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3-Formylchromone Based Topoisomerase IIα Inhibitors: Discovery of Potent Leads

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ABSTRACT

Substituted 3-formylchromones were synthesized and evaluated as inhibitors of human DNA topoisomerase IIα (hTopo-IIα) enzyme. The results of the decatenation, relaxation and DNA intercalation assays revealed that the compounds (**11b**, **12a**, **12b**, **12d**, **12e**, **13a** and **13b**) exhibited potent inhibitory activity against hTopo-IIα enzyme, and are nonintercalating agents. These compounds also possess significant *in vitro* cytotoxcity (LC_{50} ranges from 0.5-8.6μM) against Prostate (PC-3) cancerous cell line as seen in comparison to the standard drug etoposide. To further probe the plausible mode of action of 3-formylchromone derivatives, molecular docking studies have also been carried out, which showed that the compounds under investigation fitted well in the ATP binding pocket of hTopo-IIα enzyme with good docking score and makes nonbonding interactions with the crucial residues of the catalytic site.

Key Words: 3-Formylchromone; Topoisomerase IIα inhibitors; DNA non-intercalator; Cytotoxcity; Molecular Docking.

1. Introduction

The structure of DNA is prone to topological challenges arising from intertwining of its complementary strands and can generate super helical strain which impairs the vital processes (transcription, replication, chromatin assembly and chromosome segregation). The cells rely on "topoisomerases", essential enzymes, which have the ability to resolve the topological problems associated with the double helix structure of DNA without changing the underlying chemical structure of DNA. [1] Two major forms of topoisomerase enzymes (Topo-I and Topo-II) are present in all the cells and are differentiated on the basis of mechanistic and physical properties. Topo-I causes the single strand break in superhelical DNA resulting

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in relaxation, and finally relegates the nick. Single strand cut allows the controlled rotation of broken DNA around the other intact strand and thereby reducing the stress. Topo-II requires ATP for its catalytic activity and it has the ability to create a transient double strand break in the DNA, allowing a passage of one intact DNA helix through cleaved end and the break is resealed by enzyme.[1,2] Human Topo-II exists in two isoforms, α (alpha) and β (beta). Topo-IIα is required to regulate both cell cycle and growth cycle, and its level increases throughout the S phase, and peaks at G_2/M boundary. Moreover, it is present exclusively in rapidly proliferating tissues, which suggests that Topo-IIα bears the major responsibility toward events associated with DNA replication and chromosome segregation. In contrast, level of Topo-II β isoform is found to be independent of both cell cycle and growth cycles due to its presence in cell regardless of their proliferation status. [3]

DNA topoisomerases have fascinated both clinical scientists and medicinal chemists for several decades; these have been recognized as important molecular targets for a variety of pharmaceutical agents .[4] Most of the chemotherapeutic anticancer treatments rely on the use of at least one drug that inhibits topoisomerases. [5,6] Some of the clinically important anticancer drugs targeting topoisomerase type II include doxorubicin, daunorubicin, etoposide, teniposide, mitoxanthrone, amonafide, and amsacrine [7,8], whereas camptothecin derivatives target the topoisomerase type I. [9,10] Dual topoisomerase I and II inhibitors as anticancer drugs are also being investigated. [1]

Molecules targeting Topo-II are classified into two types: Topo-II catalytic inhibitors and poisons. [11] Molecules/therapeutic agents belonging to catalytic inhibitor class interfere at one of the stages involving binding of the enzyme to DNA, ATP to the DNA-enzyme complex or ATP hydrolysis. The second class Topo-II poisons act by stabilizing the cleavable complex of Topo-II with DNA by the formation of a covalent ternary complex of drug/agent-

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cleaved G segment DNA and enzyme. Previous studies indicated that Topo-II poisons may induce chromosomal translocation, which lead to specific leukaemias. [12] Consequently, in recent years efforts have been directed towards the development of Topo-II catalytic inhibitors to avoid cytotoxic effects of Topo-II poisons; some of the agents are also capable of overcoming the multidrug resistance (MDR).[13, 14]

Chromone moiety is a part of a large number of molecules of medicinal significance and is capable of interacting with a variety of proteins, enzymes and biological receptors, and many of chromone derivatives display significant anticancer activity.[15] Earlier we had reported 3-formylchromone derivatives as potential topoisomerase inhibitors, which exhibit high anticancer activity against Ehrlich Ascites cancer cells *in vitro* as well as in EAC implanted mice.[16] Some recently reported synthetic and naturally occurring chromone derivatives as Topo-II inhibitors (Fig. 1) include benzoxanthone (**1**) derivatives, which are DNA cross-linker [17], and synthetic flavonoid MHY336 (**2**); the latter arrests the cell cycle in G2/M or S phase via Topo-II dependent mechanism [18]. Dietary flavonoid fisetin (**3**), has been found to act as a dual inhibitor of Topo-I and Topo-II in cells.[19] Hecht and coworkers have demonstrated that topopyrones (**4-7**) are also strong Topo-II poisons.[20, 21] In the case of psorospermin (**8**), a natural antitumor antibiotic [22], it has been shown that it intercalates with DNA and its alkylating potential is significantly increased in the presence of Topo-II. [23]

Prompted by reported anticancer activity of molecules possessing chromone nucleus, present investigations were aimed at design, synthesis, bioevaluation and molecular modeling studies of chromone based anticancer agents, targeting human DNA Topo IIα (hTopo-IIα) enzyme. Based on our earlier design considerations [16], it was envisaged that differently substituted 3-formylchromones and its derivatives could act as potential inhibitors of this key enzyme, regulating the topological changes of DNA. Herein we report the synthesis of 3-

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formylchromones derivatives, followed by their *in vitro* evaluation using decatenation, relaxation and DNA intercalation assays to identify their hTopo-IIα inhibitory activity. *In vitro* cytotoxic activity of the potent compounds was also tested against PC-3 cancerous cell line by MTT assay. Finally, molecular docking studies were performed on investigational compounds to identify their plausible mode of interaction with hTopo-IIα enzyme.

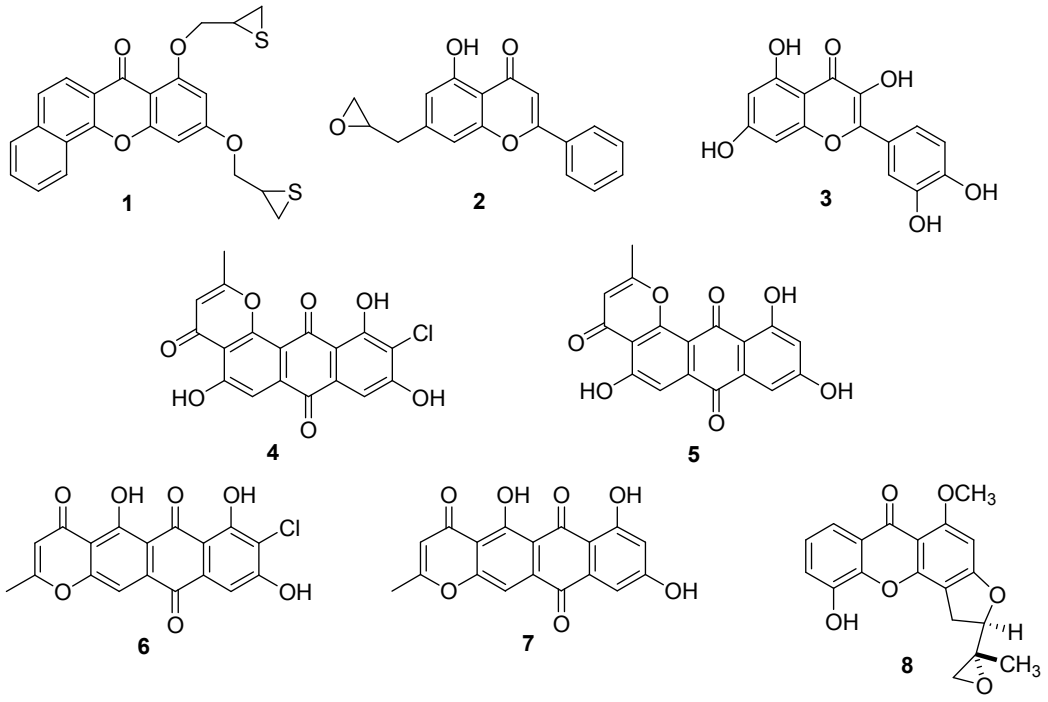

Fig. 1. Chromone based compounds as topoisomerase II inhibitors

Results and discussion

Chemistry

The synthetic approaches adopted for the preparation of the desired compounds are illustrated in scheme 1. A variety of 3-formylchromones and its derivatives (**11a-e, 12a-e and 13a-e**) were synthesized by utilizing the protocol established in our previous study [16, 24] which involve synthesis of 3-formylchromone and further derivatisation by reaction with *N*-phenylhydroxylamine yielding nitrones (**X**), which on thermal rearrangement in the presence

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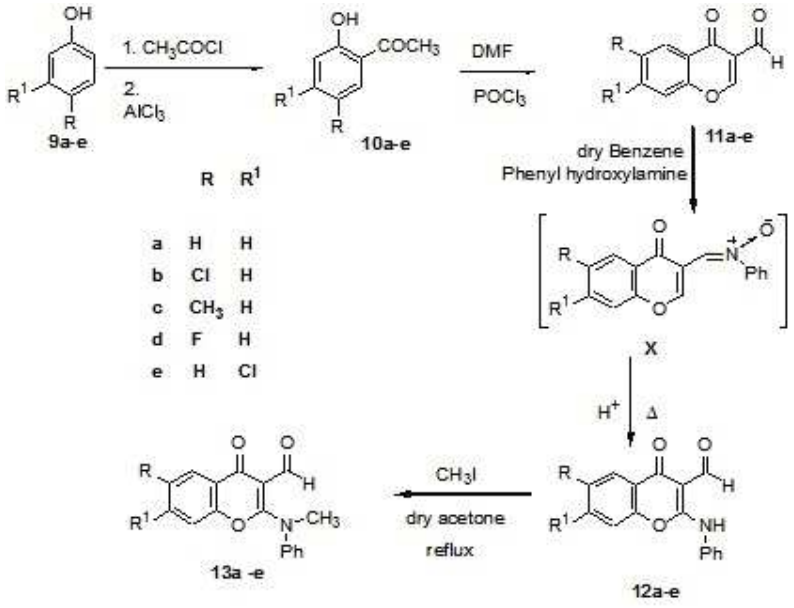
of a few drops of acetic acid, leads to the formation of 2-anilino-3-formylchromones (**12a-e**). *N*- methylation of **12a-e** was carried out with methyl iodide in the presence of anhydrous potassium carbonate by refluxing in dry acetone leading to the formation of 2-(*N*-methylanilino)-3-formylchromones (**13a-e**, Scheme 1).



Scheme 1 Synthesis of various 3-formylchromone derivatives

Pharmacology

The bioevaluation of the investigational compounds (**11a-e**, **12a-e** and **13a-e**) against hTopo-IIα enzyme was carried out to study decatenation, relaxation processes and mode of interaction (intercalating or nonintercalating). A screening kit purchased from TopoGEN, Inc. (Columbus, OH) was utilized for this purpose. To investigate the cytotoxic activity of potent hTopo-IIα inhibitors, they were tested against Prostate (PC-3) cancerous cell line by MTT assay; cell survival was measured after the treatment of cells with the investigational compounds for 48 h. Etoposide, a known Topo II inhibiting anticancer drug, was used as a standard in all the *in vitro* experimental assays.

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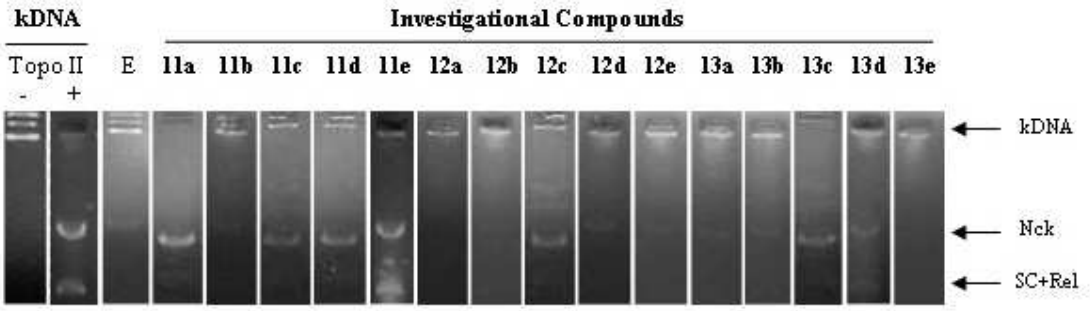
hTopo-IIα based decatenation assay

The ATP-dependent kDNA decatenation assay was performed to examine the effect of the investigational compounds on hTopo-IIα enzyme and results are presented in Fig. 2A. This assay was performed using kinetoplast DNA (kDNA) as a substrate and etoposide (known topoII-inhibiting anticancer drug) as a standard.[14, 25] Incubation of kDNA with hTopo-IIα leads to the formation of decatenated products: nicked (Nck) and supercoil (SC)/relaxed (Rel) circular DNA, which move easily in agarose gel as compared to catenated kDNA because of latter's overall size.[26] The standard drug etoposide causes the partial or moderate decatenation. In case of the investigational compounds (**11a-13e**) almost no decatenation activity was observed in the presence of compounds **11b**, **12a**, **12b**, **12d**, **12e**, **13a** and **13b**, indicating their good inhibitory activity against hTopo-IIα enzyme (Fig. 2A). The decatenation products (Nck, Rel, and SC) were quantified by analyzing the densitometric data obtained using Quantity One (BioRad) and the results were compared with standard drug etoposide (Fig. 2B). In comparison to etoposide, compounds **11b**, **12a**, **12b**, **12d**, **12e**, **13a** and **13b** have shown higher degree of inhibition of hTopo-IIα activity.

A



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B.

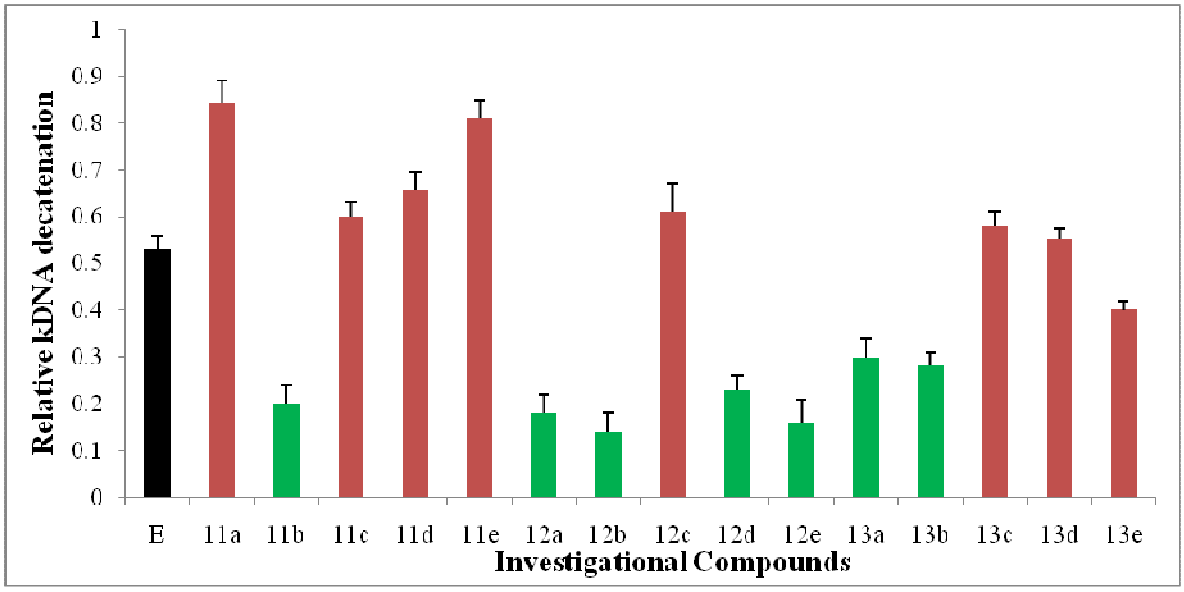

Fig. 2 (A) Effect of test compounds on Topo IIα mediated kDNA decatenation assay. kDNA was treated with hTopoIIα in the presence of either 100 μM etoposide or test compounds. Reaction mixture was incubated at 37 °C for 30 min and 1% agarose gel was run in TAE buffer **(B)** Graphical representation of decatenated products formed in kDNA decatenation assay.

hTopo-IIα based relaxation assay

Further, in order to confirm the hTopo-IIα inhibitory activity of investigational molecules, compounds **11b**, **12a**, **12b**, **12d**, **12e**, **13a** and **13b** were selected on the basis of results of decatenation assay and were subjected to relaxation assay.[14, 25] In this assay, negatively supercoiled DNA (pRYG) was used as a substrate and etoposide as positive control. When the supercoiled form of substrate plasmid DNA (pRYG) was treated with hTopo-IIα, a set of variably relaxed topoisomers was formed, which migrate more slowly as compared to supercoiled form in agarose gel. The results of relaxation assay revealed similar trends as observed in kDNA decatenation assay (Fig. 3A). Quantification of relaxed topoisomers was done by analyzing the densitometry data using Quantity One (BioRad) and the results were compared with etoposide (Fig. 3B). It was observed that compounds (**11b**, **12a**, **12b**, **12d**, **12e**, **13a** and **13b**) were found to be more potent than the standard etoposide as the degree of relaxation of the substrate pRYG plasmid DNA was less in the presence of compounds under

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investigation (Fig. 3B). The obtained results indicate that investigational compounds are more potent hTopo-IIα inhibitors as compared to the etoposide and the results obtained in relaxation assay were found to be consistent with kDNA decatenation assay.

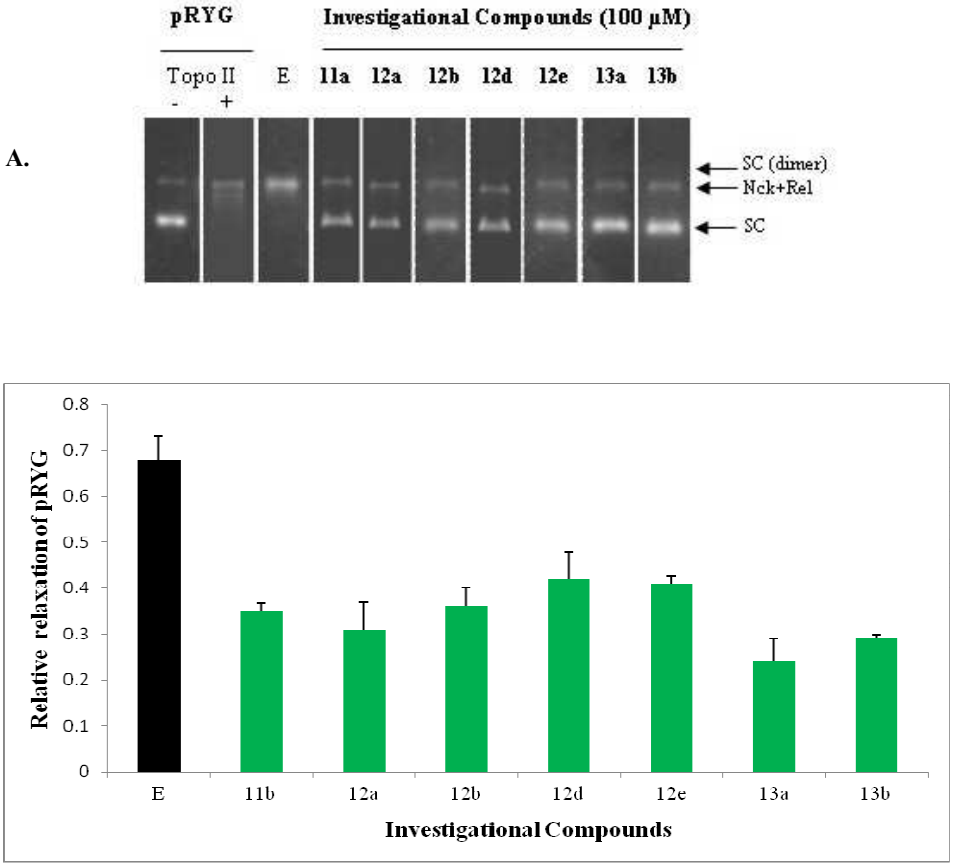

Fig. 3 (A) Effect of test compounds on Topo IIα mediated supercoiled pRYG plasmid DNA relaxation assay. Negatively supercoiled pRYG plasmid DNA was treated with hTopoIIα in the presence of either 100 µM etoposide or test compounds. Electrophoresis was carried out in 1% agarose gel in TAE buffer without ethidium bromide. **(B)** Graphical representation of relative relaxation in pRYG DNA by investigational compounds **11b**, **12a**, **12b**, **12d**, **12e**, **13a** and **13b**

DNA intercalation assay

To gain deeper insight, whether active investigational compounds are DNA intercalator or nonintercalators, DNA intercalation assay was performed with active compounds **11b**, **12a**, **12b**, **12d**,**12e**, **13a** and **13b**. In this assay negatively supercoiled small circular plasmid DNA, isolated from *E. coli* was used as a substrate.[27, 28] From the results obtained, it was found that

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in the presence of ethidium bromide (DNA intercalating agent), there was retardation in the migration of the DNA, whereas, in the case of etoposide (DNA nonintercalator) and our tested compounds, there was no such retardation, which indicates that the investigational compounds are DNA nonintercalators (Fig. 4).

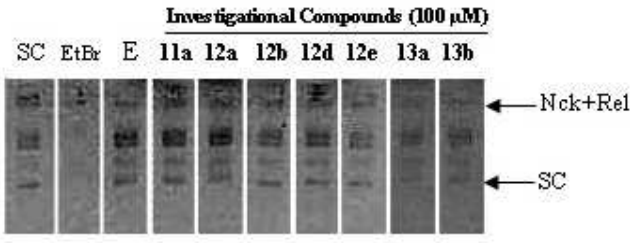

Fig. 4. DNA intercalation assay. Negatively supercoiled plasmid was incubated with 1 μg/mL ethidium bromide or 100 μM etoposide or test compounds. Electrophoresis was carried out in 1% agarose gel in TAE buffer without ethidium bromide.

Anticancer activity of investigational compounds: Cell survival assay

The cytotoxic activity of the potent investigational compounds (**11b**, **12a**, **12b**, **12d**, **12e**, **13a** and **13b**) was investigated in Prostate (PC-3) cells by MTT assay and the results are shown in Fig. 5. Cell survival was measured after treating cells with the investigational compounds for 48 h. It was observed that with an increasing concentration of the compounds, the cell viability of PC-3 cells decreased. Among the compounds tested for cytotoxic studies, compound, **13a** was found to be most active as it killed 50% of PC-3 cell at concentration of 0.5 μM, which was less than the standard drug etoposide (LC_{50}- 7.5 μM). The compounds **13b**, **12e** and **11b** were also found to possess significant cytotoxic potential as they have lower range of LC_{50} i.e. 2.1, 2.4 and 5.7 μM, respectively, as compared to the positive control, etoposide. While the other three investigational compounds **12a**, **12b** and **12d** were found to possess cytotoxic activity (LC_{50}: 8.2, 8.3 and 8.6 μM) comparable to that of etoposide.

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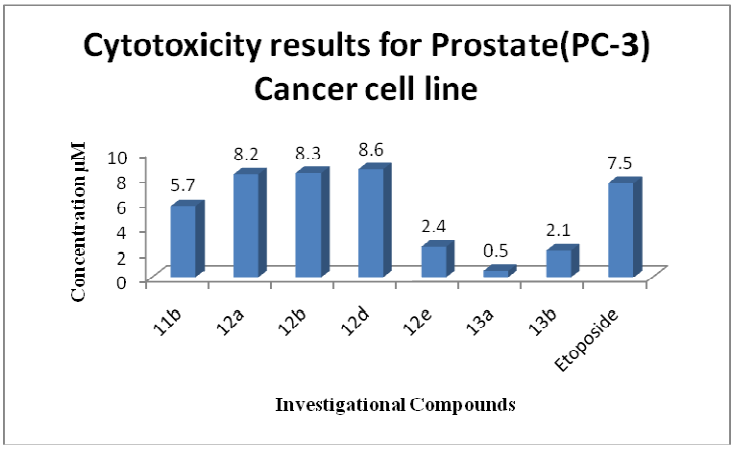

Fig. 5 MTT cell survival assay (LC_{50})

Further the IC_{50} value for the most active investigational compound (13a) (selected on the basis of results of biological assays) was determined by performing kDNA decatenation assay using concentration range from 20 µM to 100 µM and IC_{50} value for compound 13a was found to be 67.86 µM. (fig.6 A and B)

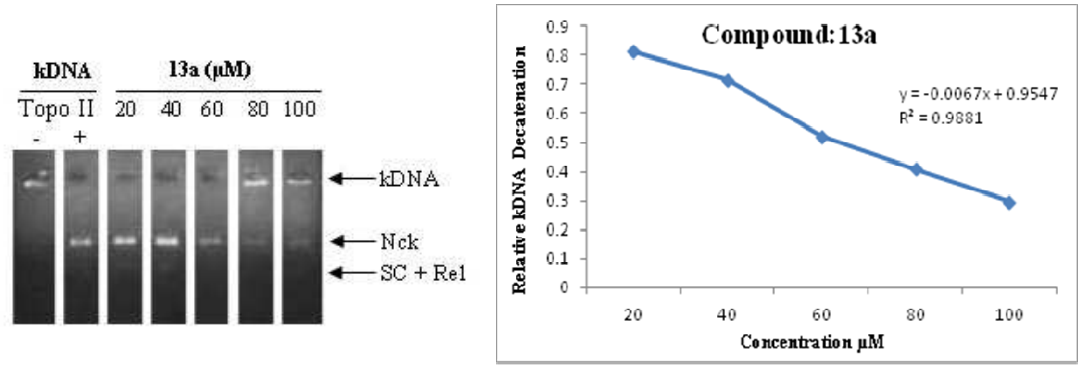

Figure.6: (A) Effect of test compound 13a on Topo IIα mediated kDNA decatenation assay. kDNA was treated with hTopoIIα in the presence of various concentrations of compounds 13a (20 µM to 100 µM). Electrophoresis was carried out in 1% agarose gel in TAE buffer **(B)** Graphical plot of decatenated products formed in kDNA decatenation assay.

Molecular modeling studies

Nowadays, molecular modeling studies are routinely used to understand the atomistic level details of protein-ligand interactions, and to identify potential inhibitors against disease target

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of interest. But accurate and reliable results can only be obtained if the ligands are considered in their most stable conformation. In present study, the basic nucleus 3-formylchromone of the synthesized compounds can exist in two forms due to the rotation of 3-formyl group assigned as 0^o and 180^o. Thus, in order to find the most stable conformation of 3-formylchromone derivatives (**11a-e**, **12a-e** and **13a-e**), the rotamers of the representative structures (**11a**, **12a** and **13a**) were considered and subjected to quantum chemical calculations. After full optimization of these species in gas phase using density functional theory (DFT) method at B3LYP/6-31+G(d,p) level, the conformations with about 180^o rotation (C-C-C-O) in 3-formyl group were named as **11α**, **12α** and **13α** and those with about 0^o rotation as **11β**, **12β** and **13β** (Fig. 7). The relative energy differences between each respective rotamer are shown in Table 1. From the table it is clear that the conformations **11α -13α** are more stable than their respective rotamers **11-13 β**. The higher stability of all the alpha conformations is attributed to the fact that intramolecular hydrogen bonding can be formed between 3-formyl-H and 4-carbonyl groups, while this type of interaction is absent in beta conformations. Moreover in beta conformations, the electrostatic repulsion between the lone pairs of oxygens in 3-formyl and 4-carbonyl groups can also makes them less stable as compared to alpha conformations. The information obtained from the quantum chemical analysis was further utilized to build the representative 3D stable structure of the compounds for molecular docking study.

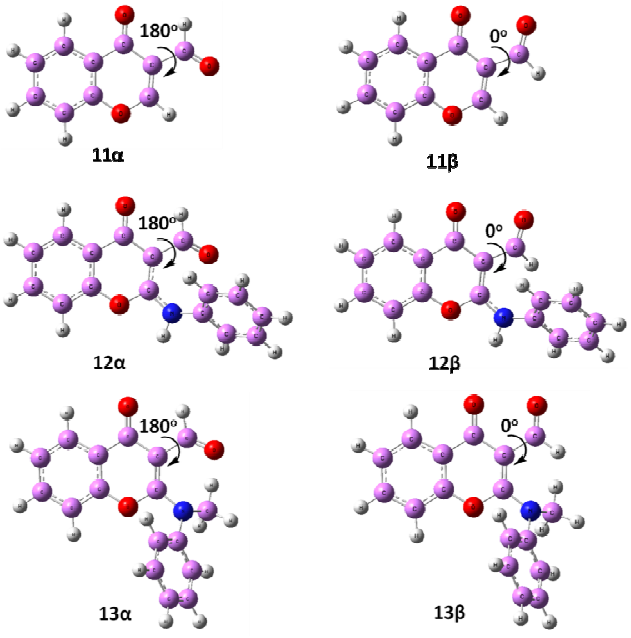

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Fig. 7 Fully optimized rotamers of 3-formylchromone derivatives in gas phase at B3LYP/6-31+G(d,p) level. Color representation of atoms is as follows: carbon-magenta, oxygen-red, nitrogen-blue, hydrogen-white.

Table 1 Relative energy difference between 3-formylchromones rotamers.

Name	B3LYP/6-31+G(d,p) Relative energy (kcal/mol)
11α	0.00
11β	5.06
12α	0.00
12β	5.17
13α	0.00
13β	7.39

From the decatenation, relaxation and DNA intercalation assays performed on synthesized 3-formylchromone derivatives, it was established that compounds **11b**, **12a**, **12b**, **12d**, **12e**, **13a** and **13b** are nonintercalating agents and possess potent hTopo-IIα inhibitory activity. In addition, these compounds also possess good cytotoxcity (LC_{50} ranges from 0.5-8.6 μM) activity against PC-3 cancerous cell line. Further the previous computational study established that several designed molecules bind to the ATPase domain of hTopo-IIα and the possible interactions between ligands and enzyme are already made available.[14] Thus, to obtain insight into the molecular mechanism of action of 3-formylchromone derivatives, molecular docking of the synthesized compounds was carried out in the ATPase domain of hTopo-IIα enzyme (PDB ID: 1ZXM). [29]

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Molecular docking studies were performed using FlexX program[30], which uses incremental construction algorithm. The docking protocol was already established as a reliable one in previous report[14], which revealed the importance of interaction with Mg^{2+} ion in the active site cavity. The detailed intermolecular non-bonding interactions (hydrogen bonding, hydrophobic and metal ion interactions) and FlexX docking score of substrate ATP and active compounds with the ATPase domain of hTopo-IIα is provided in Table 2.

Table 2. FlexX docking results for some of the synthesized compounds and substrate ATP in presence of two conserved water molecules in hTopo-IIα ATP binding site.

Compound	[a]LC_{50} (μM)	FlexX Score	Hydrogen bonding Interactions		Hydrophobic interactions	Interaction with Mg cation
			Backbone	Side chain		
ATP ***(Substrate)***		-84.06	Ser149, Arg162, Asn163, Gly164, Tyr165, Gly166, Ala167	Asn91, Asn120, Ser148, Ser149, Asn150, Lys168, Gln376, Lys378	Ile125, Ile141, Phe142, Ile217	Ionic interaction
11b	5.7	-29.10	Gly164, Tyr165, Gly166, Ala167	Ser148, Asn150, Gln376	Ile141	π interaction
12a	8.2	-27.78	Ser149	Ser148 (2), Asn150	Ile141, Phe142	π interaction
12d	8.6	-25.38	Ser149, Gly164, Tyr165, Gly166	Ser148, Asn150, Gln376, Lys378	-	π interaction
12e	2.5	-23.42	Ser149	Asn91, Ser148 (2), Asn150 (2)	Ile141, Phe142	π interaction
13a	0.5	-23.09	Ser149	Ser148, Asn150	Ile141, Phe142, Ala167	π interaction
12b	8.3	-22.00	Ser149	Ser148 (2), Asn150 (3)	Ile141, Phe142	π interaction
13b	2.1	-18.77	Ser149	Ser148 (2), Asn150 (3)	Ile141, Phe142, Ala167	π interaction

[a]The values were calculated by noting down the concentration of the compound at which 50% of the PC-3 cancerous cell gets killed (see cell survival assay).

Binding pose analysis of the active 3-formylchromone derivatives **11b**, **12a**, **12b**, **12d**, **12e**, **13a** and **13b** showed that they can fit well in the active site of ATPase domain and exhibit good docking score (Table 2). Interestingly, it was found that the best docked conformation (highest FlexX score among 30 conformations) of each of the active 3-

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formylchromones can exist in the binding pocket in a less stable structural representation (**11β**, **12β** and **13β**). This shows that during the process of molecular recognition, the compounds exhibit induced fit effect to maximize the interactions with the active site residues of protein and as a result, the energy difference between the stable (**α**) and less stable (**β)** structures can be easily overcome.

All the compounds showed key interactions with the amino acid residues of ATP binding pocket of hTopo-IIα enzyme (Table 2) as observed in the docked pose of ATP. Some of the compounds (**11b** and **12d**) formed one or more hydrogen bonding interactions with the P-loop, the so called Walker A consensus sequence (Gly161, Arg162, Asn163, Gly164, Tyr165, Gly166, and Ala167) [31] of ATPase domain. Site-directed mutagenesis studies have established the importance of these residues in the binding of substrate and catalytic inhibitors.[32-34] Binding model of the compounds suggested that chromone ring of these ligands can effectively make π interaction with the Mg^{2+} ion, which is a requisite feature to be an hTopo-IIα ATPase inhibitor.[13,25] Moreover, recent review article by Mahadevi and Sastry also showed that cation-π interactions play significant role in the process of molecular recognition.[35]

The most active compound **13a** (LC_{50} = 0.5 μM) displayed a FlexX docking score of -23.09 (Table 2) and it showed several hydrogen bonding interactions with the active site residues of ATP binding pocket. Binding model of compound **13a** is depicted in Fig. 8A. The 3-formyl group of chromone ring showed hydrogen bonding with the main chain -NH of Ser149, and 4-oxo with the side chains of Ser148 and Asn150. The 2-[methyl(phenyl)amino]-group of compound **13a** was further stabilized by hydrophobic interactions with the side chains of Ile141, Phe142 and Ala167. In addition, the chromone nucleus of compound **13a** could also form NH· · ·π interactions with the side chain of Lys168 and Gln376.

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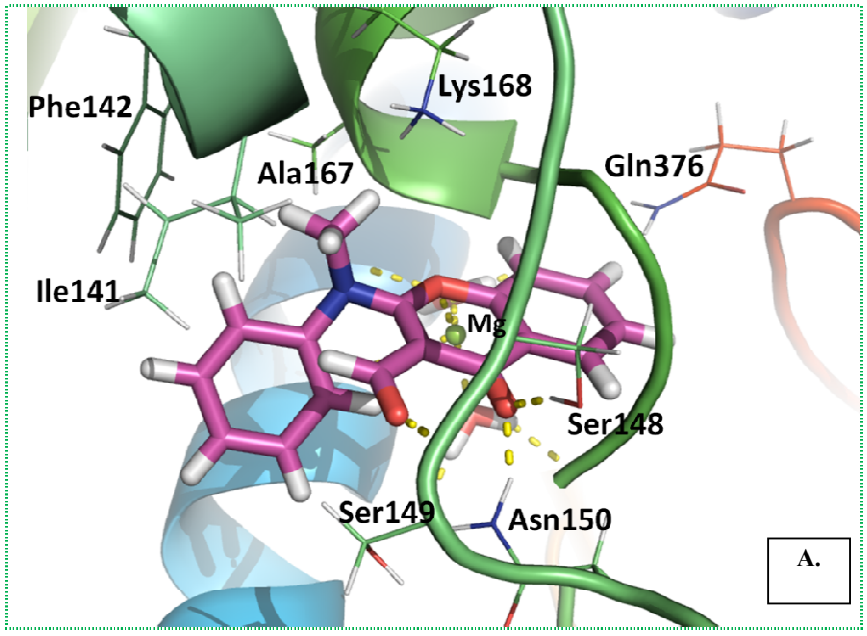
The FlexX docking score for the best docked compound **11b** (LC_{50} = 5.7 μM) was found to be -29.10. Its binding model (Fig. 8B) revealed that 3-formyl group can form intermolecular hydrogen bonding interactions with the main chain -NH of Walker A consensus sequence viz. Gly164, Tyr165, Gly166 and Ala167. Moreover, the 3-formyl group also showed hydrogen bonding with the side chain $-NH_2$ of Gln376, which is one of the amino acid residues of the QTK-loop (Gln376-Thr377-Lys378), a transducer domain. The 4-oxo group of chromone ring showed additional hydrogen bonding interactions with the side chains of Ser148 and Asn150. The chromone nucleus could also be involved in hydrophobic interaction with the Ile141 and (NH· · ·π) interaction with side chain of Lys168.

Thus, molecular docking studies performed on some of the 3-formylchromone derivatives suggested that they could act as catalytic inhibitors possibly *via* occupying the ATPase domain of hTopo-IIα enzyme.



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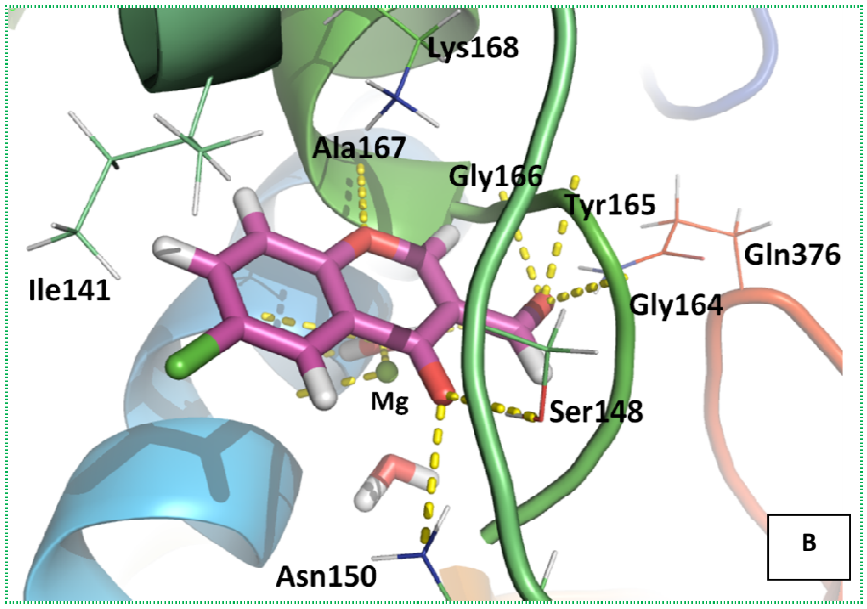

Fig. 8 Binding mode of 3-formylchromones derivatives into the active site of hTopo-IIα ATPase domain. **(A)** Most active compound **13a** and **(B)** best docked **c**ompound **11b** are displayed in magenta color with stick representation (carbon atom-magenta, oxygen atom-red, chlorine atom-green and hydrogen atom-white), magnesium ion in green sphere, two water molecules in stick representation and hydrogen bonding interactions in yellow dashed lines. Enzyme is represented in cartoon structure with side chains displayed as lines.

Conclusion

The differently substituted 3-formylchromones were synthesized and evaluated for their inhibitory action on hTopo-IIα enzyme. The results of the decatenation and relaxation assays showed that the compounds under investigations possess significant hTopo-IIα inhibitory activity. The DNA intercalation assay established that these compounds are DNA non-intercalators, thus more specific for binding to the hTopo-IIα. Screening of the compounds for their cytotoxic nature revealed that some of the compounds being more cytotoxic than the positive control etoposide (LC_{50}- 7.5 μM) and the most active compound **13a** exhibits LC_{50} value of 0.5 μM against PC-3 cancerous cell line. Molecular docking studies have revealed that these compounds act as catalytic inhibitor of hTopo-IIα and block the ATPase domain of

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the enzyme. From this comprehensive study, it can be concluded that these compounds shall serves as useful 'Leads' for further design and development of potent topoisomerase II catalytic inhibitors, useful for combating deadly disease cancer.

Experiments

Synthesis - General

General procedure for the synthesis of 3-formylchromones (11a-e)

Variously substituted phenols were treated with acetylcholride (1.1 molar equivalents); the contents were heated for one hour. The reaction mixture was further subjected to thermal Fries rearrangement using anhydrous $AlCl_3$ (2 eq). The contents were heated for 6-7 h, and subsequently were fused with dilute HCl; reaction mixture was partitioned between ethyl acetate and water. Extracted ethyl acetate was dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under reduced pressure to obtain substituted *o*-hydroxyacetophenones. To a cooled stirred solution of various substituted *o*-hydroxyacetophenones and *N*, *N*-dimethyl-formamide was added phosphorus oxychloride drop-wise with constant stirring and the reactants were allowed to stand overnight, and then treated with ice cold water to obtain yellow crystalline solid, which was filtered and washed with diethyl ether. The crude solid was recrystallized from acetone to get variously substituted 3-formylchromones.

General procedure for the conversion of chromones to 2-anilino-3-formylchromones (12a-e)

To a clear solution of chromones (10 g) in dry benzene (150 mL) was added *N*-phenylhydroxylamine (1 molar equivalent) dissolved in dry benzene (20 mL), leading to the formation of nitrone. The obtained nitrone was further subjected to refluxing under anhydrous conditions in presence of few drops of acetic acid for 5-6 h. After the conversion,

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solvent was removed under reduced pressure and 2-anilino-3-formylchrome was further recrystallized from chloroform:hexane (1:2)

General procedure for the conversion of 2-anilino-3-formylchromes to 2-(N-methylanilino)-3-formylchromones (13a-e)

2-Anilino-3-formylchromes (**12a-e**, 5.0 g) were dissolved in dry acetone (150 mL) and to the solution was added K_2CO_3 (excess) and methyl iodide (2.0 molar equivalents). The contents were refluxed with stirring under anhydrous condition and the progress of reaction was monitored by TLC. After the completion of reaction (6-7 h), contents were filtered hot and residue was washed with acetone, and, solvent was removed under reduced pressure, 2-(*N*-methylanilino)-3-formylchromone were recrystallized from chloroform:hexane (1:2).

Biological assay- General

All the reagents required for the testing of new chemical entities were purchased from TopoGEN, Inc. (Columbus, OH). The testing of the compounds was performed using a commercially available Topo-II drug screening kit. All the synthesized compounds and etoposide were dissolved in DMSO at a concentration of 1 mM as a stocked solution and stored at -20 °C. Topo I and II inhibition assay were performed as described in the supplier manual with minor modifications as per the requirement.

hTopo IIα mediated DNA decatenation assay

Topo IIα mediated DNA inhibition activity for the synthesized compounds were performed as follows. Reaction mixture containing freshly prepared 5x complete reaction assay buffer (buffer A: 0.5 M Tris-HCl (pH-8), 1.50 M sodium chloride, 100 mM magnesium chloride, 5 mM dithiothreitol, 300 μg of bovine serum albumin/mL; buffer B: 20 mM ATP in water), 150 ng catenated kDNA (substrate), 100 μM drug or test compound dissolved in DMSO followed by 2-4 units of purified hTopo-IIα were incubated at 37 °C for 30 min. The reaction

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was then terminated with addition of 10% SDS, followed by digestion with proteinase K. Further the reaction mixture was again incubated at 37 °C for 15 min. 20 μL of each sample were then subjected to 1% agarose gel electrophoresis in Tris-acetate- EDTA (TAE) buffer containing 0.5 μg/mL ethidium bromide and further destained with water for 20 min. The bands were analyzed under UV trans-illuminator and the decatenated kDNA products were quantified with QuantityOne (BioRad).

hTopo IIα mediated DNA relaxation assay

Supercoiled plasmid DNA (pRYG) was used as substrate in Topo II mediated DNA relaxation assay. Reaction mixture contained freshly prepared 5x complete buffer (A: 0.5MTris- HCl (pH-8), 1.50 M sodium chloride, 100 mM magnesium chloride, 5 mM dithiothreitol, 300 μg of bovine serum albumin/mL; buffer B: 20 mM ATP in water), 250 ng of supercoiled plasmid DNA (pRYG), followed by either test compound or standard drug (100 μM) and finally hTopo-IIα (2-4 units) in a total of 20 μL. Reaction mixture was then incubated at 37 °C for 30 min and stopped with addition of 10% SDS followed by digestion with proteinase K. Further the reaction mixture was again incubated at 37 °C for 15 min. Electrophoresis of the each sample was carried out in a 1% agarose gel in TAE buffer without ethidium bromide. Gel was stained with ethidium bromide (0.5 μg/mL) for 15 min and destained with water for 20 min. The bands were analyzed under UV trans-illuminator and quantification of the products was carried out as mentioned in decatenation assay.

DNA Intercalation Assay

In this assay, negatively supercoiled plasmid isolated from *E. coli* was used as a substrate. 250 ng plasmid was incubated with 1 μg/mL ethidium bromide, 100 μM standard drug (etoposide), or with the investigational compound at 37 °C for 20 min. Samples were then loaded in 1% agarose gel and electrophoresis was carried out in TAE buffer. Further the gel

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was stained with ethidium bromide (0.5 μg/mL) for 15 min and destained with water for 20 min and photographed using BioRad.

MTT cell survival assay

Cell lines and cell culture

PC-3 (human prostate adenocarcinoma cells) was obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in DMEM, containing 10% FBS and 1% penicillin-streptomycin. DMEM, FBS and Etoposide were obtained from Sigma Chemicals and Invitrogen. Cell cultures were maintained in flasks under standard conditions: incubation at 37 ^{o}C and 5% CO_2. All the subcultures were used prior to passage 15. Cells were routinely sub-cultured using 0.25% trypsin. For treatment, cells were cultured in the presence of increasing concentrations (1μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM) of compounds for 48 hours.

In vitro cytotoxicity measurements

All *in-vitro* experiments for cell proliferation/inhibition were performed in triplicates. For the PC-3 cell growth inhibition assay, 2.0 X 10^4 cells/well were plated in 96-well microtiter plate. After 24 hours cells were incubated in the presence and absence of increasing concentration of positive control etoposide or the test compounds (1μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM) at 37 ^{o}C and 5% CO_2. Cell proliferation was determined by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method (MTT obtained from Hi-Media). After 48 hours incubation, cells were treated with MTT solution for 4 hours in a cell culture incubator at 37 ^{o}C and 5% CO_2. MTT which is a tetrazolium salt, is converted into insoluble formazan by mitochondrial dehydrogenases in live cells. Formazan is dissolved in DMSO (Merk) and absorbance was measured at dual wavelength of 550nm and 630nm on

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ELISA plate spectrophotometer (flex station).The total number (percentage) of viable cells relative to viable cells in untreated control is calculated.

Molecular modeling

Ab initio density functional theory (DFT)[36] based geometry optimization calculations of the 3-formylchrmone rotamers were performed using GAUSSIAN09 software package.[37] The gas phase geometry of all the species were fully optimized using B3LYP (Becke3, Lee, Yang, Parr) methods with 6-31+G(d,p) basis sets without any geometrical constraint. Frequencies were computed analytically for all the optimized species to characterize stationary points as minima and to estimate the zero point vibrational energies (ZPE). The calculated ZPE values (at 298.15K) were scaled by a factor of 0.9806 for the B3LYP levels.[38]

The 3D structure of the compounds (considering stable conformation revealed from quantum chemical analysis) under study were built using SYBYL 7.1 molecular modeling package[39], installed on a Silicon Graphics Fuel Work station running IRIX 6.5. Gasteiger-Hückel partial charges were applied to the atoms of the compound and Powell method with Tripos force field was used for minimization. Finally, a database of ligands was created in SYBYL. For the purpose of docking, crystal structure of the ATPase domain of hTopo-IIα [29] bound to AMPPNP (5`-adenylyl-β,γ-imidodiphosphate, a non-hydrolyzable ATP analog, PDB ID: 1ZXM, resolution 1.86 Å) was utilized. This PDB structure composed of a homodimer, each chain having bound ligand AMPPNP and Mg^{2+} ion forming distorted octahedral coordination (3-point contacts with phosphate groups, one with Asn91 and two with conserved water molecules 927 and 928) in an active site. The enzyme structure with two conserved water molecules in the active site was subjected to protein preparation by implementing the methodology reported earlier.[14]

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Docking simulation was performed using FlexX (version 1.2)[30] programme incorporated in SYBYL 7.1 package. It was established in previous study [14] that score and poses obtained while keeping the two conserved water molecules in coordination with Mg^{2+} ion give more accurate results. Thus, in this work also we have employed the same protocol for molecular docking. For the protocol validation bound ligand AMPPNP and substrate ATP were docked initially, and then analyzed for their binding pose and hydrogen bonding interactions with the key residues. Afterwards, a database of ligands (**11b**, **12a**, **12b**, **12d**, **12e**, **13a** and **13b)** was subjected to docking by using FlexX multiple ligand docking mode. At the end of docking, 30 poses per ligand were generated which were sorted according to their FlexX score, reflecting binding affinity with the enzyme. Top most poses were visually inspected and analyzed for their interactions with Mg^{2+} ion and active site residues.

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Figures and Schemes

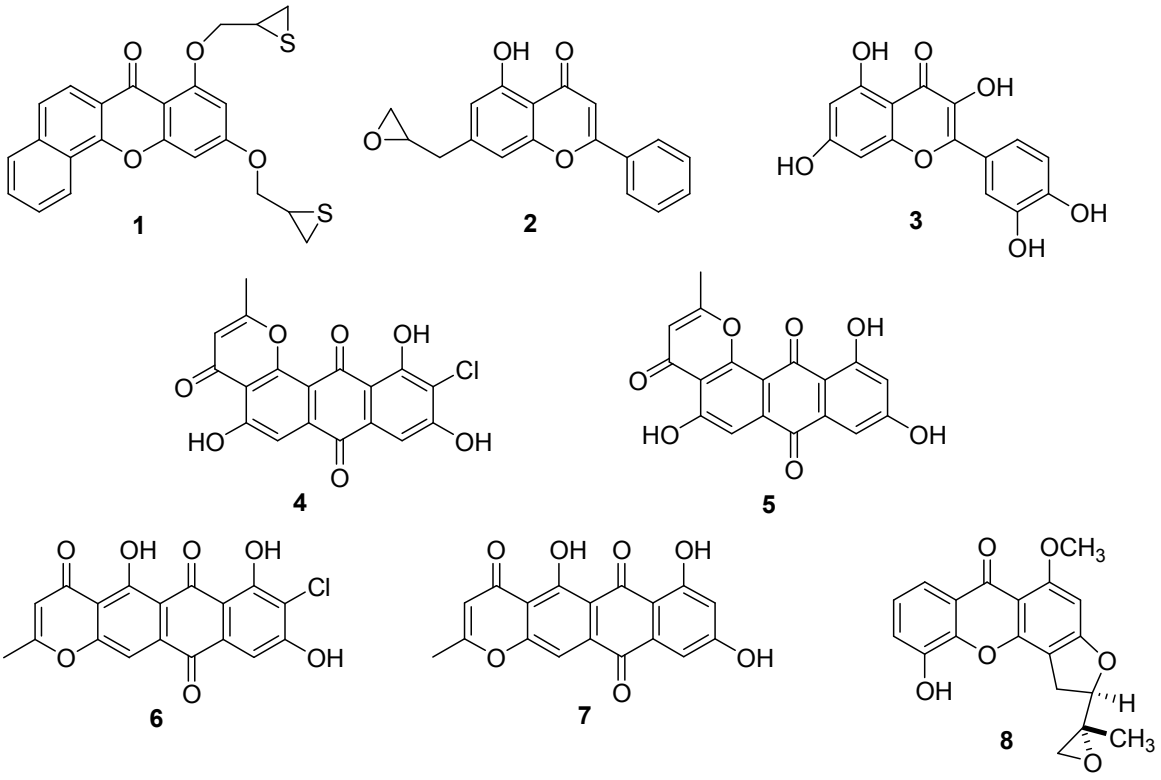

Fig. 1. Chromone based compounds as topoisomerase II inhibitors

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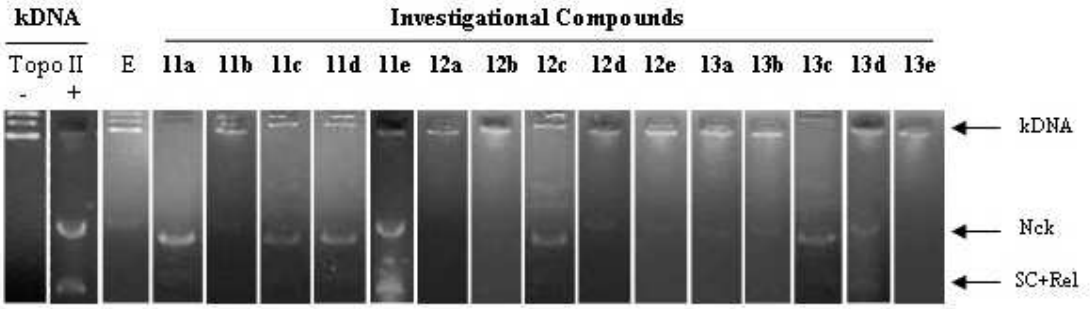
A



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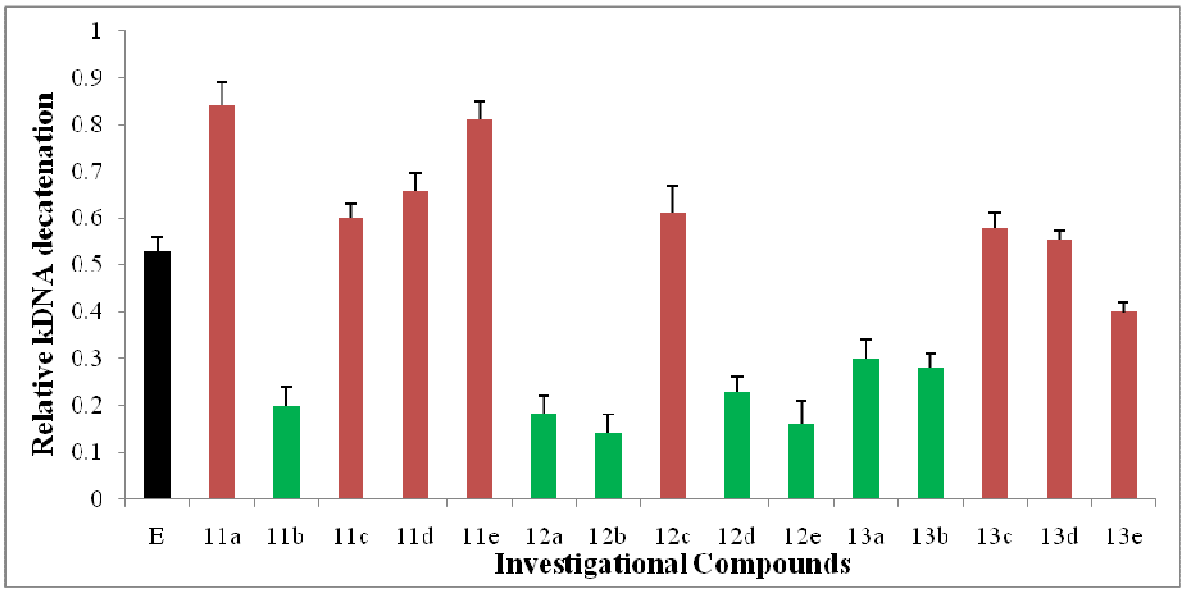

Fig. 2 (A) Effect of test compounds on Topo IIα mediated kDNA decatenation assay. kDNA was treated with hTopoIIα in the presence of either 100 μM etoposide or test compounds. Reaction mixture was incubated at 37 °C for 30 min and 1% agarose gel was run in TAE buffer **(B)** Graphical representation of decatenated products formed in kDNA decatenation assay.

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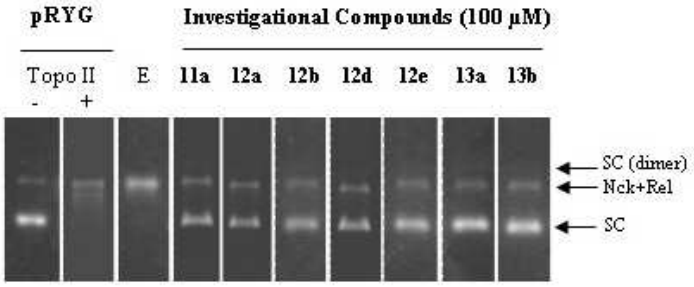

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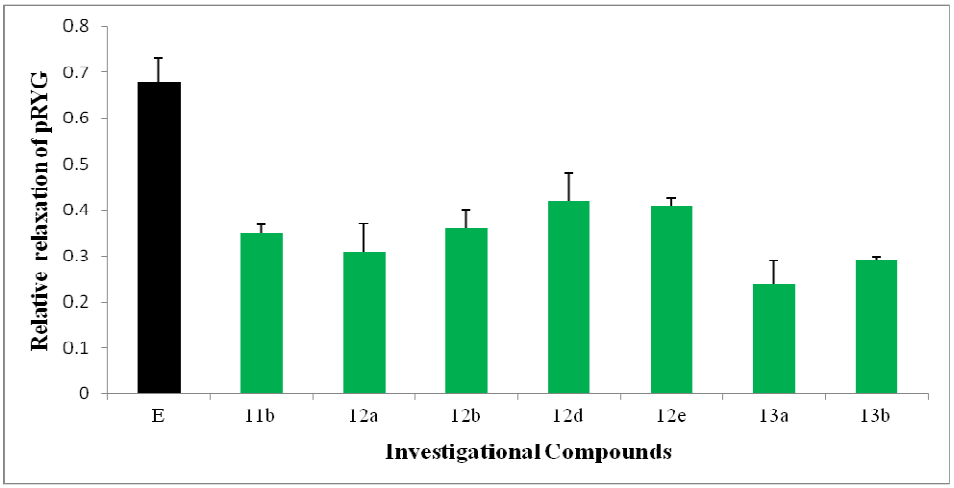

Fig. 3 (A) Effect of test compounds on Topo IIα mediated supercoiled pRYG plasmid DNA relaxation assay. Negatively supercoiled pRYG plasmid DNA was treated with hTopoIIα in the presence of either 100 µM etoposide or test compounds. Electrophoresis was carried out in 1% agarose gel in TAE buffer without ethidium bromide. **(B)** Graphical representation of relative relaxation in pRYG DNA by investigational compounds **11b**, **12a**, **12b**, **12d**, **12e**, **13a** and **13b**

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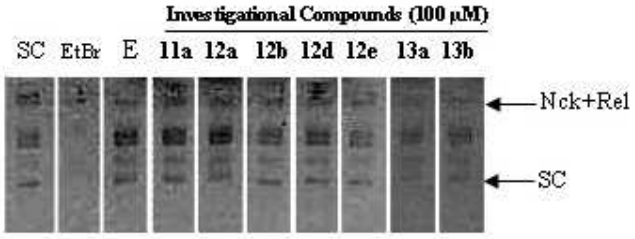

Fig. 4. DNA intercalation assay. Negatively supercoiled plasmid was incubated with 1 μg/mL ethidium bromide or 100 μM etoposide or test compounds. Electrophoresis was carried out in 1% agarose gel in TAE buffer without ethidium bromide.

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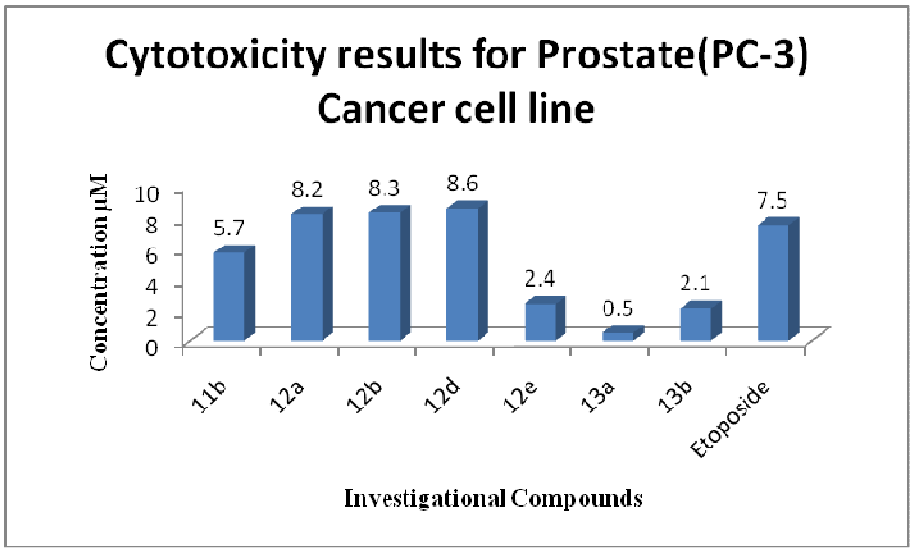

Fig. 5 MTT cell survival assay (LC_{50})

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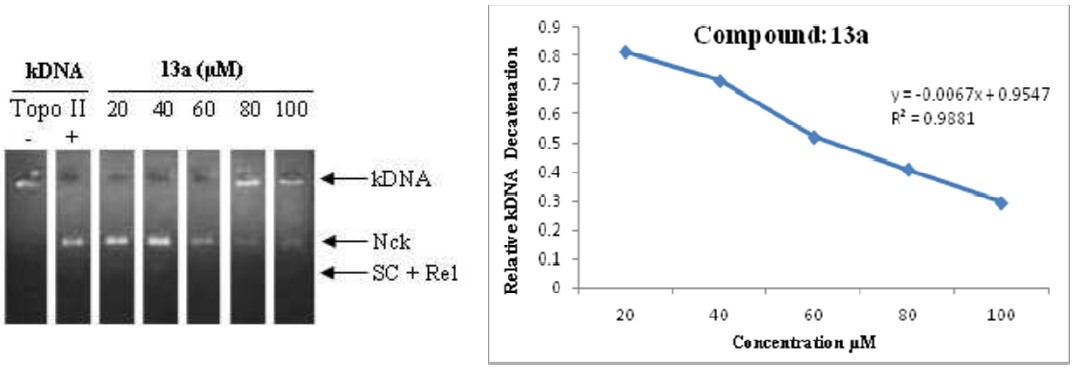

Figure.6: (A) Effect of test compound 13a on Topo IIα mediated kDNA decatenation assay. kDNA was treated with hTopoIIα in the presence of various concentrations of compounds 13a (20 μM to 100 μM). Electrophoresis was carried out in 1% agarose gel in TAE buffer **(B)** Graphical plot of decatenated products formed in kDNA decatenation

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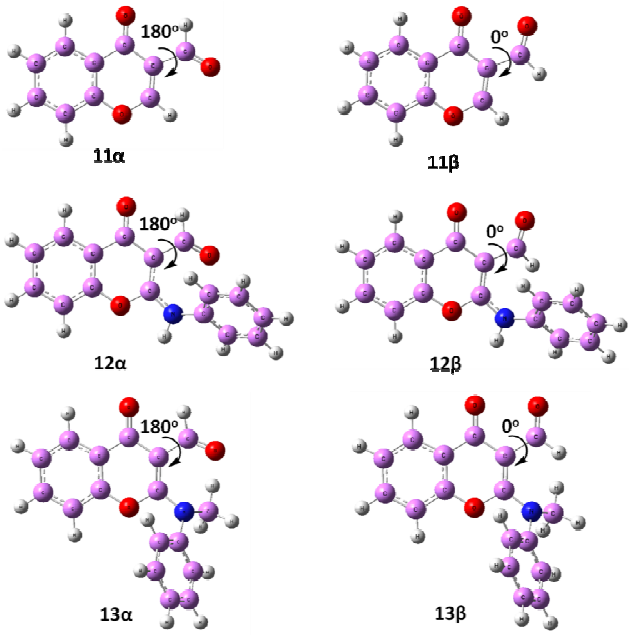

Fig. 7 Fully optimized rotamers of 3-formylchromone derivatives in gas phase at B3LYP/6-31+G(d,p) level. Color representation of atoms is as follows: carbon-magenta, oxygen-red, nitrogen-blue, hydrogen-white.

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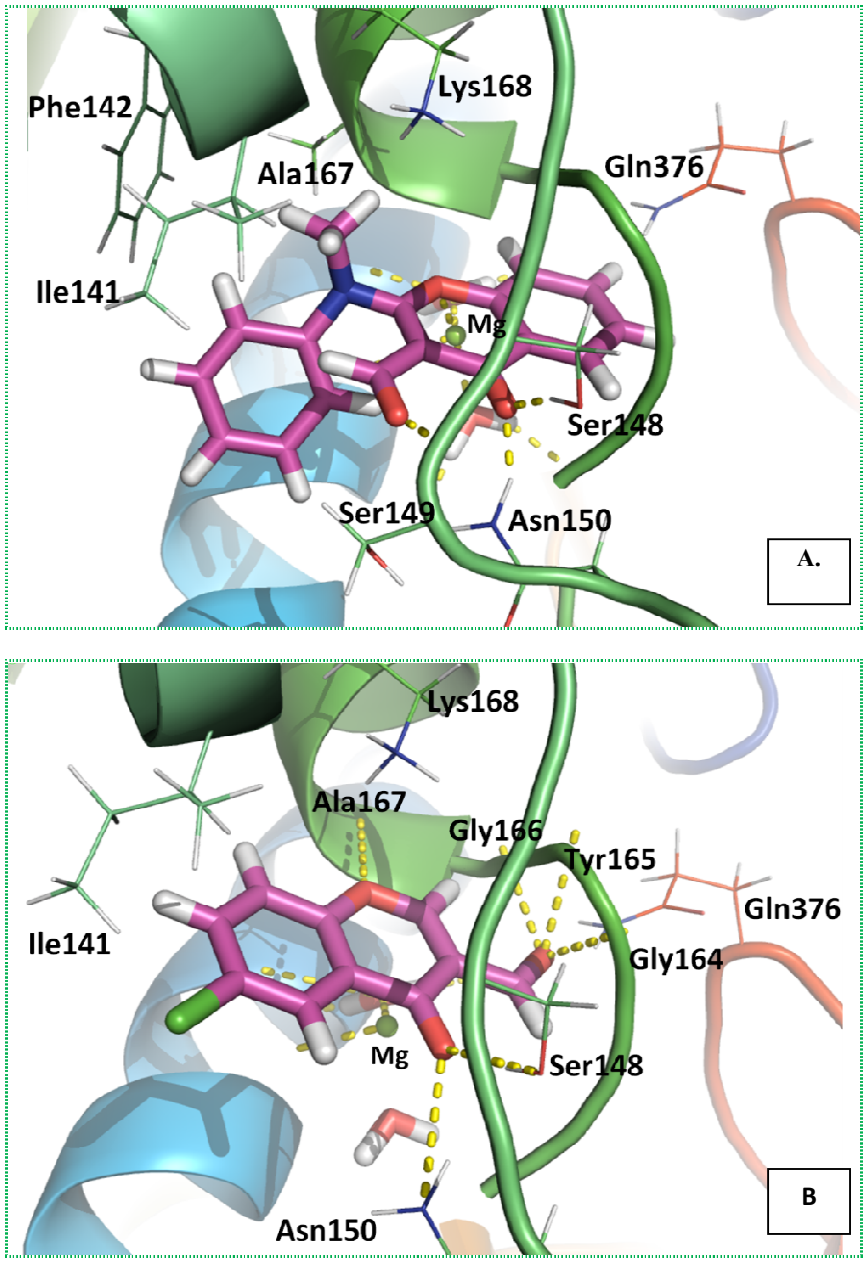

Fig. 8 Binding mode of 3-formylchromones derivatives into the active site of hTopo-IIα ATPase domain. **(A)** Most active compound **13a** and **(B)** best docked **c**ompound **11b** are displayed in magenta color with stick representation (carbon atom-magenta, oxygen atom-red, chlorine atom-green and hydrogen atom-white), magnesium ion in green sphere, two water molecules in stick representation and hydrogen bonding interactions in yellow dashed lines. Enzyme is represented in cartoon structure with side chains displayed as lines.

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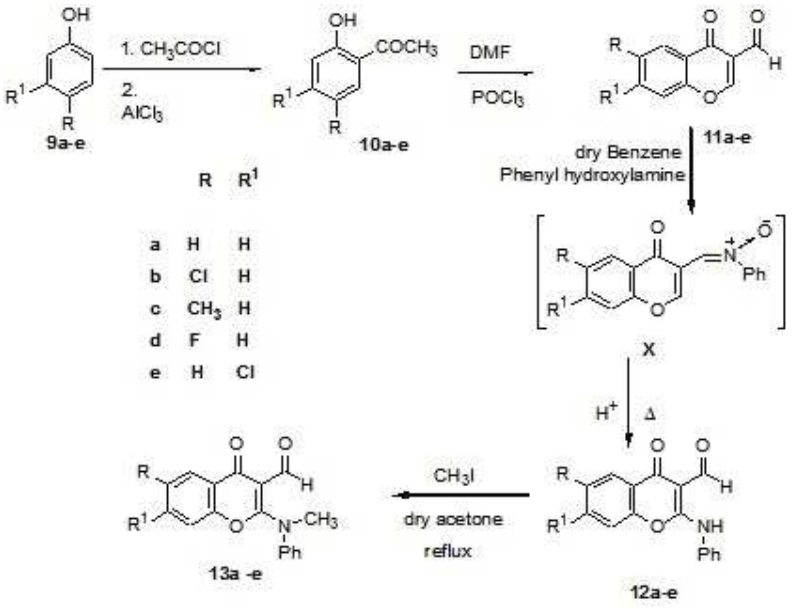

Scheme 1 Synthesis of various 3-formylchromone derivatives