Design, Synthesis, DNA Binding, and Biological Evaluation of Water-Soluble Hybrid Molecules Containing Two Pyrazole Analogues of the Alkylating Cyclopropylpyrroloindole (CPI) Subunit of the Antitumor Agent CC-1065 and Polypyrrole Minor Groove Binders

Pier Giovanni Baraldi,*,† Gianfrano Balboni,† Maria Giovanna Pavani,† Giampiero Spalluto,† Mojgan Aghazadeh Tabrizi,† Erik De Clercq,‡ Jan Balzarini,‡ Toshikazu Bando,§ Hiroshi Sugiyama,§ and Romeo Romagnoli†

Dipartimento di Scienze Farmaceutiche, Università di Ferrara, 44100 Ferrara, Italy, Rega Institute for Medical Research, Laboratory of Virologyand Chemotherapy, Minderbroedersstraat 10, B-3000 Leuven, Belgium, and Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Surugadai, Kanda, Chiyoda, Tokyo 101-0062, Japan

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We have synthesized and evaluated a series of hybrids, denoted **22**−**27**, for in vitro cytotoxic activity against a variety of cancer cell lines. These hybrids represent a molecular combination of polypyrrole minor groove binders structurally related to the natural antitumor agent distamycin A and two pyrazole analogues of the left-hand segment called cyclopropylpyrroloindole (CPI) of the potent antitumor antibiotic (+)-CC-1065. These novel water-soluble hybrids have been designed to enhance the minor groove binding ability of alkylating units **20** and **21**, which should increase their clinical appeal by overcoming the administration problems of (+)-CC-1065 derivatives. The DNA alkylating and cytotoxic activities against several tumor cell lines are reported and discussed in terms of their structural differences in relation to both the number of *N*-methyl pyrrole rings and the type of the alkylating unit tethered to the oligopeptidic frame. It may be noted that, in general, and especially for **22**−**24**, the cytotoxicity of the hybrids was much greater than that of the alkylating units alone. In only one case, compound **27**, did the hybrid have cytotoxic activity comparable to that of the alkylating unit alone against FM3A/0 cells. The broadest spectrum of activity and greatest potency was shown by the hybrid **24**, in which the alkylating unit **20** and the deformyl distamycin A are tethered by 1-methyl 2,5-dicarbonyl pyrazole, with IC_{50} values for the different tumor cell lines ranging from 7 to 71 nM. For compounds **22**−**24**, the increase of the length of the pseudopeptidic moiety from one to three *N*-methylpyrrole residues led to an increased cytotoxicity. Among the hybrids tested for their inhibitory effects on the proliferation of murine L1210 leukemia cell line, compound **24** proved to be the most active (IC_{50} = 7.4 nM), and in the sequencing gel experiments, it showed the strongest and most highly sequence-specific DNA alkylation activity. For compounds **22**−**24**, the sequence specificity of DNA alkylation appears to be affected by the modification of the number of pyrrole rings, and the correlation between cytotoxicity and alkylation pattern suggests that **24** exerts its cytotoxicity through DNA sequence-specific alkylation of the third adenine located in the sequence 5′-ACA*A*AAATCG-3′. The two other hybrids **22** and **23** were slightly less active for tumor cell proliferation, with IC_{50} values of 58 and 19 nM, respectively. With only one exception, none of the compounds was endowed with antiviral activity at subtoxic concentrations. Compound **24** inhibited the effect of vaccinia virus at a concentration that was significantly lower than its minimum cytotoxic concentration for the E_6SM host cells. These compounds gave distinct patterns of alkylation in AT-rich sequences, indicating that minor structural changes produced marked alterations in sequence selectivity.

Introduction

Many natural and synthetic anticancer agents with the ability to interact with DNA have been discovered, but most have little sequence specificity and often exhibit severe toxicity to normal tissues. For these reasons, there has been considerable interest in molecular biology and human medicine to find small molecules able to alkylate the DNA in a sequence-specific manner and modify the function of nucleic acids irreversibly. Distamycin A is a naturally occurring antibiotic characterized by the presence of *N*-methylpyrrole-2-carboxamide units ending with an amidino moiety,[1,2] which binds to the DNA minor groove, preferentially to AT-rich sequence, and in a reversible manner.[3−5] Distamycin A (**1**) can be used as a DNA sequence-selective vector for both alkylating and nonalkylating agents leading to a substantial increase of cytotoxicity against cancer cell lines in comparison to that of distamycin alone.[6−8]

(+)-CC-1065 (**2**) is a member of the class of cyclopropylindole antitumor antibiotics first isolated from *Strep-*

* Corresponding author. Tel: +39-(0)532-291293. FAX: +39-(0)-532-291296. E-mail: pgb@ifeuniv.unife.it.
† Università di Ferrara.
‡ Rega Institute for Medical Research.
§ Tokyo Medical and Dental University.

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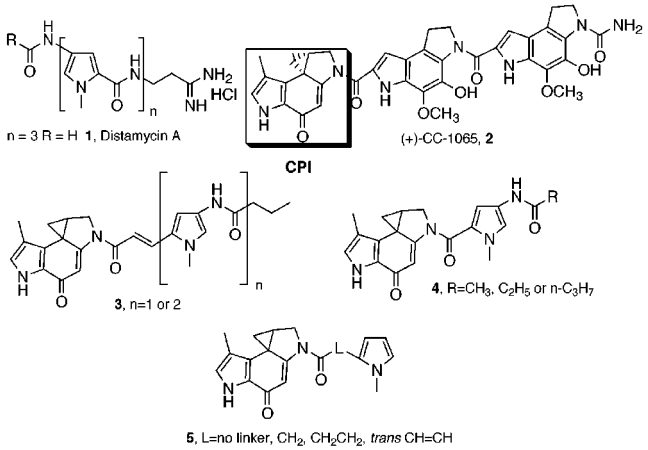
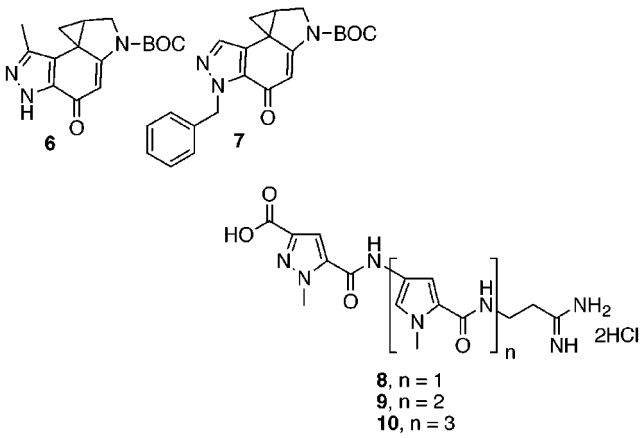
tomyces zelensis by scientists at the Upjohn Company,[9] and it is a highly potent natural agent with activity both in vitro and in experimental animals.[10−12] This activity has generated great interest in this agent as a potential anticancer drug.[13,14] Studies on the mechanism of cytotoxic action have shown that CC-1065 affords its biological activity through binding to double-stranded B-DNA within the minor groove to AT-rich sequences and selectively alkylating at the N_3 position of the 3′-adenine by its cyclopropylindole (CPI) subunit.[15−17] It was also demonstrated that synthetic enantiomer (−)-CC-1065 alkylates the adenine 5′end of the AT-rich sequence.[18] Despite its high potency and broad spectrum of antitumor activity, CC-1065 cannot be used in humans because it causes delayed death in experimental models.[19]

Lown and colleagues have synthesized a series of hybrid compounds belonging to the general structures **3**−**5**, which contain the CPI moiety (in racemic form) coupled with various lexitropsins constituted by one or two *N*-methyl pyrroles.[20,21] Among the synthesized compounds, the two most potent CPI−lexitropsin hybrids were the compounds possessing structure **3**, where the CPI unit was linked to one or two *N*-methyl pyrroles via a trans double bond. The reason for this enhanced cytotoxic activity due to the presence of a trans double bond linker on CPI compounds **3** is not well understood. In all derivatives **3**−**5**, the alkylating units were synthesized and linked in racemic form.



Recently, we have reported the synthesis and biological evaluation of two CPI pyrazole analogues, (±)-*N*-Boc-CPzI **6**[22,23] and (±)-*N*-Boc-N−BnCPzI **7**,[24] which demonstrate a cytotoxicity against L1210 leukemia cells that was comparable to and 10-fold lower than that of the reference compound *N*-Boc-CPI [IC_{50} = 330 nM for (+)-*N*-Boc-CPI vs IC_{50} = 370 nM for (±)−**6** and IC_{50} = 3064 nM for (±)−**7**, respectively]. Because of their limited sequence specificity, low affinity for DNA, and poor water solubility, it was reasoned that it may be beneficial to tether these alkylating compounds to a DNA binding vector, such as polypyrrole pseudopeptides, which can permeate cell membranes and has the potential to control specific gene expression. The vector could therefore deliver the reactive group more efficiently and in a sequence-specific manner to the DNA. Moreover, water solubility made these hybrid compounds attractive to overcome the administration problem of CC-1065 derivatives.

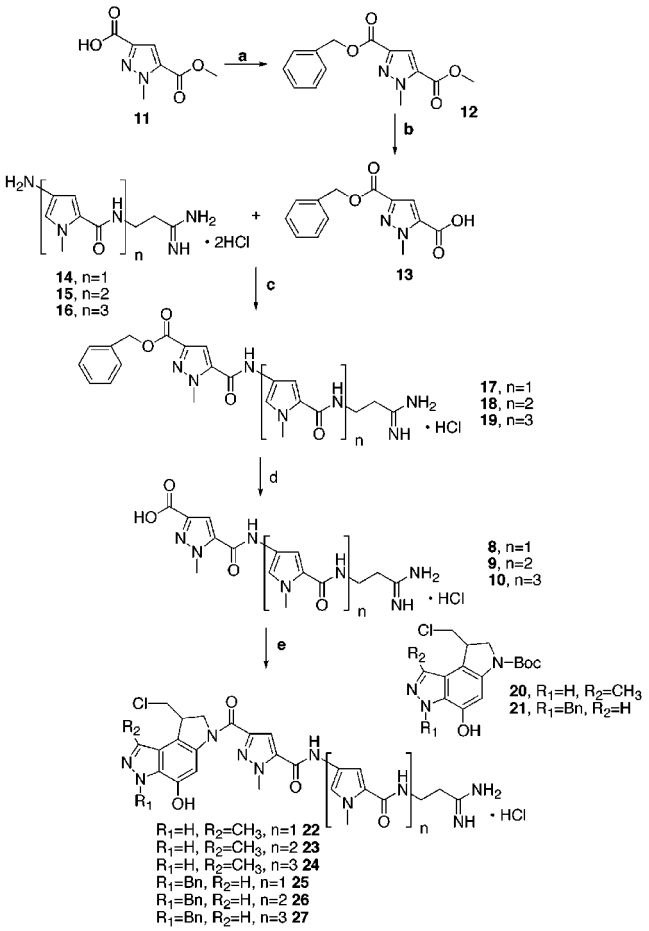
Here we wish to report the synthesis and biological evaluation of a novel series of conjugates comprising of either of these two CPI pyrazole analogues **6** and **7** and three mixed pyrazole−pyrrole compounds **8**−**10**, called lexitropsins (or information-reading oligopeptides), consisting of a varying number of pyrrole amide units (from one to three) tethered on the *N*-terminus to a 3,5-pyrazole dicarboxylic acid moiety and structurally related to the DNA minor groove binder distamycin A. The DNA binding domains chosen are structurally related to distamycin A, which is known to bind to the minor groove of DNA in AT-rich sequences. In addition, the amidino moiety would also enhance the DNA-binding affinity by providing noncovalent electrostatic DNA binding. In synthesizing these novel water-soluble hybrids, we wanted to increase the potency of pyrazole CPI analogues **20** and **21** by increasing their affinity for DNA and to determine the structure−activity relationship among the length of the oligopyrrolic frame, antitumor activity, and sequence specificity.



Chemistry

The synthesis of the alkylating units **20**[22,23] and **21**[24] has been previously reported. The key intermediate for the synthesis of lexitropsins **8**−**10** was the partially masked pyrazole **13**, which acts as a rigid linker. In fact, by the carbonyl functions present in the 3 and 5 position on the pyrazolic moiety, it was possible to join the alkylating units **20**−**21** and the polypyrrolic aminoamidines **14**−**16**. The known compound **11**[25] was transformed into the corresponding benzyl ester **12** by treatment with benzyl bromide and potassium carbonate. The latter compound was selectively deprotected by selective alkaline hydrolysis with 1 equiv of sodium hydroxide, affording the acid **13** after acidification with diluted hydrochloric acid (5% v/v). Coupling of carboxylic acid−benzyl ester **13** with the well-known amines **14**,[26] **15**,[27] and **16** (deformyl distamycin A)[1] was readily achieved with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI) and 1*H*-hydroxy-1,2,3-benzotriazole (HOBt) to give **17**, **18**, and **19**, respectively. The protecting benzyl group in **17**−**19** was cleaved off by catalytic hydrogenation (10% Pd on C) in methanol, in the presence of a few drops of diluted hydrochloric acid (5% v/v) to give compounds **8**−**10**, respectively. The final incorporation of the alkylating units **20** and **21** into the DNA-binding moieties, constituted by one, two, and three pyrrole residues (com-

Scheme 1



pounds **8**, **9** and **10**, respectively), was achieved using the classical coupling procedure mediated by EDCI after deprotection of the BOC group in (±)-seco-*N*-Boc-CPzI **20**[22,23] and (±)-seco-*N*-Boc-N−BnCPzI **21**[24] by acid hydrolysis with dry HCl in ethyl acetate. The final hybrid compounds **22**−**27** possessed improved water solubility with respect to the alkylating units **20** and **21** (which are poorly soluble in aqueous solution) and can be easily stored as the stable chlorohydrate salt after purification by preparative HPLC and lyophilization.

Results and Discussion

Antitumor Activity. In Table 1, we have reported the in vitro cytotoxic activity of compounds **22**−**27** against a panel of tumor cell lines using the alkylating units **20** and **21** as reference compounds. By previous in vitro assay on L1210 cell line,[22−24] we have observed that there was not any detectable difference between the cytotoxic activies of the seco-derivatives **20** and **21** (IC_{50} = 520 ± 6 and 2710 ± 490 nM, respectively) and that of the corresponding spiro derivatives **6** and **7** (IC_{50} = 370 ± 4 and 3064 ± 220 nM, respectively). All compounds **20**−**27** demonstrated profound inhibitory effects on the proliferation of murine leukemia (L1210), murine mammary carcinoma (FM3A), human T-lymphoblastoid (Molt/4 and CEM), and human B-lymphoblastoid (Daudi) cells.

As evident from Table 1, tethering the pyrazole CPI analogues **20** and **21** to the DNA binders **8**−**10** (to afford the hybrids **22**−**27**) resulted, with only a few exceptions, in improved cytotoxic potency against most of the tumor cell lines as compared to that of the alkylating units alone. This is likely due to the more efficient delivery of **20** and **21** to the DNA, followed by a covalent reaction with the DNA at selected sites. The results show that the hybrids **22**−**24** were about 8- to 70-fold more potent than the alkylating unit **20**. Among these, the hybrid **24** demonstrated the highest potency across the panel of tumor cell lines, especially against T- and B-lymphoblast cells, with IC_{50} values between 7.4 and 71 nM. Against L1210 cells, the tripyrrole analogue **24** was 2- to 8-fold more active than the bis and monopyrrole counterparts (compounds **23** and **22**, respectively). This is presumably due to the increased DNA binding of the "longer" compounds. The IC_{50} for **23** ranged between 19 and 45 nM with respect to the tumor cell lines L1210, Molt4, CEM, and Daudi, but the same compound was somewhat less active against FM3A (IC_{50} = 190 nM). Compound **24** was more active against L1210 cells than against the other tumor cell lines. For this series of hybrids, it is possible to correlate structure with biological activity, as previously reported,[6−8] increasing the number of pyrrole rings from one to three results in increased cytotoxic activity.

The hybrids **25**−**27** demonstrated potent cytotoxic activity against Daudi cells (IC_{50} values ranging from 11 to 100 nM). While being somewhat less toxic to the other tumor cells (IC_{50} values ranging between 70 and 19 300 nM), they are always more cytotoxic than the alkylating unit **21** alone (with only few exceptions). The FM3A cell line was substantially less sensitive to the hybrids **25**−**27**, which exhibited cytotoxic activities comparable to or 3-fold higher (IC_{50} values ranging from 4 to 19 μM) than that of the alkylating unit **21** alone. In the CEM cell line, compounds **25**−**27** demonstrated IC_{50} values between 70 and 400 nM, which were 30- to 100-fold higher than that reported for **21**, with compound **25** exhibiting potent cytotoxicity (IC_{50} = 70 nM).

A fairly marked dependence on the number of pyrrolic rings for the antiproliferative activity has been observed in the **22**−**24** series, with compound **24** comprising three pyrrolic rings proving to be the most active. The relationship between the number of pyrrole groups in the **25**−**27** series and their corresponding cytotoxicity did not seem to follow this pattern. In fact, the cytotoxicity was higher for compound **25**, which possesses only one pyrrole ring. It is interesting to note that the L1210 cell line was 50-fold more susceptible to the cytotoxic action of compound **27** than FM3A cells. For all cell lines, taken together, compounds possessing the same number of pyrrole rings and the alkylating unit **20** appeared to be more cytotoxic than those containing **21** as the alkylating agent.

Antiviral Activity. The most active compound, hybrid **24**, has been also tested for its antiviral activity against herpes simplex virus (HSV)-1 and -2, vaccinia virus (VV), vesicular stomatitis virus (VSV), herpes simplex virus-1 TK^- KOS ACV, and herpes simplex virus-1 TK^- VMW 1837 in E_6SM (embryonic skin muscle) cell cultures. Results were compared both with alkylating unit **20** alone and with the antiviral agents BVDU, ribavirin, ACG, and DHPG (Table 2). Compound **24** proved to be rather toxic to the host cells (morphology and growth), and no specific antiviral activity could be

Table 1. In Vitro Activity of Hybrids **22**−**27** and Alkylating Units **20** and **21** against the Proliferation of Murine Leukemia (L1210), Murine Mammary Carcinoma (FM3A), Human T-Lymphoblast (Molt/4 and CEM) and Human B-Lymphoblast (Daudi) Cells[a]

	IC_{50} (nM ± SE)				
compound	L1210	FM3A	Molt/4	CEM	Daudi
20	520 ± 6.6	1400 ± 40	1740 ± 50	1260 ± 30	680 ± 150
21	2710 ± 490	18300 ± 200	8550 ± 280	6720 ± 1040	7520 ± 30
22	58 ± 17	1600 ± 50	340 ± 20	230 ± 10	150 ± 40
23	19 ± 2	190 ± 6	45 ± 1	39 ± 1	22 ± 10
24	7.4 ± 0.4	31 ± 11	17 ± 4	71 ± 9	8.8 ± 0.1
25	240 ± 30	4000 ± 1000	130 ± 20	70 ± 21	11 ± 6.0
26	600 ± 90	5600 ± 1400	160 ± 60	210 ± 110	38 ± 7.0
27	400 ± 16	19300 ± 3400	310 ± 70	400 ± 50	100 ± 10

[a] IC_{50} = compound concentration required to inhibit tumor cell proliferation by 50%. Data are expressed as the mean ±SE from the dose−response curves of at least three indipendent experiments.

Table 2. Cytotoxicity and Antiviral Activity of Compounds **20** and **24** against Several Viruses in E_6^{SM} Cell Cultures

	MIC[b] (*μg*/mL)						
compound	MCC[a] (*μ*g/mL)	HSV-1 (KOS)	HSV-2 (G)	VV	VSV	HSV-1 TK-KOS ACV	HSV-1 TK-VMW 1837
24	0.8	>0.16	0.16	0.019	>0.16	>0.16	>0.16
20	8	>1.6	>1.6	>1.6	>1.6	>1.6	>1.6
BVDU	>400	0.015	240	0.077	>400	240	0.128
Ribavirin	>400	240	48	16	48	240	240
ACG	>400	0.077	0.077	>400	>400	48	0.384
DHPG	>100	0.004	0.004	>100	>100	1.92	0.019

[a] Minimum cytotoxic concentration (MCC) required to cause a microscopically detectable alteration of normal cell morphology when incubated with cells for the same duration as that required to measure antiviral activity. [b] Minimum inhibitory concentration (MIC) required to reduce virus-induced cytopathogenicity by 50%. Viral cytopathogenicity was recorded as soon as it reached completation in the control virus-infected cell cultures.

discerned at subtoxic concentrations with respect to HSV-1 and -2, VSV, HSV-1 TK^- KOS ACV, and HSV-1 TK^- VMW 1837. Furthermore, compound **24** showed a minimum inhibitory concentration (MIC) of 0.0192 *μ*g/mL against vaccinia virus replication, this concentration being about 40-fold below the toxicity threshold of the compound for the E_6SM host cells (0.8 *μ*g/mL).

The other derivatives **22**, **23**, and **25**−**27** were also evaluated for their antiviral activity against cytomegalovirus (CMV), varicella-zoster virus (VZV), herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), and vaccinia virus (VV). The compounds proved to be very toxic to human embryonic lung cells (estimated by altered morphology and inhibition of growth), and no specific antiviral activity could be discerned at subtoxic concentrations. All five hybrids were not selectively active against CMV, VZV, HSV-1 and-2, and VV—that is, they did not inhibit the cytopathic effect of the viruses at a concentration that was lower than their minimum cytotoxic concentration for the host cells.

DNA Alkylation and Molecular Modeling Studies. The sequence-selective alkylation by these hybrids **22**−**27** was assayed using the 5′-Texas Red-end-labeled 450 base pair DNA fragments. Alkylated DNA fragments were cleaved by heating at 90 °C, and the fragments analyzed using a DNA sequencer were cleaved as described previously[28] (Figure 1).

When compounds **25**−**27** were incubated at different concentrations (ranging from 0.1 to 100 *μ*M), no significant cleavage was observed by thermally induced strand cleavage of DNA fragment. For compounds **25**−**27**, the presence of a benzyl group at the azaindole moiety **21** significantly disrupted the alkylation reaction, and the effect of this substitution has presumably altered the positioning of the compound on the DNA. In the orientation that is suitable for DNA alkylation, the benzyl group needs to protrude from the minor groove, and it is assumed that this causes large energy loss. Therefore, the introduction of a benzyl group decreased the alkylating activity and also the corresponding cytotoxicity.

In contrast to the case of hybrids **25**−**27**, hybrids **22**−**24** show unique DNA sequence-selective alkylation in AT-rich sequences. High-resolution denaturating gel electrophoresis indicated that **24** selectively alkylates the third adenine of the 5′-ACA*A*AAATCG-3′ motif within a 400 bp DNA fragment (Figure 1). This compound elicited the strongest and most highly sequence-specific DNA alkylation activity. For compound **24**, DNA alkylation was observed even at 50 nM. Interestingly, DNA alkylation sites by **22**−**24** in AT-rich sequence were slightly different, as shown in inset of Figure 1a. The results clearly suggest the different orientation of the binding of these agents, as shown in Figure 1b, in which pyrrole moiety of **24** and **23** is oriented N → C with respect to the 3′ → 5′ direction of the reacting DNA strand, whereas compound **22** pyrrole moiety is oriented N → C with respect to the 5′ → 3′ direction of the reacting DNA strand. Differences in binding orientation might derived from the balance of binding preference of polypyrrole moiety and the alkylating site of **20**. The results also suggest the interesting possibility that only the 4*S* enantiomer of **24** and **23** and 4*R* isomer of **22** were used for DNA alkylation. When the Texas Red-labeled DNA fragment was incubated with increased concentrations of the hybrids **22**−**24**, the intensity of the DNA alkylation also increased without altering the selectivity. These results clearly indicate the importance of the hybrid structure **24** constructed from **20** and the lexitropsin **10** for the DNA binding.

On the basis of the results of the sequencing gel experiment for compound **24**, we constructed the bind-

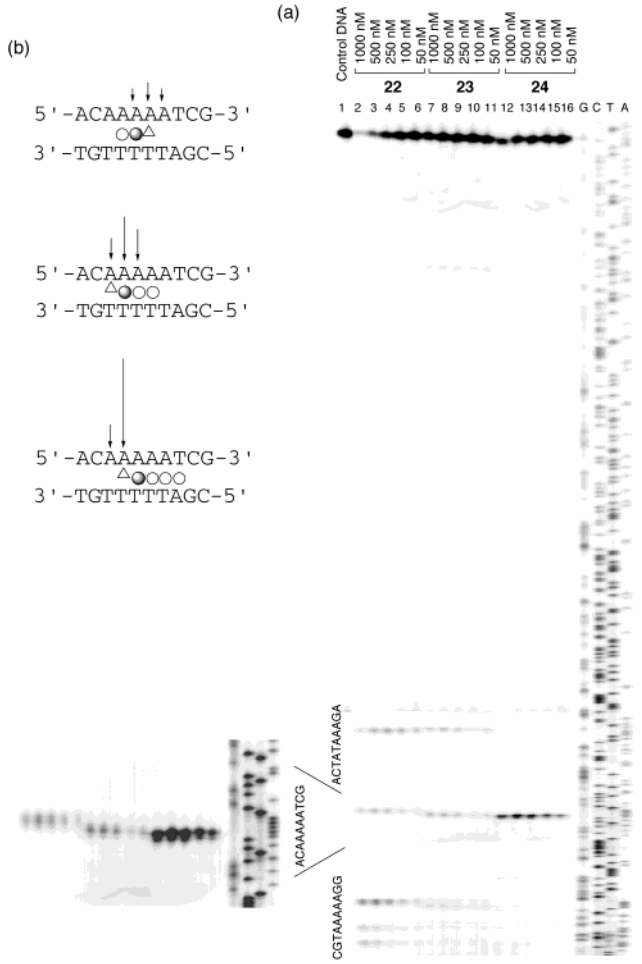


Figure 1. Thermally induced strand cleavage of 5′-Texas Red-labeled pUC 18F 780-1229 DNA fragment incubated with hybrids **22**−**24** (a) and the schematic prepresentation of binding mode of hybrids **22**−**24** (b). Lane 1: DNA control. Lanes 2−6: 1000, 500, 250, 100, and 50 nM **22**, respectively. Lanes 7−11: 1000, 500, 250, 100, and 50 nM **23**, respectively. Lanes 12−16: 1000, 500, 250, 100, and 50 nM **24**, respectively. The pyrrole rings and CPI are represented by circles and triangles, respectively.

ing model of **24** and 5′-ACAAAAATCG-3′/5′-CGATTTTTGT-3′. The initial structure of a 1:1complex was constructed by manual docking of the 4*S* isomer of **24** in the observed binding orientation to the minor groove of duplex decanucleotides in ideal B-form DNA. This was generated using the builder module of the Discover/InsightII program (MSI). The assembled structure in the presence of 18 sodium cations and 10 Å layer of water was minimized with a CFF force field (MSI) with convergence criteria of less than 0.001 kcal/Å. The energy minimized binding model is shown in Figure 2. Inspection of the minimized structure suggested that only 4*R* stereoisomer of **24** can akylate 5′ side of adenine of AT-rich sequence. Further study to evaluate the contribution of the individual enantiomers is in progress.

Conclusions

The present work demonstrated not only the molecular design and the chemical synthesis of novel CPzI-lexitropsin hybrids, but also their DNA-binding profiles and cytotoxic activities. Many of the compounds prepared in this study were shown to demonstrate pronounced cytotoxicity toward five different tumor cell lines. Results from this investigation suggest a promising approach for developing a new generation of DNA-alkylating agents based on CPI analogues and lexitropsin hybrid system that can alkylate purine bases in a sequence-selective fashion. Because of the high efficiency of alkylation, results from the present investigation suggest that these molecules should be useful in the design of compounds that target a single gene. Further studies on the generality and the optimization of this new class of DNA alkylation systems are currently in progress.

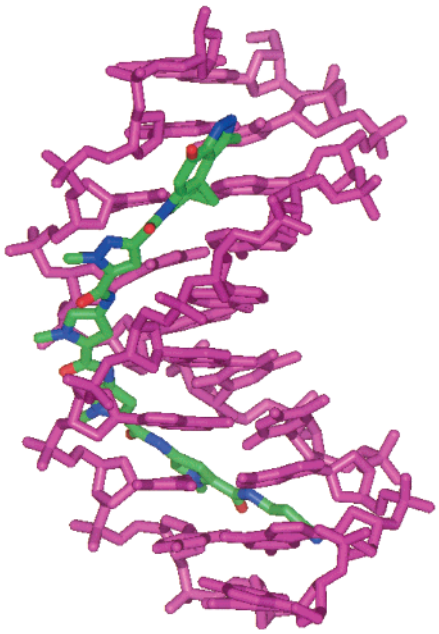


Figure 2. Energy-minimized structure of the **24**-5′-ACAAAAATCG-3′/5′-CGATTTTTGT-3′ complex. Minimization was performed in the presence of 18 sodium cations and a 10 Å layer of H_2O by CFF force field. For simplicity, hydrogen atom, sodium ions, and H_2O are not presented. DNA is drawn in purple. Compound **24** is drawn in blue and red.

Experimental Section

Chemistry. Materials and Methods. ^{1}H NMR spectra were recorded on a Bruker AC 200 spectrometer. Chemical shifts (δ) are given in parts per million upfield from tetramethylsilane as internal standard, and the spectra were recorded in appropriate deuterated solvents indicated in the procedure. Melting points (mp) were determined on a Buchi-Tottoli apparatus and are uncorrected. 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} 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 $MgSO_4$. Dioxane was distilled from calcium hydride, 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% B in 25 min at a flow rate of 30 mL/min, and 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 the 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).

1-Methyl-3-benzyloxycarbonyl-5-methoxycarbonyl Pyrazole (12). A stirred solution of acid−ester **11** (860 mg, 4.67 mmol), DBU (0.8 mL, 5.14 mmol), and benzyl bromide (1.4 mL, 10.3 mmol) in dry DMF (5 mL) was stirred at room temperature for 18 h, and the reaction mixture was concentrated in vacuo. The residue was dissolved in EtOAc (10 mL), washed with 5% HCl (3 mL), and a saturated solution of $NaHCO_3$ (3 mL). The organic phase was separated and dried, and after concentration in vacuo, the crude product was purified by flash chromatography (EtOAc/petroleum ether 1:1), yielding compound **12** as a white solid: yield 1.14 g (89%); mp 60−63 °C; ^{1}H NMR ($CDCl_3$) δ 3.89 (s, 3H), 4.26 (s, 3 H), 5.38 (s, 2H), 7.37 (m, 5 H); IR (KBr) cm^{-1} 3441, 1705, 1557, 1443, 1264, 1231, 1187, 1123, 757. Anal. ($C_{14}H_{14}N_2O_4$) C, H, N.

3-Benzyloxycarbonyl-1-methylpyrazole-5-carboxylic Acid (13). To a solution of ester **12** (548 mg, 2 mmol) in dioxane (10 mL) was added 2 N KOH (1 mL), and the mixture was stirred at r.t. for 24 h. At the end of this time, the solvent was evaporated, the crude residue was dissolved in water (8 mL), and the resulting solution was extracted with EtOAc (5 mL). The water phase was acidified to pH 1 with concentrated HCl and then extracted with EtOAc (2 × 10 mL). The recombined organic phases were dried on Na_2SO_4 and evaporated, and the residue was purified by flash chromatography (EtOAc), yielding compound **13** as a white solid: yield 458 mg (88%); mp 128−130 °C; ^{1}H NMR (DMSO-d_6) δ 4.13 (s, 3 H), 5.31 (s, 2H), 7.40 (m, 5 H), 13.6 (bs, 1H); IR (KBr) cm^{-1} 3392, 1738, 1707, 1455, 1284, 1204, 1009, 710. Anal. ($C_{13}H_{12}N_2O_4$) C, H, N.

General Procedure for the Coupling of 3-Benzyloxycarbonyl-1-methylpyrazole-5-carboxylic Acid (13) with Pyrrole−Pyrazole Oligomers (14−16). To a stirred 0.4 M solution of the pyrrole oligomer **14**−**16** in anhydrous DMF under argon atmosphere was added the Hunig's base (1 equiv) at 0 °C. After 5 min, acid **13** (1.1 equiv) was added, followed by EDCI (2 equiv) and then HOBT (1.1 equiv). The resulting mixture was stirred for 18 h as it warmed to room temperature, acidified with 10% HCl to pH 3, 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 1.5:8.5 and then 2:8) and recrystallized (CH_3OH/diethyl ether) to give **17**−**19**.

3-[1-Methyl-4-(1-methyl-3-benzyloxycarbonylpyrazole-5-carboxamido)-pyrrole-2-carboxamido]propionamidine Hydrochloride (17). Yield 52%; brown solid; mp 112−114 °C; ^{1}H NMR (DMSO-d_6) δ 2.62 (t, 2H, J = 5.8 Hz), 3.46 (m, 2H), 3.81 (s, 3H), 3.82 (m, 3H), 4.18 (s, 3H), 5.33 (s, 2H), 6.98 (s, 1H), 7.26 (s, 1H), 7.42 (m, 5H), 7.60 (s, 1H), 8.34 (t, 1H, J = 7.4 Hz), 8.68 (bs, 2H), 9.03 (bs, 2H), 10.55 (s, 1H). Anal. ($C_{22}H_{26}ClN_7O_4$) C, H, Cl, N.

3-[1-Methyl-4-[1-methyl-4-(1-methyl-3-benzyloxycarbonylpyrazole-5-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine Hydrochloride (18). Yield 52%; brown solid; mp 138−140 °C; ^{1}H NMR (DMSO-d_6) δ 2.71 (m, 2H), 3.48 (m, 2H), 3.81 (s, 3H), 3.86 (m, 3H), 4.18 (s, 3H), 5.34 (s, 2H), 6.95 (s, 1H), 7.09 (s, 1H), 7.21 (s, 1H), 7.31 (s, 1H), 7.45 (m, 5H), 7.61 (s, 1H), 8.27 (t, 1H, J = 7.4 Hz), 8.71 (bs, 2H), 9.03 (bs, 2H), 10.0 (s, 1H), 10.58 (s, 1H). Anal. ($C_{28}H_{32}ClN_9O_5$) C, H, Cl, N.

3-[1-Methyl-4-[[1-methyl-4-[1-methyl-4-(1-methyl-3-benzyloxycarbonylpyrazole-5-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]-propionamidine Hydrochloride (19). Yield 46%; brown solid; mp 195−200 °C; ^{1}H NMR (DMSO-d_6) δ 2.72 (m, 2H), 3.47 (m, 2H), 3.76−3.88 (m, 9H), 4.19 (s, 3H), 5.34 (s, 2H), 6.96 (s, 1H), 7.11 (s, 1H), 7.21 (s, 1H), 7.22 (s, 1H), 7.26 (s, 1H), 7.31 (s, 1H), 7.45 (m, 5H), 7.61 (s, 1H), 8.27 (t, 1H, J = 7.4 Hz), 8.70 (bs, 2H), 9.02 (bs, 2H), 9.96 (s, 1H),10.0 (s, 1H), 10.58 (s, 1H). Anal. ($C_{34}H_{38}ClN_{11}O_6$) C, H, Cl, N.

General Procedure for the Debenzylation of Lexitropsins (17−19). A solution of benzyluretane **17**−**19** (1 mmol) in 10 mL of a MeOH containing 0.5 mL of 5% HCl was hydrogenated over 50 mg of 10% Pd/C at 50 p.s.i for 4 h. The catalyst was removed by filtration, and the filtrate was concentrated to give a foam oil which was used without purification for the next step.

3-[1-Methyl-4-(1-methyl-3-carboxylpyrazole-5-carboxamido)-pyrrole-2-carboxamido]propionamidine Hydrochloride (8). Yield 78%; green solid; mp 205−207 °C; ^{1}H NMR (DMSO-d_6) δ 2.68 (t, 2H, J = 5.8 Hz), 3.52 (m, 2H), 3.82 (s, 3H), 4.23 (s, 3H), 6.98 (s, 1H), 7.18 (s, 1H), 7.56 (s, 1H), 8.28 (t, 1H, J = 7.2 Hz), 8.62 (bs, 2H), 9.00 (bs, 2H), 10.58 (s, 1H).

3-[1-Methyl-4-[1-methyl-4-(1-methyl-3-carboxylpyrazole-5-carboxamido) pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine Hydrochloride (9). Yield 72%; green-yellow solid; mp 187−190 °C; ^{1}H NMR (DMSO-d_6) δ 2.72 (m, 2H), 3.36 (m, 2H), 3.81 (s, 3H), 3.86 (m, 3H), 4.12 (s, 3H), 6.95 (s, 1H), 7.10 (s, 1H), 7.21 (s, 1H), 7.33 (s, 1H), 7.54 (s, 1H), 8.28 (t, 1H, J = 7.2 Hz), 8.78 (bs, 2H), 9.08 (bs, 2H), 10.0 (s, 1H), 10.60 (s, 1H).

3-[1-Methyl-4-[[1-methyl-4-[1-methyl-4-(1-methyl-3-carboxypyrazole-5-carboxamido)pyrrole-2-carboxamido]-pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine Hydrochloride (10). Yield 68%; brown solid; mp 257−260 °C; ^{1}H NMR (DMSO-d_6) δ 2.74 (m, 2H), 3.52 (m, 2H), 3.72 (s, 3H), 3.76 (s, 3H), 3.87 (s, 3H), 4.23 (s, 3H), 6.98 (s, 1H), 7.11 (s, 1H), 7.21 (s, 1H), 7.24 (s, 1H), 7.29 (s, 1H), 7.34 (s, 1H), 7.61 (s, 1H), 8.29 (t, 1H, J = 7.4 Hz), 8.70 (bs, 2H), 9.08 (bs, 2H), 9.96 (s, 1H),10.06 (s, 1H), 11.04 (s, 1H).

General Procedure for Coupling Reaction between Carboxylic Acid Lexitropsins (8−10) and Alkylating Units 20 and 21. A solution of (**20**−**21**) (0.1 mmol) in 3M HCl-EtOAc (5 mL) at 0 °C was stirred for 30 min before the solvent was removed under reduced pressure. The resulting unstable crude amine hydrochloride was dissolved in dry DMF (3 mL) and treated sequentially with the appropriate lexitropsin acid (**8**−**10**) (0.1 mmol) and EDCI (0.5 mmol, 86 mg). After 18 h of stirring, the solution was evaporated, and the crude residue was purified by semipreparative HPLC to give compounds **22**−**27**.

3-[1-Methyl-4-[1-methyl-3-[6-(3-methyl-4-chloromethyl-8-hydroxy-4,5-dihydro-6H-pyrrole-[3,2-e]-1-*H*-indazole] carboxylpyrazole-5-carboxamido]-pyrrole-2-carboxamido]propionamidine Hydrochloride (22). Yield 78%; green solid; mp 192−195 °C; ^{1}H NMR (DMSO-d_6) δ 2.59 (s, 3H), 2.64 (t, 2H, J = 6.4 Hz), 3.56 (m, 2H), 3.83 (s, 3H), 3.89−3.97 (m, 3H), 4.20 (s, 3H), 4.48−4.76 (m, 2H), 6.99 (s, 1H), 7.27 (s, 1H), 7.54 (s, 1H), 7.87 (s, 1H), 8.32 (t, 1H, J = 7.2 Hz), 8.56 (bs, 2H), 8.96 (bs, 2H), 10.29 (s, 1H), 10.57 (s, 1H), 12.21 (bs, 1H). Anal. ($C_{26}H_{30}Cl_2N_{10}O_4$) C, H, Cl, N.

3-[1-Methyl-4-[1-methyl-4-(1-methyl-3-[6-(3-methyl-4-chloromethyl-8-hydroxy-4,5-dihydro-6*H*-pyrrole-[3,2-e]-1-H-indazole]carboxylpyrazole-5-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine Hydrochloride (23). Yield 72%; green-yellow solid; mp 185−187 °C; ^{1}H NMR (DMSO-d_6) δ 2.58 (s, 3H), 2.62 (t, J = 6.2 Hz, 2H), 3.28 (m, 2H), 3.82 (s, 3H), 3.89 (m, 3H), 3.92−4.16 (m, 3H),4.23 (s, 3H), 4.42−4.64 (m, 2H), 6.98 (s, 1H), 7.12 (s, 1H), 7.22 (s, 1H), 7.38 (s, 1H), 7.60 (s, 1H), 7.92 (s, 1H), 8.18 (s, 1H), 8.22 (t, 1H, J = 7.4 Hz), 8.58 (bs, 2H), 8.98 (bs, 2H), 10.00 (s, 1H), 10.58 (s, 1H), 10.59 (s, 1H). Anal. ($C_{32}H_{36}Cl_2N_{12}O_5$) C, H, Cl, N.

3-[1-Methyl-4-[[1-methyl-4-[1-methyl-4-(1-methyl-3-[6-(3-methyl-4-chloromethyl-8-hydroxy-4,5-dihydro-6*H*-pyrrole-[3,2-e]-1-H-indazole] carboxypyrazole-5-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]-pyrrole-2-carboxamido]propionamidine Hydrochloride (24). Yield 64%; yellow solid; mp 275−278 °C; ^{1}H NMR (DMSO-d_6) δ 2.54 (s, 3H), 2.59 (m, 2H), 3.64 (m, 2H), 3.69 (m, 1H), 3.81 (s, 3H), 3.85 (s, 3H), 3.89 (s, 3H), 3.94−4.6 (m, 2H), 4.22 (s, 3H), 4.55−4.72 (m, 2H), 6.97 (s, 1H), 7.09 (s, 1H), 7.14 (s, 1H), 7.18 (s, 1H), 7.25 (s, 1H), 7.34 (s, 1H), 7.55 (s, 1H), 7.87 (s, 1H), 8.22 (t, 1H, J = 7.2 Hz), 8.50 (bs, 2H), 8.92 (bs, 2H), 9.94 (s, 1H), 10.03 (s, 1H), 10.29 (s, 1H), 10.59 (s, 1H), 12.75 (s, 1H). Anal. ($C_{38}H_{42}Cl_2N_{14}O_6$) C, H, Cl, N.

3-[1-Methyl-4-(1-methyl-3-[6-(1-benzyl-4-chloromethyl-8-hydroxy-4,5-dihydro-6H-pyrrole-[3,2-e]-1-*H*-indazole] carboxylpyrazole-5-carboxamido)-pyrrole-2-carboxamido]propionamidine Hydrochloride (25). Yield 62%; white solid; mp 247−250 °C; ^{1}H NMR (DMSO-d_6) δ 2.51 (t, 2H, J = 6 Hz), 3.50 (m, 2H), 3.84 (s, 3H), 4.02−4.16 (m, 3H), 4.20 (s, 3H), 4.42−4.74 (m, 2H), 5.71 (s, 2H), 7.00 (s, 1H), 7.15−7.29 (m, 7H), 7.54 (s, 1H), 7.92 (s, 1H), 8.36 (t, 1H, J = 7.2 Hz), 8.52 (bs, 2H), 8.94 (bs, 2H), 10.54 (s, 1H), 10.56 (s, 1H). Anal. ($C_{32}H_{34}Cl_2N_{10}O_4$) C, H, Cl, N.

3-[1-Methyl-4-[1-methyl-4-(1-methyl-3-[6-(1-benzyl-4-chloromethyl-8-hydroxy-4,5-dihydro-6*H*-pyrrole-[3,2-e]-1-H-indazole]carboxylpyrazole-5-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine Hydrochloride (26). Yield 72%; green solid; mp 262−265 °C; ^{1}H NMR (DMSO-d_6) δ 2.60 (t, J = 6.4 Hz, 2H), 3.36 (m, 2H), 3.81 (s, 3H), 3.87 (m, 3H), 3.94−4.15 (m, 3H),4.22 (s, 3H), 4.51−4.76 (m, 2H), 5.79 (s, 2H), 6.96 (s, 1H), 7.10−7.33 (m, 8H), 7.55 (s, 1H), 7.96 (s, 1H), 8.18 (s, 1H), 8.21 (t, 1H, J = 7.4 Hz), 8.52 (bs, 2H), 8.94 (bs, 2H), 10.00 (s, 1H), 10.55 (s, 1H), 10.59 (s, 1H). Anal. ($C_{38}H_{40}Cl_2N_{12}O_5$) C, H, Cl, N.

3-[1-Methyl-4-[[1-methyl-4-[1-methyl-4-(1-methyl-3-[6-(1-benzyl-4-chloromethyl-8-hydroxy-4,5-dihydro-6*H*-pyrrole-[3,2-e]-1-H-indazole] carboxypyrazole-5-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]-pyrrole-2-carboxamido]propionamidine Hydrochloride (27). Yield 72%; yellow solid; mp 234−236 °C; ^{1}H NMR (DMSO-d_6) δ 2.59 (m, 2H), 3.49 (m, 2H), 3.81 (s, 3H), 3.85 (s, 3H), 3.88 (s, 3H), 3.92−4.14 (m, 3H), 4.21 (s, 3H), 4.42−4.82 (m, 2H), 5.70 (s, 2H), 6.86 (s, 1H), 6.96 (s, 1H), 7.08 (s, 1H), 7.13−7.34 (s, 8H), 7.57 (s, 1H), 8.01 (s, 1H), 8.18 (s, 1H),8.20 (t, 1H, J = 7.2 Hz), 8.64 (bs, 2H), 8.99 (bs, 2H), 9.96 (s, 1H), 10.05 (s, 1H), 10.61 (s, 2H). Anal. ($C_{44}H_{46}Cl_2N_{14}O_6$) C, H, Cl, N.

Biological Assays. Cells. Human embryonic lung (HEL) fibroblasts and E_6SM cells were grown in minimum essential medium (MEM) supplemented with 10% inactivated fetal calf serum (FCS), 2 mM L-glutamine and 0.3% $NaHCO_3$.

Viruses. The laboratory wild-type VZV strains Oka and YS, the thymidine kinase-deficient VZV strains 07−1 and YS−R, HSV-1 (KOS), HSV-2 (G), the thymidine kinase-deficient HSV-1 strains B-2006 and VMW 1837, the CMV strains Davis and AD-169, and vaccinia virus were used in the virus inhibition assays.

Antiviral Assays. Confluent HEL cells grown in 96-well microtiter plates were inoculated with VZV at an input of 20 PFU (plaque forming units) per well or with CMV at an input of 100 PFU per well. Confluent E_6SM cells were inoculated with HSV or vaccinia virus at 100 $CCID_{50}$ (50% cell culture infective doses) per well. After a 1 or 2 h incubation period, residual virus was removed, and the infected cells were further incubated with MEM (supplemented with 2% inactivated FCS, 1% L-glutamine and 0.3% $NaHCO_3$) containing varying concentrations of the compounds. Antiviral activity was expressed as EC_{50} (50% effective concentration) or compound concentration required to reduce viral plaque formation after 5 days (VZV) or virus-induced cytopathicity (CMV after 7 days and HSV and vaccinia virus after 3 days) by 50% as compared to the untreated control.

Cytostatic Assays. Murine leukemia L1210, murine mammary carcinoma FM3A, human T-lymphocyte CEM and Molt 4/ clone 8, and human B-lymphocyte Daudi cells were seeded in 96-well microtiter plates at 250 000−300 000 cells/mL and were treated with different concentrations of the experimental drugs. Cell cultures were incubated for 2−3 days at 37 °C. At the indicated time, the cell number was determined using a Coulter counter. The 50% cytostatic concentration (IC_{50}) was defined as the compound concentration required to reduce the cell number by 50%.

Preparation of 5′-Texas Red-End-Modified 450-Base Pair DNA Fragments. The 5′-Texas Red-end-modified 450-base pair DNA fragments pUC18 F780*-1229 and pUC18 R1459−1908 (they are complementary) were prepared by the PCR method using 5′-end Texas Red modified 18mers 5′-AGAATCAGGGGATAACGCAG-3′ (pUC 18 forward 780−799) as a primer[28] and purified by filtration using Suprec-02. Their concentration was determined by ethidium bromide staining. The asterisk indicates Texas Red modification and the nucleotide numbering starts with the replication site.

High-Resolution Gel Eletrophoresis. The 5′-Texas Red labeled DNA fragment (75 nM) was alkylated by various concentration of the agents in 10 mL of 10 mM Na phosphate buffer (pH 7.0) containing 10% DMF at 23 °C for 16 h. The reaction was quenched by adding 400 ng of calf thymus DNA and heating for 5 min at 90 °C. The solvent was evaporated in vacuo. The resulting residue was dissolved in 8 mL of loading dye (formamide with fushin red), heated at 94 °C for 20 min, and then immediately cooled to 0 °C. A 2 mL of aliquot was electrophoresed on 6% denaturing polyacrylamide gel using a Hitachi 5500-S DNA sequencer.

Molecular Modeling Studies. Minimizations were performed with the Discover (MSI, San Diego, CA) program by using CFF force-field parameters. The starting structure of **24**-d(ACAAAAATCG)/d(CGATTTTTGT) complex was built using standard bond lengths and angles. The Du moiety of the assembled initial structure was energy minimized using a distance-dependent dielectric constant of $e = 4r$ (r stands for the distance between atoms i and j) and the convergence criteria with the rms gradient of less than 0.001 kcal/mol Å. Twenty Na cations were placed at bifucating positions of O−P−O angle at a distance of 2.51 Å form the phosphorus atom. The resulting complex was soaked in 10 Å layer of water. The whole system was minimized without any constraint to the stage where the rms was less than 0.001 kcal/mol Å.

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