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Concise Article

Selective Inhibition of Bacterial Topoisomerase I by alkynyl-bisbenzimidazoles†

Cite this: DOI: 10.1039/xoxxooooox

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Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxooooox

www.rsc.org/

ABSTRACT

Hoechst dyes are well known DNA binders that non-selectively inhibit the function of mammalian topoisomerase I and II. Herein, we show that Hoechst 33258 based bisbenzimidazoles (DPA 151–154), containing a terminal alkyne, are effective and selective inhibitors of E. coli. topoisomerase I. These bisbenzimidazoles displayed topoisomerase I inhibition much better than Hoechst 33342 or Hoechst 33258 with IC50 values in the range of 2.47–6.63 μM. Bisbenzimidazoles DPA 151-154 also display selective inhibition of E. coli. topoisomerase I over DNA gyrase, Human topoisomerases I and II, and effectively inhibit bacterial growth.

Introduction

New approaches for the discovery of antibacterial drugs are paramount to our efforts in the continuing fight against bacterial resistance. In this regard, enzyme inhibitors that selectively target a bacterial enzyme over their human counterpart offer unique opportunities for such selective inhibition approaches. Bacterial DNA topoisomerases[1, 2] are one such class of enzymes that help in regulating DNA topology. The cellular functions of topoisomerases include relaxing (+) and (-) supercoil in DNA as well as in introducing supercoils to their DNA substrates.[3] These functions of DNA topoisomerases can be used to develop anticancer or antibacterial agents.[2, 4] The therapeutic interest in the development of small molecules as inhibitors of DNA topoisomerase lies in their ability to act as both cleavable complex stabilizing agents as well as in their ability to bind at the ATP binding site.[2]

A number of small molecules have been discovered that poison the functions of DNA topoisomerases. These have included camptothecin[5] and its derivatives, intercalators and compounds that interact with the minor groove of B-DNA such as bisbenzimidazoles.[6-12] Benzimidazoles are important class of compounds that display a widespread range of biological activities. Halogenated monobenzimidazoles have shown antimycobacterial activity better than isoniazid.[13] Similarly, triazolyl derivatized monobenzimidazoles have displayed antimicrobial properties.[14] In comparison to abundant literature reports on the biological properties of monobenzimidazoles, studies on the antimicrobial properties of bisbenzimidazoles (particularly those modeled from Hoechst 33258) are very limited.[12, 15] Hoechst 33258 is a bisbenzimidazole compound that has been a subject of intense study for over three decades due to its binding to AT rich duplex DNA structures.[16-18]

In this report we present the synthesis, nucleic acid binding, topoisomerase I activity, and antimicrobial activity of Hoechst 33258 functionalized bisbenzimidazoles (Chart 1). We show that the addition of alkyne functionalized alkyl chain converts Hoechst 33258 from a non-selective topoisomerase (bacterial and human) inhibitor to a highly selective bacterial topoisomerase I inhibitor. The results obtained opens up a new approach to targeting bacterial topoisomerases and the potential role of a hydrophobic pocket in the DNA-*E.Coli* topoisomerase I complex.

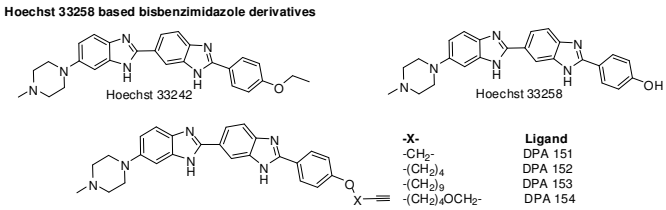


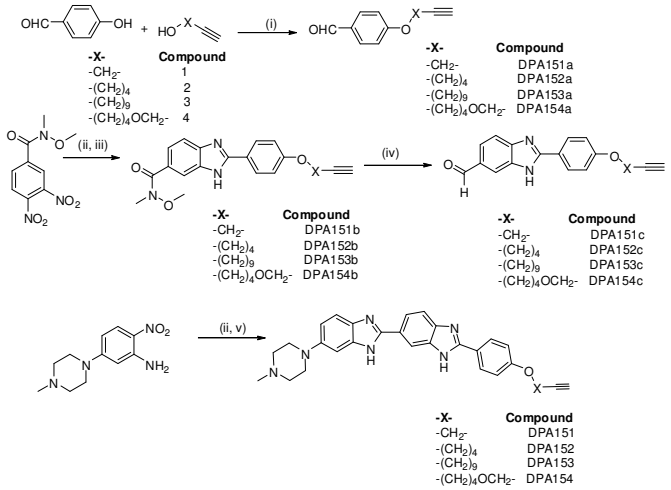
Chart 1. *Structures of compounds used in the study.*

Results and discussion

Synthesis of ligands DPA 151-154

The synthesis of the ligands (DPA 151-154) was performed using a divergent strategy[19, 20] to construct the alkyl linkers (Scheme 1). To introduce the linkers, we carried out Mitsunobu reactions of 4-hydroxy benzaldehyde with aliphatic alcohols (1-4) that terminated in the requisite alkyne functionality. The aliphatic alcohols were obtained commercially or prepared in one step from a corresponding diol. The 4-substituted benzaldehydes (DPA 151a- DPA 154a) were coupled with 3, 4-diamino-N-methoxy-N-methylbenzamide in the presence of an oxidant to yield the corresponding benzimidazoles (DPA 151b-DPA 154b). These benzimidazoles containing the

weinreb amide functionality were then easily reduced to their corresponding aldehydes (DPA 151c-DPA 154c) using lithium aluminum hydride. Coupling of these aldehydes with 4-(4-methylpiperazin-1-yl) benzene-1, 2-diamine,[21] in the presence of an oxidant resulted in the synthesis of target bisbenzimidazoles DPA 151-DPA 154 in good yields. The presence of a rather inert functional group alkyne also makes these molecules useful for further modifications using click chemistry applications. All compounds were characterized by spectroscopic techniques (NMR, IR and HRMS/MALDI-TOF, see supporting information, Figure S1-S16).



Scheme 1. *Reagent and conditions (i) PPh_3, DIAD, 1,4 dioxane, dichloromethane, rt, overnight, 50-85 %, (ii) Pd-C, H_2, ethanol,rt, 5 h, qaunt, (iii) DPA 151a-DPA 154a, ethanol, $Na_2S_2O_5$, H_2O, reflux, 12-14 h, 61-85 % (for two steps), (iv) THF- ether, LAH , -78 ^oC to 0 ^oC., 6-12 h, 55-73 %, (v) DPA 151c-DPA 154c, ethanol, $Na_2S_2O_5$, H_2O, reflux, overnight, 50-72 % (for two steps).*

Inhibition of bacterial DNA topoisomerase I

We tested the inhibitory activities of the newly synthesized bisbenzimidazoles against a few DNA topoisomerases, i.e., *E. coli* DNA topoisomerase I, *E. coli* DNA gyrase, human DNAtopoisomerase I, and human DNA topoisomerase II. To our surprise, these newly synthesized compounds showed a selective and enhanced inhibition against *E. coli* DNA topoisomerase I. Figure 1 shows the results of inhibitory assays of Hoechst 33258 and its derivative, DPA151, against *E. coli* DNA topoisomerase I. In the absence of Hoechst 33258 or DPA151, 6 nM of *E. coli* DNA topoismerase I was sufficient to relax the supercoiled plasmid DNA template, pBAD-GFPuv (lanes 1 of Figure 1A and 1C). The titration of increasing amounts of either compounds into the reaction mixtures resulted in the inhibition of *E. coli* DNA topoisomerase I. For Hoechst 33258, approximately 20 μM was needed to completely inhibit the activities of E. coli DNA topoisomerase I (lane 10 of Figure 1A). In contrast, it took only approximately 5 μM of DPA151 to fully suppress the activities of *E. coli* DNA topoisomerase I (lane 9 of Figure 1B). The IC_{50} of Hoechst 33258 and DPA151 were determined to be 19.50±1.32 and 5.50±0.50 μM, respectively (Figure 1C and Table 1). These results demonstrated that the addition of a hydrophobic group to the hydroxyl tail of Hoechst 33258 dramatically increased the inhibitory capacity against *E. coli* DNA topoisomerase I. Similar results were also obtained for DPA152, 153 and 154 in which a hydrophobic group with different lengths were added to the hydroxyl group of Hoechst 33258. Our results are summarized in Table 1 and Figure S17-19.

Inhibition of human DNA topoisomerase I

Next we examined the inhibitory activities of these newly synthesized bisbenzimidazoles against human DNA topoisomerase I, a type IB topoisomerase. Figure 2 shows our results. For Hoechst 33258 and DPA151, 50 μM of these two compounds was able to prevent about 70% (Figure S20) of supercoiled DNA template from relaxation by human DNA topoisomerase I (compare lanes 3 and 5 to lane 1 of Figure 2). However, 50 μM of other newly synthesized bisbenzimidazoles was only capable of preventing 5-15% of supercoiled DNA from relaxation (Figure 2). These results suggest that the addition of a hydrophobic tail to the hydroxyl group of Hoechst 33258 significantly reduced the inhibitory activities against human DNA topoisomerase I. In this study, we also tested the inhibitory activities of these newly synthesized compounds against

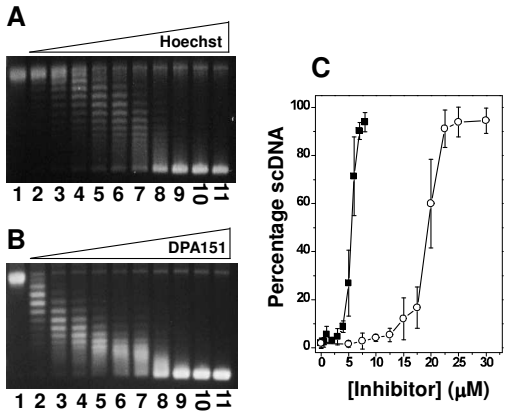


Figure 1. *Inhibitory activities of Hoechst 33258 and DPA 151 against E. coli DNA topoisomerase I. Inhibition assays against E. coli DNA topoisomerase I were performed as described under Materials and Methods. The plasmid DNA molecules were isolated and subjected to 1% agarose gel electrophoresis in the absence of chloroquine. (A) The inhibition activities of Hoechst 33258 against E. coli DNA topoisomerase I. Lanes 1 to 11 contain 0, 5, 7.5, 10, 12.5, 15, 20, 22.5, 25, 25, 30 μM of Hoechst 33258, respectively. (B)The inhibition activities of DPA 151 against E. coli DNA topoisomerase I. Lanes 1 to 11 contain 0, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10 μM of DPA151 respectively. (C) The quantification analyses of the inhibitory activities of Hoechst 33258 (open circles) and DPA 151 (closed squares) against E. coli DNA topoisomerase I. The values of IC_{50} (the half maximal inhibitory concentration) were obtained from these analyses. Standard deviation was obtained from three independent determinations. scDNA represents supercoiled DNA.*

two type II topoisomerases, E. coli DNA gyrase and human DNA topoisomerase II. Our results showed that Hoechst 33258 and the newly synthesized bisbenzimidazoles did not inhibit DNA gyrase's activities under our experimental conditions (Figure S21). For human DNA topoisomerase II, 50 μM of Hoechst 33258 was sufficient to completely inhibit its activities (lane 10 of Figure 3A). In contrast, the addition of a hydrophobic moiety to the hydroxyl group of Hoechst 33258 completely eliminated the inhibition of 50 μM of these newly synthesized compounds against human DNA topoisomerase II except DPA151 (Figure 3; 50 μM of DPA151 partially inhibit the activities of human DNA topoisomerase II). The IC50 values of Hoechst 33258 and DPA151 against human topoisomerases I and II are summarized in Table 1, and Figure S22.

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UV thermal denaturation studies

Bisbenzimidazoles are known to bind to the minor groove of AT rich DNA. The UV thermal denaturation experiments of the synthesized ligands were carried out with an AT rich DNA duplex. A 60 mer B-DNA duplex that was prepared by mixing equimolar amounts of a 60 mer homoadenine with a 60 mer homothymine polymer. The results obtained from these experiments are shown in Table 2 and the denaturation curves in Figure S23. The thermal denaturation experiments show some dependence of thermal stabilization on the length and composition of the linker present on the Hoechst 332258 derivatives DPA 151-DPA 154. As depicted in Figure S23, in the absence of ligand, the duplex dA60.dT60 exhibited a sharp hyperchroism at 62.5 ^{o}C indicating the dissociation of the duplex into single strands. The thermal denaturation of dA60.dT60 was then carried out in the presence of DPA 151 (10 μM). At this concentration, the DNA was saturated with ligand, and lower concentrations (1-5 μM) of ligand resulted in a biphasic thermal denaturation profiles. In the presence of DPA 151, a 22.9 ^{o}C thermal stabilization of DNA was observed. The thermal stabilization afforded by DPA 151 (22.9 ^{o}C) was similar to the

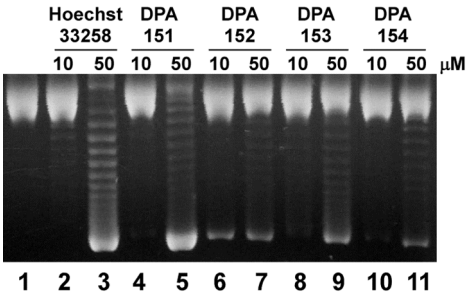

Figure 2. *Decreased inhibitions of the newly synthesized bisbenzimidazoles against human DNA topoisomerase I. Inhibition assays against human topoisomerase I were performed as described in Materials and Methods in the presence of one of the bisbenzimidazoles. Following the inhibition assays, the plasmid DNA molecules were isolated and subjected to 1% agarose gel electrophoresis in the absence of chloroquine. Lane 1 represents the relaxed plasmid DNA pBAD-GFPuv. Two different concentrations for each compound (10 and 50 μM) were used in these assays.*

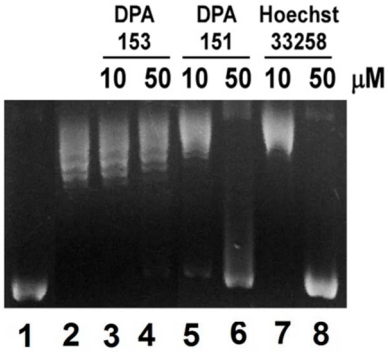

Figure 3. *Inhibitory activities of DPA151, DPA153, and Hoechst 33258 against human DNA topoisomerase II. Inhibition Assays against human DNA topoisomerase II were performed as described in Materials and Methods. The plasmid DNA molecules were isolated and subjected to 1% agarose gel electrophoresis in the absence of chloroquine. Lanes 1 and 2 are the supercoiled and relaxed plasmid DNA template pBAD-GFPuv, respectively. Two different concentrations for each compound (10 and 50 μM) were used in these assays.*

Table 1. IC_{50} values of the newly synthesized bisbenzimidazoles against E. coli DNA topoisomerase I, human DNA topoisomerase I, and human DNA topoisomerase II

Compound	IC_{50} (μM)[a] ecTopo I[b]	hTopo I[b]	hTopo II[b]
Hoechst 33258	19.50±1.32	22.86±1.55	28.92±4.45
Hoechst 33342	29.83±2.75	>50μM	-
DPA151	5.50±0.50	25.41±2.20	51.6±2.5
DPA152	4.57±0.81	>50μM	-
DPA153	2.47±0.06	>50μM	-
DPA154	6.63±0.47	>50μM	-

[a]IC_{50} was determined as described under Materials and Methods. The values are the average of at least three independent determinations. [b]ecTopo I, hTopo I, and hTopo II represent *E. coli* DNA topoisomerase I, human DNA topoisomerase I, and human DNA topoisomerase II, respectively.

thermal stabilization afforded by Hoechst 33258 and Hoechst 33342 (~24^{o}C). This thermal stabilization was, however found to be dependent on the linker length and composition of the linker. As the linker length increases to a very long carbon chain (eleven atoms) in DPA 153, a significant drop in ΔT_m was observed (23.9 ^{o}C thermal stabilization for DPA 151 and 8 oC thermal stabilization for DPA 153). Control experiments with a GC rich calf thymus DNA showed very poor (1-2 ^{o}C) thermal stabilization of the DNA by DPA 151-154 that confirmed the preference of these ligands for AT rich DNA sequences (data not shown). Surprisingly, DPA 153 is the most effective inhibitor of E. coli Topoisomerase I. The addition of a long hydrophobic linker, capable of aggregation, limits the DNA binding of the dye. However, in the presence of the topoisomerase, it is possible that DNA binding is restored as the hydrophobic pocket in the enzyme acts to free the ligand aggregation. An alternative explanation is that duplex DNA binding is not required for enzyme activity. The inhibitory activities of small molecules against these enzymes are believed to mainly stem from the binding of these ligands to the minor groove of the DNA double helix.[12, 22] However, duplex DNA binding is not a sole criterion for effective topoisomerase inhibitions as a DNA non-binder, camptothecin,[23] is a well-known DNA topoisomerase I poison.[5] We also tested cytotoxicity of these compounds against a prostate cancer cell line DU-145. Considerable variation in toxicity was observed with changes in linker length. DPA 153, the most potent Topo I inhibitor,

Table 2. A table showing the thermal denaturation temperatures of duplex DNA ($dA_{60}.dT_{60}$) in the presence of all studied ligands (10 μM each) in buffer 10 mM sodium cacodylate, 0.1 mM EDTA and 100 mM NaCl at pH 7.0.

Ligand	Tm(°C)	ΔTm (°C)
None	62.5	-
Hoechst 33258	87.1	24.6
Hoechst 33242	86.6	24.1
DPA 151	85.4	22.9
DPA 152	83.4	20.9
DPA 153	70.5	8.0
DPA 154	85.9	23.4

displayed much lower cytotoxicity (IC_{50} > 10 μM) compared to Hoechst 33242 (IC_{50} = 4.25 μM), whereas DPA 152 was nearly

twice as toxic as Hoechst 33242. These results clearly warrant further studies to understand the role of hydrophobic linkers in modulating the activity/cytotoxicity ratios (Table S1, Supporting Information).

Antibacterial activity

Compounds belonging to the bisbenzimidazole class of ligands have shown profound antibacterial effect against a variety of strains, which include methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Entercoccus faecalis*.[24, 25] To discern if these inhibitors of *E. coli* Topoisomerase I are effective at inhibiting bacterial growth, we evaluated the antibacterial effect of these compounds against both gram positive and gram negative strains, as listed in Table 3. In cases where a sharp inflection in the bacterial growth was not observed, the MIC is given as a range of values. As seen in Table 1, all four compounds are effective antibacterial compounds against a variety of strains, including the two *E.coli* strains. DPA 152 and DPA 154 show markedly improved activity against *E. faecalis* 29212. Both Hoechst dyes (33258 and 33342) are not good inhibitors of *E. faecalis* 29212. Of note also is that the molecular mass of DPA 151-154 is considerably higher than Hoechst 33342 (10-35% higher mass), implying that the antibacterial activities listed here (in μg/ml) are even better on a per mole basis for all newly synthesized compounds.

Table 3. Minimal inhibitory concentrations (MIC) of the studied ligands against various bacterial strains by microbroth dilution. S1= *S. aureus* 29213; S2= *S. aureus* 33591; S3= *E. coli* 25922; S4= *P. aeruginosa* 27853; S5= *E. coli* K 12; S6= *E. faecalis* 29212

Sample	MIC (μg/ml)					
	S1	S2	S3	S4	S5	S6
Ht 33258	≥ 32	16-32	16	16	16	16-32
Ht 33342	2-4	2-4	8-16	16	8	16-32
DPA 151	2-4	2-4	8	16	8	16-32
DPA 152	2-4	2-4	8-16	16	8-16	4
DPA 153	16	16	16	16	8-16	16-32
DPA 154	2-4	2-4	8-16	16	8-16	8

Conclusions

Overall, our results clearly show that bisbenzimidazoles (DPA 151-DPA 154) which are excellent inhibitors of E. *coli* DNA topoisomerase I, also display good antibacterial activity. Additionally, and more importantly, the *E.coli* topoisomerase I inhibition is extremely selective as DNA gyrase and mammalian topoisomerases are not inhibited. The *E. coli.* Topo I IC_{50} (2.47±0.06 μM) for DPA 153 is even better than recently reported bisbenzimidazole derivative DMA (3.8 μM).[26] It is plausible that alkynyl linkers present in our benzimdiazoles interact with the bacterial topoisomerase I enzyme leading to a stabilization of the cleavable complex. The alkynyl chains likely interact with the ternary complex as the bisbenzimidazole binds in the minor groove of DNA. Our findings suggest that the ternary complex formed by the bacterial Topoisomerase I has distinct sites for small molecule recognition, as compared to those found in DNA gyrase and mammalian topoisomerases, and these differences could be further exploited for antibacterial drug development. Further studies to investigate the mechanism of antibacterial activity and enzyme inhibition are being investigated and will be reported in due course.

Experimental section

General methods

Unless otherwise specified, chemicals were purchased from Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and used without further purification. Hoechst 33258 and Hoechst 33242 were obtained as their hydrochloride salts and used without further purification. All solvents were purchased from VWR (West Chester, PA). Silica gel (32-65 μM mesh size) was purchased from Sorbtech (Atlanta, GA). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance (300/500 MHz) Spectrometer. Chemical shift are given in ppm and are referenced to residual solvent peaks (^{1}H and ^{13}C NMR). Mass (MALDI-TOF) spectra were collected using a Bruker Microflex mass spectrometer. Ultra Violet (UV) spectra were collected on a Varian (Walnut Creek CA) Cary 100 Bio UV-Vis spectrophotometer equipped with a thermoelectrically controlled 12-cell holder.

Synthesis

*Synthesis of 4-(prop-2-ynyloxy)benzaldehyde (***DPA 151a***).* To a solution of *p*-hydroxybenzaldehyde (2.00 g, 16.3 mmol) in dry dichloromethane (30.0 mL) and 1, 4 dioxane (5.00 mL), triphenyl phosphine (6.30 g, 24.2 mmol) and propargyl alcohol (0.91 g, 16.3 mmol) were dissolved under argon and the solution was ice cooled. To this mixture, diisopropyl azodicarboxylate (DIAD) (4.80 mL, 24.2 mmol) was added dropwise over a period of 15 min at 0 ^{o}C. The contents were initially stirred at 0 ^{o}C for 30 min and then allowed to slowly warm up to room temperature and stirred overnight. Progress of the reaction was monitored using thin layer chromatography (TLC) on silica gel. The volatiles were removed under reduced pressure and the gummy residue was redissolved in ethyl acetate-hexane (80.0 mL, 1:1 *v/v*). The reaction mixture was allowed to stand overnight in the refrigerator. The precipitated solid was vacuum filtered and the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (hexanes-ethyl acetate, 100:0-70:30) to afford the desired compound as a white solid (1.3 g, 50%): R_f = 0.42 (hexanes: ethyl acetate 7:3 *v/v*) ; mp 80-81 ^{o}C; IR (neat, cm^{-1}) 3419, 2112, 1654; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.91(s, 1H), 7.87 (dd, J_1 = 8.82 Hz, J_2 = 1.94 Hz, 2H), 7.10 (dd, J_1 = 8.74 Hz, J_2 = 1.70 Hz, 2H), 4.77 (d, *J* = 2.36 Hz, 2H, -O**CH$_2$**CCH), 2.60 (t, *J* = 2.43 Hz, 1H, -OCH$_2$C**CH**); ^{13}C NMR (125 MHz, $CDCl_3$) δ 190.7, 162.3, 131.8, 130.6, 115.1, 77.8, 76.3, 55.9.

N-methoxy-N-methyl-2-(4-(prop-2-ynyloxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (**DPA 151-b**)**.** To a solution of *N*- Methoxy, *N*-methyl 3, 4 dinitrobenzamide (0.90 g, 3.52 mmol) in ethanol (20.0 mL), 150 mg of 10% Pd-C was added. Hydrogenation for 5h at the atmospheric pressure afforded corresponding diamine. The diamine was used immediately after filtration of the catalyst without further purification. 4-(prop-2-ynyloxy)benzaldehyde (0.62 g, 3.87 mmol) and sodium metabisulfite (0.37 g, 1.93 mmol) in water (1.00 mL) were added into the diamine and the reaction mixture was refluxed for 12h. The volatiles were evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane-methanol (0-10% methanol in dichloromethane) as eluent to afford the desired product as a pale yellow solid (0.55 g, 83%): R_f = 0.53 (dichloromethane : methanol 9:1, *v/v*); mp 245-246 ^{o}C; IR (neat, cm^{-1}) 2974, 2124, 1617; ^{1}H NMR (500 MHz, DMSO-d_6) δ 13.1 (s, 1H), 8.15 (d, *J* = 8.5 Hz, 2H), 7.91-7.79 (m, 1H), 7.67 (d, *J* = 8.50 Hz, 1H, 7.55 (d, *J* = 8.00 Hz, two sets of doublets, 1H), 7.50-7.46 (m, 1H), 7.18 (dd, J_1 = 2.50 Hz, J_2 = 9.00 Hz, 2H), 4.92 (d, *J* = 2.53 Hz, 2H, , -O**CH$_2$**CCH), 4.36 (d, *J* = 2.12 Hz, 1H, ,-OCH$_2$C**CH**), 3.58 (s, 3H), 3.35 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 170.1, 169.9, 159.4, 159.3, 153.6, 153.1, 145.9, 137.0, 128.6, 123.1, 119.0, 115.8,

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111.0, 79.0,62.5, 56.1, 25.9; MS (MALDI-TOF) *m/z* calcd for $C_{19}H_{17}N_3O_3$ 335.13, found 336.38 $[M+H]^+$.

2-(4-(prop-2-ynyloxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (**DPA 151-c**). To a solution of *N*-methoxy-*N*-methyl-2-(4-(prop-2-ynyloxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (0.30 g, 0.89 mmol) in tetrahydrofuran (THF)-ether (40 mL, 3:1 *v/v*), lithium aluminum hydride (0.13 g, 3.57 mmol) was added in small portions at -70 °C under argon and then the stirring was continued for 6h while allowing the slush bath to warm up to -20 °C. The reaction mixture was quenched by the addition of saturated ammonium chloride solution (50 mL). The precipitated grey solid was filtered off. The filtrate was extracted with ethyl acetate (3 × 50 mL). Organic layers were combined and then dried over sodium sulfate. Volatiles were removed under reduced pressure. The crude product was purified by column chromatography on silica gel using hexanes: ethyl acetate (1:1-2:1 *v/v*) to yield the desired compound as a light yellow solid (0.18 g, 73%): R_f = 0.76 (in ethyl acetate); mp >255 °C (dec); IR (neat, cm^{-1}) 3278, 2919, 2120, 1669; ^{1}H NMR (500 MHz, DMSO-d_6) δ 13.25 (s, br, 1H), 10.05 (s, 1H), 8.18 (d, *J* = 8.50 Hz, 2H), 8.10 (br, 1H), 7.77 (br, 1H) 7.68 (br, 1H) 7.20 (d, *J* = 9.0 Hz, 2H), 4.92 (d, *J* = 2.66 Hz, 2H, , -O**CH$_2$**CCH), 3.65 (t, *J* = 2.28 Hz, 1H, ,-OCH$_2$C**CH**); ^{13}C NMR (125 MHz, DMSO-d_6) δ 193.0, 159.6, 155.4, 154.1, 149.0, 144.2, 135.5, 131.5, 129.0, 123.4, ,119.3, 115.8, 114.2, 112.2, 79.4, 60.2, 56.1; MS (MALDI-TOF) *m/z* calcd for $C_{17}H_{12}N_2O_2$ $[M]^+$ 276.09, found 277.66 ($[M+H]^+$).

Synthesis of 6-(4-methylpiperazin-1-yl)-2'-(4-(prop-2-ynyloxy)phenyl)-1H,3'H-2,5'-bibenzo[d]imidazole (**DPA 151**). To a solution of 5-(4-methylpiperazin-1-yl)-2-nitroaniline (0.06 g, 0.27 mmol) in ethanol (8.0 mL), 10% Pd-C (40.0 mg) was added and then it was hydrogenated for 6h at the atmospheric pressure. TLC on silica gel (ethyl acetate-methanol 8:2 *v/v*) showed complete reduction of the starting material. After filtering the catalyst over a bed of celite, 2-(4-(prop-2-ynyloxy)phenyl)-3H-benzoimidazole-5-carbaldehyde (0.08 g, 0.31 mmol) was added. To this solution, $Na_2S_2O_5$ (30.0 mg, 0.16 mmol) in water (0.2 mL) was added and the mixture was refluxed for 14h . The reaction mixture was allowed to come to the room temperature. The volatiles were evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane-methanol as eluent (0-18% methanol in dichloromethane) to afford the desired product as yellow solid (85 mg, 65%): R_f = 0.23 (ethyl acetate-methanol 8:2 *v/v*); mp 252-256 °C; IR (neat, cm^{-1}) 3235, 2919, 2109; ^{1}H NMR (300 MHz, methanol-d_4) δ 8.25 (br, 1H), 8.06 (d, *J* = 8.84 Hz, 2H), 7.95 (dd, J_1 = 9.97 Hz, J_2 =1.30 Hz, 1H), 7.69 (d, *J* = 8.32 Hz, 1H),7.51 (d, *J* = 8.77 Hz, 1H), 7.15 (3H), 7.05 (s, 1H), 4.82 (d, *J* = 2.29 Hz, 2H), 3.24 (t, *J* = 4.55 Hz, 4H), 3.04 (t, *J* = 2.33 Hz, 1H), 2.72 (t, *J* = 4.64 Hz, 4Hz), 2.42 (s, 3H) (some proton peaks are masked with the solvent peaks) ; ^{13}C NMR (75 MHz, DMSO-d_6) δ 159.1, 153.2, 152.1, 145.3, 144.5, 138.6, 136.4, 135.7, 128.6, 123.5, 122.0, 120.7, 119.1, 116.5, 115.7, 111.9, 97.6, 79.4, 79.0, 56.0, 55.3, 50.7 50.2, 46.2; ESI-HRMS (*m/z*) calcd. for $C_{28}H_{27}N_6O$ 462.2246, found 463.2237.

Synthesis of 4-(hex-5-ynyloxy)benzaldehyde (**DPA 152-a**). To an ice cold solution of *p*-hydroxy benzaldehyde (1.00 g, 8.18 mmol) in dry dichloromethane (15.0 mL) and dioxane (5.0 mL), 5-Hexyn-1-ol (0.80 g, 8.18 mmol) and triphenyl phosphine (3.17 g, 12.1 mmol) were added under argon . To this solution, DIAD (2.40 mL, 12.1 mmol) was added dropwise. The reaction mixture was stirred at 0 °C for 1h followed by stirring at room temperature was 6h. Volatiles were evaporated and the crude mixture was redissolved in ethyl acetate-hexanes (80.0 mL, 1:1 *v/v*). The mixture was allowed to stand in the refrigerator for a day and the precipitated solid was vacuum filtered. The filtrate containing the crude product was concentrated under reduced pressure. The crude mixture was purified on a silica gel column using hexanes- ethyl acetate (0-25% ethyl acetate in hexanes) as eluent to yield the desired compound as colorless oil (1.4 g, 85%): R_f = 0.7 (hexanes-ethyl acetate 7:3); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.81(s, 1H), 7.82 (dd, J_1 = 8.82 Hz, J_2 = 1.98 Hz, 2H), 7.00 (dd, J_1 = 8.71 Hz, J_2 = 1.75 Hz, 2H), 4.08 (d, *J* = 6.25 Hz, 2H, -O**CH$_2$**CCH), 2.32-2.27 (m, 2H), 2.00-1.91 (m, 3H), 1.79-1.69 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 190.8, 164.1, 132.0, 130.7, 114.7, 84.8, 68.8, 67.7, 28.0, 24.8, 18.1.

Synthesis of 2-(4-(hex-5-ynyloxy)phenyl)-N-methoxy-N-methyl-3H-benzoimidazole-5-carboxamide (**DPA 152-b**). To solution of *N*-Methoxy, *N*-methyl 3, 4 dinitrobenzamide (1.00 g, 3.91 mmol) in ethanol (30.0 mL), 10% Pd-C (0.10 g) was added. Hydrogenation for 5h at atmospheric pressure yielded corresponding diamine which was used immediately after filtration of the catalyst. 4-(hex-5-ynyloxy)benzaldehyde (0.82 g, 4.10 mmol) and sodium metabisulfite (0.39 g, 2.05 mmol) in water (0.50 mL) were added into it. The reaction mixture was refluxed for 8h. Volatiles were evaporated under reduced pressure. Column chromatography on silica gel using dichloromethane-methanol (0-8% methanol in dichloromethane) as eluent afforded the desired product as pale brown oil (1.1 g, 74%): R_f = 0.75 (in dichloromethane-isopropanol 9:1 *v/v*); IR (neat, cm^{-1}) 3297 (alkyne C-H stretch), 2938 (aromatic C-H stretch), 2116 (alkyne C-C stretch), 1723; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.13 (d, br, *J* = 13.7 Hz, 2H), 7.93-7.80 (1H), 7.67 (dd, J_1 = 8.27 Hz, J_2 = 8.36 Hz, 1H), 7.48 (d, *J* = 8.62 Hz, 1H), 7.11 (d, br, *J* = 8.87 Hz, 2H), 4.07 (t, *J* = 4.64 Hz, 2H, -O**CH$_2$**CCH), 3.57 (s, 3H), 3.30 (s, 3H), 2.80 (t, *J* = 2.64 Hz, 1H, -OCH$_2$C**CH**), 2.28-2.22 (m, 2H), 1.88-1.79 (m, 2H), 1.67-1.57 (m, 2H) (Imino proton was not observed); ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.2, 160.7, 153.8, 146.0, 143.4, 137.0, 128.7, 123.0, 119.0, 115.3, 111.8, 84.7, 71.8, 67.6, 60.9, 34.1, 28.2, 25.0, 21.1, 17.9; MS (MALDI-TOF) *m/z* calcd. for $C_{22}H_{23}N_3O_3$ $[M]^+$ 377.17, found 378.34 $[M+H]^+$.

Synthesis of 2-(4-(hex-5-ynyloxy)phenyl)-3H-benzimidazole-5-carbaldehyde (**DPA 152-c**). To a stirred suspension of 2-(4-(hex-5-ynyloxy)phenyl)-*N*-methoxy-*N*-methyl-3H-benzoimidazole-5-carboxamide (0.77 g, 2.04 mmol) in dry THF (40.0 mL), lithium aluminum hydride (0.31 g, 8.17 mmol) was added in small portions at -70 °C under argon and the stirring was continued for 12h at 0 °C .TLC was used to monitor the progress of the reaction. The reaction mixture was quenched by the addition of saturated ammonium chloride solution (100 mL). The resulting grey precipitate was filtered off. The filtrate was extracted by ethyl acetate (3 × 100 mL). Organic layers were combined and dried over sodium sulfate. Volatiles were removed under reduced pressure. Column chromatography on silica gel using hexanes-ethyl acetate (1:1-2:1) as eluent afforded the desired compound as light yellow liquid (0.47 g, 72%): R_f = 0.66 (ethyl acetate:hexanes 6:4 *v/v*); mp 156-158 °C; IR (neat, cm^{-1}) 3289 (alkyne C-H stretch 2116 (alkyne C-C stretch), 1696; ^{1}H NMR (300 MHz, methanol-d_4) δ 13.20 (br, 1H), 9.89 (s, 1H), 7.96 (br, 1H) 7.87 (dd, J_1 = 8.89 Hz, J_2 = 2.01 Hz, 2H,), 7.69 (dd, J_1 = 9.8 Hz, J_2 = 1.44 Hz, 1H,), 7.56 (d, *J* = 8.32 Hz, 1H), 6.91 (dd, , J_1 = 8.92 Hz, J_2 = 2.02 Hz, 2H), 3.93-3.89 (t, *J* = 5.91 Hz, 2H, -O**CH$_2$**CCH), 2.25-2.20 (m, 3H), 1.88-1.79 (m, 2H), 1.69-1.59 (m, 2H); ^{13}C NMR (75MHz , DMSO-d_6) δ 192.4, 171.5, 161.5, 155.2, 131.7, 128.3, 123.7, 121.0, 114.6, 83.3, 68.5, 67.4, 60.1, 27.9, 24.8, 19.9, 17.4, 16.5; MS (MALDI-TOF) *m/z* calcd. for $C_{20}H_{18}N_2O_2$ $[M]^+$ 318.14, found 319.25 $[M+H]^+$.

Synthesis of 2'-(4-(hex-5-ynyloxy)phenyl)-6-(4-methylpiperazin-1-yl)-1H,3'H-2,5'-bibenzo[d]imidazole (**DPA 152**). To a solution of 5-(4-methylpiperazin-1-yl)-2-nitroaniline (0.28 g, 1.00 mmol) in ethanol (40.0 mL), Pd-C (0.10 g) was added which was followed by

hydrogenation at atmospheric pressure for 5h. Charcoal was filtered off over a bed of celite. To this solution, 2-(4-(hex-5-ynyloxy)phenyl)-3H-benzimidazole-5-carbaldehyde (0.35 g, 1.10 mmol) and a solution of $Na_2S_2O_5$ (0.10 g, 0.55 mmol) in water (0.20 mL) were added and the mixture was refluxed for 23h . The reaction mixture was allowed to come to room temperature. The filtrate was evaporated under reduced pressure. Column chromatography on silica gel using dichloromethane-methanol as eluent (0-15% methanol in dichloromethane) afforded the desired product as yellow solid (0.36 g, 72%): R_f = 0.15 (ethyl acetate:methanol 8:2 with two drops of triethylamine); mp 210-220 ºC; IR (neat, cm^{-1}) 3293 (alkyne C-H stretch), 2116 (alkyne C-C stretch), 1618; ^{1}H NMR (500 MHz, methanol-d_4) δ 8.21 (s, 1H), 8.00 (dd , J_1 = 8.60Hz, J_2 = 2.06 Hz, 2H), 7.92 (dd, J_1 = 8.64 Hz, J_2 = 2.02 Hz, 1H) 7.66 (d, J = 8.30 Hz, 1H), 7.51 (d, J = 9.00 Hz, 1H) , 7.12 (d, J = 8.80 Hz, 1H), 7.04 (d, J = 8.54 Hz, 1H), 7.02 (dd, J_1 = 8.84 Hz, J_2 = 2.06 Hz, 2H), 4.01 (t, J = 2.26 Hz, 2H, -O**CH$_2$**CCH), 3.24 (t, J = 4.62 Hz, 4H), 2.75 (t, J = 4.66 Hz, 4H), 2.53 (s, br, 1H), 2.44 (s, 3H), 2.28-2.24 (m , br, 2H), 1.93-1.86 (m, br, 2H), 1.70-1.77 (m, br, 2H) (Imino protons were not observed because of exchange with the NMR solvent); ESI-HRMS (*m/z*) calcd. for $C_{31}H_{33}N_6O$ 505.2716, found 505.2701; HPLC: t_R 2.87 min, purity 96.1% (see procedure for method details).

Synthesis of 4-(undec-10-ynyloxy)benzaldehyde (**DPA153-a**). To an ice cold solution of *p*-hydroxybenzaldehyde (0.50 g, 4.09 mmol) in dry dichloromethane-dioxane mixture (15.0 mL 2:1 *v/v*), triphenyl phosphine (1.60 g, 6.05 mmol) and 10-undecyn-1-ol (0.70 g, 6.05 mmol) were dissolved and kept at 0 ^{o}C. To this, diisopropyl azodicarboxylate (1.22 g, 6.05 mmol) was added dropwise over a period of 15 min. The contents were initially stirred at 0 ^{o}C for 30 min and then allowed to warm up to room temperature and stirred overnight. The crude mixture was concentrated and redissolved in ethyl acetate-hexanes mixture (50 mL, 1:1 *v/v*) and kept in the refrigerator for a day. The precipitated solid was filtered off and the filtrate was concentrated under reduced pressure. Column chromatography on silica gel using hexanes-ethyl acetate as eluent (0-50% ethyl acetate in hexanes) afforded the desired compound as white solid (0.58 mg, 52%): R_f = 0.54 (hexanes-ethyl acetate 7:3 *v/v*); mp 65-68 ºC; IR (neat, cm^{-1}) 3421 (alkyne C-H stretch), 2097 (alkyne C-C stretch), 1684; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.90 (s, 1H), 7.84 (dd, J_1 = 7.1 Hz, J_2 = 1.9 Hz, 2H), 7.00 (dd, J_1 = 7.1 Hz, J_2 = 1.9 Hz, 2H), 4.06 (t, J = 6.50 Hz, 2H, -O**CH$_2$**CCH), 2.23-2.18 (m, 2H), 1.96 (t, J = 2.71 Hz, 1H, -OCH$_2$C**CH**), 1.88-1.79 (m ,2H), 1.58-1.27 (12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 190.8, 164.2, 131.9, 129.7, 114.7, 84.7, 74.3 68.4, 68.1, 29.3, 29.1, 29.0, 28.7 28.4, 21.6, 18.4.

Synthesis of N-methoxy-N-methyl-2-(4-(undec-10-ynyloxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (**DPA 153-b**). To solution of *N*- Methoxy, *N*-methyl 3, 4 dinitrobenzamide (0.40 g, 1.37 mmol) in ethanol-ethyl acetate mixture (15.0 mL), 10% Pd-C (0.09 g) was added. Hydrogenation at atmospheric pressure for 5h afforded corresponding diamine which was used without purification after filtration of the catalyst. 4-(undec-10-ynyloxy)benzaldehyde (0.49 g, 1.78 mmol) and sodium metabisulfite (0.26 g, 1.37 mmol) in water (1.00 mL) were added into it and the reaction mixture was refluxed overnight. Volatiles were evaporated under reduced pressure. The crude product was purified on a silica gel column using dichlromethane-methanol (0-10% methanol in dichloromethane) as eluent to afford the desired product as slightly yellow gummy solid (0.60 g, 85%): R_f = 0.67 (dichloromethane-isopropanol 9:1 *v/v*); IR (neat, cm^{-1}) 3305 (alkyne C-H stretch) , 2112 (alkyne C-C stretch), 1614; ^{1}H NMR (300 MHz, acetone-d_6) δ 8.17 (d, J = 8.4 Hz, 2H), 8.03-7.87 (br, 1H), 7.68-7.50 (m, 2H), 7.11 (d, J = 8.7 Hz, 2H), 4.14 (t, J = 6.2 Hz, 2H, -O**CH$_2$**CCH), 3.62 (s, 3H), 3.34 (s, 3H), 2.32 (t, J = 2.5 Hz, 1H, -OCH$_2$C**CH**), 2.21-2.16 (m, 2H), 1.88-1.78 (m, 2H), 1.57-1.30 (m, 12H) (Imino proton was not observed because of exchange with the NMR solvent); ^{13}C NMR (75 MHz, acetone-d_6) δ 170.1, 160.9, 153.4, 138.9, 137.2, 128.3, 128.2, 122.7, 122.4, 118.2, 117.5, 114.7, 113.8, 101.2, 84.1, 68.9, 67.9, 60.2, 33.3, 29.7-28.2 (peaks masked with the acetone-d_6 peak), 25.8, 27.8; MS (MALDI-TOF) *m/z* calcd. for $C_{27}H_{33}N_3O_3$ 447.57, found 448.69 ([M+H]$^+$).

Synthesis of 2-(4-(undec-10-ynyloxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (**DPA 153-c**). To a solution of *N*-methoxy-*N*-methyl-2-(4-(undec-10-ynyloxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (0.80 g, 1.78 mmol) in THF-ether (80.0 mL, 3:1), lithium aluminum hydride (0.20 g, 5.28 mmol) was added at -78 ^{o}C under argon and then allowed to stir at 0 ^{o}C for 8h . The reaction mixture was quenched by the addition of saturated ammonium chloride solution (75.0 mL). The resulting grey precipitate was filtered off. The filtrate was extracted with ethyl acetate (3 × 100 mL). Organic layers were combined and dried over sodium sulfate. Volatiles were removed under reduced pressure. Column chromatography on silica gel using hexanes-ethyl acetate as eluent afforded the desired compound as a light yellow solid (0.42 g, 60%): R_f = 0.78 (ethyl acetate-hexanes 6:4); mp 126-128 ºC; IR (neat, cm^{-1}) 3305 (alkyne C-H stretch, 2108 (alkyne C-H stretch), 1503; ^{1}H NMR (300 MHz, acetone-d_6) δ 10.08 (s, 1H), 8.20 (d, J = 8.8 Hz, 2H), 8.14 (1H), 7.80 (dd, J_1 = 8.3 Hz, J_2 = 1.3 Hz, 1H), 7.72 (d, J = 8.3 Hz, 1H), 7.11 (d, J = 8.9 Hz, 2H), 4.10 (t, J = 6.5 Hz, 2H, -O**CH$_2$**CCH), 2.32 (t, J = 2.7 Hz, 1H, -OCH$_2$C**CH**), 2.21-2.15 (m, 2H), 1.87-1.78 (m, 2H), 1.56-1.37 (m, 12H) (Imino proton was not observed because of exchange with the NMR solvent); ^{13}C NMR (125 MHz, acetone-d_6) δ 191.5, 161.3, 155.3, 154.0, 149.3, 144.5, 139.7, 138.9, 135.3, 131.8, 128.4, 127.9, 123.3, 122.1, 119.0, 114.9, 113.7, 112.9, 111.3, 84.0, 68.8, 67.9, 33.5, 28.1; MS (MALDI-TOF) *m/z* calcd. for $C_{25}H_{28}N_2O_2$ 388.50, found 390.12 ([M+2H]$^+$).

6-(4-methylpiperazin-1-yl)-2'-(4-(undec-10-ynyloxy)phenyl)-1H,3'H-2,5'-bibenzo[d]imidazole (**DPA 153**). To a solution of 5-(4-methylpiperazin-1-yl)-2-nitroaniline (0.24 g, 1.02 mmol) in ethanol-ethyl acetate mixture (30.0 mL), 10% Pd-C (0.10 g) was added followed by hydrogenation at atmospheric pressure for 5h. TLC on silica gel showed complete reduction of the starting material. Charcoal was filtered over a bed of celite. To the filtrate, 2-(4-(undec-10-ynyloxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (0.40 g, 1.02 mmol) and a solution of $Na_2S_2O_5$ (0.19 g, 1.02 mmol) in water (1.0 mL) was added and the mixture was refluxed for 14h. Volatiles were removed under reduced pressure. Column chromatography on silica gel in dichloromethane-methanol (0-15% methanol in dichlromethane) afforded the pure product as yellow solid (0.29 g, 50%): R_f = 0.32 (dichlromethane-methanol 8:2); mp 170-175 ºC; IR (neat, cm^{-1}) 2919 (aromatic C-H stretch), 1614; ^{1}H NMR (300 MHz, methanol-d_4) δ 8.20 (br, 1H), 7.95-7.89 (br, 3H), 7.63 (d, J = 8.5Hz, 1H), 7.50 (d, J = 8.7 Hz, 1H), 7.10 (d, J = 2.0 Hz, 1H), 7.01 (dd, J_1 = 8.8 Hz, J_2 = 2.2 Hz, 1H), 6.93 (d, J = 8.5 Hz, 2H), 3.85 (t, J = 6.2 Hz, 2H), 3.21 (t, J = 4.5 Hz, 4H), 2.72 (t, J = 4.8 Hz, 4H), 2.42 (s, 3H), 2.19-2.11(m ,br, 3H), 1.73-1.63 (m, 2H), 1.52-1.22(br, 12H) (Imino protons were not observed because of exchange with the NMR solvent); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.7, 153.1, 148.0, 145.4, 144.6, 139.3, 136.5, 135.7, 128.6, 124.7, 122.6, 120.7, 119.0, 115.3, 115.1, 111.8, 85.02, 71.5, 71.5, 68.1, 61.5, 55.2, 46.0, 33.6, 29.3, 29.2, 29.1, 28.9, 28.5, 28.4, 25.9, 18.1; ESI-HRMS (*m/z*) calcd. for $C_{36}H_{43}N_6O$ 575.3498, found 565.3497; HPLC: t_R 8.04 min, purity 98.4% (see procedure for method details).

Synthesis 4-(4-(prop-2-ynyloxy)butoxy)benzaldehyde (**DPA 154-a**). To a solution of *p*-hydroxybenzaldehyde (4.00 g, 32.7 mmol) in

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dry dichloromethane-dioxane (40.0 mL, 3:1 *v/v*), triphenyl phosphine (12.6 g, 48.0 mmol) and 4-(prop-2-ynyloxy) butan-1-ol (4.20 g, 32.7 mmol) were dissolved and the solution was ice cooled. To this mixture, diisopropyl azodicarboxylate (9.70 g, 48.0 mmol) was added drop wise over a period of 15 min at 0 ^{o}C. The contents were initially stirred at 0 ^{o}C for 30 min and then at room temperature overnight. The solvents were removed under reduced pressure and the mixture was redissolved in ethyl acetate-hexanes (100 mL, 1:1 *v/v*) and allowed to stand in the refrigerator overnight. The precipitated solid was filtered off and the filtrate was concentrated under reduced pressure. Column chromatography on silica gel using hexanes-ethyl acetate as eluent, afforded the desired compound as pale yellow oil (6.2 g, 82%): R_f = 0.55 (hexanes-ethyl acetate 7:3 *v/v)*; IR (neat, cm^{-1}) 3289 (alkyne C-H stretch), 2116 (alkyne C-C stretch), 1688; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.84 (s, 1H), 7.79 (dd, J_1 = 1.79 Hz, J_2 = 8.81 Hz, 2H), 6.96 (d, J = 8.81 Hz, 2H), 4.13 (d, J = 2.39 Hz, 2H), 4.04 (t, J = 6.27 Hz, 2H), 3.57 (t, J = 6.42 Hz, 2H), 2.45 (t, J = 2.38 Hz, 1H), 1.92-1.87 (m, 2H), 1.79-1.74 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) 190.7, 164.1, 131.9, 129.8, 114.7, 79.9, 74.3, 69.4, 67.9, 58.0, 26.0, 25.8.

Synthesis of N-methoxy-N-methyl-2-(4-(4-(prop-2-ynyloxy)butoxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (**DPA 154-b**). To solution of *N*- Methoxy, *N*-methyl 3, 4 dinitrobenzamide (0.73 g, 2.86 mmol) in ethanol-ethyl acetate mixture (40.0 mL, 3:1 *v/v*), 10% Pd-C (0.30 g) was added. Hydrogenation at the atmospheric pressure for 5h yielded corresponding diamine which was used without further characterization after filtration of the catalyst (R_f = 0.46 in dichloromethane:methanol 9:1, *v/v*). 4-(4-(prop-2-ynyloxy)butoxy)benzaldehyde (0.70 g, 2.86 mmol) and sodium metabisulfite (0.54 g, 2.86 mmol) in water (1.00 mL) were added into it and the reaction mixture was refluxed for 6h. Volatiles were evaporated under reduced pressure. The crude product was purified by column chromatography on a silica gel using dichloromethane-methanol as eluent to afford the desired product as pale yellow gummy solid (0.70 g, 61%): R_f = 0.50 (in dichloromethane-methanol 9:1 *v/v*); IR (neat, cm^{-1}) 3231 (alkyne C-H stretch) , 2230 (alkyne C-C stretch), 1608; ^{1}H NMR (500 MHz, methanol-d_4) δ 7.98 (dd, J_1 = 8.90 Hz, J_2 = 2.00 Hz, 2H), 7.93 (br, 1H), 7.59-7.55 (m, 2H), 6.99 (dd, J_1 = 8.65, J_2 = 2.00, 2H), 4.13 (d, J = 2.39 Hz, 2H, -$CH_2O\mathbf{CH_2}CCH$), 3.97 (t, J = 6.27 Hz, 2H, -$O\mathbf{CH_2}CH_2$-), 3.61 (s, 3H), 3.55 (t, J = 6.40 Hz, 2H), 3.39 (s, 3H), 2.85 (t, J = 2.26 Hz, 1H, -$CH_2OCH_2C\mathbf{CH}$), 1.84-1.78 (m, 2H), 1.74-1.68 (m, 2H) (Imino proton was not observed because of exchange with the NMR solvent) ; ^{13}C NMR (75 MHz, methanol-d_4) δ 170.5, 161.1, 154.1, 140.5, 138.4, 128.1, 127.6, 122.7, 121.2, 114.6, 113.6, 79.5, 74.3, 69.4, 69.1, 67.5, 60.1, 57.3, 33.4, 25.74, 25.70; MS (MALDI-TOF) *m/z* calcd for $C_{23}H_{25}N_3O_4$ $[M]^+$, 407.18, found 407.10 ($[M]^+$).

Synthesis of 2-(4-(4-(prop-2-ynyloxy)butoxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (**DPA 154-c**).To a solution of *N*-methoxy-*N*-methyl-2-(4-(4-(prop-2-ynyloxy)butoxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (0.70 g, 1.71 mmol) in THF-ether (80.0 mL, 3:1), lithium aluminum hydride (0.26 g, 6.58 mmol) was added at -78 ^{o}C and then allowed to warm up and stir at 0 ^{o}C for 6h . The reaction mixture was quenched by the addition of saturated ammonium chloride solution (100 mL). The grey solid that precipitated out was filtered off. The filtrate was extracted with ethyl acetate (3 × 75 mL). Organic layers were combined and dried over sodium sulfate. Volatiles were removed under reduced pressure. The crude product was purified by column chromatography on silica gel using hexanes-ethyl acetate (0-80% ethyl acetate in hexanes) as eluent to yield the desired compound as a light yellow liquid (0.33 g, 55%); R_f = 0.65 (ethyl acetate-hexane 8:2 *v/v*); IR (neat, cm^{-1}) 2945 (aromatic C-H stretch), 2066 (alkyne C-C stretch), 1686; ^{1}H NMR (500 MHz, methanol-d_4) δ 9.99 (s, 1H), 8.08 (s, br, 1H), 8.00 (dd, J_1 = 8.82 Hz, J_2 = 1.95 Hz, 2H), 7.80 (dd, J_1 = 8.28 Hz, J_2 = 1.46 Hz, 1H), 7.67 (s, br, 1H), 7.05 (dd, J_1 = 9.06 Hz, J_2 = 2.01 Hz, 2H) , 4.18 (d, J = 2.32 Hz, 2H, -$CH_2O\mathbf{CH_2}CCH$), 4.05 (t, J = 6.32 Hz, 2H, -$O\mathbf{CH_2}CH_2$-), 3.61 (t, J = 6.27 Hz, 2H), 2.87 (t, J = 2.56 Hz, 1H, -$CH_2OCH_2C\mathbf{CH}$), 1.91-1.85 (m, 2H), 1.80-1.75 (m, 2H) (Imino proton was not observed because of exchange with the NMR solvent); ^{13}C NMR (75 MHz, methanol-d_4) δ 192.4, 161.4, 131.7, 128.3, 123.7, 120.0, 114.7, 79.4, 74.2, 69.1, 67.5, 57.3, 29.13, 25.73, 25.70 (Four aromatic carbon peaks were not observed likely because of aggregation of the molecule) ; MS (MALDI-TOF) *m/z* for $C_{21}H_{20}N_2O_3$ $[M]^+$ calcd 348.15, found 349.48 $[M+H]^+$.

Synthesis of 6-(4-methylpiperazin-1-yl)-2'-(4-(4-(prop-2-ynyloxy)butoxy)phenyl)-1H,3'H-2,5'-bibenzo[d]imidazole (**DPA 154**). To a solution of 5-(4-methylpiperazin-1-yl)-2-nitroaniline (0.21 g, 0.91 mmol) in ethanol- ethyl acetate mixture (20.0 mL), Pd-C (0.15 g) was added and the mixture was hydrogenated for 5h at the atmospheric pressure. Charcoal was filtered off. To this solution, 2-(4-(4-(prop-2-ynyloxy)butoxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (0.32 g, 0.91 mmol) and a solution of $Na_2S_2O_5$ (0.17 g, 0.91 mmol) in water (1.00 mL) were added. The mixture was refluxed for 12h following which it was allowed to come to room temperature. Volatiles were removed under reduced pressure. The crude mixture was purified by column chromatography on a silica gel column using dichloromethane-methanol as eluent (0-15% methanol in dichloromethane) which afforded the desired product as yellow solid (0.29 g, 60%): R_f = 0.40 (dichloromethane-methanol 8:2 *v/v*); mp 150-155 ºC; IR (neat, cm^{-1}) 2236 (alkyne C-C stretch), 1630, 1433; ^{1}H NMR (300 MHz, methanol-d_4) δ 8.21 (d, J = 1.11 Hz, 1H) 7.98 (dd, J_1 = 8.86 Hz, J_2 = 2.00 Hz, 2H), 7.91 (dd, J_1 = 8.46 Hz, J_2 = 1.75 Hz, 1H), 7.65 (d, J = 8.46 Hz, 1H), 7.50 (d, J = 8.86 Hz, 1H), 7.14 (d, J = 2.00 Hz, 1H), 7.04-7.00 (m, 3H), 4.17 (d, J = 2.47 Hz, 2H), 4.01 (t, J = 6.15 Hz, 2H), 3.59 (t, J = 6.14 Hz, 2H), 3.34-3.30 (Some peaks are masked with the NMR solvent signal) 3.03 (t, J = 4.63 Hz, 4H), 2.86 (t, J = 2.32 Hz, 1H), 2.66 (s, 3H), 1.88-1.72 (m, 4H) (Imino protons were not observed because of exchange with the NMR solvent); ^{13}C NMR (75 MHz, methanol-d_4) δ 161.1, 153.9, 152.4, 147.4, 138.9, 134.5, 129.9, 128.1 (two peaks), 124.2, 121.3, 121.0, 115. 1,115.0, 114.5, 112.1, 101.3, 79.4, 74.2, 71.2, 69.1, 67.5, 63.5, 57.3, 54.2, 49.3, 43.5, 25.7; ESI-HRMS (*m/z*) *m/z* calcd. for $C_{32}H_{35}N_6O_2$ 535.2821, found 535.2814; HPLC: t_R 2.46 min, purity 98.6% (see procedure for method details).

HPLC analysis

HPLC analysis of compounds DPA 151-154 was performed on HP1100 series analytical HPLC instrument. The experiments were performed on a Supelcosil LC-18S column using the following gradient conditions.

DPA 151: 40% B in A with initial hold for 2 minutes and then equilibrate at 40% B in A to 100% B over 8 minutes at a flow rate of 2.0 mL/minute; DPA 152: 60% B in A with initial hold for 2 minutes and then equilibrate at 60% B in A to 100% B over 8 minutes at a flow rate of 2.0 mL/minute; DPA 153: 60% B in A with initial hold for 2 minutes and then equilibrate at 60% B in A to 100% B over 8 minutes. This was followed by 100% B over next five minutes at a flow rate of 2.0 mL/minute; DPA 154: 60% B in A with initial hold for 2 minutes and then equilibrate at 60% B in A to 100% B over 8 minutes. This was followed by 100% B over next five minutes at a flow rate of 2.0 mL/minute. Where, A- H_2O containing 0.1% trifluoroacetic acid and B- 95:5 CH_3CN-H_2O

Nucleic acids

Nucleic acids were purchased from Integrated DNA Technologies (Coraville, IA). The concentration of the nucleic acid was determined using the extinction coefficient provided by the supplier. The DNA duplex was prepared by heating the dA_{60} and dT_{60} in equimolar ratio in buffer 10 mM sodium cacodylate, 0.1 mM EDTA and 100 mM NaCl at pH 7.0 at 95 ^{o}C for 15 minutes and then slowly allowing it to cool back to room temperature. After two days of incubation, the duplex formation was checked by UV thermal denaturation experiments. The stock solution was stored at 4 ^{o}C and diluted to desired concentrations as required.

Ultra Violet (UV) thermal denaturation experiments

All UV spectra were obtained on a 12 cell holder Cary 1E UV-Vis spectrophotometer equipped with temperature controller. Quartz cells with 1cm path length were used for all the experiments. Spectrophotometer stability and wavelength alignment were checked prior to initiation of each thermal denaturation experiment. For all experiments, the samples were prepared by diluting a stock sample. The melting of DNA with and without the ligand was performed at a heating rate of 0.2 °C/min. Samples were brought back to 20 °C after the experiment. All UV melting experiments were monitored at 260 nm. For the T_m determinations, derivatives were used. Data points were recorded every 1.0 °C. The DNA concentration was 1 μM /duplex while the ligand concentration was 10 μM. All ligand solutions were prepared in dimethyl sulfoxide (DMSO) as concentrated stock solution (6-10 mM) and diluted to desired concentrations in buffer.

Minimum Inhibitory Concentration (MIC) determination

Bacteria used in this study were *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Escherichia coli* K12, *Staphylococcus aureus* ATCC 33591, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212. All compounds were tested by the microbroth dilution method following Clinical and Laboratory Standards Institute guidelines.[1] Briefly, Mueller-Hinton broth (Difco Laboratories, Becton Dickinson) was inoculated with each organism and incubated at 37 °C with shaking to establish logarithmic growth. Following incubation, each culture was pelleted by centrifugation (3,500 x g for 5 min) and resuspended in 0.85% sterile saline solution to an optical density at 625 nm of 0.1. Samples were tested in triplicate using 96 well microplates (Corning Costar Corp. Cambridge, MA), yielding final bacterial concentrations of 5×10^5 CFU/mL, and incubated for 24 h at 37 °C. Following incubation, optical densities of each well were determined with a μQuant microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT) at 625 nm. The MIC was defined as the lowest concentration needed to completely inhibit growth as compared to no treatment controls.

Inhibition assays against E. coli DNA topoisomerase I

Inhibition assays were used to determine the activities of the newly synthesized compounds against *E. coli* DNA topoisomerase I. The reaction mixture (30 μl) contained 20 mM Tris-HCl at pH 7.9, 50 mM KAc, 10 mM $Mg(Ac)_2$, 1 mM DTT, 1 μg/mL BSA, 150 ng of the supercoiled plasmid pBAD-GFPuv, 6 nM of *E. coli* topoisomerase I, and one of the compounds at a specified concentration that ranges from 0.5 to 45 μM. All components were assembled on ice and incubated for 15 min at 37 °C. After the incubation, the reactions were terminated by extraction with an equal volume of phenol. The topological state of the DNA samples was analyzed with 1% agarose gel electrophoresis in 1×TAE buffer, pH 7.8. Following electrophoresis, the agarose gel was stained with Ethidium Bromide, destained, and photographed under UV light. The intensity of DNA topoisomers was determined using KODAK 1D Image Analysis Software. The percentage of (-) supercoiled DNA was calculated from a comparison of the intensity of the (-) supercoiled band with the total intensity of all DNA topoisomers. The IC_{50} was calculated as the amount of the drug for which 50% of the DNA was still in a (-) supercoiled state.

Inhibition assays against *E. coli* DNA gyrase

The reaction mixture (30 μl) contained 35 mM Tris-HCl at pH 7.5, 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 1.75 mM ATP, 5mM spermidine, 0.1 mg/ml BSA, 6.5% glycerol, 250 ng of the relaxed plasmid pBAD-GFPuv, 0.5 units of *E. coli* DNA Gyrase, and one of the compounds at a final concentration that ranges from 1 to 50 μM. All components were assembled on ice and incubated for 30 min at 37 °C. After the incubation, the reactions were terminated by extraction with an equal volume of phenol. The topological state of the DNA samples was analyzed with 1% agarose gel electrophoresis in 1×TAE buffer, pH 7.8. Following electrophoresis, the agarose gel was stained with Ethidium Bromide, destained, and photographed under UV light. The intensity of DNA topoisomers was determined using KODAK 1D Image Analysis Software.

Inhibition assays against human DNA topoisomerase I

The inhibition assays were used to determine the activities of the newly synthesized compounds against Human DNA topoisomerase I. The reaction mixture (25 μl) contained 20 mM Tris-HCl at pH 7.9, 50 mM KAc, 10 mM $Mg(Ac)_2$, 1 mM DTT, 1 μg/ml BSA, 250 ng of the supercoiled plasmid pBAD-GFPuv, 2 units of Human DNA topoisomerase I, and one of the compounds at a final concentration that ranges from 5 to 50 μM. All components were assembled on ice and incubated for 30 min at 37 °C. After the incubation, the reactions were terminated by extraction with an equal volume of phenol. The topological state of the DNA samples was analyzed with 1% agarose gel electrophoresis in 1×TAE buffer, pH 7.8. Following electrophoresis, the agarose gel was stained with Ethidium Bromide, destained, and photographed under UV light. The intensity of DNA topoisomers was determined using KODAK 1D Image Analysis Software. The percentage of (-) supercoiled DNA was calculated from a comparison of the intensity of the (-) supercoiled band with the total intensity of all DNA topoisomers. The IC_{50} was calculated as the amount of the drug for which 50% of the DNA was in a (-) supercoiled state.

Inhibition Assays against human DNA topoisomerase II

The reaction mixture (25 μl) contained 20 mM Tris-HCl at pH 7.9, 50 mM KAc, 10 mM $Mg(Ac)_2$, 1 mM DTT, 1 μg/mL BSA, 200 ng supercoiled plasmid pBAD-GFPuv, 4 units of Human topoisomerase II, and one of the compounds at a final concentration that ranges from 5 to 50 μM. All components were assembled on ice and incubated for 30 min at 37 °C. After the incubation, the reactions were terminated by extraction with an equal volume of phenol. The topological state of the DNA samples was analyzed with 1% agarose gel electrophoresis in 1×TAE buffer, pH 7.8. Following electrophoresis, the agarose gel was stained with Ethidium Bromide, destained, and photographed under UV light. The intensity of DNA topoisomers was determined using KODAK 1D Image Analysis Software. The percentage of (-) supercoiled DNA was calculated from a comparison of the intensity of the (-) supercoiled band with the total intensity of all DNA topoisomers. The IC_{50} was calculated as the amount of the drug for which 50% of the DNA was in a (-) supercoiled state.

Cytotoxicity experiments

DU 145 cell line was cultured according to ATCC protocols. Cells were harvested using trypsin-EDTA solution and counted

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using trypan blue exclusion. Cells were seeded at a volume of 100 µl/well in the wells of tissue culture treated 96 well plates at a density of 10×10^5 cells per well. Seeded 96 well plates were returned to incubator (37 °C, 5% CO_2) for twenty-four hours to resume exponential growth

Test compounds (Hoechst 33258, Hoechst 33342, DPA 151, DPA 152, DPA 153 and DPA 154) and known drug control (cisplatin) were diluted in appropriate culture media to the following concentrations: 40, 20, 10, 5, 2.5, 1.25 and 0.125 µM. Cell lines were then treated with 100 µl of each test compound or cisplatin in triplicate. The final concentrations of each treatment were: 20, 10, 5, 2.5, 1.25, 0.625 and 0.0625 µM. Each plate also contained wells containing untreated cells and media only as controls. After receiving treatments, the 96 well plates were returned to incubator (37 °C, 5% CO_2) for forty-eight hours.

After forty-eight hours of treatment, the treated plates were fixed with trichloroacetic acid and stained with sulforhodamine B using a modified version of the protocol described by Skehan et al.[1] In short 50 µl of cold 80% TCA was added to each well, at a final concentration of 16% TCA, and plates were incubated at 4 °C for one hour. The media and TCA solution was discarded and plates were washed four times using room temperature tap water. Plates were allowed to air dry overnight. Plates were stained with the addition of 70 µl/well of 40% (w/v) SRB in 1% (v/v) acetic acid solution. Samples were stained for fifteen minutes and then stain was discarded. Plates were then washed four times with 1% (v/v) acetic acid solution to remove unbound stain and allowed to air dry overnight at room temperature. Finally, SRB stain was solubilized by adding 150 µl of 10 mM unbuffered Tris base to each well. The absorbance of each samples at 560 nm was recorded using a Tecan plate reader and the IC_{50} was determined using Origin 4.0 software. Each study was completed in duplicate.

ACKNOWLEDGMENT

We thank the NIH for financial support (CA125724 and GM100607 to DPA and 5SC1HD063059-04 to FL). We also thank Zhao Chen (Clemson University) for help with antibacterial studies.

Notes

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[†]Electronic Supplementary Information (ESI) available: [Images of spectroscopic characterization of all newly synthesized compounds as well additional images for topoisomerase inhibition experiments]. See DOI: 10.1039/c000000x/

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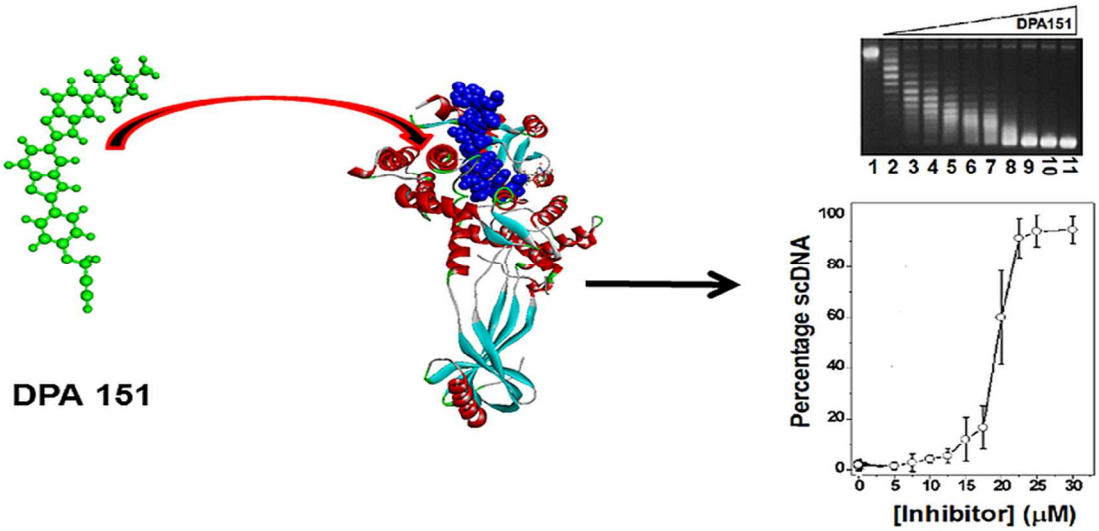

Highly selective inhibition of *E. coli* topoisomerase I by alkynyl bisbenzimidazoles