The biological macromolecules, including protein and nucleic acid, have naturally become targets for a drug design. Nucleic acids play important roles in living systems via participation in genetic information storage, replication, transcription and translation directing protein synthesis. Thus, targeting nucleic acids can selectively disrupt gene expression for treating virus-caused diseases and cancers at the genetic level (1). An understanding of the mechanism, by which minor groove binding agents interact with DNA, has led to the design of agents that can interact reversibly with the selected DNA target sequences. Due to their capability to react with DNA in a more specific manner, minor groove binders hold a great promise to treat the above mentioned diseases at the DNA level (2). Actually, these minor groove-binding agents have been extensively studied as a class of potential therapeutics exhibiting antibacterial, antiprotozoal, antitumor and/or antiviral properties. Analogues of naturally occurring antitumor agents, such as distamycin A or netropsin, which bind into the minor groove of DNA, named lexitropsins, represent a new class of anticancer compounds, currently being under investigation (3). Distamycin A, netropsin (Fig. 1) and their analogues have also been used as carriers of different active fragments, e.g., alkylating groups, with desirable result (4).

This study is a continuation of rational drug design program aiming at the development of distamycin analogues. Over the last few years, the synthesis and biological evaluation of distamycin derivatives containing benzene rings in the place of N-methylpyrrole rings have been reported (5-9). Compounds 1-6 presented here have different heterocyclic residue connected to 3-[3-(4-dimethylaminobutyrylamino)benzamido-benzamide] fragment (Fig. 2). They were obtained using solid phase syntheses (10). This novel approach to distamycin analogues preparation allows the use of several different aromatic nitro amines, as well as acyl chlorides with nitro group. It also sets a new way to obtain a lot of new compounds based on distamycin A structure as the parent molecule.

Abstract: The evaluation of a new group of distamycin analogues 1-6 as potential minor groove binders for the treatment of cancer were investigated. The activity of the new compounds against several restriction enzymes was examined. The studied compounds did not block GC-rich sequences regions of DNA but inhibited catalytic action of endonucleases in AA, AT, TT and AG restriction sites. Determination of association constants using calf thymus DNA, T4 coliphage DNA, poly(dA-dT)2, and poly(dG-dC)2 have confirmed that the tested compounds bind within minor groove of B-DNA. All of the compounds demonstrated activity against DNA topoisomerases II at the concentration 10 µM, but they did not inhibit activity of topoisomerase I. The studied derivatives were evaluated in human MCF-7 breast cancer cells and showed antiproliferative and cytotoxic effects in the range of 81.70 µM and 200.00 µM.

Keywords: distamycin analogue, DNA topoisomerase, endonucleases inhibition, cytotoxic activity, antiproliferative agents

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Application of that preparation method has been illustrated by parallel semi-automatic syntheses of three groups of distamycin analogues containing benzene units, with use of the Syncore Reactor (11). Ethidium bromide assay was used to show that these compounds bind to plasmid pBR322. The most active, in fluorescence reduction, appeared to be compounds 1-6 presented here with configuration meta-meta of benzene rings (11). The largest degree of fluorescence reduction was observed in the case of compound 6 – 37.20%, while the fluorescence reduction caused by distamycin under the same condition was 63.45% (7).

The purpose of our work was to confirm the biological activity of these new compounds. We investigated the influence of compounds 1-6 onto restriction enzymes activity, to show their specificity of DNA binding. To understand the mechanism by which compounds 1-6 interacted with DNA, the association constants of complexes drug-DNA, using calf thymus DNA, T4 coliphage DNA, poly(dA-dT)\textsubscript{2} and poly(dG-dC)\textsubscript{2}, were assessed.

The antitumor activity of DNA binding drugs is not only due to their interaction with DNA \textit{per se} but, is at least in part, the result of the inhibition of different enzymes activity. Such enzymes could be DNA topoisomerase I and II. Hence in our investigation, compounds 1-6, employing the topoisomerase I/II inhibition assay, were studied.

The final stage of the research was to determine the antiproliferative and cytotoxic effects of the new distamycin analogues 1-6 against the MCF-7 line cells.
EXPERIMENTAL

Materials

Compounds 1-6 were synthesized in the Department of Organic Chemistry of Medical University of BiałyStok. Ethidium bromide was purchased from Carl Roth GmbH, topoisomerase I and II from Amersham Pharmacia, Biotech (USA). Stock cultures of MCF-7 breast cancer were purchased from the American Type Culture Collection, Rockville, MD, USA. Dulbecco’s modified Eagle’s medium, fetal bovine serum (FBS), netropsin, streptomycin, penicillin, calf thymus DNA, poly(dA–dT) •poly(dA–dT), T4 DNA and poly(dG–dC) •poly(dG–dC) were products of Sigma. Plasmid pBR322 and restriction enzymes was purchased from Fermentas Life Science.

Endonuclease inhibition assay

Plasmid pBR322 (0.2 mg/mL) was linearized by incubation of DNA at 37°C for 1 h with 5 units of restriction endonuclease NdeI (reaction buffer R: 10 mM Tris HCl (pH 8.5 at 37°C), 10 mM MgCl₂, 100 mM KCl, 0.1 mg/mL BSA). The enzyme was inactivated by incubation for 20 min at 65°C. Afterwards, a single compound of the following: netropsin (NT) and compounds 1-6 (2 mL of 1 mM solutions) were added to all the samples, and the samples were incubated at 37°C for 1 h. After that, 5 units of next restriction nuclease: SspI, VspI, BamHI, SalI, SmaI, PstI, EcoRV, HindIII (reaction buffer recommended by the producer) were added to each sample. The mixtures were incubated at 37°C for 1 h, and the enzymatic action was terminated after 20 min at 65°C (for SspI, VspI, SalI, SmaI, EcoRV) and at 80°C (for BamHI, PstI). The reaction mixtures were subjected to electrophoresis (2 h, 70 V) on a 1.5% agarose gel, containing 1% ethidium bromide, in the standard TBE buffer. The gels were photographed under UV light (312 nm) and analyzed (Gel Doc System, InGenius, Syngen) (12).

Ethidium bromide displacement assay

Each well of 96-well plate was loaded with Tris buffer containing ethidium bromide (0.1 M Tris, 1 M NaCl, pH 8, 0.5 mM EtBr final concentration, 100 μL). Then, 15 μg DNA (calf thymus DNA, poly(dA–dT)₂, T4 DNA and poly(dG–dC)₂) as water solution (0.05 μg/μL) was added to each well. Afterwards, compounds 1-6 (1 mM solution in water; final concentrations 10, 50, 75, 100, 150 and 200 μM) were added to each well. After the incubation at 37°C for 30 min the fluorescence of each mixture was read on a fluorescence spectrophotometer Infinite M200 TECAN (ex. 546 nm, em. 595 nm) in duplicate experiments with two control wells (no drug = 100% fluorescence, no DNA = 0% fluorescence). Then, the concentrations reducing the fluorescence to 50% for each solution under scope were calculated. 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 $K_{inh}[EtBr] = K_{app}[drug]$, where $[drug]$ = the concentration of tested compound at a 50% reduction of fluorescence and $K_{inh}$ is known (14).

Relaxation assay of topoisomerase I and II

Native pBR322 plasmid DNA (0.20 μg) was incubated with 2 units of human topoisomerase I (reaction buffer: 50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.5 M NaCl, 1 mM diithiothreitol) or human topoisomerase II (reaction buffer:10 mM Tris-HCl (pH 7.9), 1 mM ATP, 50 mM KCl, 5 mM MgCl₂, 50 mM NaCl, 0.1 mM EDTA, and 15 μg/mL bovine serum albumin) in the absence or presence of varying concentrations of the tested compounds (10, 50 and 100 μM), in a final volume of 10 μL. The mixtures were incubated at 37°C for 30 min and the reactions were terminated by adding 2 μL of 10% SDS. The reaction mixtures were subjected to electrophoresis (3 h, 90 V) on a 1.0% agarose gels in TBE buffer (90 mM Tris-borate and 2 mM EDTA). The gels were stained with ethidium bromide solution (0.5 μg/mL) for 30 min and photographed under UV light (Gel Doc System, InGenius, Syngen). The DNA was visualized using 312 nm wavelength transilluminator.

Cytotoxic and antiproliferative activity

Cell culture

Human MCF-7 breast cancer cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 50 μg/mL streptomycin, 100 U/mL penicillin at 37°C. The cells were cultured 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 the cells from passages 3-7, growing as monolayer in 6-well plates (Nunc) (5 × 10⁵ cells per well).

Determination of IC₅₀ by fluorescent microscopy assay

To examine the effects of the studied compounds on MCF-7 cells proliferation, the cells were seeded in 24-well tissue culture dishes at 1 × 10⁵ cells/well with 1 mL of growth medium. The com-
pounds were dissolved in DMSO and used at concentrations of 10, 50, 100 and 150 μM. Microscopic observations of the cell monolayers were performed with a Nikon optiphot microscope. Wright-Giemsa staining was performed using the Fischer Leuko Stat Kit. After 24 h of the drug treatment, the cancer 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 of the media were removed and the 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 the cells suspension was mixed with 10 μL of the dye mix and 200 cells per sample were examined by fluorescence microscopy. Then, the amount of viable and non-viable (apoptotic and necrotic) cells at each concentration of compounds 1-6 were counted.

Cell viability of breast cancer cells cultured in the presence of the studied compounds was calculated as a percent of control cells. The experiments were performed in triplicates. Cell number was plotted versus drug concentration and IC₅₀ values were calculated from dose-response curves as the concentration of drugs that reduce the number of viable cells to 50% of control using Excel 2013 software.

Determination of IC₅₀ by flow cytometry assessment of annexin V binding

Apoptosis was determined by assessment of phosphatidylserine exposure by Annexin V binding using the Annexin V-PE Apoptosis Detection Kit according to the manufacturer’s instruction. Ungated cells (10,000) were analyzed in a flow cytometer (Beckman, Coulter, USA). Propidium iodide exclusively stains cells with a disrupted cell membrane and can be used to identify late apoptotic and dead cells.

Statistical analysis

In all experiments, the mean values for three assays ± standard deviations (SD) were calculated. The results were submitted to statistical analysis using the Student’s test. Differences were considered significant when p < 0.05.

RESULTS AND DISCUSSION

The synthetic route of compounds 1-6 was presented in a previous paper (10). These carbocyclic distamycin analogues were prepared with high purity and in good yield. The chemical structures of the new lexitropsins 1-6 were proved by NMR and LC-MS analysis.

Restriction enzyme activity assay has been employed earlier to examine the sequence selectivity of the DNA binding to lexitropsins (12), so applying the same method in our study to examine the activity of compounds 1-6 against several restriction enzymes was the logical choice.

Double-stranded closed circular medium copy plasmid pBR322, used in this experiment, present in more than 90% in the supercoiled form, was linearized by the first nuclease - NdeI. Such linear plasmid was incubated with netropsin (NT) and compounds 1-6 allowing them to bind to the DNA. The next applied restriction enzyme was able to cut of DNA at their recognition site unless it was occupied by the tested compound. Table 1 presents the applied endonucleases, their recognizable DNA sequence and the activity of the studied compounds compared to NT. The inhibition of the letter applied

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Recognized sequence</th>
<th>Compound</th>
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<tbody>
<tr>
<td></td>
<td>NT</td>
<td>1</td>
</tr>
<tr>
<td>SspI</td>
<td>AAA↓TTT</td>
<td>+</td>
</tr>
<tr>
<td>VspI</td>
<td>AT↓TAAT</td>
<td>+</td>
</tr>
<tr>
<td>BamHI</td>
<td>G↓GATCC</td>
<td>±</td>
</tr>
<tr>
<td>Sall</td>
<td>G↓TCGA</td>
<td>-</td>
</tr>
<tr>
<td>SmaI</td>
<td>CCC↓GGG</td>
<td>-</td>
</tr>
<tr>
<td>PstI</td>
<td>TGCA↓G</td>
<td>+</td>
</tr>
<tr>
<td>EcoRV</td>
<td>GAT↓ATC</td>
<td>+</td>
</tr>
<tr>
<td>HindIII</td>
<td>A↓AGCTT</td>
<td>+</td>
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</tbody>
</table>

+: inhibition of enzyme, ±: slight inhibition of enzyme, -: no effect, nm: no migration of DNA, ↓: restriction.
enzymes by NT and compounds 1-6 was identified after a process of electrophoresis. It was not seen two, specific for each enzyme, DNA fragments on the gel, but only one, corresponding to linearized plasmid pBR322. If the studied compound did not inhibit an endonuclease activity, two linear fragments of DNA were observed. Plasmid DNA did not migrate in the presence of compound 1 and remained in take-off pockets in the experimental conditions. Such results led us to a conclusion that compound 1 did not interact with the endonucleases in this process.

At the preliminary stage of the experiment, compounds 1-6 were tested against three restriction enzymes cleaving the following sequences: Smal – CCC↓CCC, SspI – AAA↓TTT and VspI – AT↓TAAT. Evidently, the studied compounds did not block GC-rich sequence but they were active against AT-rich regions.

Afterwards, the cleavage of DNA by EcoRV (GAT↓ATC) and HindIII (A↓AGCTT) in the presence of the ligands 1-6 was examined. Surprisingly, neither of the compounds was active against the first enzyme, but substances 2, 3, 5 and 6 inhibited activity of enzyme HindIII. This indicated that compounds 2-6 could bind to AA and TA-rich sequences with an embedded GC pair, but not to ATAT-box.

The observation of the products of cleavage by SalI, BamI and PstI led to assumption that the studied compounds did not bind to GT and GG recognition sites but they could block AG sequences. The obtained results confirmed DNA affinity and sequence-specificity of the studied compounds, because most of these ligands had inhibitory influence on AT-specific enzymes.

Values of association constants of different ligands of DNA permitted to qualify potential and selectivity of interactions between ligands and DNA. The binding affinities of compounds 1-6 to calf thymus DNA, T4 coliphage DNA, and synthetic polymers poly(dA-dT)2 and poly(dG-dC)2 were compared by using the ethidium displacement assay and are presented in Table 2. The DNA-binding data characterized the affinity of compounds 1-6 and gave an indication of base-sequence specificity.

The values of association constants demonstrated that each of the tested compounds can bind to all of the studied types of DNA. The affinity constants of compounds 1-6 in the range of 2.14 – 4.66 × 105 indicated moderate interactions with deoxyribonucleic acid from *calf thymus*. The high values of binding constants for T4 coliphage DNA were observed for analogues 1-6, which 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 (16). These data indicated that compounds 1-6 had interacted with GC-base pairs, although for compounds 1, 3 and 6 the binding affinity had been weaker than for AT-base pairs. Since calf thymus DNA contains random sequences and therefore fewer AT sites than poly(dA-dT)2, the selectivity of ligands 3, 5 and 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 weaker than netropsin.

Topoisomerases were an extremely useful research strategy for the potential anticancer and antimicrobial drugs. DNA minor groove binders were often topoisomerase I and/or topoisomerase II

<table>
<thead>
<tr>
<th>Ligand</th>
<th>K_{app} × 10^5 M^{-1}</th>
<th>IC_{50} [µM]^c</th>
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<tbody>
<tr>
<td>Ethidium bromide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT</td>
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<td>DST</td>
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</tr>
<tr>
<td>6</td>
<td>2.14</td>
<td>3.70</td>
</tr>
</tbody>
</table>

^a values from ref. 13; ^b values from ref. 14; ^c values from ref. 6; ^d values from ref. 15. ^e Mean values ± SD from three independent experiments done in duplicate are presented.
inhibitors. Distamycin A blocked activity of topoisomerase I (17), as well as topoisomerase II (18). The ability of compounds 1-6 to inhibit these DNA processing enzymes was also investigated.

Purified human topoisomerases I and II were incubated at increasing concentration of compounds 1-6 (10, 50 and 100 µM) in the presence of supercoiled plasmid DNA pBR322. The products were subjected to electrophoresis to separate relaxed and supercoiled circular DNA. Figure 3 shows the results of electrophoresis analysis of the examined compounds after staining with ethidium bromide.

As seen on Figure 3, the supercoiled plasmid (lane -) was relaxed by topoisomerases (lane +). Figure 3 demonstrates that at concentration of 10 µM all of the compounds have visible effect on the ability of topoisomerase II to transform supercoiled DNA into several topoisomer forms of relaxed DNA. At that concentration neither of the compounds exhibited an activity in the presence of topoisomerase I. The proof of the cleavage inhibition of DNA by the topoisomerase II is the fact that minor groove binders interact with DNA and occupy the binding sites of the enzyme (18). Probably, these compounds inhibited the catalytic activity of the topoisomerase II at the step prior to the formation of the topo-DNA complexes because they acted making it impossible access of topoisomerase to sequences in DNA selectively recognized by the drug. Under identical conditions, complete inhibition of DNA cleavage was obtained using 2 µM camptothecin (inhibitor of topoisomerase I) and 10 µM etoposide (inhibitor of topoisomerase II) (19).

The described compounds were tested for their in vitro antitumor activity in the standard human MCF-7 breast cancer cells. Their cytotoxic activity is presented in Table 2 as IC50 values. All of the tested compounds displayed concentration dependent activity. The concentrations for all the compounds that inhibited 50% of the colony were within the range of 81.70 (± 2) µM to 200.00 (± 2) µM, while IC50 value of netropsin was 5.40 (± 2) µM and distamycin 64.06 (± 2) µM. From those data we could see that the most active, against MCF-7 cells, was compound 6 with IC50 value of 81.70 ± 2 µM. The high cytotoxic properties of compound 6 also manifested themselves in highly effective DNA-binding affinity – this ligand was the most active in ethidium bromide assay with value of fluorescence reduction of 37.20%, compared to 63.45% for distamycin (11).

CONCLUSION

The target of the research was to confirm the specificity binding effectiveness of the chosen compounds to DNA in minor groove and their interactions with enzymes involved in anti-cancerous processes. Additionally, the optional study of the antiproliferative and cytotoxic activity in human MCF-7 breast cancer cells was carried out.

The investigated compounds 1-6 displayed an interesting spectrum of activity. They, except compound 1, inhibited catalytic action of endonucleases recognizing sequence of AA, AT, TT and AG. These agents 2-6 interacted with GC-rich sequences
though their binding affinity was weaker compared to their activity in AT-rich regions.

The experiment confirmed all of the compounds were bound in minor groove of DNA.

Evaluation of the DNA topoisomerases inhibition provided an additional knowledge about the mechanism of the 1-6 compounds activity. It was shown that compounds 1-6 can bind to DNA and are potent inhibitors of topoisomerase II. The observed inhibitive activity of the studied compounds against topo II, while their inactivity against topo I, indicated that the topoisomerase II inhibiting activity contributes to the cytotoxicity of the investigated compounds.

It was impossible to establish a close structure-activity relationship in this study due to insignificant differences obtained within the whole spectrum in the activity of the group. But evidently it remained clear that incorporating of the heterocyclic residue into new distamycin analogues structure have not increased the activity of the studied compounds – the most active in this study was the compound with 6-aminophenyl residue. However, the investigated analogues of distamycin with free amino groups could be used as carriers of active groups, e.g., alkylating fragments. The potential therapeutic applications of the studied compounds could be considered after their further investigation.

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