Acta Poloniae Pharmaceutica – Drug Research, Vol. 65 No. 1 pp. 135–140, 2008 ISSN 0001-6837
Polish Pharmaceutical Society

DNA-BINDING ACTIVITY AND CYTOTOXICITY OF Pt-BERENIL COMPOUNDS IN MDA-MB-231 AND MCF-7 BREAST CANCER CELLS

KRZYSZTOF BIELAWSKI[1*], ANNA BIELAWSKA[1], TOMASZ SŁODOWNIK[2], BOŻENA POPŁAWSKA[1] and URSZULA BOŁKUN-SKÓRNICKA[1]

[1] Department of Medicinal Chemistry and Drug Technology, Medical University of Białystok, Kilińskiego 1, 15-089 Białystok, Poland,
[2] Department of Pharmaceutical Technology, Medical University of Białystok, Mickiewicza 2, 15-089 Białystok, Poland

Abstract: The compounds of formula $[Pt_2Cl_4(berenil)_2]Cl_4$ and $[Pt_2Cl_2(NH_3)_2(berenil)_2]Cl_4$ were examined for cytotoxicity in breast cancer cell cultures and for inhibition of topoisomerases I and II. Evaluation of the cytotoxicity of these compounds employing a MTT assay and inhibition of [^{3}H]thymidine incorporation into DNA in both MDA-MB-231 and MCF-7 breast cancer cells demonstrated that these compounds were more active than cisplatin. The DNA-binding ability of these compounds was evaluated by an ultrafiltration method using calf thymus DNA, poly(dA-dT)$_2$ and poly(dG-dC)$_2$, indicated that these compounds show strong specificity for AT base pairs. Binding studies indicate that these compounds bind more tightly to double-stranded DNA than cisplatin. The degree to which these compounds inhibited cell growth breast cancer cells was generally consistent with their relative DNA binding affinity. Mechanistic studies revealed that these compounds act as topoisomerase II (topo II) inhibitors in plasmid relaxation assays.

Keywords: cytotoxicity; cisplatin; DNA-binding; breast cancer, DNA topoisomerase

Platinum compounds are among the most important chemotherapeutic agents for treatment of cancer and cisplatin has been the primary platinum based drug for cancer treatment (1-3). However, its high efficacy is compromised by its propensity to cause several types of dose-limiting toxicity, including severe nephrotoxicity, and by the frequent occurrence of initial and acquired resistance to treatment (1-3). Nowadays, most efforts are based on the reasonable working hypothesis that new platinum compounds which bind to DNA and affect its conformation in a different manner than cisplatin might overcome cisplatin resistance.

In this study, the compounds of formula $[Pt_2Cl_4(berenil)_2]Cl_4$ (**1**) and $[Pt_2Cl_2(NH_3)_2(berenil)_2]Cl_4$ (**2**) (Figure 1) were examined for cytotoxicity in breast cancer cell cultures. Polynuclear platinum compounds comprise a unique class of anticancer platinum agents with distinct chemical and biological properties discrete from mononuclear platinum drugs (4, 5). Berenil (1,3-bis(4'-amidinophenyl)triazene) can exhibit intercalative, as well as minor groove binding properties when it binds to both DNA and RNA duplexes, while also exhibiting a preference for DNA duplexes with unobstructed minor grooves (6, 7). Recent work on the targeting of antitumor agents to DNA by the use of DNA minor groove-binding ligands has shown that this strategy can greatly enhance both the *in vitro* cytotoxicity and the *in vivo* antitumor activity of the alkylating moiety, when compared with untargeted compounds of similar reactivity (8-10). Berenil preferentially recognizes and binds to AT-rich DNA sequences and it is strong catalytic inhibitor of mammalian DNA topoisomerase II (11). Several other widely used anticancer agents, including doxorubicin and other anthracyclines, amsacrine, etoposide and mitoxantrone, also target topoisomerase II and are thought to be cytotoxic by virtue of their ability to stabilize a covalent topoisomerase II-DNA intermediate (12, 13).

In this study, we have investigated the antiproliferative activity of $[Pt_2Cl_4(berenil)_2]Cl_4$ and $[Pt_2Cl_2(NH_3)_2(berenil)_2]Cl_4$ in both MDA-MB-231 and MCF-7 breast cancer cells and assessed their ability to act as inhibitors of topoisomerases I and II (topo I and II). The DNA-binding ability of these compounds were studied employing the ultrafiltration method using calf thymus DNA, poly(dA-dT)$_2$ and poly(dG-dC)$_2$ (14-17).

* Corresponding author: phone: 48 085 7485701, fax: 48 85 7485416, e-mail: kbiel@amb.edu.pl

EXPERIMENTAL

Reagents and materials

$[Pt_2Cl_4(berenil)_2]Cl_4$ and $[Pt_2Cl_2(NH_3)_2(berenil)_2]Cl_4$ were synthesized as reported in the literature (18). The chemical structures of the synthesized compounds were confirmed by means of their elemental analysis and ^{1}H- and ^{13}C-NMR spectra. Calf thymus DNA, homopolymers poly(dA-dT)·poly(dA-dT), and poly(dG-dC)·poly(dG-dC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (USA). Supercoiled pKMp27 DNA, topoisomerase I and II were purchased from TopoGEN (USA). Stock cultures of breast cancer MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection, Rockville, MD. Dulbecco's minimal essential medium (DMEM) and fetal bovine serum (FBS) used in cell culture were products of Gibco (USA). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc. (USA). [^{3}H]Thymidine (6.7 Ci/mmol) was the product of NEN (USA).

Ultrafiltration method

A test compound (100 mM) was mixed with calf thymus DNA (0.5 mM as base pairs) in 1.0 mL of TB buffer (10 mM Tris•HC1 (pH 8) – 1 mM EDTA containing 0.15 M NaCl). The mixture was allowed to stand at room temperature overnight. The mixture was ultrafiltered using a ultrafree-MC centrifugal filter unit (PLCC NMWL 5000, Millipore) at 20°C, then the content of the test compound in the filtrate was determined by UV-absorption measurement ($Abs._{DNA+}$) and this value was taken as the concentration of free compound ($[comp]_{DNA+}$). The same procedure was carried out with a solution of the test compound in the absence of DNA as the control ($Abs._{DNA-}$ and $[comp]_{DNA-}$, respectively). Measurements were made with a Unicam/Helios UV-vis spectrophotometer at λ_{max} = 370 nm for compounds **1-2** and berenil. Results are the means of three independent experiments. The DNA-binding ability of the test compound was defined by the following equation:

DNA binding ability (%) = $(1 - [comp]_{DNA+}/[comp]_{DNA-}) \times 100 = (1 - Abs_{DNA+}/Abs_{DNA-}) \times 100$

Cell culture

Human breast cancer MDA-MB-231 and MCF-7 cells maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 mg/mL streptomycin at 37°C. 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, counted in hemocytometers and plated at 5×10^5 cells per well of 6-well plates (Nunc) in 2 mL of growth medium (DMEM without phenol red with 10% CPSR1). Cells reached about 80% of confluency at day 3 and in most cases such cells were used for the assays.

Cytotoxic assay

To examine the effect of studied drugs on MCF-7 and MDA-MB-231cells proliferation, the cells were seeded in 24 well tissue culture dishes at 1×10^5 cells/well with 1 mL of growth medium. After 48 h ($1.8 \pm 0.1 \times 10^5$ cells/well) plates were incubated with varying concentrations of compounds **1-2** and cisplatin and 0.5 mCi of [^{3}H]thymidine for 24 h at 37°C. Cells were rinsed 3 times with PBS, solubilized with 1 mL of 0.1 M sodium hydroxide containing 1% SDS, scintillation fluid (9 mL) was added and radioactivity incorporation into DNA was measured in scintillation counter.

Cell viability assay

The assay was performed according to the method of Carmichael using 3-(4,5-di-methylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (19). Confluent cells, cultured for 48 h with various concentrations of the studied compounds in 6-well

Table 1. DNA-binding abilities of compounds **1,2** and berenil determined by ultrafiltration assay using calf thymus DNA.

Comp.	Abs. DNA(-)[a]	Abs. DNA(+)[a]	DNA-binding ability[b] (%)	Scatchard analyses		
				Binding constant (Ka) (10^3 M^{-1})	Binding sites (n) (per bp)	Correlation coefficient (R)
berenil	0.440	0.150	65.9	6.8	0.4	0.77
1	0.446	0.141	68.3	6.9	0.5	0.74
2	0.460	0.120	73.9	7.9	0.4	0.75

[a] The UV-absorption was measured at λ_{max} = 370 nm after 5 dilutions with TB buffer.
[b] DNA binding ability (%) = $(1 - Abs_{DNA+}/Abs_{DNA-}) \times 100$.

Table 2. Base pair specificity of compounds **1, 2** and brenil determined by utrafiltration assay.

Compounds	DNA-binding ability (%)[a]		Inhibition of Topo I (μM)[b]	Inhibition of Topo II (μM)[b]
	AT	GC		
berenil	80.3	43.5	200	50
1	82.4	42.7	180	20
2	80.8	44.6	150	20

[a] The concentrations of the inhibitor that prevented 50% of the supercoiled DNA from being converted into relaxed DNA (IC_{50} values) were determined by averaging the data from at least three experiments.

[b] DNA-binding ability of berenil and compounds **1, 2** was determined by ultrafiltration assay. AT refers to poly(dA-dT)$_2$ and GC to poly(dG-dC)$_2$.

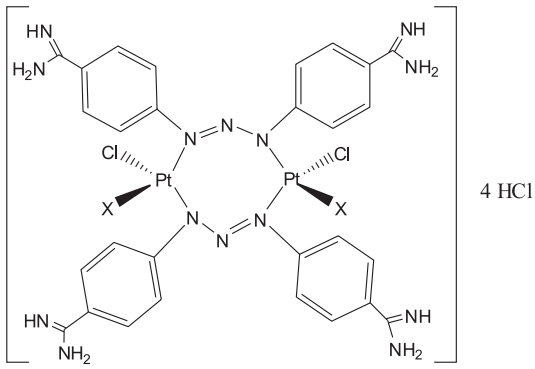
plates were washed three times with PBS and then incubated for 4 h in 1 mL of MTT solution (0.5 mg/mL of PBS) at 37°C in 5% CO_2 in an incubator. The medium was removed and 1 mL of 0.1 M HCl in absolute isopropanol was added to attached cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Cell viability of breast cancer cells cultured in the presence of ligands was calculated as a per cent of control cells.

Relaxation assay of topoisomerase I and II

Supercoiled pKMp27 DNA (0.5 mg) was incubated with 4 units of human topo I or II (TopoGEN), in relaxation buffer (50 mM Tris (pH 7.8), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ethylene diaminetetraacetic acid (EDTA)), in the presence of varying concentrations of the test compounds. Reactions were carried out at 37°C for 1 h and then terminated by the addition of sodium dodecyl sulfate (SDS) to 0.25% and proteinase K to 250 mg/mL. The reaction mixture was subjected to electrophoresis through a 0.8% agarose gel containing 0.5 mg/mL ethidium bromide in TBE buffer (90 mM Tris-borate and 2 mM EDTA). The gels were stained with ethidium bromide and photographed under UV light. For the quantitative determination of topoisomerase concentration activity, photographic negatives were scanned and the area representing supercoiled DNA, migrating as a single band at the bottom of the gel was measured using UVI-KS4000i gel documentation and analysis system (SyngenBiotech, USA). The concentrations of the inhibitor that prevented 50% of the supercoiled DNA from being converted into relaxed DNA (IC_{50} values) were determined by averaging the data from at least three experiments.

Statistical analysis

In all experiments, the mean values for six independent experiments ± standard deviations (SD) were calculated, unless otherwise indicated. The results were submitted to statistical analysis using Student t-test, accepting $p < 0.05$, as significant.



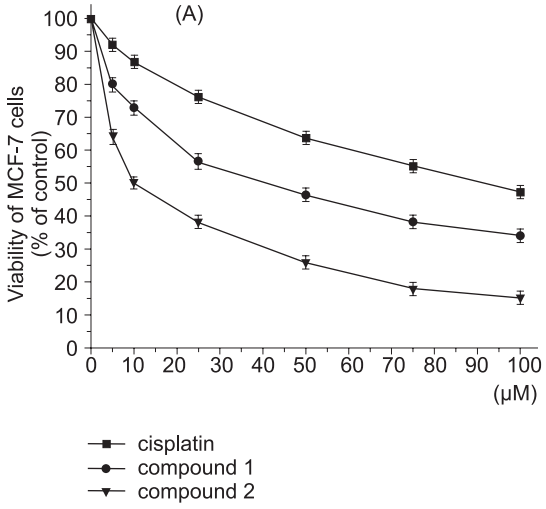
(1) X = Cl (2) X = NH_3

Figure 1. Structure of compounds **1** and **2.**

RESULTS AND DISCUSSION

The synthesis of $[Pt_2Cl_4(berenil)_2]Cl_4$ and $[Pt_2Cl_2(NH_3)_2(berenil)_2]Cl_4$ have been reported (18). However, to date no biological properties, such as cell growth in breast cancer cells or topoisomerase I and II inhibition, have been reported for these compounds.

Cell viability of breast cancer cells was measured by the method of Carmichael et al. using tetrazolium salt (19). Although growth inhibition was concentration-dependent in either cell line, it was more pronounced at shorter times, in MCF-7 than MDA-MB-231 (Figure 2). In terms of reduction in cell viability, the compounds rank in both MCF-7 and MDA-MB-231 cells in the order **2** > **1** > cisplatin. Among the derivatives, compound **1** in both MDA-MB-231 and MCF-7 proved to be more



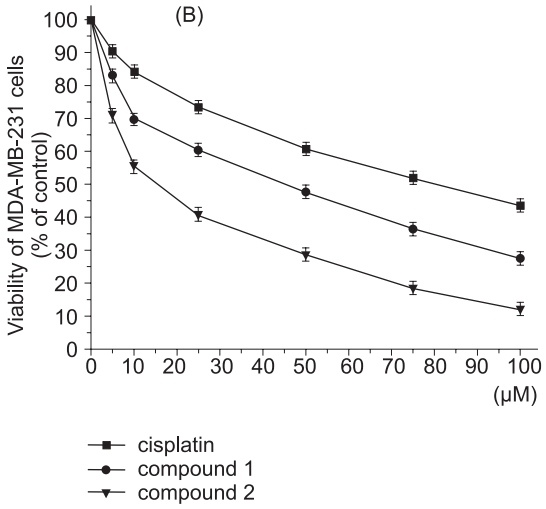
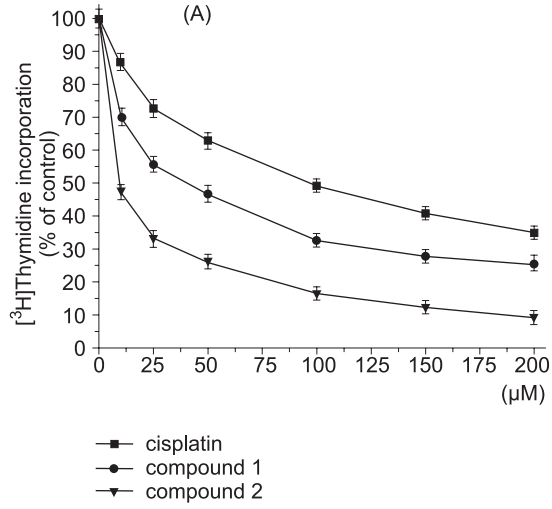


Figure 2. Viability of MCF-7 (A) and MDA-MB-231 (B) cells treated for 24 h with different concentrations of compounds **1, 2** and cisplatin. The mean values ± SD from three independent experiments done in duplicate are presented.



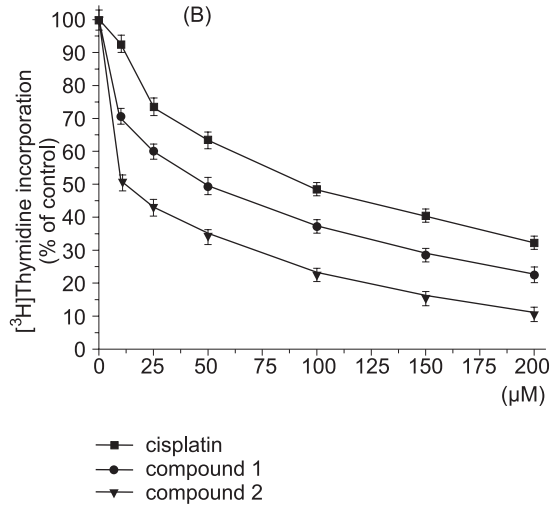


Figure 3. Cytotoxic effects of cisplatin and compounds **1, 2** on the cultured breast cancer MCF-7 (A) and MDA-MB-231 (B) cells as measured by inhibition of [^{3}H]thymidine incorporation into DNA. The mean values ± S.D. of 3 independent experiments (n = 4) done in duplicates are presented.

potent than cisplatin, with IC_{50} values of 44 ± 2 and 48 ± 2 mM, respectively, compared to 87 ± 2 and 98 ± 2 mM for cisplatin. Compound **2** is clearly much more active and showed a high level of cytotoxic potency, IC_{50} 9 ± 2 and 16 ± 2 mM in MCF-7 and MDA-MB-231, respectively.

To analyze if the inhibition in cell viability was due to decreased cell proliferation, we measured DNA synthesis in presence of compounds **1-2** and cisplatin (Figure 3). Measurement of [^{3}H]thymidine incorporation during DNA synthesis by proliferating breast cancer cells showed that these compounds inhibited DNA synthesis in a dose-dependent manner. Furthermore, the profiles of DNA synthesis obtained were similar between MCF-7 and MDA-MB-231 (Figure 3). The concentrations of **1** and **2** needed to inhibit [^{3}H]thymidine incorporation into DNA by 50% (IC_{50}) in MDA-MB-231 was found to be 48 ± 2 mM and 14 ± 2 mM, respectively, suggesting higher cytotoxic potency compared to cisplatin (IC_{50} = 98 ± 2 mM). The concentrations of **1**, **2** and cisplatin needed for 50% reduction in

[^{3}H]thymidine incorporation into DNA in breast cancer MCF-7 (IC_{50}) was found to be 40 ± 2 mM, 9 ± 2 mM and 102 ± 2 mM, respectively. Because the antiproliferative effect of compounds **1** and **2** is independent of the estrogen receptor status of the breast cancer cells, these potent inhibitors are potential pharmacological agents for the treatment of both hormone responsive and nonresponsive breast cancer cells.

To test whether cytotoxic properties were related to DNA-binding, compounds **1** and **2** were evaluated in a cell-free system. The binding affinity for a given DNA sequence is determined by a number of factors, including hydrogen bonding, electrostatic interactions and ligand conformation (20). Structures of DNA complexes of berenil, for example, reveal that it binds in the DNA minor groove at the central AATT sequence. This compound penetrate deeply into the groove and fit snugly between the walls of the groove. Its amidines form H-bonds with thymine-O2 and/or adenine-N3 acceptor groups on the edges of the bases at the floor of the groove (6, 7). The presence of the guanine NH_2 sterically impedes berenil from binding across GC pairs (20). The DNA-binding ability of compounds **1** and **2** was evaluated by the ultrafiltration method (Table 1) (14-17). The binding constants K_a and number of sites per nucleotide n were estimated from Scatchard plots using the classical Scatchard equation for independent binding sites without cooperativity (21). The order of DNA-binding under the utrafiltration assay conditions of the test compounds is **2** > **1** > berenil. As can be seen from Table 1, the binding constant (K_a) for compounds **1, 2** varies from 7.9 $\times 10^3$ M^{-1} for compound **2** to 6.8 $\times 10^3$ M^{-1} for berenil. Since calf thymus DNA is heterogeneous in base sequence and about 42% GC, the DNA-binding ability with calf thymus DNA characterizes the strength of the interaction with heterogeneous base sequences. The homopolymer DNA-binding data reported in Table 2 characterizes the affinity of compounds **1**, **2** for a more limited set of DNA-binding sites and can give an indication of base-sequence specificity for DNA-binding molecules. Compounds **1**, **2** were found to interact with a GC base pair though the binding affinities were weak compared with that for an AT base pair (Table 2). These data show that in broad terms the cytotoxic potency of **1** and **2** in cultured breast cancer MCF-7 and MDA-MB-231 cells increases in accord with their increases in DNA affinity, as shown by the binding constant values (Table 1). This activity may be related to the ability of these compounds to inhibit transcription, especially at AT-rich sequences.

Many DNA-binding agents exert their antitumor effect, at least in part, by inhibition of topo I (e.g. camptothecin) or topo II (e.g. doxorubicin) (12, 13). DNA topoisomerases are nuclear enzymes capable of resolving the topology of DNA to allow its replication (22). Hence, we examined the new compounds for their ability to interfere with the activity of these enzymes. The effect of compounds **1, 2** and berenil on the catalytic activities of purified human topo I and II was quantified by measuring the action on supercoiled plasmid DNA substrate as a function of increasing concentration of the ligands by the use of agarose gel electrophoresis. The concentrations of the inhibitor that prevented 50% of the supercoiled DNA from being converted into relaxed DNA (IC_{50} values) were determined (Table 2). These results demonstrated that **1** and **2** had topo I inhibitory activity at concentration of 150-180 mM. The compounds **1, 2** were the most potent topoisomerase II inhibitors, with 50% inhibitory concentration (IC_{50}) 20 mM, which is better than of berenil (IC_{50} = 50 mM). The topoisomerase-targeting drugs can be classified as either topo poisons, which act by stabilizing enzyme-DNA cleavable complexes leading to DNA breaks, or topo catalytic inhibitors, which act at stages in the catalytic cycle of the enzyme where both DNA strands remain intact and no DNA strand breaks occur (12, 13, 22). Compounds **1**, **2** do not promote DNA cleavage by topo I or II, in contrast to the reference classical poisons camptothecin (for topo I) and etoposide (for topo II). The intensity of the band corresponding to nicked DNA (for topo I) and linear DNA (for topo II) is not amplified in the presence of compounds **1**, **2**, implying that these compounds do not act as topo poisons. It is likely that the ability of compounds **1**, **2** to inhibit the activity of topo I and II that we have observed (Table 2) is simply due to blockade of the binding of these enzymes to DNA.

The DNA binding studies performed earlier had shown that these complexes form unique interstrand and intrastrand crosslinks by using the two terminal Pt atoms (23, 24). Therefore, these complexes represent a new class of anti-cancer agents, distinct in DNA binding and anti-tumor activity compared to the mononuclear cisplatin and are of special interest since the molecular interactions they are capable of are not possible for their mononuclear counterpart. It is probable that deregulation of DNA replication and transcription by inhibition of topoisomerase activity contribute significantly to the cytotoxicity of compounds **1**, **2** in addition to primary drug-DNA reaction products.

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