AMINO AND CHLORAMBUCIL ANALOGUES OF PENTAMIDINE – SYNTHESIS AND BIOLOGICAL EXAMINATIONS

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Abstract: The amino analogues of pentamidine with a polymethylene (n = 3 – 6) chain and their chlorambucil derivatives were synthesized. The obtained compounds revealed cytotoxic effect on MCF-7 human breast cancer cell line (IC$_{50}$ = 22 ~ 95 ± 2 µM), mainly by the induction of apoptosis. The topoisomerase I/II inhibition assay and the ethidium displacement assay with the use of pBR322 plasmid DNA were used to the study of mechanism by which the obtained compounds could act. All the compounds are able to bind with DNA and interfere *in vitro* with the activity of topoisomerase (I and II). The determination of association constants with the use of calf thymus DNA, T4 coliphage DNA, poly(dA-dT)$_2$ and poly(dG-dC)$_2$ showed that the tested compounds bind within minor groove of B-DNA, but not selectively. The alkylating activity of chlorambucil derivatives determined *in vitro* using a Preussmann test was similar to the activity of chlorambucil. The influence of all the compounds on the amidolytic activity of plasmin and trypsin was also examined. The plasmin activity was inhibited by pentamidine, chlorambucil and aromatic bis-amines (IC$_{50}$ = 0.1 ~ 8 mM), whereas the trypsin activity was influenced only by pentamidine.

Keywords: pentamidine, chlorambucil, DNA binding, topoisomerase, anticancer activity

The aromatic bisamidines, such as berenil, pentamidine, propamidine and furamidine (Figure 1), are well-known antimicrobial and antifungal drugs (1–4). The antibiotic and anticancer activity of this kind of compounds has also been described (5–7). Despite its toxicity (8), pentamidine in particular has most commonly been used to treat *Pneumocystis carinii* pneumonia (PCP) in patients with AIDS as well as for the treatment of antimony-resistant leishmaniasis (9, 10). The exact mechanism by which this drug acts *in vivo* is still elusive and the molecular targets of pentamidine have not been unambiguously identified yet. The results of NMR (11), X-ray crystallography (12), footprinting (13) and molecular modeling (14, 15) studies indicated that pentamidine and its analogues bind with the minor groove of double helix DNA through a noncovalent and nonintercalative mode, and they prefer adenine-thymine rich sequence. The interactions involve the hydrogen bonds of amidine groups of pentamidine and the bases of DNA, and van der Waals contacts by sticking the drug between minor groove walls. In addition, the positive charge of amidine groups facilitates the interactions with phosphate groups and the negative electrostatic potential of the groove. Pentamidine can interfere with the enzymatic activity of pathogen topoisomerases (16, 17) as well as is capable of binding with tRNA and inhibiting aminoacylation and translation (18). The anticancer activity of pentamidine is considered to mediate *via* inactivation of cancer cell-expressed protein tyrosine phosphatases (19). These facts contribute to the considerable interest in the development of novel drugs based on the structure of aromatic bis-amidines as potential agents for the treatment of both antiparasitic and anticancer diseases (7, 20, 21).

In the course of our investigations of new potential anticancer agents we have found that the carbocyclic analogues of netropsin and distamycin

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can act as DNA minor groove binders (22, 23), and some of them are able to inhibit the amidolytic activity of proteolytic enzymes, such as plasmin or urokinase, which are involved in cancer progression and metastasis (24). It has also been found that the amino analogues of pentamidine with tetra- and hexamethylene chain between aromatic units show the antiproliferative activity in the MCF7 cell line of mammalian tumor (Carmichael test) and the inhibitory influence on the activity of topoisomerase I and II (25) and the activity of DNA restriction enzymes (26). In this paper the amino analogues of propamidine **1** and pentamidine **3** were synthesized (Scheme 1) in order to compare the biological properties of these agents with compounds including odd polymethylene chains between aromatic rings. Additionally, the aromatic amines, also those with the even polymethylene linker, were transformed into chlorambucil derivatives **5–8** (Scheme 1) as the potentially new alkylating agents. The cytotoxic effect of all the compounds was determined by fluorescent microscopy and flow cytometric analysis. To understand the mechanism by which the prepared compounds could act, the DNA-binding properties of the compounds **1–8** were examined by the use of the ethidium displacement assay and the determination of association constants of drug-DNA complexes by the use of calf thymus DNA, T4 coliphage DNA, poly(dA-dT)$_2$ and poly(dG-dC)$_2$ (27, 28). To check whether the reported ligands can interfere with the proteolytic enzymes involved in cancer progression, the amidolytic activity of plasmin and trypsin in the presence of **1–8** was also studied. The alkylating activity of compounds **5–8** with chlorambucil moieties was determined by the use of a Preussmann test (29). The aim of this work was to examine the structure-activity relationship of aromatic bis-amines and their derivatives as DNA minor groove binders and also as the carriers of active groups (e.g., alkylating groups). Such study

could be useful in the rational design of anticancer drugs, which are structurally similar to pentamidine.

EXPERIMENTAL

Materials

N-methylmorpholine, N-hydroxybenzotriazole (HOBt), 1,3-dicyclohexylcarbodiimide (DCC) were produced by Iris Biotech GmbH (Marktredwitz, Germany), and 4-dimethylaminobenzaldehyde (DMAB), palladium/charcoal activated (10% Pd) and precoated TLC plates by Merck Chemicals (Darmstadt, Germany). Ethidium bromide was purchased from Carl Roth GmbH (Karlsruhe, Germany), recombinant human topoisomerase I and purified human DNA topoisomerase IIá were obtained from TopGEN Inc. (Port Orange, FL, USA). Stock cultures of MCF-7 were purchased from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), netropsin, pentamidine as isothionate salt, chlorambucil, calf thymus DNA, T4 coliphage DNA, poly(dA-dT)•poly(dA-dT), and poly(dG-dC)•poly(dG-dC), streptomycin, penicillin, trypsin and Bzl-L-Arg-pNA.HCl (Bzl = benzyl) were purchased from Sigma-Aldrich Sp. z o.o. (Poznań, Poland). Plasmid pBR322 was purchased from Fermentas Life Science (Vilnius, Lithuania) and the Annexin V-PE Apoptosis Detection Kit from Calbiochem, Merck (Darmstadt, Germany). Plasmin and S-2251 (H-D-Val-Leu-Lys-pNA) were obtained from Chromogenix Instrumentation Laboratory S.P.A. (Milano, Italy). Dichloromethane (DCM), dimethylformamide (DMF), methanol and benzene were produced by ChemPur (Piekary Śląskie, Poland).

Chemistry

The identifications of the final compounds were controlled by TLC (1% NH$_3$ in methanol),
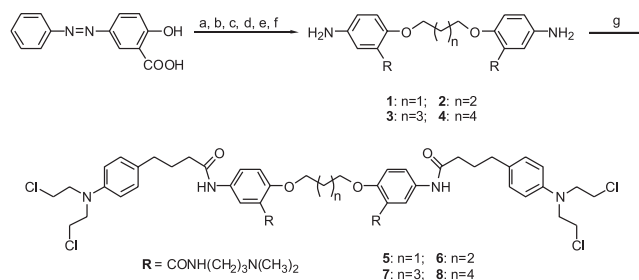
Scheme 1. Structure and synthesis of compounds **1–8**: a) MeOH, H$_2$SO$_4$, Δ; b) I(CH$_2$)$_n$I, K$_2$CO$_3$, acetone, Δ; c) 1. NaOH, DMF/H$_2$O, Δ; 2. HCl$_{aq}$; d) SOCl$_2$, benzene, Δ; e) NH$_2$(CH$_2$)$_3$N(CH$_3$)$_2$, CH$_2$Cl$_2$, r.t.; f) H$_2$, Pd/C (10%), MeOH; g) 4-{4-[bis(2-chloroethyl)amino]phenyl}butanoic acid, HOBt, DCC, N-methylmorpholine

chromatograms were visualized by UV irradiation and by spraying of DMAB solution (4-dimethyl-aminobenzaldehyde – 1 g, 36% HCl – 30 mL, EtOH – 10 mL, n-butanol – 180 mL). Thin layer chromatograms were prepared on precoated plates (Merck, silica gel 60 F$_{254}$). The structures of all the compounds were confirmed by ¹H NMR and ¹³C NMR spectra recorded on a Bruker AC 400F spectrometer, using TMS as an internal standard. The melting points were not determined because of the hygroscopic and polymorphic form of the obtained compounds. Elemental analysis of C, H, and N was performed on a Perkin Elmer 240 analyzer, while Cl was determined by Shuniger method, and satisfactory results within ± 0.4% of the calculated values were obtained.

Preparation of pentamidine amino analogues

The procedure for the synthesis and the properties of compounds **2** and **4** were described earlier (25). The analogues of pentamidine **1** and **3** were synthesized similarly. The catalytic hydrogenation of diazenyl group was carried out in methanol (with addition of 10% HClaq 36% in methanol, v/v) in the presence of Pd/C (10%). The obtained aromatic amines **1** and **3** were purified by column chromatography using the solvent system as follows: (1) C$_6$H$_6$/C$_2$H$_5$OH 1:4 v/v; (2) CH$_3$OH.

1,3-Bis{4-amino-2-[(3-dimethylaminopropyl)carbamoyl]phenyloxy}propane tetrahydrochloride (1)

Yield 53.5%, R$_f$ = 0.25; ¹H NMR (400 MHz, MeOD, δ, ppm): 1.87–1.91 (m, 4H), 2.32–2.35 (t, *J* = 6 Hz, 2H), 2.85 (s, 12H), 3.05–3.09 (m, 4H), 3.32–3.35 (m, 4H), 4.26–4.29 (t, *J* = 6.2 Hz, 4H), 6.93–6.96 (d, *J* = 8.4 Hz, 2H), 7.02–7.05 (dd, *J* = 8.4 Hz, *J* = 2.6 Hz, 2H), 7.26–7.27 (d, *J* = 2.6 Hz, 2H); ¹³C NMR (400 MHz, MeOD, δ, ppm): 26.15, 30.15, 37.34, 43.49, 56.57, 68.78, 116.55, 119.76, 124.79, 130.48, 141.20, 151.40, 169.30; Analysis: calcd. for C$_{27}$H$_{46}$Cl$_4$N$_6$O$_4$: C 49.10, H 7.02, Cl 21.47, N 12.72%; found: C 49.06, H 7.04, Cl 21.51, N 12.68%.

1,5-Bis{4-amino-2-[(3-dimethylaminopropyl)carbamoyl]phenyloxy}pentane tetrahydrochloride (3)

Yield 52.4%, R$_f$ = 0.26; ¹H NMR (400 MHz, MeOD, δ, ppm): 1.61–1.76 (m, 2H), 2.01–2.07 (m, 8H), 2.90 (s, 12H), 3.19–3.27 (m 4H), 3.40–3.51 (m, 4H), 4.26–4.29 (t, *J* = 6 Hz, 4H), 7.65–7.66 (d, *J* = 8.2 Hz, 2H), 7.85–7.87 (dd, *J* = 8.2 Hz, *J* = 2.6 Hz, 2H), 7.87–7.89, (d, *J* = 2.6 Hz, 2H); ¹³C NMR (400 MHz, MeOD, δ, ppm): 23.61, 26.09, 29.64, 37.67, 43.56, 56.63, 70.76, 115.72, 116.94, 121.49, 128.35,

130.81, 158.21, 167.54; Analysis: calcd. for C$_{29}$H$_{50}$Cl$_4$N$_6$O$_4$: C 50.59, H 7.32, Cl 20.60, N 12.21%; found: C 50.51, H 7.35, Cl 20.64, N 12.19%.

Preparation of chlorambucil derivatives

HOBt (4.5 eq.), DCC (4.5 eq.) dissolved in DMF and methylmorpholine (4.5 eq.) were added to the suspension of aromatic bis(amine) tetrahydrochloride (respectively **1–4**, 1 eq.) and 4-{4-[bis-(2-chloroethyl)amino]phenyl}butanoic acid (4.5 eq.) in dried DMF. The mixture was stirred in room temperature for 48 h. The precipitate was filtered out and the obtained solution was evaporated. The brown oil was dissolved in methanol and acidified (10% v/v HCl in methanol) to pH = 1, and the solvent was evaporated. The chlorambucil derivative was purified by column chromatography (silica gel 60, Merck, 240 mesh) with the use of chloroform and methanol (4 : 1 v/v) as an eluent. The fractions included chlorambucil derivative were collected, evaporated and rechromatographed in the same conditions.

1,3-Bis{4-[4-[4-di(2-chloroethyl)amino]phenyl]butanoylamino-2-[(3-dimethylaminopropyl)carbamoyl]phenyloxy}propane tetrahydrochloride (5)

Yield 20.4%; R$_f$ = 0.32; ¹H NMR (400 MHz, MeOD, δ, ppm): 1.94–2.00 (m, 2H), 2.24–2.34 (m, 4H), 2.50–2.67 (m, 4H), 2.97 (s, 12H), 3.15–3.33 (m, 12H), 3.41–3.54 (t, *J* = 6.18 Hz, 4H), 3.65–3.74 (m, 16H), 4.21–4.30 (t, *J* = 6.21 Hz, 4H), 6.68–6.73 (d, *J* = 8.8 Hz, 4H), 7.13–7.19 (m, 6H), 7.61–7.65 (dd, *J* = 8.3 Hz, *J* = 2.4 Hz, 2H), 7.92–7.93 (d, *J* = 2.4 Hz, 2H); ¹³C NMR (400 MHz, MeOD, δ, ppm): 25.32, 27.64, 32.63, 36.17, 38.31, 44.65, 56.89, 71.27, 78.37, 113.39, 114.68, 120.55, 123.84, 127.01, 131.03, 132.98, 146.24, 154.64, 169.25, 174.36. Analysis: calcd. for C$_{55}$H$_{80}$Cl$_8$N$_8$O$_6$: C 53.58, H 6.54, Cl 23.00, N 9.09%; found: C 53.51, H 6.56, Cl 23.05, N 9.07%.

1,4-Bis{4-[4-[4-di(2-chloroethyl)amino]phenyl]butanoylamino-2-[(3-dimethylaminopropyl)carbamoyl]phenyloxy}butane tetrahydrochloride (6)

Yield 21.7%; R$_f$ = 0.29; ¹H NMR (400 MHz, MeOD, δ, ppm): 1.97–2.02 (m, 4H), 2.33–2.40 (m, 4H), 2.61–2.70 (m, 4H), 2.87 (s, 12H), 3.05–3.22 (m, 12H), 3.39–3.50 (t, *J* = 6.2 Hz, 4H), 3.63–3.73 (m, 16H), 4.20–4.29 (t, *J* = 6.15 Hz, 4H), 6.71–6.76 (d, *J* = 8.74 Hz, 4H), 7.09–7.13 (m, 6H), 7.60–7.64 (dd, *J* = 8.35 Hz, J = 2.3, 2H), 7.96–7.97 (d, *J* = 2.3

Hz, 2H); ^{13}C NMR (400 MHz, MeOD, δ, ppm): 26.22, 26.90, 28.72, 31.65, 37.18, 37.41, 43.55, 54.79, 70.07, 79.47, 113.99, 114.51, 121.50, 123.73, 126.02, 130.73, 133.50, 146.10, 154.34, 169.20, 174.42. Analysis: calcd. for $C_{56}H_{82}Cl_8N_8O_6$: C 53.94, H 6.63, Cl 22.75, N 8.99%; found: C 53.88, H 6.64, Cl 22.80, N 8.97%.

1,5-Bis{4-[4-[4-di(2-chloroethyl)amino]phenyl]butanoylamino-2-[(3-dimethylaminopropyl)carbamoyl]phenyloxy}pentane tetrahydrochloride (7)

Yield 24.5%; R_f = 0.31; ^{1}H NMR (400 MHz, MeOD, δ, ppm): 1.65–1.75 (m, 2H), 1.73–1.91 (m, 4H), 1.93–1.98 (m, 8H), 2.37 (s, 12H), 2.45–2.55 (m, 8H), 2.64–2.72 (m, 4H), 3.42–3.50 (t, J = 6.2 Hz, 4H), 3.68–3.74 (m, 16H), 4.16–4.24 (t, J = 6.14 Hz, 4H), 6.76–6.80 (d, J = 8.8 Hz, 4H), 7.14–7.20 (m, 6H), 7.74–7.80 (dd, J = 8.5 Hz, J = 2.4, 2H), 7.86–7.88 (d, J = 2.4 Hz, 2H); ^{13}C NMR (400 MHz, MeOD, δ, ppm): 26.32, 28.40, 29.25.17, 31.45, 36.27, 38.21, 40.35, 43.45, 53.58, 58.26, 71.29, 113.34, 114.39, 122.75, 123.75, 125.62, 130.49, 131.24, 132.82, 145.08, 153.69, 166.98, 173.24. Analysis: calcd. for $C_{57}H_{84}Cl_8N_8O_6$: C 54.29, H 6.71, Cl 22.49, N 8.89%; found: C 54.26, H 6.73, Cl 22.53, N 8.86%.

1,6-Bis{4-[4-[4-di(2-chloroethyl)amino]phenyl]butanoylamino-2-[(3-dimethylaminopropyl)carbamoyl]phenyloxy}hexane tetrahydrochloride (8)

Yield 21.8%; R_f = 0.34; ^{1}H NMR (400 MHz, MeOD, δ, ppm): 1.50–1.65 (m, 4H), 1.70–1.89 (m, 4H), 1.90–1.95 (m, 8H), 2.30 (s, 12H), 2.35–2.43 (m, 8H), 2.55–2.63 (m, 4H), 3.32–3.40 (t, J = 6.18 Hz, 4H), 3.62–3.70 (m, 16H), 4.13–4.19 (t, J = 6.16 Hz, 4H), 6.64–6.68 (d, J = 8.85 Hz, 4H), 7.04–7.09 (d, J = 8.85 Hz, 4H), 7.12–7.15 (d, J = 8.5 Hz, 2H), 7.70–7.76 (dd, J = 8.5 Hz, J = 2.4 Hz, 2H), 7.80–7.84 (d, J = 2.4 Hz, 2H); ^{13}C NMR (400 MHz, MeOD, δ, ppm): 28.32, 28.68, 30.17, 30.75, 37.37, 39.01, 41.45, 45.50, 54.57, 58.20, 70.29, 113.54, 114.29, 123.75, 123.85, 125.92, 130.69, 131.64, 133.32, 146.00, 154.64, 167.91, 174.34. Analysis: calcd. for $C_{58}H_{86}Cl_8N_8O_6$: C 54.64, H 6.80, Cl 22.25, N 8.79%; found: C 54.58, H 6.82, Cl 22.28, N 8.76%.

Biological investigations
Relaxation assay of topoisomerase I and II

Topoisomerase I and II (4 units, in the standard buffer added by the producer) were incubated for 1 h at 37°C with compounds 1–8 (at concentrations of 10, 50, 100, 150 μM) in the presence of supercoiled pBR322plasmid DNA (20 ng). The reaction mixtures were subjected to electrophoresis (3 h, 50 V) in 1% agarose gel in the standard TBE buffer. The gels were stained for 30 min with ethidium bromide solution (0.5 μg/mL). The DNA was visualized on a transilluminator using 312 nm wavelength and photographed under UV light. The area representing supercoiled DNA, migrating as a single band at the bottom of gel, was measured using AlphaEaseFC gel documentation and analysis system (Alpha Innotech, USA). The concentrations of the compounds that prevented 50% of the supercoiled DNA (IC_{50} values) were determined by averaging the data from at least three experiments.

Ethidium bromide 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). pBR322 plasmid DNA (15 μg) as water solution (0.05 μg/μL) was added to each well. Then, pentamidine, chlorambucil or compound 1–8 (1 μL of 1 mM solution in water, 10 μM final concentration) was added to appropriate well. After incubating for 30 min in 25°C, the fluorescence of each well was read by the fluorescence spectrophotometer Infinite M200 TECAN (ex. 546 nm, em. 595 nm) in triplicate experiments with two control wells (no drug = 100% fluorescence, no DNA = 0% fluorescence). The results are reported as a percentage of fluorescence in relation to the controls.

Ethidium displacement assay determination of DNA-binding constants

The fluorescence of DNA solutions (calf thymus DNA, T4 coliphage DNA, poly(dA-dT)$_2$, and poly(dG-dC)$_2$) with the investigated compounds (final concentrations 10, 50, 75, 100, 150 and 200 μM) was measured by the fluorescence spectrophotometer Infinite M200 TECAN at room temperature according to the procedure described above. Then, the concentration which reduced fluorescence to 50% was determined. The data points of fluorescence intensity were fitted to the 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] = the concentration of the tested compound at 50% reduction of fluorescence and K_{EtBr} and [EtBr] are known [27, 28]. The compounds 1, 3 and 5–8 and their DNA-bound complexes showed neither optical absorption nor fluorescence at 595 nm and did not interfere with the fluorescence of unbound ethidium bromide.

Determination of alkylating properties (Preussmann test)

The tested compounds (0.01 mmol) were dissolved in 2-metoxyethyl ether (200 μL) and solution of 4-(4'-nitrobenzyl)-pyridine (NBP) in 2-metoxyethyl ether (5%, 200 μL) was added. The samples were heated at $100 \pm 0.5^{\circ}C$ for 1 h and then quickly cooled to $20^{\circ}C$. 2-Metoxyethyl ether (500 μL) and piperidine (100 μL) were added to the samples to give a total volume of 1 mL. The final concentration of the tested compounds was 10 μM. After 90 s, the absorbance was measured at $\lambda = 560$ nm in a quartz cell (1 cm). 2-Metoxyethyl ether was used as a reference solvent.

MCF-7 cell culture

MCF-7 human breast cancer cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 50 μg/mL streptomycin, 100 U/mL penicillin at $37^{\circ}C$ and at the atmosphere containing 5% CO_2. The cells were cultivated in Costar flasks and the 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 grown as monolayer in six-well plates (Nunc) (5×10^5 cells per well) and preincubated for 24 h without phenol red.

Fluorescent microscopy

The compounds were dissolved in $DMSO/H_2O$ (10:90) and used at concentrations of 5, 10, 30, 50 and 100 μM. The microscopic observations of cell monolayers were performed with a Nikon optiphot microscope. 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 experiment, all the media were removed and the cells were harvested by incubating 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 according to the following criteria: viable cells with normal nuclei (a fine reticular pattern of green stain in the nucleus and red/orange granules in the cytoplasm); viable cells with apoptotic nuclei (green chromatin which is highly condensed or fragmented and uniformly stained by the acridine orange); nonviable cells with normal nuclei (bright orange chromatin with an organized structure); and nonviable cells with apoptotic nuclei (bright orange chromatin which is highly condensed or fragmented).

Determination of IC_{50}

These values were counted from the values received by the fluorescent microscopy assay. The percentage of non-viable (apoptotic and necrotic) cells and the compound concentrations, which inhibited 50% of the colony formation (IC_{50} values), were counted. The results were analyzed using the method of the partial least squares regression.

Flow cytometry assessment of annexin V binding

Annexin V-PE Apoptosis Detection Kit was used, according to the manufacture's instruction, to determine apoptosis by the assessment of phosphatidylserine exposure by Annexin V binding. The 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 indentify late apoptotic and dead cells.

Antiamidolytic assay

The influence of compounds **1–8** on the amidolytic activity of plasmin and trypsin was determined with the use of enzyme synthetic substrates as described previously (30, 31). The IC_{50} value (the average of triplicate ± SD) was determined as the concentration of the inhibitor which decreased the absorbance (at 405 nm) of the released *p*-nitroaniline by 50% compared with the absorbance measured in the same condition without the inhibitor.

Statistical analysis

In all the experiments the mean values for three assays ± standard deviations (SD) were calculated. The results were submitted to statistical analysis using the Student's test. The differences were considered significant when $p < 0.05$. The mean values, the standard deviations and the number of measurements in the group are presented in the figures. The values of DNA association constants and IC_{50} in the cell viability test were determined by the partial least squares regression using Microsoft Office Excel 2007.

RESULTS

Chemistry

Compounds **1–8** (Scheme 1) were prepared as hydrochloride salts with good yields by standard chemical transformations. Compounds **1–4** were obtained by multi-step syntheses according to the method described previously (25). The starting material, 2-hydroxy-5-phenylazobenzoic acid, was easily transformed into methyl ester, and succes-

sively the diethers were obtained in the reaction with appropriate diiodoalkane (C_3–C_6). After alkaline hydrolysis and activation of carboxyl group these bis(phenylazobenzenecarboxylates) were condensed with N,N-dimethylpropyl-1,3-diamine. The catalytic hydrogenation of diazenyl group was carried out in methanol (with addition of 10% HCl in methanol) in the presence of Pd/C (10%) and gave bis(aminobenzamides) 1–4. The chlorambucil derivatives 5–8 were obtained by the condensation of these aromatic amines with 4-{4-[bis(2-chloroethyl)amino]phenyl}butanoic acid in DMF in the presence of HOBt, DCC and N-methylmorpholine.

Biological investigations

The flow cytometric analysis based on the detection of morphological changes, DNA fragmentation, DNA loss and membrane changes are increasingly used for the quantitative investigation of apoptosis. Apoptosis was determined by the assessment of phosphatidylserine exposure by Annexin V-PE. During an early stage of apoptosis, phosphatidylserine translocates from the interior to the exterior part of the plasma membrane and becomes exposed at the cell surface, facilitating recognition by macrophages (32, 33). The incubation of MCF-7 breast cancer cells with pentamidine, chlorambucil and compounds 1–8 induced visible
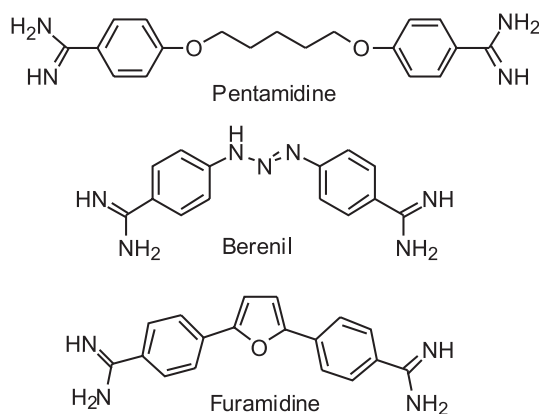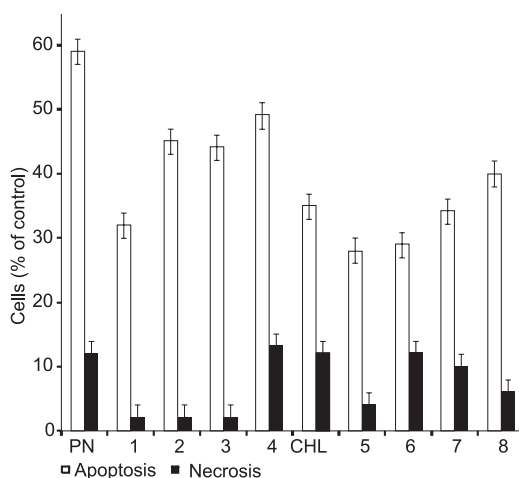

Figure 1. Structure of bisamidines

Figure 2. Morphological apoptosis evaluation in the annexin V-PE assay on MCF-7 cells treated for 24 h with 50 μM pentamidine (**PN**), chlorambucil (**CHL**) and compounds **1–8**. White columns represent annexin[+]/propidium iodide^{−} cells in the apoptotic stage and black columns represent annexin[+]/propidium iodide^{+} cells in the necrotic stage. Mean percent ± SD from three independent experiments are presented

Table 1. Antiproliferative activity of compounds **1–8** against MCF-7 breast cancer cells.

Compound	IC$_{50}$ [μM][a]	Compound	IC$_{50}$ [μM][a]
Pentamidine	14 (± 2)	Chlorambucil	29 (± 2)
1	67 (± 2)	**5**	95 (± 2)
2	72 (± 2)	**6**	91 (± 2)
3	71 (± 2)	**7**	66 (± 2)
4	22 (± 2)	**8**	79 (± 2)

[a] The results represent the mean (± SD) of three independent experiments done in duplicates.

Table 2. DNA binding effect of pentamidine, chlorambucil and compounds **1–8**.

Compound	Decrease of fluorescence [%]	Compound	Decrease of fluorescence [%]
Pentamidine	69.56	Chlorambucil	61.73
1	72.12	**5**	53.93
2	55.76	**6**	62.42
3	69.69	**7**	59.39
4	72.12	**8**	41.80

Table 3. Association constants (K$_{app}$ × 10^5 M^{-1}) and topoisomerases DNA inhibitory effect of pentamidine (**PN**) and compounds **1–8**.

Ligand	Calf thymus DNA	T4 DNA	poly(dA-dT)$_2$	poly(dG-dC)$_2$	Inhibition of topo I activity [μM][d]	Inhibition of topo II activity [μM][d]
	[K$_{app}$ × 10^5 M^{-1} ± 0.2 × 10^5 M^{-1}]					
EthBr	100[a]	100[b]	95[b]	99[b]	-	-
Nt	8.7	8.3	875	2.5	-	-
PN	7.7	4.2	9.8	3.6	> 100	> 100
1	11.9	19.1	11.4	8.0	90	80
2	10.23	11.4	8.2[c]	1.3[c]	50	50
3	19.5	11.9	8.0	7.1	50	80
4	10.68	11.8	12.2[c]	1.5[c]	30	30
5	3.64	2.8	8.1	2.5	50	30
6	6.45	3.1	12.8	10.7	30	30
7	6.1	5.7	8.0	7.9	50	50
8	3.5	5.5	14.76	13.7	90	80

[a] values from (27); [b] values from (28); [c] values from (26); Nt – netropsin; PN – pentamidine; [d] the concentrations of the inhibitor that prevented 50% of the supercoiled DNA from being converted into relaxed DNA were determined by averaging the data from at least three experiments.

phosphatidylserine exposure after 24 h of treatment. In this experiment it was discovered that apoptosis was the main pathway of the cell death. All the compounds at examined concentrations induced dose-dependent apoptosis of MCF-7 cells. The percentage of apoptotic and necrotic MCF-7 cells after the treatment with 50 μM solutions of pentamidine, chlorambucil and compounds **1–8** is shown in Figure 2. The influence of pentamidine, chlorambucil and compounds **1–8** on cell viability was also estimated by a fluorescent microscopy assay after acridine orange and ethidium bromide staining. The

results given in the fluorescent microscopy assay confirmed proapoptotic activity of the studied compounds and they were used for the determination of IC_{50} values presented in Table 1. The concentrations of the compounds that inhibited 50% of the colony formation were in the range $22 \pm 2 – 95 \pm 2$ µM, while IC_{50} of pentamidine and chlorambucil was 14.5 ± 2 µM and 29 ± 2 µM, respectively.

In order to test whether the cytotoxic properties are related to DNA-binding and topoisomerase I/II inhibition, the new compounds were evaluated in a cell-free system. The ethidium bromide assay showed that the investigated compounds can bind with plasmid DNA (Table 2). Both amino and chlorambucil derivatives interfere with the formation of the EtBr-DNA complex. Amongst the studied bisamines, compound **2** (butane derivative) caused a greater decrease of fluorescence (55.76%) compared with pentamidine (69.56%), while derivative **3** with

a pentamethylene chain had a similar potential (69.69%). Chlorambucil derivatives, except **6**, were more active than chlorambucil (61.73%), and compound **8** with a hexamethylene chain moderated the formation of the EtBr-DNA complex most intensively (41.8% decrease of fluorescence).

The determination of association constants enables the qualification of the potential and selectivity of the interactions between ligands and DNA. The binding affinities of compounds **1–8** to calf thymus DNA, T4 coliphage DNA, and synthetic polymers poly(dA-dT)$_2$ and poly(dG-dC)$_2$ are presented in Table 3. This data demonstrates that all the analyzed compounds can bind with the studied DNAs. The major groove of T4 coliphage DNA is blocked by α-glycosylation of the 5-(hydroxymethyl)cytidine residues (34) and the high value of the binding constant of ligand for T4 coliphage DNA provides evidence of its minor-groove specificity. The con-

Table 4. Alkylating activity of compounds **5–8** on comparison to chlorambucil; NBP test results.

Compound	Molar extinction coefficient (ε)	Absorbance (A)[a] 560 nm	Alkylation activity[b]
Chlorambucil	92.7	0.0927	+
5	77.8	0.0778	+
6	60.4	0.0604	+
7	73.8	0.0738	+
8	53.6	0.0536	+

[a] Average data from three determinations. [b] According to (29): (–) A < 0.05, (+) A = 0.05–0.1, (++) A = 0.1–0.5, (+++) A > 0.5.

Table 5. The effect of **1–8**, pentamidine and chlorambucil on the amidolytic activity of plasmin and trypsin.

Compound	IC_{50} [mM]	
	Plasmin S-2251	Trypsin Bzl-L-Arg-pNA.HCl
1	0.10 ± 0.01	n.i.
2	6.10 ± 0.20	n.i.
3	8.05 ± 0.07	n.i.
4	1.00 ± 0.10	n.i.
5	n.i.	n.i.
6	n.i.	n.i.
7	n.i.	n.i.
8	n.i.	n.i.
Pentamidine	< 0.01	0.10 ± 0.02
Chlorambucil	0.10 ± 0.01	n.i.

n.i. = no inhibition

stants determined for compounds **1–8** in comparison with netropsin and pentamidine revealed similar strength of binding to T4 coliphage DNA. Therefore, it can be supposed that these ligands can interact within minor groove of DNA. In this experiment all the amino compounds **1–4** were slightly more active than their chlorambucil derivatives **5–8**. It might be the consequence of less tight structures of compounds **5–8** compared to the corresponding aromatic amines. On the one hand, the presence of a flexible aliphatic chain in the chlorambucil unit can facilitate the location of the alkylating bis(2-chloroethyl)amine group near a suitable nucleic base. On the other hand, this group can hinder the fitting of chlorambucil derivatives to the shape of minor groove. Further investigations showed that compounds **1–8** are able to interact both with poly(dA-dT)₂ and poly(dG-dC)₂ with similar binding affinity. On the basis of these results, the DNA sequence selectivity of compounds **1–8** could not be definitively determined.

A number of minor-grove binding drugs inhibit the catalytic activity of the isolated topoisomerases (both I and II) (35). These data suggest that these topological enzymes read the DNA structure at least in part through the minor groove (36). The ability of compounds **1–8** to inhibit the activity of topoisomerase I and II was quantified by measuring the relaxation of the supercoiled pBR322 DNA as a function of the increasing ligand concentration by the use of agarose gel electrophoresis. The concentrations of the inhibitor that prevented 50% of the supercoiled DNA from being converted into the relaxed DNA (IC_{50} values) were determined (Table 3). These results demonstrate that in the concentration range of 30 to 90 µM, compounds **1–8** inhibited the activity of topoisomerase I (topo I) and topoisomerase II (topo II). The inhibitory effects of the investigated compounds **1–8** were similar for both topoisomerases. Significant differences in the activity of amine or chlorambucil compounds have not been observed, so it could be supposed that the chlorambucil fragment does not take part in topo-drug interactions. The inhibitory effect of **1–8** might be the consequence of DNA-binding and the hindrance in the formation of the drug-topo complex.

The aim of this work was also to determine the alkylating activity of the chlorambucil derivatives **5–8**. For this purpose a Preussmann test *in vitro* was used. All the tested compounds demonstrated their alkylating activity toward NBP molecule (Table 4). It can be observed that compounds **5–8** have low alkylation activity (+), similarly to chlorambucil. Thus, the annexation of minor groove binder frag-

ments does not reduce the alkylating properties of this known antitumor drug.

Recently we have found that some minor groove binders are capable of inhibiting the amidolytic activity of plasmin (24). Plasmin, trypsin-like serine protease, is the key enzyme for fibrinolysis and plays a significant role in numerous biological processes, such as wound healing, tissue repair, cell migration and inflammation, and it is associated with tumor invasion, metastasis and angiogenesis (37, 38). Table 5 demonstrates the effect of the studied compounds **1–8** on the plasmin and trypsin activity in comparison with pentamidine and chlorambucil. Aromatic bis-amines **1–4**, in contrast to their alkylating derivatives **5–8**, are able to influence the biological function of plasmin. The most active compound **1** ($IC_{50} = 0.1$ mM), which has the shortest trimethylene linker between aromatic units, revealed a similar activity to chlorambucil and was 80 fold more potent than compound **3**, whose molecule contains a pentamethylene chain (the same as in pentamidine). The obtained results prove that compounds **1–4** are able to inhibit plasmin activity and none of the studied compounds, except pentamidine, does not disturb the trypsin function. It can be assumed that such compounds could constitute selective inhibitors of plasmin.

DISCUSSION AND CONCLUSION

The quantitative structure-activity relationship and molecular modeling studies of pentamidine analogues confirmed that the special shape of molecule, which allows the fitting to the minor groove of DNA, and charged amidine groups, which are able to form H-bond with nucleic bases and can interact electrostatically with phosphate backbons, are needed to enhance the potency of bisamidines (39). The amidine group, however, is implicated in the toxicity of pentamidine through its affinity for imidazoline I2 receptor (40). The search for non-amidine drugs resulted in the discovery of bisamino analogues of pentamidine identified as novel DNA minor groove binders (41, 42). The results described in the paper indicate that bisamines **1–4** are able to interact with DNA, probably within minor groove, and they can inhibit the activity of topoisomerases and plasmin. The presence of the less reactive amino group in the aromatic core instead of amidine group can cause lower cytoxicity in relation to pentamidine, although additional 3-dimethylamino-propanamide group can contribute to the DNA recognition by the formation of H-bonds with nucleic bases, and by the electrostatic interaction with

negative potential of the groove (35). The incorporation of this fragment to another position of aromatic ring can possibly improve the activity of the described pentamidine analogues.

The analysis of the results obtained for bisamines **1**–**4** and their chlorambucil derivatives **5**–**8** did not allow us to define exact mechanism of their activity but the similarities in their biological properties enable us to conclude that the amino compounds can act as DNA minor groove binding agents and they could be used as specific carriers of active groups.

It is noteworthy that all the studied compounds were capable of inducing apoptosis in the cancer cell line. This fact persuade us to search for new pentamidine-like compounds with more potent anticancer activity.

The authors have declared no conflict of interest.

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