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Synthesis and Growth Inhibition Activity of α-Bromoacrylic Heterocyclic and Benzoheterocyclic Derivatives of Distamycin A Modified on the Amidino Moiety

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Abstract—The design, synthesis and in vitro activities of novel α-bromoacryloyl pyrazole, imidazole and benzoheterocyclic derivatives of distamycin A, in which the amidino moiety has been replaced by moieties of different physico-chemical features are described, and the structure–activity relationships are discussed. In spite of the relevance of these modifications on the distamycin frame, these derivatives showed significant growth inhibitory activity against mouse leukemia L1210 cells. Therefore, the presence of the amidino moiety, and in general of a basic moiety, is not an absolute requirement for biological activity of α-bromoacrylic derivatives of distamycin.

Introduction

Distamycin A **1** is a natural antibiotic belonging to the class of DNA minor groove binding drugs and characterized by an oligopeptidic pyrrolecarbamoyl frame ending with an amidino moiety.[1] This compound binds reversibly to double helical of B-DNA with high selectivity for AT-rich sequences containing at least four adenine-thymine (AT) base pairs. Van der Waals forces and hydrogen bonding play the key role for the DNA binding, while hydrophobic interactions and electrostatic binding component from the cationic end stabilise the complex.[2] Distamycin A was used as DNAminor groove sequence-selective vector of alkylating functions, leading to compounds endowed with relevant cytotoxic and antitumor activity in comparison to that, very weak, of distamycin itself.[3]

Among the cytotoxic derivatives in which a potential alkylating moiety of low chemical reactivity was tethered to the N-terminal position of the distamycin frame, the α-bromoacryloyl derivative of distamycin A **2** showed a good antiproliferative activity ($IC_{50} = 79.6 \pm 14$ nM against L1210 murine leukaemia cell line), more than two order of magnitude greater than that of **1**,[4] further improved in the case of its four rings homologue **3**.[4] The increase from three to four of the number of pyrrolic rings is associated with a 10-fold increase in growth inhibition potency. This possibly arises from a tighter DNA binding, depending on the increased multiplicity of interaction between the pyrrolecarbamoyl units and DNA AT-rich sequences. Recently, the α-bromoacrylamido derivative of four-pyrrole distamycin homologue **3** was found to bind to DNA minor groove AT-rich regions,being however unable to alkylate AT sequences. This finding may suggest that **3** represents the lead of a new class of minor groove binders.[5,6]

The relevant activity and the atypical mechanism of action of **3** stimulated the synthesis of new halogenoacrylic compounds with the aim to optimising their profile of activity and possibly to help in defining their mechanism of action. In previous papers we have described the synthesis and growth inhibition activitiesagainst L1210 murine leukaemia cell line of a series of

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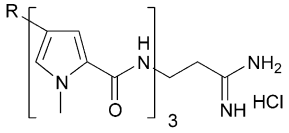
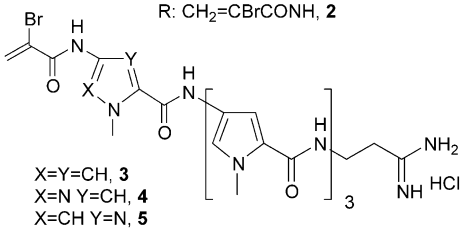
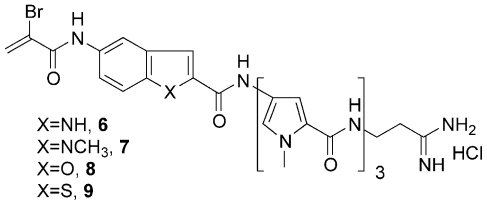
isosteric analogues of the a-bromoacrylamido derivative of four pyrrole homologue of distamycin A **3**.[7,8] The replacing of *N*-methylpyrrole directly linked to the α-halogenoacrylic with isosteric five-membered heterocyclic rings such as *N*-methyl-pyrazole and -imidazole gave the compounds **4** and **5**, respectively,[7] or with different benzoheterocyclic rings, such as indole, *N*-methylindole, benzofuran and benzothiophen, furnished the derivatives **6**, **7**, **8** and **9** respectively.[8] The isosters **4** and **5** were 2- and 7-fold less actives than **3** ($IC_{50}=6.3\pm1.3$ nM for **3** vs $IC_{50}=13.3\pm0.5$ nM and 46.9 ± 13 nM for **4** and **5**, respectively). For the derivatives **6–9**, the antileukemic activity was only slightly affected by the kind of heteroatom present in the benzoheterocyclic ring ($IC_{50}=4.1\pm1.3$ nM, 2.4 ± 0.4 nM, 6.1 ± 0.4 nM and 14.3 ± 5.9 nM for **6**, **7**, **8** and **9**, respectively).



R=NHCHO, Distamycin A, **1**
R: CH_2=$CBrCONH$, **2**

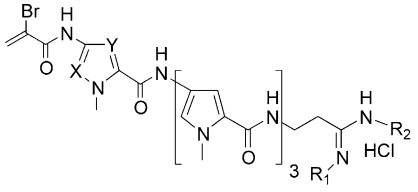


X=Y=CH, **3**
X=N Y=CH, **4**
X=CH Y=N, **5**

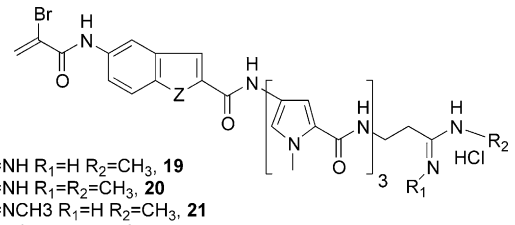


X=NH, **6**
X=NCH_3, **7**
X=O, **8**
X=S, **9**

A feature of **3** is the presence of an amidino moiety that, due to its strong basic nature (its pK_a should be about 12.5), exhibits a complete protonation in any biological conditions, and may play a key role both for DNA binding and cell or tissue bioavailability. In compound **3** the replacement of the amidino group with basic or non-basic amidino moieties of different nature led to compounds in which the potent cytotoxicity of the parent amidino derivative was fully maintained and in some cases even increased. This occured not only in the case of basic amidino-like compounds of different lipophilicity and bulk, such as *N*-methylamidine (**10**, $IC_{50}=2.7\pm0.78$ nM) and *N,N′*-dimethylamidine (**11**, $IC_{50}=1.9\pm0.18$ nM), but also by non basic amidino-derived such as cyanamidine (**12**, $IC_{50}=4.1\pm0.3$ nM). The finding that the amidino moiety of the α-bromoacryloyl derivative **3** could be replaced by other amidino-like or non basic-amidino-like moieties of different nature confirms therefore that the presence of a basic moiety is not an absolute requirement for in vitro activity against L1210 cell line.[9]

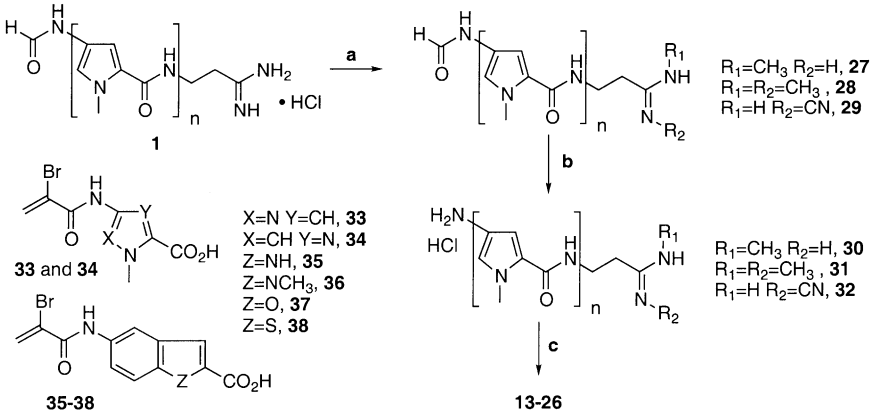


X=Y=CH R_1=H R_2=CH_3, **10**
X=Y=CH R_1=R_2=CH_3, **11**
X=Y=CH R_1=CN R_2=H, **12**
X=N Y=CH R_1=H R_2=CH_3, **13**
X=N Y=CH R_1=R_2=CH_3, **14**
X=N Y=CH R_1=CN R_2=H, **15**
X=CH Y=N R_1=H R_2=CH_3, **16**
X=CH Y=N R_1=R_2=CH_3, **17**
X=CH Y=N R_1=CN R_2=H, **18**



Z=NH R_1=H R_2=CH_3, **19**
Z=NH R_1=R_2=CH_3, **20**
Z=NCH3 R_1=H R_2=CH_3, **21**
Z=NCH3 R_1=R_2=CH_3, **22**
Z=O R_1=H R_2=CH_3, **23**
Z=O R_1=R_2=CH_3, **24**
Z=S R_1=H R_2=CH_3, **25**
Z=S R_1=R_2=CH_3, **26**

In this article we reported the synthesis and growth inhibitory activity of a new series of α-bromoacrylic derivatives, **13–26**, structurally related to compounds **4–9**, in which (a) the N-terminal pyrrole was replaced with



Scheme 1. CH_3NH_2, DMF, rt (for the synthesis of 27), CH_3NH_2, DMF 80 °C (for the synthesis of **28**) NH_2CN, NaH, DMF, rt (for the synthesis of **29**); (b) HCl in EtOH, rt; **c: 33–38,** EDC, DIPEA, DMF.

both pentatomic heteroaromatic units, such as pyrazole or imidazole rings, and benzoheterocyclic units, such as indole, *N*-methylindole, benzofurane and benzothiophene and (b) the amidino moiety was replaced by basic and non-basic groups of different electronic nature, lipophilicity and bulk. While *N*-methylamidine derivatives **13**, **16**, **19**, **21**, **23** and **25** and *N*, *N*-dimethylamidine derivatives **14**, **17**, **20**, **22**, **24** and **26** are strongly basic amidine compounds (pK_a values for the *N*-methylamidine and *N*, *N*-dimethylamidine residues are 12.37 and 12.68, respectively), cyanoamidines **15** and **18** are not basic at all ($pK_a = 0.256$ for the cyanoamidine fragment).[10]

Chemistry

The novel derivatives **13–26** were obtained following the procedure reported in the Scheme 1. The starting material utilized for the synthesis of the cited compounds was Distamycin A **1**.[1] The *N*-methylamidine **27** and *N*,*N*-dimethylamidine **28** were prepared by reacting **1**, dissolved in DMF, with 3 equiv of aq CH_3NH_2, at 25 °C or 6 equiv of aq CH_3NH_2 at 80 °C, respectively, whereas the cyanamidine **29** was prepared from Distamycin A with 3 equiv of NH_2CN sodium salt, obtained in situ with NaH in DMF. These amidino modified Distamycin A derivatives **27–29** were then transformed in the corresponding desformyl derivatives **30–32**.[9] The condensation of **30–32** with the appropriate α-bromoacryloyl heterocyclic/benzoheterocyclic-2-carboxylic acid derivative **33–38**[7,8] was performed using an excess (2 equiv) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI) as the coupling agent, in DMF as a solvent, in the presence of Hunig's base, at room temperature and with identical reaction times (18 h), to obtain the final compounds **13–26** in good yields after purification by silica gel flash-chromatography.

Results and Discussion

All the newly synthesized compounds have been firstly assayed in vitro on L1210 murine leukemia cells, in order to obtain preliminar informations on the effects on cell growth. We employed the L1210 screening system since this cell line has been claimed to be predictors of clinically useful anticancer drugs.[11–15] L1210 cells were treated continously for 48 h with increasing amounts of the tested compounds and the number of viable cells were compared with that of control untreated cells. The results obtained demonstrate that all of the tested compounds displayed growth inhibition effects, despite with different activities. The growth inhibitory activities of the synthesized compounds were expressed as nanomolar concentrations of the compound that inhibited 50% of cell proliferation (IC_{50}) and are compared with the IC_{50} values in the same cell lines of distamycin A **1**, Doxorubicin (DX) and the distamycin derivatives **2–12** (Table 1, left column).

With the only exception of distamycin A, compounds **16** and **18** ($IC_{50} = 10$,[4] 133 and 186 nM, respectively), all tested molecules exhibited strong growth inhition activities on L1210 murine leukemia cell line, with IC_{50} ranged between 2 and 80 nM. For the compounds which possess the same modified amidine terminus, the replacement of the N-terminal pyrrole ring with indole and *N*-methyl indole keep the activity substantially unchanged (compounds **10**, **19**, **21** and **11**, **20**, **22**). By comparing the activities of compounds in which the N-terminal pyrrole (compounds **10–12**) has been substituted with a pyrazole (derivatives **13–15**), it appears that the presence of a basic moiety is not an absolute requirement for in vitro activity. This confirms the finding already reported[9] which contrasts with the common opinion that electrostatic interactions between the cationic moiety and the negatively charged DNA phosphate residues may represent one of the main contributions to molecular recognition of distamycin-like derivatives. The neutral or positive role played by the modifications of the amidino moiety is confirmed also in the case of pyrazole and benzoheterocyclic derivatives **13–15** and **19–26**, respectively. These compounds maintain or even improve the cytotoxicity of the amidine parent compounds **4** and **6–9**, respectively. Moreover, in the series of pyrazolic amidino modified-derivatives **13–15** on the L1210 cell line, the non-basic cyanamidine derivative **15** is not only more active than the *N*-methylamidine and *N*,*N*-dimethylamidine derivatives **13** and **14** respectively, but also more potent than the parent amidine compound **4**. On the other hand, the imidazole *N*-methylamidine **16** and cyanamidine **18** derivatives showed a decreased activity in comparison to the parent

Table 1. In vitro activity and resistance index (R.I.) of distamycin derivatives against L1210 and L1210 murine leukemia cells resistant to the doxorubicin (DX)

Compound	IC_{50} (nM ± S.E.)		R.I[a]
	L1210	L1210/DX	
Distamycin A[4]	10,069 ± 1647	459,000 ± 5216	45.6
2[4]	79.6 ± 14	700.0 ± 109	8.8
3[4]	6.3 ± 1.34	24.0 ± 2	3.8
4[7]	13.3 ± 0.54	93.1 ± 17	7
5[7]	46.9 ± 13	726.9 ± 6.9	15.5
6[8]	4.1 ± 1.3	35.8 ± 5.6	8.7
7[8]	2.4 ± 0.39	43.6 ± 1.5	18
8[8]	6.1 ± 0.93	39.0 ± 2	6.4
9[8]	14.3 ± 5.89	48.3 ± 2	3.4
10[9]	2.7 ± 0.78	58.6 ± 6.4	21.7
11[9]	2.0 ± 0.18	26 ± 7.2	13
12[9]	4.1 ± 0.27	75.6 ± 5.71	18.3
13	10.3 ± 0.26	38.6 ± 3	3.8
14	25.6 ± 3.75	115.7 ± 3.4	4.5
15	7.7 ± 0.34	41.0 ± 4	5.3
16	155.1 ± 52.9	970.0 ± 11	6.3
17	59.7 ± 15.6	200.2 ± 4.1	3.4
18	186 ± 11.48	914.8 ± 15.2	4.9
19	4.5 ± 0.25	23.5 ± 7	5.2
20	7.6 ± 2.84	26.7 ± 7	3.5
21	4.8 ± 0.49	38.3 ± 1.11	7.9
22	2.8 ± 0.36	15.4 ± 2	5.6
23	9.5 ± 1.76	33.3 ± 9	3.6
24	6.3 ± 0.62	38.6 ± 1	6.1
25	10.5 ± 1.48	53.2 ± 4	5.1
26	17.9 ± 6.66	62.3 ± 5	3.5
Doxorubicin	21.8 ± 1.33	601 ± 37.6	27.6

IC_{50} = 50% inhibitory concentration as the mean ± SE from dose–response curves of at least three experiments.
[a]R.I (resistance index) = ratio between IC_{50} values on resistant cells

compound **5**, while, on the contrary, the activity of the imidazolic *N*,*N*′-dimethyamidine derivative **17** appears good and roughly comparable to that of the parent amidino derivative **5**. The low antiproliferative potency of imidazole derivatives **16** and **18** contrast with the significant activity of pyrazole counterparts **13** and **15** which possess the basic *N*-methylamidine and the non-basic cyanamidine moieties, respectively. These data indicate a lack of correlation between the basicity of the amidine-like structure and growth inhibition activity, where this latter depends only from the isosteric five-membered heterocyclic ring (*N*-methyl-pyrazole and -imidazole) joined to the α-bromoacrylic moiety.

Table 1 (central and right columns) shows also the growth inhibitory activities and the resistance index (R.I.) values of **1**–**26** on L1210 leukemic cell line resistant to doxorubicin (DX) and compared with that of DX. The results obtained show that the newly synthesized compounds **13**–**26** display low R.I. values, ranging from four to eight, showing that neither the modification on the amidino moiety nor the heterocycle joined to the α-bromoacryloyl moiety were involved in the resistance mechanism.

In addition, the second set of experiments was performed using as target cells two human cell lines, the K562 cell line, from a patient with chronic myelogenous leukemia in blast crisis[16] and the B-lymphoid Raji[17] cell line, from a B-lymphoma. This evaluation was undertaken in order to ascertain whether the compounds were cytotoxic to human neoplastic cells. In this case we investigated the in vitro antiproliferative activity measured after 4 days (K562 cells) and 5 days (Raji cells), when control untreated cells are in the log phase of cell growth. This is required in order to compare the activities of the same compounds on cell lines exhibiting different proliferation rates. The growth inhibitory activities of the synthesized compounds are shown in Table 2 were expressed as μmolar concentrations of the compound that inhibited 50% of cell proliferation (IC_{50}). Usually, the IC_{50} values obtained using K562 cells were lower with respect to those obtained by using Raji cells. These differences are not unexpected, since it is well known that cells from different origins and histotype or exhibiting different cell growth kinetics could display sharply different IC_{50} values when exposed to antiproliferative compounds.[18] In any case, when compounds are compared within the same cell line, the results obtained are convergent and in agreement from those obtained using the L1210 screening system.

The data reported in the Tables 1 and 2 reveal that L1210 cells are the most sensitive to these compounds in contrast to K562 and Raji cells , while the B-lymphoid Raji cell line is generally more resistant to tested derivatives. The huge differential in the activity of these compounds toward murine and human cell lines may be due, in part at least, to the fact that L1210 cells grow far more quickly than both K562 and Raji cells.

In order to determine the efficiency of interaction with DNA, arrested polymerase-chain reaction (PCR) was performed on selected compounds **3**, **9**, **11**, **13**, **24** and **25**. In a single control experiment, the antiproliferative activity of the same compounds used in PCR assays, was assayed both on K562 and Raji cells after 5 days cell culture. We have elsewhere reported that arrested PCR is a valuable tool to determine difference in the efficiency of interactions between DNA-binding drugs and target DNA molecules.[19–22] The results obtained are shown in Table 3 and suggest that the compounds tested show the ability to interact with DNA, but only at high concentrations (5–80 μM). In addition, a good relationship between efficiency in the arrested PCR

Table 2. Effects of distamycin derivatives on in vitro cell growth of human erythroleukemic K562 and B-lymphoid Raij cell lines

Compd	IC_{50} (μM ± SE)	
	K562	Raji
Distamycin A[4]	20 ± 2.2	35 ± 2.3
2[4]	1.11 ± 0.2	1.7 ± 0.13
3[4]	0.045 ± 0.013	0.046 ± 0.01
4[7]	0.51 ± 0.032	1.2 ± 0.11
5[7]	1.4 ± 0.07	3.7 ± 0.13
6[8]	0.58 ± 0.035	2.2 ± 0.14
7[8]	0.29 ± 0.05	1.2 ± 0.011
8[8]	1.2 ± 0.13	1.4 ± 0.15
9[8]	1.26 ± 0.09	1.41 ± 0.12
10[9]	0.038 ± 0.0072	0.510 ± 0.023
11[9]	0.031 ± 0.0027	0.0462 ± 33
12[9]	0.083 ± 0.0021	0.160 ± 0.023
13	0.682 ± 0.13	6.25 ± 0.23
14	0.70 ± 0.11	3.32 ± 0.28
15	2.5 ± 0.16	8.01 ± 0.36
16	1.53 ± 0.23	5.11 ± 0.35
17	1.75 ± 0.23	5.51 ± 0.48
18	2.85 ± 0.28	12.12 ± 1.7
19	0.530 ± 0.09	1.82 ± 0.23
20	0.85 ± 0.11	2.11 ± 0.22
21	0.72 ± 0.11	2.40 ± 0.29
22	0.035 ± 0.007	0.67 ± 0.0082
23	0.99 ± 0.19	2.16 ± 0.22
24	1.18 ± 0.14	1.65 ± 0.21
25	1.51 ± 0.10	1.93 ± 0.21
26	1.43 ± 0.34	7.51 ± 0.45
Doxorubicin	0.053 ± 0.0052	0.068 ± 0.0023

IC_{50} = concentration of the compound (μM) causing 50% inhibition of cell proliferation as the mean ± SE from dose–response curves of at least three experiments.

Table 3. In vitro effects of distamycin derivatives on arrested PCR and on cell growth both of K562 and Raji cell lines

Compd	Polymerase-chain reaction (PCR) IC_{50}[a] (μM)	K562 IC_{50}[b] (μM)	Raji IC_{50}[b] (μM)
Distamycin A	200	25	39
3	5	0.025	0.045
9	30	1.5	1.5
11	8	0.05	0.05
13	80	1	8
24	50	1.7	2.2
25	50	2.5	2

[a]Inhibitory concentration (μM) necessary to obtain 50% inhibition of generation of PCR product.
[b]Concentration causing 50% inhibition of growth both of human erythroleukemic K562 and B-lymphoid Raji cells.

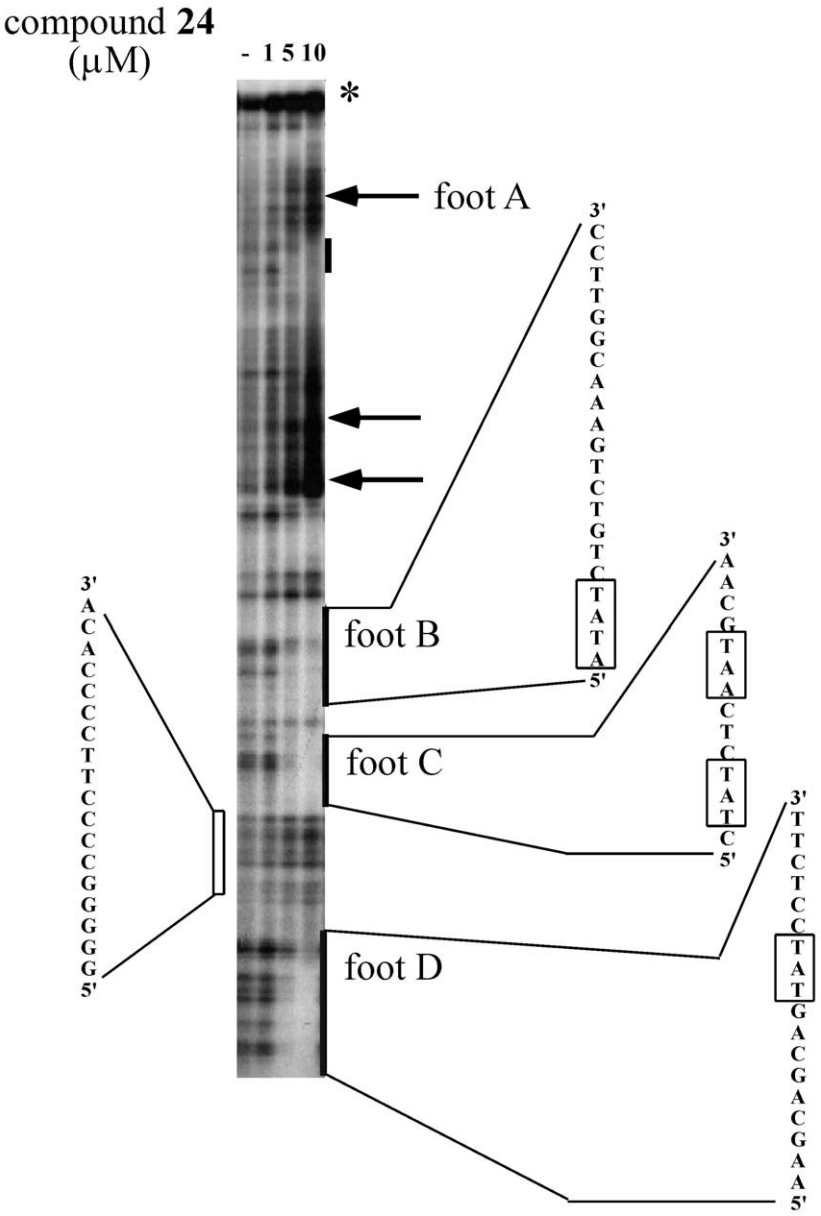


Figure 1. DNase I footprinting assay. In this experiment a ^{32}P-labelled γ-globin gene PCR product was produced using ^{32}P-labelled forward (5′-GGA ATG ACT GAA TCG GAA CAA GGC-3′) and unlabelled reverse (5′-CTG CTG AAG GGT GCT TCC TTT TAT-3′) PCR primer. An aliquot of the footprinting probe was incubated in 50 μL in the absence (−) or in the presence of 1 (a), 5 (b) and 10 (c) μM compound **24**. After 30 min incubation at room temperature the samples were treated with 1U/reaction of DNAse I (2 min), ethanol precipitated and analysed by electrophoresis on a sequencing gel. The nucleotide sequences corresponding to the DNase I footprints are indicated. ATA/TATA motifs are boxed. Arrows indicate DNase I hypersensitive sites. Asterick indicates full sized PCR products.

assay and inhibitory effects of cell growth of K562 and Raji cells was obtained (Table 3).

With respect to selectivity of the interaction with the DNA, an example of the results obtained is shown in Figure 1, depicting the DNase I footprinting[23] obtained using compound **24** at different concentrations (1, 5 and 10 μM, respectively). As clearly appreciable, when a DNA mimicking the γ-globin gene promoter is incubated with DNase I after treatment with increasing amounts of compound **24**, clear footprints are detectable (Fig. 1, right side of the panel) only at 10 μM, as well as DNase I hypersensitive regions (arrowed). Interestingly, all the footprints obtained contain TAT or TATA elements, sustaining the concept that these drugs belongs to AT-binders. Accordingly, lack of footprints is related to a lack of TAT/TATA elements (Fig. 1, left side of the panel). Similar results were obtained with the other compounds, when used at concentrations allowing efficient binding to DNA.

In conclusion, the positive role played by some modifications of the amidino moiety is confirmed also in the case of pyrazole and benzoheterocyclic derivatives, and some of these compounds maintain or even improve the growth inhibitory activity of the parent

compounds **4** and **6–9**, respectively. On the other hand, the imidazolic amidine derivatives **16** and **18** showed a decreased activity in comparison to the parent compound **5**, and this may be due to the drug's ability to enter the cell. The new synthesized compounds **13–26** showed the capability to interact with DNA at high concentrations with selectivity for AT-rich sequences. Although the mechanism of action of the α-bromoacryloyl derivatives **13–26** is still unknown, it may be hypothised that they are a new class of minor groove binders acting through a mechanism different from a direct DNA alkylation.

Experimental

Chemical materials and methods. General procedure

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 thin-layer chromatography on silica gel (precoated F_{254} Merk plates), the spots were examined with UV light and visualized with aq KMnO4. Infrared spectra were recorded on a Perkin-Elmer 1710 spectrophotometer. ^{1}H NMR spectra were recorded in the given solvent with a Bruker AC 200 spectrometer. Chemical shifts are reported as (δ) values in parts per million. The splitting pattern abbreviations are as follows: s (singlet), d (doublet), dd (double doublet), t (triplet), br (broad), and m (multiplet). 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 α-cyano-4-idroxycinnamic acid matrix. Melting points (mp) were determined using a Buchi-Tottoli apparatus and are uncorrected. All products reported showed ^{1}H NMR spectra in agreement with the assigned structures. Mass spectra were recorded on a Nermag R10, 10C spectrometer. Elemental analyses, conducted by the Mycroanalytical Laboratory of the Chemistry Department of the University of Ferrara, were within 0.4% of the theorical values calculated for C, H, Br, Cl and N. Column chromatography was carried out with Merck silica gel (230–240 mash). All compounds obtained commercially were used without further purification. Organic solutions were dried over anhydrous $MgSO_4$. Methanol was distilled from magnesium turnings, 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.

Synthesis of the 5-α-bromoacrylamido 2-benzothiophene-2-carboxylic acid (38). A solution of 5-nitro-benzothiophen-2-carboxylic acid[24] (1.15 g., 5 mmol) in dry DMF (10 mL) cooled at 0 °C was treated with CDI (1.1 g, 6.5 mmol). After 1 h at room temperature, *t*-butanol (1 mL) and DBU (0.9 mL) were added. The mixture was stirred for 2 h, poured in cooled water (50 mL) and the resulting crystalline solid collected and washed with water, affording the desired *t*-butyl-5-nitro benzothiophen-2-carboxylate as a white solid (963 mg, 69% yield); mp = 175–177 °C; IR (KBr): 1717, 1533, 1370, 1370, 1343, 1253, 1156 and 831 cm^{-}; ^{1}H NMR (CDCl3) δ: 1.63 (s, 9H), 7.96 (d, J = 8.8 Hz, 1H), 8.09 (s, 1H), 8.27 (dd, J = 8.8 and 2.2 Hz, 1H), 8.75 (d, J = 2.2 Hz, 1H).

The *t*-butyl-5-nitro benzothiophen-2-carboxylate (5 mmol) was reduced over 10% Pd/C (100 mg) in a mixture of dioxane (10 mL) and methanol (10 mL) at room temperature under 55 psi pressure. After reduction, the catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. The residue,after purification by flash-chromatography (EtOAc–petroleum ether, 1:1, v/v) was dissolved in EtOAc and precipitated with an excess of petroleum ether. The *t*-butyl-5-aminobenzothiophen-2-carboxylate was obtained as a yellow oil (1.25 g, >95%). ^{1}H NMR (CDCl3)d: 1.59 (s, 9H), 3.44 (bs, 2H), 7.44 (d, J = 8.4 Hz, 1H), 7.62 (s, 1H), 8.22 (dd, J = 8.4 and 2.2 Hz, 1H), 8.55 (d, J = 2.2 Hz, 1H).

To a solution of α-bromoacrylic acid (600 mg, 4 mmol) in dry DMF (5 mL), a solution of EDC (383 mg, 2 mmol) in dry DMF (5 mL) was slowly added. After 1 h, the solution obtained was added to a solution of *t*-butyl-5-aminobenzothiophen-2-carboxylate (500 mg, 2 mmol) in dry DMF (5 mL). The reaction was stirred at room temperature for 18 h, and then concentrated under reducedpressure. The residue was dissolved with a mixture of EtOAc (10 mL) and water (5 mL), the organic phase dried over Na_2SO_4, evaporated to dryness in vacuo and the resulting crude residue purified by flash chromatography (EtOAc–petroleum ether, 2:8, v/v) furnished *t*-butyl-5-bromoacrylamidobenzothiophen-2-carboxylate as a orange oil (587 mg, 77% yield). ^{1}H NMR ($CDCl_3$) δ: 1.61 (s, 9H), 6.17 (d, J = 1.6 Hz, 1H), 6.38 (d, J = 1.6 Hz, 1H) 7.50 (dd, J = 8.6 and 2.2 Hz, 1H), 7.81 (d, J = 8.6 Hz, 1H), 7.94 (s, 1H), 8.27 (d, J = 2.2 Hz, 1H), 8.49 (bs, 1H).

A solution of *t*-butyl-5-bromoacrylamidobenzothiophen-2-carboxylate (381 mg, 1 mmol) in CF_3COOH (1 mL), was stirred at room temperature for 3 h. The solvent was removed under reduced pressure and the resulting residue was suspended in CH_2Cl_2 (3 mL) , collected by filtration and triturated with ethyl ether (5 mL) to obtain **38** as a white powder (300 mg, 88% yield); mp = 190–195 °C; IR (KBr): 3292, 1658, 1535, 1302 and 892 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 6.33 (d, J = 3.2 Hz, 1H), 6.78 (d, J = 3.2 Hz, 1H) 7.70 (dd, J = 9 and 1.4 Hz, 1H), 7.99 (d, J = 9 Hz, 1H), 8.11 (s, 1H), 8.36 (d, J = 1.4 Hz, 1H), 10.5 (bs, 1H).

Preparation of *N*-[5-({[5-({[3-amino-3-(methylimino)-propyl]amino}carbonyl)-1-methyl-1H-pyrrol-3-yl]amino}-carbonyl)-1-methyl-1H-pyrrol-3-yl]-4-(formylamino)-1-methyl-1H-pyrrole-2-carboxamide dihydrochloride (27). To a stirred solution of distamycin A (2 g, 3.86 mmol) in DMF (50 mL) was added methylamine (40% in water) (0.32 mL, 3.86 mmol) at room temperature. The

solution was stirred for 24 h, and after this time methylamine (0.08 mL, 0.097 mL) was added. The mixture was stirred for another 5 h, then evaporated. The crude product purified by chromatography on a silica gel column, using DCM–MeOH (8:2, v/v) as eluent, furnished the compound **27** (1.5 g, 73% yield) as a brown solid; mp = 156–159 °C; IR (KBr): 3417, 1637, 1465, 1432, 1257 and 845 cm^{-1}; ^1H NMR(DMSO-d_6) d 2.61 (m, 2H), 2.78 (s, 3H), 3.50 (m, 2H), 3.81 (s, 3H), 3.86 (s, 3H), 3.87 (s, 3H), 6.93 (m, 2H), 7.06 (d, J = 1.8 Hz, 1H), 7.18 (m, 2H), 7.22 (d, J = 1.8 Hz, 1H), 8.21 (d, J = 1.6 Hz, 1H), 8.22 (m, 1H), 8.50–9.5 (bs, 3H), 9.91 (s, 1H), 9.93 (s, 1H), 10.12 (s, 1H); FAB-MS (MALDI–TOF): 497.5 $[M+1]^+$.

Preparation of 4-(formylamino) -1-methyl -*N*-[1-methyl-5-({[1-methyl-5-({[3-(methylamino) -3-(methylimino) propyl]amino} carbonyl)-1H-pyrrol-3-yl]amino}carbonyl)-1H-pyrrol-3-yl]-1H-pyrrole-2-carboxamide dihydrochloride (28). To a stirred solution of distamycin A (1.5 g, 2.89 mmol) in DMF (20 mL) heated at 80 °C were added three portions of methylamine (40% in water) (2.5 mL), waiting 1 h after the last addition. After 1 h from the addition of the last portion of methylamine the mixture was evaporated. The crude product purified by chromatography on a silica gel column, using DCM–MeOH (9:1, 8:2 and then 7:3) as eluent, furnished the compound **28** (500g, 32% yield) as a yellow solid; mp = 168–170 °C; IR (KBr): 3424, 1652, 1456, 1422, 1274, 1237 and 872 cm^{-1}; ^1H NMR (DMSO-d_6) d 2.71 (m, 2H), 2.78 (s, 3H), 3.02 (s, 3H), 3.49 (m, 2H), 3.80 (s, 3H), 3.84 (s, 3H), 3.85 (s, 3H), 6.92 (m, 2H), 7.05 (d, J = 1.8 Hz, 1H), 7.20 (m, 2H), 7.22 (d, J = 1.8 Hz, 1H), 8.12 (d, J = 1.6 Hz, 1H), 8.31 (m, 1H), 9.93 (bs, 2H), 10.12 (s, 1H); FAB-MS (MALDI–TOF): 511.1 $[M+1]^+$.

Preparation of *N*-[5-({[5-({[(3-amino-3-(cyanoimino)propyl]amino}carbonyl)-1-methyl-1H-pyrrol-3-yl]amino}carbonyl) -1-methyl -1H -pyrrol -3 -yl] -4 -(formylamino) -1-methyl-1H-pyrrole-2-carboxamide Hydrochloride (29). To a stirred solution of NH_2CN(650 mg, 15.5 mmol) in dry DMF (50 mL) was slowly added NaH (50% suspension in mineral oil) (375 mg of suspension corresponding to 15.5 mmol of pure NaH). After 30′, Distamycin A (2 g, 3.86 mmol) was dissolved in the solution and the mixture stirred for another 5 h. After this time, the solution was acidified (pH = 4) with acetic acid and then evaporated. The residue purified by column chromatography using DCM and MeOH (9:1, v/v) as eluent afforded 1.8 g. (92% yield) of compound **29** as a cream solid, mp = 123–126 °C; IR (KBr): 2256, 1648, 1442, 1427, 1256 and 845 cm^{-1}; ^1H NMR(DMSO-d_6) d 2.70 (m, 2H), 3.45 (m, 2H), 3.82 (s, 3H), 3.84 (s, 3H), 3.85 (s, 3H), 6.85 (m, 2H), 7.91 (d, J = 1.8 Hz, 1H), 7.02 (d, J = 1.8 Hz, 1H), 7.21 (d, J = 1.8 Hz, 1H), 7.22 (d, J = 1.8 Hz, 1H), 8.12 (d, J = 1.6 Hz, 1H), 8.05–8.40 (bs, 2H), 9.91 (s, 1H), 9.93 (bs, 2H), 10.08 (s, 1H); FAB-MS (MALDI–TOF): 507.6 $[M+1]+$.

General procedure for the synthesis of (30–32). To 5 mL of a solution obtained by the addition of 24 mL of ethanol (95%) to 1 mL of 36% HCl in water was added 1 equiv of compounds **27–29**, and the mixture so obtained stirred at room temperature for 24 h. After this time, the reaction was filtered, the residue washed with dry ethyl ether (5 mL) and then dried on P2O5. The crude product has been used without purification for the next reaction.

Preparation of 4-amino-*N*-[5-({[5-({[3-amino-3-(methylimino)propyl]amino}carbonyl)-1-methyl-1H-pyrrol-3-yl]amino}carbonyl)-1-methyl-1H-pyrrol-3-yl]-1-methyl-1H-pyrrole-2-carboxamide dihydrochloride(30). Brown solid, mp = 168–170 °C;IR (KBr): 3417, 1637, 1466, 1431 and 1258 cm^{-1}; ^1H NMR(DMSO-*d6*) d 2.61 (m, 2H), 2.80 (d, J = 6.0 Hz, 3H), 3.40-3.60 (m, 2H), 3.79 (s, 3H), 3.85 (s, 3H), 3.90 (s, 3H), 6.91 (d, J = 1.7 Hz, 1H), 7.05 (d, J = 1.7 Hz, 1H), 7.08 (d, J = 1.7 Hz, 1H), 7.11 (d, J = 1.7 Hz, 1H), 7.19 (d, J = 1.7 Hz, 1H), 7.25 (d, J = 1.7 Hz, 1H), 8.25 (t, J = 5.8 Hz, 1H), 8.63 (s, 1H), 9.20 (s, 1H), 9.65 (m, 1H), 10.18 (s, 1H), 10.20 (s, 3H); FAB-MS (MALDI–TOF): 470.5 $[M+1]^+$.

Preparation of 4-amino-1-methyl-*N*-[1-methyl-5-({[1-methyl-5-({[3-(methylamino)-3-(methylimino)propyl]amino}carbonyl)-1H-pyrrol-3-yl]amino}carbonyl)-1H-pyrrol-3-yl]-1H-pyrrole-2-carboxamide dihydrochloride (31). Brown solid, mp = 187–189 °C; IR (KBr): 3434, 1648, 1512, 1445 and 1287 cm^{-1}; ^1H NMR(DMSO-d_6) d 2.72 (m, 2H), 2.80 (d, J = 6.0 Hz, 3H), 3.02 (d, J = 6.0 Hz, 3H), 3.50 (m, 2H), 3.82 (s, 3H), 3.86 (s, 3H), 3.92 (s, 3H), 6.92 (d, J = 1.8 Hz, 1H), 7.02 (d, J = 1.8 Hz, 1H), 7.06 (d, J = 1.8 Hz, 1H), 7.12 (d, J = 1.8 Hz, 1H), 7.19 (d, J = 1.8 Hz, 1H), 7.24 (d, J = 1.8 Hz, 1H), 7.90 (bs, 1H), 8.38 (t, J = 5.9 Hz, 1H), 8.35(m, 1H), 9.62 (m, 1H), 9.96 (s, 1H), 10.18 (s, 1H), 10.22 (bs, 3H); FAB-MS (MALDI–TOF): 484.6 $[M+1]+$.

Preparation of 4-amino -*N*-[5-({[5-({[(3Z)-3-amino-3-(cyanoimino)propyl]amino}carbonyl)-1-methyl-1H-pyrrol-3-yl]amino}carbonyl)-1-methyl-1H-pyrrol-3-yl]-1-methyl-1H-pyrrole-2-carboxamide hydrochloride (32). Green solid, mp = 112–114¡C; IR (KBr): 3251, 2251, 1713, 1667, 1651, 1557, 1440, 1260, 1102 and 778 cm^{-1}; ^1H NMR (DMSO-d_6) d 2.70 (m, 2H), 3.55 (m, 2H), 3.82 (s, 3H), 3.85 (s, 3H), 3.91 (s, 3H), 6.88 (m, 1H), 7.02 (d, J = 1.8 Hz, 1H), 7.10 (d, J = 1.8 Hz, 1H), 7.12 (d, J = 1.8 Hz, 1H), 7.20 (d, J = 1.8 Hz, 1H), 7.28 (d, J = 1.8 Hz, 1H), 8.28 (bs, 1H), 8.35(bs, 1H), 9.91(s, 1H), 10.12 (s, 1H), 10.15 (bs, 3H);FAB-MS (MALDI–TOF): 479.5 $(M+1)^+$.

General procedure for the synthesis of compounds (13–26). A solution of amidino-modified *N*-deformyldistamycin A dihydrochloride (compounds **30–31**, 0.5 mmol) in dry DMF (5 mL) was cooled to 5 °C and *N*, *N*'diisopropylethylamine (86 μL, 0.5 mmol) was added. After 10 min, the acids **33–38** (0.6 mmol, 1.2 equiv) and then EDC (192 mg, 1 mmol) were added. The reaction mixture was stirred at room temperature for 18 h, then 2 N hydrochloric acid was added until pH = 4. The solvent was evaporated in vacuo and the crude residue purified by flash chromatography to yield an oil, which was precipitated from MeOH/diethyl ether.

***N*-Methyl-3-[1-methyl-4 [1-methyl-4 [1-methyl-4 [3(α-bromoacrylamido) pyrazole-5-carboxamido] pyrrole-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]propionamidine hydrochloride (13).** Following the general procedure, starting from **30** (200 mg, 0.365

mmol), Hunig's base (63 μL, 0.367 mmol), EDC (150 mg, 0.75 mmol) and the acid **33** (100 mg, 0.365 mmol), after work-up, the residue was purified by flash chromatography using methanol–methylene chloride 3:7 (v:v) as eluent. The resulting oil was precipitated and yielding the compound **13** as a white solid (175 mg, 63% yield); mp > 300 °C; IR (KBr): 3401, 1637, 1578, 1438, 1405 and 1261 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 2.59 (m, 2H), 2.83 (d, $J = 4.8$ Hz, 3H);3.52 (m, 2H), 3.82 (s, 3H), 3.86 (s, 3H), 3.89 (s, 3H), 4.07 (s, 3H), 6.34 (d, $J = 3.4$ Hz, 1H), 6.82 (d, $J = 3.4$ Hz, 1H), 6.94 (d, $J = 1.6$ Hz, 1H), 7.07 (d, $J = 1.6$ Hz, 1H), 7.11 (d, $J = 1.6$ Hz, 1H), 7.20 (d, $J = 1.6$ Hz, 1H), 7.26 (d, $J = 1.6$ Hz, 1H), 7.34 (d, $J = 1.6$ Hz, 1H), 7.38 (s, 1H), 8.26 (m, 1H), 8.64 (s, 1H), 9.16 (s, 1H), 9.58 (bs, 1H), 9.96 (s, 1H), 10.04 (s, 1H), 10.53 (s, 1H), 11.07 (s, 1H);FAB-MS (MALDI–TOF): 725.6 $(M+1)^+$. Anal. calcd for $C_{30}H_{36}BrClN_{12}O_5$: C, 47.41; H, 4.77; Br, 10.51; Cl, 4.66; N, 22.11. Found: C, 47.25; H, 4.61; Br, 10.23; Cl, 4.46; N, 22.02.

***N, N′*-Dimethyl-3-[1-methyl-4 [1-methyl-4 [1-methyl-4 [3-(α-bromoacrylamido) pyrazole-5-carboxamido] pyrrole-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]propionamidine hydrochloride (14).** Following the general procedure, starting from **31** (166 mg, 0.3 mmol), Hunig's base (51 μL, 0.3 mmol), EDC (120 mg, 0.6 mmol) and the acid **33** (85 mg, 0.32 mmol), after work-up, the residue was purified by flash chromatography using methanol–methylene chloride 2:8 (v:v) as eluent. The resulting oil was precipitated and yielding the compound **14** as a white solid (165 mg, 71% yield); mp > 300 °C; IR (KBr): 3392, 1647, 1560, 1432, 1403, 1257, 1109 and 888 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 2.72 (m, 2H), 2.77 (d, $J = 4.6$ Hz, 3H), 3.00 (d, $J = 4.4$ Hz, 3H), 3.34 (m, 2H), 3.84 (s, 3H), 3.86 (s, 3H), 3.98 (s, 3H), 4.05 (s, 3H), 6.33 (d, $J = 3.2$ Hz, 1H), 6.80 (d, $J = 3.2$ Hz,1H), 6.93 (s, 1H), 7.06 (s, 1H), 7.09 (s, 1H), 7.19 (s, 1H), 7.24 (s, 1H), 7.31 (s, 1H), 7.36 (s, 1H), 8.31 (m, 1H), 8.70 (q, $J = 4.8$ Hz, 1H), 9.44 (q, $J = 4.8$ Hz, 1H), 9.96 (s, 1H), 10.02 (s, 1H), 10.50 (s, 1H), 11.05 (s, 1H); FAB-MS (MALDI–TOF): 739.6 $(M+1)^+$. Anal. calcd for $C_{31}H_{38}BrClN_{12}O_5$: C, 48.10; H, 4.95; Br, 10.32; Cl, 4.58; N, 21.71. Found: C, 47.89; H, 4.73; Br, 10.13; Cl, 4.37; N, 21.37.

***N*-Cyano-3-[1-methyl-4 [1-methyl-4 [1-methyl-4 [3-(α-bromoacrylamido) pyrazole-5-carboxamido] pyrrole-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]propionamidine (15).** Following the general procedure, starting from **32** (206 mg, 0.4 mmol), Hunig's base (68 μL, 0.4 mmol), EDC (192 mg, 1 mmol) and the acid **33** (110 mg, 0.42 mmol), after work-up, the residue was purified by flash chromatography using methanol–methylene chloride 2:8 (v:v) as eluent. The resulting oil was precipitated and yielding the compound **15** as a white solid (165 mg, 71% yield); mp > 300 °C; IR (KBr): 2256, 1637, 1576, 1467, 1405, 1260, 1113 and 889 cm^{-1}; ^1H NMR(DMSO-d_6) δ: 2.48 (m, 2H), 3.34 (m, 2H), 3.80 (s, 3H), 3.85 (s, 3H), 3.87 (s, 3H), 4.05 (s, 3H), 6.30 (d, $J = 3.4$ Hz, 1H), 6.79 (d, $J = 3.4$ Hz, 1H), 6.88 (s, 1H), 7.04 (s, 1H), 7.09 (s, 1H), 7.20 (s, 1H), 7.25 (s, 1H), 7.31 (s, 1H), 7.36 (s, 1H), 8.34 (bs, 2H), 9.96 (s, 1H), 9.98 (s, 1H), 10.02 (s, 1H), 10.51 (s, 1H), 11.03 (s, 1H); FAB-MS (MALDI-TOF): 735.6 $(M+1)^+$. Anal. calcd for $C_{30}H_{32}BrN_{13}O_5$: C, 49.05; H, 4.39; Br, 10.88; N, 24.79. Found: C, 48.88; H, 4.23; Br, 10.71; N, 24.58.

***N*-Methyl-3-[1-methyl-4 [1-methyl-4 [1-methyl-4 [4(α-bromoacrylamido) imidazole-2-carboxamido] pyrrole-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]propionamidine hydrochloride (16).** Following the general procedure, starting from **30** (270 mg, 0.5 mmol), Hunig's base (86 μL, 0.5 mmol), EDC (193 mg, 1 mmol) and the acid **34** (160 mg, 0.6 mmol), after work-up, the residue was purified by flash chromatography using methanol–methylene chloride 3:7 (v:v) as eluent. The resulting oil was precipitated and yielding the compound **16** as a yellow solid (251 mg, 66% yield); mp > 300 °C; IR (KBr): 3412, 1637, 1542, 1431, 1405, 1259, 1209 and 1107 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.61 (m, 2H); 2.80 (d, $J = 4.6$ Hz, 3H), 3.48 (m, 2H), 3.82 (s, 3H), 3.86 (s, 3H), 3.87 (s, 3H), 3.99 (s, 3H), 6.32 (d, $J = 3$ Hz, 1H), 6.82 (d, $J = 2.8$ Hz, 1H), 6.94 (s, 1H), 7.08 (s, 1H), 7.19 (s, 2H), 7.25 (s, 1H), 7.29 (s, 1H), 7.55 (s, 1H), 8.24 (m, 1H), 8.59 (s, 1H), 9.14 (s, 1H), 9.56 (bs, 1H), 9.95 (s, 1H), 10.01 (s, 1H), 10.15 (s, 1H), 10.59 (s, 1H);FAB-MS (MALDI–TOF): 725.6 $(M+1)^+$. Anal. calcd for $C_{30}H_{36}BrClN_{12}O_5$: C, 47.41; H, 4.77; Br, 10.51; Cl, 4.66; N, 22.11. Found: C, 47.22; H, 4.56; Br, 10.26; Cl, 4.51; N, 21.98.

***N, N′*-Dimethyl-3-[1-methyl-4 [1-methyl-4 [1-methyl-4 [4-(α-bromoacrylamido) imidazole-2-carboxamido] pyrrole-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]propionamidine hydrochloride (17).** Following the general procedure, starting from **31** (166 mg, 0.3 mmol), Hunig's base (51 μL, 0.3 mmol), EDC (120 mg, 0.6 mmol) and the acid **34** (85 mg, 0.32 mmol), after work-up, the residue was purified by flash chromatography using methanol–methylene chloride 3:7 (v:v) as eluent. The resulting oil was precipitated and yielding the compound **17** as a white solid (174 mg, 75% yield); mp > 300 °C; IR (KBr): 3400, 1647, 1550, 1432, 1426, 1260 and 775 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 2.73 (m, 2H), 2.78 (d, $J = 4.8$ Hz, 3H), 3.01 (d, $J = 4.6$ Hz, 3H), 3.46 (m, 2H), 3.80 (s, 3H), 3.84 (s, 3H), 3.92 (s, 3H), 3.98 (s, 3H), 6.31 (d, $J = 3$ Hz, 1H), 6.81 (d, $J = 3$ Hz, 1H), 6.93 (s, 1H), 7.08 (s, 1H), 7.18 (s, 1H), 7.20 (s, 1H), 7.25 (s, 1H), 7.28 (s, 1H), 7.54 (s, 1H), 8.33 (m, 1H), 8.73 (q, $J = 4.8$ Hz, 1H), 9.50 (q, $J = 4.8$ Hz, 1H), 9.95 (s, 1H), 10.00 (s, 1H), 10.15 (s, 1H), 10.59 (s, 1H); FAB-MS (MALDI–TOF): 739.6 $(M+1)^+$. Anal. calcd for $C_{31}H_{38}BrClN_{12}O_5$: C, 48.10; H, 4.95; Br, 10.32; Cl, 4.58; N, 21.52. Found: C, 47.89; H, 4.78; Br, 10.12; Cl, 4.46; N, 21.55.

***N*-Cyano-3-[1-methyl-4 [1-methyl-4 [1-methyl-4 [4-(α-bromoacrylamido) imidazole-2-carboxamido] pyrrole-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]propionamidine (18).** Following the general procedure, starting from **32** (206 mg, 0.4 mmol), Hunig's base (68 μL, 0.4 mmol), EDC (192 mg, 1 mmol) and the acid **34** (110 mg, 0.42 mmol), after work-up, the residue was purified by flash chromatography using methanol–methylene chloride 1:9 (v:v) as eluent. The resulting oil was precipitated and yielding the compound **18** as a

white solid (170 mg, 58% yield); mp > 300 °C; IR (KBr): 3412, 2252, 1674, 1643, 1558, 1496, 1433, 1223 and 798 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 2.64 (m, 2H), 3.35 (m, 2H), 3.81 (s, 3H), 3.85 (s, 3H), 3.86 (s, 3H), 3.99 (s, 3H), 6.31 (d, $J = 3$ Hz, 1H), 6.81 (d, $J = 3.2$ Hz, 1H), 6.88 (s, 1H), 7.06 (s, 1H), 7.16 (s, 1H), 7.21 (s, 1H), 7.26 (s, 1H), 7.28 (s, 1H), 7.55 (s, 1H), 8.22 (bs, 3H), 9.94 (s, 1H), 9.99 (s, 1H), 10.19 (s, 1H), 10.58 (s, 1H); FAB-MS (MALDI–TOF): 735.6 $(M+1)^+$. Anal. calcd for C30H32BrN13O5: C, 49.05; H, 4.39; Br, 10.88; N, 24.79. Found: C, 48.88; H, 4.24; Br, 10.67; N, 24.58.

***N*-Methyl-3-[1-methyl-4 [1-methyl-4 [1-methyl-4 [5(α-bromoacrylamido) indole-2-carboxamido] pyrrole-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]propionamidine hydrochloride(19).** Following the general procedure, starting from **30** (270 mg, 0.5 mmol), Hunig's base (86 μL, 0.5 mmol), EDC (192 mg, 1 mmol) and the acid **35** (183 mg, 0.6 mmol), after work-up, the residue was purified by flash chromatography using methanol–methylene chloride 2:8 (v:v) as eluent. The resulting oil was precipitated and yielding the compound **19** as a white solid (320 mg, 83% yield); mp > 300 °C; IR (KBr): 3394, 1646, 1559, 1442, 1405, 1261, 1108 and 1062 cm^{-1}; ^1H NMR (DMSO–d_6) δ 2.60 (m, 2H), 2.79 (d, $J = 4.8$ Hz, 3H), 3.51 (m, 2H), 3.81 (s, 3H), 3.86 (s, 3H), 3.89 (s, 3H), 6.28 (d, $J = 3.0$ Hz, 1H), 6.76 (d, $J = 3.0$ Hz, 1H), 6.95 (s, 1H), 7.09 (s, 1H), 7.21 (s, 1H), 7.27 (s, 1H), 7.30 (s, 1H), 7.36 (s, 1H), 7.40 (s, 1H), 7.40 (m, 2H), 8.01 (s, 1H), 8.26 (t, $J = 5.7$ Hz, 1H), 8.60 (s, 1H), 9.14 (s, 1H), 9.53 (m, 1H), 9.96 (s, 1H), 10.06 (s, 1H), 10.21 (s, 1H), 10.44 (s, 1H), 11.73 (s, 1H); FAB-MS (MALDI–TOF): 774.7 $(M+1)^+$. Anal. calcd for $C_{34}H_{37}BrClN_{11}O_5$: C, 51.36; H, 4.69; Br, 10.05; Cl, 4.46; N, 19.38. Found: C, 51.13; H, 4.48; Br, 9.79; Cl, 4.33; N, 19.15.

***N*,*N*′-Dimethyl-3-[1-methyl-4[1-methyl-4[1H-5(α-bromoacrylamido)indole-2-carboxamido]pyrrole-2-carboxamido]-pyrrole-2-carboxamido]propionamidine hydrochloride (20).** To a solution of amino-amidine **31**(272 mg, 0.5 mmol) in anhydrous DMF (5 mL) cooled to 0 °C, Hunig's base (86 mL, 0.5 mmol) was added. After 5 min the acid **35** (186 mg, 0.6 mmol) and then EDC (192 mg,1 mmol) were added. The reaction mixture was slowly warmed to room temperature and allowed to stir for 18 h. After this time, the solution was acidified with 20% HCl at pH 4 and DMF was removed under reduced pressure The resulting solid purified by flash column chromatography with methanol–methylene chloride 2:8 (v:v) afforded a yellow oil which was precipitated from methanol-ethyl ether to give **20** as a yellow solid (288 mg, 71% yield); mp > 300 °C; IR (KBr):3400, 1641, 1578, 1435, 1252 and 1106 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.72 (t, $J = 4.8$ Hz, 2H), 2.78 (d, $J = 4.6$ Hz, 3H), 3.01 (d, $J = 4$ Hz, 3H), 3.49 (m, 2H), 3.81 (s, 3H), 3.86 (s, 3H), 3.89 (s, 3H), 6.28 (d, $J = 2.6$ Hz, 1H), 6.78 (s, 1H), 6.94 (s, 1H), 7.09 (s, 1H), 7.12 (s, 1H), 7.22 (s, 1H), 7.28 (s, 1H), 7.32 (s, 1H), 7.37 (s, 1H), 7.40 (s, 2H), 8.00 (s, 1H), 8.37 (t, $J = 4.6$ Hz, 1H), 8.84 (q, $J = 4.8$ Hz, 1H), 9.61 (q, $J = 4.8$ Hz, 1H), 9.98 (s, 1H), 10.06 (s, 1H), 10.23 (s, 1H), 10.50 (s, 1H), 11.78 (s, 1H); FAB-MS (MALDI–TOF): 774.6 $(M+1)^+$. Anal. calcd for $C_{35}H_{39}BrClN_{11}O_5$: C, 51.96; H, 4.86; Br, 9.88; Cl, 4.38; N, 19.04. Found: C, 50.72; H, 4.47; Br, 10.16; Cl, 4.47; N, 19.66.

***N*-Methyl-3-[1-methyl-4 [1-methyl-4 [1-methyl-4 [1-methyl-5(α-bromoacrylamido) indole-2-carboxamido] pyrrole-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]propionamidine hydrochloride(21).** Following the general procedure, starting from **30** (275 mg, 0.5 mmol), Hunig's base (86 μL, 0.5 mmol), EDC (192 mg, 1 mmol) and the acid **36** (194 mg, 0.6 mmol), after work-up, the residue was purified by flash chromatography using methanol–methylene chloride 2:8 (v:v) as eluent. The resulting oil was precipitated and yielding the compound **21** as a red solid (271 mg, 69% yield); mp > 300 °C; IR (KBr): 3400, 1638, 1559, 1433, 1404, 1257 and 1110 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.61 (m, 2H), 2.79 (d, $J = 4.6$ Hz, 3H), 3.49 (m, 2H), 3.81 (s, 3H), 3.85 (s, 3H), 3.88 (s, 3H), 4.02 (s, 3H), 6.29 (d, $J = 2.6$ Hz, 1H), 6.78 (d, $J = 2.6$ Hz, 1H), 6.93 (s, 1H), 7.03 (d, 1H), 7.13 (d, 1H), 7.21 (m, 2H), 7.26 (s, 1H), 7.34 (s, 1H), 7.51 (d, $J = 6.6$ Hz, 2H), 8.05 (s, 1H), 8.25 (bs, 1H), 8.60 (s, 1H), 9.16 (s, 1H), 9.56 (bs, 1H), 9.96 (s, 1H), 10.02 (s, 1H), 10.27 (s, 1H), 10.47 (s, 1H); FAB-MS (MALDI–TOF): 774.7 $(M+1)^+$. Anal. calcd for $C_{35}H_{39}BrClN_{11}O_5$: C, 51.96; H, 4.89; Br, 9.88; Cl, 4.38; N, 19.04. Found: C, 51.77; H, 4.67; Br, 9.76; Cl, 4.22; N, 18.89.

***N*,*N*′-Dimethyl-3-[1-methyl-4 [1-methyl-4 [1-methyl-4 [1-methyl - 5(α - bromoacrylamido)indole - 2 - carboxamido]-pyrrole-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido] propionamidine hydrochloride (22).** Following the general procedure, starting from **31** (166 mg, 0.3 mmol), Hunig's base (51 μL, 0.3 mmol), EDC (112 mg, 0.6 mmol) and the acid **36** (97 mg, 0.6 mmol), after work-up, the residue was purified by flash chromatography using methanol–methylene chloride 3:7 (v:v) as eluent. The resulting oil was precipitated and yielding the compound **22** as a white solid (213 mg, 86.6% yield); mp > 300 °C; IR (KBr): 3398, 1643, 1560, 1412, 1400, 1277 and 1089 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.73 (m, 2H), 2.78 (d, $J = 3.2$ Hz, 3H), 3.01 (d, $J = 3.4$ Hz, 3H), 3.43 (m, 2H), 3.81 (s, 3H), 3.85 (s, 3H), 3.89 (s, 3H), 4.02 (s, 3H), 6.29 (d, $J = 3.0$ Hz, 1H), 6.76 (d, $J = 3.0$ Hz, 1H), 6.93 (d, $J = 1.6$ Hz, 1H), 7.08 (d, $J = 1.6$ Hz, 1H), 7.12 (d, $J = 1.4$ Hz, 1H), 7.20 (d, $J = 1.6$ Hz, 1H), 7.26 (s, 1H), 7.34 (d, $J = 1.4$ Hz, 1H), 7.46 (s, 1H), 7.57 (d, $J = 4.8$ Hz, 2H), 8.06 (s, 1H), 8.31 (t, $J = 6$ Hz, 1H), 8.71 (q, $J = 4.8$ Hz, 1H), 9.46 (q, $J = 5$ Hz, 1H), 9.96 (s, 1H), 10.02 (s, 1H), 10.24 (s, 1H), 10.46 (s, 1H); FAB-MS (MALDI–TOF): 788.7 $(M+1)^+$. Anal. calcd for $C_{36}H_{41}BrClN_{11}O_5$: C, 52.23; H, 5.02; Br, 9.71; Cl, 4.31; N, 18.72. Found: C, 52.36; H, 4.89; Br, 9.55; Cl, 4.12; N, 18.56.

***N*-Methyl-3-[1-methyl-4 [1-methyl-4 [1-methyl-4 [5(α-bromoacrylamido)benzofurane-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine hydrochloride (23).** Following the general procedure starting from **30** (275 mg, 0.5 mmol), Hunig's base (86 μL, 0.5 mmol), EDC (192 mg, 1 mmol) and the acid **37** (190 mg, 0.6 mmol), after work-up, the

residue was purified by flash chromatography using methanol–methylene chloride 2:8 (v:v) as eluent to give **23** (308 mg, 78% yield); mp > 300 °C; IR (KBr):3328, 1637, 1578, 1433, 1262, 1208, 1109 and 802 cm^{-1}; ^1H NMR (DMSO-d_6) δ2.57 (t, $J = 6.6$ Hz, 2H), 2.81 (d, $J = 4.6$ Hz, 3H), 3.49 (m, 2H), 3.79 (s, 3H), 3.85 (s, 3H), 3.88 (s, 3H), 6.33 (d, $J = 3$ Hz, 1H), 6.80 (d, $J = 3$ Hz, 1H), 6.93 (d, $J = 1.8$ Hz, 1H), 7.08 (d, $J = 1.8$ Hz, 1H), 7.16 (d, $J = 1.8$ Hz, 1H), 7.19 (d, $J = 1.8$ Hz, 1H), 7.26 (d, $J = 1.8$ Hz, 1H), 7.34 (d, $J = 1.8$ Hz, 1H), 7.70 (m, 3H), 8.17 (s, 1H), 8.16 (bs, 1H), 8.58 (s., 1H), 9.14 (s., 1H), 9.54 (q, $J = 4.8$ Hz, 1H), 9.96 (s, 1H), 10.06 (s, 1H), 10.47 (s, 1H), 10.77 (s, 1H); FAB-MS (MALDI–TOF): 761.7 $(M+1)^+$. Anal. calcd for $C_{34}H_{36}BrClN_{10}O_6$: C, 51.30; H, 4.56; Br, 10.04; Cl, 4.45; N, 17.59. Found: C, 51.05; H, 4.34; Br, 9.89; Cl, 4.33; N, 17.35.

***N,N′*-Dimethyl-3-[1-methyl-4 [1-methyl-4 [1-methyl-4 [5(α-bromoacrylamido)benzofurane-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine hydrochloride (24).** Following the general procedure, starting from **31** (166 mg, 0.3 mmol), Hunig's base (51 μL, 0.3 mmol), EDC (115 mg, 0.6 mmol) and the acid **37** (93 mg, 0.3 mmol), after work-up, the residue was purified by flash chromatography using methanol–methylene chloride 3:7 (v:v) as eluent, yielding the compound **24** as a white solid (190 mg, 78% yield); mp > 300 °C; IR (KBr): 3328, 1637, 1578, 1405, 1262, 1209 and 867 cm^{-1}; ^1H NMR (DMSO-d_6) δ2.72 (m, 2H), 2.78 (d, $J = 4.6$ Hz, 3H), 3.01 (d, $J = 4.4$ Hz, 3H), 3.37 (m, 2H), 3.81 (s, 3H), 3.85 (s, 3H), 3.89 (s, 3H), 6.33 (d, $J = 3$ Hz, 1H), 6.79 (d, $J = 3$ Hz, 1H), 6.94 (s, 1H), 7.08 (s, 1H), 7.16 (s, 1H), 7.20 (s, 1H), 7.26 (s, 1H), 7.34 (s, 1H), 7.68 (m, 3H), 8.17 (s, 1H), 8.32 (bs., 1H), 8.70 (q, $J = 4.8$ Hz, 1H), 9.46 (q, $J = 4.8$ Hz, 1H), 9.96 (s, 1H), 10.05 (s, 1H), 10.45 (s, 1H), 10.75 (s, 1H); FAB-MS (MALDI–TOF): 775.7 $(M+1)^+$. Anal. calcd for $C_{35}H_{38}BrClN_{10}O_6$: C, 51.89; H, 4.73; Br, 9.86; Cl, 4.38; N, 17.29. Found: C, 51.67; H, 4.55; Br, 9.72; Cl, 4.13; N, 17.11.

***N*-Methyl-3-[1-methyl-4 [1-methyl-4 [1-methyl-4 [5(α-bromoacrylamido) benzothiopene-2-carboxamido] pyrrole-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]propionamidine hydrochloride (25).** Following the general procedure, starting from **30** (270 mg, 0.5 mmol), Hunig's base (86 μL, 0.5 mmol), EDC (192 mg, 1 mmol) and the acid **38** (200 mg, 0.6 mmol), after work-up, the residue was purified by flash chromatography using methanol–methylene chloride 2:8 (v:v) as eluent. The resulting oil was precipitated and yielding the compound **25** as a white solid (313 mg, 77% yield); mp > 300 °C; IR (KBr): 3296, 1634, 1582, 1434, 1262, 1207 and 805 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.59 (m, 2H), 2.78 (d, $J = 3$ Hz, 3H), 3.50 (m, 2H), 3.81 (s, 3H), 3.85 (s, 3H), 3.89 (s, 3H), 6.36 (s, 1H), 6.83 (s, 1H), 6.94 (s, 2H), 7.08 (s, 1H), 7.13 (s, 1H), 7.20 (s, 1H), 7.27 (s, 1H), 7.34 (s, 1H), 7.67 (d, $J = 8.8$ Hz, 1H), 8.01 (d, $J = 8.6$ Hz, 1H), 8.27 (s, 1H), 8.37 (s, 1H), 8.59 (bs, 1H), 9.15 (s, 1H), 9.55 (bs, 1H), 9.97 (s, 1H), 10.06 (s, 1H), 10.53 (s, 1H), 10.74 (s, 1H); FAB-MS (MALDI–TOF): 777.7 $(M+1)^+$. Anal. calcd for $C_{34}H_{36}BrClN_{10}O_5S$: C, 50.28; H, 4.47; Br, 9.84; Cl, 4.37; N, 17.25. Found: C, 50.22; H, 4.25; Br, 9.67; Cl, 4.15; N, 17.06.

***N*, *N′*-Dimethyl-3-[1-methyl-4 [1-methyl-4 [1-methyl-4 [5(α-bromoacrylamido) benzothiopene-2-carboxamido] pyrrole-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]propionamidine hydrochloride (26).** Following the general procedure (K), starting from **31** (111 mg, 0.2 mmol), Hunig's base (35 μL, 0.5 mmol), EDC (77 mg, 0.4 mmol) and the acid **38** (73 mg, 0.22 mmol), after work-up, the residue was purified by flash chromatography using methanol–methylene chloride 2:8 (v:v) as eluent. The resulting oil was precipitated and yielding the compound **26** as a red solid (127 mg, 77% yield); mp > 300 °C; IR (KBr): 3255, 1637, 1578, 1523, 1432, 1260 and 1206 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.72 (m, 2H), 2.78 (d, $J = 4.8$ Hz, 3H), 3.01 (d, $J = 4.4$ Hz, 3H), 3.38 (m, 2H), 3.81 (s, 3H), 3.86 (s, 3H), 3.89 (s, 3H), 6.35 (d, $J = 3.2$ Hz, 1H), 6.81 (d, $J = 3$ Hz, 1H), 6.94 (s, 1H), 7.08 (s, 1H), 7.13 (s, 1H), 7.20 (s, 1H), 7.26 (s, 1H), 7.33 (s, 1H), 7.65 (d, $J = 8.8$ Hz, 1H), 8.00 (d, $J = 8.8$ Hz, 1H), 8.24 (s, 1H), 8.27 (bs, $J = 6$ Hz, 1H), 1H), 8.36 (s, 1H), 8.66 (q, $J = 4.6$ Hz, 1H), 9.41 (q, $J = 4.6$ Hz, 1H), 9.95 (s, 1H), 10.03 (s, 1H), 10.48 (s, 1H), 10.68 (s, 1H); FAB-MS (MALDI–TOF): 791.7 $(M+1)^+$. Anal. calcd for $C_{35}H_{38}BrClN_{10}O_5S$: C, 50.88; H, 4.64; Br, 9.67; Cl, 4.29; N, 16.95. Found: C, 50.68; H, 4.52; Br, 9.34; Cl, 4.08; N, 16.77.

Biological assays

Growth inhibitory activity on murine L1210 and human K562 and Raji tumor cell lines. The murine lymphocytic L1210 leukemia,[12] the human chromic myelogenous K562,[16] and the human B-lymphoid Raji[17] 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. The murine lymphocytic leukemia cells L1210 and L1210/DX were grown in vitro as a stationary suspension culture 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 survival after drug exposure, exponentially growing L1210 cells were continously exposed to various concentrations of drugs for 48 h and surviving cells were counted in 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). Human K562 and Raji cell lines were treated with the tested compounds for 4 and 5 days respectively and then the cell number/mL determined as elsewhere reported.[18–20] All experiments were repeated at least twice. For each drug concentration, duplicate cultures were used. Vehicle or solvent controls were run with each experiment.

PCR primers and arrested PCR and arrested PCR. The sequences of γ-globin gene primers used for polymerase chain reaction (PCR) were the following: γ-globin-forward, 5′-GGA ATG ACT GAA TCG GAA CAA GGC-3′, γ-globin-reverse, 5′α-CTG CTG AAG GGT

GCT TCC TTT TAT-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 DNA; 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, 1 min; elongation, 72 °C, 1 min (30 cycles). Arrested PCR[21,22] was performed after allowing different concentrations of the tested DNA-binding drugs to interact with the pre-amplified target DNA. After PCR, the products were analysed by agarose gel electrophoresis and ethidium bromide staining as elsewhere reported. The inhibitory activity was expressed as IC_{50}, causing a 50% inhibition of the generation of PCR products.

Footprinting probes and DNase I footprinting. Footprinting probes were generated by PCR using the primers described in the previous section. For PCR-mediated amplification the target DNA was 20 ng of genomic DNA. After a first PCR round (20 cycles), samples were heated at 90 °C for 5 min and a second PCR round was performed (20 cycles) using a ^{32}P-labelled γ-globin-forward PCR primer. After PCR, each reaction, containing ^{32}P-labelled PCR products was resuspended in water, aliquots were incubated with increasing amounts of the tested compounds (0, 1, 5, 10 μM) and 1 U/reaction of DNAse I was added. After 2 min, 5 μL of loading dye (0.1% xilene-cyanol, 0.1% bromophenol blue, 0.1 M NaOH–formamide 1:2) was added and the reactions electrophoresed through a sequencing gel as described.

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