Cellular Pharmacology, Antineoplastic Activity and Low *In Vivo* Toxicity of a Carboxylato-Bridged Platinum(II) Complex bis(acetato)diammine-bis-μ-acetato diplatinum (II) Dihydrate

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Abstract: The dinuclear platinum complex bis(acetato)diammine-bis-μ-acetato diplatinum (II) dihydrate has been previously shown to exert profound cytotoxicity in diverse tumor cell lines, while being far less detrimental than the clinically applied platinum drugs against some susceptible to platinum toxicity non-malignant cellular populations. In the present study we report the investigation of the cellular accumulation kinetics and apoptosis induction of the dinuclear complex in K-562, its potent *in vivo* antineoplastic activity against L1210 leukemia and Lewis lung carcinoma tumor models and its lower nephrotoxicity, myelosuppresive potential and clastogenicity *in vivo* relative to cisplatin.

Key Words: Dinuclear platinum complexes, K-562, L-1210 leukemia, Lewis lung carcinoma, nephrotoxicity, genotoxicity.

INTRODUCTION

Cisplatin is a valuable antineoplastic drug effective for the treatment of germ cell tumors and other solid malignancies [1,2]. The significant toxicological potential of this drug, together with the intrinsic non-responsiveness of some common cancers and the development of acquired resistance during treatment have prompted intensive research efforts in pursuit of novel analogues, characterized *via* lower toxicity and/or activity in cisplatin-refractory tumors [3,4]. The second generation platinum compounds e.g. carboplatin and nedaplatin, developed *via* replacement of the chlorine leaving groups with less labile carboxylato ligands are indeed characterized with low nephrotoxicity relative to cisplatin, because the organic ligands render them less reactive *in vivo* [1,5]. Unfortunately the decreased toxicity upon the kidneys is attained at the expense of lower cytotoxic activity against malignant cells too, thus these agents generally do not have significantly improved therapeutic indices relative to cisplatin [1]. Furthermore they induce the same type of DNA-adducts as the parent drug and not surprisingly share the same spectrum of anticancer activity [1-3,5].

Considering the obvious incapacity of cisplatin-type agents to overcome the disadvantages of the prototype there has been increasing interest towards designing structurally dissimilar platinum coordination compounds, anticipated to induce different response at the cellular/molecular level and conversely to bypass the mechanisms of cellular resistance to cisplatin [5,6]. One of the most intriguing and extensively exploited innovative strategies towards development of platinum agents endowed by high antitumor activity without cross-resistance to cisplatin, has been the design of di-, tri- and polynuclear compounds [7]. An representative example is the trinuclear charged compound with polyamine bridging ligands BBR-3464 which has been shown to induce cisplatin-dissimilar spectrum of DNA adducts and to circumvent primary and acquired resistance to cisplatin in different preclinical tumor models [7,8]. Furthermore the unique long-range inter-strand crosslinks induced by this agent are less susceptible to modification by the DNA-repair machinery. The structural perturbation in the higher order DNA structure induced by BBR-3464 condition alternative recognition by the p53 proteins and furthermore the BBR-3464 platination has proved to be detrimental for the DNA binding of NFκB – a nuclear factor implicated in the resistance to cisplatin [8]. Currently BBR-3464 is under intensive clinical trial development and has demonstrated activity in tumors typically non-responsive to cisplatin [2,6-8].

Within our platinum-based drug discovery program we have developed series of platinum(II) complexes with carboxylic acids in an attempt to confer low toxicity, while attaining high antineoplastic activity. Among these compounds three dinuclear complexes – with acetate, propionate and valerate ligands have been proved superior in an *in vivo* P388 leukemia prescreen system [9]. More recently the former complex - bis(acetato)diammine-bis-μ-acetato diplatinum (II) dihydrate (BAP (**1**)), was found to significantly outclass the remaining analogues in a spectrum of human and animal tumor cell lines whereby its activity was equal-to-superior relative to cisplatin [10,11]. Furthermore this complex has demonstrated quite less pronounced propensity to induce detrimental effects upon cellular populations susceptible to platinum toxicity - renal epithelium, primary neu-

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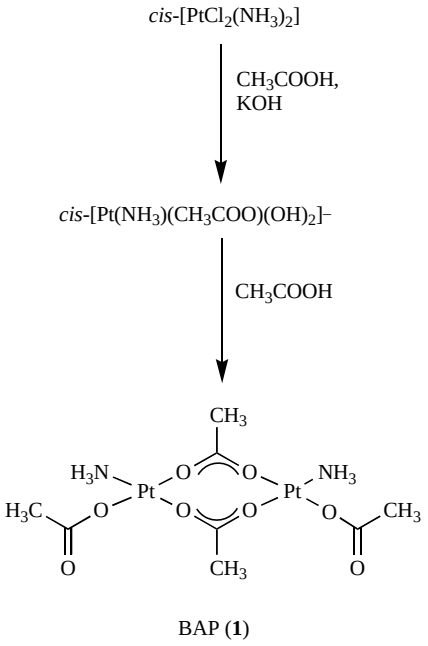
rons and long term bone marrow cell cultures as compared to the clinically applied platinum drugs cisplatin, carboplatin and oxaliplatin [12].

In the present study we aimed at: i) defining the cellular pharmacological profile of BAP in K-562 in terms of cytotoxicity, induction of apoptosis and cellular accumulation; ii) evaluation of the antineoplastic activity of the complex in L-1210 and Lewis lung carcinoma bearing mice; iii) assessment of its *in vivo* toxicological profile regarding nephrotoxicity, bone marrow function and clastogenicity.

EXPERIMENTAL SECTION

General

The synthesis and the characterization of the presented Pt(II) complex with acetic acid (Platacetat) were carried out as previously described (Fig. (**1**)) [1,2]. The behavior of the complex in solution was investigated in comparison to cisplatin. To meet this objective 0.001 mol.dm^{-3} solutions of both compounds were prepared in DMSO and their molar electric conductance was evaluated.



BAP (**1**)

Fig. (1). Schematic representation of the synthetic procedure for synthesis of the tested compound BAP(**1**). The reactions were carried out in aqueous medium.

Cells, Culture Conditions and Chemosensitivity Assay

The chronic myeloid leukemia K-562 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). The cells were grown in controlled environment – cell culture flasks with RPMI-1640 liquid medium supplemented with 10 % FBS and 2 mM L-glutamine, at 37^{o}C in an incubator 'BB 16-Function Line' Heraeus (Kendro, Hanau, Germany) with humidified atmosphere and 5% CO_2. The chemosensitivity testing after 24 h or 48 h incubation was carried out using the standard MTT-assay as previously described [13] with minor modifications [14].

Determination of the Platinum Cellular Pharmacokinetics

Aliquots of 2×10^7 K-562 cells (in 2 ml RPMI 1640) were placed in sterile petry dishes and exposed to equimetal concentrations of cisplatin or BAP, corresponding to 50 μM platinum for 30 or 120 minutes, at 37°C with constant stirring. After the exposure period the cells were spun at 2000 rpm for 5 minutes and the drug-containing medium was discarded. The treated cells were then washed thrice with PBS in order to remove residual cell surface-associated platinum complexes and aliquots were taken for counting and cell viability determination (Trypan blue dye exclusion assay). Thereafter the cells were resuspended in 1 ml mixture of conc. hydrochloric acid and conc. nitric acid (3:1) and heated at 100°C for 1h. Following the complete decomposition of the cells the volume of the samples was adjusted to 1 ml and the platinum concentration thereof was determined by electrothermal atomic absorption spectrometry (EAAS). The measurements were carried out on a Zeeman Perkin-Elmer 3030 spectrometer with an HGA-600 atomizer. The light source used was a hollow-cathode lamp for Pt. The spectral bandpass was 0.7 nm. Standard uncoated graphite tubes were used as atomizer. Sample aliquots (20 μl) were introduced into the graphite atomizer using an AS-60 auto sampler and the platinum content was quantified at 265.9 nm with an atomizing temperature of 2600°C, using argon as purge gas. Atomic absorption signals were recorded on an Anadex printer. Only peak areas were used for quantification. Calibration was performed using aqueous standard solutions, prepared by appropriate dilution of a stock standard solution (1g/L Pt, Merck, Darmstadt, Germany). The results were expressed as ng Pt/10^6 cells.

DNA-fragmentation Analysis

The characteristic for apoptosis oligonucleosomal DNA fragmentation was quantified using 'Cell Death Detection ELISA' kit (Roche Diagnostics GmbH, Germany). Aliquots of 50 000 exponentially growing K-562 cells were exposed to either BAP, cisplatin or vehicle (RPMI-1640 medium) for 12 or 24 h. The cytosolic fraction (13000×g supernatant) of 10^4 treated or untreated K-562 cells served as an antigen source in a sandwich ELISA with primary anti-histone antibody-coated microplate and a secondary anti-DNA peroxydase-conjugated antibody. The immunoassay for quantification of histone-associated DNA fragments was performed at 405 nm, according to the recommendations of the manufacturer. The results were presented as enrichment factor (EF) representing the ratio between the 405 nm absorption of the treated and the untreated control samples.

Animals

The animals used in this study – C57BL/6, DBA/2, BDF1 and ICR mice were all supplied from the National Breeding Centre at the Bulgarian Academy of Sciences (Slivnitsa, Bulgaria) and were housed at the Animal Care Facility of the Faculty of Pharmacy, MU-Sofia. The mice were kept under standard conditions: 20-22°C, 12 h alternating light/dark cycles and free access to food (Vitaprot-Ltd., Kostinbrod, Bulgaria) and water, All of the animal experiments were conducted *via* strictly adhering to approved protocols, com-

plying to the requirements of the European Convention for Protection of Vertebrate Animals used for Experimental and other Specific Purposes and to the current Bulgarian laws and regulations.

L1210 Murine Leukemia

The tumor line was obtained from the National Oncological Centre, Bulgaria and was locally maintained by serial weekly passages of 10^5 viable tumor cells i.p. in DBA/2 mice. The leukemia propagation for the chemotherapeutic experiment was performed in hybrid BDF1 mice, implanted i.p. with 10^5 viable cells. Immediately after the inoculation with the malignant cells the mice were randomly divided in the corresponding treatment groups, consisting of at least 6 animals: untreated controls, cisplatin treated - 5×0.4 mg/kg i.p.; 5×0.9 mg/kg i.p.; 5×1 mg/kg i.p. in 5 consecutive days, BAP treated – 5×1 mg/kg i.p.; 5×1.8 mg/kg i.p.5×3 mg/kg i.p.; 5×6 mg/kg i.p., in 5 consecutive days. The treatment started on the 24th h after the tumor implantation. For all groups the median survival time (MST) was determined and the T/C (%) criterion was used as merit for antineoplastic efficacy as follows: T/C<125 – inactive; T/C= 125 ÷ 150 – low activity; T/C > 150 – high activity [15].

Lewis Lung Murine Carcinoma

The tumor line was a generous gift from Professor Dimiter Todorov, from the Lab. of Oncopharmacology at the National Oncological Centre, Bulgaria. It was propagated *via* serial biweekly passages of LL tumor homogenate (1 g wet tumor tissue in 10 ml PBS), inoculated s.c. in C57BL/6 mice. The tumor propagation for the chemotherapeutic investigation was carried out in BDF1 mice following s.c. inoculation of 0.5 ml tumor homogenate in the left dorsal area. Following the tumor homogenate inoculums the animals were randomly divided in 5 experimental groups (n=10), as follows: untreated controls, cisplatin treated at doses 3×2.5 mg/kg i.p.; 3×5 mg/kg, i.p.; BAP treated at doses 3×5 mg/kg, i.p.; 3×10 mg/kg, i.p. Dosing with both cisplatin and BAP was carried out on the 1st, 5th and 9th days post implantation. The MST were determined for all groups investigated and the activity was expressed as T/C (%) values, utilizing the same criteria for efficacy as indicated above. Additionally the mean tumor diameters were determined on the 6th, 10th and 14th days post implantation *via* caliper measurements and the mean tumor volume was quantified using the formula ($V_t = (\pi/6)\times d^3$). The tumor growth inhibition (TGI (%)) was calculated as follows: TGI (%) = ((V_t*control* - V_t *treated)/* V_t *control). 100.*

Nephrotoxicity Determination

The determination of the nephrotoxic effects of BAP and cisplatin was carried out within the L1210 treatment experiments. Blood samples were taken on the 24th h after the completion of the treatment schedule and the serum levels of urea and creatinine, as markers of kidney function/damage were determined using commercially available standard kits.

Morphological Investigations

After complete treatment (L-1210 protocol) some leukemic mice were sacrificed and their kidneys removed. Thin slices thereof were fixed *via* immersion in buffered 10% formalin, dehydrated and embedded in paraffin. 3 μm slices were stained with hematoxilin and eosine and analyzed for signs of swelling, desquamation , necrosis, cytoplasmic vacuolization.

Blood Counts

Healthy ICR mice were treated with BAP (course doses 15 and 30 mg/kg) and cisplatin (course dose 5 mg/kg) as described in the L1210 experiment. The total leukocyte, lymphocyte and neutrophile counts were determined 48 h after the complete 5-day treatment course and the results were expressed as percentage of the initial (pre-treatment) values.

Cytogenetical Analysis for Chromosomal Aberrations

The cytogenetical analysis was carried out as previously described [16,17]. Healthy C57BL/6 mice were used in this study, whereby every experimental group consisted of 8-10 animals from both sexes (5♀ and 5♂). The animals were exposed to equimolar doses of cisplatin (4.5 mg/kg) and BAP (7.5 mg/kg); mitomycin C (3.5 mg/kg) was used as positive control. Following the corresponding exposure period (6,12 or 24 h post injection) the animals were treated with colchicine at a dose of 40 mg/kg, i.p. One hour later the mice were euthanized, the femurs were dissected and bone marrow was flushed therefrom and hypotonized with 0.075 M KCl for 15-18 min at 37ºC. Thereafter the extracted bone marrow was turned into single cell suspension by means of vigorous repetitive trituration through a Pasteur pipette. The cells were fixed in methanol:acetic acid (3:1) dropped on chilled slides and air-dried. The slides were stained using 5% Giemsa stain solution and were examined *via* light microscopy, without cover glass, whereby the immersion oil was poured directly onto the slides. At least 50 well spread metaphase plates were examined per animal at random. The slides were assayed for abnormalities in chromosomal structure and for determination of the frequency of chromosomal aberrations. The different types of chromosomal aberrations (chromatide and isochromatide breakage, fragmentations and exchanges were identified and scored.

The results from the cytogenetical analysis were statistically evaluated using a 3-step variant analysis (ANOVA), followed by paired Student's t-test and post hoc Dunnet test, using BMD P4V, BMD P3D and BMD P7D software [18].

Inhibition of DNA Synthesis in L1210 Leukemic Cells (*In Vivo*)

15 BDF1 mice were implanted i.p. with 10^5 viable L1210 cells and after 24h were randomly separated in three groups: solvent treated controls (0.9% NaCl, i.p.); BAP treated (at course dose of 7.5 mg/kg); cisplatin treated at course dose 4.5 mg/kg, i.p., whereby the doses of the platinum complexes are equimolar. Two h before the end of the exposure period mice were injected with methyl-^{3}H-thymidine (NEN products, Boston, MA, USA) (specific activity 20mCi/mmol) at a dose of 2μCi/g i.p. After the completion of the exposure period the animals were sacrificed and ascitic fluid was extracted thereof Smears of the ascitic fluid were prepared and covered with liquid nuclear emulsion (Ilford K2, England) and exposed 14 days at 4°C. The autoradiographs were

stained with 5% Giemsa solution. and examined on an Reichet microscope (Austria). To estimate the percentage of labeled cells at least 1000 leukemic cells were counted per animal.

Data Processing and Statistics

The experimental data were processed using Origin Plot, Microsoft EXCEL and GraphPad Prizm Software for PC. The actual statistical test utilized is denoted at the corresponding table of results.

RESULTS

Solution Chemistry

For cisplatin a λ_M value of 11.13 μS/cm was encountered, whereas for BAP the λ_M value was 11.41 μS/cm. According to the relevant literature data, the dinuclear platinum(II) complex behaves like a non-electrolyte [19].

Time-dependent Cytotoxicity of BAP and Cisplatin in K-562 Cells

K-562 cells were exposed to BAP or cisplatin for 24 or 48 h and their viability was thereafter assessed by MTT-assay. The results from the chemosensitivity testing, together with data regarding 72 h exposure from our previous paper [11] are summarized in Table **1**. As evident from the data presented, the treatment duration had a substantial impact onto the chemosensitivity to cisplatin, whereby the reduced exposure was consistent with a strong increase in the IC_{50} values. In case of BAP the shortened treatment duration has only minor influence onto the encountered effect of the compound.

Table 1. Cytotoxic Activity of BAP and Cisplatin on K-562 Cells After 24, 48 or 72h Exposure (MTT-Assay)

Exposure duration (h)	IC_{50}(μM)[a]	
	BAP	Cisplatin
24	72	132
48	50.1	75.2
72[b]	37.9[b]	36.9[b]

[a]means from at least 6 independent experiments (in triplicate)

[b]data from preceding paper [11]

Cellular Pharmacokinetics in K-562

The results from the platinum accumulation determination in K-562 cells following exposure to either BAP or cisplatin at equimetal concentrations (50 μM platinum) are summarized in Table **2**. BAP treatment for 30 minutes is associated with ca. 2 times lower accumulation of platinum as compared to cisplatin treatment. Interestingly after prolonged exposure over 120 min the platinum levels in the cisplatin-treated cells were almost 4 times higher than those achieved after BAP treatment.

DNA Fragmentation Analysis

The effects of BAP and cisplatin treatment on the DNA fragmentation in K-562 are shown in Fig. (**2**). At both concentration levels BAP caused far more pronounced enrichment of K-562 cytosole with histone-associated oligonucleosomal DNA fragments, relative to cisplatin.

Table 2. Kinetics of Pt Cellular Accumulation in K-562 Cells Following Exposure to Equimetal Oncentations of BAP or Cisplatin, Corresponding to 50 μM Platinum (EAAS, n=3)

Treatment	Intracellular platinum levels (ng/1.10^6 cells)	
	30 min exposure	120 min exposure
BAP (25 μM)	6.5**	7.7**
Cisplatin (50 μM)	11.4	28.4

**p≤0,001 vs. cisplatin (t-test)

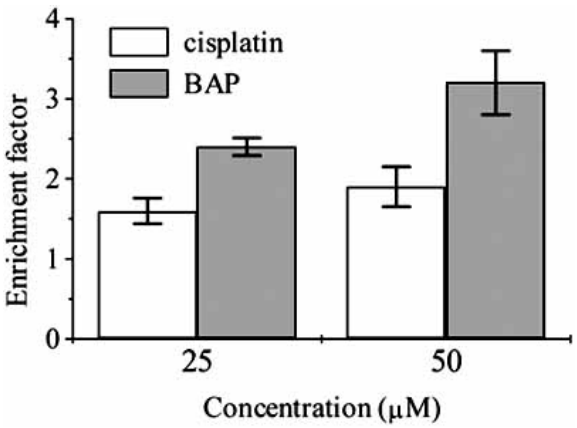


Fig. (2). Oligonucleosomal fragmentation of genomic DNA following 24 h exposure to either BAP or cisplatin (Cell Death Detection Elisa ™ Roche Diagnostics).

Antineoplastic Activity *In Vivo*

Table **3** represents the effects of tested compounds upon the survival of L1210-bearing mice. Both cisplatin and BAP

Table 3. Effects of BAP and Cisplatin on the Survival of L1210 Leukemia-Bearing Mice

Treatment group	MST	T/C (%)
Untreated control	9.0	-
BAP (5 × 1 mg/kg i.p.)	11.2	124.44
BAP (5 × 1.8 mg/kg i.p.)	14.44*	160.5
BAP (5 × 3 mg/kg i.p.)	12.0*	133.33
BAP (5 × 6 mg/kg i.p.)	10.8	120.00
Cisplatin (5 × 0.4 mg/kg i.p.)	11.16	124
Cisplatin (5 × 0.9 mg/kg i.p.)	11.34	126
Cisplatin (5 × 1 mg/kg i.p.)	14.2*	157.78

*p≤0.05 vs. the untreated control (Survival curve comparison; log rank test)

exerted anileukemic effects in this classic preclinical tumor model. The referent drug caused significant increase of the life span of leucemic mice (by ca. 58%) only at course dose of 5 mg/kg. The dinuclear platinum complex exhibited potent inhibitory effects as well causing 60% increase of the life span of leukemic mice at 9 mg/kg and 33% 15 mg/kg.

The growth kinetics of s.c. transplanted Lewis lung carcinoma, in control or treated BDF_1 mice is depicted in Fig. (**3**). Prominent inhibitory effect upon tumor propagation was established with all therapeutic regimens investigated,

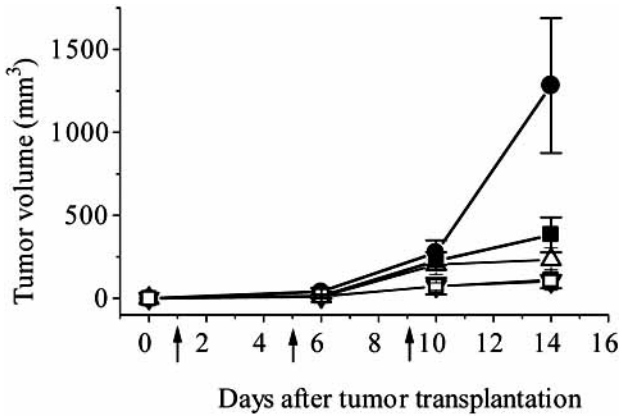


Fig. (3). Inhibition of Lewis lung carcinoma growth after treatment with BAP or cisplatin. BDF1 mice were injected with LL tumor homogenate (s.c.) and were treated thrice with either BAP (5 mg/kg (Δ); 10 mg/kg (▼)) or cisplatin (2.5 mg/kg (■); 5 mg/kg (□)), *vs*. the untreated control group (●). The arrows indicate the days when drug dosing took place. The tumor volume values represent the mean ± sd (n=10).

whereby optimal TGI was encountered with 15 mg/kg cisplatin and 30 mg/kg BAP at the final examination of tumor dimensions (on the 14th day.post implantation; Table **4**). The survival of cisplatin-treated animals, however did not parallel its potent inhibitory effects on tumor growth – in both dose levels only moderate prolongation of life span was established. Maximal survival of LL carcinoma bearing animals was documented in the group treated with 30 mg/kg BAP. It is noteworthy however that dislike cisplatin, in both BAP-based regimens no early deaths were established (survival curves not shown) and moreover there were long term survivors, sacrificed at the end of the observation period (65 day post transplantation) and excluded form the calculation of the corresponding MST values.

Inhibition of DNA Synthesis in L1210 Leukemic Cells

Both platinum complexes, led to significant inhibition of the DNA synthesis in L1210 cells, assessed 96 h after the complete treatment of leukemia bearing mice applied at equimolar doses (Table **5**). Thus cisplatin (4.5 mg/kg) caused ca. 38% inhibition of the ^{3}H-thymidin incorporation in L1210 cells. The inhibitory activity of the dinuclear platinum complex BAP (7.5 mg/kg) was even more pronounced - by 49 %, relative to the control group.

Table 5. Effects of BAP and Cisplatin Treatment on the ^{3}H-Thymidin Incorporation into L1210 Cells (*In Vivo*)

Treatment group	^{3}H-thymidin incorporation (%)[a]
Untreated control	26.4 ± 2.53
BAP (7.5 mg/kg i.p.)	13.6 ± 2.03*
Cisplatin (4.5 mg/kg i.p.)	16.3 ± 1.5*

[a]means ± sd;*p≤0.05 *vs*. the untreated control (t-test)

Nephrotoxicity Assessment

The results from the serum creatinine and urea measurements are shown in Table **6**. Cisplatin treatment is associated with a significant 8.9 fold increase in the serum urea values and 7.5 fold increase of the serum creatinine values relative to the untreated leukemic control. In mice treated with all of the BAP-based regimens the serum urea concentrations did not differ significantly from the untreated control, whereas the minor increase encountered in the creatinine levels reached statistical significance only after the course dose of 15 mg/kg.

The histological evaluation of the kidneys following 5 days consecutive treatment with cisplatin demonstrated prominent necrobiotic changes in the tubular epithelium, together with presence of protein materials within the lumen

Table 4. *In Vivo* Antineoplastic Activity of BAP and Cisplatin Against s.c. Lewis Lung Carcinoma in BDF_1 Mice

Treatment group (cumulative doses, i.p.)	MST	T/C (%)	TGI (%)	
			10 days	14 days
Untreated control	18.5	-	-	-
BAP 15 mg/kg i.p.	22.5*	121.6	35.6	81.8
BAP 30 mg/kg i.p.	28*	151.3	73.6	92.1
Cisplatin 7.5 mg/kg i.p.	23*	124.3	22.5	71.17
Cisplatin 15 mg/kg i.p.	24*	129.7	74.05	91.3

*p≤0.05 vs. the untreated control (Survival curve comparison; log rank test)

Table 6. Serum Urea and Creatinine Levels Determined 24 h After the Last Dosing with Cisplatin or BAP in L1210 Leukemic BDF1 Mice (Means of 4 Independent Experiments)

Experimental group	Dose regimen	serum urea (mmol/l)	serum creatinine (μmol/l)
L1210 control	-	7.9	42
healthy BDF_1 mice	-	9.3	41
BAP	5 × 1 mg/kg i.p.	8.3	48
BAP	5 × 3 mg/kg i.p.	8.3	56*
BAP	5 × 6 mg/kg i.p.	9.1	46
cisplatin	5 × 1 mg/kg i.p.	70.6**	315**

*p≤0.05; **p≤0.01 vs. the untreated leukemic control group (t-test)

of the tubules (Fig. (**4b**)). These data well correlate with the significantly elevated levels of serum urea and creatinine encountered in the same treatment group.

In the BAP-treated mice only after the course dose of 30 mg/kg there were some detectable changes in the histopathological findings, relative to the control (Fig **4c**). Thus there are some mild necrobiotic changes in the tubules together with single enlarged glomeruli, but far less prominent as compared to the analogous changes encountered after cisplatin treatment.

Myelotoxicity

In order to assess the myelotoxic potential of BAP in comparison to cisplatin the L1210 treatment regimen was simulated in healthy ICR mice. The white blood cell nadir was estimated 24 h after the complete 5-day treatment of mice (Table **7**). The results demonstrate that cisplatin has only moderate inhibiting activity of bone marrow, causing ca. 15% decrease of the leukocyte counts relative to the pretreatment values. The lymphocytes were decreased by 27 % and neutrophiles by 28 %. The dinuclear platinum complex

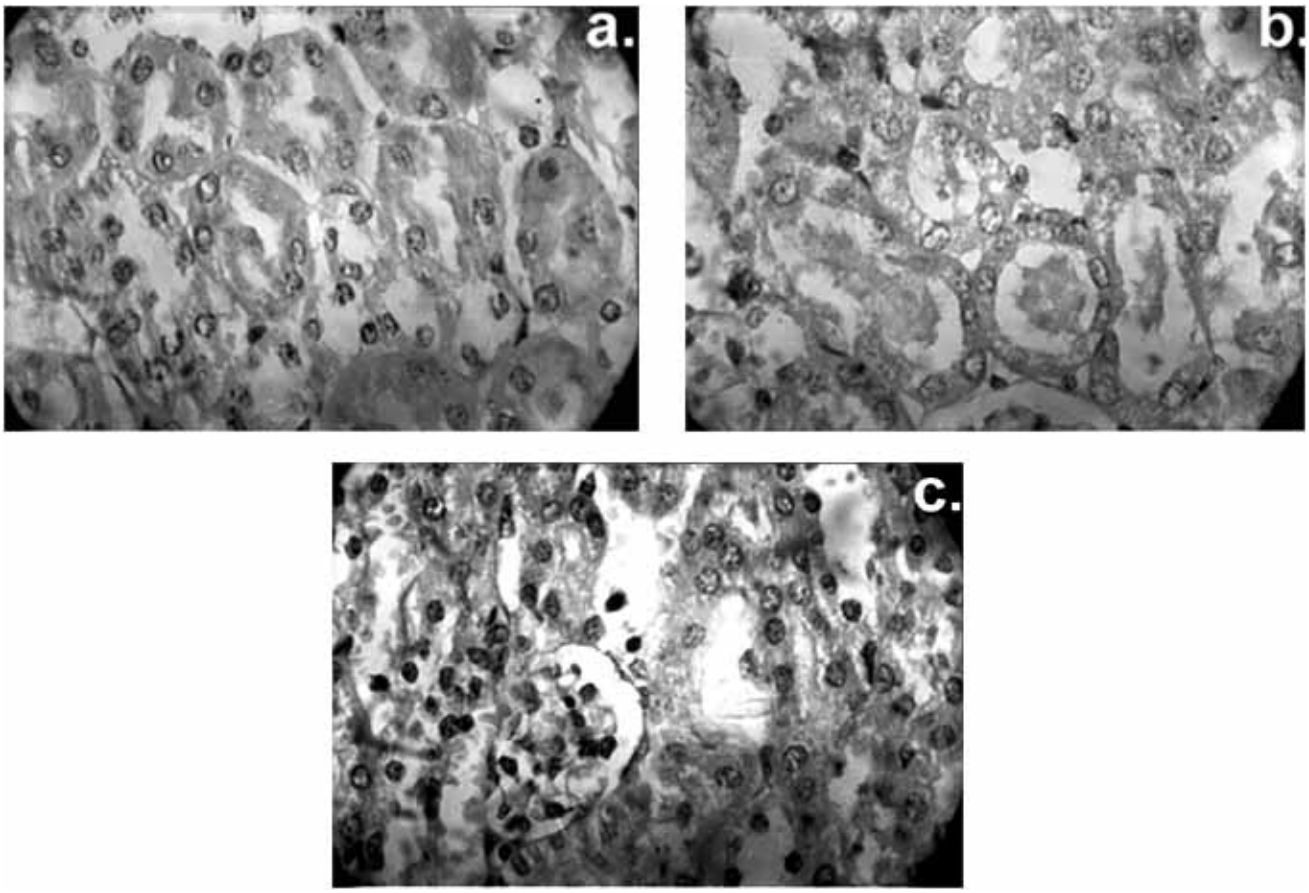


Fig. (4). Histological investigation of kidney slides, following 5 days of consecutive treatment with 5×1 mg/kg cisplatin (b), 5×6 mg/kg BAP (c), *vs*. the untreated control group (a).

Table 7. Leukocyte Blood Counts 24 h After the Complete 5 d Treatment with Cisplatin or BAP in Healthy ICR Mice (Means of 4 Independent Experiments)

Experimental group	% cell number *vs.* the pre-treatment level (n=6)		
	Leukocytes	Lymphocytes	Neutrophils
BAP (15 mg/kg)	98.6	96.4	103.6
BAP (30 mg/kg)	99.3	82.2*	85.0*
cisplatin (5 mg/kg)	84.7*	73.0*	71.9*

*p≤0.05 vs. the initial values (Student's t-test)

injected at 3 or 6 times higher total doses exerted even less pronounced myelotoxic effects – the total number of leukocytes was practically unaffected at both doses. The decrease of lymphocyte and neutriophile counts reached statistical significance only at the higher dose, but invariably the effects were less pronounced than those of cisplatin.

Clastogenic Activity

Table **8** presents the frequencies of metaphase chromosomal abnormalities in C57BL/6 mice exposed to equimolar doses of cisplatin or BAP. Cisplatin treatment led to a significant increase in the frequency of aberrant mitoses relative to the control group by factors of 5.8 (6 h post injection) 9 (12 h) and 14.3 (24 h). The established abnormalities are of the chromatide type e.g. breaks, fragments and exchanges, the former being the predominant lesions encountered. The exchanges are almost exclusively of the c/c type (with only one t/t exchange encountered at 6 h exposure). The dinuclear platinum complex under investigation exerted far less pronounced clastogenic activity, whereby there was only minor increase in the frequency of aberrant mitoses, relative to the control group. Even at the longest exposure period the frequency of aberrant metaphases was increased only 4-fold. Interestingly the spectrum of aberration also differed drastically from those induced by cisplatin – there were no fragments after BAP treatment for 6-12 h, and at the 24 h the encountered breaks were ca. 10-fold less frequent as compared to cisplatin. The frequency of chromosomal breaks was 3 fold lower at the 12 h and the c/c exchanges were the major type of established aberrations. In the BAP treated group there were t/t exchanges encountered as well. I t is noteworthy that there were no destroyed metaphases after BAP treatment.

DISCUSSION

The cellular pharmacological profile of BAP was evaluated in depth in the chronic myeloid leukemia cell line K-562. It was chosen as a model system, due to its constitutive

Table 8. Low Clastogenic Activity of BAP as Compared to Cisplatin. C57BL/6 Mice were Treated with Tested Compounds for 6, 12 or 24 h and Bone Marrow Cells were Extracted and Scored for Chromosomal Aberrations (The Referent Clastogen Mitomycin C was Used as Positive Control)

Experimental groups	Dose (mg/kg)	Interval (h)	Number of metaphases scored	Type of chromosomal aberrations						Percentage of aberrant metaphases	Destroyed metaphase plates
				Breaks	Fragments	Exchanges			Rings		
						c/c	c/t	t/t			
Control	-	-	600	-	1	12	-	-	1	2.17 ± 0.28	-
Mitomycin C	3.5	6	400	15	23	21	-	-	-	13.25 ± 2.38**	-
Mitomycin C	3.5	12	400	17	24	20	-	-	-	15.25 ± 0.81**	-
Mitomycin C	3.5	24	400	18	46	25	-	4	-	23.25 ± 0.95**	3
Cisplatin	4.5	6	600	30	23	22	-	1	-	12.66 ± 1.55**	5
Cisplatin	4.5	12	500	59	35	12	-	-	-	19.27 ± 2.37**	1
Cisplatin	4.5	24	450	44	78	17	-	-	1	31.11 ± 4.01**	33
BAP	7.5	6	450	4	-	16	-	-	-	4.44 ± 2.78++	-
BAP	7.5	12	450	16	-	13	-	2	-	6.88 ± 4.13*++	-
BAP	7.5	24	500	11	8	20	-	2	-	8.40 ± 2.63 *++	-

c/c centromer-centromeric fusions; t/t-telomer-telomeric fusions; c/t-centromer-telomeric fusions; *P≤0.05, **P≤0.01 Dunnet control group comparison test versus the untreated control group; ++P≤0.01 Dunnet control group comparison test versus cisplatin treated group;

expression of the non-receptor tyrosine kinase BCR-ABL, which endows cells with intrinsic low responsiveness to pro-apoptotic stimuli and chemotherapeutics in particular [20-22]. We have previously shown that prolonged 72 h treatment with BAP and cisplatin inhibits K-562 proliferation with practically identical IC_{50} values [11]. Hereby we demonstrate that the decrease of the exposure period is crucial for the efficacy of cisplatin, whereas BAP attains considerable cytotoxicity even after 24 h exposure. These data were paralleled *via* the quantitative assessment of the ability of tested agents to trigger apoptosis in K-562 after 24 h exposure, whereby invariably of the concentration levels BAP was more active than cisplatin.

Intriguingly, despite its superior activity in respect to both cytotoxicity and apoptosis induction BAP was found to accumulate far less efficiently in K-562, relative to cisplatin, which could be ascribed to its higher molecular mass and profound water solubility. These data clearly indicate its cisplatin-dissimilar profile of cellular effects and imply that most probably far less efficient DNA-platination by BAP is sufficient to recruit the cell death signaling pathways. Conversely the third generation platinum compound oxaliplatin has been found to be at least as effective as cisplatin cytotoxic agent, despite inducing less DNA platination in treated cells or in cell free systems [23]. The observed discrepancies between the cellular intake of BAP and its cytotoxic activity need further clarification. They could be due to sequence- or gene- specific platination causing crucial DNA-abnormalities or formation of cisplatin dissimilar-lesions with low susceptibility to the DNA-repair machinery - features well established for diverse di- and polynuclear species and other Pt complexes lacking cross-resistance to cisplatin [7,24-25].

BAP demonstrates significant antileukemic activity in L1210 leukemia – the classical screening model at the National Cancer Institute before the introduction of the more convenient and cost-effective *in vitro* cell line screen [15]. In this test system BAP reaches the efficacy of cisplatin and even higher T/C value was established at one of the dose levels. The mechanistic elucidation of the antileukemic effects demonstrate that the exposure of the implanted L1210 cells to equimolar doses of cisplatin and BAP is consistent with equal, near 50 % inhibition of DNA synthesis therein.

The profound efficacy of BAP (at 30 mg/kg total dose) in s.c. Lewis lung carcinoma is even more surprising since this is the murine allograft tumor model, which is the most refractory to chemotherapy, among these used at the NCI [15]. In our hands cisplatin, although causing comparable tumor growth inhibition proves to be far less effective in prolonging the life span of tumor bearing mice relative to the higher dose of BAP. Furthermore the presence of long term survivors in BAP-treated groups, but not in the cisplatin injected is additional implication for its superior activity in this tumor model. At the same time, these animals are excluded from the MST calculation, and conversely do not contribute to the T/C values obtained.

The toxicological studies firmly point out that BAP is endowed *via* low nephrotoxicity in compliance with the drug-design strategy. This feature could be ascribed to its high water solubility and the higher stability of the acetate ligands. Conversely the replacement of the chlorine ligands in cisplatin with the less reactive organic leaving groups is a well-established approach towards decrease of nephrotoxicity, e.g. in carboplatin and nedaplatin [1,4,5]. It is noteworthy that in both compounds the activity against malignant cells is lowered, as they require higher concentrations and exposure periods for equivalent log kill, relative to cisplatin [4,5]. Additional shortcoming of the second generation platinum drugs is their dose-limiting myelotoxicity in contrast to cisplatin, which has only moderate myelosuppressive potential [1]. In a dissimilar fashion in BAP the encountered low nephrotoxicity is accompanied with low myelosuppression, while attaining profound cytotoxic/antineoplastic efficacy in line with our previously described paper addressing the *in vitro* toxicological potential of BAP upon cultured proximal tubule epithelium, neurones and bone marrow cells [12].

Since the alkylating agents and the platinum complexes are *ab initio* genotoxic, the assessment of their ability to induce genetic/chromosomal abnormalities is an integral and inevitable part of their preclinical development [26]. The cytogenetical analysis demonstrates profound discrepancies between the clastogenicity of BAP and the referent compound cisplatin, the latter being a well established mutagen, teratogen and carcinogen [27,28]. The low clastogenicity of BAP and especially its low propensity of inducing DNA breaks imply that it has far less pronounced genotoxic potential than cisplatin - an additional virtue, demonstrating further its less pronounced deleterious effects upon non-malignant cells.

Finally, the observed cisplatin-dissimilar cellular pharmacology profile, profound antineoplastic activity and low toxicological potential of BAP favor its candidacy as potential antineoplastic agents and warrant further more detailed evaluation of this dinuclear platinum compound.

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ABBREVIATIONS

BAP	=	Bis(acetato)diammine-bis-μ-acetato diplatinum (II) dihydrate
c/c	=	Centromer-centromeric fusions
c/t	=	Centromer-telomeric fusions
EASS	=	Electrothermal atomic absorption spectrometry
EF	=	Enrichment factor
FBS	=	Fetal bovine serum
MST	=	Median survival time
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
t/t	=	Telomer-telomeric fusions
TGI	=	Tumor growth inhibition

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