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ANALOGUES OF DISTAMYCIN — SYNTHESIS AND BIOLOGICAL EVALUATION OF NEW AROMATIC OLIGOPEPTIDES, POTENTIAL ANTICANCER AGENTS

DANUTA DROZDOWSKA[1*], MAŁGORZATA RUSAK[2], TOMASZ BIELAWSKI[3], and KRYSTYNA MIDURA-NOWACZEK[1]

[1] Department of Organic Chemistry, Medical University, 2A Mickiewicza, 15-222 Białystok, Poland
[2] Department of Haematological Diagnostic, Medical University, Białystok, Poland
[3] Department of Gynaecological Endocrinology, Medical University, Białystok, Poland

Abstract: Six new aromatic oligopeptides were synthesized and evaluated for their activity in the standard cell line of the mammalian tumor MCF-7 as well as in a cell-free system employing the topoisomerase I/II inhibition assay.

Keywords: oligopeptides, analogues of distamycin; DNA topoisomerase; cytotoxicity; anticancer agent

The pyrrole-amidine antibiotics netropsin and distamycin are characterized by antiviral and oncolytic properties. These compounds represent prototypic DNA minor groove binders with a pronounced selectivity for AT-rich sequences. A vast number of netropsin and distamycin analogues, named lexitropsins and their conjugates targeting specific sequences in DNA have been designed (1). Several different aromatic oligopeptides as potent antitumor drugs have been obtained, such as the distamycin-nitrogen mustard conjugate tallimustine (2) or brostallicin (3), which were undergoing clinical trials as anticancer agents.

In addition to the interacting with specific sequences in DNA, minor groove binders can interfere with the catalytic activities of nuclear enzymes involved in the regulation of DNA topology in cells. Topoisomerases I and II are the primary targets of many anticancer drugs including the anthracyclines and camptothecins (4, 5). Certain DNA minor groove binding agents related to Hoechst 33342 or bifunctional netropsin analogues act as poisons for topoisomerase I (6, 7), and a hybrid molecule, such as distamycin – intercalator, may interference with topoisomerase II functions (8).

Our drug design program aiming at the development of potential minor groove binders to B-DNA was concentrated on synthetic distamycin and netropsin analogues, non-specific aromatic oligopeptides.

We focused on the strategy to replace the N-methylpyrrole rings of distamycin and netropsin with benzene rings, simultaneously modifying of cationic heads. The study of interaction of compounds to DNA by the ethidium displacement assay confirmed that they had the larger specificity to A-T in comparison to G-C rich regions, similarly to model netropsin and distamycin (9).

We examined that the carbocyclic analogues of distamycin with unsubstituted N-terminal NH_2 group inhibited *in vitro* activity of topoisomerase I and II (10), similarly like derivatives of netropsin with aliphatic linker (4 and 6 CH_2 groups) (11). Derivatives with N-terminal chlorambucil group exhibited activity in cultured breast cancer MCF -7 (12), and with 5-[N,N-bis(2-chlorethyl)amine]-2,4-dinitrobenzoyl group – in the face of hepatoma HEP G2 under hypoxic conditions (13).

These considerations prompted us to design a new series of potential DNA minor groove binders, inhibitors of topoisomerases. We developed the new compounds which skeleton combines the structural features of distamycin (**A**) and furamidine (**B**) (Fig. 1.). Eight of such distamycin and furamidine analogues showed antiproliferative and cytotoxic effects against both cell lines in the range 3.47 – 12.53 μM for MDA-MB-231 and 4.35 – 12.66 μM for MCF-7, and demonstrated activity against DNA topoisomerases I and II at the concentration 50 μM (14).

* Corresponding author: e-mail: Danuta.Drozdowska@umwb.edu.pl

In the course of our investigation of minor groove binding drugs, we report a synthesis and cytotoxicity of 6 analogues of distamycin **1-6** (Scheme 1) in cultured breast cancer MCF-7 cells. We also investigated the mode of an action of compounds **1**-**6** in a cell-free system employing the topoisomerase I/II inhibition assay.

EXPERIMENTAL

Chemistry

All compounds were synthesized from suitable parent substances (Merck and Aldrich). Thin-layer chromatograms were run on precoated plates (Merck, silica gel 60F-254) and visualized under UV light. Methanol with 5% NH_3 was used as a solvent system. Silica gel 60 (Merck, 230-400 mesh) was used for column chromatography. Solvents used in the experiments were dried and distilled.

Melting points were determined on Buchi 535 melting-point apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC 200F spectrometer, using TMS as an internal standard. Chemical shifts were expressed in δ values (ppm). Multiplicities of resonance peaks are indicated as singlet (s), doublet (d), triplet (t), quartet (q) and multiplet (m). The results of elemental analyses for C and H were obtained within ± 0.4 % of theoretical values.

General procedure for preparation of compounds 1-6

Compounds **1**-**6** were obtained as outlined in Scheme 1. The starting materials for synthesis, aromatic diamines $\mathbf{C_1}$-$\mathbf{C_3}$, with 4-dimethylaminopyridine were dissolved in methylene chloride and cooled. Acid chlorides $\mathbf{D_1}$ and $\mathbf{D_2}$, dissolved in methylene chloride, were added dropwise to the solutions of amines. The reaction mixtures were stirred at room temperature and the precipitated crude products were filtered off, dissolved in methylene chloride and purified by column chromatography (gradient CH_2Cl_2/MeOH).

The nitro groups of obtained compounds were reduced by catalytic hydrogenation (Pd/C) in methanol. Then, the catalyst was filtered off and the resulting solution was concentrated under reduced pressure. The crude products were purified by column chromatography using chloroform with methanol gradient.

3,5-Di(3-amino-4-methylbenzamide)-1H-1,2,4-triazole **(1)**

Starting materials: 4-dimethylaminopyridine (DMAP) (0.024 g, 0.2 mmol), 3,5-diamino-1,2,4-triazole (0.5 g, 5.045 mmol), 4-methyl-3-nitrobenzoyl chloride (2.215 g, 11.099 mmol). Product **1** (1.02 g, 2.79 mmol), m.p. 197-198°C.

3,5-Di(3-aminobenzamido)-1H-1,2,4-triazole **(2)**

Starting materials: DMAP (0.024 g, 0.2 mmol), 3,5-diamino-1,2,4-triazole (0.5 g, 5.045 mmol), 3-nitrobenzoyl chloride (2.06 g; 11.10 mmol). Product: **2** (0.22 g, 0.65 mmol), m.p. 167-168°C.

2,4-Di(3-amino-4-methylbenzamide)-1,3,5-triazine **(3)**

Starting materials: DMAP (0.024 g, 0.2 mmol), 2,4-diamino-1,3,5-triazine (0.5 g, 4.5 mmol), 4-methyl-3-nitrobenzoyl chloride (1.976 g, 9.9 mmol). Product **2** (0.32 g, 0.85 mmol), m.p. 109-110°C.

2,4-Di(3-aminobenzamido)-1,3,5-triazine **(4)**

Starting materials: DMAP (0.024 g, 0.2 mmol), 2,4-diamino-1,3,5-triazine (0.5 g, 4.5 mmol), 3-nitrobenzoyl chloride (1.84 g; 9.9 mmol). Product **4** (0.29 g, 0.83 mmol), m.p. 173-174°C.

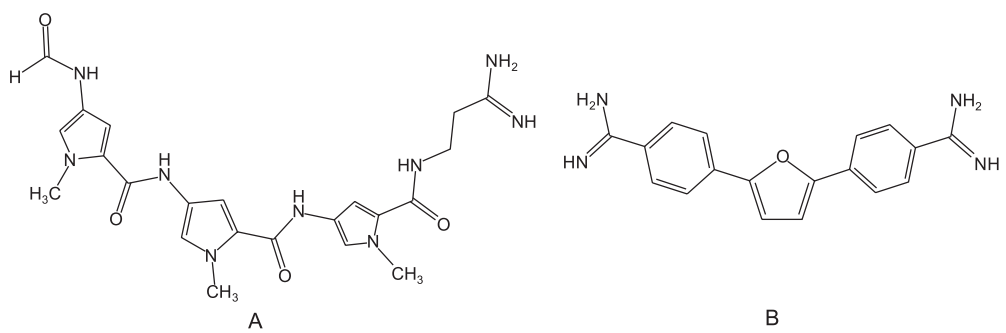
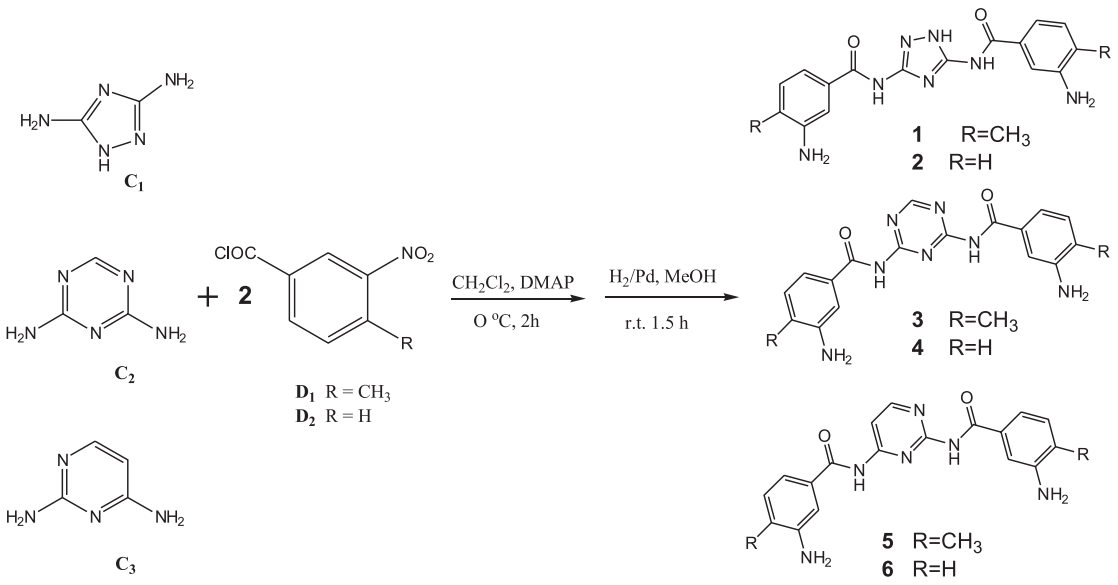


Figure 1. Structures of distamycin (**A**) and furamidine (**B**)



Scheme 1. Synthesis of compounds **1-6**

2,4-Di(3-amino-4-methylbenzamido)-pyrimidine **(5)**

Starting materials: DMAP (0.024 g, 0.2 mmol), 2,4-diaminopyrimidine (0.5 g, 4.54 mmol), 4-methyl-3-nitrobenzoyl chloride (1.99 g, 9.9 mmol). Product **5** (0.34 g, 0.90 mmol), m.p. 98-99°C.

2,4-di(3-aminobenzamide)-pyrimidine **(6)**

Starting materials: DMAP (0.024 g, 0.2 mmol), 2,4-diaminopyrimidine (0.5 g, 4.54 mmol), 3-nitrobenzoyl chloride (1.85 g; 9.98 mmol). Product **6** (0.18 g, 0. 52 mmol), m.p. 291-292°C.

Biology

Ethidium bromide was purchased from Carl Roth GmbH, topoisomerase I and II was from Amersham Pharmacia, Biotech (USA). Stock cultures of breast cancer MCF-7 and 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, streptomycin and penicillin were products of Sigma. Plasmid pBR322 was purchased from Fermentas Life Science.

Cell culture

Stock cultures of breast MCF-7 cancer cells were maintained in continuously exponential growth by weekly passage in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 50 μg/mL streptomycin, 100 U/mL penicillin at 37°C in humid atmosphere containing 5% CO_2. The cells were cultivated in Costar flasks and subconfluent cells were detached with 0.05% trypsin and 0.02% EDTA in calcium-free phosphate-buffered saline. The study was carried out using cells from passages 3-7, growing as monolayer in six-well plates (Nunc) (5×10^5 cells per well) and preincubated 24 h without phenol red.

Determination of IC_{50}

The compounds were dissolved in DMSO and used at concentrations of 10, 50, 100 and 150 μM. Microscopic observations of cells 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 washed with the medium. Then, 250 μL of cells suspension was mixed with 10 μL of the dye mixture and 200 cells per sample were examined by fluorescence microscopy. The percentage of non-viable (apoptotic and necrotic) cells was counted. The

Table 1. Physico-chemical properties of compounds **1**-**6**

Comp.	Formula Molecular weight	Yield [%]	R_f	^{1}H NMR, δ [ppm] (DMSO-d_6)	^{13}C NMR, δ [ppm] (DMSO-d_6)
1	$C_{18}H_{19}N_7O_2$ 365.39	78.59	0.84	8.67 (s, 2H), 8.19 (s, 1H), 7.37 (d, 2H), 6.91 (d, 2H), 6.38 (s, 2H), 5.60 (br s, 4H), 2.52 (s, 6H)	163.87, 157.9, 152.08, 137.32, 134.94, 132.68, 126.73, 19.92
2	$C_{16}H_{15}N_7O_2$ 337.34	51.59	0.91	7.46 (s, 2H), 7.21 (s, 1H), 7.17 (m, 2H), 7.12 (s, 2H), 6.67 (d, 4H), 5.61 (br s, 4H)	167.4, 157.65, 147.81, 134.13, 131.95, 128.05, 123.11
3	$C_{19}H_{19}N_7O_2$ 377.40	74.56	0.89	9.02 (s, 2H), 8.59 (d, 2H), 8.25 (s, 1H), 8.11 (d, 2H), 6.98 (s, 2H), 5.50 (s, 4H), 2.48 (s, 6H)	165.48, 158.01, 149.58, 142.13, 139.03, 130.98, 126.81, 19.97
4	$C_{17}H_{15}N_7O_2$ 349.35	68.03	0.48	8.75 (s, 2H), 8.64 (s, 1H), 8.32 (m, 4H), 8.26 (d, 2H), 8.1 (d, 2H), 5.68 (br, 4H)	159.58, 156.99, 145.04, 135.15, 128.91, 122.03, 118.13
5	$C_{20}H_{20}N_6O_2$ 376.41	78.26	0.45	9.63 (s, 2H), 9.04 (s, 2H), 8.75 (d, 2H), 8.29 (s, 2H), 7.57 (d, 2H), 5.46 (br s, 4H), 2.43 (s, 6H)	164.98, 159.87, 154.67, 155.23, 152.32, 142.08, 138.15, 130.69, 125.0, 19.87
6	$C_{18}H_{16}N_6O_2$ 348.36	42.62	0.88	8.82 (s, 2H), 8.22 (s, 2H), 7.85 (d, 2H), 7.77 (d, 2H), 7.38 (s, 2H), 6.81 (m, 2H), 5.94 (s, 4H)	164.78, 162.1, 150.06, 146.0, 136.14, 131.89, 128.22, 124.51, 119.98

results were submitted to statistical analysis using the method of the last squares.

Relaxation assay of topoisomerase I and II

Native pBR322 plasmid DNA (0.20 μg) was incubated with 4 units of topoisomerase I (reaction buffer: 50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.5 M NaCl, 1 mM dithiothreitol) or human topoisomerase II (reaction buffer:10 mM Tris-HCl (pH 7.9), 1 mM ATP, 50 mM KCl, 5 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, and 15 mg/mL bovine serum albumin) in the absence or presence of varying concentrations of the test compounds (10, 50, 100 or 150 μM) in a final volume of 10 μL. The mixture was incubated at 37°C for 30 min and the reaction was terminated by addition of 2 μL of 10% 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 312 nm wavelength transilluminator and photographed under UV light (Canon PowerShot G6, 7.1 megapixels).

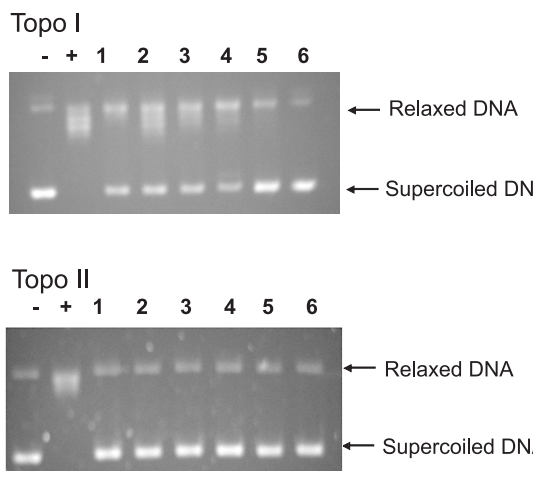


Figure 2. Inhibition of topoisomerases by compounds **1**-**6**. Native pBR322 plasmid DNA (lane -) was incubated with 2 units of topoisomerases I (Topo I) and II (Topo II) in the absence (lane +) or in the presence of compounds (**1**-**6**). The DNA was analyzed by 1% agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed under UV light

RESULTS AND DISCUSSION

We obtained six new compounds, analogues of distamycin, potential minor groove binders. Yields and some physico-chemical properties of the synthesized compounds are reported in Table 1. The *in vitro* experimental findings shown in Table 2 revealed that all of the new oligopeptides exhibit tumor cell cytotoxicity towards the standard cell line of the mammalian tumor MCF-7. The IC_{50} values of the examined compounds are in the range 183.53 –

232.50 μM and are similar to that of other minor groove binder DAPI (IC_{50} = 176 μM) and weaker than for distamycin with IC_{50} = 56.95 μM (13) or Hoechst 33258 (IC_{50} = 55 μM) (14.). IC_{50} of carbocyclic analogue of distamycin is 40.73 μM) (15).

To identify the biochemical target(s) of compounds **1-6**, we investigated their action on DNA topoisomerases using a gel electrophoresis assay. Purified topoisomerases I (Topo I) and II (Topo II) were incubated with increasing concentrations of **1-6** in the presence of supercoiled plasmid DNA, and the products were subjected to electrophoresis to separate the relaxed and supercoiled circular DNA. Figure 2 shows the results of our electrophoresis analysis at the concentration of 100 μM, after staining with ethidium bromide. The compounds at lower concentrations showed no changes in the activity of topoisomerases. The superhelical plasmid (lane **-**) was relaxed by topoisomerases (lane +). At the concentration of 100 μM, each compound inhibited both topo I and topo II ability to transform supercoiled DNA into several topoisomer forms of relaxed DNA. As can be seen (lanes **1-6**), compounds **1-6** are more active against topoisomerase II. None of topological forms of DNA is present in Topo II assay, whereas in the Topo I assay compounds **2**, **3** and **4** inhibited the enzyme activity only partially – some topoisomer forms on gel can be seen (compare lane **2**, **3**, **4** with lane +).

Under identical conditions, complete inhibition of DNA cleavage was obtained using 2 μM camptothecin (Cpt) and 10 μM etoposide (Et), the drugs which are potent inhibitors of topoisomerase I and topoisomerase II, respectively (16, 17).

CONCLUSION

We have shown that compounds **1-6** are potent inhibitors of both topoisomerase I and II. These compounds inhibited the catalytic activity if the topoisomerase is before the formation of the topo-DNA complexes. This suggests that DNA-binding may be implicated in the cytotoxicity of the compounds, possibly by inhibition of interactions between topoisomerases and their DNA targets. The obtained analogues of distamycin represent a new series of potent minor groove binders. Obviously, the mode of binding of these compounds to DNA should be investigated, whether it is non-intercalative binding to minor groove or intercalation of heteroaromatic rings.

The syntheses of these aromatic oligopeptides were simple, convenient and with a good yield. This procedure could be useful to seek other derivatives of distamycin to find the best configuration of aromatic (benzene) and heteroaromatic rings. The IC_{50} values for synthetic analogues **1-6** suggest that these binders can serve as potential carriers of strongly acting moieties, e.g. alkylating fragments. Free amino group of these compounds can be connected with such moieties.

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Table 2. Viability of MCF-7 cells treated for 24 h with different concentrations of compounds **1-6**

Concentration [μM]	Non-viable cells (% of control ± 2)[a]					
	1	**2**	**3**	**4**	**5**	**6**
10	5	12	10	9	7	10
50	11	20	16	14	10	14
100	13	26	26	20	25	25
150	41	44	36	26	36	42
IC_{50}	216.09	183.53	211.39	232.50	212.32	189.59

[a] The mean values ± SD from three independent experiments done in duplicate are presented

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