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Benzoyl Nitrogen Mustard Derivatives of Benzoheterocyclic Analogues of Netropsin: Synthesis and Biological Activity

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Abstract—Synthesis, DNA binding properties and biological activity of a series of bis-benzoheterocycle derivatives **5**–**11**, structurally related to the natural dipyrrole antitumor agent netropsin, and tethered to a benzoyl nitrogen mustard (BAM) as alkylating moiety is reported and structure–activity relationships determined. These compounds **5**–**11** have been evaluated for sequence selective alkylating properties and cytotoxicity against murine L1210 and human K562 leukaemia cells. Using as target sequence a portion of the long terminal repeat of the type-1 human immunodeficiency virus, we found that these compounds induce similar patterns of DNA fragmentation. In addition, the results obtained indicate that all synthesized compounds retain a good antiproliferative activity in the submicromolar range, and generally are more active against L1210 than K562 cells. With respect to both these cell lines, compounds **6**, **7**, **10** and **11** showed the greatest potency, ranging from 0.3 to 1 μM, while compounds **8** and **9** exhibit the lowest activity (IC_{50} = 2–12 μM). Among compounds **5**–**11**, the derivative **11** was found to be the most potent member of this class and it is 5 and 10-fold less active than the bis-pyrrole counterpart **2** against K562 and L1210 cell lines, respectively. For compound **11**, the substitution of the C-terminus benzofurane with *N*-methylindole and indole (to give the compounds **5** and **6**, respectively) led to a decrease in cytotoxicity, which is more evident against the K562 cell line. Finally, differences were found among compounds **5**–**11** in induction of K562 differentiation. Some of them (compounds **7**, **8** and **9**) are potent inducers of erythroid differentiation of K562 cells, and could be proposed for differentiation anti-cancer therapy.

Introduction

DNA minor groove alkylating agents are a relatively new class of anticancer agents reported to exhibit high cytotoxic activity in in vitro and in vivo preclinical models.[1] The increased interest on this group of compounds is related to their ability to interact in a sequence-selective fashion to DNA. This class of compounds are members of a small group of DNA-binding ligands, able to interact with the minor groove of double-stranded DNA, with strict sequence specificity. This group includes netropsin, a naturally occuring antibiotic containing two pyrrole-amide units and terminating with an amidino moiety.[2] This molecule exhibits strong antiviral activity and low antitumor capability.[3]

Nevertheless, netropsin has been shown to represent an important model for the design of new cytotoxic minor groove binder derivatives, in which the charged guanidineacetic moiety has been replaced by moieties of mild chemical reactivity with DNA.[4] These structural modifications have been proposed to both improve antitumor activity and modify DNA sequence selectivity.

For instance, the benzoyl nitrogen mustard (BAM) derivative of netropsin, which incorporates a dimethylamino propyl group in the C-terminus (compound **2**), showed very good antileukemic activity, significantly higher than that of netropsin.[5] For **2** the cytotoxic activity is the result of the combination of two moieties, which are per se almost inactive as cytotoxic agents. It should be underlined that this compound is 3-fold more cytotoxic against L1210 with respect to K562 cell line (IC_{50} = 22 and 60 nM, respectively). The synthesis and in vitro antitumor activity of heterocyclic isosters of **2**, in which the pyrrolic rings were both substituted with pyrazole[6] and imidazole[7] nucles, have been reported elsewhere[6,7] and herein further described.

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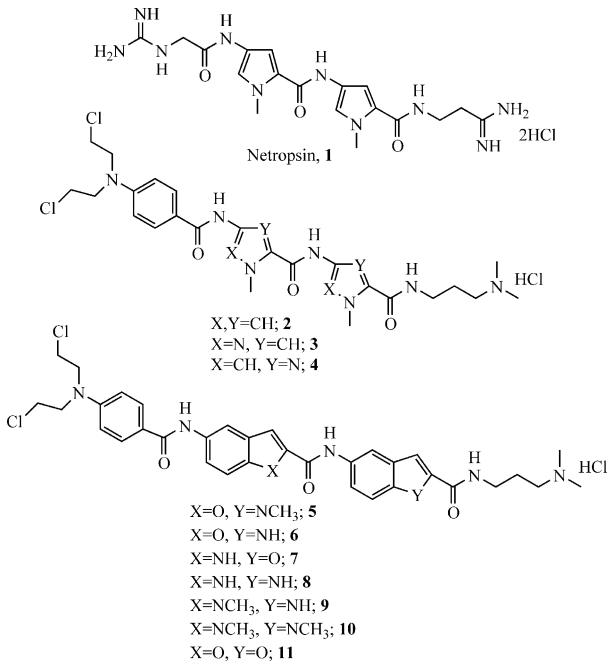
Starting from compound **2**, the replacement of pyrrole with pyrazole, to give the isoster derivative **3**, leads to effects on the antitumor activity, which is 3-fold lower against both L1210 and K562 cell lines (IC_{50} = 72 and 270 nM, respectively). The in vitro antiproliferative activity was much deeply modified by the substitution of pyrrole with imidazole. The bis-imidazole derivative **4** was 30-fold less cytotoxic, on both L1210 and K562 cell lines, than the pyrrolic counterpart **2** (IC_{50} = 469 and 1737 nM for **4** against L1210 and K562 cells, respectively).

Following these results, we report in the present study the synthesis and the biological evaluation of novel isosters of compound **2**, carrying substitutions of both pyrrolic rings by different benzoheterocycles such as indole, *N*-methyl indole and benzofuran, generating the corresponding derivatives **5**–**11**. Since pyrrole is susceptible to oxidative breakdown,[6] these new derivatives have been designed as potentially more stable minor groove binders, in order to improve the chemical stability of the polypyrrolic skeleton. The choice of the employed benzoheterocycles can be also justified by their possible importance in increasing the binding affinity to DNA and the selectivity of alkylation, as found in the case of CC-1065 analogues, such as U-71, 184, adozelesin and bizelesin.[8]



The synthesized compounds **5**–**11** have two moieties in their structure. One is a netropsin-like moiety to acquire DNA minor groove binding activity, and the other is the BAM residue for DNA alkylation.

Chemistry

The synthetic route followed for the synthesis of derivatives **2**–**11** is outlined in Scheme 1. The key step was the coupling between the acid chlorides of benzoheterocyclic carboxylic acids **12**–**17**[9] bearing the BAM moiety and the amines obtained by the catalytic hydrogenation of derivatives **24**–**29**. These condensations were performed at room temperature and with identical reaction times (18 h), in a water/dioxane mixture containing sodium bicarbonate ($NaHCO_3$) as base. Compounds **2**–**11** were obtained in acceptable yields (50–65%), after purification by silica gel flash-chromatography.

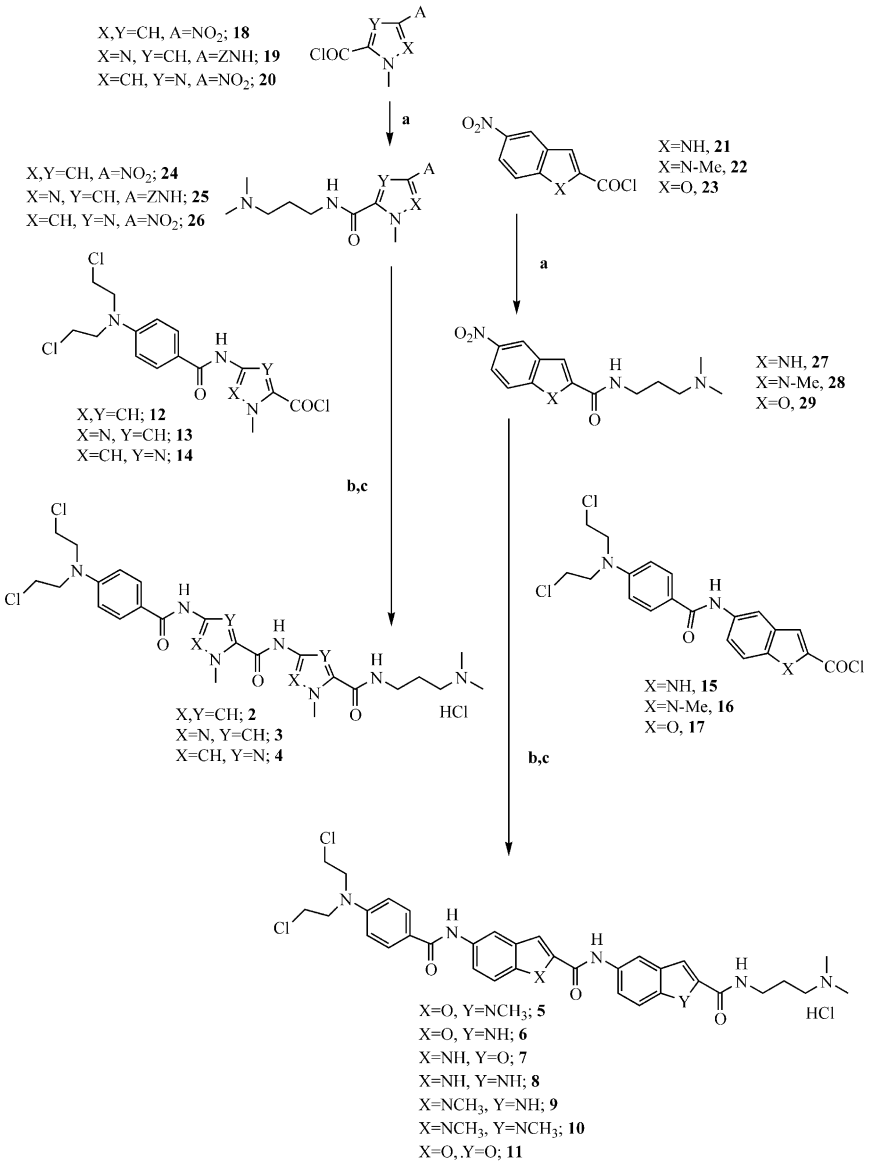
The synthetic approach employed for the preparation of dimethylammino derivatives **24**–**29** is also shown in Scheme 1. Starting from acid chlorides of the well-known nitro-acids **18**–**23**,[10] they were condensed with commercially available 3-dimethylamino-1-propylamine to give the corresponding nitro-amides **24**–**29**.[11] These latter compounds, after catalytic hydrogenation at high pressure (50 psi) with 10% Pd/C at room temperature, were transformed into the corresponding amines as hydrochloride salts in high yield and purity; since these compounds are highly unstable, they were immediately used for the coupling reaction with compounds **12**–**17.**

Biological Evaluations

All synthesized compounds **2**–**11** were evaluated in vitro for their inhibitory effects on the proliferation of both mouse L1210 and human K562 leukemia cells. The results, expressed as IC_{50} values (the concentration of test agent inhibiting cell growth by 50%), are reported in Table 1. The benzoheterocyclic derivatives **5**–**11** showed an activity ranging between 0.3 and 2 μM against murine L1210 leukaemia cell line, while for the human K562 leukaemia cell line the IC_{50} values ranged between 0.3 and 3 μM, with the notable exception of compound **8**, which exhibited very low biological activity (IC_{50} = 11 μM). Generally, with the only exception of **11**, all the benzoheterocyclic derivatives were more active against murine with respect to human leukemic cell line.

Among the benzoheterocyclic derivatives **5**–**11**, none was more active than bis-pyrrole and bis-pyrazole counterparts (compound **2** and **3**, respectively), although some agents showed activity higher than or comparable with that of the bis-imidazole derivative **4**. The results indicate that all benzoheterocyclic compounds exhibits a good antiproliferative activity in the submicromolar range, with the exception of derivatives **8** and **9**, which are the less effectives in the series, with values ranging from 2 to 11 μM. The greatest potency and broadest spectrum of activity against both human and murine leukemia cell lines have been shown by the derivatives **5**–**7**, ranging from 0.3 to 1 μM.

The most active compound in the series of derivatives **5**–**11** was compound **11**, which showed similar potency both on L1210 and K562 cell line (IC_{50} = 290 and 315 nM, respectively). In this compound, the replacement of the benzofurane linked to the dimethylamino propyl moiety with a *N*-methyl indole (compound **5**) had a slight effect on the cytostatic effect against L1210 cell line (IC_{50} = 290 and 370 nM for **11** and **5**, respectively), whereas the potency on K562 decreased by more than



Scheme 1. Reagents: a. 3-dimethylamino-1-propylamine (5 equiv.), dioxane, r.t., 18h; **b.** H_2, 10% Pd/C, 10% HCl in water (5 drops), MeOH; **c: 12-17**, $NaHCO_3$, dioxane/water (1:4, v/v).

Table 1. In vitro antiproliferative activities against murine L1210 and human K562 leukemia cell lines

Compd	IC_{50} (nM)	
	L1210	K562
2[5]	22±2.8	60±15.4
3	72±18	270±40
4	469±85	1737±338
5	370±50	1720±15
6	525±32	950±70
7	550±180	850±8
8	2020±100	11860±1402
9	2100±300	3100±14
10	345±50	3220±32
11	290±10	315±15

IC_{50} = 50% inhibitory concentration represents the mean ±S.D. from dose–response curves of at least three experiments.

5-fold (IC_{50} = 315 and 1720 nM for **11** and **5**, respectively). The same effect has been observed for compound **6**, in which the C-terminus benzofuran was replaced with an indole ring. This latter compound was 2- and 3-fold less active than the bis-benzofuran counterpart **11** against both L1210 and K562 cell lines, respectively.

For the same derivative **11**, the replacement of the benzofuran tethered to the alkylating moiety with an indole (compound **7**) halved the potency on both cell lines. This effect was found to be more dramatic after the substitution of both benzofuran rings with the same number of indole moieties (compound **8**), especially on the K562 cell line. In fact, compound **8** was almost 8- and 35-fold less active than **11** on L1210 and

K562 cell line, respectively. The same effect was not observed for the bis-*N*-methyl indole counterpart (compound **10**), which exhibit the same potency of **11** on L1210 cell line, but was considerably less active than **11** on K562 cells.

When the compound **10** was considered, the replacement of the C-teminus *N*-methyl indole by an indole (compound **9**), causes a maintenance of the same activity on K562 cells and at the same time a 6-fold reduction of the potency on L1210 cells.

In conclusion, the replacement of both pyrrolic rings of the reference compound **2** by benzoheterocycles does not lead to any improvement in terms of cytotoxic activity. No clear-cut structure–activity relationship can be observed and it is difficult to find common physicochemical features among both active and inactive reported compounds herein reported. Nevertheless, the role of heteroatom in the benzoheterocycle moiety on the antiproliferative effect is remarkable. It is noted that other factors such as low penetration into target cells, cellular distribution and metabolic deactivation may also influence the cytoxicity results, but they are not assessed in the present study.

With respect to sequence selectivity, this issue was studied by evaluating the DNA fragmentation activity, as elsewhere reported,[9c] using the upstream portion of the long terminal repeat (LTR) of the human immunodeficiency virus, type 1 (HIV-1) as experimental model system. This sequence does not exhibit A + T runs, but it is recognized by distamycin-related compounds, as suggested by several studies by our research group.[12] We analysed the most active compound **11**, and compared its activity with that of compounds **3**, **4** and **6**. Figure 1A shows that a concentration of 1–5 μM is sufficient for all the compounds to breaking down the full length HIV-1 PCR products in low molecular weight fragments. Figure 1B shows the DNA fragmentation pattern obtained using compound **11**. The fragments are generated at 1 μM concentration at the level of the sequences 5′-ACAAGGGA-3′, 5′-TGGGACT-3′ and 5′-CAGGGAGGC-3′. These DNA sequences surround the two nuclear factor KB (NF-kB) binding sites present within the HIV-1 LTR.[12a] Therefore, these results are in agreement with DNase I footprinting patterns of distamycin-related compounds, which show binding of distamycin and distamycin related compound (including netropsin) to the HIV-1 NF-kB binding sites.[12a] Figure 1C shows that no major differences are found after comparison of the fragmentation patterns obtained using compound **2**, **3**, **6** and **11**.

In order to verify whether antiproliferative activity of the studied compounds is associated with induction of differentiated functions, K562 cells were cultured with drug concentrations causing 50% inhibition of cell growth and the proportion of benzidine-positive (hemoglobin containing) cells determined after 6 days. The results obtained are shown in Table 2, suggesting that most of the compounds, with the exception of those having X = O and/or Y = NCH_3 (such as **5**, **6**, **10** and **11**) induce K562 erythroid differentiation. Analysis by cellogel acetate gel electrophoresis shows that, as found for other antitumor compounds (including cisplatin, cytosine-arabinoside, chromomycin),[13,14] Hb Portland ($\zeta_2\gamma_2$) is the major hemoglobin present in cell lysates from erythroid induced K562 cells. In conclusion, antitumor differentiation therapy could be proposed for compounds **2**–**4** and **7**–**9**.

Table 2. Induction of erythroid differentiation of human K562 leukemia cells

Compd	Erythroid differentiation Benzidine positive cells (%)
2	42 ± 4.3
3	76 ± 5.5
4	30 ± 4.1
5	No induction
6	No induction
7	31 ± 3.8
8	43 ± 5.7
9	32 ± 4.4
10	No induction
11	No induction

The indicated values represent the means ± S.D. of three independent experiments. The proportion of benzidine-positive, Hb containing cells was determined after 6 days of cell culture using a concentration of the compounds causing 50% inhibition of cell growth.

Experimental

Chemistry

All reactions were carried out under Argon atmosphere, unless otherwise described. Standard syringe techniques were applied for transferring anhydrous solvents. Reaction courses and product mixtures were routinely monitored by TLC on silica gel (precoated F_{254} Merck plates) and visualized with aqueous $KMnO_4$. Infrared spectra were recorded on a Perkin-Elmer 1710 spectrophotometer. ^{1}H NMR spectra were obtained in DMSO solutions with a Bruker AC 200 spectrometer. Chemical shifts (δ) are given in parts per million (ppm) upfield from tetramethylsilane. All products reported showed ^{1}H NMR spectra in agreement with the assigned structures. Matrix-assisted laser desorbion ionization time-of-flight (MALDI-TOF) mass spectrometry of all synthesized compounds was conducted using a Hewlett Packard G 2025 A LD-TOF instrument. The samples were analyzed in the linear mode with 28 kV accelerating voltage, mixing them with a saturated solution of a-cyano-4-idroxycinnamic acid matrix. Melting points (mp) were determined on a Buchi–Tottoli apparatus and are uncorrected. Elemental analyses were conducted by the Mycroanalytical Laboratory of the Chemistry Department of the University of Ferrara. Column chromatography was carried out using Merck silica gel (230–240 mash). All compounds obtained commercially were used without further purification. Organic solutions were dried over anhydrous Na_2SO_4. Dioxane was distilled from calcium hydride and anhydrous DMF was distilled from calcium chloride and stored over molecular sieves (3 Å). In high-pressure hydrogenation experiments, a Parr shaker on a high-pressure autoclave was used.

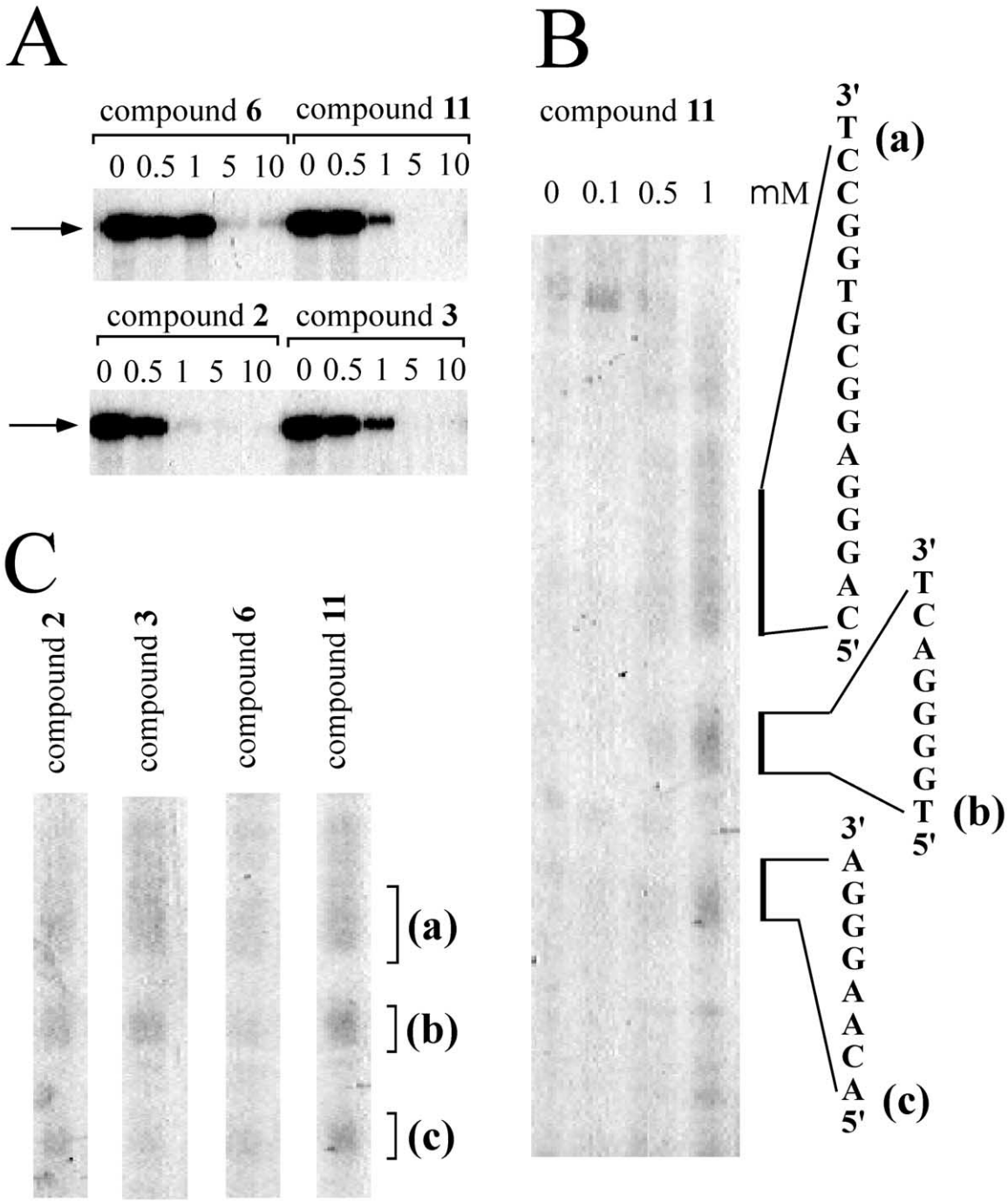


Figure 1. Effects of compounds **2**, **3**, **6** and **11** on recovery of full-length HIV-1 LTR PCR product. In this experiment the 32P-labelled ER PCR product was produced using a 32P-labelled HIV-1 LTR forward PCR primer. After production of the HIV-1 LTR PCR probe, an aliquot was incubated in 50 mL in the absence (−) or in the presence of 0.5, 1, 5 and and 10 mM compounds. After 5 h incubation at room temperature the samples were heated at 90°C for 30 min, ethanol precipitated and analysed by electrophoresed on a sequencing gel. The full lenght PCR product is arrowed. B,C. Accumulation of DNA fragment of low molecular weight. The nucleotide sequences corresponding to DNA fragmentation are indicated in B taking the reactions with compound 11 as representative examples. In panel C a comparison of reactions with 1 mM concentrations of compounds **2**, **3**, **6** and **11** is shown. Reactions with 5 and 10 mM compounds **2**, **3**, **6** and **11** were not informative, since in these conditions, as expected, very short fragments were generated, due to multiple breakages of each 32P-labelled target molecules very close to the 32P-labelled ends.

General procedure for the synthesis of compounds (25–29)
A solution of 3-dimethylaminopropylamine (3 equiv) in dioxane (5 mL) was added dropwise to a stirred solution of **19**–**23** (10 mmol) dissolved in dioxane (10 mL). The reaction mixture was stirred for 18 h, the solvent was removed in vacuo and the residue oil dissolved in EtOAc (15 mL). The organic phase was washed with water (5 mL), brine (5 mL), dried over Na_2SO_4 and concentrated. The residual was recrystallized from ethyl ether to give a powder.

3-(1-Methyl-4-benzyloxycarbonylaminopyrazole-2-carboxamido) dimethylaminopropane (25). Following the general procedure, the compound **25** was isolated as a white powder. Yield: 68%; mp = 123–125 °C; IR (KBr): 3329, 1741, 1642, 1585, 1550, 1474, 1318, 1243 and 1225 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.78 (m, 2H), 2.37 (s, 6H), 2.56 (t, $J = 7.6$ Hz, 2H), 3.54 (q, $J = 6.0$ Hz, 2H), 4.01 (s, 3H), 5.22 (s, 2H), 6.74 (s, 1H), 7.46 (m, 5H), 8.42 (bs, 1H), 8.68 (bs, 1H). FAB-MS (MALDI-TOF): 360.5 $[M+1]^+$.

3-(1-Methyl-4-nitroimidazole-2-carboxamido) dimethylaminopropane (26). Following the general procedure, the compound **26** was isolated as a yellow powder. Yield: 52%; mp = 138–140 °C; IR (KBr): 3144, 1668, 1533, 1382, 1306, 1137 and 998 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.63 (m, 2H), 2.21 (s, 6H), 2.34 (t, $J = 7.2$ Hz, 2H), 3.24 (m, 2H), 4.02 (s, 3H), 6.74 (s, 1H), 8.56 (s, 1H), 8.85 (t, $J = 5.6$ Hz, 1H). FAB-MS (MALDI-TOF): 360.4 $[M+1]^+$.

3-(5-Nitro-1*H*-indole-2-carboxamido) dimethylaminopropane (27). Following the general procedure, the compound **27** was isolated as a yellow powder. Yield: 58%; mp = 173–175 °C; IR (KBr): 3245, 1638, 1560, 1325, 1069 and 748 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.66 (m, 2H), 2.16 (s, 6H), 2.30 (t, $J = 7.2$ Hz, 2H), 3.30 (m, 2H), 7.38 (s, 1H), 7.55 (d, $J = 9.0$ Hz, 1H), 8.06 (d, $J = 9.2$ Hz, 1H), 8.70 (s, 1H), 8.83 (t, $J = 5.2$ Hz, 1H). FAB-MS (MALDI-TOF): 291.1 $[M+1]^+$.

3-(5-Nitro-1-methylindole-2-carboxamido) dimethylaminopropane (28). Following the general procedure, the compound **28** was isolated as a brown powder. Yield: 70%; mp = 124–126 °C; IR (KBr): 3126, 1658, 1546, 1498, 1416, 1305 and 1106 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.68 (m, 2H), 2.14 (s, 6H), 2.26 (t, $J = 6.8$ Hz, 2H), 3.28 (m, 2H), 4.03 (s, 3H), 7.29 (s, 1H), 7.74 (d, $J = 9.2$ Hz, 1H), 8.11 (dd, $J = 9.0$ and 1.8 Hz, 1H), 8.69 (s, 1H), 8.80 (t, $J = 5.2$ Hz, 1H). FAB-MS (MALDI-TOF): 305.4 $[M+1]^+$.

3-(5-Nitrobenzofuran-2-carboxamido) dimethylaminopropane (29). Following the general procedure, the compound **29** was isolated as a white powder. Yield: 73%; mp = 110–115 °C; IR (KBr): 3433, 1663, 1525, 1341, 1270, 1172 and 739 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.66 (m, 2H), 2.11 (s, 3H), 2.14 (s, 3H), 2.27 (t, $J = 7.0$ Hz, 2H), 3.32 (m, 2H), 7.72 (s, 1H), 7.89 (d, $J = 9.2$ Hz, 1H), 8.30 (dd, $J = 9.2$ and 2.4 Hz, 1H), 8.75 (d, $J = 2.4$ Hz, 1H), 9.03 (t, $J = 5.2$ Hz, 1H). FAB-MS (MALDI-TOF): 292.3 $[M+1]^+$.

General procedure for the synthesis of compounds (3–11). A solution of compounds **21**–**26** (1 mmol) dissolved in a mixture of methanol (10 mL) and 10% HCl in water (5 drops), was hydrogenated over 10% Pd/C at 50 p.s.i. After 2 h, the catalyst was removed by filtration through a bed of Celite and the filtrate concentrated in vacuum. The resulting foam solid was used without any purification for the next reaction.

To a cooled solution of the compound obtained by the reduction of **21**–**26** (1 mmol), in a mixture water-dioxane (10 mL, 1/4 v/v) containing $NaHCO_3$ (3 mmol), the derivative **12**–**17** (1.1 equiv) in dioxane (3 mL) was addedd dropwise. After 0.5 h at 0 °C, the reaction mixture was allowed to rise at room temperature and stirred for 18 h. After this time, the solution was carefully acidified (pH 1–2) with 10% HCl in water, evaporated and the residue purified by silica gel column chromatography (eluant dichloromethane (DCM)–MeOH 9.5:0.5 then 9:1). The final product was then purified by precipitation with a mixture methanol/ ethyl ether 1/20 (v/v).

3-(1-Methyl-4-(1-methyl-4-(4-bis (2-chloroethyl) amino benzamido)-pyrazole-2-carboxamido) pyrazole-2-carboxamido) dimethylaminopropane hydrochloride (3). Following the general procedure, the compound **3** was prepared from **25** and **13** and it was isolated as a cream solid. Yield: 61%; mp = 167–169 °C; IR (KBr): 3402, 1655, 1615, 1534, 1456, 1318, 1254 and 1116 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.82 (m, 2H), 2.68 (s, 6H), 3.01 (m, 2H), 3.23 (m, 2H), 3.79 (m, 8H), 4.01 (s, 3H), 4.04 (s, 3H), 6.86 (d, $J = 6.8$ Hz, 2H), 7.45 (s, 1H), 7.72 (s, 1H), 7.94 (d, $J = 6.8$ Hz, 2H), 8.53 (t, $J = 5.6$ Hz, 1H), 9.42 (bs, 1H), 10.1 (bs, 1H), 10.3 (bs, 1H). FAB-MS (MALDI-TOF): 594.3 $[M+1]$, 614.9 $[M+Na]^+$. Anal. calcd for $C_{26}H_{36}Cl_3N_9O_3$: C, 49.65; H, 5.77; Cl, 16.91; N, 20.04. Found: C, 49.45; H, 5.49 Cl, 16.78; N, 19.89.

3-(1-Methyl-4-(1-methyl-4-(4-bis (2-chloroethyl) amino benzamido)-imidazole-2-carboxamido) imidazole-2-carboxamido) dimethylaminopropane hydrochloride (4). Following the general procedure, the compound **4** was prepared from **26** and **14** and it was isolated as a white solid. Yield: 52%; mp = 180–184 °C; IR (KBr): 3392, 1651, 1635, 1526, 1471, 1367, 1254 and 1211 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.74 (m, 2H), 2.73 (s, 6H), 3.04 (m, 2H), 3.32 (m, 2H), 3.78 (m, 8H), 4.01 (s, 3H), 4.06 (s, 3H), 6.96 (d, $J = 6.8$ Hz, 2H), 7.23 (s, 1H), 7.58 (s, 1H), 7.88 (d, $J = 6.8$ Hz, 2H), 8.82 (t, $J = 5.8$ Hz, 1H), 10.1 (bs, 1H), 10.6 (bs, 1H), 11.28 (bs, 1H). FAB-MS (MALDI-TOF): 594.5 $[M+1]$, 633.7 $[M+K]^+$. Anal. calcd for $C_{26}H_{36}Cl_3N_9O_3$: C, 49.65; H, 5.77; Cl, 16.91; N, 20.04. Found: C, 49.48; H, 5.53 Cl, 16.68; N, 19.87.

3-(1-Methyl-5-(5-(4-*N*,*N*-bis(2-chloroethyl)aminobenzene-1-carboxamido)benzofuran-2-carboxamido)-(1*H*)-indole-2-carboxamido) propionamidine hydrochloride (5). Following the general procedure, the compound **5** was prepared from **29** and **17** and it was isolated as a brown powder. Yield: 59%; mp = 130–133 °C; IR (KBr): 3391, 1647, 1605, 1514, 1472, 1346, 1274 and 1235 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.93 (m, 2H), 2.74 (s, 3H), 2.77 (s, 3H), 3.10 (m, 2H), 3.32 (m, 2H), 3.82 (m, 8H), 3.99 (s, 3H), 6.87 (d, $J = 7.4$ Hz, 2H), 7.14 (s, 1H), 7.72 (m, 7H), 8.18 (s, 1H), 8.31 (m, 1H), 8.72 (t, $J = 5.8$ Hz, 1H), 10.1 (s, 1H), 10.3 (bs, 1H), 10.5 (s, 1H). FAB-MS (MALDI-TOF): 678.4 $[M+1]^+$, 700.3 $[M+Na]^+$, 716.2 $[M+K]^+$. Anal. calcd for $C_{35}H_{39}Cl_3N_6O_4$: C, 58.87; H, 5.50; Cl, 14.89; N, 11.77. Found: C, 58.76; H, 5.28 Cl, 14.68; N, 11.56.

3-(5-(5-(4-*N*,*N*-bis(2-chloroethyl)aminobenzene-1-carboxamido)benzofuran-2-carboxamido)-(1*H*)-indole-2-carboxamido)propionamidine hydrochloride (6). Following the general procedure, the compound **6** was prepared from

28 and **17** and it was isolated as a yellow powder. Yield: 68%; mp = 170–172 °C; IR (KBr): 3400, 1636, 1605, 1560, 1515, 1472, 1235, and 807 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.94 (m, 2H), 2.74 (s, 3H), 2.76 (s, 3H), 3.10 (m, 2H), 3.35 (m, 2H), 4.14 (m, 8H), 6.87 (d, J = 9.0 Hz, 2H), 7.16 (s, 1H), 7.66 (m, 5H), 7.92 (d, J = 8.8 Hz, 2H), 8.12 (s, 1H), 8.30 (s, 1H), 8.74 (t, J = 5.8 Hz, 1H), 10.1 (s, 1H), 10.4 (bs, 1H), 10.5 (s, 1H), 11.7 (s, 1H). FAB-MS (MALDI-TOF): 664.4 $[M+1]^+$. Anal. calcd for $C_{34}H_{37}Cl_3N_6O_4$: C, 58.33; H, 5.33; Cl, 15.19; N, 12.00. Found: C, 58.14; H, 5.08 Cl, 15.02; N, 11.77.

3-(5-(5-(4-*N*,*N*-bis(2-chloroethyl)aminobenzene-1-carboxamido)-(1H)-indole-2-carboxamido)-benzofuran-2-carboxamido)propionamidine hydrochloride (7). Following the general procedure, the compound **7** was prepared from **27** and **17** and it was isolated as a brown powder. Yield: 64%; mp = 156–158 °C; IR (KBr): 3400, 1636, 1607, 1519, 1465, 1437, 1273 and 1207 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.95 (m, 2H), 2.75 (s, 3H), 2.77 (s, 3H), 3.10 (m, 2H), 3.34 (m, 2H), 3.81 (m, 8H), 6.85 (d, J = 8.8 Hz, 2H), 7.64 (m, 8H), 8.15 (s, 1H), 8.30 (m, 1H), 8.94 (t, J = 5.8 Hz, 1H), 9.89 (s, 1H), 10.1 (bs, 1H), 10.4 (s, 1H), 11.8 (s, 1H). FAB-MS (MALDI-TOF): 664.5 $[M+1]^+$, 686.4 $[M+Na]^+$. Anal. calcd for $C_{34}H_{37}Cl_3N_6O_4$: C, 58.33; H, 5.33; Cl, 15.19; N, 12.00. Found: C, 58.21; H, 5.13 Cl, 15.00; N, 11.85.

3-(5-(5-(4-*N*,*N*-bis(2-chloroethyl)aminobenzene-1-carboxamido)-(1*H*)-indole-2-carboxamido)-(1*H*)-indole-2-carboxamido)propionamidine hydrochloride (8). Following the general procedure, the compound **8** was prepared from **27** and **15** and it was isolated as a white powder. Yield: 58%; mp = 128–132 °C; IR (KBr): 3401, 1647, 1607, 1517, 1473, 1276 and 1238 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.93 (m, 2H), 2.75 (s, 3H), 2.78 (s, 3H), 3.13 (m, 2H), 3.36 (m, 2H), 3.81 (m, 8H), 6.85 (d, J = 8.8 Hz, 2H), 7.14 (s, 1H), 7.38 (s, 1H), 7.43 (s, 2H), 7.49 (m, 2H), 7.90 (d, J = 7.2 Hz, 2H), 8.12 (s, 2H), 8.72 (t, J = 5.6 Hz, 1H), 9.87 (s, 1H), 10.0 (bs, 1H), 10.1 (s, 1H), 11.6 (s, 1H), 11.7 (s, 1H). FAB-MS (MALDI-TOF): 664.6 $[M+1]^+$, 686.7 $[M+Na]^+$. Anal. calcd for $C_{34}H_{38}Cl_3N_7O_3$: C, 58.42; H, 5.48; Cl, 15.21; N, 14.03. Found: C, 58.22; H, 5.35; Cl, 15.05; N, 13.89.

3-(1-Methyl-5-(5-(4-*N*,*N*-bis(2-chloroethyl)aminobenzene-1-carboxamido)-(1*H*)-indole-2-carboxamido)-(1*H*)-indole-2-carboxamido)propionamidine hydrochloride (9). Following the general procedure, the compound **9** was prepared from **27** and **16** and it was isolated as a yellow powder. Yield: 70%; mp = 134–137 °C; IR (KBr): 3392, 1645, 1554, 1475, 1273 and 757 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.91 (m, 2H), 2.74 (s, 3H), 2.76 (s, 3H), 3.12 (m, 2H), 3.24 (m, 2H), 3.82 (m, 8H), 4.02 (s, 3H), 6.84 (d, J = 8.8 Hz, 2H), 7.11 (s, 1H), 7.69 (m, 7H), 8.22 (s, 1H), 8.35 (m, 1H), 8.69 (t, J = 5.8 Hz, 1H), 10.1 (s, 1H), 10.2 (bs, 1H), 10.8 (s, 1H), 11.6 (s, 1H). FAB-MS (MALDI-TOF): 714.1 $[M+1]^+$. Anal. calcd for $C_{35}H_{40}Cl_3N_7O_3$: C, 58.95; H, 5.65; Cl, 14.91; N, 13.75. Found: C, 58.78; H, 5.45; Cl, 14.74; N, 13.58.

3-(1-Methyl-5-(1-methyl-5-(4-*N*,*N*-bis(2-chloroethyl)aminobenzene-1-carboxamido)-(1*H*)-indole-2-carboxamido)-(1*H*)-indole-2-carboxamido)propionamidine hydrochloride (10). Following the general procedure, the compound **10** was prepared from **28** and **16** and it was isolated as a white powder. Yield: 62%; mp = 110–112 °C; IR (KBr): 3270, 1692, 1639, 1547, 1419, 1326, 1243 and 738 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.86 (m, 2H), 2.72 (s, 3H), 2.76 (s, 3H), 3.10 (m, 2H), 3.28 (m, 2H), 3.79 (m, 8H), 3.99 (s, 3H),4.02 (s, 3H), 6.87 (d, J = 7.8 Hz, 2H), 7.10 (s, 1H), 7.68 (m, 7H), 8.19 (s, 1H), 8.31 (m, 1H), 8.69 (t, J = 5.8 Hz, 1H), 10.1 (s, 1H), 10.2 (bs, 1H), 10.5 (s, 1H). FAB-MS (MALDI-TOF): 728.1 $[M+1]^+$. Anal. calcd for $C_{36}H_{42}Cl_3N_7O_3$: C, 59.47; H, 5.82; Cl, 14.63; N, 13.48. Found: C, 59.33; H, 5.67; Cl, 14.48; N, 13.37.

3-(5-(5-(4-*N*,*N*-bis(2-chloroethyl)aminobenzene-1-carboxamido)benzofuran-2-carboxamido)-benzofuran-2-carboxamido)propionamidine hydrochloride (11). Following the general procedure, the compound **11** was prepared from **29** and **17** and it was isolated as a white powder. Yield: 72%; mp = 156–158 °C; IR (KBr): 3401, 1654, 1607, 1516, 1472 and 1239 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.68 (m, 2H), 2.74 (s, 6H), 3.02 (m, 2H), 3.35 (m, 2H), 3.82 (m, 8H), 6.87 (d, J = 7.0 Hz, 2H), 7.78 (m, 9H), 8.333 (m, 1H), 8.95 (t, J = 5.8 Hz, 1H), 10.1 (s, 1H), 10.2 (bs, 1H), 10.7 (s, 1H). FAB-MS (MALDI-TOF): 665.4 $[M+1]^+$, 688.5 $[M+Na]^+$, 704.4 $[M+K]^+$. Anal. calcd for $C_{34}H_{36}Cl_3N_5O_5$: C, 58.25; H, 5.18; Cl, 15.17; N, 9.99. Found: C, 58.03; H, 4.97; Cl, 15.01; N, 9.78.

Biological assay: growth inhibitory activity on murine L1210 and human K562 cells

The murine lymphocytic L1210 leukemia[11] and the human chronic myelogenous K562[12] cell lines were obtained from the American Type Culture Collection (ATCC). All the tested compounds were dissolved in DMSO at 1 mg/mL immediately before the use and diluted in medium before addition to the cells. Both cell lines were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% FCS (Flow, Irvine, UK), 2 mM L-glutamine (GIBCO), 10 mM β-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin. To determine the effects of the studied compounds on in vitro cell growth, exponentially growing L1210 and K562 cells were exposed to increasing concentrations of drugs and the value of cell number/mL was determined after different days of cell culture using a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL). Results were expressed as IC_{50} (dose causing 50% inhibition of cell growth in treated cultures relative to untreated controls).[13] All experiments were repeated at least twice. For each drug concentration, duplicate cultures were used.

Biological assay: erythroid differentiation of K562 cells

K562 cells containing heme or hemoglobin (Hb) were detected by specific reaction with a benzidine/hydrogen peroxide solution, as reported elsewhere.[14] The final concentration of benzidine was 0.2% in 5 M glacial acetic acid, 10% H_2O_2. In order to analyse hemoglobin production by erythroid induced K562 cells, 2 μL of

total fresh post-mitochondrial cell lysates were electrophoresed on cellulose acetate strips (Poliphor, Balerna, Swisse) in Tris–ethylenediamine–tetraacetic acid (EDTA)–borate buffer. After an electrophoresis of 30 min at 5 mA, the gels were stained with benzidine/hydrogen peroxide solution (1% benzidine in 4.3 M acetic acid, 3% H_2O_2) and photographed.[14]

Direct DNA fragmentation assay

A ^{32}P-labelled HIV-1 LTR PCR product was produced using a ^{32}P-labelled HIV-1 LTR forward PCR primer (5′-ATT TCA TCA CAT GGC CCG AG-3′) and an unlabelled HIV-1-LTR reverse primer (5′-GCA AGC TTT ATT GAG GCT-3′). Taq DNA polymerase was purchased from FINNZYMES OY (Espoo, Finland) and added at 2.5 U/25 μL final concentration. For PCR-mediated amplification the target DNA was 20 ng of pre-amplified and purified HIV-1 LTR PCR products; PCR buffer, Taq DNA polymerase and the four dNTPs were added as elsewhere described. Conditions of PCRs were: denaturation, 92 °C, 1 min; annealing, 55 °C (ER) or 62 °C (Ha-ras), 1 min; elongation, 72 °C, 1 min (25 cycles). After production of the HIV-1-LTR PCR product, an aliquot was incubated in 50 μL of 0.1×SSC in the presence of DNA-binding drugs. After 5 h incubation at room temperature, the samples were heated at 90 °C for 30 min and ethanol precipitated. Each reaction was resuspended in 5 μL of loading dye (0.1% xilene-cyanol, 0.1% bromophenol blue, 0.1 M NaOH: formamide 1:2) and electrophoresed through a sequencing gel as described.[12a]

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