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ORIGINAL ARTICLE

Carbocyclic potential DNA minor groove binders and their biological evaluation

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Abstract
The biological evaluation of carbocyclic minor groove binders **1–6** is described. The cytotoxicity of the obtained compounds was tested on MDA-MB-231 breast cancer cells. The mechanism of action of compounds **1–6** was studied employing the topoisomerase I/II inhibition assay and ethidium displacement assay using pBR322. Determination of association constants was done using calf thymus DNA, T4 coliphage DNA, poly(dA-dT)$_2$, and poly(dG-dC)$_2$. The effect of compounds **1–6** on the amidolytic activity of plasmin, trypsin, thrombin, and urokinase was also examined.

Keywords: *Pentamidine; netropsin; DNA topoisomerases; DNA-binding; antiamidolytic activity*

Introduction

The clinical significance of DNA-binding compounds can hardly be overstated, as many anticancer regimens include the compound that binds to and/or modifies DNA.

Netropsin, bis-netropsin, or bis-amidines (e.g. pentamidine) (Figure 1) have been extensively studied due to their ability to bind to the minor groove of the DNA double helix in a sequence-specific manner, and have served as models for biochemical and physical studies of drugs that bind to the DNA minor groove[1]. In particular, it has been shown that they bind DNA reversibly through hydrogen bonds, van der Waals contacts, and electrostatic interactions at sequences of four or more consecutive AT pairs, and strongly discriminate against GC pairs[2]. The rapidly increasing knowledge in molecular biology affords possibilities for the observation that this large family of sequence-specific ligands nonintercalatively binding within the minor groove of B-DNA is very important in the antitumor drug search[3]. The inhibition of many cellular processes and the cytotoxic effects of these antineoplastic agents are determined mainly by interference with the catalytic activity of important enzymes, such as DNA topoisomerases or a number of proteases[4].

A model of binding of netropsin and distamycin with B-DNA became the inspiration for seeking new compounds with a similar interaction with DNA. The concept of information-reading molecules was introduced. This class of synthetic heteroaromatic oligopeptides, projected after modeling netropsin and the other minor groove binders, received the name lexitropsins[5]. Although there has been huge progress in designing distamycin and netropsin analogues, so far it has not resulted in compounds that can be applied in therapy[6].

The present work is in conjunction with our ongoing program on the synthesis and biological studies of carbocyclic potential minor groove binders. Such lexitropsins, which are readily available, can be modified easily, and are stable under most experimental conditions. We have found that carbocyclic analogues of netropsin and distamycin (Figure 1) can be used as carriers for the groove-specific delivery of functionalized groups to DNA[7,8]. Molecular modeling of their interaction with d(CGCGAATTCGCG)$_2$ showed that their structure is effectively isohelical with the DNA minor groove, however with decreased affinity for the minor groove of AT-rich regions in comparison to netropsin and distamycin[9]. From energy analysis it appears that van der Waals and electrostatic interactions are more imortant than specific hydrogen bonds in stabilizing the ligand–duplex complexes. This has been confirmed by investigation of such distamycin analogues with a free aromatic amine group, which showed antiproliferative and cytotoxic effects against both MDA-MB-231 and MCF-7 cell lines[10].

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We present the biological evaluation of carbocyclic potential minor groove binders **1–6** with free aromatic amine groups (Figure 2). Their synthesis and activity in the standard mammalian tumor cell line MCF-7 were described earlier[11]. Here we present their antiproliferative and cytotoxic effects against MDA-MB-231 breast cancer cells. The ethidium displacement assay was used to show whether these compounds bind to plasmid pBR322. Determination of the association constants of drug–DNA complexes was done using calf thymus DNA, T4 coliphage DNA, poly(dA-dT)$_2$, and poly(dG-dC)$_2$ [12,13].

Because the antitumor activity of DNA-binding drugs is not due to the interaction with DNA *per se* but is, at least in part, the result of the inhibition of different enzymes, the mechanism of action of compounds **1–6** was also studied employing the topoisomerase I/II inhibition assay.

Plasmin and urokinase are involved in numerous biological processes, and also in tumor invasion, metastasis, and angiogenesis[14,15]. We reported earlier that some netropsin and pentamidine amino analogues inhibited the amidolytic activity of plasmin or trypsin[16]. Here we present the effect of compounds **1–6** on the amidolytic activity of serine proteases: plasmin, trypsin, thrombin, and urokinase.

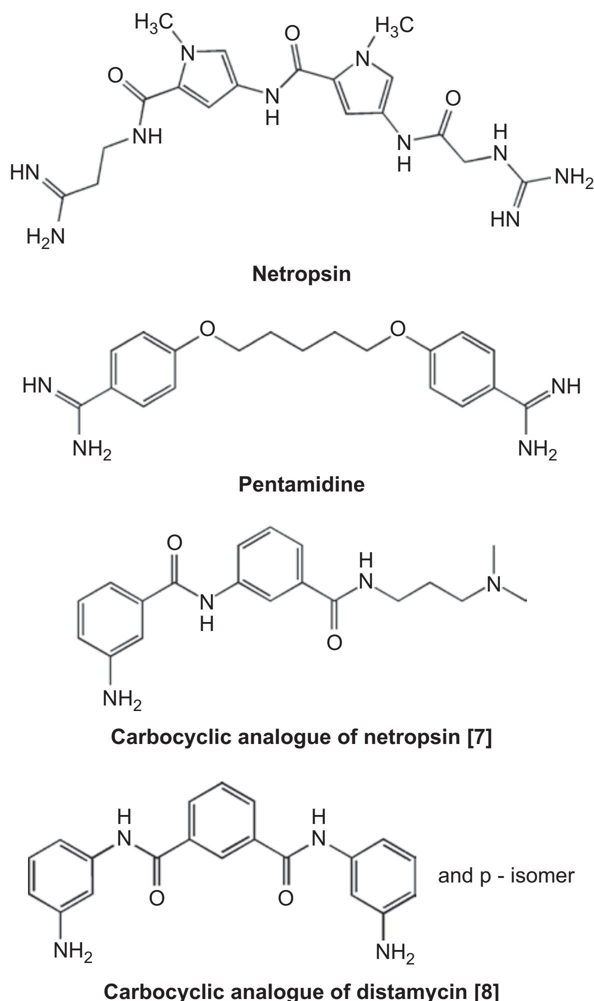

Figure 1. Structures of netropsin, pentamidine, and some carbocyclic minor groove binders.

Materials and methods

Pharmacology

Ethidium bromide was purchased from Carl Roth GmbH, and topoisomerase I (calf thymus) and II (*Escherichia coli* containing a clone of human topoisomerase II gene) from Amersham Pharmacia Biotech, and were used without further purification. Stock cultures of MDA-MB-231 were purchased from the American Type Culture Collection, Rockville, MD, USA. Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), distamycin, netropsin, pentamidine, streptomycin, and penicillin were products of Sigma. Plasmid pBR322 was purchased from Fermentas Life Sciences.

Urokinase, trypsin, and Bzl-L-Arg-pNA·HCl (Bzl = benzyl) were purchased from Sigma. Plasmin S-2444 (pyro-Glu-Gly-Arg-pNA·HCl), S-2238 (H-D-Phe-Pip-Arg-pNA), and S-2251 (H-D-Val-Leu-Lys-pNA) were obtained from Chromogenix. Thrombin was purchased from Lubelska Wytwórnia Szczepionek.

Cell culture

Human breast cancer MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 50 µg/mL streptomycin, and 100 U/mL penicillin

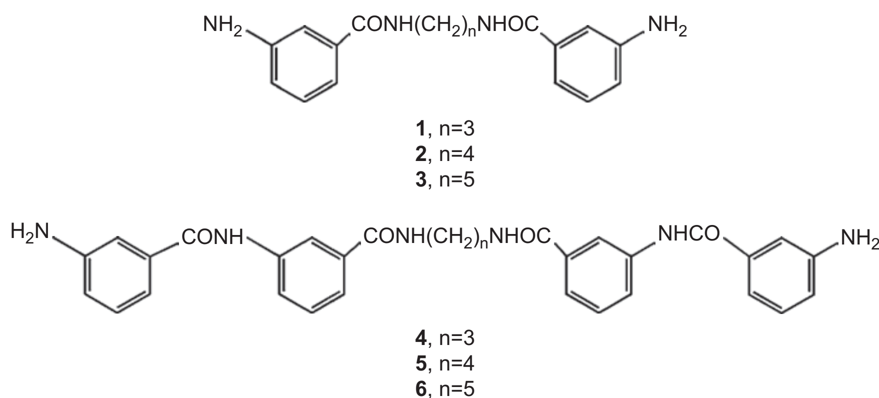

Figure 2. Structures of compounds **1–6**.

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at 37°C, in an atmosphere containing 5% CO_2. Cells were cultivated in Costar flasks, and subconfluent cells were detached with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in calcium-free phosphate-buffered saline. The study was carried out using cells growing as a monolayer in six-well plates (Nunc) (5×10^5 cells per well) and preincubated for 24 h without phenol red.

Determination of IC_{50}

The compounds were dissolved in dimethylsulfoxide (DMSO)/H_2O (10:90) and used at concentrations of 5, 10, 15, 30, and 50 μM. Microscopic observations of cell monolayers were performed with a Nikon Optiphot microscope. Wright–Giemsa staining was performed using the Fischer Leuko Stat Kit. After 24 h of drug treatment, MCF-7 cells were mixed with a dye mixture (10 μM acridine orange and 10 μM ethidium bromide, prepared in phosphate-buffered saline). At the end of each experimental time point, all the medium was removed, and cells were harvested by incubation with 0.05% trypsin and 0.02% EDTA for 1 min, and then washed with the medium. Then, 250 μL of cell suspension was mixed with 10 μL of dye mix and 200 cells per sample were examined by fluorescence microscopy. The percentage of nonviable (apoptotic and necrotic) cells was calculated, and concentrations that inhibited 50% of colony formation (IC_{50} values) were determined. The results were submitted to statistical analysis using the method of least squares.

Relaxation assay of topoisomerase I and II

Native pBR322 plasmid DNA (0.20 μg) was incubated with 4 units topoisomerase I (reaction buffer: 50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.5M NaCl, 1 mM dithiothreitol) or topoisomerase II (reaction buffer: 10 mM Tris-HCl (pH 7.9), 1 mM adenosine triphosphate (ATP), 50 mM KCl, 5 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, 15 μg/mL bovine serum albumin) in the absence or presence of varying concentrations of the test compounds (10, 50, and 100 μM) in a final volume of 10 μL. The mixture was incubated at 37°C for 30 min and the reaction was terminated by the addition of 2 μL of 10% sodium dodecyl sulfate (SDS). The reaction mixture was subjected to electrophoresis (3 h, 90 V) through a 1.0% agarose gel in TBE buffer (90 mM Tris-borate and 2 mM EDTA). The gels were stained for 30 min with ethidium bromide solution (0.5 μg/mL). The DNA was visualized using a 312 nm wavelength transilluminator and photographed under ultraviolet (UV) light. For the quantitative determination of topoisomerase activity, an area representing supercoiled DNA, migrating as a single band at the bottom of the gel, was measured using the AlphaEaseFC gel documentation and analysis system (Alpha Innotech, USA). The concentration of compound that converted 50% of the supercoiled DNA (IC_{50} values) was determined by averaging the data from at least three experiments.

Ethidium bromide assay

Each well of a 96-well plate was loaded with Tris buffer containing ethidium bromide (0.1 M Tris, 1 M NaCl, pH 8.0, 0.5 mM EtBr, final concentration, 100 μL). To each well was added 15 μg plasmid pBR322 as an aqueous solution (0.05 μg/μL). Then, to each well was added pentamidine, netropsin, or compound **1–6** (1 μL of 1 mM solution in water, 10 μM final concentration). After incubation at 25°C for 30 min, the fluorescence of each well was read on a Tecan Infinite M200 fluorescence spectrophotometer (excitation wavelength 546 nm, emission wavelength 595 nm) in duplicate experiments with two control wells (no drug = 100% fluorescence, no DNA = 0% fluorescence). Readings are reported as percentage fluorescence relative to control.

Ethidium displacement assay: determination of association constants

The fluorescence of the DNA solutions (calf thymus DNA, poly(dA-dT)$_2$, T4 DNA, and poly(dG-dC)$_2$) with the investigated compounds (at final concentrations of 10, 50, 75, 100, 150, and 200 μM) was measured on a Tecan Infinite M200 fluorescence spectrophotometer at room temperature, according to the procedure described above. Then, the concentration that reduced the fluorescence to 50% was determined. The fluorescence intensity data points were fit to theoretical curves with one or two different iterative nonlinear least-squares computer routines. The apparent binding constant was calculated from:

$$K_{EtBr}[EtBr] = K_{app}[drug]$$

where [drug] is the concentration of test compound at a 50% reduction of fluorescence and K_{EtBr} and [EtBr] are known[11,12]. Compounds **1–6** and their DNA-bound complexes showed neither optical absorption nor fluorescence at 595 nm, and did not interfere with the fluorescence of unbound ethidium.

Antiamidolytic assay

Determination of the amidolytic activity was performed as previously described[17]. A detailed description of the method is given below. Quantities of 0.2 mL of examined preparation (as control, 0.15 M NaCl), buffer, and 0.1 mL of enzyme solution were mixed together. The mixture was incubated at 37°C for 3 min then the synthetic substrate solution in the same buffer was added. After 20 min of incubation, the reaction was stopped by adding 0.1 mL of 50% acetic acid, and the absorbance of the released *p*-nitroaniline was measured at 405 nm. Every value represents the average of triplicate determination. The IC_{50} value is considered as the concentration of inhibitor that decreases the absorbance by 50%, compared with the absorbance measured under the same conditions without an inhibitor.

- enzyme: urokinase (50 units/mL), synthetic substrate: S-2444 (0.1 mL, 3 mM/L), Tris buffer: 0.6 mL (pH 8.8);
- enzyme: thrombin (1 unit/mL), synthetic substrate: S-2238 (0.2 mL, 0.75 mM/L), Tris buffer: 0.5 mL (pH 8.4);

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- enzyme: plasmin (0.4 unit/mL); synthetic substrate: S-2251 (0.2 mL, 3 mM/L), Tris buffer: 0.5 mL (pH 7.4);
- enzyme: trypsin (0.4 unit/mL), synthetic substrate: Bzl-L-Arg-pNA·HCl (0.2 mL, 8 mM/L), borane buffer: 0.5 mL (pH 7.5).

Our results were compared with the data obtained for 2-phenethyl-SO_2-D-Ser-Ala-Arg-al, the irreversible urokinase plasminogen activator (uPA) inhibitor with the same tripeptide sequence[18]. The determination methods were identical.

Statistical analysis

In all experiments, the mean values for three assays ± standard deviations (SD) were calculated.

The results were submitted to statistical analysis using Student's *t* test. Differences were considered significant when $p < 0.05$. Mean values, standard deviations, and the number of measurements in a group are presented in the tables.

Results and discussion

Antiproliferative and cytotoxic effects of compounds **1–6** in the standard human breast cancer cell line MCF-7 were investigated earlier[11]. Their MCF-7 IC_{50} values together with *in vitro* antitumor activities in estrogen-independent human breast cancer cells MDA-MB-231 are presented in Table 1. All of the tested compounds showed concentration-dependent activity. Against MDA-MB-231 cells, the compounds were more cytotoxic than pentamidine with $IC_{50} = 17.74 \pm 2\,\mu M$ and netropsin with $IC_{50} = 228.80 \pm 2\,\mu M$. The compound concentration that inhibited 50% of colony formation was in the range 8.10 ± 2 to 17.52 ± 2 µM. IC_{50} values against the MCF-7 cell line were in the range 209.8 ± 2 to 406.12 ± 2 µM, while IC_{50} of pentamidine was 14.31 ± 2 µM and of netropsin was 5.40 ± 2 µM. From these data we can see that compounds **1–6** were nearly 20 times more active against MDA-MB-231 than against MCF-7 cells.

Table 1. Antiproliferative activity of netropsin (NT), pentamidine (PN), and compounds **1–6** against breast cancer cells.

Compound	IC_{50} (µM)[a]	
	MCF-7[b]	MDA-MB-231
NT	5.40 (± 2)	228.80 (± 2)
PN	14.31(± 2)	17.74 (± 2)
1	209.80 (± 2)[b]	13.39 (± 2)
2	250.00 (± 2)[b]	8.91 (± 2)
3	222.45 (± 2)[b]	17.52 (± 2)
4	213.68 (± 2)[b]	14.92 (± 2)
5	483.12 (± 2)[b]	8.10 (± 2)
6	258.30 (± 2)[b]	10.86 (± 2)

[a]Results represent mean (± SD) of three independent experiments done in duplicate.
[b]Data from reference 11.

To test whether the cytotoxic properties were related to DNA-binding and topoisomerase I/II inhibition, the new minor groove binders were evaluated in a cell-free system. The ethidium bromide assay showed that the investigated compounds could bind to plasmid DNA, although relatively weaker than netropsin and pentamidine (Table 2). The topoisomerase DNA-inhibitory effects and binding affinities of compounds **1–6**, netropsin, and pentamidine to calf thymus DNA, T4 coliphage DNA, and synthetic polymers poly(dA-dT)$_2$ and poly(dG-dC)$_2$ are presented in Table 3. These data demonstrate that all compounds could bind to the DNAs studied. The high-binding constant values for T4 coliphage DNA for **1–6** gave evidence of their minor-groove selectivity, because the major groove of T4 coliphage DNA is blocked by α-glycosylation of the 5-(hydroxymethyl)cytidine residues[19]. The DNA-binding data reported in Table 3 characterize the affinity of compounds **1–6** for a more limited set of DNA-binding sites, and can give an indication of base-sequence specificity for DNA-binding molecules. These data indicate that compounds **1–6** interacted with a GC base pair, though the binding affinity was weak compared with that for an AT base pair. Since calf thymus DNA contains random sequences and therefore fewer AT sites than poly(dA-dT)$_2$, the selectivity of **1–6** was further demonstrated by their much weaker binding to calf thymus DNA compared to poly(dA-dT)$_2$. All of the compounds bound to AT-rich sequences similar to pentamidine, but more weak than netropsin[20].

Table 2. DNA-binding effect of netropsin (NT), pentamidine (PN). and compounds **1–6**.

Compound	NT	PN	**1**	**2**	**3**	**4**	**5**	**6**
Fluorescence (%)	74.01	69.56	89.13	92.39	93.48	81.52	76.09	89.13

Table 3. Association constants (K_{app}) and topoisomerase DNA inhibitory effects of netropsin (NT), pentamidine (PN), and compounds **1–6**.

Ligand	K_{app} ($\times 10^5\,M^{-1} \pm 0.2 \times 10^5\,M^{-1}$)				Inhibition of topo I activity (µM)[d]	Inhibition of topo II activity (µM)[d]
	Calf thymus DNA	T4 DNA	poly (dA-dT)$_2$	poly (dG-dC)$_2$		
EtBr	100[a]	100[b]	95[b]	99[b]	—	—
NT[c]	8.7[c]	8.3[c]	875[c]	2.5[c]	1	5
PN	7.7	4.2	9.8	3.6	>100	>100
1	3.2	2.5	8.4	2.2	10	50
2	4.8	1.9	6.8	1.3	20	30
3	4.4	0.9	4.8	1.2	10	30
4	2.9	6.4	3.5	1.5	30	100
5	6.3	7.9	7.1	1.5	20	80
6	3.9	3.5	3.6	2.9	40	90

[a]Values from reference 12.
[b]Values from reference 13.
[c]Values from reference 20.
[d]Concentrations of inhibitor that prevented supercoiled DNA from being converted into relaxed DNA were determined by averaging data from at least three experiments.

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A number of minor-groove binding drugs inhibit the catalytic activity of isolated topoisomerases (both I and II)[21,22]. These data suggest that these topological enzymes read the DNA structure at least in part through the minor groove[23]. The ability of compounds **1–6** to inhibit topoisomerase I and II activity was quantified by measuring the action on supercoiled pBR322 DNA substrate as a function of increasing concentration of the ligands by the use of agarose gel electrophoresis. The concentrations of inhibitors that prevented 50% of supercoiled DNA from being converted into relaxed DNA (IC_{50} values) were determined (Table 3). These results demonstrated that **1–6** had topoisomerase I (topo I) inhibitory activity in the range from 10 to 40 µM and topoisomerase II (topo II) inhibitory activity in the range from 30 to 100 µM. Compounds **1–3** were more active, as both topo I and topo II inhibitors, compared to **4–6**. Pentamidine had weak activity in this experiment; netropsin was more active than all other test compounds. The compounds that were less effective against topoisomerase I/II activity were also weaker DNA-binding ligands (Table 3).

The influence of compounds **1–6** on the amidolytic activity of urokinase, thrombin, plasmin, and trypsin is shown as IC_{50} values in Table 4. Compounds **1**, **2**, and **3** were ineffective as amidolytic activity inhibitors. None of the investigated compounds inhibited the activity of thrombin. Compounds **4–6** were inhibitors of plasmin; meanwhile, amidolytic urokinase activity was inhibited by **5** and **6**. Trypsin activity was inhibited only by compound **6**.

We were unable to establish a quantitative relationship between potency of enzyme (both topoisomerases and proteases) inhibition and cytotoxicity. The investigated compounds showed an interesting spectrum of activity. We can see that they bind to minor groove B-DNA and inhibit topo I and topo II activity. Some of them are also inhibitors of plasmin and urokinase. The differences in antiproliferative and cytotoxic effects against MCF-7 and MBA-MD-231 breast cancer cell lines demonstrate that the mechanism of action of our compounds is dependent not only on DNA-binding mode but can be partially connected with the fact that in the case of MDA-MB-231 cells, higher uPA/uPAR (urokinase plasminogen activator system) expression and higher plasminogen-binding was observed than in the MCF-7 cell line[24].

Table 4. Inhibition of amidolytic activity of proteolytic enzymes.

	IC_{50} (mM)			
Compound	Urokinase S-2444	Thrombin S-2238	Plasmin S-2251	TrypsinBzl-L-Arg-pNA·HCl
NT	0.03 ± 0.002	n.i.	0.04 ± 0.0032	n.i.
PN	0.02 ± 0.0015	n.i.	<0.01	0.10 ± 0.02
1	n.i.	n.i.	n.i.	n.i.
2	n.i.	n.i.	n.i.	n.i.
3	n.i.	n.i.	n.i.	n.i.
4	n.i.	n.i.	0.02 ± 0.0014	n.i.
5	0.01 ± 0.0008	n.i.	0.02 ± 0.0015	n.i.
6	0.014 ± 0.001	n.i.	0.01 ± 0.0008	0.02 ± 0.0016

Note. n.i., no inhibition was observed in maximum concentration (0.02 M).

The exact mechanism of action of the tested compounds and their structural investigation should be investigated in further studies.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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