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Synthesis and Mechanism Studies of 1, 3-Benzoazolyl Substituted Pyrrolo[2,3-*b*]pyrazine Derivatives as Non-intercalative Topoisomerase II Catalytic Inhibitors

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ABSTRACT:

Novel topoisomerase II (Topo II) inhibitors have gained considerable interests for development of anticancer agents. In this study, a series of 1,3-benzoazolyl-substituted pyrrolo[2,3-*b*]pyrazine derivatives were designed, synthesized, and evaluated as potential Topo II catalytic inhibitors. It was found that some of derivatives had good antiproliferative activity on seven cancer cell lines, especially on HL-60/MX2, a cancer cell line derivative from HL-60 that is resistant to Topo II poison. Topo II mediated DNA relaxation assay results showed that derivatives could significantly inhibit the activity of Topo II, and the structure-activity relationship studies indicated the importance of the alkylamino side chain and the benzoazolyl group. Further mechanism studies revealed that derivatives function as Topo II non-intercalative catalytic inhibitor and may block the ATP binding site of Topo II. Moreover, flow cytometric analysis showed that this class of compounds could induce apoptosis of HL-60 cells.

KEYWORDS: Pyrrolo[2,3-*b*]pyrazines; benzoazolyl; Topo II; non-intercalative catalytic inhibitor; anti-proliferation

INTRODUCTION

Human topoisomerase (Topo) has been recognized as an important target in anticancer drug discovery.[1–4] Two types of topoisomerase exist in humans, namely, type I topoisomerase (Topo I) and type II topoisomerase (Topo II). Both isomers are nuclear enzymes essential to resolve topological problems that occur during DNA transcription, replication, and chromosome segregation.[5–7] Topo I, introduces single-strand breaks in DNA, whereas Topo II introduces double-strand breaks and requires ATP for full activity.[8, 9] Given that the activity of Topo is essential for several cellular processes, investigating the inhibitory activities of eukaryotic Topo is widely performed in anticancer drug development.[10–12]

Topo II is the specific target of some of the most active anticancer drugs such as etoposide, doxorubicin, mitoxantrone, amonafide, and amsacrine.[13] Drugs or agents targeting Topo II based on their mode of action are classified into two types: Topo II poison and Topo II catalytic inhibitor.[14,15] Topo II poison (e.g., doxorubicin, etoposide, amsacrine, and mitoxantrone) stabilizes the Topo-DNA complex and prevents the cleaved DNA strand (s) from religation, leading to an accumulation of undesired truncated DNA. The rest of the Topo II inhibitors, aside from Topo II poisons, can be grouped as Topo II catalytic inhibitors. These catalytic inhibitors act by preventing the binding of Topo II to DNA (e.g., suramin), blocking the ATP-binding site of the enzyme (e.g., novobiocin and salvicine), or inhibiting the cleavage of DNA (e.g., merbarone and bisdioxopiperazines). A series of Topo II poisons is currently widely used in clinical treatment of cancer. However, some mutations and clinic studies have indicated that Topo II poison, related to the level of enzyme-associated DNA breaks versus recombination-repair pathways for the apoptosis, may trigger chromosomal translocations that lead to specific luckemia.[16, 17] Thus, development of Topo II catalytic inhibitors that modulate the cytotoxic effect of Topo II poison and overcome

multidrug resistance has become important in recent years.[18, 19] Several Topo II catalytic inhibitors are clinically used to increase the efficacy of other drugs, for example, aclarubicin[20] as antineoplastic agents, (S)-(+)-1,2-bis(3,5-dioxopiperazinyl)propane (ICRF-187)[21] as cardioprotectors, and novobiocin[22] as modulators.

Pyrrolo[2,3-*b*]pyrazine derivatives are a class of biologically active compounds with antibronchospastic effect and ability to inhibit important kinases, including p38 MAP kinase, cyclin-dependent kinases, and glycogen synthase kinase-3, displaying an anticancer effect.[23–26]Among the most efficient pyrrolo[2,3-*b*]pyrazine derivatives are aloisine A and B (Figure 1). Aloisines bind to the kinase ATP–binding pocket as competitive inhibitors.[27, 28] Recently, Daniel Reker et al, reported a series of aloisine derivatives as antimalarial agents. A self-organizing map was used to predict the potent targets of these compounds and the results indicated that the top-ranking targets may be kinases and DNA Topo.[29]

N-fused imidazole is a class of heterocyclic compound represented by marketed drugs such as zolpidem (a hypnotic drug) and zolimidine (an antiulcer drug). Several derivatives of benzoxazoles, benzimidazoles, and related fused heterocyclic compounds have exhibited significant antimicrobial effects and antiviral activities *in vitro*. More detailed investigations on benzimidazole derivatives revealed that these compounds constitute a new class of Topo II inhibitors (Figure 1). Studies on this class of compounds have indicated that the benzimidazole ring in the structure is critical for the activity. The 3D-QSAR study showed that the phenyl group linked to benzimiazole has a special contribution to the activity of the compound. Mechanism studies and molecular docking analysis showed that benzimiazole derivatives function as non-intercalative Topo II catalytic inhibitors and their catalytic inhibition mode may be through blocking the ATP-binding site of enzyme.[30–32]

To search for new anticancer agents that target Topo II with high potency, we introduced the 1,3-benzoazole pharmacophore to the R′ position of aloisine moiety to produce a new class of 1,3-benzoazolyl-subsituted pyrrolo[2,3-*b*]pyrazine derivatives (BPPs, Figure 2). We found that the

new derivatives were similar to adenosine (ANP). When the new scaffold was aligned with ANP in the ATP binding site of Topo II, the aloisine moiety was aligned with pruine rine, and the benzimidazolyl group was partly laminated with the glycosyl group of ANP (Figure 2). The new scaffold overlap with ANP indicated that BPPs may have potential as Topo II inhibitors. Based on this information, a series of BPPs derivatives was synthesized and bio-evaluated. The structure activity relationships and activity mechanism were investigated in this study. The results revealed that BPPs function as potential non-intercalation catalytic inhibitors of Topo II. Further study in the cellular level showed that BPPs displayed strong anti-proliferation activity and showed obvious relationship between cytotoxicity and Topo II inhibition.

■ RESULT AND DISCUSSION

Chemistry. The BPPs were synthesized following the general method, as shown in Schemes 1, and 2. A two-step nucleophilic reaction was used to build the BPP scaffold. The starting materials 5,6-dichloropyrazine-2,3-dicarbonitrile and α-azaheteroarylacetonitriles (**1a**–**e**) were used in the first step as C-nucleophiles to produce intermediates **2a**–**e**. The next step was the nucleophilic substitution of the second chlorine atom by different N-nucleophiles of the primary amines, followed by the addition of the secondary amine to the nitrile group and the formation of the pyrrolo[2,3-*b*]pyrazine cyclic system.[26–27] Compounds **3a**–**3w**, **4m**–**4u**, **5d**–**5t**, **6c**–**6u** and **7c**–**7u** were synthesized with different side chains at the N-position of pyrrole ring. Compounds **8t** and **8u** were synthesized through the route shown in Scheme 2. The 2-(cyanomethyl)benzimidazole reaction with 1,4-dibromobutane provided intermediates **8-1**, **8-2** and **8-3**, which were synthesized according to methods described previously. Compounds **3x** were synthesized through the route shown in Scheme 1 according to the reported method.[48] The structure of the BBPs is shown on Table 1.

BPPs as Topo II potential inhibitors. Several *in vitro* experiments, such as relaxation, cleavage complex, unwinding assay, and ATP competition assay, were performed for the bio-evaluation of the synthesized BPPs on their ability to inhibit Topo II and investigate their mode of action.

Topo II-mediated DNA relaxation assay was first performed to test whether this class of compounds target Topo II similar to our hypothesis, using etoposide as standard and pBR322 DNA as plasmid. Compounds **3a**–**3w** with different amine side chains were first synthesized and tested. The results are presented in Figure 3 and Table 2. Compounds **3m**, **3n**, **3t**, and **3u** almost completely inhibited Topo II activity at 50 μM, whereas the other tested compounds did not show any inhibition. In particular, compounds **3m**, **3n**, **3t**, and **3u**, n = 3 or 4, with respectively a dimethylamino or diethylamino terminal, would seem to be more potent Topo II inhibitors，while introducing a shorter (n = 2, **3c**, **3d**,) or longer side chain (n = 5, **3w**)，which had same terminal as **3m**, **3n**, **3t**, and **3u**, would decrease the inhibitory activity to Topo II. On the other hand, when the length of side chain is n = 3, compound **3q**, **3r**, **3s**, and **3v**, with respectively a alkly, hydroxyl, cyclic amino, and aromatic amino terminal groups, did not show any inhibition on Topo II at 50 μM. The results indicated that the length and terminal groups of side chain have significant impact on the compounds in inhibiting the Topo II activity and should be maintained in future structure modification.

In order to investigate the effect of benzimidazole ring on the activity, this moiety was changed in following four ways: replacement of benzimidazole to benzoxazole (**4m**, **4s**, **4t**, and **4u**) or benzothiazole (**5c**, **5m**, and **5t**); *N*-methylation of benzimidazole (**6c**, **6d**, **6m**, **6n**, **6t**, and **6u**); introduction of chlorine to the phenyl ring of benzimidazole (**7c**, **7d**, **7m**, **7n**, **7t**, and **7u**); and removing of benzimidazole group (**3x**). As shown in Figures 3D, compounds **4m**, **4t**, **5m**, **5t**, **6t**, and **6u** showed strong activity in the inhibition of Topo II at 20 μM. Comparing those compounds with same pyrrolo[2,3-*b*]pyrazine moiety, it was found that benzimidazole derivatives (**3m** and **3t**) and

benzothiazole derivatives (**5m** and **5t**) showed an equal activity in the inhibition of Topo II, but benzoxazole derivatives (**4m** and **4t)** had a weaker inhibition. It was found that *N*-methylation of benzimidazole led to a decrease of inhibitory activity on Topo II when compared the activity of methylation compounds **6m**, **6n**, **6t**, and **6u** with that of nonmethylation compounds **3m**, **3n**, **3t**, and **3u** (Figure 3C and 3D). In addition, as shown in Figure 3C, compounds **7c**, **7d**, **7m**, **7n**, **7t**, and **7u** showed a weak Topo II inhibition activity at 50 μM, indicating the introduction of chlorine to benzimidazole has an unfavorable effect on the activity. Moreover, removing of benzimidazole from the scaffold, compounds **3x** did not showed any Topo II inhibition activity at 50 μM (Figure 3C). These results revealed the importance of the benzoazolyl as a functional scaffold.

In addition, compounds **8t** and **8u**, which moved the amine side chain from pyrrolo ring to imidazole ring, also showed a moderate Topo II inhibition activity (Figures 3C and 3D).

Topo I-mediated DNA relaxation assay was performed to test whether this class of compounds also target Topo I. The result is presented in Figure S1. All of the tested BPPs did not exhibit Topo I inhibitory activity at 50 μM, indicating that BPPs selectively inhibited the activity of Topo II.

As compounds **3t** and **3u** showed the best Topo II inhibition activity, these two compounds were selected for the further mechanism studies.

BPPs as non-intercalative Topo II catalytic inhibitor. Topo II inhibitors are classified according to their ability to induce DNA double-strand breaks with (Topo II poisons) or without (Topo II catalytic inhibitors) the formation of cleaved complex, reflecting different inhibition mechanisms. Moreover, Topo II catalytic inhibitors can antagonize Topo II poison-mediated DNA damage.[33] To gain insight into the mode of action, cleavage complex assay was performed to verify whether BPPs function as Topo II poisons or catalytic inhibitors. Etoposide, a Topo II poison, exhibited its activity by stabilizing Topo II-DNA covalent complexes *in vitro* and *in vivo*, leading to the formation of linear DNA.[34, 35] In contrast to etoposide, the linear form of the DNA is not visible

in compounds **3t** and **3u** (Figure 4A). These results showed that the tested compound did not act as classical Topo II poison even at 20 μM. In addition, the linear band was reduced when the etoposide was pretreated with compounds **3t** and **3u**, which revealed its antagonizing effect on Topo II poison (Figure 4A). These observations indicate that these BPPs act as Topo II catalytic inhibitor. Furthermore, the ability of compound **3t** to induce DNA damage in cells was examined to confirm this mode of inhibition. A hallmark of DNA double–strand breaks termed γ-H_2AX was measured by western blot analysis in the HL-60 cell line.[36] Figure 4B showed that etoposide induced DNA double-strand break, whereas compound **3t** did not induce γ-H_2AX formation. Moreover, pretreatment with **3t** blocked etoposide induced γ-H_2AX accumulation, suggesting that compound **3t** antagonized Topo II poison-mediated DNA break. These finding are consistent with the results obtained from the cleave assay.

Topo II catalytic inhibitors have two types, namely, DNA intercalators and non-intercalators.[4] In order to examine whether compounds function as DNA intercalators or not, we first employed Topo I mediated DNA intercalating/unwinding assay using a pBR322 DNA as a substrate, since the reformation of supercoiled DNA in this assay is a characterization of intercalative drugs.[37] As shown in Figure 4C, the selected compounds **3t** and **3u** cannot intercalate into plasmids by transforming relaxed DNA into a supercoiled DNA substrate in the presence of Topo I even at high concentration (50 μM), unlike the action of ethidium bromide (EB), which is a classic intercalator of DNA. The ability of **3t** to interact with DNA was also confirmed by fluorescence-based EB displacement assay (Figure S2). The DNA bound form of EB has a stronger fluorescence emission than free EB, so the displacement of EB from DNA could be monitored by a decrease in the fluorescence signal.[38] As expected, the addition of the DNA intercalator mAMSA (amsacrine) was accompanied by a concomitant decrease in EB fluorescence. In contrast, the addition of increasing concentration of **3t** did not alter the fluorescence intensity of EB, indicating that **3t** was incapable of intercalating into DNA. In addition, UV-titration was also performed to measure the binding affinity of **3t** to calf

thymus DNA (CT-DNA).[39] The apparent binding constant (K_b) of **3t** to CT-DNA was 2.8×10^5 M^{-1} (Figure S3), indicating that BPPs showed weak interaction with CT-DNA. Taken together, these finding indicates that the tested compounds are non-intercalative Topo II catalytic inhibitors.

BPPs blocking the ATP-binding site of Topo II. Topo II requires ATP to carry out its essential catalytic cycle.[40] Thus, compounds blocking the ATP binding site of Topo II can affect ATP hydrolysis and influence the progression of the enzyme through catalytic functions. The ATP competition assay was performed to identify the potential action of BPP derivative **3t** to the ATPase domain of Topo II. The result showed that the inhibitory activity of **3t** reduced with the increase in ATP concentrations in Topo II-mediated DNA relaxation. As shown in Figure 5, the concentrations of ATP increased from 1 mM to 4 mM, the corresponding inhibitory activity IC_{50} value for Topo II were increased from 0.9 μM to 16.5 μM. These results indicate that BPPs may act as ATP competitor inhibitors by blocking the ATP-binding site of the enzyme.

ATP competition assay showed that the BPPs catalytic mode of inhibition may be through blocking the ATPase domain of the enzyme. Molecular docking analysis was carried out to identify the potential interaction of BPPs with the ATPase domain of Topo II.[41, 42] Compounds **3c** and **3t** were docked into the catalytic site of the ATPase domain of Topo II (PDB code: 1ZXM) by using the Surflex-dock program incorporated with the SYBYL software package (Tripos, Inc. St. Louis. MO). Both compounds can fit into the ATP binding pocket, as showed in Figure 6.

For active compound **3t** (Figure 6B), the amino group (NH_2) formed a special hydrogen bond interaction with the carboxylic group of residues Thr147, which may provide extra stability to the enzyme–ligand interactions. The benzimidazole group showed hydrophobic interaction with residues Ile141 and Ser149, whereas the pyrazine group formed a hydrophobic interaction with residue Phe142. In addition, the terminal amino group of compound **3t** can fit into the cave formed by residues Asn163, Gly164, Tyr165, Gly166, and Gln376 in the deep site of the ATPase domain. The

non–active compound **3c** (Figure 6A) formed a weak hydrogen interaction with residue Ser149; benzimidazole also displayed hydrophobic interaction with residues Ile141 and Ser149. However, the hydrogen bond interaction with residues Thr147 disappeared, and the terminal amino group cannot fit into the cave formed in the deep site of the ATPase domain. These results showed that BPPs are catalytic inhibitors that act by occupying the ATP binding pocket of the ATPase domain of Topo II and making favorable interactions with its key residues. The molecular docking analysis also explained the important contribution of the terminal side chain to the activity of compounds.

***In vitro* cytotoxic activity.** The cell proliferation inhibitory activities of the compounds are listed in Table 2 as values of IC_{50}. All substances were evaluated for anti-proliferative activities against the human acute leukemia cell line (HL-60), human cervical cancer cell line (Hela), human breast cancer cell line (MDA-MB-201), adenocarcinomic human alveolar basal epithelial cancer cell line (A549), human chronic myeloid leukemia cell (K562), human lymphoma cancer cell line (Raji), HL-60/MX2 (a cancer cell line derivative from HL-60 that is resistant to Topo II poison), and human embryonic kidney 293 cells (HEK-293, a normal cell line) using the MTT assay as described by Mosmann with modifications.[43]

Etoposide was tested as a reference compound. Most of compounds displayed significant antitumor activities with low nanomolar to micromolar IC_{50} values, as shown in Table 2. It was found that most of the compounds showed good correlation between their Topo II inhibitory activity and cytotoxicity toward seven cancer cell lines. The compounds **3m**, **3n**, **3t**, **3u**, **4m**, **4t**, **4u**, **5m**, **5t**, **6m**, **6n**, **6t**, **6u**, **8t**, and **8u**, which showed strong inhibition of Topo II, displayed promising anti-proliferative activity against all the tested seven cancer lines. Compounds **3t** and **3u**, which displayed the most significant Topo II inhibition activity, showed the best anti-proliferation activity, with an IC_{50} ranging between 0.43–11.24 μM and 0.46–10.07 μM. Meanwhile, the other tested compounds that had no effect on Topo II activity showed low or moderate activity, which could be

attributable to their lack of Topo II inhibition. The relationship between cytotoxicity and Topo II inhibition was obvious. However, some compounds, such as **3p**, **3s**, **7m**, **7n**, **7t**, and **7u**, displayed significant cytotoxicity against all the tested cancer cells, although they showed weak Topo II inhibition, suggesting these compounds may target other proteins in cancer cells.[21–27]Moreover, this class of compounds showed a cytotoxic selectivity for the HL-60 cell line, some of the tested compounds displayed a nanomolar level IC_{50}. But BPPs also had cytotoxicity toward HEK-293, which indicated these series of compounds did not display a meaningful selectivity between cancer cells and normal cells.

The HL-60/MX2, a cell line derived from HL-60 cells selected for resistance to mitoxantrone, displays atypical multidrug resistance with altered Topo II catalytic activity and reduced levels of Topo IIα and Topo IIβ proteins.[44] A reduced Topo II catalytic activity means that a decrease of DNA damage; hence, resistance to Topo II posion.[45] Topo II catalytic inhibitor kills cancer cells depending on the inhibition of Topo II; thus, cells with reduced Topo II are sensitive to Topo II catalytic inhibitors. As shown in Table 2, HL-60/MX2 resistant to etoposide, with resistant index (RI) of 56. The BPPs showed an RI range from 0.6 to 4.4, indicating that HL-60/MX2 is not cross-resistant to the BPPs. This result indicates that BPPs act as Topo II catalytic inhibitors, which consistent with the results obtained from the mechanism studies.

Apoptosis Assay by Annexin V/PI binding. Apoptosis is the process of programmed cell death involving a series of biochemical events.[46] In cancer, the apoptosis cell division ratio is altered. Many chemotherapy agents exert their anticancer effects by apoptosis.[47]To investigate the effect of BPPs on apoptosis of HL-60 cells, double staining for FITC-labeled annexin-V binding to phosphatidylserine of membrane and propidium iodide (PI) binding for cellular DNA was carried out, followed by flow cytometry. As shown in Figure 7, after treating HL-60 cell with compounds **3t**

and **3u** (0.5–2 μM) for 12 h, followed by annexin V-FITC/PI staining, the percentages of apoptotic cells greatly increased at a concentration of 2 μM. After incubation (0.25–1 μM) for 24 h, compounds **3t** and **3u** induced the major population of HL-60 cells into the late apoptotic stage (90.51%, 45.05%) at 1 μM concentration, indicating that compounds **3t** and **3u** suppressed HL-60 cell proliferation by inducing apoptosis in a dose and time-dependent manner.

■ CONCLUSION

In this study, we designed and synthesized a series of BPPs as potent Topo II inhibitors, dissected the Topo II inhibition mechanism, and anti-proliferation activity. Forty-five compounds were synthesized, which showed easy preparation and good water solubility. The Topo II inhibitory activity of these compounds was tested by relaxation and cleavage complex in vitro assays. Unwinding assay and EB displacement assay were performed with EB as positive control. The results revealed that 15 compounds, namely, **3m**, **3n**, **3t**, **3u**, **4m**, **4t**, **4u**, **5m**, **5t**, **6m**, **6n**, **6t**, **6u**, **8t**, and **8u**, acted as potent DNA non-intercalating Topo II catalytic inhibitors. The ATP competition assay and molecular docking analysis suggested that the catalytic mode of inhibition of the compounds may be through blocking the ATP-binding site of the enzyme. The Topo II-DNA cleave assay and western blot analysis revealed that these compounds can antagonize Topo II poison-mediated DNA break.

Most of the compounds showed good correlation between their Topo II inhibitory activity and cytotoxicity toward seven cancer cell lines. Investigation of the structure-activity relationship indicated that the length of the side chain and terminal amino groups are important in enhancing both enzyme inhibition and cytotoxicity of BPPs. Furthermore, flow cytometric analysis showed that this class of compounds can significantly induce HL-60 cells apoptosis in a dose and time-dependent manner.

The result indicated that in addition to the 1,3-benzoazole compounds (reported Topo II inhibitor) with aloisines (kinases inhibitor and predicted Topo II inhibitor) exhibited significant Topo II inhibitory activity and anti-proliferation activity. Since Topo II is important target for cancer chemotherapy, these findings may provide advance opportunities for the design and development new chemotherapy agents.

■ EXPERIMENTAL SECTION

Chemistry. ^{1}H and ^{13}C NMR spectra were recorded using TMS as the internal standard in DMSO-d_6 or $CDCl_3$ with a Bruker BioSpin GmbH spectrometer at 400 and 101 MHz, respectively. The chemical shifts are reported in parts per million (ppm) relative to residual $CHCl_3$ (δ = 7.26, ^{1}H; δ = 77.0, ^{13}C) and DMSO-d_6 (δ = 2.50, ^{1}H; δ = 39.5, ^{13}C) in the corresponding deuterium agents. High-resolution mass spectra (HRMS) were recorded on Shimadzu LCMS-IT-TO. The melting point (Mp) were determined using an SRS-OptiMelt automated melting point instrument without correction. The purities of synthesized compounds were confirmed by analytical HPLC performed with a dual pump Shimadzu LC-20AB system equipped with an Ultimate XB-C18 column and eluted with methanol/water (90%) at a flow rate of 0.7 mL min^{-1} and the purities were proved to be higher than 95%. All the reagents were commercially available. The compounds BPPs were prepared following the reported method [28, 29]as mentioned in Scheme 1, and 2. All the products were identified by ^{1}H and ^{13}C NMR and HRMS spectrometry.

General procedure for the synthesis of intermediates 2a, **2b**, **2c**, **2d**, **and 2e.** A solution of 5, 6-dichloropyrazine-2, 3-dicarbonitrile (5.0 mmol) and α-Azaheteroarylacetonitriles (5.0 mmol) in DMF (7mL) was stirred at 40 °C for 3 h. The mixture was left standing at 25 °C for 16 h. The

formed precipitate was filtered off, washed with water and DMF, and dried *in vacuo* to give bright yellow product.

General procedure for the synthesis of compound BPPs. A solution of **2a**, **2b**, **2c**, **2d** or **2e** (0.5 mmol), primary amines (0.55 mmol) and TEA (1.0 mmol) in DMF (3 mL) was stirred at 60 °C for 3 h. The mixture was concentrated in vacuo and the residue taken up in water (100 mL). The precipitate was filtered off, taken up in MeOH (50mL), and stirred at 65 °C for 30 min. The precipitate was filtered off and dried *in vacuo*.

2-(1-Methyl-1*H*-benzo[*d*]imidazol-2-yl)acetonitrile (1d). A suspension of 2-(cyanomethyl)benzimidazol (3.14 g, 20 mmol) and K_2CO_3 (2.76 g, 20 mmol) in DMF (10 mL) was treated with Me_2SO_4 (2.52 mL, 20 mmol) and stirred at 40 °C for 5 h. K_2CO_3 (2.76 g, 20 mmol) and Me_2SO_4 (2.52 mL, 20 mmol) were added. The mixture was stirred at 40 °C for 5 h, poured on brine (100 mL), and extracted with EtOAc. The combined org. phases were kept standing at 4 °C over night, the formed precipitated was filtered off, and the filtrate was concentrated *in vacuo*. The residue were purified by CC (SiO_2; EA:PA = 1:3) to yield **1d** (0.71 mg, 18%) as a brown solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.79 – 7.68 (m, 1H); 7.36 – 7.29 (m, 3H), 4.09 (s, 1H), 3.87 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 146.2, 142.2, 136.4, 122.9, 122.3, 119.3, 116.8, 110.6, 30.3, 17.9.

2-(1-(4-Bromobutyl)-1*H*-benzo[*d*]imidazol-2-yl)acetonitrile (8-1). A suspension of 2-(cyanomethyl)benzimidazol (1.57g, 10 mmol) and cesium carbonate (50 mmol) in Me_2O (50 ml) was treated with 1,4-dibromobutane (50 mmol), removed Me_2O, poured on brine (150 ml), extracted with EtOAc, the org. phases was concentrated *in vacuo*. The residue were purified by CC (SiO_2; EA:PA = 1:3) to yield **8-1** (1.51g, 50%). ^{1}H NMR (400 MHz, DMSO-d_6) : 7.93 (d, J = 8.1 Hz, 1H), 7.71 (dd, J = 6.2, 2.7 Hz, 1H), 7.59 – 7.49 (m, 2H), 4.42 (t, J = 7.4 Hz, 2H), 3.66 (t, J = 6.5 Hz, 3H), 3.52 (s, 2H), 2.00 – 1.92 (m, 2H), 1.89 – 1.69 (m, 2H).

5-((1-(4-Bromobutyl)-1*H*-benzo[*d*]imidazol-2-yl)(cyano)methyl)-6-chloropyrazine-2,3-dicarbonitrile (8-2). A solution of 5, 6-dichloropyrazine-2, 3-dicarbonitrile (5.0 mmol) and **8-1**(5.0 mmol) in DMF (7mL) was stirred at 40 °C for 3 h. The mixture was left standing at 25 °C for 16 h. The formed precipitate was filtered off, washed with water and DMF, and dried *in vacuo* to give bright yellow product. ^{1}H NMR (400 MHz, DMSO-d_6) : 14.28 (s, 1H), 7.93 (d, J = 7.9 Hz, 1H), 7.72 (dd, J = 6.2, 2.3 Hz, 1H), 7.59 – 7.49 (m, 2H), 4.42 (t, J = 7.3 Hz, 2H), 3.66 (t, J = 6.5 Hz, 2H), 2.01 – 1.93 (m, 2H), 1.89 – 1.71 (m, 2H).

6-Amino-7-(1-(4-bromobutyl)-1*H*-benzo[*d*]imidazol-2-yl)-5-methyl-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (8-3). A solution of **8-2** (0.5 mmol), methylamine (0.55 mmol) and TEA (1.0 mmol) in DMF (3 mL) was stirred at 60 °C for 3 h. The mixture was concentrated in vacuo and the residue taken up in water (100 mL). The precipitate was filtered off, taken up in MeOH (50mL), and stirred at 65 °C for 30 min. The precipitate was filtered off and dried *in vacuo*. ^{1}H NMR (400 MHz, DMSO-d_6): 9.26 (s, 2H), 7.62 (dd, J = 8.7, 7.1 Hz, 2H), 7.24 (dd, J = 12.4, 6.3 Hz, 2H), 4.77 – 4.57 (m, 2H), 3.70 (s, 3H), 3.60 (t, J = 6.4 Hz, 2H), 1.94 – 1.52 (m, 4H).

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-methyl-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3a). Methylamine and **2a** were used as reactants to give **3a**.Yield: 83%.Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.89 (s, 1H), 9.15 (s, 2H), 7.63 (d, J = 5.6 Hz, 1H), 7.59 (dd, J = 5.6, 3.2Hz, 1H), 7.20 – 7.14 (m, 2H), 3.68 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.5, 147.0, 142.8, 141.2, 139.3, 134.2, 126.2, 122.0, 118.2, 117.7, 117.0, 116.4, 112.1, 84.3, 28.0. HR ESI-MS $(M + H)^+$ m/z = 315.1092 (Cacld for $C_{16}H_{10}N_8$: 315.1101), HPLC purity: 97.8%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-ethyl-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile(3b). Ethylamine and **2a** were used as reactants to give **3b**. Yield: 91%.Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6): 11.97 (s, 1H), 9.24 (s, 2H), 7.68 – 7.58 (m, 2H), 7.24 – 7.18 (m, 2H), 4.30 (q, J = 7.1 Hz, 2H), 1.32 (t, J = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.1,

146.5, 142.2, 140.2, 138.8, 133.7, 125.7, 121.5, 117.8, 117.2, 116.5, 115.9, 111.8, 83.8, 35.82, 13.61. HR ESI-MS $(M + H)^+$ *m/z* = 327.1118 (Cacld for $C_{17}H_{12}N_8$: 327.1112), HPLC purity: 99.4%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(2-(dimethylamino)ethyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile(3c). *N*, *N*-Dimethylamine and **2a** were used as reactants to give **3c**.Yield: 91%.Yellow solid. Mp >270 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.94 (s, 1H), 9.50 (s, 2H), 7.68 – 7.54 (d, *J* = 10.4 Hz, 2H), 7.22 – 7.14 (m, *J* = 41.9, 5.8, 3.0 Hz, 2H), 4.48 (s, 2H), 3.13 – 2.92 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.8, 146.3, 142.0, 140.7, 138.8, 133.7, 125.6, 121.5, 117.6, 117.2, 116.4, 115.8, 111.7, 83.9, 55.7, 44.0, 38.1. HR ESI-MS $(M + H)^+$ *m/z* = 372.1664 (Cacld for $C_{19}H_{17}N_9$: 372.1680),HPLC purity: 95.9%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(2-(diethylamino)ethyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile(3d). *N*, *N*-Diethylamine and **2a** were used as reactants to give **3d**. Yield: 88%. Yellow solid. Mp >243 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.97 (s, 1H), 9.70 – 9.10 (br s, 2H), 7.70 – 7.58 (m, 2H), 7.21 – 7.13 (m, 2H), 4.37 (q, *J* = 7.0 Hz, 2H), 2.76 (d, *J* = 21.7 Hz, 2H), 0.86 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.9, 146.4, 142.2, 140.9, 138.8, 133.7, 125.6, 121.5, 117.6, 117.2, 116.4, 115.8, 111.7, 83.9, 50.4, 46.3, 25.5 11.3. HR ESI-MS $(M + H)^+$ *m/z* = 400.1983 (Cacld for $C_{21}H_{21}N_9$: 400.1993), HPLC purity: 99.8%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(2-(pyrrolidin-1-yl)ethyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3e). 1-Pyrrolidineethanamine and **2a** were used as reactants to give **3e**.Yield: 86%. Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.97 (s, 1H), 9.32 (br s, 2H), 7.69 – 7.55 (m, 2H), 7.22 – 7.08 (m, 2H), 4.39 (t, *J* = 5.8 Hz, 2H), 2.86 (t, *J* = 5.7 Hz, 2H), 2.58(s, 4H), 1.69 (s, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.4, 146.9, 142.7, 141.2, 139.3, 134.2, 126.2, 122.1, 118.2, 117.7, 117.0, 116.4, 112.3, 84.5, 53.9, 53.8, 41.0, 23.6. HR ESI-MS $(M + H)^+$ *m/z* = 398.1826 (Cacld for $C_{21}H_{19}N_9$: 398.1836), HPLC purity: 97.6%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(2-(4-methylpiperazin-1-yl)ethyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3f). 1-Methyl-4-(2-aminoethyl)piperazine and **2a** were used as

reactants to give **3f**. Yield: 77%. Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.98 (s, 1H), 9.45 (s, 2H), 7.70 – 7.56 (m, 2H), 7.23 – 7.12 (m, 2H), 4.38 (t, J = 5.6 Hz, 2H), 2.71 (t, J = 5.6 Hz, 2H), 2.35 – 2.20 (br s, 4H), 2.14 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.9, 146.5, 142.3, 140.9, 138.9, 133.8, 125.7, 121.6, 117.7, 117.3, 116.5, 115.9, 111.8, 83.9, 55.3, 54.4, 52.4, 45.4. HR ESI-MS $(M + H)^+$ *m/z* = 427.2083 (Cacld for $C_{22}H_{22}N_{10}$: 427.2102), HPLC purity: 99.8%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(2-morpholinoethyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3g). *N*-ethyl-2-morpholinoethanamine and **2a** were used as reactants to give **3g**.Yield: 77%. Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.96 (s, 1H), 9.40 (s, 2H), 7.76 – 7.49 (m, 2H), 7.18 (dd, J = 5.7, 3.2 Hz, 2H), 4.37 (d, J = 5.3 Hz, 2H), 3.51 (s, 4H), 2.70 (t, J = 5.3 Hz, 2H), 2.59 (s, 4). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.2, 147.0, 142.8, 141.4, 139.3, 134.3, 126.3, 122.0, 118.2, 117.7, 117.0, 116.4, 112.3, 84.4, 66.5, 56.3, 53.6, 39.0. HR ESI-MS $(M + H)^+$ *m/z* = 414.1773 (Cacld for $C_{21}H_{19}N_9O$: 414.1785), HPLC purity: 97.1%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-phenethyl-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile(3h). Phenylethylamine and **2a** were used as reactants to give **3h**.Yield: 89%. Yellow solid; Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.96 (s, 1H), 9.25 (s, 2H), 7.70 – 7.58 (m, 2H), 7.28 – 7.15 (m, 7H), 4.52 (t, J = 7.4 Hz, 2H), 3.05 (t, J = 7.3 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.6, 146.9, 142.7, 140.9, 139.0, 137.8, 134.2, 129.5, 128.7, 127.1, 126.1, 122.1, 122.0, 118.2, 117.7, 116.9, 116.3, 112.3, 84.3, 42.4, 34.0. HR ESI-MS $(M + H)^+$ *m/z* = 405.1548 (Cacld for $C_{23}H_{16}N_8$: 405.1571), HPLC purity: 98.3%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(4-methoxyphenethyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3i). 2-(4-Methoxy-phenyl) ethylamine and **2a** were used as reactants to give **3i**. Yield: 87%.Yellow solid; Mp >196 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.98 (s, 1H), 9.25 (s, 2H), 7.21 – 7.14 (m, 2H), 7.11 (d, J = 8.6 Hz, 2H), 6.79 (d, J = 8.6 Hz, 2H), 4.47 (t, J = 7.3 Hz, 2H), 3.69 (s, 3H), 2.98 (t, J = 7.3 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.5, 157.6,

146.8, 140.8, 139.1, 130.4, 129.7, 126.1, 122.0, 118.2, 116.8, 116.3, 114.2, 84.2, 49.0, 42.5, 33.2. HR ESI-MS $(M + H)^+$ *m/z* = 435.1657 (Cacld for $C_{24}H_{18}N_8O$: 435.1676), HPLC purity: 97.5%.

5-(2-(1*H*-indol-2-yl)ethyl)-6-amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3j). 3-(2-Aminoethyl)indole and **2a** were used as reactants to give **3j**.Yield: 91%.Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.97 (s, 1H), 10.85 (s, 1H), 9.27 (s, 2H), 7.71 – 7.58 (m, 2H), 7.53 (d, J = 7.8 Hz, 1H), 7.32 (d, J = 8.1 Hz, 1H), 7.24 – 7.11 (m, 3H), 7.05 (t, J = 7.2 Hz, 1H), 6.94 (t, J = 7.2 Hz, 1H), 4.62 – 4.51 (m, 2H), 3.17 (t, J = 7.3 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.8, 147.0, 142.8, 141.0, 139.3, 136.5, 134.3, 127.5, 126.0, 123.7, 122.1, 122.0, 121.5, 118.8, 118.4, 118.3, 117.7, 116.9, 116.4, 112.3, 111.8, 110.4, 84.4, 41.92, 24.1. HR ESI-MS $(M + H)^+$ *m/z* = 444.1658 (Cacld for $C_{25}H_{17}N_9$: 444.1680), HPLC purity: 99.9%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-propyl-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3k). 1-Propylamine and **2a** were used as reactants to give **3k**.Yield: 83%.Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.97 (s, 1H), 9.22 (s, 2H), 7.68 – 7.58 (m, 2H), 7.21 – 7.15 (m, 2H), 4.21 (t, J = 7.4 Hz, 2H), 2.03 – 1.46 (m, 2H), 0.93 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.8, 147.0, 142.8, 141.0, 139.3, 134.3, 126.0, 123.7, 121.8, 118.4, 117.7, 116.9, 116.4, 112.3, 84.4, 41.92, 24.1, 15.8. HR ESI-MS $(M + H)^+$ *m/z* = 341.1278 (Cacld for $C_{18}H_{14}N_8$: 341.1269, HPLC purity: 97.6%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(2-hydroxyethyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3l). 2-Ethylaminoethanol and **2a** were used as reactants to give **3l**.Yield: 90%.Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.00 (s, 1H), 9.19 (s, 2H), 7.83 – 7.50 (m, 2H), 7.32 – 7.05 (m, 2H), 5.04 (s, 1H), 4.36 (t, J = 5.3 Hz, 2H), 3.78 (t, J = 5.9 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.4, 146.9, 142.7, 141.4, 139.3, 134.2, 126.2, 122.0, 121.9, 118.2, 117.7, 116.9, 116.4, 112.3, 84.4, 58.8, 44.0. HR ESI-MS $(M + H)^+$ *m/z* = 345.1185 (Cacld for $C_{17}H_{12}N_8O$: 345.1207), HPLC purity: 99.6%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(3-(dimethylamino)propyl)-5*H*-pyrrolo[2, 3-*b*] pyrazine-2,3-dicarbonitrile (3m). *N*, *N*-Dimethyl-1,3-propanediamine and **2a** were used as reactants to give **3m**.Yield: 90%. Yellow solid. Mp >218 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.96 (s, 1H), 9.19 (br s, 2H), 7.70 – 7.55 (m, 2H), 7.22 – 7.12 (m, 2H), 4.25 (t, J = 6.7 Hz, 2H), 2.29 (t, J = 6.6 Hz, 2H), 2.16 (s, 6H), 1.96 – 1.84 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.9, 146.4, 142.2, 140.6, 138.9, 133.7, 125.7, 121.5, 117.8, 117.2, 116.5, 115.9, 111.8, 83.9, 55.1, 44.7, 38.7, 25.7. HR ESI-MS $(M + H)^+$ m/z = 386.1827 (Cacld for $C_{20}H_{19}N_9$: 386.1836), HPLC purity: 98.5%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(3-(diethylamino)propyl)-5*H*-pyrrolo[2,3-*b*]pyraz ine-2,3-dicarbonitrile (3n). *N*, *N*-Diethyl-1,3-propanediamine and **2a** were used as reactants to give **3n**.Yield: 92%. Yellow solid. Mp >232 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.94 (s, 1H), 9.20 (br s, 2H), 7.72 – 7.58 (m, 2H), 7.22 – 7.10 (m, 2H), 4.26 (t, J = 7.0 Hz, 2H), 2.45 (m, 6H), 1.94 – 1.80 (m, 2H), 0.92 (t, J = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.7, 146.5, 142.1, 140.5, 138.9, 133.5, 125.7, 121.5, 117.8, 117.4, 116.5, 115.9, 111.8, 83.9, 49.2, 45.6, 25.4, 11.2. HR ESI-MS $(M + H)^+$ m/z = 414.2138（Cacld for $C_{22}H_{23}N_9$: 414.2149), HPLC purity: 99.5%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(3-morpholinopropyl)-5*H*-pyrrolo[2,3-*b*]pyrazine -2,3-dicarbonitrile (3o). *N*-(3-Aminopropyl)pyrrolidine and **2a** were used as reactants to give **3o**.Yield: 88%. Yellow solid. Mp >196 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.98 (s, 1H), 9.23 (s, 2H), 7.70 – 7.59 (m, 2H), 7.23 – 7.14 (m, 2H), 4.33 (t, J = 6.4 Hz, 2H), 3.39 (s, 4H), 2.35 (t, J = 6.3 Hz, 2H), 2.23 (t, J =6.8Hz, 4H), 2.00 – 1.85 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.2, 147.0, 142.8, 141.4, 139.5, 134.3, 126.0, 122.2, 122.0, 118.2, 117.7, 117.0, 116.5, 112.3, 84.5, 68.9, 54.7, 52.9, 23.9. HR ESI-MS $(M + H)^+$ m/z = 428.1929 (Cacld for $C_{22}H_{21}N_9O$: 428.1942), HPLC purity: 97.8%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(3-(4-methylpiperazin-1-yl)propyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3p). 3-(4-Methylpeperazin-1-yl)propylamine and **2a** were used as reactants to give **3p**.Yield: 75%. Yellow solid. Mp >272 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.98 (s, 1H), 9.23 (s, 2H), 7.72 – 7.61 (m, 2H), 7.19 –7.13 (m, 2H), 4.31 (t, J = 6.2 Hz, 2H), 2.33 (t, J = 6.1 Hz, 2H), 2.23 (s, 4H), 2.12 (s, 4H), 2.03 (s, 3H), 1.98 – 1.88 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.3, 147.1, 142.8, 141.4, 139.6, 134.3, 126.1, 122.1, 122.0, 118.2, 117.7, 117.0, 116.5, 112.3, 84.6, 54.8, 52.8, 46.1, 24.6. HR ESI-MS $(M + H)^+$ m/z = 441.2231 (Cacld for $C_{23}H_{24}N_{10}$: 441.2258), HPLC purity: 98.4%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-butyl-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3q). Butylamine and **2a** were used as reactants to give **3q**. Yield: 88%.Yellow solid. Mp >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.99 (s, 1H), 9.23 (s, 2H), 7.68 – 7.58 (m, 2H), 7.20 – 7.15 (m, 2H), 4.26 (t, J = 7.3 Hz, 2H), 1.77 – 1.65 (m, 2H), 1.40 – 1.30 (m, 2H), 0.93 (t, J = 7.4 Hz, 3H). 13C NMR (101 MHz, DMSO-d_6) δ: 158.3, 146.9, 142.7, 141.4, 139.3, 134.2, 126.2, 122.0, 121.9, 118.2, 117.7, 117.0, 116.4, 112.3, 84.4, 43.9, 30.5, 19.7, 14.0. HR ESI-MS $(M + H)^+$ m/z = 355.1428 (Cacld for $C_{19}H_{16}N_8$: 355.1425), HPLC purity: 99.2%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(3-hydroxypropyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3r). Propanolamine and **2a** were used as reactants to give **3r**. Yield: 83%.Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.99 (s, 1H), 9.20 (s, 2H), 7.83 – 7.52 (m, 2H), 7.36 – 7.03 (m, 2H), 4.68 (s, 1H), 4.32 (t, J = 7.2 Hz, 2H), 3.51 (t, J = 6.1 Hz, 2H), 1.95 – 1.87 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.5, 146.6, 142.2, 140.5, 138.9, 133.4, 125.5, 121.6, 121.5, 117.8, 117.2, 116.6, 115.9, 83.8, 58.0, 38.2, 31.0. HR ESI-MS $(M + H)^+$ m/z = 357.1199 (Cacld for $C_{18}H_{14}N_8O$: 357.1218), HPLC purity: 97.9%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(3-(piperidin-1-yl)propyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3s). 1-Piperidinepropanamine and **2a** were used as reactants to give **3s**. Yield: 79%. Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.96 (s, 1H), 9.26 (br s,

2H), 7.68 – 7.58 (m, 2H), 7.22 – 7.14 (m, 2H), 4.29 (t, J = 6.3 Hz, 2H), 2.30 (t, J = 6.1 Hz, 2H), 2.20 (s, 4H), 2.02 – 1.80 (m, 2H), 1.38-1.22 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.0, 146.5, 142.3, 140.9, 139.0, 133.8, 125.5, 121.5, 117.7, 117.2, 116.5, 115.9, 111.8, 84.0, 54.9, 53.6, 39.2, 25.2, 24.3, 23.9. HR ESI-MS (M + H)$^+$ m/z = 426.2128 (Cacld for $C_{23}H_{23}N_9$: 426.2149, HPLC purity: 99.6%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(4-(dimethylamino)butyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3t). *N*, *N*-Dimethylbutylamine and **2a** were used as reactants to give **3t**. Yield: 87%.Yellow solid. Mp >202 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.96 (s, 1H), 9.19 (br s, 2H), 7.71– 7.63 (m, 2H), 7.24 – 7.14 (m, 2H), 4.25 (t, J = 7.3 Hz, 2H), 2.29 (t, J = 7.2 Hz, 2H), 2.14 (s, 6H), 1.80 – 1.70 (m, 2H), 1.52 – 1.44 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.8, 146.9, 142.7, 141.0, 139.3, 134.2, 126.3, 122.0, 118.3, 117.7, 117.0, 116.4, 112.3, 84.3, 58.7, 45.4, 41.0, 26.3, 24.1. HR ESI-MS (M + H)$^+$ m/z = 398.1866 (Cacld for $C_{21}H_{21}N_9$: 398.1847), HPLC purity: 97.1%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(4-(diethylamino)butyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3u). *N*, *N*-Diethylbutylamine and **2a** were used as reactants to give **3u**. Yield: 87%.Yellow solid. Mp>214 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.98 (s, 1H), 9.66 (br s, 2H), 7.68 – 7.58 (m, 2H), 7.23 – 7.13 (m, 2H), 4.32 – 4.26 (m, 2H), 2.48 – 2.34 (m, 6H), 1.79 – 1.69 (m, 2H), 1.50 – 1.40 (m, 2H), 0.93 (t, J = 6.1 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.8, 146.9, 140.9, 139.2, 126.2, 122.0, 118.3, 117.7, 117.0, 116.4, 112.4, 100.0, 84.3, 52.2, 46.9, 41.0, 26.5, 24.1, 12.4. HR ESI-MS (M + H)$^+$ m/z = 427.1275 (Cacld for $C_{23}H_{25}N_9$): 426.2160), HPLC purity: 99.3%.

6-Amino-7-(1*H*-benzo[*d*]imidazol-2-yl)-5-(3-(aniline)propyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3v). *N*-phenylpropane-1,3-diamine and **2a** were used as reactants to give **3v.** Yield: 91%. Mp >212 °C (decomp). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.99 (s, 1H), 9.24 (s, 2H), 7.73 – 7.55 (m, 2H), 7.19-7.15 (m, 2H), 7.05 (t, J = 7.8 Hz, 2H), 6.58-6.52 (m, 3H), 5.55 (s, 1H), 4.37 (t, J

= 6.8 Hz, 2H), 3.09 (t, *J* = 6.2 Hz, 2H), 2.12 – 1.95 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.8, 149.1, 146.9, 140.9, 139.2, 129.3, 126.2, 122.0, 118.3, 116.9, 116.5, 116.3, 112.5, 84.3, 40.7, 28.1. HR ESI-MS $(M + H)^+$ *m/z* = 414.2162 (Cacld for $C_{22}H_{23}N_9$): 414.2149), HPLC purity: 97.0%.

6-Amino-7-(1H-benzo[*d*]imidazol-2-yl)-5-(5-(dimethylamino)pentyl)-6,7-dihydro-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (3w). *N*, *N*-dimethylpentane-1,5-diamine and **2a** were used as reactants to give **3w**. Yield: 88%. Mp > 232°C (decomp). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.94 (s, 1H), 9.26 (br s, 2H), 7.66-7.58 (m, 2H), 7.21-7.13 (m, 2H), 4.22 (t, *J* = 6.8 Hz, 2H), 2.18 (t, *J* = 7.0 Hz, 2H), 2.10 (s, 6H), 1.81 – 1.67 (m, 2H), 1.49-1.41 (m, 2H), 1.38 – 1.25 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.8, 146.9, 141.0, 139.2, 126.3, 122.1, 118.3, 117.1, 116.4, 84.3, 59.3, 45.6, 41.1, 28.3, 27.0, 24.1. HR ESI-MS $(M + H)^+$ *m/z* = 434.1847 (Cacld for $C_{24}H_{19}N_9$): 434.1836), HPLC purity: 96.9%.

6-Amino-5-(4-(dimethylamino)butyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3,7-tricarbonitrile (3x). The **3x** were synthesized following the reported method.[48] A solution of **2f** (0.5 mmol) and *N*, *N*-dimethylbutyldiamine (1.0 mmol) in DMF (3 ml) was stirred at 60 °C for 5 h. It was then cooled, acidified with acetic acid (0.5 ml), the precipitate filtered off, and recrystallized from dioxane. Yield: 70%. Yellow solid. Mp > 208°C (decomp). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.26 (br s, 2H), 4.22 (t, *J* = 6.8 Hz, 2H), 2.66 (t, *J* = 6.0 Hz, 2H), 2.44 (s, 6H), 2.03 – 1.95 (m, 2H), 1.82 – 1.75 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 151.7, 141.0, 139.4, 132.6, 126.3, 118.5, 117.6, 115.8, 88.5, 61.3, 50.0, 47.0, 28.6, 25.5. HR ESI-MS $(M + H)^+$ *m/z* = 308.3476 (Cacld for $C_{15}H_{16}N_8$: 308.3411), HPLC purity: 96.2%.

6-Amino-7-(benzo[*d*]oxazol-2-yl)-5-(3-(dimethylamino)propyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (4m). *N*, *N*-Dimethyl-1,3-propanediamine and **2b** were used as reactants to give **4m**.Yield: 38%.Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.24 (br s, 2H), 7.65 – 7.55 (m, 2H), 7.30 – 7.20 (m, 2H), 4.22 (t, *J* = 6.7 Hz, 2H), 2.35 – 2.25(m, 8H), 2.10 – 2.00 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.8, 146.5, 142.2, 140.6, 138.6, 133.7, 125.7, 121.5, 117.7,

117.2, 116.5, 116.0, 111.7, 83.8, 55.0, 44.7, 38.7, 25.7. HR ESI-MS $(M + H)^+$ *m/z* = 387.1648 (Cacld for $C_{20}H_{18}N_8O$: 387.1676), HPLC purity: 99.6%.

6-Amino-7-(benzo[*d*]oxazol-2-yl)-5-(3-(piperidin-1-yl)propyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (4s). 1-Piperidinepropanamine and **2b** were used as reactants to give **4s**. Yield: 40%.Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.29 (br s, 2H), 7.81 (d, J = 6.8 Hz, 1H), 7.70 (d, J =6.8 Hz, 1H), 7.41 – 7.30 (m, 2H), 4.29 (t, J = 6.3 Hz, 2H), 2.31 (d, J = 5.9 Hz, 2H), 2.26 – 2.18 (m, 4H), 1.978 – 1.87(m, 2H), 1.36 – 1.25 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 159.5, 158.7, 149.5, 141.7, 141.2, 126.5, 125.1, *124*.6, 119.5, 118.7, 116.6, 116.3, 111.0, 82.2, 55.2, 54.1, 25.7, 24.6, 24.4. HR ESI-MS $(M + H)^+$ *m/z* = 427.1987 (Cacld for $C_{23}H_{22}N_8O$: 427.1989), HPLC purity: 98.1%.

6-Amino-7-(benzo[*d*]oxazol-2-yl)-5-(4-(dimethylamino)butyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (4t). *N*, *N*-Dimethylbutylamine and **2b** were used as reactants to give **4t**.Yield: 45%.Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.40 (br s, 2H), 7.71 (dd, J = 6.8, 2.8, 1H), 7.67(dd, J =6.0, 2.0Hz, 1H), 7.40 – 7.32(m, 2H), 4.35 – 4.18 (m, 2H), 2.66 (t, J = 6.0 Hz, 2H), 2.44 (s, 6H), 2.03 – 1.95 (m, 2H), 1.82 – 1.75 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 159.3, 156.7, 151.7, 141.0, 139.4, 132.6, 126.3, 125.8, 124.0, 122.0, 121.0, 118.5, 116.3, 115.8, 88.5, 57.5, 44.0, 40.3, 25.5, 22.7. HR ESI-MS $(M + H)^+$ *m/z* = 401.1816 (Cacld for $C_{21}H_{20}N_8O$: 401.1833), HPLC purity: 95.3%.

6-Amino-7-(benzo[*d*]oxazol-2-yl)-5-(4-(diethylamino)butyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (4u). *N*, *N*-Diethylbutylamine and **2b** were used as reactants to give **4u**.Yield: 43%.Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.37 (br s, 2H),7.85 – 7.76 (m, 1H), 7.70 (d, J = 6.9 Hz, 1H), 7.42 – 7.30 (m, 2H), 4.26 (t, J = 7.2 Hz, 2H), 2.59 (s, 6H), 1.84 – 1.66 (m, 2H), 1.57 – 1.45 (m, 2H), 1.00 (t, J = 7.0 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 159.9, 157.5, 148.8, 140.8, 140.6, 139.3, 126.2, 124.7, 124.1, 119.0, 118.2, 116.1, 115.8, 110.5, 81.5, 51.3,

46.2, 40.6, 25.6, 22.7, 10.8. HR ESI-MS $(M + H)^+$ *m/z* = 429.2123 (Cacld for $C_{23}H_{24}N_8O$: 429.2146), HPLC purity: 98.6%.

6-Amino-7-(benzo[*d*]thiazol-2-yl)-5-(2-(dimethylamino)ethyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (5c). *N*, *N*-Dimethylethylamine and **2c** were used as reactants to give **5c**.Yield: 91%.Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.31 (br s, 2H), 8.08 (d, *J* = 7.9 Hz, 1H), 7.98 (d, *J* = 7.9 Hz, 1H), 7.50 (td, *J* = 8.0, 1.2 Hz, 1H), 7.36 (td, *J* = 8.0, 1.2 Hz, 1H), 4.35 (t, *J* = 5.6 Hz, 2H), 2.74 – 2.66 (m, 2H), 2.27 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 159.8, 158.0, 152.2, 141.8, 140.0, 133.3, 126.8, 124.4, 122.4, 121.4, 118.9, 116.7, 116.2, 89.1, 57.0, 45.4. HR ESI-MS $(M + H)^+$ *m/z* = 387.1156 (Cacld for $C_{19}H_{16}N_8S$): 387.1146, HPLC purity: 95.4%.

6-Amino-7-(benzo[*d*]thiazol-2-yl)-5-(3-(dimethylamino)propyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (5m). *N*, *N*-Dimethyl-1,3-propanediamine and **2c** were used as reactants to give **5m**. Yield: 93%.Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.24 (br s, 2H), 8.11 (d, *J* = 7.4 Hz, 1H), 7.98 (d, *J* = 7.4 Hz, 1H), 7.52 (dt, *J* = 8.0, 1.2 Hz, 1H), 7.37 (dt, *J* = 8.0, 1.2 Hz, 1H), 4.34 (t, *J* = 6.7 Hz, 2H), 2.92 – 2.80 (m, 2H), 2.54 (s, 6H), 2.12 – 2.02 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 159.4, 157.0, 151.8, 141.2, 139.7, 132.6, 126.4, 125.8, 124.0, 122.0, 121.0, 118.5, 116.4, 115.6, 88.6, 54.3, 43.2, 38.4, 24.2. HR ESI-MS $(M + H)^+$ *m/z* = 403.1440 (Cacld for $C_{20}H_{18}N_8S$: 403.1448), HPLC purity: 96.8%.

6-Amino-7-(benzo[*d*]thiazol-2-yl)-5-(4-(dimethylamino)butyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (5t). *N*, *N*-Dimethylbutylamine and **2c** were used as reactants to give **5t**.Yield: 87%.Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.14 (br s, 2H), 8.12 (d, *J* = 7.4 Hz, 1H), 7.99 (d, *J* = 8.1 Hz, 1H), 7.51 (td, *J* =8.0, 1.2 Hz, 1H), 7.37 (td, *J* = 8.0, 1.2 Hz, 1H), 4.28 (t, *J* = 7.1 Hz, 2H), 2.60 – 2.54 (m, 2H), 2.33 (s, 6H), 1.80 – 1.70 (m, 2H), 1.58 – 1.50 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 159.5, 156.8, 151.6, 141.0, 139.5, 126.3, 125.8, 125.3, 124.0, 122.0, 121.0, 118.5, 117.9, 116.3, 115.8, 88.5, 57.5, 44.0, 40.3, 25.5, 22.8. HR ESI-MS $(M + H)^+$ *m/z* = 417.1598 (Cacld for $C_{21}H_{20}N_8S$: 417.1604), HPLC purity: 99.2%.

6-Amino-5-(2-(dimethylamino)ethyl)-7-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (6c). *N*, *N*-Dimethylamine and **2d** were used as reactants to give **6c**. Yield: 83%. Yellow solid. Mp >258 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.29 (br s, 2H), 7.64 (d, J = 6.9 Hz, 1H), 7.58 (d, J = 7.2 Hz, 1H), 7.33 – 7.20 (m, 2H), 4.38 (s, 2H), 4.01 (s, 3H), 2.69 (s, 2H), 2.26 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 159.3, 148.2, 142.9, 141.1, 139.6, 136.3, 125.9, 122.4, 122.2, 118.5, 117.7, 116.9, 116.4, 110.5, 84.4, 57.2, 45.5, 32.8. HR ESI-MS (M + H)$^+$ m/z = 386.1821 (Cacld for $C_{20}H_{19}N_9$: 386.1836), HPLC purity: 97.6%.

6-Amino-5-(2-(diethylamino)ethyl)-7-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (6d). *N*, *N*-Diethylamine and **2d** were used as reactants to give **6d**. Yield: 89%. Yellow solid. Mp >239 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.23 (br s, 2H), 7.67 (d, J = 7.3 Hz, 1H), 7.60 (d, J = 7.4 Hz, 1H), 7.33 – 7.21 (m, 2H), 4.75 (s, 2H), 4.00 (s, 3H), 3.47 (s, 2H), 3.40 (s, 4H), 1.25 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.6, 147.8, 142.8, 141.7, 140.8, 136.2, 126.2, 122.5, 122.3, 118.6, 117.6, 116.9, 116.3, 110.6, 84.8, 47.1, 32.7, 9.0. HR ESI-MS (M + H)$^+$ m/z = 414.2133 (Cacld for $C_{22}H_{23}N_9$: 414.2149), HPLC purity: 96.9%.

6-Amino-5-(3-(dimethylamino)propyl)-7-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (6m). *N*, *N*-Dimethyl-1,3-propanediamine and **2d** were used as reactants to give **6m**. Mp >204 °C (decomp).Yield: 91%. Yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.28 (br s, 2H), 7.65 (dd, J = 6.8, 1.5 Hz, 1H), 7.59 (dd, J = 6.6, 1.4 Hz, 1H), 7.29 – 7.16 (m, 2H), 4.38 (t, J = 5.9 Hz, 2H), 4.01 (s, 3H), 2.68 (t, J = 5.7 Hz, 2H), 2.25 (s, 6H), 2.24 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 159.3, 148.2, 143.0, 141.0, 139.8, 136.3, 125.9, 122.4, 122.2, 118.6, 117.8, 117.0, 116.4, 110.6, 84.4, 55.5, 45.2, 32.7, 26.2. HR ESI-MS (M + H)$^+$ m/z = 400.2003 (Cacld for $C_{21}H_{21}N_9$: 400.1993), HPLC purity: 98.0%.

6-Amino-5-(3-(diethylamino)propyl)-7-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (6n). *N*, *N*-Diethyl-1,3-propanediamine and **2d** were used as reactants to give **6n**. Yield: 76%. Yellow solid. Mp >192 °C (decomp).^{1}H NMR (400 MHz,

DMSO-d_6) δ: 9.24 (br s, 2H), 7.66 (d, J = 7.2 Hz, 1H), 7.60 (d, J = 8.1 Hz, 1H), 7.30 – 7.23 (m, 2H), 4.28 (t, J = 6.9 Hz, 2H), 3.99 (s, 3H), 2.46 – 2.38 (m, 6H), 1.95 – 1.82 (m, 2H), 0.94 (t, J = 7.0 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 159.0, 148.1, 143.0, 140.9, 139.8, 136.3, 125.9, 122.6, 122.4, 118.6, 117.8, 117.0, 116.4, 110.5, 84.4, 49.5, 46.1, 32.7, 25.8, 11.5. HR ESI-MS $(M + H)^+$ m/z = 428.2306 (Cacld for $C_{23}H_{25}N_9$: 428.2306), HPLC purity: 96.5%.

6-Amino-5-(4-(dimethylamino)butyl)-7-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (6t). *N*, *N*-Dimethylbutylamine and **2d** were used as reactants to give **6t**. Yield: 92%. Yellow solid. Mp >228 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.03 (br s, 2H), 7.66 (dd, J = 6.8, 1.4 Hz, 1H), 7.59 (d, J = 7.2 Hz,1H), 7.32 – 7.13 (m, 2H), 4.26 (t, J = 7.2 Hz, 2H), 3.99 (s, 3H), 2.27 (t, J = 7.1 Hz, 2H), 2.13 (s, 6H), 1.76 – 1.68 (m, 2H), 1.53 – 1.45 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.6, 148.1, 142.9, 140.7, 139.6, 136.3, 125.9, 122.4, 122.2, 118.5, 117.8, 117.0, 116.4, 110.5, 84.3, 58.8, 45.5, 41.0, 32.7, 26.4, 24.2. HR ESI-MS $(M + H)^+$ m/z = 414.2141 (Cacld for $C_{22}H_{23}N_9$: 414.2149), HPLC purity: 97.1%.

6-Amino-5-(4-(diethylamino)butyl)-7-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (6u). *N*, *N*-Diethylbutylamine and **2d** were used as reactants to give **6u**. Yield: 88%. Yellow solid. Mp >239 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.97 (br s, 2H), 7.67 (dd, J = 6.9, 1.5 Hz, 1H), 7.60 (dd, J = 6.8, 1.2 Hz, 1H), 7.32 – 7.22 (m, 2H), 4.28 (t, J = 7.3 Hz, 2H), 4.01 (s, 3H), 2.49 – 2.43 (m, 6H), 1.80 – 1.66 (m, 2H), 1.51 – 1.43 (m, 2H), 0.95 (t, J = 6.7 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.6, 148.1, 142.9, 140.7, 139.6, 136.3, 125.9, 122.4, 122.2, 118.6, 117.8, 116.9, 116.4, 110.5, 84.3, 52.1, 46.7, 41.1, 32.7, 26.5, 24.0, 12.1. HR ESI-MS $(M + H)^+$ m/z = 442.2451 (Cacld for $C_{24}H_{27}N_9$: 442.2462), HPLC purity: 99.8%.

6-Amino-7-(6-chloro-1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-5-(2-(dimethylamino)ethyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (7c). *N*, *N*-Dimethylamine and **2e** were used as reactants to give **7c**. Yield: 81%. Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.16 (s, 1H), 9.42 (br s, 2H), 7.67-7.61 (m, 2H), 7.20 (dd, J = 8.4, 1.9 Hz, 1H), 4.38 (t, J = 5.7 Hz, 2H), 2.68 (t, J

= 5.4 Hz, 2H), 2.24 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.4, 141.2, 139.5, 126.2, 126.0, 122.1, 118.5, 116.9, 116.4, 83.9, 55.5, 45.2, 26.2. HR ESI-MS $(M + H)^+$ *m/z* = 406.1280 (Cacld for $C_{19}H_{16}N_9Cl$: 406.1290), HPLC purity: 99.2%.

6-Amino-7-(6-chloro-1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-5-(2-(diethylamino)ethyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (7d). *N*, *N*-Diethylamine and **2e** were used as reactants to give **7d**. Yield: 80%. Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.16 (s, 1H), 9.42 (br s, 2H), 7.68 – 7.60 (m, 2H), 7.20 (dd, *J* = 8.4, 1.9 Hz, 1H), 4.38 (t, *J* = 5.7 Hz, 2H), 2.76 (t, *J* = 5.4 Hz, 2H), 2.52 – 2.44 (m, 4H), 0.84 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.2, 148.2, 141.1, 139.4, 126.4, 126.2, 122.1, 118.5, 116.9, 116.4, 83.9, 49.6, 46.1, 25.8, 11.6. HR ESI-MS $(M + H)^+$ *m/z* = 432.1443 (Cacld for $C_{21}H_{20}N_9Cl$: 432.1457), HPLC purity: 97.1%.

6-Amino-7-(6-chloro-1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-5-(3-(dimethylamino)propyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (7m). *N*, *N*-Dimethyl-1,3-propanediamine and **2e** were used as reactants to give **7m**. Yield: 95%. Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.10 (s, 1H), 9.06 (s, 2H), 7.60 (s, 2H), 7.18 (d, *J* = 7.1 Hz, 1H), 4.23 (s, 2H), 2.30 (s, 2H), 2.17 (s, 6H), 1.89 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.4, 141.2, 139.5, 126.3, 126.1, 122.1, 118.5, 117.0, 116.4, 84.0, 55.5, 45.2, 39.2, 26.2. HR ESI-MS $(M + H)^+$ *m/z* = 420.1438 (Cacld for $C_{20}H_{18}N_9Cl$: 420.1446), HPLC purity: 97. 6%.

6-Amino-7-(6-chloro-1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-5-(3-(diethylamino)propyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (7n). *N*, *N*-Diethyl-1,3-propanediamine and **2e** were used as reactants to give **7n**. Yield: 73%. Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.06 (s, 1H), 9.05 (BR s, 2H), 7.61 (m, 2H), 7.18 (dd, *J* = 8.5, 2.0 Hz, 1H), 4.25 (t, *J* = 6.9 Hz, 2H), 2.49 – 2.39 (m, 6H), 1.98 – 1.74 (m, 2H), 0.92 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 158.2, 141.2, 139.5, 126.2, 126.0, 122.1, 118.5, 116.9, 116.4, 84.0, 49.6, 46.1, 25.8, 11.6. HR ESI-MS $(M + H)^+$ *m/z* = 448.1744 (Cacld for $C_{22}H_{22}N_9Cl$: 448.1759), HPLC purity: 98.9%.

6-Amino-7-(6-chloro-1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-5-(4-(dimethylamino)butyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (7t). *N*, *N*-Dimethylbutylamine and **2e** were used as reactants to give **7t**. Yield: 90%. Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.08 (s, 1H), 9.13 (s, 2H), 7.68-7.62 (m, 2H), 7.19 (dd, J = 8.5, 1.9 Hz, 1H), 4.24 (t, J = 7.1 Hz, 2H), 2.27 (t, J = 7.0 Hz, 2H), 2.13 (s, 6H), 1.77 – 1.69 (m, 2H), 1.50 – 1.44 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.7, 140.9, 139.2, 126.2, 126.0, 122.1, 118.5, 116.9, 116.3, 83.8, 58.6, 45.4, 40.8, 26.2, 24.1. HR ESI-MS $(M + H)^+$ m/z = 434.1589 (Cacld for $C_{21}H_{20}N_9Cl$: 434.1603), HPLC purity: 98.0%.

6-Amino-7-(6-chloro-1-methyl-1*H*-benzo[*d*]imidazol-2-yl)-5-(4-(diethylamino)butyl)-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (7u). *N*, *N*-Diethylbutylamine and **2e** were used as reactants to give **7u**. Yield: 88%. Yellow solid. Mp >300 °C.^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.03 (s, 1H), 9.20 (br s, 2H), 7.70 – 7.56 (m, 2H), 7.19 (dd, J = 8.5, 1.9 Hz, 1H), 4.24 (t, J = 7.1 Hz, 2H), 2.45 – 2.37 (m, 6H), 1.77 – 1.69 (m, 2H), 1.50 – 1.39 (m, 2H), 0.93 (t, J = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 157.8, 141.0, 139.2, 126.2, 126.1, 122.1, 118.4, 116.9, 116.3, 83.8, 52.0, 46.6, 40.9, 26.4, 23.8, 11.9. HR ESI-MS $(M + H)^+$ m/z = 462.1899 (Cacld for $C_{23}H_{24}N_9Cl$: 462.1916), HPLC purity: 99.0%.

6-Amino-7-(1-(4-(dimethylamino)butyl)-1*H*-benzo[*d*]imidazol-2-yl)-5-methyl-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (8t). Dimethylamine and **8-3** were used as reactants to give **8t**. Yield: 91%. Mp >190 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.01 (br s, 2H), 7.66 – 7.61 (m, 2H), 7.30 – 7.19 (m, 2H), 4.72 – 4.61 (m, 2H), 3.71 (s, 3H), 2.12 (t, J = 7.2 Hz, 2H), 2.02 (s, 6H), 1.79 – 1.68 (m, 2H), 1.42 – 1.35 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 159.8, 147.8, 142.8, 140.9, 139.1, 135.4, 125.6, 122.4, 122.2, 118.5, 117.6, 116.9, 116.3, 110.7, 84.5, 58.9, 45.5, 45.0, 28.2, 24.5. HR ESI-MS $(M + H)^+$ m/z = 414.2137 (Cacld for $C_{22}H_{23}N_9$: 414.2149), HPLC purity: 95.7%.

6-Amino-7-(1-(4-(diethylamino)butyl)-1*H*-benzo[*d*]imidazol-2-yl)-5-methyl-5*H*-pyrrolo[2,3-*b*]pyrazine-2,3-dicarbonitrile (8u). Diethylamine and **8-3** were used as reactants to give **8u**. Yield: 86%. Mp >212 °C (decomp).^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.14 (br s, 2H), 7.74 – 7.56 (m, 2H), 7.34 – 7.16 (m, 2H), 4.81 – 4.56 (m, 2H), 4.35 (s, 3H), 3.60 (t, J = 5.6 Hz, 2H), 3.44 (s, 4H), 1.88 – 1.70(m, 4H), 1.06 (t, J = 6.7 Hz, 6H).^{13}C NMR (101 MHz, DMSO-d_6) δ: 159.8, 147.8, 142.7, 140.8, 139.0, 135.3, 125.6, 122.4, 122.3, 118.5, 117.7, 116.9, 116.3, 110.6, 84.3, 56.5, 45.3, 44.4, 29.6, 28.2, 27.8, 19.0. HR ESI-MS $(M + H)^+$ m/z = 442.2454 (Cacld for $C_{24}H_{27}N_9$: 442.2462), HPLC purity: 95.4%.

Biological Assay. The testing of new chemical entities was performed using a commercially available. Purified Topo II purchased from TopoGEN, Inc. pBR322 DNA purchased from Takara. Topo I purchased from Takara. A stock solution of epotoside of concentration, 10 mM in DMSO, was prepared and stored at -20 °C. Synthesized compounds were also stored at -20 °C. The assay protocols followed were same as mentioned in the supplier manual, except the concentration of the reagents which were varied according to the requirement.

Topo II-mediated DNA relaxation assay. We used the Topo II assay kit from TopoGEN to determine the effects of drugs on DNA relaxation catalyzed by Topo II. Relaxation assays were carried out according to the manufacturer's instructions with minor modifications. The assay was performed in a final volume of 20 μL in Topo II reaction buffer (1 × Topo II buffer = 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM ATP, 0.5 mM DTT, and 30 $\mu g.ml^{-1}$ BSA) with 0.2 μg pBR322 DNA. Compounds were included in the reactions at a constant solvent volume. Reactions were initiated by addition of 1 U human Topo II, and incubated for 30 min at 37 °C. Reaction was terminated with 5 × stop buffer (5 μL per 20 μL reaction volume). Stop buffer contained 5% sarcosyl, 0.02% bromophenol blue and 25% glycerol. Reaction products were

analyzed on a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) and electrophoresis for 1.5 h at 75 V. Gels were stained for 30 min in an aqueous solution of Ged Red (0.5 $\mu g.ml^{-1}$). DNA bands were visualized through transillumination with UV light and then photographed with an Alpha Innotech digital imaging system.

Topo I-mediated DNA relaxation assay. The effects of drugs on DNA relaxation catalyzed by DNA Topo I (TaKaRa, Kyoto, Japan) was determined by measuring the relaxation of supercoiled pBR322 DNA using camptothecin as a positive control. The reaction mixture was prepared according to the provided protocol, and incubated at 37 °C for 30 min. The reactions were terminated by the addition of dye solution containing 1% SDS, 0.02% bromophenol blue and 25% glycerol. The mixtures were applied to 1% agarose gel and subjected to electrophoresis for 1 h at 90 V, in TAE buffer (40 mM Tris-acetate, 2 mM EDTA). Gels were stained for 30 min in an aqueous solution of Ged Red (0.5 $\mu g.ml^{-1}$). DNA bands were visualized by transillumination with UV light and then photographed with an Alpha Innotech digital imaging system.

Topo II-DNA cleavage reaction assays. In brief, Topo II (6 units), 0.1 μg negatively supercoiled pBR322 DNA, and 20 μM BPPs derivatives (or etoposide, 100 μM) were employed in a total of 20 μL of Topo II buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM ATP, 0.5 mM DTT, and 30 $\mu g.ml^{-1}$ BSA). After incubating for 6 min at 37 °C to reach the cleavage religation equilibrium, cleavage intermediates were trapped by adding 2 μL of 1% SDS, followed by 2 μL of 250 mM NaEDTA, pH 8.0. Proteinase K was added (2 μL of 0.8 $mg.ml^{-1}$), and reactions were incubated for 30 min at 45 °C to digest the Topo II. Samples were mixed with 2 μL of agarose gel loading buffer (30% sucrose, 0.5% bromophenol blue, and 0.5% xylene cyanole FF in 10 mM Tris-HCl, pH 7.9), heated at 72 °C for 2 min, and subjected to electrophoresis in a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) for 1 h at 75 V. Gels were stained for 30 min in an

aqueous solution of Ged Red (0.5 μg.ml^{-1}), and kept on electrophoresis for 30-45 min at 75 V. Cleavage was monitored by the conversion of negatively supercoiled plasmid to nicked DNA. DNA bands were visualized by UV light, photographed with an Alpha Innotech digital imagingsystem.

DNA unwinding assays. The ability of BPPs derivatives to unwind plasmid DNA was determined as described by Fortune.et al.34 with modification. Relaxed pBR322 plasmid DNA utilized in unwinding assays was generated by treating negatively supercoiled pBR322 with Topo I in Topo I reaction bufer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, and 30 μg.ml^{-1} BSA) prior to the addition of other reaction components. Assay mixtures contained 0.1 μg relaxed pBR322 plasmid DNA, Topo I (1 units), and compounds in 20 μL of Topo I reaction buffer. Following a 10 min incubation of DNA and drug at room temperature, Topo I was added, and reactions were incubated for 30 min at 37 °C. Reactions were stopped by adding an equal volume of phenol chloroform. Aqueous samples (20 μL) were removed from the reactions, and 3 μL of stop solution (0.77% SDS, 77 mM NaEDTA, pH 8.0) followed by 2 μL of agarose gel loading buffer (30% sucrose, in 10 mM Tris-HCl, pH 7.9) was added to each. Samples were subjected to electrophoresis in a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) for 1 h at 90 V. DNA bands were stained with an aqueous solution of Ged Red (0.5 μg.ml^{-1}), visualized with UV light, and photographed with an Alpha Innotech digital imaging system.

EB displacement assay. In brief, increasing concentration of **3t** or mAMSA were added to samples contained 20 nM pBR322 DNA plasmid and 2.5 μM EB in a fluorescence buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA). Fluorescence emission spectra (λ_{max} 595 nm; excitation 510 nm) were obtained each drug concentration and compared with those obtain using mAMSA, a Topo II poison that is known to intercalate DNA and displace EB at high drug concentration.

UV-vis titration. Absorbance titration experiments were performed as previously described.35 Binding assays were carried out in DPBS (2.67 mM KCl, 1.47 mM KH_2PO_4, 137.93 mM NaCl, 8.06 mM Na_2HPO_4, pH 7.4). A solution of CT-DNA (Sigma-Aldrich) in DPBS gave a ratio of UV-vis absorbance of 1.8–1.9 at 260 and 280 nm, indicating that the DNA was sufficiently free of protein. The CT-DNA concentration was determined by UV absorbance at 260 nm using a molar absorptivity constant of 13 200 M (bp) $^{-1}$ cm $^{-1}$. Compound **3t** (5 μM) was prepared in DPBS with 1% DMSO in the presence or absence of increasing concentrations of CT-DNA (0–25 μM). Absorption spectra were recorded in the 300–500 nm spectral range after equilibration at room temperature for 20 min using UV-visible spectrophotometer UV-2450 (Shimadzu Instruments, Inc.).

Molecular docking analysis. The structures of Topo II were generated based on their X-ray structures (PDB code: 1ZXM) using SYBYL software package (Tripos, Inc. St. Louis, MO). The residues were corrected for physiological pH. In Topo II, Mg ion and its two binding water molecules were conserved. Molecular docking was carried out with Surflex-dock. For this protein, the protocol that characterizes the binding site of the receptor was generated using a ligand-based approach. All other parameters accepted default settings. The docking results were visualized using the Discovery Studio software package.

MTT assay. The growth inhibitory effects of BPPs derivatives toward cancer cell lines, were evaluated by using the MTT assay as described by Mosmann with modifications.[39] The cells were plated at a density of 5000 per well in 96-well microplates, and allowed to incubate overnight. BPPs derivatives were added to the wells at increasing concentrations (0-50 μM). After 48 h, each well was treated with 20 μL 2.5 mg.ml^{-1} MTT solution, and the cells were further incubated at 37 °C for 4 h. At the end of the incubation, the untransformed MTT was removed, and 100 μL DMSO was

added. The microplates were well shaken to dissolve the formazan dye, and the absorbance at 570 nm was measured using a microplate-reader (Bio-Tek).

Apoptosis analysis. HL-60 cells (5.0×10^5 cells per ml) were incubated in the presence of BIPPs for an additional 12 h and 24 h, then harvested and washed twice with cold 1 × PBS, resuspended in 1 × binding buffer, and then stained with 5 μL FITC Annexin V and 5 μL propidium iodide (KeyGEN BioTECH, China) for 15 min in the dark. The stained cells were analyzed by flow cytometry (BD, FACSCalibur, USA) within 1 h. The experiments were repeated three times.

Western blot assay. Briefly, cell lysates were prepared in RIPA buffer (25 mM HEPES [pH 7.8], 0.5 M NaCl, 5 mM EDTA, 1.5% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS, and 5 mM EDTA). Samples were subjected to SDS-PAGE and transferred to a PVDF membrane (Immobilon P, Millipore). Membranes were incubated with the indicated primary antibodies and secondary antibodies and visualized by Tanon 5200.

■ ASSOCIATED CONTENT

Supporting Imformation

Experimental procedures for the Topo I mediated pBR322 DNA relaxation assay, the UV-vis titration assay, the ^{1}H NMR, ^{13}C NMR spectra, HPLC spectra and HMRS spectra of the target compounds. This material is available free of charge via the internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest

■ ABBREVIATIONS

ANP, adenosine; BPPs, pyrrolo[2,3-*b*]pyrazine derivatives; CT-DNA, calf thymus DNA; 3D-QSAR, three dimensional quantitative structure activity relationship; EB, ethidium bromide; FITC, fluorescein isothiocyanate; γ-H_2AX, hallmark of DNA double–strand breaks; mAMSA, amsacrine;

MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide; PI, propidium iodide; Topo, toisomerase; Topo I, topoisomerase I; Topo II, topoisomerase II.

■ REFERENCES

1. Bailly, C. Contemporary challenges in the design of Topoisomerase II inhibitors for cancer chemotherapy. *Chem. Rev*. **2012**, *112*, 3611-3640.
2. Pommier, Y. DNA Topoisomerase I inhibitors: chemistry, biology, and interfacial inhibition. *Chem. Rev.* **2009**, *109*, 2894–2902.
3. Pommier, Y. Drugging topoisomerases: lessons and challenges. *ACS Chem. Biol*. **2013**, *8*, 82-95.
4. Nitiss, J. L. Targeting DNA topoisomerase II in cancer chemotherapy. *Nature Rev. Cancer*. **2009**, *9*, 338–350.
5. Vos, S. M.; Tretter, E. M.; Schmidt, B. H.; Berger, J. M. All tangled up: how cells direct, manage and exploit topoisomerse function. *Nat. Rev. Mol. Cell Biol*. **2011**, *12*, 827–841.
6. Wang, J. C. DNA Topoisomerases. Ann. Rev. *Biochem*. **1996**, *65*, 635–692.
7. Nitiss, J. L. Investigating the biological function of DNA topoisomerases in eukaryotic cells. *Biochem. Biophys. Acta*. **1998**, *1400*, 63–81.
8. Wang, J. C. Cellular roles of DNA topoisomerases: a molecular perspective. *Nature Rev. Mol. Cell Biol*. **2002,** *3*, 430–440.
9. Goto, T.; Wang, J. C. Yeast DNA topoisomerase II: an ATP dependent type II topoisomerase that catalyzes the catenation, decatenation, unknotting, and relaxation of double-stranded DNA rings. *J. Biol.Chem*. **1982**, *257*, 5866–5872.
10. Zhuo, S. T.; Li, C. Y.; Hu, M. H.; Chen, S. B.; Yao, P. F.; Huang, S. L.; Ou, T. M.; Tan, *J*. H.; An, L. K.; Li, D.; Gu, L. Q.; Huang, Z. S. Synthesis and biological evaluation of

benzo[a]-phenazine derivatives as a dual inhibitor of topoisomerase I and II. *Org. Biomol. Chem*. **2013**, *11*, 3989–4005.

11. Kiselev, E.; Sooryakumar, D.; Agama, K.; Cushman, M; Pommier, Y. Optimization of the lactam side chain of 7-azaindenoisoquinoline topoisomerase I inhibitors and mechanism of action studies in cancer cells. *J. Med. Chem.*, **2014**, *57*, 1289–1298.
12. Rahman, A.M.; Park, S.E.; Kadi, A.A.; Kwon, Y.J. Fluorescein hydrzones as novel non-intercalative topoisomerase catalytic inhibitors with low DNA Toxicity. *J. Med. Chem.* **2014**, *57*, 9139-9151.
13. Atwell, G. J.; Rewcastle, G. W.; Baguley, B. C.; Denny, W. A. Potential antitumor agents. In vivo solid-Tumor activity of Derivatives of N-[2-(Dimethy1amino)ethyl]acridine-4-carboxamide. *J. Med. Chem.* **1987**, *30*, 664–669.
14. Wilstermann, A. M.; Osheroff, N. Stabilization of eukaryotic topoisomerase II-DNA cleavage complexes. *Curr, Top. Med. Chem.* **2003**, *3*, 321-338.
15. Vos, S. M.; Tretter, E. M.; Schmidt, B. H.; Berger, J. M. All tangled up: how cells direct, manage and exploit topoisomerase function. *Nat. Rev. Mol. Cell Biol.* **2011**, *12*, 827-841.
16. McClendon, A. K.; Osheroff, N. DNA topoisomerase II, genotoxicity, and cancer. *Mutat. Res*. **2007**, *623*, 83–97.
17. Winick, N. J.; McKenna, R. W.; Shuster, J. J.; Schneider, N. R.; Borowitz, M. J.; Bowman, W. P.; Jacaruso, D.; Kamen, B. A.; Buchanan, G. R. Secondary acute myeloid leukemia in children with acute lymphoblastic leukemia treated with etoposide. *J. Clin. Oncol.* **1993**, *11*, 209–217.
18. Huang, H.; Chen, Q.; Ku, X.; Meng, L.; Lin, L.; Wang, X.; Zhu, C.; Wang, Y.; Chen, Z.; Li, M.; Jiang, H.; Chen, K.; Ding, J.; Liu, H. A series of α-heterocyclic carboxaldehyde thiosemicarbazones inhibit topoisomerase IIα catalytic activity. *J. Med. Chem.* **2010**, *53*, 3048–3064.

19. Jimenez-Alonso, S.; Orellana, H. C.; Braun, A. E.; Ravelo, A. G.; Sacau, E. P.; Machin, F. Design and synthesis of a novel series of pyranonaphthoquinones as topoisomerase II catalytic inhibitors. *J. Med.Chem.* **2008**, *51*, 6761–6772.

20. Yoshida, M.; Maehara, Y.; Sugimachi, K. MST-16, a novel bisdioxopiperazine anticancer agent, ameliorates doxorubicin-induced acute toxicity while maintaining antitumor efficacy. *Clin.Cancer.Res*. **1999**, *5*, 4295–4300.

21. Weiss, G.; Loyevsky, M.; Gordeuk, V. R. Dexrazoxane (ICRF-187). *Gen. Pharm*. **1999**, *32*, 155–158.

22. Larsen, A. K.; Escargueil, A. E.; Skladanowski, A. Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacol. Ther*. **2003**, *99*, 167–181.

23. Trejo, A.; Arzeno, H.; Browner, M.; Chanda, S.; Cheng, S.; Comer, D. D. Design and synthesis of 4-azaindoles as inhibitors of p38 MAP kinase. *J. Med. Chem*. **2003**, *46*, 4702–4713.

24. Arya, K.; Tomar, P.; Singh, J. Design, synthesis and biological evaluation of novel spiroindole-pyridothiazine analogs as antiproliferative agent. *RSC Adv.* **2014**, *4*, 3060–3064.

25. Mettey, Y.; Gompel, M.; Thomas, V.; Garnier, M.; Leost, M.; CeballosPicot, I. Aloisines, a new family of CDK/GSK-3 inhibitors. SAR study, crystal structure in complex with CDK2, enzyme Selectivity, and cellular effects. *J. Med. Chem*. **2003**, *46*, 222–236.

26. Mei*J*er, L.; Flajolet, M. Pharmacological inhibitors of glycogen synthase kinase-3. Trends. *Pharmacol. Sci*. **2004**, *25*, 471–480.

27. Mapelli, M.; Massimilinao, L.; Crovace, C.; Seeliger, M. A.; Tsai, L. H.; Meijer, L.; Musacchio, A. Mechanism of CDK5/p25 binding by CDK Inhibitors. *J. Med. Chem*. **2005**, *48*, 671–679.

28. Dubinina, G. G.; Platonov, M. O.; Golovach, S. M.; Borysko, P. O.; Tolmachov, A.O.; Volovenko, Y. M. Novel 5, 7-disubstituted 6-amino-5H-pyrrolo[3,2-b]pyrazine-2, 3-dicarbonitriles, the promising protein kinase inhibitors with antiproliferative activity. *Eur. J. Med. Chem.* **2006**, *41*, 727–737.

29. Reker, D.; Seet, M.; Pillong, M.; Koch, C. P.; Schneider, P.; Witschel, M. C.; Rottmann, M.; Freymond, C.; Brun, R.; Schweizer, B.; Illarionov, B.; Bacher, A.; Fischer, M.; Diederich, F. A; Schneider, G. Deorphaning pyrrolopyrazines as potent multi-target antimalarial agents. *Angew. Chem. Int. Ed.* **2014**, *53*, 7079 –7084.

30. Tekiner-Gulbas, B.; Temiz-Arpaci, O.; Yildiz, I.; Aki-Sener, E.; Yalcin, I. 3D-QSAR study on heterocyclic topoisomerase II inhibitors using CoMSIA. *SAR QSAR Environ. Res*. **2006**, *17*, 121–132.

31. Pinar, A.; Yurdakul, P.; Ylildiz, I.; Temiz-Arpaci, O.; Acan, N. L.; Aki-Sener, E.; Yalcin, I. Some fused heterocyclic compounds as eukaryotictopoisomerase II inhibitors. *Biochem. Biophys. Res. Commun*. **2004**, *317*, 670–674.

32. Baviskar, A. T.; Madaan, C.; Preet, R.; Mohapatra, P.; Jain, V.; Agarwal, A.; Sankar, K.; Guchhait; Chanakya, N.; Kundu; Uttam, C.; Banerjee; Bharatam, P. V. N-fused imidazoles as novel anticancer agents that inhibit catalytic activity of topoisomerase IIα and induce apoptosis in G1/S phase. *J. Med. Chem*. **2011**, *54*, 5013–5030.

33. Tanabe, K.; Ikegami, Y.; Ishida, R.; Andoh, T. Inhibition of topoisomerase II by antitumor agents bis (2,6-dioxopiperazine) derivatives. *Cancer Res*. **1991**, *51*, 4903–4908.

34. Glisson, B. S.; Smallwood, S. E.; Ross, W. E. Characterization of VP-16–induced DNA damage in isolated nuclei from L1210 cells. *Biochim. Biophys. Acta*. **1984**, *783*, 74–79.

35. Friche, E.; Danks, M. K.; Schmidt, C. A.; Beck, W. T. Decreased DNA topoisomerase II in daunorubicin-resistant Ehrlich Ascites tumor cells. *Cancer Res.* **1991**, *51*, 4211-4218.

36. Kawatani, M.; Takayama H.; Muroi, M.; Kimura, S.; Maekawa, T.; Osada, H. Identification of a small-molecule inhibitor of DNA topoisomerase II by proteomic profiling. *Chemistry & Biology*. **2011**, *18*, 743–751

37. Burres, N. S.; Sazesh, S.; Gunawardana, G. P.; Clement, J. J. Antitumor activity and nucleic acid binding properties of dercitin, a new acridine alkaloid isolated from a marine dercitus species sponge. *Cancer Res.* **1989**, *49*, 5267–5274.

38. Janson T. B., Kang, Z.L., Austin, C.A., Kurz, E.U. Salicylate, a catalytic inhibitor of Topoisomerase II, inhibitors DNA cleavage and selective for the α isoform. *Mol Parmacol*, **2014**, *85*, 198-207.

39. Yalowich, J. C.; Wu, X.; Zhang, R.; Kanagasabai, R.; Hornbaker, M.; Hasinoff, B. B. The anticancer thiosemicarbazones Dp44mT and triapine lack inhibitory effects as catalytic inhibitors or poisons of DNA topoisomerase II*α*. *Biochem. Pharmacol*. **2012**, *84*, 52–58.

40. Baldi, M. I.; Benedetti, P.; Mattoccia, E.; TocchiniValentini, G. P. In vitro catenation and decatenation of DNA and a novel eucaryotic ATP-dependent topoisomerase. *Cell*, **1980**, *20*, 461–467.

41. Wei, H.; Ruthenburg, A. J.; Bechis, S. K.; Verdine, G. L. Nucleotide-dependent domain movement in the ATPase domain of a human type IIA DNA topoisomerase. *J Biol Chem.* **2005,** *280*, 37041-37047.

42. Classen, S.; Olland, S.; Berger, J. M. Structure of the topoisomerase II ATPase region and its mechanism of inhibition by the chemotherapeutic agent ICRF-187. *Proc Natl Acad Sci.* **2003**, *100*, 10629-10634.

43. Mosmann, T. J. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*. **1983**, *65*, 55–63.

44. Ishida, R.; Miki, T.; Narita, T.; Yui, R.; Sato, M.; Utsumi, K.R.; Tanabe, K.; Andoh, T. Inhibition of intracellular topoisomerase II by antitumor bis(2,6-dioxopiperazine) derivatives: mode of cell growth inhibition distinct from that of cleavable complex-forming type inhibitors. *Cancer Res*. **1991**, *51*, 4909–4916.

45. Indran, I. R.; Tufo, G.; Pervaiz, S.; Brenner, C. Recent advances in apoptosis, mtiochondria and drug resistance in cancer cells. *Biochim. Biophys. Acta*. **2011**, *1807*, 735–745.

46. Kerr, J. F.; Wyllie, A. H.; Currie, A. R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*. **1972**, *26*, 239–257.

47. Woo, J. T.; Kawatani, M.; Kato, M.; Shinki, T.; Yonezawa, T.; Kanoh, N.; Nakagawa, H.; Takami, M.; Lee, K.H.; Stern, P. H. Reveromycin A, an agent for osteoporosis, inhibits bone resorption by inducing apoptosis specifically in osteoclasts. *Proc. Natl. Acad. Sci. U. S. A*. **2006**, *103*, 4729–4734.

48. Volovenko, Y.M., Dubinna, G.G. Synthesis of 6-amino-5-R^2-7-(6-R^1-4-OXO-3,4-dihydro-2-quinazolyl)-5-pyrrolo[2,3-*b*]pyrazine-2,3-di-carbonitriles. *Chem. Heter. Comp.* **2002**, *38*, 219-225.

FIGURE AND SCHEME LEGENDS

Figure 1. Structure of aloisines and reported benzoazolyl Topo II inhibitors

Figure 2. Design of novel agents as potential Topo II inhibitors

Figure 3. Topo II inhibitory activities of BPPs. (A), (B), (C), (D) , (E), and (F), lane **D**: pBR322 DNA; lane **T**: pBR322 DNA + Topo II; lane **E**: pBR322 DNA + Topo II + etoposide (100 μM); other lanes : pBR322 DNA + Topo II + BPPs

Figure 4. (A) Effects of compounds **3t** and **3u** on Topo II-DNA cleavage complexes formation. Lane **1**-**2**: control group of supercoiled pBR322 DNA without or with Topo II; Lane **3**-**5**: effect of etoposide (100 μM) and tested compound (20 μM) on Topo II with supercoiled pBR322 DNA; Lane **6**-**7**: pretreatment of tested compounds (20 μM) antagonizes the etoposide-enhanced DNA cleavage. The positions of supercoiled DNA (S), relaxed DNA (R), linear DNA (L) and nicked DNA (N) are indicated. (B) Evaluation of γ-H_2AX expression was measured by western blot. HL-60 cells were treated with etoposide and compound **3t** alone, or with both of them for 1 h. (C) The unwinding assay of BPPs derivatives. Lane **D**: pBR322 DNA; lane **T**: pBR322 DNA + Topo I; other lane: pBR322 DNA + Topo I + BPPs or EB with different concentration.

Figure 5. (A), (B), and (C) The effects of compound **3t** on DNA relaxation catalyzed by Topo II at 1 mM, 2 mM and 4 mM ATP respectively.

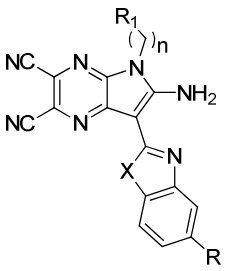
Figure 6. Schematic representation of the proposed binding modes of **3c** and **3t** with the catalytic site of the ATPase domain of Topo II (PDB code: 1ZXM) (A: compound **3c**, B: compound **3t**).

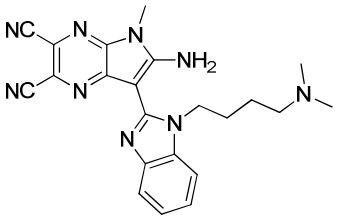
Figure 7. Apoptotic cells were detected with Annexin V/PI double staining after incubation with compound **3t** and **3u** for 12 h and 24 h. The lower left quadrants represent live cells, the lower right quadrants are for early apoptotic cells, upper right quadrants are for late apoptotic cells, while the upper left quadrants represent cells damage during the procedure.

Scheme 1. Synthetic route and structure of **3a**–**3x**, **4m**–**4u**, **5c**–**5t**, **6c**–**6u** and **7c**–**7u.** Reagents and conditions: (a) DMF, 40 °C, 3h; (b) $R_1(CH_2)_nNH_2$, TEA, DMF, 60 °C, 2 h; (c) TEA, DMF, r.t., 5h; (d) *N*, *N*-dimethylbutyldiamine, DMF, 60 °C, 5h.

Scheme 2. Synthetic route and structure of **8t** and **8u.** Reagents and conditions: (a) 1,4-dibromobutane, Cs_2CO_3, acetone, 50 °C, 3 h; (b) 5,6-dichloropyrazine-2,3-dicarbonitrile, DMF, 40 °C, 4 h; (c) CH_3NH_2, TEA, DMF, 60 °C, 2 h. (d) dimethylamine or diethylamine, K_2CO_3, DMF, 65 °C, 8 h.

Table 1. Structures of synthesized target compounds





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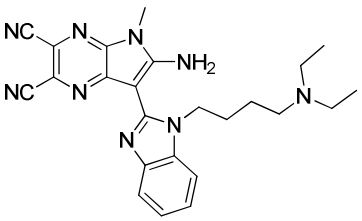

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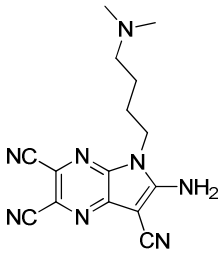


3x

Compd.	n	X	R	R_1	Compd.	n	X	R	R_1
3a	0	NH	H	CH_3	**3v**	3	NH	H	Ph-NH
3b	1	NH	H	CH_3	**3w**	5	NH	H	N $(CH_3)_2$
3c	2	NH	H	N $(CH_3)_2$	**4m**	3	O	H	N $(CH_3)_2$
3d	2	NH	H	N $(CH_2CH_3)_2$	**4s**	3	O	H	$N(CH_2)_5$
3e	2	NH	H	$N(CH_2)_4$	**4t**	4	O	H	N $(CH_3)_2$
3f	2	NH	H	$N(CH_2)_4NCH_3$	**4u**	4	O	H	N $(CH_2CH_3)_2$
3g	2	NH	H	$N(CH_2)_4O$	**5c**	2	S	H	N $(CH_3)_2$
3h	2	NH	H	Ph	**5m**	3	S	H	N $(CH_3)_2$
3i	2	NH	H	*p*-MeO-Ph	**5t**	4	S	H	N $(CH_3)_2$
3j	2	NH	H	indole	**6c**	2	NCH_3	H	N $(CH_3)_2$
3k	2	NH	H	CH_3	**6d**	2	NCH_3	H	N $(CH_2CH_3)_2$
3l	2	NH	H	OH	**6m**	3	NCH_3	H	N $(CH_3)_2$
3m	3	NH	H	N $(CH_3)_2$	**6n**	3	NCH_3	H	N $(CH_2CH_3)_2$
3n	3	NH	H	N $(CH_2CH_3)_2$	**6t**	4	NCH_3	H	N $(CH_3)_2$
3o	3	NH	H	$N(CH_2)_4O$	**6u**	4	NCH_3	H	N $(CH_2CH_3)_2$
3p	3	NH	H	$N(CH_2)_4NCH_3$	**7c**	2	NH	Cl	N $(CH_3)_2$
3q	3	NH	H	CH_3	**7d**	2	NH	Cl	N $(CH_2CH_3)_2$
3r	3	NH	H	OH	**7m**	3	NH	Cl	N $(CH_3)_2$
3s	3	NH	H	$N(CH_2)_5$	**7n**	3	NH	Cl	N $(CH_2CH_3)_2$
3t	4	NH	H	N $(CH_3)_2$	**7t**	4	NH	Cl	N $(CH_3)_2$
3u	4	NH	H	N $(CH_2CH_3)_2$	**7u**	4	NH	Cl	N $(CH_2CH_3)_2$

Table 2. Data of inhibition of cancer cell proliferation and Topo II by BPPs

Compd.	IC_{50} (μM)									Topo II inhibition[b]
	Hela	MDA-MB-201	A549	K562	Raji	HL-60	HL-60/MX2	RI[a]	HEK-293	
3a	>50	>50	>50	>50	>50	>50	>50		>50	-
3b	>50	>50	>50	>50	>50	>50	>50		>50	-
3c	8.88	43.46	>50	6.34	8.32	2.22	3.78	1.7	9.73	-
3d	11.58	25.43	>50	2.49	8.95	1.88	7.15	3.8	12.22	-
3e	>50	>50	>50	6.48	>50.	4.76	>50		18.26	-
3f	>50	9.11	40.48	6.55	>50	4.3	6.49	1.5	10.89	+
3g	>50	>50	>50	>50	2.88	9.04	>50		22.31	-
3h	>50	>50	>50	>50	15.39	>50	>50		>50	-
3i	>50	>50	>50	>50	2.78	>50	>50		>50	-
3j	>50	>50	>50	>50	>50	>50	>50		>50	-
3k	>50	>50	1.97	>50	>50	45.23	>50		>50	-
3l	>50	>50	>50	>50	5.52	>50	>50		>50	-
3m	25.85	1.82	10.38	1.73	1.23	0.87	2.39	2.7	8.36	+++
3n	3.15	28.9	25.33	1.8	1.87	0.73	1.82	2.5	7.87	+++
3o	>50	>50	>50	7.72	14.37	>50	5.57		>50	-
3p	15.64	4.52	5.75	1.26	2.62	0.75	3.32	4.4	6.26	-
3q	>50	>50	>50	>50	>50	>50	>50		>50	-
3r	>50	>50	>50	>50	>50	>50	>50		>50	-
3s	6.68	6.23	6.11	3.46	3.38	1.68	1.01	0.6	6.72	-
3t	11.24	0.53	8.24	1.43	2.56	0.43	1.78	4.1	4.16	+++
3u	10.07	0.64	5.75	1.44	2.64	0.46	1.55	3.3	5.37	+++
3v	26.32	N.D.[C]	N.D.	N.D.	N.D.	6.92	N.D.		N.D.	+
3w	10.82	N.D.	N.D.	N.D.	N.D.	3.42	N.D.		N.D.	++
3x	>50	N.D.	N.D.	N.D.	N.D.	12.86	N.D.		N.D.	-
4m	11.17	5.64	7.17	5.9	1.94	0.39	1.31	3.3	5.88	+++
4s	12.21	32.23	8.67	>50	3.19	3.82	5.45	1.4	19.23	-

4t	1.95	6.13	5.47	1.38	9.2	0.64	0.96	1.5	6.24	+++
4u	6.42	2.71	9.39	1.98	2.61	0.45	1.72	3.8	3.96	+++
5c	>50	>50	>50	8.0	1.96	1.67	>50		11.84	-
5m	0.72	2.93	8.87	1.8	2.99	0.51	1.31	2.5	4.29	+++
5t	18.72	2.74	35.78	1.33	10.13	0.83	2.14	2.6	5.31	+++
6c	>50	>50	>50	>50	46.52	>50	4.68		>50	--
6d	>50	>50	>50	>50	2.08	8.8	3.25	0.4	25.36	--
6m	4.32	3.67	11.66	3.56	0.94	2.12	11.32	5.3	8.41	+++
6n	12.38	6.75	10.7	3.27	2.22	2.81	4.47	1.6	7.24	+++
6t	0.46	1.79	6.15	1.45	0.32	0.74	2.58	3.5	3.87	+++
6u	1.61	2.89	1.72	1.01	0.92	0.70	1.73	2.5	3.16	+++
7c	>50	>50	>50	11.26	11.82	>50	>50		>50	-
7d	>50	>50	>50	>50	31.53	>50	9.29		>50	-
7m	16.49	28.1	>50	10.69	3.11	2.93	5.31	1.8	11.65	+
7n	24.38	23.17	>50	4.83	1.62	3.47	2.46	0.7	8.42	+
7t	3.12	3.28	12.07	1.93	0.95	1.48	0.58	0.4	4.73	+
7u	3.64	2.84	2.27	1.27	0.72	0.82	1.77	2.2	3.86	+
8t	10.12	6.48	11.22	11.26	1.07	2.27	2.81	1.2	7.13	+++
8u	11.83	6.95	13.1	3.18	0.6	2.86	3.92	1.3	8.82	+++
Etoposide	32.45	31.33	6.74	0.51	1.68	0.16	9.02	56.4	0.69	+++

[a] The values express the ration between IC_{50} determined in resistant (HL-60/MX2) and non-resistant cell line (HL-60) .

[b] The relative Topo II inhibitory potencies of the BPPs are present as follow: -, no detectable activity at 50 μM ; +, weak activity at 50 μM ; ++, weak activity at 20 μM; +++, strong activity at 20 μM.

[c]No detected.

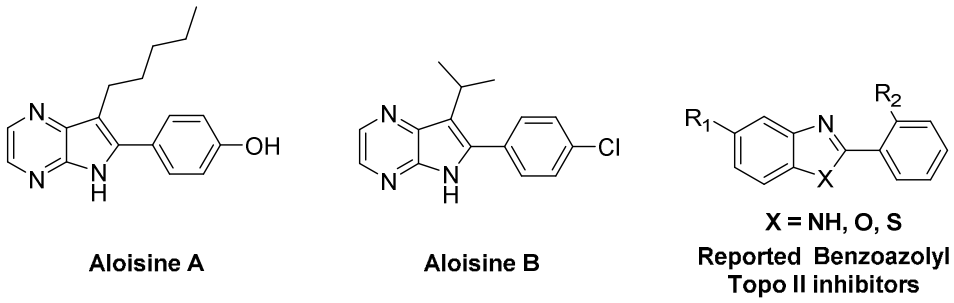


Figure 1

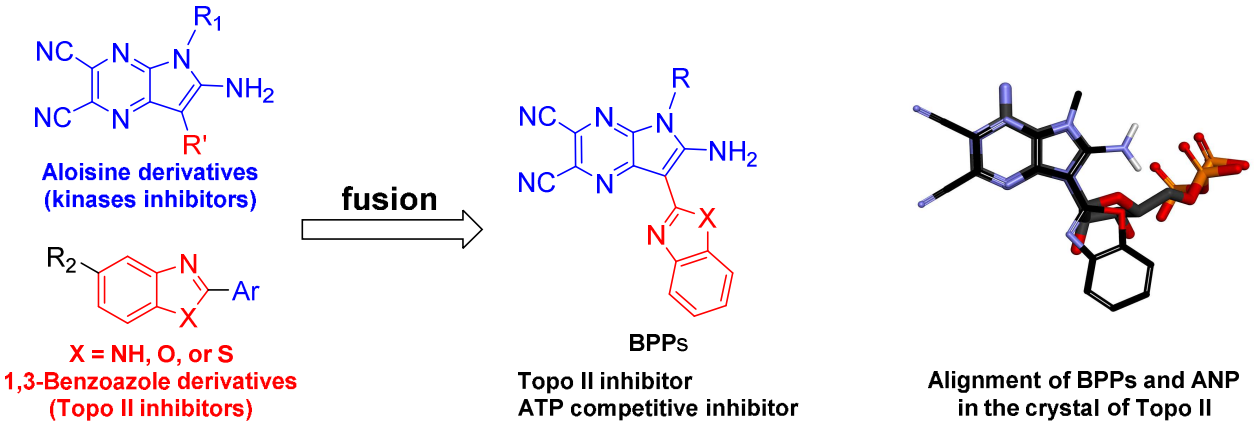


Figure 2

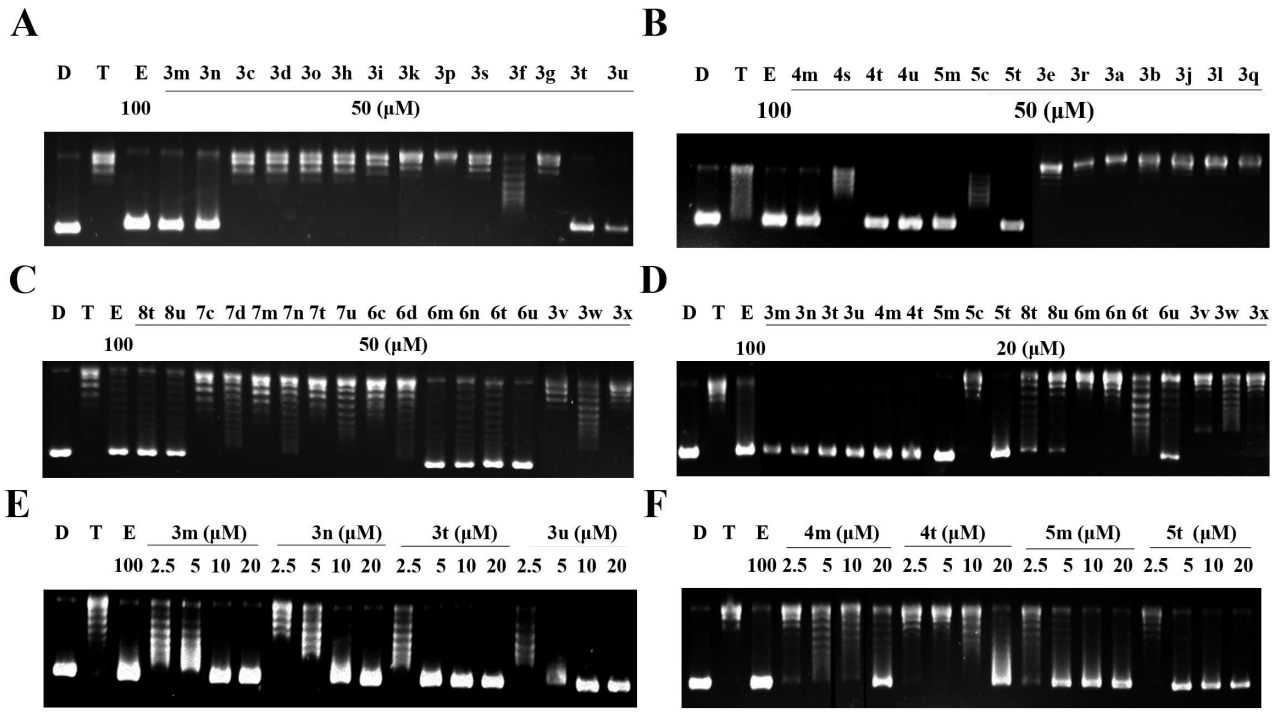


Figure 3

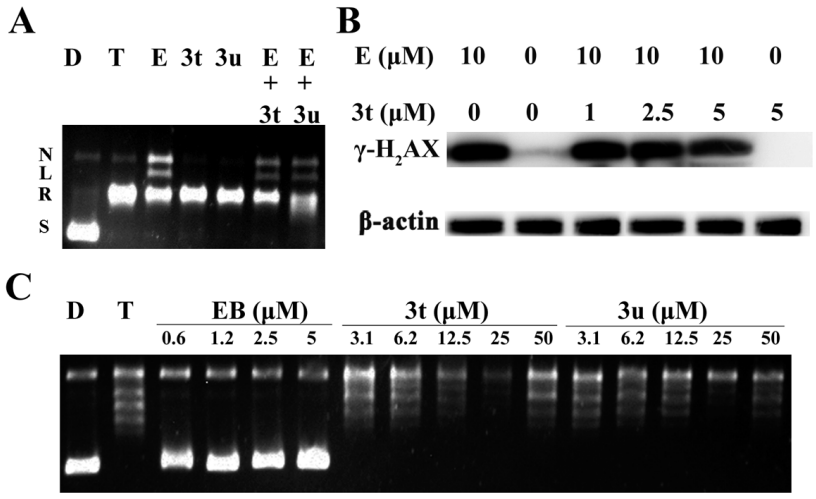


Figure 4

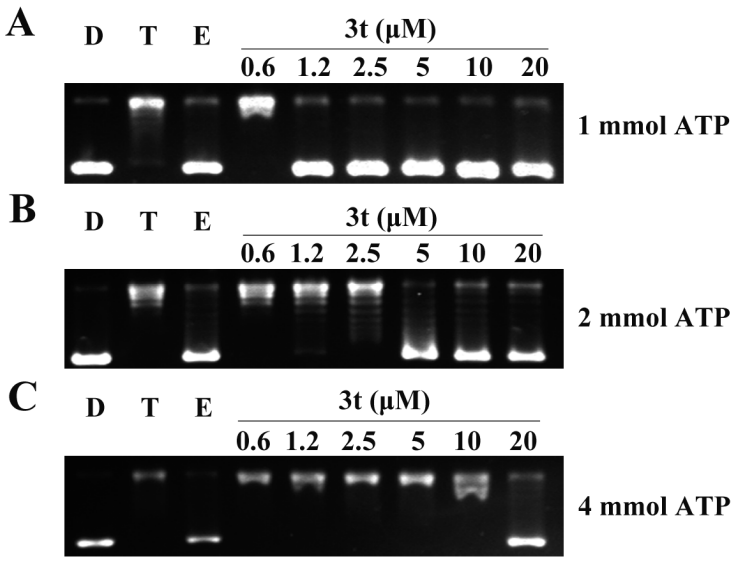


Figure 5

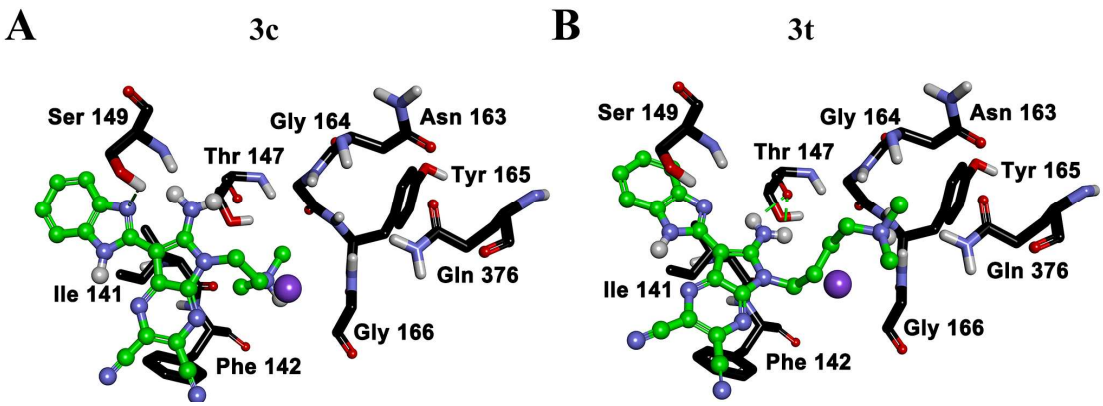


Figure 6

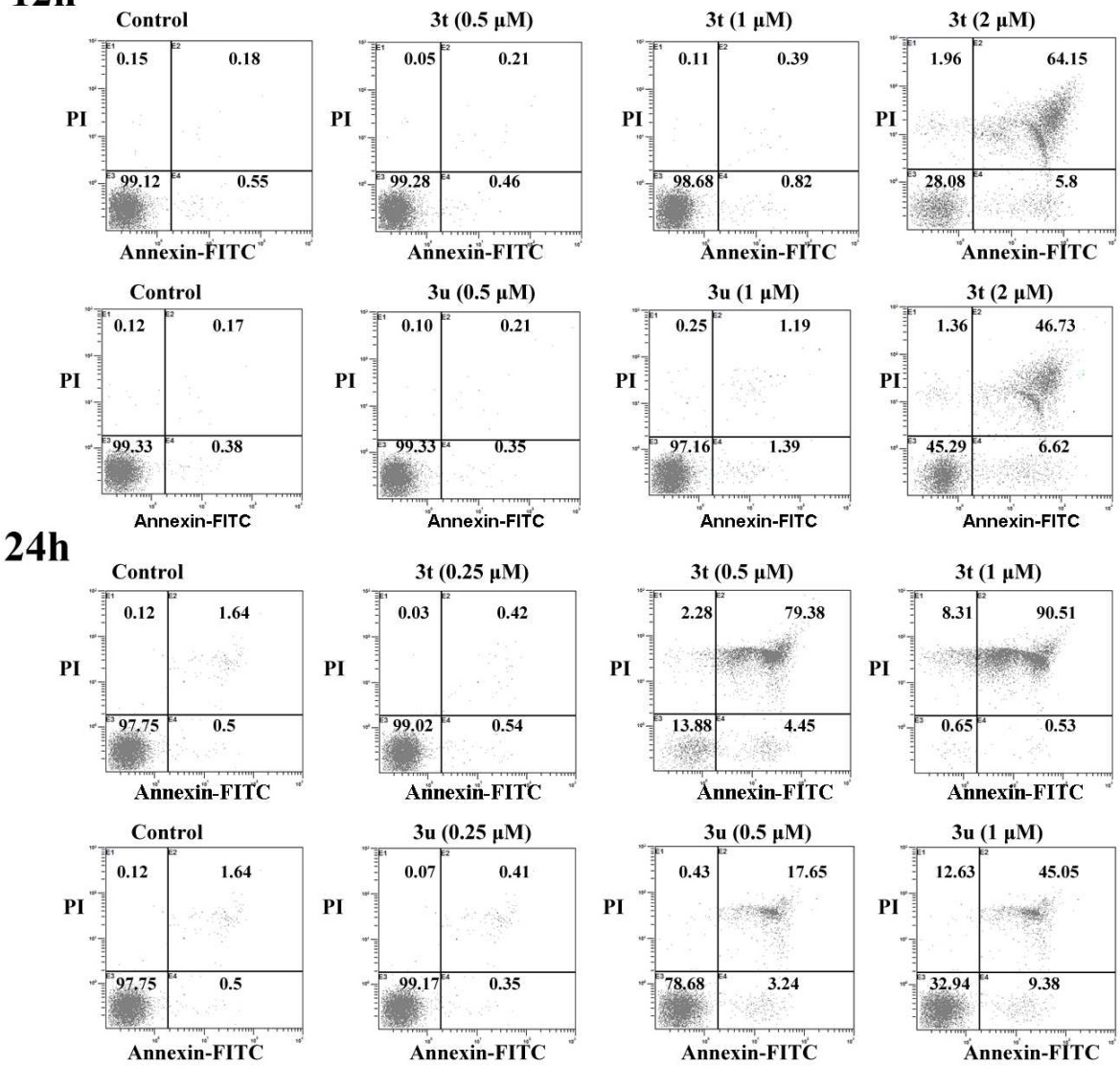
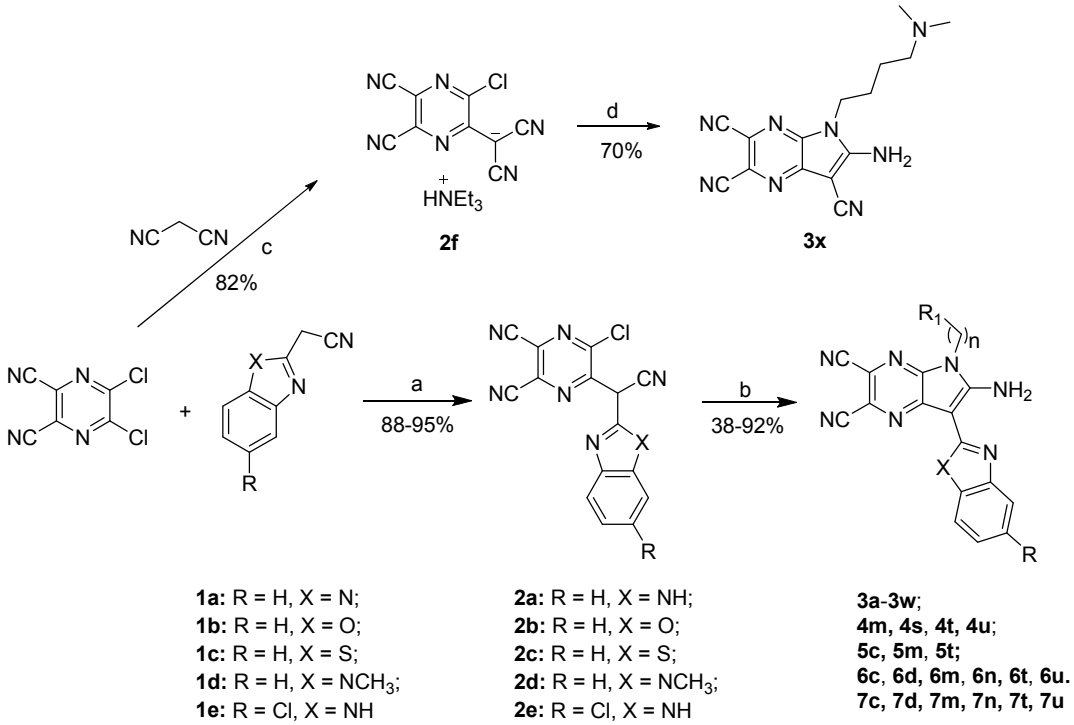
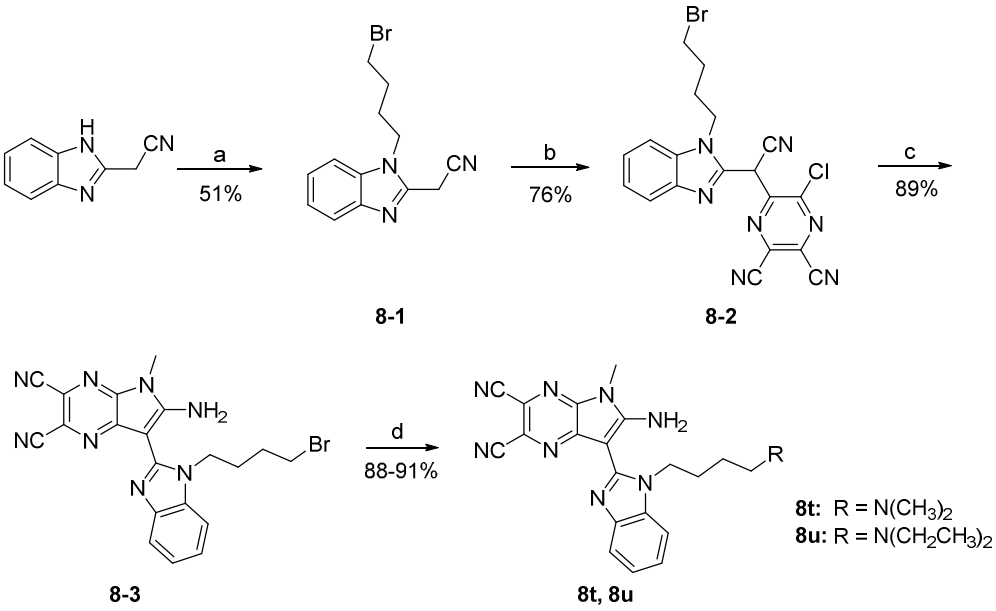

12h
Control
3t (0.5 μM)
3t (1 μM)
3t (2 μM)
0.15
0.18
99.12
0.55
0.05
0.21
99.28
0.46
0.11
0.39
98.68
0.82
1.96
64.15
28.08
5.8
Control
3u (0.5 μM)
3u (1 μM)
3t (2 μM)
0.12
0.17
99.33
0.38
0.10
0.21
99.33
0.35
0.25
1.19
97.16
1.39
1.36
46.73
45.29
6.62
24h
Control
3t (0.25 μM)
3t (0.5 μM)
3t (1 μM)
0.12
1.64
97.75
0.5
0.03
0.42
99.02
0.54
2.28
79.38
13.88
4.45
8.31
90.51
0.65
0.53
Control
3u (0.25 μM)
3u (0.5 μM)
3u (1 μM)
0.12
1.64
97.75
0.5
0.07
0.41
99.17
0.35
0.43
17.65
78.68
3.24
12.63
45.05
32.94
9.38
PI
Annexin-FITC

Figure 7

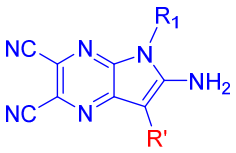


Scheme 1

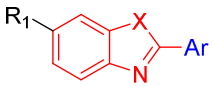


Scheme 2

For Table of Contents Only

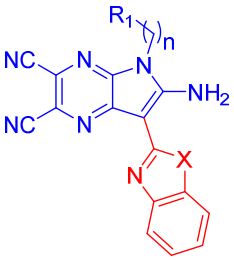


Aloisine derivatives
(Kinases inhibitor)



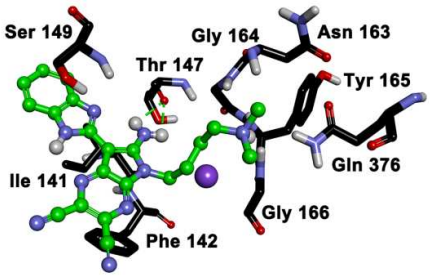
1,3-Benzoazole derivatives
(Topo II inhibitors)
$IC_{50} \approx 20$ μM

fusion



n = 3 or 4; X = NH, O, S;
R_1 = dimethylamino or diethylamino

Topo II catalyic inhibitors



Compound 3t occupy
ATP binding pocket of Topo II
$IC_{50} \approx 2.5$ μM