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Synthesis of imine-pyrazolopyrimidinones and their mechanistic interventions on anticancer activity

Ashish T. Baviskar [b], Uttam C. Banerjee [b], Mukesh Gupta [c], Rajveer Singh [c], Sunil Kumar [c], Manish K. Gupta [c], Sanjeev Kumar [d], Satish K. Raut [f], Madhu Khullar [f], Sandeep Singh [e], Raj Kumar [a,c,*,1]

[a] Laboratory for Drug Design and Synthesis, Centre for Chemical and Pharmaceutical Sciences, Central University of Punjab, 151 001 Bathinda, India
[b] Department of Pharmaceutical Technology (Biotechnology), National Institute of Pharmaceutical Education and Research (NIPER), Mohali, S.A.S. Nagar, Sec 67, 160 062 Punjab, India
[c] Molecular Modeling and Pharmacoinformatics Lab, Indo-Soviet Friendship College of Pharmacy, Moga, 141 001 Punjab, India
[d] Centre for Biosciences, Central University of Punjab, 151 001 Bathinda, India
[e] Centre for Genetic Diseases and Molecular Medicine, Central University of Punjab, 151 001 Bathinda, India
[f] Department of Experimental Medicine and Biotechnology, PGIMER, 160 012 Chandigarh, India

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ABSTRACT

Design, synthesis and anticancer activity of a series of imine-pyrazolopyrimidinones is reported for the first time. Compounds **9d**, **9n** and **9o** in the series show encouraging in vitro anticancer activity with low micromolar IC_{50} values against prostate (PC3) and breast (MCF7) cancer cell lines. Some notions about structure–activity relationships and plausible mechanism of biological activity are presented.

1. Introduction

Cancer is a complex, multifactorial disease characterized by uncontrolled growth of abnormal cells in the body.[1] It is comprised of more than hundred diseases having common features of excessive proliferation, angiogenesis and may also harbour metastatic properties. It is a leading cause of deaths worldwide after cardiovascular diseases.[2] As per WHO, 70% of all cancer deaths occurred in low and medium income countries in 2008, are predicted to continue rising with an estimate of 13.1 million deaths in the year 2030.[3] Although, several anticancer drugs are available, the failure to achieve the desired therapeutic efficacy with existing agents due to multi-drug resistance, toxicity or poor bioavailability warrants evaluation of newer compounds.[4–6]

Naturally occurring stilbenes[7,8] especially trans-stilbenes including resveratrol[9] and piceatannol[10] are being explored as anticancer agents. However, the limited bioavailability of the these stilbenes due to glucuronidation and sulfation regardless of their greaterabsorption[11] has prompted the researchers to rely on their alterations/modifications[12] such as acetylation,[13] methylation[14] of resveratrol or hybridization of stilbene-coumarin.[15] The published success stories on a single molecule with multiple pharamcophores[16–18] having beneficial and reduced or unwanted side effects encouraged us to rationally design a hybrid pharmacophore (Fig. 1). The hybrid pharamcophore is designed as a template for anticancer agent with substitution of one of the aromatic rings of stilbenes/imines (**A**) with pyrazolo[3,4-*d*]pyrimidinone (**B**).

Pyrazolo[3,4-*d*]pyrimidines/pyrimidinones have been already reported as anticancer agents,[19] through a variety of investigated mechanisms such as inhibition of EGFR,[20] IGF-1R[21] or dual Src/Abl.[22]

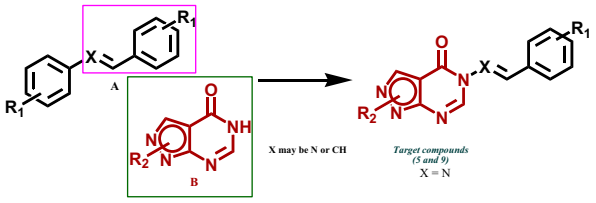

Figure 1. Design of the target compounds **5** and **9**.

* Corresponding author. Fax: +91 163 623 6564.
E-mail address: raj.khunger@gmail.com (R. Kumar).
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2. Results and discussion

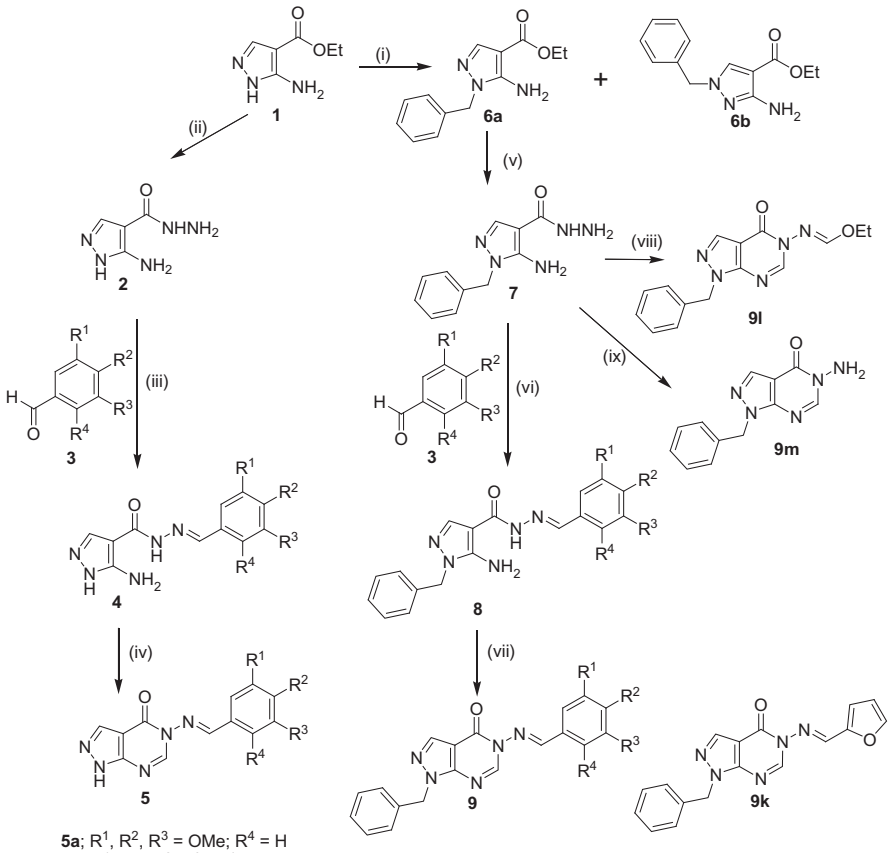
2.1. Synthesis

Preparation of the target compounds **5a–f** and **9a–p** was accomplished by means of the synthetic procedures shown in Scheme 1. Briefly, nucleophilic substitution of ester of **1** was carried out with hydrazine hydrate to afford hydrazide **2** which was condensed with substituted or unsubstituted benzaldehyde (**3**) to give hydrazone derivatives **4**.

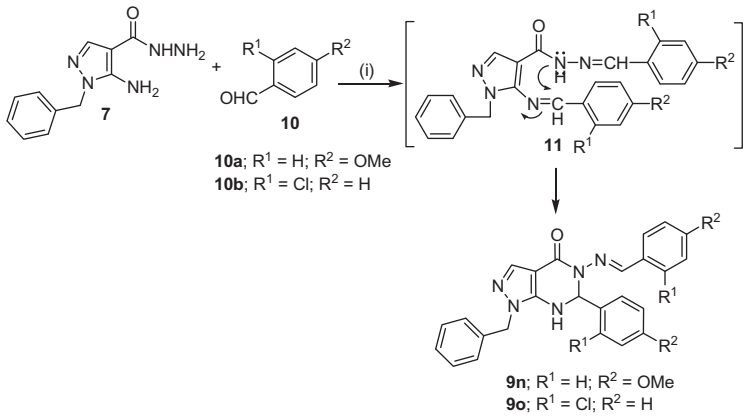
Heating **4** with $CH(OEt)_3$ afforded ring cyclized target compounds **5**. For the synthesis of desired compounds **9a–p**, **1** was treated with benzyl chloride to afford a mixture of two regio-isomers (**6a** and **6b**). The major isomer **6a** was isolated by crystallization from ethyl acetate and treated with hydrazine hydrate to yield **7**. Condensation of **7** with various benzaldehyde derivatives **3** resulted into **8** which was further ring cyclized to afford **9a–k** using $CH(OEt)_3$ under acid catalyzed heating conditions. **7** when treated with $CH(OEt)_3$, **9l** was obtained whereas a different product **9m** was isolated when **7** was heated with $CH(OEt)_3$ under acidic conditions. The position of benzyl group in case of **6a** and **6b** was confirmed using UV spectroscopy (**6a**; λ_{max} (MeOH) = 266 nm and **6b**; λ_{max} (MeOH) = 254 nm). Treatment of **7** with anisaldehyde **10a** or 2-chlorobenzaldehyde **10b** resulted into condensation followed by ring cyclisation to afford **9n** and **9o**, respectively, via formation of intermediate **11** (Scheme 2). Reduction of imine of **9a** using $NaBH_4$ in methanol afforded **9p** (Scheme 3). The products were purified either by crystallization or column chromatography using EtOAc/hexane as elution solvents. All new compounds were fully characterized by ^1H and ^{13}C NMR, mass spectral, and elemental micro-analytical data.

2.2. Antiproliferative activity

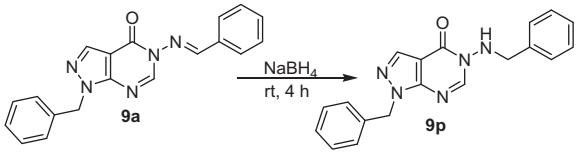
In order to determine the antiproliferative potential of the target compounds **5a–f**, **9a–k**, and **9n–p** were screened in vitro against six cancer cell lines, including THP-1 (leukemia), IGROV-1 (ovary), HeLa (cervix), PC3 (prostate), MCF7 (breast fibroadenoma), H460 (liver). The IC_{50} values for tested compounds are summarized in Supplementary Table 1 (see Supplementary data).[23] The compounds that showed the highest in vitro anticancer activity in prostate, breast and liver cancer lines are collected in Table 1. Etoposide (Etop)[24] was used as the positive control. These compounds when further tested on normal human cells, little or no significant cytotoxicity towards human buccal cavity cells (less than 20% apoptosis) at highest concentration of 100 μM in comparison to etoposide



Scheme 1. Reagents and conditions: (i) BnCl, NaH, MeCN, rt, 12 h; (ii) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux, 12 h; (iii) MeOH, reflux, 4 h; (iv) $CH(OEt)_3$, MeCN, reflux, 8 h; (v) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux, 10 h; (vi) EtOH, reflux, 4 h; (vii) $CH(OEt)_3$, MeCN, reflux, 4 h; (viii) $CH(OEt)_3$, reflux, 12 h; (ix) $CH(OEt)_3$, MeCN, H_2SO_4 (1 drop), reflux, 7 h.



Scheme 2. Reagents and conditions: (i) EtOH, reflux, 4 h.



Scheme 3. Synthesis of **9p**.

Table 1
Anticancer activity of **9d**, **9n** and **9o**

Cd	PC3 Prostate	MCF7 Breast	H460 Liver
IC_{50} (μM)[a]			
9d	10.1	29.7	34.3
9n	17.6	18.1	35.7
9o	18.1	20.1	39.8
Etop	18.2	20.9	<30

[a] Values are derived from averaging three independent experiments and each experiment was done in triplicate.

(30% apoptosis) was observed. This overall suggests that the compounds are cytotoxic to cancer cells only (Fig. 2).

2.3. Structure–activity relationships

Some general notions about structure–activity relationships emerged from these studies on the tested compounds (see Supplementary Table 1 in Supplementary data):[23] (a) removal of benzyl group from **9g**, **9j**, **9d**, **9e** and **9f** resulted in decrease of anticancer activity as exemplified by **5a**, **5c**, **5d**, **5e** and **5f**, respectively, (b) reduction or removal of iminic bond diminishes the activity (**9a** > **9p** and **9l** > **9m**), (c) addition of phenyl ring on iminic carbon enhances the cytotoxic activity (**9l** < **9a**) and (d) on phenyl ring, in general the activating groups such as methoxy and hydroxy potentiated the activity (**9a** < **9d**, **9e** and **9g**) whereas the deactivating groups such as cyano and chloro resulted in dilution of the activity (**9a** > **9b** and **9c**).

2.4. Mechanistic interventions on anticancer activity

2.4.1. Cell cycle analysis and DNA damage studies

In order to execute preliminary mechanistic studies on anticancer activity, we focused on exploring the stage(s) of the cell cycle being affected by selected compounds **9d** and **9o**. To this end, we performed flow cytometry on PC3 cells treated with 10 μM of **9d** and **9o** for 24 h, and the results are collected in Figure 3A. Initial cell cycle analysis confirmed that these compounds lead to increased cell numbers in G2/M phase (13.8% vs 48.1% and 52.2%). Figure 3A shows that these compounds induce apoptosis through G2/M arrest.

Many of the drugs also induce free radicals as well as alter various other signaling cascades inside the cancer cells. We evaluated our compounds for any oxidant potential using spectrophotometric and fluorescence microscopy analysis of H2DCFDA. The results indicate that these compounds increased ROS levels at low concentrations while at 5 μM there was more than 1.5 fold increase in free radicals (Fig. 3B and C). Further evaluation for DNA damage was indicative that these compounds also cause DNA damage at low concentrations. Comet analysis revealed that there was almost 9% decrease in comet head indicating potential increase in DNA damage (Fig. 3D and E). Taken together biological analyses of these compounds indicate their selective cytotoxic potential to cancer cells and induce free radical stress as well as DNA damage in these cancer cells. These findings are suggestive of multiple pathways are being affected by these compounds.

2.4.2. Compounds were selective TopoIIα inhibitors

It has been formerly reported that cell cycle arrest at G2/M phase is also correlated with higher topoisomerase IIα (TopoIIα) expression in rapidly proliferating cells[25] whereas inhibition of topoisomerase activity is also associated with increased DNA damage.[26] It was planned to screen **9d**, **9n** and **9o** against topoisomerase enzymes (TopoIIα and TopoI). The effect of the compounds on human topoisomerase IIα was examined using ATP-dependent kDNA decatenation assay, Kinetoplast DNA (kDNA) was used as a substrate whereas etoposide (TopoII inhibitor), was used as standard (Fig. 4A and B). Incubation of kDNA with human topoisomerase IIα showed two decatenated products: nicked kDNA (Nck) and supercoiled (SC)/relaxed (Rel) circular DNA. In agarose gel, catenated kDNA appears at the top as it cannot enter into the gel because of its overall size while other decatenated products move into.

The samples containing etoposide showed partial or moderate decatenation, same as reported in the literature because of its known reversible inhibition of topoisomerase IIα.[27,28] Compounds **9d**, **9n** and **9o** were found to be most active against human topoisomerase IIα. Almost no decatenation activity was observed in the presence of these compounds. In comparison to etoposide, compounds **9d**, **9n** and **9o** had shown higher inhibition of topoisomerase IIα activity. Compounds under investigation were also subjected to topoisomerase II dependent relaxation (Fig. 4C and D) in which a negatively supercoiled DNA (pRYG) was measured using agarose gel electrophoresis. Results obtained were in tune

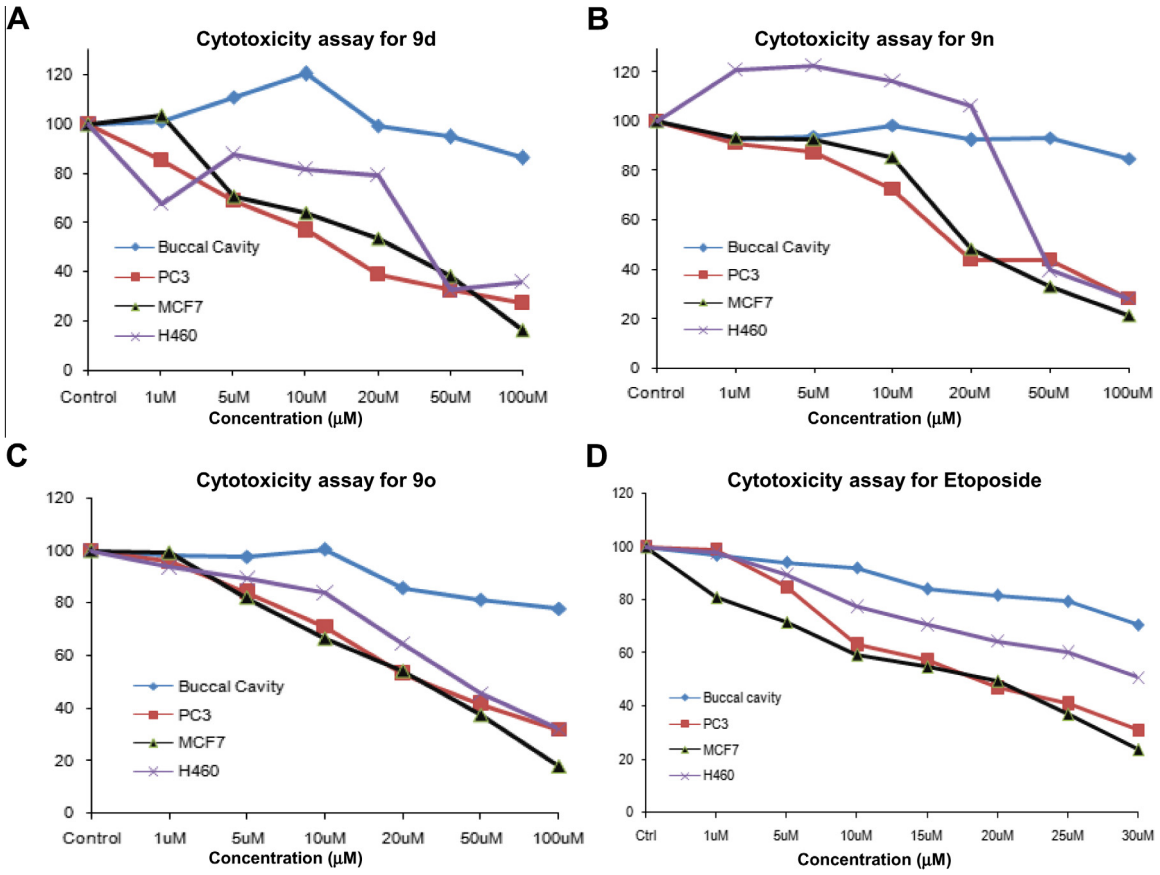


Figure 2. Cytotoxicity (MTT) assay for normal and cancerous cell lines. Compounds **9d** (A), **9n** (B), **9o** (C) were tested in normal buccal cavity (blue bars), prostate cancer PC3 (red bars), breast cancer MCF7 (black bars) and liver cancer H460 (violet bars) cell lines. (D) Etoposide was used as positive control to compare the IC_{50} values in all four cell lines. All the experiments were done in triplicate as three independent experiments (total 9 replicates per treatment) and values were averaged to plot the graphs.

with as observed in kDNA decatenation assay. It was detected that supercoiled form of substrate pRYG plasmid DNA was reduced by topoisomerase II and appeared as a set of variably relaxed topoisomers that migrate more slowly as compared to supercoiled form. It was noticed that compounds **9d**, **9n**, and **9o** were found to be more potent topoisomerase IIα inhibitors in comparison to etoposide (IC_{50} = 78.4 μM) as indicated by the calculated IC_{50} value 46.5 μM (Fig. 4E) of the **9d** (see Supplementary data).[23] Further, the tested compounds (**9d**, **9n**, and **9o**) were tuned up to be weak inhibitors of TopoI as compared to camptothecin (C; a standard hTopoI inhibitor) in topoisomerase I mediated relaxation assay indicating their selectivity towards TopoIIα (Fig. 4).

2.4.3. Compounds were catalytic TopoIIα inhibitors

Since our compounds expressed topoisomerase II inhibitory activity and also indicated genotoxicity through generation of ROS, so we next planned to answer the question whether these compounds are catalytic inhibitors of Topo II or the poisons. We performed the DNA relaxation assay by setting up competition between ATP and our compound **9d**.[23] Nuclear lysate from HeLa cells was used as source of topoisomerase and incubated with varying concentration of **9d** with constant amount of ATP. Our results signposted that compound **9d** was able to inhibit the relaxation induced by topoisomerase in increasing concentration, thus indicating that our compounds are catalytic inhibitors of topoisomerase activity while also induce free radicals and DNA damage through secondary mechanisms (see Supplementary data).[23]

2.4.4. Molecular modeling

As **9d** emerged to be a potent catalytic inhibitor of TopoIIα, we were interested to identify its binding interactions with ATPase domain of hTopoIIα[29] in the active site by molecular docking simulation using the GOLD software.[30] Overall binding mode of **9d** with binding site residue of ATPase domain of hTopoIIα[29] was found similar to that observed with co-crystalized AMPPNP.[27,29] The major interactions of **9d** with topoisomerases IIα include H-bonds, polar and van der Waals interactions. The highly conserved Walker A motif at ATP binding site of topoisomerases IIα is composed of Arg162, Asn163, Gly164, Tyr165, Gly166 and Ala 167. The studies revealed that the Walker A motif is crucial for binding of catalytic inhibitors acting on ATPase domain. The ring A of **9d** gets positioned in the vicinity of Walker A motif and involved in two H-bonds and van der Waals interactions (Fig. 5). In this, the oxygen atom of OCH_3 (ring A) shows a H-bond interaction with backbone NH of Arg162.The other H-bond is observed between phenolic oxygen and NH ofGly166. The other residues Glu87, Gly160, Asn163, Gly164, Tyr165 and Gln376 are involved in polar and/or van der Waals interactions with ring A. The ring-A also showed an effective cation-π interaction with the Mg^{2+}. The Mg^{2+} ion is an important for catalytic activity at the ATPase domain of hTopoIIα.[25,29] The nitrogen (at intermediate chain) is involved in polar interactions with carbonyl function of Ile141 and Thr147. The carbonyl function at ring B oriented towards Ser148 and Ser149 and shows many polar interactions. The ring B also shows arene-hydrogen interaction with the side chain of Ile141. The guanidine group of Arg98 functions as a backbone acceptor to the methylene group present in

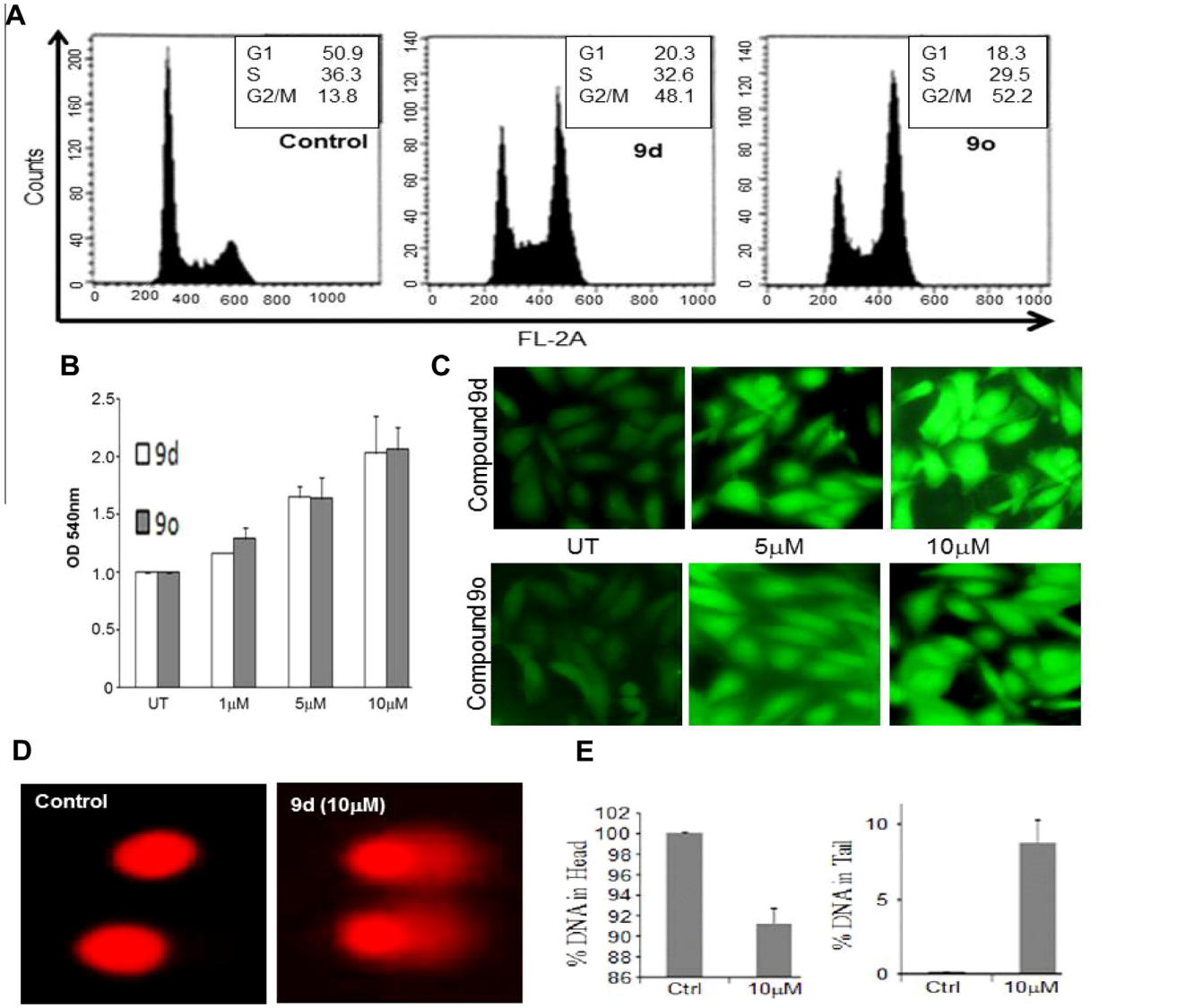


Figure 3. (A) FACS analysis for cell cycle profiling of control and drug treated PC3 cells. The first graph shows untreated control cells, the middle showing PC3 cells treated with 5 μM of **9d** and the right side graph shows PC3 cells treated with 10 μM of compound **9o**. (B) Spectrophotometric analysis of DCFDA intensity in PC3 cells treated with compound **9d** (white bars) and **9o** (grey bars) to evaluate the levels of free radicals in cells. (C) PC3 cells treated with **9d** and **9o** compounds were stained with DCFDA and captured under fluorescent microscope. The green color represents DCFDA staining. (D) Comet assay pictures showing formation of comet in treated PC3 cells. (E) Comets were analyzed for percentage of DNA in head and tail. Total 30 cells were scored using Cometscore15 software Version 1.0.1.0 and all the values were averaged and standard errors were calculated.

between ring C and D. The ring D lies parallel to the side chain carbonyl group of Asn95 and showed a unique lone pair-π interaction.[31] The other residues surrounding to the ring D include Asn120 and Lys123. Thus the present study indicates that **9d** is a catalytic inhibitor acting via occupying the ATP binding pocket of ATPase domain of hTopoIIα and making favorable interactions with its key residues.

3. Conclusions

In conclusion, we have designed and synthesized some compounds that show encouraging anticancer activity in vitro with low micromolar IC_{50} values compared to etoposide against prostate and breast cancer cell lines tested and with low toxicity to the normal cells. Our preliminary mechanistic studies of biological activity of **9d**, **9n**, and **9o** suggest their effect on the G2/M phase of the cell cycle. The experiments suggested that these compounds provoke several stress pathways in the cancer cells including elevated ROS levels, thus causing DNA damage. The compounds were also found to be potent and selective catalytic inhibitors of hTopoIIα inhibitors indicated by ATP-dependent decatenation and relaxation assays which was further supported by molecular modeling studies. We propose that anticancer activity expressed by the compounds is a result of hijacking/interfering of multiple pathways through regulation of free radicals, DNA damage and catalytic-TopoIIα activity concurrently in cancer cells. Further detailed investigations of SARs as well as explorations of antitumor activity are presently in progress.

4. Experimental

4.1. Chemistry

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.

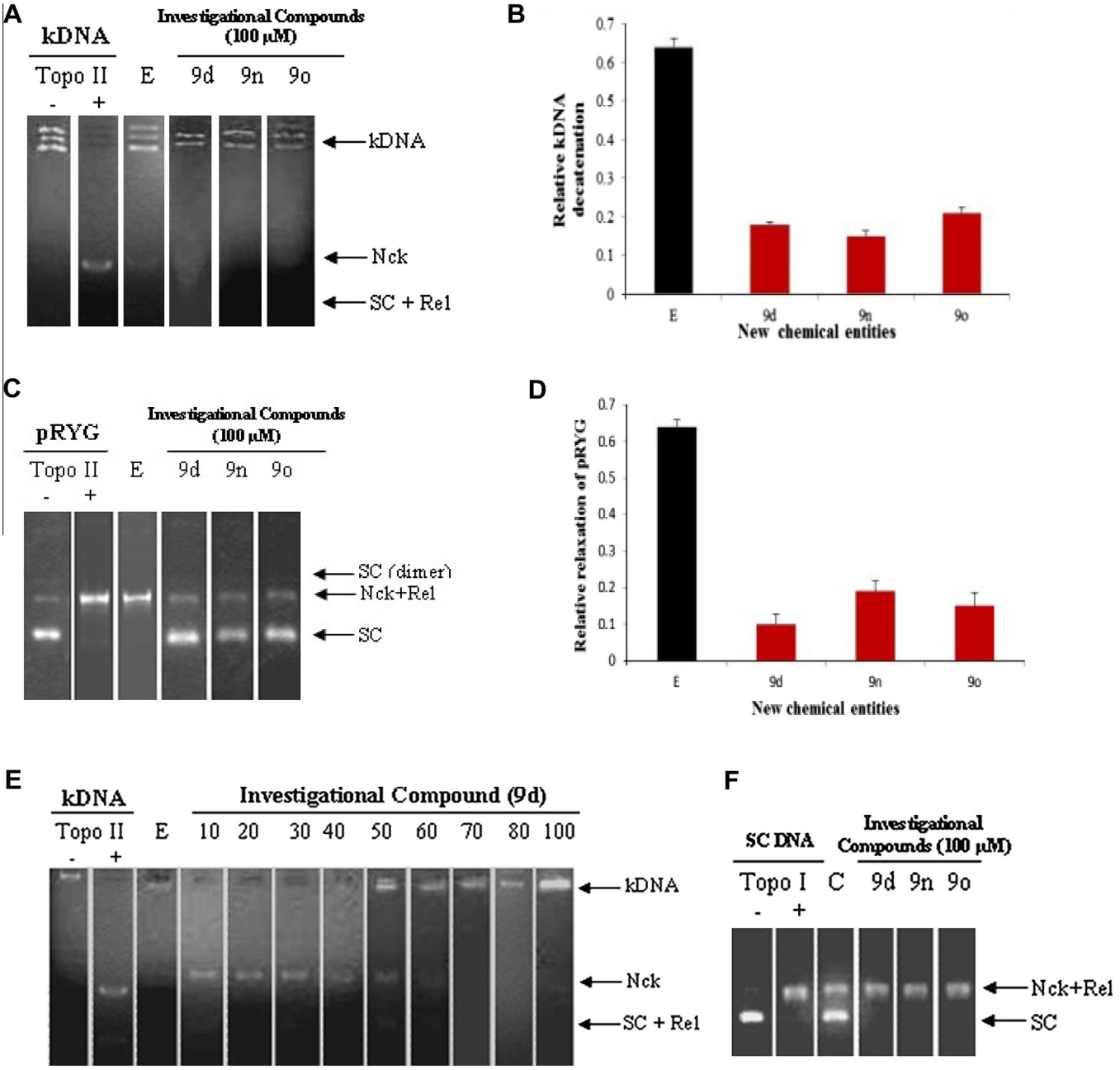


Figure 4. (A). Effect of test compounds on TopoIIα mediated kDNA decatenation assay. (B) Graphical representation of decatenated products formed in TopoIIα mediated kDNA decatenation assay. (C) Effect of test compounds on TopoIIα mediated supercoiled plasmid pRYG DNA relaxation assay. (D) Graphical representation of relaxed products formed in TopoIIα mediated supercoiled plasmid pRYG DNA relaxation assay. (E) Inhibition of topoisomerase IIα activity by compound **9d** at various concentrations ranging from 10 to 100 μM in kDNA decatenation assay. (F) Effect of test compounds on Topol mediated supercoiled plasmid DNA relaxation assay.

Products were characterized by by spectroscopic data (IR, ^{1}H NMR, ^{13}C NMR and MS spectra). NMR experiments 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. The purity of the all the tested compounds was assessed by reverse phase analytical HPLC. All the target compounds were unreported and their physical data is presented as below:

4.1.1. Ethyl 5-amino-1-benzyl-1H-pyrazole-4-carboxylate (6a)

A solution of 3-amino-4-carbethoxy pyrazole (**1**, 4 g, 1 mmol, 1 equiv) in acetonitrile (60 mL) and sodium hyride (1.5 g, 2 equiv) was stirred in a 250 mL round bottom flask for 1 h under ice cold conditions. Benzylchloride (6 mmol, 1.5 equiv) was added to the reaction mixture and further stirred at room temperature for 12 h. The mixture was extracted with ethyl acetate (3 × 10 mL). The organics were washed, dried on anhydrous Na_2SO_4, evaporated on a rotary evaporator to afford the crude product. The crude product was recrystallized (MeOH) to get pure compound **6a**. Yield: 60%, white solid; mp: 161–162 °C; IR (KBr): 3465, 3301, 1693, 1605 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.24 (t, 3H, J = 6.96 Hz, CH_3), 4.17 (q, 2H, J = 6.92 Hz, OCH_2), 5.35 (s, 2H, CH_2), 7.35–7.24 (m, 5H), 8.08 (s, 1H, CH); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 55.09, 59.52, 98.09, 127.61, 127.78, 128.17, 128.25, 128.72, 128.93, 133.63, 137.28, 157.07, 164.14.

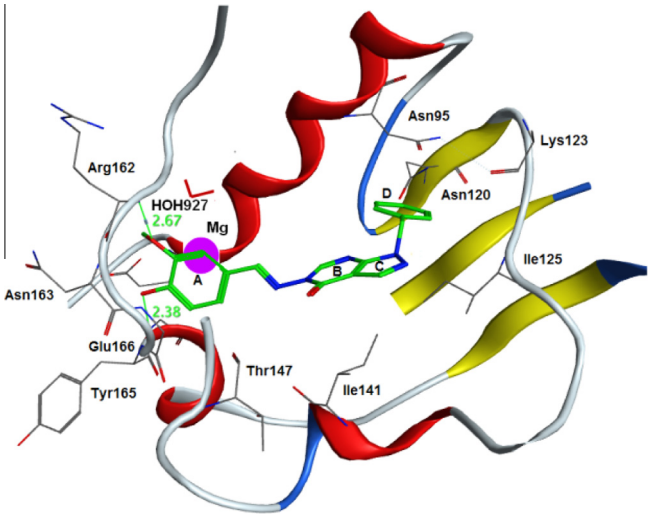


Figure 5. Docked conformation of **9d** at the ATPase domain of hTopoIIα.

4.1.2. 5-Amino-1-benzyl-1H-pyrazole-4-carbohydrazide (7)

A solution of **6a** (200 mg, 1 mmol, 1 equiv) in ethanol (10 mL) and hydrazine hydrate 98% (2 mL, 5 equiv) was refluxed in 25 mL round bottom flask for 10 h. White crystals of **7** obtained on cooling were filtered, washed with diethyl ether and dried. Yield: 80%, White crystalline; IR (KBr): 3465, 3301, 1693, 1605 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 4.20 (s, 2H, D_2O exchangeable NH_2), 5.04 (s, 2H,D_2O exchangeable NH_2), 5.35 (s, 2H, CH_2), 7.35–7.26 (m, 3*H*), 7.20 (d, *J* = 7.64 Hz, 2H), 7.92 (s, 1H, C*H*), 9.01 (s, 1H, D_2O exchangeable N*H*); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 55.17, 99.64, 128.11, 128.94, 130.09, 137.56, 156.75, 164.91.

4.1.3. Synthesis of 5a–5f

A solution of 3-amino-4-carbethoxy pyrazole (**1**, 200 mg, 1 mmol, 1 equiv) in ethanol (2 mL) and hydrazine hydrate 98% (2 mL, 4 equiv) was refluxed for 12 h in 25 mL round bottom flask. White crystals were filtered off, washed with diethyl ether and dried to afford the **2** which was used for the next step as follows:

4.1.4. 5-(3,4,5-Trimethoxybenzylideneamino)-1H-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (5a)

A solution of **2** (30 mg, 2 mmol, 1 equiv) in methanol (5 mL) and 3,4,5-trimethoxy benzaldehyde (54 mg, 1.3 equiv) was refluxed for 4 h in 10 mL round bottom flask. The reaction mixture was then dried to remove methanol under vacuum at rotary evaporator to give **4**. Then 2 mL of triethylorthoformate was added and the reaction mixture was refluxed for 8 h. White crystals obtained on cooling were filtered off, washed with diethyl ether and dried to afford **5a**. Yield: 59%, Yellowish white solid; mp: 169–171 °C. IR (KBr): 3177, 3088, 1701, 1611 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 3.76 (m, 3H, –OCH_3), 3.86 (m, 6H, –OCH_3), 7.26 (m, 2H, C*H*), 8.28 (s, 1H, C*H*), 8.48 (s, 1H, N–C*H*), 9.03 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 56.49, 60.70, 105.85, 106.44, 107.15, 128.20, 141.51, 148.16, 153.65, 166.68. Anal. Calcd for $C_{15}H_{15}N_5O_4$: C, 54.71; H, 4.59; N, 21.27. Found: C, 54.68; H, 4.61; N, 21.32.

4.1.5. 5-(2,3,4-Trimethoxybenzylideneamino)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (5b)

A solution of **2** (30 mg, 2 mmol, 1 equiv) in methanol (5 mL) and 2,3,4-trimethoxy benzaldehyde (54 mg, 1.3 equiv) was refluxed for 4 h in 10 mL round bottom flask. The reaction mixture was then dried to remove methanol under vacuum to give **4**. Then 2 mL of triethylorthoformate was added and the reaction mixture was refluxed for 4 h (TLC). The crude compound obtained was purified by column chromatography to give **5b** using ethyl acetate:hexane (1:3) as eluents. Yield: 75%, white solid; mp: 212–215 °C; IR (KBr): 3192, 1709, 1592 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 3.79 (s, 3H, –OCH_3), 3.89 (m, 6H, –OCH_3) , 7.01 (d, 1H, *J* = 8.5 Hz, C*H*), 7.78 (d, 1H, *J* = 8.5 Hz, C*H*), 8.36 (s, 1H, C*H*), 8.44 (s, 1H, N–C*H*), 9.14 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 56.61, 61.01, 62.64, 105.94, 109.19, 118.85, 122.39, 141.93, 148.32, 154.60, 157.70, 162.43. Anal. Calcd for $C_{15}H_{15}N_5O_4$: C, 54.71; H, 4.59; N, 21.27. Found: C, 54.79; H, 4.65; N, 21.24.

4.1.6. 5-(3,4-Dimethoxybenzylideneamino)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (5c)

A solution of **2** (50 mg, 3 mmol, 1 equiv) in methanol (5 mL) and 3,4-dimethoxy benzaldehyde (76 mg, 1.3 equiv) was refluxed for 4 h in a 10 mL round bottom flask. After 4 h, methanol was distilled off to give **4**. Triethylorthoformate (2 mL) and acetonitrile (1 mL) were added to crude residue. The reaction mixture was refluxed for 5 h (TLC). The excess of triethylorthoformate and acetonitrile were distilled off and the crude compound obtained was purified by column chromatography to give **5c** using ethyl acetate:hexane (1:1.5) as eluents. Yield: 70%, white solid; mp: 252–256 °C; IR (KBr): 3300, 1693, 1580 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 3.84 (s, 3H, –OCH_3), 3.97 (s, 3H, –OCH_3), 7.11 (d, 1H, *J* = 8.1 Hz, C*H*), 7.41 (d, 1H, *J* = 8.1 Hz, C*H*), 7.51 (s, 1H, C*H*), 8.25 (s, 1H, C*H*), 8.43 (s, 1H, N–C*H*), 8.94 (s, 1H, N=C*H*);^{13}C NMR (100 MHz, DMSO-d_6): δ = 56.04, 56.21, 105.90, 109.71, 111.97, 125.03, 125.46, 148.07, 149.59, 153.15; MS (APCI): *m*/*z* = 300.33 $[M+1]^+$, *m*/*z* = 164.13 $[M+1]^+$ a fragment peak. Anal. Calcd for $C_{14}H_{13}N_5O_3$: C, 56.18; H, 4.38; N, 23.40. Found: C, C, 56.21; H, 4.41; N, 23.49.

4.1.7. 5-(3-Methoxy-4-hydroxybenzylideneamino)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (5d)

A solution of **2** (30 mg, 2 mmol, 1 equiv) in methanol (5 mL) and 3-methoxy-4-hydroxy benzaldehyde (42 mg, 1.3 equiv) was refluxed for 4 h in 10 mL round bottom flask. The reaction mixture was then dried to remove methanol under vacuum to give **4**. Then 2 mL of triethylorthoformate was added and the reaction mixture was refluxed for 4 h (TLC). The crude compound obtained was purified to give **5d** by column chromatography using ethyl acetate:hexane (1:3) as eluents. Yield: 78%, brown solid; mp: 239–241 °C; IR (KBr): 3113, 1708, 1601 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 3.84 (s, 3H, –OCH_3), 6.93 (d, 1H, *J* = 7.9 Hz, C*H*), 7.31 (d, 1H, *J* = 7.9 Hz, C*H*), 7.50 (s, 1H, C*H*), 8.27 (s, 1H, C*H*), 8.43 (s, 1H, N–C*H*), 8.84 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 56.49, 60.70, 105.85, 106.44, 107.15, 128.20, 141.51, 148.16, 153.65, 166.68. Anal. Calcd for $C_{13}H_{11}N_5O_3$: C, 54.74; H, 3.89; N, 24.55. Found: C, 54.77; H, 3.93; N, 24.62.

4.1.8. 5-(3-Hydroxy-4-methoxybenzylideneamino)-1*H*-pyrazolo[5,4-*d*]pyrimidin-4(5*H*)-one (5e)

A solution of **2** (30 mg, 2 mmol, 1 equiv) in methanol (5 mL) and 3-hydroxy-4-methoxy benzaldehyde (42 mg, 1.3 equiv) was refluxed for 4 h in 10 mL round bottom flask. The reaction mixture was then dried to remove all methanol under vacuum to give **4**. Then 2 mL of triethylorthoformate was added and the reaction mixture was refluxed for 4 h (TLC). The crude compound obtained was purified by column chromatography to give **5e** using ethyl acetate:hexane (1:3) as eluents. Yield: 63%, light brown solid; mp: 242–244 °C; ^1H NMR (400 MHz, DMSO-d_6): δ = 3.85 (s, 3H, –OCH_3), 7.08 (d, 1H, *J* = 7.6 Hz, C*H*), 7.28 (d, 1H, *J* = 7.6 Hz, C*H*),

7.40 (s, 1H, C*H*), 8.26 (s, 1H, C*H*), 8.43 (s, 1H, N–C*H*), 8.85 (s, 1H, N=C*H*). Anal. Calcd for $C_{13}H_{11}N_5O_3$: C, 54.74; H, 3.89; N, 24.55. Found: C, 54.80; H, 3.92; N, 24.58.

4.1.9. 5-((Benzo[*d*][1,3]dioxol-6-yl)methyleneamino)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (5f)

A solution of **2** (30 mg, 2 mmol, 1 equiv) in methanol (5 mL) and piperonal (38 mg, 1.2 equiv) was refluxed for 4 h in 10 mL round bottom flask. The reaction mixture was then dried to remove methanol under vacuum to give **4**. Then 2 mL of triethylorthoformate was added and the reaction mixture was refluxed for 6 h (TLC). The crude compound obtained was purified by column chromatography to give **5f** using ethyl acetate:hexane (1:3) as eluents. Yield: 71%, white solid; mp: 251–252 °C; IR (KBr): 3435, 3190, 1718, 1576 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 6.14 (s, 2H, O–CH_2–O), 7.10 (d, 1H, *J* = 7.45, C*H*), 7.40 (d, 1H, *J* = 7.45, C*H*), 7.49 (s, 1H, C*H*), 8.28 (s, 1H, C*H*), 8.43 (s, 1H, N–C*H*), 8.92 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 101.92, 105.27, 105.89, 108.58, 126.43, 126.56, 147.40, 148.04, 151.07, 166.25; MS (APCI): *m*/*z* = 284.13 $[M+1]^+$, *m*/*z* = 164.33 $[M+1]^+$ a fragment peak. Anal. Calcd for $C_{13}H_9N_5O_3$: C, 55.13; H, 3.20; N, 24.73. Found: C, 55.18; H, 3.25; N, 24.79.

4.1.10. 1-Benzyl-5-(benzylideneamino)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9a)

A solution of **7** (200 mg, 8 mmol, 1 equiv) in ethanol (5 mL) and benzaldehyde (136 mg, 1.5 equiv) was refluxed for 4 h in 50 mL round bottom flask. The reaction mixture was then dried to remove all ethanol under vacuum to give **8**. Then 2 mL of triethylorthoformate was added and the reaction mixture was refluxed for 4 h. White crystals obtained on cooling were filtered off, washed with diethyl ether and dried to afford **9a**. Yield: 79%, white solid; mp: 207–209 °C; IR (KBr): 3425, 3106, 1683, 1591 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.45 (s, 2H, CH_2), 7.82 (m, 2H), 7.52 (m, 3H), 7.38 (m, 5H), 8.05 (s, 1H, CH), 8.29 (s, 1H, N–C*H*), 9.47 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 57.75, 107.91, 128.50, 128.56, 128.76, 128.87, 128.92, 129.15, 132.02, 133.19, 134.29, 149.21, 156.77, 157.28, 162.36; MS (APCI): *m*/*z* = 330.20 $[M+1]^+$, *m*/*z* = 227.47 $[M+1]^+$ a fragment peak. Anal. Calcd for $C_{19}H_{15}N_5O$: Calcd C, 69.29; H, 4.59; N, 21.26. Found: C, 69.35; H, 4.63; N, 21.24.

4.1.11. 5-(4-Chlorobenzylideneamino)-1-benzyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9b)

A mixture of **7** (200 mg) in ethanol (5 mL) and 4-chloro benzaldehyde (157 mg, 1.3 equiv) was refluxed for 4 h in 10 mL. round bottom flask. The reaction mixture was dried to remove all ethanol under vacuum to give **8**. Crude product was dissolved in 2 mL acetonitrile and 2 mL of triethylorthoformate and the reaction mixture was refluxed for 4 h (TLC). White crystals obtained on cooling were filtered, washed with diethyl ether and dried to afford **9b**. Yield: 78%; brownish solid; Molecular formula: $C_{19}H_{14}ClN_5O$; Molecular weight: 363; mp: 240–241 °C; IR (KBr, cm^{-1}): 1720, 1635, 1610, 740; ^1H NMR (300 MHz, $CDCl_3$): δ = 5.46 (s, 2H, CH_2), 7.39 (m, 5H, C*H*), 7.44 (d, 2H, *J* = 7.8, C*H*), 7.77 (d, 2H, *J* = 7.5, C*H*), 8.05 (s, 1H, C*H*), 8.29 (s, 1H, N–C*H*), 9.53 (s, 1H, N=C*H*); MS (APCI): *m*/*z* = 364 $[M+1]^+$, *m*/*z* = 227.47 $[M+1]^+$ a fragment peak. Anal. Calcd for $C_{19}H_{14}ClN_5O$: Calcd C, 62.73; H, 3.88; N, 19.25. Found C, 62.80; H, 3.86; N, 19.28.

4.1.12. 5-(4-Cyanobenzylideneamino)-1-benzyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9c)

A solution of **7** (30 mg, 1 mmol, 1 equiv) in ethanol (5 mL) and 4-cyano benzaldehyde (21 mg, 1.2 equiv) was refluxed for 4 h in 10 mL round bottom flask. The reaction mixture was then dried to remove all ethanol under vacuum to give **8**. Then 2 mL of triethylorthoformate was added and the reaction mixture was refluxed for 4 h (TLC). The crude compound obtained was purified by column chromatography to give **9c** using ethyl acetate:hexane (1:2) as eluents. Yield: 58%, Creamish white solid; mp: 226–230 °C; IR (KBr): 3442, 3114, 2224, 1694, 1585 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 5.52 (s, 2H, CH_2), 7.37 (m, 5H, C*H*), 8.02 (d, 2H, *J* = 7.8 Hz, C*H*), 8.08 (d, 2H, *J* = 7.8 Hz, C*H*), 8.46 (s, 1H, C*H*), 8.87 (s, 1H, N–C*H*), 9.39 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, DMSO-d_6): 56.76, 107.07, 128.52, 128.61, 129.14, 129.44, 131.02, 133.40, 136.52, 137.51, 148.36, 155.81, 162.08; MS (APCI): *m*/*z* = 355.27 $[M+1]^+$, *m*/*z* = 227.53 $[M+1]^+$ a fragment peak. Anal. Calcd for $C_{20}H_{14}N_6O$: C, 67.79; H, 3.98; N, 23.72. Found: C, 67.87; H, 3.93; N, 23.77.

4.1.13. 5-(3-Methoxy-4-hydroxybenzylideneamino)-1-benzyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9d)

A solution of **7** (30 mg, 1 mmol, 1 equiv) in ethanol (5 mL) and 3-methoxy-4-hydroxy benzaldehyde (26 mg, 1.3 equiv) was refluxed for 4 h in 10 mL round bottom flask. The reaction mixture was then dried to remove all ethanol under vacuum to give **8**. Then 2 mL of triethylorthoformate was added and the reaction mixture was refluxed for 4 h (TLC). The crude compound obtained was purified by column chromatography to give **9d** using ethyl acetate:hexane (1:2) as eluents. Yield: 60%, Yellowish white solid; mp: 199–201 °C; IR (KBr): 3263, 1686, 1595 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 3.96 (s, 3H, –OCH_3), 5.45 (s, 2H, CH_2), 6.91 (d, 1H, *J* = 8.16 Hz, C*H*), 7.21 (m, 1H, C*H*), 7.38 (m, 5H, C*H*), 7.50 (s, 1H, C*H*), 8.05 (s, 1H, C*H*), 8.26 (s, 1H, N–C*H*), 9.14 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, $CDCl_3$): 56.10, 57.75, 107.90, 108.25, 114.48, 125.29, 125.35, 128.58, 128.63, 128.93, 129.15, 134.27, 147.12, 148.65, 149.80, 156.49, 157.40, 163.66; MS (APCI): *m*/*z* = 376.13 $[M+1]^+$, *m*/*z* = 227.40 $[M+1]^+$ a fragment peak. Anal. Calcd for $C_{20}H_{17}N_5O_3$: C, 63.99; H, 4.56; N, 18.66. Found: C, 63.83; H, 4.66; N, 18.69.

4.1.14. 5-(3-Hydroxy-4-methoxybenzylideneamino)-1-benzyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9e)

A solution of **7** (30 mg, 1 mmol, 1 equiv) in ethanol (5 mL) and 3-hydroxy-4-methoxy benzaldehyde (26 mg, 1.3 equiv) was refluxed for 4 h in 10 mL round bottom flask. The reaction mixture was then dried to remove all ethanol under vacuum to give **8**. Then 2 mL of triethylorthoformate was added and the reaction mixture was refluxed for 4 h (TLC). The crude compound obtained was purified by column chromatography to give **9e** using ethyl acetate:hexane (1:2) as eluents. Yield: 65%, brown solid; mp: 208–210 °C; IR (KBr): 3453, 1685, 1596 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 3.96 (s, 3H, –OCH_3), 5.45 (s, 2H, CH_2), 5.81 (s, 1H, D_2O exchangeable O*H*), 6.91 (d, 1H, *J* = 8.2 Hz, C*H*), 7.28 (d, 1H, *J* = 8.2 Hz, C*H*), 7.38–7.36 (m, 5H, C*H*), 7.50 (s, 1H, C*H*), 8.04 (s, 1H, C*H*), 8.26 (s, 1H, N–C*H*), 9.24 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, $CDCl_3$): 57.06, 57.75, 107.95, 110.33, 113.04, 122.86, 126.58, 128.57, 128.63, 128.92, 129.15, 134.29, 146.06, 149.03, 150.01, 156.67, 157.37, 162.66; MS (APCI): *m*/*z* = 376.20 $[M+1]^+$, *m*/*z* = 227.47 $[M+1]^+$ a fragment peak. Anal. Calcd for$C_{20}H_{17}N_5O_3$: C, 63.99; H, 4.56; N, 18.66. Found: C, 63.85; H, 4.60; N, 18.61.

4.1.15. 5-((Benzo[*d*][1,3]dioxol-6-yl)methyleneamino)-1-benzyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9f)

A solution of **7** (30 mg, 1 mmol, 1 equiv) in ethanol (5 mL) and piperonal (23 mg, 1.2 equiv) was refluxed for 4 h in 10 mL round bottom flask. The reaction mixture was then dried to remove all ethanol under vacuum to give **8**. Then 2 mL of triethylorthoformate was added and the reaction mixture was refluxed for 8 h. White crystals obtained on cooling were filtered off, washed with diethyl ether and dried to afford **9f**. Yield: 68%, white solid; mp: 241–

243 °C; IR (KBr): 3053, 1687, 1596 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 5.51 (s, 2H, CH_2), 6.15 (s, 2H, –O–CH_2–O), 7.08 (d, 1H, J = 7.84 Hz, C*H*), 7.38–7.36 (m, 6H, C*H*), 7.46 (s, 1H, C*H*), 8.37 (s, 1H, C*H*), 8.82 (s, 1H, N–C*H*), 8.98 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, DMSO-d_6): 56.72, 102.43, 106.26, 107.18, 109.08, 126.77, 127.27, 128.50, 128.59, 129.13, 130.61, 136.58, 148.00, 148.59, 151.43, 155.55, 157.64, 165.51; MS (APCI): m/z = 374.27 $[M+1]^+$, m/z = 227.47 $[M+1]^+$ a fragment peak. Anal. Calcd for $C_{20}H_{15}N_5O_3$: C, 64.34; H, 4.05; N, 18.76. Found: C, 64.31; H, 4.09; N, 18.81.

4.1.16. 5-(3,4,5-Trimethoxybenzylideneamino)-1-benzyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9g)

A solution of **7** (30 mg, 1 mmol, 1 equiv) in ethanol (5 mL) and 3,4,5-trimethoxy benzaldehyde (32 mg, 1.3 equiv) was refluxed for 4 h in 10 mL round bottom flask. The reaction mixture was then dried to remove all ethanol under vacuumto give **8**. Then 2 mL of triethylorthoformate was added and the reaction mixture was refluxed for 4 h (TLC). The crude compound obtained was purified by column chromatography to give **9g** using ethyl acetate:hexane (1:2) as eluents. Yield: 75%, white solid; mp: 191–194 °C; IR (KBr): 3434, 1693, 1609, 1591 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 3.92 (s, 9H, –OCH_3), 5.45 (s, 2H, CH_2), 7.08 (s, 2H, C*H*), 7.38–7.36 (m, 5H, C*H*), 8.05 (s, 1H, C*H*), 8.29 (s, 1H, N–C*H*), 9.32 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, $CDCl_3$): 56.26, 57.77, 61.02, 105.59, 107.86, 128.29, 128.58, 128.75, 128.95, 129.16, 134.26, 141.50, 149.00, 153.53, 156.69, 157.27, 162.46;MS (APCI): m/z = 420.13 $[M+1]^+$, m/z = 227.40 $[M+1]^+$ a fragment peak. Anal. Calcd for $C_{22}H_{21}N_5O_4$: C, 63.00; H, 5.05; N, 16.70. Found: C, 63.08; H, 5.10; N, 16.72.

4.1.17. 5-(2,3,4-Trimethoxybenzylideneamino)-1-benzyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9h)

A solution of **7** (30 mg, 1 mmol, 1 equiv) in ethanol (5 mL) and 2,3,4-trimethoxy benzaldehyde (32 mg, 1.3 equiv) was refluxed for 4 h in 10 mL round bottom flask. The reaction mixture was then dried to remove all ethanol under vacuumto give **8**. Then 2 mL of triethylorthoformate was added and the reaction mixture was refluxed for 4 h (TLC). The crude compound obtained was purified by column chromatography to give **9h** using ethyl acetate:hexane (1:2) as eluents. Yield: 73%, Yellowish white solid; mp: 155–157 °C; IR (KBr): 3433, 3069, 1694, 1592 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 3.88 (s, 3H, –OCH_3), 3.93 (s, 3H, –OCH_3), 3.96 (s, 3H, –OCH_3), 5.45 (s, 2H, CH_2), 6.78 (d, 1H, J = 8.9 Hz, C*H*), 7.37–7.34 (m, 5H, C*H*), 7.84 (d, 1H, J = 8.9 Hz, C*H*), 8.05 (s, 1H, C*H*), 8.24 (s, 1H, N–C*H*), 9.37 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, $CDCl_3$): 56.16, 57.72, 60.97, 62.14, 107.89, 107.92, 119.27, 122.11, 128.58, 128.89, 129.13, 134.30, 141.86, 148.44, 154.67, 156.29, 157.35, 157.51, 160.51; MS (APCI): m/z = 420.20 $[M+1]^+$, m/z = 227.53 $[M+1]^+$ a fragment peak. Anal. Calcd for $C_{22}H_{21}N_5O_4$: C, 63.00; H, 5.05; N, 16.70. Found; C, 63.04; H, 5.11; N, 16.76.

4.1.18. 5-(3,4-Dimethoxybenzylideneamino)-1-benzyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9i)

A solution of **7** (40 mg, 1 mmol, 1 equiv) in ethanol (5 mL) and 3,4-dimethoxy benzaldehyde (43 mg, 1.5 equiv) was refluxed for 4 h in 10 mL round bottom flask. The reaction mixture was then dried to remove all ethanol under vacuum to give **8**. Then 2 ml of triethylorthoformate was added and the reaction mixture was refluxed for 4 h (TLC). The crude compound obtained was purified by column chromatography to give **9i** using ethyl acetate:hexane (1:2) as eluents. Yield: 76%, white solid; mp: 209–211 °C; IR (KBr): 3425, 3106, 1683, 1591 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 3.95 (s, 3H, –OCH_3), 3.96 (s, 3H, –OCH_3), 5.45 (s, 2H, CH_2), 6.91 (d, 1H, J = 8.4 Hz, C*H*), 7.27 (d, 1H, J = 8.4 Hz, C*H*), 7.38 (m, 5H, C*H*), 7.52 (s, 1H, C*H*), 8.04 (s, 1H, C*H*), 8.27 (s, 1H, N–C*H*), 9.21 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 107.92, 108.50, 110.61, 124.68, 125. 82, 128.56, 128.90, 129.12, 134.29, 148.74, 149.51, 152.72, 156.62, 157.40, 163.21; MS (APCI): m/z = 390.27 $[M+1]^+$, m/z = 227.47 $[M+1]^+$ a fragment peak. Anal. Calcd for $C_{21}H_{19}N_5O_3$: C, 64.77; H, 4.92; N, 17.98. Found: C, 64.72; H, 4.98; N, 18.10.

4.1.19. 5-(2,4,5-Trimethoxybenzylideneamino)-1-benzyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9j)

For the synthesis of compound-**5**, mixture of **2** (200 mg) in ethanol (5 mL) and 2,4,5-trimethoxy benzaldehyde (168 mg, 1.3 equiv) was refluxed for 4 h in 10 mL. round bottom flask. The reaction mixture was dried to remove all ethanol under vacuum to give **8**. Crude product was dissolved in 2 mL acetonitrile and 2 mL of triethylorthoformate and the reaction mixture was refluxed for 4 h (TLC). White crystals obtained on cooling were filtered, washed with diethyl ether and dried to afford **9j**. Yield: 70%; color: white solid; mp: 228–230 °C; IR (KBr, cm^{-1}): 1710, 1635, and 1620; ^1H NMR (300 MHz, $CDCl_3$): δ = 3.86 (s, 3H, –OCH_3), 3.92 (s, 3H, –OCH_3), 3.96 (s, 3H, –OCH_3), 5.45 (s, 2H, CH_2), 6.49 (s, 1H, C*H*), 6.91 (s, 1H, C*H*), 7.37 (7.36–7.38, m, 5H, C*H*), 8.23 (s, 1H, C*H*), 8.45 (s, 1H, N–C*H*), 9.35 (s, 1H, N=C*H*); MS (APCI): m/z = 420 $[M+1]^+$ m/z = 227.47 $[M+1]^+$ a fragment peak. Anal. Calcd for$C_{22}H_{21}N_5O_4$: C, 63.00; H, 5.05; N, 16.70. Found: C, 63.09; H, 5.03; N, 16.68.

4.1.20. 5-((Furan-2-yl)methyleneamino)-1-benzyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9k)

A solution of **7** (30 mg, 1 mmol, 1 equiv) in ethanol (5 mL) and 2-furaldehyde (15 mg, 1.2 equiv) was refluxed for 4 h in 10 mL round bottom flask. The reaction mixture was then dried to remove all ethanol under vacuum to give **8**. Then 2 mL of triethylorthoformate was added and the reaction mixture was refluxed for 4 h (TLC). The crude compound obtained was purified by column chromatography to give **9k** using ethyl acetate:hexane (1:2) as eluents. Yield: 55%, brown solid; mp: 158–161 °C; IR (KBr): 3427, 3092, 1692, 1607 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.45 (s, 2H, CH_2), 6.34 (m, 1H, C*H*), 7.07 (m, 1H, C*H*), 7.38–7.36 (m, 5H, C*H*), 7.69 (m, 1H, C*H*), 8.21 (s, 1H, C*H*), 8.40 (s, 1H, N–C*H*), 9.42 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, $CDCl_3$): 112.41, 117.94, 128.58, 128.76, 128.96, 129.17, 146.33, 149.31, 150.24; MS (APCI): m/z = 320.20 $[M+1]^+$, m/z = 227.40 $[M+1]^+$ a fragment peak. Anal. Calcd for $C_{17}H_{13}N_5O_2$: C, 63.94; H, 4.10; N, 21.93. Found: C, 63.97; H, 4.13; N, 21.97.

4.1.21. Synthesis of 9l

A solution of **7** (400 mg, 1 mmol, 1 equiv) in triethylorthoformate (2 mL) was refluxed for 12 h (TLC). The crude compound obtained was purified by column chromatography to give **9l** using ethyl acetate:hexane (1:5) as eluents. Yield: 57%, Creamish white solid; mp: 201–203 °C; IR (KBr): 1703, 1617, 1584 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 1.34 (t, J = 7.04 Hz, 3H, CH_3), 4.32 (q, J = 6.99 Hz, 2H, OCH_2), 5.49 (s, 2H, CH_2), 7.36–7.32 (m, 5H), 8.15 (s, 1H, CH), 8.50 (s, 1H, N–C*H*), 8.78 (s, 1H, N=C*H*); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 14.40, 56.67, 64.64, 107.16, 128.43, 128.52, 129.10, 130.10, 136.17, 148.08, 155.55, 157.93, 168.74; MS (APCI): m/z = 298.27 $[M+1]^+$, m/z = 227.47 $[M+1]^+$ a fragment peak. Anal. Calcd for $C_{15}H_{15}N_5O_2$: C, 60.60; H, 5.09; N, 23.56. Found: C, 60.68; H, 5.23; N, 23.53.

4.1.22. Synthesis of 5-amino-1,5-dihydro-1-benzyl-pyrazolo[3,4-*d*]pyrimidin-4-one (9m)

A solution of **7** (400 mg, 1 mmol, 1 equiv) in acetonitrile (2 mL) and triethylorthoformate (1 mL) was refluxed for 7 h. One drop of H_2SO_4 was also added. White crystals obtained on cooling were

filtered off, washed with diethyl ether and dried to afford **9m**. Yield: 54%, white solid; mp: 184–186 °C; IR (KBr): 3328, 1697, 1586 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 5.47 (s, 2H, D_2O exchangeable NH_2), 5.60 (s, 2H, CH_2), 7.30–7.35 (m, 6H), 8.17 (s, 1H, C*H*), 8.72 (s, 1H, N–C*H*); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 56.65, 106.67, 128.42, 128.51, 129.09, 129.56, 136.67, 150.91, 158.31, 158.59. Anal. Calcd for $C_{12}H_{11}N_5O$: C, 59.74; H, 4.60; N, 29.03. Found: C, 59.58; H, 4.30; N, 29.21.

4.1.23. Synthesis of 5-(4-methoxybenzylideneamino)-1-benzyl-6,7-dihydro-6-(4-methoxyphenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9n)

A mixture of **7** (200 mg) in ethanol (5 mL) and anisaldehyde (1.6 mL, 1.3 equiv) was refluxed for 4 h in 10 mL round bottom flask. White crystals obtained on cooling were filtered, washed with diethyl ether and dried to afford **9n**. Yield: 70%; color: white solid; mp: 161–163 °C; IR (KBr, cm^{-1}): 3200, 1730, and 1635; ^1H NMR (300 MHz, $CDCl_3$): δ = 3.84 (s, 3H, –OCH_3), 3.92 (s, 3H, –OCH_3), 5.27 (s, 2H, CH_2), 6.92 (d, 2H, *J* = 8.1 Hz, CH), 7.05 (d, 2H, *J* = 7.8, CH), 7.26 (s, 1H, CH), 7.37 (m, 5H,CH), 7.74 (d, 2H, *J* = 8.1 Hz, CH), 7.90 (s, 2H, *J* = 7.8 Hz, CH), 8.11 (s, 1H, CH), 9.06 (s, 1H, N=C*H*), 11.53 (s, D_2O Exchangeable proton NH); MS (APCI): *m*/*z* = 468 $[M+1]^+$.Anal. Calcd for $C_{27}H_{25}N_5O_3$: C, 69.36; H, 5.39; N, 14.98. Found; C, 69.41; H, 5.63; N, 14.84.

4.1.24. Synthesis of 5-(2-chlorobenzylideneamino)-1-benzyl-6,7-dihydro-6-(2-chlorophenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9o)

A mixture of **7** (200 mg) in ethanol (5 mL) and 2-chlorobenzaldehyde (1.57 mL, 1.3 equiv) was refluxed for 4 h in 10 mL round bottom flask. White crystals obtained on cooling were filtered, washed with diethyl ether and dried to afford **9o**. Yield: 76%; color: white solid; mp: 231–233 °C; IR (KBr, cm^{-1}): 3100, 1720, and 1630; ^1H NMR (300 MHz, DMSO-d_6): δ = 5.18 (s, 2H, CH_2), 7.38 (m, 12H, C*H*), 7.45 (m, 2H, C*H*), 8.97 (s, 1H, C*H*), 9.36 (1H, N=C*H*), 11.63 (s, 1H, D_2O exchangeable proton N*H*); MS (APCI): *m*/*z* = 477 $[M+1]^+$. Anal. Calcd for$C_{27}H_{25}N_5O_3$: C, 69.36; H, 5.39; N, 14.98. Found: C, 69.26; H, 5.32; N, 14.79.

4.1.25. 1-Benzyl-5-(benzylamino)-1H-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9p)

To a solution of **9a** (30 mg, 1 mmol, 1 equiv) in ethanol (5 mL) was added sodium borohydride (2 equiv). The reaction mixture was stirred at rt for 4 h (TLC). The crude compound obtained was purified by column chromatography to give **9p** using ethyl acetate:hexane (1:1) as eluents. Yield: 50%, semi-solid; IR (KBr): 3476, 2923, 2853, 1726, 1636 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 4.11 (s, 2H, CH_2), 5.46 (s, 2H, CH_2), 6.84 (s, 1H, D_2O exchangeable proton N*H*), 7.29–7.33 (m, 10H, C*H*), 7.84 (s, 1H, N=C*H*); ^{13}C NMR (75 MHz, $CDCl_3$): 54.42, 56.68, 106.99, 127.99, 128.55, 128.84, 129.11, 129.36, 129.60, 129.92, 136.61, 137.51, 151.20, 157.77, 158.28; MS (APCI): *m*/*z* = 332.33 $[M+1]^+$. Anal. Calcd for $C_{19}H_{17}N_5O$: C, 68.87; H, 5.17; N, 21.13. Found: C, 68.93; H, 5.23; N, 21.09.

4.2. Biology

4.2.1. Cell culture and treatments

All the cell lines were procured from National cell repository situated at NCCS, Pune. MCF7 (breast adenocarcinoma), PC3 (prostate), H460 (liver), THP-1 (leukemia), IGR-OV-1 (ovarian) and HeLa (cervix) cell lines representing different human cancers were grown in DMEM media supplemented with 10% fetal bovine serum (FBS) and antibiotic solution (1× Penstrip, all the reagents from Invitrogen). Buccal cavity cells were harvested and culture according to the earlier described protocol by Weisberg et al.[32,33] Briefly the mouth was rinsed atleast 2 h before with mouthwash. Cells were harvested in PBS and washed three times with antibiotic containing PBS to remove the contaminants. Cells were cultured in DMEM media with 10% FBS, 50 U/mL penicillin G, 50 μg/mL streptomycin sulfate and 1.25 μg/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 (5000 cells per well of 96-well plate and 100,000 cells per well of 6 well plate). Afterwards cells were washed once with sterile 1× PBS and cultured with serum free media for 8 h for synchronization. The compounds as well as etoposide (positive control) were dissolved in cell culture grade DMSOupto concentration of 100 mM and further dilutions were done in serum free DMEM media. The total amount of media per well (200 μL per well of 96 well plate and 2 mL per well for six well plates) was kept constant and all the treatment volumes were accommodated within these ranges only.

4.2.2. 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

MTT assay was carried out in 96-well plates where total volume of media was 200 μl/well. Briefly cells after the treatments were washed with 1× PBS and were mixed with 100 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 100 μl of DMSO to dissolve the crystals followed by ELISA readings at 570 nm. The results were then plotted in graphs to calculate IC_{50} and dose dependent response to the treatment.

4.2.3. Analysis of total reactive oxygen species

Free radicals were measured using DCFDA dye (Sigma) in two different ways; using fluorescence microscopy and spectrophotometry. For this assay cells were treated for short periods (2 h). For microscopy cells were seeded in 6-well plate on sterile cover slips at a density of 100,000 cells per well followed by described treatments. Afterwards cells were incubated with DCFDA dye at 37 °C for 30 min in dark followed by fixation in 1% paraformaldehyde. The cover slips were then mounted upside down on slides using mounting media and observed under fluorescent microscope (Olympus).[34] For spectrophotometric analysis, cells were taken into solution using trypsin and washed with 1× PBS. Staining was done by incubating the cells with respective dye in 1× PBS on ice for 20 min. Cells were than washed three times with 1× cold PBS and OD was taken in spectrophotometer. The measurement parameters are: for DCFDA, 488 nm excitation and 540 nm emission.[34]

4.2.4. Cell cycle analysis

Cells were resuspended in 500 μl of ice cold 1× PBS (with 0.1% glucose) followed by addition of 5 mL of chilled 70% ethanol and incubated for 30 min on ice. Cells were than washed twice with 10 mL of ice cold PBS. Cells were then resuspended in 300 μl of propidium iodide (Sigma, 69 μM in 38 mM sodium citrate pH 7.4) followed by 20 μl of RNase (10 mg/mL solution in water). Samples were then incubated at 37 °C for 45 min and observed using FACS Calibur (BD). The analysis was done using FACS DIVA software.

4.2.5. Single cell gel electrophoresis, SCGE or Comet assay

DNA fragmentation caused by the essential oil in the breast cancer cells was determined by single cell gel electrophoresis according to the modified method of Olive and Banath.[35] In brief, 1 mL suspension of 15×10^5 cells/mL was mixed with 1 mL of 1% low-gelling-temperature agarose at 40 °C and coated on the slides precoated with 1% agarose. These were kept in the lysis solution

(1.2 M NaCl, 100 mM Na_2EDTA, 0.1% SDS, 0.26 M NaOHwith pH >13) overnight at 4 °C in dark. Rinsed with rinsing solution (0.03 M NaOH, 2 mM Na_2EDTA with pH 12.3) 2–3 times and submerged in the same solution in the Comet Assay Electrophoresis unit (Sci. Plas, UK). Electrophoresis was done in rinse solution for 30 min at a voltage of 15 V and 40 mA current. Cells were analyzed by examining at 50 comet images from each slide under fluorescent microscope (Olympus Magnus). Scoring of the comets obtained was done by using Cometscore15 software Version 1.0.1.0.

4.2.6. Topoisomerase assays

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 topoisomerase 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. TopoI and II inhibition assay were performed as described in the supplier manual with minor modifications as per the requirement.

4.2.7. hTopoIIα mediated DNA decatenation assay

hTopoIIα mediated DNA inhibition activity for the synthesized compounds were performed as follow. Reaction mixture containing freshly prepared 5× 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 human topoisomerase II 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 distained with water for 20 min. The bands were analyzed under UV trans-illuminator and the decatenated kDNA products were quantified with QuantityOne (BioRad).

4.2.8. TopoIIα mediated DNA relaxation assay

Supercoiled plasmid DNA (pRYG)[36,37] was used as substrate in TopoII mediated DNA relaxation assay. Reaction mixture contained freshly prepared 5x complete buffer ((A) 0.5M 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), 250 ng of supercoiled plasmid DNA (pRYG), followed by either test compound or standard drug (100 μM) and finally topoisomerase II (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.

4.2.9. hTopoI mediated DNA relaxation assay

Reaction mixture containing freshly prepared 5× complete buffer (100 mM Tris–HCl (pH 7.9), 10 mM EDTA, 1.5 M Nacl, 1% Bovine Serum Albumin, 1 mM Spermidine and 50% glycerol), 250 ng Supercoiled DNA, 1 unit of topoisomerase I and 100 μM test or camptothecin in total volume of 20 μl. Reaction mixture was then incubated at 37 °C for 30 min and stopped by addition of 10% SDS. 50 μg/mL proteinase K was added to the reaction mixture and further incubated at 37 °C for 15 min. 1% Agarose gel was run until the dye front was at the bottom of the gel. Gel were then stained with ethidium bromide and destained with water for 20 min and photo documented the results using QuantityOne (BioRad).

4.3. Molecular docking

Three dimensional (3D) X-ray structures of human topoisomerase I (PDB entry: 1K4T;[38] resolution: 2.10 Å) and the ATPase domain of human topoisomerase IIα (PDB entry: 1ZXM;[29] resolution: 1.86 Å) were procured from protein data bank. The topoisomerase IIα (1ZXM) is a homodimer consisting of two identical subunits, each contains AMPPNP (5′-adenylyl-β,γ-imidodiphosphate) and Mg^{2+} ion at the ATP binding pocket. The structure of **9d** was drawn in MOE2011.10 and energy minimized using the MMFF94x force field, to a gradient less than 0.001 kcal/mol.[39] A flexible docking was carried out using GOLD 5.0.1[30] in the active site of ATPase domain of hTopoIIα. The GOLD performs genetic algorithm based ligand docking to optimize the conformation of ligand at the receptor binding site. It utilizes Gold Score fitness function to evaluate the various conformations of ligand at the binding site and comprises of four components: protein–ligand hydrogen bond energy, protein–ligand van der Waals (vdw) energy, ligand internal vdw energy, and ligand torsional strain energy. The compound was docked ten times and each pose was ranked according to the Gold Score. To validate the adopted docking procedure, the co-crystallized structure of AMPPNP was extracted from the pdb file (1ZXM)[29] and further docked at the ATPase binding site of topoisomerases IIα. The RMSD value of 0.432 Å between co-crystalized and docked conformation of AMPPNP validates and ensures the reliability of the adopted docking procedure.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.07.016.

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