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CARBOCYCLIC ANALOGUES OF LEXITROPSIN – DNA AFFINITY AND ENDONUCLEASE INHIBITION

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Abstract: A DNA-binding affinity and the effect on restriction enzymes activity of seven carbocyclic mono- and bis-lexitropsins and two analogues of pentamidine with unsubstituted N-terminal amine group were investigated. DNA association constants (K_{app}) show that DNA affinity of mono-compounds is much weaker than netropsin and distamycin. Bis-analogues of netropsin bind DNA more strongly than mono-ligands, but without sequence-selectivity. Only pentamidine derivatives reveal preference to AT-rich sequence. The studied compounds can inhibit catalytical action of endonucleases recognizing sequence of four AT base pairs following one another.

Keywords: netropsin, bis-netropsin, DNA-binding, inhibition of endonuclease

The rapidly increasing knowledge in molecular biology affords possibilities for observation that most of antitumor drugs belong to the large family of sequence-specific ligands nonintercalatively binding within minor groove of B-DNA (Figure 1). This interaction results in occupancy of natural nucleic sequences targeted by enzymes or in DNA distortion nearby these sites. The cytotoxic effect of these antineoplastic agents and inhibition of many cellular processes is determined mainly by interference with catalytic activity of important regulatory proteins, such as topoisomerase I and II and a number of proteases (1). Most of the minor groove DNA-binding drugs, such as netropsin, pentamidine, Hoechst 33258 exhibit high sequence selectivity. They occupy the binding sites containing a run of at least four AT nucleic pairs. The complex DNA-drug is stabilized by hydrogen bonds, ionic charge attractions, as well as Van der Waals interactions (1). Lexitropsins are synthetic analogues of *Streptomyces* antibiotics – netropsin and distamycin A – and instead of N-methylpyrrole rings contain different aromatic rings. This replacement changes DNA sequence selectivity of these compounds in comparison with netropsin and distamycin (2, 3). The extension of molecules, both by increasing the number of amide unit and by the formation of dimers from the shorter "oligopeptides", gives compounds displaying higher DNA affinity and biological activity. The carbocyclic lexitropsins in comparison with netropsin and distamycin show decreased toxicity, increased antibacterial, antiviral and antitumor activity (4) and similar AT specificity, although they reveal reduced DNA affinity (5, 6). Moreover, bis-arylamines and bisdiazonium salts cleave DNA with much sequence-selectivity (7, 8).

In the course of our investigation of carbocyclic lexitropsins (9, 10) the effect of compounds **1**-**9** (Figure 2) on the activity of different restriction endonucleases (11, 12) and their influence on ethidium bromide-DNA complexes were studied (13). The presented compounds inhibit catalytical activity of topoisomerase I and II and show antiproliferative effect in the standard cell line of mammalian tumor MCF7 (10).

EXPERIMENTAL

Materials

Ethidium bromide, pBR322 and pUC19, calf thymus DNA, homopolymers poly(dA-dT)$_2$, and poly(dG-dC)$_2$, were purchased from Sigma, whereas restriction enzymes – from MBI Fermentas. Compounds **1**-**9** were prepared as hydrochloride salts (10). The stock solutions of these ligands were adjusted to pH range 7.5-8.0: 10 mg of substance was dissolved in 200 μL of DMSO and 600 μL of distilled H_2O, 0.1 M NaOH was added to given solu-

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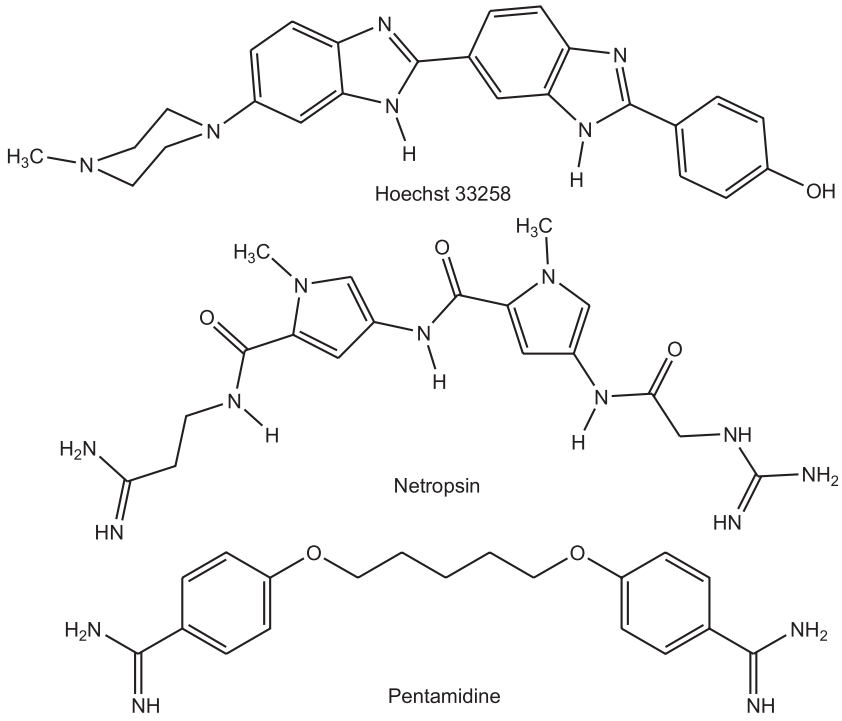

Figure 1. DNA minor groove binders.

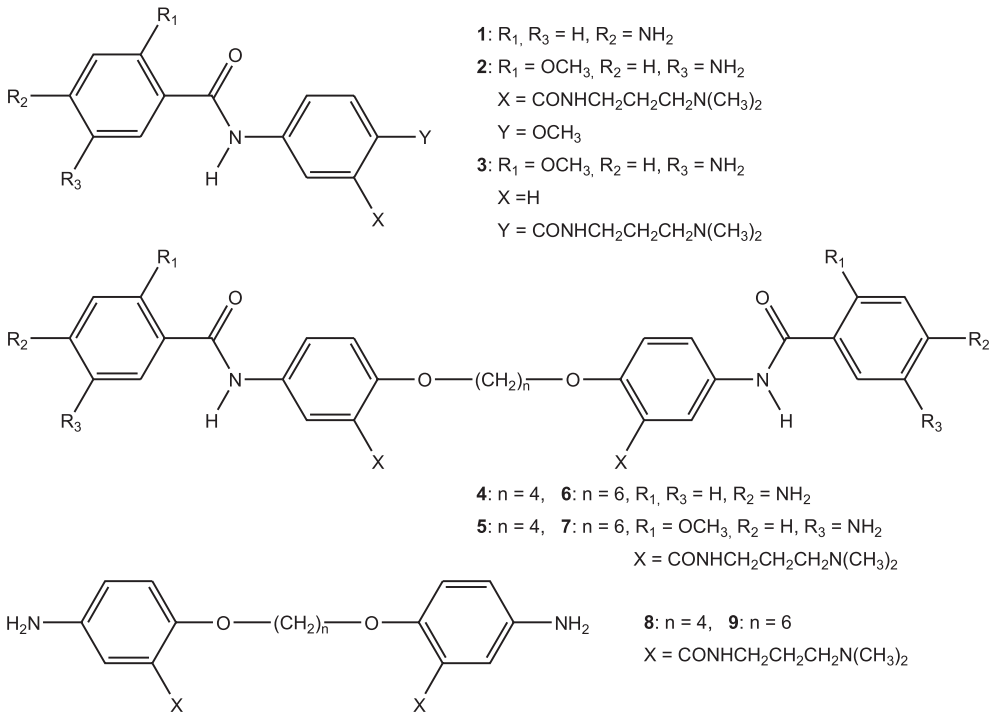

Figure 2. Structure of compounds **1**-**9**.

tion until pH=7.5, and the appropriate volume of distilled water was added to make a 10 mM solution of the tested compounds.

Ethidium displacement assay

The fluorescence was measured on Hitachi spectrophotometer F-250 FL at room temperature. DNA solution (25 μL A_{260} = 2) was added to 2 mL of ethidium bromide buffer (5.0×10^{-6} M in 10 mM Tris-HCl, pH 7.4, and 75 mM NaCl) and fluorescence was measured at λ = 546 nm excitation and 595 nm emission. The stock solutions (10 mM) of tested compounds were added titrimetrically to the ethidium bromide-DNA complexes, and the fluorescence was measured after each addition until the fluorescence was reduced to 50%. The apparent binding constants were calculated from:

$$K_{EtBr}(EtBr) = K_{app}(drug),$$

where (drug) = the concentration of tested compound at a 50% reduction of fluorescence and K_{EtBr} and (EtBr) are known. The binding constants of ethidium bromide to calf thymus DNA, poly(dA-dT)$_2$ and poly(dG-dC)$_2$ are 1.0×10^7 M^{-1}, 9.5×10^6 M^{-1}, and 9.9×10^6 M^{-1}, respectively (13).

Endonuclease inhibition assay

Plasmid pBR322 (0.2 mg) or pUC19 (0.2 mg) was linearized with 5 units of ScaI (reaction buffer: 10 mM Bis-Tris propane-HCl, pH 6.5, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/mL BSA) or with 5 units of HindIII (reaction buffer: 10 mM Tris HCl, pH 8.5, 10 mM $MgCl_2$, 100 mM KCl, 0,1 mg/mL BSA) by incubation at 37°C for 1 h. After the disactivation of the enzymes at 65°C for 20 min, compounds **1**-**9** (2 mL of 1 mM) were added, and the samples were incubated at 37°C for 1 h. After that 5 units of restriction nuclease (reaction buffer recommended by the producer) were added to each sample. The mixture was incubated at 37°C for 1 h, and the enzymatic action was terminated at 80°C after 20 min. The reaction mixture was subjected to electrophoresis (4 h, 50 V) on 1% agarose gel, containing 1% of ethidium bromide, in the standard TBE buffer. The gels were photographed under UV light (312 nm, UVP Transilluminator). The final concentration of compounds **1**-**9** was 200 mM.

RESULTS AND DISCUSSION

Compounds **1** and **3**, the benzene analogues of netropsin, in which p-aminobenzoic and 5-amino-2-methoxybenzoic acid are coupled by amide bonds, differ in the sequence of di- and tri-substituted benzene rings. Molecular modelling shows that compound **2** is isohelical with DNA minor groove (14). Ligands **1** and **3** may adopt a crescent conformation like **2**, although the presence of p-substituted benzene rings in their molecules slightly changes the radius of this shape in comparison with **2**, and probably it could influence their DNA affinity. Compounds **4**-**7** are the analogues of bis-netropsin, where both **1** and **2** are linked by aliphatic tetra- and hexamethylene chains. The flexible linker should permit them a better fit and antiparallel orientation of their subunits within the minor groove (15, 16). Compounds **8** and **9** are similar to aromatic bis-amidines and they were used mainly as semisubstrates to synthesis of **4**-**7**.

Table 1. Association constants ($K_{app} \times 10^5$ M^{-1}) of ligands **1-9** with polynucleotides.

Ligand	Calf thymus DNA	Poly(dA-dT)$_2$	Poly(dG-dC)$_2$
Ethidium bromide[a]	100.0	95.0	99.0
Netropsin[a]	8.7	875.0	2.5
Distamycin[a]	7.5	340.0	2.0
1	8.7	13.7	7.4
2[b]	3.0	14.3	1.7
3	12.8	24.0	20.8
4	71.7	75.9	111.4
5	148.3	82.9	73.9
6	not determined	200.5	171.1
7	277.5	211.4	252.2
8	not determined	8.2	1.3
9	0.2	12.2	1.5

[a] data from (13), [b] data from (9). The error for compounds 1, 3-9 is ± 0.2×10^5 M^{-1}.

Table 2. Influence of compounds **1**-**9** on endonuclease activity.

Enzyme	Recognized sequence	Ligands									
		Netropsin	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
VspI	ATTAAT	+	+	+	+	+	+	+	+	+	+
SmaI	CCCGGG	-	-	-	-	-	-	-	-	-	-
EcoRI	GAATTC	+	+	+	±	+	nm	+	nm	-	±
NdeI	CATATG	+	-	-	-	+	-	+	-	-	±
HindIII	AAGCTT	-	-	+	-	nm	nm	nm	nm	±	+
PaeI	GCATGC	-	-	-	-	-	-	-	-	-	-
PstI	CTGCAG	+	-	-	+	nm	nm	-	nm	-	-

+: inhibition of enzyme, ±: slight inhibition of enzyme, nm: no DNA migration

In order to identify the DNA affinity of reported compounds the DNA-binding to calf thymus DNA, poly(dA-dT)$_2$ and poly(dG-dC)$_2$ was studied. The respective DNA association constants (K_{app}) of compounds **1**, **3** and **4**-**9** were determined by means of well established ethidium bromide displacement experiment (13), and were compared to those of netropsin, distamycin and **2** (9). Compound **2** and its derivative with a chlorambucil moiety show antiproliferative and cytotoxic effect on cultured breast cancer MCF-7 cells. These compounds bind to AT-rich sequences with high selectivity, though more weakly than the extensively studied minor groove binders, such as netropsin and distamycin (9, 17). In the case of compound **6** and **8,** IC_{50} of fluorescence of ethidium bromide-calf thymus DNA complexes were not achieved and association constants were not determined. The data in Table 1 demonstrate that all the compounds can bind to the studied DNAs more weakly than netropsin and distamycin. Compounds **1** and **3** have better binding affinity but lower selectivity between AT and GC sequences than **2**. Ligands **8** and **9** show similar sequence selectivity in comparison with compound **2**, which is, however, high compared to that of compounds **1**, **3** and **4**-**7**, whose strength of binding to poly(dA-dT)$_2$ and to poly(dG-dC)$_2$ is equal. On the other hand, ligands **4**-**7** as dimers of **1** and **2**, as expected, bind to DNA stronger than parent compounds. The presence of p-substituted benzene rings instead of tri-substituted ring in mono-compounds slightly intensifies the DNA-ligand interactions, however, does not improve the sequence selectivity. In the case of bis-compounds, the relationship between their structure and DNA binding can not be estimated. The fact that compounds **4**-**7** are more potent than **1** and **2** indicates that DNA affinity of the studied compounds is proportional to the number of repeating benzene carboxamide units. This is confirmed by the effect of these compounds on the activity of topoisomerase I and II (10).

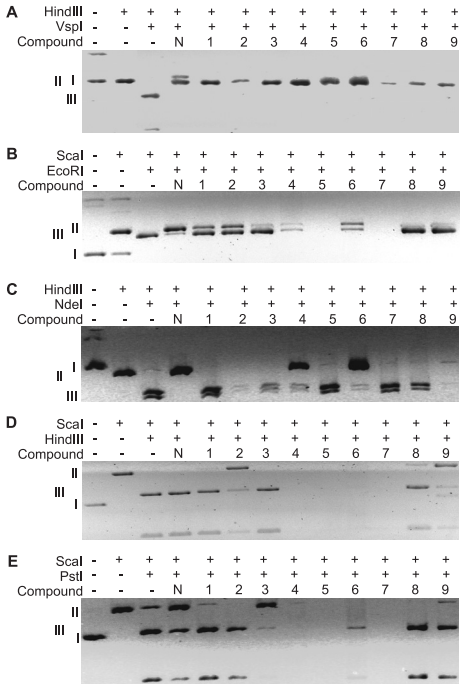

Figure 3. Effect of compounds **1**-**9** on activities of selected nucleases.

A. Cleavage of pBR322 by VspI. Lane 1: DNA, lane 2: linearized DNA by HindIII, lane 3: DNA + HindIII + VspI, lane 4: DNA + HindIII + netropsin (N) + VspI, lanes 5-13: DNA + HindIII + compounds **1**-**9**, respectively + VspI.

B. Cleavage of pUC19 by EcoRI. Lane 1: DNA, lane 2: linearized DNA by ScaI, lane 3: DNA + ScaI + EcoRI, lane 4: DNA ScaI + netropsin (N) + EcoRI, lanes 5-13: DNA + ScaI + compounds **1**-**9**, respectively + EcoRI.

C. Cleavage of pBR322 by NdeI. Lane 1: DNA, lane 2; linearized DNA by HindIII, lane 3: DNA + HindIII + NdeI, lane 4: DNA + HindIII + netropsin (N) + NdeI, lanes 5-13: DNA + HindIII + compounds **1**-**9**, respectively + NdeI.

D. Cleavage of pUC19 by HindIII. Lane 1: DNA, lane 2: linearized DNA by ScaI, lane 3: DNA + ScaI + HindIII, lane 4: DNA + ScaI + netropsin (N) + HindIII, lanes 5-13; DNA + ScaI + compounds **1**-**9**, respectively + HindIII.

E. Cleavage of pUC19 by PstI. Lane 1: DNA, lane 2: linearized DNA by ScaI, lane 3: DNA + ScaI + PstI, lane 4: DNA + ScaI + netropsin (N) + PstI, lanes 5-13: DNA + ScaI + compounds **1**-**9**, respectively + PstI.

Moreover, the influence of compounds **1**-**9** at concentration of 200 mM on the cleaving activity of restriction enzymes using a gel electrophoresis assay was established (11, 12). Figure 3 demonstrates selected results of the experiment. DNA, mainly as the supercoiled circle (form I) is linearized to form II by nuclease ScaI (lanes 2 on Fig. 3B, 3D and 3E) or HindIII (lanes 2 on Fig. 3A and 3C). In the presence of the second enzyme (lanes 3 on Fig. 3A-E) DNA is cleaved, and form III is the most visible. Table 2 shows the applied endonucleases, their recognizable DNA sequence and the activity of studied compounds compared with netropsin. The inhibition of the second enzymes by netropsin and compounds **1**-**9** was identified when form II of DNA was visible. In some cases DNA in the presence of compounds **4**-**7** did not migrate and remained in take-off pockets. Such results were considered to have no influence on endonuclease activity.

At the preliminary stage of the experiment, ligands were tested against two restriction enzymes: SmaI (data not shown on Fig. 3) and VspI cleaving the sequence of six G/C or A/T base pairs, respectively. Evidently, the studied compounds do not block GC-rich sequence. At the next stage, the cleavage of DNA by EcoRI and NdeI (four A, T base pairs) in the presence of compounds **1**-**9** was examined. Substances **1**, **2**, **4**, **6** (and **3**, **9** in a certain to insignificant extent) interfere with EcoRI activity, whereas compounds **4** and **6** affect NdeI activity. These results show that the studied compounds could reveal preference to continuous rather than alternating sequence of A and T. The observation of the products of DNA cleavage by PaeI (data not shown on Fig. 3) and HindIII leads to an assumption that **2**, **9**, and **8** could bind to AT-rich sequence with an embedded GC pair, but not to an inverted configuration of these nucleic pairs. In the case of PstI which recognizes the sequence of all nucleic bases following one another, only compound **3** is the one inhibiting the activity of this enzyme.

The obtained results indicate weak DNA affinity and sequence-specificity of studied compounds, although, most of these ligands have inhibitory influence on AT-specific enzymes. However, on the basis these results, it can not be affirmed that the inhibition of nuclease activity by the studied compounds arises from their DNA-binding or from simultaneous occupancy of nucleic sequences targeted by restriction enzymes.

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