Hybrid molecules based on distamycin A as potential antitumor agents

Pier Giovanni Baraldi,*[a] Abdel Naser Zaid,[b] Delia Preti, [a] Francesca Fruttarolo, [a] Mojgan Aghazadeh Tabrizi, [a] Antonietta Iaconinoto, [a] Maria Giovanna Pavani, [a] Maria Dora Carrion, [a] Carlota Lopez Cara, [a] and Romeo Romagnoli [a]

[a] *University of Ferrara, Dipartimento di Scienze Farmaceutiche, Via Fossato di Mortara 17/19, 44100 Ferrara, Italy*

[b]*An-Najah National University - Coolege of Pharmacy – Nablus*

E-mail: baraldi@unife.it

Abstract

Many natural and synthetic anticancer agents with the ability to interact with DNA have been discovered, but most of them have relatively low therapeutic index. This is probably related to the fact that these derivatives cause DNA damage in an unspecific manner, inducing unselective growth inhibition and death, both in neoplastic and in highly proliferative normal tissues. For these reasons, there has been considerable interest in finding small molecules able to alkylate the DNA with a much higher degree of sequence specificity and to modify the function of nucleic acids irreversibly. Analogues of naturally occurring antitumor agents, such as distamycin A, which bind in the minor groove of DNA, represent a new class of anticancer compounds currently under investigation. Distamycin A has driven researcher's attention not only for the biological activity, but also for its non intercalative binding to the minor groove of double-stranded B-DNA, where it forms strong reversible complex preferentially at the nucleotide sequences consisting of 4-5 adjacent AT base pairs. The pyrrole-amide skeleton of distamycin A has also been used as DNA sequence selective vehicle for the delivery of alkylating functions to DNA targets, leading to a sharp increase of its cytotoxicity, in comparison to that, very weak, of distamycin itself. 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.

Keywords: Minor groove binders, distamycin A, alkylating agents, DNA target

Contents

1. Introduction

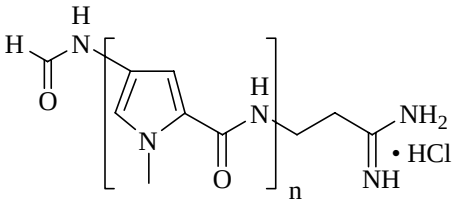
A number of innovative research approaches aimed to target the malignant abnormalities of tumor cells are in development. The targets of these approaches include pathways for oncogenes, tumor suppressor genes, components of the cell cycle, regulation of apoptosis and telomerase. Cytotoxic agents will continue to represent an essential part of the therapy for several years to come, possibly in combination with novel agents. This implies a need for new cytotoxics, with greater or broader activity and lower toxicity. The putative mode of action of many antitumor agents involves DNA damage, however most of DNA-interacting agents have only a limited degree of sequence specificity, which imply that they may hit all the cellular genes. For these reasons, there is currently interest in the study and development of low molecular weight sequence-selective agents interacting with double-stranded DNA. These molecules are frequently based on natural products and have been investigated for their ability to interact selectively with the minor groove of DNA.[1] One of the most studied minor groove binders is distamycin A.

Distamycin A **1** is a naturally occurring antibiotic agent isolated in 1962 from the cultures of *Streptomyces distallicus* active against some viruses, Gram-positive bacteria and protozoa (but inactive as antitumor agent).[2] As reported in the Figure 1, distamycin A **1** is characterized by the presence of an oligopeptidic pyrrolecarbamoyl frame ending with an amidino moiety, which reversibly binds to the minor groove of DNA by hydrogen bonds, van der Waals contacts and electrostatic interactions with a strong preference for adenine-thymine (AT) rich sequences containing at least four AT base pairs.[3]

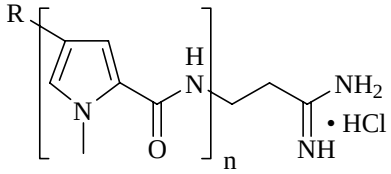
The tetrapyrrole distamycin A homologue **2**, although exerting very low cytotoxic activity on L1210 leukaemia cell line, is always almost 20-fold more active than the distamycin A and increasing the number of pyrrole units of the oligopeptidic frame increases the sequence specificity for longer tracts of AT-rich DNA, as a result of the greater availability of hydrogen bonding and van der Waals surface.

The synthesis of different analogues of distamycin A allows to establish some important molecular requirements for bioactivity. With the objective to identify novel promising candidates, distamycin A **1** and its four pyrroles homologue **2** have been used as DNA minor groove sequence-selective vector of alkylating functions, in which the formyl group has been substituted by benzoyl nitrogen mustard (BAM), chlorambucil (CHL), halogenoacryloyl,

epoxycarbonyl moieties, leading to compounds (with general formula **3**) endowed with relevant cytotoxic and antitumor activity than distamycin and **2** themselves, respectively.[4]



1 (Distamycin A), n=3
2, n=4



3, n=3 or 4
R= 4-[$(ClCH_2CH_2)_2N$]C_6H_4CONH, $CH_2(O)CHCONH$
4-[$(ClCH_2CH_2)_2N$]$C_6H_4(CH_2)_3CONH$, $(ClCH_2CH_2)_2N$
CH_2=CBrCONH, CH_2=CClCONH, .

Figure 1. Chemical structures of Distamycin A (1), its homologue (2) and their derivatives with an alkylating moiety (3)

2. Hybrid compounds between cytotoxics and distamycin A

In the last ten 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.[5] The nature of antitumor agents and therefore also the rationale that led to these compounds were different. Our strategy is represented by hybrid molecules, the so called combilexins, combining derivatives of naturally occurring alkylating agent or moiety, with a DST-like minor groove binder with the aim of combining high DNA affinity and sequence selectivity. In general the interaction with DNA tends to be dominated by the minor groove binding moiety, i.e. the conjugates bind to the minor groove with preferential interaction with TA-rich sequences.

2.1 PBD-distamycin A hybrids

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

The complex is stabilized by hydrogen bonds from C9-OH, N10 and the end of the acrylamide tail to base pair edges on the floor of the minor groove. X ray and footprinting studies on covalent DNA-PBD adduct have demonstrated a high sequence-specificity for G-C rich DNA regions, in particular for X-G-X triplets (X=purine).[7]

Both our[8,9] and Lown's group[10] have reported the synthesis, biological activity and DNA binding properties of novel hybrids (**6-9**), consisting respectively of one, two, three or four pyrrole amide units linked to a pyrrolo [2,1-*c*][1,4] benzodiazepine **10**, through a spacer arm, in order to study the structure-activity relationship between length of the oligopyrrolic frame, antiproliferative activity and sequence specificity (Figure 2).

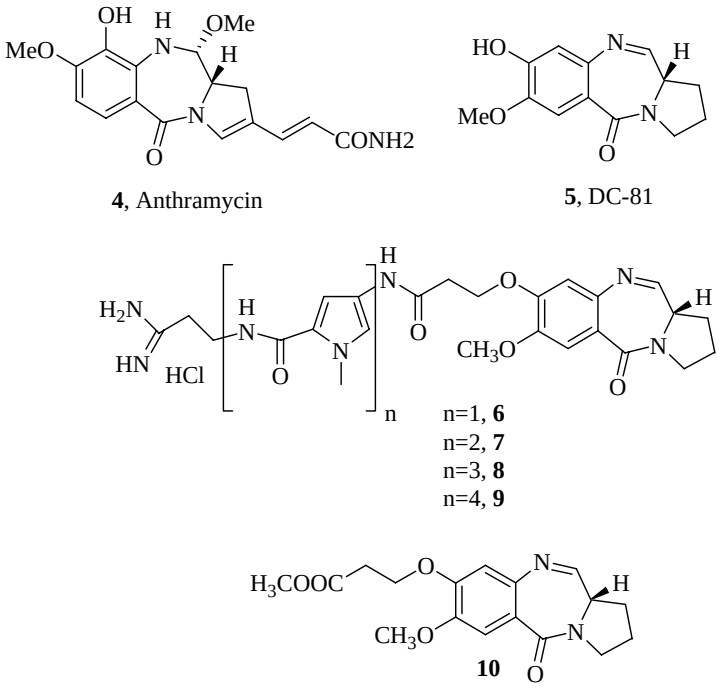


Figure 2. Chemical structures of natural compounds anthramycin (4) and DC-81 (5), hybrid conjugates (6-9) and PBD derivative (10)

The rationale that led to the synthesis of this series of pyrrolo[2,1-*c*][1,4]benzodiazepine-lexitropsin conjugates 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) **10**, with the aim to obtain new derivatives which could result more cytotoxic than the parent compounds. In these new hybrids (**6-9**), we conjugated the capability of PBD **10** to covalently bind to GC-rich sequences, with that of distamycin's different recognition pattern. The antiproliferative activity of the hybrids has been evaluated *in vitro* by using both the human chronic myeloid leukaemia K562 and the T-lymphoblastoid Jurkat cell lines. The results obtained are summarized in Table 1 and compared to antiproliferative effects of the natural product distamycin A, and the PBD methyl ester **10.**

Table 1. *In vitro* biological effects of distamycin A (1), DC-81 (5), PBD methyl ester 10 and PBD-polypyrrolic hybrids 6-9 on K562 and Jurkat cell lines

Compound	IC_{50} (μM)	
	Jurkat	K562
Distamycin A (1)	20	12
DC-81 (5)	2.2	1
10	3	1.5
6	80	>100
7	50	6
8	0.8	0.7
9	0.07	0.04

IC_{50}= compound concentration required to inhibit tumor cell proliferation by 50%.

The results obtained demonstrate that the hybrids (**6-9**) exhibit different DNA-binding activity with respect to both distamycin A **1** and PBD **10**. In addition, a direct relationship was found between number of pyrrole rings present in the hybrids (**6-9**) and stability of drugs/DNA complexes. With respect to antiproliferative effects, it was found that the increase in the length of the polypyrrole backbone led to an increase of in vitro antiproliferative effects, i.e. the hybrid **9** containing the four pyrroles distamycin analogue, was more active than (**8**) both against K562 (IC_{50} μM, 0.07 vs. 0.8, respectively) and Jurkat (IC_{50} μM, 0.04 vs. 0.7, respectively) cell lines. On regard of the above mentioned derivatives **8** and **9**, it can be observed that they are much more active than distamycin A (**1**). Only derivatives **8** and **9** retain a higher antiproliferative activity when compared to PBD **10** alone. In fact, compounds **6**, 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, **7**, containing two pyrrole moieties, exhibited to some extent antiproliferative activity on the Jurkat cell line (IC_{50} 6μM), being scarcely active on K562 cells (IC_{50} 50 μM). DC-81 was found to retain antiproliferative activities similar to those exhibited by compound **10**.

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 A-T rich sequences of DNA, is crucial for antiproliferative effects of PBD-distamycin related compounds. Accordingly, the tri- and tetrapyrrole hybrids **8** and **9** are the most potent antiproliferative compounds of this series, exhibiting probably higher binding affinities with respect to the mono- and dipyrrole conjugates **6** and **7**, due to additional amido hydrogen bonds and van der Waals interactions.

These data 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 additional binding sites than

distamycin, as well as to an increase in the stability of drugs/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.

The PBD-distamycin hybrid **8** is also able to inhibit the DNA binding of the transcription factor Sp1.[11] The results obtained demonstrate that treatment of Sp1 target DNA with PBD-distamycin hybrid **8** renders the site unrecognizable by nuclear proteins. These data are in our opinion of interest, since transcription factors belonging to the Sp1 superfamily are very important for the control of transcription of cellular and viral genes, including the oncogenes Ha-ras and c-myc, the collagen-α1 (I) gene and the human immunodeficiency type 1 virus (HIV-1).[12]

The hybrid compounds **6-9** are also strong inhibitors of *in vitro* and *ex in vivo* transcription directed by the long terminal repeat (LTR) of HIV-1.[13] The higher inhibition of HIV-1 LTR directed transcription by these hybrid molecules was observed for the derivatives with three and four pyrrole moiety (compounds **8** and **9**, respectively) and it was mainly due to an increase in the stability of drugs/DNA complexes. With respect to the possible use of these compounds for therapeutic anti-HIV approaches, *in vivo* toxicity should be carefully analysed. As we have reported before, unfortunately, compounds **8** and **9** exhibit high antiproliferative activity, and therefore are expected to be toxic when administrated to cells. However, compounds containing lower numbers of pyrrole moieties could still exhibit biological activity, displaying at the same time low antiproliferative effects. In this context, the two pyrroles hybrid **7** appears of great interest, since it exhibits inhibitory effects on HIV-1 driven transcription, but low inhibitory effects on cell growth of Jurkat cells, and, therefore, could be proposed in further experiments (including bioavailability, gene expression profiling and in vivo toxicity) aimed at developing novel anti-HIV agents.

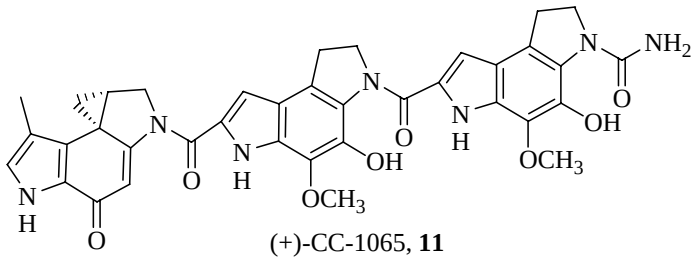
The potential utility of the hybrids **7-9** as future chemotherapeutics for the AIDS therapy was confirmed by their ability as RNA-binding drugs, which can interrupt protein/TAR-RNA interactions and Tat–induced LTR-driven HIV-transcription. [14] Tat is a regulatory protein that is required to induce high-level transcription of the HIV-1 genome after binding to a structured TAR-RNA. Tat-induced transcription of the HIV-genome is strongly enhanced by the interaction of the human cyclin T1 with the loop region of structured TAR-RNA. For this reason, inhibition of this complex assembly may represent a new target for anti-HIV compounds. Among the active molecules, compound **7** appears to be of interest, since it exhibits the lowest cytotoxic profile.

2.2 CPI-distamycin A hybrids

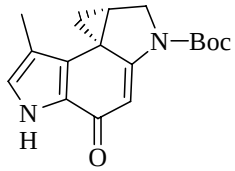
It has also been evaluated the synthesis of a series of hybrids which represent a molecular combination of polypyrrole minor-groove binders structurally related to the natural antitumor agent distamycin A, and two pyrazole analogues of the left hand segment called cyclopropylpyrroloindole (CPI) of the potent antitumor antibiotic (+)-CC-1065.[15]

(+)-CC-1065 (**11**) (Figure 3) is a member of the class of cyclopropylindole antitumor antibiotics firstly isolated from *Streptomyces zelensis* by scientists at the Upjohn Company,[16] and

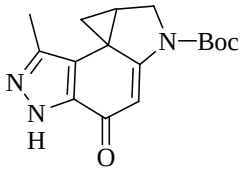
it is a highly potent natural agent with activity both *in vitro* and in experimental animals.[17,18] Studies on the mechanism of cytotoxic action have shown that CC-1065 affords its biological activity through binding to double-stranded B-DNA within the minor groove at AT-rich sequences and selectively alkylating at the N3 position of the 3'-adenine by its cyclopropylindole (CPI) subunit **12**.[19] 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.[20]



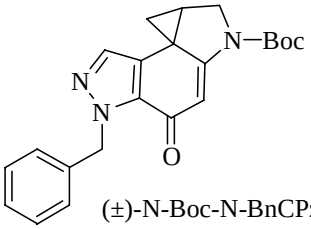
(+)-CC-1065, **11**



(+)-N-Boc-CPI, **12**



(±)-N-Boc-CPzI, **13**

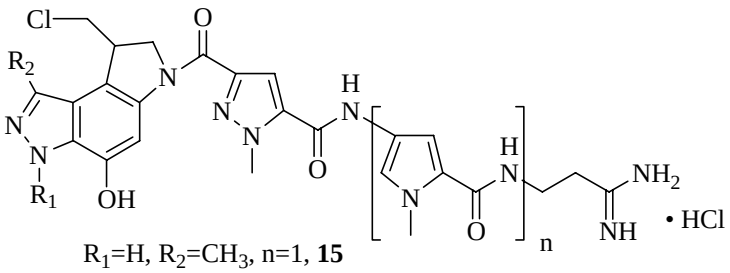


(±)-N-Boc-N-BnCPzI, **14**

Figure 3. Chemical structures of natural compounds (+)-CC-1065 (11), (+)-*N*-Boc-CPI (12), (±)-*N*-Boc-CPzI (13) and (±)-*N*-Boc-N-BnCPzI (14)

Several years ago our group has synthesized two CPI pyrazole analogues named (±)-*N*-Boc-CPzI **13**[21,22] and (±)-*N*-Boc-N-BnCPzI **14**,[23] which demonstrate a cytotoxicity against L1210 leukemia cells that was comparable to or 10-fold lower than, respectively, that of the reference compound *N*-Boc-CPI **12** [IC_{50}=330 nM for (+)-*N*-Boc-CPI vs. IC_{50}=370 nM for (±)-**13** and IC_{50}=3064 nM for (±)-**14**]. 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.

In synthesizing these novel water-soluble hybrids, we wanted to increase the potency of pyrazole CPI analogues **13** and **14** by increasing their affinity for DNA and to determine the structure-activity relationship between the length of the oligopyrrolic frame, antitumor activity and sequence specificity.



R_1=H, R_2=CH_3, n=1, **15**
R_1=H, R_2=CH_3, n=2, **16**
R_1=H, R_2=CH_3, n=3, **17**
R_1=Bn, R_2=H, n=1, **18**
R_1=Bn, R_2=H, n=2, **19**
R_1=Bn, R_2=H, n=3, **20**

Figure 4. Chemical structures of hybrid compounds 15-20 comprising either two CPI pyrazole analogues (13 and 14) and three mixed pyrazole-pyrrole lexitropsins structurally related to distamycin A

These hybrids compounds **15-20** have obtained coupling the two N-Boc deprotected CPI pyrazole analogues **13** and **14** with three mixed pyrazole-pyrrole compounds 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 (Figure 4).

As evident from Table 2, it was found that tethering the pyrazole CPI analogues **13** and **14** to the DNA-binding lexitropsins afforded, with few exceptions, conjugate molecules that showed enhanced cytotoxic activity against five different cancer cell lines *in vitro*.

The results show that the hybrids **15-17** were about 8- to 70-fold more potent than the alkylating unit **13**. Among these, the hybrid **17** 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 **17** was 2- to 8-fold more active than the bis and monopyrrole counterparts (compounds **16** and **15**, respectively). This is presumably due to the increased DNA binding of the 'longer' compounds. The IC_{50} for **16** 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 **17** 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, increasing the number of pyrrole rings from one to three results in increased cytotoxic activity.

Table 2. *In vitro* activity of alkylating units 13 and 14 and hybrids 15-20 against the proliferation of five different cancer cell lines

	IC_{50} (nM±S.E.)				
Compound	L1210	FM3A	Molt/4	CEM	Daudi
13	520±6.6	1,400±40	1,740±50	1,260±30	680±150
14	2710±490	18,300±200	8,550±280	6,720±1,040	7,520±30
15	58+17	1600±50	340±20	230±10	150±40
16	19±2	190±6	45±1	39±1	22±10
17	7.4±0.4	31±11	17±4	71±9	8.8±0.1
18	240±30	4,000±1000	130±20	70±21	11±6.0
19	600±90	5600±1400	160±60	210±110	38±7.0
20	400±16	19,300±3,400	310±70	400±50	100±10

IC_{50}= compound concentration required to inhibit tumor cell proliferation by 50%.

The hybrids **18-20** 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 **14** alone (with only few exceptions). The FM3A cell line was substantially less sensitive to the hybrids **18-20**, which exhibited cytotoxic activities comparable or 3-fold higher (IC_{50} values ranging from 4 to 19 μM) than that of the alkylating unit **14** alone. In the CEM cell line, compounds **18-20** demonstrated IC_{50} values between 70-400 nM, which was 30- to 100-fold higher than that reported for **14**, with compound **18** exhibiting potent cytotoxicity (IC_{50} of 70 nM).

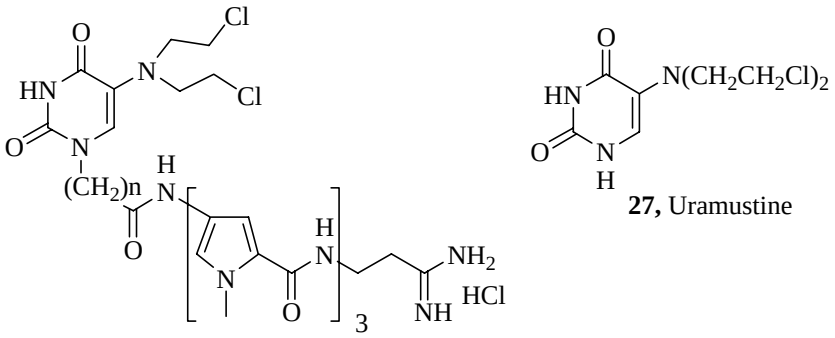
A fairly marked dependence on the number of pyrrolic rings for the antiproliferative activity has been observed in the **15-17** series with compound **17** comprising three pyrrolic rings proving to be the most active. The relationship between the number of pyrrole groups in the **18-20** series and their corresponding cytotoxicity did not seem to follow this pattern. In fact, the cytotoxicity was higher for the compound **18**, 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 **20** than FM3A cells. For all cell lines, taken together, compounds possessing the same number of pyrrole rings and the alkylating unit **13** appeared to be more cytotoxic than those containing **14** as the alkylating agent.

In addition, high-resolution denaturating gel electrophoresis experiments have been performed on the hybrid compounds **15-20**. When compounds **18-20** were incubated at different concentrations (ranging from 0.1 μM to 100 μM), no significant cleavage was observed by thermally induced strand cleavage of DNA fragment. For these derivatives, the presence of a benzyl group at the azaindole moiety 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 **18**-**20**, hybrids **15-17** show unique DNA sequence-selective alkylation in AT-rich sequences. High-resolution denaturating gel electrophoresis indicated that **17** selectively alkylates the third adenine of the 5'-ACAAAAATCG-3' motif within a 400 bp DNA fragment, the strongest and most highly sequence-specific DNA alkylation activity observed. This compound elicited the strongest and most highly sequence-specific DNA alkylation activity. For compound **17**, DNA alkylation was observed even at 50 nM. 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.

2.3 Uramustine-distamycin A hybrids

An approach, which has proved to be particularly successful, is represented by a novel series of hybrid molecules **21-26**, namely a molecular combination of the natural antibiotic distamycin A and the antineoplastic agent uramustine. Uramustine (uracil mustard) **27** is an inexpensive oral alkylating agent that has been effective in the treatment of patients with lymphosarcoma,[24,25] chronic lymphatic leukaemia[26] and thrombocythemia.[27] Uramustine interacts in GC rich regions being able to alkylate guanine-N7 in 5'-PyGCC-3' (Py=pyrimidine) sequences.[28-30]



27, Uramustine

n=1, **21**
n=2, **22**
n=3, **23**
n=4, **24**
n=5, **25**
n=6, **26**

Figure 5. Hybrids between Uramustine and Distamycin A

This homologous series **21-26** consisted of the minor groove binder distamycin A joined to uramustine (uracil mustard) by suitable aliphatic carboxylic acid moieties containing a flexible polymethylene chain which is variable in length [$(CH_2)_n$, where n=1 up to 6]. [31] A flexible polymethylene spacer was chosen, allowing the nitrogen mustard of uramustine to interact more closely with the DNA target. All the hybrid compounds in this series exhibit enhanced activity compared both to distamycin A and uramustine, giving IC_{50} values in the range 7.26-0.07 μM on human leukemic K562 cells, with maximal activity shown when n=6 (Table 3). Compounds **21-23** with methylene linkers of 1-3, respectively, gave similar IC_{50} values in the low micromolar range. The distance between the uramustine and distamycin frame is crucial for the cytotoxicity, in fact as the size of the linker spacer increases from n=4 to 6, the cytotoxic activity is also enhanced, with IC_{50} values in the range 0.07-0.14μM. Compound **26** with the longest linker length in the series was the most active compound with an IC_{50} value greater than 1000 times lower than that for distamycin in this same screen. In summary, there was a large increase in cytotoxicity across the homologous series that can not be explained entirely by changes in mustard reactivity and may be related to altering orientation of the mustard with respect to the DNA.

Table 3. *In vitro* activity of Distamycin A, Uramustine and hybrid compounds 21-26 against K562 human leukemia cell line

Compound	*in vitro* IC_{50} (μM±S.E.)
Distamycin	>100
Uramustine	5.1±0.6
21	4.06±1.03
22	2.54±2.23
23	7.26±5.88
24	0.11±0.02
25	0.14±0.05
26	0.07±0

IC_{50}= compound concentration required to inhibit tumor cell proliferation by 50%

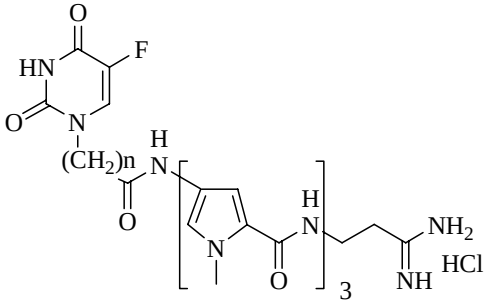
DNase I footprinting experiments showed that selective covalent binding of uramustine-distamycin hybrids **21-26** to A+T rich DNA sequences with a non-covalent binding specificity is identical to that observed for distamycin and tallimustine. The compounds **24-26** were more effective at producing footprints than **21-23**. These corresponded to the sequences 5'-TTTTTG, AAAACG, and TTTTTA, respectively, with the alkylation on the last base (G/A). All six derivatives bound at these sequences, but the relative extent of alkylation at these sites differed between compounds.

In the series of compounds **21-26** progressive enhancement in cytotoxic potency with the length of the polymethylene chain does not correspond to increased alkylation intensity. Instead, this observation is tentatively attributed to enhanced cellular uptake due to increased lipophilicity as the polymethylene chain extends. Two consequences can be derived from our study: a) the distamycin moiety acts binding to A/T reach DNA sequence and, consequently, is responsible for the alkylation regioselectivity found in footprinting studies; b) the higher flexibility due to a longer linker between the distamycin and uracil moieties allows the formation of complexes with the mustard moiety situated more deeply into the minor groove and, hence, with a better alkylating properties.

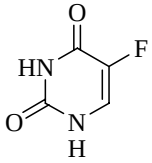
2.4 5-Fluorouracil-distamycin A hybrids

In some cases the hybrid drug approach apparently failed to present practical significant advantages in terms of activity, while in some cases these derivatives even lost the activity of the antitumor moiety, as occurred e.g. in a case concerning the tethering of anticancer drug 5-fluorouracil to a DST-like frame (Figure 5, compounds **28-33**).[32] 5-Fluorouracil (5-FU, compound **34**) is a well known antimetabolite, used for the treatment of several malignances (e.g. breast cancer, tumors of the gastrointestinal tract and other solid tumors),[33] which express, after intracellular glycosylation, its antimetabolic potential by irreversible alkylation of thymidylate synthase (TS).[34]

This homologous series **28-33** consisted of the oligopeptides distamycin A joined to 5-fluorouracil by aliphatic carboxylic acid moieties containing a polymethylene chain [$(CH_2)_n$, where n=1 up to 6). In these derivatives, 5-FU contains an C-6 electrophilic center, which can be positioned near nucleophilic centers on DNA, such as N3 of adenine in the minor groove or N7 of guanine in the major groove. A flexible polymethylene spacer was chosen, to allow 5-FU to accommodate closer to the DNA target. The hybrids **28-33** were tested *in vitro* against the human chronic myeloid leukemia K562 cell line and compared to distamycin A.



n=1, **28**
n=2, **29**
n=3, **30**
n=4, **31**
n=5, **32**
n=6, **33**



34, 5-Fluorouracil

Figure 6. Hybrid compounds between 5-Fluorouracil and Distamycin A

The derivative **28,** which possess the shorter spacer, is completely inactive. With respect to compounds **29-33**, it is interesting to note that the modification in the length of the polymethylene spacer [$(CH_2)_n$, with n=2-6] between the pyrrole backbone of the distamycin and the 5-FU do not have major effects on the antitumor activity, which appear to be either less or comparable with that of distamycin A (Table 4).

Table 4. *In vitro* antiproliferative activity of hybrids 28-33 against K562 human leukemia cells

Compound	*in vitro* IC_{50} (μM)
Distamycin	20
28	>200
29	23
30	82
31	153
32	50
33	50

IC_{50}= compound concentration required to inhibit tumor cell proliferation by 50%

Only compound **29** shows a cytotoxic activity comparable than that distamycin A, and increasing the length of the polymethylene chain from three to six does not induce large changes in the activity. In fact, the compounds **30-33** proved to be the less active with IC_{50} values of 82, 153, 50 and 50 μM, respectively. For these compounds the *in vitro* antitumor activity was not influenced by the length of the -$[CH_2]_n$ linker, being comparable to that of the parent compound distamycin A. Arrested polymerase-chain experiments demonstrated selective binding of the 5-fluorouracil-distamycin hybrids to A+T rich DNA sequences.

3. Conclusions

In the last years several hybrid compounds in which known antitumor moieties have been tethered to distamycin frame, have been described in the literature and are still an interesting class of DNA ligands which might have a therapeutic role in cancer. The nature of antitumor agents and therefore also the rationale that led to these compounds were different. In general the interaction with DNA tends to be dominated by the minor groove binding moiety, i.e. the conjugates bind to the minor groove with preferential interaction with AT-rich sequences. Until the end of last century, the hybrid approach did not receive much attention, because it failed to present practical significant advantages in terms of activity. In fact, in some cases these hybrid derivatives even lost the activity of the antitumor moiety, as occurred when the antimetabolite 5-fluorouracil has been joined to distamycin-like frame (Figure 5, compounds **28-33**). The present

work demonstrated the validity of hybrids approach between distamycin A (as minor groove binder) and derivatives of naturally occurring antitumor antibiotics with DNA-alkylating properties. It may be noted that, in general, the cytotoxicity of these hybrid derivatives was much greater than that of the alkylating units alone. The hybrid approach with the nitrogen mustard derivative uramustine has proved so far to be particularly advantageous in terms of activity and represent an important model for the design of new cytotoxic minor groove binders. It was, in fact, disclosed the possibility of obtaining potent agents by combining moieties of mild cytotoxic activity with a DNA binding-frame derived from distamycin A, acting as a sequence-selective vector. Also in this case the interaction with DNA tends to be dominated by the minor groove binding moiety distamycin A.

Acknowledgments

We wish to thank Ministero Università e Ricerca Scientifica (MIUR) for generous financial support for this work.

References and Notes

1. D'Incalci, M.; Sessa, C. *Exp. Opin. Invest. Drugs* **1997**, *6*, 875.
2. Arcamone, F.; Penco, S.; Orezzi, P.G.; Nicolella, V.; Pirelli, A. *Nature* **1964**, *203*, 1064.
3. Pelton, J.G., Wemmer, D.E. *J. Am. Chem. Soc.* **1990**, *112*, 1393.
4. Baraldi, P.G.; Bovero, A.; Fruttarolo, F.; Preti, D.; Tabrizi, M.A.; Pavani, M.G.; Romagnoli, R. *Med. Chem. Rev.* **2004,** *24*, 475.
5. Tietze, L.F.; Bell, H.P.; Chandrasekhar, S. *Angew. Chem. Int. Ed.* **2003**, *42*, 3996-4008.
6. Thurston, D.E.; Bose, D.S. *Chem. Rev.*, **1994**, *94*, 433.
7. Kopka, M.L.; Goodsell, D.S.; Baikalov, I.; Grzeskowiak, K.; Cascio, D.; Dickerson, R.E. *Biochemistry*, **1994**, *33*, 13593.
8. Baraldi, P.G.; Cacciari, B.; Guiotto, A.; Leoni, A.; Romagnoli, R.; Spalluto, G.; Mongelli, N.; Thurston, D.E.; Bianchi, N.; Gambari, R. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3019.
9. Baraldi, P.G.; Balboni, G.; Cacciari, B.; Guiotto, A.; Manfredini, S.; Romagnoli, R.; Spalluto, G.; Howard, P.H.; Thurston, D.E.; Bianchi, N.; Rutigliano, C.; Mischiati, C.; Gambari, R. *J. Med. Chem.* **1999**, *42*, 5131.
10. Damayanthi, Y.; Praveen Reddy, B.S.; Lown, J.W. *J. Org. Chem.* **1999**, *64*, 290-292.
11. Baraldi, P.G.; Cacciari, B.; Guiotto,A.; Romagnoli, R.; Spalluto, G.; Leoni, A.; Bianchi, N.; Feriotto, G.; Rutigliano, C.; Mischiati, C.; Gambari, R. *Nucleosides, Nucleotides & Nucleic Acids* **2000**, *19*, 1219.
12. Roebuck, K.A.; Saifuddin, M. *Gene Expr.* **1999**, *8*, 67.

13. Borgatti, M.; Rutigliano, C.; Bianchi, N.; Mischiati, C.; Baraldi, P.G.; Romagnoli, R.; Gambari, R. *Drug Dev. Res.* **2003**, *60*, 173.
14. Mischiati, C.; Finotti, A.; Sereni, A.; Boschetti, S.; Baraldi, P.G.; Romagnoli, R.; Feriotto, G.; Jeang, K.T.; Bianchi, N.; Borgatti, M.; Gambari, R. *Biochem. Pharmacol.* **2004**, *67*, 401.
15. Baraldi, P.G.; Balboni, G.; Pavani, M.G.; Spalluto, G.; Tabrizi, M.A.; De Clercq, E.; Balzarini, J.; Bando, T.; Sugiyama, H.; Romagnoli, R. *J. Med. Chem.*, **2001**, *44*, 2536**.**
16. Hanka, L.J.; Dietz, A.; Gerpheide, S.A.; Kuentzel, S.L.; Martin, D.G. *J. Antibiot.* **1978**, *31*, 1211.
17. Martin, D.G.; Biles, C.; Gerpheide, S.A.; Hanka, L.J.; Kroeger, W.C.; Mc Govren, J.P.; Mizsk, S.A.; Neil, G.L.; Stewart, J.C.; Visser, J. *J. Antibiot.* **1981**, *34*, 1119.
18. Bhuyan, B.K.; Newell, K.A.; Crampton, S.L.; Von Hoff, D.D. *Cancer Res.* **1982**, *42*, 3532.
19. Reynolds, V.L.; Molineaux, I.J.; Kaplan, D.J.; Swenson, D.H.; Hurley, L.H. *Biochemistry*, **1985**, *24*, 6220.
20. Mc Govren, J.P.; Clarke, G.L.; Pratt, E.A.; De Koning, T.F. *J. Antibiot.* **1984**, *37*, 63.
21. Baraldi, P.G.; Cacciari, B.; Pineda de Las Infantas, M.J.; Romagnoli, R.; Spalluto, G.; Cozzi, P.; Mongelli, N. *Anti-Cancer Drug Des.* **1997**, *12*, 67.
22. Baraldi, P.G.; Cacciari, B.; Romagnoli, R.; Spalluto, G.; Gambari, R.; Bianchi, N.; Passadore, M.; Ambrosino, P.; Mongelli, N.; Cozzi, P.; Geroni, C. *Anti-Cancer Drug Design*, **1997**, *12*, 555.
23. Baraldi, P.G.; Cacciari, B.; Guiotto, A.; Romagnoli, R.; Spalluto, G.; Zaid, A.N.; Capolongo, L.; Cozzi, P.; Geroni, C.; Mongelli, N. *Il Farmaco*, **1997**, *52*, 717.
24. Berry, D.H.; Sutow, W.W.; Vietti, T.J.; Fernbach, D.J.; Sullivan, M.P.; Haggard, M.E.; Lane, D.M. *J. Clin. Pharmacol. New Drugs*, **1972**, *12*, 169.
25. Fernbach, D.J.; Haddy, T.B.; Holcomb, T.M.; Lusher, J.; Sutow, W.W.; Vietti, T.J. *Cancer Chemother. Rep.* **1968**, *52*, 287.
26. Sokal, J.E. *Cancer*, **1972**, *30*, 1275.
27. Robertson, J.H. *Blood*, **1970**, *35*, 288.
28. Doweyko, A.M.; Mattes, W.B. *Biochemistry* **1992**, *31*, 9388.
29. Hartley, J.A.; Bingham, J.P.; Souhami, R.L. *Nucleic Acids Res.*, **1992**, *20*, 3175.
30. Mattes, W.B.; Hartley, J.A.; Kohn K.W. *Nucleic Acids Res.* **1986**, *14*, 2971.
31. Baraldi, P.G.; Romagnoli, R.; Entrena Guadix, A.; Pineda de las Infantas, M.J.; Gallo, M.A.; Espinosa, A.; Martinez, A.; Bingham, J.P.; Hartley, J.A. *J. Med. Chem.* **2002**, *45*, 3630.
32. Baraldi, P.G.; Romagnoli, R.; Martinez, A.; Pineda de las Infantas, M.J.; Gallo, M.A.; Espinosa, A.; Rutigliano, C.; Bianchi, N.; Gambari, R. *Med. Chem. Res.*, **2001**, *10*, 390.
33. Ozaki, S. *Med. Res. Rev.*, **1996**, *16*, 51.
34. Santi, D.V.; Mc Henry, C.S.; Somer, H. *Biochemistry*, **1974**, *13*, 471.