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Cinnamoyl nitrogen mustard derivatives of pyrazole analogues of tallimustine modified at the amidino moiety: design, synthesis, molecular modeling and antitumor activity studies

Pier Giovanni Baraldi,[a,*] Italo Beria,[b] Paolo Cozzi,[b] Cristina Geroni,[b] Antonio Espinosa,[c] Miguel A. Gallo,[c] Antonio Entrena,[c] John P. Bingham,[d] John A. Hartley[d] and Romeo Romagnoli[a]

[a]*Dipartimento di Scienze Farmaceutiche, Università di Ferrara, 44100 Ferrara, Italy*
[b]*Pharmacia Italia S.p.A., Discovery Research Oncology, 20014 Nerviano, Milan, Italy*
[c]*Departamento de Química Farmacéutica y Orgánica, Facultad de Farmacia, Universidad de Granada, 18071, Spain*
[d]*Cancer Research UK Drug–DNA Interactions Research Group, Department of Oncology, Royal Free and University College Medical School, UCL, 91 Riding House Street, London W1W 7BS, UK*

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Abstract—The design, synthesis and in vitro activities of a series of cinnamoyl nitrogen mustard pyrazole analogues of tallimustine **8**–**13**, in which the amidino moiety has been replaced by moieties of different physico-chemical features are described, and the structure–activity relationships are discussed.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 amidino moiety, these derivatives showed significant growth inhibitory activity against mouse leukemia L1210 cells. A selected series of compounds have been evaluated for their sequence selective alkylating properties and cytotoxicity against human K562 leukemia cells. Therefore, the presence of the amidino moiety, and in general of a basic moiety, is not an absolute requirement for biological activity. Our preliminary results indicated that the compounds of this series have a pattern of alkylation similar to that of tallimustine, but they seem to be less reactive overall in alkylating naked DNA.

1. Introduction

DNA minor groove binders can acts as suitable carriers for alkylating agents, giving rise to compounds with cytotoxic properties. Tallimustine **1** (FCE 24517, PNU-152241), a distamycin A[1] derivative in which the formyl group has been replaced by a benzoyl nitrogen mustard moiety (BAM), is a potent cytotoxic agent, which exhibits a broad spectrum of antitumor activity in experimental tumor models.[2] Previous studies have indicated that tallimustine retains the AT preference of distamycin A and appears to possess a high preference for alkylation of adenines located in the 5′-TTTTGA-3′ sequence.[3] The mechanism by which the alkylation induced by tallimustine leads to cytotoxicity remains unclear. While the cytotoxicity of BAM is related to the ability to form interstrand cross-links in DNA[4] with consequent inhibition of DNA replication and transcription, the mechanism of antitumor action of tallimustine, although it is not fully elucidated yet, may be due to its ability to inhibit the binding of some transcription factors to their consensus sequences in DNA, thereby preventing transcription.[3,5] Tallimustine was selected as a candidate antineoplastic drug in view of its strong activity against a series of experimental tumors. Unfortunately, tallimustine showed a severe myelotoxicity that probably impaired the achievement of an effective therapeutic doses and its Phase II clinical development was discontinued.[6]

Nevertheless, tallimustine has represented an important model for the design of new cytotoxic minor groove binders derived from distamycin, where the formyl group has been replaced by moieties of mild chemical

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* Corresponding author. Tel.: +39-0532-291293; fax: +39-0532-2912-96; e-mail: brlpgv@unife.it

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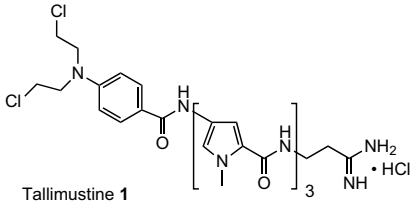
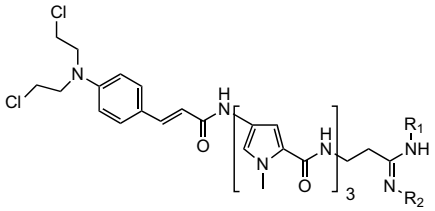
reactivity. The cinnamic nitrogen mustard derivative of distamycin A (PNU-157911, **2**)[7] a vinylogue of tallimustine, shows very good antileukemic activity, significantly superior to that of tallimustine. Compound **2** appears significantly more cytotoxic than tallimustine in accordance with its increased chemical reactivity due to the long-range nitrogen-carbonyl conjugation via the vinylic double bond. For compound **2**, as in the case of tallimustine, the cytotoxic activity is the result of the combination of two moieties, which are per se relatively inactive as cytotoxics.

A feature of **2** is the presence of an amidino moiety that, due to its strong basic nature (its calculated pK_a should be about 12), exhibits a complete protonation in any biological conditions, and may play a key role both in the DNA binding and cell or tissue bioavailability. In the case of compound **2** the replacement of the amidino group with basic or nonbasic amidino moieties of different nature led to compounds **3**–**6**, in which the potent cytotoxicity of the parent amidino derivative was fully maintained and in some cases even increased.[8] The finding that the amidino moiety of cinnamic mustard derivative **2** could be replaced by other basic amidino-like or nonbasic-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 leukemia cells.[8]



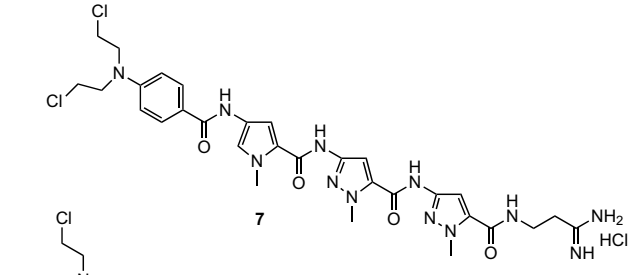
Tallimustine **1**



R_1=H HCl, R_2=H; PNU-157911, **2**
R_1=H HCl, R_2=CH_3; **3**
R_1=CH_3 HCl, R_2=CH_3; **4**
R_1=H, R_2=CN; **5**
R_1=H, R_2=OH; **6**

We have now investigated the possibility of incorporating azole moieties other than the original pyrroles into the structure of **2**. We have published recently the synthesis and cytotoxicity of a series of isosteric tallimustine derivatives containing one or more pyrazolic rings, which replace the pyrroles of tallimustine.[9,10] Among the synthesized compounds, the tallimustine isoster **7**, in which two pyrrole units near the amidine terminus were replaced by the same number of pyrazolic rings, showed on L1210 fourfold reduced cytotoxicity with respect to tallimustine but superior in vivo antileukemic activity.[10a] The cinnamic acid mustard derivative of **7** (compound **8**) appears to be 20-fold more cytotoxic than **7** (IC50 = 14.2 ± 0.52 vs 306 ± 56 nM for **8** and **7**, respectively) and maintains an in vitro potency equivalent to that of tallimustine's vinylogue **2**.

In the present study we report the synthesis and growth inhibitory activity of a new series of cinnamic nitrogen mustard derivatives, **9**–**13**, structurally related to compound **8**, in which the amidino moiety was replaced by basic and nonbasic groups of different electronic nature, lipophilicity and bulk. While *N*,*N*-dimethylamidine and imidazolin-2-yl derivatives (compounds **9** and **10**, respectively) are strongly basic amidine compounds (calculated pK_a values for the *N*,*N*-dimethylamidine and imidazolin-2-yl residues are 12.68 and 11.0, respectively), cyanoamidine, amidoxime, and amide (compounds **11, 12** and **13**) are not basic at all (calculated pK_a = 0.256 for the cyanoamidine fragment).[11]



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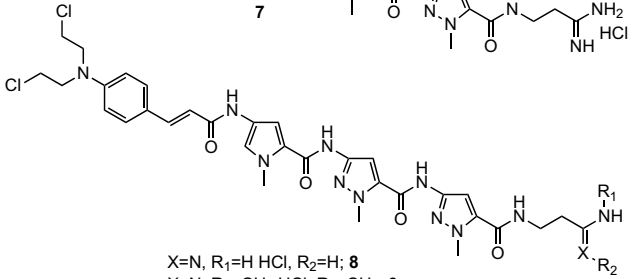
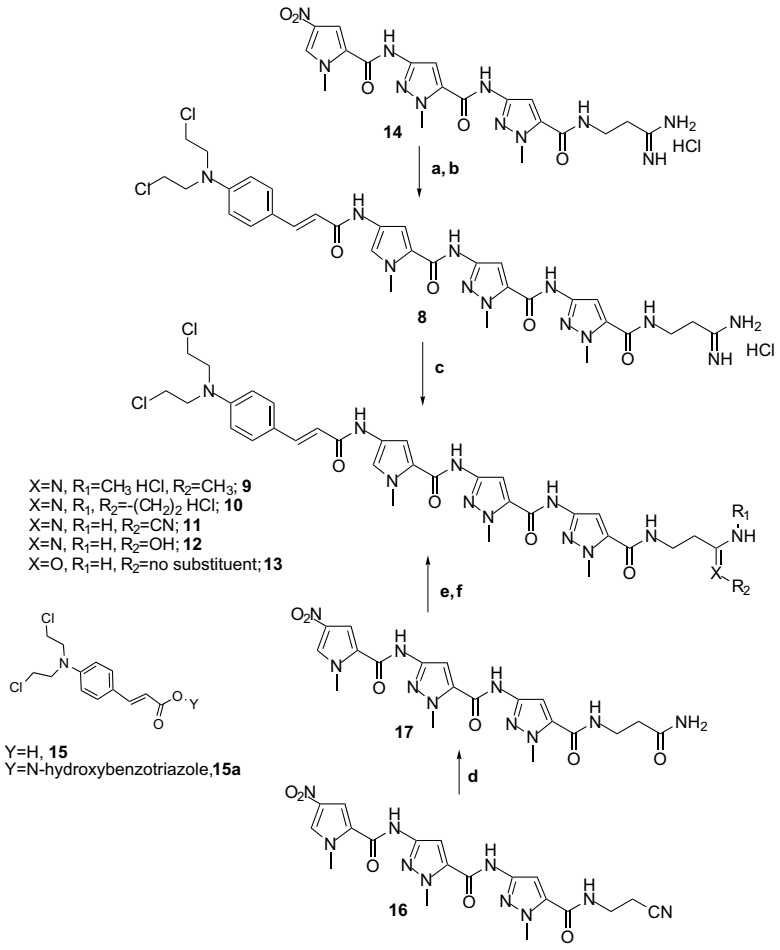


X=N, R_1=H HCl, R_2=H; **8**
X=N, R_1=CH_3 HCl, R_2=CH_3; **9**
X=N, R_1, R_2=-$(CH_2)_2$ HCl; **10**
X=N, R_1=H, R_2=CN; **11**
X=N, R_1=H, R_2=OH; **12**
X=O, R_1=H, R_2=no substituent; **13**

2. Chemistry

The novel derivatives **8**–**13** were obtained following the procedure reported in the Scheme 1. The key intermediate utilized for the synthesis of the compounds **9**–**12** was the cinnamoyl nitrogen mustard derivative **8**, characterized by the presence of the amidino moiety. Compound **8** was synthesized starting from the nitroamidine **14**,[9] which was transformed into the corresponding amine after catalytic hydrogenation with 10% Pd/C at room temperature and then immediately coupled with the activated ester **15a** of *N*,*N*-dichloroethyl cinnamic acid mustard **15**.[12] The cinnamoyl nitrogen mustard derivatives characterized by a modified amidino moiety, as dimethylamidine, imidazoline, cyanamidine, and amidoxime, corresponding to derivatives **9**–**12**, were prepared by a common procedure consisting in the direct reaction of the amidino derivative **8** with



Scheme 1. Reagents and conditions: (a) H_2, 10% Pd/C, 2–3 drops of 10% HCl, MeOH/dioxane/water; (b) **15a**, $NaHCO_3$, DMF, 18 h, rt; (c) CH_3NH_2, DMF, 80 °C (for the synthesis of **9**); $NH_2CH_2CH_2NH_2$, CH_3CN/water, rt (for the synthesis of **10**); NH_2CN, NAH, DMF, rt (for the synthesis of **11**); NH_2OH, HCl, TEA, DMF, 70 °C (for the synthesis of **12**); (d) 37% HCl, 40 °C, 1 h; (e) H_2, 10% Pd/C, 2–3 drops of 10% HCl, dioxane/MeOH; (f) **15a**, $NaHCO_3$, DMF, 18 h, rt.

the appropriate amino derivative. In detail, the *N*,*N*-dimethylamidine **9** was prepared by reacting **8**, with 6 equiv of aqueous CH_3NH_2 in DMF at 80 °C; imidazolin-2-yl derivative **10** was prepared from **8** and 3 equiv of ethylendiamine in CH_3CN/H_2O (2/1, v/v) at room temperature; the cyanamidine **11** was prepared from **8** with 3 equiv of NH_2CN sodium salt, obtained in situ with NaH in DMF, whereas the amidoxime **12** was prepared from **8** and 3 equiv of NH_2OH free base, obtained in situ from NH_2OH HCl and TEA, in DMF at 70 °C. The compound **13** was synthesized in a different way. The nitro-nitrile **16**,[9] was first transformed into the corresponding nitro-amide **17** by acid hydrolysis of the cyano moiety with a 37% HCl solution in water at 40 °C for 1 h, then catalytic hydrogenation of **17** and final condensation with the activated ester **15a**, furnished the amide derivative **13**.

3. Results and discussion

Tallimustine, cinnamoyl mustard derivatives **2**–**6**, and all the newly synthesized compounds **8**–**13** have been firstly assayed in vitro on L1210 murine leukemia cells, in order to obtain preliminary information on cell growth effects. We employed the L1210 screening system since this cell line has previously been shown to be a predictor of clinically useful anticancer drugs.[13–17] The results obtained demonstrate that all tested compounds displayed growth inhibition effects (Table 1, left column).

In the series of pyrazolic amidino modified-derivatives **9**–**13**, all tested compounds exhibited growth inhibition activities on L1210 murine leukemia cell line, with IC50 values ranging between 5 and 100 nM. Only the *N*,*N*-dimethylamidine derivative **9** maintains, or even improves, the cytotoxicity in comparison to the amidine parent compound **8**, while, in contrast the imidazolin-2-yl derivative **10** showed a decreased activity in comparison to the compound **8**. The relatively low antiproliferative potency of imidazolin-2-yl derivative **10** contrasts with the higher activity of compounds **9** and **13**, which possess the basic *N*,*N*-dimethylamidine and the nonbasic carbamoyl moieties, respectively. These data indicate a lack of correlation between the basicity of the amidine-like structure and growth inhibition activity.

Table 1. In vitro activity and resistance index of derivatives **1**–**13** against L1210 murine leukemia and its subline resistant to doxorubicin (DX)

Compound	IC_{50} (nM ± SE)[a]		RI[b]
	L1210	L1210/DX	
Tallimustine	68.5 ± 6.61	2638 ± 567	38.5
2	9.54 ± 2.71	128.3 ± 23.1	13.4
3	5.04 ± 2.07	29.5 ± 3.40	5.8
4	8.12 ± 3.17	73.5 ± 19.5	9.0
5	7.21 ± 1.87	91.2 ± 16.2	12.6
6	57.3 ± 28.8	1297 ± 108	22.6
7	306 ± 56	9367 ± 1231	30.6
8	14.2 ± 2.13	109 ± 16.4	7.68
9	5.33 ± 1.73	131 ± 36.2	24.5
10	74.5 ± 9.73	628 ± 65.2	8.43
11	98.1 ± 23.7	>700	>7
12	94.3 ± 19.5	323 ± 22.3	3.39
13	39.7 ± 5.67	>1500	>37.5

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

By comparing the activities of these amidino-modified compounds, it appears that the presence of a basic moiety is not an absolute requirement for in vitro activity. This confirms the finding already reported in the series of close tallimustine analogs.[8] Within the group, however the compounds characterized by the presence of a nonbasic modified amidino moiety (derivatives **11**–**13**) are less active that the basic amidino **8** and *N*,*N*-dimethylamidino **9**. The carbamoyl derivative **13** is not only the compound more potent in the series of nonbasic derivatives, but it is also more active than the strongly basic imidazolin-2-yl derivative **10**, a fact that confirm again the lack of correlation between basicity and activity in the series.

Comparing the compounds of the pyrrole and pyrazole series which possess the same modified amidine terminus (**4** vs **9**, **5** vs **11**, and **6** vs **12**), only for the *N*,*N*-dimethylamidine derivatives, wherein the last two C-terminal pyrolic rings were replaced with pyrazoles, keep the activity unchanged (compounds **4** and **9**, respectively), while the others pyrazole-tallimustine analogues (compounds **11** and **12**) are substantially less active then the pyrrole counterparts **5** and **6**, respectively.

Table 1 also shows the growth inhibitory activities and the resistance index (RI) values of **1**–**13** in a L1210 leukemic cell line with acquired resistance to doxorubicin (DX). The results obtained show that the newly synthesized compounds **8**–**13** display different RI values and for compound **13** the resistance index exceeds 30-fold, showing that carbamoyl replacement has contributed to the reduced resistance.

A second set of cytotoxicity experiments was performed on a selected series of compounds against the human K562 leukemia cell line, using the MTT assay. This evaluation was undertaken in order to ascertain whether the compounds were cytotoxic on human neoplastic cells. In this case we investigated the in vitro antiproliferative activity at 4 days following a 1 h exposure. The growth inhibitory activities of the synthesized compounds are shown in Table 2. The IC50 values obtained using K562 cells were higher than those obtained using L1210 cells, this reflects the different cell origin, the different exposure time to test agent and the different assay employed.

Table 2. In vitro activity of tallimustine and compounds **2** and **7**–**13** against K562 human leukemia cells

Compound	IC_{50} (μM ± SE)[a]
Tallimustine	2.38 ± 0.53
2	1.30 ± 0.28
7	0.74 ± 0.24
8	0.57 ± 1.04
9	0.89 ± 0.16
10	5.82 ± 2.82
11	1.31 ± 0.12
12	5.72 ± 0.34
13	3.52 ± 0.22

[a] IC_{50} = 50% inhibitory concentration represents the mean from dose-response curves of at least three experiments.

The data with the novel compounds on K562 cells followed the same general trend as that observed on L1210 cells. Compounds **8** and **9**, with sub-μM IC_{50} values, were more active than **10**–**13** and tallimustine. Compounds **10**–**13** gave IC_{50} values in the 1–6 μM range.

The sequence selectivity of alkylation by a selected series of compounds was determined using a Taq polymerase stop assay, and representative results for compounds **8**, **9**, and **12** are shown in Figure 1. All the compounds gave evidence of dose dependant alkylation in the DNA fragment studied. A high degree of sequence selectivity was observed compared to the nitrogen mustard chlorambucil, which alkylates at guanine-N7 positions in the DNA major groove. All the compounds showed a strong preference for alkylation at the sequence 5′-TTTTG<u>A</u> (with the alkylated base underlined) as indicated by the arrow in the figure. The degree of selectivity for this site was, however, less than that observed for tallimustine, which showed little evidence of alkylation at other sites within the sequence studied. In addition the compounds required higher concentrations than tallimustine to obtain a similar level of overall alkylation to naked DNA.

Molecular modeling of new tallimustine analogues performed over the 5′-CTCACTCAAAAGGCGGTAATAC-3′ DNA sequences (see experimental section for details) demonstrate that in all the cases, there exists a hydrogen bonds pattern formed between the NH moieties of the ligand and the adenine N3 and thymine O2 atoms, which is similar to the pattern described for distamycin.[18] Figure 2 shows as example a general view of the compound **8** inserted into the minor groove of the studied DNA sequence as well as a schematic representation of the hydrogen bonds pattern. Similar representations of the distamycin complex are included for comparison purpose.

A common feature in all these compounds is the situation of the mustard moiety inside the minor groove.

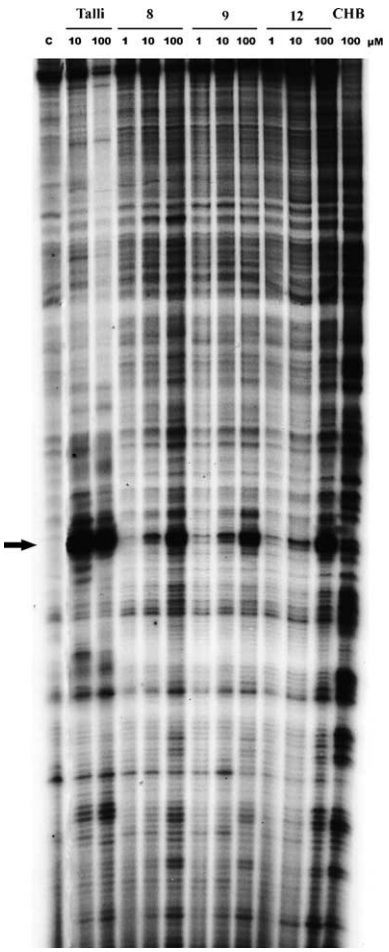


Figure 1. Covalent modification by tallimustine and compounds **8**, **9**, and **12** on a fragment of plasmid pUC18 DNA measured using the Taq polymerase stop assay. Lane C is control unalkylated DNA, and CHB is DNA alkylated with the nitrogen mustard chlorambucil. The major site of alkylation at the sequence 5′-TTTTGA is indicated by an arrow.

Ethylene group is a rigid spacer that does not allow a big flexibility in this part of the molecules. On the other hand, the *p*-substitution in the benzene ring introduce a high degree of symmetry in the nitrogen mustard, allowing only a significant movement in the bis(2-chloroethyl)amino group. Our calculations indicate that in the more stable conformations of all these complexes, one chloroethyl group is situated near to the N3 nitrogen atom of **A17**, in a very suitable disposition for the subsequent alkylation reaction. Figure 3 shows, as an example, such disposition for the complex of compound **8**.

These findings can explain the observed results in the Taq polymerase stop assay, where the major site of alkylation is the sequence 5′-TTTTTGA. It seems that in all these compounds, like in other described previously,[19] the modified distamycin moiety acts as a carrier for the binding to AT rich zones of DNA and allows an excellent situation of the nitrogen mustard moiety for the further alkylation process.

The nature of the amidino moiety conditions the biological activity of these compounds since this group also

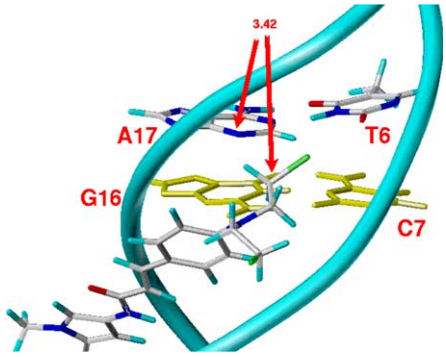


Figure 3. Detailed view of the nitrogen mustard moiety inside the DNA minor groove in the complex of compound **8**. It can be seen the appropriate situation of one chloromethyl group for a further alkylation of N3 nitrogen atom of **A17**. This disposition is similar in all the studied complexes.

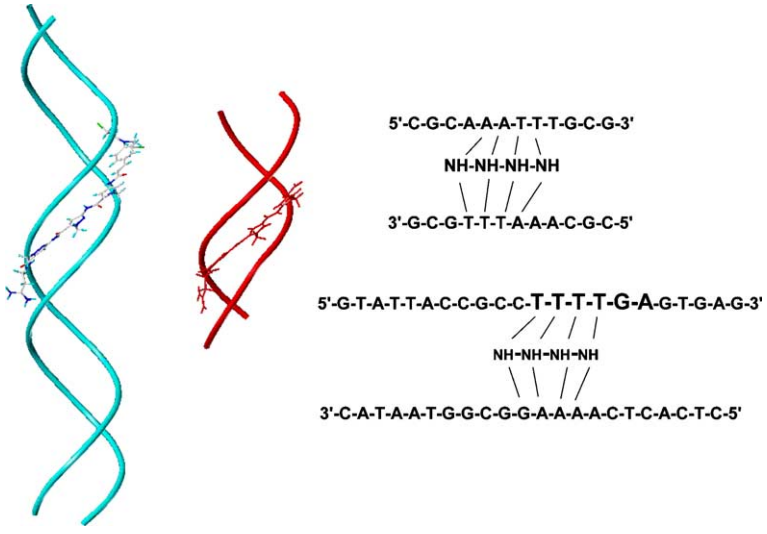


Figure 2. Left: General view of the compound **8** inserted into the minor grove of the DNA double helix 5′-GTATTACCGCCTTTTGAGTGAG-3′ represented as a cyan tube compared with the crystal structure of the distamycin complex. Right: Schematic representation of the hydrogen bonds patterns in both complexes: NH symbolizes each amide nitrogen of the molecule. The preferred alkylation sequence (TTTGA) is highlighted.

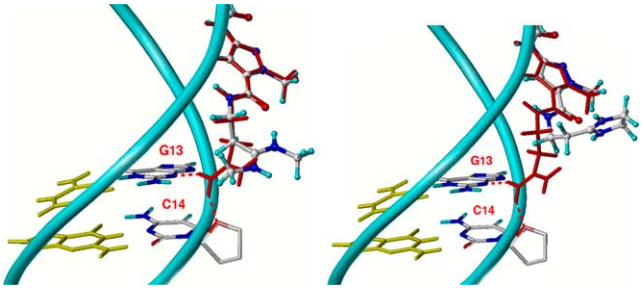


Figure 4. Detailed views of the interaction between the DNA and the amidino moiety in compounds **8**–**10**. Compound **8** (red) forms two hydrogen bonds with the N3 atom of **G13** and the ribose fragment of **C14**. Compound **9** (left) forms an hydrogen bond with the carbonyl group of the last amide and insert one methyl group inside the minor groove. In compound **10** (right) the pyrazoline moiety is situated totally outside of the minor groove.

affects the stability of the DNA–ligand complexes. Compounds **8**–**10** bear a basic and protonated moiety that differs in the substituents situated over the nitrogen atoms. In compound **8** there are no substituents and hence is similar to tallimustine or to distamycin. In facts, our calculations indicate that the amidino moiety of this molecule interacts with the DNA by forming two hydrogen bonds with the N3 atom of G13 and the ribose moiety of **C14** (Fig. 4). These interactions are equivalents to those of distamycin and/or tallimustine and stabilize the complex giving place to an active compound.

Compound **9** bears two methyl groups over the amidino fragment that could give place to steric interaction with the DNA. Our results indicate that in this compound only one methyl group can be inserted inside the minor groove avoiding the formation of the hydrogen bonds (Fig. 4). Nevertheless, this methyl group still stabilizes the complex due to the nonbonded interaction with the DNA backbone and/or bases. In compound **10**, the amidino group is also disubstituted by an ethylene group giving place to a more rigid and bulky protonated imidazoline moiety. In this case, we only found complexes in which this imidazoline moiety is situated totally outside of the minor groove (Fig. 4) and, hence, it does not exist any kind of additional stabilization of the complex. For this reason, the biological activity of compound **10** drops drastically when compared with compounds **8** or **9**.

In the other hand, compounds **11**–**13** are characterized by the substitution of the amidino moiety by a nonbasic group. Consequently, in these compounds the tail group it is not protonated in physiological conditions and do not bear a positive charge like in the previous compounds **8**–**10**. In all these compounds still exists a NH_2 terminal group that is inserted inside the minor groove and forms the same hydrogen bonds as compound **8**. Figure 5 shows a detailed view of these complexes and it can be seen that the geometry is totally equivalent to that complex of compound **8** and, in principle, these findings cannot explain the lost of activity of these molecules. Nevertheless, a careful study of the docking energies (Table 3) indicates that it exist a very important difference between the calculated values for compounds **8**–**10** in one hand and for **11**–**13** in the other. These differences are more significant in the electrostatic components and can be attributed to the lack of the positive charge in the tail of compounds **11**–**13**. Hence, it can be concluded that instead of the appropriate geometry and hydrogen bonding of the tail fragments in compounds **11**–**13**, these molecules forms weaker complexes with DNA and, consequently, their biological activity are considerably lower.

The substitution of two pyrrole rings by pyrazoles do not produce any significant modification in the geometry of the complexes since this part of both rings are orientated outside the minor groove, exposed to the solvent. Table 3 shows the calculated docking energy for the complex of compounds **4**, **5**, and **6**, and it can be seen that the stability of these complexes are very similar to those of compounds **9**, **11**, and **12**, respectively. Hence, the difference in biological activities between similar compounds of both series must be due to another reason.

Substitution of a CH group by a N atom increase the polarity of this part of the molecule and, probably, the interaction with the water molecules surrounding the complex in physiological conditions. These additional interactions with the solvent could slightly weaken the complex in the pyrazole series, specially in compounds **11** and **12** that, due to the lack of a positive charged tail, forms weaker complexes. This reason could explain the observed smaller biological activities of compounds **11** and **12** compared with **5** and **6**. Instead of that, compound **9** has a protonated disubstituted amidino moiety

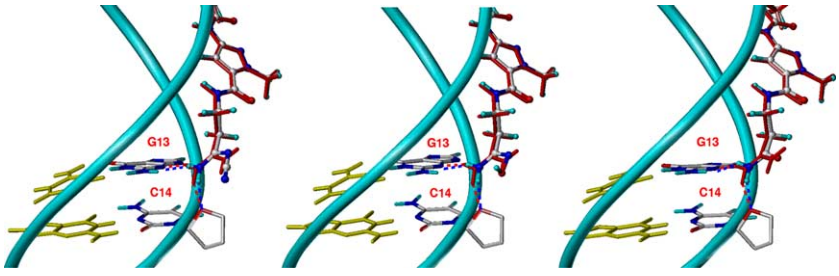


Figure 5. Detailed views of the interaction between the DNA and the tail moiety in compounds **11**–**13**. Compound **8** (red) are represented as reference. In all these compounds, the NH_2 group forms two hydrogen bonds (blue dashed lines) with the N3 atom of G13 and the ribose fragment of C14 similar to those of compound **8** (red dashed lines).

Table 3. Docking Energies (kcal/mol) as calculated by SYBYL defining the DNA and the ligand as aggregates, respectively

Compound	VDW	Elec.	Total
4	−82.35	−80.25	−162.60
5	−79.79	−34.64	−114.43
6	−79.45	−32.90	−112.36
8	−74.52	−98.48	−173.00
9	−80.74	−82.42	−163.16
10	−72.88	−94.36	−167.25
11	−78.37	−36.94	−115.31
12	−78.55	−35.99	−114.55
13	−76.67	−36.26	−112.93

and forms a stronger complex with DNA. Interactions with the solvent do not weaken too much this complex and for this reason its biological activity is very similar to that of compound **4**.

4. Conclusions

In conclusion, the positive role played by some modifications of the amidino moiety is confirmed also in the case of pyrazole isosters of cinnamoyl mustard derivatives of distamycin, and some of these compounds maintain or even improve the growth inhibitory activity of the parent compounds **8**. On the L1210 cell line, only the *N*,*N*-dimethylamidine derivative **9** showed a cytotoxicity better than that of **8**, while the others amidino-modified derivatives **10**–**13** are less potent. The novel compounds **8**–**13** showed the capability to interact with DNA with a sequence selectivity for certain AT-rich sequences although the level of overall alkylation to naked DNA did not correlate directly with in vitro cytotoxicity. Like in other described compounds, distamycin moiety acts as a carrier for the regioselective DNA binding of these new molecules, situating the nitrogen mustard moiety in a very suitable disposition for the further alkylation process. The positive charged tail it is not necessary for the biological activity but more strong complexes are formed when it is present. The substitution of pyrrole by pyrazole diminished the biological activity probably due to additional interaction with solvent, especially in those compounds lacking the positive charged tail.

5. Experimental section

5.1. Chemical materials and methods

5.1.1. 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} Merck plates), the spots were examined with UV light and visualized with aqueous $KMnO_4$. 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 desorption 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 theoretical values calculated for C, H, and N. Column chromatography was carried out 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.

5.1.2. 3-[1-Methyl-3-[1-methyl-3-[1-methyl-4[4-*N*,*N*-bis-(2-chloroethyl) aminocinnamoyl]aminopyrrole-2-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxamido]-propionamidine hydrochloride (8). A solution of **14** (266 mg, 0.51 mmol) in 20 mL of a mixture MeOH/dioxane/water (2:1:1; v/v) containing few drops of aqueous 10% HCl was hydrogenated over 100 mg of 10% Pd/C at 50 psi for 18 h. The catalyst was removed by filtration, the filtrate was concentrated to give a residue, which was used without purification for the next step.

The crude amine above prepared was dissolved in anhydrous DMF (7 mL) containing $NaHCO_3$ (75 mg, 0.75 mmol) and at this solution was added **15a** (150 mg, 0.51 mmol) dissolved in 5 mL of dry DMF. The reaction mixture was stirred at room temperature for 12 h, and the evaporation of the DMF under reduced pressure gave a solid, which was purified by column chromatography using methylene chloride–MeOH as eluant (9/1 and then 8/2 v/v). The compound **8** was obtained as a orange solid (280 mg, 72% yield); mp = 245–247 °C; IR (KBr): 3270, 1654, 1559, 1519, 1458, and 1179 cm^{-1}; ^{1}H NMR(DMSO-d_6) δ 2.63 (t, J = 7.2 Hz, 2H), 3.56 (m, 2H), 3.76 (m, 8H), 3.87 (s, 3H), 4.02 (s, 3H), 4.05 (s, 3H), 6.26 (d, J = 15.8 Hz, 1H), 6.80 (d, J = 8.2 Hz, 2H), 7.03 (s, 1H), 7.31 (s, 1H), 7.46 (m, 5H), 8.63 (s, 2H), 8.81 (t, J = 7.2 Hz, 1H), 9.01 (s, 2H), 10.2 (s, 1H), 10.7 (s, 1H), 11.2 (s, 1H); FAB-MS (MALDI-TOF): 725.28 [M]$^+$. Anal. Calcd for $C_{32}H_{39}Cl_3N_{12}O_4$: C, 50.43; H, 5.16; N, 22.06. Found: C, 50.23; H, 5.01; N, 21.89.

5.1.3. *N,N'*-Dimethyl-3-[1-methyl-3-[1-methyl-3-[1-methyl-4[4-*N,N*-bis(2-chloroethyl) aminocinnamoyl]aminopyrrole-2-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxamido]propionamidine hydrochloride (9). To a stirred solution of **8** (153 mg, 0.2 mmol) in DMF (2 mL) heated at 80 °C were added three portions of methylamine (40% in water) (0.25 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 **9** (90 mg, 57% yield) as a yellow solid; mp = 205–207 °C; IR (KBr): 3244, 1662, 1560, 1520, 1438, 1396, and 1179 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.62 (t, $J = 7.2$ Hz, 2H), 2.78 (d, $J = 4.6$ Hz, 3H), 3.01 (d, $J = 4.6$ Hz, 3H), 3.53 (t, $J = 7.2$ Hz, 2H), 3.77 (m, 10H), 3.87 (s, 3H), 4.02 (s, 3H), 4.05 (s, 3H), 6.56 (d, $J = 16$ Hz, 1H), 6.81 (d, $J = 8.2$ Hz, 2H), 7.03 (s, 1H), 7.44 (m, 5H), 9.62 (m, 2H), 10.1 (s, 1H), 10.6 (s, 1H), 11.2 (s, 1H). FAB-MS (MALDI-TOF): 753.39 $[M]^+$. Anal. Calcd for $C_{34}H_{43}Cl_3N_{12}O_4$: C, 51.68; H, 5.49; N, 21.27. Found: C, 51.48; H, 5.33; N, 21.05.

5.1.4. 3-[1-Methyl-3-[1-methyl-3[1-methyl-4-[4-*N,N*-bis-(2-chloroethyl) aminocinnamoyl]aminopyrrole-2-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxamido]-ethyl-2-(4,5-dihydro-1*H*-imidazol-2-ine) hydrochloride (10). To a stirred solution of **8** (153 mg, 0.2 mmol) in 3 mL of a mixture CH_3CN/water (2:1, v/v) was added ethylendiamine (40 μL, 0.6 mmol) and the mixture was stirred for 18 h at room temperature. After this time, the mixture was evaporated and the crude product purified by chromatography on a silica gel column, using DCM–MeOH (9:1, v/v) as eluent, furnished the compound **10** (104 mg, 66% yield) as a yellow solid; mp = 195–197 °C; IR (KBr): 3398, 1653, 1559, 1459, 1398, and 1179 cm^{-1}; ^1H NMR(DMSO-d_6) δ2.72 (t, $J = 7.2$ Hz, 2H), 3.56 (m, 2H), 3.78 (m, 8H), 3.82 (m, 4H), 3.87 (s, 3H), 4.02 (s, 3H), 4.05 (s, 3H), 6.56 (d, $J = 15.6$ Hz, 1H), 6.80 (d, $J = 8.4$ Hz, 2H), 7.03 (s, 1H), 7.44 (m, 6H), 8.82 (t, $J = 6.2$ Hz, 1H), 10.1 (s, 1H), 10.7 (br s, 3H), 11.2 (s, 1H). FAB-MS (MALDI-TOF): 753.39 $[M+2]^+$. Anal. Calcd for $C_{34}H_{41}Cl_3N_{12}O_4$: C, 51.81; H, 5.24; N, 21.33. Found: C, 51.48; H, 5.33; N, 21.05.

5.1.5. *N*-Cyano-3-[1-methyl-3-[1-methyl-3-[1-methyl-4[4-*N,N*-bis(2-chloroethyl) aminocinnamoyl]aminopyrrole-2-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxamido]propionamidine (11). To a stirred solution of NH_2CN (30 mg, 0.72 mmol) in dry DMF (1 mL) was slowly added NaH (50% suspension in mineral oil) (70 mg of suspension corresponding to 1.5 mmol of pure NaH). After 30′, compound **8** (110 mg, 0.14 mmol) was dissolved in the solution and the mixture stirred for other 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 89.3 mg (85% yield) of compound **11** as a yellow solid; mp 188–190 °C; IR (KBr): 3256, 2256, 1634, 1577, 1465, 1402, 1260, and 1110 cm^{-1}; ^1H NMR(DMSO-d_6) δ 2.77 (t, $J = 7.2$ Hz, 2H), 3.44 (m, 2H), 3.87 (m, 8H), 3.92 (s, 3H), 4.01 (s, 3H), 4.05 (s, 3H), 6.55 (d, $J = 15.4$ Hz, 1H), 6.81 (d, $J = 9.0$ Hz, 2H), 7.06 (d, $J = 15.4$ Hz, 1H), 7.43 (m, 6H), 8.41 (br s, 2H), 8.77 (br s, 1H), 10.0 (s, 1H), 10.6 (s, 1H), 11.1 (s, 1H). FAB-MS (MALDI-TOF): 750.33 $[M+1]^+$. Anal. Calcd for $C_{33}H_{37}Cl_2N_{13}O_4$: C, 52.80; H, 4.97; N, 24.26. Found: C, 52.67; H, 4.78; N, 24.07.

5.1.6. *N*-Hydroxy-3-[1-methyl-3-[1-methyl-3-[1-methyl-4[4-*N,N*-bis(2-chloroethyl) aminocinnamoyl]aminopyrrole-2-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carboxamido]propionamidine (12). To a stirred solution of **8** (153 mg, 0.2 mmol) in DMF (5 mL) heated at 70 °C, was added hydroxylamine hydrochloride (69.5 mg, 1 mmol) dissolved in a mixture of water (1 mL) and TEA (140 μL, 1 mmol). The mixture was stirred for 30′, 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 **12** (86 mg, 68% yield) as a yellow solid; mp 195–196 °C; IR (KBr): 3247, 1654, 1560, 1520, 1439, 1398, and 1179 cm^{-1}; ^1H NMR(DMSO-d_6) δ 2.82 (t, $J = 7.4$ Hz, 2H), 3.43 (m, 2H), 3.77 (m, 7H), 3.87 (s, 3H), 4.02 (s, 3H), 4.08 (s, 3H), 6.57 (d, $J = 15.6$ Hz, 1H), 6.80 (d, $J = 8.6$ Hz, 2H), 7.02 (s, 1H), 7.44 (m, 6H), 8.61 (m, 1H), 8.82 (m, 1H), 9.01 (m, 2H), 10.1 (s, 1H), 10.6 (s, 1H), 10.8 (br s, 1H), 11.1 (s, 1H). FAB-MS (MALDI-TOF): 741.7 $[M+1]^+$. Anal. Calcd for $C_{32}H_{38}Cl_2N_{12}O_5$: C, 51.82; H, 5.16; N, 22.66. Found: C, 51.66; H, 5.03; N, 22.48.

5.1.7. 3-[1-Methyl-3[1-methyl-3[1-methyl-4[4-nitropyrrole-2-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carbamoyl]ethylamide (17). A suspension of the nitronitrile **16** (235 mg, 0.5 mmol) in 37% hydrochloric acid (3 mL) was heated at 40 °C for 1 h. The solution was cooled at rt, evaporated in vacuo and the residue purified by crystallization with acetone furnished the final compound **17** as a yellow solid; mp 190–191 °C; NMR (DMSO-d_6) δ 2.34 (t, $J = 7.0$ Hz, 2H), 3.37 (m, 2H), 3.97 (s, 3H), 4.01 (s, 3H), 4.05 (s, 3H), 5.23 (br s, 2H), 7.26 (s, 1H), 7.56 (s, 1H), 7.80 (s, 1H), 8.23 (s, 1H), 8.64 (t, $J = 6.4$ Hz, 1H), 11.0 (s, 1H), 11.2 (s, 1H). FAB-MS (MALDI-TOF): 488.3 $[M+2]^+$.

5.1.8. 3-[1-Methyl-4[1-methyl-3[1-methyl-3[4-*N,N*-bis(2-chloroethyl)aminocinnamoyl]aminopyrrole-2-carboxamido]pyrazole-5-carboxamido]pyrazole-5-carbamoyl]ethylamide (13). A suspension of **17** (194 mg, 0.40 mmol) in 10 mL of a mixture MeOH/dioxane (1:1; v/v) containing few drops of aqueous 10% HCl was hydrogenated over 50 mg of 10% Pd/C at 50 psi for 18 h. The catalyst was removed by filtration, the filtrate was concentrated to give a residue, which was used without purification for the next step.

The crude amine above prepared was dissolved in anhydrous DMF (5 mL) containing $NaHCO_3$ (50 mg, 0.6 mmol) and at this solution were added **15a** (0.44 mmol) dissolved in 3 mL of dry DMF. The reac-

tion mixture was stirred at room temperature for 3 h, and the evaporation of the DMF under reduced pressure gave a solid, which was purified by column chromatography using methylene chloride–MeOH as eluant (9/1 and then 8/2 v/v). The compound **13** was obtained as a yellow solid (151 mg, 52% yield); mp 220–223 °C; IR (KBr): 3326, 2929, 1626, 1575, 1436, 1244, and 1180 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.32 (t, $J = 7.2$ Hz, 2H), 3.42 (m, 2H), 3.77 (m, 8H), 3.87 (s, 3H), 4.01 (s, 3H), 4.05 (s, 3H), 6.56 (d, $J = 15.6$ Hz, 1H), 6.82 (t, $J = 10.0$ Hz, 2H), 7.02 (s, 1H), 7.42 (m, 8H), 8.64 (t, $J = 7.2$ Hz, 1H), 10.0 (s, 1H), 10.5 (s, 1H), 11.1 (s, 1H); FAB-MS (MALDI-TOF): 725.28 $[M]^+$. Anal. Calcd for $C_{32}H_{37}Cl_2N_{11}O_5$: C, 52.90; H, 5.13; N, 21.2. Found: C, 52.77; H, 5.00; N, 20.89.

6. Molecular modeling

Molecular modeling of new tallimustine analogues **8**–**13** as well as compounds **4**–**6** was performed using SYBYL6.9 molecular modeling package.[20] The experimental X-ray three-dimensional structure of distamycin[21] complexed with the DNA dodecamer 5′-CGCAAATTTGCG-3′ (PDB code: 2DND) was used as template with the object of reproducing the distamycin hydrogen bonds pattern in the interaction with DNA.

Distamycin moiety was extracted from the complex and compounds **8**–**13** were built by addition of the appropriated standard fragments from the SYBYL libraries. Hydrogen atoms were added and partial atomic charges were calculated with AM1[22,23] hamiltonian implemented in MOPAC.[24] Geometry was partially optimized using the Powell[25] method and the Tripos force field[26] ($\varepsilon = 1$, distance dependent) until the gradient was smaller than 0.1 kcal/mol Å. During this optimization, the geometry of the distamycin analogue moiety was keeping rigid in order to relax steric interactions without losing the bowed shape of the molecule needed for the interaction with DNA.

In order to reproduce the Taq DNA polymerase stop results obtained for compounds **8**–**13**, a DNA double helix with the sequence 5′-CTCACTCAAA GGCGGTAATAC-3′ was build using the biopolymer module of SYBYL. All hydrogen atoms have been added to this dimer and partial atomic charges were read from the Kollman all-atom dictionary. This dimer was superimpose over the DNA dodecamer in the distamycin complex crystal structure using the backbone of appropriate base pairs as fitting.

Complexes of compounds **8**–**13** were built by manually docking of each ligand inside the minor grove of the DNA sequence and the geometry was optimized as described above after removing all restriction in the ligand molecule. These optimized complexes shows the distamycin hydrogen bonds pattern,[18] formed between the NH moieties of the ligand and the adenine N3 and thymine O2 atoms, and were used for the subsequent stages. In order to investigate the more favored orientation of the ligands into the DNA minor groove, two conformational searches were performed. In one of them, all bonds of the nitrogen mustard moiety were rotated to investigate the orientation of such fragment inside the minor grove. In the other one, all bonds of the amidino moiety (compound **8**) or the modified fragments was rotated. The more stable conformers in each search were selected, fully minimized after the elimination of all restrictions and compared each other to identify those complexes that are energetically and geometrically different. Once the most stable complex is identified for each compound, docking energies are calculated as the difference between the total steric energy of the complex and the sum of the energies of both the DNA and the ligand.

7. Biological assays

7.1. Growth inhibitory activity on murine L1210 and L1210/DX cell lines

The murine lymphocytic L1210 leukemia was 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 its subline resistant to doxorubicin (L1210/DX) were grown in vitro as a stationary suspension culture in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum, 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 continuously 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 IC50 (dose causing 50% inhibition of cell growth in treated cultures relative to untreated controls). All experiments were repeated at least three times. For each drug concentration, duplicate cultures were used. Vehicle or solvent controls were run with each experiment.

7.2. Growth inhibitory activity against the human K562 cell line

The K562 human chronic myeloid leukemia cells were maintained in RPM1 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine at 37 °C in a humidified atmosphere containing 5% CO_2 and were incubated with a specified dose of drug for 1 h at 37 °C in the dark. The incubation was terminated by centrifugation (5 min, 300 g) and the cells were washed once with drug-free medium. Following the appropriate drug treatment, the cells were transferred to 96-well microtitre plates, 10^4 cells per well, eight wells per sample. Plates were then kept in the dark at 37 °C in a humidified

atmosphere containing 5% CO_2. The assay is based in the ability of viable cells to reduce a yellow soluble tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma Chemical Co.) to an insoluble purple formazan precipitate. Following incubation of the plates for 4 days (to allow control cells to increase in number by 10-fold) 20 mL of a 5 mg/mL solution of MTT in phosphate buffered saline was added to each well and the plates further incubated for 5 h. The plates were then centrifuged for 5 min at 300 g and the bulk of the medium pipetted from the cell pellet leaving 10–20 mL per well. DMSO (200 mL) was added to each well and the samples agitated to ensure complete mixing. The optical density was then read at a wavelength of 550 nm on a Titertek Multiscan ELISA plate reader, and the dose-response curve was constructed. For each curve, an IC50 value was read as the dose required to reduce the final optical density to 50% of the control value.

7.3. Taq DNA polymerase stop assay[27]

Plasmid pUC18 DNA was linearized with HindIII to provide a stop for the *Taq* downstream from the primer. The oligodeoxynucleotide primer (5′CTCACT CAAAGGCGGTAATAC) was 5′-end labeled prior to amplification using T4 polynucleotide kinase and [γ-^{32}P]-ATP (5000 Ci/mmol, Amersham. UK). The labeled primers were purified by elution through Bio-Rad spin columns. Linear amplification of DNA was carried out in a total volume of 100 μL containing 0.5 μg of template DNA, 5 pmol of labeled primer, 200 μM of each dNTP, 10 U *Taq* polymerase, 50 mM KCl, 10 mM Tris–HCl, pH 9.0, 0.1% Triton X-100, 2.5 mM $MgCl_2$. Where appropriate template DNA had been reacted with test agent for 1 h at 37 °C and then precipitated with ethanol. After an initial denaturation at 94 °C for 4 min, the cycling conditions were as follows: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, for a total of 30 cycles. After being amplified, the samples were ethanol precipitated and washed with 70% ethanol. Samples were dissolved in formamide loading dye, heated for 2 min at 90 °C, cooled on ice, and electrophoresed at 2500–3000 V for 3 h on a 80 cm × 20 cm × 0.4 mm 6% acrylamide denaturing sequencing gel (Sequagel, National Diagnostics). The gels were dried, and X-ray film was exposed to the gels (Hyperfilm, Amersham. UK).

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