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Discovery and Mechanistic Study of Tailor-Made Quinoline Derivatives as Topoisomerase 1 Poison with Potent Anticancer Activity

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KEYWORDS: Topoisomerase 1, Camptothecin, poison, ADME, FRAP, γH2AX

ABSTRACT

To overcome chemical limitations of camptothecin (CPT), we report design, synthesis and validation of quinoline based novel class of topoisomerase I (Top1) inhibitors and establish compound **28** (N-(3-(1H-imidazol-1-yl)propyl)-6-(4-methoxyphenyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine) exhibits the highest potency in inhibiting human Top1 activity with a IC_{50} value of 29 nM. Compound **28** traps Top1-DNA cleavage complexes (Top1cc) both in the in vitro cleavage assays and in live cells. Point mutation of Top1-N722S fails to trap compound **28**-induced Top1cc due to its inability to make hydrogen bond with compound **28**. Unlike CPT, compound **28** shows excellent plasma serum stability and is not a substrate of P-glycoprotein 1 (permeability glycoprotein; Pgp) advancing its potential anticancer activity. Finally, we provide evidence that compound **28** overcomes the chemical instability of CPT in human breast adenocarcinoma cells through generation of persistent and less reversible Top1cc-induced DNA double strand breaks as detected by γH2AX foci immunostaining after 5h of drug removal.

INTRODUCTION

Topoisomerases are ubiquitous enzymes, which are indispensable for relaxing the topological constraints arising during the DNA metabolic processes of replication, transcription, and chromatin remodeling.[1–4] Human topoisomerase 1 (HTop1) creates a single-stranded nick onto the DNA by a nucleophilic attack on the DNA phosphodiester bond to form a "cleavage complex" in which the enzyme is covalently linked to the 32 end of the broken DNA strand (Top1 cleavage complexes; Top1cc). This is then followed by a "controlled rotation" of the broken scissile strand around the intact strand resulting in the relaxation of the DNA super helical tension. Eventually, the 5' end of the scissile strand mediates a nucleophilic attack on the phosphotyrosyl-DNA phosphodiester to religate the DNA and release the enzyme which ends the catalytic cycle of the enzyme.[5,6] Trapped Top1cc's generate detrimental lesions which lead to the formation of DNA double-strand breaks (DSBs) upon collision with ongoing replication forks and/or transcription machinery and are accountable for the killing of proliferating malignant cancer cells.[2,7–9]

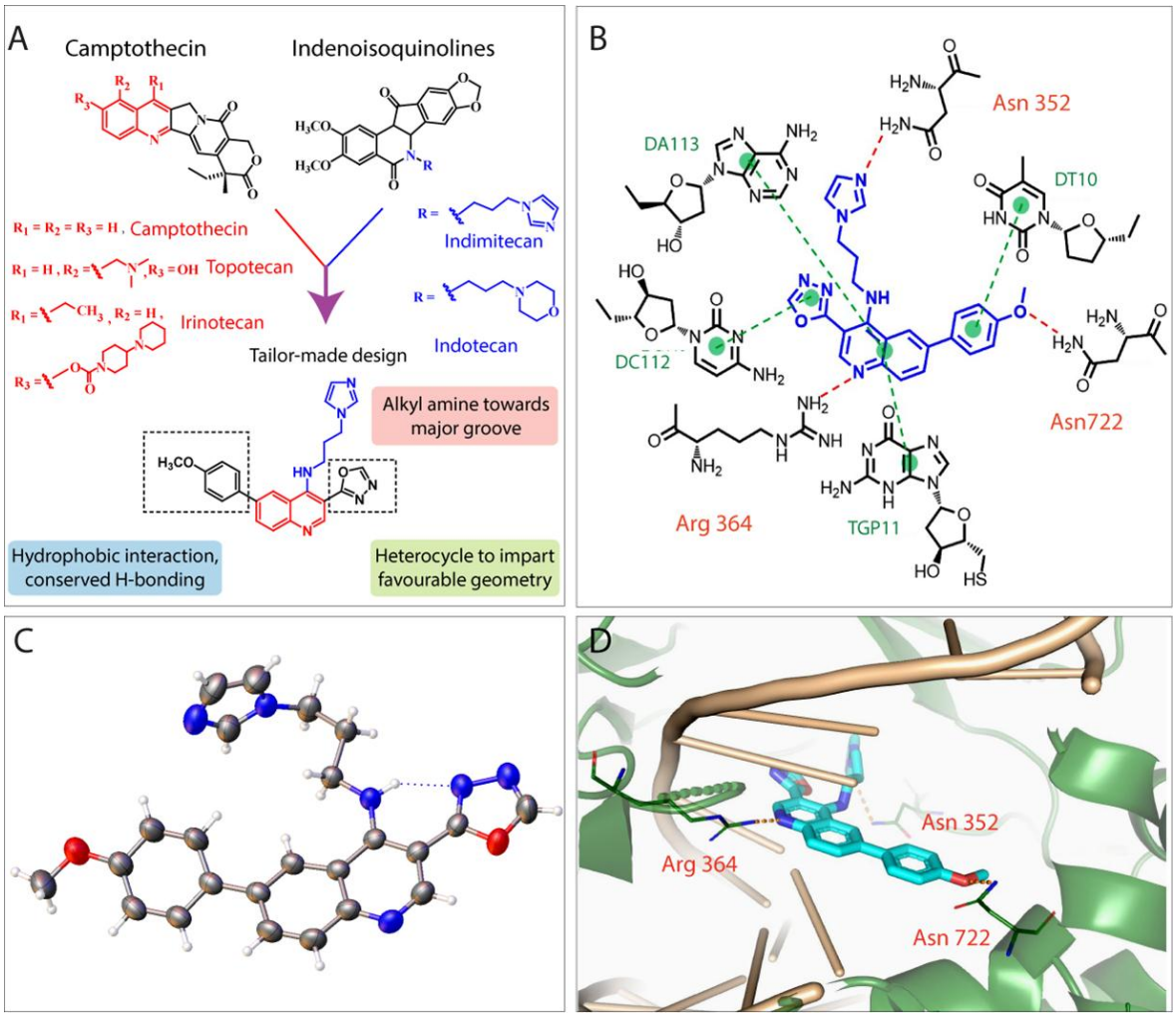


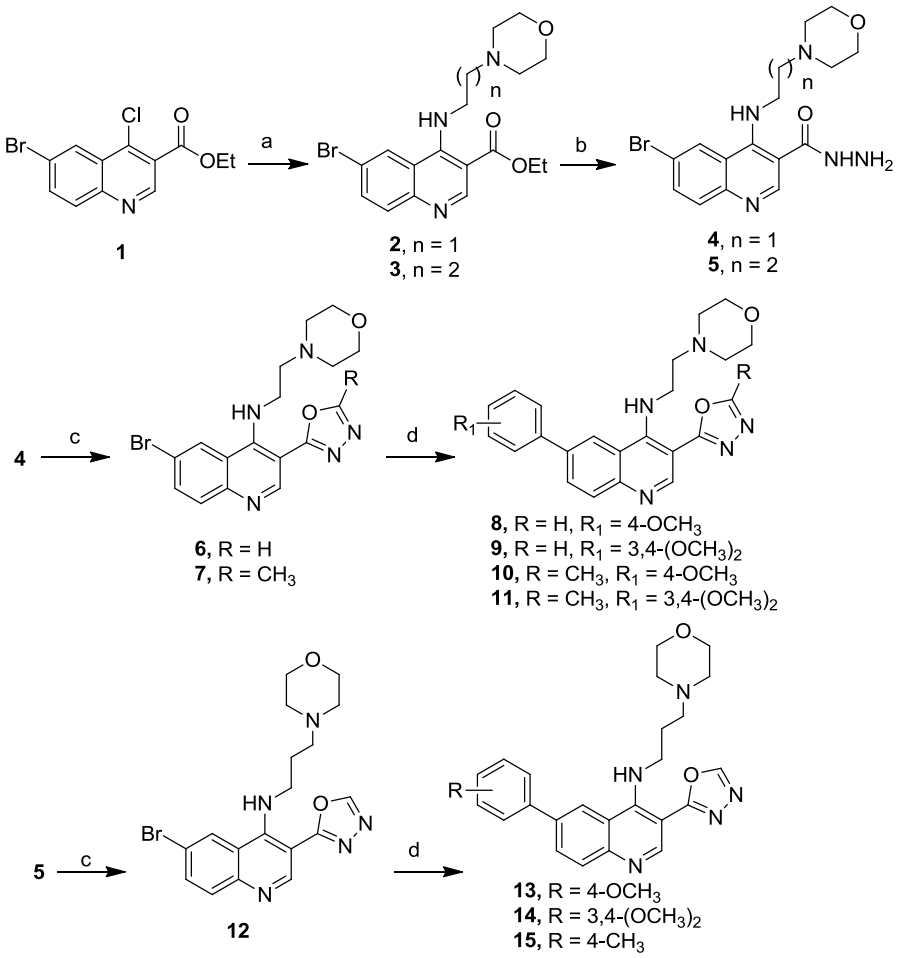
Figure 1. **(A)** The design of tailor-made analog conceived from the structural features of known ligand and analysis of the active site; **(B)** 2D binding interactions of compound **28** with the important amino acid residues and base pairs. Red dotted lines imply hydrogen bond and green dotted lines indicate π-π stacking interaction; **(C)** X-ray crystal structure of compound **28**; **(D)** Hypothetical binding model of the ternary complex of human Top1-DNA-**28**. The structure was generated from the crystal structure of Top1-DNA-CPT (PDB ID: 1T8I).

There are two types of topoisomerase inhibitors, which include catalytic inhibitors and poisons.[2,5,10,11] Catalytic inhibitors directly bind with the free topoisomerase and inhibit its catalytic activity.[12,13] While, poisons reveal their anticancer activity by selectively trapping the Top1-DNA covalent cleavage

complexes[9,14,15] (Top1cc stabilizes the Top1-DNA cleavage complex) inside cells which include camptothecin (CPT)[16] and its clinical derivatives as well as several non-CPT Top1 poisons including indenoisoquinolines,[17,18] indolocarbazoles,[19] and thiohydantoin[20] derivatives. Clinical derivatives of camptothecin like topotecan and irinotecan are used for advanced colorectal carcinomas and ovarian cancers. However, CPT and its derivatives suffer from dose-limiting toxicity resulting in severe diarrhea and neutropenia rapidly inactivated in plasma due to hydrolysis of lactone E-ring and binding of the ensuing hydroxyl acid to plasma proteins and finally accumulate resistance in the Topoisomerase I (Top1) gene.[6,15] CPT was also found to be a substrate of Pgp.[21] Moreover, irinotecan is a prodrug, which requires a carboxylesterase enzyme to convert into an active metabolite.[22] Recent advances in developing non-CPT Top1-targeted drug leads to the discovery of indenoisoquinolines (indotecan and indimitecan) which are in clinical trials for treatment in adults with solid tumors and lymphomas.[15,22,23]

Here we report design, synthesis, mechanism study and validation of a novel class of Top1 poison based on quinoline core, identified through the structural features of known ligands (CPT, topotecan, irinotecan and indenoisoquinolines) binding through network of interactions in the active site of human Top1 enzyme as revealed by cocrystal structures[18,24–29] (Figure 1A) for potent anticancer activity. Compound **28** mediated induction of Top1cc formation in live cells was substantiated by fluorescence recovery after photobleaching (FRAP) assays. We also show Top1-N722[30,31] is critical for the compound's *in vivo* trapping of Top1cc in cancer cells treated with compound **28**. We provide compelling *in vitro* ADME, biochemical and cellular evidence that advocate for compound **28** as a potential anticancer agent.

Scheme 1[a]



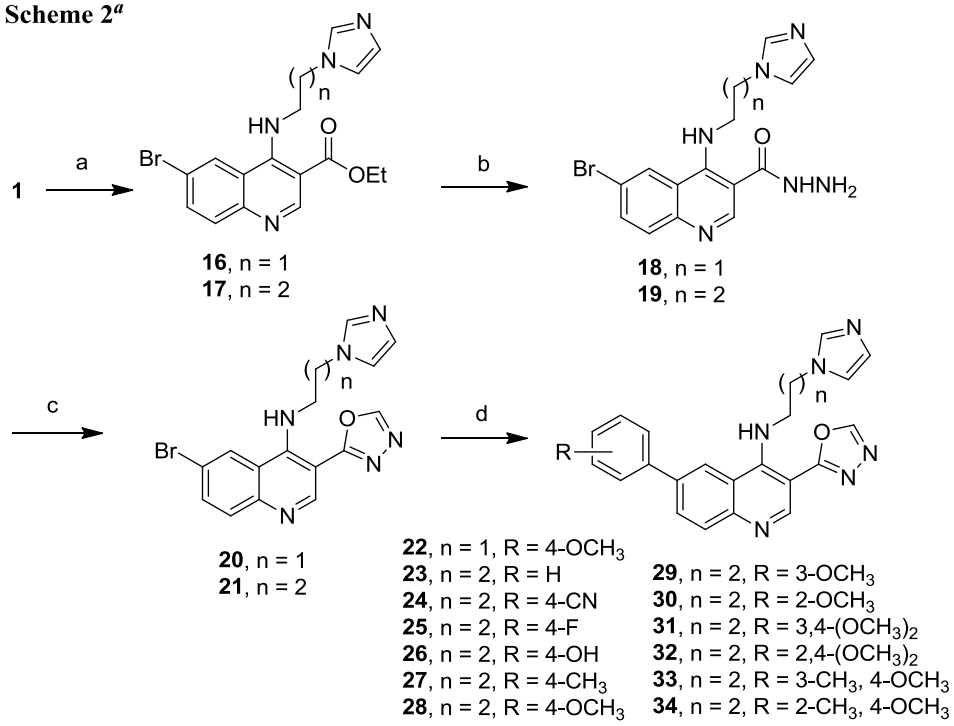
[a]Reagents and conditions: (a) 2-Morpholinoethanamine (for **2**) or 3-morpholinopropan-1-amine (for **3**), DIPEA, 1,4-dioxane, rt, 12 h; (b) Hydrazine monohydrate, EtOH, rt, 8-12 h; (c) Triethyl orthoformate, 110 ºC, 12 h (for **6** and **12**) or triethyl orthoacetate, EtOH, reflux, 8 h (for **7**); (d) 2-(4-Methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (for **8**, **10** and **13**), (3,4-dimethoxyphenyl)boronic acid (for **9**, **11** and **14**), *p*-tolylboronic acid (for **15**), $Pd(PPh_3)_4$, 2M Na_2CO_3, 1,4-dioxane, 100 ºC, 8-12 h.

CHEMISTRY

Compound **1** was treated with suitable aminoalkyl amines in the presence of a base (DIPEA) in 1,4-dioxane solvent to afford compound **2** and **3** in excellent yield. Compound **2** or **3** was dissolved in

ethanol and treated with the solution of hydrazine monohydrate to obtain compound **4** and **5** respectively. Compound **6** was prepared by heating compound **4** with triethyl orthoformate at 110 ºC for 12 hours. Similarly, compound **4** was refluxed with triethyl orthoacetate in ethanol for 8 hours to obtain **7**. Compound **4** was subjected to Suzuki coupling reaction with suitable boronic acids to get compound **8**-**11**. Similar Suzuki reaction of **5** with the respective boronic acid provided compound **13**-**15** (Scheme 1). Similar reaction sequence (Scheme 2) was followed for imidazole analogs. Finally, the Suzuki coupling was performed to produce a series of compounds (**22**-**34**).

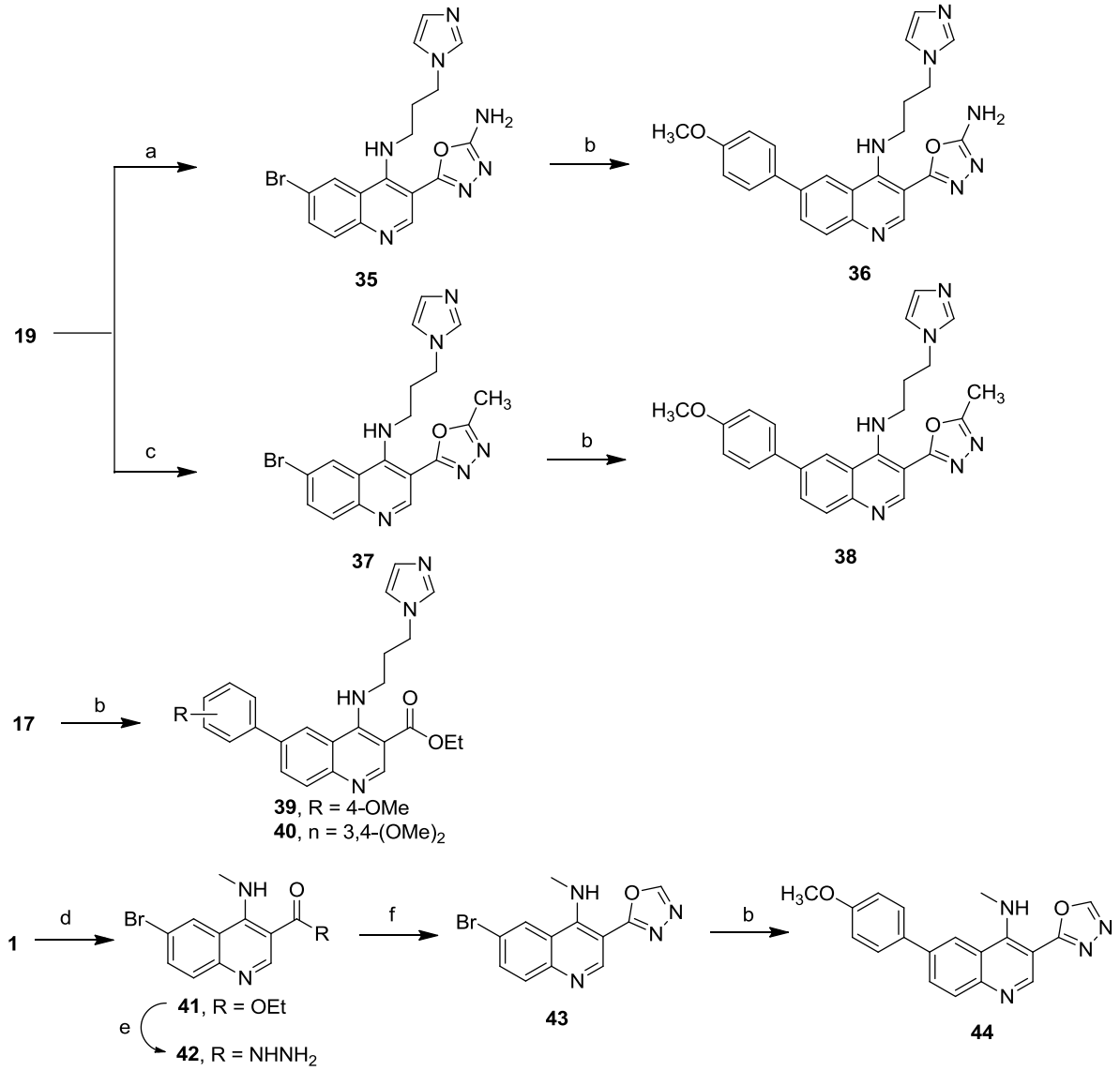
Scheme 2[a]



[a]Reagents and conditions: (a) 2-(1*H*-Imidazole-1-yl)ethanamine (for **16**) or 1-(3-aminopropyl)imidazole (for compound **17**), DIPEA, 1,4-dioxane, rt (for **17**) or 100 ºC (for **16**), 12 h; (b) Hydrazine monohydrate, EtOH, rt, 8-12 h; (c) Triethyl orthoformate, 100 ºC, 12 h; (d) Various boronic acids, $Pd(PPh_3)_4$, 2M Na_2CO_3, 1,4-dioxane, 100 ºC, 7-24 h.

In scheme 3, compound **19** was converted to oxadiazol-2-amine derivative **35** and oxadiazol-5-methyl derivative **37** by treating with cyanogen bromide in methanol and triethyl orthoacetate in ethanol respectively under refluxing condition. Suzuki reaction of **35** and **37** with 2-(4-methoxyphenyl)boronic ester provided compound **36** and **38** respectively. Similar Suzuki reaction of **17** with the respective boronic acid provided compound **39** and **40**. By following the similar reaction sequence in scheme 1, compound **1** was converted to methylamine derivative **41** followed by hydrazine derivative **42**. Subsequent oxadiazole formation with triethyl orthoformate leads to compound **43**, which on Suzuki coupling with 2-(4-methoxyphenyl)boronic ester provided compound **44**. All compounds subjected to assay were checked for > 95% purity by HPLC.

Scheme 3



[a]Reagents and conditions: (a) Cyanogen bromide, MeOH, reflux, 4 h;(b) 2-(4-Methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (for **36**, **38**, **39** and **44**) or (3,4-dimethoxyphenyl)boronic acid (for **40**), $Pd(PPh_3)_4$, 2M Na_2CO_3, 1,4-dioxane, 100 ºC, 6-12 h. (c) Triethyl orthoacetate, EtOH, reflux, 6 h; (d) Methylamine, DIPEA, THF, 60 ºC, 16 h; (e) Hydrazine monohydrate, EtOH, rt, 12 h; (f) Triethyl orthoformate, 140 ºC, 14 h.

RESULTS AND DISCUSSION

Design of Top1 Inhibitor: The basis of design has been depicted in figure 1B or 1A. As CPT and its clinically approved derivatives topotecan and irinotecan suffer from several limitations, we embarked on the discovery of 'non-CPT' Top1 poisons[21,32] with improved efficacy. We initiated our design from the quinoline core, which is also shared by CPT and its clinically approved derivatives topotecan and irinotecan (Figure 1A). The substitution pattern on quinoline core was strategically positioned at C-3, C-4 and C-6 to avail the conserved hydrogen bond interactions, hydrophobic interactions such as π-π stacking and to attain the requisite geometry to be able to stabilize the Top1-DNA cleavage complex (Figure 1B).[5] In the indenoisoquinoline series, the importance of suitable alkyl amino chain containing a heteroatom has been previously validated as it is capable of serving as a hydrogen-bond acceptor into the major groove of the ternary complex.[33–35] Also, the dimethylamine group in topotecan is predicted to project into the major groove to attain similar interactions. We presumed that similar alkyl amino chain at the C-4 position of quinoline core will project in the major groove towards interacting residue Asn352. The alkyl chain attached heterocycle installed at C-4 can serve a similar purpose and attain similar interactions as observed in indenoisoquinoline and CPT derivatives.[36] The crystal structures of the ternary complex of CPT and indenoisoquinoline revealed the importance of polycyclic rings with flat planar geometry that can accommodate in the interface of the Top1-DNA cleavage complexes and block the relegation reaction. We envisioned that the strategically placed C-6 substituted aromatic ring along with a heterocycle at C-3 position would impart requisite geometry and suitable curvature essential to attain the conserved hydrogen bond interactions as well as hydrophobic interactions such as π-π stacking for stabilizing Top1-DNA cleavage complex (Figure 1B). As a next logical step, we explored the nature of substitution on the aromatic ring at C-6 and on heterocycles at C-3 that can modulate top1 inhibition to identify potent Top1 poison.

Related to Top1 inhibition, half maximal inhibitory concentration (IC_{50}) values for the designed analogs were investigated in plasmid DNA relaxation inhibition assays by using either recombinant Top1 or MCF7 cellular lysates as a source of Top1 (Table 1). We used both biochemical as well as cellular studies to validate the mechanism of Top1 inhibition. Further insight into the ability of the potent top1 inhibitor (compound **28**) to trap mutant Top1 at residue Asn722 justified by FRAP assays in live cells. The accumulation and disappearance of DNA damage by the identified compounds were measured by immunological staining with phosphorylated histone H2AX (γH2AX) under confocal microscopy. Subsequently, *in vitro* ADME studies were conducted to determine the metabolic stability and efflux property of these analogs.

Morpholine analogs. We synthesized the first batch of compounds keeping alkyl substituted morpholine group at the C-4 position, 4-methoxy and 3,4-dimethoxy phenyl groups at the C-6 position and oxadiazole group at C-3 (Scheme 1). The choice of substitution pattern at C-4 and C-6 position was inspired from the structural features present in CPT derivatives and indenoisoquinoline derivatives (indotecan and indimitecan) (Figure 1A) established as Top1 poison and are currently under clinical trials.[15,23,34,37] Previous reports have suggested that installation of such moiety resulted in improved top1 inhibitory activity.[33,34] Compound **8** with two carbon morpholine group at C-4 and the 4-methoxy group at C-6 and compound **9** with two carbon morpholine group at C-4 and 3,4-dimethoxy phenyl group at C-6 did not show significant Top1 inhibitory activity in the plasmid DNA relaxation assays at 10 μM concentration (Table 1). Although 4-methoxy derivative **8** and 3,4-dimethoxy phenyl derivative **9** did not show significant inhibition at 10 μM but at higher concentration (100 μM) compound **8** showed 50% inhibition as compared to **9** with 20% inhibition. Installation of a methyl group at C-5" position of oxadiazole ring in **8** and **9** resulted in compound **10** and **11** (Scheme 1) respectively which also failed to show 50% Top1 inhibition (Table 1) at 10 μM concentration. Thereafter, we increased the chain length

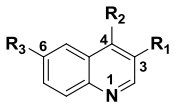
connected to the morpholine group at C-4 from two carbons to three carbons. Indeed, increasing the chain length in compound **13** with the 4-methoxyphenyl group at C-6 showed marked increase in Top1 inhibitory activity with a IC_{50} value of 1.79 μM against recombinant human Top1.[20,38–40] However, **14** with 3,4-dimethoxy phenyl group failed to inhibit at least 50% Top1 relaxation activity (Table 1) at 10 μM concentration. An initial comparison between **8**, **9**, **13** and **14** suggests that combination of three carbon chain length at C-4 with the mono substitution of 4-methoxyphenyl group at C-6 reinforces Top1 inhibitory activity. The importance of 4-methoxyphenyl group at C-6 was further substantiated by replacement of the $–OCH_3$ group present at the C-6 substituted phenyl ring by a methyl group ($-CH_3$) in compound **15** leading to loss of activity. Taken together, the initial studies indicate that morpholine substituted propylamine at C-4 and 4-methoxyphenyl group at the C-6 position are two important features that can trigger Top1 inhibition in quinoline core.

Imidazole analogs. As both morpholine and imidazole group are present in indotecan, indimitecan and indenoisoquinolines derivatives, therefore, we replaced the C-4 substituents containing morpholine moiety with imidazole in our newly synthesized analogs and investigated in Top1 inhibition. A series of compounds was prepared bearing imidazole group with varying chain length at C-4, substituted phenyl group at C-6 and oxadiazole ring at C-3 (Scheme 2). Based on our previous observation which showed the importance of morpholine substituted propylamine at C-4, it seemed prudent to keep the chain length of the flexible aminoalkyl group to three carbon linker. A series of compounds was prepared by substituting the C-4' position of the phenyl group at C-6 with cyano (**24**), fluoro (**25**), hydroxyl (**26**), methyl (**27**) and methoxy (**28**) group (Scheme 2). Compounds with cyano (**24**), fluoro (**25**) and hydroxyl (**26**) modifications failed to show significant Top1 inhibitory activity in plasmid DNA relaxation assays at 10 μM (Table 1). Remarkably, compound **28** bearing 4-methoxy group at C-4' showed the highest ability to inhibit recombinant Top1 activity with the IC_{50} of 29 nM in the plasmid DNA relaxation assay

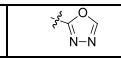
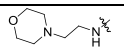
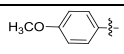
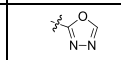
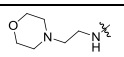
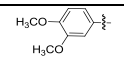
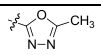
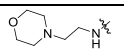
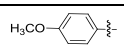
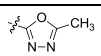
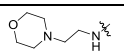
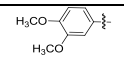
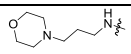
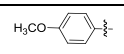
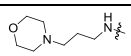
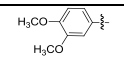
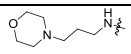
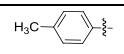
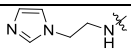
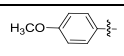
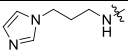
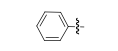
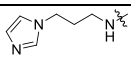
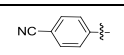
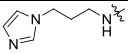
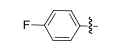
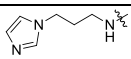
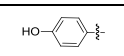
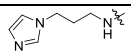
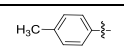
(Table 1).[12] Notably, compound **27** with 4-methyl substitution at C-4' position of the phenyl group also showed Top1 inhibitory activity with an IC_{50} value of 1.06 μM in Top1 inhibition. Installing the unsubstituted phenyl group at C-6 in **23** resulted in a sharp decrease in Top1 inhibitory activity (40% inhibition at 10μM). A direct comparison of the activities of morpholine (**13** and **15**) and imidazole series (**23** and **27**) shows that the imidazole substituted propylamine at C-4 along with the 4-methoxyphenyl group at C-6 position confers increased Top1 inhibitory activity.

For a better understanding, the activity of compound **28**, hypothetical binding models of **28**-Top1-DNA ternary complexes were constructed with the human Top1 crystal structure (PDB code 1T8I). The oxygen atom of the methoxy group makes hydrogen bonding interaction with Asn722 in the C-terminal domain of Top1. Asn722 is an important amino acid responsible for binding of the ligands with Top1 cleavage complexes.[41] The nitrogen atom in the quinoline ring also forms a hydrogen bond with Arg364 and the three carbon alkyl chain length is ideal for imidazole ring to attain hydrogen bond interaction with Asn352. The aminopropylamino chain at C-4 and 4-methoxyphenyl group substitution at the C-6 position of quinoline ring make contacts with the Top1cc and DNA may attribute to Top1 inhibition (Figure 1B and 1D). In keeping with the docking analysis (Figure 1B and 1D), the X-ray crystal structure of compound **28** (Figure 1C) reveals that it has favorable geometry to fit into the 3′ end of the broken DNA strand covalently linked with Top1 and the free 5'-OH end to inhibit Top1 religation activity.[42]

Table 1. Recombinant HTop1 Plasmid DNA Relaxation Inhibition Assays in MCF7 Cell Line



Comp	R_1	R_2	R_3	Top1 inhibition IC_{50} (μM)		Activity index[a]
				In vitro	*Ex vivo*	
CPT				0.025	2.5	++++
Topotecan				0.021	1.8	++++

8				>10	NT[b]	+
9				>10	NT	+
10				>10	NT	+
11				>10	NT	+
13				1.79 ± 0.62	2.51 ± 0.39	+++
14				>10	NT	+
15				>10	NT	+
22				>10	NT	+
23				>10	NT	+
24				>10	NT	+
25				>10	NT	+
26				>10	NT	+
27				1.06±0.417	3.75±0.102	+++
28				0.029±0.004	2.74±0.314	++++
29				1.054±0.792	4.11±0.339	+++
30				>10	NT	+
31				>10	NT	+
32				>10	NT	+
33				2.36±0.884	3±0.98	++
34				>10	NT	+
36				>10	NT	+
38				>10	NT	+
39				>10	NT	+
40				>10	NT	+
44				>10	NT	+

[a]Compound-induced *in vitro* inhibition of Top1 with scores given according to the following system based on the activity of camptothecin: + = IC_{50} > 10 μM; ++ = IC_{50} in between 2 μM and 10 μM; +++ = IC_{50} in between 100 nM and 2 μM; ++++ = IC_{50} < 100 nM. [b]NT = cell line not tested.

To further demonstrate the importance of substitution at C-4' in compound **28**, we synthesized positional analogs of **28** having $–OCH_3$ group at C-2' (**30**) and at C-3' (**29**) position of the phenyl ring (Scheme 2) and were tested for Top1 inhibitory activity. Interestingly, changing the position of $–OCH_3$ group from C-4' to C-3' results in a slight decrease in Top1 inhibition as compound **29** shows the IC_{50} value of 1.05 μM (Table 1). These findings were noteworthy as **29** showed that suitable substitution at C-3' position can also confer Top1 inhibition. However, compound **30** failed to show any significant Top1 inhibition at 10μM. These results underline the importance of suitable substitution at C-4' and C-3'of the phenyl group at the C-6 position of quinoline core required for Top1 inhibition. The result prompted us to revisit the di-substitution pattern on the phenyl group at the C-6 position. In the next series of di-substituted compounds (**31**, **32**, **33** and **34**), the position of $–OCH_3$ group was fixed at C-4' and another $–OCH_3$ or $–CH_3$ was introduced at C-2' and C-3' position (Scheme 2). Compound **31** and **32** with dimethoxy substitution on the phenyl group at C-6 failed to show significant Top1 inhibition at 10 μM (Table 1). A similar result was obtained for morpholine analog **14**. Interestingly, replacing one of the $–OCH_3$ groups at 3' position with $–CH_3$ in **33** provided best top1 inhibition (IC_{50} value 2.36 μM) among di-substituted phenyl group at C-6 (Table 1).

In the next development, keeping the optimized features at C-4 and C-6 position constant, a limited survey of C-5" position of oxadiazole ring was performed by incorporating small hydrophilic and hydrophobic groups to determine the nature of substituents that can be accommodated for modulation of Top1 inhibition. Compound **36** with hydrophilic $–NH_2$ group resulted in a drastic fall in the inhibitory activity. Installation of small hydrophobic $–CH_3$ group at C-5" resulted in a similar drop in Top1 activity (compound **38**). On a similar note, substituting aminopropylamino chain at C-4 with methylamine in compound **44** fails to show any Top1 inhibitory activity at 10 μM. The result validated

the importance of aminopropylamino side chain at C-4 position of quinoline core for providing significant impact on Top1 inhibition.

Our efforts to develop an acceptable hypothetical binding model that rationalize the structure-activity relationships of all the molecules resulting from minor changes in the structure proved difficult. However, molecular modeling indicated that depending on the nature of substituents at C-3, C-4 and C-6 position of the quinoline ring, it might be capable of forming hydrogen bond interactions with important residues such as Arg364, Asn722 and Asn352 as well as stacking interactions with DA113, DC112, TGP11 and DT10 in the ternary complex. In representative compound **22** with the flexible two-carbon linker, the molecular modeling indicated that the heteroatom present in the heterocycles were unable to form a hydrogen bond with Asn352 in the major groove (Figure S2). The result signifies the importance of the length of the flexible chain containing heterocycles. Analysis of the various poses of disubstituted compound **31** and **33** within the ternary complex revealed that the 3,4-dimethoxy phenyl group is not orientated in the same plane as observed in compound **28**. As a result compound **31** was unable to form hydrogen bond with Arg364 and Asn722 along with the loss in π-π stacking interactions. Interestingly, compound **33** was found to attain the stacking interactions with DA113, DC112, TGP11, and DT10 along with hydrogen bond with Arg364, which signifies the importance of specific substitutions at the C-6 position of quinoline ring.

The structure-activity relationship signifies the importance of a perfect balance of substitution patterns at C-3, C-4 and C-6 position in our design. All these results together vindicate the robustness of our design and rationalize the highest activity of compound **28** against Top1 in plasmid relaxation activity (Table 1). Compound **28** was selected for further lead assessment and mechanistic insight.

Table 2. Solubility, plasma stability and Log D results of potent compounds at pH 7.4

Comp.	Aq. Sol (μg/mL)	Plasma Stability[a]	LogD @ pH 7.40
CPT	2.5	15.70	1.74*
13	28.08±0.11	83.38	3.26±0.01
28	30.09±0.66	93.34	2.48±0.04

[a]Mean % remaining at 2 hrs in human Plasma, *Log P value

Table 3. Caco-2 permeability of compound **13** and **28**

Comp.	P_{app} (10^{-6} cm/sec)		Efflux Ratio
	Apical to Basal	Basal to Apical	
13	10.89	10.98	1.02
28	7.42	11.55	1.60

***In vitro* ADME study**. Compounds **13** and **28** with similar structural features except for the nature of weak basic group present at the C-4 position were evaluated in a panel of in vitro ADME assays (Table 2 and 3). Both these compounds were found to have moderate aqueous solubility as compared to CPT. It is noteworthy to mention that both **13** and **28** demonstrated admirable human plasma stability even after 2 hours, whereas CPT due to its unstable lactone was not stable. The most active compound **28** was found to be highly stable in human plasma with 93% present after 2 hours. The in vitro LogD values of **13** and **28** are 3.26 and 2.48 respectively, which are considered to be ideal for oral absorption.[43-45] As CPT is known to be a substrate of Pgp, we examined the Caco-2 permeability for these compounds as well as the efflux ratio to evaluate whether **13** and **28** are Pgp substrates.[46,47] To our great satisfaction the efflux ratio of **13** and **28** are found to be < 2, which signify that these compounds are not Pgp substrates. We conclude that unlike CPT, our 'tailor-made' compounds **13** and **28** are highly plasma stable with ideal LogD value for oral absorption and are not Pgp substrates. Based on ADME study[48,49] (Table 2 and 3) and human Top1 plasmid DNA relaxation inhibition assays (Table 1), we further extend our study with compound **28** to get mechanistic insight into Top1 inhibition and evaluate its role in the anticancer activity.

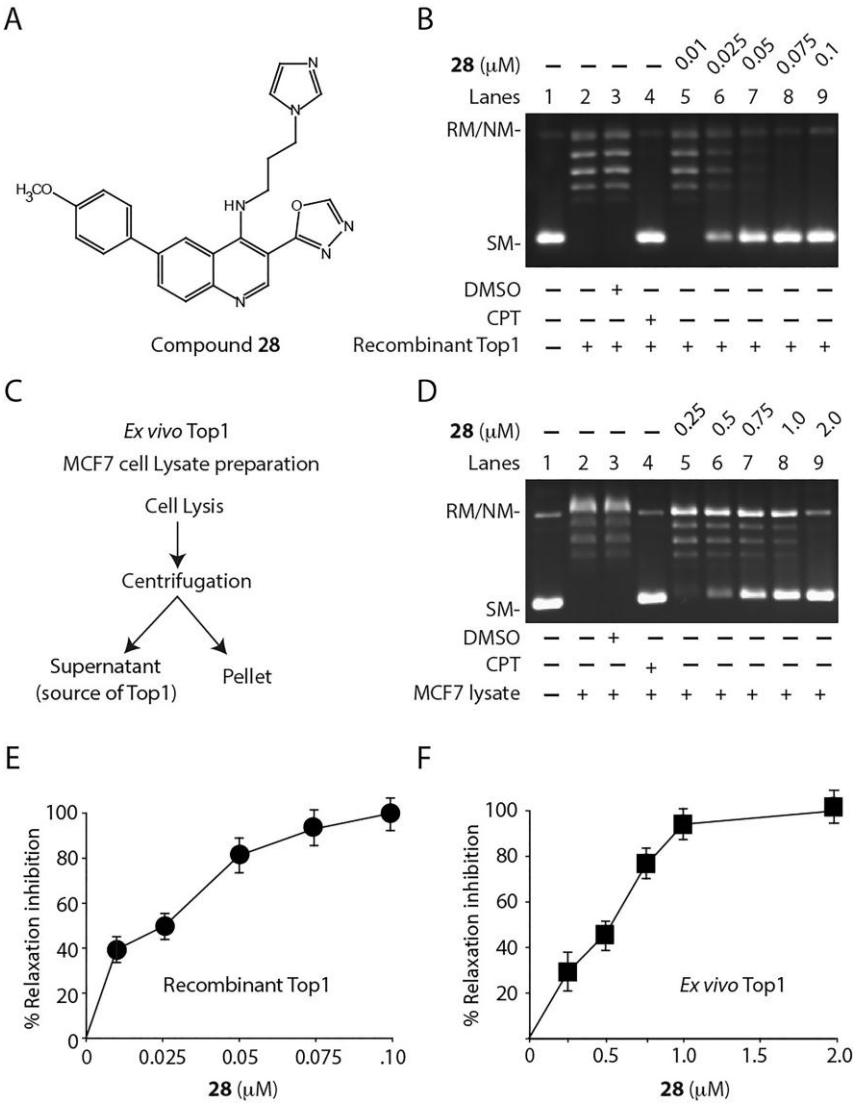


Figure 2. Inhibition of Topoisomerase 1-induced plasmid DNA relaxation activity by compound **28**. (A) Most active compound **28**. (B) Relaxation assay of supercoiled plasmid DNA using recombinant HTop1 at 3:1 molar ratio. Lanes 1 and 9, pBS (SK+) DNA (90 fmol); lanes 2, pBS (SK+) DNA (90 fmol) incubated with 30 fmol of recombinant Top1; lane 3, same as lane 2 additionally Top1 was incubated with 2% DMSO; lane 4, same as lane 2 but incubated simultaneously with 2 μM CPT; lanes 5−9, same as lane 2 but was incubated with variable concentrations of compound **28** (as per indication) at 37 °C for 30 min. (C) Schematic representation for MCF7 whole cell lysate preparation used as the source of endogenous human Top1 for ex vivo Top1 relaxation assays. (D) Relaxation of supercoiled pBS (SK+) DNA by Top1 activity from MCF7 cellular extract (each reaction volume contains 0.1 μg

protein). Lanes 1 and 10, pBS (SK+) DNA (0.3 μg); lane 2, same as lane 1 but pBS (SK+) DNA (0.3 μg) was incubated with MCF7 cell lysates; lanes 3 and 12, same as lane 2 but incubated with 2% DMSO; lanes 4−9, same as lane 2 but MCF7 whole cell lysates were incubated simultaneously with 5 μM CPT or with varying concentrations of **28** (as indicated) together with plasmid DNA at 37 °C for 30 min. Positions of supercoiled monomer (SM) and nicked and relaxed monomer (NM/RL) are indicated. (E and F) Quantitative representation of Top1 DNA relaxation inhibition (%) at variable concentrations of **28** as showed in panel B and D. Recombinant Top1 (E) or endogenous Top1 from cellular extracts (F). All the experiments were performed in triplicate and expressed as the mean ± SD.

Compound 28 poisons human Top1-DNA cleavage complexes. To examine the specificity of compound **28** (Figure 2A) for Top1, we used both recombinant Top1 enzyme (Figure 2B) and endogenous Top1 from the whole cell extracts of human breast adenocarcinoma (MCF7) cells[12,20] (Figure 2C and 2D). When recombinant Top1 and compound **28** were added simultaneously in the plasmid DNA relaxation assays (Figure 2B, lanes 5-9), 85-90 % inhibition of Top1 could be achieved at 0.1 μM concentration of compound **28** (Figure 2B, lane 7). Next, the cellular extracts were utilized as a source of Top1 (Figure 2B) for the plasmid DNA relaxation assays (*ex-vivo*) (Figure 2C). The utility of using whole cell extract lies in the fact that the Top1 enzyme in the extract is conserved in its native structure amongst a plethora of other cellular proteins. The *ex-vivo* relaxation inhibition assays with compound **28** efficiently inhibited Top1 activity when the cellular extracts were incubated with compound **28** (Figure 2D, lanes 5-9) and the quantification in figure 2E-F. Taken together, all these results indicate that compound **28** selectively inhibits human Top1, both as a recombinant enzyme (Figure 2B) and as an endogenous protein (Figure 2D), without being impaired by the pool of proteins contained in the whole cell extracts.

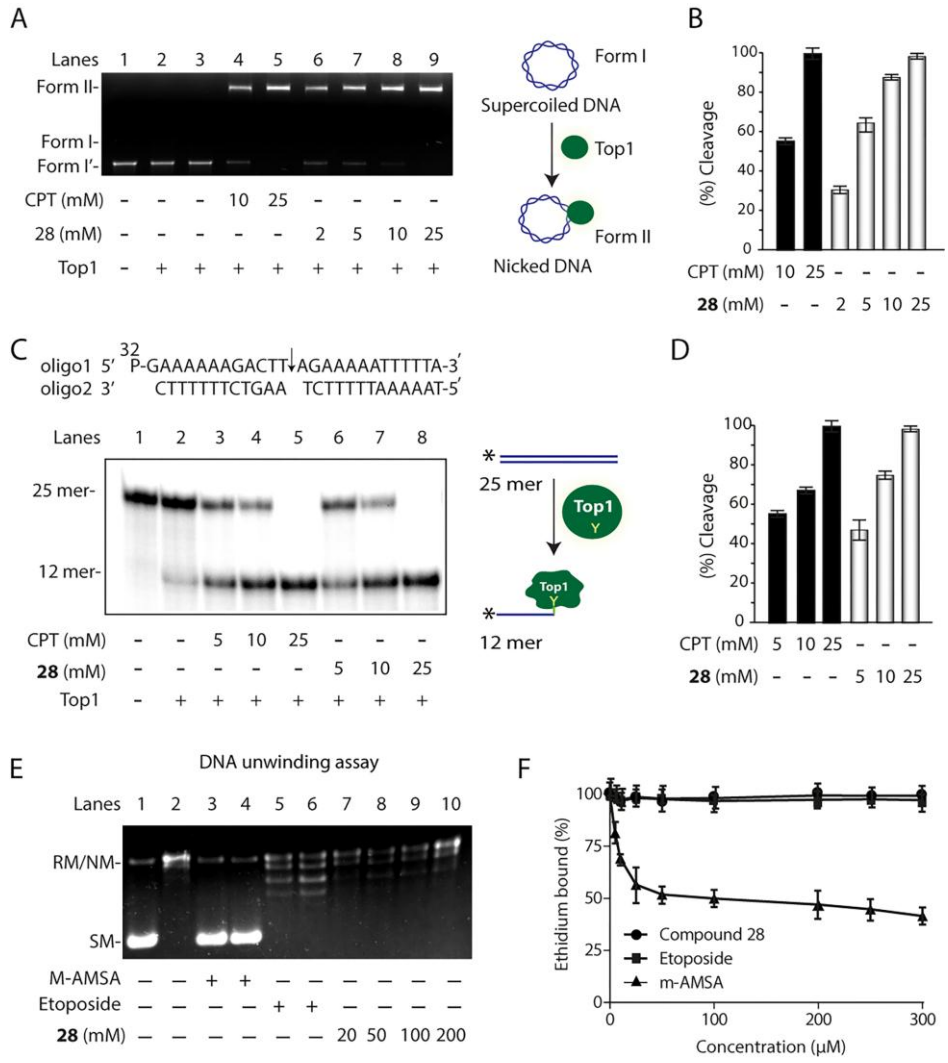


Figure 3. Compound **28** mediated trapping of Top1-DNA cleavage complexes. (A) Representative gel image depicting plasmid DNA cleavage mediated by Top1 in the presence of CPT or compound **28**. Lane 1, 60 fmol of pBS (SK+) supercoiled DNA. Lanes 2−9, same as lane 1 but incubated with equal amounts of recombinant human Top1 (100 fmol) at the indicated concentrations of CPT or compound **28** or only DMSO at 37 °C for 30 min. Positions of the supercoiled substrate (form I) and nicked monomers (form II) are indicated. Schematic representation of Top1 mediated nicked DNA formation. (B) Representative gel showing Top1-mediated 25 mer duplex oligonucleotide cleavage in the presence of CPT and compound **28**. Lane 1, 15nM 5'-^{32}P-end labeled 25-mer duplex oligo as indicated above. Lane 2, same as lane 1 but incubated with recombinant Top1 (0.2 μM). Lanes 3−5, same as lane 2 but

incubated with an indicated concentration of CPT. Lanes 6−8, same as lane 2 but incubated with an indicated concentration of compound **28**. Positions of uncleaved oligonucleotide (25-mer) and the cleavage product (12-mer oligonucleotide complexed with residual Top1) are indicated. Schematic representation of the formation of 12-mer oligonucleotide attached with Top1 in presence of Top1 poison. Quantitative measurement of cleavage complex (Top1cc) formation (%) by CPT and compound **28** either by supercoiled DNA (C) or through oligo cleavage assay (D). All the experiments were performed three times and expressed as the mean ± SD. (E) Compound **28** is not a DNA intercalator. Compound **28**-DNA interaction as investigated by agarose gel electrophoresis in Top1 unwinding assays. Lane 1, 50 fmol of pBS (SK^+) DNA. Lane 2, relaxed pBS (SK^+) DNA generated by an excess of Top1. Lanes 3–6, same as lane 2, but incubated with 50 and 200 μM of m-AMSA and etoposide, respectively. Lanes 7–10, same as lane 2, but incubated with 20, 50, 100, 200 μM of compound **28** as indicated. **(F)** Fluorescence-based ethidium bromide displacement assay. All Samples contained 1 μM of EtBr and 5 nM CT DNA. Graphical representation of EtBr bound (%) of increasing concentration (0–300 μM) of compound **28**, m-AMSA and etoposide were added as indicated. EtBr fluorescence was monitored with excitation wavelength at 510 nm and emission at 590 nm.

As both CPT and indenoisoquinolines stabilize Top1-DNA cleavable complexes (Top1cc) to inhibit Top1 activity,[5,9,14,15,38,39,50] the mechanism of Top1 inhibition with compound **28** was investigated in the plasmid DNA cleavage assays. Closed circular DNA (form I) get converted to nicked circular DNA (form II) by Top1 in the presence of specific inhibitors and are referred to as "cleavage complex" (Figure 3A, right panel). Figure 3A shows that compound **28** also stabilizes Top1cc formation like CPT, suggesting that compound **28** acts as a Top1 poison. We further confirmed that compound **28** is capable of stabilizing Top1cc in single turnover equilibrium cleavage assays (Figure 3B, lanes 3-8) by allowing recombinant Top1 to react with 25-mer duplex oligonucleotides modified to harbor preferred

Top1 cleavage sites in the presence of indicated concentrations of compound **28** or CPT consistent with plasmid DNA cleavage assays (Figure 3A). In addition, quantification of cleavage assays (Figure 3C-D) suggests that the extent or rate of Top1-DNA cleavage complex formation (% cleavage) with compound **28** at indicated concentration is similar to CPT (Figure 3C and 3D). Cumulatively, our data are indicative that compound **28** is able to stabilize Top1 cleavage complexes and inhibit the religation activity with similar efficacy as that of CPT.

To investigate the ability of the compound **28** to intercalate into DNA, we carried out both Top I unwinding assays and EtBr displacement assays.[12,40] Topoisomerase unwinding assay is based on the ability of the intercalating compounds to unwind the DNA duplex and thereby change the DNA twist. Figure 3E clearly shows that in the presence of strong intercalative drug such as m-AMSA, a net negative supercoiling of the relaxed substrate DNA was induced at 50 and 200 μM concentration (Figure 3E, lanes 3 and 4). However, under similar conditions, non-intercalative compounds like etoposide failed to show such effect at 50 and 200 μM concentrations (Figure 3E, lanes 5 and 6). Compound **28** had no effect on the topological state of the relaxed plasmid DNA at the indicated concentrations (Figure 3E, lanes 7-10), suggesting compound **28** is not a DNA intercalator (Figure 3). Next, we further confirm that compound **28** is not a DNA intercalator by performing EtBr displacement assays. Figure 3F shows that the intercalative drug m-AMSA has the capability to dislodge the bound fluorophore (EtBr) at 50 μM concentration. However, non-intercalative drug such as etoposide was unable to do so. Under similar condition compound **28** induces no displacement of fluorophore even at high concentration (200 μM) compared with the very low IC50 (29 nM) of topoisomerase I inhibition. Taken altogether, our data suggest that compound **28** is not a DNA intercalator.

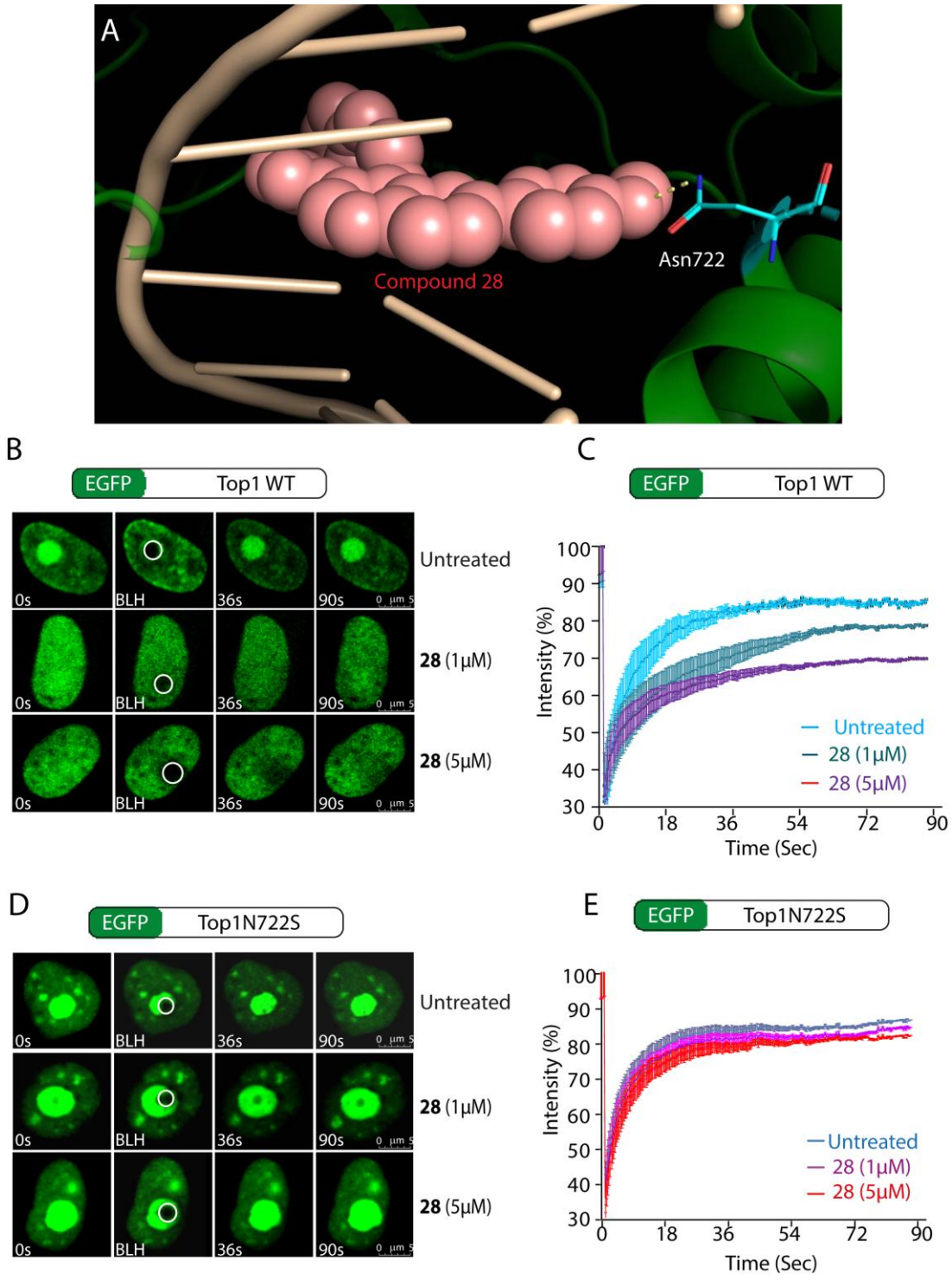


Figure 4. Compound **28** mediated stabilization of Top1-DNA cleavage complex in live cells. **(A)** Space-filling model showing conservative H-bonding interaction between Asn722 and **28**. **(B)** Compound **28** accumulates immobile/bound Top1 in the nucleus. Representative images depicting the fluorescence recovery after photobleaching (FRAP) of enhanced green fluorescence tagged HTop1

(EGFP-Top1WT) transiently expressed in MCF7 cells. Cells were treated with indicated concentration of **28** for 10 min and were analyzed by live cell spinning disk confocal microscopy and photobleaching. A sub-nuclear spot (ROI) indicated by a circle was bleached (BLH) for 30 ms and photographed at regular intervals of 3 ms thereafter. Successive images taken for ~90 s after bleaching illustrate fluorescence return into the bleached areas. **(D)** Top1N722 residue is critical for **28**-induced nuclear dynamics. Representative images showing the FRAP of EGFP–Top1N722S transiently expressed in MCF7 cells. Cells were treated with **28** (indicated concentration) for 10 min and were analyzed by live cell spinning disk confocal microscopy and FRAP experiments were carried out in a similar way as with the EGFP-Top1WT). Right panels (C and E) quantification of FRAP data showing mean curves of Top1 variants in the presence and absence of **28**. Error bars represent mean ± S.E. (n = 15).

Compound 28 traps Top1 cleavage complexes in live cancer cells. To obtain direct evidence for compound **28** mediated trapping of Top1cc (Figure 4A) in live cells, we ectopically expressed a green fluorescent tagged human Top1 (EGFP-Top1) in MCF7 cells and tested the nuclear mobility of Top1 under live cell confocal microscopy combined with fluorescence recovery after photobleaching (FRAP) technology as described previously.[12,51] In absence of compound **28**, FRAP recovery of EGFP-Top1 was fast (~85–90% suggesting a large mobile population and a smaller (~10–15%) immobile population of EGFP-Top1 (Figure 4B, and the quantification in 4C; untreated). This data implies that Top1 is mostly mobile and binds transiently with the DNA (reversible Top1cc) and are freely exchanged in the nuclear compartments.

In the presence of compound **28**, the fluorescence recovery of EGFP-Top1 was markedly impeded (~55–65%) with increasing concentration of compound **28** (Figure 4B, and the quantification in 4C; (+) compound **28**; 1 and 5 μM). These data suggest that compound **28** traps Top1cc on DNA in live cells like CPT,[10,18] leading to a subsequent increase in bound/immobile fraction of EGFP-Top1 (Figure 4B-

C), which is consistent with **28**-mediated stabilization Top1cc in the *in vitro* cleavage assays (Figure 3). The molecular modeling indicates compound **28** possibly makes hydrogen bonding interaction with Asn722 located in the catalytic domain of HTop1 may contribute to the stabilization of Top1 cleavage complexes with the nicked DNA (Figure 4A). To investigate this possibility, we tested the ability of compound **28** to trap mutant Top1 at residue Asn722 (EGFP-Top1^{N722S}) in live cells by using FRAP kinetic analysis (see Figure 4D and E, panel (+) **28**). Figure 4E suggests that the FRAP recovery of EGFP–Top1^{N722S} was unaffected in the presence of increasing dosage of **28** (Figure 4D-4E) and was similar to no drug treatment condition (Figure 4E), suggesting **28** failed to trap Top1cc when Asn722 is mutated to Ser. Taken together, this data indicates that Asn722 is critical for compound **28** mediated trapping of human Top1cc.

Compound 28 accumulates persistent DNA double-strand breaks (DSBs) compared to CPT. As compound **28** stabilizes Top1cc *in vitro* (Figure 3) and in live cells (Figure 4), we investigated the accumulation and disappearance of DSBs in MCF7 cells treated with compound **28** by measuring γH2AX foci formation under confocal microscopy (Figure 5), as γH2AX is a well-defined marker for Top1-mediated DSBs.[52–57]

Under similar condition, we detected a time-dependent increase in γH2AX foci formation in cells treated with compound **28** for 3h and 5h comparable with CPT-induced γH2AX foci at similar time periods, suggesting that both compound **28** and CPT generate similar levels DSBs at indicated time periods in MCF7 cells (Figure 5B and 5C).

CPT-induced γH2AX have a short half-life as they are reversible immediately after a wash,[52,54] which is consistent with the reversal of Top1cc intermediates within minutes after washing out CPT.[58] Next to test the ability of the compound **28** to induce more persistent and less reversible DNA breaks, we investigated the disappearance of γH2AX in cells treated with compound **28** and compared it with CPT after subsequent wash and culture in the drug-free medium for indicated time periods (Figure 5A). In

Figure 5B, the right panel shows faster disappearance of γH2AX foci after washing out CPT from media at indicated time periods (see the quantification in Figure 5C). In contrast, cells showed (3-4 fold) more persistent γH2AX foci (Figure 5B, right panel and the quantification in Figure 5C) even after washing out compound **28** at the indicated time. Figure 5C further indicates that γH2AX foci were persistent for 5h after drug removal in compound **28** treated cells. Taken together our data suggest that compound **28** generates more persistent and less reversible Top1cc-induced DSBs compared to CPT.

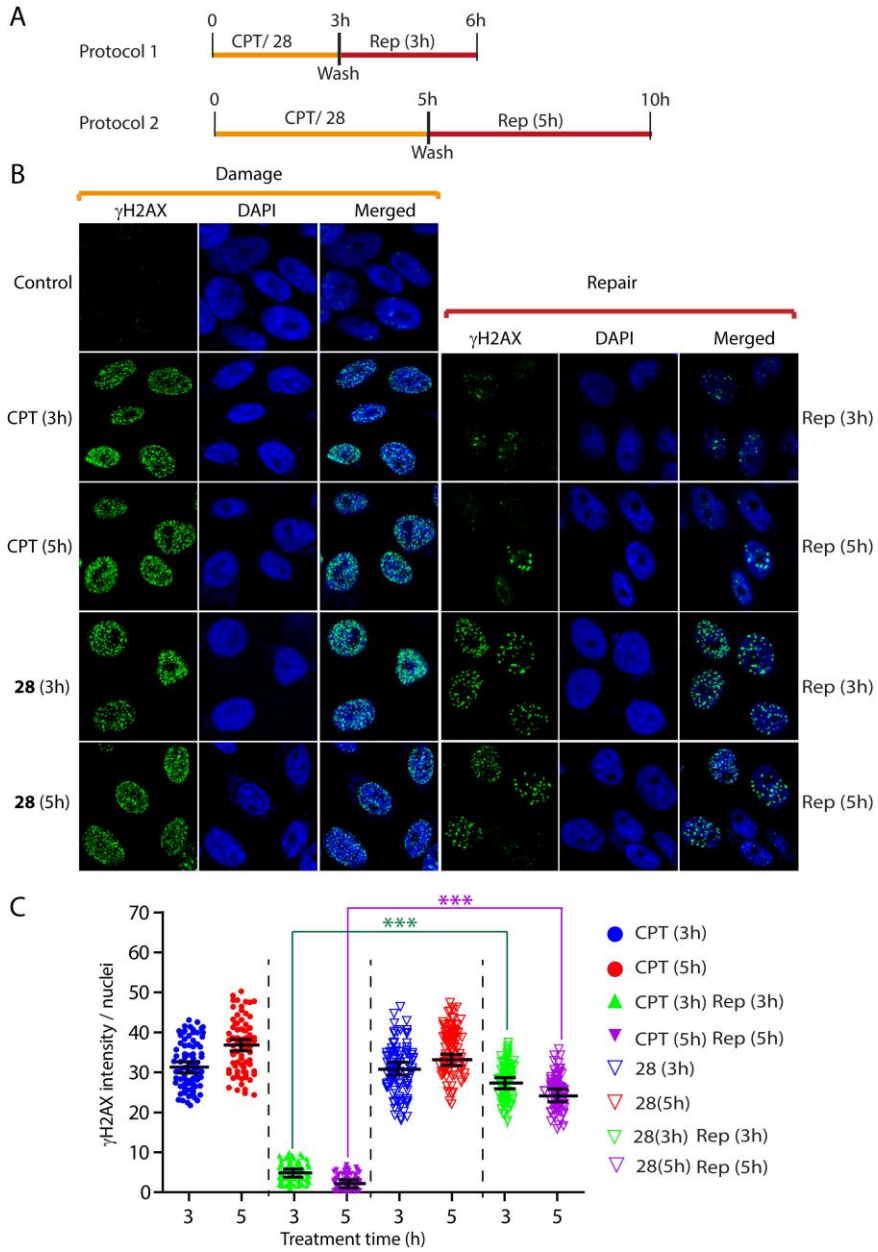


Figure 5. Compound **28** generates persistent and less reversible DNA double-strand breaks as detected by γH2AX staining (A) Overview for the protocol of drug treatment and reversal in MCF7 cells. (B) Time-dependent accumulation of γH2AX foci formation in MCF7 cells treated with compound **28** or CPT for 3h and 5h and reversal of γH2AX foci after drug removal for indicated times. (C) Quantification of γH2AX intensity per nucleus after treatment and post the removal of indicated inhibitors (Compound **28** or CPT) obtained from immunofluorescence confocal microscopy was calculated for 35–40 cells (mean ± S.E.M.) and plotted as a function of time (h).

Compound 28 exhibits potent anticancer activity. Compound **28** was evaluated for its cytotoxicity in the cancer cell lines from different tissue origin.[52] Cytotoxicity assays were performed in human breast adenocarcinoma cell lines (MCF7), human cervical cancer cell lines (HeLa), human colon carcinoma cell lines (HCT116), human ovarian adenocarcinoma cell lines (NIH:OVCAR-3) as well as non-cancerous human embryonic kidney (HEK293) cells with variable concentrations of compound **28**. Table S2 (Supporting Information) indicates that compound **28** revealed cytotoxicity in cancerous cells including MCF7 (IC_{50}: 2.74 μM), HeLa (IC_{50}: 2.61 μM), HCT116 (IC_{50}: 2.34 μM), NIH:OVCAR-3 (IC_{50}: 2.35 μM) cells compared to the non-cancerous cells like HEK293 (IC_{50}: 8.34 μM).

TDP1 hydrolyzes the phosphodiester bond at a DNA 3'-end linked to a tyrosyl moiety of stalled Top1-DNA covalent complexes, therefore, TDP1-/- cells are hypersensitive towards Top1 poisons.[51–54,59,60] Further evidence for the poisoning of Top1cc with compound **28** and hypersensitivity in DNA repair-deficient cells, we have performed cytotoxicity assays with TDP1-/- (IC_{50}: 1.02 μM) and TDP1+/+ MEFs (IC_{50}: 2.91 μM) cells in the presence of compound **28**. Table S2 indicates that TDP1-/- MEFs cells were hypersensitive to compound **28**, further providing evidence that compound **28** induces cytotoxicity by stabilizing Top1cc in cells. Taken together all data confirms that compound **28** is a potent Top1 poison and is a potential candidate as an anticancer chemotherapeutic agent.

CONCLUSION

As CPT and its clinically approved drugs suffer from several limitations, there is a great interest in the development of 'non-CPT' Top1 poisons as anticancer agents. In the present study, we successfully report a novel class of Top1 poisons based on quinoline core through an understanding of the structural features of ligands essential for binding in the active site. The design signifies the importance of a perfect balance of substitution patterns at C-3, C-4 and C-6 position of quinoline core. Most potent compound **28** stabilizes Top1cc *in vitro* and in live cells to inhibit the religation activity without intercalating to the DNA with similar efficacy as that of CPT. The mechanistic insight through FRAP assay of compound **28** mediated trapping of human Top1cc revealed the significance of Asn722, an important amino acid residue at the active site. Unlike CPT, compound **28** generates less reversible DNA double-strand breaks due to the accumulation of irreversible Top1cc-induced DSBs. *In vitro* ADME study revealed that unlike CPT and its derivatives, compound **28** is highly plasma stable with ideal LogD value for oral absorption and is not a Pgp substrate. Summarily, we provide compelling evidence that advocate for compound **28** as a potential anticancer agent.

EXPERIMENTAL SECTION

Chemistry. General Methods. All starting materials, reagents, and solvents were purchased from commercial suppliers and used without further purification. Air-sensitive reactions were carried out under dry nitrogen or argon atmosphere. Solvents were distilled before use and also dried using standard methods. TLC was performed on silica gel plates (Merck silica gel 60, F_{254}) and the spots were visualized under UV light (254 nm and 365nm) or by charring the plate dipped in ninhydrin or $KMnO_4$ or vanillin solution. For purification of the compounds along with manual column flash chromatography was also performed with RediSepRf silica gel columns on the Teledyne ISCO CombiFlashRf system using 230-400 mesh silica gel. ^{1}H NMR was recorded at 300 MHz (Bruker-DPX), 400 MHz (Jeol) and

600 MHz (Bruker-Avance) frequency and ^{13}C NMR spectra were recorded at 75 MHz (Bruker-DPX), 100 MHz (Jeol) and 150 MHz (Bruker-Avance) frequency in $CDCl_3$ or CD_3OD or DMSO-d_6 solvent using TMS as the internal standard. Chemical shifts were measured in parts per million (ppm) referenced to 0.0 ppm for tetramethylsilane and 7.260 ppm for $CHCl_3$. The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br. = broad. Coupling constants, *J* was reported in Hertz unit (Hz). High-resolution mass spectra, HRMS (m/z) were measured using EI (Jeol-JMS 700mass spectrometer), ESI (Q-Tof Micro mass spectrometer) techniques and ESI (LTQ OrbitrapXLmass spectrometer). The purity of the selected compounds were analyzed by Hitachi HPLC using column Xtimate C18 (4.6 x 150 mm 5.0 µm,).The HPLC purity of all the compounds subjected to biological assay is > 95%.

Ethyl 6-bromo-4-chloroquinoline-3-carboxylate (1). Ethyl 6-bromo-4-hydroxyquinoline-3-carboxylate (2 g, 6.75 mmol) was taken in $POCl_3$ (15 mL) in ice-cold condition. The reaction mixture was allowed to come to room temperature and heated for 2 hours at 100 ºC. The reaction mixture was poured into crushed ice and neutralized with a saturated sodium bicarbonate solution. The organic part was extracted with ethyl acetate, washed with water and brine, concentrated and dried. The solid was purified by silica gel flash column chromatography, eluting with 15% ethyl acetate in hexane to give compound **1** as a white crystalline solid (1.8 g, 85% yield). ^1H NMR (300 MHz, $CDCl_3$) δ 9.2 (s, 1H), 8.58 (d, *J* = 2.4 Hz, 1H), 8.02 (d, *J* = 9.3 Hz, 1H), 7.92 (dd, *J* = 9.0, 1.8 Hz, 1H), 4.51 (q, *J* = 7.5 Hz, 2H), 1.47 (t, *J* = 7.5 Hz, 3H). MS (ESI) *m/z* $[M+Na]^+$ 338.16.

General procedure A: Ethyl 6-bromo-4-(2-morpholinoethylamino)quinoline-3-carboxylate (2). Compound **1** (1 g, 3.18 mmol) was dissolved in 1,4-dioxane (5 mL) under N_2 atmosphere. To the reaction mixture dry DIPEA (1.11 mL, 6.36 mmol) and 4-(2-aminoethyl)morpholine (0.63 mL, 4.77 mmol) were added respectively. The reaction mixture was allowed to stir for 12 hours at room temperature. It was poured into 50 mL water. The solid obtained was filtered and purified by column

chromatography to give compound **2** as a white solid (1.1 g, 85% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.51 (br. s, -NH), 9.1 (s, 1H), 8.4 (d, J = 1.5 Hz, 1H), 7.83 (d, J = 9.0 Hz, 1H), 7.73 (dd, J = 8.7, 1.8 Hz, 1H), 4.41 (q, J = 7.2 Hz, 2H), 3.92-3.87 (m, 2H), 3.78 (t, J = 4.5 Hz, 4H), 2.71 (t, J = 6.0 Hz, 2H), 2.57 (t, J = 4.2 Hz, 4H), 1.44 (t, J = 7.5 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 168.0, 155.7, 152.0, 149.8, 134.0, 131.6, 128.2, 120.9, 117.2, 103.8, 67.0, 60.7, 58.0, 53.3, 45.4, 14.4. MS (ESI) *m/z* $[M+H]^+$ 410.35. HRMS (EI) *m/z* $[M]^+$ calculated for $C_{18}H_{22}BrN_3O_3$ 407.0845; found 407.0852.

Ethyl 6-bromo-4-(3-morpholinopropylamino)quinoline-3-carboxylate (3). Compound **1** (1 g, 3.18 mmol) was dissolved in 1,4-dioxane (5 mL) under N_2 atmosphere. To the reaction mixture dry DIPEA (1.11 mL, 6.36 mmol) and 3-morpholinopropan-1-amine (0.63 mL, 4.77 mmol) were added respectively and the reaction was performed according to general procedure **A**. The solid obtained was filtered and dried to give compound **3** as a white solid (1.2g, 90% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.23 (br. s, -NH), 9.06 (s, 1H), 8.36 (d, J = 2.1 Hz, 1H), 7.81 (d, J = 9.0 Hz, 1H), 7.72 (dd, J = 9.0, 2.1 Hz, 1H), 4.38 (q, J = 7.2 Hz, 2H), 3.87-3.81 (m, 2H), 3.69 (t, J = 4.5 Hz, 4H), 2.50 (t, J = 7.2 Hz, 2H), 2.44 (t, J = 4.5 Hz, 4H), 1.99-1.90 (m, 2H), 1.42 (t, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.7, 156.1, 152.0, 149.8, 134.3, 131.6, 128.3, 120.9, 117.5, 103.5, 67.0, 61.0, 56.0, 53.8, 46.9, 27.8, 14.4. ESI-MS m/z $[M+H]^+$ 422.30. HRMS *m/z* $[M+H]^+$ calculated for $C_{19}H_{25}BrN_3O_3$ 422.1079; found 422.1072.

General procedure B: 6-Bromo-4-(2-morpholinoethylamino)quinoline-3-carbohydrazide (4). Compound **2** (1 g, 2.45 mmol) was dissolved in ethanol (10 mL). To the solution hydrazine hydrate (10 mL) was added. The reaction mixture was stirred for 10 hours at room temperature. Ethanol was removed under vacuum. The residue was then dissolved in $CHCl_3$ and the organic layer was washed with water and brine, dried and concentrated to give compound **4** as a yellow solid (0.6 g, 62% yield). Mp > 250 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.64 (s, 1H), 8.27 (d, J = 1.5 Hz, 1H), 7.84 (d, J = 8.7 Hz, 1H), 7.73 (dd, J = 8.7, 1.5 Hz, 1H), 3.77 (t, J = 4.8 Hz, 4H), 3.70-3.65 (m, 2H), 2.68 (t, J = 6.3 Hz, 2H),

2.54 (t, J = 4.2 Hz, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.2, 152.3, 149.1, 148.3, 133.7, 131.2, 126.6, 121.2, 118.2, 107.8, 67.0, 57.7, 53.2, 44.1, 31.6, 22.7. ESI-MS m/z $[M+H]^+$ 394.41. HRMS *m/z* $[M+H]^+$ calculated for $C_{16}H_{21}BrN_5O_2$ 394.0878; found 398.0874.

6-Bromo-4-(3-morpholinopropylamino)quinoline-3-carbohydrazide (5). Compound **3** (1 g, 2.37 mmol) was dissolved in ethanol (10 mL). To the solution hydrazine hydrate (10 mL) was added. The reaction was performed according to general procedure **B**. The residue was dissolved in $CHCl_3$ and the organic layer was washed with water and brine, dried and concentrated to give compound **5** (0.85 g, 88% yield) as a pale yellow solid. Mp > 250 ºC. ^{1}H NMR (300 MHz,CD_3OD) δ 8.48 (d, J = 2.1 Hz, 1H), 8.40 (s, 1H), 7.82 (dd, J = 8.7 Hz, 1H), 7.74 (d, J = 9.0 Hz, 1H), 3.76 (t, J = 4.5 Hz, 4H), 3.71 (t, J = 4.8 Hz, 2H), 3.56 (t, J = 6.6 Hz, 2H), 2.52-2.50 (m, 4H), 1.96-1.87 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 169.1, 149.9, 148.6, 146.7, 134.5, 133.4, 129.8, 121.0, 118.5, 108.2, 66.3, 57.0, 53.6, 53.4, 46.3, 44.9, 25.4. ESI-MS m/z $[M+H]^+$ 408.20. HRMS *m/z* $[M+H]^+$ calculated for $C_{17}H_{23}BrN_5O_2$ 408.1035; found 408.1030.

6-Bromo-N-(2-morpholinoethyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (6). Compound **4** (1 g, 2.54 mmol) was taken in triethyl orthoformate (5 mL, 30.06 mmol) and the mixture was heated for 12 hours at 110 ºC. The reaction mixture was allowed to come to room temperature. Excess hexane was added to the mixture. A precipitation formed which was filtered and purified by column chromatography, eluting with 1% methanol in $CHCl_3$ to give compound **6** as a light yellow solid (0.4 g, 39% yield). Mp 180 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.22 (br. s, -NH), 9.03 (s, 1H), 8.47 (m, 2H),7.86 (d, J = 8.7 Hz, 1H), 7.75 (dd, J = 8.7, 1.8 Hz, 1H), 4.00-3.95 (m, 2H), 3.77 (t, J = 4.5 Hz, 4H), 2.74 (t, J = 6.0 Hz, 2H), 2.58 (t, J = 4.5 Hz, 4H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 163.5, 151.4, 151.0, 149.2, 148.7, 134.0, 131.7, 128.0, 120.5, 117.8, 99.2, 67.0, 57.9, 53.3, 45.8. MS (ESI) *m/z* $[M+H]^+$ 404.37. HRMS (EI) *m/z* $[M]^+$ calculated for $C_{17}H_{18}BrN_5O_2$ 403.0644; found 403.0635.

6-Bromo-3-(5-methyl-1,3,4-oxadiazol-2-yl)-N-(2-morpholinoethyl)quinolin-4-amine (7). Compound **4** (0.15 g, 0.38 mmol) was dissolved in ethanol (3 mL) and to the solution triethyl orthoacetate (3 mL, 16.37 mmol) was added. The reaction mixture was refluxed for 8 hours. The reaction mixture was allowed to come to room temperature and solvent was evaporated. Excess hexane was added to the mixture. A precipitation formed which was filtered and purified by column chromatography, eluting with 3% methanol in $CHCl_3$ to give compound **7** (0.14 g, 88% yield) as a brown solid. Mp 230 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.17 (br. s, -NH), 8.99 (s, 1H), 8.48 (d, J = 1.8 Hz, 1H), 7.86 (d, J = 8.7 Hz, 1H), 7.74 (dd, J = 8.7, 2.1 Hz, 1H), 4.00-3.94 (m, 2H), 3.78 (t, J = 4.5 Hz, 4H), 2.75 (t, J = 6.0 Hz, 2H), 2.68 (s, 3H), 2.59 (t, J = 4.5 Hz, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.8, 162.2, 151.3, 148.6, 133.8, 131.7, 128.0, 120.7, 117.8, 99.7,67.1, 58.1, 53.4, 45.9, 11.1. ESI-MS m/z [M+H$^+$] 418.37. HRMS *m/z* [M+H]$^+$ calculated for $C_{18}H_{21}BrN_5O_2$ 418.0878; found 408.0880.

General procedure C. 6-(4-Methoxyphenyl)-N-(2-morpholinoethyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (8). Compound **6** (0.15 g, 0.37 mmol) was dissolved in 1,4-dioxane (5 mL) under Ar atmosphere. 2-(4-Methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.17 g, 0.74 mmol) was added to the mixture. 2(M) Na_2CO_3 solution (0.5 mL) was added to the reaction mixture and argon was purged for 15 minutes. $Pd(PPh_3)_4$ (0.035 g, 0.03 mmol) was added to the mixture and argon purging was performed for 15 minutes. The mixture was heated for 12 hours at 100 ºC. 1,4-Dioxane was removed under vacuum, the residue dissolved in $CHCl_3$ and the organic layer was washed with water and brine, dried and concentrated. The residue was purified by silica gel column chromatography to produce compound **8** as a white solid (0.09 g, 56% yield). Mp 156 ºC. ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.05 (s, 1H), 8.49 (s, 1H), 8.45 (d, J = 1.2 Hz, 1H), 8.06 (d, J = 8.4 Hz, 1H), 7.94 (dd, J = 8.4, 1.2 Hz, 1H), 7.62 (d, J = 9.0 Hz, 2H), 7.05 (d, J = 8.4 Hz, 2H), 4.10-4.07 (m, 2H), 3.89 (s, 3H), 3.77 (t, J = 4.8 Hz, 4H), 2.76 (t, J = 6.0 Hz, 2H), 2.58 (br. s, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.7, 159.4, 152.6, 150.9, 149.2, 148.0, 136.9, 132.8, 130.1, 130.0, 128.2, 122.9, 119.5, 114.4, 98.9, 67.0, 58.0, 55.4, 53.2, 46.0.

MS (ESI) *m/z* $[M+H]^+$ 432.42. HRMS (FAB) *m/z* $[M+H]^+$ calculated for $C_{24}H_{26}N_5O_3$ 432.2035; found 432.2053. HPLC purity 99.55%.

6-(3,4-Dimethoxyphenyl)-N-(2-morpholinoethyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (9). Compound **6** (0.10 g, 0.25 mmol) was taken along with 3,4-dimethoxyphenylboronic acid (0.091 g, 0.5 mmol) in 1,4-dioxane (5 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.023 g, 0.02 mmol) were added to the mixture and the reaction was performed according to procedure **A**. The reaction mixture was heated at 90 ºC for 12 hours. The residue was purified by column chromatography, eluting with 5% methanol in $CHCl_3$ to give compound **9** as a yellow solid (0.035 g, 31% yield). Mp 116 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.21 (br. s, -NH), 9.06 (s, 1H), 8.51 (s, 1H), 8.45 (d, J = 1.5 Hz, 1H), 8.07 (d, J = 8.7 Hz, 1H), 7.93 (dd, J = 8.7, 1.8 Hz, 1H), 7.25 (dd, J = 8.7, 1.8 Hz, 1H), 7.19 (d, J = 2.1 Hz, 1H), 7.03 (d, J = 8.4 Hz, 1H), 4.11-4.06 (m, 2H), 4.01 (s, 3H), 3.98 (s, 3H), 3.77 (t, J = 4.5 Hz, 4H), 2.76 (t, J = 6.0 Hz, 2H), 2.58 (t, J = 4.5 Hz, 4H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 163.7, 152.9, 151.0, 149.4, 149.1, 148.9, 147.8, 137.5, 133.4, 130.5, 129.8, 123.3, 119.7, 119.5, 111.7, 110.6, 99.1, 67.0, 58.1, 56.1, 53.3, 46.1. MS (ESI) *m/z* $[M+H]^+$461.51. HRMS (EI) *m/z* $[M]^+$ calculated for $C_{25}H_{27}N_5O_4$ 461.2063; found 461.2051. HPLC purity 99.27%.

6-(4-Methoxyphenyl)-3-(5-methyl-1,3,4-oxadiazol-2-yl)-N-(2-morpholinoethyl)quinolin-4-amine (10). Compound **7** (0.075 g, 0.18 mmol) was taken along with 2-(4-methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.17 g, 0.36 mmol) in 1,4-dioxane (5 mL). 2(M) Na_2CO_3 solution (0.23 mL) and $Pd(PPh_3)_4$ (0.017g, 0.014 mmol) were added to the mixture and the reaction was performed according to procedure **A**. The residue was purified by silica gel column chromatography, eluting with 2% methanol in $CHCl_3$ to afford compound **10** as an off-white solid (0.025 g, 31% yield). Mp 160 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.14 (br. s, -NH), 8.99 (s, 1H), 8.44 (d, J = 1.5 Hz, 1H), 8.05 (d, J = 8.7 Hz, 1H), 7.92 (dd, J = 8.7, 1.5 Hz, 1H), 7.63 (d, J = 8.7 Hz, 2H), 7.05 (d, J = 8.7 Hz, 2H), 4.09-4.04 (m, 2H), 3.90 (s, 3H), 3.76 (t, J = 4.5 Hz, 4H), 2.75 (t, J = 6.0 Hz, 2H), 2.69 (s, 3H), 2.57 (t,

J= 4.2 Hz, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.0, 162.0, 159.5, 152.6, 147.8, 137.0, 133.0, 130.1, 128.3, 123.0, 119.7, 116.2, 114.5, 99.5, 67.1, 58.2, 55.4, 53.3, 46.2, 11.0. MS (ESI) *m/z* $[M+H]^+$ 446.50. HRMS (EI) *m/z* $[M]^+$ calculated for $C_{25}H_{27}N_5O_4$ 445.2114; found 445.2112. HPLC purity 96.96%.

6-(3,4-dimethoxyphenyl)-3-(5-methyl-1,3,4-oxadiazol-2-yl)-N-(2-morpholinoethyl)quinolin-4-amine (11). Compound **7** (0.065 g, 0.16 mmol) was taken along with 3,4-dimethoxyphenylboronic acid (0.073 g, 0.4 mmol) in 1,4-dioxane (5 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.015 g, 0.013 mmol) were added to the mixture and the reaction was performed according to general procedure A. Purification was done by silica gel column chromatography, eluting with 3% methanol in $CHCl_3$ to get compound **11** as a light yellow solid (0.03 g, 41% yield). Mp 180 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.11 (br. s, -NH), 8.99 (s, 1H), 8.42 (s, 1H), 8.05 (d, *J* = 8.7 Hz, 1H), 7.90 (dd, *J* = 8.4, 1.5 Hz, 1H), 7.24 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.17 (d, *J* = 1.5 Hz, 1H), 7.01 (d, *J* = 8.4 Hz, 1H), 4.08-4.03 (m, 2H), 3.99 (s, 3H), 3.96 (s, 3H), 3.75 (t, *J* = 4.5 Hz, 4H), 2.74 (t, *J* = 6.0 Hz, 2H), 2.68 (s, 3H), 2.56 (t, *J* = 4.5 Hz, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.0, 162.0, 152.6, 149.4, 149.0, 148.0, 137.2, 133.6, 130.2, 130.1, 123.3, 119.7, 111.7, 110.6, 99.6, 67.1, 58.3, 56.1, 53.3, 46.2, 11.0. MS (ESI) *m/z* $[M+H]^+$ 476.58. HRMS (EI) *m/z* $[M]^+$ calculated for $C_{25}H_{27}N_5O_4$ 475.2220; found 475.2226. HPLC purity 96.38%.

6-Bromo-N-(3-morpholinopropyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (12). Compound **5** (1 g, 2.45 mmol) was taken in triethyl orthoformate (5 mL, 30.06 mmol) and the mixture was heated for 12 hours at 110 ºC. The crude mixture was purified by column chromatography, eluting with 4% methanol in $CHCl_3$ to give compound **12** as a yellow solid (0.25 g, 25% yield). Mp 144 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.03 (s, 1H), 8.82 (br. s, -NH), 8.49 (s, 1H), 8.46 (d, *J* = 0.9 Hz, 1H), 7.86 (d, *J* = 8.7 Hz, 1H), 7.76 (dd, *J* = 9.0, 1.8 Hz, 1H), 3.99-3.93 (m, 2H), 3.70 (t, *J* = 4.5 Hz, 4H), 2.54 (t, *J* = 6.9 Hz, 2H), 2.45 (t, *J* = 3.9 Hz, 4H), 2.08-1.99 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.8, 151.6, 151.0,

149.1, 148.7, 134.1, 131.7, 128.0, 120.4, 118.0, 98.9, 66.9, 55.7, 53.7, 46.9, 27.7. MS (ESI) *m/z* $[M+H]^+$ 418.31. HRMS *m/z* $[M+H]^+$ calculated for $C_{18}H_{21}BrN_5O_2$ 418.0878; found 418.0862.

6-(4-Methoxyphenyl)-N-(3-morpholinopropyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (13). Compound **12** (0.10 g, 0.24 mmol) was taken along with 2-(4-methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.11 g, 0.48 mmol) in 1,4-dioxane (5 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.022 g, 0.019 mmol) were added to the mixture and the reaction was performed according to procedure A. The residue was purified by silica gel column chromatography to produce compound **13** as a grey solid (0.037 g, 35% yield). Mp 134 ºC. ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.06 (s, 1H), 8.86 (br. s, -NH), 8.51 (s, 1H), 8.47 (d, J = 1.2 Hz, 1H), 8.08 (d, J = 9.0 Hz, 1H), 7.96 (dd, J = 9.0, 1.8 Hz, 1H), 7.64 (d, J = 9.0 Hz, 2H), 7.07 (d, J = 8.4 Hz, 2H), 4.13-4.10 (m, 2H), 3.91 (s, 3H), 3.69 (t, J = 4.2 Hz, 4H), 2.57 (t, J = 7.2 Hz, 2H), 2.47 (s, 4H), 2.12-2.07 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.1, 159.6, 153.0, 150.9, 149.2, 147.9, 137.2, 133.0, 130.4, 130.2, 128.3, 123.2, 119.5, 114.6, 98.8, 66.8, 55.8, 55.5, 53.7, 47.3, 27.9. MS (ESI) *m/z* $[M+H]^+$ 446.39. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{25}H_{28}N_5O_3$ 446.2192; found 446.2190. HPLC purity 98.23%.

6-(3,4-Dimethoxyphenyl)-N-(3-morpholinopropyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (14). Compound **12** (0.10 g, 0.24 mmol) was taken along with 3,4-dimethoxyphenylboronic acid (0.087 g, 0.48 mmol) in a mixture of 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.022 g, 0.02 mmol) were added to the mixture and the reaction was performed according to procedure A. The residue was purified by silica gel column chromatography to afford compound **14** as white solid (0.033 g, 30% yield). Mp 182 ºC. ^{1}H NMR (300 MHz, CD_3OD) δ 9.08 (s, 1H), 8.93 (s, 1H), 8.58 (s, 1H), 8.08 (dd, J = 9.0, 1.5 Hz, 1H), 7.97 (d, J = 8.7 Hz, 1H), 7.35-7.34 (m, 2H), 7.12 (d, J = 9.3 Hz, 1H), 4.09 (t, J = 6.9 Hz, 2H), 3.97 (s, 3H), 3.92 (s, 3H), 3.64 (t, J = 4.8 Hz, 4H), 2.60 (t, J = 6.9 Hz, 2H), 2.49 (t, J = 3.9 Hz, 4H), 2.11-2.07 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.2, 152.9, 150.9, 149.6, 149.4, 149.0, 148.2, 137.3, 133.5, 130.4, 123.4, 119.6, 119.5, 111.7, 110.5,

98.8, 66.9, 56.1, 55.8, 53.7, 47.3, 28.1. MS (ESI) *m/z* $[M+H]^+$ 476.37. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{25}H_{28}N_5O_3$ 476.2298; found 476.2291. HPLC purity 99.67%.

N-(3-Morpholinopropyl)-3-(1,3,4-oxadiazol-2-yl)-6-(p-tolyl)quinolin-4-amine (15). Compound **12** (0.10 g, 0.24 mmol) was taken along with *p*-tolylboronic acid (0.049 g, 0.36 mmol) in 1,4-dioxane (5 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.023 g, 0.02 mmol) were added to the mixture and the reaction was performed according to procedure **A**. The reaction took 8 hours to complete. The residue was purified by silica gel column chromatography to produce compound **15** as white solid (0.039 g, 38% yield). Mp 168 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.04 (s, 1H), 8.82 (br. s, -NH), 8.48 (s, 2H), 8.05 (d, J = 8.7 Hz, 1H), 7.95 (d, J = 8.4 Hz, 1H), 7.58 (d, J = 7.8 Hz, 2H), 7.32 (d, J = 7.5 Hz, 2H), 4.11-4.05 (m, 2H), 3.64 (t, J = 4.5, 4H), 2.52 (t, J = 7.2 Hz, 2H), 2.44-2.42 (m, 7H), 2.09-2.01 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.1, 152.9, 150.8, 148.1, 143.5, 137.7, 137.4, 130.4, 129.8, 127.1, 123.7, 123.6, 66.9, 55.8, 53.7, 47.3, 28.0, 21.1. MS (ESI) *m/z* $[M+H]^+$ 430.41. HRMS *m/z* $[M+H]^+$ calculated for $C_{25}H_{28}N_5O_2$ 430.2243; found 430.2240**.** HPLC purity 98.89%.

Ethyl 4-(2-(1H-imidazol-1-yl)ethylamino)-6-bromoquinoline-3-carboxylate (16). Compound **1** (0.5 g, 1.59 mmol) was dissolved in 1,4-dioxane (2.5 mL) and DMF (1 mL) under N_2 atmosphere. To the reaction mixture dry DIPEA (0.55 mL, 3.18 mmol) and 2-(1*H*-imidazol-1-yl)ethanamine (0.27 g, 2.39 mmol) were added respectively. The reaction mixture was heated for 12 hours at 100 ºC. 1,4-Dioxane and DMF were removed under vacuum, the residue dissolved in $CHCl_3$/ MeOH mixture and the organic layer was washed with water and brine, dried and concentrated. The residue was purified by silica gel column chromatography, eluting with 4% methanol in $CHCl_3$ to produce compound **16** as a yellow solid (0.35 g, 57% yield). Mp 218 ºC. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.79 (s, 1H), 8.45 (s, 1H), 8.35 (br. s, -NH), 7.85 (dd, J = 9.0, 1.8 Hz, 1H), 7.76 (d, J = 9 Hz, 1H), 7.58 (s, 1H), 7.13 (s, 1H), 6.80 (s, 1H), 4.33-4.30 (m, 4H), 3.89-3.88 (m, 2H), 1.32 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 168.4, 155.8, 151.6, 149.7, 134.5, 131.9, 127.5, 120.7, 118.2, 105.1, 61.3, 49.6, 47.5, 14.3.

MS (ESI) *m/z* $[M+H]^+$ 389.29. HRMS *m/z* $[M+H]^+$ calculated for $C_{17}H_{18}BrN_4O_2$ 389.0613, found 389.0609; $[M+Na]^+$ calculated 411.0433, found 411.0425.

Ethyl 4-((3-(1H-imidazol-1-yl)propyl)amino)-6-bromoquinoline-3-carboxylate (17). Compound **1** (1 g, 3.18 mmol) was dissolved in 1,4-dioxane (5 mL) under N_2 atmosphere. To the reaction mixture dry DIPEA (1.11 mL, 6.36 mmol) and 3-(1*H*-imidazol-1-yl)propan-1-amine (0.57 mL, 4.77 mmol) were added respectively. The reaction mixture was allowed to stir for 12 hours at room temperature. It was poured into 50 mL water. The solid obtained was filtered and dried to give compound **17** as a white solid (1.2 g, 94% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.29 (t, J = 4.8 Hz, -NH), 9.10 (s, 1H), 8.23 (d, J = 2.4 Hz, 1H), 7.82 (d, J = 9.0 Hz, 1H), 7.73 (dd, J = 9.0, 1.8 Hz, 1H), 7.46 (s, 1H), 7.06 (s, 1H), 6.90 (s, 1H), 4.41 (q, J = 7.2 Hz, 2H), 4.15 (t, J = 7.2 Hz, 2H), 3.78-3.75 (m, 2H), 2.28-2.23 (m, 2H), 1.43 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 168.9, 156.0, 151.8, 149.8, 137.1, 134.5, 131.7, 130.0, 128.0, 120.7, 118.7, 117.8, 103.8, 61.2, 45.3, 43.8, 32.4, 14.3. MS (ESI) *m/z* $[M+H]^+$ 403.32. HRMS *m/z* $[M+H]^+$ calculated for $C_{18}H_{20}BrN_4O_2$ 403.0769; found 403.0787.

General procedure D: 4-((2-(1H-Imidazol-1-yl)ethyl)amino)-6-bromoquinoline-3-carbohydrazide (18). Compound **16** (1 g, 2.58 mmol) was dissolved in ethanol (10 mL). To the solution hydrazine hydrate (10 mL) was added. The reaction mixture was stirred for 10 hours at room temperature. Ethanol was removed under vacuum. The residue was dissolved in $CHCl_3$ and the organic layer was washed with water and brine, dried and concentrated to give compound **18** (0.82 g, 85% yield) as a yellow solid. Mp > 250 ºC. ^{1}H NMR (600 MHz, CD_3OD) δ 8.40 (s, 1H), 8.31 (d, J = 1.8 Hz, 1H), 7.78 (dd, J = 9.0, 1.8 Hz, 1H), 7.71 (d, J = 8.4 Hz, 1H), 7.59 (s, 1H), 7.09 (s, 1H), 6.91 (s, 1H), 4.31 (t, J = 6.0 Hz, 2H), 3.83 (t, J = 6.0 Hz, 2H), 1.81 (s, -CONHN**H**$_2$, 2H). ^{13}C NMR (150 MHz, CD_3OD) δ 168.6, 149.7, 149.2, 146.6, 137.3, 133.5, 129.8, 127.8, 124.8, 121.0, 119.4, 118.9, 109.0, 46.1, 46.0. MS (ESI) *m/z* $[M+H]^+$375.05.HRMS *m/z* $[M+H]^+$ calculated for $C_{15}H_{16}BrN_6O$ 375.0569; found 375.0575.

4-(3-(1H-Imidazol-1-yl)propylamino)-6-bromoquinoline-3-carbohydrazide (19). Compound **17** (1 g, 2.48 mmol) was dissolved in ethanol (10 mL). To the solution hydrazine hydrate (10 mL) was added and the reaction was performed according to general procedure **D**. The residue was dissolved in $CHCl_3$ and the organic layer was washed with water and brine, dried and concentrated to give compound **19** as a light green solid (0.75 g, 78% yield). Mp > 250 ºC. ^{1}H NMR (300 MHz, CD_3OD) δ 9.68 (br. s, -NH), 8.59 (d, J = 1.2 Hz, 1H), 8.31 (s, 1H), 7.78 (dd, J = 8.7, 1.5 Hz, 1H), 7.72 (d, J = 8.7 Hz, 1H), 7.60 (s, 1H), 7.42 (t, J = 5.1 Hz, -CON**H**), 7.16 (s, 1H), 6.88 (s, 1H), 4.52 (br. s, -CONHN**H**$_2$), 4.02 (t, J = 6.9 Hz, 2H), 3.32-3.26 (m, 2H), 2.10-2.01 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ167.8, 150.5, 147.7, 147.0, 137.3, 132.6, 131.2, 128.4, 125.0, 121.0, 119.3, 117.7, 109.5, 43.8, 42.2, 30.8. MS (ESI) *m/z* $[M+H]^+$ 389.28. HRMS *m/z* $[M+H]^+$ calculated for $C_{16}H_{18}BrN_6O$ 389.0725; found 389.0724.

General procedure E: N-(2-(1H-Imidazol-1-yl)ethyl)-6-bromo-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (20). Compound **18** (1 g, 2.67 mmol) was taken in triethyl orthoformate (5 mL, 30.06 mmol) and the mixture was heated for 12 hours at 100 ºC. The residue was purified by column chromatography, eluting with 4% methanol in $CHCl_3$ to give compound **20** as a yellow solid (0.22 g, 43% yield). Mp 197 ºC. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.42 (s, 1H), 8.78 (s, 1H), 8.50 (d, J = 2.4 Hz, 1H), 8.14 (t, J = 5.4 Hz, -NH), 7.89 (dd, J = 9.0, 2.4 Hz, 1H), 7.82 (d, J = 9.0 Hz, 1H), 7.55 (s, 1H), 7.09 (s, 1H), 6.78 (s, 1H), 4.27 (t, J = 6.0 Hz, 2H), 3.86-3.84 (m, 2H). ^{13}C NMR (150 Hz, $CDCl_3$) 163.5, 151.4, 151.3, 149.1, 148.4, 137.5, 134.5, 132.1, 130.1, 127.2, 120.4, 119.0, 118.7, 100.7, 49.9, 47.5. MS (ESI) *m/z* $[M+H]^+$ 385.27. HRMS *m/z* $[M+H]^+$ calculated for $C_{16}H_{14}BrN_6O$ 385.0412; found 385.0409.

N-(3-(1H-imidazol-1-yl)propyl)-6-bromo-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (21). Compound **19** (1 g, 2.57 mmol) was taken in triethyl orthoformate (5 mL, 30.06 mmol) and the mixture was heated for 12 hours at 110 ºC. The reaction was carried out according to general procedure **E**. The crude mixture was purified by column chromatography, eluting with 3% methanol in $CHCl_3$ to afford compound **21** as a yellow solid (0.2 g, 19% yield). Mp 147 ºC. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.40

(s, 1H), 8.77 (s, 1H), 8.56 (d, *J* = 1.8 Hz, 1H), 8.18 (br. s, -NH), 7.87 (dd, *J* = 9.0, 1.8 Hz, 1H), 7.81 (d, *J* = 9.0 Hz, 1H), 7.56 (s, 1H), 7.11 (s, 1H), 6.87 (s, 1H), 4.03 (t, *J* = 7.2 Hz, 2H), 3.43-3.40 (m, 2H), 2.13-2.08 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 163.9, 151.4, 151.2, 149.2, 148.5, 134.5, 131.9, 127.8, 120.3, 118.5, 99.4, 45.7, 32.3, 29.7. MS (ESI) *m/z* $[M+H]^+$ 399.37. HRMS *m/z* $[M+H]^+$ calculated for $C_{17}H_{16}BrN_6O$ 399.0569; found 399.0565. HPLC purity 97.38%.

General procedure F. N-(2-(1H-Imidazol-1-yl)ethyl)-6-(4-methoxyphenyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (22). Compound **20** (0.07 g, 0.18 mmol) was dissolved in a mixture of 1,4-dioxane (4 mL) and DMF (1 mL) under argon atmosphere. 2-(4-Methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.043 g, 0.18 mmol) was added to the mixture. 2(M) Na_2CO_3 solution (0.24 mL) was added to the reaction mixture and argon was purged for 15 minutes. $Pd(PPh_3)_4$ (0.017 g, 0.014 mmol) was added to the mixture and argon purging was performed for 15 minutes. The mixture was heated for 12 hours at 100 ºC. 1,4-Dioxane was removed under vacuum, the residue dissolved in $CHCl_3$ and the organic layer was washed with water and brine, dried and concentrated. The residue was purified by silica gel column chromatography to afford compound **22** as a white solid (0.02 g, 27% yield). Mp 213 ºC. ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.05 (s, 1H), 8.62 (t, *J* = 4.2 Hz, 1H), 8.52 (s, 1H), 8.21 (d, *J* = 1.8 Hz, 1H), 8.08 (d, *J* = 9.0 Hz, 1H), 7.95 (dd, *J* = 9.0, 1.8 Hz, 1H), 7.58 (d, *J* = 8.4 Hz, 2H), 7.46 (s, 1H), 7.06 (d, *J* = 8.4 Hz, 2H), 6.98 (s, 1H), 6.91 (s, 1H), 4.36-4.33 (m, 4H), 3.90 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 163.8, 159.7, 152.6, 151.2, 149.4, 147.7, 138.0, 137.5, 132.6, 130.7, 130.1, 128.4, 122.2, 119.6, 118.7, 114.7, 100.7, 55.5, 50.1, 47.6. MS (ESI) *m/z* $[M+H]^+$ 413.16. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{23}H_{21}N_6O_2$ 413.1726; found 413.1732. HPLC purity 98.37%.

N-(3-(1H-imidazol-1-yl)propyl)-3-(1,3,4-oxadiazol-2-yl)-6-phenylquinolin-4-amine (23). Compound **21** (0.08 g, 0.20 mmol) was taken along with phenylboronic acid (0.049 g, 0.40 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.25 mL) and $Pd(PPh_3)_4$ (0.018 g, 0.016 mmol) were added to the mixture and the reaction was performed according to procedure B. The residue

was purified by silica gel column chromatography, eluting with 4% methanol in $CHCl_3$ to produce compound **23** as a white solid (0.032 g, 40% yield). Mp 177 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.11 (s, 1H), 8.82 (br. s, -NH), 8.53 (s, 1H), 8.38 (s, 1H), 8.09 (d, J = 9.0 Hz, 1H), 7.98 (d, J = 8.4 Hz, 1H), 7.63 (d, J = 7.5 Hz, 2H), 7.53 (t, J = 7.5 Hz, 2H), 7.46-7.41 (m, 2H), 7.04 (s, 1H), 6.91 (s, 1H), 4.22 (t, J = 6.9 Hz, 2H), 4.03-3.98 (m, 2H), 2.41-2.32 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.2, 152.7, 151.1, 149.9, 148.2, 140.4, 138.0, 137.1, 130.8, 130.7, 129.9, 129.2, 127.9, 127.3, 123.6, 119.3, 118.8, 99.2, 46.0, 44.0, 32.5. MS (ESI) *m/z* $[M+H]^+$ 397.43. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{23}H_{21}N_6O$ 397.1777; found 397.1771. HPLC purity 99.53%.

4-(4-(3-(1H-Imidazol-1-yl)propylamino)-3-(1,3,4-oxadiazol-2-yl)quinolin-6-yl)benzonitrile (24). Compound **21** (0.08 g, 0.20 mmol) was taken along with 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzonitrile (0.092 g, 0.40 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.25 mL) and $Pd(PPh_3)_4$ (0.018 g, 0.016 mmol) were added to the mixture and the reaction was performed according to procedure B. The residue was purified by silica gel column chromatography, eluting with 4% methanol in $CHCl_3$ to afford compound **24** as a bright yellow solid (0.035 g, 41% yield). Mp 238 ºC. ^{1}H NMR (300 MHz, $CDCl_3$+1 drop CD_3OD) δ 8.86 (s, 1H), 8.56 (s, 1H), 8.22 (s, 1H), 7.88 (d, J = 8.7 Hz, 1H), 7.78 (dd, J = 8.7, 1.5 Hz, 1H), 7.64 (d, J = 8.4 Hz, 2H), 7.56 (d, J = 8.1 Hz, 2H), 7.31 (s, 1H), 6.77 (d, J = 4.8 Hz, 2H), 4.06 (t, J = 6.9 Hz, 2H), 3.76 (m, 2H), 2.24-2.15 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.1, 152.6, 151.2, 150.5, 148.8, 144.8, 137.2, 135.8, 133.0, 131.2, 130.2, 130.0, 127.9, 124.3, 119.4, 118.8, 118.7, 111.5, 99.5, 46.0, 43.9, 32.3. MS (ESI) *m/z* $[M+H]^+$ 422.04. HRMS (ESI) *m/z* $[M]^+$ calculated for $C_{24}H_{20}N_7O$ 422.1729; found 4221747. HPLC purity 96.02%.

N-(3-(1H-imidazol-1-yl)propyl)-6-(4-fluorophenyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (25). Compound **21** (0.08 g, 0.20 mmol) was taken along with 4-fluorophenylboronic acid (0.056 g, 0.40 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.25 mL) and $Pd(PPh_3)_4$

(0.018 g, 0.016 mmol) were added to the mixture and the reaction was performed according to procedure B. It took 8 hours for the completion of the reaction. The residue was purified by silica gel column chromatography, eluting with 4% methanol in $CHCl_3$ to produce compound **25** as a pale yellow solid (0.025 g, 30% yield). Mp 218 ºC. ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.09, (s, 1H), 8.80 (t, J = 4.8 Hz, -NH), 8.53 (s, 1H), 8.29 (d, J = 1.8 Hz, 1H), 8.07 (d, J = 9.0 Hz, 1H), 7.90 (dd, J = 9.0, 1.8 Hz, 1H),7.58-7.55 (m, 2H), 7.46 (s, 1H), 7.20 (t, J = 9.0 Hz, 2H), 7.03 (s, 1H), 6.89 (s, 1H), 4.22 (t, J = 6.6 Hz, 2H), 3.98-3.96 (m, 2H), 2.37-2.33 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.2, 152.6, 151.1, 149.8, 148.2, 137.1, 137.0, 130.8, 130.6, 130.0, 128.9, 123.4, 119.4, 118.8, 116.2, 116.1, 99.3, 46.0, 43.9, 32.4. ESI-MS m/z $[M+H]^+$ 415.16. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{23}H_{20}FN_6O$ 415.1682; found 415.1682. HPLC purity 96.60%.

4-(4-((3-(1H-Imidazol-1-yl)propyl)amino)-3-(1,3,4-oxadiazol-2-yl)quinolin-6-yl)phenol (26). Compound **21** (0.10 g, 0.25 mmol) was taken along with 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenol (0.11 g, 0.5 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.023 g, 0.02 mmol) were added to the mixture and the reaction was performed according to procedure B. After 7 hours the residue was purified by silica gel column chromatography, eluting with 10% methanol in $CHCl_3$ to get compound **26** as a pale yellow solid (0.04 g, 39% yield). Mp > 250 ºC. ^{1}H NMR (300 MHz, CD_3OD) δ 9.09 (s, 1H), 8.93 (s, 1H), 8.44 (d, J = 1.5 Hz, 1H), 8.02 (dd, J = 8.7, 1.8 Hz, 1H), 7.94 (d, J = 8.7 Hz, 1H), 7.65 (s, 1H), 7.57 (d, J = 8.7 Hz, 2H), 7.15 (s, 1H), 6.97 (s, 1H), 6.97-6.94 (m, 3H), 4.27 (t, J = 6.9 Hz, 2H), 3.98 (t, J = 6.6 Hz, 2H), 2.42-2.33 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$ + 1 drop CD_3OD) δ 164.0, 157.3, 152.5, 151.3, 148.7, 147.4, 138.1, 136.9, 131.3, 130.7, 129.6, 129.3, 128.2, 122.5, 119.2, 118.9, 116.1, 99.0, 46.0, 44.4, 32.1. ESI-MS m/z ($M+H^+$) 413.31. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{23}H_{21}N_6O_2$ 413.1726; found 413.1724. HPLC purity 99.09%.

N-(3-(1H-imidazol-1-yl)propyl)-3-(1,3,4-oxadiazol-2-yl)-6-p-tolylquinolin-4-amine (27). Compound **21** (0.07 g, 0.18 mmol) was taken along with p-tolylboronic acid (0.037 g, 0.27 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.23 mL) and $Pd(PPh_3)_4$ (0.017 g, 0.014 mmol) were added to the mixture and the reaction was performed according to procedure B. After 8 hours the residue was purified by silica gel column chromatography, eluting with 6% methanol in $CHCl_3$ to afford compound **27** as a yellow solid (0.037 g, 51% yield). Mp 228 ºC. ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.07 (s, 1H), 8.77 (t, J = 4.2 Hz, -NH), 8.51 (s, 1H), 8.33 (d, J = 1.8 Hz, 1H), 8.05 (d, J = 9.0 Hz, 1H), 7.94 (dd, J = 9.0, 1.8 Hz, 1H), 7.51 (d, J = 7.8 Hz, 2H), 7.46 (s, 1H), 7.32 (d, J = 7.8 Hz, 2H), 7.04 (s, 1H), 6.90 (s, 1H), 4.21 (t, J = 7.2 Hz, 2H), 3.99-3.96 (m, 2H), 2.43 (s, 3H), 2.36-2.32 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.2, 152.6, 151.0, 149.7, 148.0, 137.9, 137.4, 137.1, 130.7, 130.6, 129.9, 127.1, 123.2, 119.3, 118.8, 99.2, 46.0, 44.0, 32.5, 21.2. MS (ESI) *m/z* $[M+H]^+$ 411.65. HRMS *m/z* $[M+H]^+$ calculated for $C_{24}H_{23}N_6O$ 411.1933; found 411.1930. HPLC purity 99.67%.

N-(3-(1H-imidazol-1-yl)propyl)-6-(4-methoxyphenyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (28). Compound **21** (0.10 g, 0.25 mmol) was taken along with 2-(4-methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.12 g, 0.5 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.023 g, 0.02 mmol) were added to the reaction mixture and the reaction was performed according to procedure B. The residue was purified by silica gel column chromatography, eluting with 7% methanol in $CHCl_3$ to afford compound **28** as a yellow solid (0.035 g, 33% yield). Mp 202 ºC. ^{1}H NMR (300 MHz, CD_3OD) δ 9.15 (s, 1H), 8.97 (s, 1H), 8.53 (s, 1H), 8.50 (s, 1H), 8.30 (d, J = 8.7 Hz, 1H), 7.98 (d, J = 8.4 Hz, 1H), 7.70 (d, J = 8.7 Hz, 2H), 7.50 (s, 1H), 7.32 (s, 1H), 7.10 (d, J = 8.4 Hz, 2H), 4.43 (t, J = 7.2 Hz, 2H), 4.13 (t, J = 6.3 Hz, 2H), 3.89 (s, 3H), 2.53-2.45 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.2, 159.6, 152.6, 151.0, 149.4, 147.8, 137.6, 137.1, 132.8, 130.6, 130.5, 129.9, 128.3, 122.8, 119.4, 118.8, 114.6, 99.2, 55.4, 46.0, 44.0, 32.5. MS (ESI) *m/z*

$[M+H]^+$ 427.52. HRMS (EI) *m/z* $[M]^+$ calculated for $C_{24}H_{22}N_6O_2$ 426.1804; found 426.1810. HPLC purity 98.44%.

N-(3-(1H-imidazol-1-yl)propyl)-6-(3-methoxyphenyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (29). Compound **21** (0.10 g, 0.25 mmol) was taken along with (3-methoxyphenyl)boronic acid (0.06 g, 0.38 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.023 g, 0.02 mmol) were added to the reaction mixture and the reaction was performed according to procedure B. The residue was purified by silica gel column chromatography, eluting with 9% methanol in $CHCl_3$ to get compound **29** as a pale yellow solid (0.052 g, 49% yield). Mp 96 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.07 (s, 1H), 8.78 (t, J = 4.0 Hz, -NH), 8.50 (s, 1H), 8.34 (d, J = 2.0 Hz, 1H), 8.05 (d, J = 8.8 Hz, 1H), 7.93 (dd, J = 8.4, 2 Hz, 1H), 7.44 (d, J = 4.0 Hz, 1H), 7.41 (d, J = 8.0 Hz, 1H), 7.18 (dd, J = 7.6, 1.2 Hz, 1H), 7.14 (m, 1H), 7.01 (s, 1H), 6.94 (dd, J = 8.4, 1.6 Hz, 1H), 6.89 (s, 1H), 4.19 (t, J = 6.8 Hz, 2H), 3.99-3.95 (m, 2H), 3.88 (s, 3H), 2.37-2.30 (m, 2H).^{13}C NMR (150 MHz, $CDCl_3$) δ 164.2, 160.2, 152.6, 151.1, 149.9, 148.2, 141.8, 137.8, 130.8, 130.6, 130.3, 129.9, 123.7, 119.7, 119.3, 118.9, 113.5, 112.7, 99.2, 55.4, 46.0, 44.0, 32.5. MS (ESI) *m/z* $[M+H]^+$ 427.37. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{24}H_{23}N_6O_2$ 427.1882; found 427.1880. HPLC purity 95.44%.

N-(3-(1H-Imidazol-1-yl)propyl)-6-(2-methoxyphenyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (30). Compound **21** (0.08 g, 0.2 mmol) was taken along with (2-methoxyphenyl)boronic acid (0.06 g, 0.4 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.018 g, 0.02 mmol) were added to the reaction mixture and the reaction was performed according to procedure B. The residue was purified by silica gel column chromatography, eluting with 8% methanol in $CHCl_3$ to get compound **30** as a white solid (0.024 g, 23% yield). Mp 90 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.07 (s, 1H), 8.82 (br. s, -NH), 8.50 (s, 1H), 8.39 (s, 1H), 8.03 (d, J = 6.6 Hz, 1H), 7.89 (d, J = 6.3 Hz, 1H), 7.44 (s, 1H), 7.39-7.35 (m, 2H), 7.09 (t, J = 5.4 Hz, 1H), 7.04-7.00 (m, 2H), 6.88 (s, 1H), 4.18 (t, J = 5.1 Hz, 2H), 3.97-3.93 (m, 2H), 3.80 (s, 3H), 2.33-2.30 (m, 2H). ^{13}C NMR (125

MHz, $CDCl_3$) δ 164.3, 156.5, 152.6, 151.0, 149.3, 147.9, 135.2, 133.3, 130.8, 129.6, 129.4, 126.1, 121.3, 118.7, 111.6, 98.8, 55.7, 45.7, 44.0, 32.5. MS (ESI) *m/z* $[M+H]^+$ 427.29. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{24}H_{23}N_6O_2$ 427.1882; found 427.1889**.** HPLC purity 96.15%.

N-(3-(1H-Imidazol-1-yl)propyl)-6-(3,4-dimethoxyphenyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (31). Compound **21** (0.10 g, 0.25 mmol) was taken along with 3,4-dimethoxyphenylboronic acid (0.091 g, 0.5 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.023 g, 0.02 mmol) were added to the mixture and the reaction was performed according to procedure **B**. After 24 hours the residue was purified by silica gel column chromatography, eluting with 7% methanol in $CHCl_3$ to afford compound **31** as a off-white solid (0.04 g, 35% yield). Mp 205 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.09 (s, 1H), 8.72 (br. s, -NH), 8.53 (s, 1H), 8.31 (s, 1H), 8.07 (d, *J* = 8.7 Hz, 1H), 7.94 (dd, *J* = 8.7, 1.2 Hz, 1H), 7.46 (s, 1H), 7.18-7.14 (m, 2H), 7.02 (d, *J* = 7.8 Hz, 2H), 6.90 (s, 1H), 4.22 (t, *J* = 6.9 Hz, 2H), 4.02-3.99 (m, 2H), 3.98 (s, 3H), 3.96 (s, 3H), 2.39-2.31 (m, 2H).^{13}C NMR (75 MHz, $CDCl_3$) δ 164.2, 152.6, 151.0, 149.6, 149.5, 149.2, 148.0, 137.9, 137.1, 133.3, 130.7, 130.6, 129.9, 123.0, 119.7, 119.4, 118.8, 111.8, 110.6, 99.3, 56.1, 45.9, 43.9, 32.5. MS (ESI) *m/z* $[M+H]^+$ 457.45. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{25}H_{25}N_6O_3$ 457.1988; found 457.1992. HPLC purity 95.37%.

N-(3-(1H-imidazol-1-yl)propyl)-6-(2,4-dimethoxyphenyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (32). Compound **21** (0.07 g, 0.18 mmol) was taken along with 2,4-dimethoxyphenylboronic acid (0.064 g, 0.36 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.23 mL) and $Pd(PPh_3)_4$ (0.017 g, 0.014 mmol) were added to the mixture and the reaction was performed according to procedure B. The crude product was purified by silica gel column chromatography, eluting with 7% methanol in $CHCl_3$ to afford compound **32** as a yellow solid (0.035 g, 44% yield). Mp 140 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.07 (s, 1H), 8.80 (br. s, -NH), 8.52 (s, 1H), 8.37 (d, *J* = 1.2 Hz, 1H), 8.02 (d, *J* = 8.4 Hz, 1H), 7.88 (dd, *J* = 9, 1.8 Hz, 1H), 7.47 (s, 1H), 7.30 (d, *J* = 8.4 Hz, 1H), 7.03 (s,

1H), 6.90 (s, 1H), 6.66-6.61 (m, 2H), 4.21 (t, J = 6.9 Hz, 2H), 4.00-3.93 (m, 2H), 3.89 (s, 3H), 3.80 (s, 3H), 2.37-2.28 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.2, 160.8, 157.5, 152.5, 150.9, 149.1, 147.8, 134.9, 133.1, 131.2, 129.3, 125.6, 122.3, 118.7, 105.1, 99.2, 98.8, 55.6, 55.5, 45.6, 43.9, 32.4. MS (ESI) *m/z* $[M+H]^+$ 457.51. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{25}H_{25}N_6O_3$ 457.1988; found 457.1985. HPLC purity 97.51%.

N-(3-(1H-imidazol-1-yl)propyl)-6-(4-methoxy-3-methylphenyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (33). Compound **21** (0.07 g, 0.18 mmol) was taken along with 4-methoxy-3-methylphenylboronic acid (0.058 g, 0.36 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.23 mL) and $Pd(PPh_3)_4$ (0.017 g, 0.014 mmol) were added to the mixture and the reaction was performed according to procedure B. The residue was purified by silica gel column chromatography, eluting with 7% methanol in $CHCl_3$ to produce compound **33** as a pale yellow solid (0.031 g, 40% yield). Mp 191 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.07 (s, 1H), 8.75 (br. s, -NH), 8.52 (s, 1H), 8.29 (s, 1H), 8.04 (d, J = 8.7 Hz, 1H), 7.93 (dd, J = 8.7, 1.2 Hz, 1H), 7.46 (s, 1H), 7.43-7.41 (m, 2H), 7.03 (s, 1H), 6.96 (d, J = 9.3 Hz, 1H), 6.90 (s, 1H), 4.21 (t, J = 6.9 Hz, 2H), 4.02-3.96 (m, 2H), 3.90 (s, 3H), 2.39-2.34 (m, 2H), 2.33 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.3, 157.8, 152.6, 151.0, 149.5, 147.9, 137.9, 137.1, 132.4, 130.7, 130.5, 130.0, 129.5, 127.4, 125.6, 122.7, 119.4, 118.8, 110.4, 99.2, 55.5, 46.0, 44.0, 32.6, 16.6. MS (ESI) *m/z* $[M+H]^+$ 441.41. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{25}H_{25}N_6O_2$ 441.2039; found 441.2038. HPLC purity 96.92%.

N-(3-(1H-imidazol-1-yl)propyl)-6-(4-methoxy-2-methylphenyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (34). Compound **21** (0.08 g, 0.20 mmol) was taken along with 4-methoxy-2-methylphenylboronic acid (0.067 g, 0.40 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.30 mL) and $Pd(PPh_3)_4$ (0.018 g, 0.016 mmol) were added to the mixture and the reaction was performed according to procedure B. After 7 hours the residue was purified by silica gel column chromatography, eluting with 6% methanol in $CHCl_3$ to produce compound **34** as a yellow gummy

liquid (0.040 g, 45% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.07 (s, 1H), 8.76 (br. s, -NH), 8.52 (d, J = 1.8 Hz, 1H), 8.06 (s, 1H), 8.00 (d, J = 7.2 Hz, 1H), 7.66 (d, J = 8.4 Hz, 1H), 7.41 (s, 1H), 7.17 (d, J = 6.6 Hz, 1H), 6.98 (s, 1H), 6.84-6.83 (m, 3H), 4.16 (t, J = 4.8 Hz, 2H), 3.88-3.87 (m, 2H), 3.84 (s, 3H), 2.28 (m, 2H), 2.26 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.2, 159.6, 152.4, 151.1, 149.3, 148.0, 138.4, 137.1, 136.8, 133.5, 133.2, 131.0, 129.7, 129.6, 125.6, 118.9, 118.8, 116.0, 111.5, 99.0, 55.3, 45.8, 43.9, 32.4, 20.9. MS (ESI) *m/z* $[M+H]^+$ 441.15. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{25}H_{25}N_6O_2$ 441.2039; found 441.2040. HPLC purity 96.32%.

5-(4-(3-(1H-imidazol-1-yl)propylamino)-6-bromoquinolin-3-yl)-1,3,4-oxadiazol-2-amine (35). Compound **19** (0.5 g, 1.28 mmol) was dissolved in methanol (5 mL) and to it cyanogen bromide (0.163 g, 1.54 mmol) was added. The mixture was refluxed for 4 hours. Excess solvent was evaporated and the organic part was extracted with $CHCl_3$, washed with saturated $NaHCO_3$ solution, dried and concentrated to give compound **35** (0.1 g, 19% yield). Mp > 250 ºC. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.67, (s, 1H), 8.43 (s, 1H), 8.25 (t, J = 3.6 Hz, -NH), 7.81 (dd, J = 9.0, 1.8 Hz, 1H) 7.78 (d, J = 8.4 Hz, 1H), 7.55 (s, 1H), 7.12 (s, 1H), 6.86 (s, 1H), 4.06 (t, J = 7.2 Hz, 2H), 3.59-3.56 (m, 2H), 2.14-2.09 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.4, 157.0, 149.5, 148.9, 148.1, 133.6, 132.5, 132.0, 128.9, 127.2, 124.2, 120.6, 117.9, 100.2, 45.0, 43.8, 32.1. MS (ESI) *m/z* $[M+H]^+$ 414.23. HRMS *m/z* $[M+H]^+$ calculated for $C_{17}H_{17}BrN_7O$ 414.0678; found 414.0671.

5-(4-(3-(1H-imidazol-1-yl)propylamino)-6-(4-methoxyphenyl)quinolin-3-yl)-1,3,4-oxadiazol-2-amine (36). Compound **35** (0.07 g, 0.17 mmol) was taken along with 2-(4-methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.08 g, 0.34 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.016 g, 0.014 mmol) were added to the mixture and the reaction was performed according to procedure B. The residue was purified by silica gel column chromatography, eluting with 6% methanol in $CHCl_3$ to produce compound **36** as a brown solid (0.017 g, 23% yield). Mp 215 ºC. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.72 (s, 1H), 8.49 (s, 1H),

8.09 (d, *J* = 8.4 Hz, 1H), 7.96 (d, *J* = 8.4 Hz, 1H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.57 (s, 1H), 7.43-7.40 (m, 2H), 7.12 (d, *J* = 9.0 Hz, 2H), 4.26 (t, *J* = 7.2 Hz, 2H), 3.85 (s, 3H), 3.82-3.80 (m, 2H), 2.30-2.25 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.5, 159.8, 156.8, 137.2, 136.5, 132.1, 130.3, 128.8, 122.1, 121.5, 119.4, 115.1, 99.8, 55.8, 45.8, 45.1, 31.3. MS (ESI) *m/z* $[M+H]^+$ 442.29. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{24}H_{24}N_7O_2$ 442.1991; found 442.1991. HPLC purity 97.91%.

N-(3-(1H-imidazol-1-yl)propyl)-6-bromo-3-(5-methyl-1,3,4-oxadiazol-2-yl)quinolin-4-amine (37). Compound **19** (1 g, 2.57 mmol) was dissolved in ethanol (5 mL) and to the solution triethyl orthoacetate (5 mL, 27.27 mmol) was added. The reaction mixture was refluxed for 6 hours. The residue was purified by column chromatography, eluting with 4% methanol in $CHCl_3$ to give **37** as a yellow gummy compound (0.25 g, 24% yield). Mp 105 ºC. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.71 (s, 1H), 8.50 (d, *J* = 1.8 Hz, 1H), 8.16 (t, *J* = 4.8 Hz, -NH), 7.84 (dd, *J* = 9.0, 1.8 Hz, 1H), 7.78 (d, *J* = 8.4 Hz, 1H), 7.53 (s, 1H), 7.10 (s, 1H), 6.85 (s, 1H), 4.04 (t, *J* = 7.2 Hz, 2H), 2.59 (s, 3H), 2.13-2.08 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.2, 162.4, 151.2, 149.1, 148.4, 134.2, 131.9, 127.7, 120.4, 118.4, 99.9, 45.7, 44.0, 32.4, 11.1. MS (ESI) *m/z* $[M+H]^+$ 413.24. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{18}H_{18}BrN_6O$ 413.0725; found 413.0718.

N-(3-(1H-imidazol-1-yl)propyl)-6-(4-methoxyphenyl)-3-(5-methyl-1,3,4-oxadiazol-2-yl)quinolin-4-amine (38). Compound **37** (0.10 g, 0.24 mmol) was taken along with 2-(4-methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.11 g, 0.5 mmol) in 1,4-dioxane (4 mL) and DMF (1 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.023 g, 0.02 mmol) were added to the mixture and the reaction was performed according to procedure B. It took 6 hours to complete the reaction. The residue was purified by silica gel column chromatography, eluting with 4% methanol in $CHCl_3$ to afford compound **38** as a white solid (0.033 g, 31% yield). Mp 224 ºC. ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.02 (s, 1H), 8.73 (t, *J* = 4.8 Hz, -NH), 8.29 (d, *J* = 1.8 Hz, 1H), 8.04 (d, *J* = 9.0 Hz, 1H), 7.91 (dd, *J* = 8.4, 1.8 Hz, 1H), 7.55 (d, *J* = 8.4 Hz, 2H), 7.45 (s, 1H), 7.04 (d, *J* = 8.4 Hz, 2H), 7.03 (s, 1H),

6.90 (s, 1H), 4.20 (t, J = 7.2 Hz, 2H), 3.97-3.93 (m, 2H), 3.88 (s, 3H), 2.69 (s, 3H), 2.34-2.30 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.4, 162.2, 159.6, 152.3, 149.4, 147.8, 137.5, 137.1, 132.9, 130.5, 130.3, 129.9, 128.3, 122.7, 119.5, 118.8, 114.6, 99.8, 55.5, 45.9, 44.0, 32.5, 11.1. MS (ESI) *m/z* $[M+H]^+$ 441.72. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{25}H_{25}N_6O_2$ 441.2039; found 441.2140. HPLC purity 95.01%.

Ethyl 4-((3-(1H-imidazol-1-yl)propyl)amino)-6-(4-methoxyphenyl)quinoline-3-carboxylate (39). Compound **17** (0.1 g, 0.25 mmol) was taken along with 2-(4-methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.12 g, 0.51 mmol) in 1,4-dioxane (5 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.023 g, 0.02 mmol) were added to the mixture and the reaction was performed according to procedure B. The reaction was completed in 6 hours. The residue was purified by silica gel column chromatography to afford compound **39** as a white solid (0.035 g, 33% yield). Mp 190 ºC. ^{1}H NMR (600 MHz, CD_3OD) δ 8.91 (s, 1H), 8.38 (s, 1H), 8.06 (d, J = 8.4 Hz, 1H), 7.87 (d, J = 8.4 Hz, 2H), 7.61 (d, J= 7.2 Hz, 2H), 7.24 (s, 1H), 7.07-7.06 (m, 3H), 4.42 (q, J = 7.2 Hz, 2H), 4.26 (t, J = 6.6 Hz, 2H), 3.95 (t, J = 6.6 Hz, 2H), 3.86 (s, 3H), 2.35-2.32 (m, 2H), 1.43 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, CD_3OD) δ 167.7, 160.1, 157.2, 147.9, 138.1, 131.6, 128.0, 125.6, 123.1, 118.9, 114.3, 102.7, 61.3, 54.5, 48.2, 45.6, 31.2, 13.1. MS (ESI) *m/z* $[M+H]^+$ 431.08. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{25}H_{27}N_4O_3$ 431.2083; found 431.2083.HPLC purity 98.92%.

Ethyl 4-((3-(1H-imidazol-1-yl)propyl)amino)-6-(3,4-dimethoxyphenyl)quinoline-3-carboxylate (40). Compound **17** (0.1 g, 0.25 mmol) was taken along with 3,4-dimethoxyphenylboronic acid (0.091 g, 0.5 mmol) in 1,4-dioxane (5 mL). 2(M) Na_2CO_3 solution (0.3 mL) and $Pd(PPh_3)_4$ (0.023 g, 0.02 mmol) were added to the mixture and the reaction was performed according to procedure B. After 6 hours the residue was purified by silica gel column chromatography to get compound **40** as a white solid (0.038 g, 33% yield). Mp 162 ºC. ^{1}H NMR (300 MHz, CD_3OD) δ 8.98 (s, 1H), 8.54 (s, 1H), 8.24-8.22 (m, 2H), 7.95 (d, J = 8.7 Hz, 1H), 7.41 (s, 1H), 7.33-7.31 (m, 2H), 7.23 (s, 1H), 7.14 (d, J =

9.0 Hz, 1H), 4.48 (q, *J* = 6.9 Hz, 2H), 4.37 (t, *J* = 6.9 Hz, 2H), 4.13 (t, *J* = 6.3 Hz, 2H), 3.95 (s, 3H), 3.92 (s, 3H), 2.51-2.42 (m, 2H), 1.47 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, CD_3OD) δ 167.0, 157.8, 149.9, 149.7, 145.9, 139.2, 132.9, 131.9, 123.7, 123.0, 120.4, 119.8, 118.5, 112.1, 110.8, 102.6, 61.8, 55.4, 55.1, 45.6, 45.1, 30.7, 13.1. MS (ESI) *m/z* $[M+H]^+$ 461.1117. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{26}H_{29}N_4O_4$ 461.2189; found 461.2192. HPLC purity 98.80%.

Ethyl 6-bromo-4-(methylamino)quinoline-3-carboxylate (41). Compound **1** (0.5 g, 1.59 mmol) was dissolved in THF (3mL) under N_2 atmosphere in a sealed tube. To the reaction mixture dry DIPEA (0.6 mL, 3.18 mmol) and methylamine (0.7 mL, 15.9 mmol) were added respectively. The reaction mixture was heated for 16 hours at 60 ºC. Organic part was extracted with chloroform. The crude was purified by column chromatography to obtain compound **41** (0.2 g, 41% yield). Mp 134 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.48 (br. s, -NH), 9.07 (s, 1H), 8.48 (d, *J* = 1.8 Hz, 1H), 7.81 (d, *J* = 9.0 Hz, 1H), 7.72 (dd, *J* = 8.7, 2.1 Hz, 1H), 4.38 (q, *J* = 7.2 Hz, 2H), 3.50 (d, *J* = 5.4 Hz, 3H), 1.42 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 168.8, 156.4, 151.8, 149.8, 134.3, 131.5, 128.4, 120.5, 117.2, 102.8, 60.9, 35.4, 14.3. ESI-MS m/z 309.02 $[M+H]^+$ HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{13}H_{14}BrN_2O_2$ 309.0238; found 309.0236.

6-Bromo-4-(methylamino)quinoline-3-carbohydrazide (42). Compound **41** (0.5 g, 1.62 mmol) was dissolved in ethanol (8 mL). To the solution hydrazine hydrate (8 mL) was added. The reaction mixture was stirred for 12 hours at room temperature. Ethanol was removed under vacuum. The residue was then dissolved in $CHCl_3$ and the organic layer was washed with water and brine, dried and concentrated to give compound **42** as a yellow solid (0.34 g, 73% yield). Mp > 250 ºC. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.59 (s, 1H), 8.51 (d, *J* = 1.8 Hz, 1H), 8.26 (s, -CONH-, 1H), 7.74 (dd, *J* = 9.0, 1.8 Hz, 1H), 7.69 (d, *J* = 9.0 Hz, 1H), 2.91 (d, *J* = 4.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$ + 1 drop CD_3OD) δ 150.3, 149.9, 133.3, 129.6, 124.8, 118.5, 31.3. ESI-MS m/z 295.24 ($M+H^+$). HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{11}H_{12}BrN_4O$ 295.0194; found 295.0192.

6-Bromo-N-methyl-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (43). Compound **42** (0.5 g, 1.7 mmol) was taken in triethyl orthoformate (5 mL, 30.06 mmol) and the mixture was heated for 14 hours at 140 ºC. The residue was purified by column chromatography, eluting with 4% methanol in $CHCl_3$ to afford compound **43** as a yellow solid (0.15 g, 29% yield). Mp 220 ºC. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.02 (s, 1H), 8.57 (s, 1H), 8.48 (s, 1H), 7.86 (d, J = 9.0 Hz, 1H), 7.76 (dd, J = 9.0, 1.5 Hz, 1H), 3.62 (d, J = 5.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.1, 151.0, 148.6, 134.2, 131.8, 128.2, 120.3, 117.9, 98.6, 35.7. ESI-MS m/z 304.86 $[M+H]^+$ HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{12}H_9BrN_4O$ 305.0038; found 305.0029.

6-(4-Methoxyphenyl)-N-methyl-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine (44). Compound **43** (0.1 g, 0.33 mmol) was taken along with 2-(4-methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.12 g, 0.51mmol) in 1,4-dioxane (5 mL). 2(M) Na_2CO_3 solution (0.4 mL) and $Pd(PPh_3)_4$ (0.03 g, 0.03 mmol) were added to the mixture and the reaction was performed according to procedure B. The residue was purified by silica gel column chromatography to afford compound **40** as a white solid (0.038 g, 35% yield). Mp 155 ºC. ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.03 (s, -NH), 8.57 (s, 1H), 8.48 (s, 1H), 8.05 (d, J = 8.4 Hz, 1H), 7.93 (d, J = 8.4 Hz, 1H), 7.62 (d, J = 7.8 Hz, 2H), 7.05 (d, J = 7.8 Hz, 2H), 6.79 (s, 1H), 3.89 (s, 3H), 3.71 (d, J = 4.8 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.2, 159.5, 153.2, 150.8, 147.9, 137.0, 133.0, 130.3, 130.2, 128.3, 123.3, 119.3, 116.1, 114.8, 114.6, 98.3, 55.5, 36.0. MS (ESI) *m/z* $[M+H]^+$ 333.55.HRMS *m/z* $[M+H]^+$ calculated for $C_{19}H_{17}N_4O_2$ 333.1351; found 333.1338. HPLC purity 95.14%.

Recombinant human topoisomerase 1 and plasmid DNA relaxation assay. The recombinant human Top1 was purified from Sf-9 insect cells which were infected with the recombinant baculovirus (a kind gift from Prof. James. J. Champoux) as described previously.[20,35] The type 1 DNA topoisomerases are assayed by the decreased mobility of the relaxed isomers of supercoiled pBS (SK+) DNA in 1% agarose gel. The relaxation assay was carried out with recombinant human Top1 or the

whole cell extracts of MCF7 cells as a source of endogenous Top1, diluted in the relaxation buffer with supercoiled plasmid DNA as described previously.[12,38,40]

Cleavage assay. Plasmid DNA cleavage assay was carried out as described previously.[20,35] Equilibrium Cleavage assays with a 25-mer duplex of an oligonucleotide containing a Top1 binding motif were labeled and annealed as described previously.[38,40] Samples were analyzed by 12 % sequencing gel electrophoresis, dried and exposed on PhosphorImager screens and imaged with Typhoon FLA 7000 (GE Healthcare, UK).

Analysis of compound 28-DNA intercalation. The ability of the drug to intercalate into plasmid DNA was determined by Top1 unwinding assay.[12,40] Assays were performed with 50 fmol of pBluscript (SK+) DNA in the presence or absence of compound **28**, m-AMSA and etoposide. Relaxed DNA was prepared by treatment of the supercoiled plasmid DNA with an excess of topoisomerase I, followed by proteinase K digestion at 37°C, phenol/chloroform extraction and ethanol precipitation. After incubation at 37°C for 15 min, reactions were terminated and electrophoresed on to 1% agarose gel as described above. The DNA band was stained with 0.5 µg/ml of EtBr and visualized by UV light as described above.

Second, an ethidium-displacement fluorescence assay[40] was employed to determine whether the compound **28** binds in the minor groove of DNA. Fluorescence emission spectra (λ_{max}= 590 nm, excitation wavelength 510 nm) were obtained at 25°C. The assays contained 1 µM EtBr, 0–300 mM of the compound **28** and 5 nM calf thymus (CT) DNA in 2 ml of fluorescence buffer.

Cell culture and transfection. Human cancerous cell lines like MCF7, HeLa, HCT116, NIH: OVCAR-3 and HEK293 were obtained from the Developmental Therapeutics Program as kind gift from Dr. Yves Pommier (NIH/NCI/USA), TDP1+/+ and TDP1-/- primary MEF (Mouse Embryonic Fibroblasts) cells, kind gift from Dr Cornelius F Boerkoel (University of British Columbia, Canada)

were cultured as described previously.[51,53] Plasmid DNAs were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Photobleaching experiments. Photobleaching experiments were performed as described formerly[20,50,52] using AndorSpining disc inverted confocal laser-scanning microscope equipped with a 60X/1.42 NA oil-immersion objective (Olympus) and with a CO2-controlled on-stage heated environmental chamber set to 37ºC. FRAP analyses were carried out with living MCF7 cells ectopically expressing EGFP-human Top1 constructs (wild-type and N722S)[50] were grown on chamber cover glass (Genetix, India) and drug treated as indicated. For FRAP analysis, a subnuclear spot was bleached for 30 ms by solid-state laser line (488 nm for EGFP) adapted to the fluorescent protein of interest and FRAP curves were generated individually normalized to the pre-bleach signal as described previously.[50,52,53]

Immunocytochemistry and confocal microscopy. Immunofluorescence staining and confocal microscopy were performed as described by previously.[51–53] After treatment, MCF7 cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Primary antibody against γH2AX (cell signaling, USA) was detected using anti-mouse IgG secondary antibodies labeled with Alexa 488 (Invitrogen). Cells were mounted in anti-fade solution with DAPI (Vector Laboratories, Burlingame, CA) and examined using a laser scanning confocal microscope Leica TCS SP8 confocal laser-scanning microscope with a × 63 oil objective. The γH2AX intensity per nucleus was determined with Adobe Photoshop 7.0 by measuring the fluorescence intensities normalized to the number of cell count.[51]

Cell survival assay. Cell survival was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as discussed previously.[12,20] The percent inhibition of viability for each concentration of the compounds was calculated with respect to the control and IC_{50} values were estimated.

Molecular docking study. The molecular docking experiment was performed for selected compounds with LibDock in Discovery Studio 4.1 client. The ligand centroid coordinates of the ternary complex for docking were defined using the ligand in the Top1-DNA camptothecin crystal structure (PDB code 1T8I) as the centre of the binding pocket (x = 21.386168, y = -2.148207, z = 28.116527). The docking study suggests the probable binding poses of these inhibitors in the binding site in Top1-DNA cleavage complex. The best poses for every ligand were selected based on the hydrogen bond interaction with Arg364, Asn722, Asp533, and π-π hydrophobic interactions. The entire complex was subsequently subjected to minimization using a Full minimization method executing 2000 steps which maintained the RMS gradient of 0.01kcal mol^{-1}. The best binding pose of compound **28** is depicted in figure 1D.

Aqueous solubility assay. 5 µL of 20 mM DMSO stock from the stock plate was added to the reaction deep well plate containing 495 µL of pH 7.4 pION buffer which includes DMSO control and the samples were mixed and incubated for 18 hours. The plate was sealed well during the incubation process. The DMSO content in the sample was <1.0%. The concentration in deep well plates was 200 µM. The working stock plate was prepared by adding 4 µL of 20 mM stock (including DMSO control) to 996 µL of acetonitrile. 75 µL of working stock was added to 75 µL of blank buffer and read on spectrophotometer as a reference plate at 240 nm. At the end of the incubation period, a filter plate was used to vacuum filter 100 µL of sample from the storage plate. This step wets the filters and the filtrate was discarded. Another 200 µL of the sample from a deep well plate was vacuum filtered into a new filter collection plate. 75 µL of the filtrate from the filter collection plate was transferred to a UV sample plate. 75 µL of acetonitrile was added to this UV plate. The solution was mixed and the spectrum was read using the UV spectrophotometer at 240 nm. Plates were read on spectramax using SoftMax Pro software version 5.3.

Human Plasma stability assay. The stock solution of 10 mM in DMSO was prepared and stored at 4 ºC. 25 µM stock of test compound was prepared in acetonitrile: water by diluting from the previously prepared 10 mM stock (i.e. 2.5 µL of 10 mM stock solution was added to 997.5 µL of acetonitrile: water (50:50). The frozen plasma was thawed at room temperature and centrifuged at 1400x RCF 4 ^{0}C, for 15 minutes. Approximately 90% of the clear supernatant fraction was transferred to a separate tube and then was used for the assay.

For 0 min samples, plasma was heat inactivated at 56 ºC for 45 min. 3 µL of 25 µM test compound was added to 72 µL of heat inactivated plasma. A 25 µL aliquot of the mixture was taken and crashed with 200 µL of acetonitrile containing internal standard and further processed along with other time points. A final working stock of 1 µM was prepared by diluting in plasma for other time point samples (i.e. 8 µL of 25 µM acetonitrile: water stock was added to 192 µL of plasma). 200 µL of plasma containing the test compound was incubated for 2 hrs at 37 ºC in a shaker water bath with gentle shaking. 25 µL aliquot of the sample at 0, 15, 30, 60 and 120 min was precipitated immediately with 200 µL of acetonitrile containing internal standard and centrifuged at 4000x RCF, 4 ºC for 20 minutes. 150 µL of supernatant was diluted with 150 µL of water and analyzed on LC-MS/MS.

Lipophilicity assay. 1.56 g $NaH_2PO_4.2H_2O$ was dissolved in 0.5 L water in a 1 L beaker. The volume of the solution was made up to 1 L after adjusting pH to 7.4 using NaOH solution. Equal volumes of sodium phosphate buffer (10 mM, pH 7.4) and n-octanol were added to a separation funnel and mixed thoroughly. The two layers were allowed to separate for 2 days and then dispensed in two separate glass bottles. 10 mM stock solution was prepared in 100% DMSO and stored at 4 ºC. 500 µL of the organic phase (1-octanol) was added to each well of a 2 mL deep well plate, followed by 500 µL of buffer and 15 µL of the test substance was added. The plate was vortexed for 1 hr on a plate shaker at 1200 rpm. The samples were allowed to equilibrate for 20 min after incubation and then centrifuged at 4000 rpm for 30 min for complete phase separation and analysed by LC-UV.

Caco-2 permeability assay. 5 mL of 100 mM sodium pyruvate, 5 mL of 100xnon essential amino acids, 5 mL of pen-strep were added to 100 mL of heat-inactivated fetal bovine serum to 385 mL of DMEM aseptically and mixed thoroughly. One vial of Hank's balanced salt (Sigma-H1387) was dissolved in 900 mL of milli Q water and adjusted the pH to 7.4. The volume was made up to 1000 mL with the same. The solution was filter sterilized and stored at 4 ºC. Stock solution of test compound (10 mM) was prepared in DMSO. 10 mM stock was diluted with HBSS Buffer to a final concentration of 10 µM.

Revival of Caco-2 cells: As per SOP-BIO-IA-TCL-013-00

Sub culturing of Caco-2 cells: As per SOP-BIO-TCL-013-00

250 µL of DMEM was added to the basal compartment of 96 well multi-screen Caco-2 plate and seeded 12000 cells/well (0.16 x 106 cells/ml) in all the apical wells required and one well with only media as blank without cells, placed the Caco-2 plate in CO_2 incubator at 37 ºC for proliferation of cells. On the day of assay, the medium was removed and washed twice with HBSS Buffer. The medium was incubated with HBSS buffer for 30 min in an incubator and wells with TEER values > 230 ohm.cm^2 were selected for the incubation. 75 µL of the test compound was added to apical wells and 250 µL of HBSS buffer with 2% BSA was added to basal wells. 25 µL of basal samples were collected at 120 min and processed as mentioned below. 250 µL of the test compound was added to basal wells and 75 µL of HBSS buffer with 2% BSA was added to apical wells. 25 µL of apical samples were collected at 120 min and processed as stated below. Single point calibration curve in HBSS buffer with 2% BSA was used. Donor samples were diluted 1:1 with HBSS containing 2% BSA and receiver samples were diluted with 1.1 HBSS buffer. It was precipitated with 200 µL of acetonitrile containing internal standard and vortexed for 5 min @ 1000 rpm, centrifuged at 4000 rpm for 10 min. Finally, 100 µL of supernatant was diluted with 200 µL of water and submitted for LC-MS/MS analysis.

ASSOCIATED CONTENT

Supporting Information

Supporting Information includes the following things.

a) X-ray crystal data of compound **28**,

b) Cytotoxicity data of compound **28** and CPT in various cell lines,

c) Molecular docking analysis of compound **22**, **27**, **31**, and **33**,

c) ^{1}H NMR and ^{13}C NMR spectra of compounds **1**-**44**,

d) HPLC chromatogram of tested compounds,

e) Molecular formula strings.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

HTop1, Human topoisomerase 1; CPT, Camptothecin; Top1cc, Topoisomerase 1-DNA cleavage complexes, IC_{50}, The half maximal inhibitory concentration; FRAP, Fluorescence Recovery After Photobleaching, Pgp, permeability glycoprotein, ADME, absorption, distribution, metabolism, and excretion; DSBs, DNA double-strand breaks; DIPEA, N,N-Diisopropylethylamine, pBS (SK+) DNA, pBluescript plasmid DNA, EGFP, Enhanced green fluorescent protein

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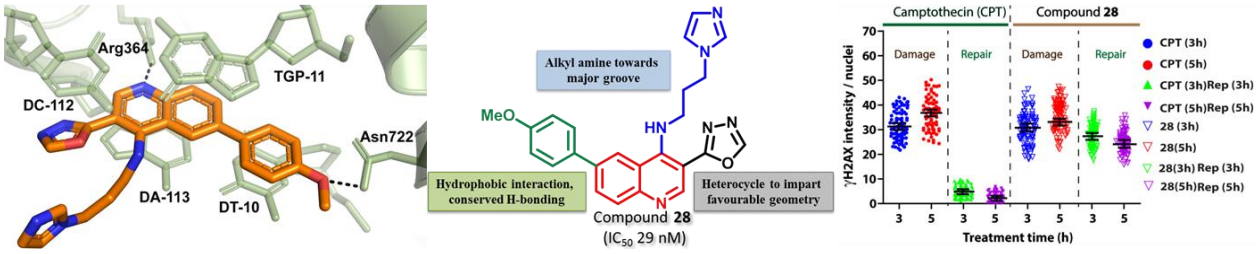
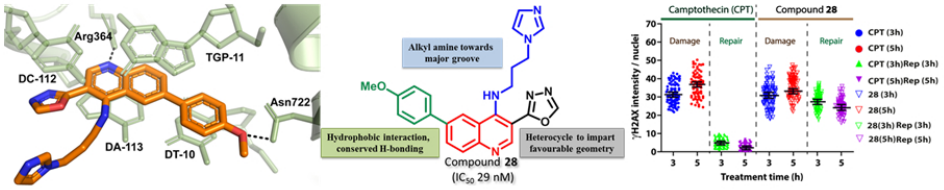
TABLE OF CONTENTS GRAPHICS





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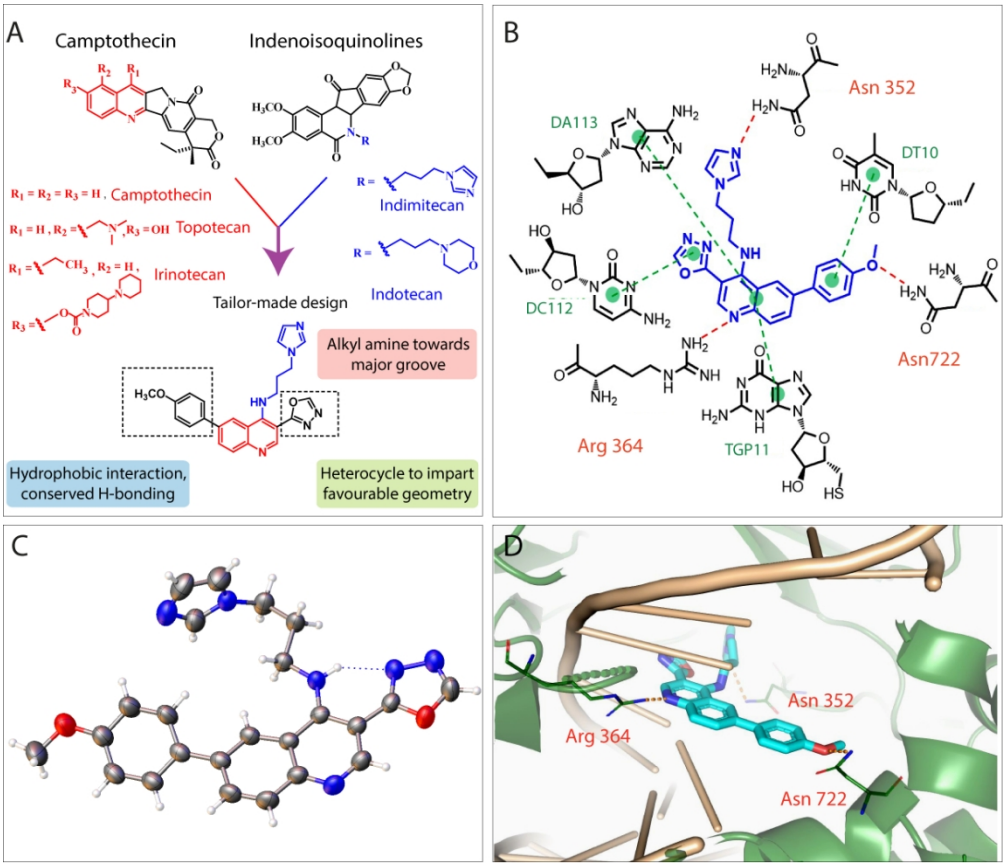
A
Camptothecin
Indenoisoquinolines
R1 = R2 = R3 = H , Camptothecin
R1 = H , R2 = , R3 = OH Topotecan
R1 = , R2 = H , Irinotecan
R3 =
R =
Indimitecan
R =
Indotecan
Tailor-made design
Alkyl amine towards major groove
Hydrophobic interaction, conserved H-bonding
Heterocycle to impart favourable geometry
B
Asn 352
DA113
DT10
DC112
Asn722
Arg 364
TGP11
C
D
Asn 352
Arg 364
Asn 722

Figure 1

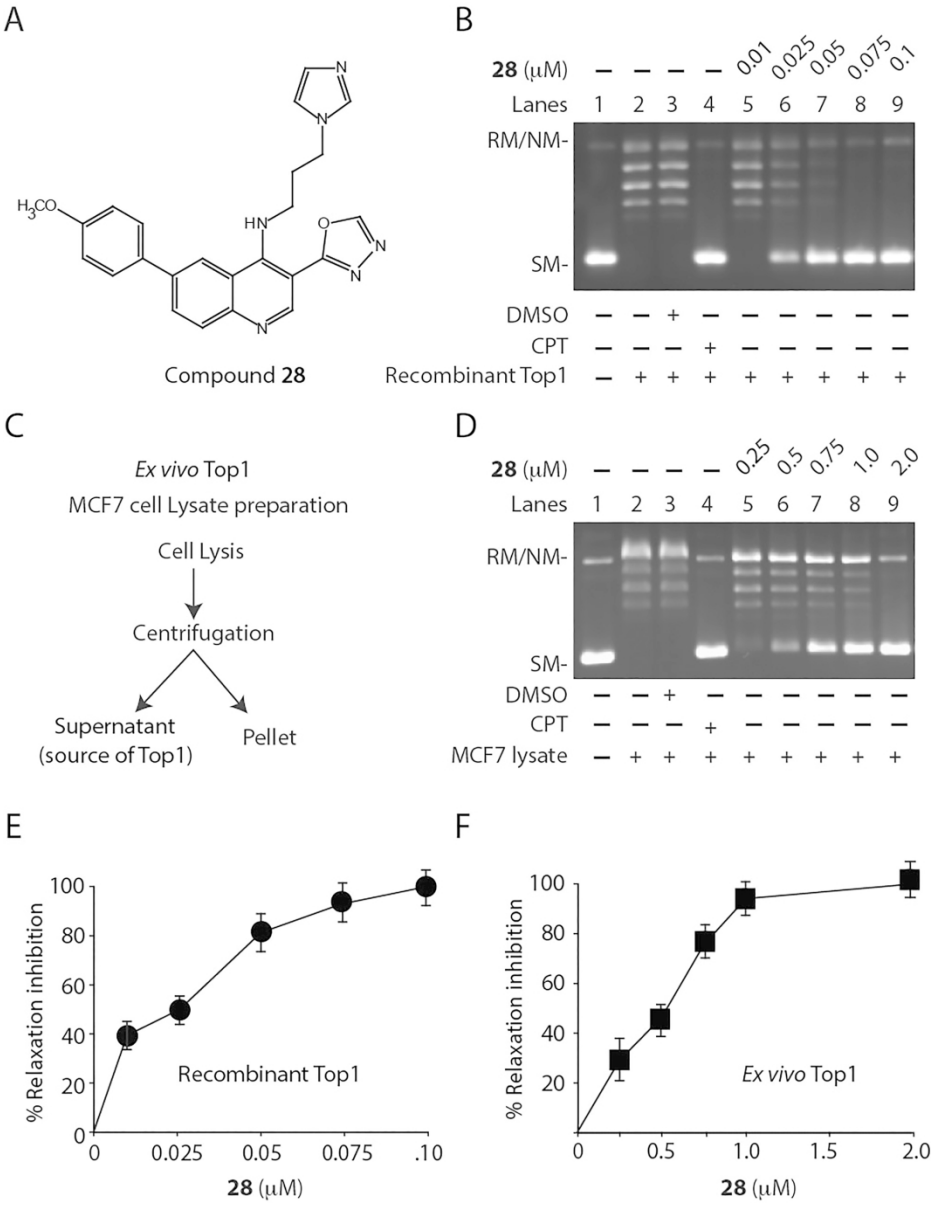

Figure 2

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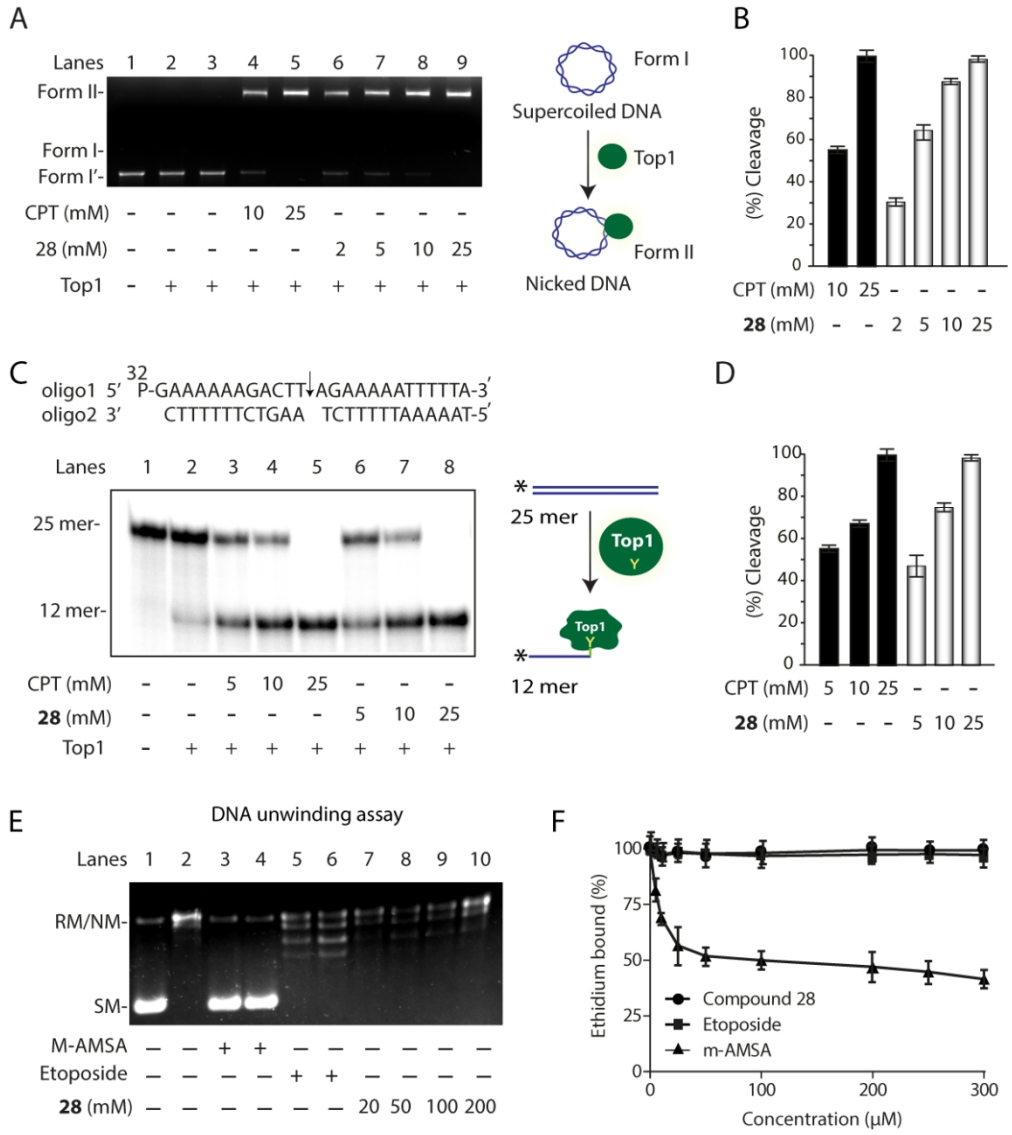

A
Lanes 1 2 3 4 5 6 7 8 9
Form II-
Form I-
Form I'-
CPT (mM) - - - 10 25 - - - -
28 (mM) - - - - - 2 5 10 25
Top1 - + + + + + + + +
Form I
Supercoiled DNA
Top1
Form II
Nicked DNA
B
(%) Cleavage
CPT (mM) 10 25 - - - -
28 (mM) - - 2 5 10 25
C
oligo1 5' 32P-GAAAAAAGACTT↓AGAAAAATTTTTA-3'
oligo2 3' CTTTTTTCTGAA TCTTTTTAAAAAT-5'
Lanes 1 2 3 4 5 6 7 8
25 mer-
12 mer-
CPT (mM) - - 5 10 25 - - -
28 (mM) - - - - - 5 10 25
Top1 - + + + + + + +
25 mer
Top1
12 mer
D
(%) Cleavage
CPT (mM) 5 10 25 - - -
28 (mM) - - - 5 10 25
E
DNA unwinding assay
Lanes 1 2 3 4 5 6 7 8 9 10
RM/NM-
SM-
M-AMSA — — + + — — — — — —
Etoposide — — — — + + — — — —
28 (mM) — — — — — — 20 50 100 200
F
Ethidium bound (%)
Compound 28
Etoposide
m-AMSA
Concentration (μM)

Figure 3

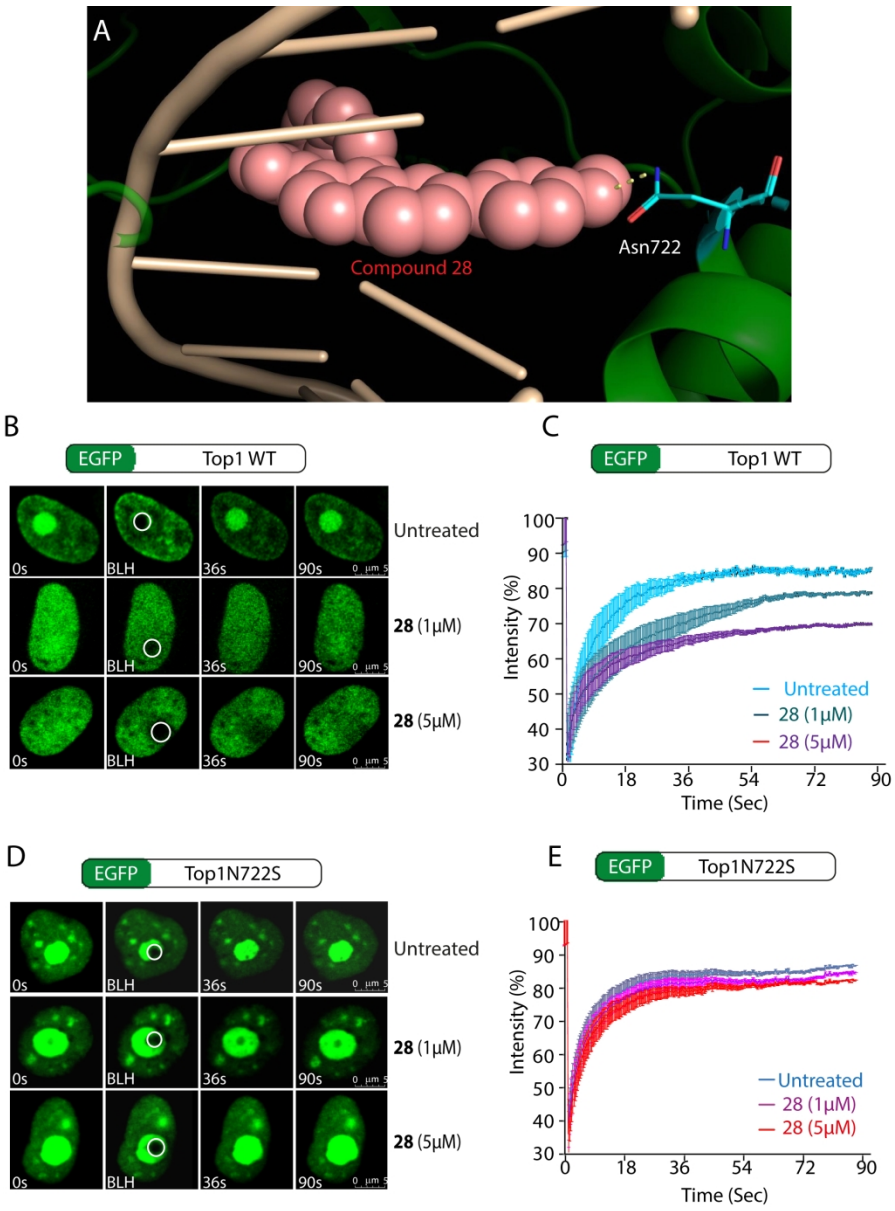
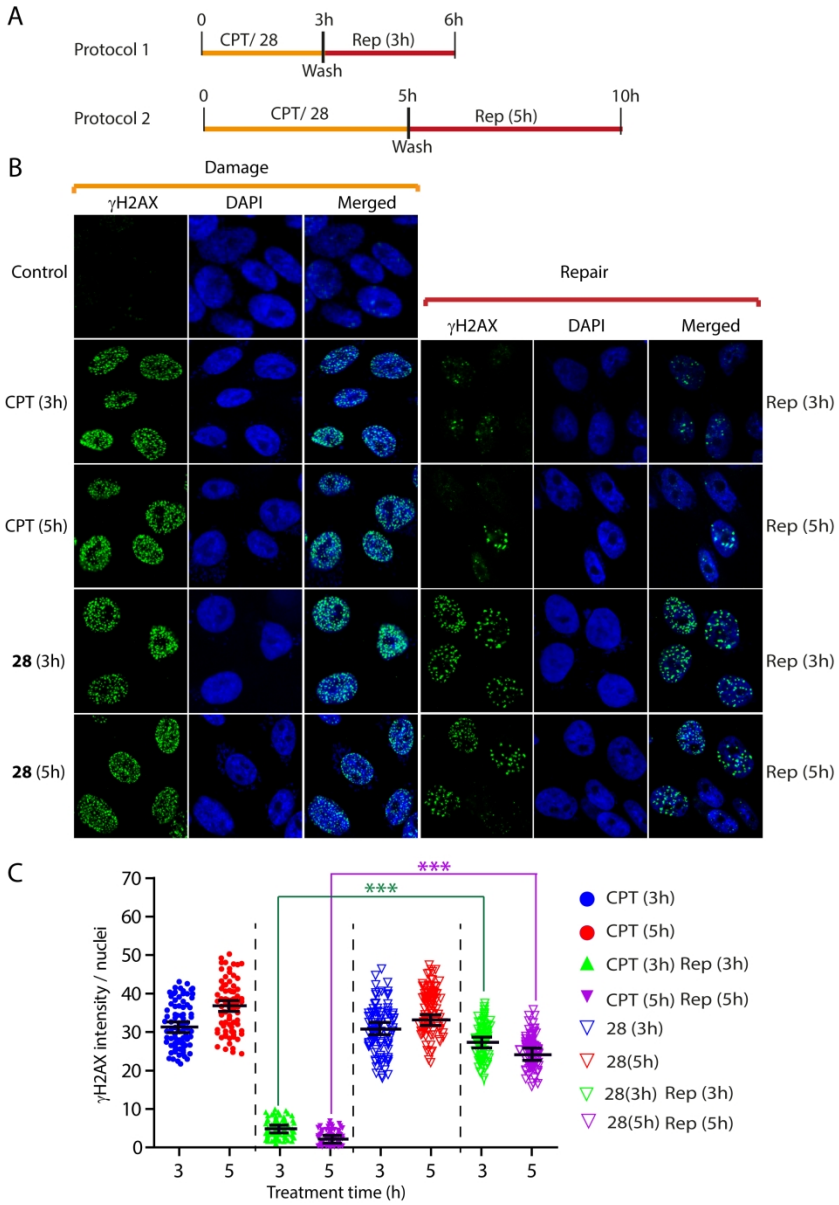

A
Compound 28
Asn722
B
EGFP
Top1 WT
Untreated
28 (1μM)
28 (5μM)
0s
BLH
36s
90s
C
EGFP
Top1 WT
Intensity (%)
Time (Sec)
Untreated
28 (1μM)
28 (5μM)
D
EGFP
Top1N722S
Untreated
28 (1μM)
28 (5μM)
E
EGFP
Top1N722S
Intensity (%)
Time (Sec)
Untreated
28 (1μM)
28 (5μM)

Figure 4


A
Protocol 1
0
3h
6h
CPT/ 28
Rep (3h)
Wash
Protocol 2
0
5h
10h
CPT/ 28
Rep (5h)
Wash
B
Damage
γH2AX
DAPI
Merged
Repair
Control
CPT (3h)
CPT (5h)
28 (3h)
28 (5h)
Rep (3h)
Rep (5h)
C
γH2AX intensity / nuclei
Treatment time (h)
***
***
CPT (3h)
CPT (5h)
CPT (3h) Rep (3h)
CPT (5h) Rep (5h)
28 (3h)
28(5h)
28(3h) Rep (3h)
28(5h) Rep (5h)