Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 1611–1618

BIOORGANIC & MEDICINAL CHEMISTRY

Benzoyl and Cinnamoyl Nitrogen Mustard Derivatives of Benzoheterocyclic Analogues of the Tallimustine: Synthesis and Antitumour Activity

Pier Giovanni Baraldi,[a,*] Romeo Romagnoli,[a] Maria Giovanna Pavani,[a] Maria del Carmen Nunez,[a] John P. Bingham[b] and John A. Hartley[b]

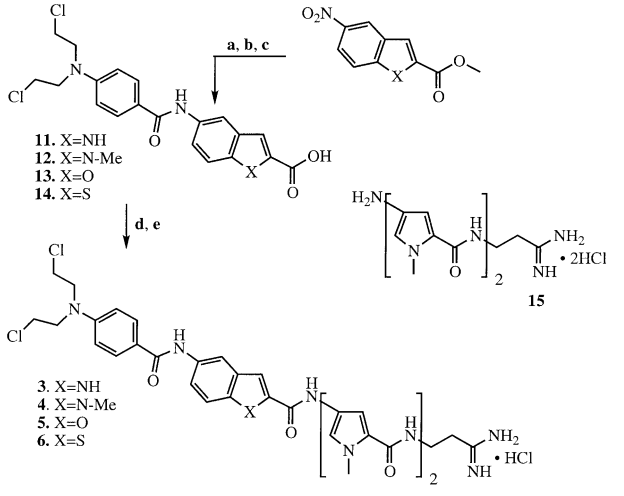
[a]*Dipartimento di Scienze Farmaceutiche, Università di Ferrara, Via Fossato di Mortara 17/19, 44100 Ferrara, Italy*
[b]*CRC Drug-DNA Interactions Research Group, Department of Oncology, Royal Free and University College Medical School, 91 Riding House Street, London W1W 7BS, UK*

Received 9 October 2001; accepted 30 November 2001

Abstract—A series of benzoyl and cinnamoyl nitrogen mustards tethered to different benzoheterocycles and to oligopyrroles structurally related to netropsin consisting of two pyrrole-amide units and terminating with an amidine moiety have been synthesised and a structure–activity relationship determined. Derivatives **3**–**10** have been evaluated for their sequence selective alkylating properties and cytotoxicity against human K562 leukaemia cells. They are 2- to 50-fold less cytotoxic than tallimustine, with compound **8** being the most potent member of this series. Among tallimustine isosters, the compounds with an indole **3** or benzothiophene **6** are 4-fold less cytotoxic than tallimustine, while the compounds with an *N*-methyl indole or benzofuran showed a 7- and 14-fold reduced cytotoxic potency, respectively. Our preliminary results indicate that these derivatives preferentially bind to AT-rich sequence with a sequence selectivity similar to tallimustine.

Introduction

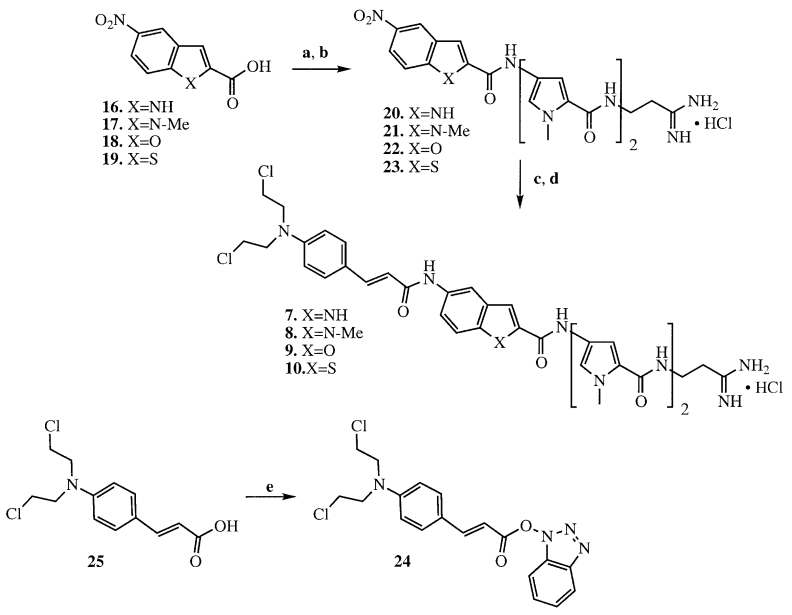
Minor groove alkylating agents are a relatively new class of anticancer agents reported to possess high cytotoxic activity in in vitro and in vivo preclinical models.[1] Tallimustine **1**,[2] a benzoic acid nitrogen mustard (BAM) derivative of distamycin A, is a minor groove alkylating agent which binds selectively to AT-rich sequence of DNA, showing excellent activity against a series of murine transplanted solid tumours and human xenographs[3] and it has proven active in a wide range of tumour cell lines resistant to alkylating agents.[2,4] Tallimustine retains the AT preference of distamycin A and appears to possess a high preference for alkylation of 3′-adenine-N3 located in 5′-TTTTPuA sequences in the minor groove of DNA.[5] 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[6] with consequent inhibition of DNA replication and transcription; the mechanism of antitumour action of tallimustine, although it is not yet fully elucidated, may be due to its ability to inhibit the binding of some transcription factors to their consensus sequences in DNA, thereby preventing transcription.[5,7]



Scheme 1. H_2, 10% Pd/C, MeOH; **b**.*p*-[bis-(2-chloroethyl)amino]-benzoyl chloride, TEA, dioxane; **c**. KOH 2 N, dioxane, rt; **d**. $SOCl_2$, dioxane; **e**. **15**, $NaHCO_3$, dioxane/water (1/4, v/v).

*Corresponding author. Tel.: +39-0532-291293; fax: +39-0532-291296; e-mail: pgb@dns.unife.it

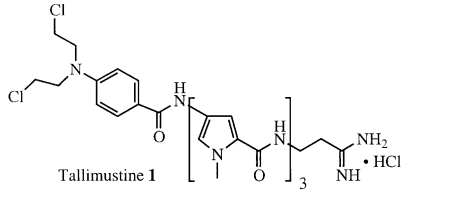
0968-0896/02/$ - see front matter
PII: S0968-0896(01)00425-4

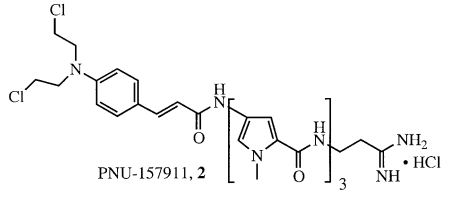


Scheme 2. $SOCl_2$, 1,4-dioxane, reflux, 2 h; **b**. **15**, dioxane/water (1/4, v/v) 18 h, rt; **c**. H_2, 10% Pd/C, 2–3 drops of 10% HCl, MeOH/water; **d**. **24**, $NaHCO_3$, DMF, 18 h, r.t.; **e**. HOBT, DCC, DMF, 70 °C, 4 h.

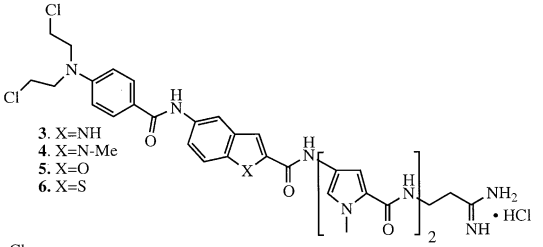
Unfortunately, tallimustine showed a severe myelotoxicity that probably impaired the reaching of effective therapeutic doses and its Phase II clinical development was discontinued.[8]

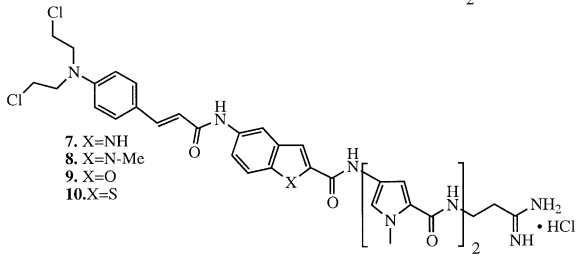
Nevertheless, tallimustine has represented an important model for the design of new cytotoxic minor groove binders derived from distamycin, where the formyl group has been replaced by moieties of mild chemical reactivity with the DNA. Among these, a cinnamoyl nitrogen mustard derivatives of distamycin A (PNU-157911, **2**)[9] a vinylogue of tallimustine, has been disclosed by Pharmacia & Upjohn and shows very good antileukemic activity, significantly superior to that of tallimustine ($IC_{50} = 7.2$ ng/mL compared with 50.3 ng/mL for tallimustine against L1210 leukemia). Compound **2** appears significantly more cytotoxic than tallimustine in accordance with its increased chemical reactivity.[9] For **2**, as in the case of tallimustine, the cytotoxic activity is the result of the combination of two moieties, which are per se substantially inactive as cytotoxics.





Following these results, in the present study we report the synthesis and the biological evaluation of novel isosters of tallimustine and **2**, showing the substitution of the N-terminal pyrrole ring tethered to benzoyl and cinnamoyl nitrogen mustard alkylating moiety by different benzoheterocycles such as indole, *N*-methyl indole, benzothiopene and benzofuran, to give the corresponding derivatives **3**–**6** and **7**–**10**, respectively. The compounds **7**–**10** are vinylogues of **3**–**6**, respectively, and are characterised by an increased distance between the alkylating moiety and the DNA–polypyrrolic binding frame via the vinylic double bond. Since pyrrole is susceptible to oxidative breakdown,[10] these new derivatives have been prepared as potentially more stable minor groove binders aimed to improved the relative instability of the polypyrrolic skeleton. The choice of these benzoheterocycles can be also justified due to their importance in increasing the binding affinity to DNA and the selectivity of alkylation of CC-1065 analogues such as U-71, 184, adozelesin and bizelesin.[11]





Chemistry

The synthetic route followed for the synthesis of derivatives **3**–**6** is outlined in Scheme 1. The key step was the coupling between the acid chlorides of benzoheterocyclic carboxylic acids **11**–**13**[12] and **14** bearing the BAM moiety and the known amino-amidine **15**.[9,13] These condensations were performed at room temperature and with identical reaction times (18 h), in a water/dioxane mixture containing sodium bicarbonate ($NaHCO_3$) as base. Compounds **3**–**6** were obtained in acceptable yields (50–65%), after purification by silica gel flash-chromatography.

The synthetic approach employed for the preparation of derivatives **7**–**10** is shown in Scheme 2. Starting from the amino-amidine **15**,[13] it was condensed with the acid chlorides of the well-known nitro-acids **16**–**19**.[12] The corresponding nitro-amidines **20**–**23** so obtained, after catalytic hydrogenation with 10% Pd/C at room temperature, were transformed into the corresponding amines in high yield and purity, which were unstable and therefore were immediately coupled with the active ester **24** of cynnamic acid mustard **25**[9] to give the derivatives **7**–**10**. The compound **24** is readily synthesised from equimolar amounts of the cynnamic acid mustard **25** and 1-hydroxy-1,2,3-benzotriazole (HOBT) by employing *N*,*N*′-dicyclohexylcarbodiimide (DCC) as coupling reagent.

Table 1. In vitro activities against K562 human leukemia of the tallimustine and compounds **3**–**10**

Compd	In vitro IC_{50} (μM)
Tallimustine	2.38 ± 0.5
3	8.23 ± 4.08
4	15.5 ± 0.5
5	31.96 ± 15.08
6	9.0 ± 4.51
7	> 100
8	3.88 ± 2.1
9	8.58 ± 4.41
10	27.0 ± 13

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

Biological Evaluation

Tallimustine and all synthesised compounds **3**–**10** were evaluated in vitro for their cytotoxic activities against human K562 leukemia cells using the MTT assay. The results, expressed as IC_{50} values (the concentration of test agent inhibiting growth by 50%), are reported in Table 1.

None of the compounds **3**–**10** was more active than tallimustine, although some had similar activity. Among the derivatives, compounds **3**, **6**, **8** and **9** proved to be only slightly less potent than tallimustine, with IC_{50} values of 8.23, 9.00, 3.88 and 8.58 μM, respectively, compared to 2.38 μM for tallimustine. In contrast, compound **7**, which contains the benzofuran tethered to the cinnamoyl nitrogen mustard, is clearly much less active and showed a very low level of cytotoxic potency ($IC_{50} > 100$ μM).

Comparing tallimustine with the derivatives **3**–**6** which share the BAM moiety, the derivatives **3** and **6**, in which the pyrrole closest to the BAM moiety has been replaced by indole and benzofuran, respectively, are approximately 4 times less cytotoxic than tallimustine, while compounds containing a *N*-methyl indole (**4**) or benzothiopene (**5**) are 7 and 15 times less potent.

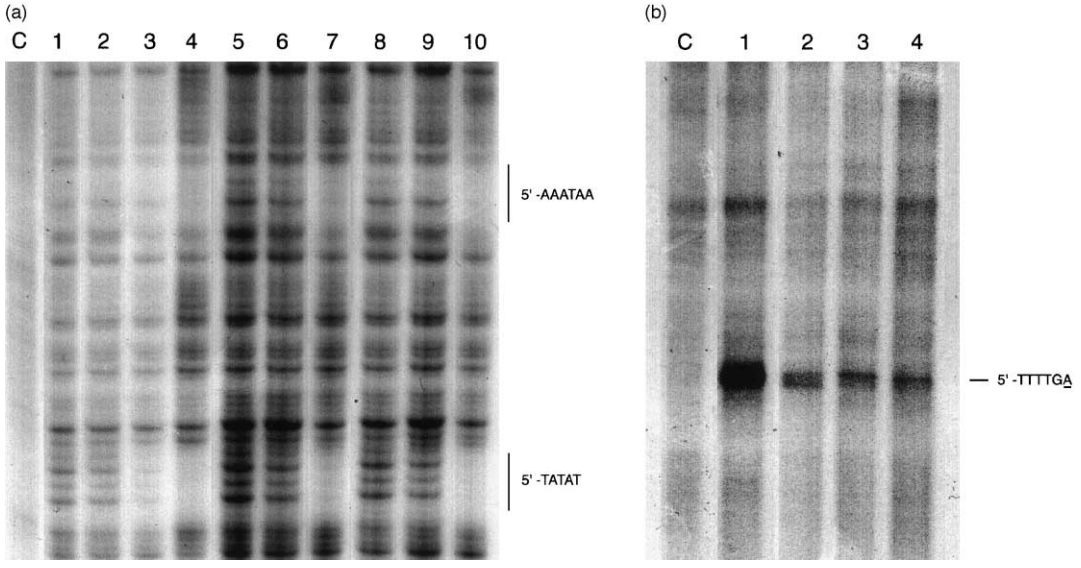


Figure 1. (a) DNAse I footprinting of compounds **4**, **6**, and **8** on a fragment of plasmid pUC18 DNA. Lane C is control, non-DNAse I treated DNA. Lane 1 is DNAse I alone; lanes 2–4 are DNA treated with **8** at 0.01, 0.1 and 1 μM, respectively; lanes **5**–**7** are treated with **6** at 0.01, 0.1 and 1 μM, respectively; lanes **8**–**10** are treated with **4** at 0.01, 0.1 and 1 μM, respectively. The two major sites of footprinting are indicated. (b) Covalent modification by compounds **4**, **6**, and **8** on a fragment of plasmid pUC18 DNA measured using the Taq polimerase stop assay. Lane C is control DNA; lanes 1–4 are DNA treated with tallimustine, **4**, **6** and **8** at 10 μM. The major site of alkylation at the sequence 5′-TTTTGA is indicated.

Comparing compounds with the same oligopeptidic frame, for the compounds **3** versus **7** (containing indole) and **6** versus **10** (containing benzofuran), the introduction of a vinylic double bond between the phenyl nitrogen mustard and the carboxamido tethered to the benzoheterocycle, a decrease in cytotoxicity of >12- and 3-fold, respectively, was observed. In contrast, as was observed with tallimustine and **2**, for derivatives **4** versus **8** (containing *N*-methyl indole) and **5** versus **9** (containing benzothiophene), the substitution with cinnamoyl nitrogen mustard led to an increase in the cytotoxicity of 4-fold.

DNA sequence-specific non-covalent interaction of compounds **3**–**10** was evaluated by Dnase I footprinting experiments. Figure 1a shows a representative of such experiments.

All the compounds **3**–**10** showed evidence of non-covalent binding by footprinting to two AT-rich sequences in the minor groove (AAATAA and TATAT), which were also sites of non covalent binding for distamycin and tallimustine.

Sequence-specific DNA alkylation produced by the compounds **3**–**10** tested was evaluated using the *Taq* polymerase stop assay (Fig. 1b). As can be seen from Figure 1b, at the concentration of 10 μM tallimustine showed sequence specific alkylation, with preferential alkyation at a 5′-TTTTGA sequence. Similar alkylation was also observed for several of the compounds, but with lower intensity compared to tallimustine. The least active compound **7** did not give any evidence of alkylation in the same DNA sequence at 100 μM, and the fact that this compound is 50 times less cytotoxic than tallimustine is likely to result from its reduced capacity to interact with DNA. For the other compounds in the series, alkylation was evident within this DNA sequence and the sequence specificity observed with tallimustine was preserved.

In conclusion, for both tallimustine and **2**, replacement of the N-terminal pyrrole by a benzoheterocycle does not impart any improvement in terms of cytotoxicity activity and DNA-binding capability. No clear-cut structure–activity relationship can be observed and it is difficult to find common physico-chemical features both among active and inactive reported compounds. It is noted that other factors such as low penetration into cell, cellular distribution and metabolic deactivation may also influence the cytoxicity result, but they are not assessed in the present study.

Experimental

Chemistry

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

Synthesis of 5-[4-*N*,*N*-bis(2-chloroethyl)aminobenzene-1-carboxamido]benzothiophene-2-carboxylic acid (14). A solution of methyl 5-nitrobenzothiophene-2-carboxylate[14] (3 mmol, 711 mg) in methanol (15 mL) was hydrogenated over 10% Pd/C at 50 psi. After 2 h, the catalyst was removed by filtration through a bed of Celite and the filtrate concentrated in vacuum. The resulting foam solid (615 mg, 95% of yield) was used without any purification for the next reaction. ^{1}H NMR ($CDCl_3$) δ 3.44 (bs, 2H), 4.00 (s, 3H), 7.98 (d, $J=9$ Hz, 1H), 8.38 (dd, $J=9$ and 2 Hz, 2H), 8.77 (d, $J=1.6$ Hz, 1H). FAB-MS (MALDI-TOF): 208.4 $[M+1]^+$.

p-*N*,*N*-bis(2-Chloroethyl)aminobenzoyl chloride[15] (603 mg, 2.3 mmol) dissolved in dry dioxane (5 mL) was added dropwise at room temperature to a stirring solution of methyl 5-aminobenzothiophene-2-carboxylate (435 mg, 2.1 mmol) and dry triethylamine (0.32 mL, 2.3 mmol) in dry dioxane (10 mL). The reaction mixture was allowed to stir overnight. After TLC analysis indicated that all of the starting material had disappeared, the reaction mixture was concentrated under reduced pressure to a brown solid, which was dissolved in EtOAc and washed with water twice (10 mL each). The organic layer was dried (Na_2SO_4), concentrated and the resulting pure product was precipitated from EtOAc/hexane, to give methyl 5-[4-*N*,*N*-bis(2-chloroethyl)aminobenzene-1-carboxamido]benzothiophene-2-carboxylate as a brown powder which was dried in vacuum at room temperature. Yield: 430 mg (85%); mp 200 °C; ^{1}H NMR ($CDCl_3$) δ 3.78 (m, 8H), 3.94 (s, 3H), 6.72 (d, $J=7.8$ Hz, 2H), 7.59 (d, $J=9$ Hz, 1H), 7.82 (t, $J=8$ Hz, 3H), 7.99 (s, 1H), 8.35 (s, 1H), 10.1 (s, 1H). FAB-MS (MALDI-TOF): 451.2 $[M+1]^+$.

To a well-stirred solution of methyl 5-[4-*N*,*N*-bis(2-chloroethyl)aminobenzene-1-carboxamido]benzothiophene-2-

carboxylate (370 mg, 0.82 mmol) in 5 mL of dioxane was treated with 2 N KOH in water (2 mL) and stirred at room temperature for 1 h. The clear solution was evaporated to remove dioxane, diluted with water (5 mL), cooled on an ice-water bath and acidified with aqueous hydrochloric acid to pH 2. The aqueous suspension was extracted with ethyl acetate (2×10 mL) and the organic layers were combined, dried (Na_2SO_4) and concentrated. The resulting residue was precipitated from ethyl acetate with hexane to give the product **14** as a brown powder. Yield: 330 mg (92%). mp > 300 °C. ^{1}H NMR (DMSO-d_6): δ ^{1}H NMR ($CDCl_3$) δ 3.80 (m, 8H), 6.86 (d, J = 8.6 Hz, 2H), 7.94 (m, 4H), 8.09 (s, 1H), 8.51 (s, 1H), 10.17 (s, 1H), 13.03 (bs, 1H). FAB-MS (MALDI-TOF): 436.2 $[M+1]^+$.

General procedure for the synthesis of the acyl chlorides of the nitro-acids (11–14). A suspension of **11**–**14** (1 mmol) in dry dioxane (3 mL) containing $SOCl_2$ (0.5 mL) was boiled under reflux for 2 h. The solvent was evaporated and the residue was used without any purification for the next reaction.

General procedure for the synthesis of compounds (3–6). To a cooled solution of the amino-amidine **15** (1 mmol), in a mixture of water–dioxane (10 mL, 1/4 v/v) containing $NaHCO_3$ (2 mmol), the acyl chloride of the compounds **11**–**14** (1 mmol) in dioxane (3 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. After this time, the solution was carefully acidified (pH 1–2) with 10% HCl in water, evaporated, and the residue purified by silica gel column chromatography [eluant dichloromethane (DCM)/MeOH 9.5/0.5 then 9/1)].

3-[1-Methyl-4[1-methyl-4[5-[4-*N*,*N*-bis(2-chloroethyl)-aminobenzene-1-carboxamido] indole-2-carboxamido]-pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine hydrochloride (3). Following the general procedure, starting from **11** (100 mg, 0.24 mmol), it was transformed in the corresponding acyl chloride and added to the amino-amidine **15** (100 mg, 0.24 mmol), after workup, the residue was purified by flash chromatography using methanol–methylene chloride 1/9 (v/v) as eluant. The resulting oil, precipitated by a mixture methanol/ethyl ether 1/20 (v/v), yielded the compound **3** as a brown solid (143 mg, 77% yield); mp 200–203 °C; ^{1}H NMR (DMSO-d_6) δ 2.64 (m, 2H), 3.49 (m, 2H), 3.82 (m, 8H), 3.81 (s, 3H), 3.88 (s, 3H), 6.85 (d, J = 9 Hz, 2H), 6.96 (s, 1H), 7.10 (s, 1H), 7.42 (m, 5H), 7.91 (d, J = 8.6 Hz, 2H), 8.11 (s, 1H), 8.29 (t, J = 5.6 Hz, 1H), 8.77 (bs, 2H), 9.08 (bs, 2H), 9.89 (s, 1H), 10.05 (s, 1H), 10.49 (s, 1H), 11.70 (s, 1H). FAB-MS (MALDI-TOF): 734.6 $[M+1]^+$. Anal. calcd. for $C_{35}H_{39}Cl_3N_{10}O_4$: C, 54.59; H, 5.10; Cl, 13.81; N, 18.19. Found: C, 54.48; H, 5.02; Cl, 13.68; N, 18.04.

3-[1-Methyl-4[1-methyl-4[1-methyl-5-[4-*N*,*N*-bis(2-chloroethyl)aminobenzene-1-carboxamido]indole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido] propionamidine hydrochloride (4). Following the general procedure, starting from **12** (260 mg, 0.6 mmol), it was transformed in the corresponding acyl chloride and added to the amino-amidine **15** (200 mg, 0.5 mmol), after work-up, the residue was purified by flash chromatography using methanol/methylene chloride 1/9 (v/v) as eluant. The resulting oil, precipitated by a mixture of methanol/ethyl ether 1/20 (v/v), yielded the compound **4** as a brown solid (256 mg, 65% yield); mp 193–195 °C; ^{1}H NMR (DMSO-d_6) δ 2.66 (m, 2H), 3.66 (m, 2H), 3.78 (m, 8H), 3.81 (s, 3H), 3.88 (s, 3H), 4.02 (s, 3H), 6.86 (d, J = 8.8 Hz, 2H), 6.96 (s, 1H), 7.12 (s, 1H), 7.22 (s, 2H), 7.35 (s, 1H), 7.54 (d, J = 6 Hz, 2H), 7.91 (d, J = 8.8 Hz, 2H), 8.17 (s, 1H), 8.23 (t, J = 5.6 Hz, 1H), 8.70 (bs., 2H), 9.04 (bs., 2H), 9.93 (s, 1H), 10.01 (s, 1H), 10.45 (s, 1H). FAB-MS (MALDI-TOF): 748.6 $[M+1]^+$. Anal. calcd for $C_{36}H_{41}Cl_3N_{10}O_4$: C, 55.14; H, 5.25; Cl, 13.56; N, 17.86. Found: C, 55.03; H, 5.11; Cl, 13.45; N, 17.77.

3-[1-Methyl-4[1-methyl-4[5-[4-*N*,*N*-bis(2-chloroethyl)aminobenzene-1-carboxamido] benzofuran-2-carboxamido]-pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine hydrochloride (5). Following the general procedure, starting from 13 (105 mg, 0.25 mmol), it was transformed in the corresponding acyl chloride and added to the amino-amidine **15** (100 mg, 0.25 mmol), after workup, the residue was purified by flash chromatography using methanol/methylene chloride 2/8 (v/v) as eluant. The resulting oil, precipitated by a mixture methanol/ethyl ether 1/20 (v/v), yielded the compound **5** as a white solid (100 mg, 52% yield); mp 205–210 °C; ^{1}H NMR (DMSO-d_6) δ 2.63 (m, 2H), 3.48 (m, 2H), 3.82 (m, 8H), 3.81 (s, 3H), 3.82 (s, 3H), 6.86 (d, J = 9 Hz, 2H), 6.96 (s, 1H), 7.15 (s, 1H), 7.21 (s, 2H), 7.34 (s, 1H), 7.68 (m, 3H), 7.90 (d, J = 8.6 Hz, 2H), 8.29 (s, 1H), 8.61 (bs, 2H), 8.98 (bs, 2H), 10.03 (s, 1H), 10.09 (s, 1H), 10.73 (s, 1H). FAB-MS (MALDI-TOF): 735.6 $[M+1]^+$. Anal. calcd for $C_{35}H_{38}Cl_3N_9O_5$: C, 54.52; H, 4.97; Cl, 13.79; N, 16.35. Found: C, 54.41; H, 4.78; Cl, 13.64; N, 16.25.

3-[1-Methyl-4[1-methyl-4[5-[4-*N*,*N*-bis(2-chloroethyl)aminobenzene-1-carboxamido] benzothiophene-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido] propionamidine hydrochloride (6). Following the general procedure, starting from **14** (180 mg, 0.41 mmol), it was transformed in the corresponding acyl chloride and added to the amino-amidine **15** (160 mg, 0.41 mmol), after workup, the residue was purified by flash chromatography using methanol/methylene chloride 2/8 (v/v) as eluant. The resulting oil, precipitated by a mixture methanol/ethyl ether 1/15 (v/v), yielded the compound **6** as a brown solid (186 mg, 58% yield); mp 188–190 °C; ^{1}H NMR (DMSO-d_6) δ 2.66 (m, 2H), 3.36 (m, 2H), 3.78 (m, 8H), 3.81 (s, 3H), 3.87 (s, 3H), 6.86 (d, J = 9 Hz, 2H), 6.96 (s, 1H), 7.13 (s, 1H), 7.23 (s, 1H), 7.35 (s, 1H), 7.94 (m, 4H), 8.29 (s, 1H), 8.50 (s, 1H), 8.73 (bs, 2H), 9.05 (bs, 2H), 10.04 (s, 1H), 10.17 (s, 1H), 10.78 (s, 1H). FAB-MS (MALDI-TOF): 751.5 $[M+1]^+$. Anal. calcd for $C_{35}H_{38}Cl_3N_9O_4S$: C, 53.40; H, 4.87; Cl, 13.51; N, 16.01; S, 4.07: Found: C, 53.26; H, 4.75; Cl, 13.38; N, 15.89; S, 3.95.

General procedure for the synthesis of compounds (20–23). The nitroacid **16**–**19** (2 mmol) was refluxed in a mixture of dioxane (5 mL) and thionyl chloride (4 mL)

of for 1 h. The reaction mixture was concentrated in vacuo and the resulting residue was coevaporated with toluene. The resulting acid chloride was used without further purification.

To a cooled solution of **15** (2 mmol) and $NaHCO_3$ (336 mg, 4 mmol) in a mixture of 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 and the residue purified by column chromatography using methylene chloride/methanol as eluant (9/1).

3-[1-Methyl-4[1-methyl-4[5-nitro-indole-2-carboxamido]-pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine hydrochloride (20). Following the general procedure, starting from the nitro-acid **16** (206 mg, 1 mmol), it was transformed into the corresponding acyl chloride and coupled with the amino-amidine **15** (404 mg, 1 mmol). After workup, the nitro-amidine **20** was obtained as a yellow solid (358 mg, 67% yield); mp > 300 °C; ^{1}H NMR (DMSO-d_6) δ 2.63 (m, 2H), 3.49 (m, 2H), 3.82 (s, 3H), 3.88 (s, 3H), 6.96 (s, 1H), 7.11 (s, 1H), 7.22 (s, 1H), 7.38 (s, 1H), 7.62 (s, 2H), 8.08 (d, J = 8.8 Hz, 1H), 8.26 (s, 1H), 8.67 (bs, 2H), 8.75 (s, 1H), 9.02 (bs, 2H), 10.04 (s, 1H), 10.75 (s, 1H), 12.48 (s, 1H). FAB-MS (MALDI-TOF): 521.5 $[M+1]^+$. Anal. calcd for $C_{24}H_{26}ClN_9O_5$: C, 51.85; H, 4.71; Cl, 6.38; N, 22.67; Found: C, 51.74; H, 4.61; Cl, 6.23; N, 22.54.

3-[1-Methyl-4[1-methyl-4[1-methyl-5-nitro-indole-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]propionamidine hydrochloride (21). Following the general procedure, starting from the nitro-acid **17** (216 mg, 1 mmol), it was transformed into the corresponding acyl chloride and coupled with the amino-amidine **15** (404 mg, 1 mmol). After workup, the nitro-amidine **21** was obtained as a yellow solid (283 mg, 53% yield); mp 167–170 °C; ^{1}H NMR (DMSO-d_6) δ 2.66 (m, 2H), 3.42 (m, 2H), 3.54 (s, 3H), 3.76 (s, 3H), 4.08 (s, 3H), 6.97 (s, 1H), 7.01 (s, 1H), 7.13 (s, 1H), 7.22 (s, 1H), 7.36 (s, 1H), 7.51 (s, 1H), 7.97 (m, 1H), 8.12 (m, 1H), 8.28 (t, J = 7.2 Hz, 1H), 8.72 (bs, 2H), 9.06 (bs, 2H), 10.04 (s, 1H), 10.78 (s, 1H). FAB-MS (MALDI-TOF): 535.5 $[M+1]^+$. Anal. calcd for $C_{25}H_{28}ClN_9O_5$: C, 52.68; H, 4.95; Cl, 6.22; N, 22.12. Found: C, 52.53; H, 4.78; Cl, 6.11; N, 22.01.

3-[1-Methyl-4[1-methyl-4[5-nitro-benzofuran-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]-propionamidine hydrochloride (22). Following the general procedure, starting from the nitro-acid **18** (207 mg, 1 mmol), it was transformed into the corresponding acyl chloride and coupled with the amino-amidine **15** (404 mg, 1 mmol). After work-up, the nitro-amidine **22** was obtained as a yellow solid (243 mg, 46% yield); mp 188–190 °C; ^{1}H NMR (DMSO-d_6) δ 2.62 (m, 2H), 3.49 (m, 2H), 3.82 (s, 3H), 3.89 (s, 3H), 6.96 (s, 1H), 7.11 (s, 1H), 7.22 (s, 1H), 7.38 (s, 1H), 7.62 (s, 1H), 8.07 (m, 1H), 8.11 (t, J = 7.2 Hz, 1H), 8.26 (s, 1H), 8.68 (bs, 2H), 8.75 (s, 1H), 9.02 (bs, 2H), 10.04 (s, 1H), 10.78 (s, 1H). FAB-MS (MALDI-TOF): 522.5 $[M+1]^+$. Anal. calcd for $C_{24}H_{25}ClN_8O_6$: C, 51.76; H, 4.52; Cl, 6.37; N, 20.12. Found: C, 51.58; H, 4.42; Cl, 6.21; N, 22.01.

3-[1-Methyl-4[1-methyl-4[5-nitro-benzothiophene-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]propionamidine hydrochloride (23). Following the general procedure, starting from the nitro-acid **19** (223 mg, 1 mmol), it was transformed into the corresponding acyl chloride and coupled with the amino-amidine **15** (404 mg, 1 mmol). After work-up, the nitro-amidine **23** was obtained as a yellow solid (311 mg, 54% yield); mp 210–212 °C; ^{1}H NMR (DMSO-d_6) δ 2.74 (m, 2H), 3.50 (m, 2H), 3.82 (s, 3H), 3.88 (s, 3H), 6.97 (s, 1H), 7.15 (s, 1H), 7.23 (s, 1H), 7.36 (s, 1H), 8.31 (m, 2H), 8.52 (s, 1H), 8.11 (t, J = 7.2 Hz, 1H), 8.69 (bs, 2H), 8.95 (s, 1H), 9.03 (bs, 2H), 10.04 (s, 1H), 11.05 (s, 1H). FAB-MS (MALDI-TOF): 538.6 $[M+1]^+$. Anal. calcd for $C_{24}H_{25}ClN_8O_5S$: C, 50.30; H, 4.40; Cl, 6.19; N, 19.55; S, 5.60. Found: C, 50.14; H, 4.28; Cl, 6.02; N, 22.01; S, 5.35.

Synthesis of 1-hydroxybenzotriazolyl-*N*,*N*-bis(2-chloroethyl)aminocinnamoate (24). A solution of **25** (2 mmol, 596 mg), HOBt (2 mmol, 270 mg), DCC (2 mmol, 412 mg) in 10 mL of dry DMF was heated at 70 °C for 4 h. After this time, the mixture was filtered, removing the DCU formed. The filtered containing **24** was added dropwise to the appropriate amine.

General procedure for the synthesis of compounds (7–10). A solution of **20–23** (2 mmol) in 15 mL of a mixture MeOH/water (4:1 v/v) containing a few drops of aqueous 10% HCl 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 residue which was used without purification for the next step.

The crude amine above was prepared was dissolved in anhydrous DMF (7 mL) containing $NaHCO_3$ (2 mmol, 168 mg) and at this solution was added **24** (808 mg, 2 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).

3-[1-Methyl-4[1-methyl-4[5-[4-*N*,*N*-bis(2-chloroethyl)-aminocinnamoyl]aminoindole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine hydrochloride (7). Following the general procedure, starting from **20** (273 mg, 0.5 mmol), after catalytic hydrogenation, this compound was coupled-to **24** (202 mg, 0.5 mmol). The usual workup furnished the compound **7** as a brown powder (386 mg, 48.6%). mp 202–205 °C; ^{1}H NMR (DMSO-d_6) δ 2.68 (m, 2H), 3.43 (m, 2H), 3.76 (m, 8H), 3.77 (s, 3H), 3.83 (s, 3H), 6.36 (d, J = 15.4 Hz, 1H), 6.65 (s, 1H), 6.78 (d, J = 8.4 Hz, 2H), 6.91 (s, 1H), 7.05 (s, 1H), 7.12 (s, 1H), 7.28 (s, 1H), 7.48 (m, 5H), 7.75 (d, J = 15.4 Hz, 1H), 8.02 (s, 1H), 8.08 (s, 1H), 8.34 (bs, 2H), 8.68 (bs, 2H), 10.04 (s, 1H), 10.75 (s, 1H), 12.48 (s, 1H). FAB-MS (MALDI-TOF): 760.7 $[M+1]^+$. Anal. calcd for $C_{37}H_{41}Cl_3N_{10}O_4$: C, 55.82; H, 5.19; Cl, 13.36; N, 17.59. Found: C, 55.67; H, 5.04; Cl, 13.17; N, 17.43.

3-[1-Methyl-4[1-methyl-4[1-methyl-5-[4-*N*,*N*-bis(2-chloroethyl)aminocinnamoyl]amino indole-2-carboxamido]pyrrole-2-carboxamido] pyrrole-2-carboxamido]propionamidine hydrochloride (8). Following the general procedure, starting from **21** (267 mg, 0.5 mmol), after catalytic hydrogenation, this compound was coupled to **24** (202 mg, 0.5 mmol). The usual workup furnished the compound **8** as a yellow powder (310 mg, 48%); mp > 217–220 °C; ^{1}H NMR (DMSO-d_6) δ 2.72 (m, 2H), 3.34 (m, 2H), 3.71 (m, 8H), 3.82 (s, 3H), 3.88 (s, 3H), 4.03 (s, 3H), 6.33 (d, J = 15.4 Hz, 1H), 6.65 (s, 1H), 6.80 (d, J = 8.4 Hz, 2H), 6.91 (s, 1H), 7.03 (s, 1H), 7.10 (s, 1H), 7.48 (m, 6H), 7.70 (d, J = 15.4 Hz, 1H), 8.01 (s, 1H), 8.12 (s, 1H), 8.52 (bs, 2H), 9.12 (bs, 2H), 10.04 (s, 1H), 10.78 (s, 1H). FAB-MS (MALDI-TOF): 774.7 $[M+1]^+$. Anal. calcd. for $C_{38}H_{43}Cl_3N_{10}O_4$: C, 56.33; H, 5.35; Cl, 13.13; N, 17.29; Found: C, 56.18; H, 5.18; Cl, 13.02; N, 17.05.

3-[1-Methyl-4[1-methyl-4[5-[4-N,N-bis(2-chloroethyl)-aminocinnamoyl]amino benzofuran-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine hydrochloride (9). Following the general procedure, starting from **22** (273 mg, 0.5 mmol), after catalytic hydrogenation, this compound was coupled to **24** (202 mg, 0.5 mmol). The usual workup furnished the compound **9** as a yellow powder (386 mg, 48%); mp 200–203 °C; ^{1}H NMR (DMSO-d_6) δ 2.68 (m, 2H), 3.48 (m, 2H), 3.72 (m, 8H), 3.80 (s, 3H), 3.88 (s, 3H), 6.45 (d, J = 15.4 Hz, 1H), 6.54 (s, 1H), 6.62 (d, J = 7.2 Hz, 2H), 6.84 (s, 1H), 7.06 (s, 1H), 7.22 (s, 1H), 7.28 (s, 1H), 7.45 (m, 5H), 7.78 (d, J = 15.4 Hz, 1H), 8.07 (s, 1H), 8.11 (s, 1H), 8.34 (bs, 2H), 8.46 (bs, 2H), 10.04 (s, 1H), 10.55 (s, 1H). FAB-MS (MALDI-TOF): 761.7 $[M+1]^+$. Anal. calcd for $C_{37}H_{40}Cl_3N_9O_5$: C, 55.75; H, 5.06; Cl, 13.34; N, 15.81. Found: C, 55.62; H, 4.89; Cl, 13.16; N, 15.69.

3-[1-Methyl-4[1-methyl-4[5-[4-*N*,*N*-bis(2-chloroethyl)-aminocinnamoyl]amino benzothiophene-2-carboxamido]-pyrrole-2-carboxamido]pyrrole-2-carboxamido] propionamidine hydrochloride (10). Following the general procedure, starting from **23** (285 mg, 0.5 mmol), after catalytic hydrogenation, this compound was coupled to **24** (202 mg, 0.5 mmol). The usual workup furnished the compound **10** as a yellow powder (412 mg, 51%); mp > 300 °C; ^{1}H NMR (DMSO-d_6) δ 2.56 (m, 2H), 3.33 (m, 2H), 3.68 (m, 8H), 3.81 (s, 3H), 3.88 (s, 3H), 6.32 (d, J = 15.4 Hz, 1H), 6.58 (s, 1H), 6.68 (d, J = 7.2 Hz, 2H), 6.92 (s, 1H), 7.05 (s, 1H), 7.20 (s, 1H), 7.28 (s, 1H), 7.36 (m, 5H), 7.75 (d, J = 15.4 Hz, 1H), 8.02 (s, 1H), 8.06 (s, 1H), 8.34 (bs, 2H), 8.56 (bs, 2H), 10.04 (s, 1H), 10.75 (s, 1H). FAB-MS (MALDI-TOF): 777.7 $[M+1]^+$. Anal. calcd for $C_{37}H_{40}Cl_3N_9O_4S$: C, 54.65; H, 4.96; Cl, 13.08; N, 15.50; S,3.94. Found: C, 54.61; H, 4.89; Cl, 13.01; N, 15.43; S, 3.85.

Biological evaluation

DNA footprinting. The oligodeoxynucleotide primer (5′ gtcggttaggagagctccacttg) was 5′-end labelled using T4 polynucleotide kinase and [γ-23P]-ATP (5000 Ci/mmol, Amersham, UK) and then purified down a Bio-Rad spin column. PCR amplification was carried out to produce the target 447bp Human Topoisomerase IIα promotor region for footprinting. Amplification was carried out in a final volume of 50 μl containing 4 pmols of both the labelled primer and 'complementary' primer (5′ ctgtccagaaagccggcactcag), 10 ng pCAT557 plasmid (Promega) containing the topo2a promotor (10 ng/μl) (courtesy of Dr. B. Tolner), 120 μM of each dNTP, 2.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 9), 50 mM KCl, 0.1% Triton X-100 and 10 u *Taq* polymerase. After an initial denaturation at 94 °C for 3 min, the cycling conditions were as follows: 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min for a total of 30 cycles. This was followed by a final step of 72 °C for 5 min. Amplified samples were then run down a 2% agarose gel and purified.

This singly end-labelled DNA was then used at a concentration of approx 100 cps per sample and drug-treated for 2.5 h (room temperature) in 50 mM KCl,1 mM $MgCl_2$, 500 μM EDTA, 500 μM DTT and 20 mM Hepes pH7.9 in a final volume of 50 μL. After treatment the samples were immediately cleaved by the addition of a 3 μL solution containing 0.5 u DNAseI and 83.3 mM $MgCl_2$ and $CaCl_2$. Cleavage was terminated by the addition of stop mix 100 μL 3M NaOAC and ethanol precipitated. Samples were washed, dried, resuspended in 5μl formamide dye and run down a 6% sequencing gel (Sequagel, National Diagnostics). The gels were dried, and X-ray film was exposed to the gels (Hyperfilm, Amersham, UK).

Taq DNA polymerase stop assay.[16] Plasmid pUC18 DNA was linearised with HindIII to provide a stop for the *Taq* downstream from the primer. The oligodeoxynucleotide primer (5′CTCACTCAAAGGCGGTAATAC) was 5′-end labelled prior to amplification using T4 polynucleotide kinase and [γ-^{32}P]-ATP (5000Ci/mmol, Amersham, UK). The labelled 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 labelled primer, 200 μM of each dNTP, 10U *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).

Cytotoxicity assay. 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-2H-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. 200 mL DMSO was added to each well and the samples agitated to ensure complete mixing. The optical density was then read at a wavelength of 550 nm on a Titertek Multiscan ELISA plate reader, and the dose-response curve was constructed. For each curve, an IC_{50} value was read as the dose required to reduce the final optical density to 50% of the control value.

Acknowledgements

We wish to thank Pharmacia & Upjohn for providing distamycin A and Ministero Università e Ricerca Scientifica (MURST) (60%) for generous financial support of this work. Maria del Carmen Núñez is the recipient of a Fundación Ramón Areces fellowship.

References and Notes

1. (a) Marchini, S.; Broggini, M.; Sessa, C.; D'Incalci, M. *Exp. Opin. Invest. Drugs* **2001**, *10*, 1703. (b) Baraldi, P. G.; Cacciari, B.; Spalluto, G.; Romagnoli, R. *Exp. Op. Ther. Pat.* **2000**, *6*, 891. (c) Cozzi, P.; Mongelli, N. *Curr. Pharm. Des.* **1998**, *4*, 181.
2. Arcamone, F. M.; Animati, F.; Barbieri, B.; Configliacchi, E.; D'Alessio, R.; Geroni, C.; Giuliani, F. C.; Lazzari, E.; Menozzi, M.; Mongelli, N. *J. Med. Chem.* **1989**, *32*, 774.
3. Pezzoni, G.; Grandi, M.; Biasoli, G.; Capolongo, L.; Ballinari, D.; Giuliani, F. C.; Barbieri, B.; Pastori, A.; Pesenti, E.; Mongelli, N.; Spreafico, F. *Br. J. Cancer* **1991**, *64*, 1047.
4. Capolongo, L.; Melegaro, G.; Broggini, M.; Mongelli, N.; Grandi, M. *Br. J. Cancer* **1993**, *86*, 916.
5. (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. *Nucl. Acids Res.* **1995**, *23*, 81.
6. Sunters, A.; Springer, C. J.; Bagshawe, K.; Souhami, R. L.; Hartley, J. A. *Biochem. Pharmacol.* **1992**, *44*, 59.
7. Bellorini, M.; Moncellin, V.; D'Incalci, M.; Mongelli, N.; Mantovani, R. *Nucl. Acids Res.* **1995**, *23*, 1657.
8. (a) Sessa, C.; Pagani, O.; Zurlo, M. G.; Jong, D. E.; Hoffmann, C.; Lassus, M.; Marrari, P.; Strolin-Benedetti, M.; Cavalli, F. *Ann. Oncol.* **1994**, *5*, 901. (b) Ghielmini, M.; Bosshard, G.; Capolongo, L.; Geroni, C.; Pesenti, E.; Torri, V.; D'Incalci, M.; Cavalli, F.; Sessa, C. *Br. J. Cancer* **1997**, *75*, 878.
9. Cozzi, P.; Beria, I.; Biasoli, G.; Caldarelli, M.; Capolongo, L.; Geroni, C.; Mongelli, N. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2985.
10. Lee, H. H.; Boyd, M.; Gravatt, G. L.; Denny, W. A. *Anti-Cancer Drug Des.* **1991**, *6*, 501.
11. (a) Boger, D. L.; Boyce, C. W.; Garbaccio, R. M.; Golberg, J. A. *Chem. Rev.* **1997**, *97*, 787. (b) Zhou, Q.; Duan, W.; Simmons, D.; Shayo, Y.; Raymond, M. A.; Dorr, R. T.; Hurley, L. H. *J. Am. Chem. Soc.* **2001**, *123*, 4865. (c) Wang, Y.; Yuan, H.; Ye, W.; Wright, S. C.; Wang, H.; Larrick, J. W. *J. Med. Chem.* **2000**, *43*, 1541.
12. Baraldi, P. G.; Romagnoli, R.; Beria, I.; Cozzi, P.; Geroni, G.; Mongelli, N.; Bianchi, N.; Mischiati, C.; Gambari, R. *J. Med. Chem.* **2000**, *43*, 2675.
13. Xie, G.; Gupta, R.; Lown, J. W. *Anti-Cancer Drug Des.* **1995**, *10*, 389.
14. Zambias, R. A.; Hammond, M. L. *Synth. Commun.* **1991**, *21*, 959.
15. Elderfield, R. C.; Liao, T. K. *J. Org. Chem.* **1961**, *26*, 4996.
16. Ponti, M.; Forrow, S. M.; Souhami, R. L.; D'Incalci, M.; Hartley, J. A. *Nucl. Acids Res.* **1991**, *19*, 2929.