In Vitro Cytotoxicity of GC Sequence Directed Alkylating Agents Related to Distamycin

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Imidazole containing analogues 7, 10, and 17 of distamycin wherein the C-terminus contain a dimethylamino moiety have been shown to selectively bind to the minor groove of GC-rich sequences. Accordingly, these agents were employed as vectors for the delivery of a variety of alkylating agents to GC-rich sequences. The alkylating agents are attached to the N-terminus of these vectors thus providing the benzoyl N-mustards (8, 15, and 18 that contain one, two, and three imidazole units, respectively) and substituted acetamides 11–14. Results from the ethidium displacement assay for the formamides 7, 10, and 17 and mustards 15 and 18 showed that these agents bind to calf thymus DNA, poly(dA.dT), poly(dG.dC), and also to coliphage T4 DNA, thus confirming their binding in the minor groove. The reduced binding constants of these compounds for poly(dA.dT) while still binding as strongly, or more strongly, to poly(dG.dC) than distamycin provided evidence for their acceptance of GC sequences. Selectivity for GC-rich sequences was also indicated by CD titration studies. Titration of 10, 15, 17, and 18 to poly(dA.dT) produced weak drug-induced CD bands at ~330 nm; however, interaction of these agents to poly(dG.dC) in equimolar drug concentrations gave strong bands in this region. Results from dialysis and cross-link gel experiments provided evidence of alkylation and cross-linking of DNA by the mustards which could explain their enhanced cytotoxicity over the formamido analogues. The bifunctional N-mustard-containing analogues 15 and 18 are significantly more cytotoxic than the monoalkylating acetamides 11–14. The mustards also exhibited significant activity against cell lines derived from solid tumors such as melanomas, ovarian cancers, CNS cancers, and small cell lung cancer.

Introduction

Lack of selectivity is a challenging problem in the use of drugs for the treatment of cancer.[1] Many anticancer and antiviral drugs interfere with nucleic acid metabolism such as the replication and transcription of DNA by binding to double-stranded DNA.[1a,b] Although some of these agents exhibit limited DNA sequence preference, this is not generally believed to be sufficient to account for their selectivity against cancer versus normal cells.[1,2] Consequently, there is an intense effort in the development of sequence-selective DNA binding agents which can be used as vectors for the delivery of DNA interactive agents thereby producing new compounds that might be more effective clinically for the treatment of cancer.[2] There is a particular interest in developing ligands with GC base pair sequence selectivity because most of the known DNA minor groove binding compounds are AT specific. This interest also stems from the observation that regions of high GC content are commonly found in genomes of mammals, including humans, and that a functional role of GC-rich sequences is suggested by their frequent occurrence in genes associated with proliferation, including a number of oncogenes.[3,4]

The DNA sequence (AT) and minor groove binding agent distamycin (1) has received much attention,[5] and recently its derivative FCE-24517 (2) has been shown to exhibit anticancer activity against a broad spectrum of murine cancers and human small cell lung carcinoma and melanoma xenografts implanted in nude mice including those resistant to melphalan (L-PAM).[6] This compound

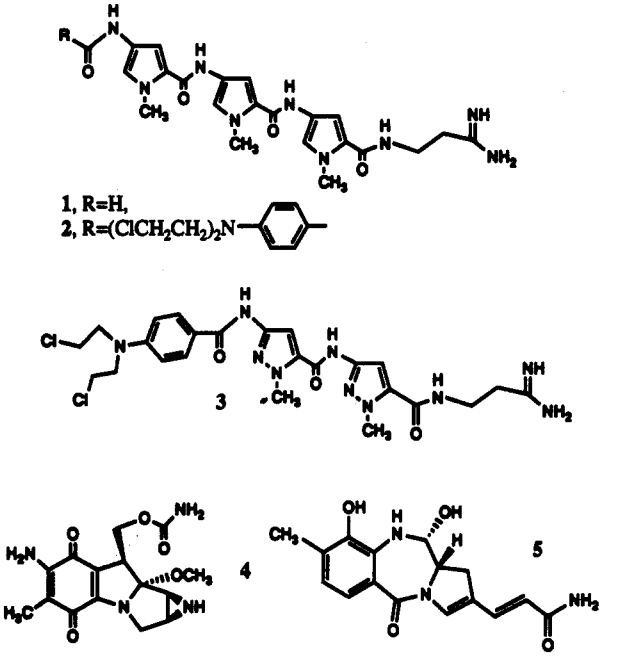

Figure 1.

is believed to exert its biological activity by binding to AT-rich sequences of DNA followed by alkylation of adenine-N3.[6c] Currently FCE-24517 is undergoing phase I clinical trials in Europe.[6b] A pyrazole containing and AT sequence-selective alkylating agent 3 has also been demonstrated to have good anticancer activity; however, this compound is quite chemically unstable.[7]

Directed alkylation in the minor groove of long GC sequences of DNA is virtually unexplored. A number of naturally occurring anticancer agents such as mitomycin

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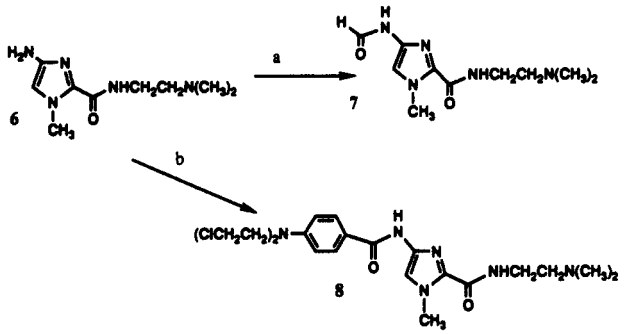
Scheme I[a]



[a] (a) Acetic formic anhydride; (b) *p*-[bis(2-chloroethyl)amino]benzoyl chloride, triethylamine.

C 4[8] and anthramycin 5[9a] exert their biological activity by alkylation of GC sites in the minor groove. Recently, a polymethylene-linked pyrrolo[2,1-*c*][1,4]benzodiazepine (DSB-120) was shown to form DNA interstrand cross-links with G-2-NH_2 groups preferentially at 5′-PuGATCPy or 5′-PyGATCPu sequences.[9b,c]

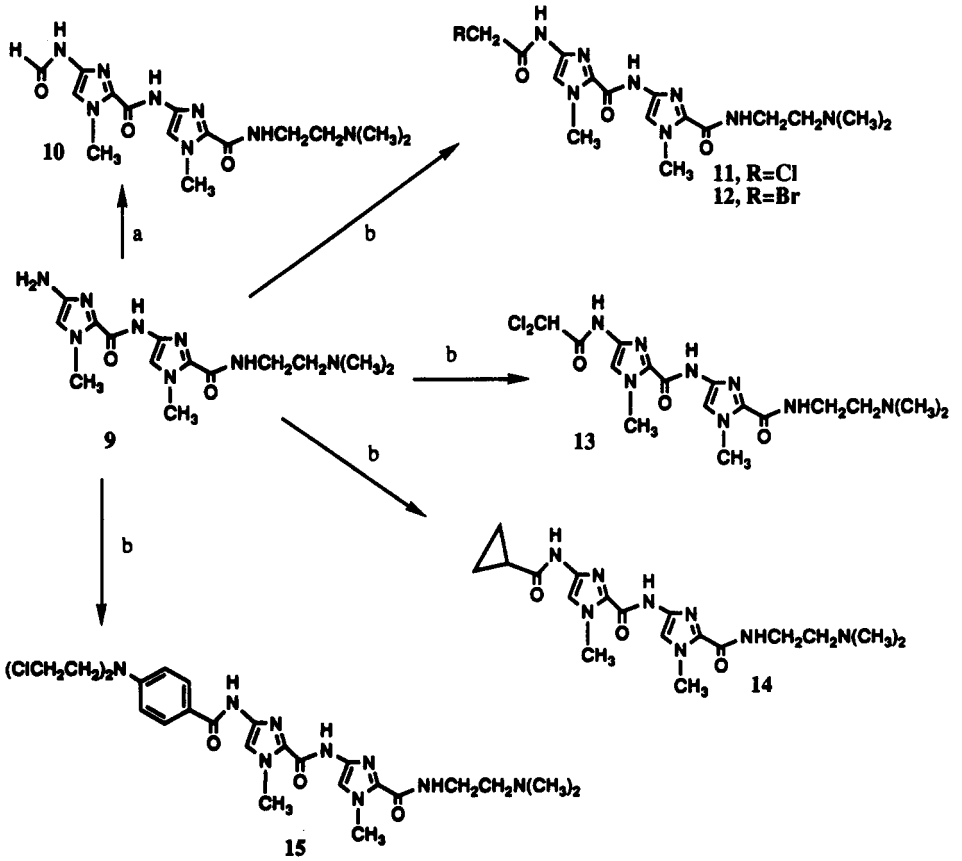
Our approach to the development of GC sequence-selective interactive agents uses the naturally occurring oligopeptides netropsin and distamycin[10] that bind specifically to AT sequences as models. The firm and sequence specific binding is a net result of van der Waals contacts, hydrogen bonding,[11] and electrostatic interactions[12] between the ligand and DNA.[2c] It has been demonstrated that replacement of the DNA contacting CH group of the pyrrole moieties by –N–, such that the heterocycle is capable of providing space and accepting a hydrogen bond from the protruding exocyclic 2-amino group of guanine in the minor groove could alter the strict preference for AT to permit GC recognition. Accordingly, imidazole analogues of netropsin have increased selectivity for GC-rich sequences.[13] Therefore, it appears that imidazole-containing analogues of distamycin are well suited for the development of agents that can target GC-rich sequences of DNA.

In this paper, we report the synthesis, DNA binding properties, and cytotoxicity of a series of imidazole-containing analogues of distamycin that are conjugated to alkylating agents including the benzoyl N-mustard moiety. The synthesis of C-terminus-modified analogues, wherein a dimethylamino moiety is substituted for the parent amidine group, provides a number of advantages. First, compounds containing the modified terminus are chemically stable, and thus the synthetic methodology is readily adaptable to the preparation of further analogues. Second, they are not hygroscopic and are easy to handle. Third, the dimethylamino group is uncharged, and thus products and intermediates can be readily purified by column chromatography or recrystallization. Finally, with a pK_a of ~9.3, this moiety would be protonated at physiological pH of 7.4 to provide favorable electrostatic attraction to the negative electrostatic potential of the DNA. Furthermore, the dimethylamino moiety has been shown to have a similar sequence selectivity to the amidinium group of distamycin.[14]

Results and Discussion

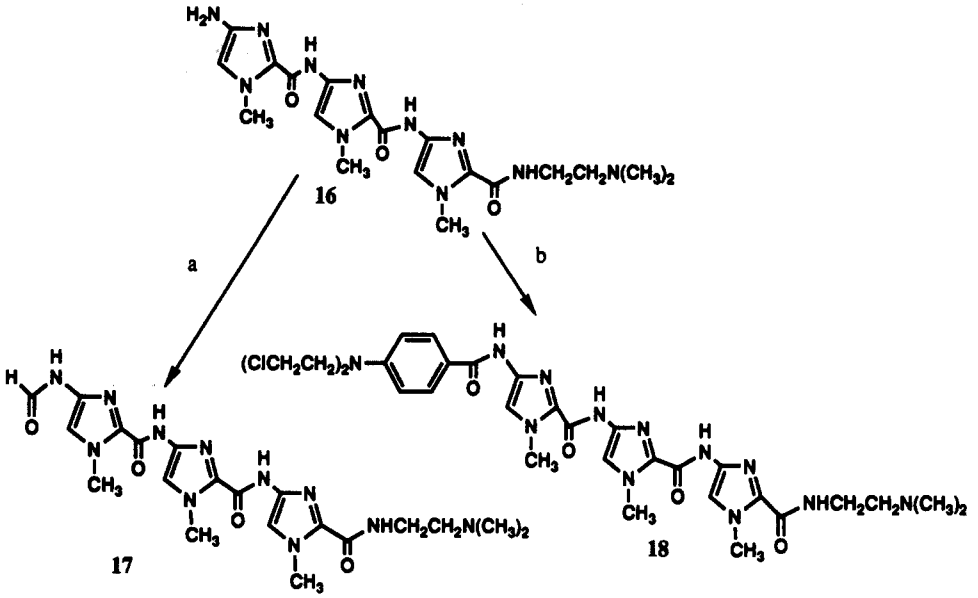
Synthesis. The synthetic approach for the proposed minor groove GC sequence selective alkylating agents that contain one, two, and three imidazole moieties are given in Schemes I, II, and III, respectively. The synthetic strategy used for the previously described formamido

Scheme II[a]



[a] (a) Acetic formic anhydride; (b) appropriate acid chloride, triethylamine.

Scheme III[a]



[a] (a) Acetic formic anhydride; (b) *p*-[bis(2-chloroethyl)amino]benzoyl chloride, triethylamine.

analogues **7**, **10**, and **17**[15] was used for the preparation of the alkylating agents in this study. In the synthesis of the monoimidazole analogues **8** as depicted in Scheme I, reaction of amine **6**[15] with *p*-[bis(2-chloroethyl)amino]-benzoyl chloride[16] gave the desired product in 27% yield.

In the preparation of the diimidazole analogues as shown in Scheme II, amine **9**[15] was coupled with the appropriate α-substituted acetyl chloride in the presence of triethylamine to give compounds **11–14** in 25, 5, 47, and 11%, respectively. The benzoyl mustard **15** was synthesized from the reaction of *p*-[bis(2-chloroethyl)amino]benzoyl chloride[16] with amine **9** in 27% yield.

The three-imidazole-containing mustard **18** was synthesized (see Scheme III) by condensation of amine **16**[15] with *p*-[bis(2-chloroethyl)amino]benzoyl chloride in the presence of triethylamine in 44% yield. The structures of the prepared compounds were confirmed by analyses of their ^{1}H- and ^{13}C-NMR and IR spectra. The ^{1}H-NMR spectra of compounds **8**, **15**, and **18** showed multiplets at 3.68 and 3.82 ppm and two sets of doublets (J = 8.1 Hz) at 6.74 and 7.94 ppm which indicated the presence of the bis(2-chloroethyl)amino moiety. Furthermore, two positive signals at 40.2 ppm (CH_2Cl) and 53.3 ppm (NCH_2) were observed in the ^{13}C-NMR "attached proton test" experiment which confirmed the presence of the bis(2-chloroethyl)amino moiety.[7]

DNA Sequence and Groove Binding Selectivity. The apparent binding constants (K_{app}) of drugs to DNA can be estimated and compared by measuring the loss of ethidium fluorescence as a function of added drug.[17] The drug concentration which produces 50% inhibition of fluorescence is approximately inversely proportional to the apparent binding constant (K_{app}).[18] Under our experimental conditions, these studies provide relative rather than absolute values of K_{app} which reflect primarily noncovalent interactions, and the data are compared to those of distamycin.

The apparent binding constants, K_{app}, of distamycin and compounds **7**, **10**, **15**, **17**, and **18** to calf thymus DNA, T4 coliphage DNA, poly(dA.dT), and poly(dG.dC) are presented in Table I. The values of K_{app} for **7** and **10** are lower than that of distamycin, possibly due to the lower number of amide moieties in **7** and **10** (2 and 3, respectively, versus 4) and van der Waals contacts as compared to distamycin. The values of K_{app} of **10** and **15** for poly(dG.dC) are approximately the same as those for poly(dA.dT), while the K_{app} of poly(dG.dC) for distamycin is about 2 orders of magnitude lower than that for the K_{app} of poly(dA.dT). For the triimidazole analogues **17** and **18**, the K_{app} values are comparable to those of the parent distamycin for calf thymus and T4 coliphage DNA. However, compounds **17** and **18** bind significantly weaker to poly(dA.dT) but slightly stronger to poly(dG.dC) than distamycin, thus indicating that changing the pyrrole moieties in distamycin to imidazole groups increases the acceptance of GC base pairs by these compounds. Furthermore, these data suggest that increasing the number of imidazole groups and hence the number of base pairs that the drugs can recognize increases the DNA binding constant which has also been shown for polypyrrole analogues.[19]

Table I. Association Constants K_{app} ($\pm 0.05 \times 10^5$ M^{-1}) of Compounds and Polynucleotides

compound	calf thymus	T4	poly(dA.dT)	poly(dG.dC)
EtBr	100[18]	100[29]	95[29]	99[29]
distamycin	7.74	6.50	348	2.03
7	0.25			0.02
10	0.32	0.40	0.17	0.23
15	4.56	2.36	4.75	4.55
17	7.74	6.67	5.94	6.13
18	5.91	1.68	9.50	4.95

The apparent binding constants to T4 coliphage DNA give an indication of the groove selectivity of these compounds. The major groove of T4 coliphage DNA is blocked by α-glycosylation of the 5-(hydroxymethyl)-cytidine residues; therefore, the only place available for nonintercalating agents to bind is the minor groove.[20] These results are in good agreement with distamycin and its analogues which has been shown through X-ray[11] and ^{1}H-NMR data[13] to bind in the minor groove. The values of K_{app} (T4 coliphage) for the imidazole analogues **15**, **17**, **18**, and distamycin are close; thus it is reasonable to assume that these compounds are binding in the minor groove of DNA.

Circular Dichroism Studies. Interaction of achiral molecules to optically active DNA can cause changes in the CD spectrum, such as the appearance of ligand-induced band(s) and/or alteration in the original CD spectrum.[21] The primarily noncovalent component of the interaction of the drugs to a number of DNA [calf thymus, poly(dA.dT) and poly(dG.dC)] were analyzed by CD titrations studies using Zimmer's methods.[21,22] The results show that compounds **10**, **15**, **17**, and **18** bind to the DNAs as indicated by the appearance of drug-induced CD bands at about 290–340 nm, because the drugs alone do not exhibit any CD spectra. The induced CD band is presumably due to the UV absorption π to π^* transition of the drug in the drug:DNA complex. Specifically, at equimolar concentrations, ligands **10** and **17** gave stronger Cotton effect bands at 295–340 nm for poly(dG.dC) than for poly(dA.dT) at 320 nm, thus indicating a preference for GC sequences.[22] Independent MPE footprinting studies of **10** and **17** on the Bam H1/Sal 1 fragment pBR322 plasmid DNA, which will be published separately, showed that they bind to specific 5′-(G.C)$_3$(A.T) and 5′-(G.C)$_4$(A.T) sequences, respectively.[15]

The CD titration experiments on the benzoyl N-mustards **15** and **18** were also performed with the same three polynucleotides. Titration of **15** to poly(dG.dC) gave rise to a negative band at 320 nm (0.8 mdeg, $r' = 0.2$) and an isodichroic point at 297 nm. However, equimolar titration of this compound to poly(dA.dT) gave a weaker negative band at 300 nm (0.4 mdeg, $r' = 0.2$). In addition, titration of **15** to poly(dA.dT) also gave a positive band at 350 nm (1.8 mdeg, $r' = 0.2$) and an isodichroic point at 315 nm. Titration of this compound to calf thymus DNA revealed, at low r' values, a weak positive band at 340 nm (0.8 mdeg, $r' = 0.2$), a negative band at 310 nm (0.2 mdeg, $r' = 0.2$), and an isodichroic point at 320. However at higher drug concentrations ($r' > 0.4$), the positive band at 340 nm disappeared, and the negative band at 315 nm intensified (to ~3 mdeg), dramatically indicating a different mode of binding of the drug to the DNA.

Titration of mustard **18** to poly(dA.dT) caused minor changes in the CD spectrum, suggesting that there was minimal interaction of the drug with this DNA even when the concentration of the drug was raised to $r' = 1.0$ (see Figure 2a). However, titration of this drug to poly(dG.dC) produced a negative Cotton effect at 333 nm (0.8 mdeg, $r' = 0.2$) along with a slight decrease in the positive band at 275 nm, and an isodichroic point at 283 nm was observed (see Figure 2B). It should be noted that the ellipticity of the induced-CD band(s) increases with additional increments of drug. Finally, titration of **18** to calf thymus DNA produced a weak negative band at 335 nm (0.5 mdeg, $r' = 0.2$) and a decrease in the positive band at 285 nm, and an isodichroic point at 300 nm was recorded (see Figure 2C). The above data obtained from titration of the drugs into the three DNAs provide evidence that these compounds show significant acceptance, and for **18** a distinct preference for GC-rich sequences as indicated by the larger induced band for poly(dG.dC) than poly(dA.dT) at equal drug concentrations (see Figure 3 for compound **18**). In addition, the appearance of the positive and negative bands at ~270 and ~250 nm in the titration spectra suggest that the conformation of the DNA duplex in the drug: DNA complexes remains in the B-form. Therefore interaction of these drugs with DNA causes only minor conformational changes to the double helix.

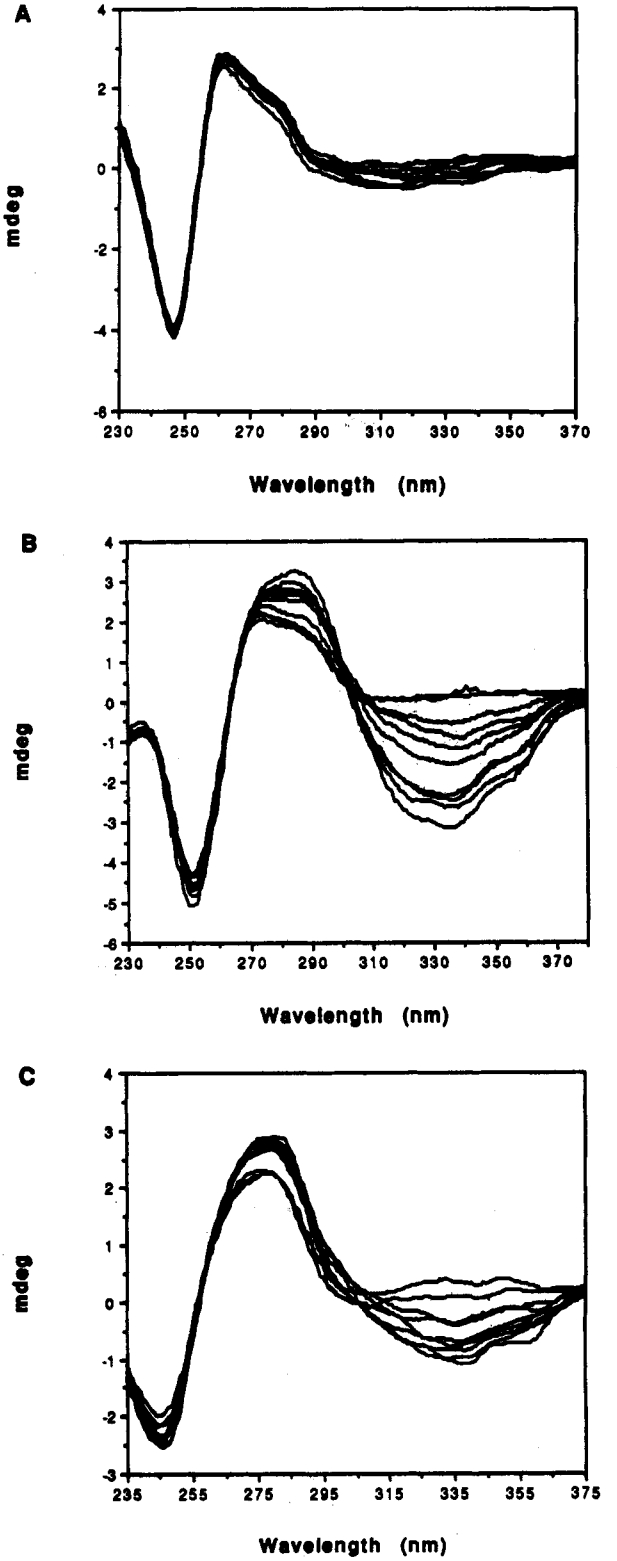


Figure 2. Titration of the triimidazole analogue **18** to (a) poly(dA.dT), (b) poly(dG.dC), and (c) calf thymus DNA. The plots correspond to r' values of 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 for calf thymus and poly(dA.dT). For poly(dG.dC), the r' values are 0, 0.05, 0.1, 0.22, 0.35, 0.5, 0.6, 0.7, 0.9, and 1.0.

DNA Alkylation. All DNA minor groove binding agents that exhibit significant anticancer properties have been shown to interact covalently with DNA, including FCE 24517 **2**, anthramycin **5**, mitomycin C **4**, and others.[23] In order to determine whether binding of these drugs, especially the N-mustards **15** and **18**, to DNA was

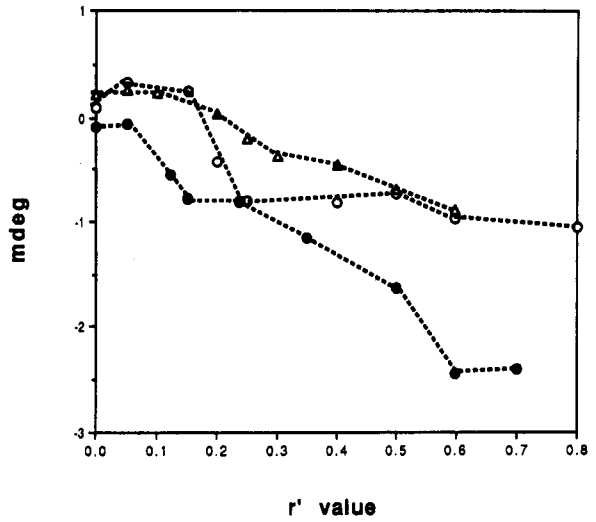


Figure 3. Variation of ellipticity (mdeg) of the ligand-induced bands (300–340 nm) of various DNAs as a function of r' for compound 18–poly(dG.dC) (●), –calf thymus DNA (○), and –poly-(dA.dT) (△).

reversible, preincubated solutions containing calf thymus DNA and drugs at $r' = 0.25$ were dialyzed against buffer. The UV absorption spectrum of the dialysate was recorded periodically to determine the concentration of each compound outside the dialysis bag. After 20 h of dialysis, the data shows that 55 ± 2 and 45 ± 2% of 15 and 18 were still retained in the dialysis bag, while only 35 ± 2% of 17 was left in the bag. Thus under these conditions, compound 15 and 18 appear to bind to DNA more strongly suggesting the formation of an irreversible adduct (alkylation). Preliminary studies designed to measure DNA interstrand cross-linking of compounds 15 and 18 using an agarose gel assay,[24] in which linear plasmid DNA was incubated with the drugs for 2 h at 37 °C, showed evidence of cross-link formation at 100 μM drug concentration. In contrast compound 17 did not form any cross-links even at 1 mM. For comparison conventional nitrogen mustards such as L-PAM produce cross-links at 10 μM under identical conditions.

In Vitro Cytotoxicity Data. The compounds of this study were screened for cytotoxicity activity against a large number of human cancers. Compounds 10, 13, 14, and 17 showed no activity against the growth of K562 (human chronic myeloid leukemia) cells with IC_{50} values of >500 μM. Compounds containing a monohaloacetyl moiety group, 11, 12, showed negligible activity against this cell line with IC_{50} values of 350 and 400 μM, respectively. However, the benzoyl mustards demonstrated significant activity with IC_{50} values of 12.5 and 0.3 μM for 15 and 18, respectively. Under identical conditions, the IC_{50} value for the major groove cross-linking drug L-PAM was 35 μM. It is worthy to note that the cytotoxicity of 18 is generally comparable to that reported for the AT sequence selective agent FCE24517.[6c]

Compounds 8, 15, 17, and 18 were further tested for cytotoxic properties by the National Cancer Institute, Bethesda, MD.[25] The monoimidazole benzoyl mustard 8 and the formamido analogue 17 demonstrated little or no activity against the panel of cancers even at a concentration of 10^{-4} M. In contrast the benzoyl mustards 15 and 18 showed significant activity (see Table II) with the triimidazole analogue being at least 10-fold more potent that the diimidazole counterpart, presumably due to the increased DNA binding affinity of the "longer" compound. Of interest, however, is the selectivity of compound 15 for all the melanoma lines contained in the screen.

Table II. Cytotoxicity Data (LC50 Values in 10^{-6} M) of 15 and 18 against a Panel of Cancers Determined by NCI, Bethesda, MD

cell line	15	18
	Leukemia	
HL-60(TB)	7.84	0.72
K-562	17.2	0.58
Molt-4	18.1	0.98
	Non-Small Cell Lung Cancer	
EKVS	47.5	0.88
HOP-62	52.3	1.17
NCl-H23	47.1	0.96
	Small Cell Lung Cancer	
DMS-114	5.83	0.54
DMS-273		0.99
	Colon Cancer	
COLO-205	5.88	0.97
HCC-2998	33.7	0.68
KM12	47.0	0.96
SW-620	9.95	0.79
	CNS Cancer	
SF-268	50.0	0.77
SNB-75	41.0	0.61
U251	6.73	0.50
	Melanoma	
MALME-3M	6.14	2.63
M19-MEL	5.64	0.56
SK-MEL-2	7.17	0.65
SK-MEL-28	5.69	0.91
	Ovarian Cancer	
OVCAR-3	56.0	0.72
OVCAR-4	34.2	0.61
SK-OV-3	52.9	0.83
	Renal Cancer	
RXF-393	50.3	0.66
SN12C	15.6	0.61

In preliminary studies on the effects of these compounds on the cell cycle, mustard 15 was shown to block in the late S/G2-M phase of SW-626 cells after 48-h recovery from drug treatment (100 μM for 1 h).[26] Compound 18 demonstrated similar effects on the cell cycle of L1210 cells, suggesting that these agents exert their activity by interacting with DNA.[27] Furthermore, it is clear that for this class of compounds to exhibit any significant cytotoxicity, they must contain an alkylating group, providing strong indication that the activity of agents of this type is due to both the alkylating group and the oligopeptide moiety.[6c,7,28]

Experimental Section[15]

Melting points were determined on a Mel-Temp apparatus and are uncorrected. The ^1H- and ^{13}C-NMR spectra were recorded on a Varian VXR 300S spectrometer. The spectra were recorded in appropriate deuterated solvents indicated in the procedure with tetramethylsilane as internal standard and line positions are recorded in ppm from reference signal. Infrared spectra were recorded on a Perkin-Elmer 1600 FT spectrophotometer with the solvent indicated in the procedure, and only the principle sharply defined bands were reported in wavenumbers. Mass spectra were recorded on a Hewlett-Packard 5988A GC-MS. FAB and high-resolution mass spectra were performed by the University of South Carolina, Columbia Analytical Laboratory. Fluorescence assays were performed on an Aminco-Bowman spectrophotofluorometer and the Sequoia-Turner 450 fluorometer. UV–vis spectra were obtained on a Hewlett-Packard 8452A diode array spectrophotometer. Carbon and hydrogen analysis was performed by Midwest Microlab, Indianapolis, IN.

Analytical thin-layer chromatography was accomplished on silica gel GF, containing a 254-nm indicator, with a thickness of 0.25 mm, using the solvent system indicated in the procedure. Column chromatography was performed using 230–400-mesh silica gel and the solvent system indicated in the procedure.

Commercial grade solvents and reagents were used without further purification with the following exceptions: triethylamine, acetic anhydride, formic acid, deuterated chloroform ($CDCl_3$), deuterated dimethyl sulfoxide (DMSO-d_6), and methylene chloride were dried over molecular sieves, 3 Å. Ether was dried over sodium. THF was dried by distillation over sodium and benzophenone.

Biochemicals (DNA) were purchased from Pharmacia, and DNA solutions of $2A_{260}$ were made as follows.

T4 coliphage DNA: 10 units of DNA were dissolved in 1 mL of 10 mM sodium phosphate (pH 7.1) and 0.25 mM EDTA buffer to give a $10A_{260}$ solution. A 0.3-mL portion of the stock solution was diluted to 1.5 mL with 10 mM sodium phosphate (pH 7.1) and 0.25 mM EDTA buffer to give a $2A_{260}$ solution.

Calf thymus DNA: 100 units of DNA were dissolved in 5 mL of 10 mM sodium phosphate (pH 7.1) and 0.25 mM EDTA buffer to give a $20A_{260}$ solution. A 1.5-mL portion of the stock solution was diluted to 15 mL with 10 mM sodium phosphate (pH 7.1) and 0.25 mM EDTA buffer to give a $2A_{260}$ solution.

Poly(dA.dT) and poly(dG.dC) DNA: 100 units of DNA were dissolved in 10 mL of 10 mM sodium phosphate (pH 7.1) and 0.25 mM EDTA buffer to give a $10A_{260}$ solution. A 3-mL portion of the stock solution was diluted to 15 mL with 10 mM sodium phosphate (pH 7.1) and 0.25 mM EDTA buffer to give a $2A_{260}$ solution.

4-[4-[Bis(2-chloroethyl)amino]benzamido]-*N*-[2-(dimethylamino)ethyl]-1-methylimidazole-2-carboxamide (8). *p*-[Bis(2-chloroethyl)amino]benzoyl chloride was prepared by dissolving *p*-[bis(2-chloroethyl)amino]benzoic acid (328 mg, 1.25 mmol) in benzene (5 mL) and thionyl chloride (5 mL) and heating to reflux under a drying tube for 1.25 h.[16] The excess thionyl chloride and solvent were then removed under reduced pressure and the residue coevaporated with dry CH_2Cl_2 (10 mL, twice).

To a stirred solution of amine **6** [prepared from the reduction of the nitro group of the corresponding starting material (301 mg, 1.25 mmol)] in dry CH_2Cl_2 (30 mL) with dry triethylamine (192 μL, 1.38 mmol), cooled to −20 °C, was added the above acid chloride dissolved in dry CH_2Cl_2 (10 mL) dropwise under an atmosphere of argon. The mixture was kept at −20 °C for an additional 15 min and then allowed to stir at 23 °C (19.5 h). After TLC analysis indicated that all of the starting material was gone, the reaction mixture was concentrated under reduced pressure to a yellow solid, which was purified by column chromatography (silica gel) with 2% methanol in chloroform as eluent to give **8** as a bright yellow powder: yield 152 mg (0.334 mmol, 27%); mp 43–56 °C; TLC (10% MeOH/$CHCl_3$) R_f 0.35; ^{1}H NMR ($CDCl_3$) δ 2.29 (s, 6 H, NMe_2), 2.51 (t, 5.7, 2 H, CH_2NMe_2) 3.47 (q, 5.7, 2 H, NCH_2C), 3.68 (t, 7.1, 4 H, chloroethyl), 3.82 (t, 7.1, 4 H, chloroethyl), 4.05 (s, 3 H, imidazole-1-Me), 6.72 (d, 8.7, 2 H, phenyl), 7.48 (br s, 1 H, NH), 7.51 (s, 1 H, imidazole), 7.81 (d, 8.7, 2 H, phenyl), 8.19 (s, 1 H, NH); IR (Nujol): ν 3440, 1670, 1605, 1536 cm^{-1}; MS (FAB, NBA) *m/e* (relative intensity) 455 (M + H^+, 35). Anal. ($C_{20}H_{28}N_6O_2Cl_2\cdot 2H_2O$) C, H.

4-[[4-(Chloroacetamido)-1-methylimidazol-2-yl]carboxamido]-*N*-[2-(dimethylamino)ethyl]-1-methylimidazole-2-carboxamide (11). A solution of amine **9** (180 mg, 0.54 mmol) in dry CH_2Cl_2 (15 mL) and dry triethylamine (84 μL, 0.60 mmol) under an atmosphere of argon was cooled to −20 °C, and a solution of chloroacetyl chloride (48 μL, 0.603 mmol) dissolved in dry CH_2Cl_2 (3 mL) was added dropwise. The mixture was kept at −20 °C for an additional 15 min and then allowed to stir overnight at 23 °C (17 h). The mixture was concentrated to a light yellow foam which was dissolved in water (40 mL) and saturated $NaHCO_3$ (20 mL). The aqueous phase was extracted with CH_2Cl_2 (100 mL, 3×). The combined organic layers were then washed with saturated NaCl (100 mL), dried (Na_2SO_4), and concentrated to a yellow oil which was purified by column chromatography (silica gel) using a stepwise gradient of methanol (1–7%) in chloroform as eluent. The resulting pure product was precipitated from CH_2Cl_2 with ether and hexane, and the off-white powder of **11** was dried in vacuo at 23 °C: yield 55.3 mg (0.14 mmol, 25%); mp 206 °C dec; TLC (10% MeOH/$CHCl_3$) R_f 0.27; ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 6 H, NMe_2), 2.50 (t, 6.1, 2 H, CH_2NMe_2), 3.50 (q, 6.1, 2 H, NCH_2C), 4.05 (s, 3 H, imidazole-1-Me), 4.10 (s, 3 H, imidazole-1-Me), 4.20 (s, 2 H, chloroacetyl), 7.35 (s, 1 H, imidazole), 7.45 (s, 1 H, imidazole), 7.55 (t, 6.1, 1 H, NH), 8.70 (br s, 1 H, NH), 9.25 (br s, 1 H, NH); IR (Nujol) ν 3382, 1663, 1535 cm^{-1}; UV (EtOH) λ_{max} 208, 304 nm; MS (FAB, TFA-NBA) *m/e* (relative intensity) 411 (M + H^+, 1), 307 (38). Anal. ($C_{16}H_{23}ClN_8O_3\cdot 2H_2O$) C, H.

4-[[4-(Bromoacetamido)-1-methylimidazol-2-yl]carboxamido]-*N*-[2-(dimethylamino)ethyl]-1-methylimidazole-2-carboxamide (12). The procedure is similar to that for the synthesis of **11** except bromoacetyl chloride was used. The resulting pure product was precipitated from CH_2Cl_2 with ether and hexane, and the yellow powder of **12** was dried in vacuo at 23 °C: yield 13.8 mg (0.030 mmol, 5%); mp 197–201 °C dec; TLC (10% MeOH/$CHCl_3$) R_f 0.26; ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 6 H, NMe_2), 2.51 (t, 5.7, 2 H, CH_2NMe_2), 3.47 (q, 5.7, 2 H, NCH_2C), 4.04 (s, 3 H, imidazole-1-Me), 4.07 (s, 3 H, imidazole-1-Me), 4.20 (s, 2 H, bromoacetyl), 7.40 (s, 1 H, imidazole), 7.45 (s, 1 H, imidazole), 7.55 (t, 5.7, 1 H, NH), 8.65 (br s, 1 H, NH), 9.23 (br s, 1 H, NH); IR (Nujol) ν 3420, 1664, 1534 cm^{-1}; UV (ethanol) λ_{max} 206, 304 nm; MS (FAB, NBA) *m/e* (relative intensity) 455 (M + H^+, 2), 338 (10), 307 (20); HRMS (FAB-NBA) *m/e* 455.1164 ($C_{16}H_{24}N_8O_3Br$ requires 455.1157).

4-[[4-(Dichloroacetamido)-1-methylimidazol-2-yl]carboxamido]-*N*-[2-(dimethylamino)ethyl]-1-methylimidazole-2-carboxamide (13). The procedure is similar to that for the synthesis of **11** except dichloroacetyl chloride was used. The excess liquid was drawn off, and the light yellow solid of **13** was dried in vacuo at 23 °C: yield 112.8 mg (0.253 mmol, 47%); mp 195 °C dec; TLC (10% MeOH/$CHCl_3$) R_f 0.30; ^{1}H NMR ($CDCl_3$) δ 2.30 (s, 6 H, NMe_2), 2.55 (t, 6.5, 2 H, CH_2NMe_2), 3.55 (q, 6.5, 2 H, NCH_2C), 4.03 (s, 3 H, imidazole-1-Me), 4.08 (s, 3 H, imidazole-1-Me), 6.15 (br s, 1 H, dichloroacetyl), 7.40 (s, 1 H, imidazole), 7.45 (s, 1 H, imidazole), 7.90 (br s, 1 H, NH), 9.15 (br s, 1 H, NH), 9.35 (br s, 1 H, NH); ^{13}C NMR ($CDCl_3$) δ 35.5, 35.8, 36.5, 45.1, 57.9, 66.1, 113.6, 115.3, 134.9, 135.4, 155.7, 175.9; IR (Nujol) ν 3490, 1660, 1529 cm^{-1}; UV (H_2O) λ_{max} 200 (ε 5.35 × 10^4 cm^{-1} M^{-1}), 214 (ε 5.47 × 10^4 cm^{-1} M^{-1}), 306 nm (ε 2.01 × 10^4 cm^{-1} M^{-1}); MS (FAB, TFA-NBA) *m/e* (relative intensity) 445 (M^+, 100). Anal. ($C_{16}H_{22}Cl_2N_8O_3\cdot{}^1/_2H_2O$) C, H.

4-[[4-(Cyclopropanecarboxamido)-1-methylimidazol-2-yl]carboxamido]-*N*-[2-(dimethylamino)ethyl]-1-methylimidazole-2-carboxamide (14). The procedure is similar to that for the synthesis of **11** except cyclopropanecarbonyl chloride was used. The resulting pure product was precipitated from CH_2Cl_2 with ether and hexane, and the yellow powder of **14** was dried in vacuo at 23 °C: yield 31 mg (0.080 mmol, 11%); mp 133 °C dec; TLC (10% MeOH/$CHCl_3$) R_f 0.22; ^{1}H NMR ($CDCl_3$) δ 0.7 (m, 2 H, $iPrCH_2$), 1.18 (m, 2 H, $iPrCH_2$), 1.65 (m, 1 H, iPrCH), 2.40 (s, 6 H, NMe_2), 2.69 (t, 5.9, 2 H, CH_2NMe_2), 3.68 (q, 5.9, 2 H, NCH_2C), 4.05 (s, 3 H, imidazole-1-Me), 4.10 (s, 3 H, imidazole-1-Me), 7.35 (s, 1 H, imidazole), 7.43 (s, 1 H, imidazole), 7.90 (br s, 1 H, NH), 8.45 (br s, 1 H, NH), 9.3 (br s, 1 H, NH); IR (Nujol) ν 3383, 1656, 1534 cm^{-1}; UV_{max} (ethanol) λ_{max} 214, 306 nm; MS (FAB, TFA-NBA) *m/e* (relative intensity) 403 (M + H^+, 100). Anal. ($C_{18}H_{26}N_8O_3\cdot 1.25H_2O$) C, H.

4-[[4-[4-[Bis(2-chloroethyl)amino]benzamido]-1-methylimidazol-2-yl]carboxamido]-*N*-[2-(dimethylamino)ethyl]-1-methylimidazole-2-carboxamide (15). The procedure is similar to that for the synthesis of **8** except amine **9** was used. The resulting pure product of **15** was isolated as an off-white powder: yield 216 mg (0.37 mmol, 27%); mp 165 °C dec; TLC (10% MeOH/$CHCl_3$) R_f 0.40; ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 6 H, NMe_2), 2.53 (t, 6.3, 2 H, CH_2NMe_2), 3.50 (q, 6.3, 2 H, NCH_2C), 3.68 (t, 6.9, 4 H, chloroethyl-CH_2), 3.82 (t, 6.9, 4 H, chloroethyl-CH_2), 4.02 (s, 3 H, imidazole-1-Me), 4.06 (s, 3 H, imidazole-1-Me), 6.75 (d, 8.7, 2 H, phenyl), 7.39 (s, 1 H, imidazole), 7.58 (s, 1 H, imidazole), 7.83 (t, 6.3, 1 H, NH), 7.94 (d, 8.7, 2 H, phenyl), 8.71 (br s, 1 H, NH), 9.31 (br s, 1 H, NH); ^{13}C NMR ($CDCl_3$) δ 35.6, 36.7, 40.2, 45.2, 53.3, 57.9, 111.2, 113.3, 114.6, 122.0, 129.4, 133.5, 135.4, 136.9, 149.0, 158.8, 159.5, 163.9; IR (Nujol) ν 3420, 1654, 1606, 1518, 1185, 668 cm^{-1}; UV (H_2O) λ_{max} 216 (ε 3.41 × 10^4

cm^{-1} M^{-1}), 316 nm (ϵ 2.26 × 10^4 cm^{-1} M^{-1}); MS (FAB, TFA-NBA) *m/e* (relative intensity) 578 (M^+, 80). Anal. ($C_{25}H_{33}Cl_2N_9O_3 \cdot {}^1/_2H_2O$) C, H.

4-[[4-[[4-[Bis(2-chloroethyl)amino]benzamido]-1-methylimidazol-2-yl]carboxamido]-1-methylimidazol-2-yl]carboxamido]-*N*-[2-(dimethylamino)ethyl]-1-methylimidazole-2-carboxamide (18). The procedure is similar to that for the synthesis of 8 except amine 16 was used. The resulting pure product 18 was isolated as an off-white powder: yield 115.8 mg (0.222 mmol, 44%); mp 134–146 °C dec; TLC (10% MeOH/$CHCl_3$) R_f 0.53; ^1H NMR ($CDCl_3$) δ 2.32 (s, 6 H, NMe_2), 2.54 (t, 5.7, 2 H, CH_2NMe_2) 3.52 (q, 5.7, 2 H, NCH_2C), 3.69 (t, 7.1, 4 H, chloroethyl), 3.83 (t, 7.1, 4 H, chloroethyl), 4.05 (s, 3 H, imidazole-1-Me), 4.09 (s, 6 H, imidazole-1-Me), 6.76 (d, 8.7, 2 H, phenyl), 7.43 (s, 1 H, imidazole), 7.49 (s, 1 H, imidazole), 7.60 (br s, 1 H, NH), 7.61 (s, 1 H, imidazole), 7.87 (d, 8.7, 1 H, phenyl), 8.36 (s, 1 H, NH), 9.30 (s, 1 H, NH), 9.33 (s, 1 H, NH); ^{13}C NMR ($CDCl_3$) δ 35.6, 36.6, 40.1, 45.2, 53.3, 57.9, 111.3, 122.0, 129.2, 135.4, 135.7, 136.8, 146.0, 146.6, 149.2, 155.8, 159.1, 159.2, 170.5; IR (Nujol) ν 3440, 1665, 1605, 1536 cm^{-1}; UV (ethanol) λ_{max} 204, 310 nm; MS (FAB, NBA) *m/e* (relative intensity) 702 ($M + H^+$, 10). Anal. ($C_{30}H_{38}Cl_2N_{12}O_4 \cdot 3H_2O$) C, H.

Ethidium Displacement Assay. To 2 mL of an ethidium bromide buffer solution (10 mM Tris, 1 mM EDTA, 1.3 μM EtBr, pH 7.4)[18] was added 25 μL of a $2A_{260}$ DNA solution, and the maximum fluorescence was measured (excitation wavelength = 546 nm, emission wavelength = 600 nm) at ambient temperature. Aliquots of a 10 mM stock drug solution (1 mg of drug to be tested was dissolved with 1 molar equiv of 0.1 M HCl and then diluted with appropriate volume of distilled water to make a 10 mM solution) were then added to the fluorescing solution and the fluorescence measured after each addition until a 50% reduction of fluorescence occurs. If the 10 mM stock solution lowers the percent fluorescence too quickly, the stock solution was further diluted to 1 mM prior to titration. The apparent binding constant was then calculated from the equation: K_{EtBr}[EtBr] = K_{app}[drug], where [drug] = the concentration of drug that gives a 50% reduction of fluorescence and K_{EtBr}, the binding constant for ethidium bromide.[29]

Dialysis Experiment. A solution of $2A_{260}$ calf thymus DNA (0.5 mL) was diluted to 2 mL with 10 mM sodium phosphate and 0.25 mM EDTA buffer, pH 7.1. Four aliquots (3 μL each) of a 1 mM stock drug solution in distilled water were added to the DNA solution, and a UV spectrum was recorded after each addition. The drug/DNA solution was left for 24 h at 23 °C, and another UV spectrum was recorded. The drug/DNA solution was then transferred into a dialysis bag (<MW 2000) then it was suspended in 30 mL of 10 mM sodium phosphate and 0.25 mM EDTA buffer, pH 7.1, at ambient temperature. After 20 h, a spectrum of the buffer outside the dialysis bag was recorded. The absorbance of the drug peak at 304–316 nm was converted to concentration by the Beer-Lambert equation, and the percent of the drug remaining in the bag was calculated.

CD Titration Studies. The experiments were performed with a continuous flow of nitrogen purging the polarimeter. A 1-mm path length jacketed cell was used, and all experiments were done at 23 °C. Initially, DNA (0.02 mmoles (bp), 130 μL) was added to the cuvette, and the spectrum of the DNA was collected. Aliquots of drug were then added and the spectra collected. The concentration of the aqueous drug solutions (as the hydrochloride salt) were 1 mM, and amounts of the drug added correspond to a drug to base pair ratio (0.02, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.50, 0.60, 0.80, 1.00).

The scan parameters were set and standardized for all experiments and were as follows: the spectra were collected from 400 to 220 nm at 23 °C. The sensitivity was set at 1 mdeg with a scan speed of 200 nm/min. Three scans were accumulated and automatically averaged by the computer. The λ_{max} and ellipticity (mdeg) for each spectrum were collected from the raw scans, and the final plots were smoothed by the noise reduction program on the computer.

Cytotoxicity Studies. The K562 human chronic myeloid leukemia cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine at 37 °C in a humidified atmosphere containing 5% CO_2 and were incubated with a specified dose of drug for 1 h at 37 °C in the dark. The incubation was terminated by centrifugation (5 min, 300*g*) and the cells washed once with drug-free medium.

Following the appropriate drug treatment, the cells were transferred to 96-well microtitre plates, 10^4 cells/well, 8 wells/sample. Plates were then kept in the dark at 37 °C in a humidified atmosphere containing 5% CO_2. The assay is based in the ability of viable cells to reduce a yellow soluble tetrazolium salt, 3-(4,5-dimethylthiazole-2,5-diyl)diphenyltetrazolium bromide (MTT, Sigma Chemical Co.) to an insoluble purple formazan precipitate.[30] Following incubation of the plates of 5–6 days (to allow control cells to increase in the number by 10-fold), 20 μL of a 5 mg/mL solution of MTT in phosphate-buffered saline was added to each well and the plates further incubated for 5 h. The plates were then centrifuged for 5 min at 300*g* and the bulk of the medium pipetted from the cell pellet leaving 10–20 μL/well. DMSO (200 μL) was added to each well and the samples agitated to ensure complete mixing. The optical density was then read at a wavelength of 550 nm on a Titertek Multiscan ELISA plate reader and the dose-response curve constructed. For each curve, an IC_{50} value was read as the dose required to reduce the final optical density to 50% of the control value.

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