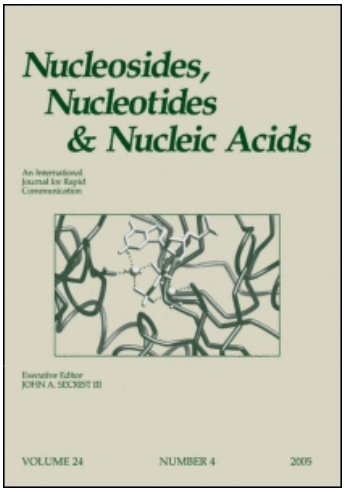

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

[2,1-*c*] [1,4]Benzodiazepine (PBD)-Distamycin Hybrid Inhibits DNA Binding to Transcription Factor Sp1

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[a] Dipartimento di Scienze Farmaceutiche, Università di Ferrara, Ferrara, Italy [b] Dipartimento di Biochimica e Biologia Molecolare, Università di Ferrara, Ferrara, Italy

To cite this Article Baraldi, P. G. , Cacciarit, B. , Guiotto, A. , Romagnoli, R. , Spalluto, G. , Leoni, A. , Bianchi, N. , Feriotto, G. , Rutigliano, C. , Mischiati, C. and Gambari, Roberto(2000) '[2,1-*c*] [1,4]Benzodiazepine (PBD)-Distamycin Hybrid Inhibits DNA Binding to Transcription Factor Sp1', Nucleosides, Nucleotides and Nucleic Acids, 19: 8, 1219 — 1229
To link to this Article: DOI: 10.1080/15257770008033045
URL: http://dx.doi.org/10.1080/15257770008033045

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(8), 1219–1229 (2000)

[2,1-c][1,4]BENZODIAZEPINE (PBD)-DISTAMYCIN HYBRID INHIBITS DNA BINDING TO TRANSCRIPTION FACTOR Sp1

P. G. Baraldi*†, B. Cacciari†, A. Guiotto†, R. Romagnoli†, G. Spalluto†, A. Leoni†, N. Bianchi§, G.Feriotto§, C.Rutigliano§, C.Mischiati§ and Roberto Gambari§

†*Dipartimento di Scienze Farmaceutiche, Università di Ferrara, 44100 Ferrara, Italy,*
§*Dipartimento di Biochimica e Biologia Molecolare, Università di Ferrara, 44100 Ferrara, Italy*

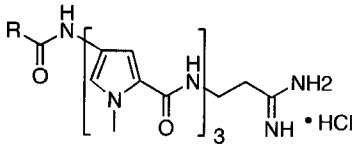
ABSTRACT: We designed and synthesized the hybrid **6**, prepared combining the minor groove binders distamycin A and pyrrolo [2,1-*c*][1,4] benzodiazepine (PBD) **4**, related to the natural occurring anthramycin (**2**) and DC-81 (**3**). In this paper, the effects of the compound **6** on molecular interactions between DNA and transcription factor Sp1 were studied. The results obtained demonstrate that PBD-distamycin hybrid is a powerful inhibitor of Sp1/DNA interactions.

It is well established that anticancer and antiviral agents which bind and interact to the B-form of DNA can alter the binding activity of transcription factors, thereby acting as selective modulators of the transcription of a variety of genes involved in growth survival, malignant properties of cancer cells and expression of viral genes[1].

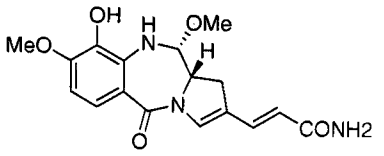
In the last years a large number of compounds have been discovered, both natural and synthetic, which bind in a sequence-dependent manner with the minor groove of DNA. Among these minor groove binders, one of the most important is distamycin A **1**[2], a naturally occurring antibiotic, characterized by the presence of an oligopeptide N-methylpyrrolecarbamoyl frame ending with an amidino moiety. This compound has driven researcher's attention not only for its biological activity, but also for its non intercalative binding to the minor groove of double-stranded B-DNA, where it form strong reversible complex preferentially at the nucleotide sequences consisting of four or more adjacent AT base pairs[3]. It is widely known that distamycin has been used as selective vehicle of alkylating functions [as benzoic acid mustard (BAM) analogues, epoxycarbonyl, halogenoacryloyl or nitrogen mustard] able to react with biological nucleophiles, leading to a sharp increase of its cytotoxicity and antiviral activity, in comparison to that, very weak, of distamycin. In the last few years, several hybrid compounds,

in which known antitumor compounds or simple active moieties of known antitumor and antiviral agents have been tethered to distamycin frame have been designed, synthetized and tested[4].

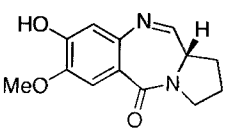
The pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) group[5], which includes the natural compounds anthramycin (**2**)[6] and DC-81 (**3**), owes its DNA-interactive ability and resultant biological effects to an N10-C11 carbinolamine/imine moiety in the central B-ring which is capable of covalently binding to the C2-NH_2 of guanine residues in the minor groove of DNA. X ray and footprinting studies on covalent DNA-PBD adducts have demonstrated a high sequence-specificity for G-C rich DNA regions, in particular for X-G-X triplets (X=purine)[7].



1, Distamycin A



2, Anthramycin



3, DC-81

Recently, we[8a,b] and the Lown's group[8c] have described an example in which distamycin A was used as DNA vector for pyrrolo [2,1-*c*][1,4] benzodiazepine **4**, leading to the synthesis of conjugate **6**. The rationale that led to the preparation of **6** was to tether the distamycin A frame, which plays the role of pure minor groove binder, to the minor groove alkylating moiety represented by the pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) **4**, with the aim to obtain a new derivative which could result more cytotoxic than the parent compounds. In the new hybrid **6**, we conjugated the capability of PBD **4** to covalently bind to GC-rich sequences with the distamycin different DNA recognition pattern.

In this paper we report a study focused on the ability of compound **6** to inhibit the DNA binding of the transcription factor Sp1[9]. It should be underlined that Sp1 is very important for the control of transcription of cellular and viral genes. Accordingly, G+C selective DNA-binding drugs, such as mithramycin and chromomycin, suppress molecular interactions between the transcription factor Sp1 and viral and eukaryotic promoters[10], leading to alteration of the expression of Sp1-regulated transcriptional units, including the oncogenes Ha-ras and c-myc, the collagen-α1(I) gene and the human immunodeficiency type 1 virus (HIV-1). With respect to this point, it should be pointed out that distamycin is not able to inhibit Sp1/DNA interactions[11]. The results described in this paper demonstrate that treatment of Sp1 target DNA with PBD-distamycin hybrid **6** renders the site unrecognizable by Sp1 nuclear proteins.

Chemistry

The synthesis of the hybrid **6** has been previously reported[8a,b], and obtained by the coupling between the N10-Troc protected PBD **4** and deformyldistamycin A[2a] **5** and subsequent cleavage of the Troc[12] group with Cd/Pb couple in aqueous NH_4Ac[13] (Scheme 1). Using the same cleavage methodology, the methyl ester of the compound **4** was converted to **7**.

Before the choice of 2,2,2-trichloroethylchloroformate (Troc) as amino protecting group, at the beginning we have used the diethyl thioacetal **8**, bearing allyloxycarbonyl function (Alloc)[14] as amino protective group. Allyl carbamates have been used in various field of chemistry, and although other methods are known for removal of this protective group[15], the deprotection is most frequently carried out under relatively mild conditions using a palladium (0) catalyst in the presence of a nucleophile[16]. Starting from the well-known compound **8**[17], after protection of the amino group as Alloc to yield **9**, the subsequent cyclization using $HgCl_2/HgO$ in aqueous acetonitrile at room temperature[18] produced **10**. Acid hydrolysis of the ester **10** afforded the acid **11** which was coupled with the amine moiety of deformyldistamycin A **5** to obtain **12** (Scheme 2). Deprotection reaction upon use of standard conditions [10% $Pd(PPh_3)_4$, 0.6 equiv. PPh_3, excess of nucleophile as *n* -BuSnH, THF, rt.] lead to rather disappointing results and very poor yield of **6**. The compound **12** has been evaluated *in vitro* by using L1210 leukaemia cell line, and it is 3-fold more active than distamycin A (IC_{50}, 1.8 μg/mL for **12** vs. 5.2 μg/mL for distamycin A).

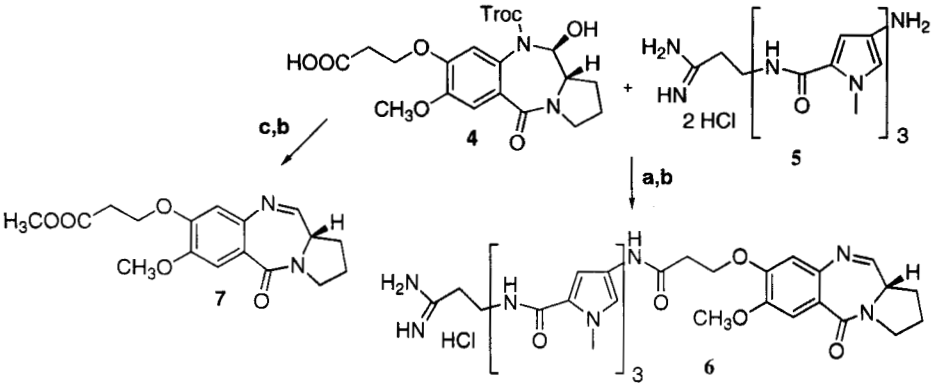
RESULTS AND DISCUSSION

Effects of distamycin A, PBD and PBD-distamycin hybrid on in vitro cell proliferation of K562 and Raji tumor cell lines. Fig.1 shows the effects of distamycin A **1**, PBD **7**, PBD-distamycin hybrids **6** and the combined use of **1** plus **7** on cell growth of human leukemic K562 (Fig.1A) and B-lymphoid Raji (Fig.1B) cells. In this experiment cells were treated for 7 days in the presence of the indicated compounds and the cell number/ml determined. As clearly evident, the PBD-distamycin hybrid **6** was always found to be more active than distamycin A **1** and PBD **7**, either used alone or in combination. In fact, the results obtained clearly show that distamycin A **1** inhibits K562 and Raji cell growth only when added at 5 μM and 3 μM, respectively.

Treatment with PBD **7** or a combined use of distamycin A plus PBD **7** gives very similar results, i.e. inhibition of K562 and Raji cell growth only at about 1.2 μM. By sharp contrast, the PBD-distamycin hybrid **6** is much more effective as antiproliferative agent. 0.2 μM and 0.15 μM final concentrations of compound **6** are indeed sufficient to strongly inhibit K562 and Raji cell growth.

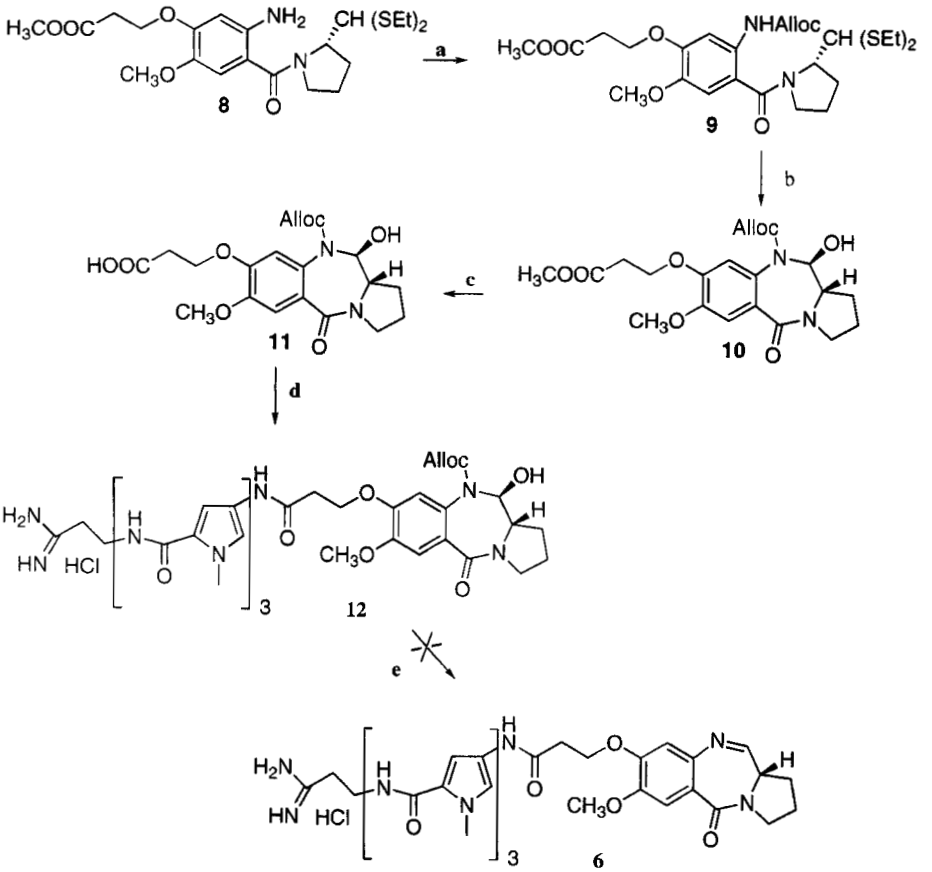
Effects of distamycin, PBD and PBD-distamycin hybrid on binding of nuclear proteins to the Sp1 mer. The effects of distamycin A, PBD **7** and PBD-distamycin hybrid **6** on molecular interactions between nuclear proteins and ^{32}P-labelled Sp1 mer were studied by

Scheme 1



Reagents[a]: **a**: EDCI (2 eq.), DIPEA, dry DMF; **b**: 10% Cd/Pb couple, CH_3OH, 1M NH_4Ac; c: CH_2N_2, Et_2O, r.t.

Scheme 2



Reagents: **a**: Alloc-Cl, pyridine; **b**: $HgCl_2$, $CaCO_3$, MeCN-H_2O; **c**: HCl 10%, THF; **d**: **5** (1 eq.), EDCI, DIPEA, dry DMF; **e**: PPh_3, $Pd(PPh_3)_4$, n-Bu_3SnH

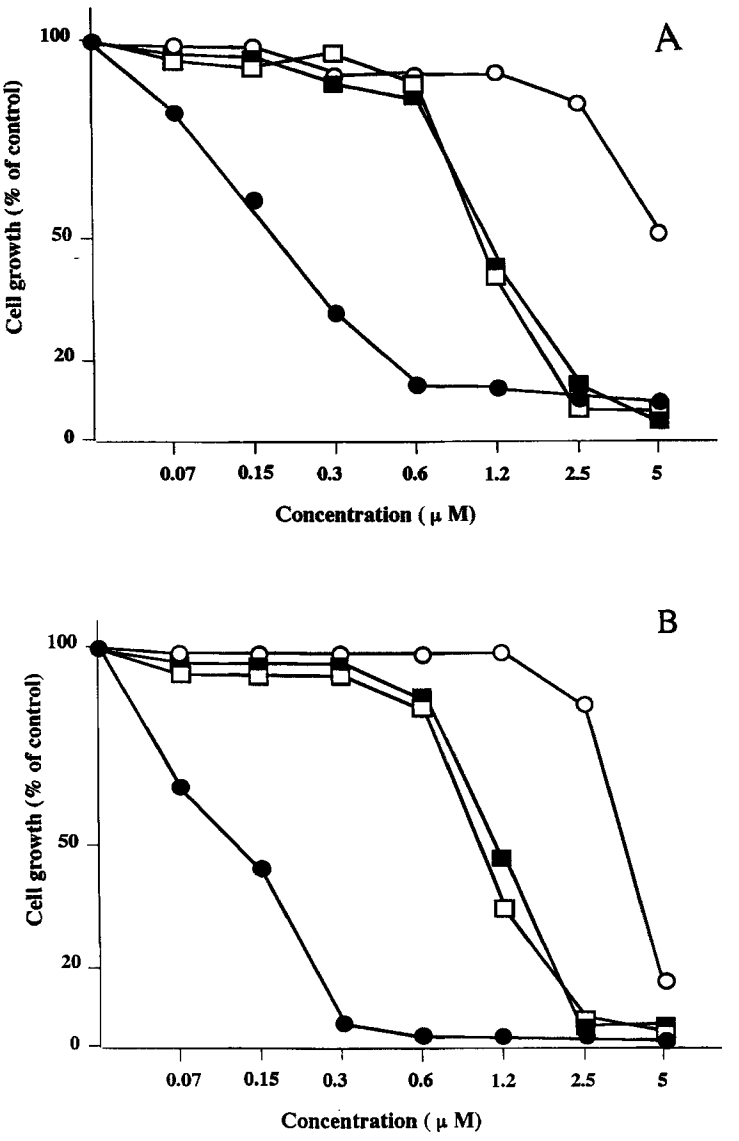


Fig.1. Effects of distamycin A **1**, PBD **7** and the PBD-distamycin hybrid **6** on *in vitro* cell proliferation of human erythroleukemic K562 (A) and B-lymphoid Raji cell lines. Cells were cultured in the absence or in the presence of the indicated concentrations of distamycin A **1** (open cyrcles), PBD **7** (open squares), PBD **7** plus distamycin A **1** (closed squares) or PBD-distamycin hybrid **6** (closed cyrcles). After 7 days of cell culture the cell number/ml was determined and the values obtained in treated cell cultures were compared to those of control cells.

filter binding assays. The results are reported in Fig.2 and clearly show that distamycin A and PBD **7**, even when used at 50 μM concentration, are not active in inhibiting the binding of ^{32}P-labelled Sp1 mer to nuclear proteins isolated from K562 cells and immobilized on the filter after electrophoretic separation (Fig.2A). By contrast, the PBD-distamycin hybrid **6** fully blocks the Sp1/DNA interaction when added at 10 μM concentration (Fig.2B). These results have been confirmed by gel-shift experiments (data not shown).

Conclusions. In this paper we have reported a study focused on the ability of PBD-distamycin hybrid **6** to inhibit the DNA binding of the transcription factor Sp1. The results obtained demonstrate that treatment of Sp1 target DNA with PBD-distamycin hybrid **6** renders the site unrecognizable by nuclear proteins. These data are in our opinion of interest, since transcription factors belonging to the Sp1 superfamily are very important for the control of transcription of cellular and viral genes, including the oncogenes Ha-ras and c-myc, the collagen-α1(I) gene and the human immunodeficiency type 1 virus (HIV-1) [1].

EXPERIMENTAL SECTION

Chemistry

^{1}HNMR spectra were recorded on a Bruker AC 200 spectrometer. Chemical shifts (δ) are given in ppm upfield from tetramethylsilane as internal standard, and the spectra were recorded in appropriate deuterated solvents indicated in the procedure. Infrared spectra were recorded on a Perkin-Elmer 257 spectrophotometer with the solvent indicated in the procedure. All products reported showed ^{1}H-NMR spectra in agreement with the assigned structures. All reactions were carried out under an inert atmosphere of dry nitrogen, unless otherwise described. Standard syringe techniques were applied for transferring dry solvents. Reaction courses and product mixtures were routinely monitored by TLC on silica gel (precoated F_{254} Merk plates) and visualized with aqueous $KMnO_4$. Flash-chromatography was performed using 230-400 mesh silica gel and the solvent system indicated in the procedure. All commercially available compounds 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, triethylamine was dried over molecular sieves of 3 Å and dry 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.

(2*S*)-N-[4'-[2''-carbomethoxyethyloxy-5'-methoxy-2'-(Alloc) carbonylamino benzoyl]]pyrrolidine-2-carboxaldehyde diethyl thioacetal (9). To a solution of **8** (1 g., 2.19 mmol) in 15 mL of THF cooled at 0°C were added pyridine (0.18 mL) and allylchloroformate (Alloc-Cl, 0.23 mL). The reaction mixture was stirred at 0°C for 2 hours, then heated to room temperature and extracted with 10% HCl (2x50 mL), satd. $NaHCO_3$ aq. (2x50 mL) and brine (50 mL). The organic phase was dried with Na_2SO_4 and evaporated to

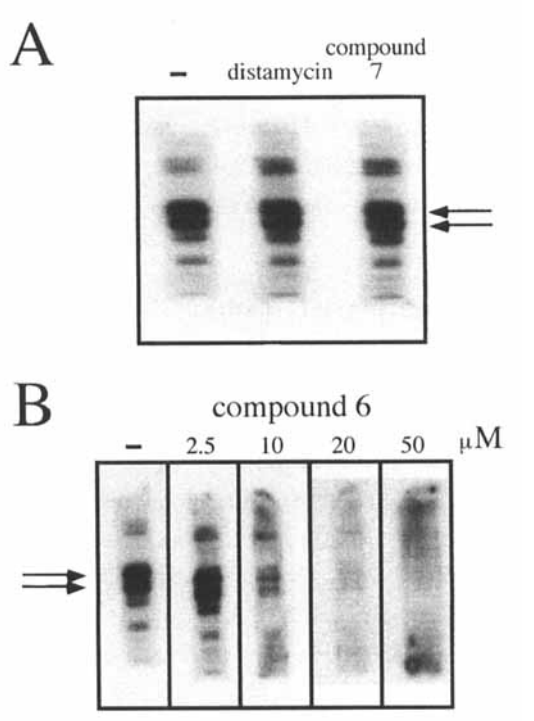


Fig.2. Filter binding assay. Nuclear factors isolated from K562 cells (10 μg/lane) were separated by polyacrylamide gel electrophoresis and blotted onto nitrocellulose filter. Each lane was cutted and independent binding reactions were performed for 1 hour in the absence (-) or in the presence of 50 μM distamycin A **1** (A), 50 μM PBD **7** (A) or 2.5-50 μM PBD-distamycin hybrid **6** (B). After binding the filters were washed and autoradiographed. Specific binding of ^{32}P-labelled Sp1 mer to K562 nuclear factors is arrowed.

yield a yellow oil, which after purification by flash chromatography, afforded **9** as a yellow oil. Yield: 800 mg., (67%). ^{1}H-NMR ($CDCl_3$): δ 1.23 (m, 6H), 1.7-2.03 (m, 4H), 2.24 (m, 1H), 2.74 (m, 4H), 2.86 (t, J=6.8 Hz, 2H), 3.58 (m, 2H), 3.71 (s, 3H), 3.78 (s, 3H), 4.33 (t, J=6.9 Hz, 2H), 4.61-4.74 (m, 3H), 5.26 (d, J=12 Hz, 1H), 5.39 (d, J=12 Hz, 1H), 5.89 (m, 1H), 6.88 (s, 1H), 7.89 (s, 1H), 9.01 (s, 1H).

(11*S*,11a*S*)-8-(2'-Carbomethoxyethyloxy)-11-hydroxy-7-methoxy-10-N-Alloc-1,2,3,10,11,11a-hexahydro-5H-pyrrolo [2,1-*c*] [1,4] benzodiazepin-5-one (10). A suspension of the amino thioacetal **9** (850 mg, 1.57 mmol), $HgCl_2$ (940 mg, 4 mmol) and $CaCO_3$ (390 mg, 3.9 mmol) in CH_3CN-water (5:1 v/v, 18 ml) was stirred at room temperature for 12 h. until TLC (AcOEt-petroleum ether, 1:1 v/v) indicated which the starting material was disappeared. After this time, at the solution was added AcOEt (20 mL) and washed

with aqueous NaHCO3 (1x20 mL) and brine (1x20 mL) and the combined aqueous layer was back-extracted with AcOEt (2x20 mL). The combined organic phase was evaporated and the residue purified by flash chromatography (CHCl3) to yield **10** as a white solid. Yield: 497 mg., (73%). ^{1}H-NMR (DMSO-d6): δ 2.02-2.12 (m, 4H), 2.82 (t, J=6 Hz, 2H), 3.42 (m, 2H), 3.64 (s, 3H), 3.79 (s, 3H), 4.17 (m, 2H), 4.43 (bs, 1H), 4.58 (d, J=12 Hz, 1H), 4.62 (d, J=12 Hz, 1H), 5.11 (m, 2H), 5.48 (m, 1H), 5.82 (bs, 1H), 6.53 (m, 1H), 6.82 (s, 1H), 7.08 (s, 1H).

(11*S*,11a*S*)-8-(2'-Carboxyethyloxy)-11-hydroxy-7-methoxy-10-N-Alloc-1,2,3,10,11,11a-hexahydro-5H-pyrrolo[2,1-*c*] [1,4]benzodiazepin-5-one (11). 2 mL of 10% HCl were added to a solution of **10** (50 mg., 0.11 mmol) in 10 mL of THF. The resulting mixture was stirred at room temperature for 96 hours, then was diluted with 20 mL of ethyl acetate and extracted with water (2x20 mL). The organic layer was dried with Na_2SO_4 and evaporated at reduced pressure. The resulting oil residue was purified by flash chromatography (CHCl3-MeOH, 9:1 v/v) to afford **11** as colourless oil. Yield: 35 mg, (72%). ^{1}H-NMR (DMSO-d6): δ 1.89-2.04 (m, 4H), 2.79 (t, J=6 Hz, 2H), 3.53 (m, 2H), 3.81 (s, 3H), 4.10 (m, 3H), 4.42 (bs, 1H), 4.61 (m, 2H), 5.12 (m, 2H), 5.41 (m, 1H), 5.77 (bs, 1H), 6.53 (m, 1H), 6.81 (s, 1H), 7.08 (s, 1H).

3-[1-Methyl-4-[1-methyl-4-[1-methyl-4-[(11*S*,11a*S*)-8-(2'-carboxamido ethyloxy)-11-hydroxy-7-methoxy-10-N-Alloc-1,2,3,10,11,11a-hexahydro-5H-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one]-pyrrole-2-carboxamido]pyrrole-2-carboxamido]-pyrrole-2-carboxamido]-propionamidine hydrochloride (12). To a stirred solution deformyldistamycin A **5** (52.6 mg, 0.1 mmol) in anhydrous DMF (1 mL) under argon atmosphere the Hunig's base (17 μL, 0.1 mmol) was added at 0°C. After 5 min, **11** (42 mg, 0.1 mmol) followed by EDCI (27.4 mg, 0.2 mmol) were added. The resulting mixture was stirred overnight as it warmed to room temperature, acidified with 20% HCl to pH=5, and then evaporated to dryness in vacuum. The residue was dissolved into a small volume of MeOH and then ethyl ether was added to precipitate the crude product as a brown solid: this procedure was repeated 5 times. The solid residue was purified by column chromatography (CH_2Cl_2/CH_3OH 20%) and recrystallised (CH_3OH/diethyl ether) to give **12** as a brown solid (52.6 mg, 58% yield), m.p. 256-258°C (dec.).^{1}H NMR (*d6* -DMSO) δ: 1.88 (m, 5H), 2.58 (t, J=6 Hz, 2H), 2.82 (m, 2H), 3.32 (m, 5H), 3.77 (s, 3H), 3.80 (s, 3H), 3.86 (s, 6H), 4.17 (m, 2H), 4.42 (m, 1H), 4.62 (d, J=12 Hz, 1H); 5.11 (m, 1H), 5.48 (dd, J=5 and 7 Hz, 1H), 5.82 (bs, 1H), 6.52 (m, 1H), 6.83 (s, 1H), 6.95 (s, 2H), 7.07 (s, 2H), 7.19 (s, 2H), 7.24 (s, 1H), 8.25 (t, J=3.6 Hz, 1H), 8.65 (s, 2H), 9.00 (s, 2H), 9.95 (m, 2H), 10.12 (s, 1H).

Attempt for the deprotection of Alloc from the hybrids 11. Preparation of activated hybrid 6. Alloc-protected hybrid **12** (0.084 mmol) was dissolved into a mixture of THF (1 mL) and water (1 mL) and at this solution was added PPh_3 (22 mg, 0.084 mmol),

$Pd(PPh_3)_4$ (18 mg, 0.014 mmol) and n-Bu_3SnH (2.2 equiv.) was added portionwise: no significative products were detectable on TLC during reaction progress.

Molecular and Cellular Biology

Cell lines and culture conditions. The murine leukaemia L1210 cells and the human leukemic K562 and B-lymphoid Raij cell lines were maintained in RPMI 1640 (Flow Laboratories) in 10% fetal bovine serum (FBS, CELBIO), 5% CO_2 supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin[19]. Cell growth was studied by determining the cell number/ml after different days of *in vitro* cell culture [19]. The concentration of DNA binding drugs causing 50% inhibition of cell proliferation (IC_{50}) was determined by comparing the cell number/ml of cell cultures treated with DNA-binding drugs to the value of untreated control cell cultures.

Filter binding assay. The filter binding assay[20] was performed by using double stranded Ha-ras Sp1 mer (5'-GGGGGCGGGGGCGGGGCGGGG-3'). Nuclear factors from K562 cells (10 μg) were isolated and separated by electrophoresis on a 10% SDS-polyacrylamide gel as described[21]. After blotting to nitrocellulose filters (Hybond C, Amersham Pharmacia), each lane was cutted and the strips were renaturated by immersion in guanidinium hydrochloride solutions at concentrations progressively decreasing, and incubated in the presence of ^{32}P-labelled Sp1 mer. Binding reactions were performed as described elsewhere[20] in binding buffer (5% glycerol, 20 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 0.01% Triton X-100) containing 20 μg of poly (dIdC)·poly (dIdC) (Pharmacia, Uppsala, Sweden), 0.25 ng of labelled oligonucleotide, in a total volume of 1 ml. After 1 hr of incubation, the strips were separately washed in binding buffer (3 times, 10 min each) and exposed to Kodak XAR-5 film at -80°C.

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. R.G. is granted by CNR Target Project on Biotechnology, ISS (AIDS-1998), by PRIN-98 and by Progetto per la Ricerca Finalizzata 1998 (Ministero della Sanità). N.B. is the recipient of a FIRC fellowship.

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