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Structure–Activity Relationship of a Series of C-Terminus Modified Aminoalkyl, Diaminoalkyl- and Anilino-containing Analogues of the Benzoic Acid Mustard Distamycin Derivative Tallimustine: Synthesis, DNA Binding and Cytotoxicity Studies

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Abstract—As part of our investigations into the design of more cytotoxic analogues of the experimental anticancer drug tallimustine, **1**, C-terminus modified aminoalkyl-, **2a–c**, diaminoalkyl-, **3**, and anilino-containing, **4**, derivatives have been synthesized. Compounds **2a–c** differ by 2, 3, or 4 methylene units in the C-terminus, respectively. Results from an ethidium displacement study on poly(dA–dT), poly(dG–dC), calf thymus DNA and T4 coliphage DNA showed that compounds **2–4** interact in the minor groove of the polynucleotides with a preference for poly(dA–dT) over poly(dG–dC). Compound **4** bound more weakly to the DNAs than **2a–c** and **3**. Using a CD dilution assay compounds **2a–c** and **3** were demonstrated to bind irreversibly to calf thymus DNA. The sequence selectivity by which compounds **2–4** alkylate DNA was demonstrated using a Taq polymerase stop assay. All the compounds alkylated preferentially at the 3′-purine residue in a 5′-TTTTGPu-3′ sequence (Pu = A or G). This observed sequence specificity is similar to that of tallimustine and a related compound **5**. At an equimolar concentration the aminoalkyl compounds **2a–c** (**2b** > **2a** > **2c**), and diaminoalkyl compound **3** were more efficient at alkylating these sequences than the anilino compound **4**. Following a one hour exposure of human chronic myeloid leukemia K562 cells, compounds **2b** and **3** have lower IC_{50} values (1.64 μM and 3.03 μM, respectively) than tallimustine (5 μM) and similar values to a related compound **5** (2.2 μM). The order of cytotoxicity for all the compounds is **2b** > **5** > **3** > **2a** > **1** > **2c** = **4**. These results indicate that the cytotoxicities of these compounds are related to their relative ability to alkylate the consensus DNA binding sequence. © 1997 Elsevier Science Ltd.

Tallimustine, (FCE24517, **1** Fig. 1), is a benzoic acid mustard derivative of the naturally occurring oligopeptide distamycin,[1] that has been shown to be active against a wide range of human and mouse tumors, including ones that are resistant to cisplatin.[1,2] Currently, **1** is undergoing clinical evaluation for the treatment of cancer in Europe.[3] Data from biochemical pharmacology studies have indicated that **1** exerts its biological activity by interacting with DNA,[4] presumably by alkylating at specific sequences.[5] Recent reports have shown that compound **1** and the related compound **5** (Fig. 1) react covalently with the 3′-purine residue within the consensus sequence 5′-TTTTGPu-3′ where Pu is either an A or G residue.[5,6a] We have also demonstrated that with agents of this type the compounds with the most enhanced sequence specificities for alkylation were also the most cytotoxic.[6]

The primary goal of this study was to systematically investigate the influence of the C-terminal group on the biological activity of **1**. In this study, three types of structural modifications were made. First, the ethylamidinium group of **1** was replaced with an aminoalkyl moiety, in which the number of methylene groups were varied from 2 to 4 to give compounds **2a–c**, respectively. With these compounds, the optimal number of methylene groups at the C-terminus required for maximum DNA binding and cytotoxicity was established.

In the second aspect of this study, compound **3** was designed on the basis that cytotoxic agents bearing diamino tethers can be significantly more reactive with DNA and have more potent anticancer activities than their parent compounds. For example, a chlorambucil-spermidine conjugate was shown to be approximately 1000-fold more effective in producing interstrand crosslinks in naked DNA than chlorambucil itself. Moreover, the spermidine conjugate was approximately 35 times more cytotoxic against ADJ/PC6 plasmacytoma cells, and fourfold more active against the same tumor in vivo than chlorambucil.[7] The improved potency of the chlorambucil-spermidine conjugate was attributed to its enhanced DNA reactivity, in addition to its increased accumulation in the tumor cells, presumably due to its preferential transport by a diamine uptake system.[7]

Almost all analogues of tallimustine have an aliphatic amine group in the C-terminus.[1,6] Compound **4** was therefore designed to investigate the influence of an

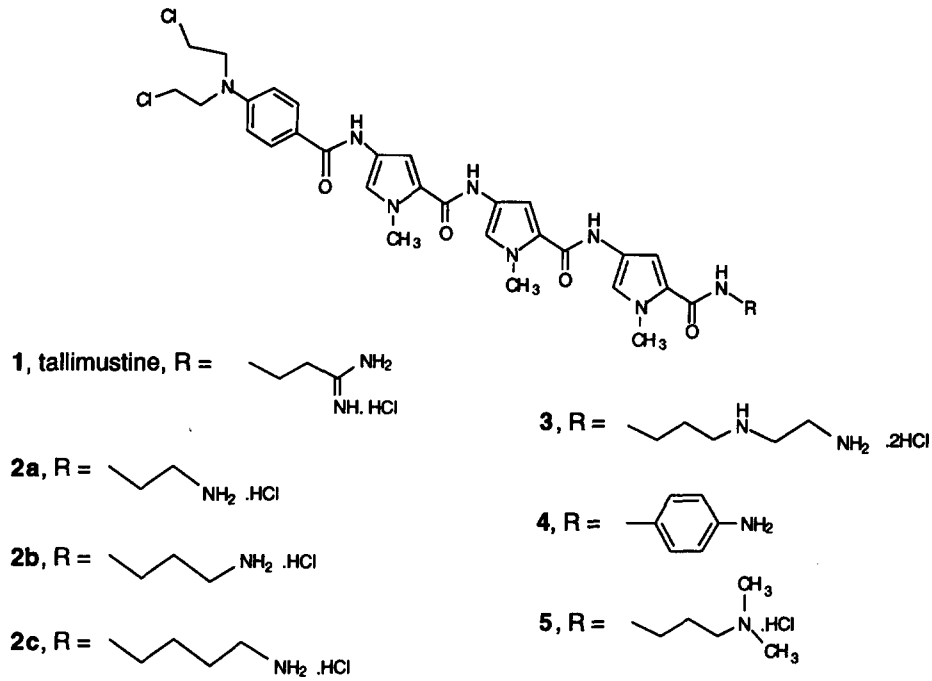


Figure 1. Structures of tallimustine **1**, the aminoalkyl analogues **2a–c**, the diaminoalkyl derivative **3**, the anilino derivative **4**, and analogue **5**.

aromatic group at the C-terminus on the DNA binding and cytotoxic properties of this class of compounds.

Results and discussion

Synthesis

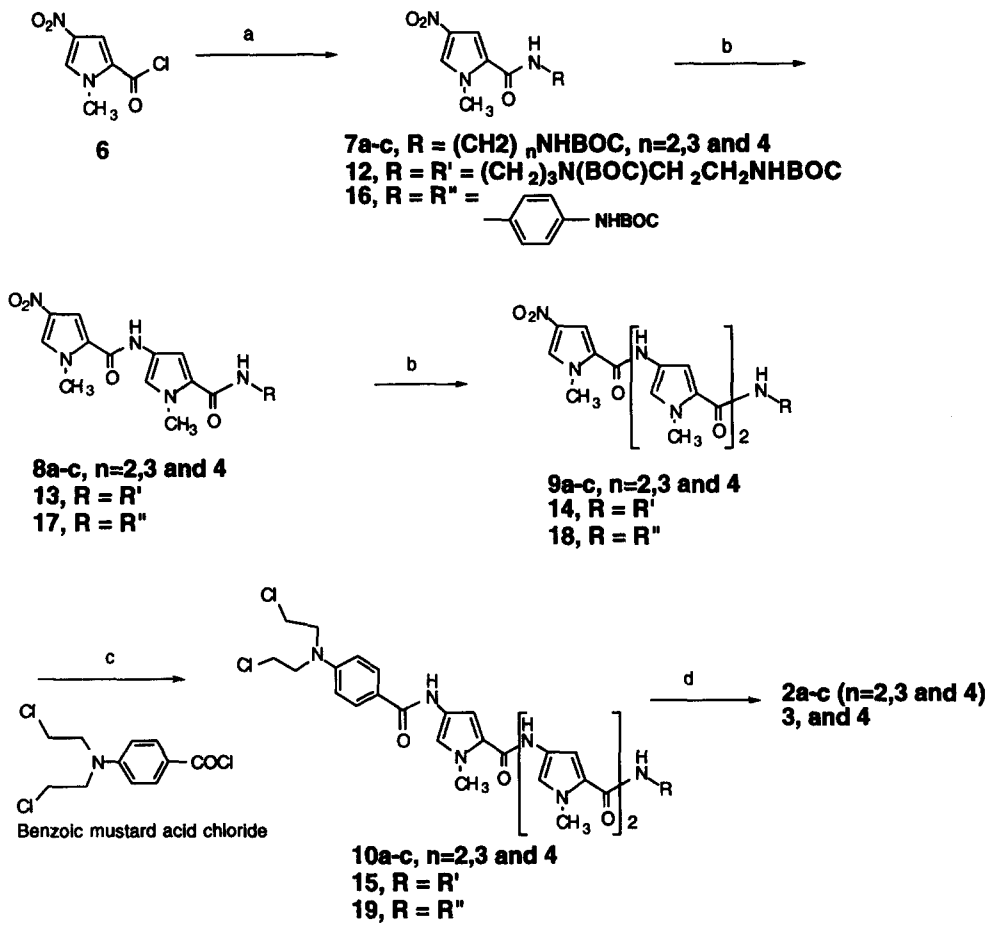
The synthetic approach for making compounds **2a–c**, **3**, and **4** is depicted in Scheme 1. Reaction of the pyrrole acid chloride **6** with *N*-BOC-diaminoalkanes, wherein the number of methylene units were 2, 3, and 4, gave amide **7a–c** in good yields (75–100%). Catalytic hydrogenation of the nitro group of **7a–c** followed by coupling with acid chloride **6** gave compounds **8a–c** (66–76% yield). Subsequent hydrogenation of **8a–c** and reaction of the amine intermediate with acid chloride **6** gave agents **9a–c** (50–60% yield). Reduction of the nitro group of compounds **9a–c** followed by coupling with benzoic mustard acid chloride, or *p*-(*N,N*-bis-(2-chloroethyl)amino)benzoyl chloride, in the presence of triethylamine gave compound **10a–c** (21–42% yield). The BOC group was removed by treatment of compounds **10a–c** with methanolic hydrochloric acid to afford the desired compounds **2a–c** in quantitative yields.

The strategy for the preparation of compounds **3** and **4** was identical to that used for the synthesis of compounds **2a–c**, except a diBOC protected triamine **11**[8] was used for the synthesis of compound **3**, and *N*-BOC-1,4-phenylenediamine was used in the synthesis of compound **4**. The percent yields for each of the reactions in the synthesis of compounds **3** and **4** were similar to those of the above reactions, and were in the range of 46–100%. The structures of all compounds have been characterized by IR, ^{1}H NMR, and mass spectrometric analyses including accurate mass measurements.

DNA binding studies

The apparent DNA binding constants, K_{app}, of compounds **2a–c**, **3**, and **4** to poly(dA–dT), poly(dG–dC), calf thymus DNA, and T4 DNA were determined using an ethidium displacement assay,[10] and the results are given in Table 1. The rank order of binding constants to the four polynucleotides was **2b** > **5** > **3** > **2a** > **2c** > **4**, with compound **4** being one to two orders of magnitude lower than **2b**. The low DNA binding constants for compound **4** was presumably due to its lack of a positive charge at physiological pH of 7.4 since the pK_a values of anilines are typically around 3–4. In contrast, compounds **2a–c**, **3**, and **5** would be protonated at physiological pH (pK_a of amines **9–10**). The positive charge(s) on these molecules provides electrostatic attraction to the negative charges on the DNA. All the compounds given in Table 1 demonstrate some degree of preference for poly(dA–dT) over poly(dG–dC), although for **2–5** the preference was less than that seen with distamycin. The binding of all the compounds to both calf thymus DNA and coliphage T4 DNA suggested that they bind to the minor groove because the major groove of the latter oligonucleotide is blocked by α-glycosylation.[11] Furthermore, for the aminoalkyl compounds **2a–c**, these results revealed that the optimum number of methylene groups on the C-terminus for maximal DNA binding affinity was 3 (**2b**).

The interactions of compounds **2a–c** and **3** to poly(dA–dT), poly(dG–dC), and calf thymus DNA were also



Scheme 1. (a) *N*-BOC-diaminoalkane (n = 2–4), or **11** $H_2N(CH_2)_3$ $N(BOC)CH_2CH_2NHBOC$, or *N*-BOC-1,4-phenylenediamine, pyridine, methylene chloride; (b) i, H_2, 5% Pd–C, ii, acid chloride **6**, pyridine, CH_2Cl_2; (c) i, H_2, 5% Pd–C, ii, benzoic mustard acid chloride, Et_3N, CH_2Cl_2; methanolic HCl.

investigated using circular dichroism studies. In these studies, positive DNA induced ligand bands were observed for the titration of these compounds to poly(dA–dT) (Table 2). Weak DNA-induced ligand bands were observed with calf thymus DNA and poly (dG–dC) for **3**, with negligible effects on both these DNAs with **2a–c** and **4**. Titration spectra of compound **2b** to poly(dA–dT) and poly(dG–dC) are shown in Figure 2A and B, respectively. Since compounds **2–4** alone did not produce any CD spectrum, the appearance of DNA induced ligand bands is indicative of their binding to the DNAs. The results for **2b** show a dose-dependent increase in the ellipticity of the DNA induced ligand band at 340–345 nm, but this was not the case for poly(dG–dC), thereby reinforcing the AT-sequence preference of compounds **2–4**. Since all of the CD studies were done under identical conditions at room temperature and using comparable concentrations of DNA and drugs, and assuming that these compounds bind to the DNAs in a similar orientation, the data suggest that all compounds given in Table 2 demonstrate a preference for binding reversibly to AT rich sequences.

The ability of compounds **2–4** to react irreversibly with calf thymus DNA was studied using a CD assay.[1,6] The

Table 1. Apparent DNA binding constants, K_{app} ($\times 10^5$ M^{-1}) compounds **2–4**

Compound	Poly(dG–dC)	Poly(dA–dT)	CT-DNA	T4-DNA
2a	14.3±0.3	14.5±0.1	37.4±0.2	9.2±1.4
2b	52.5±0.5	83.7±0.5	217±0.5	91.2±0.5
2c	2.6±0.8	2.8±0.1	1.7±1	4.6±0.2
3	23.4±0.01	42.2±0.22	37.4±0.16	63.7±0.62
4	0.79±0.05	2.4±0.1	0.89±0.04	0.41±0.01
5[a]	26.0	71.0	37.0	87.0
Distamycin[a]	2.0	350	7.7	6.5

[a]Data from ref 6b.

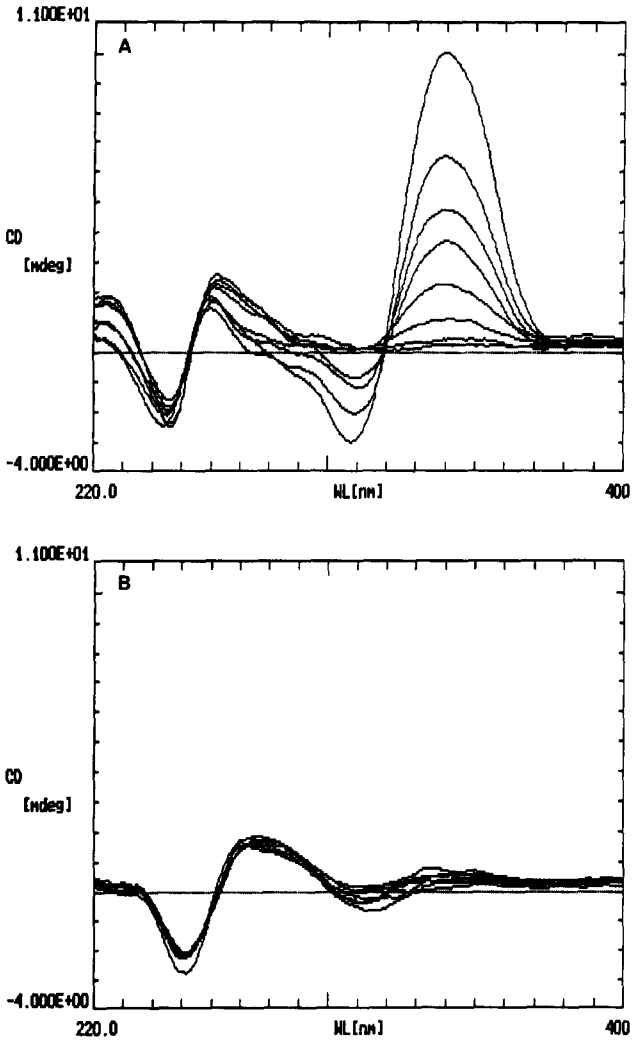


Figure 2. Part A shows the CD titration Spectra of compound **2a** to poly(dA–dT), r' = 0, 0.025, 0.050, 0.10, 0.15, 0.20, 0.30, 0.40. Part B depicts the CD spectra of compound **2a** to poly(dG–dC), r' = 0, 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.60.

results show that after overnight treatment of the polynucleotides with the drugs, r' of 0.20–0.35, at 37 °C, the compounds gave evidence of covalent binding. The values of r' correspond to the number of moles of drug to the number of moles of DNA base pairs. The percent irreversible binding for these compounds are given in Table 2, compared to compound **5**. Despite the lower apparent binding constant of **4** to calf thymus DNA this compound produced a significant level of irreversible binding to DNA under the conditions employed.

Sequence specificity of alkylation

The sequence specificity of covalent binding for **2a–c**, **3**, and **4** was determined using a Taq polymerase stop assay.[12] Figure 3 shows the results in an AT-rich region of pBR322 DNA. All five compounds showed a similar sequence specific alkylation pattern with a preference for the 3′G residue in the sequence 5′-TTTTGG. Consistent with the K_{app} values, compound **2b** was more efficient at alkylating this site at an equivalent dose than **2a** or **2c**. Similarly, compound **4** was less efficient than **3**. Sequence selective binding was also demonstrated at the sequence 5′-TTTTGA (data not shown), and this consensus sequence for minor groove alkylation is identical to that observed previously for **1**[5] and **5**[6a].

Although compounds **1–5** have different C-terminal groups their sequence specificity in producing covalent lesions on DNA is strikingly similar. The molecular recognition of **1** and its analogues to the 5′-TTTTGPu-3′ sequence may be dictated by the structural complementarity between the drug and DNA, and not the sequence per se. The 5′-TTTTGPu sequence has been shown to adopt a unique conformation in which the minor groove of the T4 tract is compressed, the AT base pairs are propeller twisted, and the minor groove at the TG step is decompressed thereby causing a bend along the sequence.[6a] Studies to elucidate the structure and conformation of the adduct of an analogue of **1** with an oligonucleotide are currently in progress.

In vitro cytotoxicity

The ability of compounds **2–4** to inhibit the growth of chronic human myeloid leukemia K562 cells in culture

Table 2. Ellipticity of the DNA induced ligand bands from CD Titration studies of compounds **2a–c**, **3**, and distamycin

Compound	Poly(dG–dC)	Poly(dA–dT)	Calf-thymus DNA	% Irreversible binding to CT DNA[a]
2a (r' = 0.2)[b]	negligible	1.20 (345)[c]	nd[d]	53±0.5%
2b (r' = 0.2)	negligible	5.0 (345)	nd	67±1.2
2c (r' = 0.2)	negligible	1.3 (345)	nd	41±0.7
3 (r' = 0.4)	0.58 (338)[c]	2.10 (340)	1.32 (340)	39±2
4 (r' = 0.15)	negligible	0.7 (340)	negligible	54±1
5[e]	−0.3 (308)	−4.5 (308)	nd	17±5
Distamycin (r' = 0.20)	negligible	1.99 (330)	0.66 (329)	nd

[a]The CD dilution studies were done using r' of 0.35.
[b]r' is the number of moles of the ligand to the number of moles of DNA base pairs.
[c]Wavelength (nm) of the DNA induced ligand band.
[d]nd = not determined.
[e]Data from ref 6b.

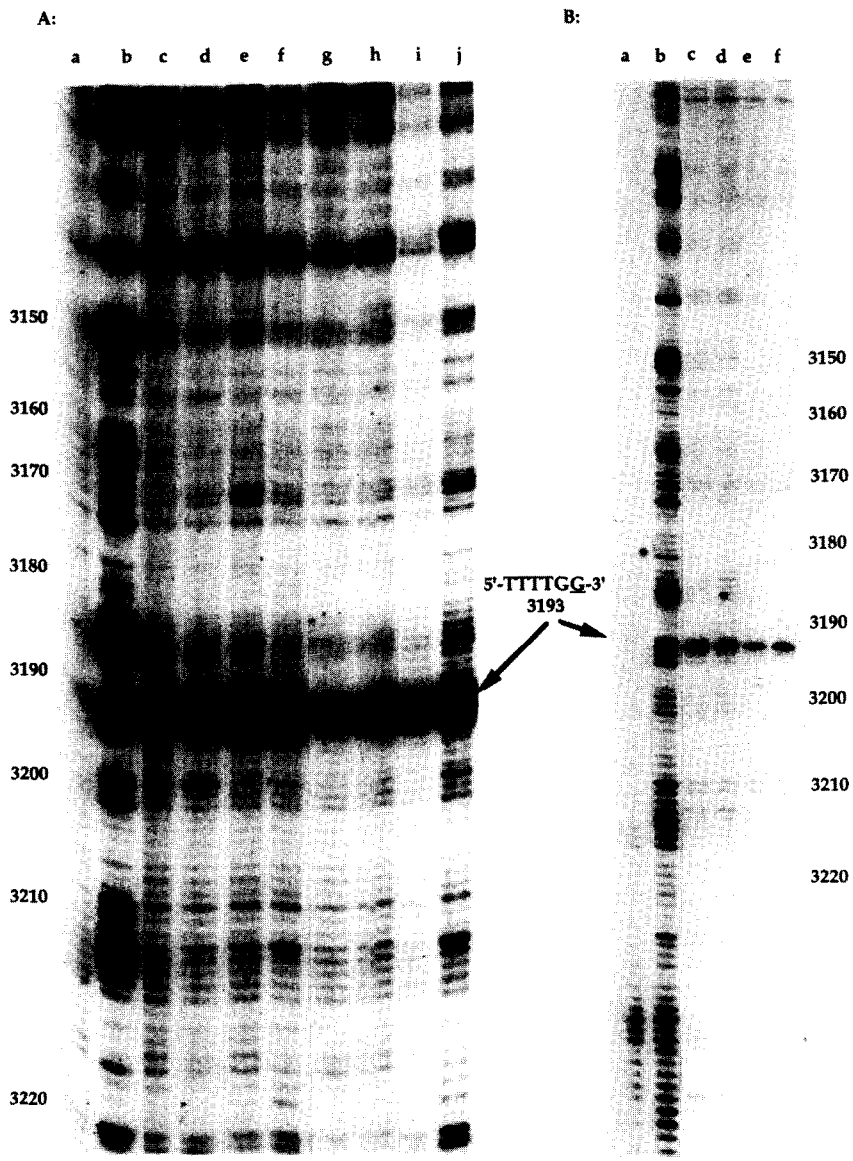


Figure 3. *Taq* polymerase gel examining DNA damage on the top strand of an AT-rich region of pBR322 DNA (defined as SRM in materials and methods). (A) Lane a, control; b, 100 μM CHL (chlorambucil); c, 1 μM **2a**; d, 5 μM **2a**; e, 1 μM **2b**; f, 5 μM **2b**; g, 1 μM **2c**; h, 5 μM **2c**; i, 1 μM **5**; j, 5 μM **5**. (B) Lane a, control; b, 100 μM CHL; c, 1 μM **3**; d, 5 μM **3**; e, 1 μM **4**, f, 5 μM **4**.

was measured using the MTT assay,[9] following a 1 h exposure of the compounds to the cells. The IC_{50} values are presented in Table 3, and they are compared to **1** and **5**. Compound **2b** was the most cytotoxic, and compound **4** the least cytotoxic of the agents. Neither the diamino- nor anilino- substitution (compounds **3** and **4**, respectively) altered the cytotoxicity markedly from that of **1** and **5**. In particular the greater-than-tenfold decreased apparent binding constant of **4** to naked DNAs was not reflected in an equivalent loss of cytotoxicity. The decreased cytotoxicity of **4** over **2b** and **3** was more related to its decreased efficiency of alkylation to the 5′-TTTTGPu sequence. In the aminoalkyl series of compounds, the order of cytotoxicity is **2b** > **2a** > **2c** which correlated with their binding constants and their relative covalent reactivity with the consensus sequence for alkylation.

In conclusion, the cytotoxicities of the series of C-terminus modified aminoalkyl, diaminoalkyl-, and anilino-containing analogues of the benzoic acid mustard derivative tallimustine were related to their relative ability to alkylate the consensus DNA binding sequence 5′-TTTTGPu.

Experimental

***N*-(2-Butoxycarboxamidoethyl)-1-methyl-4-nitropyrrole-2-carboxamide 7a.** To a suspension of 1-methyl-4-nitropyrrole-2-carboxylic acid (2.74 g, 16.12 mmol) was added thionyl chloride (12 mL). The reaction mixture was heated to reflux under a drying tube for 10 min at which time the excess thionyl chloride was removed under presssure and the acid chloride was

Table 3. In vitro cytotoxicities of compounds **1–5** against human chronic myeloid leukemia K562 cells

Drug	IC_{50} (μM)[a]
1	5.0±1
2a	4.71±1.5
2b	1.64±0.55
2c	15.1±4.5
3	3.03±0.85
4	15.1±2.5
5	2.2±0.5

[a]Drug incubation time was 1 h, 37 °C.

coevaporated twice with dry CH_2Cl_2 (5 mL each) yielding 1-methyl-4-nitropyrrole-2-carbonyl chloride **6** as an off-white powder.

To a cooled (0 °C, ice bath) and stirring solution of *N*-BOC-ethylenediamine (2.0 g, 12.4 mmol) in dry pyridine (20 mL, 0.248 mol), and dry CH_2Cl_2 (30 mL) was added dropwise a solution of the acid chloride **6** in dry CH_2Cl_2 (30 mL). The reaction was kept at 0 °C for an additional 15 min and then allowed to stir under a drying tube at room temperature for 3 h. The reaction mixture was concentrated under reduced pressure to a dark brownish oil which was suspended in water (50 mL) and extracted three times with $CHCl_3$ (75 mL each). The organic layers were combined, dried (Na_2SO_4), and concentrated under reduced pressure to a yellow solid. The product was precipitated from $CHCl_3$, and the white solid was collected as pure product, while the filtrate was purified by silica gel column chromatography (1% MeOH/$CHCl_3$) to give a white solid (3.72 g, 74%). Mp 158–164 °C. TLC (5% MeOH/$CHCl_3$) R_f = 0.46. ^{1}H NMR ($CDCl_3$) δ 1.46 (s, 9H), 3.39 (q, 6.0, 2H), 3.46 (q, 6.0, 2H), 3.99 (s, 3H), 4.95 (t br, 5.20, 1H), 7.16 (d, 1.80, 1H), 7.26 (t br, 6.0, 1H), 7.53 (d, 1.80, 1H). IR (neat) ν 3346, 3123, 2931, 1685, 1643, 1536, 1493, 1418, 1333, 1274 cm^{-1}. MS (FAB, NBA/TFA) *m/z* (relative intensity) 313 ($M+H^+$, 30). HRMS (FAB, NBA/TFA) *m/z* for $C_{13}H_{21}N_4O_5$: calcd 313.1512, found 313.1508.

***N*-(3-Butoxycarboxamidopropyl)-1-methyl-4-nitropyrrole-2-carboxamide 7b.** The procedure was similar to that for **7a**, except *N*-butoxycarbonyl-1,3-diaminopropane (1.5 g, 8.59 mmol) was used. Total yield: 2.80 g, 100%. Mp 115–116 °C. TLC (5% MeOH/$CHCl_3$) R_f = 0.26. ^{1}H NMR ($CDCl_3$) δ 7.54 (d, 1.80, 1H), 7.38 (s br, 1H), 7.25 (d, 1.8, 1H), 4.80 (t br, 1H), 4.00 (s, 3H), 3.42 (q, 6.30, 2H), 3.24 (q, 6.30, 2H), 1.69 (quintet, 6.0, 2H), 1.47 (s, 9H). IR (neat) ν 3346, 3128, 2576, 2921, 1692, 1649, 1556, 1529, 1420, 1360, 1311, 1267, 1169, 750 cm^{-1}. MS (FAB, NBA) *m/z* (relative intensity) 327 ($M+H^+$, 15). HRMS (FAB) *m/z* for $C_{14}H_{23}N_4O_5$: calcd 327.1668, found 327.1684.

***N*-(4-Butoxycarboxamidobutyl)-1-methyl-4-nitropyrrole-2-carboxamide 7c.** The procedure was similar to that for **7a**, except *N*-butoxycarbonyl-1,4-diaminobutane (2.5 mL, 13.1 mmol) was used. Total yield: 2.42 g, 90.9%. Mp 99–101 °C. TLC (10% MeOH/$CHCl_3$) R_f = 0.50. ^{1}H NMR ($CDCl_3$) δ 1.46 (s, 9H), 1.61 (m, 4H), 3.19 (q, 6.0, 2H), 3.45 (q, 6.0, 2H), 3.99 (s, 3H), 4.80 (br s, 1H), 6.85 (s br, 1H), 7.24 (d, 2.0, 1H), 7.54 (d, 2.1, 1H). IR (neat) ν 3651, 2975, 2932, 1692, 1654, 1311, 1163, 744 cm^{-1}. MS (FAB, NBA) *m/z* (relative intensity) 341 ($M+H^+$, 40). HRMS (FAB) *m/z* for $C_{15}H_{25}N_4O_5$: calcd 341.1825, found 341.1810.

***N*-(2-Butoxycarboxamidoethyl)-1-methyl-4-[1-methyl-4-nitropyrrole-2-carboxamido]pyrrole-2-carboxamide 8a.** A solution of **7a** (2.0 g, 6.4 mmol) in chilled methanol (−20 °C, 100 mL) was hydrogenated over 5% Pd on carbon (500 mg) at room temperature and atmospheric pressure for 6 h. The catalyst was removed by suction filtration. The filtrate was concentrated, and the residue was coevaporated with dry CH_2Cl_2 (5 mL, twice) to give the amine as an orange foam which was used directly in the next step.

To a cooled (0 °C, ice bath) and stirred solution of the amine in dry pyridine (10.35 mL, 0.128 mol), and dry CH_2Cl_2 (20 mL) was added dropwise a solution of the acid chloride **6** [prepared from 1-methyl-4-nitropyrrole-2-carboxylic acid (1.42 g, 0.083 mol) and thionyl chloride (8 mL)] in dry CH_2Cl_2 (10 mL). The reaction was kept at 0 °C for an additional 15 min and then allowed to stir under a drying tube at room temperature for 3 h. The reaction mixture was concentrated under reduced pressure to a dark brownish oil which was suspended in water (50 mL) then extracted four times with $CHCl_3$ (75 mL each). The organic layers were combined, dried (Na_2SO_4), and concentrated under reduced pressure to a dark brown solid. The crude product was suspended in $CHCl_3$ (10 mL) and the yellow solid was filtered as product, while the filtrate was purified by silica gel column chromatography (1–5% MeOH/$CHCl_3$). Total yield: 1.86 g, 66.8%. Mp 220–225 °C. TLC (5% MeOH/$CHCl_3$) R_f= 0.53. ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 9H), 3.32 (q, 5.0, 2H), 3.47 (q, 5.0, 2H), 3.91 (s, 3H), 4.04 (s, 3H), 5.58 (s br, 1H), 6.76 (d, 1.5, 1H), 7.01 (s br, 1H), 7.23 (d, 1.5, 1H), 7.56 (d, 1.5, 1H), 7.61 (d, 1.5, 1H), 9.67 (s, 1H). IR (neat) ν 3380, 3134, 2931, 2354, 1691, 1664, 1568, 1504, 1445, 1392, 1322, 1247 cm^{-1}. MS (FAB, NBA/TFA) *m/z* (relative intensity) 435 ($M+H^+$, 8). HRMS (FAB, NBA/TFA) *m/z* for $C_{19}H_{27}N_6O_6$: calcd 434.1914, found 434.1912.

***N*-(3-Butoxycarboxamidopropyl)-1-methyl-4-[-1-methyl-4-nitropyrrole-2-carboxamido]pyrrole-2-carboxamide 8b.** The procedure was similar to that used for **8a**. Total yield: 4.03 g, 74.4%. TLC (8% MeOH/$CHCl_3$) R_f = 0.23. Mp 165 °C. ^{1}H NMR ($CDCl_3$) δ 9.47(s, 1H), 7.52 (s, 1H), 7.47 (s, 1H), 7.17 (s, 1H), 6.81 (t br, 1H), 6.66 (s, 1H), 5.18 (s,1H), 3.97 (s, 3H), 3.84 (s, 3H), 3.32 (q, 6.0, 2H), 3.13 (q, 5.4, 2H), 1.61 (quintet, 5.7, 2H), 1.38 (s, 9H). IR (neat) ν 3815, 3694, 3564, 3303, 3117, 2921, 2355, 1725, 1681, 1632, 1513, 1305, 1278, 1164, 663 cm^{-1}. MS (FAB, NBA) *m/z* (relative intensity) 449 ($M+H^+$, 50). HRMS (FAB, NBA) *m/z* for $C_{20}H_{29}N_6O_6$: calcd 449.2148 found 449.2126.

***N*-(4-Butoxycarboxamidobutyl)-1-methyl-4-[-1-methyl-4-nitropyrrole-2-carboxamido]pyrrole-2-carboxamide 8c**. The procedure was similar to that used for **8a**. Total Yield: 2.30 g, 75.7%. Mp 193 °C. TLC (10% MeOH/$CHCl_3$) R_f = 0.35. ^1H NMR ($CDCl_3$ and a few drops of DMSO-d_6) δ 1.44 (s, 9H), 1.59 (m, 4H), 3.14 (q, 6.1, 2H), 3.35 (q, 6.3, 2H), 3.90 (s, 3H), 4.04 (s, 3H), 5.01 (br s, 1H), 6.60 (t br, 1H), 6.75 (d, 1.8, 1H), 7.18 (d, 1.5, 1H), 7.56 (d, 2.1, 1H), 7.62 (d, 1.8, 1H), 9.74 (s, 1H). IR (Nujol) ν 3346, 3139, 2844, 1676, 1534, 1485, 1316, 1283, 1158, 869, 738 cm^{-1}. MS (FAB, NBA) *m/z* (relative intensity) 463 ($M+H^+$, 15). HRMS (FAB, NBA) *m/z* for $C_{21}H_{31}N_6O_6$: calcd 463.2305 found 463.2262.

***N*-(2-Butoxycarboxamidoethyl)-1-methyl-4-[-1-methyl-4-{1-methyl-4-nitropyrrole-2-carboxamido}pyrrole-2-carboxamido]pyrrole-2-carboxamide 9a**. A solution of **8a** (1.44 g, 3.3 mmol) in chilled methanol (−20 °C, 150 mL) was hydrogenated over 5% Pd on carbon (700 mg) at room temperature and atmospheric pressure overnight. Standard workup gave the amine as a brown foam which was used directly in the next step.

To a cooled (0 °C, ice bath) and stirred solution of the amine in dry pyridine (5.33 mL, 0.066 mol), and dry CH_2Cl_2 (20 mL) was added dropwise a solution of the acid chloride **6** [prepared from 1-methyl-4-nitropyrrole-2-carboxylic acid (0.729 g, 4.29 mmol) was slowly added thionyl chloride (6 mL)] in dry CH_2Cl_2 (10 mL). The reaction was kept at 0 °C for an additional 15 min and then allowed to stir under a drying tube at room temperature for 3 h. Concentration of the reaction mixture gave a dark brownish oil which was then suspended in water (50 mL) and extracted four times with $CHCl_3$ (75 mL each). The organic layers were combined, dried (Na_2SO_4), and concentrated under reduced pressure to a brown solid which was then purified by silica gel column chromatography (1–3% MeOH/$CHCl_3$). Total yield: 1.07 g, 58.1%. Mp 183 °C. TLC (8% MeOH/$CHCl_3$) R_f = 0.27. ^1H NMR ($CDCl_3$) δ 1.43 (s, 9H), 3.30 (q, 5.0, 2H), 3.46 (q, 5.4, 2H), 3.89 (s, 3H), 3.94 (s, 3H), 4.04 (s, 3H), 5.40 (t br, 4.2, 1H), 6.66 (d, 1.3, 1H), 6.83 (d, 1.2, 1H), 6.95 (t br, 1H), 7.23 (d, 1.60, 1H), 7.27 (d, 1.8, 1H), 7.52 (d, 1.5, 1H), 7.60 (d, 1.6, 1H), 8.46 (s, 1H), 9.78 (s, 1H). IR (neat) ν 3364, 2923, 2359, 1682, 1641, 1517, 1435, 1312 cm^{-1}. MS (FAB, NBA/TFA) *m/z* (relative intensity) 557 ($M+H^+$, 20). HRMS (FAB, NBA/TFA) *m/z* for $C_{25}H_{32}N_8O_7$: calcd 556.2394, found 556.2378.

***N*-(3-Butoxycarboxamidopropyl)-1-methyl-4-[1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamide 9b**. The procedure was similar to that used for **9a**. Total yield: 1.67 g, 60.2%. Mp 136–143 °C. TLC (8% MeOH/$CHCl_3$) R_f = 0.23. ^1H NMR ($CDCl_3$) δ 10.21 (s, 1H), 8.52 (s, 1H), 7.60 (d, 1.5, 1H), 7.50 (s br, 1H), 7.33 (s, 1H), 7.21 (s, 1H), 6.86 (t br, 1H), 6.60 (s, 1H), 6.43 (s, 1H), 4.87 (t br, 1H), 4.05 (s, 3H), 3.93 (s, 3H), 3.86 (s, 3H), 3.45 (q, 5.1, 2H), 3.24 (q, 5.3, 2H), 1.72 (quintet, 5.0, 2H), 1.45 (s, 9H). IR (neat) ν 3323.1, 3118, 2974, 1682, 1651, 1646, 1539, 1513, 1436, 1308, 1251, 1164, 1113 cm^{-1}. MS (FAB, NBA) *m/z* (relative intensity) 570 ($M+H^+$, 50). HRMS (FAB, NBA) *m/z* for $C_{26}H_{34}N_8O_7$: calcd 570.2550, found 570.2549.

***N*-(4-Butoxycarboxamidobutyl)-1-methyl-4-[1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamide 9c**. The procedure was similar to that used for **9a**. Total yield: 1.79 g, 58.3%. Mp 137–146 °C. TLC (10% MeOH/$CHCl_3$) R_f = 0.43. ^1H NMR ($CDCl_3$) δ 1.43 (s, 9H), 1.58 (m 4H), 3.15 (q, 6.6, 2H), 3.45 (q, 6.9, 2H), 3.84 (s, 3H), 3.93 (s, 3H), 4.05 (s, 3H), 4.66 (t br, 1H), 6.28 (t br, 1H), 6.34 (d, 1.2, 1H), 6.62 (d, 1.8, 1H), 7.07 (d, 1.5, 1H), 7.23 (d, 1.6, 1H), 7.28 (d, 1.5, 1H), 7.52 (br s, 1H), 7.60 (d, 1.5, 1H), 8.69 (s, 1H). IR (neat) ν 3346, 2964, 2921, 2844, 1681, 1517, 1436, 1392, 1359, 1310, 1256, 1163, 1114, 815 cm^{-1}. MS (FAB, NBA) *m/z* (relative intensity) 584 ($M+H^+$, 25). HRMS (FAB, NBA/TFA) *m/z* for $C_{27}H_{36}N_8O_7$: calcd 584.2707, found 584.2711.

***N*-(2-Butoxycarboxamidoethyl)-1-methyl-4-[1-methyl-4-(1-methyl-4-{*p*-*N*,*N*-bis-(2-chloroethyl)benzamido}-pyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamide 10a**. *p*-*N*,*N*-Bis(2-chloroethyl)aminobenzoyl chloride was prepared by dissolving *p*-*N*,*N*-bis(2-chloroethyl)aminobenzoic acid (0.623 g, 2.4 mmol) in benzene (5 mL) and thionyl chloride (5 mL) and heating to reflux under a drying tube for 50 min. The excess thionyl chloride and solvent were removed under reduced pressure and the residue coevaporated with dry CH_2Cl_2 (5 mL, twice).

A solution of **9a** (1.02 g, 1.8 mmol) in chilled methanol (−20 °C, 150 mL) was hydrogenated over 5% Pd on carbon (500 mg) at room temperature and atmospheric pressure for 14 h. Removal of the catalyst followed by concentration of the filtrate and coevaporating the residue with dry CH_2Cl_2 (5 mL, twice) gave an amine as a brown foam.

The above acid chloride dissolved in dry CH_2Cl_2 (40 mL) was added dropwise to a chilled (0 °C, ice bath) and stirring solution of the amine and dry triethylamine (0.3 mL, 2.16 mmol) in dry CH_2Cl_2 (20 mL). The reaction mixture was allowed to stir in the ice bath for 15 min and then at room temperature for 5 h. Concentration of the reaction under reduced pressure produced a brown foam. The foam was suspended in water (40 mL), then extracted four times with $CHCl_3$ (75, 50, 50 mL). The organic layers were combined, dried (Na_2SO_4), concentrated to give a tan brown foam which was purified by silica gel column chromatography (1–6% MeOH/$CHCl_3$). Total yield: 0.305 g, 22%. Mp 175–190 °C. TLC (10% MeOH/$CHCl_3$) R_f = 0.30. ^1H NMR (DMSO-d_6) δ 1.39 (s, 9H), 3.06 (q, 5.3, 2H), 3.20 (q, 5.3, 2H), 3.78 (m, 8H), 3,80 (s, 3H), 3.85 (s, 3H), 3.87 (s, 3H), 6.83, (t, 8.0, 2H), 6.85 (d, 1.2, 1H), 7.05 (d, 1.2, 1H), 7.07 (d, 1.8, 1H), 7.19 (d, 1.5, 1H), 7.25 (d, 1.5, 1H), 7.31 (d, 1.8, 1H), 7.85 (d, 8.0, 2H), 7.96 (t br, 1H), 9.91 (s, 1H), 9.95 (s, 1H), 10.02 (s, 1H). IR (neat) ν 3380, 2922, 2355, 1643, 1518, 1436, 1398, 1251, 1169

cm^{-1}. MS (FAB, NBA/TFA) *m/z* (relative intensity) 769 ($M+H^+$, 10). HRMS (FAB, NBA/TFA) *m/z* for $C_{36}H_{45}N_9O_6{}^{35}Cl_2$: calcd 769.2870, found 769.2865.

***N*-(3-Butoxycarboxamidopropyl)-1-methyl-4-[1-methyl-4-(1-methyl-4-[*p*-*N*,*N*-bis-(2-chloroethyl)aminobenzamido]pyrrole-2-carboxamido)pyrrole-2-carboxamido]-pyrrole-2-carboxamide 10b**. The procedure was similar to that used for **10a**. Total yield: 1.88 g, 41.5%. Mp 160–172 °C. TLC (10% MeOH/$CHCl_3$) R_f = 0.28. ^1H NMR (DMSO-d_6) δ 1.38 (s, 9H), 1.58 (q, 6.0, 2H), 2.95 (q, 6.0, 2H), 3.16 (q, 6.6, 2H), 3.76–3.83 (m, 8H), 3.80 (s, 3H), 3.85 (s, 3H), 3.87 (s, 3H), 6.78 (t br, 1H), 6.82 (d, 9.0, 1H), 6.86 (d, 1.2, 2H), 7.05 (d, 1.2, 1H), 7.07 (d, 1.8, 1H), 7.18 (d, 1.2, 1H), 7.25 (d, 1.5, 1H), 7.31 (d, 1.8, 1H), 7.85 (d, 9.0, 2H), 7.96 (t br, 1H), 9.90 (s, 1H), 9.95 (s, 1H), 10.01 (s, 1H). IR (neat) ν 3335, 2932, 1687, 1638, 1600, 1518, 1436, 1256, 1169, 755 cm^{-1}. MS (FAB, NBA) *m/z* (relative intensity) 783 ($M+H^+$, 4). HRMS (FAB, NBA) *m/z* for $C_{37}H_{47}N_9O_6{}^{35}Cl_2$: calcd 783.3026, found 783.3021.

***N*-(4-Butoxycarboxamidobutyl)-1-methyl-4-[1-methyl-4-(1-methyl-4-[*p*-*N*,*N*-bis-(2-chloroethyl)aminobenzamido]pyrrole-2-carboxamido)pyrrole-2-carboxamido]-pyrrole-2-carboxamide 10c**. The procedure was similar to that used for **10a**. Total yield: 0.758 g, 33.3%. Mp 110–115 °C. TLC (10% MeOH/$CHCl_3$) R_f = 0.30. ^1H NMR ($CDCl_3$) δ 1.45 (s, 9H), 1.54 (m, 4H), 3.16 (q, 5.4, 2H), 3.38 (q, 6.0, 2H), 3.69 (t, 6.6, 4H), 3.80 (t, 5.7, 4H), 3.93 (s, 3H), 3.95 (s, 3H), 4.04 (s, 3H), 4.75 (s br, 1H), 6.47 (s br, 1H), 5.99 (d, 1.2, 1H), 6.61 (d, 1.2, 1H), 6.86 (d, 8.0, 2H), 6.76 (d, 1.5, 1H), 7.08 (d, 1.2, 1H), 7.12 (d, 1.2, 1H), 7.20 (s br, 1H), 7.32 (s br, 1H), 7.42 (s br, 1H), 7.59 (d, 1.5, 1H), 7.96 (d, 8.0, 2H). IR (neat) ν 3389, 2932, 1681, 1632, 1556, 1534, 1512, 1436, 1392, 1305, 1251, 1201, 1163, 1103 cm^{-1}. MS (FAB, NBA/TFA) *m/z* (relative intensity) 742 ($M+H^+$-C_4H_9, 5).

***N*-(2-Aminoethyl)-1-methyl-4-[1-methyl-4-(1-methyl-4-[*p*-*N*,*N*-bis-(2-chloroethyl)benzamido]pyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamide hydrochloride 2a**. To a chilled (0 °C, ice bath) and stirring solution of **10a** (0.0585 g, 0.0761 mmol) in dry MeOH (5 mL), under a drying tube, was bubbled in HCl (g) for 10 min. The solution was stirred for another 2 h in the ice bath. At that time the reaction mixture was degassed under a vacuum line. The the reaction was then concentrated under reduced pressure and the residue was coevaporated with dry CH_2Cl_2 (5 mL, twice). The resulting tan residue was **2a** as a hydrochloride salt. Total yield: 53 mg, 100%. Mp 160 °C. TLC (30% MeOH/$CHCl_3$ and a drop of concd NH_4OH) R_f = 0.27. ^1H NMR (DMSO-d_6) δ 2.73 (m, 2H), 3.26 (m, 2H), 3.78 (m, 8H), 3.81 (s, 3H), 3.85 (s, 3H), 3.87 (s, 3H), 6.82 (d, 8.4, 2H), 6.90 (d, 1.8, 1H), 7.05 (d, 1.8, 1H), 7.07 (d, 1.2, 1H), 7.18 (d, 1.8, 1H), 7.24 (d, 1.8, 1H), 7.30 (d, 1.40, 1H), 7.84 (d, 8.5, 2H), 7.98 (t br, 1H), 9.89 (s, 1H), 9.95 (s, 1H), 10.01 (s, 1H). IR (KBr) ν 3400, 3280, 2942, 2354, 1643, 1605, 1517, 1436, 1403, 1267 cm^{-1}. UV (water) λ_{max} 310 nm (ε = 24988). MS (FAB, NBA) *m/z* (relative Intensity) 670 ($M+H^+$, 5). HRMS *m/z* for $C_{31}H_{38}N_9O_4{}^{35}Cl_2$: calcd 670.2424, found 670.2451.

***N*-(3-Aminopropyl)-1-methyl-4-[1-methyl-4-(1-methyl-4-[*p*-*N*,*N*-bis-(2-chloroethyl)benzamido]pyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamide hydrochloride 2b**. The procedure was similar to that used for **2a**. Total yield: 152 mg, 100%. Mp 195–208 °C. TLC (30% MeOH/$CHCl_3$ and a drop of concd NH_4OH) R_f = 0.22. ^1H NMR (DMSO-d_6) δ 1.79 (quintet, 5.0, 2H), 2.80 (q, 5.0, 2H), 3.25 (q, 5.0, 2H), 3.82 (s, 3H), 3.77–3.83 (m, 8H), 3.85 (s, 3H), 3.87 (s, 3H), 6.85 (d, 9.0, 2H), 6.98 (d, 1.8, 1H), 7.07 (d, 1.8, 1H), 7.09 (d, 1.2, 1H), 7.21 (d, 1.8, 1H), 7.25 (d, 1.8, 1H), 7.30 (d, 1.4, 1H), 7.88 (d, 9.0, 2H), 7.94 (s br, 2H), 8.20 (t br, 1H), 9.93 (s, 1H), 9.96 (s, 1H), 10.04 (s, 1H). IR (KBr) ν 3324, 2899, 1638, 1600, 1513, 1468, 1376, 1262 cm^{-1}. UV (water) λ_{max} 312 nm (ε = 37720). MS (FAB, NBA) *m/z* (relative intensity) 684 ($M+H^+$, 2). HRMS *m/z* for $C_{32}H_{40}N_9O_4{}^{35}Cl_2$: calcd 684.2580, found 684.2579.

***N*-(4-Aminobutyl)-1-methyl-4-[1-methyl-4-(1-methyl-4-[*p*-*N*,*N*-bis-(2-chloroethyl)benzamido]pyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamide hydrochloride 2c**. The procedure was similar to that used for **2a**. Total yield: 228 mg, 100%. TLC (30% MeOH/$CHCl_3$ and a drop of concd NH_4OH) R_f = 0.26. Mp 220 °C. ^1H NMR ($CDCl_3$ plus a few drops of DMSO-d_6) δ 1.57 (m, 4H), 2.70 (m, 2H), 3.20 (br q, 4.5, 2H), 3.80 (m, 8H), 3.83 (s, 3H), 3.84 (s, 3H), 3.87 (s, 3H), 6.84 (d, 9.0, 2H), 6.92 (d, 1.4, 1H), 7.07 (d, 1.4, 1H), 7.09 (d, 1.2, 1H), 7.16 (d, 1.2, 1H), 7.24 (d, 1.2, 1H), 7.31 (d, 1.2, 1H), 7.70 (s br, 3H), 7.86 (d, 9.0, 2H), 8.08 (t br, 4.5, 1H), 9.91 (s, 1H), 9.97 (s, 1H), 10.02 (s, 1H). IR (KBr) ν 3367, 3259, 3128, 2942, 1632, 1599, 1512, 1305, 1262, 1201, 1153, 1115, 1055, 807 cm^{-1}. UV (water) λ_{max} 310 nm (ε = 2.11×10^5 M^{-1} cm^{-1}). MS (FAB, NBA) *m/z* (relative intensity) 698 ($M+H^+$, 4). HRMS *m/z* for $C_{33}H_{42}O_4{}^{35}Cl_2$: calcd 698.2737, found 698.2725.

3-[(*N*-Butoxycarbonyl)-N-(2-butoxycarboxamidoethyl)-aminopropyl]-1-methyl-4-nitropyrrole-2-carboxamide 12. The procedure was similar to that used for **7a** except compound **11** was used. Total yield: 828.2 mg, 75.6%. TLC (10% MeOH/$CHCl_3$) R_f = 0.34. ^1H NMR ($CDCl_3$) δ 1.43 (s, 9H), 1.50 (s, 9H), 1.76 (br s, 2H), 3.34 (m, 8H), 4.00 (s, 3H), 4.92 (br s, 1H), 7.31 (s, 1H), 7.56 (s, 1H), 7.84 (s, 1H). IR ($CHCl_3$-cast) ν 3335, 3128, 2953, 1681, 1523, 1414, 1360, 1310 1251, 1164, 750 cm^{-1}. MS (FAB, NBA) *m/z* (relative intensity) 470 ($M+H^+$, 22). HRMS (FAB, NBA) *m/z* for $C_{21}H_{36}N_5O_7$ calcd 470.2615, found 470.2629.

3-[(*N*-Butoxycarbonyl)-N-(2-butoxycarboxamidoethyl)-aminopropyl]-1-methyl-4-[1-methyl-4-nitropyrrole-2-carboxamido]pyrrole-2-carboxamide 13. The procedure was similar to that used for **8a**. Total yield: 1.31 g, 88%. TLC (10% CH_3OH/$CHCl_3$) R_f=0.33. ^1H NMR ($CDCl_3$) δ 1.53 (s, 9H), 1.50 (s, 9H), 1.73 (m, 2H), 3.28 (m, 8H), 3.91 (s, 3H), 4.04 (s, 3H), 4.85 (br s, 1H), 6.56 (s, 1H), 7.12(s, 1H), 7.33 (s, 1H), 7.60 (s, 1H), 8.68 (s br,

1H), 8.73 (s br, 1H). IR ($CHCl_3$-cast) ν 3332, 3128, 2974, 1669, 1508, 1425, 1362, 1312, 1237, 1150, 787, 713 cm^{-1}. MS (FAB, NBA) *m/z* (relative intensity) 591.5 (M^+, 16). HRMS (FAB, NBA) *m/z* for $C_{27}H_{41}N_7O_8$: calcd 591.3017, found 591.3015.

3-[(*N*-Butoxycarbonyl)-N-(2-butoxycarboxamidoethyl)-aminopropyl]-1-methyl-4-[1-methyl-4-[1-methyl-4-nitropyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamide 14. The procedure was similar to that used for **9a**. Total yield: 216.4 mg, 62%. Mp 112–118 °C. TLC (10% $CH_3OH/CHCl_3$) R_f = 0.57. ^1H NMR ($CDCl_3$) δ 1.41 (s, 9H), 1.46 (s, 9H), 1.80 (m, 2H), 3.28 (m, 8H), 3.80 (s, 3H), 3.88 (s, 3H), 4.01 (s, 3H), 4.98 (s, 1H), 6.46 (s, 1H), 6.62 (s, 1H), 7.18 (s, 1H), 7.33 (s, 1H), 7.57 (s, 1H), 7.61 (s, 1H), 7.62 (s, 1H), 8.62 (s, 1H), 9.05 (s, 1H). IR ($CHCl_3$) ν 3323, 3128, 2974, 1656, 1562, 1462, 1436, 1400, 1364, 1307, 1251, 1164, 741, 676, 571 cm^{-1}. MS (FAB, NBA) *m/z* (relative intensity) 713 (M^+, 12). HRMS (FAB, NBA) *m/z* for $C_{33}H_{47}N_9O_9$: calcd 713.3490, found 713.3484.

3-[(*N*-Butoxycarbonyl)-N-(2-butoxycarboxamidoethyl)-aminopropyl]-1-methyl-4-[1-methyl-4-[1-methyl-4-[*p*-*N*,*N*-bis-(2-chloroethyl)aminobenzamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamide 15. The procedure was similar to that used for **10a**. Total yield: 244.2 mg, 63%. Mp 89–95 °C. TLC (5% $CH_3OH/CHCl_3$) R_f = 0.70. ^1H NMR ($CDCl_3$) δ 1.40 (s, 9H), 1.42 (s, 9H), 1.70 (m, 2H), 3.24 (m, 8H), 3.59 (m, 4H), 3.73 (m, 4H), 3.78 (s, 6H), 3.80 (s, 3H), 5.10 (s, 1H), 6.54 (d, 8.1, 2H), 6.60 (s, 1H), 6.62 (s, 1H), 6.70 (s, 1H), 6.86 (s, 1H), 7.14 (s, 1H), 7.15 (s, 1H), 7.20 (s, 1H), 7.79 (d, 7.8, 2H), 8.10 (s, 1H), 8.56 (s, 1H), 9.04 (s, 1H). IR ($CHCl_3$-cast) ν 3314, 2954, 1643, 1518, 1431, 1251, 1169 cm^{-1}. MS (FAB, NBA) *m/z* (relative intensity) 926 (M^+, 3). MS (FAB, NBA) *m/z* for $C_{42}N_{10}H_{56}O_8{}^{35}Cl_2$: calcd 926.3937, found 926.3970.

3-[*N*-(2-Aminoethyl)aminopropyl]-1-methyl-4-[1-methyl-4-[1-methyl-4-[*p*-*N*,*N*-bis-(2-chloroethyl)aminobenzamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]-pyrrole-2-carboxamide dihydrochloride 3. The procedure was similar to that used for **2a**. Total yield: 0.093g, 99%. Mp 190–200 °C. TLC (10% $CH_3OH/CHCl_3$ and three drops glacial acetic acid) R_f = 0.27. ^1H NMR(DMSO-d_6) δ 1.84 (quintet, 7.0, 2H), 2.89 (m, 2H), 3.16 (m, 4H), 3.26 (q, 6.9, 2H), 3.76 (m, 8H), 3.80 (s, 3H), 3.84 (s, 3H), 3.85 (s, 3H), 6.81 (d, 8.4, 2H), 6.93 (d, 1.8, 1H), 7.05 (d, 1.8, 1H), 7.07 (d, 1.8, 1H), 7.18 (d, 1.5, 1H), 7.23 (d, 1.8, 1H), 7.29 (d, 1.8, 1H), 7.85 (d, 8.4, 2H), 8.18 (t br, 6.9, 1H), 8.29 (s, 3H), 9.24 (s br, 2H), 9.90 (s, 1H), 9.94 (s, 1H), 10.01 (s, 1H). IR ($CHCl_3$-cast) ν 2953, 1632, 1599, 1518, 1436, 1398, 1261 cm^{-1}. UV (water) λ_{max} 308 nm ($\varepsilon = 4.58 \times 10^3$ cm^{-1} M^{-1}). MS (FAB, NBA) *m/z* (relative intensity) 727 ($M+H^+$, 8). MS (FAB, NBA) *m/z* for $C_{34}N_{10}H_{45}O_4{}^{35}Cl_2$: calcd 727.3015, found 727.3007.

***N*-(4-Butoxycarboxamido)phenyl-1-methyl-4-nitropyrrole-2-carboxamide 16**. The procedure was similar to that used for **7a** except *N*-BOC-1,4-phenylenediamine (1.00 g, 4.81 mmol) was used. The desired product was crystallized from chloroform to give a yellow solid. Total yield: 3.23 g, 100%. Mp 160–180 °C. TLC (5% $CH_3OH/CHCl_3$) R_f = 0.48. ^1H NMR ($CDCl_3$) δ 1.52 (s, 9H), 4.03 (s, 3H), 6.79 (s, 1H), 7.35 (d, 9.0, 2H), 7.54 (d, 1.8, 1H), 7.57 (d, 9.0, 2H), 7.59 (d, 1.8, 1H), 8.98 (s, 1H). IR ($CHCl_3$ cast) ν 3346, 2921, 1692, 1556, 1496, 1409, 1316, 1251, 1158, 1059 cm^{-1}. MS (FAB/NBA, TFA) *m/z* (relative intensity) 360 (M^+, 12), 361 ($M+H^+$, 7). HRMS (FAB/NBA, TFA) *m/z* for $C_{17}H_{20}N_4O_5$: calcd 360.1435, found 360.1438.

***N*-(4-Butoxycarboxamido)phenyl-1-methyl-4-[1-methyl-4-nitropyrrole-2-carboxamido]pyrrole-2-carboxamide 17**. The procedure was similar to that used for **8a**, except the product was crystallized from chloroform to give a yellow solid. Total yield: 1.94 g, 70%. Mp 200 °C. TLC (5 % $CH_3OH/CHCl_3$) R_f = 0.40. ^1H NMR ($CDCl_3$) δ 1.53 (s, 9H), 3.95 (s, 3H), 4.05 (s, 3H), 7.00 (d, 1.8, 1H), 7.29 (d, 1.8, 1H), 7.39 (d, 8.7, 2H), 7.59 (d, 1.8, 1H), 7.65 (d, 1.8, 1H), 7.68 (d, 9.0, 2H), 8.80 (s, 1H), 9.40 (s, 1H), 9.96 (s, 1H). IR ($CHCl_3$ cast) ν 3335, 3117, 3921, 1692, 1626, 1588, 1512, 1496, 1430, 1392, 1316, 1240, 1163, 1120, 1054 cm^{-1}. MS (FAB/NFA, TFA) *m/z* (relative intensity) 482 (M^+, 20), 483 ($M+H^+$, 20). HRMS (FAB, NBA/TFA) *m/z* for $C_{23}H_{26}N_6O_6$: calcd 482.1914, found 482.1900.

***N*-(4-Butoxycarboxamido)phenyl-1-methyl-4-[1-methyl-4-[1-methyl-4-nitropyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamide 18**. The procedure was similar to that used for **17**. The product was isolated as a yellow solid (1.41 g, 58%). Mp 240–245 °C (dec). TLC (10% $CH_3OH/CHCl_3$) R_f = 0.46. ^1H NMR ($CDCl_3$) δ 1.51 (s, 9H), 3.91 (s, 3H), 3.94 (s, 3H), 4.04 (s, 3H), 7.10 (d, 1.5, 1H), 7.14 (d, 1.8, 1H), 7.25 (d, 1.8, 1H), 7.27 (d, 1.8, 1H), 7.39 (d, 9.0, 2H), 7.59 (d, 9.0, 2H), 7.63 (d, 1.8, 1H), 7.88 (d, 1.8, 1H), 8.79 (s, 1H), 9.49 (s, 1H), 9.82 (s, 1H), 10.17 (s, 1H). IR ($CHCl_3$-cast) ν 3400, 3128, 1692, 1665, 1643, 1656, 1528, 1458, 1430, 1398, 1370, 1305, 1229, 1158, 1109, 1054 cm^{-1}. MS (FAB/NBA, TFA) *m/z* (relative intensity) 604 (M^+, 3) 605 ($M+H^+$, 3). HRMS (FAB, NBA/TFA) *m/z* for $C_{29}H_{32}N_8O_7$: calcd 604.2359, found 604.2394.

***N*-(4-Butoxycarboxamido)phenyl-1-methyl-4-[1-methyl-4-[1-methyl-4-[*p*-*N*,*N*-bis-(2-chloroethyl)aminobenzamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]-pyrrole-2-carboxamide 19**. The procedure was similar to that used for **10a**. Total yield: 0.88 g, 46%. Mp 242 °C. TLC (10% $CH_3OH/CHCl_3$) R_f = 0.31. ^1H NMR ($CDCl_3$) δ 1.53 (s, 9H), 3.74 (m, 4H), 3.82 (m, 4H), 3.88 (s, 3H), 3.92 (s, 3H), 3.93 (s, 3H), 6.80 (d, 9.0, 2H), 7.11 (d, 1.8, 1H), 7.14 (d, 1.8, 1H), 7.16 (d, 1.8, 1H), 7.24 (d, 1.4, 1H), 7.28 (d, 1.4, 1H), 7.32 (d, 1.8, 1H), 7.38 (d, 9.0, 2H), 7.59 (d, 9.0, 2H), 7.90 (d, 9.0, 2H), 9.02 (s, 1H), 9.66 (s,1H), 9.90 (s br, 2H), 9.96 (s, 1H). IR ($CHCl_3$-cast) ν 3302, 3139, 3308, 2964, 1708, 1643, 1605, 1556, 1512, 1463, 1430, 1398, 1365, 1305, 1251, 1158, 1103, 1049 cm^{-1}. MS (FAB/NBA, TFA) *m/z* (relative intensity) 817 (M^+, 2) 818 ($M+H^+$, 2). HRMS (FAB,

NBA/TFA) *m/z* for $C_{40}H_{45}N_9O_6{}^{35}Cl_2$: calcd 817.2870, found 817.2839.

***N*-*p*-Aminophenyl-1-methyl-4-[1-methyl-4-[1-methyl-4-[*p*-*N*,*N*-bis-(2-chloroethyl)aminobenzamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamide 4.** The procedure was similar to that used for **2a**. Total yield: 1.00 g, 100%. Mp 235 °C (dec). TLC (10% $CH_3OH/CHCl_3$) R_f = 0.59. ^1H NMR (DMSO-d_6) δ 3.72 (m, 4H), 3.83 (m, 4H), 3.88 (s br, 2H), 3.92 (s, 3H), 3.93 (s, 3H), 3.94 (s, 3H), 6.74 (d, 8.7, 2H), 7.15 (s, 2H), 7.25 (s, 2H), 7.30 (s, 1H), 7.33 (s, 1H), 7.36 (d, 9.0, 2H), 7.87 (d, 9.0, 2H), 7.93 (d, 8.7, 2H), 9.77 (s, 1H), 9.80 (s, 1H), 9.88 (s, 1H), 9.92 (s, 1H). IR (Nujol) ν 3389, 1637, 1605, 1550, 1512, 1463, 1430, 1403, 1310, 1251, 1201, 1109, 1060 cm^{-1}. UV (water) λ_{max} 312 nm (ε = 4118.6 cm^{-1} M^{-1}). MS (FAB/NBA, TFA) *m/z* (relative intensity) 717 (M^+, 1) 718 ($M+H^+$, 2) HRMS (FAB, NBA/TFA) *m/z* for $C_{35}H_{37}N_9O_4{}^{35}Cl_2$: calcd 717.2345, found 717.2309.

Ethidium displacement assay

To 2 mL of an ethidium bromide solution (10 mM Tris, 1 mM EDTA, 1.3 μM ethidium bromide) at pH 7.4 was added 25 μL of $2A_{260}$ DNA solution in 10 mM sodium phosphate and 0.25 mM EDTA at pH 7.2 and the fluorescence was measured (excitation wavelength = 520 nm, emission wavelength = 620 nm). Aliquots of a 1 mM stock drug solution (1 mg of drug to be tested at room temperature, 1 equiv of 1 M HCl, and the appropriate volume of distilled water to make a 1 mM solution) were added to the solution and the fluorescence was measured after each addition until the fluorescence was reduced to 50%. The apparent binding constants were calculated using the equation: K_{EtBr}[EtBr] = K_{app}[drug], where [drug] = the concentration of drug at a 50% reduction of fluorescence and K_{EtBr} and [EtBr] are known. The concentration of ethidium bromide is 1.3 μM. The binding constants of ethidium bromide to calf thymus DNA, T4 coliphage DNA, poly(dA–dT) and poly(dG–dC) are 1.0×10^7 M^{-1}, 1.0×10^7 M^{-1}, 9.5×10^6 M^{-1}, and 9.9×10^6 M^{-1}, respectively.[6b]

CD titration studies

A $2A_{260}$ DNA solution in 10 mM sodium phosphate and 0.25 mM EDTA (154 μM (bp), 130 μL) at pH 7.2 was added to the cell (1 mm path length) and a CD spectrum was recorded on a Jasco model J710 instrument. Aliquots of a drug solution were added and each of the resulting spectrum was collected. The drug solutions were 1 mM and the amounts of drugs added corresponded to the r' values of 0, 0.025, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.40, 0.60, 0.80, and 1.0, where r' is the number of moles of ligand to the number moles of DNA base pairs. All CD experiments were done at room temperature, and were scanned from 400 to 220 nm. The sensitivity was set at 1 mdeg and the scan speed was set at 500 nm min^{-1}. Three scans were accumulated and automatically averaged by the computer. The λ_{max} mdeg for each spectrum was collected from the raw scans and the final plots were smoothed by the 'box-car' noise reduction program on the computer.

Irreversible binding to DNA

Solutions of $2A_{260}$ calf thymus DNA in 10 mM sodium phosphate and 0.25 mM EDTA (154 μM (bp), 130 μL) at pH 7.2 were treated with freshly prepared 1 mM drug solutions that corresponded to an r' value of 0.35. The solutions were kept at 37 °C for 15 h. At that time, the reaction mixture was transferred to a CD cell (1 mm path length) and scanned. When the spectrum was saved, 65 μL of the solution in the cell was replaced with 1% SDS (65 μL), and after agitating the cell another scan was made. The undissociated fraction (or per cent irreversible binding) was calculated by the formula below: percent irreversible binding = $(2\Delta A^*/\Delta A) \times 100$, where A is the starting CD ellipticity and A* is the ellipticity after dilution with 1% SDS.

Taq polymerase stop assay

The procedure employed was previously described by Ponti et al. (1991). Prior to drug/DNA incubation, plasmid pBr 322 DNA was linearised with an appropriate restriction enzyme to provide a stop for the *Taq* downstream from the primer. The oligodeoxynucleotide primers were 5′-end labelled prior to amplification using T4 polynucleotide kinase and [γ-^{32}P]-ATP (5000 Ci/mmol, Amersham. UK). The labelled primers were purified by elution through Bio-Rad spin columns. The synthetic primer 5′-GCAGCAGATTACGCGCAGAA-3′, identified as SCA, binds to the complementary strand at position 3090–3109 and was used to examine the alkylation on the bottom strand. The primer 5′-GCATTGGTAACTGTCAGACC-3′, identified as SRM, binds in the sequence 3303-3284 and was used to examine the top strand. Linear amplification of DNA was carried out in a total volume of 100 μL containing 0.5 μg of template DNA, 50 pmol of labelled primer, 250 μM of each dNTP, 1 U Red Hot *Taq* polymerase, 20 mM $(NH_4)_2SO_4$. 75 mM Tris–HCl, pH 9.0, 0.01% Tween, 2.5 mM $MgCl_2$, and 0.01% gelatin. After an initial denaturation at 94 °C for 4 min, the cycling conditions were as follows: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, for a total of 30 cycles. After being amplified, the samples were ethanol precipitated and washed with 70% ethanol.

Samples were dissolved in formamide loading dye, heated for 2 min at 90 °C, cooled on ice, and electrophoresed at 2500–3000 V for 3 h on an 80 cm × 20 cm × 0.4 mm, 6% acrylamide denaturing sequencing gel (Sequagel, National Diagnostics). The gels were dried, and X-ray film was exposed to the gels (Hyperfilm, Amersham. UK). Densitometry was carried out on a Bio-Rad GS-670 imaging densitometer.

Cytotoxicity studies

The K562 human chronic myeloid leukemia cells were maintained in RPM1 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 per well, eight wells per 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-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT, Sigma) to an insoluble purple formazan precipitate.[9] Following incubation of the plates for 4 days (to allow control cells to increase in the number by tenfold) 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 per well. 200 mL DMSO 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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