally isolated from plants of the *Asclepiadacea* family,[4] and are well-known to exhibit diverse pharmacological activities such as antitumor,[5] anti-inflammatory,[6] anti-microbial,[7] antifungal,[8] antiarthritis,[9] antilupus,[10] antiviral[11] and anti-angiogenic activities.[12] The most noteworthy biological property among these is the intense cytotoxic activity against various cancer cell lines, including multidrug-resistant cell lines.[13] This can be attributed to the inhibition of the enzymes involved in DNA synthesis[14] and intercalation of the phenanthrene core between the DNA base pairs.[15] Tylophorine and tylocrebrine (**Figure 1**) are the representative natural alkaloids from this class.

On the other hand 1,2,3-triazoles are regarded as privileged building blocks for the synthesis of bioconjugates because of their high stability, selectivity and less adverse reactions.[16] They are highly stable under basic and acid hydrolysis including oxidative and reductive conditions. Moreover, this heterocycle is the bioisostere of amide and is capable of interacting with biomolecular targets through hydrogen-bonding.[17] This attractive chromophore displays a wide variety of activities like antibacterial, antifungal, antiallergic, anti-HIV, antitubercular, anticancer, antiviral, antimalarial and anticonvulsant profile.[18] Additionally, it can also interact with DNA and acts as a supporting motif for DNA targeting drugs.[19] The Cu(I)-catalyzed azide-alkyne cycloaddition or 'click reaction' can rapidly yield bioactive molecules linked through 1,2,3-triazoles[20] with high atom economy and has been found wide range of applications in combinatorial synthesis,[21] bio-conjugate chemistry[22] and material science.[23]

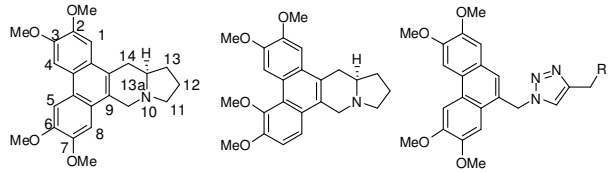

Figure 1. Structures of Tylophorine, Tylocrebrine and the newly synthesized C-9 linked 1,2,3-triazolo-phenanthrene hybrids.

The Structure Activity Relationships (SARs) revealed that the substitution of a polar pharmacophore at C9-position of the phenanthrene ring is of prime importance for potential antitumor activity.[24] Hence, the incorporation of the polar 1,2,3-triazole ring at C9-position might effectively enhance the antitumor activity. As part of our scientific contributions toward bioactive scaffolds,[25] herein we have designed and synthesized a new series of 1,2,3-triazolo-phenanthrene hybrids by using diverse azide and alkyne building blocks. The newly synthesized compounds **7a–o**

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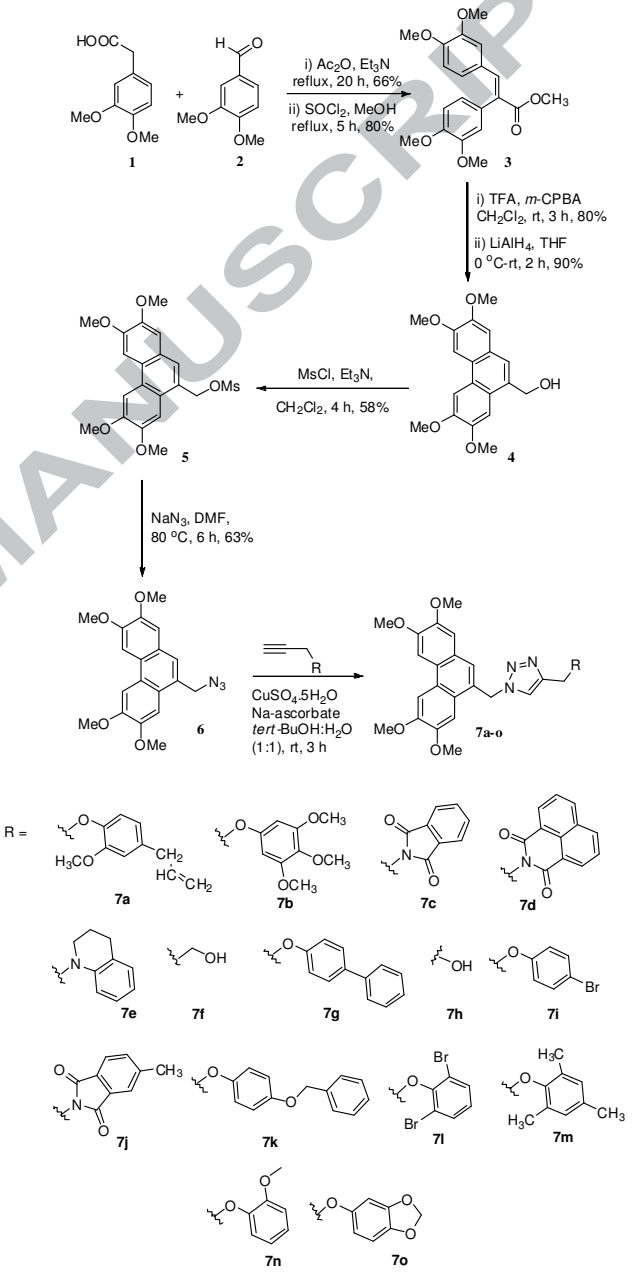
were further evaluated for their *in vitro* cytotoxicity, cell growth inhibition and DNA interaction.

The synthetic route for the preparation of 1,2,3-triazolo-phenanthrene hybrids **7a–o** was outlined in **Scheme 1**. The azide partner **6** was synthesized from 2,3,6,7-tetramethoxy phenanthren-9-yl methanol (**4**). The intermediate **4** was prepared from the commercially available starting materials **1** and **2** *via* the conventional four step sequence according to the reported procedure.[26] Initially, Perkin condensation of 3,4-dimethoxybenzaldehyde (**2**) and 3,4-dimethoxy phenyl acetic acid (**1**) resulted in the formation of 2,3-bis(3,4-dimethoxyphenyl)acrylic acid. The acid was converted into its methyl ester **3** followed by *m*-CPBA/TFA mediated intramolecular oxidative cyclization to give the tricyclic phenanthrene ester. The ester functionality was reduced to alcohol **4** by $LiAlH_4$. The alcohol **4** was mesylated by using mesyl chloride in the presence of Et_3N to give **5**. As this intermediate is highly unstable, then it was immediately taken-up for azidation reaction by using NaN_3, refluxing in DMF to afford 9-(azidomethyl)-2, 3, 6, 7-tetramethoxyphenanthrene (**6**). Next for the synthesis of new 1,2,3-triazolo-phenanthrene hybrids, different substituted alkyne building blocks *i.e., N*- and *O*-linked alkynes were prepared from propargylation of substituted amines and phenols respectively. Finally, we tailored these azides and alkynes by employing 'CuAAC' reaction to obtain the new hybrids **7a–o** in overall 63–90% yields. All the newly synthesized compounds **7a–o** were characterized by IR, HRMS, ^1H and ^{13}C NMR spectroscopy.

The newly synthesized 1,2,3-triazolo-phenanthrene hybrids **7a–o** were evaluated for their *in vitro* cytotoxicity on nine different cancer cell lines such as lung (A549), prostate (PC-3 and DU145), breast (BT-549 and 4T1), gastric (HGC-27), cervical (HeLa) and triple negative breast cancer (MDA-MB-231 and MDA-MB-453) by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[27] The IC_{50} (μM) values (concentration required to inhibit 50% of the tumor cells) of the tested compounds and the reference drug 5-fluorouracil (5-FU) are listed in **Table 1**. It is noticeable from the initial screening that the hybrids **7c**, **7d**, **7j** and **7o** displayed broad range of activity against majority of the tested human cancer cell lines with IC_{50} values ranging from 1.5±0.09 μM to 18.9±0.81 μM (**Table 1**). Among them, one of the compounds **7d** with a napthalimide substitution at C9-position linked through 1,2,3-triazolo moiety was found to be more active on all the cancer cell lines except A549 and exhibited highest potency on DU145 cell line (IC_{50} 1.5±0.09 μM). It was also observed that, the derivatives **7j** and **7o** were active against A549 cells with IC_{50} values of 12.3±0.42 and 14.5±0.61 μM respectively. Compounds **7c**, **7d**, **7e**, **7g, 7j, 7l** and **7o** displayed activity less than 20 μM in PC-3 cells, among which the compound **7j** possessing 1,2,3-triazolo methyl phthalimide substitution proved to be best with an IC_{50} of 7.12±0.73 μM, followed by compound **7c** with a 1,2,3-triazolo pthalimide substitution (IC_{50} 7.6±0.32 μM) and then compound **7d** having 1,2,3-triazolo napthalimide substitution (IC_{50} 8.5±0.82 μM). Except **7e**, **7g**, **7h**, and **7m**, all other derivatives were found to be active on HGC-27 cell line and **7d** being the most active (IC_{50} 3.5±0.24 μM) followed by **7i** with the substitution of 4-bromo phenyloxy group (IC_{50} 3.9±0.16 μM). Derivatives **7b**, **7c**, **7h**, and **7o** showed moderate activity on MDA-MB-231 triple negative breast cancer cells with IC_{50} values ranging from 6.8±0.13 to 19.2±0.46 μM. Interestingly, compound **7o** with a triazolo-benzodioxolo substitution was found to be active on most of the tested cancer cell lines. Compound **7b** with a trimethoxy phenyl substitution proved to be selectively cytotoxic against HGC-27 and MDA-MB-231 cell lines with IC_{50} values of 14.8±0.37 μM and 19.2±0.46 μM respectively. It could be easily observed from the *in vitro* cytotoxicity data that the hybrids with napthalimide and phthalimide groups at C9-position of triazolo-phenanthrene scaffold elevated the biological response. On the other hand, compound **7o** with a triazolo-benzodioxolo substitution also displayed a broad range of activities on all the tested cancer cell lines indicating the importance of methylene dioxy group as a bioactive scaffold. In view of the encouraging results, the most active compound **7d** was taken-up for further studies like cell growth inhibition and DNA interaction.



Scheme 1. Synthesis of various C9-linked 1,2,3-triazolo-phenanthrene hybrids **7a–o**.

Table 1. Cytotoxic activity (IC_{50} in µM)[a] of 1,2,3-triazolo-phenanthrene hybrids **7a–o**

Compound	A549[b]	PC-3[c]	DU145[d]	HGC-27[e]	HeLa[f]	BT-549[g]	MDA-MB-231[h]	MDA-MB-453[i]	4T1[j]
7a	>20	>20	>20	7.6±0.21	7.12±0.71	>20	>20	>20	7.3±0.52
7b	>20	>20	>20	14.8±0.37	>20	>20	19.2±0.46	>20	>20
7c	>20	7.6±0.32	2.6±0.34	7.5±0.18	12.6±0.31	>20	11.8±0.29	>20	11.3±0.46
7d	>20	8.5±0.82	1.5±0.09	5.1±0.36	2.9±0.19	3.7±0.22	4.2±0.14	5.1±0.37	4.2±0.29
7e	>20	13.9±0.38	>20	>20	>20	>20	>20	>20	>20
7f	>20	>20	>20	14.2±0.29	17.2±0.42	>20	>20	>20	>20
7g	>20	12.5±0.51	>20	>20	>20	>20	>20	>20	10.8±0.21
7h	>20	>20	>20	>20	18.3±0.51	8.2±0.21	6.8±0.13	13.5±0.43	>20
7i	>20	>20	>20	3.9±0.16	3.8±0.32	>20	>20	>20	>20
7j	12.3±0.42	7.12±0.73	1.87±0.12	3.5±0.24	2.7±0.31	3.9±0.31	4.6±0.25	6.5±0.41	10±0.35
7k	>20	>20	>20	9.6±0.25	21.1±1.12	14.3±0.21	>20	>20	9.3±0.32
7l	>20	14.1±0.61	>20	3.6±0.13	5.15±1.14	>20	>20	>20	7.4±0.21
7m	>20	>20	>20	>20	17.6±0.42	12.4±0.26	>20	12.6±0.25	6.2±0.24
7n	>20	>20	>20	9.1±0.22	11.2±1.23	>20	>20	>20	>20
7o	14.5±0.61	13.2±0.45	16.7±0.72	9.5±0.41	>20	18.1±0.38	12.6±0.27	18.9±0.81	6.5±0.23
5-FU[k]	1.60±0.10	1.2±0.01	2.18±0.33	14.72±0.42	1.86±0.02	-	4.33±0.02	-	-
Antofine[l]	0.022±0.07	-	0.025±0.05	-	-	-	0.012±0.02	-	-

[a]50% inhibitory concentration after 48 h of compound treatment; [b]Lung cancer cells; [c, d]prostate cancer cells; [e]Gastric cancer cells; [f]cervical cancer cells; [h,i]triple negative breast cancer; [g, j] Breast cancer cells; [k]5-fluorouracil: standard for MTT assay. [l]antofine: reference compound.

Phase contrast microscopic studies were performed to know whether the treatment with these compounds could lead to reduced cell viability and apoptosis induction.[28] DU145 prostate cancer cells were treated with 2 µM, 4 µM and 8 µM concentrations of active compound **7d**. Cells were observed and images were captured by phase contrast microscope. It can be known from **Figure 2** that the increased concentration of compound led to the decreased viable cells in comparison to control as observed by morphological changes such as cell detachment and nuclear shrinkage.

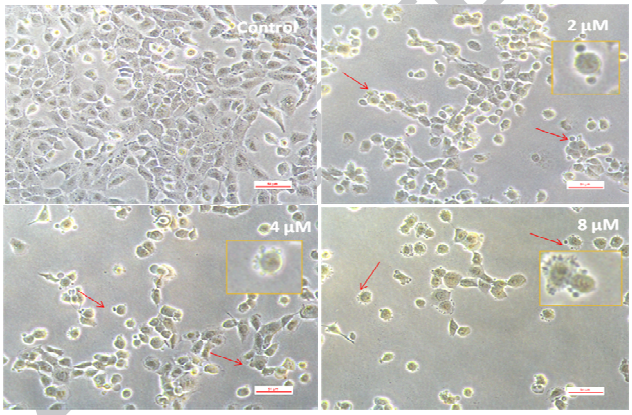


Figure 2. Effect of compound **7d** on morphology and cell viability of DU145 cells.

In order to distinguish the apoptotic, necrotic and live cells, Acridine orange/ethidium bromide (AO/EB) fluorescent staining assay was performed.[29] AO permeates the intact cells and stain the nuclei green, whereas EB penetrates only membrane disintegrated cells and stain the nucleus red.

It can be observed from **Figure 3** that the control cells showed green colour due to their normal morphology. Fluorescence microscopic images of cells treated with compound **7d** at 8.0 µM and clearly showed the altered morphological characteristics such as membrane blebbing, chromatin condensation, cell shrinkage and apoptotic body formation, suggesting that the compound **7d** induced cell death on DU145 cells.

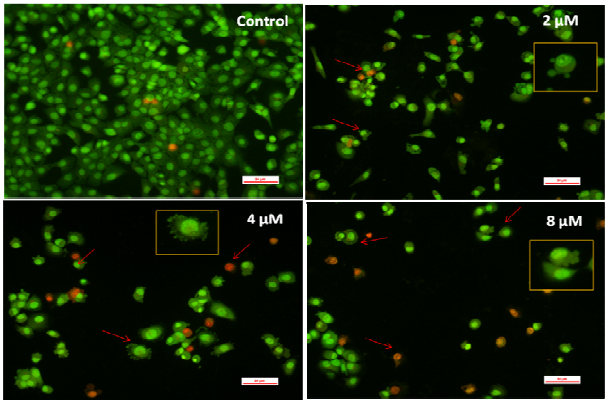


Figure 3. AO/EB staining in DU145 cells treated with various concentrations of compound **7d**. Apoptotic features such as apoptotic bodies, membrane blebbing and dead cells were clearly observed.

DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain that effectively binds to A-T rich regions in DNA and reveals the nuclear damage or chromatin condensation. DAPI permeates the live cell membrane less efficiency. Therefore, the intensity of staining in live cells is less whereas apoptotic cells are stained bright due to the presence of condensed nucleus which is a typical apoptotic characteristic. Therefore, it was considered of interest to study the effect of compound **7d** on DU145 cells by using DAPI staining.[30] DAPI stains the nucleus bright blue by forming a fluorescent complex with chromatin. As observed from **Figure 4**, the nuclear structure of control cells was intact; whereas compound **7d** treated DU145 cells exhibited horse-shoe shaped and condensed nuclei, indicating the characteristic features of apoptosis induction.

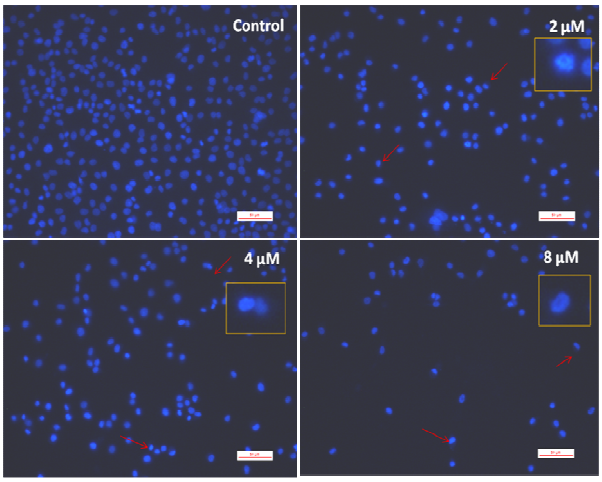


Figure 4. Nuclear morphology in DU145 cells stained with DAPI. DU145 cells treated with compound **7d** for 24 h were stained with DAPI. The images were captured with fluorescence microscope with a DAPI filter.

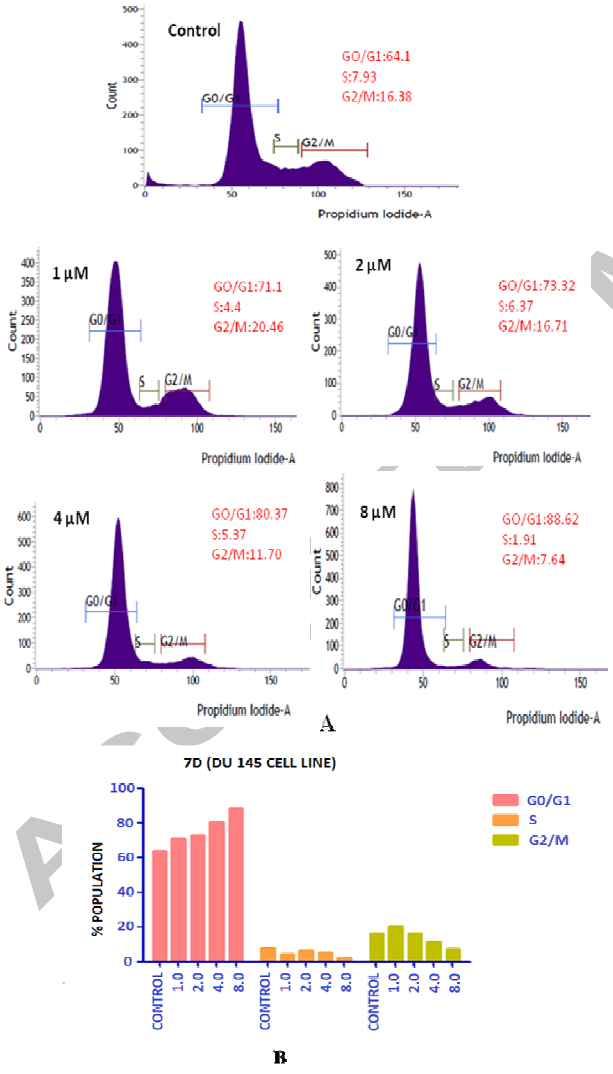


Figure 5 (**A** and **B**)**.** Cell cycle analysis of DU145 cancer cells treated with compound **7d** for 24 h. The cell cycle distribution was analysed by using propidium iodide staining method and analysed by flow cytometry. Each bar represents mean ± SD from three independent experiments.

Majority of the cytotoxic compounds exhibit their growth inhibitory potential by arresting specific phase of a cell cycle. Thus blockade of cell cycle progression by cytotoxic agents has been considered as an effective strategy to develop potential chemotherapeutic agent. Therefore, we studied the effect of compound **7d** on distribution of cell population in different phases of cell cycle by flow cytometry analysis.[31] DU145 cells were treated with compound **7d** at 1 µM, 2 µM, 4 µM and 8 µM for 24 h, and ethanol fixed cells were stained with propidium iodide and further subjected to flow cytometry analysis. The results from **Figure 5** represents that the ratio of DU145 cells in G0/G1 phase increased from 64.1% in control to 71.1% at 1 µM, 73.3% at 2 µM, 80.3% at 4 µM and 88.6% at 8 µM respectively and simultaneous decreased number of cells in G2/M phase. Therefore, these results were clearly indicated that compound **7d** exerts G0/G1 phase arrest in DU145 cells.

The generation of ROS is a typical characteristic of many chemotherapeutic agents by initiating oxidative damage to the mitochondrial permeability and membrane potential. Hence, DCFDA staining method[32] has been used to assess the generation of intracellular reactive oxygen species (ROS) of compound **7d** on DU145 cells. The treatment of compound **7d** for 6 h resulted in enhanced DCFDA fluorescence in a dose dependent manner, indicating the capability of compounds in accumulating ROS (**Figure 6**). On the other hand, decreased fluorescence intensity was observed when DU145 cells treated with N-acetyl cysteine (NAC) prior to compound treatment thus indicating the compounds induced cytotoxicity by ROS generation. Moreover, H_2O_2 treatment of DU145 cells led to the increased fluorescence when compared to the control due to the generation of radicals.

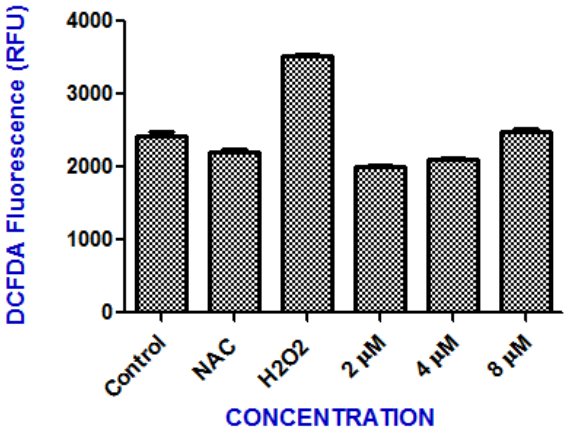


Figure 6. Effect of compound **7d** on Reactive oxygen species (ROS) levels. Dose dependent increment of fluorescence observed compared to control. Each bar represents mean ± SD from three independent experiments.

Enhanced ROS generation can lead to oxidative stress thereby cause loss in mitochondrial membrane potential. Therefore, it is of high importance to study the effect of compound **7d** on mitochondrial membrane potential (DΨm). Lipophilic cationic JC-1 dye was used to determine the membrane potential.[33] Normal polarised mitochondria stains red due to formation of J-aggregates, whereas depolarised mitochondria of apoptotic cells stains green because of J-monomers. DU145 cells were treated with 2 µM, 4 µM and 8 µM of compound **7d** for 24 h and stained with JC-1 dye. The results from **Figure 7** clearly displayed the dose dependent increase in the green fluorescence, thus indicating the loss of mitochondrial membrane potential (DΨm) by the compound **7d.**

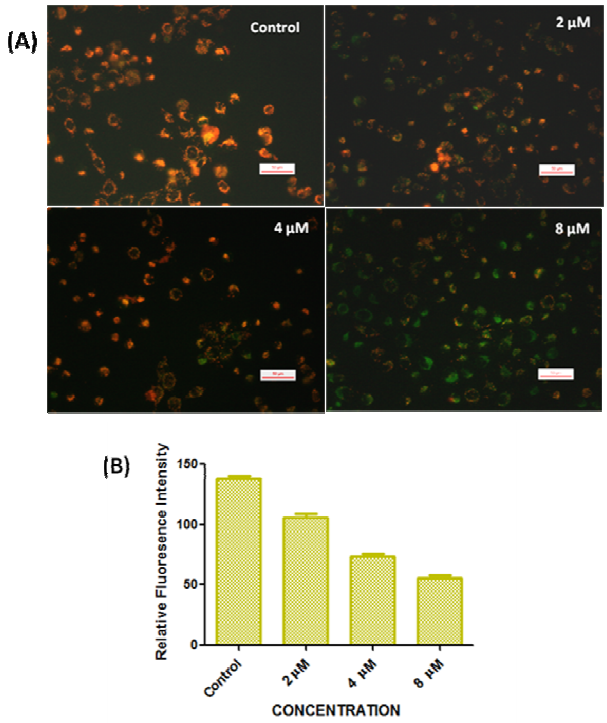


Figure 7. (**A**) Effect of **7d** on Mitochondrial membrane potential. DU145 cells were treated with 2 µM, 4 µM and 8 µM of **7d.** Cells staining orange (JC-1 aggregates) have a healthy DΨm, while cells staining green (JC-1 monomers) are cells with a disrupted DΨm. (**B**) Quantitative measurement of fluorescence intensity (JC-1 aggregates) was measured by spectrofluorometry.

Relative viscosity experiments were performed for the most potent compounds **7d** and **7j** to study their form of interaction with DNA.[34] A substantial increase in the viscosity of DNA solutions can be observed upon intercalation of small molecules like Ethidium bromide, between the base pairs of DNA; whereas only slight change in viscosity occurs in case of groove binders such as Hoechst 33258. The relative viscosity of the complex solutions has increased considerably on gradually increasing the concentration of the compounds **7d** and **7j**. This indicates that the synthesized triazolo-phenanthrene hybrids bind to DNA by intercalation. Among the hybrids, **7d** showed equivalent increase in viscosity to that of Ethidium bromide upon its interaction with DNA, signifying considerable DNA binding affinity (**Figure 8**).

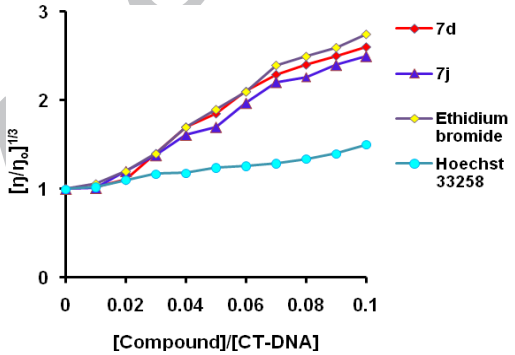


Figure 8. Relative viscosity experiment of hybrids **7d** and **7j** with CT-DNA. Ethidium Bromide and Hoechst 33258 were used as controls. Data represents mean from three individual experiments.

Molecular docking simulations of the synthesized compounds were performed using the DNA duplex with the sequence $d(GAAGCTTC)_2$ using XP Glide 6.9 (Schrödinger 2015-4).[35] The perfectly planar insertion of the tricyclic phenanthrene ring system between the G–C base pairs in the intercalation site was observed in the docked poses for one of the best scored derivatives, **7d** (**Figure 9**). Further, the 1,2,3-triazole moiety participates in hydrogen bonding as well as in orienting the molecule so that the naphthalimide scaffold lies in the minor groove. The docked poses were also stabilized electronically by π–π stacking, dipole-dipole interactions and hydrogen-bonds between the carbonyl oxygens of the naphthalimide and the DNA base pairs in the minor groove. Such a dual mode of binding *i.e.*, intercalation and minor groove binding, effectively enhances the DNA binding affinity of these scaffolds. The results thus obtained from docking studies were consistent with the *in vitro* cytotoxicity as well as relative viscosity data.

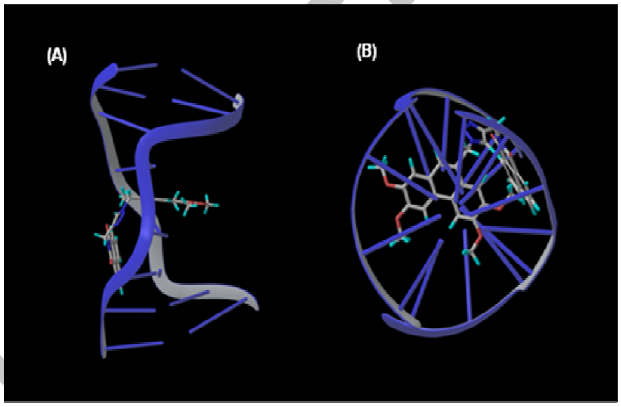


Figure 9. Side view (**A**) and top view (**B**) of the intercalation of **7d** between G–C base pairs of $d(GAAGCTTC)_2$.

In conclusion, a new series of C9-linked 1,2,3-triazolo-phenanthrene hybrids has been synthesized by employing 'click' reaction. These compounds were evaluated for their *in vitro* cytotoxic potential against various human cancer cell lines *viz.* lung (A549), prostate (PC-3 and DU145), breast (BT-549 and 4T1), gastric (HGC-27), cervical (HeLa) and triple negative breast cancer (MDA-MB-231 and MDA-MB-453). From the initial screening, it was observed that some of the compounds were active on the tested cancer cell lines below 20 µM. The cytotoxicity profile indicated that compound **7d** and **7j** showed broad range of activity on majority of the cancer cell lines at below 10 µM. The compound **7d** on DU145 cells resulted G0/G1 phase of cell cycle arrest in a dose dependent manner. AO/EB, DAPI staining, ROS study and JC-1 staining clearly showed the distinctive apoptotic features indicating that the compound inhibited cell proliferation by apoptosis. Further compounds **7d** and **7j** led to increased viscosity suggesting that these compounds bind to DNA by intercalation. Molecular docking study also supports the DNA interaction of these compounds. Overall, the current study established that these new 1,2,3-triazolo-phenanthrene hybrids have the potential to be advanced as DNA interactive cytotoxic agents for the treatment of prostate cancer.

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