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ARTICLE

Synthesis and biological evaluation of new 2, 5-dimethylthiophene/furan based *N*-acetyl pyrazolines as selective topoisomerase II inhibitors

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Based on the reported pharmacophores as topoiomerase inhibitors, 2,5-dimethylthiophen/furan based N-acetyl pyrazolines were designed and envisaged as topoisomerase inhibitors. The target compounds were synthesized and tested *in vitro* against human topoisomerases in decatenation, relaxation, cleavage complex and DNA intercalation assay. Out of 29 compounds, three (**10**, **11** and **29**) showed potent and selective toposiomerse II inhibitory activity with no intercalation with DNA. Further, molecular docking studies also endorsed them as ATP dependent topoisomerase II catalytic inhibitors. These compounds exerted potential anticancer effects on breast, colon, lung and prostate cancer cell lines at low micromolar level as compared to etoposide and low toxicity to normal cells. Apart from the topoisomerase II inhibition, these compounds also induced the reactive oxygen species (ROS) level in cancer cells. The cell cycle analyses showed their apoptotic effect at G1 phase.

Introduction

Human DNA topoisomerases I and II (hTopoI and hTopoII) are the validated drug targets in oncology due to their involvement in central DNA processing steps which include replication, transcription, translation, and recombination.[1] hTopoI catalyses the temporary breakage of DNA, one strand at a time, and the succeeding re-joining of the strands.[2] Whereas TopoII is a dimeric and ATP – dependant enzyme belonging to the GHKL family.[3, 4] It is involved in many functional roles related to the management of higher-order DNA structure, modulation of topological state, chromosome segregation, and chromatin condensation.[5] It catalyses the transient cleavage of a DNA duplex (G-segment; a transient DNA-enzyme complex is formed) to yield a double-stranded gap through which another duplex (T-segment) is passed. The catalytic process involves the opening and closing of molecular 'gates' in hTopoII structure, which is regulated by ATP binding. The binding of a ligand at the ATPase domain of hTopoII leads to the inhibition of ATP regulated opening and closing of molecular gates and hence suppresses the activity of DNA hTopoII. An ATP makes three point attachments with the Mg^{2+} cation in an octahedral fashion. Further, most of the catalytic hTopoII inhibitors occupy the highly conserved Walker A motif composed of residues Arg162, Asn163, Gly164, Tyr165, Gly166 and Ala 167 of ATPase domain.[6-9] It has been evident that rapidly proliferating tumour cells express the enzyme TopoI/TopoII at 25-300 times higher levels than those of quiescent cells recommending them as druggable targets for new anticancer agents.[10] Topoisomerase I inhibitors comprise of derivatives from plant extract camptothecin. Irinotecan (CPT-11), which is a semi-synthetic derivative of camptothecin, is approved in the United States for the treatment of colorectal cancer. Other TopoI inhibitors includes 9-aminocamptothecin (9-AC), topotecan, etc.,[2] Various compounds such as doxorubicin (anthracycline),[11] etoposide (epipodophyllotoxin),[12] (-) epigallocatechin-3-gallate (catechin,[13-15] and genistein (isoflavone)[16, 17] are called TopoII poisons as they exert potent cytotoxic effect by stabilizing the DNA-enzyme complex.[18] Some synthetic purines and their analogues[19-21] and naturally occurring compounds such as flavones (myricetin),[22] and isolflavone (daidzein) block the enzyme[23-25] before DNA cleavage or in the last steps of catalytic cycle after resealing of DNA break and are called catalytic TopoII inhibitors.[26] Based on our previous experiences in discovery and development of anticancer agents.[27-32] We rationally designed target molecules as topoisomerase

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Electronic Supplementary Information (ESI) available: [NMR spectra of selected compounds are provided.]. See DOI: 10.1039/x0xx00000x

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inhibitors keeping in view of shape and structural features of chalcones,[33, 34] flavones,[22] isoflavones[25] and compound 1[35] (Fig. 1) with the following considerations: (i) possess two aromatic or heteroaromatic rings (A and B) joined by central linker; which could be made rigid by introducing N-acetyl pyrazoline moiety (ii) based on fact that thienyl/furyl group (at ring A or B) with methyl substitution increase the topoisomerase I and II inhibitory mediated anticancer activity, and (iii) are easy to synthesize.

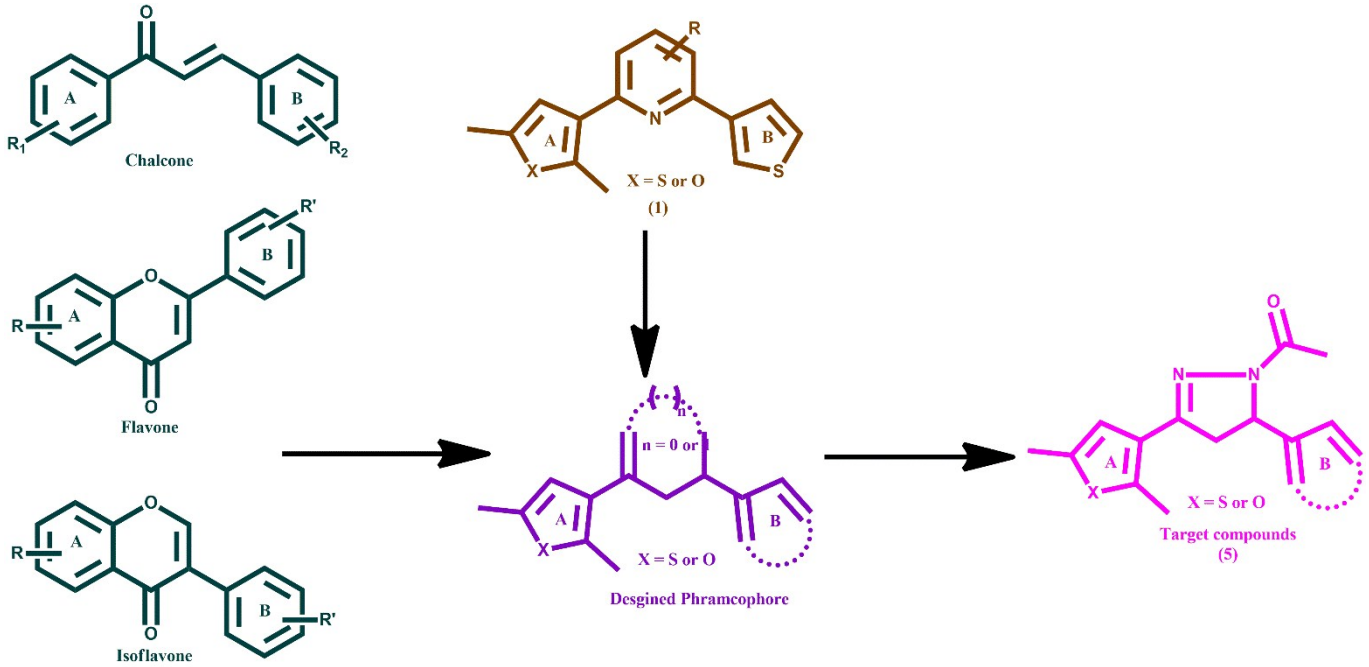


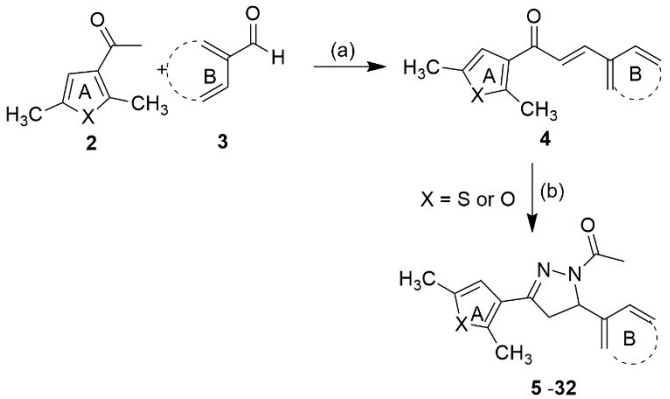
Figure 1 Design of new topoisomerase inhibitors (**5**-**32**).

Further, the limited bioavailability of the anticancer chalcones and flavoids/isoflavonsdue to glucuronidation and sulfation regardless of their greater absorption[36-39]and their Michael acceptor property have impelled the researchers to design the new anticancer agents. The present work involves the synthesis of new 2,5-dimethylthiophen/furan based *N*-acetyl pyrazolines. Their anticancer effects and mechanistic interventions as selective topoisomerases II inhibitors are reported and discussed.

Results and discussion

Synthesis

Synthesis of target compounds (**5**-**32**) (Fig.2) was accomplished by reaction of 1,3-diaryl propenone with hydrazine hydrate and acetic acid. 1,3-diaryl propenones(**4**) (*E*-isomer) were in turn synthesised via Clasien-Schmidt condensation of aryl aldehydes (**3**) with aryl ketones (**2**), in presence of ethanol under basic conditions.[28] Aryl aldehydes containing phenolic hydroxyl groupwere condensed with aryl ketone in the presence of excess base (NaOH) that yeilded sodium salt of 1,3-diarylpropenones and were neutralised with hydrochloric acid. All the final compounds were unreported and were fully characterized by melting point, IR, NMR and elemental analyses.



Scheme 1 Reagents and conditions: (a) 5% NaOH, ethanol, rt, 4-5 h, 65-82% (b) $NH_2NH_2{\cdot}H_2O$, CH_3COOH, reflux, 4-6 h, 75-90%.

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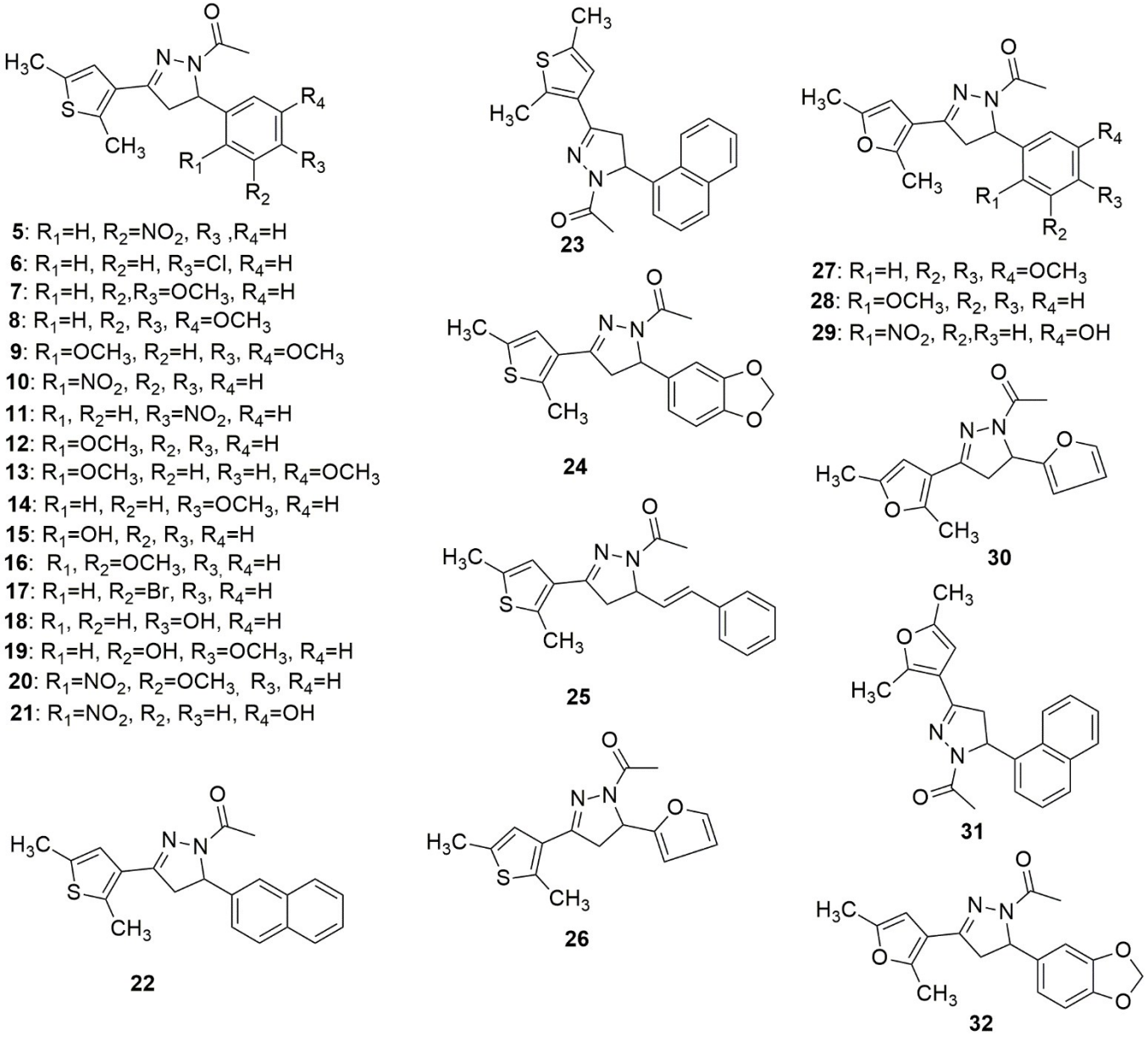

Figure 2 Chemical structures of the synthesized target compounds.

Interestingly reaction of 3-acetyl-2,5-dimethylthiophene either with vanillin or 2,3 – dihydroxybenzaldehyde or 2,3,4 – trihydroxybenzaldehyde yielded a retro product (**33**).

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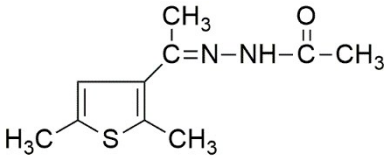

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Biological Studies

Compounds were selective and catalytic TopoIIα inhibitors

To study the topoisomerase inhibitory potential, the investigational compounds (**5**-**33**) were screened for inhibition of hTopoIIα mediated kDNA decatenation.[29, 31] Kinetoplast DNA (kDNA) was used as a substrate with etoposide (TopoII inhibitor) used as standard (Figure 3). Incubation of kDNA with hTopoIIα formed two decatenated products. In agarose gel, catenated kDNA appeared at the top as it cannot enter into the gel due to its overall larger size while other decatenated products moved into. The samples containing etoposide showed moderate decatenation, same as reported in the literature because of its known reversible inhibition of topoisomerase IIα. Although compounds **5, 6, 7, 10, 11, 13, 15, 20, 26** and **29** were found relatively active than etoposide against hTopoIIα, **10**, **11** and **29** being the most potent, were selected for further mechanistic studies. Relatively less decatenation was observed in the presence of active compounds.

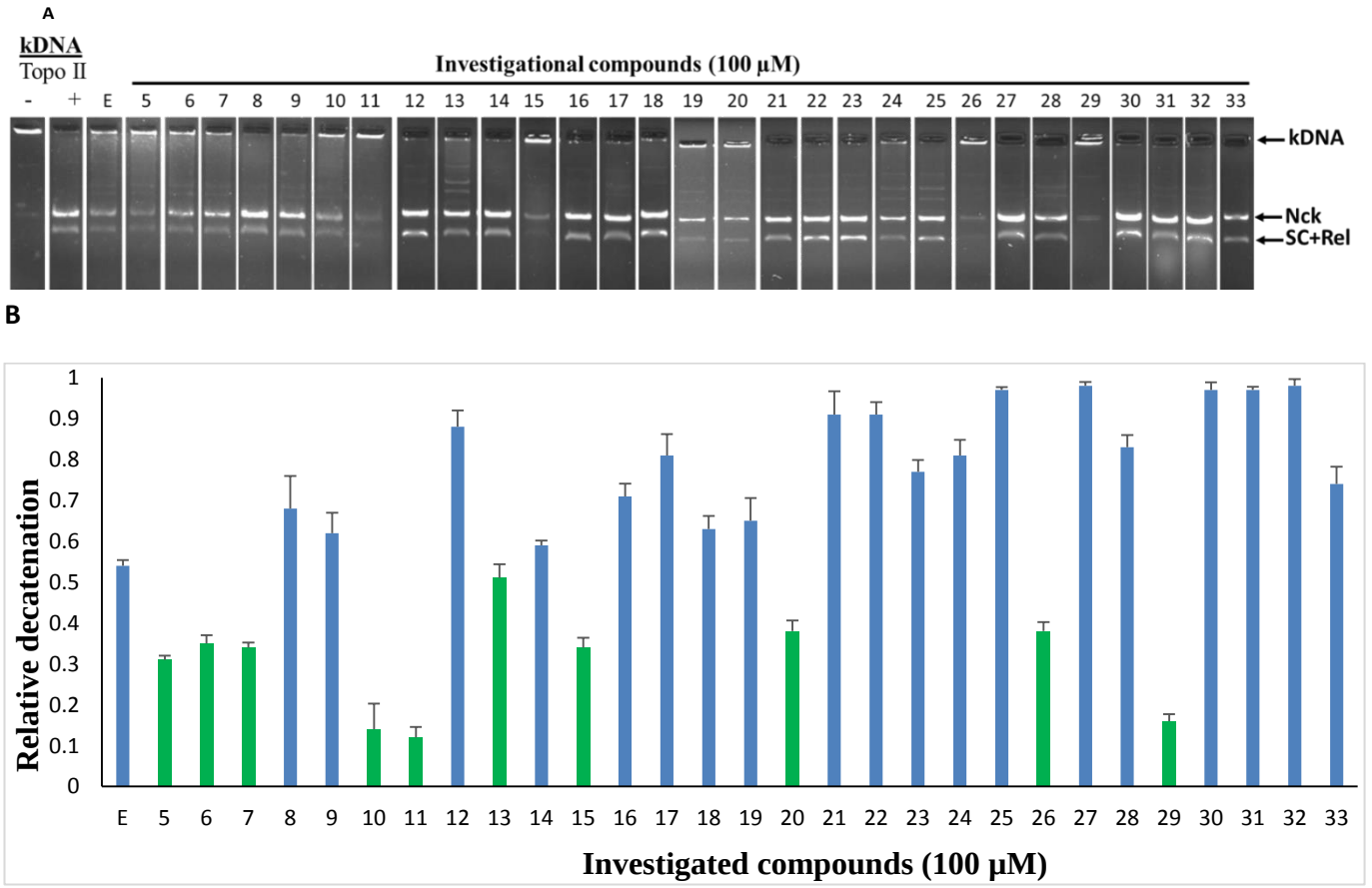

Figure 3 A. Decatenation assay: kDNA (intact kinetoplast DNA) was used as substrate, decatenation products formed were Nck (nicked), Rel (relaxed), and SC (supercoiled) DNA. kDNA was treated with hTopoIIα in presence of either 100 µM etoposide or investigational compounds (**5-33**)B.Quantification of product formed in kDNAdecatenation assay.

The representative compound **11** was screened for inhibition of hTopoI mediated relaxation of supercoiled DNA (SC DNA). In this study negatively supercoiled DNA was used as substrate and camptothecin was used as a reference standard(known TopoI inhibitor). Incubation of SC DNA with human topoisomerase-I resulted in the formation of Nck (nicked), Rel (relaxed), and SC (supercoiled) DNA isoforms. In the presence of compound **11**, no inhibition of hTopoI mediated relaxation of supercoiled DNA was observed as in case of camptothecin (Figure 4).

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This indicates that compounds were selective hTopoIIα inhibitors.

Further, It is known in literature that hTopoIIα poisons like etoposide, stabilizes a topoII-cleaved DNA complex (cleavage complex) which generally appears as linear band (Lin).[40] In the presence of **11** no such linearband was observed (Figure 5) indicating thatinvestigated compounds were catalytic hTopoIIα inhibitors.

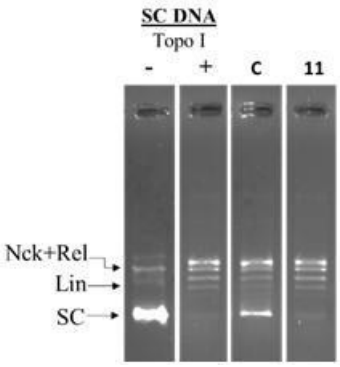

Figure 4 Topoisomerase-I relaxation assay: Negatively supercoiled DNA was treated with hTopo-I in the presence of either 100 µM camptothecin (C) or compound 11.

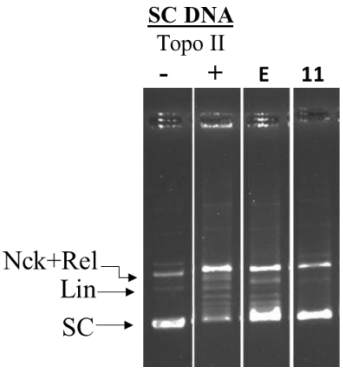

Figure 5 hTopo-IIα DNA cleavage complex assay: Negatively supercoiled DNA (pUC19) was treated with hTopo-IIα then immediately incubated with 100 µM etoposide or investigated compound**11**.

In order to find out the possibility of DNA intercalation by the investigational compounds, DNA intercalation assay was performed. DNA intercalators like ethidium bromide retards the movement of SC DNA in the gel electrophoresis. In the presence of representative compounds **10, 11** and **29** no such retardation of DNA movement was observed (Figure 6).

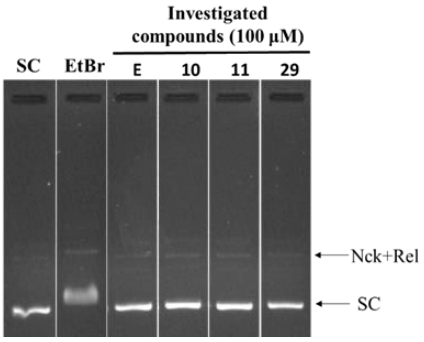

Figure 6.DNA intercalation assay: SC DNA (pUC19) was incubated with 1 µg/ml of ethidium bromide, 100 µM of investigated compounds **10**,**11**and **29**.

Thus the investigational compounds were found to be non DNA intercalating agents.

Structure-activity relationships (SAR)

Some general trends observed with respect to structure–activity relationships emerged from TopoII decatenation assay (Figure 3) with compounds are as follows: (a) Compounds having 2,5-dimethylfuran as ring A were found to be more active against TopoII than corresponding 2,5-dimethylthiophene analogues (compare **8**, **23**, **24** and **26** with **27**, **31**, **32** and **30**, respectively); (b) ring size greater than phenyl on ring B (compounds **22**-**25**, **31** and **32**) was not found to be tolerable for TopoII inhibitory activity; (c) nitro substituted compounds (**5**, **10**, **11**, **20** and **29**) at phenyl on ring B exhibited greater TopoII inhibitory activity (except **21**) than methoxy substituted compounds (**7**-**9**, **12**-**14** and **16**-**19**) irrespective of their position(s).

Molecular Docking Study

As the compounds **10, 11** and **29** emerged to be effective inhibitors of TopoII, the compounds were docked into the active site to identify their interactions with ATPase domain of hTopoII (PDB entry: 1ZXM).[6] The favorable interactions between the ligand molecules and receptor were scored using Glide (GLIDE 6.1 module of Schrödinger Suite).Validation of the docking protocol involved re-docking the phosphoraminophosphonic acid-adenylate ester (ANP), an ATP analog, into the crystal structure of ATPase domain of hTopoII. The validation results indicated a good agreement between the positioning of ligand on docking and the already bound ligand. The docked ANP displayed a binding pose similar to the co-crystallized ligand with a root mean square deviation of 0.024 Å. The binding model of ANP with ATPase domain displayed that the amino group of adenine moiety was involved in the hydrogen bonding with the side chain carbonyl oxygen of Asn120. Also, the hydroxyl group of ribose unit exhibited hydrogen bond interaction with the side of Ser149. Remarkably, the oxygen atoms in the three phosphate groups showed hydrogen bonding with the backbone residues; Asn163, Tyr165, Gly166, Ala167 and with the side chains

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residues; Glu87, Asn91, Ser148, Lys168, and Gln376. Furthermore, the superimposition of the co-crystallized ligand with docked ANP revealed that the two occupy the ATP binding site in a similar manner. ANP made three point attachments with the Mg^{2+} cation, hence forming an octahedral geometry. The highly conserved Walker A motif composed of residues Arg162, Asn163, Gly164, Tyr165, Gly166 and Ala 167 is essential for binding of catalytic inhibitors that act on ATPase domain.[31] Further, the docking studies revealed that the *R* configuration of compounds **10**, **11** and **29** showed better glide score than the ones with *S* configuration. Compound **29*R*** showed the best docking score of -6.033. The superimposition of the co-crystallized ANP with **29*R*** revealed that the nitrophenol ring overlapped with the phosphate groups and the dimethyl furan ring of ANP overlapped with the adenine moiety (Figure 7A). The interaction model of the top scoring compound, **29*R*** at ATPase binding domain revealed that the oxygen atom of the nitro group was involved in hydrogen bonding with the backbone amino group of Ala167 (d=1.8Å) and the side chain ammonium ion of Lys168 (d=2.3Å). Moreover, the hydroxyl group of the nitrophenol ring showed hydrogen bond interaction with the side chain amino acid residue Ser149 (d=2.3Å). A salt bridge was formed between the magnesium ion (d=2.0Å) and the oxygen. The compound could show hydrophobic interactions with Tyr34, Ile141, Ile217, Phe142 and Ile125 and polar interactions with Thr147, Asn150, Asn163, Ser148, Asn91, Asn120 and Asn95 (Figure 7B). The docking results for compound **11*R***also showed favorable interactions with the key residues and a docking score of -4.931. The detailed study of its binding model revealed that the oxygen atom of nitro group formed hydrogen bond with the backbone amino group of the Ala167 (d=2.7Å) and the nitrogen atom of the pyrazole ring formed hydrogen bond with HOH933 (d=2.3Å). Additionally, the compound was also involved in salt bridge formation with the magnesium ion (d=2.0Å; Figure 7C). The binding model of compound **10*R*** indicated that the compound showed interactions with Lys168 and HOH933 (d=2.3Å). The docking score of compound **10R** was computed to be -4.252 (Figure 7D). The interactions of compounds **10**, **11** and **29** collected in the Table1.

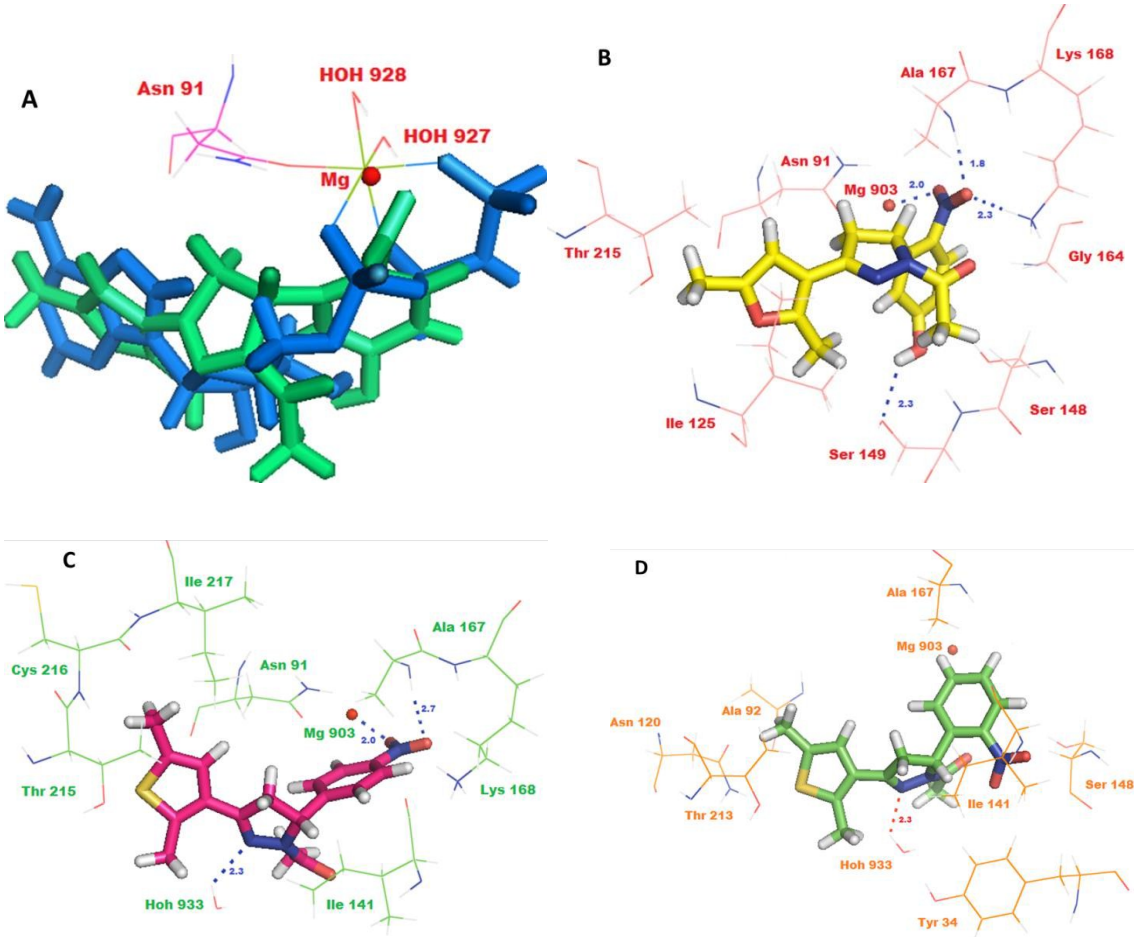

Figure 7A. Docking pose of **29*R*** (green) at the binding site of AMPPNP (blue) in ATPase domain of TopoIIα; B. Docking pose of **29R** (B), **11R** (C) and **10R** (D) showing the interactions with important amino acid residues in ATPase domain of TopoIIαCompound.

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Table 1. Docking parameter and some important key interactions observed withamino acid residues of Topo II active site and inhibitors

Cd	Glide score	Interactions	Interactions with Mg^{2+}
29*R*	-6.033	Lys168, Ala167, Ser149	Salt bridge
11*R*	-4.931	Ala167, HOH933	Salt bridge
10*R*	-4.252	Lys168, HOH933	No interaction
29*S*	-4.157	Lys168, Ser149, Phe142	Metal coordination
11*S*	-3.38	Asn120, HOH931	Arene-cation
10*S*	-3.158	Lys168, HOH924	No interaction

Antiproliferative activity

In order to determine the antiproliferative potential of compounds **10, 11, 29**(which showed best selective topo-IIα inhibitors) were screened *in vitro* against a panel of five cancer cell lines, MCF-7 (breast), HCT-116 (colon) wild type as well as p53 null type, H460 (lung), and PC-3 (prostate). We tested the compounds on p53 null type to ascertain whether the mode of inhibition is p53 independent or not. Overall the results indicate that the mode of action of the synthetic compounds is independent of p53. Since approximately 50% of the total cancer are mutated worldwide, thus the significance of the compounds increases further as their activity is independent of p53 status in the cancer cells.

The compounds **10, 11, 29** showed significant antiproliferative effectsin the aforesaid cancer lines.The results are summarized in Table 2.Etoposide (Etop)[41]was used as the positive control. The compounds did not show significant cytotoxicity towards human peripheral blood cells (hPBMCs) that served as normal control.This preferential cytotoxicityof the compounds to cancer cells could be due to intracellular accumulation of the compounds as well as their metabolites.[42]Our results indicate that when HCT116 P53 null cell line was exposed to higher doses (25μM) of our compounds, there is massive cell death. It has been shown earlier that DNA damage leads to mitotic catastrophe in p53 null cells.[43] Considering our compounds being topoisomerase inhibitors, it is assumed that the mode of apoptosis in p53 null cells at higher doses might be due to mitotic catastrophe.

Table 2 Antiproliferativeactivity of the compounds underinvestigations

Cd	MCF-7 (breast)	HCT-116WT (colon)	HCT-116 (colon) (p53 null type)	H460 (lung)	PC-3 (prostate)
			IC_{50} (μM)[a]		
10	3.1±0.3	3.5±0.6	4.1±0.6	5.0±0.3	4.9±0.5
11	2.5±0.2	5.6±0.2	4.3±0.3	3.5±0.4	6.1±0.3
29	2.7±0.5	8±0.8	5.1±0.3	3.9±0.2	7.6±0.2
Etop	20.933	....[b]	....[b]	>30.33	18.233

[a]Values are derived from averaging three independent experiments and each experiment was done in triplicate.[b]Not tested

Compounds induced Reactive oxygen species (ROS) in cancer cells

Since many anticancer compounds bring about changes in ROS status in the cells, It was hypothesized to checkwhether the anticancer effect of compounds is mediated by free radicals or not? Compounds**10, 11** and **29** were used for the ROS study using DHE based fluorescent detection system and utilising MCF-7 cancer cell line. The results showed that there is concentration dependent increase in ROS levels in the treated cells (Figure 8). Increase in ROS levels is generally associated with changes in mitochondrial membrane potential,[28] which triggers its depolarization and lead to release of cytochrome-c into cytoplasm, thus inducing apoptosis. JC-1 dye was used to measure membrane potentialof the mitochondria (Figure 9), the results showed that there is steady decrease in OD590/OD527 ratio indicating increased mitochondrial membrane depolarization.

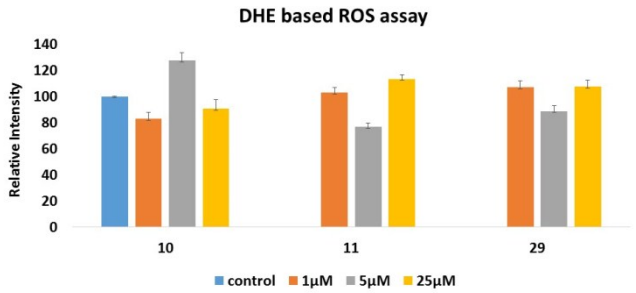

Figure 8.DHE based assay to measure intra-cellular reactive oxygen species (ROS)induced by **10**, **11** and **29**, respectively.

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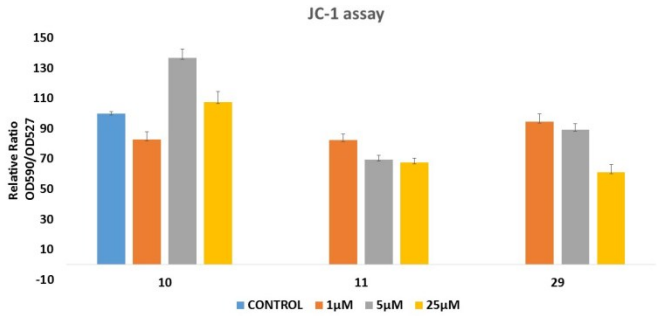

Figure 9JC-1 dye based assay to measure mitochondrial membrane potential altered by **10**, **11** and **29**,respectively.

Cell Cycle Analysis

Cell cycle analysiswas performed using flow cytometry by means of procedure as described previously.[44]The compound showed significant G1Phase arrest from 49.4% in control to 71.8% and 76.8% at concentration of 5 and 25 µl respectively (Figure.10). The predominant arrest of G0/G1Phase is attributed to the selective topoII catalytic inhibitors that might interfere with the binding between DNA and topoisomerase rendering inhibition of DNA replication, thus leading to cell cycle arrest at G0/G1Phase.

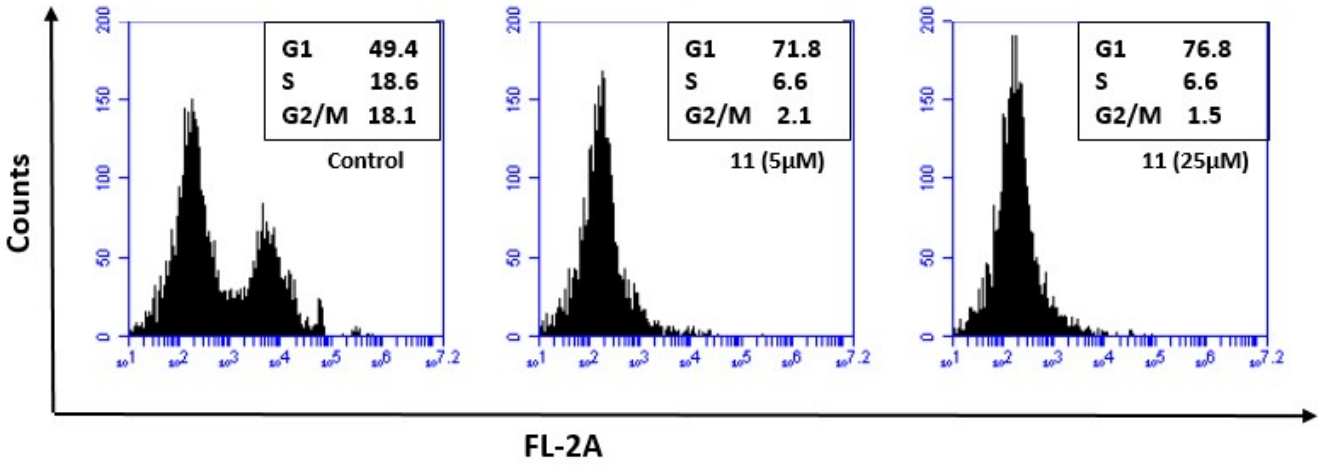

Figure 10 Cell cycle analysis using flow cytometry. The study was conducted for compound **10** using HCT-116 wild type cell line that showed profound G1 phase arrest.

Conclusions

In summary, a series of new 2,5-dimethylthiophene/furan based *N*-acetyl pyrazolines were designed as topoisomerase inhibitors through rational approach and synthesised. The target compounds were evaluated for decatenation, relaxation, cleavage complex and DNA intercalation assayagainst human topoisomerases. Compounds **10**, **11** and **29**exhibited potent, selective and catalytic toposiomerse II inhibitory activity with no intercalation with DNA which was further supported by their molecular modeling study. These compounds, in addition proved their broad anticancer potential (low micromolarIC_{50})*in vitro*against breast, colon, lung and prostate cancer cell lines with almost no toxicity to normal cells.Furthermore the compounds showed significant activity against HCT-116 colon cancer cells which are p53 null (HCT116 p53 null type) indicating that the anitcancer potential of these compounds is independent of p53. It thus signifies the importance of these compounds as in almost 50% of of human cancers are p53 mutant and these compounds can act in both p53 wild type as well as mutant type cancer cells. The compounds were able to induce cell cycle arrest at G1 phase. In addition, compounds were found to increase ROS levels as well as mitochondrial membrane depolarization, which might induce apoptosis, independent of their TopoII specific activity. These compounds could serve aspromising leads for future antitumor drug discovery.

Experimental

The reagents for the synthesis of compounds were purchased from Sigma-aldrich, Loba and CDH, India and used without further purification. All yields refer to isolated products after purification. Products were characterized by spectroscopic data (IR, ^{1}H NMR, ^{13}C NMR and MS spectra). NMR experments were measured in $CDCl_3$/ DMSO-d_6 relative to TMS (0.00 ppm). IR (KBr pallets) spectra were recorded on a Fourier transform infrared (FT-IR) Thermo spectrophotometer. Melting points were determined in open capillaries and were uncorrected. All the target compounds were unreported and their physical data is presented as below.

Synthesis of the compounds

General procedure for synthesis of chalcone (4)[28]

A mixture of aryl ketone (1 mmol) and an appropriate aryl aldehydes (1 mmol) was dissolved in 10 mL of ethanol in 25 mL conical flask. To this mixture, sodium hydroxide (5%, 2 mL) was added and the reaction mixture was stirred at room temperature for about 4-5 h. Solid was obtained after filtration which was recrystallized from ethanol to afford the chalcone.In case of phenolic aryl aldehydes, reaction was carried out in

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excess of base (30%, 5 mL) and after the completion of reaction, the reaction mixture was poured onto crushed ice. It was then neutralized with hydrochloric acid (5 %). Thus we avoided the extra step required to protect the hydroxyl group of these phenolic aryl aldehydes. Solid was obtained after filtration which was recrystallized from ethanol to afford the pure product.

General procedure for synthesis of pyrazolines(5-32)[28]

To a solution of an appropriate chalcone (1 mmol) in 20 mL of acetic acid, hydrazine hydrate 80% (1.5 mmol) was added in 50 mL round bottom flask. Then the mixture was refluxed for about 4-6 h. The mixture was then poured in ice water to get crude pyrazoline derivatives. Solid thus obtained was srecrystallized from ethanol to afford the pure product.

Synthesis of 1-[3-(2,5-dimethylthiophen-3-yl)-5-(3-nitro-phenyl)-4,5-dihydropyrazol-*1H*-1-yl]ethanone (5)

Yellow solid; yield 76%; mp: 138 – 140 °C: IR (KBr cm-1): 2917 (C-H), 1665 (C=O), 1645 (C=N), 1560 (C=C), 1524 (NO_2 asymmetric), 1349 (NO_2 symmetric), 1204 (C-N), 1141 (C-S). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0): δ = 8.08 - 8.14 (2H, m), 7.48 - 7.59 (2H, m), 6.73 (1H, s), 5.58 (1H, dd, J = 4.8 and 12.00 Hz), 3.79 (1H, dd, J = 11.7 and 17.7 Hz), 3.09 (1H, dd, J = 4.8 and 17.7 Hz), 2.66 (3H, s), 2.40 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.0, 15.9, 21.9, 44.5, 58.4, 99.9, 120.8, 122.7, 125.3, 127.4, 129.9, 132.0, 136.3, 143.9, 144.1, 150.8, 168.3. MS (EI): 343.1 (m/z). Anal. Calcd for $C_{15}H_{15}N_5O_4$: C, 54.71; H, 4.59; N, 21.27. Found: C, 54.67; H, 4.63; N, 21.32.

Synthesis of 1-[5-(4-chlorophenyl)-3-(2,5-dimethyl-thiophen-3-yl)-4,5-dihydropyrazol-*1H*-1-yl]ethanone (6)

Yellow solid; yield 71%; mp: 128 – 130 °C; IR (KBrcm^{-1}): 3014 (C-H Stretch), 1675 (C=O), 1635 (C=N Stretch), 1580 (C=C aromatic), 1204 (C-N stretch), 1161 (C-S stretch), 769 (C-Clstretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 7.28 (2H, d, J = 8.7 Hz), 7.15 (2H, d, J = 8.4 Hz), 6.73 (1H, s), 5.46 (1H, dd, J = 4.5 and 11.17 Hz), 3.66 (1H, dd, J = 12.00 and 17.7 Hz), 3.06 (1H, dd, J = 4.5 and 17.7 Hz), 2.64 (3H, s), 2.40 (3H, s), 2.36 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.0, 15.9, 21.9, 44.6, 58.4, 125.4, 127.1, 127.7, 129.0, 133.3, 136.1, 139.3, 140.4, 151.0, 168.7. MS (EI): 332.5 (m/z). Anal. Calcd for $C_{17}H_{17}ClN_2OS$: C, 61.34; H, 5.15; N, 8.42; S, 9.63. Found:C, 61.66; H, 5.23; N, 8.30; S, 9.86.

Synthesis of 1-[5-(3,4-dimethoxy-phenyl)-3-(2,5-dimethyl-thiophen-3-yl)-4,5-dihydropyrazol-*1H*-1-yl]ethanone (7)

Yellow solid; yield 75%; mp: 115 – 118 °C IR (KBr cm-1): 2860 (C-H Stretch), 1667 (C=O), 1615 (C=N Stretch), 1562 (C=C aromatic), 1250 (C-O stretch), 1219 (C-N stretch), 1181 (C-S stretch). ^{1}H NMR (300 MHz, CDCl3, TMS = 0) δ = 6.75 - 6.81 (4H, m), 5.45 (1H, dd, J = 4.5 and 11.7 Hz), 3.86 (3H, s), 3.84 (3H, s), 3.68 (1H, dd, J =12.00 and 17.7 Hz), 3.10 (1H, dd , J = 4.2 and 17.4 Hz), 2.65 (3H, s), 2.40 (3H, s), 2.37 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.0, 15.8, 22.0, 44.8, 55.8, 55.9, 58.8, 109.0, 111.4, 117.5, 125.5, 128.0, 134.6, 136.0, 139.0, 148.0, 149.1, 151.2, 168.7. MS (EI): 328.1 (m/z). Anal. Calcd for $C_{19}H_{22}N_2O_3S$: C, 63.66; H, 6.19; N, 7.82; S, 8.95. Found: C, 63.59; H, 6.33; N, 7.67; S, 8.84.

Synthesis of 1-[3-(2,5-dimethyl-thiophen-3-yl)-5-(3,4,5-trimethoxy-phenyl)-4,5-dihydro- pyrazol-*1H*-yl]- ethanone (8)

Yellow solid; yield 79%; mp: 160 – 162 °C: IR (KBr cm^{-1}): 3056 (C-H Stretch), 1654 (C=O), 1624 (C=N Stretch), 1571 (C=C aromatic), 1260 (C-O stretch), 1156 (C-N stretch), 1081 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 6.75 (1H, s), 6.41 (2H, s), 5.43 (1H, dd, J = 4.5 and 11.7 Hz), 3.83 (6H, s), 3.80 (3H, s), 3.70 (1H, dd, J =12 and 17.7 Hz), 3.08 (1H, dd, J = 4.8 and 17.7 Hz), 2.65 (3H, s), 2.40 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.0, 15.8, 21.9, 44.0, 54.7, 56.2, 56.5, 56.7, 98.1, 110.6, 121.3, 125.5, 128.3, 135.9, 138.7, 143.1, 149.1, 150.4, 152.0, 168.6. MS (EI): 328.5 (m/z). Anal. Calcd for $C_{20}H_{24}N_2O_4S$: C, 61.83; H, 6.23; N, 7.21; S, 8.25. Found:C, 61.77; H, 6.41; N, 7.19; S, 8.42.

Synthesis of 1-[3-(2,5-dimethyl-thiophen-3-yl)-5-(2,4,5-trimethoxy-phenyl)-4,5-dihydro-pyrazol-*1H*-1-yl]-ethanone (9)

Light brown solid; yield 79%; mp: 132 – 135 °C: IR (KBr cm^{-1}): 3090 (C-H Stretch), 1670 (C=O), 1644 (C=N Stretch), 1551 (C=C aromatic), 1250 (C-O stretch), 1148 (C-N stretch), 1050 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 6.74 (1H, s), 6.57 (1H, s), 6.52 (1H, s), 5.66 (1H, m), 3.86 (3H, s), 3.81 (3H, s), 3.78 (3H, s), 3.64 (1H, dd, J = 12 and 17.4 Hz), 2.98 (1H, dd, J = 4.2 and 17.4 Hz), 2.64 (3H, s), 2.39 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.0, 15.81, 21.9, 44.0, 54.7, 56.2, 56.4, 56.7, 98.17, 110.64, 121.31, 125.5, 128.2, 135.9, 138.7, 143.1, 149.1, 150.4, 152.0, 168.6. MS (EI): 358.4 (m/z). Anal. Calcd for $C_{20}H_{24}N_2O_4S$: C, 61.83; H, 6.23; N, 7.21; S, 8.25. Found: C, 61.73; H, 6.32; N, 7.11; S, 8.10.

Synthesis of 1-[3-(2,5-dimethylthiophen-3-yl)-5-(2-nitro-phenyl)-4,5-dihydropyrazol-*1H*-1-yl]ethanone (10)

Yellow solid; yield 82%; mp: 131 – 133 °C; IR (KBr cm^{-1}): 2937 (C-H Stretch), 1668 (C=O), 1639 (C=N Stretch), 1570 (C=C aromatic), 1514 (NO_2 asymmetric stretch), 1339 (NO_2symmetric stretch), 1219 (C-N stretch), 1147 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 8.10 (1H, d, J = 8.1Hz), 7.59 (1H, t, J = 7.2 Hz), 7.43 (1H, t, J = 7.5 Hz), 7.24 (1H, m), 6.74 (1H, s), 6.04 (1H, dd, J = 4.5 and 11.4 Hz), 3.99 (1H, dd,J = 11.7 and 18.00 Hz), 3.08 (1H, dd, J = 4.8 and 18.00 Hz), 2.39 (3H, s), 2.21 (3H, s), 2.02 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.9, 15.7, 21.9, 43.4, 57.5, 110.1, 121.7, 122.7, 125.4, 128.4, 129.9, 132.1, 135.3, 141.9, 143.1, 150.1, 168.5. MS (EI): 343.2 (m/z). Anal. Calcd for $C_{17}H_{17}N_3O_3S$: C, 59.46; H, 4.99; N, 12.24; S, 9.34. Found:C, 59.35; H, 5.12; N, 12.35; S, 9.39.

Synthesis of 1-[3-(2,5-dimethylthiophen-3-yl)-5-(4-nitro-phenyl)-4,5-dihydropyrazol-1-*1H*-yl]ethanone (11)

Yellow solid; yield 77%; mp: 178 – 181 °C; IR (KBr cm^{-1}): 2957 (C-H Stretch), 1655 (C=O), 1635 (C=N Stretch), 1549 (C=C aromatic), 1517 (NO_2 asymmetric stretch), 1339 (NO_2 symmetric stretch), 1219 (C-N stretch), 1157 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 8.18 (2H, d, J = 8.4 Hz), 7.39 (2H, d, J = 8.4 Hz), 6.73 (1H, s), 5.56 (1H, dd, J = 4.8 and 12.00 Hz), 3.77 (1H, dd, J = 12.00 and 17.4 Hz), 3.08 (1H, dd, J = 4.8 and 17.7 Hz), 2.65 (3H, s), 2.40 (3H, s), 2.39 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.0, 15.9, 21.8, 44.4, 58.5, 124.3, 125.2, 126.6, 127.3, 136.3, 139.6, 147.3, 148.9,

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150.8, 168.8. MS (EI): 343.1 (m/z). Anal. Calcd for $C_{17}H_{17}N_3O_3S$: C, 59.46; H, 4.99; N, 12.24; S, 9.34. Found:C, 59.39; H, 5.10; N, 12.41; S, 9.29

Synthesis of 1-[3-(2,5-dimethyl-thiophen-3-yl)-5-(2-methoxy-phenyl)-4,5-dihydro-pyrazol- *1H*-1-yl]ethanone (12)

Yellow solid; yield 72%; mp: 120 – 122 °C IR (KBr cm^{-1}): 2980 (C-H Stretch), 1667 (C=O), 1625 (C=N Stretch), 1547 (C=C aromatic), 1260 (C-O stretch), 1227 (C-N stretch), 1169 (C-S stretch). 1H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 7.23 (1H, t, J = 7.5 Hz), 6.97 (1H, d, J = 7.5 Hz), 6.87 - 6.90 (2H, m), 6.72 (1H, s), 5.76 (1H, dd, J = 4.2 and 11.4 Hz), 3.85 (3H, s), 3.67 (1H, dd, J =11.7 and 17.7 Hz), 2.95 (1H, dd, J = 4.2 and 17.4 Hz), 2.63 (3H, s), 2.42 (3H, s), 2.38 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.8, 15.7, 21.7, 44.3, 55.7, 58.5, 110.4, 116.5, 123.5, 127.0, 133.6, 136.5, 138.0, 147.0, 148.1, 151.0, 167.7. MS (EI): 328.1 (m/z). Anal. Calcd for $C_{18}H_{20}N_2O_2S$: C, 65.83; H, 6.14; N, 8.53; S, 9.76. Found: C, 65.91; H, 6.20; N, 8.71; S, 9.62.

Synthesis of 1-[5-(2,5-dimethoxyphenyl)-3-(2,5-dimethyl-thiophen-3-yl)-4,5-dihydropyrazol-*1H*-1-yl]ethanone (13)

Yellow solid; yield 78%; mp: 135 – 137 °C; IR (KBr cm^{-1}): 2882 (C-H Stretch), 1664 (C=O), 1627 (C=N Stretch), 1577 (C=C aromatic), 1261 (C-O stretch), 1228 (C-N stretch), 1172 (C-S stretch); ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 6.71- 6.79 (3H, m), 6.56 (1H, s) 5.73 (1H, d, J = 8.1Hz), 3.81 (3H, s), 3.61 – 3.71 (4H, m), 2.93 (1H, d, J = 16.2 Hz), 2.62 (3H, s), 2.41 (3H, s), 2.38 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.9, 15.8, 21.9,43.9, 54.5, 55.6, 56.0, 111.8, 111.9, 112.4, 125.5, 128.2, 130.6, 135.8, 138.8, 150.2, 151.9, 153.7, 168.6. MS (EI): 358.1 (m/z). Anal. Calcd for $C_{19}H_{22}N_2O_3S$: C, 63.66; H, 6.19; N, 7.82; S, 8.95. Found:C, 63.59; H, 6.32; N, 7.63; S, 9.01.

Synthesis of 1-[3-(2,5-dimethylthiophen-3-yl)-5-(4-methoxy-phenyl)-4,5-dihydropyrazol-*1H*-1-yl]ethanone (14)

Light brown solid; yield 75%; mp: 98 – 100 °C; IR (KBr cm^{-1}): 2992 (C-H Stretch), 1668 (C=O), 1632 (C=N Stretch), 1517 (C=C aromatic), 1267 (C-O stretch), 1239 (C-N stretch), 1157 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 7.15 (2H, d, J = 8.1 Hz), 6.83 (2H, d, J 8.4 Hz), 6.75 (1H, s), 5.45 (1H, dd, J = 3.9 and 11.7 Hz), 3.77 (3H, s), 3.63 – 3.72 (1H, m), 3.09 (1H, dd, J = 3.9 and 17.4 Hz), 2.64 (3H, s), 2.40 (3H, s), 2.35 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.0, 15.8, 22.0, 44.6, 55.5, 58.5, 114.2, 125.5, 126.9, 134.2, 136.0, 139.0, 140.1, 151.1, 158.0, 168.1. MS (EI): 328.3 (m/z). Anal. Calcd for $C_{18}H_{20}N_2O_2S$: C, 65.83; H, 6.14; N, 8.53; S, 9.76. Found:, 65.92; H, 6.23; N, 8.67; S, 9.53.

Synthesis of 1-[3-(2,5-dimethylthiophen-3-yl)-5-(2-hydroxy-phenyl)-4,5-dihydro-pyrazol- *1H*-1-yl]ethanone (15)

Brown solid; yield 77%; mp: 139 – 141 °C; IR (KBr cm^{-1}): 2995 (C-H Stretch), 1656 (C=O), 1641 (C=N Stretch), 1569 (C=C aromatic), 1257 (C-O stretch), 1212 (C-N stretch), 1161 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 7.21 (1H, m), 6.96 – 7.00 (2H, m), 6.84 – 6.89 (2H, m), 5.74 (1H, dd, J = 3 and 11.7 Hz), 3.64 – 3.76 (2H, m), 3.45 (1H, dd, J = 3 and 17.7 Hz), 2.66 (3H, s), 2.45 (3H, s), 2.38 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.8, 15.5, 21.6, 43.4, 57.3, 115.5, 121.3, 125.2, 126.4, 127.2, 136.3, 139.6, 147.3, 148.9, 150.8, 154.3, 167.4. MS (EI): 314.5 (m/z). Anal. Calcd for $C_{17}H_{18}N_2O_2S$: C, 64.94; H, 5.77; N, 8.91; S, 10.20. Found:C, 64.76; H, 5.89; N, 8.71; S, 10.34.

Synthesis of 1-[5-(2,3-dimethoxyphenyl)-3-(2,5-dimethyl-thiophen-3-yl)-4,5-dihydro-pyrazol-*1H*-1-yl]ethanone (16)

Yellow solid; yield 71%; mp: 127 – 129°C; IR (KBr cm^{-1}): 2991 (C-H Stretch), 1672 (C=O), 1634 (C=N Stretch), 1551 (C=C aromatic), 1256 (C-O stretch), 1239 (C-N stretch), 1172 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 6.97 (1H, d, J = 6.9 Hz), 6.81 (1H, d, J = 6.9 Hz) 6.73 (1H, s), 6.68 (1H, d, J = 7.2 Hz) 5.70 (1H, d, J = 8.1 Hz), 3.90 (3H, s), 3.85 (3H, s), 3.62 – 3.72 (1H, m), 3.03 (1H, d, J = 14.7 Hz), 2.63 (3H, s), 2.38 (6H, bs). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.0, 15.8, 22.0, 44.2, 54.7, 55.7, 60.4, 111.5, 118.0, 124.4, 125.5, 128.1, 135.7, 135.9, 138.8, 145.7, 151.8, 152.8, 168.6. MS (EI): 328.6 (m/z). Anal. Calcd for $C_{19}H_{22}N_2O_3S$: C, 63.66; H, 6.19; N, 7.82; S, 8.95. Found:C, 63.59; H, 6.33; N, 7.91; S, 8.71.

Synthesis of1-[5-(3-bromophenyl)-3-(2,5-dimethyl-thiophen-3-yl)-4,5-dihydro-pyrazol- *1H*-1-yl]-ethanone (17)

Yellow solid; yield 70%; mp: 103 – 105 °C; IR (KBr cm^{-1}): 2968 (C-H Stretch), 1667 (C=O), 1594 (C=N Stretch), 1567 (C=C aromatic), 1229 (C-N stretch), 1199 (C-S stretch), 715 (C-Br stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 7.33 – 7.38 (2H, m), 7.13 – 7.21 (2H, m), 6.73 (1H, s), 5.44 (1H, dd, J = 4.5 and 12 Hz), 3.70 (1H, dd, J = 11.7 and 17.7 Hz), 3.06 (1H, dd, J = 4.5 and 17.7 Hz), 2.64 (3H, s), 2.40 (3H, s), 2.38 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.0, 15.9, 21.9, 44.6, 58.5, 122.9, 124.3, 125.4, 127.7, 128.6, 130.5, 130.7, 136.1, 139.3, 144.2, 151.0, 168.7. MS (EI): 376.1, 378.3 (m/z). Anal. Calcd for $C_{17}H_{17}BrN_2OS$: C, 54.12; H, 4.54; N, 7.42; S, 8.50. Found:C, 54.09; H, 4.72; N, 7.38; S, 8.44.

Synthesis of 1-[3-(2,5-dimethylthiophen-3-yl)-5-(4-hydroxy-phenyl)-4,5-dihydro-pyrazol-*1H*-1-yl]ethanone (18)

Yellow solid; yield 75%; mp: 153 – 155 °C; IR (KBr cm^{-1}): 2987 (C-H Stretch), 1661 (C=O), 1582 (C=N Stretch), 1577 (C=C aromatic), 1254 (C-O stretch), 1235 (C-N stretch), 1179 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 6.95 (2H, d, J = 9 Hz), 6.71 (1H, m), 6.53 (2H, d, J = 9 Hz), 5.42 (1H, dd, J = 4.5 and 11.7 Hz), 3.66 (1H, dd, J = 11.7 and 17.7 Hz), 3.09 (1H, dd, J = 4.2 and 17.7 Hz), 2.62 (3H, s), 2.39 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.1, 15.8, 21.6, 42.4, 59.5, 116.2, 126.3, 126.9, 127.1, 136.3, 139.6, 147.3, 150.8, 157.2, 168.9. MS (EI): 314.3 (m/z). Anal. Calcd for $C_{17}H_{18}N_2O_2S$: C, 64.94; H, 5.77; N, 8.91; S, 10.20. Found: C, 64.81; H, 5.89; N, 8.70; S, 10.39.

Synthesis of 1-[3-(2,5-dimethylthiophen-3-yl)-5-(3-hydroxy-4-methoxy-phenyl)-4,5-dihydro–pyrazol-*1H*-1-yl]ethanone (19)

Creamy solid; yield 76%; mp: 115 – 118 °C; IR (KBr cm^{-1}): 3010 (C-H Stretch), 1656 (C=O), 1627 (C=N Stretch), 1537 (C=C aromatic), 1251 (C-O stretch), 1225 (C-N stretch), 1165 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 6.73 – 6.78

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(4H, m), 5.40 (1H, dd, J = 4.2 and 11.7 Hz), 3.84 (3H, s), 3.65 (1H, dd, J = 12 and 18 Hz), 3.07 (1H, dd, J = 4.5 and 17.7 Hz), 2.63 (3H, s), 2.54 (3H, s), 2.36 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.1, 15.8, 21.6, 43.4, 56.3, 58.6, 112.5, 113.8, 119.5, 126.6, 127.3, 133.4, 135.8, 139.6, 146.3, 148.1, 151.8, 167.2. MS (EI): 344.4 (m/z). Anal. Calcd for $C_{18}H_{20}N_2O_3S$: C, 62.77; H, 5.85; N, 8.13;; S, 9.31. Found:C, 62.82; H, 5.71; N, 8.39;; S, 9.41

Synthesis of 1-[3-(2,5-dimethylthiophen-3-yl)-5-(3-methoxy-2-nitro-phenyl)-4,5-dihydro-pyrazol-*1H*-1-yl]-ethanone (20)

Yellow solid; yield 68%; mp: 158 – 160 °C; IR (KBr cm^{-1}): 2977 (C-H Stretch), 1660 (C=O), 1639 (C=N Stretch), 1543 (C=C aromatic), 1523 (NO_2 asymmetric stretch), 1331 (NO_2 symmetric stretch), 1253 (C-O stretch), 1229 (C-N stretch), 1173 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 7.36 (1H, t, J = 8.1 Hz), 6.95 (1H, d, J = 9 Hz), 6.72 - 6.76 (2H, m), 5.43 (1H, dd, J = 5.4 and 12.0 Hz), 3.89 (3H, s), 3.71 (1H, dd, J = 12.0 and 18.0 Hz), 3.13 (1H, dd, J = 5.4 and 18.0 Hz), 2.63 (3H, s), 2.39 (3H, s), 2.37 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.9, 15.9, 21.7, 44.5, 55.1, 56.5, 99.9, 111.0, 117.0, 125.4, 131.9, 135.5, 136.1, 138.5, 139.7, 141.1, 151.2, 168.7. MS (EI): 373.4 (m/z). Anal. Calcd for $C_{18}H_{19}N_3O_4S$: C, 57.89; H, 5.13; N, 11.25; S, 8.59. Found:C, 57.92; H, 5.29; N, 11.12; S, 8.31.

Synthesis of 1-[3-(2,5-dimethylthiophen-3-yl)-5-(5-hydroxy-2-nitro-phenyl)-4,5-dihydropyrazol-*1H*-1-yl]-ethanone (21)

Brown solid; yield 65%; mp: 210 – 212 °C;IR (KBr cm^{-1}): 2996 (C-H Stretch), 1651 (C=O), 1627 (C=N Stretch), 1531 (C=C aromatic), 1523 (NO_2 asymmetric stretch), 1337 (NO_2 symmetric stretch), 1243 (C- O stretch), 1234 (C-N stretch), 1162 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 7.96 (1H, m), 6.55 – 6.76 (3H, s), 6.10 (1H, m), 3.97 (1H, m), 2.94 (1H, m), 2.64 (3H, s), 2.47 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.5, 15.4, 21.5, 42.9, 54.0, 116.7, 122.7, 125.0, 125.5, 127.4, 127.7, 129.2, 135.1, 138.0, 151.5, 152.6, 167.9. MS (EI): 359.1 (m/z). Anal. Calcd for $C_{17}H_{17}N_3O_4S$: C, 56.81; H, 4.77; N, 11.69; S, 8.92. Found:C, 56.72; H, 4.92; N, 11.71; S, 9.12.

Synthesis of 1-[3-(2,5-dimethyl-thiophen-3-yl)-5-naphthalen-2-yl-4,5-dihydro-pyrazol-1H-1-yl]ethanone (22)

Dark brown semisolid; yield 67%; IR (KBr cm-1): 3010 (C-H Stretch), 1665 (C=O), 1645 (C=N Stretch), 1580 (C=C aromatic), 1202 (C-N stretch), 1135 (C-S stretch). ^{1}H NMR (300 MHz, CDCl3, TMS = 0) δ = 7.67 – 7.78 (5H, m), 7.31 – 7.44 (2H, m), 6.75 (1H, s), 5.67 (1H, bs), 3.77 (1H, m), 3.15 (1H, m), 2.66 (3H, s), 2.40 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.0, 15.9, 22.0, 44.8, 59.2, 123.4, 124.4, 125.5, 125.9, 126.2, 127.6, 128.1, 129.0, 132.8, 133.3, 136.1, 139.17, 139.22, 149.1, 151.3, 168.8. MS (EI): 348.4 (m/z). Anal. Calcd for $C_{21}H_{20}N_2OS$: C, 72.38; H, 5.79; N, 8.04; S, 9.20. Found:C, 72.41; H, 5.81; N, 8.12; S, 9.31.

Synthesis of 1-[3-(2,5-dimethyl-thiophen-3-yl)-5-naphthalen-1-yl-4,5-dihydro-pyrazol-1H-1-yl]-ethanone (23)

Brown semisolid; yield 65%; IR (KBr cm-1): 3015 (C-H Stretch), 1677 (C=O), 1635 (C=N Stretch), 1561 (C=C aromatic), 1223 (CN stretch), 1149 (C-S stretch). ^{1}H NMR (300 MHz, CDCl3, TMS = 0) δ = 7.94 (1H, d, J = 7.8 Hz), 7.86 (1H, d, J = 6 Hz), 7.73 (1H, d, J = 9 Hz) 7.40 - 7.56 (3H, m), 7.18 (1H, d, J = 6.9 Hz), 6.62 (1H, s), 6.23 (1H, dd, J = 3.3 and 11.1 Hz), 3.87 (1H, dd, J = 12 and 17.4 Hz), 3.04 (1H, dd, J = 3.9 and 17.1 Hz), 2.64 (3H, s), 2.50 (3H, s), 2.33 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.9, 15.9, 22.0, 44.5, 56.5, 123.0, 125.5, 126.3, 127.9, 128.1, 129.1, 129.6, 134.3, 136.0, 136.1, 139.2, 143.6, 151.9, 168.9. MS (EI): 348.1 (m/z). Anal. Calcd for $C_{21}H_{20}N_2OS$: C, 72.38; H, 5.79; N, 8.04; S, 9.20. Found:C, 72.41; H, 5.81; N, 8.29; S, 9.17.

Synthesis of 1-(5-(benzo[*d*][1,3]dioxol-5-yl)-3-(2,5-dimethylthiophen-3-yl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethanone (24)

Yellow solid; yield 68%; mp: 142 – 144 °C; IR (KBr cm^{-1}): 2961 (C-H stretch), 1679 (C=O stretch), 1652 (C=N Stretch), 1559 (C=C aromatic), 1259 (C-O stretch), 1229 (C-N stretch), 1127 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 6.70 - 6.76 (3H, m), 6.67 (1H, s), 5.91 (2H, s), 5.41 (1H, dd, J = 5.2 and 11.7 Hz), 3.66 (1H, dd, J =11.7 and 17.4 Hz), 3.05 (1H, dd, J = 5.2 and 17.4 Hz), 2.64 (3H, s), 2.40 (3H, s), 2.36 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.0, 15.9, 22.0, 44.8, 58.8, 101.1, 106.0, 108.4, 119.1, 125.4, 127.9, 136.0, 136.1, 139.1, 146.9, 148.0, 151.1, 168.7. MS (EI): 342.3 (m/z). Anal. Calcd for $C_{18}H_{18}N_2O_3S$: C, 63.14; H, 5.30; N, 8.18; S, 9.36. Found:C, 63.11; H, 5.23; N, 8.18; S, 9.27

Synthesis of 1-[3-(2,5-dimethylthiophen-3-yl)-5-styryl-4,5-dihydro-pyrazol-*1H*-1-yl]ethanone (25)

Dark brown solid; yield 70%; mp: 108 – 110 °C: IR (KBr cm^{-1}): 2980 (C-H Stretch), 1660 (C=O), 1642 (C=N Stretch), 1595 (C=C aromatic), 1211 (C-N stretch), 1142 (C-S stretch);1H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 7.22 – 7.37 (6H, m), 6.87 (1H, s), 6.18 (1H, dd, J = 7.5 and 15.6 Hz), 5.49(1H, m), 3.50 (1H, m), 3.42 (1H, m), 2.77 (3H, s), 2.41 (3H, s), 2.37 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.6, 15.7, 21.6, 43.6, 58.1, 126.1, 127.2, 127.9, 128.4, 128.8, 129.1, 133.8, 134.6, 136.7, 139.7, 151.8, 167.2. MS (EI): 324.3 (m/z). Anal. Calcd for $C_{19}H_{20}N_2OS$: C, 70.34; H, 6.21; N, 8.63; S, 9.88. Found:C, 70.21; H, 6.39; N, 8.74; S, 10.01

Synthesis of 1-[3-(2,5-dimethyl-thiophen-3-yl)-5-furan-2-yl-4,5-dihydro-pyrazol-*1H*-1-yl]-ethanone (26)

Brown solid; yield 67%; mp: 145 – 148 °C; IR (KBr cm^{-1}): 3012 (C-H Stretch), 1667 (C=O), 1639 (C=N Stretch), 1542 (C=C aromatic), 1263 (C-O stretch), 1236 (C-N stretch), 1157 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 7.29 (1H, s), 6.78 (1H, s), 6.31 – 6.30 (2H, bs), 5.60 (1H, dd, J = 4.8 and 11.4 Hz), 3.53 (1H, dd, J = 11.7 and 17.4 Hz), 3.37 (1H, dd, J = 4.8 and 17.4 Hz), 2.64 (3H, s), 2.41 (3H, s), 2.34 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.02, 15.8, 21.9, 40.5, 52.4, 107.4, 110.5, 125.5, 127.8, 136.0, 139.1, 141.8, 151.3, 152.2,

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168.81. MS (EI): 288.3 (m/z). Anal. Calcd for $C_{21}H_{20}N_2O_2$: C, 75.88; H, 6.06; N, 8.43. Found:C, 75.92; H, 6.31; N, 8.24.

Synthesis of 1-[3-(2,5-dimethyl-furan-3-yl)-5-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-pyrazol-*1H*-1-yl]-ethanone (27)

Yellow solid; yield 70%; mp: 156 – 158 °C; IR (KBr cm^{-1}): 3024 (C-H Stretch), 1665 (C=O), 1617 (C=N Stretch), 1561 (C=C aromatic), 1251 (C-O stretch), 1167 (C-N stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 6.40 (2H, s), 6.13 (1H, s), 5.43 (1H, dd, J = 4.5 and 11.7 Hz), 3.82 (6H, s), 3.80 (3H, s), 3.59 (1H, dd, J =11.7 and 17.7 Hz), 2.99 (1H, dd, J = 4.8 and 17.7 Hz), 2.47 (3H, s), 2.38 (3H, s), 2.26 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.5, 15.4, 20.36, 21.4, 55.8, 55.9, 58.1, 128.2, 135.3, 138.3, 140.1, 151.54, 167.8. MS (EI): 372.1 (m/z). Anal. Calcd for $C_{20}H_{24}N_2O_5$: C, 64.50; H, 6.50; N, 7.52. Found:C, 64.21; H, 6.61; N, 7.88.

Synthesis of 1-[3-(2,5-dimethylfuran-3-yl)-5-(2-methoxy-phenyl)-4,5-dihydro pyrazol-*1H*-1-yl]ethanone (28)

Dark Yellow solid; yield 79%; mp: 152 – 155 °C; IR (KBr cm^{-1}): 3011 (C-H Stretch), 1661 (C=O), 1629 (C=N Stretch), 1561 (C=C aromatic), 1249 (C-O stretch), 1136 (C-N stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 7.25 (1H, m), 6.96 (1H, d, J = 7.5 Hz), 6.86 - 6.90 (2H, m), 6.10 (1H, s), 5.74 (1H, dd, J = 4.5 and 11.7Hz), 3.84 (3H, s), 3.59 (1H, dd, J = 11.7 and 17.7Hz), 2.85 (1H, dd, J = 4.5 and 17.4 Hz), 2.45 (3H, s), 2.39 (3H, s), 2.23 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.8, 15.8, 21.7, 44.1, 56.3, 58.4, 112.1, 115.1, 123.3, 124.2, 125.6, 127.2, 137.3, 139.2, 147.1, 145.9, 151.7, 168.6. MS (EI): 312.4 (m/z). Anal. Calcd for $C_{18}H_{20}N_2O_3$: C, 69.21; H, 6.45; N, 8.97. Found:C, 69.30; H, 6.52; N, 9.02.

Synthesis of 1-[3-(2,5-dimethylfuran-3-yl)-5-(5-hydroxy-2-nitro-phenyl)-4,5-dihydro-pyrazol-*1H*-1-yl]ethanone (29)

Black solid; Yield 70%; mp: 212 – 215 °C IR (KBr cm^{-1}): 3015 (C-H Stretch), 1659 (C=O), 1631 (C=N Stretch), 1529 (C=C aromatic), 1531 (NO_2 asymmetric stretch), 1342 (NO_2 symmetric stretch), 1239 (C-O stretch), 1229 (C-N stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 7.78 (1H, d, J = 9 Hz), 6.44 (1H, s), 6.31 (1H, d, J = 8.7 Hz), 6.04 – 6.10 (2H, m), 3.92 (1H, dd, J = 11.4 and 17.7 Hz), 2.99 (1H, dd, J = 4.5 and 18 Hz), 2.53 (3H, s), 2.47 (3H, s), 2.24 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.5, 15.8, 21.7, 44.3, 58.3, 113.2, 114.6, 124.2, 125.3, 126.7, 128.3, 137.3, 139.6, 147.4, 148.9, 156.8, 168.8. MS (EI): 343.1 (m/z). Anal. Calcd for $C_{17}H_{17}N_3O_5$: C, 59.47; H, 4.99; N, 12.24. Found:C, 59.39; H, 5.05; N, 12.38.

Synthesis of 1-[3-(2,5-Dimethylfuran-3-yl)-5-furan-2-yl-4,5-dihydropyrazol-*1H*-1-yl]ethanone (30) To the solution of 1-Black solid; yield 75%; mp: 98 – 100 °C; IR (KBr cm^{-1}): 3022 (C-H Stretch), 1657 (C=O), 1629 (C=N Stretch), 1531 (C=C aromatic),1252 (C-O stretch), 1248 (C-N stretch).1H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 7.29 (1H, s), 6.30 (2H, s), 6.14 (1H, s),5.60 (1H, dd, J = 4.5 and 11.4 Hz), 3.45 (1H, dd, J = 11.7 and 17.4 Hz), 3.28 (1H,dd, J = 4.5 and 17.1 Hz), 2.49 (3H, s), 2.32 (3H, s), 2.26 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.3, 15.8, 21.7, 44.3, 58.1, 122.3, 125.3, 126.7, 127.3, 136.3, 139.6, 146.3, 150.9, 151.6, 167.9. MS (EI): 272.4 (m/z). Anal. Calcd for $C_{15}H_{16}N_2O_2S$: C, 62.48; H, 5.59; N, 9.71; S, 11.12. Found:C, 62.33; H, 5.61; N, 9.62; S, 11.21.

Synthesis of 1-[3-(2,5-dimethyl-furan-3-yl)-5-naphthalen-1-yl-4,5-dihydro-pyrazol-1H-1-yl]ethanone (31)

Brown semisolid; yield 65%; IR (KBr cm^{-1}): 2998 (C-H Stretch), 1671 (C=O), 1625 (C=N Stretch), 1553 (C=C aromatic), 1272 (C-O stretch), 1235 (C-N stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 7.22 – 7.78 (7H, m), 6.37 (1H, s), 6.12 (1H, m), 3.79 (1H, dd, J = 11.7 and 17.1 Hz), 2.96 (1H,dd, J = 4.2 and 17.4 Hz), 2.49 (3H, s), 2.35 (3H, s), 2.28 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.1, 15.8, 21.9, 44.7, 59.1, 122.4, 125.4, 125.4, 125.8, 126.4, 127.7, 128.2, 129.1, 132.9, 134.3, 136.1, 138.1, 139.2, 148.1, 151.3, 168.8. MS (EI): 332.3 (m/z). Anal. Calcd for $C_{15}H_{16}N_2O_3$: C, 66.16; H, 5.92; N, 10.29. Found: C, 66.22; H, 6.01; N, 10.38.

Synthesis of 1-(5-(benzo[*d*][1,3]dioxol-5-yl)-3-(2,5-dimethylfuran-3-yl)-4,5-dihydro-1H-pyrazol-1-yl)ethanone (32)

Light Yellow solid; yield 77%; mp: 145 – 148 °C; IR (KBr cm^{-1}): 2982 (C-H stretch), 1671 (C=O stretch), 1639 (C=N Stretch), 1547 (C=C aromatic), 1263 (C-O stretch), 1224 (C-N stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 6.71 - 6.72 (2H, m), 6.68 (1H, m), 6.12 (1H, s), 5.90 (2H, s), 5.41 (1H, dd, J = 4.5 and 11.7 Hz), 3.58 (1H, dd, J =11.7 and 17.7 Hz), 2.96 (1H, dd, J = 4.5 and 17.4 Hz), 2.47 (3H, s), 2.34 (3H, s), 2.25 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 14.1, 14.9, 21.9, 44.8, 57.8, 108.1, 110.0, 115.4, 120.1, 125.5, 128.9, 136.2, 136.4, 139.3, 147.9, 148.2, 152.1, 168.8. MS (EI): 326.1 (m/z). Anal. Calcd for $C_{18}H_{18}N_2O_4$: C, 66.25; H, 5.56; N, 8.58. Found:C, 66.18; H, 5.63; N, 8.49.

Synthesis of acetic acid [1-(2,5-dimethyl-thiophen-3-yl)-ethylidene]-hydrazide (33)

Brown solid; yield 72%; mp: 146 – 148 °C; IR (KBr cm^{-1}): 2981 (C-H Stretch), 1665 (C=O), 1627 (C=N Stretch), 1597 (C=C aromatic), 1260 (C-N stretch), 1187 (C-S stretch). ^{1}H NMR (300 MHz, $CDCl_3$, TMS = 0) δ = 8.91 (1H, NH, D_2O exchangeable proton), 6.83 (1H, s), 2.54 (3H, s), 2.40 (3H, s), 2.32 (3H, s), 2.16 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ = 15.0, 15.2, 15.8, 20.7, 125.6, 134.2, 135.3, 136.4, 145.4, 173.6. MS (EI): 210.4 (m/z). Anal. Calcd for $C_{10}H_{14}N_2O_S$: C, 57.11; H, 6.71; N, 13.32; S, 15.25. Found:C, 57.09; H, 6.88; N, 13.21; S, 15.39.

Topoisomerase Assays

All the reagents required for the testing of investigational compounds were purchased from TopoGEN, Inc. (Columbus, OH). The testing of the compounds was performed using a commercially available hTopo-IIα drug screening kit. All the synthesized compounds and etoposide were dissolved in DMSO at a concentration of 1 mM as a stock solution and stored at -20 °C.[45, 46]

hTopo IIα mediated DNA decatenation assay

Synthesized compounds were screened for hTopo IIα mediated DNA decatenation inhibition 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

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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 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 I mediated DNA relaxation assay

Synthesized compounds were screened for hTopo I mediated DNA relaxation inhibition as follows. Reaction mixture containing freshly prepared 10X reaction assay buffer (100 mM Tris-HCl (pH-7.9), 10 mM EDTA, 1.5 M sodium chloride, 1% BSA, 1mM spermidine, 50% glycerol) 250 ng supercoiled DNA (substrate), 100 μM camptothecin or investigational compound dissolved in DMSO followed by addition of purified hTopo-I were incubated at 37 °C for 30 min. The reaction was then terminated with addition of 10% SDS, followed by digestion with proteinase K. Further the reaction mixture was again incubated at 70 °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 nicked and relaxed products were quantified with Quantity One (BioRad)

Molecular Docking

The molecular docking studies were executed using GLIDE 6.1 module of Schrödinger Suite. The co-crystal structure of human topoisomerase IIα with ANP (PDB code-1ZXM)[6]was retrieved from protein data bank. The *R* and *S* configurations of compounds **10**, **11** and **29** were sketched in ChemBio3D Ultra 12.0 and the energy minimization was performed using MM2 force field. The ligands were prepared and neutralized using the LIGPREP module. The protein preparation step involved pre-processing and filling up missing side chain and loops using PRIME. The metal binding states were generated and the protein was then optimized and minimized using the PREP WIZ module. The grid box was generated at the ATP binding site, around the centroid of the bound ligand. The docking was carried out by the GLIDE XP modules.

Cell Culture and Treatment

All the cell lines were acquired from National cell repository, NCCS, Pune. Cell lines signifying different human cancers were grown in DMEM media added with 10% fetal bovine serum (FBS) and antibiotic solution (1% Penstrip, all the reagents from Invitrogen). Cells were cultured in DMEM media with 10% FBS, 50 U/mL penicillin G, 50lg/mL streptomycin sulfate and 1.25lg/mL amophotericin B (fungizone). The cells were incubated at 37˚C with 5% CO_2 and 95% humidity conditions. For experiments, cells were seeded in equal numbers after trypan blue cell counting (8000 cells per well of 96-well plate). The compounds were dissolved in cell culture grade DMSO up to concentration of 100 mM and further dilutions were done in serum free DMEM media. The total amount of media per well (100µL per well of 96 well plate) was kept constant and all the treatment volumes were accommodated within these ranges only.

3-(4, 5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

MTT assay was carried out using 96-wellplate; each well was filled by 100 µL media to which cell were treated and subsequently washed with 1% PBS and were mixed with 10 µL/mL well of MTT (5 mg in 10 mL of 1%PBS) and incubated at room temperature in dark for 4 h to allow formation of formazan crystals. Each well was then mixed with 100µL of DMSO to dissolve the crystals followed by readings using microplate reader at 570 nm. The results were then represented as mean ± S.D obtained from three independent experiments.

Reactive oxygen species (ROS) and mitochondrial membrane integrity assays

MCF-7 cancer cells were seeded in 96-well plate and appropriate treatments of synthetics were given. 24hrs post treatments, cells were processed for JC-1 or DHE staining. For DHE staining, cells were washed by 1X PBS followed by staining with DHE at 37^{o}C for 30 minutes. Afterwards cells were washed again with ice-cold 1X PBS and finally 100µl of 1X PBS was added to each well followed by measuring OD using microplate reader. For JC-1 assay, dye was directly added to the media and cells were incubated at 37^{o}C for 30 minutes followed by washing with 1X PBS to remove extra dye. OD was measured using microplate reader (emission=527 and 590).

Cell Cycle Analysis

Cell cycle analysis was performed using flow cytometry (BD Accuri). Cellular samples (HCT-116 wild type previously treated with compound **11** for 24h) were prepared for staining by transferring 1 X 10^5 to 1 X 10^6 cells to each tube. Then cell were centrifuged at 1200 rpm for 5 min and washed with 1X PBS. The cells were then suspended using chilled ethanoland the cell concentration thereby raised to 5 X 10^5 to 1 X 10^6 cells/mL. The cells were then incubated for for 3 hrs at -20° C. after three hours cells were treated with ribonuclease A (100 µg/mL), to ensure only DNA not RNA gets stained and were further incubated for 30 min.Then they were subjected to centrifugation at 2500 rpm and washed once with 1X

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PBS.Propidium iodide 200 µl (from 50 µg/ml stock solution) was added to each tube incubated for 30 minutes at room temperature in the dark and further analysis was done using the flow cytometry instrument.

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Graphical Abstract

Synthesis and biological evaluation of new 2, 5-dimethylthiophene/furan based *N*-acetyl pyrazolines as selective topoisomerase II inhibitors

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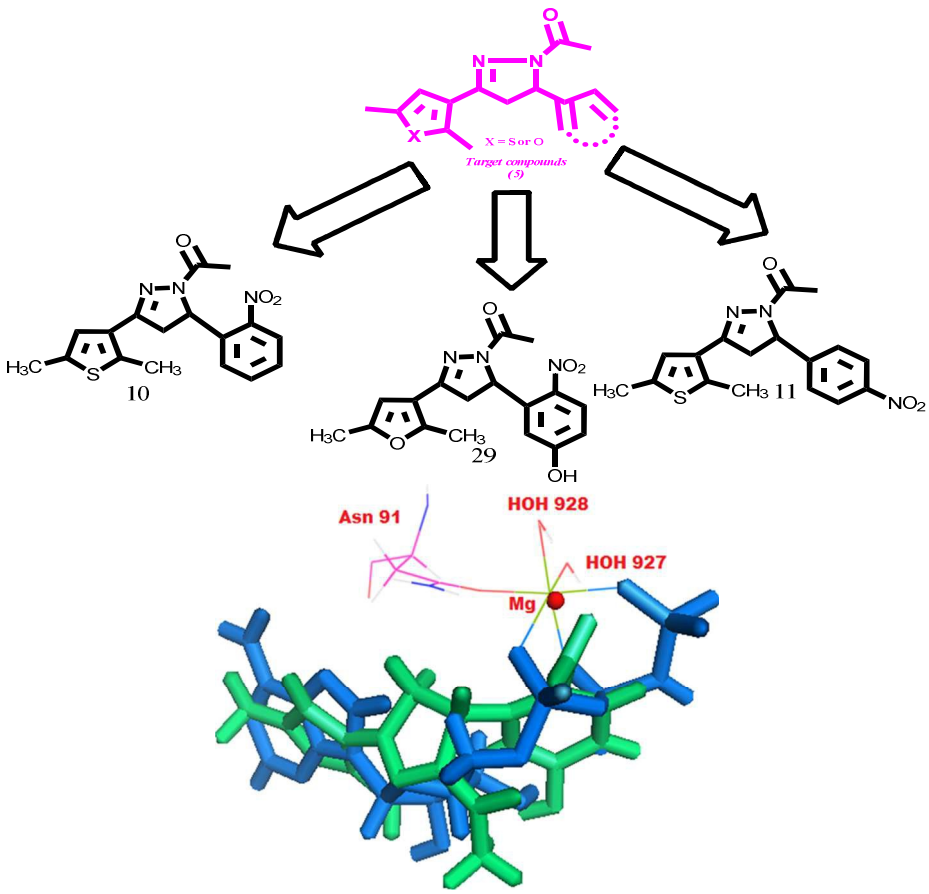

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