Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 251–262

BIOORGANIC & MEDICINAL CHEMISTRY

Novel Benzoyl Nitrogen Mustard Derivatives of Pyrazole Analogues of Distamycin A: Synthesis and Antileukemic Activity

Pier Giovanni Baraldi,[a,*] Paolo Cozzi,[b] Cristina Geroni,[b] Nicola Mongelli,[b] Romeo Romagnoli[a] and Giampiero Spalluto[a]

[a]*Dipartimento di Scienze Farmaceutiche, Università di Ferrara, 44100 Ferrara, Italy*
[b]*Pharmacia and Upjohn, Oncology, Preclinical Research, Milano, Italy*

Received 23 June 1998; accepted 4 September 1998

Abstract—The design and synthesis of novel benzoic acid mustard (BAM) derivatives of distamycin A bearing one or more pyrazole rings replacing the pyrrole rings of the latter are described. In vitro and in vivo activities against L1210 leukemia are reported and discussed. Some of these compounds show an activity profile comparable to tallimustine **1**. All the compounds bearing the pyrazole ring close to the BAM moiety show reduced cytotoxicity in comparison to derivatives characterized by the BAM linked to a pyrrole: the same effect has not been observed when occurring at the amidine terminus of the oligopeptidic frame.

Introduction

Tallimustine **1** (FCE 24517),[1] a distamycin A derivative in which the formyl group has been replaced by a benzoyl nitrogen mustard moiety (BAM), is a potent cytotoxic agent which exhibits a broad spectrum of antitumor activity in a series of experimental tumor models.[2] This compound retains the AT preference of distamycin A and appears to possess a high preference for alkylation of 3′-adenine-N3 atom located in the sequence 5′-TTTTGA-3′.[3] The potent anti-tumor activity of tallimustine may be due to its ability to inhibit the binding of not well characterized protein factor(s) recognizing this sequence of the DNA.[4]

We have recently published[5] the synthesis of three tallimustine analogues **2–4** which contain either one or two pyrazoles instead of pyrroles and the encouraging activity of these compounds prompted us to complete this series of derivatives, considering other pyrrole–pyrazole combinations **5–7** in order to verify a possible relationship between the number of pyrazoles, their position and cytotoxicity. Since polypyrrole compounds are susceptible to oxidative breakdown,[6] all these pyrazolo tallimustine-like derivatives were prepared as potentially more stable DNA minor groove binders aimed to improve the relative instability of the polypyrrolic frame.[7] Moreover, in the case of tripyrazole analogue **7**, we have also substituted the alkyl amidine side chain at the C-terminus either with a dimethylaminopropylamino (compound **8**) or a *p*-[bis(2-chloroethyl) amino] phenylamino moiety (compound **9**). Finally, we have also investigated two four-ring homologues **10** and **11** of the compound **3**, both obtained by insertion of a pyrrole and pyrazole ring, respectively, on the N-teminus of the tripeptidic frame.

Chemistry

Compounds **2–4** were prepared as previously reported.[5] For the preparation of the compounds **5** and **6** two different convergent approaches were employed. Starting from the known amino-amidine **12**,[8] condensation with the acid **13**[6] by 1-ethyl-3[3-(dimethylamino)propyl]-carbodiimide hydrochloride (EDC) as coupling agent afforded the dipeptide **14** in 89% yield. Catalytic hydrogenation of **14**, followed by condensation with the mustard derivatives **17** and **18** in presence of EDC, gave the tripeptides **5** and **6** in 73 and 78% yield, respectively (Scheme 1).

The syntheses of the acids **17** and **18** were performed by coupling methyl 1-methyl-4-aminopyrrole-2-carboxylate[9] and methyl 1-methyl-3-aminopyrazole-5-carboxylate,[6] respectively, with *p*-[bis-(2-chloroethyl)amino]benzoyl chloride,[10,11] and subsequent alkaline hydrolysis of esters **15** and **16**, in 76 and 78% yield, respectively.

In the second approach, the BAM moiety was introduced at the last step, after the preparation of tripeptidic frame, as described in Scheme 2. The unstable

Key words: Anti-tumor compounds; DNA, leukemia; substituent effects.
*Corresponding author. Tel: +39-(0)532-291293; fax: +39-(0)532-291296; e-mail: pgb@ifeuniv.unife.it

0968-0896/99/$ - see front matter
PII: S0968-0896(98)00205-3

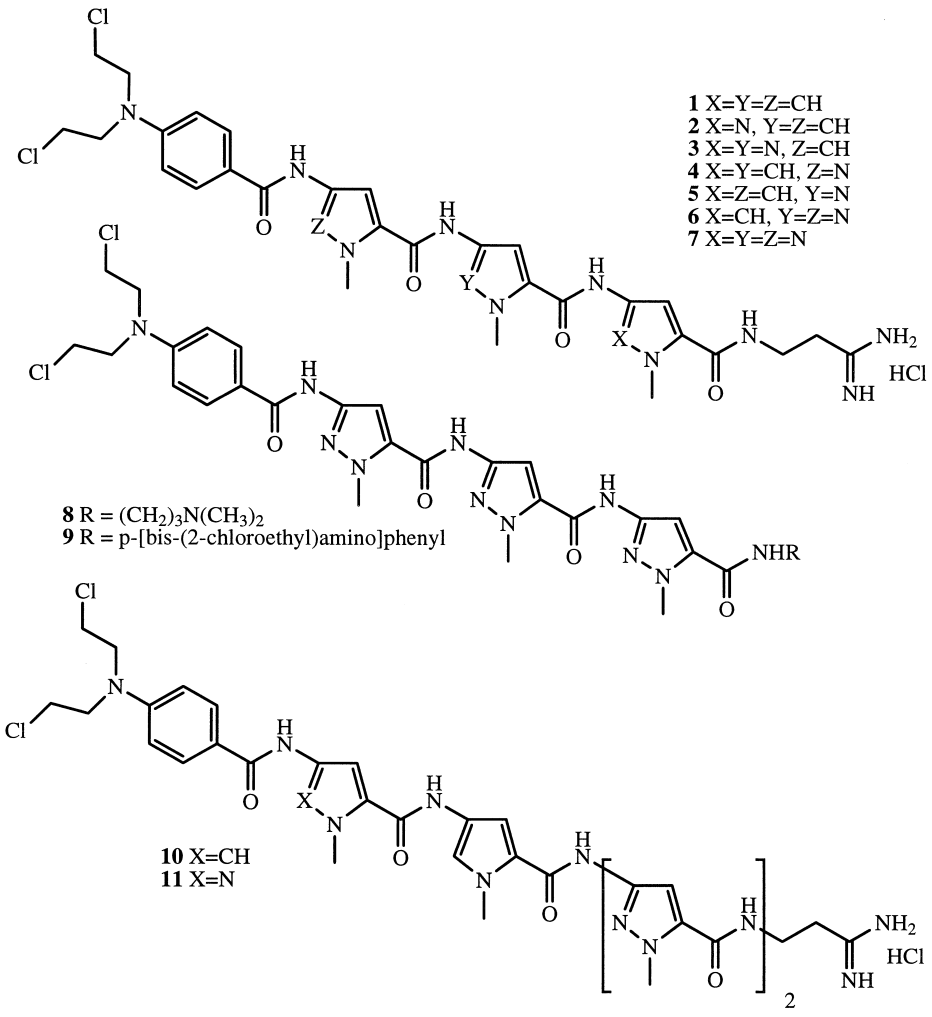


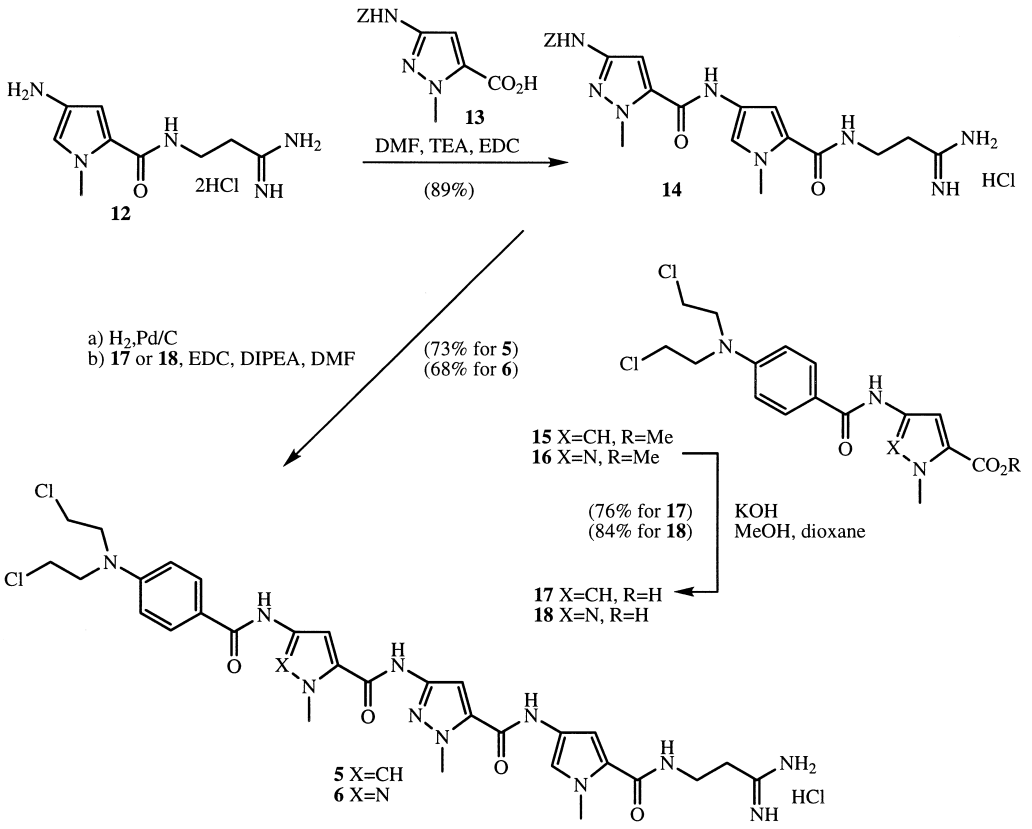
Chart 1.

amino-nitrile intermediate **19**[12] was condensed with the acid **13** to afford **20** in 87% yield, which after a reduction–condensation process with the acids **21**[12] and **22** led to the corresponding derivatives **23** and **24** in 71% and 73% yield, respectively. Treatment of **23** and **24** under Pinner reaction conditions[13] (HCl in anhydrous ethanol and then ammonia), afforded the corresponding nitro amidines **25** and **26** in 93 and 89% yield, respectively. The subsequent catalytic reduction of the nitro group gave the relative stable amine intermediates, which without isolation or purification, were directly coupled with 4-[bis(2-chloroethyl)amino]benzoyl chloride to give **5** and **6** in 58 and 68% yield, respectively.

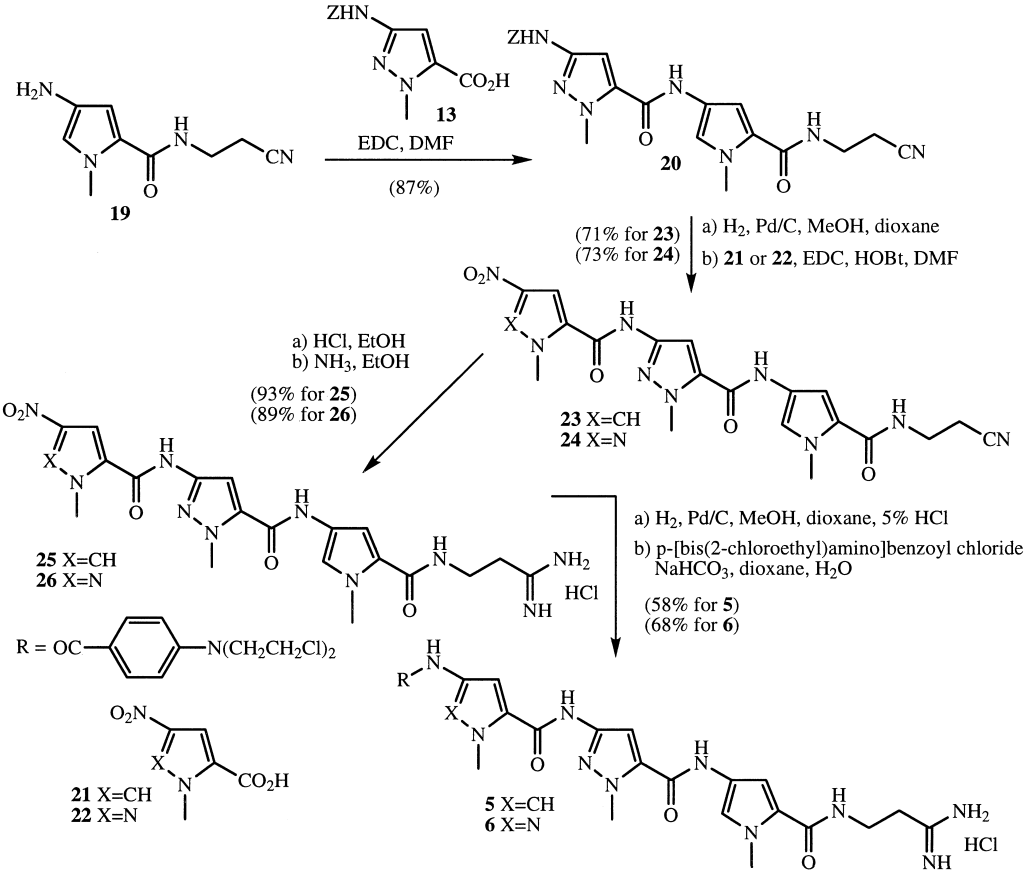
For the syntheses of compounds **3, 10**, and **11**, the nitrile **27** was employed as starting material. The treatment of **27** under Pinner reaction conditions gave the corresponding nitro-amidine **28** in 93% yield, which after a reduction–condensation process in the presence of EDC with the pyrrole and pyrazole derivatives **17** and **18**, led to the tetrapeptides **10** and **11**, respectively, with 53 and 46% yield, respectively (Scheme 3).

We have employed three different routes for the synthesis of the pyrazole-tallimustine analogue **7**. In the Scheme 4 were reported two initial approaches to the synthesis of **7**, in which the amidino group was introduced at an early stage and that proved to be unsatisfactory owing to the high sensitivity and instability of the amino-amidines **34** and **35**. The compound **34** was obtained after catalytic reduction of the nitro-amidine **32** prepared by Pinner reaction from nitrile **29**. Attempted condensation of **34** and the acid **18** with EDC did not supply the desire product.

In the second attempt, the nitrogen mustard was tethered to the pyrazole tripeptide **35**, which was prepared by catalytic hydrogenation of the nitro-amidine **33** obtained submitting to Pinner reaction the nitro-nitrile **31**. Latter compound was prepared by condensation between the known compound **30**[6] and the acid **13** in 83% yield. Condensation of **35** and *p*-[bis-(2-chloroethyl)amino]benzoyl chloride gave only a mixture of undetectable products, therefore we resolved to introduce the amidine side chain at the last step (Scheme 5). Starting from the already synthesized ester **36**,[6] catalytic removal of the benzyloxycarbonyl (Cbz or Z) protecting group and subsequent condensation with **18** provided the tripeptide **38** in 75% yield. In order to effect condensation with the β-aminopropionamidine moiety (prepared as already reported[14]) the ester **38** was converted quantitatively into the carboxylic acid **39** by



Scheme 1.



Scheme 2.

alkaline hydrolysis. Latter compound was subsequently coupled by the corresponding *N*-hydroxysuccinimide ester, with preformed β-aminopropionamidine dihydrobromide to give desired compound **7** in 93.5% yield. The same strategy was followed for the synthesis of C-terminus-modified analogues, in which a dimethylaminopropylamino (compound **8**) or a *p*-[bis-(2-chloroethyl)amino]anilino (compound **9**) moiety replaced the parent amidine group (Scheme 5): these compounds were obtained in 68 and 66% yield, respectively.

Results and Discussion

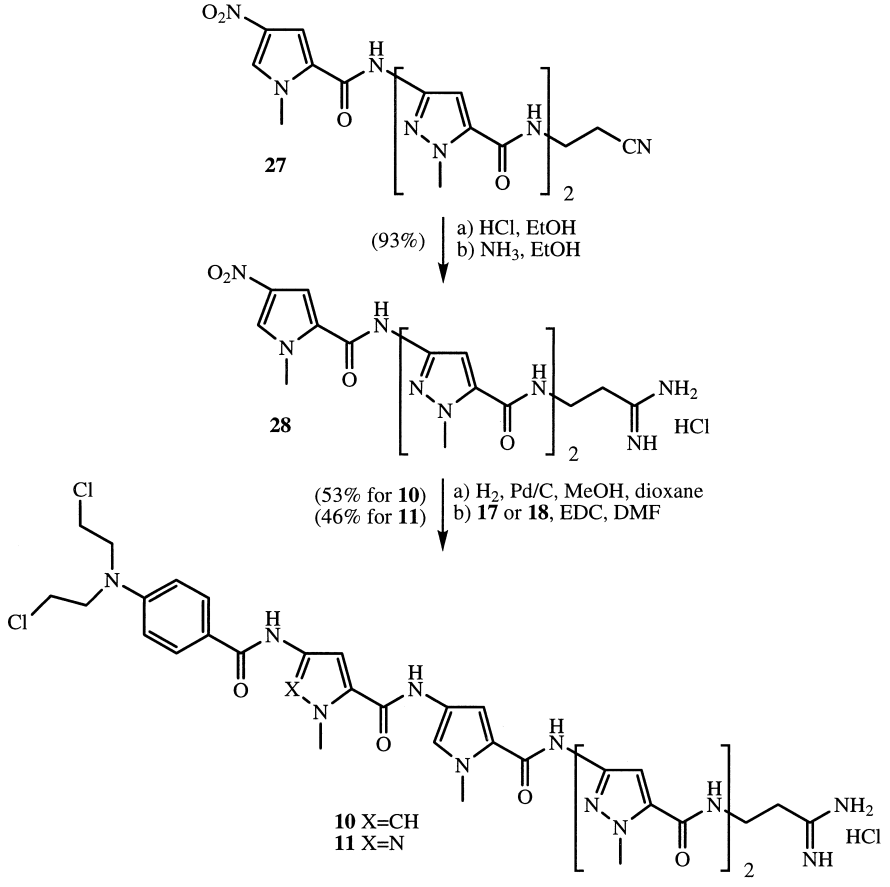
All the synthesized compounds (**5–11**) were assayed in vitro against L1210 murine leukemia cell lines, L1210 cells resistant to tallimustine (L1210/tallimustine) and to doxorubicin (L1210/DX) (obtained from NCI, Bethesda, MD). Only some representative compounds were tested in vivo against L1210 murine leukemia and M5076 murine reticulosarcoma as solid tumor model. All the results for the compounds tested both in vivo and in vitro against L1210 murine leukemia were given in Tables 1 and 2.

The cytotoxicities and the anti-leukemic activities of all synthesized compounds were evaluated as previously described and compared with that of tallimustine.[15]

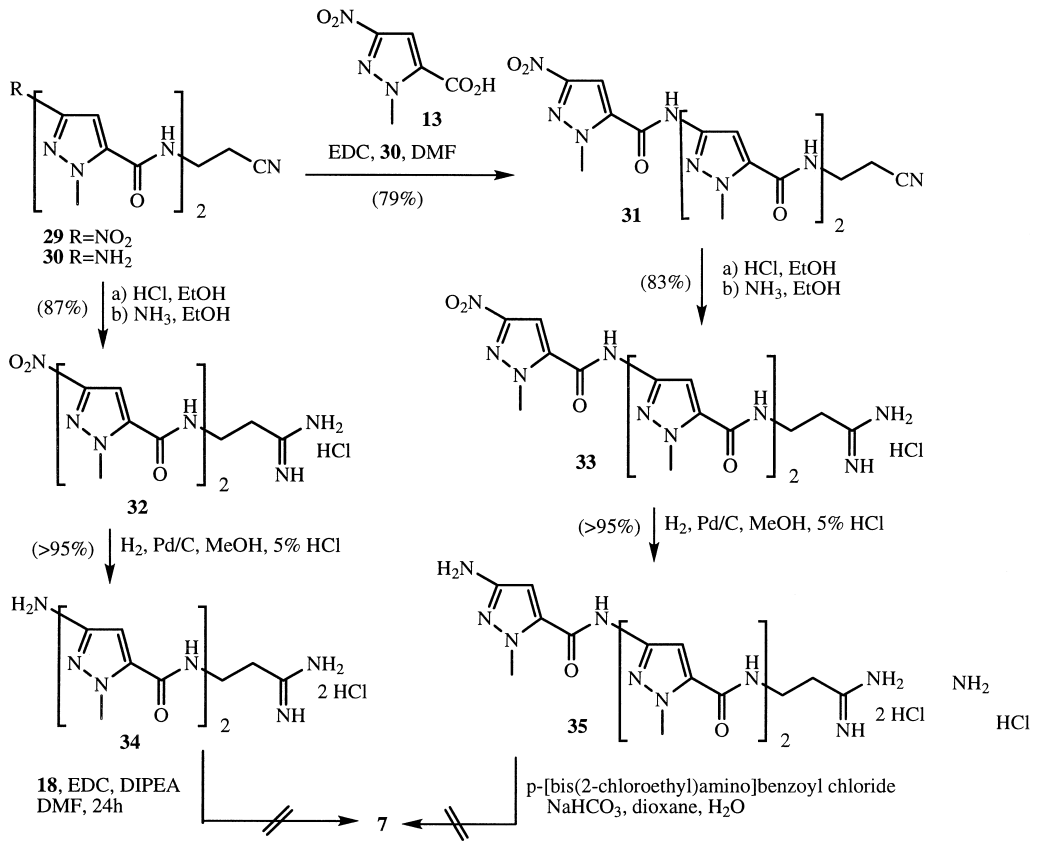
For the compound **3**, the increased number of pyrrole units in the oligopeptidic frame led to an increase of cytotoxicity. In fact, the introduction of pyrrole ring between the benzoic acid mustard and the N-terminal pyrrole of **3**, to give the homologue **10**, yielded an increase of 10-fold cytotoxicity with respect to the parent compound **3** (225.2 and 20.10 ng/mL for **3** and **10**, respectively). Moreover, this increase in the number of pyrrole units led to an increase of in vivo potency, but failed to improve anti-leukemic activity. In fact, at their optimal doses (O.D.) (12.5 and 6.25 mg/Kg for **3** and **10**, respectively) the less potent compound **3** was more effective than the pyrrole homologue **10** producing an increase of the median survival time (T/C% 213 versus 167 for **3** and **10**, respectively). On the contrary, the pyrazole homologue **11** showed threefold less potent antiproliferative activity than the parent compound **3** (225.2 and 608.3 ng/mL for **3** and **11**, respectively).

The tripyrazole analogue **7** resulted to be about 20-fold less cytotoxic in vitro than the tripyrrole counterpart tallimustine (1325 versus 50.3 ng/mL), and substantially devoid of antileukemic activity in vivo (O.D. = 12.5 mg/Kg, T/C% = 100 versus O.D. = 6.25 mg/Kg, T/C% = 125).

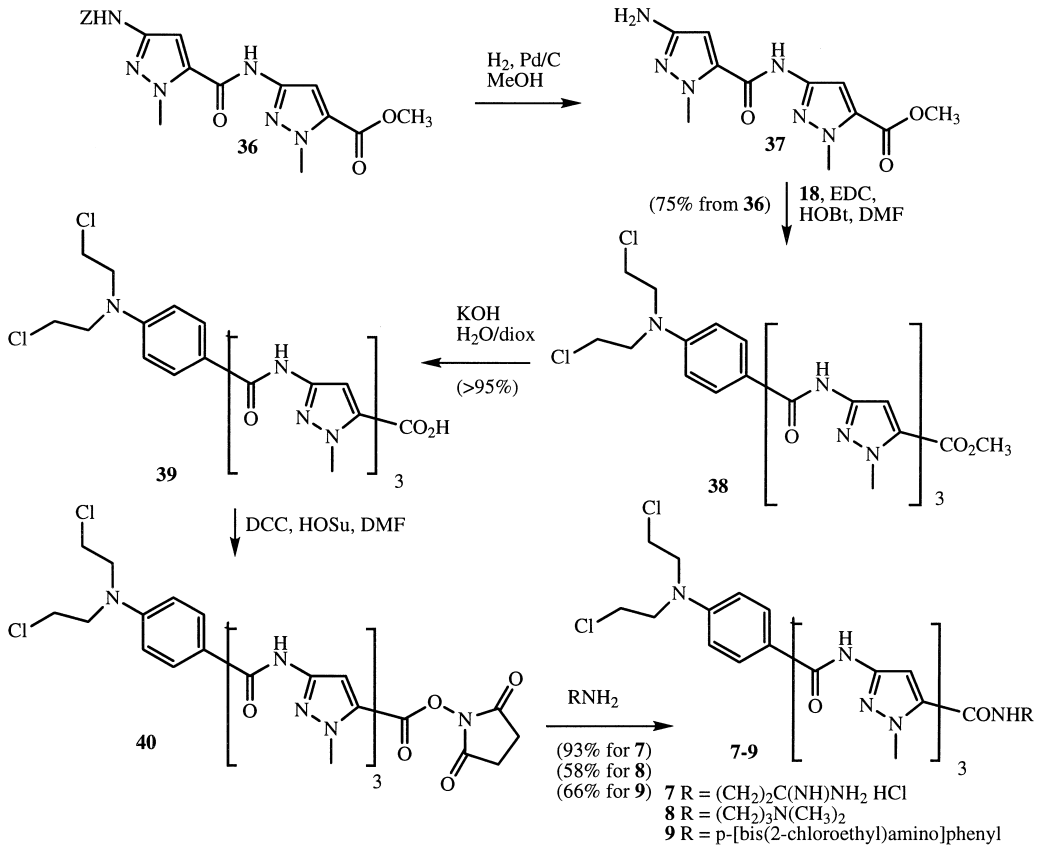
In the design of the compound **8**, an analogue of **7** that contains a dimethylaminopropyl group (extimated pK_a 9.3) at the C-terminus instead of strongly basic propionamidino moiety (extimated pK_a 12), this function should be protonated at physiological pH of 7.4 and thus it would be attracted to DNA as the amidine moiety.



Scheme 3.



Scheme 4.



Scheme 5.

Table 1 In vivo and in vitro activity against L1210 murine leukemia

Compound	in vitro L1210		in vivo L1210
	IC_{50}[a] (ng/mL)	O.D.[b] (mg/Kg)	%[c] T/C
1	50.3	6.25	125
2	35.00	6.25	213
3	225.20	12.50	213
4	1398.00	12.50	218
5	78.60	n.d[d]	n.d
6	1986.80	n.d	n.d
7	1325.00	12.50	100
8	3000.00	n.d	n.d
9	50000	n.d	n.d
10	20.10	6.25	167
11	608.30	n.d	n.d

[a]IC_{50} = 50% inhibitory concentration represents the mean from dose–response curves of at least three experiments.
[b]O.D. = optimal dose; optimal non toxic dose $< LD_{10}$.
[c]%T/C = median survival time of treated versus untreated mice×100.
[d]n.d. = not determined.

Table 2 In vitro activity of compounds **1–11** against L1210 leukemia cells, L1210 resistants to the doxorubicin (DX) and tallimustine

Compound	L1210	L1210/DX		L1210/tallimustine	
	IC_{50} (ng/mL)	IC_{50} (ng/mL)	R.I.	IC_{50} (ng/mL)	R.I
1	50.3	638.80	28.02	528.80	21.7
2	35.00	915.00	26.14	1015.00	29
3	225.20	6894.20	30.6	3089.50	13.72
4	1398.00	7980.00	5.71	2658.00	1.9
5	78.60	533.97	6.79	n.d	n.d
6	1986.80	3262.50	1.64	n.d	n.d
7	1325.00	11100.00	8.38	5800.00	4.36
8	3000.00	14500.00	4.83	4450.00	1.48
9	50000	50000	1	50000	1
10	20.10	2465.80	122.6	2550.70	127
11	608.30	36585.5	60.14	106752.5	175.5

IC_{50} = 50% inhibitory concentration represents the mean from dose–response curves of at least three experiments. R.I (resistance index) = ratio between IC_{50} values on resistant cells and sensitive cells.

Nevertheless, this derivative **8** was twofold less active than the corresponding compound **7** bearing the amidino moiety (IC_{50} ng/mL, 1325 versus 3000). Introduction of a second benzoyl nitrogen mustard moiety, as for the compound **9**, in place of the charged amidine end group on the C-terminus proved to be completely ineffective (IC_{50} ng/mL, 1325 for **7** versus 5000 for **9**).

Among the hybrids pyrrole–pyrazole tripeptidics synthesized, the two tallimustine isosters **2** and **5**, in which the pyrrole near the amidine terminus and the central pyrrole were replaced by corresponding pyrazole rings respectively, showed cytotoxicity L1210 equivalent to tallimustine (IC_{50} ng/mL; 35 and 78.6 ng/mL versus 50.3 ng/mL, respectively). Compound **3**, in which two pyrrole units near the amidine terminus was replaced with the same number of pyrazole rings, was less active than tallimustine (IC_{50} ng/mL, 225 versus 50.3) but as compound **2** showed superior antileukemic activity in vivo (O.D. = 6.25 mg/Kg, T/C% = 213 for **2** and O.D. = 12.5 mg/Kg, T/C% = 213 for **3** versus O.D. = 6.25 mg/Kg, T/C% = 125 for tallimustine). The derivatives **2** and **3** exhibited also significant activity in vivo against M 5076 murine reticulosarcoma, although they were less potent than the tallimustine (O.D = 1.56 mg/Kg, T/C% = 150 for tallimustine **1**, O.D = 6.25 mg/Kg, T/C% = 225 for **2**; O.D = 12.5 mg/Kg, T/C% = 185 for **3**). These data reveal that **2** was the best compound of this series, possessing a very high activity both against L1210 murine leukemia and M 5076 solid tumor model.

The compounds **4** and **6**, in which one or two pyrrole units near the BAM moiety were replaced by corresponding pyrazole rings, respectively, were substantially inactive (IC_{50} ng/mL; 1398 and 1986.8, respectively).

The anti-leukemic activity of derivatives of this series was strictly dependent from the position of the pyrazole ring, and appeared lower when pyrazole was close to alkylating moiety.

The cytoxicities of synthesized compounds on resistant leukaemic cell lines were reported in the Table 2. The results obtained showed that only **10** and **11** were inactive (resistance index (R.I.) from 180 to 60), whereas the other compounds presented low R.I. (from 30 to 1). It may be noted that, while the compound **10** showed a potent cytostatic activity against L1210 leukemia (IC_{50} 20.1 ng/mL), it was completely inactive against L1210/Dx and L1210/tallimustine (IC_{50} 2465.8 and 2550.7 ng/mL, respectively).

Conclusions

In this series of pyrrole–pyrazole tallimustine analogue **2–7**, we have found that the pyrrole position was critical for the activity. The presence of this heterocycle nearby benzoyl nitrogen mustard moiety was necessary for activity both in vitro and in vivo. This was confirmed for the compound **3**, where increasing the number of pyrrole rings by the introduction of a pyrrole nearby to the alkylating moiety gave the corresponding derivative **10** which was much more active than the parent compound **3**. On the contrary, the opposite effect was obtained with the incorporation of a pyrazole ring, as in compound **11**, which caused a decrease of L1210 activity in comparison **3** and **10**.

Experimental

Materials and methods

All reactions were carried out under argon atmosphere, unless otherwise described. Standard syringe techniques were applied for tranferring anhydrous solvents. Reaction courses and product mixtures were routinely monitored by TLC on silica gel (precoated F_{254} Merk plates) and visualized with aqueous $KMnO_4$. ^1H NMR spectra were obtained in DMSO solutions with a Bruker AC 200 spectrometer. Chemical shifts (δ) are given in ppm upfield from tetramethylsilane. Melting points (mp) were determined on a Buchi–Tottoli apparatus and are uncorrected. All products reported showed ^1H NMR

spectra in agreement with the assigned structures. Elemental analyses were conducted by the Mycroanalytical Laboratory of the Chemistry Department of the University of Ferrara. 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.

3-[1-Methyl-4-(1-methyl-3-benzyloxycarbonylaminopyrazole-5-carboxamido)-pyrrole-2-carboxamido] propioniamidine hydrochloride (14). To a solution of amino-amidine **12** (706 mg, 2.5 mmol) in anhydrous DMF (3 mL), cooled to 0 °C, were added triethylamine (TEA) (0.35 mL, 2.5 mmol), the acid **13** (675 mg, 0.25 mmol) and then EDC (960 mg, 5 mmol). 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 2 and DMF was removed under reduced pressure The resulting solid purified by flash column chromatography with CH_2Cl_2/EtOH/H_2O (10/10/0.8, v/v) afforded a yellow oil which was crystallized from methanol/ethyl ether to give **14** as a white solid. Yield 1.12 g (2.22 mmol, 89%); mp 213 °C; ^{1}H NMR (DMSO-d_6) δ 2.63 (t, J = 6 Hz, 2H), 3.50 (m, 2H), 3.82 (s, 3H), 3.97 (s, 3H), 5.16 (s, 2H), 6.96 (s, 1H), 7.11 (s, 2H), 7.25 (s, 1H), 7.39 (m, 5H), 8.33 (t, J = 6 Hz, 1H), 8.77 (s, 2H), 9.02 (s, 2H), 10.24 (s, 1H), 10.43 (s, 1H); Anal calcd for $C_{22}H_{27}N_8O_4Cl$: C, 52.54; H, 5.41; N, 22.28; Cl, 7.05. Found: C, 52.48; H, 5.32; N, 22.20; Cl, 6.98.

Methyl 1-methyl-4-[4-bis-(2-chloroethyl)aminophenylamido]-pyrrole-2-carboxylate (15). To a solution of *p*-*N*,*N*-bis(2-chloroethyl)aminobenzoyl chloride (700 mg, 2.5 mmol) in anhydrous dioxane (5 mL) was added dropwise to a mixture of methyl 1-methyl-4-aminopyrrole-2-carboxylate[9] (385 mg, 2.5 mmol) and triethylamine (TEA) (0.35 mL, 2.5 mmol) in anhydrous dioxane (10 mL) cooled to 0 °C. The reaction mixture was allowed to stir at room temperature overnight and then concentrated under reduced pressure to a brown solid, which was dissolved in EtOAc and washed with water (2×10 mL). The organic layer was dried (Na_2SO_4), concentrated, and the resulting residue precipitated from EtOAc/petroleum ether to give **15** as a fine brown powder. Yield 736 mg (1.87 mmol, 74%); mp 175 °C; ^{1}H NMR (DMSO-d_6) δ 3.34 (m, 8H), 3.78 (s, 3H), 4.10 (s, 3H), 6.8 (d, J = 6.8 Hz, 2H), 6.88 (s, 1H), 7.46 (s, 1H), 7.79 (d, J = 6.4 Hz, 2H), 9.98 (s, 1H); anal. calcd for $C_{18}H_{21}N_3O_3Cl_2$: C, 54.28; H, 5.31; N, 10.55; Cl, 17.8. Found: C, 54.16; H, 5.12; N, 10.41; Cl, 17.68.

Methyl 1-methyl-3-[4-bis-(2-chloroethyl)aminophenylamido]-pyrazole-5-carboxylate (16). Following the same procedure reported for the synthesis of **15**, starting from *p*-*N*,*N*-bis(2-chloroethyl)aminobenzoyl chloride (1.6 g, 5.17 mmol), methyl 1-methyl-3-aminopyrazole 5-carboxylate[6] (802 mg, 5.17 mmol), and anhydrous triethylamine (0.85 mL, 5.17 mmol), after workup compound **16** was obtained as a yellow powder. Yield 1.49 g (3.72 mmol, 72%); mp 155–157 °C; ^{1}H NMR (DMSO-d_6) δ 3.66 (d, J = 6.2 Hz, 4H), 3.77 (d, J = 6.2 Hz, 4H), 3.88 (s, 3H), 4.02 (s, 3H), 6.67 (d, J = 9 Hz, 2H), 7.40 (s, 1H), 7.78 (d, J = 9 Hz, 2H), 8.82 (s, 1H); anal. calcd for $C_{17}H_{20}N_4O_3Cl_2$: C, 51.14; H, 5.05; N, 14.03; Cl, 17.76. Found: C, 51.05; H, 4.97; N, 13.89; Cl, 17.68.

1-Methyl-4-[4-bis-(2-chloroethyl)aminophenylamido]-pyrrole-2-carboxylic acid (17). To a well-stirred solution of **15** (300 mg, 0.75 mmol) in 3 mL of dioxane was added 2 N aqueous KOH (0.75 mL) and the resulting mixture stirred at room temperature for 2 h. The clear solution was evaporated to remove dioxane, diluted with water (5 mL), cooled on an icewater bath and acidified with 10% HCl to pH 2. The suspension was extracted with EtOAc (2×10 mL) and the organic layers were combined, dried (Na_2SO_4), and concentrated. The resulting residue was precipitated from EtOAc/hexane to give the product **17** as a brown powder. Yield 219 mg (0.57 mmol, 76%); mp 185–187 °C; ^{1}H NMR (DMSO-d_6) δ 3.34 (m, 8H), 3.78 (s, 3H), 6.63 (s, 1H), 6.80 (d, J = 6.8 Hz, 2H), 7.28 (s, 1H), 7.79 (d, J = 6.8 Hz, 2H), 9.98 (s, 1H), 12.3 (bs, 1H); anal. calcd for $C_{17}H_{19}N_3O_3Cl_2$: C, 53.14; H, 4.98; N, 10.94; Cl, 18.4. Found: C, 52.6; H, 4.1; N, 10.1; Cl, 17.9.

1-Methyl-3-[4-bis-(2-chloroethyl)aminophenylamido]-pyrazole-5-carboxylic acid (18). Following the same procedure reported for the synthesis of **17**, starting from **16** (500 mg, 1.25 mmol) and 2 N aqueous KOH (1 mL, 2 equiv), after workup the acid **18** was obtained as brown solid. Yield 404 mg (1.05 mmol, 84%); mp 233–235 °C; ^{1}H NMR (DMSO-d_6) δ 2.50 (m, 4H), 3.78 (m, 4H), 4.03 (s, 3H), 6.82 (d, J = 9 Hz, 2H), 7.12 (s, 1H), 7.92 (d, J = 9 Hz, 2H), 10.66 (s, 1H), 13.25 (bs, 1H); anal. calcd for $C_{16}H_{18}N_4O_3Cl_2$: C, 49.9; H, 4.71; N, 14.5; Cl, 18.4. Found: C, 49; H, 4.1; N, 13.7; Cl, 17.7.

3-[1-Methyl-4-(1-methyl-3-benzyloxycarbonylaminopyrazole-5-carboxamido)-pyrrole-2-carboxamido]propionitrile (20). To a solution of amino-nitrile **19** (384 mg, 2 mmol) and acid **13** (522 mg, 2 mmol) in DMF (5 mL) at 0 °C was added EDC (786 mg, 4 mmol). The reaction mixture was allowed to warm to room temperature and then stirred for 24 h. The solvent was removed under reduced pressure, the residue taken up in EtOAc (10 mL) and then washed once with 5% aq HCl. The organic layer was dried on Na_2SO_4 and solvent removed under reduced pressure. The product purified by crystallization (EtOAc/petroleum ether) afforded **20** as a yellow powder. Yield 760 mg (1.74 mmol, 87%); mp 232 °C; ^{1}H NMR (DMSO-d_6) δ 2.68 (t, J = 6.2 Hz, 2H), 3.58 (d, J = 6.4 Hz, 2H), 3.81 (s, 3H), 4.01 (s, 3H), 5.22 (s, 2H), 6.97 (s, 1H), 7.10 (s, 2H), 7.23 (s, 1H), 7.39 (m, 5H), 8.38 (t, J = 6 Hz, 1H), 10.33 (s, 1H), 10.52 (s, 1H); anal. calcd for $C_{22}H_{23}N_7O_4$: C, 58.79; H, 5.16; N, 21.81. Found: C, 58.67; H, 5.07; N, 21.74.

1-Methyl-3-nitropyrazole-5-carboxylic acid (22). Following the same procedure reported for the synthesis of **17**, starting from methyl 1-methyl-3-nitropyrazole-5-carboxylate[16] (600 mg, 3.24 mmol) and 2 N aqueous KOH

(3.5 mL, 6.48 mmol), after workup the compound **22** was obtained as a yellow powder. Yield 537 mg (3.14 mmol, 97%); mp 170–172 °C; ^{1}H NMR (DMSO-d_6) δ 4.27 (s, 3H), 7.38 (s, 1H); anal. calcd for $C_5H_5N_3O_4$: C, 35.1; H, 2.95; N, 24.56. Found: C, 35.01; H, 2.88; N, 24.48.

3-[1-Methyl-4-[1-methyl-3-(1-methyl-4-nitropyrrole-2-carboxamido)pyrazole-5-carboxamido]pyrrole-2-carboxamido]-propionitrile (23). A solution of **20** (868 mg, 2 mmol) in 15 mL of a mixture MeOH/dioxane (2/1, v/v) was hydrogenated over 100 mg of 10% Pd/C at 50 psi for 3 h. The catalyst was removed by filtration, the filtrate was concentrated to give a green oil which was used without purification for the next step. This crude amine was dissolved in anhydrous DMF (7 mL) and at this solution were added **21** (340 mg, 2 mmol), HOBt (270 mg, 2 mmol), and EDC (384 mg, 2 mmol). The reaction mixture was stirred at room temperature for 12 h, and the evaporation of the DMF under reduced pressure gave a brown solid which was suspended in a mixture EtOAc (10 mL) and 10% aq HCl. This suspension was stirred for 2 h and then filtered, rinsed with water, to give **23** as a pale-yellow solid. Yield 663 mg, 71%; mp 211–213 °C; ^{1}H NMR (DMSO-d_6) δ 2.62 (t, J = 6 Hz, 2H), 3.41 (t, J = 6 Hz, 2H), 3.85 (s, 3H), 4.03 (s, 3H), 4.12 (s, 3H), 7.24 (s, 1H), 7.29 (s, 1H), 7.46 (s, 1H), 7.72 (s, 1H), 7.89 (s, 1H), 8.15 (bs, 1H), 10.92 (s, 1H), 11.28 (s, 1H); anal. calcd for $C_{20}H_{21}N_9O_5$: C, 51.39; H, 4.53; N, 26.97. Found: C, 51.29; H, 4.48; N, 26.89.

3-[1-Methyl-4-[1-methyl-3-(1-methyl-3-nitropyrazole-5-carboxamido)pyrazole-5-carboxamido]pyrrole-2-carboxamido]propionitrile (24). Starting from **20** (651, 1.5 mmol), **22** (391 mg, 1.5 mmol), HOBt (405 mg, 3 mmol), and EDC (576 mg, 3 mmol), the reaction was carried out via a similar procedure as described above for **23** to afford **24** as a white solid. Yield 512 mg, 73%; mp 255–256 °C; ^{1}H NMR (DMSO-d_6) δ 2.62 (t, J = 6 Hz, 2H), 3.41 (t, J = 6 Hz, 2H), 3.85 (s, 3H), 4.03 (s, 3H), 4.12 (s, 3H), 7.29 (s, 1H), 7.46 (s, 1H), 7.72 (s, 1H), 7.89 (s, 1H), 8.15 (bs, 1H), 8.56 (s, 2H), 8.99 (s, 2H), 10.92 (s, 1H), 11.28 (s, 1H); anal. calcd for $C_{19}H_{20}N_{10}O_5$: C, 48.72; H, 4.3; N, 29.9. Found: C, 48.67; H, 4.25; N, 29.79.

3-[1-Methyl-4-[1-methyl-3-(1-methyl-4-nitropyrrole-2-carboxamido)pyrazole-5-carboxamido]pyrrole-2-carboxamido]-propionamidine hydrochloride (25). To a cooled suspension (0 °C) of **23** (467 mg, 1 mmol) in absolute ethanol (200 mL) was bubbled with anhydrous HCl for 20 min. The reaction mixture was stirred at 0 °C for an additional hour and at room temperature for 10 min. Removal of the solvent under reduced pressure gave a solid residue, which was redissolved in absolute ethanol (150 mL). The mixture was cooled (0 °C) and anhydrous ammonia was bubbled inside for 10 min. After 24 h at room temperature, the solvent was removed in vacuo to give a white solid, which was washed with ethyl ether (twice), filtered, and dried under high vacuum at 40 °C to give amidine **25**. Yield 519 mg, 93%; mp 287–289 °C; ^{1}H NMR (DMSO-d_6) δ 2.53 (t, J = 6 Hz, 2H), 3.44 (t, J = 6 Hz, 2H), 3.82 (s, 3H), 4.00 (s, 3H), 4.08 (s, 3H), 7.32 (bs, 2H), 7.56 (s, 1H), 7.74 (s, 1H), 7.86 (s, 1H), 8.21 (bs, 1H), 8.78 (bs, 2H), 8.98 (bs, 2H), 10.98 (s, 1H), 11.16 (s, 1H); anal. calcd for $C_{20}H_{25}N_{10}O_5Cl$: C, 46.11; H, 4.84; N, 26.89; Cl, 6.81. Found: C, 46.02; H, 4.73; N, 26.80; Cl, 6.73.

3-[1-Methyl-4-[1-methyl-3-(1-methyl-3-nitropyrazole-5-carboxamido)pyrazole-5-carboxamido]pyrrole-2-carboxamido]propionamidine hydrochloride (26). According to the procedure reported for the Pinner reaction on the compound **23**, starting from **24** (940 mg, 2 mmol), the amidine **26** was obtained in 89% yield (932 mg): mp 232 °C; ^{1}H NMR (DMSO-d_6) δ 2.61 (t, J = 6 Hz, 2H), 3.44 (t, J = 6 Hz, 2H), 4.02 (s, 3H), 4.08 (s, 3H), 4.24 (s, 3H), 7.24 (s, 1H), 7.31 (s, 1H), 7.63 (s, 1H), 7.97 (s, 1H), 8.84 (bs, 1H), 8.86 (bs, 2H), 9.04 (bs, 2H), 11.32 (s, 1H), 11.58 (s, 1H); anal. calcd for $C_{19}H_{24}N_{11}O_5Cl$: C, 43.72; H, 4.63; N, 29.52; Cl, 6.79. Found: C, 43.64; H, 4.55; N,29.45; Cl, 6.68.

3-[1-Methyl-4-[1-methyl-4-[1-methyl-4-[4-bis(2-chloroethyl)-aminophenylamido]-pyrrole-2-carboxamido]pyrazole-5-carboxamido]pyrrole-2-carboxamido] propionamidine hydrochloride (5). Procedure A. A solution of **14** (500 mg, 1 mmol) in 15 mL of a mixture methanol/dioxane (1/1, v/v) and 5% aq HCl (6 drops) was hydrogenated at 50 psi over 10% Pd/C using a Parr hydrogenator for 4 h. The catalyst was removed by filtration, the yellow filtrate concentrated, and the residue used immediately in the following step. The above amine dissolved in 5 mL of anhydrous DMF was treated at 0 °C with *N*-ethyldiisopropylamine (DIPEA or Hunig's base) (192 μL, 1 mmol) and after 5 min, with **17** (385 mg, 1 mmol) and then EDC (384 mg, 2 mmol). The reaction mixture was stirred for 18 h at room temperature. The solvent was evaporated to dryness in vacuo, and the resulting residue was purified by flash column chromatography with CH_2Cl_2/MeOH (4/1, v/v) to give **5** as a brown solid. Yield 536 mg, 0.73 mmol, 73%; mp 254–256 °C; ^{1}H NMR (DMSO-d_6) δ 2.56 (m, 2H), 3.32 (m, 2H), 3.49 (m, 8H), 3.79 (s, 3H), 3.82 (s, 3H), 4.04 (s, 3H), 6.83 (d, J = 8.8 Hz, 2H), 6.98 (s, 1H), 7.15 (s, 1H), 7.24 (s, 1H), 7.42 (s, 2H), 7.84 (d, J = 8.8 Hz, 2H), 8.29 (bs, 1H), 8.51 (s, 2H), 8.94 (s, 2H), 10.07 (s, 1H), 10.44 (s, 1H), 10.64 (s, 1H); anal. calcd for $C_{31}H_{38}N_{11}O_4Cl_3$: C, 50.65; H, 5.21; N, 20.96; Cl, 14.47. Found: C, 50.58; H, 5.11; N, 20.88; Cl, 14.40.

Procedure B. The compound **25** (260 mg, 0.5 mmol) was submitted to hydrogenation following the same procedure reported for **14**. After the workup, the amine was used immediately in the following step. The above amine was dissolved in 5 mL of a mixture dioxane/water (4/1, v/v) was treated at 0 °C with $NaHCO_3$ (168 mg, 2 mmol) and *p*-*N*,*N*-bis(2-chloroethyl)aminobenzoyl chloride (140 mg, 0.5 mmol). The reaction mixture was stirred for 3 h at room temperature. After TLC analysis indicated that all of the starting material had disappeared, the reaction was acidified to pH 5 with 5% aq HCl and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography with CH_2Cl_2/MeOH (4/1, v/v) to give **5** as a brown solid. Yield 213 mg, 0.29 mmol, 58%.

3-[1-Methyl-4-[1-methyl-3-[1-methyl-3-[4-bis(2-chloroethyl)-aminophenylamido]-pyrazole-5-carboxamido]pyrazole-5-carboxamido]pyrrole-2-carboxamido] propionamidine hydrochloride (6). According to the procedure A reported for the synthesis of **5**, starting from **14** (400 mg, 0.78 mmol), Hunig's base (133 μL, 0.78 mmol), **18** (300 mg, 0.78 mmol), and EDC (300 mg, 1.56 mmol), after workup the resulting residue was purified by flash column chromatography with CH_2Cl_2/MeOH (5/1, v/v) to give **6** as a yellow solid. Yield 390 mg, 0.53 mmol, 68%; mp 278–280 °C; ^{1}H NMR (DMSO-d_6) δ 2.63 (m, 2H), 3.50 (m, 2H), 3.79 (s, 3H), 3.83 (s, 3H), 4.07 (s, 3H), 4.43 (m, 8H), 6.82 (d, J = 8.6 Hz, 2H), 6.99 (s, 1H), 7.26 (s, 1H), 7.45 (s, 1H), 7.57 (s, 2H), 7.93 (d, J = 8.8 Hz, 2H), 8.32 (bs, 1H), 8.66 (s, 2H), 9.00 (s, 2H), 10.48 (s, 1H), 10.64 (s, 1H), 11.19 (s, 1H); anal. calcd for $C_{30}H_{38}N_{12}O_4Cl_3$: C, 48.89; H, 5.2; N, 22.8; Cl, 14.43. Found: C, 48.78; H, 5.11; N, 22.72; Cl, 14.31.

According to the procedure B reported for the synthesis of **5**, starting from **26** (390 mg, 0.75 mmol), $NaHCO_3$ (252 mg, 3 mmol) and *p*-*N*,*N*-bis (2-chloroethyl)aminobenzoyl chloride (210 mg, 0.75 mmol), after workup and flash column chromatography with CH_2Cl_2/MeOH (4/1, v/v), **6** was obtained as a brown solid. Yield 375 mg, 0.51 mmol, 68%.

3-[1-Methyl-3-[1-methyl-3-(1-methyl-4-nitropyrrole-2-carboxamido)pyrazole-5-carboxamido]pyrazole-5-carboxamido] propionitrile (27). Following the same procedure reported for the synthesis of **23**, starting from 1-methyl-3-(1-methyl-3-aminopyrazole-5-carboxamido)-pyrazole-5-carboxamidopropionitrile **30**[6] (1.26 g, 4 mmol), 1-methyl-3-nitropyrrole-5-carboxylic acid (680 mg, 4 mmol), HOBt (540 mg, 4 mmol) and EDC (800 mg, 4 mmol) dissolved in DMF (20 mL), after the workup the product **27** was obtained as a white solid. Yield 1.34 g, 1.3 mmol, 72%; mp 288–290 °C; ^{1}H NMR (DMSO-d_6) δ 2.78 (t, J = 6.2 Hz, 2H), 3.45 (t, J = 6.2 Hz, 2H), 3.97 (s, 3H), 4.03 (s, 3H), 4.07 (s, 3H), 7.34 (s, 1H), 7.58 (s, 1H), 7.81 (s, 1H), 8.21 (s, 1H), 8.95 (bs, 1H), 10.98 (s, 1H), 11.22 (s, 1H); anal. calcd for $C_{19}H_{20}N_{10}O_5$: C, 48.72; H, 4.3; N, 29.9. Found: C, 48.61; H, 4.19; N, 29.82.

3-[1-Methyl-3-[1-methyl-3-(1-methyl-4-nitropyrrole-2-carboxamido)pyrazole-5-carboxamido]pyrazole-5-carboxamido] propioniamidine hydrochloride (28). Following the same procedure reported for the synthesis of **25**, starting from the compound **27** (500 mg, 1.07 mmol), after workup the amidine **28** was obtained as a green solid. Yield: 519 mg, 0.92 mmol, 93%; mp 290–291 °C; ^{1}H NMR (DMSO-d_6) δ 2.50 (t, J = 6.1 Hz, 2H), 3.38 (t, J = 6.2 Hz, 2H), 3.87 (s, 3H), 4.01 (s, 3H), 4.05 (s, 3H), 7.40 (bs, 2H), 7.56 (s, 1H), 7.80 (s, 1H), 7.95 (s, 1H), 8.24 (bs, 1H), 8.88 (bs, 2H), 9.16 (bs, 2H), 10.98 (s, 1H), 11.16 (s, 1H); anal. calcd for $C_{19}H_{24}N_{11}O_5Cl$: C, 43.72; H, 4.63; N, 29.52; Cl, 6.79. Found: C, 43.67; H, 4.58; N, 29.44; Cl, 6.72.

3-[1-Methyl-3-[1-methyl-3-[1-methyl-4-[4-bis(2-chloroethyl)-aminophenylamido)]pyrrole-2-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxamido] propionamidine hydrochloride (3). Following the procedure B reported for the synthesis of **5**, starting from **28** (230 mg, 0.44 mmol), $NaHCO_3$ (130 mg, 1.32 mmol) and *p*-*N*,*N*-bis(2-chloroethyl)aminobenzoyl chloride (308 mg, 1.1 mmol), after workup and flash column chromatography with CH_2Cl_2/MeOH (40/10, v/v), **3** was obtained as a white solid. Yield 216 mg, 0.295 mmol, 67%; mp 108–110 °C; ^{1}H NMR (DMSO-d_6) δ 2,63 (m, 2H), 3.54 (m, 2H), 3.79 (m, 8H), 3.87 (s, 3H), 4.02 (s, 3H), 4.05 (s, 3H), 6.83 (d, J = 9 Hz, 2H), 7.16 (s, 1H), 7.31 (s, 1H), 7.41 (s, 1H), 7.54 (s, 1H), 7.86 (d, J = 9 Hz, 2H), 8.65 (bs, 2H), 8.82 (m, 1H), 9.02 (bs, 2H), 10.07 (s, 1H), 10.55 (s, 1H), 11.15 (s, 1H); anal. calcd for $C_{30}H_{37}N_{12}O_4Cl_3$: C, 48.95; H, 5.07; N, 22.83; Cl, 14.45. Found: C, 48.87; H, 4.98; N, 22.75; Cl, 14.38.

3-[1-Methyl-3-[1-methyl-3-[1-methyl-4-[1-methyl-4-[4-bis-(2-chloroethyl)aminobenzenamido-pyrrole-2-carboxamido]-pyrrole-2-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxamido] propionamidine hydrochloride (10). Following the procedure A reported for the synthesis of **5**, starting from the nitro-amidine **28** (300 mg, 0.516 mmol), DIPEA (88 μL, 0.516 mmol), **17** (270 mg, 0.69 mmol) and then EDC (240 mg, 1.2 mmol), after workup and flash column chromatography with CH_2Cl_2/MeOH (50/10, v/v), **10** was obtained as a white solid. Yield 234 mg, 0.273 mmol, 53%; mp 257–260 °C; ^{1}H NMR (DMSO-d_6) δ 2.3–2.6 (m, 4H), 3.4–3.5 (m, 8H), 3.79 (s, 3H), 3.81 (s, 3H), 3.84 (s, 3H), 3.86 (s, 3H), 6.83 (d, J = 7 Hz, 2H), 6.96 (s, 1H), 7.07 (s, 1H), 7.09 (s, 1H), 7.18 (s, 1H), 7.24 (s, 1H), 7.30 (s, 1H), 7.84 (d, J = 7 Hz, 2H), 8.24 (m, 1H), 8.52 (s, 2H), 8.94 (s, 2H), 10.01 (s, 1H), 10.03 (s, 1H), 10.55 (s, 1H), 11.15 (s, 1H); anal. calcd for $C_{36}H_{43}N_{14}O_5Cl_3$: C, 50.39; H, 5.05; N, 22.85; Cl, 12.39. Found: C, 50.28; H, 4.96; N, 22.78; Cl, 12.29.

3-[1-Methyl-3-[1-methyl-3-[1-methyl-4-[1-methyl-4-[4-bis-(2-chloroethyl)aminobenzenamido]-pyrrole-2-carboxamido]-pyrrole-2-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxamido] propionamidine hydrochloride (11). Following the procedure A reported for the synthesis of **5**, starting from the nitro-amidine **28** (500 mg, 0.86 mmol), DIPEA (0.2 mL), **18** (415 mg, 1.06 mmol) and EDC (370 mg, 1.84 mmol), after workup and flash column chromatography with CH_2Cl_2/MeOH (40/20, v/v), **11** was obtained as a white solid: Yield 317 mg, 0.37 mmol, 46%; mp 220–223 °C; ^{1}H NMR (DMSO-d_6) δ 2.63 (t, J = 6 Hz, 4H), 3.55 (m, 8H), 3.63 (t, J = 6 Hz, 4H), 3.79 (s, 3H), 3.88 (s, 3H), 4.02 (s, 3H), 4.07 (s, 3H), 6.82 (d, J = 9 Hz, 2H), 7.17 (s, 1H), 7.31 (s, 1H), 7.42 (s, 1H), 7.47 (s, 1H), 7.54 (s, 1H), 7.94 (d, J = 8.8 Hz, 2H), 8.66 (bs, 2H), 8.81 (m, 1H), 9.03 (b, 2H), 10.30 (s, 1H), 10.60 (s, 1H), 10.66 (s, 1H), 11.41 (s, 1H); anal. calcd for $C_{35}H_{42}N_{15}O_5Cl_3$: C, 48.93; H, 4.93; N, 24.45; Cl, 12.38. Found: C, 48.86; H, 4.87; N, 24.33; Cl, 12.29.

3-[1-Methyl-4-[1-methyl-4-[1-methyl-3-[4-bis(2-chloroethyl)-aminophenylamido]-pyrazole-5-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido] propionamidine hydrochloride (4). Following the procedure A reported for the synthesis of **5**, starting from 1-methyl-4-(1-methyl-4-aminopyrrole-2-carboxamido)-pyrrole-2-carboxamidopropionamidine hydrochloride (496 mg, 1.36 mmol),

DIPEA (0.23 mL, 1.36 mmol), **18** (520 mg, 1.36 mmol) and then EDC (522 mg, 2.72 mmol), after workup the residue was dissolved in a small volume of methanol and then ethyl ether was added to precipitate the crude product as a brown solid. The procedure was repeated five times to give **4** as a yellow solid. Yield 700 mg, 0.952 mmol, 70%; mp 210–215 °C; ^{1}H NMR (DMSO-d_6) δ 2.49 (m, 2H), 2.61 (m, 2H), 3.38 (m, 4H), 3,49 (m, 4H), 3.61 (s, 3H), 3.66 (s, 3H), 4.06 (s, 3H), 6.82 (d, J = 9 Hz, 2H), 6.96 (s, 1H), 7.10 (s, 1H), 7.20 (s, 1H), 7.32 (s, 1H), 7.47 (s, 1H), 7.95 (d, J = 9.1 Hz, 2H), 8.24 (bt, 1H), 8.63 (bs, 2H), 8.99 (bs, 2H), 10.00 (s, 1H), 10.07 (s, 1H), 10.49 (s, 1H); anal. calcd for $C_{31}H_{38}N_{11}O_4Cl_3$: C, 50.65; H, 5.21; N, 20.96; Cl, 14.47. Found: C, 50.58; H, 5.11; N, 20.87; Cl, 14.39.

3-[1-Methyl-3-(1-methyl-3-nitropyrazole-5-carboxamido)-pyrazole-5-carboxamido] propionitrile (29). The nitroacid **13** (350 mg, 2.24 mmol) was refluxed in 4 mL of thionyl chloride for 1 h. The reaction mixture was concentrated in vacuo and the resulting residue was co-evaporated with toluene. The resulting acid chloride was used without further purification.

To a cooled solution of 1-methyl-3-aminopyrazole-5-carboxamidopropionitrile[6] (295 mg, 1.53 mmol) and $NaHCO_3$ (252 mg, 3 mmol) in a mixture water/dioxane (10 mL, 1/4, v/v), the above acid chloride in dioxane (5 mL) was added dropwise. After 2 h at 0 °C, the reaction mixture was allowed to rise to room temperature and stirred for 18 h. Solvent was evaporated, the solid dissolved in EtOAc (15 mL) and washed successively with 5% HCl (5 mL), saturated aq $NaHCO_3$ (5 mL) and brine. The organic layer was dried (Na_2SO_4), evaporated under reduced pressure and the residue purified by cristallization (EtOAc/petroleum ether) give **29** as a brown solid. Yield 439 mg, 1.27 mmol, 81%; mp 254 °C; ^{1}H NMR (DMSO-d_6) δ 2.77 (t, J = 5.6 Hz, 2H), 3.44 (m, 2H), 4.04 (s, 3H), 4.23 (s, 3H), 7.36 (s, 1H), 7.96 (s, 1H), 8.97 (m, 1H), 11.48 (s, 1H); anal. calcd for $C_{13}H_{14}N_8O_4$: C, 49.09; H, 4.07; N, 32.36. Found: C, 49.00; H, 3.98; N, 32.25.

3-[1-Methyl-3-[1-methyl-3-(1-methyl-3-nitropyrazole-5-carboxamido)pyrazole-5-carboxamido]pyrazole-5-carboxamido] propionitrile (31). Following the same procedure reported for the synthesis of **20**, starting from **30**[6] (2 g, 6.4 mmol), **13** (1 g, 6.4 mmol) and EDC (1.7 g, 8.32 mmol), after work-up, the product **31** was obtained as a white solid. Yield 2.37 g (5.06 mmol, 79%), mp 173–175 °C. ^{1}H NMR (DMSO-d_6): δ 2.76 (t, J = 6 Hz, 2H), 3.47 (m, 2H), 4.03 (s, 3H), 4.08 (s, 3H), 4.23 (s, 3H), 7.33 (s, 1H), 7.64 (s, 1H), 7.96 (s, 1H), 8.95 (m, 1H), 11.26 (s, 1H), 11.41 (s, 1H); anal. calcd for $C_{18}H_{19}N_{11}O_5$: C, 46.06; H, 4.08; N, 32.82; Found: C, 45.98; H, 4.00; N, 32.73.

3-[1-Methyl-3-(1-methyl-3-nitropyrazole-5-carboxamido)-pyrazole-5-carboxamido] propionamidine hydrochloride (32). According to the general procedure reported for the preparation of **25**, starting from **29** (600 mg, 1.73 mmol), after work-up, the nitroamidine **32** was obtained as a green solid. Yield 600 mg (1.5 mmol, 87%), mp 176–178 °C. ^{1}H NMR (DMSO-d_6): δ 2.79 (t, J = 6.2 Hz, 2H), 3.51 (m, 2H), 4.03 (s, 3H), 4.22 (s, 3H), 7.34 (s, 1H), 7.97 (s, 1H), 8.68 (bs, 4H), 9.01 (m, 1H), 11.51 (s, 1H); anal. calcd for $C_{13}H_{18}N_9O_4Cl$: C, 39.09; H, 4.54; N, 31.53; Cl, 8.87. Found: C, 38.99; H, 4.47; N, 31.46; Cl, 8.81.

Attempted formation of 3-[1-Methyl-3-[1-methyl-3-[1-methyl-3-[4-bis(2-chloroethyl)aminophenylamido]-pyrazole-5-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxamido] propionamidine hydrochloride (7). Following the procedure A reported for the synthesis of **5**, starting from the nitro-amidine **32** (200 mg, 0.5 mmol), DIPEA (86 μL, 0.5 mmol), acid **18** (160 mg, 0.61 mmol) and EDC (192 mg, 1 mmol) after work-up, no significant products were detectable on TLC.

3-[1-Methyl-3-[1-methyl-3-(1-methyl-3-nitropyrazole-5-carboxamido)pyrazole-5-carboxamido]pyrazole-5-carboxamido] propionamidine hydrochloride (33). According to the general procedure reported for the preparation of **25**, starting from **31** (470 mg, 1 mmol), after work-up, the nitroamidine **33** was obtained as a yellow solid. Yield 500 mg (0.83 mmol, 83%), mp 265–267 °C. ^{1}H NMR (DMSO-d_6): δ 2.72 (t, J = 6.2 Hz, 2H), 3.54 (m, 2H), 4.01 (s, 3H), 4.07 (s, 3H), 4.12 (s, 3H), 7.13 (s, 1H), 7.31 (s, 1H), 7.59 (s, 1H), 8.73 (m, 1H), 8.82 (s, 2H), 9.15 (s, 2H), 11.17 (s, 1H), 11.3 (s, 1H); anal. calcd for $C_{18}H_{23}N_{12}O_5Cl$: C, 41.35; H, 4.43; N, 32.14; Cl, 6.78. Found: C, 41.27; H, 4.35; N, 32.07; Cl, 6.71.

Attempted formation of 3-[1-Methyl-3-[1-methyl-3-[1-methyl-3-[4-bis(2-chloroethyl)aminophenylamido]-pyrazole-5-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxamido] propionamidine hydrochloride (7). Using the procedure B reported for the synthesis of **5**, starting from the nitro-amidine **33** (522 mg, 1 mmol), $NaHCO_3$ (168 mg, 2 mmol) and *p*-[bis-(2-chloroethyl)amino]-benzoyl chloride (270 mg, 1 mmol), the reaction was stirred for 30 min at 0 °C and then at room temperature: no significant products were detectable on TLC during reaction progress.

Methyl 3-[1-Methyl-3-[1-methyl-3-[1-methyl-3-[4-bis(2-chloroethyl)aminophenylamido]-pyrazole-5-carboxamido]-pyrazole-5-carboxamido]pyrazole-5-carboxylate (38). Using the same procedure reported for the synthesis of **23**, starting from **36** (385 mg, 1 mmol), the acid **18** (462 mg, 1.2 mmol), HOBt (135 mg, 1 mmol) and EDC (192 mg, 1 mmol) after usual work-up and flash column chromatography with EtOAc, **38** was obtained as a white solid. Yield 485 mg (0.75 mmol, 75%), mp 256 °C. ^{1}H NMR (DMSO-d_6): δ 3,79 (m, 8H), 3.85 (s, 3H), 4.02 (s, 3H), 4.03 (s, 3H), 4.04 (s, 3H), 6.82 (d, J = 8.8 Hz, 2H), 7.16 (s, 1H), 7.57 (s, 1H), 7.60 (s, 1H), 7.93 (d, J = 8.6 Hz, 2H), 10.64 (s, 1H), 11.15 (s, 1H), 11.28 (s, 1H); anal. calcd for $C_{27}H_{30}N_{10}O_5Cl_2$: C, 50.24; H, 4.68; N, 21.7; Cl, 10.98; Found: C, 50.17; H, 4.61; N, 21.62; Cl, 10.89.

3-[1-Methyl-3-[1-methyl-3-[1-methyl-3-[4-bis(2-chloroethyl)-aminophenylamido]-pyrazole-5-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxylic acid (39). Using the same procedure reported for the synthesis of **17**, starting

from **38** (129 mg, 0.2 mmol) and 2N aqueous KOH (0.25 mL), after work-up the compound **39** was obtained as a white solid. Yield 126 mg (0.2 mmol, 100%), mp 280 °C. ^{1}H NMR (DMSO-d_6): δ 3,79 (m, 8H), 4.02 (s, 3H), 4.03 (s, 3H), 4.06 (s, 3H), 6.82 (d, J = 8.4 Hz, 2H), 7.04 (s, 1H)), 7.57 (s, 1H), 7.58 (s, 1H), 7.93 (d, J = 9.1 Hz, 2H), 10.6 (s, 1H), 11.11 (s, 1H), 11.13 (s, 1H); anal. calcd for $C_{26}H_{28}N_{10}O_5Cl_2$: C, 49.45; H, 4.47; N, 22.18; Cl, 11.23. Found: C, 49.37; H, 4.41; N, 22.09; Cl, 11.17.

***N*-Succinimidyl-[1-Methyl-3-[1-methyl-3-[1-methyl-3-[4-bis(2-chloroethyl)aminophenylamido]-pyrazole-5-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxylate (40).** To a mixture of **39** (640 mg, 1 mmol) and *N*-hydroxysuccinimide (200 mg, 1.46 mmol) in 10 mL of anhydrous DMF cooled to 0 °C was added DCC (400 mg, 1.8 mmol) in a one portion and the stirring was continued for 24 h at rt. The dicyclohexylurea formed was filtered off and washed with cold DMF. The filtered was concentrated to small volume (3 mL) and this solution, containing the active ester, was used for the next step without purification.

3-[1-Methyl-3-[1-methyl-3-[1-methyl-3-[4-bis(2-chloroethyl)-aminophenylamido]-pyrazole-5-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxamido] propionamidine hydrochloride (7). To a solution of *p*-aminopropionamide dihydrobromide (74.6 mg, 0.3 mmol) and $NaHCO_3$ (25 mg, 0.3 mmol) in 6 mL of a mixture dioxane/water (3/1, v/v) cooled to 0 °C, was added dropwise the solution of **40** (73.2 mg, 0.1 mmol) in DMF (3 mL). The reaction mixture was stirred at rt for 18 h, acidified to pH 5 with 5% aqueous HCl and concentrated in vacuo. The residue was purified by flash column chromatography with MeOH/CH_2Cl_2 (1/2, v/v) to give **7** as a white solid. Yield 70 mg (0.093 mmol, 93.5%), mp 177–180 °C; ^{1}H NMR (CD_3OD) δ 2.57 (m, 2H), 3.53 (m, 2H), 3.79 (m, 8H), 4.02 (s, 3H), 4.06 (s, 3H), 4.08 (s, 3H), 6.81 (d, J = 8.6 Hz, 2H), 7.31 (s, 1H), 7.57 (s, 1H), 7.59 (s, 1H), 7.93 (d, J = 8.4 Hz, 2H); anal. calcd for $C_{29}H_{36}N_{13}O_4Cl_3$: C, 47.33; H, 4.93; N, 24.76; Cl, 14.27. Found: C, 47.20; H, 4.81; N, 24.68; Cl, 14.15.

3-[1-Methyl-3-[1-methyl-3-[1-methyl-3-[4-bis(2-chloroethyl)-aminophenylamido]-pyrazole-5-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxamido]dimethylaminopropane (8). To an ice-cooled solution of **40** (73.2 mg, 0.1 mmol) in 5 mL of anhydrous DMF was added *N*,*N*-dimethylaminopropylamine (40 μL, 0.3 mmol 0.1 mmol) in DMF (3 mL) and the mixture was stirred at rt for 18 h. Concentration of the solution provided a residue which was purified by flash column chromatography with MeOH/CH_2Cl_2 (2/1, v/v) to give **8** as a yellow solid. Yield 42 mg (0.058 mmol, 58%), mp 220–222 °C; ^{1}H NMR (DMSO-d_6) δ 1.67 (t, J = 7 Hz, 2H), 2.38 (m, 2H), 3.15 (s, 3H), 3.17 (s, 3H), 3.26 (m, 2H), 3.85 (m, 8H), 4.07 (s, 3H), 4.10 (s, 3H), 4.13 (s, 3H), 6.82 (d, J = 9 Hz, 2H), 7.28 (s, 1H), 7.57 (s, 1H), 7.59 (s, 1H), 7.95 (d, J = 8.8 Hz, 2H), 8.65 (t, J = 6.4 Hz, 1H), 10.64 (s, 1H), 11.13 (s, 1H), 11.18 (s, 1H); anal. calcd for $C_{31}H_{40}N_{12}O_4Cl_2$: C, 52.03; H, 5.63; N, 23.49; Cl, 9.91. Found: C, 51.94; H, 5.54; N, 23.38; Cl, 9.80.

[1-Methyl-3-[1-methyl-3-[1-methyl-3-[4-bis(2-chloroethyl)-aminophenylamido]-pyrazole-5-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxamido] 4-bis(2-chloroethyl)-aminoanilino (9). To a solution of 4-[bis(2-chloroethyl)amino]anilino hydrochloride[17] (88.4 mg, 0.1 mmol) and $NaHCO_3$ (25 mg, 0.3 mmol) in 6 mL of a mixture dioxane/water (3/1, v/v) cooled to 0 °C, was added dropwise the solution of **40** (73.2 mg, 0.1 mmol) in DMF (3 mL). The reaction mixture was stirred at rt for 18 h and concentrated in vacuo. The residue was purified by flash column chromatography with EtOAc to give **9** as a white solid. Yield 147 mg, 0.198 mmol, 66.5%; mp 255–258 °C; ^{1}H NMR (DMSO-d_6) δ 3.79 (m, 8H), 4.05 (s, 3H), 4.07 (s, 3H), 4.09 (s, 3H), 4.12 (m, 8H), 7.49 (s, 1H), 7.57 (s, 1H), 7.58 (d, J = 8.6 Hz, 2H), 7.60 (s, 1H), 7.96 (d, J = 8.4 Hz, 2H), 10.1 (s, 1H), 10.7 (s, 1H), 11.2 (s, 1H), 11.3 (s, 1H); anal. calcd for $C_{36}H_{40}N_{12}O_4Cl_4$: C, 51.07; H, 4.76; N, 19.85; Cl, 16.75. Found: C, 50.96; H, 4.68; N, 19.76; Cl, 16.68.

Biological testing in vitro. All the tested hybrid compounds were dissolved in DMSO at 1 mg/mL immediately before use and diluted in medium prior to adding cells. The murine lymphocytic leukemia cells L1210, L1210/Dx and L1210/tallimustine 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 mg/mL streptomycin.

To determine survival after drug exposure, exponentially growing L1210 cells were continously exposed to various concentrations of drugs for 48 h. The antiproliferative activity of the drugs was evaluated by counting surviving cells 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). All experiments were repeated at least twice. For each drug concentration, duplicate cultures were used. Vehicle or solvent controls were run with each experiment.

Biological testing in vivo. Inbred DBA/2 and C57BL/6N, first generation hybrid (C57BL/6No × DBA/2No) B6D2F1 adult female mice were used for the evaluation of the antitumor activity. Mice were 2–3 months of age and weighed 20–22 g at the time of tumor implantation. All mice were supplied by Charles River Italia (Calco, Como, Italy). Animal health was monitored by serological testing; the animals were free of infectious pathogens, including mouse hepatitis virus, Sendai virus and *Mycoplasma pulmonis*, during the course of experimentation. L1210 murine leukemia (originally obtained from the National Cancer Institute, Frederick, MD) was maintained by continuous i.p. passage (10^6 cells/mouse) in syngenic DBA/2N mice.

All drug solutions were prepared immediately before use and given intravenously (i.v.) in a volume of 10 mL/Kg of body weight. The vehicle used in preparation of solutions consisted of 10% Tween 80 and 90% saline.

L1210 leukemia (10^5 cells/mouse, CDF1 mice) was implanted i.v. The solid tumor M5076 was inoculated i.v. (10^6 cells/mouse) in CDF1 mice. A dose response was determined in all experiments. Toxicity was evaluated on the basis of the gross autopsy findings and the weight loss, mainly in terms of reduction of spleen and liver size.

Acknowledgements

We wish to thank Pharmacia & Upjhon for the financial support of this work.

References and Notes

1. Arcamone, M. A.; Animati, F.; Barbieri, B.; Configliacchi, E.; D'Alessio, R.; Geroni, C.; Giuliani, F. C.; Lazzari, E.; Menozzi, M.; Mongelli, N.; Penco, S.; Verini, M. A. *J. Med. Chem.* **1989**, *32*, 774.
2. (a) Pezzoni, G.; Grandi, M.; Basoli, G.; Capolongo, L.; Ballinari, D.; Giuliani, F. C.; Barbieri, B.; Pastori, A; Pesenti, E.; Mongelli, N. *Br. J. Cancer* **1991**, *64*, 1047. (b) Di Marco, A.; Gaetani, M.; Orezzi, P.; Scotti, T.; Arcamone, F. *Cancer Chemother. Rep.* **1962**, *18*, 15.
3. (a) Broggini, M.; Erba, E.; Ponti, M.; Ballinari, D.; Geroni, C.; Spreafico, F.; D'Incalci, M. *Cancer Res.* **1991**, *51*, 199. (b) Broggini, M.; Coley, H. M.; Mongelli, N.; Pesenti, E.; Wyatt, M. D.; Hartley, J. A.; D'Incalci, M. *Nucleic Acids Res.* **1995**, *23*, 81. (c) Fontana, M.; Letsingi, M.; Mondello, C.; Braghetti, A.; Montecucco, A.; Ciarrocchi, G. *Anti-Cancer Drug Des.* **1994**, *7*, 131.
4. Broggini, M.; D'Incalci, M. *Anti-Cancer Drug Des.* **1994**, *9*, 373.
5. Baraldi, P. G.; Beria, I.; Cacciari, B.; Capolongo, L.; Cozzi, P.; Mongelli, N.; Romagnoli, R.; Spalluto, G. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1247.
6. Lee, H. H.; Boyd, M.; Gravatt, G. L.; Denny, W. A. *Anti-Cancer Drug Des.* **1991**, *6*, 501.
7. For a review on DNA minor-groove binders see: (a) Zunico, F.; Animati, F.; Capranico, G. *Curr. Pharm. Des.* **1995**, *1*, 83. (b) Cozzi, P.; Mongelli, N. *Curr. Pharm. Des.* **1998**, *4*, 181.
8. Lee, M.; Krowichi, K.; Hartley, J. A.; Pon, R. T.; Lown, J. W. *J. Am. Chem. Soc.* **1988**, *110*, 3641.
9. (a) Huang, L.; Morgan, R.; Lown, J. W. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1751. (b) Bialer, M.; Yagen, B.; Mechoulam, R. *Tetrahedron* **1978**, *34*, 2389. (c) Xie, G.; Gupta, R.; Lown, J. W. *Anti-Cancer Drug Des.* **1995**, *10*, 389.
10. Bailly, C.; Pommery, N.; Houssin, R.; Henichart, J. *J. Pharm. Sci.* **1989**, *78*, 910.
11. Elderfield, R. C.; Liao, T. K. *J. Org. Chem.* **1961**, *26*, 4996.
12. Lown, J. W.; Krowicki, K. J. *J. Org. Chem.* **1985**, *50*, 3774.
13. Pinner, A.; Klein, F. *Chem. Ber.* **1877**, *10*, 1889.
14. Grehn, L.; Ragnarsson, U. *J. Org. Chem.* **1981**, *46*, 3492.
15. Geroni, C.; Pesenti, E.; Tagliabue, G.; Ballinari, D.; Mongelli, N.; Broggini, M.; Erba, E.; D'Incalci, M.; Spreafico, F.; Grandi, M. *Int. J. Cancer* **1993**, *68*, 916.
16. Baraldi, P. G.; Cacciari, B.; Romagnoli, R.; Spalluto, G. P. *Molecules* **1998**, *3*, 46.
17. Everett, J. L.; Ross, W. C. J. *J. Chem. Soc.* **1949**, *121*, 2674.