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Synthesis and Biological Evaluation of Distamycin Analogues – New Potential Anticancer Agents

Danuta Drozdowska[1], Malgorzata Rusak[2], Wojciech Miltyk[3], and Krystyna Midura-Nowaczek[1]

[1] Department of Organic Chemistry, Medical University, Białystok, Poland
[2] Department of Haematological Diagnostics, Medical University, Białystok, Poland
[3] Laboratory of Drug Analysis, Medical University, Białystok, Poland

Eight of analogues of distamycin, potential minor-groove binders, were synthesized and tested for *in-vitro* cytotoxicity towards human breast cancer cells MCF-7 and MDA-MB-231. The method of synthesis is simple and convenient. All of the compounds **1**–**8** showed antiproliferative and cytotoxic effects against both cell lines in the range 3.47 to 12.53 μM for MDA-MB-231 and 4.35 to 12.66 μM for MCF-7. All compounds demonstrated activity against DNA topoisomerases I and II at a concentration of 50 μM. The ethidium bromide assay showed that these compounds bind to plasmid pBR322, yet weaker than distamycin. Further investigations concerning the mechanism of cytotoxicity are now in progress, but the IC_{50} values suggest that synthetic distamycin analogues with a free amino group, **3**–**4** and **7**–**8**, can serve as potential carriers of strong acting elements, *e.g.* alkylating groups.

Keywords: Cytotoxic activity / Anticancer activity / Distamycin analogue / DNA binding / DNA topoisomerase

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Introduction

The rapidly increasing knowledge in molecular biology makes it possible to observe that the large family of sequence-specific ligands non-intercalatively binding within the minor groove of B-DNA is very important in antitumour drugs searching [1, 2]. This interaction results in the occupancy of natural nucleic sequence targeted by enzymes or in DNA distortion nearby these sites. The cytotoxic effect of these antineoplastic agents and the inhibition of many cellular processes is determined mainly by interference with the catalytic activity of important regulatory proteins, such as topoisomerase I and II and a number of proteases [1]. Most of the minor-groove DNA-binding drugs, especially netropsin and distamycin, exhibit high sequence selectivity.

From the DNA-binding model of netropsin and distamycin came the inspiration to search for new compounds with similar interaction with DNA. We searched a lot of compounds which do not intercalate with DNA, have a high base-pair specificity, an isohelical shape similar to the minor groove of B-DNA [3]. The class of synthetic heteroaromatic oligopeptides, projected after the models of netropsin and distamycin, received the name lexitropsins [4]. Although a huge progress in designing lexotropins with an extremely selective mode of operation was made, we did not obtain compounds ready to be applied in therapy [5].

We focused on the strategy to replace the *N*-methylpyrrole rings of distamycin and netropsin with benzene rings, simultaneously modifying the cationic heads. The carbocyclic analogues of distamycin with an unsubstituted *N*-terminal group NH_2 inhibited *in-vitro* activity of topoisomerase I and II [6], in the same fashion as the derivatives of netropsin with an aliphatic linker (four and six groups of CH_2) [7]. Studying the interaction of compounds with DNA by the ethidium-displacement assay confirmed that they had a larger specificity for AT in comparison to GC-rich regions, similarly to netropsin and distamycin [8, 9]. These carbocyclic analogues of netropsin and distamycin served as carriers to other

Correspondence: Danuta Drozdowska (Bartulewicz), Department of Organic Chemistry, Medical University, 15-222 Białystok, Poland.
E-mail: ddrozd@amb.edu.pl
Fax: +48 85 748-5416

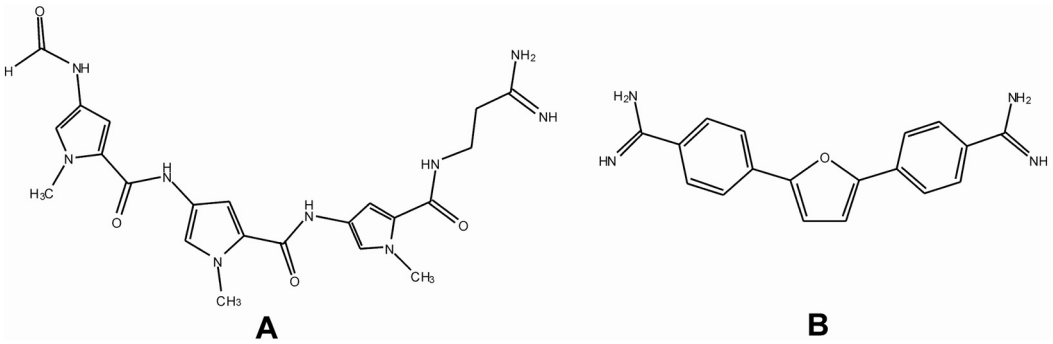


Figure 1. Structures of distamycin **A** and furamidine **B**.

groups. We obtained derivatives with a *N*-terminal chlorambucyl group which exhibited activity in cultured breast cancer MCF-7 [10], and with a 5-[*N*,*N*-bis(2-chloroethylo)amine]-2,4-dinitrobenzoyl group – in the face of hepatoma HEP G2 in hypoxic conditions [11].

This paper is in continuation of rational drug design program aiming to develop distamycin analogues, potential minor-groove binders, and inhibitors of topoisomerases. We developed new compounds with skeletons that combine the structural features of distamycin **A** and furamidine **B** (Fig. 1).

The purpose of this work was the synthesis of eight new compounds containing benzene connected with heteroaromatic rings, in different configurations. Preliminary evaluation of their biological properties – antiproliferative and cytotoxic activity in MCF-7 and MDA-MB-231 cell lines and the capacity to inhibit human topoisomerases I and II *in vitro* were planned. We also investigated the binding of compounds **1**–**8** to plasmid DNA employing the ethidium-bromide assay [12].

Results and discussion

Chemistry

The analogues **1**–**8** were synthesized by simple acylation of the aromatic amines as shown in Scheme 1. The starting materials for synthesis, isophtaloyl chloride $\mathbf{B}_1$ and terephtaloyl chloride $\mathbf{B}_2$ were dissolved in methylene chloride and cooled. Aromatic amines $\mathbf{A}_1$–$\mathbf{A}_4$, dissolved in methylene chloride and mixed with TEA (triethylamine), were added dropwise to the solution of chlorides. The reaction mixtures were stirred at room temperature for 2 h. The precipitated crude products **1**, **2**, **5**, and **6** were filtered off and washed with chloroform.

The nitro group of compounds $\mathbf{A}_3\mathbf{B}\mathbf{A}_3$ and $\mathbf{A}_4\mathbf{B}\mathbf{A}_4$ were reduced by catalytic hydrogenation (Pd / C) in methanol. The reaction mixture was stirred for 1.5 h at room temperature and at atmospheric pressure. Then, a black precipitate of catalyst was filtered off and the resulting solution was concentrated under reduced pressure. The crude products **3**, **4**, **7**, and **8** were purified by column chromatography in chloroform with a methanol gradient.

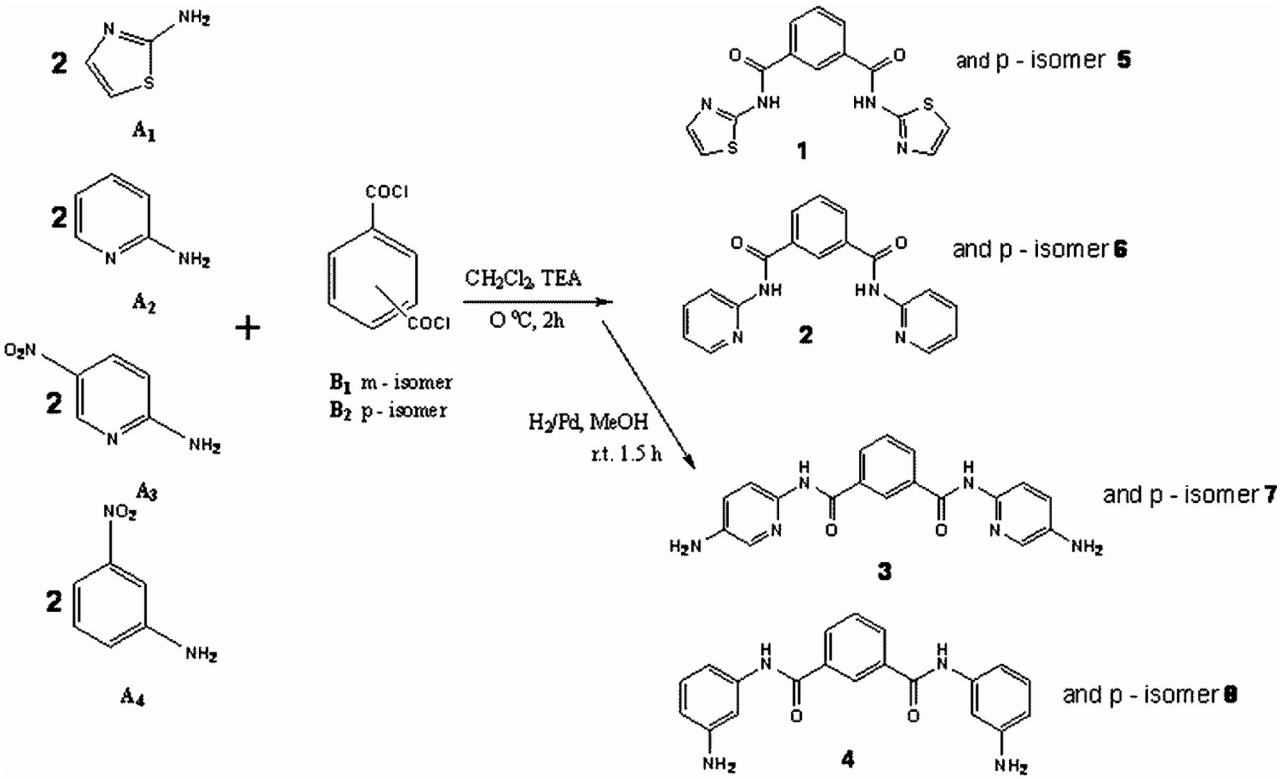
Pharmacology

The described compounds were tested for their *in-vitro* antitumour activity in the human breast cancer cells, standard MCF-7 and estrogen-independent MDA-MB-231. Their cytotoxic activity as percentage of nonviable cells is shown in Tables 1 and 2. All of the tested compounds showed concentration-dependent activity.

Fluorescent microscopy assay showed morphological changes of cells to determine the apoptosis process. After the incubation of MCF-7 and MDA-MB-231 breast cancer cells with the tested compounds, the cells were dyed. Acridine orange (fluorescent DNA-binding dye) intercalates into DNA, making it appear green, and binds to RNA, staining it red-orange. Ethidium bromide is only taken up by nonviable cells; its fluorescence overpowers that of the acridine orange, making the chromatin of necrotic cells appear orange [13].

Two hundred cells per sample were examined by fluorescence microscopy, according to the following criteria: viable cells with normal nuclei (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 acridine orange); nonviable cells with a normal nuclei (bright orange chromatin with organized structure); and nonviable cells with apoptotic nuclei (bright orange chromatin which is highly condensed or fragmented).

In this experiment, we have found that all analyzed compounds induced concentration-dependent apoptosis (Fig. 2). Only compound **2**, **3**, and **4** at the concentration



Scheme 1. Synthesis of compounds **1** – **8**.

Table 1. Viability of MCF-7 cells treated for 24 h with different concentrations of compounds **1** – **8.**

Concentration (μM)	Nonviable cells (% of control ± 2)[a]							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
5	36[a]	23	30	24	20	44	20	40
10	56	44	56	36	52	70	28	52
15	70	68	68	63	68	76	84	76
30	91	100	93	100	99	100	100	98
50	100	100	100	100	100	100	100	100
IC_{50}	**6.38**	**11.74**	**8.32**	**11.67**	**10.99**	**5.83**	**12.66**	**4.35**

[a] Mean values ± SD from three independent experiments done in duplicate are presented.

Table 2. Viability of MDA-MB-231 cells treated for 24 h with different concentrations of compounds **1** – **8**.

Concentration (μM)	Nonviable cells (% of control ± 2)[a]							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
5	34[a]	21	49	35	49	32	44	39
10	58	60	55	47	55	46	48	49
15	61	65	60	56	52	68	62	52
30	83	98	67	79	69	86	87	73
50	96	100	72	98	85	100	100	99
IC_{50}	**8.79**	**9.81**	**5.76**	**12.53**	**3.47**	**10.62**	**8.07**	**12.53**

[a] Mean values ± SD from three independent experiments done in duplicate are presented.Figure

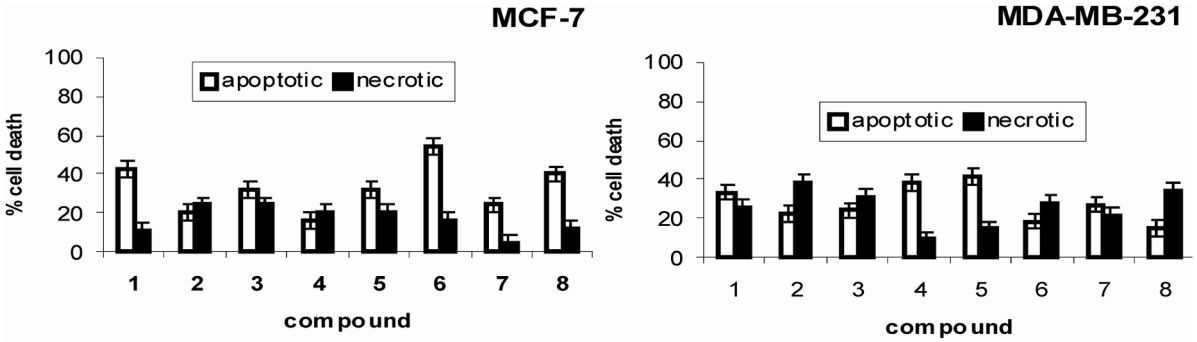


Figure 2. Morphological apoptosis evaluation in the cytotoxic assay on MCF-7 and MDA-MB-231 cells treated for 24 h with 10 μM of the examined compounds **1**–**8**. White columns represent cells in the apoptotic stage and black columns represent cells in the necrotic stage. Mean percentages ± S.D. from three independent experiments are presented.

Table 3. DNA-binding effect of distamycin and compounds **1**–**8**.

Compound:	EtBr	DNA-EtBr	DST	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
% fluorescence:	0	100	63.45	92.15	92.54	92.69	91.47	85.67	81.34	92.03	93.14

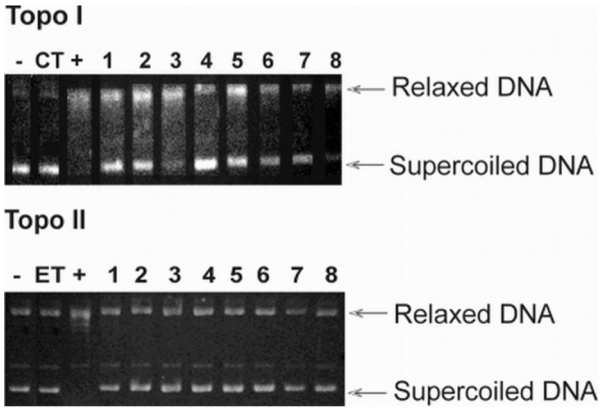


Figure 3. Inhibition of topoisomerases by compounds **1**–**8**. Native pBR322 plasmid DNA (lane –) was incubated with four units of topoisomerases I (Topo I) and II (Topo II) in the absence (lane +) or in the presence of control (line CT–camptothecin or ET–etoposide) or drug (lanes **1**–**8**). The DNA was analyzed by 1% agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed under UV light.

10 μM caused increased necrotic cell death in MCF-7 cells. Apoptosis induced by **1** and **5**–**8** was definitely stronger. We also observed that the percentage of necrotic cells was increased in case MDA-MB-231 cells. Only compound **1**, **4**, and **5** induced apoptotic death of cells.

Purified topoisomerases I and II were incubated with increased concentrations of compounds **1-8** (5, 10, 15, 30, and 50 μM) in the presence of supercoiled plasmid DNA. The products were subjected to electrophoresis to separate relaxed and supercoiled circular DNA. Figure 3 shows the results of our electrophoresis analysis of examined compounds at 50 μM concentration, after staining with ethidium bromide. The lower concentrations did not show any changes. As can be seen in Fig. 3, the superhelical plasmid (lane 1) was relaxed by topoisomerases (lane 3), controls (CT-camtothecin or ET-etoposide, respectively) inhibited activity of topoisomerases entirely (lane 2). Figure 3 demonstrates that at a concentration of 50 μM compounds **3** and **8** have a very small effect on the ability of topo I to transform supercoiled DNA into several topoisomer forms of relaxed DNA. At this concentration, other compounds inhibited the activity on topoisomerase I. As we can see, all of the compounds **1**–**8** showed inhibitory activity against topoisomerase II.

Figure 3 also shows that the investigated compounds **1**–**8** are more effective against topoisomerase II. At the concentration of 50 μM, all of them inhibit topoisomerase I activity only partially.

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

The ethidium bromide assay showed that the investigated compounds can bind to DNA, although relatively weaker than distamycin (Table 3).

Conclusion

We obtained eight new compounds, potential minor groove binders, and analogues of distamycin. Their syntheses were simple, convenient, and allowed to get the compounds in a good yield. This procedure could be useful to seek other derivatives of distamycin to find the best

configuration of aromatic, benzene, and hetero-aromatic rings.

The *in-vitro* experiment findings revealed that all of new lexitropsins exhibit sufficient tumor cell cytotoxicity towards the standard cell line of the mammalian tumour MCF-7 and estrogen-independent MDA-MB-231 breast cancer cells. The most interesting compound seems to be **1** with a time-dependent reduction in proliferation observed in both cell lines at concentrations: 6.38 μM for MCF-7 and 8.79 μM for MDA-MB-231 cells, respectively. Compound **5** with IC_{50} 10.99 μM for MCF-7 and 3.47 μM for MDA-MB-231 cells, respectively, is also interesting. All of the investigated compounds are more potent than chlorambucil which MCF-7 IC_{50} averages of 24.6 μM [6].

Evaluation of the topoisomerase inhibition provided additional insight into the structure-activity relationship associated with these compounds. All of the compounds inhibited the activity of DNA topoisomerases, but we can see more activity against topoisomerase II. It would be interesting to investigate the DNA-binding mode of these compounds in detail and to determine the topoisomerase IC_{50}, especially for compounds **3** and **8** and to determine the compounds binding constants for binding selectivity.

We have shown that compounds **1** – **8** can bind to DNA and are potent inhibitors of both topoisomerase I and II. These compounds inhibit the catalytic activity of the topoisomerase at a step prior to the formation of the topo-DNA complexes. This suggests that DNA binding may be implicated in the cytotoxicity of the compounds, possibly by inhibiting the interactions between topoisomerases and their DNA targets. Moreover, there might be other possible targets, such as other enzymes, involved in DNA metabolism and / or transcription factors because their activities were inhibited by some minor-groove binders [15 – 17]. Further biological studies of the DNA-binding mode and other biological properties will be described in due course.

The obtained analogues of distamycin with free amino groups can also be used as carriers for active groups, *e.g.* alkylating fragments. Their therapeutic applications could be considered after further investigation.

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Experimental

Chemistry

Compounds were synthesized with suitable parent substances (Merck and Aldrich, Germany). Thin-layer chromatograms were prepared on precoated plates (Merck, silica gel 60F-254) and visualized with UV. 5% NH_{3aq} in methanol 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 Büchi 535 melting-points apparatus (Büchi, Switzerland) and are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AC 200F spectrometer (Bruker, Germany), using TMS as an internal standard. Chemical shifts are expressed in δ value (ppm). Multiplicity of resonance peaks are indicated as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). The results of the elemental analyses for C and H were within ± 0.4% of the theoretical values. Syntheses of all new compounds are given below.

N,N'-Bis-thiazol-2-yl-isophthalamide ***1***

To a cooled solution of 2-aminothiazole (2 g, 19.97 mmol) in CH_2Cl_2 (50 mL) was added triethylamine (2.77 mL, 19.97 mmol). The isophthaloyl chloride (2.03 g, 9.98 mmol) in CH_2Cl_2 (50 mL) was then added dropwise during 0.5 h. Then, the reaction mixture was stirred at room temperature over a period of 3 h. The obtained beige precipitate was filtered off and washed with $CHCl_3$. After recrystallisation from $CHCl_3$ and drying, we obtained 2.67 g of compound **1** (80.1%), m.p.: 306 – 307°C; R_f = 0.72; ^{1}H-NMR (D_2O) δ [ppm]: 8.31 (s, 4H), 7.31 (d, 2H), 7.58 (d, 2H); ^{13}C-NMR (D_2O) δ [ppm]: 166.62, 164.52, 137.40, 135.53, 135.52, 129.42, 128.29, 114.00; $C_{14}H_{10}N_4O_2S_2$: 330.39.

N,N'-Di-pyridin-2-yl-isophthalamide ***2***

To a cooled solution of 2-aminopyridine (2 g, 21.25 mmol) in CH_2Cl_2 (30 mL) was added triethylamine (2.95 mL, 21.15 mmol). The isophthaloyl chloride (2.16 g, 10.63 mmol) in CH_2Cl_2 (40 mL) was then added dropwise during 0.5 h. Then, the reaction mixture was stirred at room temperature over a period of 3 h. The obtained yellow precipitate was filtered off and washed with $CHCl_3$. After recrystallisation from $CHCl_3$ and drying, we obtained 0.31 g of compound **2** (59.14%), m.p.: 296 – 297°C; R_f = 0.82; ^{1}H-NMR (DMSO-d_6) δ [ppm]: 12.67 (br, 2H), 8.82 (s, 1H), 8.32 (d, 2H), 7.72 (t, 1H), 7.41 (m, 6H), 7.28 (t, 2H); ^{13}C-NMR (DMSO-d_6) δ [ppm]: 164.55, 156.27, 146.32, 144.66, 137.53, 132.12, 131.94, 129.08, 120.86, 114.01; $C_{18}H_{12}N_4O_2$: 318.33.

N,N'-Bis-(5-amino-pyridin-2-yl)-isophthalamide ***3***

To a cooled solution of 2-amino-5-nitropyridine (2 g, 14.38 mmol) in CH_2Cl_2 (60 mL) was added triethylamine (2.95 mL, 21.15 mmol). The isophthaloyl chloride (1.46 g, 7.19 mmol) in CH_2Cl_2 (40 mL) was then added dropwise during 0.5 h. Then the reaction mixture was stirred at room temperature over a period of 20 h. The obtained beige precipitate was filtered off and washed with $CHCl_3$. After recrystallisation from $CHCl_3$ and drying, we obtained 2.11 g of the nitro-compound (71.92%). Catalytic hydrogenation of the nitrocompound (0.2 g; 0.57 mmol) was carried out in methanol (20 mL) in the presence of Pd / C (10%). The aromatic amine was purified using the solvent system CH_2Cl_2 / MeOH (gradient). After evaporating of fractions with amine, we obtained compound **3** (0.124 g, 0.39 mmol) (68.42%), m.p.: 319 – 320°C; R_f = 0.85; ^{1}H-NMR (DMSO-d_6) δ [ppm]: 10.15 (br, 2H), 8.47 (s, 1H), 8.11 (m, 4H), 7.62 (t, 3H), 6.97 (d; 2H), 5.65 (br, 4H); ^{13}C-NMR (DMSO-d_6) δ [ppm]: 164.69, 139.61, 139.60, 135.55, 131.92, 131.49, 128.78, 128.56, 109.89, 108.46; $C_{18}H_{16}N_6O_2$: 348.36.

N,N′-Bis-(3-amino-phenyl)-isophthalamide ***4***

To a cooled solution of 3-nitroaniline (2 g, 14.48 mmol) in CH_2Cl_2 (50 mL) was added triethylamine (2.01 mL, 14.48 mmol). The isophthaloyl chloride (1.47 g, 7.24 mmol) in CH_2Cl_2 (50 mL) was then added dropwise during 0.5 h. Then, the reaction mixture was stirred at room temperature over a period of 20 h. The obtained beige precipitate was filtered off and washed with $CHCl_3$. After recrystallisation from $CHCl_3$ and drying, we obtained the nitro-compound (4.31 g, 11.45 mmol) (79.07%). Catalytic hydrogenation of the nitro-compound (0.2 g, 0.53 mmol) was carried out in methanol (20 mL) in the presence of Pd / C (10%). The aromatic amine was purified using the solvent system CH_2Cl_2 / MeOH (gradient). After evaporating of fractions with amine, we obtained compound **4** (0.12 g, 0.39 mmol) (70.59%), m.p. 285 – 286°C; R_f = 0.92; ^{1}H-NMR (DMSO-d_6) δ [ppm]: 10.48 (br, 2H), 8.49 (s, 1H), 8.14 (d, 2H), 7.62 (t, 1H), 7.20-6.86 (m, 8H), 6.35 (br, 4H); ^{13}C-NMR (DMSO-d_6) δ [ppm]: 164.67, 139.61, 139.60, 135.55, 131.92, 131.49, 128.77, 128.57, 109.89, 108.49; $C_{18}H_{16}N_6O_2$: 348.36.

N,N′-Bis-thiazol-2-yl-terephthalamide ***5***

The procedure was analogous to that for compound **1** with terephtaloic chloride (2.03 g, 9.98 mmol) in CH_2Cl_2 (50 mL). Yield: 92.18% (3.04 g, 9.20 mmol), m.p.: 344 – 345°C; R_f = 0.65; ^{1}H-NMR (DMSO-d_6) δ [ppm]: 10.19 (br, 2H), 8.20 (s, 4H), 7.57 (d, 2H), 7.32 (d, 2H); ^{13}C-NMR (DMSO-d_6) δ [ppm]: 166.65, 164.56, 137.38, 135.55, 128.32, 114.04; $C_{14}H_{10}N_4O_2S_2$: 330.39.

N,N′-Di-pyridin-2-yl-terephthalamide ***6***

The procedure was analogous to that for compound **2** with terephtaloic chloride (2.16 g, 10.63 mmol) in CH_2Cl_2 (40 mL). Yield: 61.71% (2.09 g, 6.56 mmol), m.p.: 252 – 253°C; R_f = 0.74; ^{1}H-NMR (DMSO-d_6) δ [ppm]: 10.97 (br, 2H), 8.39 (d, 2H), 8.13 (d, 2H), 7.65 (s, 4H), 7.20 (m, 4H; ^{13}C-NMR (DMSO-d_6) δ [ppm]: 166.69, 152.01, 147.97, 137.54, 137.34, 128.84, 119.99, 114.79; $C_{18}H_{12}N_4O_2$: 318.33.

N,N′-Bis-(5-amino-pyridin-2-yl)-terephthalamide ***7***

The procedure was was analogous to that for compound **3** with terephtaloic chloride (1.46 g, 7.19 mmol) in CH_2Cl_2 (40 mL). Obtained nitro-compound: yield: 88.76% (2.61 g, 6.90 mmol).

Hydrogenation of the nitro-compound (0.5 g, 1.32 mmol) gave compound **7** (0.14 g, 0.40 mmol) (30.30%), m.p. 209 – 211°C; R_f = 0.90; ^{1}H-NMR (DMSO-d_6) δ [ppm]: 10.13 (br, 2H), 8.05 (s, 2H), 7.99 (d, 2H), 7.12 (s, 4H), 6.93 (d, 2H), 6.31 (d, 4H); ^{13}C-NMR (DMSO-d_6) δ [ppm]: 164.53, 139,53, 137.52, 129,15, 127.57, 109.92; $C_{18}H_{12}N_4O_2$: 318.33.

N,N′-Bis-(3-amino-phenyl)-terephthalamide ***8***

The procedure was analogous to that for compound **4** with terephtaloic chloride (1.47 g, 7.24 mmol) in CH_2Cl_2 (50 mL). Obtained nitro-compound: yield: 59.39% (1.62 g, 4.30 mmol).

Hydrogenation of the nitro-compound (0.2 g, 0.53 mmol) gave compound **8** (0.13 g, 0.37 mmol) (69.81%), m.p.: 285 – 286°C; R_f = 0.92; ^{1}H-NMR (DMSO-d_6) δ [ppm]: 10.48 (br, 2H), 8.09 (s, 4H), 7.72 (s, 4H), 6.35 (br, 4H); ^{13}C-NMR (DMSO-d_6) δ [ppm]: 164.22, 141.63, 136.96, 129.14, 127.65, 122.26, 116.02; $C_{18}H_{16}N_6O_2$: 348.36.

Pharmacology

Ethidium bromide was purchased from Carl Roth GmbH, Germany, topoisomerase I and II 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 (DMEM), fetal bovine serum (FBS), distamycin, streptomycin, and penicillin were products of Sigma (Germany). Plasmid pBR322 was purchased from Fermentas Life Science (Germany).

Cell culture

Human breast cancer MDA-MB-231 and MCF-7 cells maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 50 μg/mL streptomycin, 100 U/mL penicillin at 37°C, atmosphere containing 5% CO_2. Cells were cultivated in Costar flasks and subconflluent 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 pre-incubated 24 h without phenol red.

Determination of IC_{50}

The compounds were dissolved in DMSO and used at concentrations of 5, 10, and 15 μM. Microscopic observations of cell monolayers were performed with a Nikon optiphot microscope. Wright – Giemsa staining was performed using the Fisher Leuko Stat Kit (Fisher Scientific). 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 of the media 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 cell suspension was mixed with 10 μL of the dye mix and 200 cells per sample were examined by fluorescence microscopy and we counted the percentage of nonviable (apoptotic and necrotic) cells. The results were submitted to statistical analysis using the method of the smallest squares.

Relaxation assay of topoisomerase I and II

Native pBR322 plasmid DNA (0.20 μg) was incubated with 4 unit 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 μg/mL bovine serum albumin) in the absence or presence of varying concentrations of the test compounds (5, 10, 15, 30 or 50 μM), as well camptothecin (2 μM) or etoposide respectively (10 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 hour, 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 visualisated using 312 nm wavelength transilluminator and photographed under UV light (Canon PowerShot G6, 7.1 mLn megapixels).

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). To each well was added 15 μg plasmid

pBR322 as water solution (0.05 μg/μL). Then, to each well was added distamycin A or compound **1** – **8** (1 μL of a 1 mM solution in water, 10 μM final concentration). After incubation at 25°C for 30 min, the fluorescence of each well was read on a Multilabel Reader Victor 3V (ex.: 355 nm, em.: 615 nm) in duplicate experiments with two control wells (no drug = 100% fluorescence, no DNA = 0% fluorescence). Fluorescence readings are reported as% fluorescence relative to the controls.

Statistical analysis

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

The results were submitted to statistical analysis using the method of the smallest squares, accepting coefficient of determination in the range $0.9600 < R^2 < 1$. Mean values, the standard deviations and the number of measurements in the group are presented in the figures.

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