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2″-Substituted 5-Phenylterbenzimidazoles as Topoisomerase I Poisons

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Abstract—5-Phenylterbenzimidazole (**1**) is active as a topoisomerase I poison (topo I) and is cytotoxic to human tumor cells. No cross-resistance was observed for **1** when it was evaluated against the camptothecin-resistant cell line, CPT-K5. Derivatives of **1** substituted at the 2″-position, however, did exhibit cross-resistance to this cell line. The basis for the resistance of this cell line towards CPT is that it possesses a mutant form of topo I. These results suggest that substituents at the 2″-position may be in proximity to the wild-type enzyme. Therefore, we hypothesized that terbenzimidazoles with 2″-substituents could be capable of interacting with the enzyme and thereby influence activity within this class of topo I poisons. 5-Phenylterbenzimidazoles with a hydroxy, hydroxymethyl, mercapto, amino, *N*-benzoylaminomethyl, chloro, and trifluoromethyl group at the 2″-position were synthesized. In addition, several 2″-ethyl-5-phenylterbenzimidazoles were prepared containing either a methoxy, hydroxy, amino, or *N*-acetylamino group at the 2-position of the ethyl side-chain. These 2″-substituted 5-phenylterbenzimidazoles were evaluated as topo I poisons and for cytotoxic activity. The presence of a strong electron-withdrawing group at the 2″-position, such as a chloro or trifluoromethyl group, did enhance both topo I poisoning activity and cytotoxicity. Studies on the relative DNA binding affinity of **1** to its 2″-amino and 2″-trifluoromethyl derivatives did exhibit a correlation with their relative differences in biological activity.

Introduction

The chemotherapeutic action of a number of anticancer agents has been linked to their ability to inhibit nuclear DNA topoisomerases. DNA topoisomerases are enzymes that are present in the nuclei of cells and catalyze the breaking and rejoining of DNA strands, thus regulating the topological state of DNA.[1–4] Recent studies suggest that topoisomerases are also involved in controlling template supercoiling during RNA transcription and helicase movement.[5–7] Topoisomerases have been classified into type I or II depending on their ability to produce transient protein-bridged single-strand or double-strand DNA breaks. There are a diversity of topoisomerase II (topo II) poisons that have potent activity and are available clinically, e.g., etoposide (VP-16), teniposide (VM-26), mitoxantrone, doxorubicin and daunomycin. Crystal structures of human topoisomerase I (topo I) in covalent and non-covalent complexes with DNA have been identified.[8] These findings have aided in constructing several design principles for camptothecin analogues. All of the topo I poisons approved for clinical use are analogues of camptothecin (CPT).[9] There is substantial evidence that camptothecin binds reversibly to the putative covalent reaction intermediate, the cleavable complex.[10,11] It has been well established that stabilization of the cleavable complex is critically linked to the cytotoxicity and antitumor activity of these CPT analogues.

Hoechst 33258, 2′-(4-hydroxyphenyl)-5-(4-methylpiperazinyl)-2,5′-bi-1*H*-benzimidazole (NSC 32291, pibenzimol), and Hoechst 33342, 2′-(4-ethoxyphenyl)-5-(4-methylpiperazinyl)-2,5′-bi-1*H*-benzimidazole, represent a structurally-unique class of topo I poisons.[12–14] These agents bind to the minor groove of DNA and have been shown to bind with AT+topo I specificity.[15,16] More recently, terbenzimidazoles have been identified as topo

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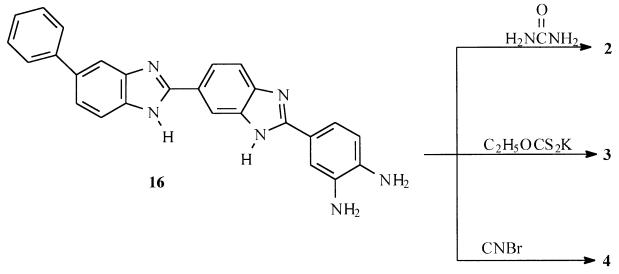
I poisons.[17] In a study with 5-phenyl-2″-substituted terbenzimidazoles, alkyl groups at the 2″-position retained activity as topo I poisons and exhibited good cytotoxicity against RPMI 8402, a human lymphoblastoma cell line.[18] Several terbenzimidazoles were also evaluated in CPT-K5 cells, a camptothecin-resistant variant of RPMI 8402. The basis for resistance of this cell line towards CPT has been postulated to be associated with the fact that it possesses a mutant form of topo I.[19] Studies in our laboratory indicated that several terbenzimidazole derivatives unsubstituted at the 2″-position did not exhibit significant cross-resistance in CPT-K5 cells.[17] However, all the terbenzimidazole derivatives with varied alkyl and aryl groups at the 2″-position evaluated for cytotoxic activity were cross-resistant in CPT-K5 cells (unpublished results). These results raise the possibility that substituents at the 2″-position may be in close enough proximity to interact with specific functional groups on the wild-type enzyme. Based upon these results, we hypothesized that terbenzimidazoles with 2″-substituents capable of interacting with the enzyme would significantly influence activity within this class of topo I poisons. Several 5-phenyl-2″-substituted terbenzimidazoles with varied physicochemical properties were synthesized. The resulting analogues (listed in Table 1) were evaluated for their relative topo I poisoning activities and cytotoxicities.

Chemistry

Synthetic methods for the preparation of **1**, **7**, **10** and **15** have been previously described.[18] The methods used for the preparation of **2**–**4** are outlined in Scheme 1. Condensation of **16** with urea/DMF gave **2** in 88% yield. Treatment of **16** with the potassium salt of ethylxanthic acid in ethanol provided **3** in 69% yield. Cyanogen bromide was reacted with **16** to give **4** in 47% yield.

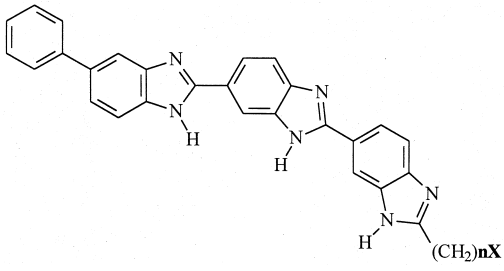
Compounds **5**, **6**, **8**, **9**, **11** and **14** were prepared as shown in Scheme 2. Compound **17** was synthesized from 2-(3,4-dinitrophenyl)-5-phenylbenzimidazole.[18] Reaction of **17** with the corresponding aldehydes **18**–**23** in nitrobenzene at 145 °C gave the desired terbenzimidazoles. Treatment of **11** with BBr_3 provided **12**.[20] The *N*-acetyl moiety on compound **14** was hydrolyzed by treatment with 2 N HCl to give the amine **13** in quantitative yield.

Aldehydes **19**–**23** were prepared by reduction and hydrolysis of their corresponding 5-cyanobenzimidazoles using Ni–Al in formic acid as outlined in Scheme 3. Several of these 5-cyanobenzimidazoles **26**–**30** were prepared using the Phillips procedure.[21,22] Compound **24** was synthesized from the commercially available 4-amino-3-nitrobenzonitrile by hydrogenation-reduction using Pd/C catalyst. Compound **25** was synthesized by condensation of **24** with urea in DMF in 81% yield. 2-Trifluoromethyl-5-cyanoterbenzimidazole (**26**) was prepared by refluxing a solution of **24** and trifluoroacetic acid

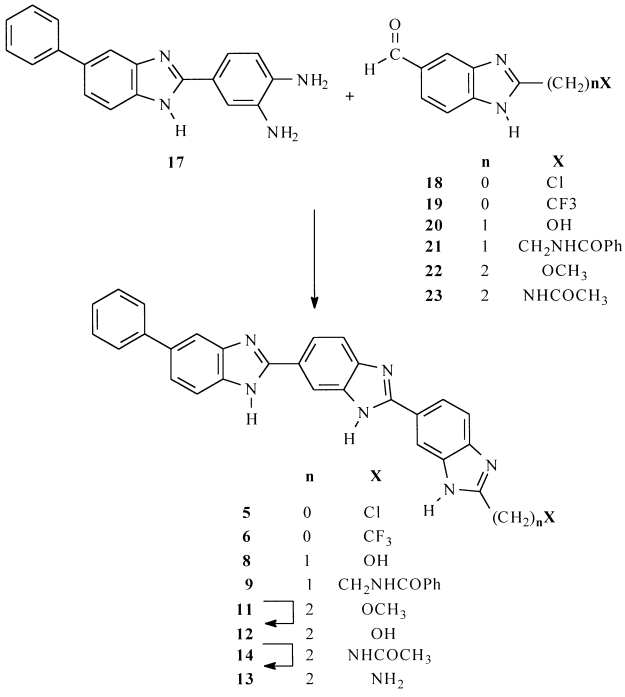


Scheme 1.

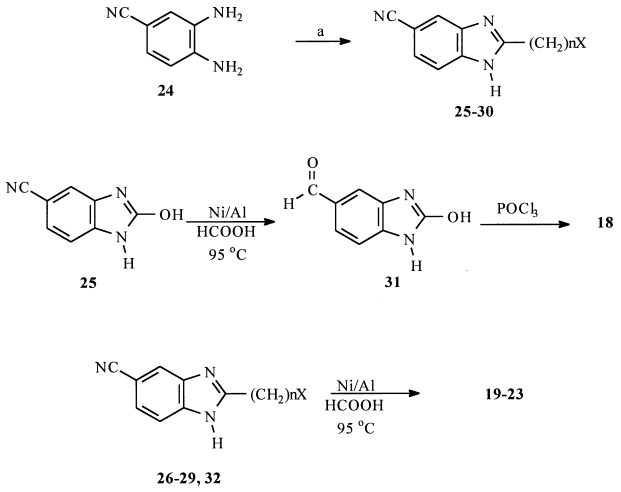
Table 1.



Compound	*n*	X
1	0	H
2	0	OH
3	0	SH
4	0	NH
5	0	Cl
6	0	CF_3
7	1	H
8	1	OH
9	1	$NHCOC_6H_5$
10	2	H
11	2	OCH_3
12	2	OH
13	2	NH_2
14	2	$NHCOCH_3$
15	2	CH_3



Scheme 2.



Scheme 3.

(neat). Reaction of **24** with glycolic acid in 4 N HCl gave 2-hydroxymethyl-5-cyanobenzimidazole (**27**) in 66% yield. The method of Lions and Hughes[23] was followed to prepare the 5-cyano-2-[(*N*-benzoyl)aminomethyl]benzimidazole **28** in 86% yield. Hippuric acid was finely ground along with **24** and heated to obtain a melt. Further heating at 165 °C for 3 h gave the product. Compound **24** was refluxed with 3-methoxypropionic acid in 4 N HCl according to Phillips' procedure to give the 5-cyano-2-(2-methoxyethyl)benzimidazole (**29**).

Reaction of **24** with β-alanine in 6 N HCl gave the 5-cyano-2-(2-aminoethyl)benzimidazole (**30**). The amino group was protected as the acetamide to prevent self-condensation upon reduction of nitrile to the aldehyde. 5-Cyano-2-(2-acetamidoethyl)benzimidazole (**32**) was formed from **30** with acetic anhydride/TEA and used as the precursor to form the 5-formyl analogue. Table 2 gives a summary of the yields of the cyano derivatives. The nitriles **25**–**30** and **32** were reduced in good yields to the 5-formyl derivatives (**18**–**23**) using HCOOH/Ni–Al catalyst. Table 3 gives a summary of the aldehydes obtained from their corresponding cyano derivatives. Aldehydes **19**–**23** were further used for the condensation reaction with **17** as shown in Scheme 2. Compound **31** was converted to **18** using $POCl_3$ in 62% yield, which was then used for the condensation reaction.

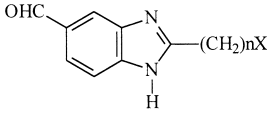
Results and Discussion

Terbenzimidazoles represent a structurally unique class of topo I poisons. Comparative biological and biophysical data have suggested that formation of a ternary enzyme–DNA–terbenzimidazole complex may involve alignment of the terbenzimidazole molecule such that its 2″-end is in close proximity to the enzyme. Analogues of 5-phenylterbenzimidazole with various 2″-substituents that may potentially interact with the enzyme and further stabilize the cleavable form of the ternary complex were synthesized. Comparative assays were performed on these analogues to assess their relative cytotoxicities and potencies as topo I poisons. Cytotoxicity was tested against the human lymphoblast cell line RPMI 8402 and its camptothecin-resistant variant CPT-K5 cells. The relative topo I poisoning activities and cytotoxicities in RPMI 8402 cells of these terbenzimidazole analogues **1**–**15** are summarized in Table 4.

Table 2. Summary of the cyano derivatives and their yields prepared from compound **24**

No.	*n*	X	a	Conditions	Yield (%)
25	0	OH	H_2NCONH_2	150 °C, DMF	81
26	0	CF_3	CF_3COOH	Reflux	88
27	1	OH	$CH_2OHCOOH$	4 N HCl, reflux	66
28	1	NHCOPh	$HOOCCH_2NHCOPh$	160 °C, fuse	86
29	2	OCH_3	$HOOCCH_2CH_2OCH_3$	4 N HCl, reflux	50
30	2	NH_2	$HOOCCH_2CH_2NH_2$	6 N HCl, reflux	46

Table 3. Summary of the 5-formylbenzimidazoles synthesized and the yields obtained from their corresponding cyano derivatives



No.	*n*	X	Yield (%)
18	0	Cl	34[a]
19	0	CF_3	41
20	1	OH	61
21	1	NHCOPh	26
22	2	OCH_3	65
23	2	$NHCOCH_3$	80

[a]This represents the overall yield from **25**.

Topo I poisoning activity

Inspection of the data in Table 4 reveals that analogues **1**–**15** were all active as topo I poisons. Quantitative comparisons of the relative topo I poisoning efficiencies of terbenzimidazoles are frequently confounded by the fact that the induced DNA cleavage pattern can differ from compound to compound. An example of this effect can be seen in the DNA cleavage pattern observed for **8** as compared to **11** in the representative gel shown in Figure 1. Specifically, some cleavage bands induced by **11** are not sufficiently intense in the cleavage pattern induced by **8** to quantify accurately. In addition, strong DNA binding by terbenzimidazoles can inhibit DNA binding by topo I, thereby resulting in diminished enzyme-mediated cleavage. Inhibition of DNA cleavage is frequently observed within this class of topo I poisons at high concentrations of the test compound. This effect can be seen in Figure 1. Specifically, as the concentration of **1** is increased from 1 to 10 μM, a decrease in the intensity of its major DNA cleavage product (denoted with an arrow) is observed. Despite these confounding effects, a dominant DNA fragment band (as indicated with an arrow in Figure 1) typically results from the interaction of most of the terbenzimidazoles studied to date. The relative intensity of this band at concentrations of compound that do not inhibit cleavage was used to provide an assessment of the relative potency of these terbenzimidazoles as topo I poisons.

Table 4. Biological activity of 2″-substituted terbenzimidazoles

Compound	Relative topo I-mediated DNA cleavage[a]	Cytotoxicity RPMI 8402[b] (IC_{50}, μM)
1	1.0	0.27
2	2.0	0.20
3	1.0	0.12
4	5.0	> 10
5	0.1	0.07
6	0.5	0.07
7	2.0	0.11
8	0.5	0.54
9	2.0	9.5
10	2.0	0.11
11	1.0	0.29
12	2.0	2.55
13	1.0	9.26
14	1.0	0.1
15	0.5	0.06

[a]Topoisomerase I cleavage values are reported in relative effective concentration (REC) values, as compared to compound **1**, whose value is assumed to be arbitrarily 1.0, that produce a similar degree of DNA cleavage. Cleavage is calculated from the intensity of the specific band indicated by an arrow in Figure 1. Campothecin is approximately 10 times more potent than **5** based upon comparisons of DNA cleavage at the lower concentrations of drug where detectable levels are initially observed. As the DNA fragmentation pattern and DNA binding affinity are very different between terbenzimidazoles and camptothecin, one should interpret this comparison with caution (see Experimental).

[b]Cytotoxicity values, as their IC_{50} values in μM, are reported after 4 days of continuous exposure. Camptothecin has an IC_{50} of 0.006 μM toward RPMI 8402 and an IC_{50} of 60 μM toward CPT-K5 cells.

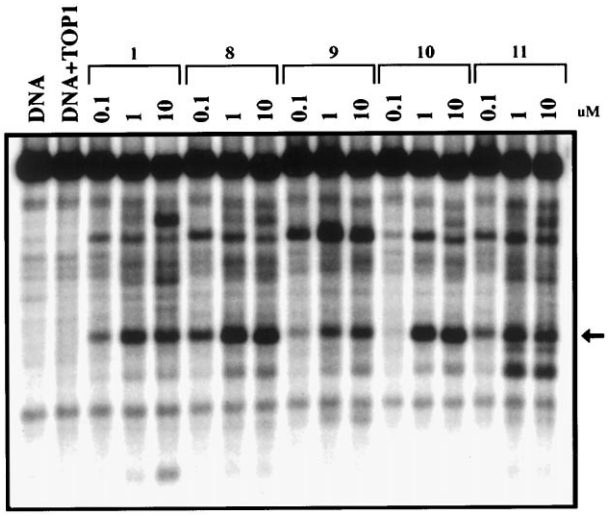


Figure 1. Representative gel illustrating stimulation of enzyme-mediated DNA cleavage by **1** and **8**–**11** using human topoisomerase I. The leftmost lane is the DNA control without topoisomerase I. The second lane from left is the control with topoisomerase I alone. The rest of the lanes are with topoisomerase I and serially (10-fold each) diluted **1**, **8**, **9**, **10** and **11**.

The 2″-hydroxy derivative **2** had approximately half of the potency of **1** as a topo I poison. The 2″-mercapto derivative **3** possessed similar intrinsic activity to that of **1**. The presence of an NH_2 group at the 2″-position of **4** substantially reduced topo I poisoning activity relative to **1**. When the amino moiety was separated by two methylene groups from the benzimidazole ring as in **13** the negative influence of the amino group with respect to topo I poisoning activity was diminished. In the case of the *N*-acetyl analogue of the 2-aminoethyl derivative **14** similar topo I poisoning activity to **1** was observed. The 2″-methoxyethyl analogue **11** and the 2″-(2-hydroxyethyl) derivative **12** of 5-phenylterbenzimidazole had similar activity to **1** as a topo I poison. The reason for the slight increase in activity observed for the 2″-hydroxymethyl analogue **8** is not clear.

The 2″-chloro derivative **5** and the 2″-trifluoromethyl derivative **6** were among the more potent topo I poisons evaluated, having greater potency than either 5-phenylterbenzimidazole **1** or its 2″-methyl derivative **7**. Previous studies by Kim et al.[24] demonstrated that the presence of a methyl, ethyl, or *n*-propyl substituent at the 2″-position of the 5-phenylterbenzimidazole as in **7**, **10** and **15** resulted in relative potencies as a topo I poisons that ranged from one-half to twice that observed for the 2″-unsubstituted analogue **1**.[18] In aggregate, these data suggest that steric factors alone do not significantly alter activity. The presence of functional groups capable of participating in hydrogen bonding interactions did not result in a significant enhancement in topoisomerase I poisoning activity.

Increased potency as topoisomerase I poisons was observed for those terbenzimidazoles that possessed electron withdrawing substituents at the 2″-position, such as a trifluoromethyl group. The strong electron withdrawing effect of the trifluoromethyl group may influence the binding of **6** to either the DNA or the enzyme by altering the hydrogen bonding capacity of the terminal benzimidazole. This altered binding affinity, in turn, may contribute to its enhanced topo I poisoning efficiency.

DNA binding studies

Spectroscopic and calorimetric techniques were used to characterize the relative DNA binding strengths of compounds **1**, **4** and **6** to assess the impact of differing 2″-substituents on terbenzimidazole–DNA recognition. The poly(dA)·poly(dT) duplex was chosen as the DNA target in these studies, since our previous studies[15,16] have revealed that terbenzimidazoles exhibit high affinities for A·T-rich tracts in duplex DNA.

Figure 2 shows the UV melting curves for the poly (dA)·poly(dT) duplex in the absence and presence of compounds **1**, **4** and **6** at the concentrations required to saturate the host DNA. Note that the presence of each of the three terbenzimidazole analogues enhances the thermal stability of poly(dA)·poly(dT) duplex. These analogue-induced changes in duplex thermal stability are consistent with all three ligands binding to the host duplex, with a preference for the duplex versus the single-stranded state.[25,26] Further inspection of Figure 2 reveals that the extent of binding-induced enhancement in duplex thermal stability and/or the concentration of ligand required to saturate the host duplex differ from analogue to analogue. Specifically, the same concentrations of compounds **1** and **6** are required to saturate the

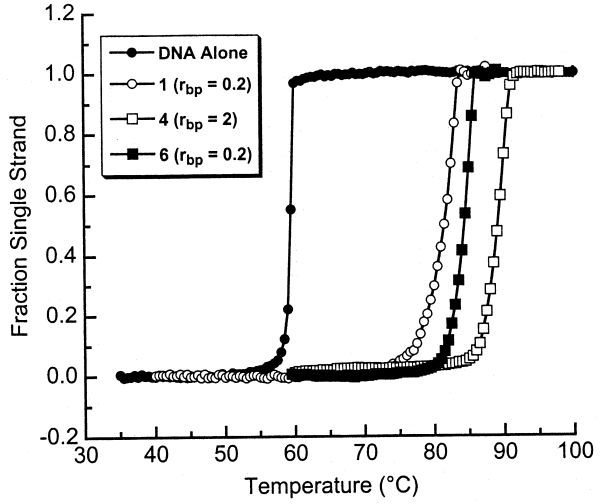


Figure 2. UV melting profiles for the poly(dA)·poly(dT) duplex (filled circles) and its complexes with compounds **1** (open circles), **4** (open squares) and **6** (filled squares) at the indicated (total ligand to base pair) (r_{bp}) ratios. The DNA concentration was 10 μM in base pair and the solution conditions were 10 mM sodium cacodylate (pH 7.0), 25 mM NaCl and 0.1 mM disodium EDTA. For clarity of presentation, the melting profiles, which were acquired at 260 nm, were normalized by subtraction of the upper and lower baselines to yield plots of fraction single strand versus temperature.[29,30]

host poly(dA)·poly(dT) duplex. However, compound **6** binding enhances the thermal stability (T_m) of poly(dA)·poly(dT) by 24.5 °C, while compound **1** binding results in a duplex thermal enhancement of only 21.8 °C. Ten times more of compound **4** is required to saturate the host poly(dA)·poly(dT) duplex than either compound **1** or **6**. However, the binding of compound **4** enhances the thermal stability of the host duplex to a greater extent ($\Delta T_m = 29.4$ °C) than either compound **1** or **6**. Thus, as measured by differences in ΔT_m, the poly(dA)·poly(dT) duplex is able to distinguish between compounds **1**, **4** and **6**, which differ only with respect to their 2″-substituents.

We used the ΔT_m method described below to assess the relative affinities of compounds **1**, **4** and **6** for the host poly(dA)·poly(dT) duplex. Measured ligand-induced changes in the thermal stability of the poly(dA)·poly(dT) duplex (see Fig. 2) were used in conjunction with the 6 base pair binding site size (n) revealed by the X-ray crystallographic studies[27] to estimate apparent ligand–DNA association constants at T_m (K_{T_m}) from the expression:[25]

$$\frac{1}{T_m^\circ} - \frac{1}{T_m} = \frac{R}{n(\Delta H_{WC})} \ln(1 + K_{T_m} L) \quad (1)$$

In this equation, T_m° and T_m are the melting temperatures of the ligand-free and ligand-saturated duplexes, respectively; ΔH_{WC} is the enthalpy change for the melting of a Watson–Crick base pair in the absence of bound ligand (a value we determined independently for the poly(dA)·poly(dT) duplex using differential scanning calorimetry (DSC));[28] and L is the free ligand concentration at T_m. We estimated the value of L using the following relationship:

$$L = L_{tot} - \frac{[bp]}{2n} \quad (2)$$

where L_{tot} is the total ligand concentration and [*bp*] is the total concentration of DNA base pairs.

Table 5 summarizes the K_{T_m} values that we have calculated using eqs (1) and (2). Inspection of these data reveals that the apparent binding affinities of compounds **1**, **4** and **6** for the poly(dA)·poly(dT) host duplex follow the hierarchy **6** > **1** > **4**. Note the agreement between this hierarchy and that noted above for the topo I poisoning activities of the three compounds (see Table 4). Thus, the differential topo I poisoning activities of the terbenzimidazole analogues studied here may be due, at least in part, to their differential DNA binding properties. Contrary to our initial hypothesis, the greater impact of 2″-substituents in regard to stabilization of the cleavable complex may not be mediated by an enhancement enzyme interaction, but rather by further increasing the binding interaction with DNA.

Cytotoxicity

Several of the 5-phenylterbenzimidazole derivatives that were among the more potent topo I poisons did exhibit greater cytotoxicity toward RPMI 8402 cells. The more potent topo I poisons, **5**, **6** and **15**, were among the most cytotoxic derivatives. Compounds **4** and **9** were significantly less potent than **1** as a topo I poison and each was also substantially less cytotoxic against RPMI 8402 cells. These associations between the subcellular assay and the cytoxicity assay are interesting, but differences between compounds in regard to cellular absorption or metabolism can significantly reduce the probability of there being an absolute correlation between these observed pharmacological activities. The decreased cytotoxicity of **12** and **13** relative to **1** against RPMI 8402 cells, for example, would not have been anticipated on the basis of their intrinsic topo I poisoning activity. In CPT-K5 cells, the campthothecin-resistant variant of RPMI 8402 cells, no significant cross-resistance was observed for compounds **1**–**3**. The IC_{50} values of **1**–**3** in this cell line were 1.7, 0.6 and 0.7 μM, respectively. We did, however, observe significant cross-resistance for compounds **4**–**15** in CPT-K5 cells. In each instance the IC_{50} observed in this cell line was at least 6 μM and frequently > 10 μM. These data support the hypothesis that the primary site of action associated with the cytotoxicity of these ter-

Table 5. ΔT_m-Derived binding affinities of compounds **1**, **4** and **6** for the poly(dA)·poly(dT) duplex

Compound	T_m° (°C)[a]	T_m (°C)	$K_{T_m} \times 10^8$ (M^{-1})
1	59.7	81.5	2.8
4	59.7	89.1	1.1
6	59.7	84.2	5.8

[a] T_m values were derived from the UV melting profiles shown in Figure 2, as described previously.[29,30] Solution conditions were 10 mM sodium cacodylate (pH 7.0), 25 mM NaCl and 0.1 mM disodium EDTA. Binding constants were determined using eqs 1 and 2, as well as a calorimetrically determined duplex-to-single strand transition enthalpy (ΔH_{WC}) of 10.4 kcal/mol base pair.[28]

benzimidazoles is topo I. In a comparison of the cytotoxicity of these compounds in another part of camptothecin-sensitive and camptothecin-resistant cell lines, U937 and U937/CR, however, no similar cross-resistance was observed in the cytotoxicity assays performed with **1**–**15**. In the case of CPT-K5 and U937/CR, there are different mutant forms of topo I.[19,31] The basis for this difference in response could be linked to the specific topo I mutants that exist in each cell type. Despite the fact that the target enzyme is the same, cross-resistance of one mutant form of the enzyme to one series of compounds (camptothecin-like) may not necessarily be observed with a distinctly different series of compounds (terbenzimidazoles). In addition, one cannot preclude that other mechanisms, such as drug uptake, could be linked to resistance.

It is our view that in the case of CPT-K5 cells the specific mutant form of topo I imparts cross-resistance to terbenzimidazoles. Compound **1**, which lacks any 2″-substitution, does not exhibit cross-resistance to the CPT-K5 cells. It is evident that the presence of a carbon, chlorine, nitrogen, or sulfur atom at the 2″-position results in a very precipitous loss in cytotoxicity specifically against CPT-K5 cells. Cytotoxicity studies revealed that, with the exception of compounds **2** and **3**, 2″-substituted terbenzimidazoles exhibit a strong cross-resistance in this camptothecin-resistant variant cell line. It is presumed that a single point mutation of aspartate to glycine (Asp533Gly) in CPT-K5 cells makes the terbenzimidazoles resistant to this cell line.[19]

These 2″-substituted terbenzimidazoles may be unable to interact with the mutant form of the enzyme in an appropriate fashion to stabilize the cleaved form of the ternary complex. Such may not be the case with the mutant form of topo I in U97/CR cells.

These studies confirm that substituents at the 2″-end can influence both topo I poisoning activity and cytotoxicity of terbenzimidazoles. These data also suggest that the presence of electronegative substituents at the 2″-position is associated with enhanced topo I poisoning activity and cytotoxicity. There was no evidence to specifically suggest that this enhanced activity was related to an increased interaction with enzyme. The increase in potency for such substituted terbenzimidazoles may be based, in part, on enhanced binding to DNA that could stabilize the cleavable ternary complex comprised of enzyme, drug, and DNA. While cross-resistance is observed for 2″-substituted terbenzimidazoles with CPT-K5 cells, this is not observed for all camptothecin-resistant tumor cells.

Experimental

Melting points were determined with a Thomas–Hoover Unimelt capillary melting point apparatus. Column chromatography refers to flash chromatography conducted on SiliTech 32–63 μm (ICN Biomedicals, Eschwegge, Ger.) using the solvent systems indicated. Radial chromatography refers to the use of a Model 8924 chromatotron (Harrison Research, CA). Infrared spectral data (IR) were obtained on a Perkin–Elmer 1600 Fourier transform spectrophotometer and are reported in cm^{-1}. Proton (^1H NMR) and carbon (^{13}C NMR) nuclear magnetic resonance were recorded on a Varian Gemini-200 Fourier transform spectrometer. NMR spectra (200 MHz ^1H and 50 MHz ^{13}C) were recorded in the deuterated solvent indicated with chemical shifts reported in δ units downfield from tetramethylsilane (TMS). Coupling constants are reported in hertz (Hz). A few drops of CF_3COOH improved ^{13}C NMR spectra by allowing for increased solubility and formation of the protonated form of the terbenzimidazoles, thereby eliminating tautomeric differences among carbon atoms. Mass spectra were obtained from Washington University Resource for Biomedical and Bio-organic Mass Spectrometry within the Department of Chemistry at Washington University, St. Louis, MO. The purity of all compounds for which HRMS data are provided was determined by analytical reverse-phase HPLC. Compounds were analyzed using both of the following conditions: (method A) a Vydac C-18 column (The Separations Group) using methanol:H_2O (87:13) with a flow rate of 1 mL/min; (method B) a Microsorb C-8 column (Rainin Instrument Co., Inc.) using methanol:0.1 M potassium phosphate buffer (pH 7.0) (70:30) with a flow rate of 1 mL/min. HPLC analyses were performed with a Hewlett–Packard 1090 liquid chromatograph equipped with a diode array UV detection monitoring at 254 and 335 nm. The % purity of these compounds was calculated from the peak area assuming that the extinction coefficient of the compound of interest and the impurity is the same. On the basis of these analyses, all the compounds were found to be 98.0–99% pure in these systems. Combustion analyses were performed by Atlantic Microlabs, Inc., Norcross, GA, and were within ±0.4% of the theoretical value. The syntheses of **1**, **7** and **15** have been detailed in the literature.[18]

5-Phenyl-2-[2′(2″-hydroxybenzimidazol-5″-yl)benzimidazol-5′-yl]benzimidazole (2). 5-Phenyl-2-[2′-(3,4-diaminophenyl)benzimidazol-5′-yl]benzimidazole (29.7 mg, 0.07 mmol) and urea (6 mg, 0.1 mmol) were dissolved in DMF (0.4 mL). The mixture was refluxed at 150 °C for 7 h. The cooled reaction mixture when concentrated in vacuo provided 27.6 mg (88%) of pure yellow solid: mp >280 °C; IR (KBr) 3382, 3133, 1693, 1475, 1444, 1279; ^1H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.3 (d, 1H, J = 8.14 Hz), 7.46–7.80 (m, 3H), 7.80 (d, 2H, J = 8.1 Hz), 7.89–8.01 (m, 4H), 8.05–8.14 (m, 2H), 8.32 (d, 1H, J = 8.3 Hz), 8.63 (s, 1H), 11.35 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 108.1, 109.6, 111.8, 114.0, 114.6, 114.6, 115.3, 120.2, 122.5, 125.5, 125.8, 127.4, 128.1, 129.3, 130.8, 131.6, 132.7, 132.9, 135.2, 135.3, 139.2, 139.6, 149.3, 153.1, 155.7; HRMS (FAB) calcd for $C_{27}H_{19}N_6O$ (MH^+) 443.1620, found 443.1625.

5-Phenyl-2-[2′-(2″-mercaptobenzimidazol-5″-yl)benzimidazol-5′-yl] benzimidazole (3). 5-Phenyl-2-[2′-(3,4-diaminophenyl)benzimidazol-5′-yl]benzimidazole (59 mg, 0.14 mmol) and ethylxanthic acid potassium salt (25 mg,

0.16 mmol) were refluxed in ethanol (1 mL) and distilled water (0.1 ml) overnight. The cooled reaction mixture was acidified to pH 3 with glacial acetic acid, volume condensed in vacuo and purified directly by column chromatography. Elution with (40–100%) ethyl acetate/*n*-hexanes provided 69% of yellow solid; mp >260 °C; IR (KBr) 3089, 2926, 2851, 1712, 1624, 1549, 1449, 1380, 1274, 1186, 1079; ^{1}H NMR NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.33–7.42 (m, 2H), 7.48–7.63 (t, 4H), 7.71–7.82 (m, 4H), 7.89 (s, 1H), 8.04–8.06 (m, 1H), 8.1–8.24 (m, 1H), 8.42(d, 1H, J = 2.4 Hz); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 107.4, 107.2, 109.2, 109.7, 111.8, 111.7, 113.3, 113.5, 114.6, 114.6, 114.7, 115.6, 125.3, 127.4, 128.2, 129.3, 131.6, 132.9, 133.3, 139.1, 136.6, 139.6, 149.5, 152.9, 171.1; HRMS (FAB) calcd for $C_{27}H_{19}N_6S$ (MH^+) 459.1392, found 459.1403.

5-Phenyl-2-[2′-(2″-aminobenzimidazol-5″-yl)benzimidazol-5′-yl] benzimidazole (4). 5-Phenyl-2-[2′-(3,4-diaminophenyl)benzimidazol-5′-yl]benzimidazole (66 mg, 0.16 mmol) was dissolved in DMF (0.2 ml) and methanol (1 mL), and was added to cyanogen bromide (10% solution in water, 0.33 ml, 0.63 mmol). The reaction mixture was stirred overnight at room temperature. The mixture was concentrated under reduced pressure. The compound was recrystallized from methanol to give 47% of pale brown solid: mp >260 °C; IR (KBr) 3352, 3052, 2926, 1680, 1624, 1574, 1461, 1261,679; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.46–7.62 (m, 3H), 7.79 (d, 2H, J = 7.3 Hz), 7.87–7.96 (m, 2H), 7.99–8.01 (m, 2H), 8.20–8.26 (m, 2H), 8.31 (s, 1H), 8.72 (d, 2H, J = 9 Hz); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 107.4, 110.9, 111.8, 111.8, 114.7, 115.1, 115.2, 115.9, 123.6, 125.6, 126.8, 127.5, 127.53, 127.57, 127.8, 128.2, 129.4, 130.9, 131.9, 133.1, 138.7, 139.7, 150.1, 152.1, 153.4; HRMS (FAB) calcd for $C_{27}H_{20}N_7$ (MH^+) 442.1780, found 442.1776.

5-Phenyl-2-[2′(2″-chlorobenzimidazol-5″-yl)benzimidazol-5′-yl]benzimidazole (5). 2-(3,4-Diaminophenyl)-5-phenylbenzimidazole (185 mg, 0.62 mmol) was heated with the 5-formyl-2-chlorobenzimidazole (110 mg, 0.62 mmol) in nitrobenzene (5 mL) at 145 °C overnight. Nitrobenzene was removed using a Kugelrohr and the compound purified by flash column chromatography. (1–5%) Methanol/ethyl acetate provided 45% pure yellow compound: mp >260 °C; IR (KBr) 3165, 2943, 1687, 1584, 1438, 1316, 1150; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.43–7.59 (m, 4H), 7.76–7.95 (m, 4H), 8.06 (s, 1H), 8.12–8.19 (m, 2H), 8.29 (m, 1H), 8.52 (s, 1H), 8.64 (m, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 106.7, 111.8, 112.5, 114.7, 115.8, 115.9, 118.2, 118.7, 119.6, 122.8, 123.9, 124.8, 125.7, 127.5, 129.4, 131.8, 133.1, 134.7, 137.2, 138.9, 139.5, 139.7, 141.5, 142.3, 149.8, 153.6; HRMS (FAB) calcd for $C_{27}H_{18}N_6Cl$ (MH^+) 461.1281, found 461.1278.

5-Phenyl-2-[2′(2″-trifluoromethylbenzimdazol-5″-yl)benzimidazol-5′-yl]benzimidazole (6). 5-Phenyl-2-[2′-(3,4-diaminophenyl)benzimidazol-5′-yl]benzimidazole (0.16 g, 0.32 mmol) was stirred with the 5-formyl-2-trifluoromethylbenzimidazole (0.12 g, 0.54 mmol) in nitrobenzene (4 mL) at 145 °C overnight. Nitrobenzene was removed using a Kugelrohr and the compound purified by flash column chromatography. (1–16%) Methanol/ethyl acetate provided 40% pure yellow compound: mp >280 °C; IR (KBr) 3047, 2927, 1698, 1626, 1543, 1440, 1287, 1158; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.44–7.59 (m, 3H), 7.79 (d, 2H, J = 7 Hz), 7.87–8.01 (m, 2H), 8.05–8.09 (m, 2H), 8.15–8.19 (m, 1H), 8.29–8.35 (m, 2H), 8.70 (m, 2H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 111.8, 114.7, 114.9, 115.9, 117.5, 117.6, 118.4, 118.41, 119.6, 120.5, 124.1, 124.8, 125.7, 125.74, 127.5, 128.2, 129.4, 131.7, 133.0, 134.9, 137.4, 139.0, 139.0, 139.1, 139.7, 140.3, 149.8, 153.5; HRMS (FAB) calcd for $C_{28}H_{18}N_6F_3$ (MH^+) 495.1545, found 495.1543.

5-Phenyl-2-[2′-(2″-hydroxymethylbenzimidazol-5″-yl)]-benzimidazol-5′-yl]benzimidazole (8). 5-Phenyl-2-[2′-(3,4-diaminophenyl)benzimidazol-5′-yl]benzimidazole (178 mg, 0.6 mmol) and 5-formyl-2-hydroxymethyl benzimidazole (104.2 mg, 0.6 mmol) were condensed in nitrobenzene (10 mL) overnight at 145 °C. Nitrobenzene was removed from the reaction mixture with a Kugelrohr and the compound was purified directly by column chromatography. Elution with (1–18%) methanol/ethyl acetate provided 62% of yellow solid : mp >260 °C; IR (Nujol) 3406, 2922, 2725, 1631, 1553, 1461, 1377; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 5.10 (s, 2H), 7.45–7.59 (m, 3H), 7.79 (d, 2H, J = 7 Hz), 8.23–8.28 (m, 1H), 8.47 (d, 1H, J = 8.6 Hz), 8.68 (s, 2H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 55.8, 111.7, 113.7, 114.6, 115.3, 115.8, 116.2, 118.3, 123.8, 124.5, 125.2, 125.6, 127.5, 127.5, 127.5, 128.1, 129.4, 131.6, 131.9, 132.9, 133.5, 137.7, 138.9, 139.7, 140.1, 150.3, 153.4, 157.6; HRMS (FAB) calcd for $C_{28}H_{21}N_6O$ (MH^+) 457.1777, found 457.1774.

5-Phenyl-2-[2′-[2″-[2-(*N*-benzoyl)aminomethyl]benzimidazol-5″-yl]benzimidazol-5′-yl]benzimidazole (9). 5-Phenyl-2-[3,4-diaminophenyl]benzimidazole (75 mg, 0.25 mmol) and 5-formyl-2-[(*N*-benzoyl)aminomethyl]-benzimidazole (70 mg, 0.25 mmol) were stirred together in nitrobenzene (6 mL) at 14 °C overnight. Nitrobenzene was removed with a Kugelrohr and the compound was loaded on a column. (2–20%) Methanol/ethyl acetate gave 45% of a yellowish compound: mp >260 °C; IR (KBr) 3204, 1637, 1542, 1442, 1384, 1292, 1026, 818; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.46–7.66 (m, 6H), 7.80 (d, 2H, J = 7 Hz), 7.87–8.1 (m, 7H), 8.21–8.25 (m, 1H), 8.43–8.48 (m, 1H), 8.67 (s, 2H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 36.7, 107.4, 111.7, 114.5, 114.6, 115.3, 115.5, 116.0, 119.5, 122.5, 124.8, 125.6, 126.7, 127.4, 127.8, 128.0, 128.1, 128.6, 129.3, 131.6, 131.9, 132.1, 132.8, 133.2, 134.1, 136.2, 137.8, 139.1, 139.7, 149.7, 152.6, 155.6, 167.6; HRMS (FAB) calcd for $C_{35}H_{26}N_7O$ (MH^+) 560.2199, found 560.2209.

5-Phenyl-2-[2′-[2″-(2-methoxyethyl)benzimidazol-5″-yl]-benzimidazol-5′-yl]benzimidazole (11). 5-Phenyl-2-[3,4-diaminophenyl]benzimidazole (170 mg, 0.35 mmol) and 5-formyl-2-(2-methoxyethyl)benzimidazole (120 mg, 0.25 mmol) were heated together in nitrobenzene (5 mL) for 15 h at 145 °C. Nitrobenzene was removed with a Kugelrohr and the compound was loaded onto a column. (1–12%) Methanol/ethyl acetate gave 80% pure

pale yellow product; mp >280 °C; IR (KBr) 3157, 2933, 1629, 1551, 1441, 1385, 1288, 1108, 818, 760, 698; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 3.33 (s, 3H), 3.45 (t, 2H), 3.89 (t, 2H), 7.45–7.59 (m, 3H), 7.79 (d, 2H, J = 7 Hz), 7.86–7.99 (m, 2H), 8.06–8.14 (m, 3H), 8.26–8.31 (dd, 1H, J = 1.3, 8.4 Hz), 8.44–8.49 (dd, 1H, J = 1,8.8 Hz), 8.72 (s, 2H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) 27.7, 58.3, 68.2, 111.7, 113.7, 114.6, 115.2, 115.7, 116.2, 118.5, 123.9, 124.2, 125.3, 125.6, 127.5, 128.1, 129.4, 131.7, 131.7, 132.9, 133.5, 137.2, 138.7, 139.7, 150.2, 153.2, 155.3; HRMS (FAB) calcd for $C_{30}H_{25}N_6O$ (MH^+) 485.2089, found 485.2089.

5-Phenyl-2-[2′-[2″-(2-hydroxyethyl)benzimidazol-5″-yl]-benzimidazol-5′-yl]benzimidazole (12). 5-Phenyl-2-[2′-[2″-(2-methoxyethyl)benzimidazol-5″-yl]benzimidazol-5′-yl]benzimidazole (30 mg, 0.06 mmol) was suspended in freshly distilled ethyl acetate (30 mL) to which was added 10 equivalents of BBr_3 (0.62 mL, 1.0 M) at −78 °C. After stirring at room temperature overnight, the reaction was quenched by adding water (10 mL). The mixture was basified to pH 9.0 with ammonium hydroxide and extracted with ethyl acetate and dried (anhyd. Na_2SO_4) and concentrated in vacuo. The crude mixture was separated on a chromatotron, eluting with (0–30%) methanol:ethyl acetate to give 10.6 mg (0.02 mmol) of the product in 33% yield as a brown solid: mp 260 °C; IR (KBr) 3401, 3150, 2876, 1632, 1540, 1442, 1388, 1268, 1013, 823; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 3.35 (t, 2H), 3.98 (t, 2H), 7.42–7.56 (m, 3H), 7.76 (d, 2H, J = 7.7 Hz), 7.85–7.98 (m, 2H), 8.06–8.19 (m, 3H), 8.33 (d, 1H, J = 8.5 Hz), 8.44 (d, 1H, J = 8.8 Hz), 8.73 (s, 2H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 30.8, 58.1, 111.7, 114.4, 114.5, 115.3, 116.0, 116.02, 118.3,119.4, 122.4, 124.7, 124.8, 125.4, 125.7, 127.4, 128.1, 129.3, 131.6, 131.6, 132.8, 134.0, 135.3, 137.8, 139.1, 139.6, 149.7, 152.6, 156.2; HRMS (FAB) calcd for $C_{29}H_{23}N_6O$ (MH^+) 471.1933, found 471.1935.

5-Phenyl-2-[2′-[2″-(2-aminoethyl)benzimidazol-5″-yl]benzimidazol-5′-yl] benzimidazole (13). 5-Phenyl-2-[2′-(2″-(2-acetamidoethyl)benzimidazol-5″-yl)]benzimidazol-5′]-benzimidazole (35 mg, 0.067 mmol) was hydrolyzed with 2 N HCl (5 mL) at 110 °C for 3 h. The cooled reaction mixture was basified with ammonium hydroxide to pH 9 and purified by column chromatography. Elution with 50:50 methanol:ethyl acetate provided 75% yellow solid: mp >260 °C; IR (Nujol) 3375, 2719, 1553, 1461, 1377; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 3.45–3.52 (m, 4H), 7.45–7.56 (m, 2H), 7.79 (d, 2H, J = 7 Hz), 7.88–8.00 (m, 2H), 8.07–8.27 (m, 5H), 8.42 (d, 1H, J = 8.9 Hz), 8.68 (d, 2H, J = 5 Hz); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 25.5, 36.4, 111.7, 113.8, 114.6, 115.3, 115.8, 116.2, 118.3, 123.8, 123.9, 124.8, 125.6, 127.5, 128.2, 129.4, 131.7, 133.0, 133.1, 134.8, 137.7, 138.8, 139.7, 140.1, 150.3, 153.4, 153.6; HRMS (FAB) calcd for $C_{29}H_{23}N_7$ (MH^+) 470.2093, found 470.2089.

5-Phenyl-2-[2′-(2″-(2-acetamidoethyl)benzimidazol-5″-yl)]benzimidazol-5′]benzimidazole (14). 5-Phenyl-2-[2′-(3,4-diaminophenyl)benzimidazol-5″-yl]benzimidazole (124 mg, 0.41 mmol) and 5-formyl-2-(2-acetamidoethyl)-benzimidazole (95 mg, 0.41 mmol) were condensed in nitrobenzene (7 mL) overnight at 145 °C. Nitrobenzene was removed with the aid of a Kugelrohr and the compound purified directly by flash column chromatography. Elution with (1–15%) methanol/ethyl acetate provided 69% of yellow solid: mp >260 °C; IR (KBr) 3064, 2954, 2862, 1654, 1556, 1442, 1286; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 1.81 (s, 3H), 3.18–3.35 (m, 2H), 3.61–3.64 (m, 2H), 7.45–7.6 (m, 3H), 7.79 (d, 2H, J = 7 Hz), 7.87–8.00 (m, 2H), 8.07–8.30 (m, 4H), 8.46 (d, 1H, J = 8.5 Hz), 8.71 (s, 2H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 22.6, 27.9, 36.4, 111.7, 113.7, 114.6, 115.2, 115.7, 116.2, 118.6, 124.0, 125.2, 125.7, 127.5, 127.6, 128.1, 128.2, 129.4, 131.6, 131.8, 132.9, 133.7, 137.1, 138.9, 139.5, 139.7, 150.2, 153.24, 155.4, 158.5, 170.3; HRMS (FAB) calcd for $C_{31}H_{25}N_7O$ (MH^+) 512.2199, found 512.2201.

5-Phenyl-2-[2′-(3,4-diaminophenyl)benzimidazol-5′-yl]-benzimidazole (16). A solution of 5-phenyl-2-[2′-(3,4-dinitrophenyl)benzimidazol-5′-yl]benzimidazole (75 mg, 0.16 mmol) in ethyl acetate (50 mL) was reduced by hydrogenation over 10% Pd/C (15 mg) for 90 min. The resulting solution was passed through a bed of Celite and the ethyl acetate was removed to give the diamine, which was used further without purification.

5-Phenyl-2-[2′-(3,4-dinitrophenyl)benzimidazol-5′-yl]benzimidazole. 2-(3,4-Diaminophenyl)-5-phenyl-benzimidazole (0.48 g, 1.59 mmol) and 3,4-dinitrobenzaldehyde (0.28 g, 1.45 mmol) were stirred together in 12 mL nitrobenzene and the mixture was heated overnight at 145 °C. The cooled reaction mixture was then purified directly by column chromatography and elution with 10–95% ethyl acetate/hexanes gave 0.44 g (0.92 mmol, 63%) of a yellow solid: mp >260 °C; IR (KBr) 3045, 2893, 1615, 1548, 1432, 1363, 1276; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.37–7.41 (m, 1H), 7.51–7.54 (m, 3H), 7.73–7.87 (m, 4H), 7.90–7.96 (m, 1H), 8.18–8.29 (m, 1H), 8.45–8.51 (m, 2H), 8.74 (dd, 1H, J = 1.7, 8.48 Hz), 8.97 (d, 1H, J = 1.5 Hz); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 111.6, 114.6, 117.8, 123.9, 125.6, 127.5, 128.2, 128.5, 129.4, 129.6, 131.4, 132.3, 132.9, 134.6, 134.9, 138.7, 139.7, 142.5, 142.8, 150.5, 150.9, 157.6, 158.3; HRMS (FAB) calcd for $C_{26}H_{17}N_6O_4$ (MH^+) 477.1311, found 477.1307.

2-(3,4-Diaminophenyl)-5-phenylbenzimidazole (17). 2-(3,4-Dinitrophenyl)-5-phenylbenzimidazole[18] (0.2 g, 0.05 mmol) was dissolved in ethyl acetate (50 mL) and reduced by hydrogenation using Pd/C 10% (50 mg) as the catalyst for 1.5 h. The catalyst was removed by filtration through Celite. The ethyl acetate was concentrated in vacuo and the residue was dried to yield 0.13 g of crude solid in 80% yield (0.44 mmol). This crude solid was further used without purification: ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 4.65 (brs, 2H), 5.01 (brs, 2H), 6.62 (d, 1H, J = 8 Hz), 7.41–7.48 (m, 8H), 7.69 (d, 2H, J = 7 Hz).

5-Formyl-2-chlorobenzimidazole (18). A mixture of 200 mg (1.23 mmol) of 5-formyl-2-hydroxybenzimidazole and $POCl_3$ (4 mL) was heated under reflux overnight. Upon cooling to ambient temperature, the solution was added to a mixture of ice and water (20 mL) with vigorous

stirring. The acidic, aqueous solution was basified to pH 10 by the addition of concentrated NH_4OH and extracted with ethyl acetate. The extracts were dried (anhyd Na_2SO_4) and concentrated in vacuo. The residue was purified by column chromatography using (45%) ethyl acetate/hexanes to give 133 mg (0.74 mmol) white colored product in 62% yield: mp 165–168 °C; IR (KBr) 3105, 2813, 1703, 1620, 1439, 1283, 991; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.66–7.70 (d, 1H, J = 8.5 Hz), 7.78–7.83 (dd, 1H, J = 1.5, 8.4 Hz), 8.15 (d, 1H, J = 1.5 Hz), 10.15 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 115.01, 118.2, 118.6, 123.5, 131.7, 139.1, 142.1, 192.8) HRMS (EI) calcd for $C_8H_5N_2OCl$ (m/z) 180.0090, found 180.0084.

5-Formyl-2-trifluoromethylbenzimidazole (19). 5-Cyano-2-trifluoromethylbenzimidazole (196 mg, 0.93 mmol) was refluxed with HCOOH (14 mL), H_2O (5 mL) and Ni–Al (0.9 g) catalyst for 6 h. The hot reaction mixture was filtered through Celite and concentrated in vacuo. The solution was basified with 2 N NaOH. The solution was extracted with ethyl acetate, dried (anhyd Na_2SO_4) and concentrated in vacuo to give the crude aldehyde. Purification was achieved by column chromatography. Elution with (90:10) hexanes:ethyl acetate gave 41% of pure white compound: mp 178–179 °C; IR (KBr) 3210, 2737, 1699, 1552, 1328, 1187, 986; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.87–7.95 (m, 2H), 8.34 (s, 1H), 10.11 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3 COOH) δ 106.6, 112.4, 116.7, 118.1, 121.9, 123.8, 124.0, 133.0, 192.7; HRMS (EI) calcd for $C_9H_5N_2OF_3$ m/z 214.0354, found 214.0348.

5-Formyl-2-hydroxymethylbenzimidazole (20). Ni–Al (1.2 g) was added to a solution of 5-cyano-2-hydroxymethylbenzimidazole (0.17 g, 0.97 mmol) in formic acid (17 mL) and water (1.6 mL). The reaction mixture was heated at 95 °C for 5 h. The hot mixture was filtered through a bed of Celite and the reaction flask and Celite bed rinsed with water and then methanol. The combined solution was concentrated in vacuo to dryness. After addition of water to this residue, a white precipitate was formed. The pH of this suspension was adjusted to 9 by dropwise addition of 2 N NaOH. The product was obtained by extraction with ethyl acetate. The ethyl acetate extract was dried (anhyd Na_2SO_4) and concentrated in vacuo to give 0.11 g (0.63 mmol) of a pure white product (61% yield): mp 189–190 °C; IR (KBr) 3309, 2924, 1674, 1619, 1434, 1291, 1073, 808; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 5.07 (s, 2H), 7.94 (d, 1H, J = 8.7 Hz), 8.04–8.09 (dd, 1H, J = 1.3, 8.5 Hz), 8.32 (s, 1H), 10.4 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 55.8, 115.0, 116.8, 126.0, 131.5, 133.9, 135.1, 192.3; HRMS (EI) calcd for $C_9H_8N_2O_2$ m/z 176.0586, found 176.0586.

5-Formyl-2-[(*N*-benzoyl)aminomethyl]benzimidazole (21). 5-Cyano-2-[(*N*-benzoyl)aminomethyl]benzimidazole (0.33 g, 1.2 mmol) was dissolved in formic acid (18 mL). Water (5 mL) and Ni–Al (1.14 g) were added. The mixture was heated under nitrogen at 95 °C for 6 h and while hot passed through a Celite bed. The bed was washed with methanol and the washings concentrated in vacuo. The pH was adjusted with 2 N NaOH to 9.0 and extracted with ethyl acetate. The extractions were dried (anhyd Na_2SO_4) and concentrated in vacuo. The residue was purified over column chromatography using (50–100%) ethyl acetate/hexanes to give 26% of pure cream colored product: mp 108–109 °C; IR (KBr) 3314, 2933, 1687, 1646, 1539, 1289, 806; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 4.99 (d, 1H, J = 5 Hz), 7.54–7.63 (m, 4H), 7.93–8.07 (m, 3H), 8.34 (s, 1H), 10.15 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) 36.8, 115.1, 117.2, 125.8, 127.9, 128.7, 132.3, 132.3, 133.2, 133.7, 155.8, 167.5, 192.5; HRMS (EI) calcd for $C_{16}H_{13}N_3O_2$ m/z 279.1008, found 279.1003.

5-Formyl-2-(2-methoxyethyl)benzimidazole (22). 5-Cyano-2-(2-methoxyethyl)benzimidazole (0.12 g, 0.61 mmol) was mixed in formic acid (9 mL) and water (1 mL), to which was added Ni–Al (0.6 g). The mixture was heated at 95 °C for 5.5 h. The mixture was passed through a Celite bed while hot and the bed was washed with methanol and the filtrate concentrated. After neutralization with 2 N NaOH and extraction with $CHCl_3$, the extracts were dried (anhyd Na_2SO_4) and concentrated in vacuo. The residue was purified by column chromatography using a gradient of 15–100% ethyl acetate/hexanes to give 80 mg (0.39 mmol) white colored product in 65% yield: mp 84–84 °C; IR (Nujol) 2846, 2728, 1692, 1290, 1109, 816; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 3.31 (s, 3H), 3.44 (t, 2H), 3.86 (t, 2H), 7.79–8.09 (m, 2H), 8.36 (s, 1H), 10.16 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 27.7, 58.3, 68.1, 114.9, 116.9, 125.9, 131.6, 133.9, 135.2, 155.8, 192.4; anal. calcd for $C_{11}H_{12}N_2O_2 \cdot 1/4\ H_2O$: C, 64.69; H, 5.92; N, 13.71; found: C, 64.76; H, 6.02; N, 13.60.

5-Formyl-2-(2-acetamidoethyl)benzimidazole (23). 5-Cyano-2-(2-acetamidoethyl)benzimidazole (94.6 mg, 0.41 mmol) was refluxed in HCOOH (5.9 mL), water (0.5 mL) and Ni–Al (0.4 g) catalyst for 6 h. The mixture was filtered hot through Celite bed and the bed washed with methanol. The washings were concentrated in vacuo. The concentrate was basified with 2 N NaOH to pH 9 and extracted with ethyl acetate. The ethyl acetate layer was dried (anhyd Na_2SO_4) and purified by flash column chromatography using 0–15% methanol/ethyl acetate to give 80% (70.10 mg, 0.3 mmol) of pure white product: mp 220–221 °C; IR (KBr) 3222, 3047, 1656, 1575, 1439, 1289, 1110, 1058, 816; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 1.79 (s, 3H), 3.29 (t, 2H), 3.54–3.64 (q, J = 6.7, 12.6 Hz 2H), 7.97–8.09 (m, 2H), 8.19 (t, 1H), 8.38 (s, 1H), 10.17 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 22.7, 27.9, 36.8, 114.9, 116.9, 125.8, 131.7, 133.8, 135.3, 155.9, 170.1, 192.5; anal. calcd for $C_{12}H_{13}N_3O_2$: C, 62.32; H, 5.67; N, 18.17; found: C, 62.10; H, 5.7; N, 17.92.

5-Cyano-2-hydroxybenzimidazole (25). 3,4-Diaminobenzonitrile (0.55 g, 4.14 mmol) was heated with urea (0.27 g, 4.5 mmol) in DMF (5 mL) for 6 h at 150 °C. The cooled solution was concentrated in vacuo to give a yellow colored solid. The compound was purified by column chromatography. Elution with (70–100%) ethyl acetate/hexanes gave 81% (0.53 g, 3.35 mmol) of pure compound: mp 162–163 °C; IR (KBr) 3235, 2924, 2214, 1645, 1610, 1388, 1332; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ

7.05–7.09 (d, 1H, $J=8$ Hz), 7.31–7.4 (m, 2H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 109.34, 111.61, 112.39, 118.13, 120.03, 126.01, 130.20, 133.94; HRMS (EI) calcd for $C_8H_5N_3O$ m/z 159.0433, found 159.0431.

5-Cyano-2-trifluoromethylbenzimidazole (26). 3,4-Diaminobenzonitrile (0.2 g, 1.5 mmol) was refluxed with trifluoroacetic acid (0.3 mL) for 6 h. The mixture was neutralized with 2 N NaOH and extracted with ethyl acetate. The ethyl acetate layer was dried (anhyd Na_2SO_4) and concentrated in vacuo. Elution with (0–10%) ethyl acetate/hexanes gave 88% of pure white compound: mp 182–183 °C; IR (KBr) 3086, 1560, 1402, 1316, 1204, 979, 824; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.72–7.77 (1H, dd, $J=1.5$, 8.6 Hz), 7.88 (d, 1H, 8.42), 8.35 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 106.5, 106.6, 112.4, 117.3, 118.1, 119.4, 123.5, 123.8, 127.5; anal. calcd for $C_9H_4N_3F_3$: C, 51.20; H, 1.91; N, 19.90; found: C, 51.45; H, 2.03; N, 19.68.

5-Cyano-2-hydroxymethylbenzimidazole (27). 3,4-Diaminobenzonitrile (0.2 g, 1.50 mmol) was heated with glycolic acid (0.18 g, 2.37 mmol) in HCl (1.5 mL of 4 N) for 2 h. After neutralization with 2 M sodium carbonate the product was extracted into ethyl acetate, dried (anhyd Na_2SO_4) and concentrated in vacuo to give a white colored solid. The compound was purified by column chromatography. Elution with (75–100%) ethyl acetate/hexanes gave 66% (133 mg, 0.76 mmol) of pure compound: mp 172–173 °C; IR (Nujol) 3350, 2928, 2219, 1621, 1536, 1302, 1217, 1036, 815; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 4.88 (s, 2H), 7.52–7.57 (dd, 1H, $J=1.5$, 8.4 Hz), 7.69 (d, 1H, $J=8.4$ Hz), 7.95 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 55.8, 108.0, 115.7, 118.6, 119.5, 129.0, 131.5, 134.4; HRMS (EI) calcd for $C_9H_7N_3O$ (m/z)173.0590, found 173.0590.

5-Cyano-2-[(*N*-benzoyl)aminomethyl]benzimidazole (28). 3,4-Diaminobenzonitrile (250 mg, 1.88 mmol) was finely ground in a mortar with (0.34 g, 1.92 mmol) hippuric acid and intimately mixed and then carefully fused for 3 h. The temperature was then raised to 160 °C when water was evolved as bubbles. After cooling the glassy mass was dissolved in ethyl acetate and purified by column chromatography. Ethyl acetate/hexanes (50–100%) yielded (0.29 g, 1.05 mmol) 86% of pale buff colored compound: mp 131–132 °C; IR (Nujol) 2826, 2221, 1635, 1313, 1019, 814; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3 COOH) δ 4.97 (d, 2H, $J=5$ Hz), 7.52–7.61 (m, 3H), 7.91–7.99 (m, 4H), 8.37 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 36.8, 107.7, 115.7, 118.8, 119.7, 127.8, 128.6, 128.6, 128.7, 128.8, 132.2, 132.2, 133.2, 135.0,156.1, 167.5; HRMS (EI) calcd for C_{16} H_{12} N_4 O m/z 276.1011, found 276.1012.

5-Cyano-2-(2-methoxyethyl)benzimidazole (29). 3,4-Diaminobenzonitrile (0.2 g, 1.5 mmol) was refluxed with 2-methoxy propionic acid (0.22 mL, 2.25 mmol) in 4 N HCl (2.5 mL) for 7 h. After neutralization with 2 N NaOH the mixture was extracted with ethyl acetate and the extracts dried (anhyd Na_2SO_4) and concentrated in vacuo. The product was purified on column chromatography using (70–100%) ethyl acetate/hexanes to give 50% yield (0.15 g, 0.76 mmol) of pure white product: mp 122–123 °C; IR (KBr) 2875, 2225, 1624, 1544, 1454, 1288, 1215, 1106, 824; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 3.29 (s, 3H), 3.42 (t, 2H), 3.84 (t, 2H), 7.87–8.00 (m, 2H), 8.40 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 27.7, 58.2, 68.0, 106.8, 115.6, 118.3, 119.4, 129.0, 131.3, 134.3, 156.1; anal. calcd for $C_{11}H_{11}N_3O{\cdot}1/2\ H_2O$: C, 62.84; H, 5.75; N, 19.97; found: C, 62.71; H, 5.70; N, 18.83.

5-Cyano-2-(2-aminoethyl)benzimidazole (30). A solution of 3,4-diaminobenzonitrile (1.0 g, 7.52 mmol) and β-alanine (1.0 g, 11.3 mmol) in HCl (8 mL, 6 N) was refluxed for 24 h. At this time the diamine was barely detectable by TLC. After adjusting the pH to 7.0 with 2 N NaOH, the compound was directly loaded on the column. Elution with (75–100%) ethyl acetate/hexanes and further with 1–25% methanol/ethyl acetate gave 46% (0.65 g, 3.49 mmol) of pure yellow compound: mp 105–106 °C; IR (KBr) 3438, 2855, 2734, 2212, 1625, 1569, 1483, 1457, 1385, 1222, 1153; ^{1}H NMR (CD_3OD) δ 3.09–3.13 (m, 4H), 7.49–7.66 (m, 2H), 7.91 (d, 1H, $J=1.5$ Hz); ^{13}C NMR δ 33.13, 41.0, 106.3, 116.6, 121.0, 121.2, 127.1, 140.4, 142.6, 158.8; HRMS (EI) calcd for $C_{10}H_{10}N_4$ m/z186.0905, found 186.0906.

5-Formyl-2-hydroxybenzimidazole (31). 5-Cyano-2-hydroxybenzimidazole (0.4 g, 2.52 mmol) was dissolved in formic acid (30 mL) and water (8 mL). Ni–Al (2.26 g) catalyst was added to it. The mixture was heated under nitrogen at 95 °C for 10 h and passed through a Celite bed while hot. The bed was washed with methanol and the filtrate concentrated in vacuo. The pH is adjusted with 2 N NaOH to 9.0 and extracted with ethyl acetate. The extractions were dried (anhyd Na_2SO_4), concentrated in vacuo, and the residue purified over column chromatography using (65–90%) ethyl acetate/hexanes to give 55% (224 mg, 1.38 mmol) of pure white product: mp 155–156 °C; IR (KBr) 3184, 1677, 1630, 1477, 1388, 1328, 1282; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.09–7.13 (d, 1H, $J=8.3$ Hz), 7.40 (s, 1H), 7.55–7.60 (d, 1H, $J=8.3$ Hz), 10.15 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 108.2, 108.8, 125.5, 130.1, 130.4, 135.7, 155.7, 192.2; HRMS (EI) calcd for $C_8H_6N_2$ O_2 m/z 162.0429, found 162.0430.

5-Cyano-2-(2-acetamidoethyl)benzimidazole (32). 5-Cyano-2-(2-aminoethyl)benzimidazole (0.4 g, 2.15 mmol) was refluxed in dry THF (10 mL), acetic anhydride (0.4 mL) and triethylamine (0.4 mL) for 4 h. The mixture was neutralized with 2 N NaOH and extracted with ethyl acetate to give the crude acetamide. The acetamide was purified using flash column chromatography. Elution with (0–15%) methanol/ethyl acetate gave 70% of pure white compound: mp 218–219 °C; IR (KBr) 3233, 3053, 2225, 1653, 1573, 1442, 1374, 1303, 1058, 815; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 1.78 (s, 3H), 3.26 (t, 2H), 3.53–3.62 (m, 2H), 7.89–7.95 (dd, 1H, $J=1.4$, 8.5 Hz) 8.00 (d, $J=8.2$ Hz, 1H), 8.45 (d, 1H, $J=1.4$ Hz); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 22.6, 27.8, 36.7, 108.1, 115.5, 118.6, 119.3, 129.0, 131.3, 134.3, 156.2, 170.2; anal. calcd for $C_{12}H_{12}N_4O.1/4H_2O$: C, 61.92; H, 5.41; N, 24.07; found: C, 62.60; H, 5.41; N, 23.82.

Topoisomerase I-mediated DNA cleavage assays

Human topo I was expressed in *E. coli* and isolated as a recombinant protein using a T7 expression system as previously described.[32] DNA topoisomerase I was purified from calf thymus gland as reported previously.[33] Plasmid YepG was purified using the QIAGEN Plasmid MAXI kit. DNA end labeling was performed by digesting YEpG with B*am*H I, followed by end-filling using Klenow polymerase. The topo I-mediated cleavage assay was performed by incubating topo I with labeled DNA in the presence of different concentrations of drugs or solvent. Reaction was carried out at 23 °C for 15 min and subsequently stopped by addition of SDS–proteinase K, followed by 37 °C incubation for 1 h. DNA fragments were separated on an agarose gel followed by autoradiography. Topo I-mediated DNA cleavage values are reported as REC, Relative Effective Concentration, i.e. concentrations relative to **1**, whose value is arbitrarily assumed as 1.0. Compound **1** has approximately 50% of the potency of Hoechst 33342 as a topo I poison. Terbenzimidazoles tend to form distinct DNA cleavage bands in the presence of topo I. Cleavage was calcd on the intensity of the strongest specific band. The DNA cleavage pattern for camptothecin is distinctly different from terbenzimidazoles, making comparisons of relative potency difficult. An estimation of the relative potency of camptothecin was made by comparing the doses of compound **5** and camptothecin that induced fragmentation of approximately 10% of the labeled DNA.

MTT-microtiter plate tetrazolinium cytotoxicity assay (RPMI 8402, CPT-K5, U937, U/CR cells)

The cytotoxicity was determined by the MTT assay.[34–36] The human lymphoblast RPMI 8402 and its camptothecin-resistant variant cell line, CPT-K5, were provided by Dr. Toshiwo Andoh (Aichi Cancer Center Research Institute, Nagoya, Japan). Cells were maintained in suspension at 37 °C and with 5% CO_2 in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (0.1 mg/mL). The cytotoxicity assay was performed using a 96-well microtiter plate with 2000 cells/well, in 200 µl of growth medium. Cells were exposed continuously for 4 days to different drug concentrations, and MTT assays were performed at the end of the fourth day. Each assay was performed with a control that did not contain any drug. All assays were performed at least twice in 4 replicate wells.

DNA binding affinity measurements

Poly(dA)·poly(dT) was purchased from Amersham-Pharmacia Biotech (Piscataway, NJ). The concentration of poly(dA)·poly(dT) was determined spectrophotometrically using an extinction coefficient at 260 nm (ϵ_{260}) of 6000 L/mol base·cm.[37] All UV absorbance experiments were conducted on an AVIV model 14DS spectrophotometer equipped with a thermoelectrically controlled cell holder. A quartz cuvette with a 1 cm pathlength was used in all the absorbance studies. Absorbance versus temperature profiles were acquired at 260 nm in buffer containing 10 mM sodium cacodylate (pH 7.0), 25 mM NaCl, and 0.1 mM disodium EDTA. The temperature was raised in 0.5 °C increments and the samples were allowed to equilibrate for 1 min at each temperature setting prior to acquisition of the absorbance reading (averaged over a 5 s period). For each optically detected transition, the melting temperature (T_m) was determined as described previously.[29,30] The DNA concentration was 10 µM in base pair, while the ligand concentrations ranged from 0 to 20 µM.

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