Comparative assessment of the cytotoxic effects of carboxylato-bridged dinuclear platinum (II) complexes against human tumor cell lines*

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The cytotoxic effects of a series of carboxylato-bridged dinuclear platinum (II) complexes with acetate (BAP), propionate (BPP) and valerate (BVP) ligands were evaluated in a panel of human tumor cell lines. BAP proved to be the most potent antineoplastic agent, whose cytotoxic effect reached and even outclassed that of the referent drug cisplatin. This compound also exerted substantial efficacy against a broader spectrum of tumor models including the multidrug-resistant HL-60/Dox cell line. In the latter case, BAP showed lower resistance index than cisplatin. BAP was furthermore found to induce apoptosis in different cell lines as evidenced by DNA-laddering and 'Cell-death' ELISA. Our experimental data give us reason to conclude that the dinuclear Pt(II) complex with acetate ligands is perspective for further detailed pharmacological and toxicological evaluation as an antineoplastic drug candidate.

Key words: dinuclear platinum complexes, acetate, cytotoxicity, apoptosis

The square planar complex cis-diamminedichloroplatinum (II) (cisplatin,cis-DDP) is an essential antineoplastic agent, whose introduction as a clinical chemotherapeutic agent in the early 70's of the last century had a revolutionary impact on the response rate and long-term survival of patients with testicular malignancies [2–4]. Cis-DDP is also widely used for the therapeutic management of a variety of solid tumors such as head and neck squamous cell carcinoma, ovarian cancer, non-small cell lung carcinoma, cervical cancer, prostate cancer etc. [3, 5, 15, 17]. Notwithstanding the clinical efficiency of cis-DDP, this drug exerts severe dose-limiting adverse effects such as nephrotoxicity, peripheral neuropathy, ototoxicity and debilitating nausea and vomiting, that seriously limit the possibilities for application of this drug in effective high dose regimens [4, 8, 12, 13]. The unique toxicological profile of cis-DDP, together with the occurrence of primary and acquired resistance, by virtue of diverse mechanisms, have solicited enormous interest towards creation of platinum coordination compounds with lesser toxicity and broader antitumor spectrum in comparison to the prototype drug [1, 6]. To meet this objective a plethora of several thousand platinum agents has been synthesized and studied for antineoplastic activity during the last three decades [3, 16, 21]. Only few of the cis-DDP analogues however, proved to be suitable for therapeutic utilization, namely: carboplatin, nedaplatin (available in Japan only), and oxaliplatin, the latter endowed by prominent efficiency in colorectal carcinoma [4, 14]. Despite of the certain advantages of these newer generation platinum drugs, esp. regarding toxicity and quality of life issues, they are by no means ideal substitutes for cis-DDP and they are characterized by a modified hall-mark toxicity rather than by lower toxicological potential. Thus carboplatin and nedaplatin exhibit profound myelossuppressive effects, whereas oxaliplatin induces significant neurotoxicity, including severe idiosyncratic peripheral neuropathy [4, 13, 14].

Considering the obvious incapacity of cisplatin-like drugs to overcome the disadvantages of the prototype some innovative strategies towards development of non-classic platinum analogues, such as: monofunctional complexes, *trans*-geometry complexes, polynuclear complexes etc. have emerged [16, 21]. Among these cisplatin-dislike agents special atten-

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tion has been paid towards the polynuclear compounds, whereby two or more platinum centers are bridged via a suitable liking group. Such agents have been shown to exhibit alternative mode of DNA-binding and to retain considerable activity in cis-DDP-resistant tumor models [7, 9, 19–21]. A trinuclear cationic complex BR3464 has entered clinical trial and is currently under phase II stage of investigation [16, 21].

The objective of the present study is a comparative evaluation of the cytotoxic effects of three carboxylato-bridged dinuclear platinum (II) complexes with organic ligands in a panel of human tumor cell lines.

Material and methods

Drugs, chemicals and solutions. The novel dinuclear platinum complexes under investigation namely: bis(acetato)diammine-bis-μ-acetato diplatinum (II) dihydrate (BAP), potassium dichloropropionate ammine-bis–propionato diplatinate (II) (BPP) and potassium chloro-bis(valerato)amminebis-μ-valerato diplatinate (II) (BVP) (Fig. 1) were synthesized, characterized and purified as previously described [18]. Agarose, ethanol, formic acid, 2-propanol, methanol, EDTA, ethidium bromide, sodium chloride, Tris hydrochloride, Triton^R X-100, L-glutamine) were purchased from AppliChem GmbH, Darmstadt, Germany. Fetal calf serum (FCS) and powdered RPMI 1640 medium were purchased from Sigma-Aldrich GmbH, Steinheim, Germany. The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was supplied from Merck, Darmstadt, Germany. The referent cytotoxic drug cis-DDP was used as a commercially available sterile dosage form for clinical application (Platidiam^R, Lachema, Czech Republic).

Stock solutions of cis-DDP, BAP, BPP and BVP were freshly prepared in purified water and were consequently sterilized via antibacterial filtration through 'Milipore' syringe filters (0.25μ m). These were diluted to the desired extent with RPMI-1640 medium.

Cell lines and culture conditions. A panel of human tumor cell lines with different cell type and origin were used (Tab. 1). All human tumor cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). The leukemic cells were grown as suspension type cultures in controlled environment – cell culture flasks at 37 °C in an incubator 'BB 16-Function Line' Heraeus (Kendro, Hanau, Germany) with humidified atmosphere and 5% CO_2. Cells were kept in log phase by supplementation with fresh medium after removal of cell suspension aliquots, two or three times a week. For all cell lines RPMI-1640 liquid medium supplemented with 10% FBS and 2 mM L-glutamine was used. The urinary bladder carcinoma cell lines 5637 and EJ were grown as monolayer cultures and were reset by trypsinisation two times per week.

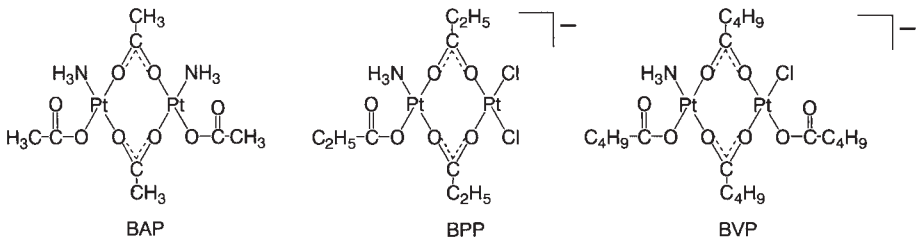

Figure 1. Chemical structures of the investigated carboxylato-bridged dinuclear platinum (II) complexes.

Table 1. IC_{50} values of BAP, BPP, BVP and the referent antineoplastic agent cis-DDP in the panel of human tumor cell lines

Cell line	Cell type	IC_{50} value (μM)			
		Cis-DDP	BAP	BPP	BVP
HL-60	Acute myeloid leukemia	8.3	2.1	93.8	140.9
HL-60/Dox	Acute myeloid leukemia	32.9	2.7	n.d.	n.d.
SKW-3	Chronic T-cell leukemia	11.2	8.4	44.2	80.6
CML-T1	Chronic myeloid leukemia	6.2	7.5	137.2	142.1
BV-173	Pre-B-cell lymphoma	10.4	36.4	136.1	180.4
HD-MY-Z	Hodgkin lymphoma	10.4	19.5	>200	>200
U-937	Histiocytic lymphoma	7.0	17.5	n.d.	n.d.
K-562	Chronic myeloid leukemia	36.9	37.9	n.d.	n.d.
5637	Urinary bladder carcinoma	9.2	16.2	n.d.	n.d.
Ej	Urinary bladder carcinoma	5.3	79.8	n.d.	n.d.

Cytotoxicity assessment (MTT-dye reduction assay). The cell viability was assessed using the standard MTT-dye reduction assay as described by MOSMANN [11] with minor modifications [10]. In brief: exponentially growing cells were seeded in 96-well flat-bottomed microplates (100 μl/well) at a density of $1x10^5$ cells per ml and after 24 h incubation at 37C they were exposed to various concentrations of the tested compounds for 48 h. For each concentration at least 6 wells were used. After the incubation with the test compounds 10 μl MTT solution (10 mg/ml in PBS) aliquots were added to each well. The microplates were further incubated for 4 h at 37 °C and the formazan crystals formed were dissolved through addition of 100 μl/well 5% formic acid (in 2-propanol). The absorption was measured using an ELISA reader (Uniscan-Titertec) at 580 nm. Cell survival fractions were calculated as percentage of the untreated control. In addition IC_{50} values were

derived from the concentration-response curves. Resistance indices were calculated from the data for the multidrug-resistant HL-60/Dox cells as the ratio between the IC_{50} for HL-60/Dox and the IC_{50} for HL-60. These were utilized as merit for the resistance encountered.

DNA fragmentation analysis. Horizontal gel-electrophoresis of cytosolic DNA, isolated from treated SKW-3 and K-562 cells was performed in order to test the ability of the compounds under investigation to trigger apoptosis. The method was executed as previously described with some modifications [10] In brief: cell suspension aliquots (10 ml) of SKW-3 and K-562 cells (1 treatment group and 1 control, respectively) at a density of $0.5x10^6$ cells/ml were transferred in sterile plastic Petry dishes. The treatment groups of both cell lines were exposed to 100 μM BAP for 24 h (SKW-3) and 72 h (for K-562, due to their known relative resistance to pro-apoptotic stimuli). After the incubation period the cells were pelleted and washed in PBS. Cell pellets were re-dispersed in 0.25 ml PBS and lysed through addition of 0.5 ml buffer containing 0.5% Triton X-100, 20 mM Tris-HCl and 1 mM EDTA (pH=7.4). Samples were incubated on ice for 5 min and thereafter spun at 13 000 rpm for 20 min. The supernatants were transferred into fresh 2 ml Eppendorf safe lock tubes and then 0.937 ml 2-propanol as well as 0.187 ml 6 M solution of NaCl were added to each sample. The tubes were gently agitated and incubated at –20 °C for 12 h in order to allow precipitation of the water-soluble DNA. The samples were centrifuged for 20 min at 13 000 rpm, the supernatants were decanted and DNA was washed in 1 ml ice cold 70% ethanol and then air dried. After that DNA was re-dissolved in 20 μl distilled water and analyzed by gel electrophoresis in 0.8% agarose gel and then stained with ethidium bromide. Finally DNA was visualized using an UV transilluminator and photographed with a fixed digital camera (Bio Doc IT^{TM} system).

'*Cell-death detection' ELISA.* This method allows semi-quantitative determination of the characteristic for the apoptotic process histone-associated mono- and oligonucleosomal DNA-fragments using 'sandwitch' ELISA. The determination was conducted as recommended by the manufacturer of the kit exploited (Roche Diagnostics).

Results

All of the investigated platinum (II) complexes, as well as the referent antineoplastic agent cis-DDP elicited concentration-dependent cytotoxic effects in the investigated panel of cell lines (Fig. 2–9 and Tab. 1).

As evident from the concentration response curves from the comparative evaluation of the platinum complexes against the myeloid HL-60 cells (Fig. 2), BAP proved to be the most active cytotoxic agent. It reached 50% inhibition of the tumor cell proliferation at approx. 4 times lower concentration than the referent drug cis-DDP did (IC_{50} values of 2.1 μM and 8.3 μM, respectively). The complex with propionate ligands caused far less prominent cell proliferation inhibition in respect to both relative potency (IC_{50}=93.8 μM) and maximal efficacy (approx. 23% vital cells at 200 μM). The cytotoxic efficacy of the valerate complex was even lesser with an IC_{50} value of 141μM.

Accordingly on the lymphoid SKW-3 cells the complex with acetate ligands BAP and the referent drug cis-DDP with IC_{50} values 8.4 μM and 11.3 μM, respectively again proved to be superior, in comparison to the remaining both analogues (Fig. 3). While the cytotoxic potential of BAP and cis-DDP were practically identical, their maximal effects differed significantly – approx. 13% vital cells at 43 μM for BAP and 20% vital cells at 100 μM for cis-DDP, respectively. The propionate complex exhibited lower cytotoxicity with IC_{50} of 44.2 μM. Its maximal effects were comparable to that of the

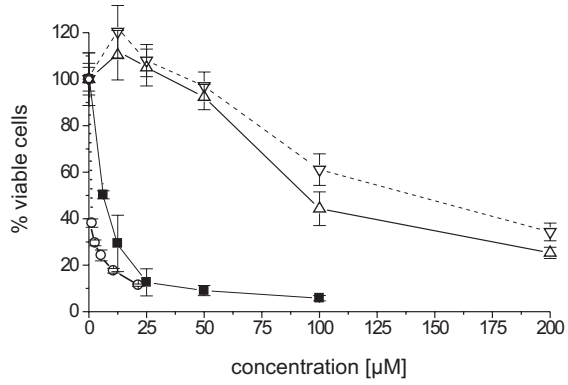

Figure 2. Cytotoxic effects of BAP (○), BPP (△), BVP (▽) and cis-DDP (■) on HL-60 cells, following 48 h treatment, as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments, whereas the error bars represent the corresponding standard deviations.

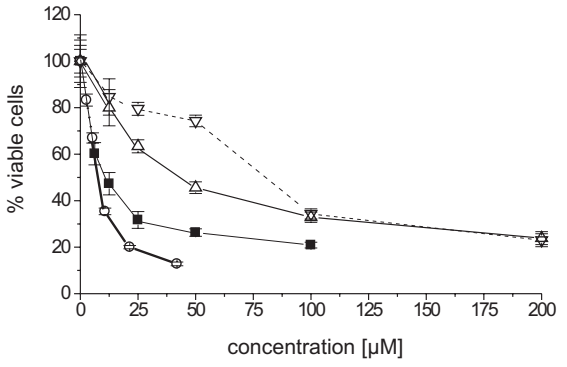

Figure 3. Cytotoxic effects of BAP (○), BPP (△), BVP (▽) and cis-DDP (■) on SKW-3 cells, following 48 h treatment, as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments, whereas the error bars represent the corresponding standard deviations.

referent drug, but was achieved at twice higher concentration (approx. 24% vital cells at 200 μM). The last dinuclear platinum complex BPV proved to be the least potent with IC_{50} value almost 8 times higher than that of the referent antineoplastic agent. The maximal efficacy of that compound was analogous to that of BPP (23% vital cells at 200 μM).

The experimental data obtained from CML-T1 treatment demonstrated that BAP by far outclassed the other dinuclear analogues (Fig. 4). Its IC_{50} value was similar to that of the referent drug cis-DDP (6.2 μM and 7.5 μM, respectively). The dinuclear analogue, however, reached more pronounced maximal efficacy with a total eradication of malignant cells at concentrations exceeding 50 μM, whereas even at 100 μM cis-DDP caused less prominent inhibition of the tumor cell proliferation (ca. 21% viable cells). The propionate and acetate complexes induced far less pronounced effects in CML-T1 cells with IC_{50} values of 137.2 μM and 142 μM, respectively. They actually reached the maximal efficacy, encountered with BAP but at 4-times higher concentrations.

The assessment of the cytotoxic effects of tested compounds on BV-173 cells showed, that BAP exerted significant, concentration dependent cytotoxicity with a IC_{50} value obtained of 36.4 μM. The referent agent cis-DDP caused prominent cell proliferation inhibition of these cells with an IC_{50} value of 10.4 μM. Despite the higher relative potency of cis-DDP in comparison to BAP, both compounds induced similar maximal tumor growth inhibition at the highest concentrations. BPP and BPV caused far less pronounced cytotoxicity on BV-173 cells with IC_{50} values 136.2 μM and 180.3 μM, respectively (Fig. 5).

The experimental data from the investigations on the lymphoid HD-MY-Z cells revealed, that the dinuclear complex BAP caused cell proliferation inhibition to an extent, comparable to the efficacy of the referent drug cis-DDP (IC_{50} values of 19.6 μM and 10.4 μM, respectively) (Fig. 6, Tab. 1). The other dinuclear coordination compounds BPP and BVP exerted only marginal cytotoxicity and failed to induce 50% cell proliferation inhibition throughout the concentration range under investigation (12.5–200 μM).

The apparent superiority of BAP over its longer aliphatic chain analogues in the preliminary cytotoxicity assessment program was furthermore confirmed in experiments utilizing a broader spectrum of cell lines, namely the multidrug-resistant HL-60/Dox, the CML-derived K-562, the histiocytic lymphoma U-937, and the urrinary bladder carcinoma 5637 and EJ cells (Fig. 7–11).

Both BAP and cis-DDP elicited prominent cytotoxic effects in U-937, although in contrast to the overal superior potency of the novel compound in this case cis-DDP displayed higher cytotoxic activity as evidenced by the IC_{50} values obtained.

PAB exhibited profound cytotoxic effect upon the multidrug resistant HL-60/Dox with a IC_{50} value of 2.7 μM and a resistance index of ca. 1.3. Interestingly the referent complex cis-DDP displayed much lower potency against HL-60/Dox with approximately 10-fold higher IC_{50} value than the dinuclear complex. The resistance index encountered in case of cis-DDP (3.96) was approximately 3-fold greater than that of BAP.

BAP exerted prominent cytotoxic effect upon K-562 cells with a practically equal potency relative to cis-DDP, on the basis of the encountered IC_{50} values. With regard to the maximal efficacy BAP outclassed the referent drug, causing more prominent cell survival inhibition at the higher concentrations employed.

The novel dinuclear Pt(II) complex with acetate ligands exerted strong cell-growth inhibitory activity on the urinary bladder carcinoma cell line 5637. Although its relative potency was lower than hat of cis-DDP (IC_{50} values of 16.2 and 9.2 μM, respectively) the encountered maximal efficacy was comparable to that of the referent drug.

The other urinary bladder carcinoma-derived cell line EJ, however, displayed far more pronounced sensitivity to cis-DDP as compared to BAP (IC_{50} values of 5.3 and 79.8 μM, respectively).

The DNA isolated from the cytosolic fraction of SKW-3 and K562 cells exposed to BAP (for 24 h and 72 h, respectively) demonstrated oligonucleosomal DNA fragmentation which is a hallmark of the programmed cell death (Fig. 12).

Accordingly, BAP treatment of HL-60 and U-937 cells led to a significant elevation of the enrichment factor (corresponding to the level of histone-assotiated DNA fragments), as compared to the untreated control group which furthermore evidences for the ability of this complex to induce apoptosis (Fig. 13).

Discussion

In the majority of polynuclear platinum complexes developed so far, the present platinum centers are bridged via N,N-coordination mode utilizing polyamines or heterocyclic compounds as bridging ligands [7, 9, 19]. In a dissimilar fashion the hereby presented series of dinuclear Pt(II) complexes are bridged by the deprotonated carboxylic anions, namely valerate, propionate and acetate, through the abundant oxygen atoms. Such structural peculiarity characterizes the presented compounds as an unique class of platinum coordination compounds. The experimental data retrieved from the cytotoxicity screening program show that all of the three novel dinuclear platinum compounds, despite of the differences in respect to relative potency, exert antineoplastic activity and since they cause more than 50% inhibition of the malignant cell proliferation, they could be considered as biologically active. On the basis of the IC_{50} values obtained it could be drawn out, that the complex with acetate ligands proved to be the most potent cytotoxic agent with relative potency equal or even exceeding that of the referent drug cis-DDP. The increase of the alkyl chain in the organic ligands of BPP and BVP is invariably related to to a pronounced loss of efficacy. The apparent trend towards decline in

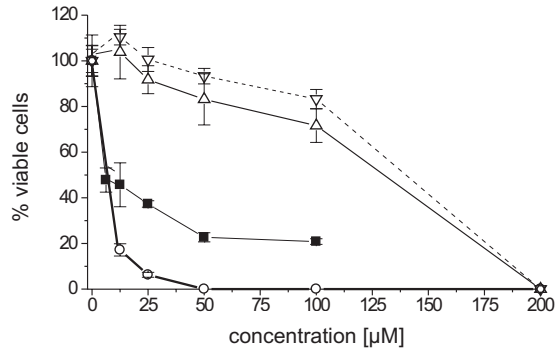

Figure 4. Cytotoxic effects of BAP (○), BPP (△), BVP (▽) and cis-DDP (■) on CML-T1 cells, following 48 h treatment, as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments, whereas the error bars represent the corresponding standard deviations.

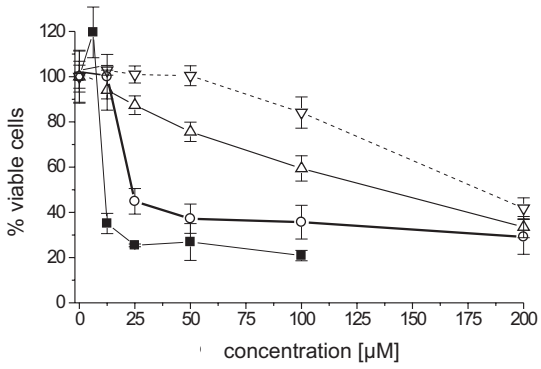

Figure 5. Cytotoxic effects of BAP (○), BPP (△), BVP (▽) and cis-DDP (■) on BV-173 cells, following 48 h treatment, as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments, whereas the error bars represent the corresponding standard deviations.

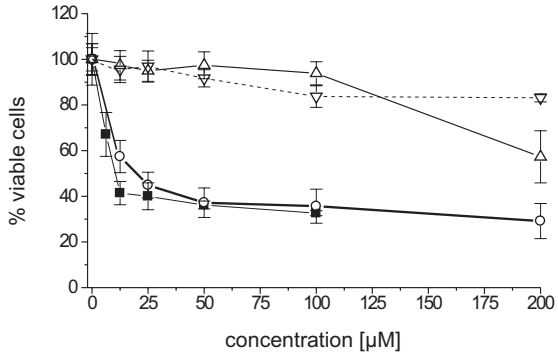

Figure 6. Cytotoxic effects of BAP (○), BPP (△), BVP (▽) and cis-DDP (■) on HD-MY-Z cells, following 48 h treatment, as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments, whereas the error bars represent the corresponding standard deviations.

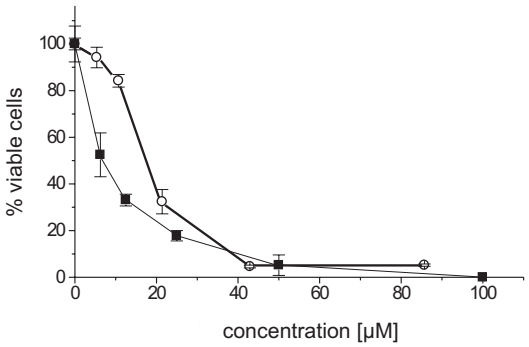

Figure 7. Cytotoxic effects of BAP (○) and cis-DDP (■) on U-937 cells, following 48 h treatment, as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments, whereas the error bars represent the corresponding standard deviations.

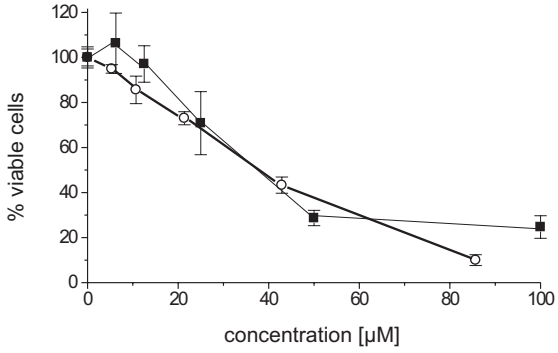

Figure 8. Cytotoxic effects of BAP (○) and cis-DDP (■) on K-562 cells, following 72 h treatment, as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments, whereas the error bars represent the corresponding standard deviations.

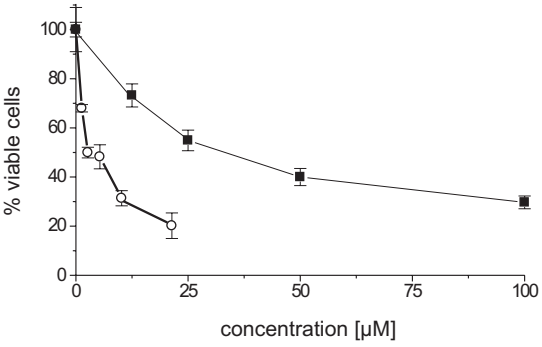

Figure 9. Cytotoxic effects of BAP (○) and cis-DDP (■) on HL-60/Dox cells, following 48 h treatment, as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments, whereas the error bars represent the corresponding standard deviations.

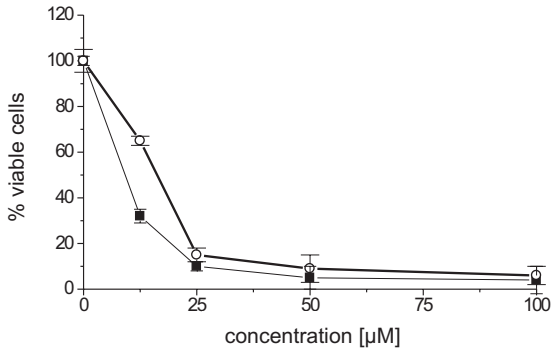

Figure 10. Cytotoxic effects of BAP (○) and cis-DDP (■) on 5637 cells, following 48 h treatment, as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments, whereas the error bars represent the corresponding standard deviations.

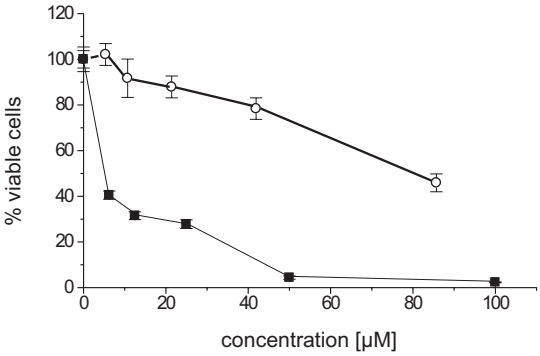

Figure 11. Cytotoxic effects of BAP (○) and cis-DDP (■) on Ej cells, following 48 h treatment, as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments, whereas the error bars represent the corresponding standard deviations.

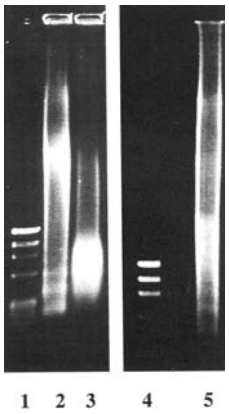

Figure 12. DNA fragmentation induced by BAP treatment in SKW-3 cells (lane 2) and K-562 cells (lane 5); lanes 1 and 4 – size marker; lane 3 – untreated control.

cytotoxic activity with increasing the alkyl side chain could be generally ascribed to the positive induction effect of the latter and the corresponding decrease in the polarity of the Pt-O bonds. The lower cytotoxic efficacy of BPP and BPV in comparison to PAB could be also attributable to the ionic nature of the former complexes, which most probably hampers their transmembrane transport and cellular accumulation.

The observed high cyto-

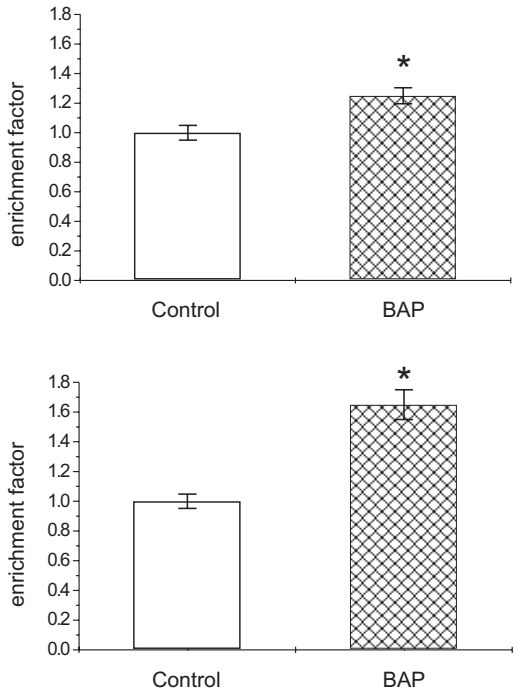

Figure 13. Increase in the levels of histone-associated DNA fragments (expressed as enrichment factor in arbitrary units), following 24 h BAP treatment in HL-60/Dox (at 10 μM (plot A)) or U937 (at 20 μM (plot B)) as assessed by 'Cell death detection' ELISA.

toxic efficacy of BAP in the preliminary cytotoxicity screening was additionally supported via the encountered effects of this compound against a broader spectrum of malignant cells lines. The considerable efficacy of BAP in the multidrug-resistant HL-60/Dox cells is of particular interest, especially taking into consideration the differing indices of resistance for cisplatin and BAP. HL-60/Dox cells are characterized by expression of MRP-1 – a member of the ATP-binding cassette protein family, which are known to interfere with cisplatin cellular efflux and resistance [1, 3, 21]. Since MRP-1 expels the xenobiotics after glutathione (GSH) conjugation, it could be hypothesized that the lower index of resistance of BAP as compared to cisplatin is an outcome of the lower propensity of the former to interact with thiols. Such lower reactivity to glutathione is well documented for complexes with organic leaving groups (incl. carboxylic anions) [2, 16, 21] and in this case it could be ascribed to the abundance of acetate ligands in the structure of BAP.

Preliminary data characterize the novel complex with acetate ligands as apoptosis-inducing agent as evidenced by the established oligonucleosomal DNA fragmentation, following BAP treatment. Thus, as well documented for cisplatin and analogues [1, 2, 21], it appears that the induction of apoptosis is an important component of the cytotoxic mode of action of this novel class of dinuclear platinum complexes too. Interestingly BAP was found to trigger apoptosis in the

human chronic myeloid leukemia K-562, which is known for its low responsiveness towards pro-apoptotic stimuli, conditioned via the expression of the non-receptor tyrosine kinase bcr-abl [10].

Our experimental data give us reason to conclude that the dinuclear Pt(II) complex with acetate ligands, by virtue of the encountered prominent antineoplastic efficacy *in vitro*, ability to induce apoptosis and partial circumvention of cross-resistance to cisplatin could be considered as an attractive candidate for further thorough pharmacological and evaluation as a possible antineoplastic drug.

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