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Selective Inhibition of Bacterial Topoisomerase I by alkynyl-bisbenzimidazoles†
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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 alkyn, 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 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. These functions of DNA topoisomerases can be used to develop anticancer or antibacterial agents. 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.

A number of small molecules have been discovered that poison the functions of DNA topoisomerases. These have included camptothecin and its derivatives, intercalators and compounds that interact with the minor groove of B-DNA such as bisbenzimidazoles. Benzimidazoles are important class of compounds that display a widespread range of biological activities. Halogenated monobenzimidazoles have shown antitumor activity better than isoniazid. Similarly, triazolyl derivatized monobenzimidazoles have displayed antimicrobial properties.

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. 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.

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 alkyn 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.

Results and discussion

Synthesis of ligands DPA 151-154

The synthesis of the ligands (DPA 151-154) was performed using a divergent strategy 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 alkyn 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
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 DNA topoisomerase 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 topoisomerase 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 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 IC_{50} values of Hoechst 33258 and DPA151 against human topoisomerases I and II are summarized in Table 1, and Figure S22.
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 33258 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 °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 °C thermal stabilization of DNA was observed. The thermal stabilization afforded by DPA 151 (22.9 °C) was similar to the thermal stabilization afforded by Hoechst 33258 and Hoechst 3342 (~24°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 ΔTm was observed (23.9 °C thermal stabilization for DPA 151 and 8 °C thermal stabilization for DPA 153). Control experiments with a GC rich calf thymus DNA showed very poor (1-2 °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>
<thead>
<tr>
<th>Compound</th>
<th>ecTopo I IC₅₀ (µM)</th>
<th>hTopo I IC₅₀ (µM)</th>
<th>hTopo II IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoechst 33258</td>
<td>19.50±1.32</td>
<td>22.86±1.55</td>
<td>25.62±1.45</td>
</tr>
<tr>
<td>Hoechst 3342</td>
<td>29.83±2.75</td>
<td>&gt;50µM</td>
<td>&gt;50µM</td>
</tr>
<tr>
<td>DPA151</td>
<td>5.50±0.50</td>
<td>&gt;50µM</td>
<td>51.6±2.5</td>
</tr>
<tr>
<td>DPA152</td>
<td>4.57±0.81</td>
<td>&gt;50µM</td>
<td>-</td>
</tr>
<tr>
<td>DPA153</td>
<td>2.47±0.06</td>
<td>&gt;50µM</td>
<td>-</td>
</tr>
<tr>
<td>DPA154</td>
<td>6.63±0.47</td>
<td>&gt;50µM</td>
<td>-</td>
</tr>
</tbody>
</table>

IC₅₀ was determined as described under Materials and Methods. The values are the average of at least three independent determinations. ecTopo I, hTopo I, and hTopo II represent E. coli DNA topoisomerase I, human DNA topoisomerase I, and human DNA topoisomerase II, respectively.

Table 2. A table showing the thermal denaturation temperatures of duplex DNA (dA₆₀.dT₆₀) 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.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Tm (°C)</th>
<th>∆Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>62.5</td>
<td>-</td>
</tr>
<tr>
<td>Hoechst 33258</td>
<td>87.1</td>
<td>24.6</td>
</tr>
<tr>
<td>Hoechst 33242</td>
<td>86.6</td>
<td>24.1</td>
</tr>
<tr>
<td>DPA 151</td>
<td>85.4</td>
<td>22.9</td>
</tr>
<tr>
<td>DPA 152</td>
<td>83.4</td>
<td>20.9</td>
</tr>
<tr>
<td>DPA 153</td>
<td>70.5</td>
<td>8.0</td>
</tr>
<tr>
<td>DPA 154</td>
<td>85.9</td>
<td>23.4</td>
</tr>
</tbody>
</table>

displayed much lower cytotoxicity (IC₅₀ > 10 µM) compared to Hoechst 33242 (IC₅₀ = 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 Enterococcus 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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1</td>
</tr>
<tr>
<td>Ht 33258</td>
<td>≥32</td>
</tr>
<tr>
<td>DPA 152</td>
<td>2-4</td>
</tr>
<tr>
<td>DPA 153</td>
<td>2-4</td>
</tr>
<tr>
<td>DPA 154</td>
<td>2-4</td>
</tr>
</tbody>
</table>

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 IC₅₀ (2.47±0.06 µM) for DPA 153 is even better than recently reported bisbenzimidazole derivative DNA (3.8 µM).26 It is plausible that alkynyl linkers present in our benzimidazoles 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). 1H NMR and 13C 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 (1H and 13C 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 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, disopropyl azodicarboxylate (DIAD) (4.80 mL, 24.2 mmol) was added dropwise over a period of 15 min at 0 °C. The contents were initially stirred at 0 °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 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%); Rₖ = 0.42 (hexanes: ethyl acetate 7:3 v/v); mp 80-81 °C; IR (neat, cm⁻¹) 3419, 2112, 1653; 1H NMR (500 MHz, CDCl₃) δ 9.99 (s, 1H), 7.87 (dd, J = 8.82 Hz, J = 1.94 Hz, 2H), 7.10 (dd, J = 8.74 Hz, J = 1.70 Hz, 2H), 4.77 (d, J = 2.36 Hz, 2H, -OCH₂CH₂), 2.60 (t, J = 9.91 Hz, 2H), -OCH₃, 2.12 Hz, 1H, -OH-C₇H₇; 13C NMR (125 MHz, CDCl₃) δ 190.7, 162.3, 131.8, 130.6, 115.7, 77.8, 76.3, 55.9.

N-methoxy-N-methyl-2-(4-(prop-2-ynyloxy)phenyl)-1H-benz[d][1,2]azaborine-6-carboxamide (DPA 151-b). To a solution of N-Methoxy-N-methyl, 3-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 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%); Rₖ = 0.05 (dichloromethane : methanol 9:1, v/v); mp 245-246 °C; IR (neat, cm⁻¹) 2974, 2921, 2146; 1H NMR (500 MHz, DMSO-d₆) 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 = 2.50 Hz, J = 9.00 Hz, 2H), 4.92 (dd, J = 2.53 Hz, 2H, -OCH₂C₇H₇), 4.36 (d, J = 2.12 Hz, 1H, -OCH₃), 3.58 (s, 3H), 3.35 (s, 3H), 3.35 (s, 3H), 3.35 (s, 3H), 3.35 (s, 3H), 3.35 (s, 3H), 3.35 (s, 3H); 13C NMR (125 MHz, DMSO-d₆) δ 170.1, 169.9, 159.4, 159.3, 153.6, 153.2, 154.9, 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 caleld for C_{19}H_{32}N_{2}O_{5} 335.13, found 336.38 [M+H]^+.

2-(4-prop-2-ynoxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (DPA 151-c). To a solution of 1H NMR (300 MHz, CDCl_3) δ 9.81 (s, 1H), 7.82 (dd, J = 8.82 Hz, J = 1.98 Hz, 2H), 7.00 (dd, J = 8.71 Hz, J = 1.75 Hz, 2H), 4.08 (d, J = 6.25 Hz, 2H, -OCH_2CH), 2.32-2.27 (m, 2H), 2.00-1.91 (m, 3H), 1.79-1.69 (m, 2H); ^13C 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-ynoxy)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-ynoxy)benzaldehyde (0.82 g, 4.10 mmol) and sodium metabsulfite (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 (8-8% methanol in dichloromethane) as eluent afforded the desired product as pale yellow oil (1.71 g, 74%). Rf = 0.75 (in dichloromethane-isopropanol 9:1 v/v); IR (neat, cm^-1) 3297 (alkyne C-H stretch), 2938 (alkyne C-H stretch), 2116 (alkyne C-C stretch), 1723; ^1H NMR (300 MHz, CDCl_3) δ 8.13 (br, d, J = 13.7 Hz, 2H), 7.93-7.80 (1H), 7.67 (dd, J = 8.27 Hz, J = 8.36 Hz, 1H), 7.48 (d, J = 8.62 Hz, 1H), 7.11 (d, J = 8.87 Hz, 2H), 4.07 (t, J = 4.64 Hz, 2H, -OCH_2CH), 3.57 (s, 3H), 3.30 (s, 3H), 2.80 (t, J = 6.24 Hz, 1H, -OCH_2CH), 2.28-2.22 (m, 2H), 1.88-1.79 (m, 2H), 1.67-1.57 (m, 2H) (Imino proton not observed); ^13C NMR (75 MHz, CDCl_3) δ 170.2, 160.7, 153.8, 146.0, 143.3, 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 caleld for C_{15}H_{23}N_{3}O_{3} [M^+] 377.17, found 378.34 [M^+].

Synthesis of 2-(4-(hex-5-ynoxy)phenyl)-3H-benzoimidazole-5-carboxaldehyde (DPA 152-c). To a stirred suspension of 2-(4-(hex-5-ynoxy)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 mixture was refluxed for 14h . The reaction mixture was allowed to come to the room temperature. The volatiles were evaporated under reduced pressure. Column chromatography on silica gel using dichloromethane-methanol (8-8% methanol in dichloromethane) as eluent afforded the desired product as yellow solid (85 mg, 65%); Rf = 0.23 (ethyl acetate-methanol 8:2 v/v); mp 252-256 °C; IR (neat, cm^-1) 3235, 2919, 2109; ^1H NMR (300 MHz, CDCl_3) δ 8.16 (t, J = 8.84 Hz, 2H), 7.95 (dd, J = 9.97 Hz, J = 3.30 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, 4H), 2.42 (s, 3H) (some proton peaks are masked with the solvent peaks) ; ^13C NMR (75 MHz, DMSO-d_6) δ 159.1, 153.2, 152.1, 145.3, 144.8, 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) caleld for C_{12}H_{13}N_{2}O_{5} 462.2246, found 463.2237.

Synthesis of 4-(hex-5-ynoxy)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 (1.17 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%): Rf = 0.7 (hexanes-ethyl acetate 7:3); ^1H NMR (300 MHz, CDCl_3) δ 9.81 (s, 1H), 7.82 (dd, J = 8.82 Hz, J = 1.98 Hz, 2H), 7.00 (dd, J = 8.71 Hz, J = 1.75 Hz, 2H), 4.08 (d, J = 6.25 Hz, 2H, -OCH_2CH), 2.32-2.27 (m, 2H), 2.00-1.91 (m, 3H), 1.79-1.69 (m, 2H); ^13C 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.

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MedChemComm

J. Name., 2012, 00, 1-3 | 5

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hydrogenation at atmospheric pressure for 5h. Charcoal was filtered off under a bed of celite. To this solution, 2-(4-hexyl-5-ynyloxy)phenyl)-3H-benzimidazole-5-carbaldehyde (0.35 g, 1.10 mmol) and a solution of Na2S2O4 (0.10 g, 0.55 mmol) in water (0.20 mL) were added and the mixture was refluxed for 23 h. 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%); Rf = 0.15 (ethyl acetate:methanol 8:2 with two drops of triethylamine); mp 210-220 °C; IR (neat, cm⁻¹) 3293, 1701, 160, 1581, 1480, 1452, 1446, 1430, 1397, 1383, 1351, 1318, 1284, 1279, 1233, 1211, 1190, 1149, 1137, 1129, 1113, 84.0, 68.8, 67.9, 33.5, 28.1; MS (MALDI-TOF) m/z calcd. for C19H15N2O2 344.104, found 344.138.

Synthesis of 2-(4-(undec-10-ynyloxy)phenyl)-1H-benz[d]imidazole-6-carbaldehyde (DPA 153-c). To a solution of N-methoxy-N-methyl-2-(4-(undec-10-ynyloxy)phenyl)-1H-benz[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 °C under argon and then allowed to stir at 0 °C for 8 h. 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%); Rf = 0.78 (ethyl acetate-hexanes 6:4); IR (neat, cm⁻¹) 3305 (alkyne C-C stretch), 1684; ¹H NMR (300 MHz, CDCl3) δ 9.87 (s, 1H), 8.50 (t, J = 8.3 Hz, 1H), 7.11 (d, J = 8.9 Hz, 2H), 4.10 (t, J = 6.5 Hz, 2H, -OCH2CH2), 2.32 (t, J = 2.7 Hz, 1H, -OCH2CH2), 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); ES-HRMS (m/z) calcd. for C38H27N3O2 589.1980, found 589.2012.

Synthesis of 4-(4-(undec-10-ynyloxy)benzylidene)pyridine (DPA 153-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 in the ice bath for 20 min. To this, disisopropyl azodicarboxylate (1.22 g, 6.05 mmol) was added dropwise over a period of 15 min. The contents were initially stirred at 0 °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-hexane 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%); Rf = 0.54 (hexanes-ethyl acetate 8:2 with two drops of triethylamine); mp 65-68 °C; IR (neat, cm⁻¹) 3293; ¹H NMR (300 MHz, acetone-d6) δ 8.04 min, purity 98.4% (see procedure for method details).
dry dichloromethane-dioxane (40.0 mL, 3:1 v/v), triphenylphosphine (12.6 g, 48.0 mmol) and 4-(prop-2-ynoxy)butan-1-ol (4.20 g, 32.7 mmol) were dissolved and the solution was ice cooled. To this mixture, disopropyl azodicarboxylate (9.70 g, 48.0 mmol) was added drop wise over a period of 15 min at 0 °C. The contents were initially stirred at 0 °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 mixture (40.0 mL, 3:1 v/v) and the reaction mixture was refluxed for 6 h. Volatiles were removed under reduced pressure. 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 dichloromethane-methanol as eluent (0-15% methanol in dichloromethane) which afforded the desired compound as a pale yellow solid (0.32 g, 0.91 mmol) and a solution of NaOH (0.17 g, 0.91 mmol) in water (1.00 mL) were added into it and the reaction mixture was refluxed for 6 h. Volatiles were evaporated under reduced pressure. The crude material was purified by column chromatography on silica gel using dichloromethane-methanol as eluent to afford the desired product as a pale yellow solid (0.70 g, 2.86 mmol) and sodium methanol as eluent to afford the desired product as a pale yellow liquid (0.33 g, 15%).

**Synthesis of** N-methoxy-N-methyl-2-(4-(4-(prop-2-ynoxy)butoxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (DPA 154). To a solution of N-Methoxy-N-methyl-3, 4 dienobenzoamide (0.73 g, 2.86 mmol) in ethanol-ethyl acetate (20.0 mL), Pd-C (0.26 g, 32.7 mmol) was added and the mixture was hydrogenated for 5 h at atmospheric pressure. Charcoal was filtered off. The solution was reduced under reduced pressure. The crude mixture was purified by column chromatography on silica gel using ‘dichloromethane-methanol’ as eluent (0-15% methanol in dichloromethane) which afforded the desired product as a pale yellow solid (0.29 g, 60%): Rf = 0.46 in dichloromethane-methanol 9:1, v/v).

**Synthesis of** N-methoxy-N-methyl-2-(4-(4-(prop-2-ynoxy)butoxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (DPA 154). To a solution of N-Methoxy-N-methyl-3, 4 dienobenzoamide (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 5 h yielded corresponding diamine which was used without further characterization after filtration of the catalyst (Rf = 0.46 in dichloromethane-methanol 9:1, v/v). 2-(4-(prop-2-ynoxy)butoxy)benzaldehyde (0.70 g, 2.86 mmol) and sodium methanolate (0.54 g, 2.86 mmol) in water (1.00 mL) were added into it and the reaction mixture was refluxed for 6 h. 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%); Rf = 0.50 (in dichloromethane-methanol 9:1 v/v); IR ( neat, cm⁻¹) 3231 (alkyne C-H stretch), 2230 (alkyne C=C stretch), 190.7, 164.1, 154.1, 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-ynoxy)butoxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (DPA 154). To a solution of N-Methoxy-N-methyl-3, 4 dienobenzoamide (0.73 g, 2.86 mmol) in ethanol-ethyl acetate mixture (20.0 mL), Pd-C (0.26 g, 32.7 mmol) was added and the mixture was hydrogenated for 5 h at atmospheric pressure. Charcoal was filtered off. The solution was reduced under reduced pressure. The crude mixture was purified by column chromatography on silica gel using ‘dichloromethane-methanol’ as eluent (0-15% methanol in dichloromethane) which afforded the desired product as a pale yellow solid (0.29 g, 60%): Rf = 0.46 (in dichloromethane-methanol 9:1, v/v); mp 150-155 °C; IR ( neat, cm⁻¹) 2236 (alkyne C=C stretch), 1630, 1433; 1H NMR (300 MHz, methanol-d₄) δ 8.21 (d, J = 1.11 Hz, 1H), 7.98 (dd, J = 8.46 Hz, J = 2.00 Hz, 2H), 7.91 (dd, J = 8.46 Hz, J = 1.75 Hz, 1H), 7.65 (d, J = 8.46 Hz, 1H), 7.50 (d, J = 18.68 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 (q, 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); 13C NMR (75 MHz, methanol-d₄) 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) calculated for C₁₉H₁₉N₅O₄ [M⁺] with 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 Supercosil 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 - H₂O containing 0.1% trifluoroacetic acid and B - 95:5 CH₃CN-H₂O

**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 $dA_0$ and $dT_0$ in equimolar ratio in buffer 10 mM sodium cacodylate, 0.1 mM EDTA and 100 mM NaCl at pH 7.0 at 95 °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 °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 using the extinction coefficient provided by the supplier.

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 35391, Pseudomonas aeruginosa ATCC 27853, and Enterococcus faecalis ATCC 29212. All compounds were tested by the broth dilution method following Clinical and Laboratory Standards Institute guidelines. 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 g 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 5×10^8 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, 200 ng supercoiled plasmid pBAD-GFPuv, 6 nM of E. coli topoisomerase I, and one of the compounds at a specified 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.

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, 200 ng 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.

Inhibition assays against human DNA topoisomerase II

The inhibition assays were used to determine the activities of the newly synthesized compounds 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 DNA 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.

Cytotoxicity experiments

DU 145 cell line was cultured according to ATCC protocols. Cells were harvested using trypsin-EDTA solution and counted.
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 × 10^3 cells per well. Seeded 96 well plates were returned to incubator (37 °C, 5% CO₂) 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 and 0.625 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% CO₂) 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. 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 solubilized by adding 150 µl of 10 mM unbuffered Tris base to each well. The absorbance of each samples was recorded using a Tecan plate reader and the IC₅₀ was determined using Origin 4.0 software. Each study was completed in duplicate.

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Notes

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1Electronic 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/

References

17. B. Willis; D. P. Arya Biochemistry 2006, 45, 10217-10232.
Highly selective inhibition of *E. coli* topoisomerase I by alkynyl bisbenzimidazoles