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Quantitative structure–activity relationships on 5-substituted terbenzimidazoles as topoisomerase I poisons and antitumor agents

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Abstract

Several 5-substituted terbenzimidazoles were synthesized and evaluated as mammalian topoisomerase I poisons and for cytotoxicity against a human lymphoblastoma cell line, RPMI-8402. No correlation was observed between topoisomerase I poisoning activity and the Hansch π value or the σ_{meta} and σ_{para} values associated with each substituent. These data suggest that electronic effects and relative lipophilicity of substituents at the 5-position of these terbenzimidazoles do not have a significant effect upon intrinsic topoisomerase I poisoning activity. There was, however, a good correlation between the relative π values for the various subtituents evaluated and cytotoxic activity. Experimentally determined log P values did not correlate well with either cytotoxicity or π values. Capacity factors (log k′) as determined by high pressure liquid chromatography did correlate well with the π values of varied substituents and cytotoxicity. These data indicate that the relative lipophilic activity of substituents at the 5-position of these terbenzimidazoles can strongly influence relative cytotoxic activity.

Keywords: Antitumor, QSAR, structure–activity, topoisomerase, terbenzimidazoles.

1. Introduction

Topoisomerases are nuclear enzymes that regulate topological and conformational changes in DNA critical to cellular processes such as replication and transcription [1–3]. Mammalian topoisomerase poisons have been recognized as effective cancer chemotherapeutics [4–7]. Several bibenzimidazoles and terbenzimidazoles have recently been identified as topoisomerase I poisons [8–14]. Despite exhibiting potent activity as topoisomerase poisons, specific terbenzimidazoles, such as compounds **1** and **2** (Fig. 1), did not exhibit significant cytotoxicity as reflected in their effect upon the human lymphoblast cell line, RPMI 8402. In contrast, the presence of a 5-phenyl or 5-pyridyl substituent on these terbenzimidazoles, as in **3** and **4**, resulted in analogs that retained activity as topoisomerase I poisons and possessed significant cytotoxicity against several tumor cell lines [12]. The basis for the lack of cytotoxicity observed for **1** and **2** has been attributed to poor penetration into cells [12,14].

Previous studies have established that varied substituents could be accommodated at the 5-position of these terbenzimidazoles with retention of activity as topoisomerase I poisons [12,14]. These data prompted the present quantitative structure-activity relationship (QSAR) study. Pharmacological data in earlier studies did suggest that lipophilicity could influence the cytotoxic activity of terbenzimidazoles. Several

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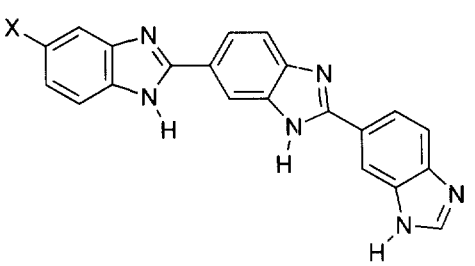
5-substituted terbenzimidazoles with varying Hansch π values [15] were selected for synthesis to evaluate the influence of lipophilicity in enhancing the cytotoxic potential of 5-substituted terbenzimidazoles. It has been hypothesized that electronic effects could alter the ability of these terbenzimidazoles to stabilize the ternary enzyme-DNA complex. To investigate whether specific electronic parameters could modify biological activity, 5-substituted terbenzimidazoles with varying electronic properties were, therefore, selected for synthesis and pharmacological evaluation. The present study examines for several 5-substituted terbenzimidazoles the relationship between these physicochemical parameters and topoisomerase I poisoning activity as well as relative cytotoxicity in human lymphoblast cells.

2. Chemistry

A group representing 14 5-substituted terbenzimidazoles was selected for synthesis and biological evaluation on the basis of their varied electronic effects as indicated by their differing σ values (Table 1). The compounds selected for incorporation in this study also display a cross section of π values representing differing degrees of lipophilicity and are listed in Table 1 in order of highest to lowest π value. Both σ_{para} and σ_{meta} values relative to the NH at position 1 were taken into consideration because of the tautomeric nature of benzimidazoles. As shown in Fig. 2, the functional group R can be viewed as *para* to the -NH of the primary benzimidazole ring in one tautomeric form while *meta* to the -NH in the other. It is important in the selection of compounds for synthesis and biological evaluation that the π and σ values not be highly correlated with each other. Two-dimensional Craig plots of π and σ_{meta} as well as π and σ_{para} verified the divergence of these parameters [16].



1, X = H **3**, X =

2, X = CN **4**, X =

Fig. 1.

Methods for the preparation of **5**, **6**, and **7** have been previously described [12,14]. Scheme 1 outlines the general procedure used for the preparation of compounds **8**, **9**, **10**, **11**, **12**, and **13**. Treatment of 5-methoxyterbenzmidazole (**12**) with BBr_3 in CH_2Cl_2 provided 5-hydroxyterbenzimidazole (**14**). Catalytic hydrogenation of **13** using 10% Pd/C provided 5-aminoterbenzimidazole (**15**).

3. Results and discussion

The Hansch π and σ values as well as the relative topoisomerase I poison activity and cytotoxicity in the RPMI 8402 cells of the terbenzimidazoles evaluated in this study are listed in Table 1. No σ_{para} values were found in the literature for the naphthyl substituents associated with the isomeric terbenzimidazole derivatives, **5** and **6**. In the case of the 5-piperidinyl terbenzimidazole, **9**, no σ_{meta} values were located in the literature for the piperidinyl moiety. While experimental π values for 5-(1-naphthyl) and 5-(2-naphthyl) substituents were unavailable, calculated π values could be obtained for 5 and 6 using Rekker's method [17].

Topoisomerase I poisoning activity is assessed in a subcellular assay that reflects the ability of the drug to stabilize the ternary cleavable complex. The assay uses isolated enzyme and plasmid DNA (see Experimental). There is approximately a 15 min time period wherein the added drug is permitted to interact with a mixture of DNA and enzyme prior to the initiation of gel electrophoresis to measure the extent of DNA fragmentation. In this measurement of the intrinsic potency as a topoisomerase I poison, factors such as drug stability and potential to cross cellular membranes are usually inconsequential. Steric factors, electronic effects, and the presence of localized area of hydrophilicity or lipophilicity would be anticipated as being among the principal physicochemical parameters that could influence topoisomerase I poisoning activity. With the exception of **5**, **6**, and **9** for which either σ_{para} or σ_{meta} values were not available, a multiple regression analysis of the remaining eleven compounds in Table 1 was performed to determine if there was a correlation between topoisomerase I poisoning and either π or σ values. As seen in the matrix provided in Chart 1, topoisomerase I activity did not correlate well with π or σ values. The highest correlation was 0.37 for σ_p. The multiple regression model as shown in Eq. (1) had a low correlation coefficient and an F value that is significantly lower than the tabulated value representing a model of significance $p > 0.05$. These results indicate that there is no definitive relationship between these physiochemical parameters and topoisomerase I activity.

$$\text{Log } 1/\text{topo I} = -0.124 + 0.098\pi + 2.05\sigma_p - 0.912\sigma_m$$

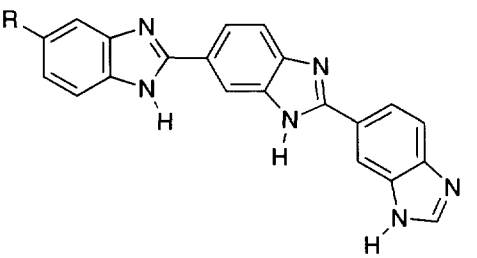
$$n = 11,\ S.E. = 0.573,\ F = 0.61,$$

$$\text{Table } F(3, 7, 0.05) = 4.35$$

$$r = 0.46,\ r^2 \text{ (Variance in Log 1/topo I explained by regression)} = 21\% \quad (1)$$

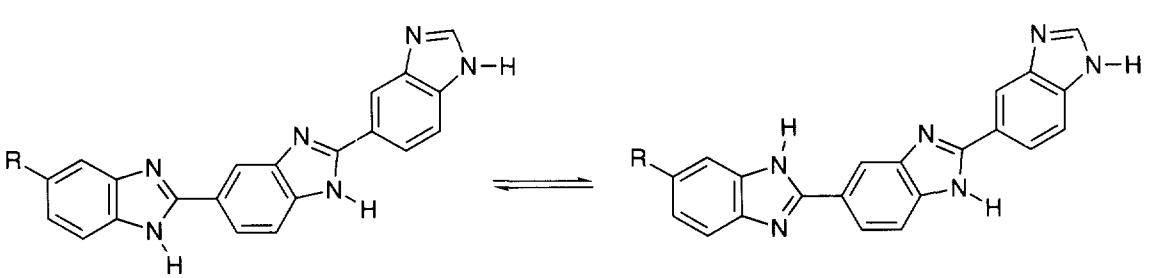
The relationship between physicochemical parameters and cytotoxicity, expressed as IC_{50}, for these eleven compounds was also examined. Cytotoxicity, as shown in the matrix listed in Chart 2, did not correlate well with σ values. In contrast, however, π values did correlate better with the cytotoxicity with a correlation coefficient of 0.70 (Chart 2). The correlation slightly improved by addition of terms based on σ_m and σ_p to the π terms bringing the $r = 0.76$. However, the F ratio

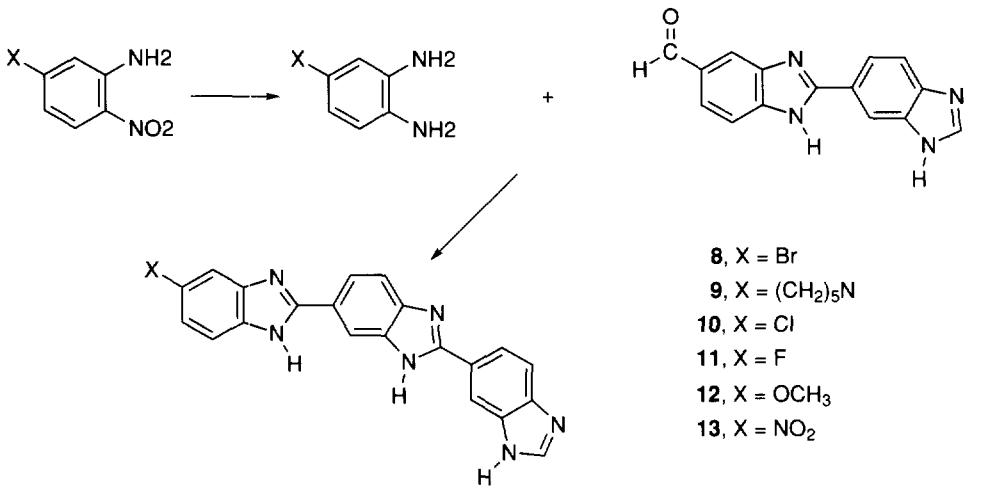
Table 1
Physicochemical parameters and biological results associated with 5-substituted terbenzimidazoles[a]



Code	R	π	σ_p	σ_m	Topo[b]	Cytotoxicity[c]
5	1-Naphthyl	3.19[d]	NA[e]	0.06	10	1.26
6	2-Naphthyl	3.19[d]	NA[e]	0.06	10	0.16
3	Phenyl	1.96	−0.01	0.06	1	0.19
7	Propyl	1.45	−0.17	−0.06	0.5	15.31
8	Br	0.86	0.23	0.39	1	1.63
9	Piperidine	0.85	−0.57	NA[e]	0.5	0.63
10	Cl	0.71	0.23	0.37	1	1.30
11	F	0.14	0.06	0.34	0.05	1.70
2	H	0.00	0.00	0.00	1	5.00
12	OCH_3	−0.02	−0.27	0.12	0.05	0.79
13	NO_2	−0.28	0.78	0.71	0.5	113.9
1	CN	−0.57	0.66	0.56	0.1	133.4
14	OH	−0.67	−0.37	0.12	0.5	150.3
15	NH_2	−1.23	−0.66	−0.16	1	61.64

[a]Hansch π and σ values were used except where otherwise noted [15].
[b]Details provided in the Experimental section. Values provided reflect relative effective concentrations needed to produce a similar degree of DNA fragmentation.
[c]Values represent the IC_{50} values (μM).
[d]Calculated value using Rekker's method [17].
[e]NA indicates that values were not available in the literature.





8, X = Br
9, X = $(CH_2)_5N$
10, X = Cl
11, X = F
12, X = OCH_3
13, X = NO_2

Scheme 1.

was still too small to validate the relationshiop between these parameters and cytotoxicity.

$$\text{Log } 1/IC_{50} = -1.504 + 0.914\pi + 2.265\sigma_p - 1.868\sigma_m$$
$$n = 11,\ S.E. = 0.799,\ F = 3.20,$$
$$\text{Table } F(3, 7, 0.05) = 4.35 \quad (2)$$
$$r = 0.76,\ r^2 \text{ (Variance in Log } 1/IC_{50} \text{ explained by regression)} = 58\%$$

In an effort to improve the correlation between the biological activities and the physicochemical parameters, an attempt was made to remove outliers. The 5-propylterbenzimidazole analog, **7**, which possesses substantial streic flexibility, is an outlier. It is possible that the flexible nature of the propyl group in terms of conformation may represent a detriment to its pharmacological activity. Removal of **7**, did improve the correlation as seen in equations 3, 4, and 5 and in the correlation matrix which showed the $r = 0.84$ for π.

$$\text{Log } 1/IC_{50} = -0.960 + 1.089\pi + 0.267\sigma_p - 0.957\sigma_m$$
$$n = 10,\ S.E. = 0.589,\ F = 8.07,$$
$$\text{Table } F(3, 6, 0.025) = 6.60,\ F(3.6, 0.01) = 9.78 \quad (3)$$
$$r = 0.90,\ r^2 \text{ (Variance in Log } 1/IC_{50} \text{ explained by regression)} = 80\%$$

$$\text{Log } 1/IC_{50} = -0.902 + 1.078\pi - 0.802\sigma_m$$
$$n = 10,\ S.E. = 0.547,\ F = 14.06,$$
$$\text{Table } F(2, 7, 0.01) = 9.55 \quad (4)$$
$$r = 0.90,\ r^2 \text{(Variance in Log } 1/IC_{50} \text{ explained by regression)} = 80\%$$

$$\text{Log } 1/IC_{50} = -0.656 + 1.016\pi - 1.165\sigma_p$$
$$n = 10,\ S.E. = 0.572,\ F = 12.53,$$
$$\text{Table } F(2, 7, 0.01) = 9.55 \quad (5)$$
$$r = 0.88,\ r^2 \text{(Variance in Log } 1/IC_{50} \text{ explained by regression)} = 78\%$$

Two variable multiple regression equations with either the variables π and σ_m or π and σ_p gave better correlations than the three variable equation with all three variables π, σ_m, and σ_p. Equation (3) (which included π, σ_p, σ_m) had a correlation coefficient of 0.90 but F ratio was significant only at the 2.5% level. Equations (4) and (5), on the other hand, had correlation coefficients of 0.90 and 0.88, respectively, as well as F value that was significant at the 1% level.

However, results of the T test on the coefficients of the above equations showed σ_m [Eq. (4)] and σ_p [Eq. (5)]

	Log 1/topo I	π	σ_p	σ_m
Log 1/topo I	1.00			
π	-0.04	1.00		
σ_p	0.37	-0.11	1.00	
σ_m	0.24	0.12	0.90	1.00

Chart 1.

	Log $1/IC_{50}$	π	σ_p	σ_m
Log $1/IC_{50}$	1.00			
π	0.70	1.00		
σ_p	-0.19	-0.11	1.00	
σ_m	-0.12	0.12	0.90	1.00

Chart 2.

to have T values of −1.64 and −1.90, respectively (Table T test = 1.895, $p = 0.1$). These T values were not significant even at the 10% level. The coefficient π, on the other hand, had a T value of 4.817 [Eq. (4)] and 5.240 [Eq. (5)], which were significant at the 1% level. Equation (6), a one variable simple linear equation, best defines the relationship between these physicochemical properites of these terbenzimidazoles and their cytotoxic activity.

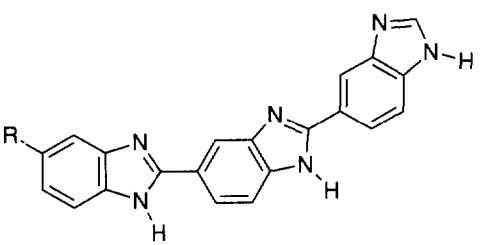
$$\begin{gathered}\text{Log } 1/IC_{50} = -0.949 + 0.996\pi \\ n = 10,\ S.E. = 0.232,\ T = 4.294, \\ \text{Table } T\,(p = 0.01) = 3.335\ F = 18.44, \\ \text{Table } F\,(1, 8, 0.01) = 11.26 \\ r = 0.84,\ r^2\ (\text{Variance in Log } 1/IC_{50} \\ \text{explained by regression}) = 70\%\end{gathered} \tag{6}$$

These data indicate that the critical parameter associated with the cytotoxicity is their π values. We included in these analyses, **5** and **6**, using calculated π values according to Rekker's method [17] and **9**, employing the Hansch π value associated with the 5-piperidinyl group. The addition of **6** and **9** to this QSAR study did not have a significant effect upon correlation observed in Eq. (6). Including these two compounds provided a correlation coefficient of 0.829 and F value of 22.06, which was significant at the 1% level and a T value of 4.697 for π, which was also significant at the 1% level. The addition of **5** did have a negative effect upon the correlation observed in Eq. (6). In the instance where **5**, **6**, and **9** were taken into consideration, a correlation coefficient of 0.761 was obtained.

Electronic parameters (σ_{para} and σ_{meta}) did not contribute to the cytotoxicity and to the topoisomerase I activity of these compounds whereas lipophilicity (π) did significantly correlate with the cytotoxicity of these 5-substituted terbenzimidazoles. Based on these results, we decided to expand this study by further investigating the effects of lipophilicity on the cytotoxicity of the 5-substituted terbenzimidazoles by applying two experimentally determined lipophilic parameters: the partition coefficient, log P_{oct}; and the capacity factor, log k′.

Table 2 summarizes the experimentally determined log k′ and log P values of compounds in this study, with the exception of the propyl analog, **7**, together with their reported or calculated π values. Similar experimental conditions to those previously described for determination of relative hydrophobicity constants by reversed-phase HPLC were employed in this study [18,19].

Table 2
Lipophilic parameters of 5-substituted terbenzimidazoles



Compound	R	π^a	Log k′[b]	Log P[c]
5	1-Naphthyl	3.19[d]	0.509	1.76
6	2-Naphthyl	3.19[d]	0.448	1.61
3	Phenyl	1.96	0.173	1.43
8	Br	0.86	0.011	2.31
9	1-Piperidinyl	0.85	0.088	0.89
10	Cl	0.71	−0.014	1.45
11	F	0.14	−0.259	3.05
2	H	0.00	−0.316	2.42
12	OCH_3	−0.02	−0.253	1.73
13	NO_2	−0.28	−0.159	1.21
1	CN	−0.57	−0.262	2.28
14	OH	−0.67	−0.573	1.17
15	NH_2	−1.23	−0.546	1.46

[a]Hansch π values obtained from literature [15].
[b]Capacity factors determined by HPLC.
[c]Average of triplicates.

As shown in Fig. 3, there is a very good correlation between the π values and the capacity factors determined by HPLC, giving the Eq. (7), where

$$\text{Log k}' = 0.2304 - 0.2328 \text{ with an } r^2 \text{ of } 0.925 \quad (7)$$

Log P values were determined experimentally under standard experimental conditions [20]. The log P values obtained did not correlate with either the π values or their pharmacological activity. One explanation for this may be that the activity of these compounds may be related to the importance of a localized increase in lipophilicity at the 5-position of the molecule. Thus, the π values may tend to correlate better than the overall lipophilicity with cytotoxicity. In addition, there was a great variation in the log P values ranging from 0.89 to 3.05 for these terbenzimidazoles which intuitively was unexpected. In studies performed by Dr D. Pilch (personal communication) it has been observed that terbenzimidazoles such as **3** can self-associate and form several different types of aggregates depending upon concentration. This physical property could significantly contribute to the unreliability of these experimentally determined log P values. There are many factors that could affect this physical phenomenon, including charge and electronic properties of the 5-substituent. Alteration of the p*K*a of the compound could increase or decrease the association constant as well as alter the fluorescence of these compounds.

The lack of convenience and reliability of these log P determinations prompted our investigation into utilizing the reverse phase HPLC for obtaining the lipophilic index of these terbenzimidazoles. The major advantages of this method are that it is not affected by self-aggregation and that small difference in the purity of the compounds do not radically alter the results.

Using log k′ as the lipophilicity parameter, the correlation between log 1/IC50 and log k′ was examined. Equation (8) was obtained where

$$\text{Log } 1/IC_{50} = 2.500 \log k' - 0.358 \quad (8)$$

which had a correlation of $r^2 = 0.56$ (Fig. 4). Removing the 5-(1-naphthyl) analog, **5**, resulted in Eq. (9) which had an improved $r^2 = 0.63$.

$$\text{Log } 1/IC_{50} = 2.971 \log k' - 0.239 \quad (9)$$

This 1-naphthyl derivative, **5**, like the propyl analog, **7**, appears to be an outlier. Its relative cytotoxicity is significantly less than that of its 2-naphthyl isomer, **6**. In contrast to **6**, there is a greater energy barrier associated with the 5-(1-naphthyl) isomer adopting a conformation wherein it resides within the same plane as the benzimidazole ring to which it is attached. This is not surprising in view of the potential of one of the peri-hydrogens on the 1-naphthyl analog (H-8), to sterically interact with H-4 or H-6 on the terbenzimidazole. This suggests that, although lipophilicity of the 5-substituents can play a major role in determining cytotoxicity of these 5-substituted terbenzimidazoles, other factors such as steric effects may also influence activity.

4. Experimental

Melting points were determined with a Thomas–Hoover Unimelt capillary melting point apparatus.

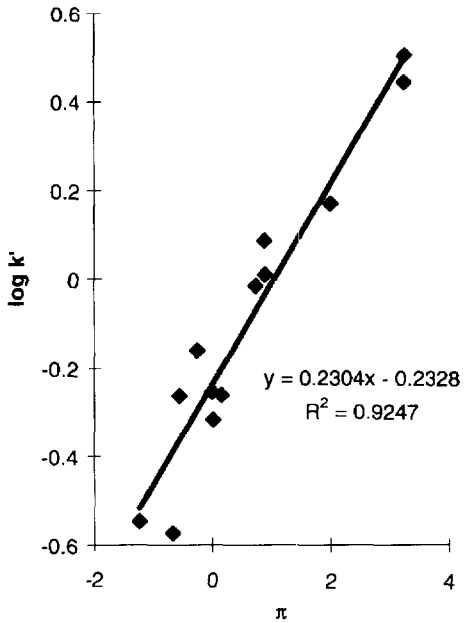


Fig. 3. Plot of π versus log k′.

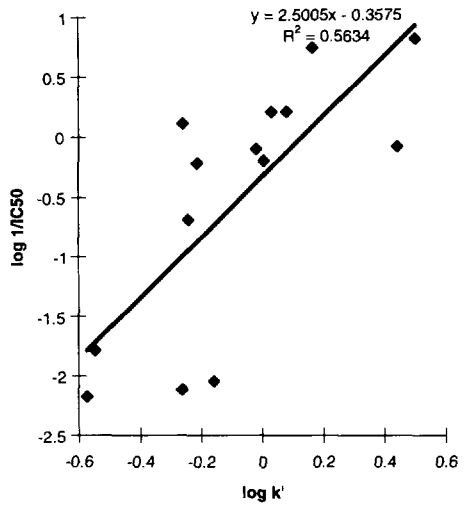


Fig. 4. Plot of log k′ versus log $1/IC_{50}$.

Column chromatography refers to flash chromatography conducted on SiliTech 32–63 μm, (ICN Biomedicals, Eschwegge, Ger.) using the solvent systems indicated. 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 Bioorganic 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) (95:05) 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 335 nm. The % purities of these compounds were calculated from the peak area assuming that the extinction coefficient of the compound of interest and the impurity are 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 $\pm 0.4\%$ of the theoretical value. Methods for the preparation of **1**,[12] **2**,[12] **3**,[12] **4**,[12] **5**,[14] **6**,[14] and **7**[12] have been previously described.

*4.1 General procedures for the preparation of 5-substituted-2[2'-(benzimidazol-5''-yl)benzimidazol-5'-yl]benzimidazole: 5-Bromo-2[2'-(benzimidazol-5''-yl)benzimidazol-5'-yl]benzimidazole (**8**)*

2-Nitro-4-bromoaniline (170 mg, 0.78 mmol) was reduced by refluxing in absolute ethanol (20 ml) with $SnCl_2$ (1.50 g, 7.91 mmol) overnight under N_2. When the reaction was complete, it was basified to pH 11 with 2 N NaOH, and then extracted with ether. After removing the solvent in vacuo, 4-bromo-1,2-phenylenediamine was obtained. Without further purification, the crude diamine (138 mg, 0.73 mmol) and 5-formyl-2-(benzimidazo-5'-yl)benzimidazole (119 mg, 0.45 mmol) were stirred in nitrobenzene (5 ml) at 145°C overnight. The cooled reaction mixture was then purified directly by column chromatography. Elution with 0–10 % methanol/ethyl acetate provided 127 mg (66%) of a brownish-white solid: mp > 280°C; IR (KBr) 3101, 1626, 1547, 1440, 1294, 1140; ^1H NMR δ 7.24 (dd, 1H, $J = 7.0, 1.5$), 7.57 (d, 1H, $J = 9.0$), 7.71–7.80 (m, 3H), 8.04–8.18 (m, 2H), 8.39 (s, 2H), 8.50 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 114.1, 115.8, 116.2, 116.4, 117.0, 118.6, 123.5, 125.3, 126.2, 128.7, 128.9, 131.8, 132.0, 132.3, 133.1, 134.4, 138.3, 140.6, 151.1, 153.4; HRMS (FAB) calcd for $C_{21}H_{14}N_6Br$ (MH^+), 429.0463, found, 429.0454.

*4.1.1 5-(1-Piperidinyl)-2[2'-(benzimidazol-5''-yl) benzimidazol-5'-yl]benzimidazole (**9**)*

2-Nitro-5-(1-piperidinyl)aniline [21] (400 mg, 1.8 mmol) was reduced by hydrogenation with Pd/C (80 mg) in ethyl acetate (50 ml) overnight. After passing through a bed of celite and concentrating in vacuo, 4-(1-piperidinyl)-1,2-phenylenediamine was obtained. Without further purification, the crude diamine (341 mg, 1.8 mmol) and 5-formyl-2-(benzimidazo-5'-yl)benzimidazole (200 mg, 0.76 mmol) in nitrobenzene (10 ml) provided 104 mg (32%) of a brownish white solid: mp > 280°C; IR (KBr) 3118, 2923, 2821, 1626, 1436, 1287, 1123; ^1H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 1.70 (m, 2H), 1.89 (m, 4H), 3.58 (m, 4H), 7.67 (d, 1H, $J = 9.0$), 7.84–7.823 (m, 5H), 8.46 (d, 1H, $J = 9.0$), 8.60 (s, 1H), 8.73 (s, 1H), 9.66 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 21.5, 24.1, 54.9, 107.5, 111.8, 114.1, 115.3, 115.4, 115.9,116.1, 117.9, 120.4, 123.4, 125.2, 132.0, 133.4, 135.4, 139.5, 140.1, 148.1, 151.8, 153.1; HRMS (FAB) calcd for $C_{26}H_{24}N_7$ (MH^+) 434.2093, found 434.2076.

*4.1.2 5-Chloro-2[2'-(benzimidazol-5''-yl)benzimidazol-5'-yl]benzimidazole (**10**)*

2-Nitro-5-chloroaniline (152 mg, 0.88 mmol) was reduced by refluxing in absolute ethanol (20 ml) with $SnCl_2$ (1.68 g, 8.86 mmol) overnight under N_2 to obtain 4-chloro-1,2-phenylenediamine. Without further purification, the crude diamine (125 mg, 0.88 mmol) and 5-formyl-2-(benzimidazo-5'-yl)benzimidazole (160 mg, 0.61 mmol) in nitrobenzene (5 ml) provided 167 (71%) of a brownish-white solid: mp > 280°C; IR (KBr) 3103, 2826, 1427, 1293; ^1H NMR δ 7.24 (dd, 1H, $J = 8.25$, 2.0), 7.60–7.87 (m, 4H), 8.07–8.17 (m, 2H), 8.40 (s, 2H), 8.49 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 114.3, 114.4, 115.3, 115.5, 115.6, 116.1, 118.5, 123.0, 125.4, 125.5, 125.6, 129.4, 132.3, 132.9, 133.0, 135.2, 138.9, 140.8, 151.8, 153.5; HRMS (FAB) calcd for $C_{21}H_{14}N_6Cl$ (MH^+) 385.0968, found 385.0954.

*4.1.3 5-Fluoro-2[2′-(benzimidazol-5″-yl)benzimidazol-5′-yl]benzimidazole (**11**)*

2-Nitro-4-fluoroaniline (99 mg, 0.63 mmol) was reduced by hydrogenation with Pd/C (20 mg) in ethyl acetate (50 ml) for 3 h. After passing through a bed of celite and concentrating in vacuo, 4-fluoro-1,2-phenylenediamine was obtained. Without further purification, the crude diamine (80 mg, 0.63 mmol) and 5-formyl-2-(benzimidazo-5′-yl)benzimidazole (84 mg, 0.32 mmol) in nitrobenzene (5 ml) provided 85 mg (72%) of a brownish-white solid: mp > 280°C; IR (KBr) 3050, 2817, 1625, 1553, 1429, 1131; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.41–7.52 (m, 1H), 7.76 (dd, 1H, J = 8.5, 2.0), 7.88–7.95 (m, 1H), 8.03–8.20 (m, 3H), 8.48 (dd, 1H, J = 9.0, 1.5), 8.61 (s, 1H), 8.73 (s, 1H), 9.71 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 100.7, 101.2, 112.4, 113.9, 114.1, 114.7, 115.7, 115.9, 116.2, 118.2, 120.3, 123.5, 125.4, 129.2, 131.7, 132.9, 133.2, 140.4, 151.3, 153.5, 162.8; HRMS (FAB) calcd for $C_{21}H_{14}N_6F$ (MH+) 369.1264, found 369.1262.

*4.1.4 5-Methoxy-2[2′-(benzimidazol-5″-yl) benzimidazol-5′-yl]benzimidazole (**12**)*

2-Nitro-4-methoxyaniline (168 mg, 1.0 mmol) was reduced by hydrogenation with Pd/C (30 mg) in ethyl acetate (50 ml) for 3 h. After passing through a bed of celite and concentrating in vacuo, 4-methoxy-1,2-phenylenediamine was obtained. Without further purification, the crude diamine (136 mg, 0.99 mmol) and 5-formyl-2-(benzimidazo-5′-yl)benzimidazole (131 mg, 0.5 mmol) in nitrobenzene (5 ml) provided 140 mg (74%) of a brownish-white solid: mp > 280°C; IR (KBr) 3405, 3093, 2940, 1625, 1429, 1269, 1138; ^{1}H NMR δ 3.83 (s, 3H), 6.85 (dd, 1H, J = 8.5, 2.0), 7.12 (s, 1H), 7.51 (d, 1H, J = 8.5), 7.73 (s, 1H), 7.79 (d, 1H, J = 8.5), 8.05 (d, 1H, J = 8.5), 8.15 (d, 1H, J = 7.5), 8.38 (d, 1H, J = 7.5), 8.39 (s, 1H), 8.48 (s, 1H): ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 56.2, 96.5, 113.8, 115.0, 115.7, 115.8, 115.9, 116.4, 117.8, 122.9, 125.2, 125.2, 126.1, 126.3, 131.8, 133.0, 133.1, 139.0, 141.2, 149.4, 153.7, 158.4; HRMS (FAB) calcd for $C_{22}H_{17}N_6O$ (MH$^+$) 381.1464, found 381.1451.

*4.1.5 5-Nitro-2[2′-(benzimidazol-5″-yl)benzimidazol-5′-yl]benzimidazole (**13**)*

4-Nitrophenylenediamine (87 mg, 0.57 mmol) and 5-formyl-2-(benzimidazo-5′-yl)benzimidazole (100 mg, 0.38 mmol) in nitrobenzene (5 ml) provided 70 mg (47%) of a brownish-white solid: mp > 280°C; IR (KBr) 3118, 1626, 1549, 1436, 1330, 1287, 1133; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.89 (d, 1H, J = 9.0), 8.02 (d, 1H, J = 8.5), 8.15 (d, 1H, J = 9.0), 8.23 (dd, 1H, J = 9.0, 2.0), 8.35 (dd, 1H, J = 8.5, 1.5), 8.46 (dd, 1H, J = 9.0, 1.5), 8.57 (d, 1H, J = 2.0), 8.66 (s, 1H), 8.78 (s, 1H), 9.65 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 112.0, 114.1, 114.3, 114.7, 114.9, 115.6, 115.9, 118.7, 123.4, 124.3, 124.6, 124.8, 132.3, 133.4, 134.0, 137.2, 138.7, 142.7, 143.1, 152.3, 155.9; HRMS (FAB) calcd for $C_{21}H_{14}N_7O_2$ (MH$^+$) 396.1209, found 396.1209.

*4.1.6 5-Hydroxy-2[2′-(benzimidazol-5″-yl) benzimidazol-5′-yl]benzimidazole (**14**)*

Compound **12** (88 mg, 0.29 mmol) was dissolved in freshly distilled CH_2Cl_2 (50 ml) and cooled to −78°C. BBr_3 (1.0 M CH_2Cl_2 solution, 0.58 ml) was slowly added. Temperature was brought to room temperature overnight under N_2. The reaction was quenched with H_2O and CH_2Cl_2 removed in vacuo. After extracting the reaction mixture with ethyl acetate, the H_2O layer was neutralized to pH 7.0 and extracted with ethyl acetate. The ethyl acetate fractions were put together and concentrated in vacuo after drying in Na_2SO_4. Separation on a silica-gel column eluting with 10–20% methanol/ethyl acetate gave 68 mg (64%) of brownish solid: mp > 280°C; IR (KBr) 3409, 3104, 2973, 2813, 1702, 1622, 1419, 1244, 1149; ^{1}H NMR δ 6.69 (dd, 1H, J = 8.5, 2.0), 6.94 (s, 1H), 7.40 (d, 1H, J = 8.0), 7.69–7.82 (m, 2H), 8.01 (s, 1H), 8.10 (d, 1H, J = 8.0), 8.19–8.50 (m, 3H), 9.18 (brs, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 98.3, 114.2, 114.8, 115.3, 115.8, 116.2, 116.3, 123.3, 125.3, 125.4, 126.4, 131.8, 133.2, 139.9, 140.3, 145.7, 148.4, 150.4, 153.3, 156.7, 161.6; HRMS (FAB) calcd for $C_{21}H_{15}N_6O$ (MH$^+$) 367.1310, found 367.1309.

*4.1.7 5-Amino-2[2′-(benzimidazol-5″-yl)benzimidazol-5′-yl]benzimidazole (**15**)*

Compound **13** (35 mg, 0.09 mmol) was reduced by hydrogenation with 10% Pd/C (7 mg) in ethyl acetate:methanol (1:1, 50 ml) at 45 psi for 24 h. After passing through a bed of celite, and concentrating in vacuo, the crude mixture was separated on a silica-gel column eluting with 10–20% methanol/ethyl acetate. 10 mg of brownish solid (31%) was obtained: mp > 280°C; IR (KBr) 3421, 3223, 2961, 2918, 1624, 1421, 1373; ^{1}H NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 7.46 (d, 1H, J = 9.0), 7.84 (s, 1H), 7.93 (d, 1H, J = 8.5), 8.06–8.18 (m, 2H), 8.30 (dd, 1H, J = 9.0, 2.0) 8.54 (dd, 1H, J = 9.0, 1.5), 8.75 (s, 1H), 8.84 (s, 1H), 9.72 (s, 1H); ^{13}C NMR (DMSO-d_6 + 3 drops CF_3COOH) δ 110.7, 114.4, 115.4, 115.5, 115.6, 115.9, 116.2, 116.3, 118.9, 120.4, 124.9, 125.5, 131.6, 133.3, 133.4, 134.1, 137.3, 138.7, 140.0, 150.7, 153.1; HRMS (FAB) calcd for $C_{21}H_{16}N_7$ (MH$^+$) 366.1471, found 366.1484.

4.2 Determination of capacity factor, k′

The capacity factor, k′, was experimentally-determined under similar conditions to those previously described [18,19]. A Hewlett-Packard 1090 liquid chromatograph equipped with diode array UV detection was

used for obtaining the capacity factors, k′, for compounds used in the QSAR study and for determining compound homogeneity of compounds for which HRMS data are provided. The samples used in this study were prepared by dissolving about 1 mg of the compound in 100 ml of methanol (HPLC grade). The Pipes buffer was prepared by dissolving 1.52 g of PIPES (purchased from Aldrich) in 1.0 l of 18 mΩ water and adjusting the pH to 7.4 with 1 N KOH using a pH meter. Each sample in methanol was injected (50 μl) on a Vydac C-18 column at a flow rate of 1.0 ml/min employing a mobile phase of 30% Pipes buffer (5 mM, pH 7.4) in methanol. The detector wavelength was 330 nm. The mobile phase hold-up time, t_0 was determined by injecting the mobile phase. Each sample was injected three times and the average of the three retention times (t_R) were used in calculating the capacity factor, k′, from the equation

$$k' = (t_R - t_0)/t_0$$

4.3 Determination of partition coefficient, log P

Partition coefficients were performed using similar methods to those previously detailed [20]. Pipes buffer was prepared by dissolving 1.52 g in 1.0 l of 18 mΩ water and adjusting the pH to 7.4 as described previously. The octanol used in this study was presaturated with the Pipes buffer by stirring overnight at 37°C and then separating the layers in a separatory funnel. Both the saturated octanol and buffer were kept in a 37°C water bath throughout the study. Analysis was done on a Shimadzu RF 5000U spectrofluorophotometer using excitation wavelength of 340 nm and emission wavelength of 380 nm. The bandwidth was adjusted from 5 nm to 10 nm depending on the analysis. Before performing the partition coefficient study, the solubility of the compounds in octanol and water had to be determined so that partitioning is not affected by saturation of the compounds in either layers. However, because sufficient amount of compounds were not available, only compounds **1** and **8** were used in determining the solubility. Compounds **1** and **8** were each saturated in 2 ml of octanol and 2 ml of Pipes buffer separately in different reactivials by continuously adding the compounds until there was excess of solid that did not dissolve. The excess solid was filtered off with a glass fiber and the octanol or buffer solutions were analyzed by fluorescence. The calibration curves for both the octanol and buffer solutions were obtained with known concentrations of the compounds. Several trial and errors were repeated to obtain the calibration curve for the range that fits the solubility of the compounds. Because the fluorescence behavior of these terbenzimidazoles were very different in octanol from that in methanol or buffer, it was important that separate calibration curves were obtained. The initial studies were not reproducible and this was attributed to the compounds sticking onto the surface of the reactivials. After the glassware used in the study was siliconized with Aquasil® (purchased from Pierce), the results were reproducible. Based on the solubility studies of the two compounds, stock solutions of each of the compounds used in the QSAR study were prepared by accurately weighing about 1 mg of the compounds and dissolving in 100 ml of HPLC grade methanol in a volumetric flask. 300 μl of each stock solution was evaporated in reactivials, and 1.5 ml of Pipes buffer (5 mM, pH 7.4), and 0.5 ml of octanol saturated with the buffer were added to the vials and stirred for 24 h at 37°C. The phases were separated and 200 μl of the octanol layer was diluted to 50 ml with methanol while 0.5 ml of buffer layer was diluted to 2 ml with Pipes buffer. Additional dilutions were made as required. Concentrations of both phases were determined by measuring the fluorescence and using the standard curves obtained for each compound in both phases. Standard curves for each compound for both the octanol layer and the buffer layer were obtained by preparing separate standard solutions from the stock solutions. The standard solutions for the octanol layer were prepared by taking 200 μl each of the stock solution, evaporating off the methanol with nitrogen gas, and diluting with 0.2% octanol in methanol to the desired concentrations. The standard solutions for the buffer layer were prepared similarly except that buffer was used for the dilutions. The partitioning study was done in triplicates for all the compounds and log P was determined from the average concentrations according to the equation

$$\log P = \log [c]o/[c]w$$

where [c] = concentration of compounds analyzed, o = octanol phase, w = aqueous phase.

4.4 QSAR calculations

QSAR-PC [22] was used for the carrying out regression analyses. Data files were constructed using DATAWELL where π, σ_p, and σ_m were chosen as the variables. The correlation matrices were obtained by using ALLREGRESS and multiple linear regression QSAR computations were preformed using REGRESS.

4.5 Chromatography

The retention times needed to calculate k′ were determined on a Vydac C18 column at a flow rate of 1.0 ml/min employing a mobile phase of 30% Pipes buffer (5 mM, pH 7.4) in MeOH. The void volume was determined by injecting methanol.

4.6 Topoisomerase I-mediated DNA cleavage assays

Human topoisomerase I was expressed in *E. coli* and purified as previously described [23]. Plasmid YEpG was also purified by the alkali lysis method followed by phenol deproteination and CsCl/ethidium isopycnic centrifugation as described [24,25]. The end-labeling of the plasmid was accomplished as previously described [25]. The cleavage assays were performed as previously reported [8]. The drug and DNA in the presence of topoisomerase I was incubated for 30 min at 37°C. After development of the gels, 24 h exposure was typically used to obtain autoradiographs outlining the extent of DNA fragmentation.

4.7 Cytotoxicity assay

The cytotoxicity as listed for several of the compounds which were prepared as part of this study was determined using Human lymphoblast RPMI 8402 cells and the MTT-microtiter plate tetrazolium cytotoxicity assay (MTA) [26–28]. The cytotoxicity assay was performed using 2000 cells/well, in 200 μl of growth medium, which were grown in suspension at 37°C in 5% CO_2 and maintained by regular passage in RPMI medium supplemented with 10% heat inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (0.1 mg/ml). The cells were exposed continuously for 4 days to different drug concentrations, and were assayed at the end of the fourth day. Each assay was performed with a control which did not contain any drug. All assays were performed at least twice in six replica wells.

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