Synthesis, in Vitro Antiproliferative Activity, and DNA-Binding Properties of Hybrid Molecules Containing Pyrrolo[2,1-*c*][1,4]benzodiazepine and Minor-Groove-Binding Oligopyrrole Carriers

Pier Giovanni Baraldi,*[,†] Gianfranco Balboni,[†] Barbara Cacciari,[†] Andrea Guiotto,[†] Stefano Manfredini,[†] Romeo Romagnoli,[†] Giampiero Spalluto,[†] David E. Thurston,[‡] Philip W. Howard,[‡] Nicoletta Bianchi,[§] Cristina Rutigliano,[§] Carlo Mischiati,[§] and Roberto Gambari[§]

Dipartimento di Scienze Farmaceutiche e Dipartimento di Biochimica e Biologia Molecolare, Università di Ferrara, 44100 Ferrara, Italy, and CRC Gene Targeted Drug Design Research Group, School of Pharmacy and Biomedical Science, University of Portsmouth, St. Michael's Building, White Swan Road, Portsmouth, Hampshire PO1 2DT, United Kingdom

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The synthesis, biological activity, and DNA-binding properties of a series of four hybrids prepared by combining polypyrrole minor groove binders and pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) **13**, related to the naturally occurring anthramycin (**3**) and DC-81 (**4**), have been described, and structure−activity relationships have been discussed. These hybrids **22**−**25** contain from one to four pyrrole units, respectively. To investigate sequence selectivity and stability of drug/DNA complexes, DNase I footprinting and arrested polymerase chain reaction (PCR) were performed on human c-myc oncogene, estrogen receptor gene, and human immunodeficiency virus type 1 long terminal repeat (HIV-1 LTR) gene sequences. The antiproliferative activity of the hybrids has been tested in vitro on human myeloid leukemia K562 and T-lymphoid Jurkat cell lines and compared to antiproliferative effects of the natural product distamycin A **1**, its tetrapyrrole homologue **17**, DC 81 (**4**), and the PBD methyl ester **12**. The results obtained demonstrate that the hybrids **22**−**25** exhibit different DNA-binding activity with respect to both distamycin A **1** and PBD **12**. In addition, a direct relationship was found between number of pyrrole rings present in the hybrids **22**−**25** and stability of drug/DNA complexes. With respect to antiproliferative effects, it was found that the increase in the length of the polypyrrole backbone leads to an increase of in vitro antiproliferative effects, i.e., the hybrid **25** containing the four pyrroles is more active than **22**, **23**, and **24** both against K562 and Jurkat cell lines.

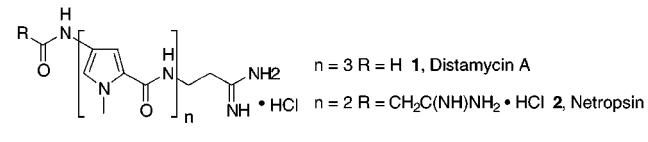
Introduction

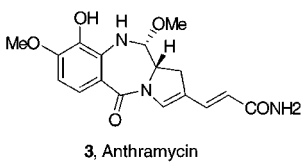
The investigation of the biological consequences of DNA modifications, due to compounds endowed with the property of interacting directly with the DNA, provides vital information on the general molecular recognition process for DNA-interactive antitumor agents.

Distamycin A **1**[1] and netropsin **2**[2] are two naturally occurring antibiotics, characterized by the presence of an oligopeptide (*N*-methylpyrrole)carbamoyl frame ending with an amidino moiety. Both compounds have driven researcher's attention not only for their biological activity but also for their nonintercalative binding to the minor groove of double-stranded B-DNA, where they form strong reversible complex preferentially at the nucleotide sequences consisting of 4−5 adjacent AT base pairs.[3] Electrostatic forces, van der Waals interactions, and hydrogen bonds are all involved in the stabilization of the complex between these compounds and DNA.

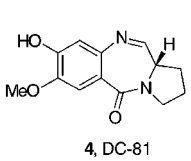
Distamycin and netropsin have been also used as DNA sequence selective vehicles for the delivery of alkylating functions to DNA targets, leading to a sharp increase of their cytotoxicity, in comparison to the very weak cytotoxicity of distamycin and netropsin themselves. In the past few years, several hybrid compounds, in which known antitumor compounds or simple active moieties of known antitumor agents have been tethered to distamycin and netropsin frames, have been designed, synthesized, and tested.[4]

The pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) group,[5] which includes the natural compounds anthramycin (**3**)[6] and DC-81 (**4**), 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]





3, Anthramycin



4, DC-81

* To whom correspondence should be addressed: Dipartimento di Scienze Farmaceutiche, Università di Ferrara, Via Fossato di Mortara 17-19, 44100 Ferrara, Italy. Tel +39-(0)532-291293; FAX +39-(0)532-291296; E-mail pgb@ifeuniv.unife.it.

† Dipartimento di Scienze Farmaceutiche, Università di Ferrara.

‡ University of Portsmouth.

§ Dipartimento di Biochimica e Biologia Molecolare, Università di Ferrara.

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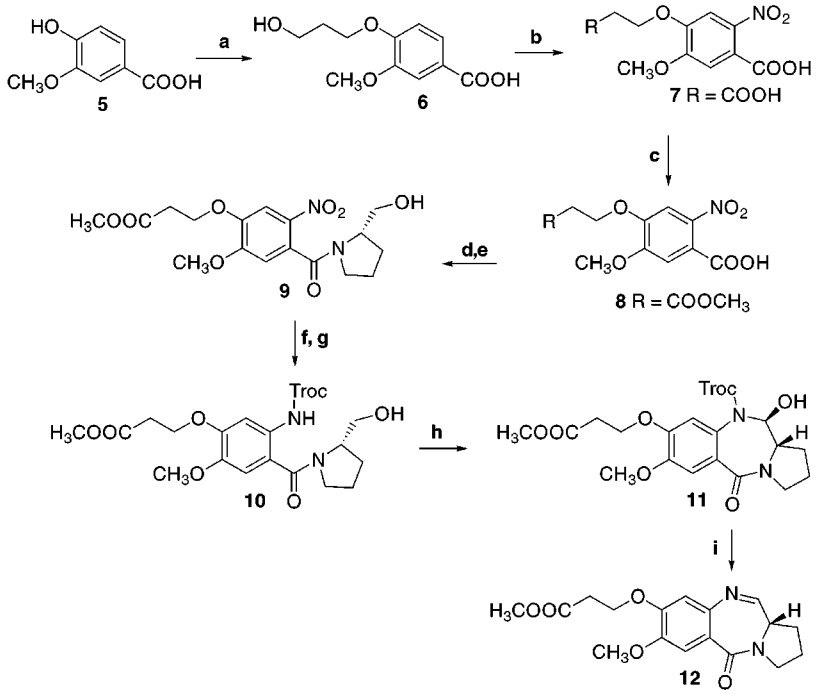
Scheme 1[a]



[a] Reagents: (a) 3-bromopropanol, aq. NaOH, reflux; (b) HNO_3 70%; (c) CH_3OH, tosyl chloride (0.1 equiv); (d) $(COCl)_2$, dry CH_2Cl_2; (e) 2(*S*)-(+)-pyrrolidinemethanol, Et_3N, dry CH_2Cl_2; (f) H_2/Pd(C), CH_3OH; (g) Troc-Cl, pyridine, dry CH_2Cl_2; (h) Swern oxidation; (i) 10% Cd/Pb couple, CH_3OH, 1 M NH_4Ac.

In this paper we report the synthesis and in vitro activity of novel hybrids **22**−**25**, consisting respectively of one, two, three, or four pyrrole amide units linked to a pyrrolo[2,1-*c*][1,4]benzodiazepine **12**, through a spacer arm, in order to study the structure−activity relationship between length of the oligopyrrolic frame, antiproliferative activity, and sequence specificity. We have described an example in which distamycin A was used as DNA vector for PBD **12**, leading to the synthesis of hybrid **24**,[8a] and recently Lown and co-workers have reported the synthesis of a new series of pyrrolo[2,1-*c*]-[1,4]benzodiazepine−lexitropsin conjugates.[8b] The rationale that led to the preparation of **24** 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) **12**, with the aim to obtain a new derivative that would be more cytotoxic than the parent compounds. In the new hybrid **24**, we conjugated the capability of PBD **12** to covalently bind to GC-rich sequences with that of distamycin's different recognition pattern.

Chemistry

The synthesis of the DC-81-related PBD **1**, prepared according to a previously reported procedure,[8] has been carried out by modification of a general strategic route for PBD synthesis starting from vanillic acid.[9] The reaction of vanillic acid **5** with 3-bromopropanol gave the compound **6**. Nitration of **6** with nitric acid and simultaneous oxidation of the alcoholic function afforded the nitro acid **7**, which by chemoselective esterification in the presence of *p*-toluenesulfonic acid yielded the nitro monoester **8** (61% yield, three steps). Coupling with (2*S*)-(+)-pyrrolidinylmethanol (via acid chloride), to give **9**, followed by catalytic hydrogenation (Pd on C) of the nitro group and protection of the newly formed amino functionality with 2,2,2-trichloroethylchloroformate (Troc-Cl),[10] achieved compound **10** (50% yield, four steps) without any proof of racemization at *pro*-C11a. Oxidation of **10** under Swern conditions gave the N10-Troc-protected PBD **11** (82%) which was converted to **12** by cleavage of the Troc group with Cd/Pb couple in aqueous NH_4Ac[11] (Scheme 1).

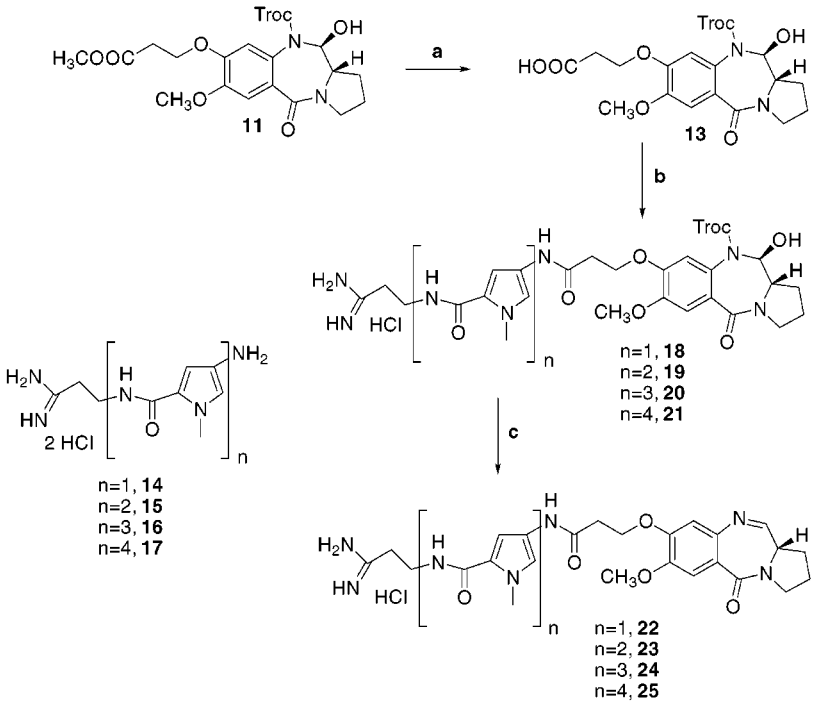
The final assembly of the desired hybrids **22**−**25** was achieved by a two step pathway and is reported in Scheme 2. Acidic hydrolysis of the ester **11** afforded the acid **13**. This was coupled with the appropriate aminopoly(pyrrolamide) moieties **14**,[12] **15**,[13] **16**,[1a] and **17**[14] in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) as condensing agent, affording in moderate yields the corresponding Troc-protected hybrid molecules **18**−**21**, respectively, which were purified by flash chromatography. The deprotection of the Troc group with the previously described methodology afforded the free N10-C11 imine moiety of the target molecules **22**−**25**, which can be easily stored as the stable chlorohydrate salt after purification by preparative HPLC and immediate lyophilization.

Results and Discussion

In Vitro Antiproliferative Effects. The effects of the four synthesized compounds **22**−**25**, pyrrolo[2,1-*c*]-[1,4]benzodiazepine (PBD) **12**, DC 81 (**4**), distamycin A **1**, and its tetrapyrrole homologue **17** on tumor cell growth were evaluated in vitro by using both the human chronic myeloid leukemia K562 and the T-lymphoblastoid Jurkat cell lines. The results obtained are summarized in Table 1.

The increase in the length of the polypyrrole backbone led to an increase of in vitro antiproliferative effects, i.e., the hybrid **25** containing the four pyrroles is more

Scheme 2[a]



[a] Reagents: (a) THF, HCl 10%; (b) 14, 15, 16, or 17 (1 equiv), EDCl, DIPEA, dry DMF; (c) 10% Cd/Pb couple, CH_3OH, 1 M NH_4Ac.

Table 1. In Vitro Biological Effects on K562 and Jurkat Cell Lines: PCR Experiments on Human Estrogen Receptor, Human c-myc Oncogene, and HIV-1 LTR

	IC_{50}[a] (μM)		polymerase chain reaction (PCR) IC_{50}[b] (μM)		
compound	Jurkat	K562	c-myc	ER	HIV-1 LTR
distamycin A (**1**)	20	12	25	5	50
DC-81	2.2	1	nd[c]	nd	nd
17	25	12	12	0.5	20
12	3	1.5	>250	>250	>250
22	80	>100	4	3	1
23	50	6	6	2	0.8
24	0.8	0.7	2.5	0.8	2
25	0.07	0.04	2	0.2	2

[a] IC_{50}, 50% inhibitory concentration represents the mean from dose-response curves of at least three experiments. [b] IC_{50}, inhibitory concentration (micromolar) necessary to obtain 50% inhibition of generation of PCR products of human c-myc, estrogen receptor (ER), and HIV-1 LTR sequences. [c] nd, not determined.

active than **24** against both K562 (IC_{50} 0.07 μM vs 0.8 μM) and Jurkat (IC_{50} 0.04 μM vs 0.7 μM) cell lines. With regard to the above-mentioned derivatives **24** and **25**, it can be observed that they are much more active than distamycin A (**1**) and **17**, containing the same number of pyrrole units, respectively. Only derivatives **24** and **25** retain a higher antiproliferative activity when compared to PBD **12** alone.

In fact, compounds **22**, containing only one *N*-methylpyrrole unit, showed negligible inhibitory activity or no activity at all on K562 and Jurkat cell proliferation, with IC_{50} values of 80 and >100 μM, respectively. On the other hand, **23**, containing two pyrrole moieties, exhibited to some extent antiproliferative activity on the Jurkat cell line (IC_{50} 6 μM), being scarcely active on K562 cells (IC_{50} 50 μM). DC-81 was found to retain antiproliferative activities similar to those exhibited by compound **12**.

Taken together, these findings are consistent with the hypothesis that a tighter DNA binding, depending on the multiplicity of interactions between the pyrrole carbamoyl units and AT-rich sequences of DNA, is crucial for antiproliferative effects of PBD−distamycin-related compounds. Accordingly, the tri- and tetrapyrrole hybrids **24** and **25** are the most potent antiproliferative compounds of this series, exhibiting probably higher binding affinities with respect to the mono- and dipyrrole conjugates **22** and **23**, due to additional amido hydrogen bonds and van der Waals interactions.

Effects on Polymerase Chain Reaction. To investigate the relationship between antiproliferative effects and DNA-binding activity, polymerase chain reaction[15] experiments were first performed. The human estrogen receptor (ER) gene,[16] the c-myc oncogene[17] and the human immunodeficiency virus type 1 long terminal repeat (HIV-1 LTR)[18a] were chosen as molecular model systems, as they are suitable for use in determining sequence selectivity of DNA-binding compounds when amplified by PCR.[18] We have recently published the nucleotide sequence of a 3.2 b genomic region located upstream of the human estrogen receptor sequence[19] originally designated exon 1, and demonstrated that this region contains AT rich sequences recognized by distamycin A and distamycin analogues.[15c,18a] The human c-myc oncogene sequences and the HIV-1 LTR, on the other hand, contain GC-rich regions and therefore have a low-efficiency interaction with distamycin, being on the other hand efficiently recognized by GC selective binders, such as chromomycin and mithramycin.[20] The (A + T)/(G + C) ratio of the estrogen receptor product is 3.46, while the (A + T)/(G + C) ratios of c-myc and HIV-1 LTR PCR products are 0.607 and 0.79, respectively.

Figure 1 shows representative examples of PCR performed with distamycin A **1**, PBD **12**, and compound **24**. Experimental conditions were employed that allow determination of differential sequence selectivity and

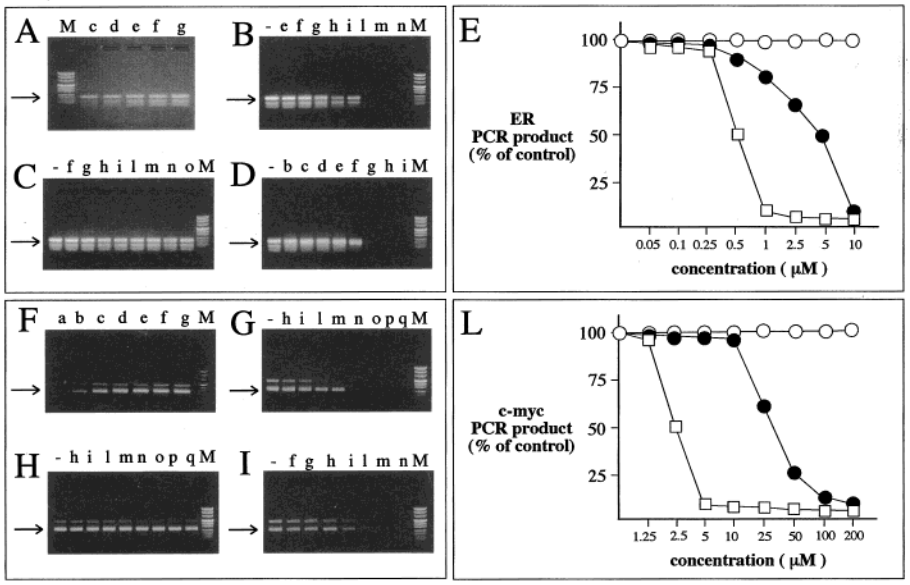


Figure 1. Panels A and F: Relationship between the number of PCR cycles and ER (A) and c-myc (F) PCR products obtained. The number of PCR cycles was 10 (a), 15 (b), 20 (c), 25 (d), 30 (e), 35 (f), or 40 (g). Panels B−D and G−I: Effects of distamycin (B, G), compound **12** (C, H), and compound **24** (D, I) on PCR-mediated amplification of estrogen receptor (ER) (B−D) and c-myc (G−I) gene sequences. Target DNAs were 20 ng of purified c-myc PCR products and 20 ng of pBLCAT8ERCAT1 plasmid, as required. Before PCR, target DNA was incubated in the absence (−) or in the presence of 0.005 (a), 0.01 (b), 0.025 (c), 0.05 (d), 0.1 (e), 0.25 (f), 0.5 (g), 1 (h), 2.5 (i), 5 (l), 10 (m), 25 (n), 50 (o), 100 (p), and 200 (q) μM DNA-binding drugsμM DNA-binding drugs. After PCR (20 cycles), 10 μL of each PCR mixture was analyzed by agarose gel electrophoresis. M = molecular weight marker (pUC mix, MBI, Fermentas, Vilnius, Lithuania). ER and c-myc PCR products are indicated by arrows. Specificity of the ER and c-myc PCR products has been assessed by Southern blotting and hybridization with ^{32}P-labeled probes[21] as well as isolation of the arrowed PCR products and direct sequencing (data not shown). Panels E and L: Densitometric quantification of the gels shown in panels B−D (E) and G−I (L). (●) Distamycin; (○) compound **12**; (□) compound **24**.

activity of DNA-binding drugs exhibiting closely related chemical structures. In these experimental conditions (20 PCR cycles), the PCR reactions are clearly in the log phase of production of the amplified sequences (Figure 1A,F). Table 1 displays all the results obtained with compounds **12**, **22**−**25** and distamycin A **1**.

In accord with an already published report,[21] distamycin A **1** inhibits ER PCR product (Figure 1B) but exhibits a lower capacity to inhibit c-myc (Figure 1G) and HIV-1 LTR (Table 1) PCR products, thus confirming a selective binding of distamycin to AT-rich gene sequences (see Table 1 for the complete set of results). By contrast, **12** does not effectively inhibit c-myc, HIV-1, and ER polymerase chain reactions (Figure 1C,H and Table 1), in agreement with previously published results showing that the kinetic of binding to DNA of this compound is very slow.[22] When compounds **22** and **23** were added to the PCR mixture, sequence-selective binding typical of distamycin was less evident (Table 1). In these experimental conditions, indeed, both **22** and **23** showed an inhibitory activity on PCR-mediated amplification of ER gene sequences which was similar to that of distamycin (Table 1). On the contrary, the inhibitory activity of **22** and **23** on the generation of c-myc PCR products was much higher than that of distamycin (Table 1). These biochemical effects are presumably due to both the presence of the PBD group and the presence of mono- and dipyrrole carriers instead of the three pyrroles, leading to an alteration of the affinity for GC and AT base pairs.

When **24** was added to PCR mixtures (Figure 1D,I; Table 1), two clear effects were observed. First, this compound inhibits the generation of c-myc and HIV-1 PCR products to an extent similar to that of compounds **22** and **23** (Table 1). Second, higher efficiency in the inhibition of the generation of ER PCR products was observed, when the activity is compared to that of compounds **22**, **23**, and distamycin. The derivative **25** inhibits c-myc and HIV-1 PCR amplification with an efficiency similar to that of compounds **22**, **23**, and **24**. On the contrary, this derivative is much more active than distamycin, **22**, **23**, and **24** in inhibiting generation of ER PCR products. These results suggest that the hybrid molecules containing PBD and mono- and oligopyrrole carriers also bind, unlike distamycin, to GC-rich DNA sequences. On the other hand, the presence of four pyrrole rings leads to a much stronger binding to the poly(A−T) stretch of ER, in accord with the proposal that an increase in the number of the pyrrole moieties could increase the acceptance for AT base pairs. Although **24** and **25** cause suppression of PCR-mediated amplification of c-myc sequences at similar concentrations (IC_{50} = 2.5 and 2 μM, respectively), the 50% inhibition of PCR-mediated amplification of ER sequences occurs, for **25**, at 0.2 μM, which is a concentration almost 4-fold lower than that exhibited by **24** (IC_{50} = 0.8 μM), as reported in Table 1. The finding that PBD-carrying distamycin analogues efficiently inhibit the generation of ER, c-myc, and HIV-1 PCR products suggests that these molecules are stable enough to produce informative results under the PCR experimental conditions used in the present paper. In addition, the finding that **24** and **25** inhibit ER PCR products at micromolar concentrations much lower than those required to inhibit PCR-mediated amplification of c-myc and HIV-1 LTR regions strongly suggest that our experimental conditions are suitable to determine differential sequence-selective recognition of the DNA-

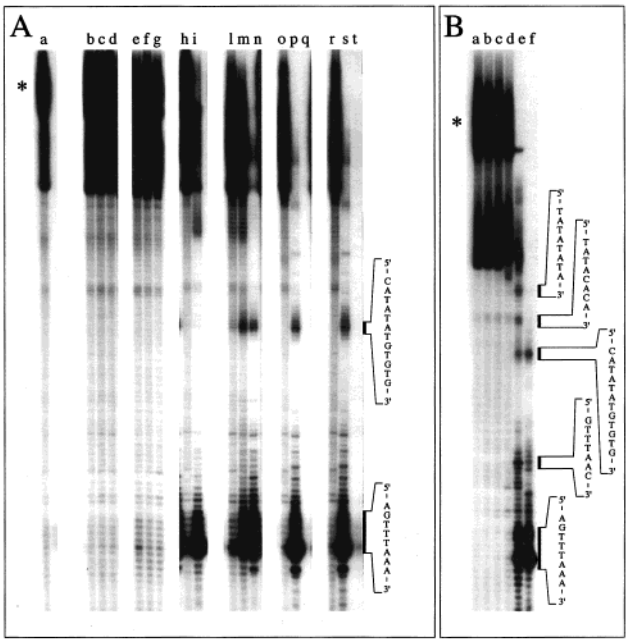


Figure 2. Analysis of the arrested sites of ER PCR performed in the absence (A, lane a) or in the presence of PBD **12** (A, lanes b−d; B, lanes a−c), distamycin A **1** (A, lanes e−g; B, lanes d−f), **22** (A, lanes h and i), **23** (A, lanes l−n), **24** (A, lanes o−q) and **25** (A, lanes r−t). Preamplified ER PCR product was incubated for 5 min in the absence (A, lane a) or in the presence of 10 (A, lanes b, e, l, o, and r), 15 (A, lanes c, f, h, m, p, and s), 20 (A, lanes d, g, i, n, q, and t), 25 (B, lanes a and d), 50 (B, lanes b and e), or 100 (B, lanes c and f) μM DNA-binding drugs. ^{32}P-Labeled ER reverse PCR primer was added, and after 20 PCR cycles the products obtained were analyzed by polyacrylamide gel electrophoresis. The asterisk indicates full-sized PCR products. The nucleotide sequences of the major sites of arrest are indicated.

binding drugs analyzed. However, it should be pointed out that the PCR-arrested experiments described in Figure 1 and Table 1 do not give information at the level of recognized nucleotide sequences; therefore, the nucleotide sequences of the arrested sites were determined by using a ^{32}P-labeled ER reverse primer and analyzing the PCR products with a sequencing gel. In addition, DNase I footprinting assays were performed with HIV-1 LTR and c-myc footprinting probes with the aim to further characterize the effects of PBD on sequence recognition of PBD−distamycin hybrid molecules.

Analysis of the Nucleotide Sequences of Sites of Arrest of ER PCR. Figure 2 shows the polyacrylamide gel electrophoretic analysis of arrested PCR performed with a ^{32}P-labeled ER reverse primer. Target DNA was a preamplified ER PCR product. In these experimental conditions it is known that distamycin causes site-specific arrest of polymerase chain reactions.[15d] It should be noted that untreated PCR products show a major band indicating full-sized products of *Taq* DNA polymerase (arrow, Figure 2). In addition, shorter products are present, presumably due to pausing of the DNA synthesis reaction occurring when the reported ER specific primers are used.[15d] This phenomenon has been also described in other experimental systems.[15e] As clearly evident, in the 10−20 μM range, no appreciable sites of arrest were detectable when PCR was performed in the presence of compounds **12** (Figure 2A, lanes b−d) or distamycin A **1** (Figure 2A, lanes e−g). Incubation with **22** leads to the generation of one major arrested site (5′-AGTTTAAA-3′; Figure 2A, lanes h and i), while addition of compounds **23** (Figure 2A, lanes l−n), **24** (Figure 2A, lanes o−q), and **25** (Figure 2A, lanes r−t) to the PCR mixture generate two major arrested sites, 5′-AGTTTAAA-3′ and 5′-CATATATGTGTG-3′.

When incubation was performed in the presence of 25−100 μM compound **12** (Figure 2B, lanes a−c) or distamycin A **1** (Figure 2B, lanes d−f), arrested sites were evident only with distamycin A **1**. In addition to the two arrest sites found with compounds **23**−**25**, distamycin A generated an arrest site at the level of nucleotide sequences 5′-TATATATA-3′, 5′-TATACACA-3′, and 5′-GTTTAAC-3′.

Taken together, these results suggest that PBD−distamycin hybrids exhibit similar patterns of sequence recognition, although some differences, after comparison to distamycin A **1**, can be observed. As expected from the results shown in Figure 1 and Table 1, addition of compound **12** does not generate any arrest site.

DNase I Footprinting Experiments. DNase I footprinting experiments were performed with HIV-1 LTR and c-myc gene sequences as footprinting probes. The HIV-1 LTR was analyzed first, as it contain nucleotide sequences that are recognized by distamycin and distamycin analogues (for instance the NF-kB and TATA box binding sites). In the first DNase I footprinting experiment we used a footprinting probe produced by PCR with a ^{32}P-labeled HIV-1-F primer. Before DNase I treatment, this ^{32}P-labeled probe was incubated for 5 min in the absence (Figure 3A, lanes indicated by −) or in the presence of distamycin A **1** (Figure 3A, lanes a−d) or compound **24** (Figure 3A, lanes e−i). As expected, distamycin A generates a footprint (5′-GGGACTTTCCGCTGG-3′) at the level of the NF-kB binding site. In addition, the results obtained suggest that the footprint generated by **24** is larger than that generated by distamycin. Moreover, a hypersensitive site detected in the presence of distamycin (Figure 3A, asterisk) was not observed in the presence of compound **24**.

Similar conclusions were obtained from the second experiment (Figure 3B), which was performed with a footprinting probe produced by PCR using a ^{32}P-labeled HIV-1-R primer. While preincubation with distamycin generates, as expected, a major footprint (5′-TGCATATAAG-3′) at the level of the TF-IID binding site (Figure 3B, lane a), preincubation with compounds **22**−**25** generated two additional footprints (5′-CAGAT-3′ and 5′-TTTGCCTGTACT-3′; Figure 3B, lanes b and c). On the contrary, PBD **12** was found to be inactive in protecting the HIV-1 LTR probe from DNase I cleavage (Figure 3B, lane f).

In agreement with the data shown in Figure 3, Figure 4 shows that, in contrast to PBD **12**, distamycin A **1** and **24** produce appreciable footprints in DNase I footprinting experiments[18] conducted with the PCR product of the c-myc promoter as footprinting probe. However, the footprints generated by compound **24** are larger than those generated by distamycin A **1**. In particular, the major footprints of distamycin were 5′-AGG-3′, 5′-GGCAGGGCTTCTCAGAGG-3′, 5′-TGGCGGGA-3′, and 5′-GAGAAG-3′, while compound **24** produced footprints at the level of the c-myc sequences 5′-AGG-3′,5′-AGGGCAGGGCTTCTCAGAGGCTTGGC-

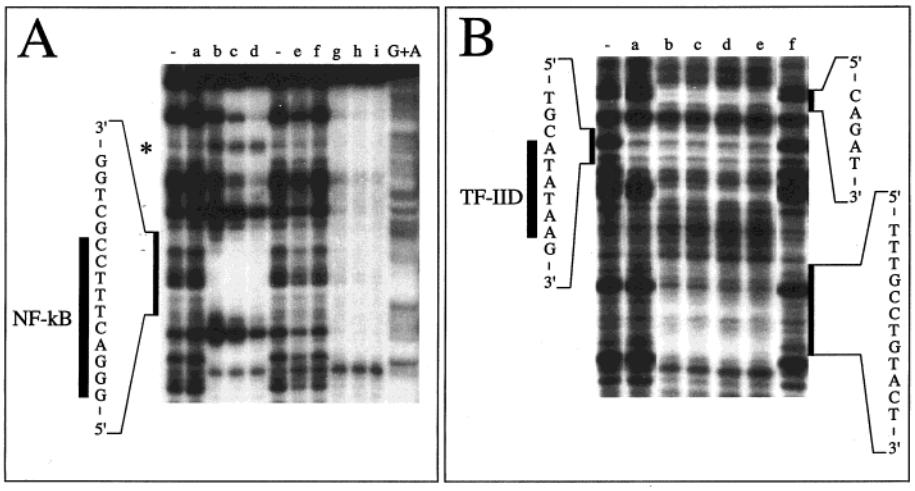


Figure 3. DNase I footprinting using HIV-1 LTR footprinting probes generated by PCR with either HIV-1-F (A) or HIV-1-R (B) ^{32}P-labeled PCR primers. ^{32}P-End-labeled HIV-1 LTR footprinting probe (150 ng) was incubated (panel A) in the absence of DNA-binding drugs (−) or in the presence of distamycin A **1** (lanes a−d) or compound **24** (lanes e−i) (a, 5 μM; b, 10 μM; c, f, 15 μM; d, g, 15 μM; e, h, 25 μM; i, 50 μM). In panel B the preincubation was performed in the absence of DNA-binding drugs (−) or in the presence of 25 μM distamycin A **1** (a), compound **22** (b), compound **23** (c), compound **24** (d), compound **25** (e), and PBD **12** (f). After 10 min of incubation, 1 units of DNase I was added for 2 min. The DNase I footprinting reactions were then stopped and layered onto a sequencing gel. The footprints generated are shown. The nucleotide sequences recognized by the DNA-binding proteins NF-kB and TF-IID are indicated.

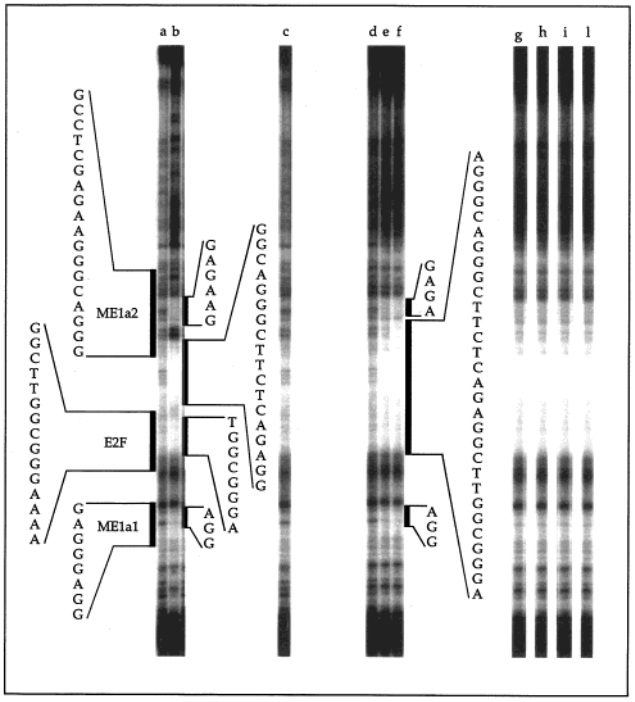


Figure 4. DNase I footprinting using the c-myc footprinting probe. 150 ng of ^{32}P-End-labeled c-myc PCR product (150 ng) was incubated in the absence of DNA-binding drugs (a) or in the presence of (b) 100 μM distamycin, (c) 100 μM compound **12**, (d) 10 μM compound **24**, (e) 20 μM compound **24**, (f−l) 50 μM compound **22** (g), **23** (h), **24** (f,i), and **25** (l). After 10 min of incubation, 1 unit of DNase I was added for 2 min. The DNase I footprinting reactions were then stopped and layered onto a sequencing gel. The footprints generated by distamycin and compound **25** are shown on the right side of lanes b and f, respectively. The nucleotide sequences recognized by the DNA-binding proteins ME1a2, E2F, and ME1a1 are indicated in the left side of the panel.

GGGA-3′, and 5′-GAGA-3′ (compare lanes b and f of Figure 4). Distamycin A **1** did not produce appreciable DNase I footprints when used at 20 μM concentrations (data not shown). When compounds **22**−**25** were compared, similar DNase I footprinting was obtained, suggesting that, as shown in Figure 3B, changes in the number of pyrrolic rings do not produce significant changes in sequence recognition (Figure 4, lanes g, h, i, and l).

Stability of DNA/Drug Complexes. The lack of correlation between antiproliferative activity of compounds **22**−**24** and the data obtained from the arrested PCR assay and the DNase I footprinting prompted us to investigate possible differences in the stability of drug/DNA complexes. This is a reasonable hypothesis, since a tighter DNA binding could depend on the increased multiplicity of interactions between the increased number of pyrrolecarboxyl units and target DNA sequences.

Therefore, to determine the stability of drug/DNA complexes, c-myc and ER PCR products were incubated in the presence of 50 μM concentrations of **22**−**25** for 10 min; after this incubation period, drug/DNA complexes were filtered 1−6 times through a Microcon-30 Amicon filter and PCR was conducted with the retentates as template DNA. This method allows us to discriminate DNA-binding drugs with respect to stability of drug/DNA complexes.[23] Representative results of this experiment are shown in Figure 5 (panels A and B) and demonstrate a differential stability of molecular complexes generated after binding of **22** and **25** to target c-myc DNA; the complete sets of the results obtained are shown in Figure 5C,D. As is clearly evident, compounds **22** and **23** are not able to inhibit PCR-mediated amplification of both c-myc and ER gene sequences after two (for compound **22**) and three (for compound **23**) washes of preformed **22**/DNA and **23**/DNA complexes. Five and six washes of **24**/c-myc DNA and **24**/ER DNA complexes, on the contrary, are necessary to allow PCR-mediated amplification of c-myc and ER gene sequences, respectively. Finally, no amplification of c-myc and ER gene sequences was obtained even after six washes of **25**/c-myc DNA and **25**/ER DNA

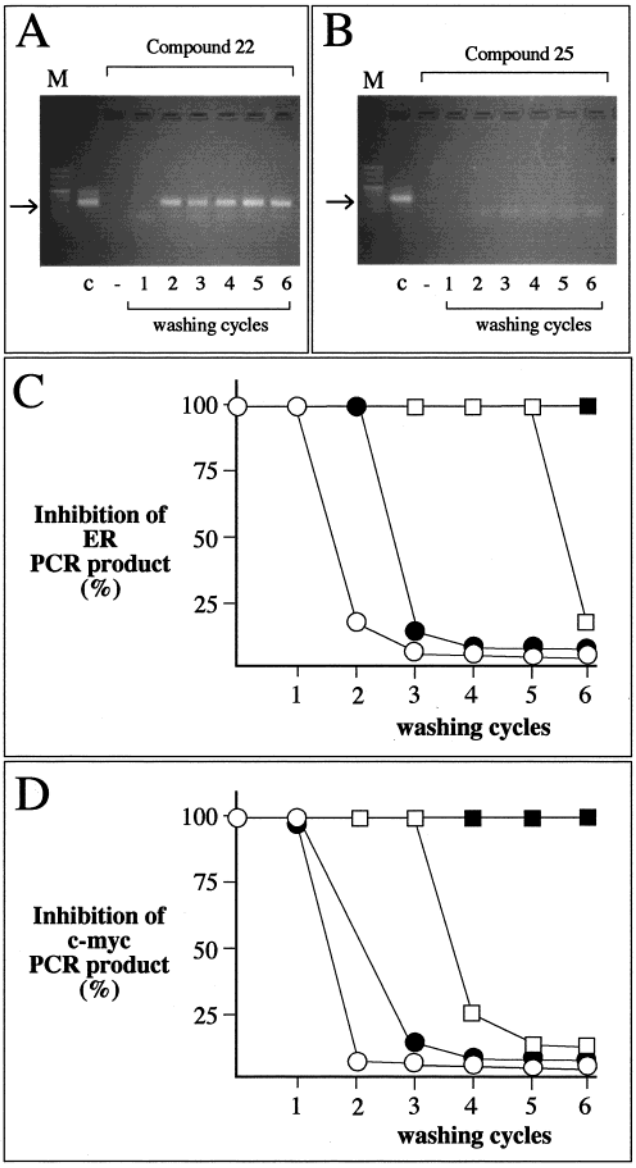


Figure 5. Stability of DNA/drug complexes. Panels A and B: c-myc PCR product (10 ng) was incubated for 10 min with 50 μM compound **22** (A) or **25** (B). Aliquots were untreated (−) or washed once (1), twice (2), or three to six (3−6) times by centrifugations through Microcon-30. The final retentate was amplified by adding c-myc PCR primers, Taq DNA polymerase, and dNTPs. After PCR, 10 μL of each PCR mixture was analyzed by agarose gel electrophoresis. M = molecular weight marker (pUC mix, MBI, Fermentas, Vilnius, Lithuania); c = control PCR reactions performed in the absence of DNA-binding drugs. Specific PCR products are indicated by arrows. Panels C and D: Inhibition of generation of ER (C) and c-myc (D) PCR products by compound **22** (■), **23** (□), **24** (●), and **25** (○). PCR was performed on the retentates obtained after the indicated washing through Microcon-30 ultrafiltration devices of c-myc target DNA preincubated for 10 min with the indicated DNA-binding drugs.

complexes. Taken together, these results give strong evidence for a direct relationship between the stability of drug/DNA complexes, the number of pyrroles of the carrier molecule, and the antiproliferative effect on tumor cell lines.

Conclusions

The data presented in this paper suggest that the higher antiproliferative activity of hybrid molecules containing pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) and minor-groove oligopyrrole carriers containing three and four pyrrole moieties is due to the recognition of more binding sites than distamycin, as well as to an increase in the stability of drug/DNA complexes. The results that we observed for our compounds encourage us to carry on these studies with the aim of developing ligands with GC base pair sequence selectivity. To do so, the synthesis of a series of imidazole-containing analogues[24] of both netropsin and distamycin conjugated to PBD analogues will be performed.

Experimental Section

Chemistry: Materials and Methods. ^{1}HNMR spectra were recorded on a Bruker AC 200 spectrometer. Chemical shifts (δ) are given in parts per million (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. Mass spectra were performed with a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) Hewlett-Packard G 2025 A instrument. Melting points (mp) were determined on a Buchi-Tottoli apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer polarimeter 241. All products reported showed ^{1}H NMR spectra in agreement with the assigned structures. Elemental analyses were conducted by the Microanalytical Laboratory of the Chemistry Department of the University of Ferrara. 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} Merck plates) and visualized with aqueous $KMnO_4$. Flash chromatography was performed with 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.

HPLC separations were conducted with a Waters Delta Prep 3000 A reversed-phase column (30 × 3 cm; 15 mm). The compounds were eluted with a gradient of 0−60% solvent B in 25 min at a flow rate of 30 mL/min; the mobile phases were solvent A [10% (v/v) acetonitrile in 0.1% TFA] and solvent B [60% (v/v) acetonitrile in 0.1% TFA]. The pure products were converted into the corresponding hydrochloride forms by addition of 0.1 N HCl aqueous solution to the mobile phase containing the pure product. Analytical HPLC analyses were performed on a Bruker liquid chromatography LC 21-C instrument using a Vydac 218 TP 5415 C18 column (250 × 4 mm, 5 mm particle size) and equipped with a Bruker LC 313 UV variable-wavelength detector. Recording and quantification were accomplished with a chromatographic data processor coupled to an Epson computer system (QX-10).

3-Methoxy-4-(3′-hydroxypropyloxy)benzoic Acid (6). To a stirred solution of NaOH (48 g, 87 mmol) in 22 mL of water was added first vanillic acid **5** (7.34 g, 43.6 mmol), followed by 3-bromopropanol (7 g, 46.8 mmol). The resulting mixture was stirred at reflux temperature for 5 h. The reaction mixture was then acidified with 10% HCl, and the precipitate obtained was filtered and recrystallized (2-butanone) to give the compound **6** as white crystals. Yield: 9.1 g (92%). Mp 162−165 °C. ^{1}H NMR (DMSO-d_6): δ 1.85 (m, 2H), 3.54 (t, J = 6 Hz, 2H), 3.79 (s, 3H), 4.07 (t, J = 6 Hz, 2H), 4.58 (br s, 1H), 7.02 (d, J = 8.4 Hz, 1H), 7.43 (d, J = 1.9 Hz, 1H), 7.54 (dd, J = 8.4 and 1.9 Hz, 1H), 12.7 (br s, 1H). IR (KBr), cm^{-1}): 3300−2800, 1677, 1278, 1228, 764, 646. Anal. ($C_{11}H_{14}O_5$): C, H.

4-[(2′-Carboxyethyl)oxy]-3-methoxy-2-nitrobenzoic Acid (7). The acid **6** (3 g, 13.27 mmol) was added in small portions to 15 mL of 70% HNO_3 cooled to 0 °C and the resulting mixture was stirred at 0 °C for 30 min, then for 1 h at room temperature. The resulting dark red syrup was poured into 100 g of crushed ice and then extracted with ethyl acetate (3 × 50 mL). The combined organic fractions were re-extracted with water (2 × 50 mL), dried with Na_2SO_4, and evaporated at reduced pressure. The resulting solid was recrystallized from ethyl acetate/ethyl ether to afford the compound **7** as yellow crystals. Yield: 2.9 g. (77%). Mp = 178−180 °C. ^{1}H NMR (DMSO-d_6): δ 2.77 (t, J = 6 Hz, 2H), 3.92 (s, 3H), 4.35 (t, J = 6 Hz, 2H), 7.23 (s, 1H), 7.53 (s, 1H). IR (KBr, cm^{-1}) 3000−2800, 1713, 1534, 1343, 1290, 1221, 1046, 868. Anal. ($C_{11}H_{11}N0_8$): C, H, N.

4-[[2′-(Methoxycarbonyl)ethyl]oxy]-3-methoxy-2-nitrobenzoic Acid (8). The nitrobenzoic acid derivative **7** (1 g, 3.5 mmol) and the *p*-toluenesulfonic acid (66 mg, 0.35 mmol) were dissolved in 20 mL of methanol. The solution was stirred at room temperature for 12 h, with monitoring by TLC (CH_2Cl_2/CH_3OH 20%) of the disappearance of the starting material. The solvent was then removed at reduced pressure, and the yellow oil thus obtained was triturated in 30 mL of water. The yellow solid that formed was filtered and recrystallized from ethyl acetate/petroleum ether to give **8** as a pale yellow solid. Yield: 0.9 g (86%). Mp 138 °C. ^{1}H NMR ($CDCl_3$): δ 2.93 (t, J = 6 Hz, 2H), 3.76 (s, 3H), 3.83 (s, 1H), 3.97 (s, 3H), 4.39 (t, J = 6 Hz, 2H), 7.22 (s, 1H), 7.45 (s, 1H). IR (KBr, cm^{-1}) 3450, 2950, 1718, 1540, 1285, 1213, 1053, 875, 767. Anal. ($C_{12}H_{13}NO_8$): C, H, N.

(2*S*)-N-[4′-[[2″-(Methoxycarbonyl)ethyl]oxy]-5′-methoxy-2′-nitrobenzoyl]-2-(hydroxymethyl)pyrrolidine (9). Oxalyl chloride (0.88 mL, 10 mmol) and few drops of DMF were added to a solution of 4-[[2′-(methoxycarbonyl)ethyl]oxy]-3-methoxy-2-nitrobenzoic acid **8** (2 g, 6.68 mmol) in 50 mL of dry CH_2Cl_2. The mixture was stirred at room temperature until complete dissolution occurred and then added dropwise over 1 h to a solution of 2*S* -(+)-pyrrolidinylmethanol (0.68 g, 6.68 mmol) and TEA (1.84 mL, 13.36 mmol) in 50 mL of CH_2Cl_2, cooled at −20 °C. The resulting reaction mixture was stirred at −20 °C for 1 further hour, and then the temperature was allowed to rise to room temperature and the solution was extracted with 1 N HCl (2 × 50 mL), water (50 mL), and brine (50 mL). The organic phase was dried with Na_2SO_4 and evaporated at reduced pressure. The crude product, after purification by flash chromatography (ethyl acetate), afforded the compound **9** as pale yellow oil. Yield: 1.42 g. (56%). ^{1}H NMR ($CDCl_3$): δ 1.7−2.3 (m, 5H), 2.92 (t, J = 6 Hz, 2H), 3.17 (dd, J = 6 and 8 Hz, 2H), 3.75 (s, 3H), 3.8−3.9 (m, 2H), 3.96 (s, 3H), 4.38 (t, J = 6 Hz, 2H), 4.58 (br s, 1H), 6.80 (s, 1H), 7.76 (s, 1H). IR (neat, cm^{-1}) 3400−3200, 2950, 1738, 1621, 1520, 1434, 1279, 1060, 875, 796. $[\alpha]^D_{25}$ = −75 ($CHCl_3$, c = 0.5). Anal. ($C_{17}H_{22}N_2O_8$): C, H, N.

(2*S*)-2-Hydroxymethyl-*N*-[4′-[[2″-(methoxycarbonyl)-ethyl]oxy]-5′-methoxy-2′-[[[trichloroethyl)oxy]carbonyl]-amino]benzoyl]pyrrolidine (10). In a solution of **9** (1.42 g, 3.7 mmol) in 50 mL of methanol was suspended 0.15 g of 10% Pd/C, and the resulting mixture was agitated in a Parr apparatus (60 psi). At the end of the reaction (12 h), the mixture was filtered over Celite and the solvent was removed at reduced pressure.The residue was dissolved, together with 0.6 mL of pyridine, in 40 mL of dry CH_2Cl_2. The solution was cooled at 0 °C and 2′,2′,2′-trichloroethyl chloroformate (Troc-Cl) (0.56 mL, 4.07 mmol), dissolved in 10 mL of dry CH_2Cl_2, was slowly added. When the addition ended the reaction mixture was stirred at 0 °C for 2 h, then heated to room temperature and extracted with 10% HCl (2 × 50 mL), saturated aqueous $NaHCO_3$ (2 × 50 mL), and brine (50 mL). The organic phase was dried with Na_2SO_4 and evaporated to yield a brown oil, which after purification by flash chromatography afforded **10** as a colorless oil. Yield: 1.6 g (82%). ^{1}H NMR ($CDCl_3$): δ 1.73−1.9 (m, 5H), 2.17 (m, 1H), 2.89 (t, J = 6 Hz, 2H), 3.53 (m, 2H), 3.73 (s, 3H), 3.82 (s, 3H), 3.86 (m, 1H), 4.36 (t, J = 6 Hz, 2H), 4.45 (br s, 1H), 4.81 (d, J = 12 Hz, 1H), 4.84 (d, J = 12 Hz, 1H), 6.86 (s, 1H), 7.73 (s, 1H), 9.01 (s, 1H). IR (neat, cm^{-1}) 3400−3200, 2950, 1731, 1600, 1520, 1270, 1121, 1024, 817, 721. $[\alpha]^D_{25}$ = −68.6 ($CHCl_3$, c = 0.88). Anal. ($C_{20}H_{24}Cl_3N_2O_8$): C, H, Cl, N.

(11*S*,11a*S*)-8-[[2′-(Methoxycarbonyl)ethyl]oxy]-11-hydroxy-7-methoxy-10-*N*-[[(trichloroethyl)oxy]carbonyl]-1,2,3,10,11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (11). To a cooled solution (−40 °C) of oxalyl chloride (0.37 mL, 4.25 mmol, 1.4 equiv) in 10 mL of dry CH_2Cl_2 was added a solution of DMSO (0.73 mL, 2.8 equiv) in 10 mL of dry CH_2Cl_2 dropwise over 30 min, with the temperature kept below −20 °C. The mixture was stirred for a further 15 min, and then the alcohol **10** (1.6 g, 3 mmol) dissolved in 25 mL of dry CH_2Cl_2 was added dropwise over 1 h. At the end of addition the mixture was stirred at −45 °C for 60 min, then a solution of TEA (1.67 mL, 4 eq.) in 15 mL of dry CH_2Cl_2 was added dropwise and the mixture was allowed to warm to room temperature.The reaction mixture was diluted with water (50 mL) and extracted with 1 N HCl (2 × 25 mL), saturated aqueous $NaHCO_3$ (2 × 25 mL), and then brine (50 mL). The organic solution was dried over Na_2SO_4 and evaporated under vacuum. The residue was purified by flash chromatography (EtOAc/petroleum ether = 1/1) to give **11** as a white foam. Yield: 1.29 g, 82%. ^{1}H NMR ($CDCl_3$): δ 2.01−2.12 (m, 5H), 2.89 (t, J = 6 Hz, 2H), 3.57 (m, 2H), 3.73 (s, 3H), 3.90 (s, 3H), 4.12 (br s, 1H), 4.24 (d, J = 12 Hz, 1H), 4.32 (t, J = 6 Hz, 2H), 5.23 (d, J = 12 Hz, 1H), 5.65 (d, J = 9.8 Hz, 1H), 6.84 (s, 1H), 7.25 (s, 1H). IR (neat, cm^{-1}) 3400−3200, 2950, 1731, 1604, 1516, 1435, 1276, 1060. FAB-MS (MALDI-TOF): 527 [M + 2]$^+$. $[\alpha]^D_{25}$ = +84.8 ($CHCl_3$, c = 1.26). Anal. ($C_{20}H_{29}Cl_3N_2O_8$): C, H, Cl, N.

(11a*S*)-8-[[2′-(Methoxycarbonyl)ethyl]oxy]-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (12). Two milliliters of a 1 M solution of NH_4Ac was added to a solution of **11** (0.1 g, 0.19 mmol) in THF (2 mL). The mixture was vigorously stirred while addition of Cd/Pb couple (0.13 g, 5 equiv) occurred. The slurry was stirred for further 2 h at room temperature and monitored by TLC (ethyl acetate). Upon the disappearance of the starting material the mixture was dropped in 50 mL of ethyl acetate, dried with Na_2SO_4, filtered, and evaporated at reduced pressure. The crude product was purified by flash chromatography (ethyl acetate) to afford **12** as a pale yellow oil. Yield: 40 mg (64%). ^{1}H NMR ($CDCl_3$): δ 1.8−2.4 (m, 4H), 2.91 (t, J = 6.6 Hz, 2H), 3.73 (s, 3H), 3.62 (m, 3H), 3.92 (s, 3H), 4.35 (t, J = 6.6 Hz, 2H), 6.86 (s, 1H), 7.5 (s, 1H), 7.7 (d, J = 5 Hz, 1H). IR (neat, cm^{-1}) 3326, 2959, 1739, 1602, 1506, 1456, 1260, 1022, 792. FAB-MS (MALDI-TOF): 333 [M + 1]$^+$. $[\alpha]^D_{25}$ = +117.6 (CH_3OH, c = 0.33). Anal. ($C_{18}H_{22}N_2O_5$): C, H, N.

(11*S*,11a*S*)-8-[(2′-Carboxyethyl)oxy]-11-hydroxy-7-methoxy-10-*N*-[[(trichloroethyl)oxy]carbonyl]-1,2,3,10,11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (13). Four milliliters of 10% HCl was added to a solution of **12** (0.2 g, 0.38 mmol) in 10 mL of THF. The resulting mixture was stirred at room temperature for 48 h and then was diluted with 20 mL of ethyl acetate and extracted with water (2 × 20 mL). The organic layer was dried with Na_2SO_4 and evaporated at reduced pressure. The product **13** was an oil, used without further purification for the next reaction. Yield: 0.16 g (84%). ^{1}H NMR ($CDCl_3$): δ 2.05−2.17 (m, 5H), 2.90 (t, J = 6 Hz, 2H), 3.53 (m, 2H), 3.62 (br s, 1H), 3.69 (s, 3H), 4.24 (d, J = 12 Hz, 1H), 4.29 (m, 3H), 5.21 (d, J = 12 Hz, 1H), 5.64 (d, J = 9.8 Hz, 1H), 6.85 (s, 1H), 7.27 (s, 1H). IR (neat, cm^{-1}) 3420, 1718, 1603, 1437, 1277, 1034, 772, 712. FAB-MS (MALDO-TOF): 513 [M + 2]$^+$. $[\alpha]^D_{25}$ = +74.1 ($CHCl_3$, c = 0.97). Anal. ($C_{19}H_{21}Cl_3N_2O_8$): C, H, Cl, N.

General Procedure for the Coupling of (11*S*,11a*S*)-8-[(2′-carboxyethyl)oxy]-11-hydroxy-7-methoxy-10-*N*-[[(trichloroethyl)oxy]carbonyl]-1,2,3,10,11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (13) with Pyrrole Oligomers (14−17). To a stirred 0.2 M solution of the opportune pyrrole oligomer **14**−**17** in anhydrous DMF under argon atmosphere was added the Hunig's base (1 equiv) at 0 °C. After 5 min, **13** (1.1 equiv) was added, followed by EDCI

(2 equiv). 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 a 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 recrystallized (CH_3OH/diethyl ether) to give **18**−**21**.

3-[1-Methyl-4-[[2′-[[(11*S*,11a*S*)-11-hydroxy-7-methoxy-10-*N*-[[(trichloroethyl)oxy]carbonyl]-1,2,3,10,11,11a-hexahydro-5*H*-5-oxopyrrolo[2,1-*c*][1,4]benzodiazepin-8-yl]oxy]ethyl]carboxamido]pyrrole-2-carboxamido]propionamidine Hydrochloride (18). Yellow solid, mp 51−53 °C. ^{1}H NMR (DMSO-d_6): δ 1.8−2.0 (m, 5H), 2.7 (t, 2H), 3.0−3.5 (m, 9H), 3.76 (s, 3H), 3.78 (s, 3H), 4.24 (br s, 2H), 4.61 (d, J = 12 Hz, 1H); 5.09 (d, J = 12 Hz, 1H); 5.49 (d, J = 9.4 Hz, 1H), 6.79 (d, J = 1.4 Hz, 1H), 6.85 (s, 1H), 7.07 (s, 1H), 7.151 (d, J = 1.4 Hz, 1H); 8.32 (t, 1H), 8.76 (s, 1H), 9.08 (s, 1H), 10.14 (s, 1H). FAB-MS (MALDI-TOF): 704 $[M + 1]^+$. $[\alpha]^D_{25}$ = +29.8 (MeOH, c = 1.24). Anal. ($C_{28}H_{35}Cl_4N_7O_8$): C, H, Cl, N.

3-[1-Methyl-4-[1-methyl-4-[[(2′-[[(11*S*,11a*S*)-11-hydroxy-7-methoxy-10-*N*-[[(trichloroethyl)oxy]carbonyl]-1,2,3,-10,11,11a-hexahydro-5H-pyrrolo[2,1-*c*][1,4]benzodiazepin-8-yl]oxy]ethyl]carboxamido]pyrrole-2-carboxamido]-pyrrole-2-carboxamido]propionamidine Hydrochloride (19). Gray solid, mp 165−167 °C. ^{1}H NMR (DMSO-d_6): δ 1.9 (m, 5H), 2.61 (t, 2H), 2.78 (t, 2H), 3.2 (m, 2H), 3.4 (m, 3H), 3.77 (s, 3H), 3.8 (s, 3H), 3.83 (s, 3H), 4.27 (m, 2H), 4.61 (d, J = 12 Hz, 1H), 5.1 (d, J = 12 Hz, 1H), 5.5 (m, 1H), 6.86 (s, 1H), 6.92 (s, 2H), 7.07 (s, 1H), 7.19 (s, 2H), 8.3 (t, 1H), 8.68 (s, 2H), 9.02 (s, 2H), 9.94 (s, 1H), 10.1 (s, 1H). FAB-MS (MALDI-TOF): 826 $[M + 2]^+$. $[\alpha]^D_{25}$ = +25.7 (MeOH, c = 0.75). Anal. ($C_{34}H_{41}Cl_4N_9O_9$): C, H, Cl, N.

3-[1-Methyl-4-[1-methyl-4-[1-methyl-4-[[2′-[[(11*S*,11a*S*)-11-hydroxy-7-methoxy-10-*N*-[[(trichloroethyl)oxy]carbonyl]-1,2,3,10,11,11a-hexahydro-5*H*-5-oxopyrrolo[2,1-*c*][1,4]benzodiazepin-8-yl]oxy]ethyl]carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine Hydrochloride (20). Gray solid, mp 230−232 °C (dec). ^{1}H NMR ($CDCl_3$): δ 1.8−2.0 (m, 5H), 2.62 (t, J = 6 Hz, 2H), 2.78 (m, 2H), 3.49 (m, 5H), 3.77 (s, 3H), 3.80 (s, 3H), 3.83 (s, 6H), 4.26 (m, 2H), 4.61 (d, J = 12 Hz, 1H); 5.09 (d, J = 12 Hz, 1H), 5.49 (dd, J = 5 and 7 Hz, 1H), 6.80−7.24 (m, 8H), 8.25 (t, J = 4 Hz, 1H), 8.69 (s, 2H), 9.01 (s, 2H), 9.95 (m, 2H), 10.12 (s, 1H). FAB-MS (MALDI-TOF): 949 $[M + 2]^+$. $[\alpha]^D_{25}$ = +74.1 ($CHCl_3$, c = 0.97). Anal. ($C_{40}H_{47}Cl_4N_{11}O_{10}$): C, H, Cl, N.

3-[1-Methyl-4-[1-methyl-4-[1-methyl-4-[1-methyl-4-[[2′-[[(11*S*,11a*S*)-11-hydroxy-7-methoxy-10-*N*-[[(trichloroethyl)oxy]carbonyl]-1,2,3,10,11,11a-hexahydro-5*H*-5-oxopyrrolo[2,1-*c*][1,4]benzodiazepin-8-yl]oxy]ethyl]carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]-pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine Hydrochloride (21). Brown solid, mp >300 °C. ^{1}H NMR (DMSO-d_6): δ 1.8−2.0 (m, 9H), 2.62 (t, 2H), 3.0−3.3 (m, 6H), 3.47 (m, 2H), 3.74−3.84 (m, 15H), 4.23 (m, 2H), 4.61 (d, J = 12 Hz, 1H), 5.1 (d, J = 12 Hz, 1H), 5.49 (dd, J = 5 and 7 Hz, 1H), 6.84−7.25 (m, 10H), 8.63 (br s, 1H), 8.99 (s, 1H), 9.96 (s, 1H), 10.11 (s, 1H). $[\alpha]^D_{25}$ = +16.4 (MeOH, c = 0.67). Anal. ($C_{46}H_{53}Cl_4N_{13}O_{11}$): C, H, Cl, N.

General Procedure for the Deprotection of Troc from the Hybrids (18−21): Preparation of Activated Hybrids (22−24). Troc-protected hybrids (**18**−**21**) (0.081 mmol) were dissolved into a mixture of THF (1 mL) and 1 M NH_4Ac (1 mL). Ten percent Cd/Pb couple (55 mg, 0.45 mmol of Cd) was added portionwise, and the mixture was vigorously stirred until no starting material was detected by TLC ($CHCl_3$/CH_3OH/HCl, 7/3/0.1). The reaction mixture was filtered over Celite, and the residue was washed with ethyl acetate aliquots. The organic solution was evaporated and the crude product was purified by semipreparative HPLC to give **22**−**25**, respectively.

1-Methyl-4-[[2′-[[(11*S*,11a*S*)-11-hydroxy-7-methoxy-11,-2,3,11a-tetrahydro-5*H*-5-oxopyrrolo[2,1-*c*][1,4]benzodiazepin-8-yl]oxy]ethyl]carboxamido]pyrrole-2-carboxamido]propionamidine Hydrochloride (22). White solid, mp 227−230 °C. ^{1}H NMR (DMSO-d_6): δ 1.7 (m, 2H), 2.6 (m, 2H), 2.8 (m, 2H), 3.4−3.8 (m, 8H), 3.9 (s, 3H), 3.92 (s, 3H), 4.7 (s, 1H), 6.7 (s, 1H), 6.8 (s, 1H), 7.07 (s, 1H), 7.22 (s, 1H), 8.3 (br s, 1H), 8.7 (s, 1H), 8.77 (br s, 2H), 9.07 (br s, 2H), 9.5 (s, 1H). $[\alpha]^D_{25}$ = +6.31 (MeOH, c = 0.52). T_R (retention time) = 8.14 min. Anal. ($C_{25}H_{32}ClN_7O_5$): C, H, Cl, N.

3-[1-Methyl-4-[1-methyl-4-[[2′-[[(11*S*,11a*S*)-11-hydroxy-7-methoxy-1,2,3,11a-tetrahydro-5*H*-5-oxopyrrolo[2,1-*c*]-[1,4]benzodiazepin-8-yl]oxy]ethyl]carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine Hydrochloride (23). Yellow solid, mp 265−266 °C. ^{1}H NMR (DMSO-d_6): δ 1.9 (m, 5H), 2.61 (t, 2H), 2.78 (t, 2H), 3.2 (m, 2H), 3.4 (m, 3H), 3.77 (s, 3H), 3.8 (s, 3H), 3.83 (s, 3H), 4.27 (m, 2H), 6.86 (s, 1H), 6.92 (s, 2H); 7.07 (s, 1H), 7.19 (s, 2H), 8.3 (t, 1H), 8.68 (s, 2H), 9.02 (s, 2H), 9.94 (s, 1H), 10.1 (s, 1H). $[\alpha]^D_{25}$ = +30.8 (CH_3OH, c = 0.5). MS (MALDI-TOF): 633 $[M + 1]^+$. T_R (retention time) = 10.35 min. Anal. ($C_{31}H_{38}ClN_9O_6$): C, H, Cl, N.

3-[1-Methyl-4-[1-methyl-4-[1-methyl-4-[[2′-[[(11*S*,11a*S*)-11-hydroxy-7-methoxy-1,2,3,11a-tetrahydro-5*H*-5-oxopyrrolo[2,1-*c*][1,4]benzodiazepin-8-yl]oxy]ethyl]carboxamide]pyrrole-2-carboxamido]pyrrole-2-carboxamido]-pyrrole-2-carboxamido]propionamidine Hydrochloride (24). White solid, mp 263−264 °C (dec). ^{1}H NMR (DMSO-d_6): δ 1.9 (m, 4H), 2.6 (t, J = 6.4 Hz, 2H), 2.78 (m, 2H), 3.4 (m, 4H), 3.73 (s, 3H), 3.77 (s, 3H), 3.80 (s, 3H), 3.84 (s, 3H), 4.3 (m, 2H), 5.7 (s, 1H); 6.9−7.3 (m, 8H), 8.2 (t, J = 7.3 Hz, 2H), 8.68 (s, 2H), 9.01 (s, 2H),9.94 (s, 1H), 9.95 (s, 1H), 10.1 (s, 1H). $[\alpha]^D_{25}$ = −11.2 (MeOH, c = 0.16). MS (MALDI-TOF): 756 $[M + 2]^+$. T_R (retention time) = 10.94 min. Anal. ($C_{37}H_{44}ClN_{11}O_7$): C, H, Cl, N.

3-[1-Methyl-4-[1-methyl-4-[1-methyl-4-[1-methyl-4-[[2′-[[(11*S*,11a*S*)-11-hydroxy-7-methoxy-1,2,3,11a-tetrahydro-5*H*-5-oxopyrrolo[2,1-*c*][1,4]benzodiazepin-8-yl]oxy]ethyl]carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine Hydrochloride (25). White solid, mp 228−230 °C (dec). ^{1}H NMR (DMSO-d_6): δ 1.8−2.0 (m, 7H), 2.63 (t, 2H), 3.0−3.3 (m, 6H), 3.48 (m, 2H), 3.72−3.85 (m, 15H), 4.24 (m, 2H), 6.85−7.25 (m, 8H), 8.23 (t, J = 7.3 Hz, 1H), 8.46 (br s, 2H), 8.64 (br s, 2H), 8.98 (s, 1H), 9.94 (s, 1H), 10.1 (s, 1H). $[\alpha]^D_{25}$ = −79 (MeOH, c = 1.2). MS (MALDI-TOF): 878 $[M + 2]^+$. T_R (retention time) = 13.97 min. Anal. ($C_{43}H_{50}ClN_{13}O_8$): C, H, Cl, N.

Inhibition of in Vitro Tumor Cell Growth. The human chronic myeloid leukemia K562 and the human lymphoblastoid Jurkat cell lines were grown in vitro as a stationary suspension culture in RPMI 1640 medium (Gibco−BRL) supplemented with 10% fetal calf serum (FCS) (Gibco−BRL), 2 mM L-glutamine (Gibco−BRL), 100 units/mL penicillin, and 100 mg/mL streptomycin. To determine survival after drug exposure, exponentially growing K562 and Jurkat cells were cultured in the presence of various concentrations of the drugs and the antiproliferative activity of the drugs was evaluated after 4 days by counting surviving cells in a model ZBI Coulter counter (Coulter Electronics, Hialeah, FL). Results were expressed as IC_{50} (dose causing 50% inhibition of cell growth in treated cultures relative to untreated controls). All experiments were repeated at least twice. For each drug concentration, duplicate cell cultures were used. Vehicle or solvent controls were run with each experiment. All the compounds tested were dissolved in DMSO at 1 mg/mL immediately before the use and diluted just before addition to the cell culture.

Target DNA, Oligonucleotide Primers, and Polymerase Chain Reaction. The sequences of the primers used for polymerase chain reaction[15a] were the following: ER forward, 5′-GACGCATGATATACTTCACC-3′; ER reverse, 5′-GCAGAATCAAATATCCAGATG-3′; c-myc forward, 5′-CGTGGGGAAAGAAAAAAGTCC-3′; c-myc reverse, 5′-TGCCTCTCGCTGGAATTACTACAG-3′; HIV-1-F (forward), 5′-ATTTCATCA-

CATGGCCCGAG-3′; HIV-1-R (reverse), 5′-GCAAGCTTTAT-TGAGGCT-3′. The nucleotide sequence of a 3.2 kb genomic region located upstream of the human estrogen receptor (ER) gene sequence originally designated exon 1 was investigated and described in our laboratory.[16,19] This region contains nucleotide sequences target of distamycin A and distamycin analogues.[15b] The primers for PCR amplification of the human c-myc oncogene are able to amplify the promoter region spanning the 554−826 nucleotides stretch.[17] The primers for PCR amplification of HIV-1 LTR are able to amplify a sequence containing both GC-rich (the Sp1 binding sites) and AT-rich (TF-IID binding sites) sequences. *Taq* DNA polymerase was purchased from Dynazyme and added at 2.5 units/25 μM final concentration. When Dynazyme *Taq* DNA polymerase was used, distamycin A was found to inhibit ER amplification when present at 2−10 μM final concentrations, depending upon the type of target DNA (genomic DNA, recombinant plasmid, or PCR product). For PCR-mediated amplification, the target DNA was 20 ng of preamplified and purified c-myc and HIV-1 LTR PCR products and 20 ng of pBLCAT8ERCAT1.[16] PCR buffer, *Taq* DNA polymerase, and the four dNTPs were added as elsewhere described.[15c] Conditions of PCR were denaturation, 92 °C, 1 min; annealing, 55 °C (ER) and 62 °C (c-myc and HIV-1), 1 min; elongation, 72 °C, 1 min. The effects of DNA-binding drugs were analyzed after incubating target DNA at room temperature, for 5 min, with increasing amounts of the compounds, as reported in the text, followed by polymerase chain reaction. Amplified DNA was analyzed by electrophoresis on 2% agarose gels. For sequence analysis of arrested PCR, we first prepared ER target DNA by PCR. After incubation for 5 min at 37 °C with the DNA-binding drugs, sample was heated at 90 °C for 5 min and PCR was performed with a ^{32}P-labeled ER reverse PCR primer. After PCR, each reaction was resuspended in 5 μL of loading dye (0.1% xylene cyanol, 0.1% bromophenol blue, and 0.1 M NaOH/formamide 1/2) and electrophoresed through a sequencing gel.

DNase I Footprinting. The c-myc reverse or HIV-1-F or HIV-1-R primers (150 ng) were 5′-end-labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP, purified by Sephadex G50 chromatography, precipitated, resuspended, and used together with the required primers to produce PCR-generated DNA fragments of the c-myc promoter or HIV-1 LTR. The ^{32}P-labeled PCR fragments were resuspended to have 100 000 cpm/footprinting reaction. The footprinting reaction was carried out in 50 μL containing 5′-end-labeled DNA, 5% glycerol, 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and 0.01% Triton X-100. Fifty microliters of 10 mM $MgCl_2$, and 5 mM $CaCl_2$ was added 1 min before the addition of DNase I (Promega Corp., Madison, WI) at 1 unit/μL stock solution, stored in aliquots at −20 °C, and diluted in 10 mM Tris-HCl pH 8 to working concentrations immediately before use. DNA was incubated with the DNA-binding drugs for 5 min at room temperature before the reaction with DNase I.[18b] The footprinting reactions were blocked at room temperature by adding 95 μL of 200 mM NaCl, 30 mM EDTA, 1% sodium dodecyl sulfate, and 100 μg/mL yeast RNA. Reactions were phenol-extracted and precipitated by adding 2.5 volumes of ethanol. The pellets were washed in 70% ethanol, air-dried, resuspended in 5 μL of loading dye (96% formamide and 6 mg/mL bromophenol blue), denatured for 2 min at 90 °C, ice-cooled for 1 min, and layered onto a 6% polyacrylamide (acrylamide/bisacrylamide ratio 19:1), 7 M urea sequencing gel. Control footprinting experiments were performed in the absence of DNA-binding drugs. Molecular weight markers were obtained by G + A sequencing reactions of the footprinting probes.[18b]

Stability of Drug/DNA Complexes. The stability of drug/DNA complexes was determined by an ultrafiltration/PCR-based method.[23] Briefly, 50 ng of PCR products containing either the c-myc or the ER gene regions was incubated in a 500 μL reaction mixture in the presence of 50 μM DNA-binding drugs in 1× PCR buffer. Control reactions were prepared containing only target DNAs and buffer. Aliquots (50 μL) of each reaction were then pipetted into sample reservoirs of Microcon-30 (Amicon) microconcentrators and the assemblies were centrifuged at 5000 rpm for 5 min in a Beckman microfuge. This procedure was performed once (1×) or repeated once (2×), twice (3×), or three to five times (4×, 5× and 6×) in order to wash for increasing lengths of time the generated drug/DNA complexes. After the desired washing steps and after removal of each assembly from the centrifuge, the retentate was amplified by PCR, by adding the c-myc or the ER forward and reverse PCR primers, as required, and dNTPs and *Taq* DNA polymerase (Dynazyme). Amplified DNA was analyzed by electrophoresis on 2% agarose gels.

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Supporting Information Available: ^{1}H NMR and IR spectral data for compounds **6**−**13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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