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QUARTERLY

Molecular modelling, synthesis and antitumour activity of carbocyclic analogues of netropsin and distamycin — new carriers of alkylating elements★

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A series of netropsin and distamycin analogues was synthesised and investigated by molecular modelling. The lowest-energy conformations of four carbocyclic lexitropsins, potential carriers of alkylating elements, were obtained using the HyperChem 4.0 program, and compared with the DNA-lexitropsin crystal structures from the Brookhaven National Laboratory Protein Data Bank. A method for synthesis of carbocyclic lexitropsins was elaborated, with the use of a nitro group or azobenzene as precursors for the aromatic amino group. The influence of methoxy group in *ortho* position with respect to amide groups on the activity of the new compounds was investigated. All of the compounds tested showed high antitumour activity in the standard cell line of mammalian tumour MCF-7.

Distamycin A (**1**) and netropsin (**2**) (Fig. 1) are the most intensly studied representatives of a series of basic, polypyrrole oligopeptide natural products with antitumour activity and wide-ranging antiviral activity [1]. These compounds bind selectively to AT-rich sequences in the minor groove of DNA. So far, over 20 high-resolution structures (NMR and X-ray) have been reported for a variety of oligonucleotide sequences complexed with netropsin, distamycin and related minor groove binders [2].

Netropsin and distamycin serve as models for studying DNA minor-groove binders. The

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Abbreviations: DMAP, 4-(dimethylamino)pyridine; Me_2SO, dimethylsulphoxide; DAPI, 4′,6′-diamidino-2-phenylindole; PDB, Protein Data Bank; TMS, tetramethylsilane.

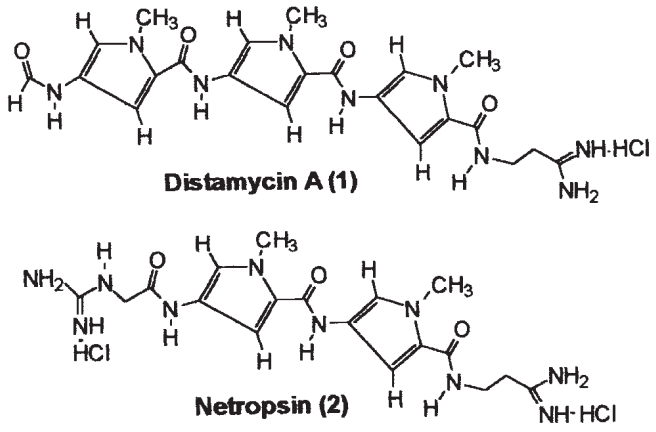


Figure 1. Structures of netropsin and distamycin hydrochlorides.

name *lexitropsin* [3] has been applied to the compounds that bind to specific DNA pairs, essentially reading the base-pair sequences.

Many strategies of design of new distamycin A and netropsin analogues have been elaborated [4–8].

We concentrated on the strategy that consists in replacing the N-methylpyrrole rings in netropsin and distamycin with other rings. By substituting imidazole, thiazole, triazole, pyrazole, or oxazole heterocycles for *N*-methylpyrrole rings of netropsin, one can obtain drugs capable of binding to sequences containing one or two G·C pairs embedded in an AT sequence [9–13]. Substitution of the terminal formamido-methylpyrrole group of distamycin by a pyridine group permits its interaction with a G·C base pair [14]. The ability of distamycin to bind pure AT sequences is retained by its pyridine analogues [15, 16].

The replacement of heterocyclic rings by carbocyclic rings [17] yields lexitropsins which in comparison with distamycin have reduced affinity to A·T pairs, increased affinity to G·C pairs [18] exhibit lower toxicity and increased antibacterial, antiviral and antitumour activity [19]. It is worth noting that benzene derivatives are readily available. They can be easily modified, and are stable under most of experimental conditions [20, 21].

Netropsin, distamycin and their analogues, are excellent carriers of compounds of known antitumour or antiviral activity [22–25].

We report here molecular modelling, synthesis and evaluation of antitumour activity of a new series of benzene analogues of netropsin and distamycin.

MATERIALS AND METHODS

Molecular modelling

Computational analysis and molecular modelling were performed on a PC Pentium 75 using the program HyperChem (version 4.0) [26]. Structures 5A, 7A, 8B and 10B (Fig. 2) were built within the HyperChem and energy-minimized with MM+ (molecular modelling) force field, *in vacuo*. Molecular energies were considered to be at minimum when the energy gradient was less than 0.08 kJ/mol (per one iteration) [26]. The conjugate gradient method was used for minimization. Two typical non-intercalating groove-binding compounds, distamycin and pyridine-2-carboxamidenetropsin, were also investigated and used as templates for molecular superimposition.

Syntheses of carbocyclic analogues of distamycin A and netropsin

Compounds 5A and 7A. The starting material for the synthesis was 3-nitrobenzoyl chloride (**1A**). The transformation of this compound to desired products (**5A** and **7A**) is outlined in Fig. 3. Treatment of **1A** dissolved in methylene chloride with 3-dimethylaminopropylamine in dry pyridine with DMAP gave the appropriate *N*-(3-nitrobenzoyl)-*N*′,*N*′-dimethyl-1,3-propanediamine, **2A**. The precursor nitro group was reduced by catalytic hydrogenation at room temperature and at atmospheric pressure to yield the desired amine **3A**. An examination of several systems that could be used in catalytic hydrogenation of nitro group revealed that the chloroplatinic acid-sodium borohydride system to give the best results.

Acylation of amine **3A** with acid chloride **1A** in the presence of DMAP in pyridine and methylene chloride at room temperature gave *N*-[3-(3-nitrobenzamide)benzoyl]-*N*′,*N*′-dimethyl-1,3-propanediamine, **4A**. Catalytic reduction of the nitro group of **4A** to the amine **5A** proceeded satisfactorily, under the reaction conditions similar to those described for the synthesis of **3A**. The reaction of acid chloride **1A** and amine **5A** under conditions similar to those described for the synthesis of **4A** gave the tripeptide **6A** in good yield. Catalytic hydrogenation of **6A** gave the desired amine **7A**.

Elementary analyses: Anal. Calcd. for **5A** ($C_{19}H_{26}N_4O_2Cl_2$): C, 55.21; H, 6.34; N 13.56; O, 7.74; Cl, 17.15. Found: C, 55.20; H, 6.30; N, 13.59; O, 7.71; Cl, 17.22. Anal. Calcd. for **7A** ($C_{26}H_{31}N_5O_3Cl_2$): C, 58.65; H, 5.87; N, 13.15; O, 9.01; Cl, 13.35. Found: C, 58.60; H, 5.83; N, 13.19; O, 8.98; Cl, 13.35

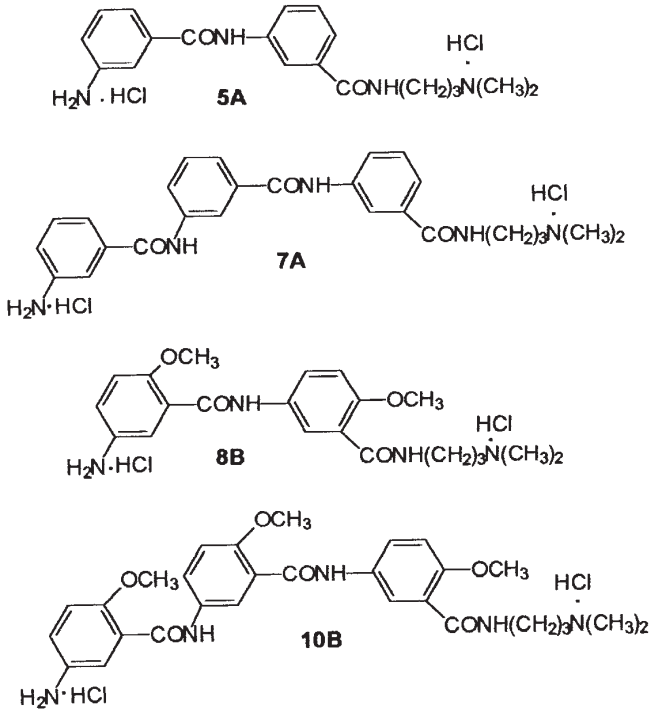


Figure 2. The structures of new carbocyclic analogues of netropsin and distamycin A (in the form of bis-hydrochlorides).

Compounds 8B and 10B. These were prepared as previously described [20, 27].

Analyses

Melting points were determined on Buchi 535 apparatus and are uncorrected. ^1H NMR (200 MHz) and ^{13}C NMR (50 MHz) spectra were recorded on a Bruker AC 200 F spec-

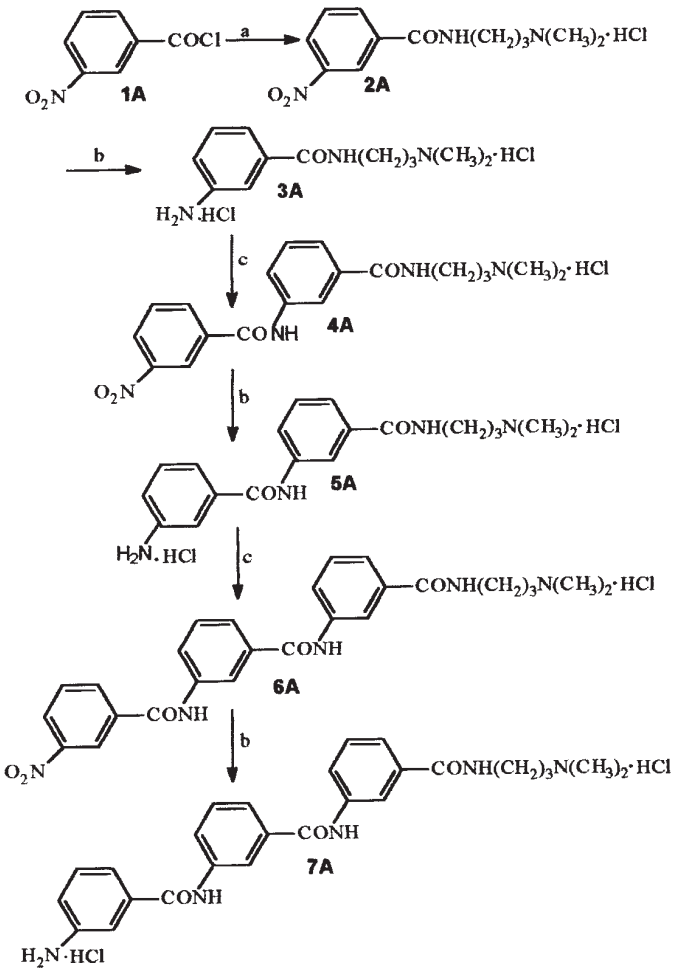


Figure 3. Synthesis of carbocyclic analogues of netropsin — 5A and 7A.

a, DMAP, Py/CH_2Cl_2, *N,N*-dimethylpropane-1,3-diamine; b, H_2/Pt, MeOH, HCl; c, DMAP, Py/CH_2Cl_2, 1A

trometer, using tetramethylsilane (TMS) as an internal standard. Chemical shifts are expressed in δ values (ppm) and coupling constants are given in *J* (Hertz). Multiplicity of resonance peaks is indicated as singlet (s), doublet (d), triplet (t), quartet (q), broad singlet (bs), and multiplet (m).

Thin-layer chromatograms were prepared on precoated plates (Merck, silica gel 60F-254). Solvent systems (all proportions by volume):

Compounds **5A** and **8A**: (A), methanol/25% ammonia (99:1).

Compounds **8B** and **10B**: (A), pyridine/ethyl acetate/acetic acid/water (5:5:1:3); (B), butanol/pyridine/water (6:4:3); (C), solvent (A)/solvent (B) (1:1).

All compounds were visualized by short-wave ultraviolet light.

Silicagel 60 (230–400 mesh ASTM) was used for column chromatography.

Antitumour activity of carbocyclic lexitropsins

Cells. Human breast cancer cell line (MCF-7) was purchased from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's Modified Eagle's Medium supplemented with 2 mM glutamine, 8% heat-inactivated foetal bovine serum, 50 μg/ml streptomycin and 100 U/ml penicillin. Cells were grown in 75 cm^3 flasks in a culture incubator at 37°C in humid atmosphere containing 5% CO_2. Cells were cultured in Costar flasks and when subconfluent detached with 0.05% trypsin and 0.02% EDTA in calcium-free phosphate buffered saline.

Drug solutions. Compounds **5A 7A**, **8B** and **10B** were dissolved in sterile water and used at concentrations of 1, 10, 100 and 1000 $\mu g/cm^3$.

Cell morphology. Microscopic observations of the cell monolayers were performed during 24 h with a Nikkon optiphot microscope. Cell morphology was evaluated by examination of a centrifuged cell preparation stained with Wright-Giemsa. Micrographs were taken at 250× magnification. Necrotic cells were identified as those which exhibited the key apoptotic features of chromatin compaction, nuclear fragmentation, cell shrinkage, and separation of the cells.

Determination of IC_{50}. MCF-7 grown in 6-well plates were stained during evaluation the time course of drug action with a dye mixture (10 μM acridine orange and 10 μM ethidium bromide), that was prepared in phosphate buffered saline. Acridine orange (fluorescent DNA-binding dye) intercalates into DNA, making it appear green, and binds to RNA, staining it red-orange. Ethidium bromide is taken up only by nonviable cells, its fluorescence overwhelming that of the acridine orange and making the chromatin of lysed cells appear orange. At the appropriate time point, 250 μl of the cell suspension was mixed with 10 μl of the dye mix and 200 cells per sample were examined by fluorescence microscopy.

Statistics

The data were analysed by Statgrafts 7.0 program for Windows package.

RESULTS

Molecular modelling

The lowest-energy conformations of the four carbocyclic lexitropsins (Fig. 4), potential carriers of alkylating elements, were compared with data from the Brookhaven National Laboratory Protein Data Bank (Table 1). The PDB data were edited to remove water and extraneous ions, and visualized by HyperChem. The

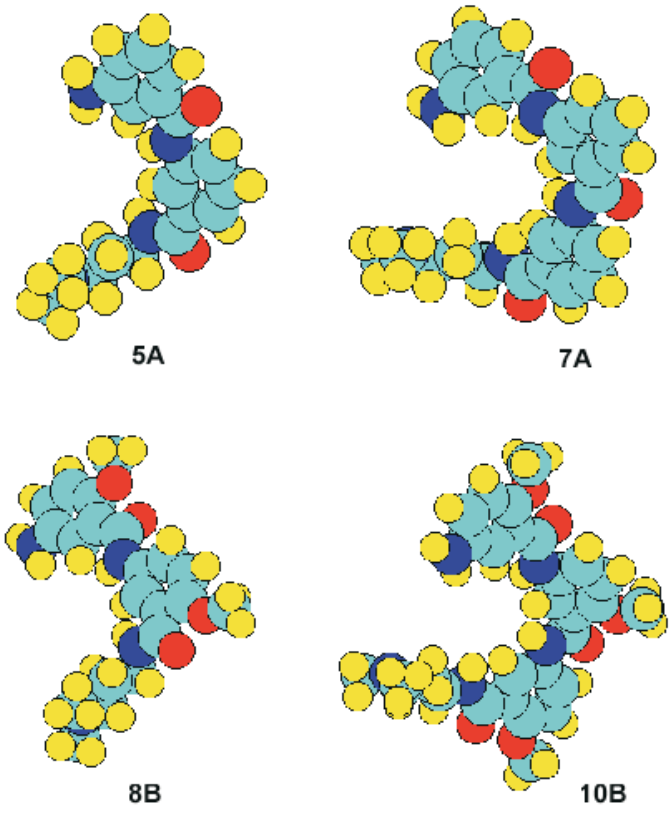


Figure 4. Energy minimized conformations of new lexitropsins.

ligands were modified manually to include double bonds, atomic net charges, and hydrogen atoms to convert them into the appropriate format for single point energy computations. Figure 5 shows the naturally curved shape of the ligands after these modifications.

Carbocyclic analogues of distamycin A and netropsin

The structures of **5A**, **7A**, **8B** and **10B** were proved by elementary analysis and ^{1}H and ^{13}C NMR spectra. Table 2 shows physicochemical data for the new compounds (in the form of hydrochlorides) (Table 2). Chromatographic methods and careful analysis of NMR spectra confirmed the structures and purity of all four compounds. Location signals of ^{1}H NMR and ^{13}C NMR were similar to those given in the literature [28–32].

Antitumour activity of carbocyclic lexitropsins

The percentage of nonviable cells for each drug concentration used (Table 3) and IC_{50} values (Table 4) were determined.

DISCUSSION

The MM+ energy-minimized conformations of compounds **5A, 7A, 8B** and **10B** correspond to a crescent-shaped structure (Fig. 4). It is reasonable to suppose that these conformations should match the convex surface of the minor groove of DNA.

Of the examined compounds, **5A** and **8B** are the most similar in shape to DNA minor groove binders. To illustrate this similarity, the minimized conformation of **5A** was superimposed on the X-ray derived structures of netropsin, distamycin and DAPI (Fig. 6). The structures of these three DNA binders and major part of **5A** molecule overlap, thus we can suppose that **5A** (and **8B,** too) have a molecular shape that fits to minor groove of DNA. On the other hand, the larger curvature of **7A** and **10B** seems to hinder their fit to the DNA minor groove.

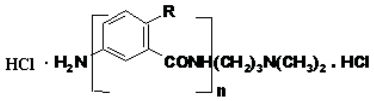
The performed theoretical studies clearly show also that the molecular dimensions of the presence of two benzene units is optimal for the activity of the investigated class of compounds. A further increase in the number of benzene rings to target for longer DNA se-

Table 1. Complexes of minor groove binders with DNA fragments (nucleotide oligomers)

No.	PDB ID code[a]	Ligand	Fragment of DNA
1	101d	Netropsin	[CGCGAATT(Br)CGCG]
2	102d	Propamidine	[CGCAAATTTGCG]
3	159d	Distamycin	[ICICICIC]
4	166d	Pentamidine	[CGCGAATTCGCG]
5	195d	Netropsin	[CGCGTTAACGCG]
6	1d30	DAPI	[CGCGAATTCGCG]
7	1d63	Berenil	[CGCAAATTTGCG]
8	1d64	Pentamidine	[CGCGATTCGCG]
9	1d86	Netropsin	[CGCGAATTCGCG]
10	1dne	Netropsin	[CGCGATATCGCG]
11	1prp	Propamidine	[CGCGAATTCGCG]
12	2dbe	Berenil	[CGCGAATTCGCG]
13	2dnd	Distamycin	[CGCAAATTTGCG]

[a]available, for example, at the web-sites: www.pdb.bnl.gov or zatoka.icm.edu.pl

Table 2. Physicochemical properties of carbocyclic lexitropsins (in the form of dihydrochlorides)



No.	R	n	Yield [%]	m.p. [°C]	R_f	^{1}H NMR, δ [ppm] (solvent)	^{13}C NMR, δ [ppm] (solvent)
5A	H	2	80.39	–	0.46 (A)	1.93 (m, 2H, C-CH_2-C) 2.72 (s, 6H, $N(CH_3)_2$) 3.32 (t, 2H, $CH_2N(CH_3)_2$) 3.73 (t, 2H, $CONHCH_2$) 5.30 (s, 2H, NH_2) 7.12 – 8.77 (m, 8H, Ar-H) 9.16 (s, 1H, CONH) 10.28 (s, 1H, CONH) (CD_3OD)	25.75 (C-CH_2-C) 37.16 ($CONHCH_2$) 43.14 ($N(CH_3)_2$) 56.30 ($CH_2N(CH_3)_2$) 120.93; 123.31; 125.05; 126.97; 128.39; 129.78; 134.30; 135.58; 137.24; 139.57; 147.93; 149.28 (12 × C_{Ar}) 165.90; 170.04 (2 × CONH) (CD_3OD)
7A	H	3	69.60	–	0.39 (A)	2.07 (m, 2H, C-CH_2-C) 2.89 (s,6H, $N(CH_3)_2$) 3.19 (t, 2H, $CH_2N(CH_3)_2$) 3.53 (t, 2H, $CONHCH_2$) 6.93 – 8.19 (m, 8H, Ar-H) ($CDCl_3/CD_3OD$)	25.28 (C-CH_2-C) 36.75 ($CONHCH_2$) 43.15 ($N(CH_3)_2$) 55.94 ($CH_2N(CH_3)_2$) 114.71; 117.98; 119.47;120.11; 120.53; 123.76; 124.15;124.81; 125.06; 129.63; 129.87; 132.01; 134.29; 134.72; 135.90; 139.08; 139.21; 147.33 (18 × C_{Ar}) 1657.90;168.48;169.72 (3 x CONH) $CDCl_3/CD_3OD$)
8B	OCH_3	2	81.25	224–226	0.28 (C)	2.21 (m, 2H, C-CH_2-C) 2.47 (NH_2) 2.83 (s, 6H, $N(CH_3)_2$) 3.18 (t, 2H, $CH_2N(CH_3)_2$) 3.63 (t, 2H, $CONHCH_2$) 3.99; 4.09 2(s,3H,OCH_3) 6.67-8.41 (m, 6H, Ar_A-H Ar_B-H) 8.78; 9.66 2(s,1H, CONH) ($CDCl_3$)	24.85 (C-CH_2-C) 36.72 ($CONHCH_2$) 42.90 ($N(CH_3)_2$) 55.46 ($CH_2N(CH_3)_2$) 56.41, 56.82 (2 x OCH_3) 106.67; 112.08. 121.15. 122.34; 122.80; 123.76; 125.71; 127.10; 130.92; 146.98; 154.34; 158.97 (12 × C_{Ar}) 162.58; 165.71 (2 × CONH) ($CDCl_3$)
10B	OCH_3	3	41.30	222–224	0.25 (C)	1.94 (m, 2H, C-CH_2-C) 2.72 (s, 6H, $N(CH_3)_2$) 3.05 (t, 2H, $CH_2N(CH_3)_2$) 3.35 (t, 2H, $CONHCH_2$) 3.38 (2H, NH_2) 3.83 (s, 3H, OCH_3) 3.87 (s, 3H, OCH_3) 3.88 (s, 3H, OCH_3) 7.01-8.25 (m, 9H, Ar_A-H, Ar_B-H, Ar_C-H) 8.43 (t, 1H, CONH) 10.15 (s, 2H, 3CONH) (d_6-Me_2SO)	24.04 (C-CH_2-C) 36.32 ($CONHCH_2$) 41.83 ($N(CH_3)_2$) 54.22 ($CH_2N(CH_3)_2$) 56.15; 56.18; 56.49 (3×OCH_3) 112.23 ; 112.30;113.45; 118.21; 120.50; 121.26; 122.12; 123.03; 123.32; 123.66; 124.78; 128.32; 132.09; 136.09; 150.48; 152.54; 153.06 (18 C Ar) 163.96; 63.98; 165.07 (3 × CONH) (d_6-Me_2SO)

Table 3. Antitumour activity of potential carriers of alkylating groups, expressed as percentage of non-viable MCF-7 mammal tumour cells

Compound	Concentration [μg/cm3]			
	1	10	100	1000
5A	52%	100%	100%	100%
7A	2%	14%	30%	98%
8B	40%	45%	8%(?)	62%
10B	20%	62%	98%	100%

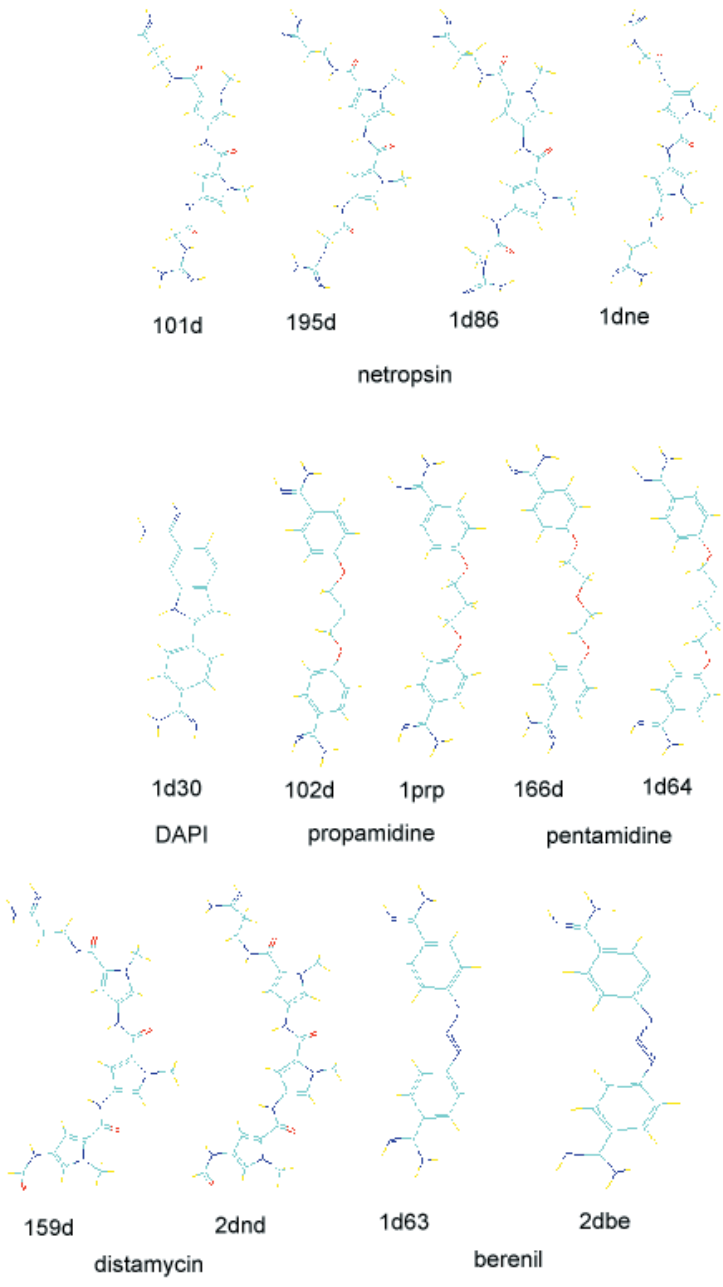


Figure 5. Naturally curved shapes of ligands.

The structures were extracted from the respective PDB file and adapted for further analysis according to the procedure described in the text.

quences seems not to be feasible due to the phasing problem [33] which arises because of the lack of dimensional correspondence between oligopeptides and oligonucleotides.

The two benzene rings of **5A** are slightly twisted so that each ring can adopt a parallel position with respect to the enclosing walls of the DNA minor groove. On the basis on either the valence bond or molecular orbital theory, one would have expected these two aromatic rings to form a common delocalized electronic system, with coplanar rings. In elongated molecules this tendency towards coplanarity could be opposed by the short-range interactions exerted by the walls of the minor groove, which twist aromatic rings as the groove snakes up and forms the helix cylinder. The actual geometry of the inter-ring bond reflects a balance or trade off between delocalization dependent flattening and groove-wall twisting. The predicted binding of **5A** within the minor groove should have a minor influence on the DNA global structure, as the minor groove in B-DNA is intrinsically wider in GC than in AT regions [34]. Changing the amidine tail of distamycin to a (dimethylamino)propyl group causes a decrease in binding to DNA [35]. This moiety, with p*K*a of about 9.3, would be protonated at physiological pH of 7.4 to provide a favorable electrostatic attraction to the negative electrostatic charge of the DNA [36]. The methoxy group in the *ortho* position to the amide group probably serves as a spacial hindrance in binding to DNA. It is obvious that the **5A**···DNA interaction model presented here should be vali-

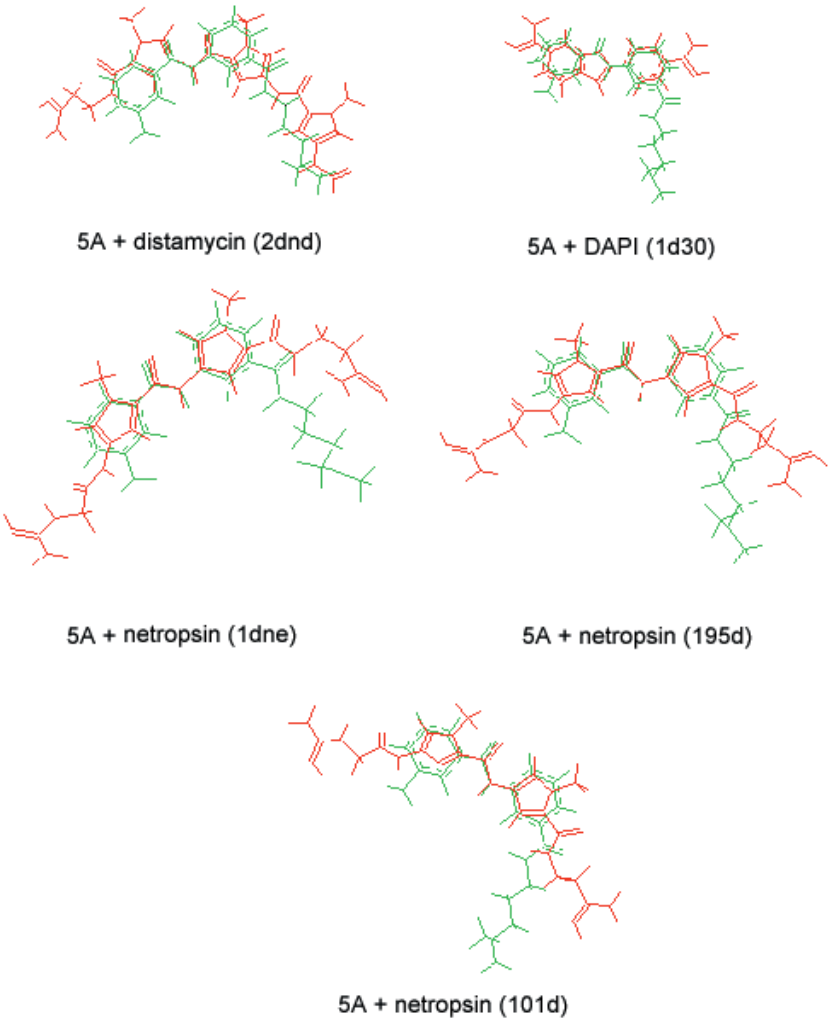


Figure 6. Superimposition of compound 5A (green) and ligands (red).

The DNA binders appear in the conformations adapted from the PDB files of the respective crystal structures.

dated in the course of more detailed investigations using a variety of physico-chemical methods.

Table 4. Drug concentrations which inhibit by 50% colony formation by MCE-7 cells

Compound	IC_{50} [$\mu g/cm^3$]	[10^{-6} M]
5A	10.10	24.43
7A	21.69	40.73
8B	43.36	91.59
10B	65.59	105.35

On the basis of molecular modelling it seems that the structure of benzene oligopeptides might be a useful starting framework for synthesis of selective DNA minor groove binding molecules. Although some structure-activity relationships point to a positive correlation between DNA binding and antitumour activity, it is usually accepted that DNA binding is a necessary, but not a sufficient, condition for antitumour activity [37]. Our design is based on the assumption that an ideal antitumour agent should consist of two essential moieties: (i) a recognition unit which serves as a carrier and (ii) a functional moiety capable of modifying DNA. Here we have described a procedure for synthesis of potential carriers of alkylating groups.

The advantage of exchange of the amidinium moiety (normally present in netropsin) by the dimethylamino group was described earlier [27]. We have observed that the presence of methoxy groups on the convex face of **8B** and **10B** would not prevent their binding to DNA but has some influence on the facility of preparation and stability of the compound obtained. The elaborated method of synthesis of amide bonds, with the use of nitro- and azobenzene-groups, allows building carbocyclic oligonucleotides with any desired number of benzene rings.

The DNA minor groove binding ligands such as distamycin and netropsin can act as suitable carriers for the alkylating functional groups, and therefore they should be good candidates as effective antitumour agents. We believe that the carbocyclic analogues of netropsin, **5A, 7A, 8B** and **10B** containing terminal free amine groups can be used as vectors for delivery of the DNA interacting agents, thereby producing new, possibly more effective, agents for the treatment of cancer. The application of these compounds as carriers will be the subject of a separate publication.

The IC_{50} values for four binders to minor groove of DNA suggest also that these binders can serve as potential carriers of alkylating groups. The drug concentration which inhibits 50% of colony formation, is in the range 10.10–65.59 $\mu g/cm^3$ and 24.43–10.35 μM, whereas IC_{50} for chlorambucil studied in the same cell line, is 24.6 μM [38]. This also points to the possibility that compounds **5A, 7A, 8B** and **10B** can be effective antitumour agents.

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