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Synthesis of novel pyrazole derivatives as promising DNA-binding agents and evaluation of antitumor and antitopoisomerase I/II activities

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Abstract

Molecules bearing pyrazole nucleus present diverse biological properties such as antitumor and anti-inflammatory activities that can be associated with DNA interactions. This study aimed to the synthesis of new pyrazol derivatives and evaluated their ability to interact with the DNA and antitumor and topoisomerase inhibition activities. All derivatives were successfully synthesized, and their structures were elucidated by ^{1}HNMR and HRMS (ESI^{+}). Antiproliferative inhibition assays, UV titration assays, fluorescence titration assays, circular dichroism (CD) assays, KI quenching studies, topoisomerase inhibitory activity assays and molecular docking were evaluated for these compounds. Especially, compounds 5e and 5q showed higher antitumor activity with IC_{50} values <13 μM for the tested cell lines. However, compounds 5e and 5q did not inhibit the topoisomerase activity evaluated by relaxation assay. These results show that the pyrazole nucleus contributes to the incorporation of molecules into the DNA. Moreover, it was highlighted that positive charges are relevant for the design of promising antitumor and DNA binding compounds.

Keywords: pyrazole; DNA; Antitumor; Antitopoisomerase

Cancer has always been a dreadful disease and continues to attract extensive research investigations. An estimated 8.2 million deaths are reported in 2012 worldwide, the annual number of new cases due to cancer is projected to rise from 14.1 million in 2012 to 21.6 million by 2030 with an increase in the death rate by 60%.[1] Various targets have been identified to restrain cancer among these DNA happens to be the most explored one. DNA is an important biological material whose base sequence controls the heredity of life and is the primary target for most anticancer and antiviral therapies.[2] The DNA binders are classified into three categories based on their mode of interaction, such as I) intercalators II) groove binders III) and combilexins.[3]

Topoisomerase is a vital nuclear enzyme involved in various DNA processes and also important targets for inhibiting the cell proliferation. These are nuclear enzymes that transiently break one or two strands of DNA allowing to solve various DNA topological problems associated with DNA replication, transcription, recombination, and other vital cellular processes.[4-5] DNA topoisomerases are generally classified into topoisomerase I (topo I) and topoisomerase II (topo II) depending on their mechanism of action, making either single- or double-stranded breaks, respectively. [6]

To date, some topoisomerase inhibitors, such as camptothecin (CPT)[7] and its derivatives that target Topo I and etoposide (VP-16)[8], doxorubicin[9] and mitoxantrone[10] which target Topo II, have been clinically used as potent anticancer drugs.

Pyrazole has shown a variety of biological and pharmacological properties. As potential antitumor compounds, they have shown good antitumor activity, multiple targets and high selectivity in vitro.[11-12] Lansiaux A. et al. studied a correlation between DNA binding affinity and nuclear uptake, and have found cationic drugs can be delivered selectively to the cell nucleus. It has been postulated that they gain entry into the cell via the human organic cation transporter 1(hOCT1).[13] Based on this information, our research group had synthesized new 1,3,5-triphenyl pyrazole derivatives which different cationic groups were used as side chains. Therefore, these compounds may easily cross the cell membrane and accumulate in the nucleus and enhance anticancer activity. These compounds were evaluated for their DNA interaction ability, giving the binding mode and affinity with DNA molecule. In addition, it was also analyzed the antiproliferative activity on tumor cells and inhibition of the topoisomerase enzyme.

Results and discussion

Chemistry The synthetic strategy for the preparation of 1,3,5-triphenyl pyrazole is outlined in the Chart 1. Using Claisene-Schmidt condensation reaction[14-17] compound **1** were synthesized. Reactions were base catalyzed, without any protection of hydroxyl groups. In the first step, 20% NaOH aqueous solution was added to the solution of equimolar amounts of aryl ketone and aryl aldehyde in ethanol to obtain compound **1**. Next, compound **2** was prepared by oxidation of compound **1**. In the next step, compound **3** was synthesized in quantitative yield by the treatment of phenylhydrazine and compound **2** in ethanol. Compound **4** was synthesized by compound **3** and dibromo alkane in the presence of pure water, KOH and TBAB. Finally, compound **4** and aliphatic amine were reacted in the presence of KI, K_2CO_3 and acetonitrile reflux for 7 h to give the crude product. The crude

product was dissolved in acetone, and then HCl was added into the solution to adjust the pH to 2-3, and white precipitates precipitated, then filtered to get the final product (**5a-5r**). All the compounds were confirmed by ^{1}HNMR and HRMS (ESI^{+}).

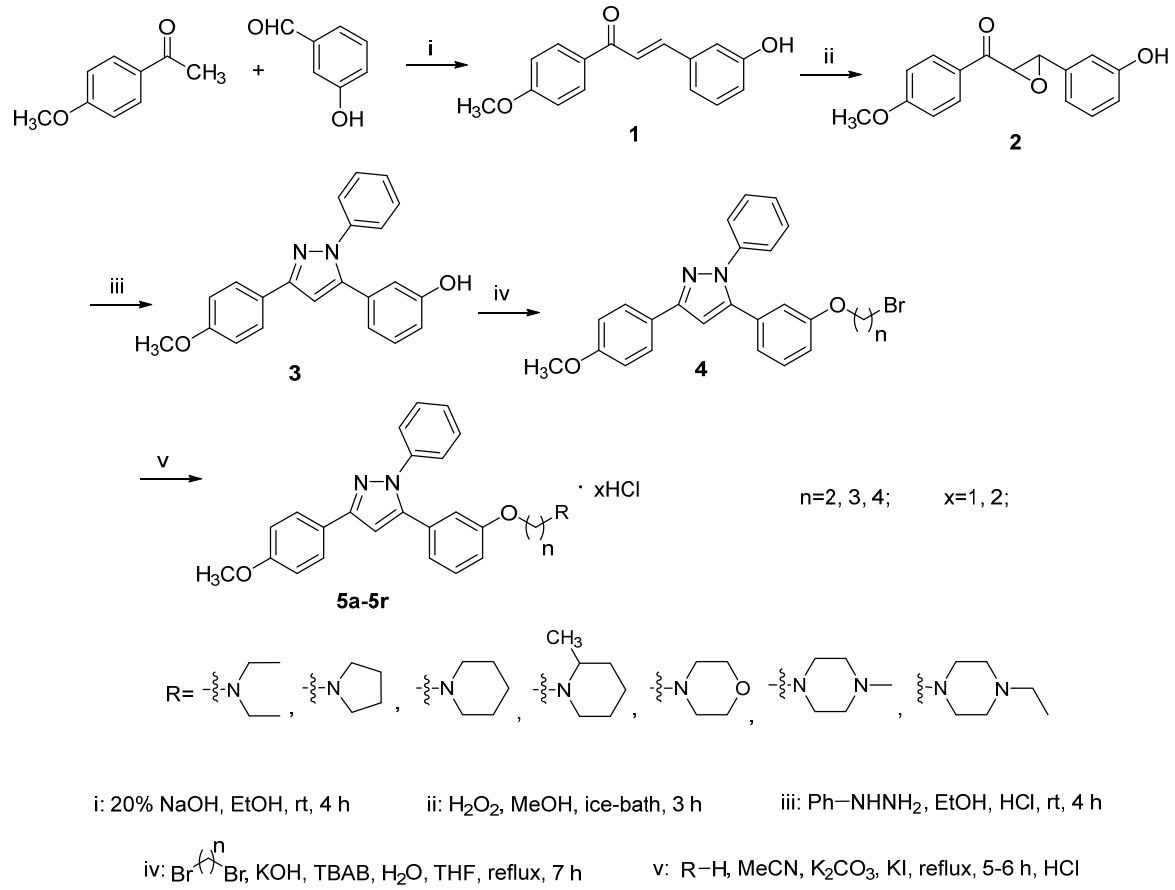
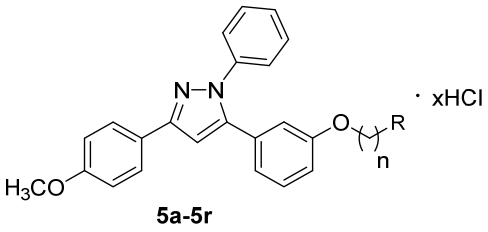
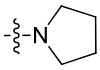
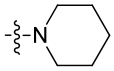
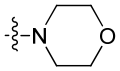
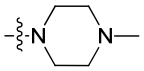
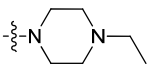
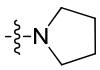
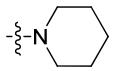
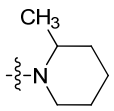
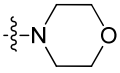
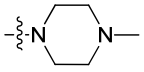
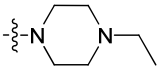
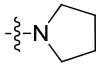
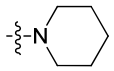


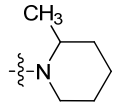
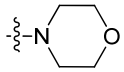
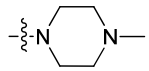
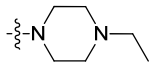
Chart 1. Synthesis of compounds **5a-5r**.

Table 1
Structure of compounds **5a-5r**.



5a-5r

Compounds	n	R	yield/%	melting-point/°C
5a	2		18.6	163-165
5b	2		64.0	211-213
5c	2		64.8	236-238
5d	2		51.5	248-250
5e	2		48.2	226-228
5f	2		36.3	224-226
5g	3		20.8	185-187
5h	3		69.2	224-226
5i	3		47.2	195-197
5j	3		55.4	203-205
5k	3		54.0	202-204
5l	3		35.4	208-210
5m	4		58.2	212-214
5n	4		68.1	213-215

5o	4		59.6	195-197
5p	4		37.4	204-206
5q	4		33.1	207-209
5r	4		56.2	66-68

Biological activity

Antiproliferative activity The antitumor potency of target compounds against four human cancer cell lines including MCF-7 (breast cancer), MGC-803 (gastric cancer), HeLa (cervical cancer), and Huh-7 (liver cancer) cells were evaluated by MTT assay. Camptothecin and etoposide were used as positive controls. The results were shown in Table 2. By comparing the IC_{50} values, compounds **5e** and **5q** showed higher antitumor activity with IC_{50} values <13 μM for all tested cell lines. Compound **5f** showed good inhibitory activity selectivity for MGC-803 as well as Huh-7, and compounds **5k** selectivity for Huh-7. Furthermore, compounds **5d** had relatively weak inhibitory activity in the four cancer cell lines. The preliminary results are promising, and some of these compounds may be potential candidates for new anticancer agents.

Table 2
The IC_{50} values of compounds **5a-5r** against various human cancer cells.

Compounds	IC_{50} (μM)			
	MCF-7	MGC-803	Hela	Huh-7
camptothecin	2.86±1.25	4.35±0.47	3.26±0.30	1.28±0.50
etoposide	1.56±0.75	2.61±0.74	3.02±0.80	1.35±0.66
5a	>60	26.48±1.13	30.11±3.60	49.18±1.13
5b	30.96±1.76	46.14±1.32	34.05±1.65	31.85±1.32
5c	52.65±5.72	>60	38.37±0.95	>60
5d	>60	>60	>60	>60
5e	12.62±2.53	8.72±2.17	12.16±2.81	5.46±2.83
5f	18.97±2.00	11.66±3.46	17.32±1.81	7.67±2.11

5g	48.35±1.68	>60	32.27±1.80	>60
5h	47.89±1.70	>60	>60	>60
5i	>60	29.56±4.36	>60	39.93±2.94
5j	>60	30.80±1.78	>60	46.80±5.63
5k	43.92±1.67	16.39±2.40	21.65±1.50	9.12±2.47
5l	>60	46.01±2.33	57.62±1.33	>60
5m	>60	50.24±1.53	50.03±5.60	21.67±3.23
5n	>60	28.37±1.98	51.01±1.17	54.52±3.46
5o	58.35±1.72	17.50±1.22	>60	48.13±6.20
5p	>60	36.01±0.87	53.25±1.50	57.72±1.51
5q	12.97±1.36	6.86±0.84	9.28±2.76	9.12±2.47
5r	>60	23.46±2.00	20.01±2.69	>60

Each value represents the Mean ± S.D. from three different experiments, performed in triplicate. Camptothecin and etoposide were used as positive controls.

DNA binding studies

To find out the nature of the interaction of active congeners **5e** and **5q** with DNA, biophysical studies such as UV-visible spectroscopy, fluorescence spectroscopy and circular dichroism spectroscopy were carried out.

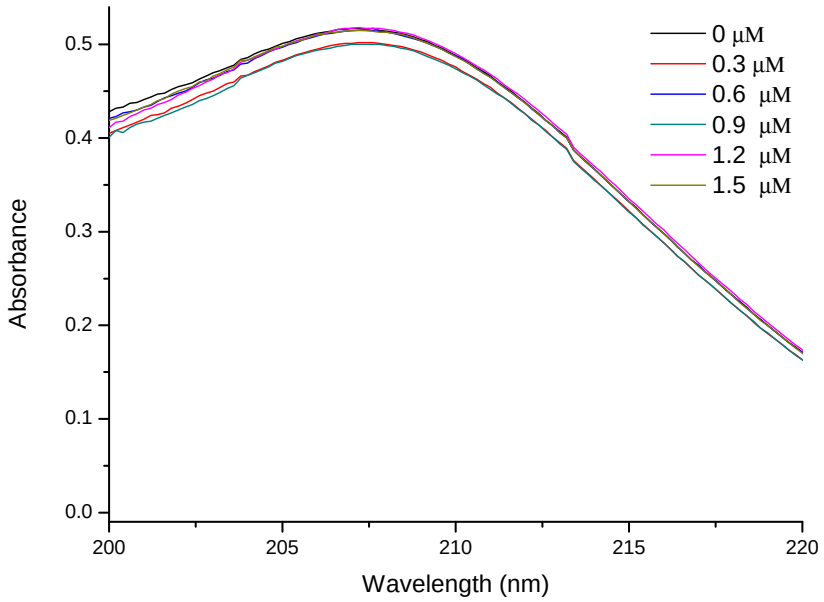
UV-visible Absorption Spectroscopy To examine the binding nature of active congeners **5e** and **5q** to DNA, the UV absorption spectra of these congeners in the presence and absence of increasing concentrations of ctDNA were examined. UV-visible studies are useful in understanding the mode of binding of these compounds with DNA. In general, hypochromism combined with a red shift is considered as an indication of intercalation of small molecules into DNA due to the stacking of the chromophore pairs. The hyperchromism originates from the breaking of the DNA secondary structure with contraction in the axis of the helix; while hypochromism results from the stabilization of the double stranded DNA either by intercalation or electrostatic effect and groove binding[18]. The maxima absorption wavelength of **5e** and **5q** in the absence of ctDNA was 207 and 208 nm, respectively. Absorption spectra of **5e** and **5q** showed that as the concentration of ctDNA increases, the curve presents hypochromism without any red or blue shift. Hypochromic effect without any red or blue shift can be suggested that the groove binding is the most susceptible mode of **5e** and **5q** with ctDNA.[19].(Fig. 1) K_b of the compounds were determined using the following Formula:

$[DNA] / (\varepsilon_A-\varepsilon_F) = [DNA] / (\varepsilon_B-\varepsilon_F) + 1 / K_b (\varepsilon_B-\varepsilon_F)$

where [DNA] is the concentration of ctDNA. In the formula, ε_A = the apparent absorption

coefficient, ε_F = the extinction coefficient of the free compound, ε_B = the extinction coefficient of the compound when fully bound to DNA, respectively. In plots of [DNA] / ($\varepsilon_A - \varepsilon_F$) versus [DNA], K_b is given by the ratio of the slope to the intercept.(table 3)

A) 5e (0.15 μM)



B) 5q (0.15 μM)

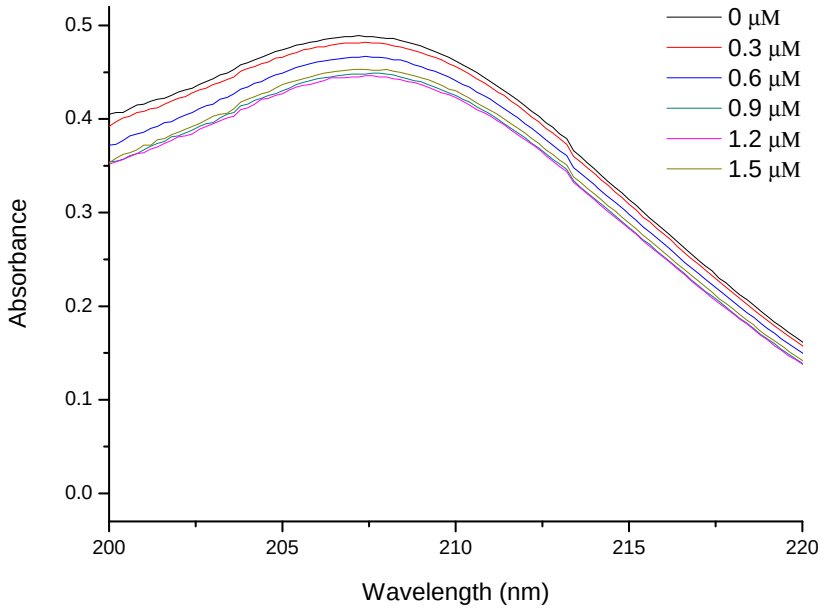


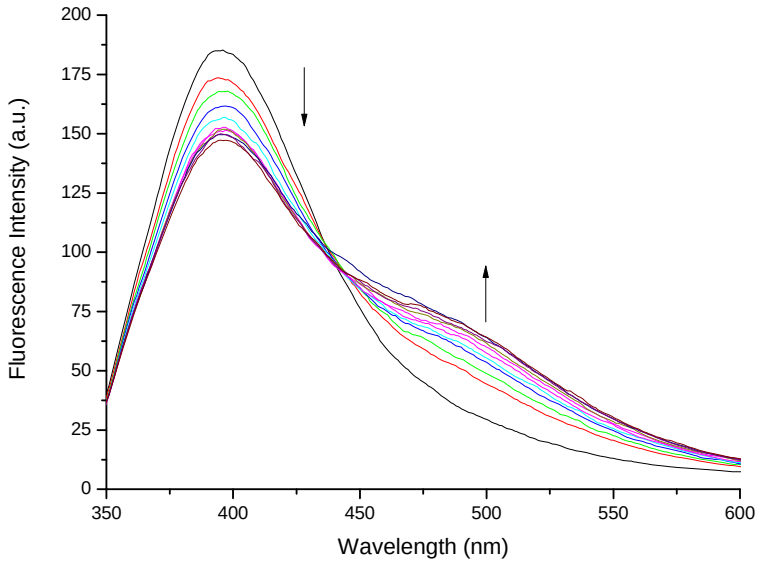
Fig. 1. Absorbance changes after interaction of **5e** (0.15 μM) and **5q** (0.15 μM) with ctDNA. ([ctDNA]=0, 0.3, 0.6, 0.9, 1.2, 1.5 μM)
(Color figure can be accessed in the online version.)

Table 3
DNA binding data for compounds.

compound	λ (nm)	Change in abs.	Bathochromism (nm)	K_b (M^{-1})
5e	207	Hypochromism	0	$1.98(\pm0.04)\times10^4$
5q	208	Hypochromism	0	$3.88(\pm0.06)\times10^4$

Fluorescence titrations To further evaluate the ctDNA interaction, the corresponding binding parameters were also determined by fluorescence spectroscopy. This Fluorescence titrations assay can provide information on the nature and affinity of binding on ctDNA.[20] On addition of equal increments of ctDNA solution to the solution of **5e** and **5q**, the hypochromicity of fluorescence emission intensity at 396 nm was observed, which probably indicates a non-intercalative mode of binding such as electrostatic binding mode (surface binding or groove binding) that leads to effective protection of **5e** and **5q** molecules by ctDNA. However, the less intense fluorescence peaks intensity of **5e** at 480-510 nm exhibited gradual enhancement with the addition of ctDNA. The enhancement in the fluorescence peaks at high wavelength may be due to a partial intercalating portion of **5e** with ctDNA. The fluorescence spectra of **5e** and **5r** were shown in Fig. 2.

A) 5e



B) 5q

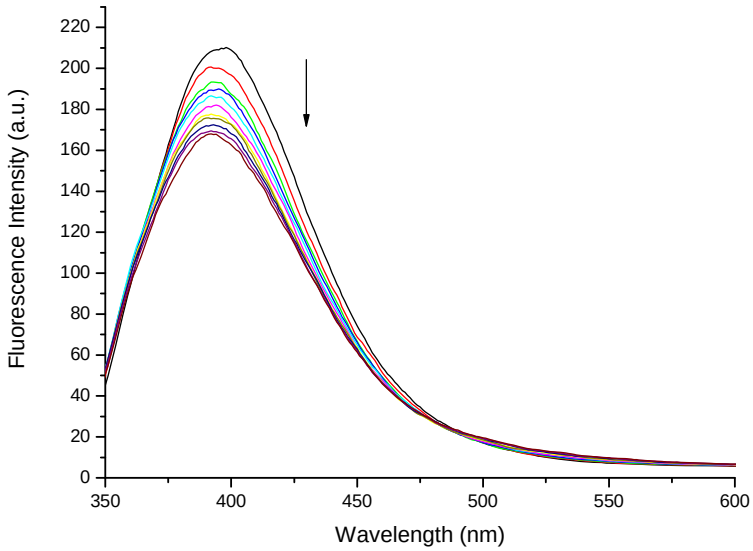
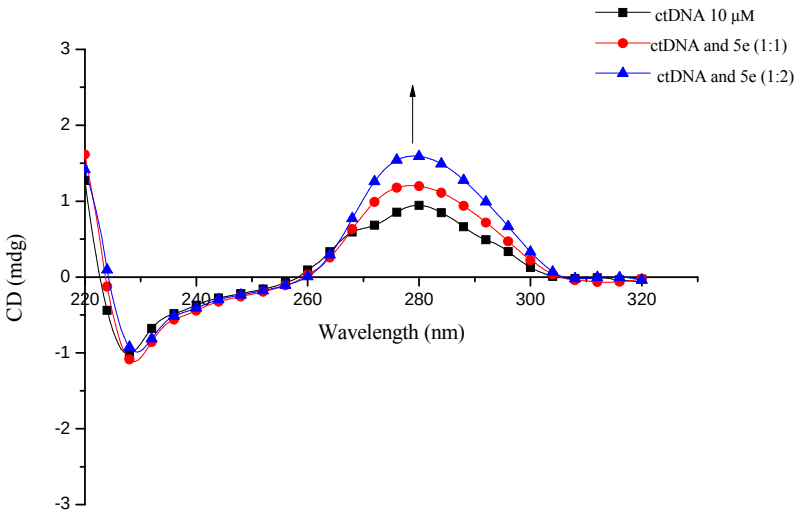
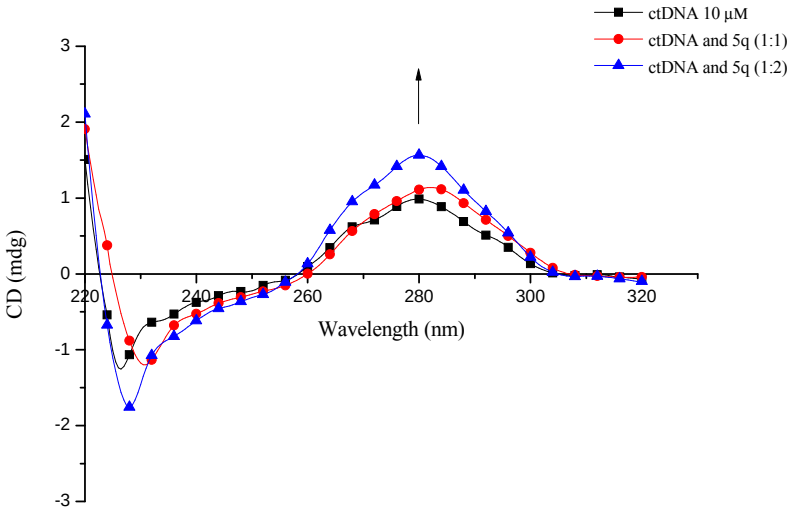


Fig. 2. Fluorescence spectra depicts the interaction of compounds **5e** (10 μM) and **5q** (10 μM) with increasing concentrations of ctDNA at 25°C.
([ctDNA]=0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μM)
(Color figure can be accessed in the online version.)

Circular dichroism studies Circular dichroism (CD) has been found to be a reliable technique for detecting DNA minor groove binding by small molecules. CD monitors the interaction of a compound to the double helix; thus, a large induced CD signal implies minor groove binding because in this interaction, ligands would be in proximity to the chiral sugars. Conversely, intercalators induce little or no induced CD signal, as they bind far from the chiral sugars.[21] We observed CD spectra of several of the new compounds to determine if they are binding in the DNA minor groove. Fig. 3 showed that the CD spectra for the interaction between ctDNA and two of the compounds (**5e** and **5q**). All of the two compounds showed very strong induced CD signals which demonstrate the ctDNA minor groove binding of these compounds. (Fig. 3)



(A)



(B)

Fig. 3. Circular dichroism spectroscopy of **5e** and **5q** with ctDNA.

KI quenching studies Iodide quenching experiments were conducted on the same fluorescence spectrophotometer described previously. Iodide ions can effectively quench the fluorescence intensity of the small molecule in an aqueous medium.[22-23] In the presence of DNA, iodide ions are repelled by similarly charged phosphate backbone of DNA and any small molecule that penetrates inside DNA helix is well protected from being quenched by

these negatively charged quenchers. However, electrostatically bounded molecules, as well as groove binders, are not well protected from anionic quenchers even in the presence of DNA environment and are readily quenched by KI.[24] The quenching was investigated by gradually adding KI to compound and compound–ctDNA complex solutions to measure the relative accessibilities of the free and bound compound. Varying concentrations of KI (0–20 mM) were added to the compound (10 μM) and compound–ctDNA (containing 10 μM compound and 40 μM ctDNA) solutions, respectively. The quenching constants in the absence and presence of ctDNA were calculated and then compared to analyze the interaction mode of the compound with ctDNA.

Fig. 4 revealed the quenching behaviors of KI in the compound and compound–ctDNA systems. It was seen that the Ksv values of **5e** were 15.48 and 16.77 L mol^{-1}, and that the Ksv values of **5q** were 20.57 and 24.82 L mol^{-1} without and with ctDNA, which indicated that the double helix of ctDNA could not protect the compound from the anionic quencher. Therefore, the interaction mode of the compound with ctDNA should be groove binding.

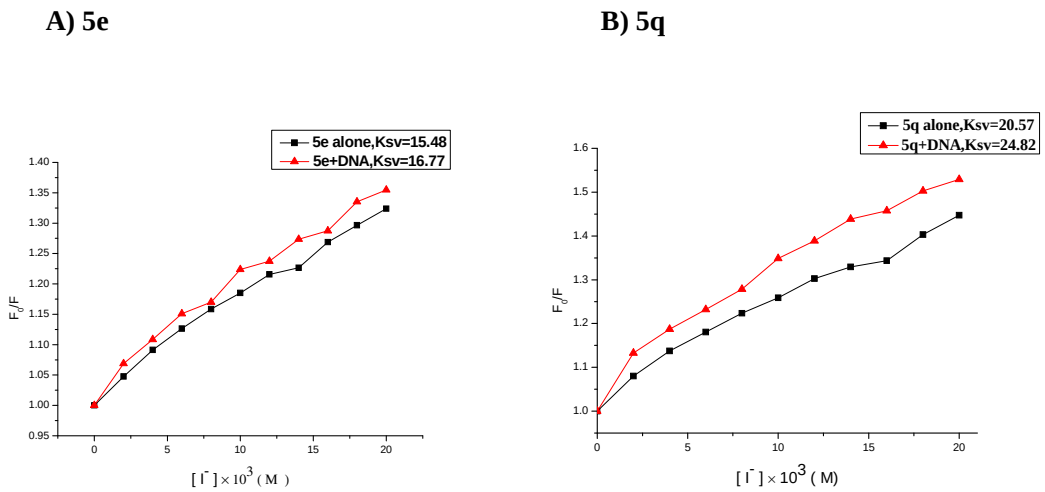


Fig. 4 KI quenching studies. Stern–Volmer plot to calculate the KI quenching constant in absence and presence of DNA. Slight increase in K_{SV} value is obtained in case of groove binding.

Molecular docking studies To further substantiate the biological activity of these compounds, molecular docking studies were performed to characterize the interaction of 1,3,5-triphenyl pyrazole with DNA. The 3D co-crystal structures of DNA (PDB: 1DNH) is selected as the receptor for molecular docking study. The results from the molecular docking indicated that these compounds bind to the minor groove of DNA sequences. Compounds **5e** and **5q** are complementary to that of the DNA groove, and a similar binding affinity was observed in comparison with the Niacin 33258 (the original ligands of 1DNH). The methoxy

groups and the alkylamine groups on both ends of the ligand were involved in extensive and dynamic H-bonds to the DNA bases. Molecular docking and fluorescence studies also support this claim.

(A) Niacin 33258 (B) compound 5e (C) compound 5q

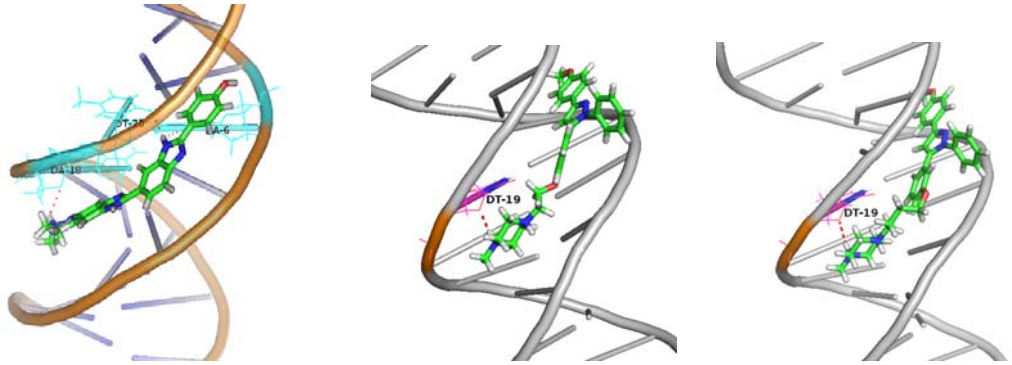
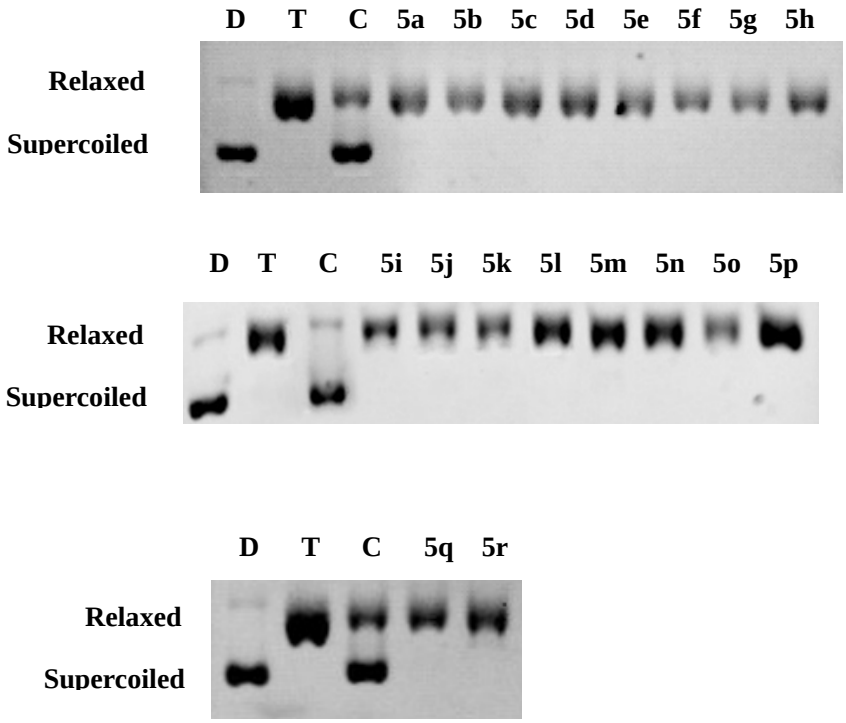


Fig 5 View of Niacin 33258 (A) compound **5e** (B) and **5q** (C) docked in the DNA binding domain

Antitopoisomerase activity The enzymes which control the topology of DNA are called topoisomerases and play a crucial role in several important processes such as DNA replications, transcription, segregation and recombination.[25] The two major classes of topoisomerase type I (topo I) and type II (topo II), are mainly classified by cleavage of single-stranded or double-stranded DNA, respectively.[26] Generally, in various tumor cells, topoisomerase is expressed much more than in normal cells, so the modulating of topoisomerase levels could be an important treatment for cancer.[27] Well-known topoisomerase I and II inhibitors, camptothecin and etoposide, were used as positive controls. The reaction products of topoisomerase I and II relaxation assays were analyzed by electrophoretic mobility and developed in ethidium bromide in the presence of UV light. The inhibitory activities were evaluated at 100 μM for all compounds. Only those compounds with moderate to significant activity were tested further at 20 μM.

Antitopoisomerase I activity All of the compounds showed no inhibitory activity on topo I at 100 μM concentrations. Fig 6 showed that the DNA fraction of super helicoidal was completely relaxed by the enzyme topo I.

A) 100 μM



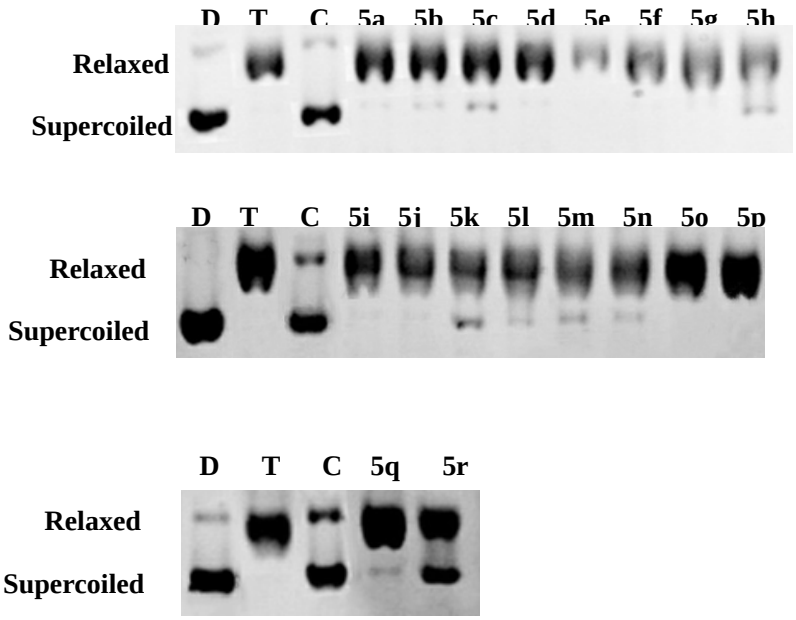
Lane D: pBR322 only,
Lane T: pBR322 + topoisomerase I,
Lane C: pBR322 + topoisomerase I + Camptothecin,
Lane 5a-5r: pBR322 + topoisomerase I + compounds **5a-5r,**

Fig 6. Topoisomerase I inhibitory activity of compounds **5a-5r**

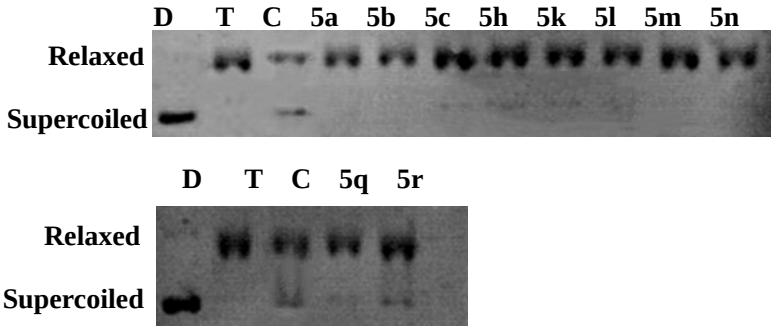
Antitopoisomerase II activity As shown in Fig 7, most of the compounds (**5c**, **5h**, **5k**, **5l**, **5m**, **5n**, **5q**, **5r**) showed topoisomerase II inhibitory activity at 100 μM. Noteworthy, the compounds of **5r** (48.2% and 13.3% at 100 and 20 μM, respectively) showed better topoisomerase II inhibitory activity.

It was suggested that the cytotoxic activity of **5e** was not due to topo II inhibition and the cytotoxic activity of **5q** showed lower topoisomerase II inhibitory activity. However, according to research findings, pyrazole compounds have a wide range of biological targets.[28-30] In future analysis, other target molecules will be investigated for better elucidation of the action mechanism of the new pyrazole derivatives synthesized in this work.

A) 100 μM



B) 20 μM



Lane D: pBR322 only,
Lane T: pBR322 + topoisomerase II,
Lane C: pBR322 + topoisomerase II + Etoposide,
Lane 5a-5g: pBR322 + topoisomerase II + compounds **5a-5r**,

Fig 7. Topoisomerase II inhibitory activity of compounds **5a-5r**

Conclusion

In conclusion, a novel series of 1,3,5-triphenyl pyrazole compounds were synthesized, and their antiproliferative properties were evaluated against four human cancer cell lines. Especially, the compounds **5e** and **5q** showed higher antitumor activity with IC_{50} values <13 μM for the tested cell lines. Compound **5e** and **5q** did not inhibit the topoisomerase activity evaluated by relaxation assay. However, the DNA binding studies disclosed that they are DNA minor groove binding and the docking studies correlated with DNA binding studies.

The findings give us an insight into the DNA binding mechanism and the anticancer activity of these 1,3,5-triphenyl pyrazole compounds. Overall, these studies are very important to understand the role of 1,3,5-triphenyl pyrazole compounds for the cytotoxic activity and DNA binding ability. And the positive charges can be used as a strategy for increasing the DNA binding affinity and anticancer activity of these compounds. It is suggested that these compounds may act as potential anticancer agents.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials.

General chemistry methods, synthesis procedures, spectral data, biological assays, molecular docking are given in Supplementary materials.

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