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Investigations on Cytotoxic and Antiproliferative Effects *In Vitro* of a Newly Synthesized Mixed Ligand Copper (II) Complex

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The aim of the study was to evaluate cytotoxic and antiproliferative activities *in vitro* of a newly synthesized mixed ligand Cu(II) complex Cu_2(BAMP)(dipy)Cl_4. The permanent cell line LSCC-SF(Mc29), established from a transplantable chicken hepatoma induced by the myelocytomatosis virus Mc29, was used in the experiments. The effects of the compound on cell viability and proliferation were studied by neutral red uptake cytotoxicity test (NR), trypan blue dye exclusion technique (TB), colony-forming assay and single cell gel electrophoresis. It was found that Cu_2(BAMP)(dipy)Cl_4 expressed significant antitumour properties *in vitro*. Thus, applied at concentrations ≥ 10 μg/ml the compound inhibited completely colony-forming ability of chicken hepatoma cells in semisolid medium. The IC_{50} (concentrations that reduced cell viability/proliferation by 50%) established by NR and TB were found to be in the range 1 — 10 μg/ml. The ability of Cu_2(BAMP)(dipy)Cl_4 to induce DNA damages in the treated cells was also observed.

Key words: copper, Mannich bases, pyrazolone, cytotoxic/antiproliferative activity, tumour cells.

Introduction

The potential antineoplastic activitiy of different metals and metal compounds have been under special interest during the recent years because of the following main reasons: 1) It has been found that the disturbed balance in the essential metal metabolism of mammals results in increased susceptibility to infections and malignancies; 2) Being involved in the regulation of some definite processes of the animal organisms several metals are biological response modifiers [6, 19]. Copper is an essential element involved in many biochemical reactions supporting life. It is a part of the active site of at least 60 enzymes and takes part in many key biological processes including cellular respiration, DNA and RNA replication, maintenance of cell membrane integrity, and sequestration of free radicals. This metal is also important for the normal functioning of the immune system [3, 12, 18]. Different copper containing compounds have been reported to express antineoplastic prop-

erties *in vitro* and *in vivo* [4, 8, 11, 14, 15, 16]. It was found in our previous investigations that some Cu(I, II), Co(II), Fe(II, III) and Ni(II) complexes with Mannich type ligands - N,N′-bis(4-antipyrylmethyl)-piperazine (BAMP) and N,N′-tetra-(antipyryl-1-methyl)-1,2-diaminoethane (TAMEN), exhibited cytotoxic and antiproliferative effects on several human and animal tumour cell lines [7, 9, 10]. In order to continue the investigations in this field the aim of the study presented here was to evaluate the antitumour activity *in vitro* a newly synthesized mixed ligand copper (II) complex containing the above-mentioned Mannich base BAMP as well as 2,2-dipyridyl as a coligand.

Materials and Methods

Compounds. The copper (II) complex Cu_2(BAMP)(dipy)Cl_4 was obtained by direct synthesis between $CuCl_2.2H_2O$, 2,2-dipyridyl and the Mannich base N,N′-bis(4-antipyrylmethyl)-piperazine (BAMP) in ethanol. The green microcrystalline product was filtered off, washed with ethanol and dried over $CaCl_2$ in air. Yield: 48 %. Analytical data were obtained by a Perkin-Elmer Model 240C elemental analyzer. Electrical conductivities were determined on a WTW LF-340. Infrared spectra of the solid complex (KBr pellet) were recorded on an IR BIO-RAD FTS 135 spectrometer. Magnetic measurements were carried out on polycrystalline samples with a Faraday-type magnetometer.

For biological experiments, the compound was dissolved in dimethylsulfoxide (DMSO, Serva) and then diluted in culture medium. The final concentration of DMSO in the stock solution of Cu_2(BAMP)(dipy)Cl_4 (10 mg/ml) is 10%. The stock solutions were stored at 4°C and used no more than two weeks after their preparation.

Cell lines and cultivation. The permanent cell line LSCC-SF(Mc29), established from a transplantable chicken hepatoma induced by the myelocytomatosis virus Mc29, was used in the experiments [2]. Cells were grown as monolayer cultures in a combination of medium H-199 and Minimum Essential medium (AppliChem, Germany), supplemented with 5-10% fetal bovine serum (Cambrex, Belgium), 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures were maintained at 37°C in a humidified CO_2 incubator.

Neutral red uptake cytotoxicity assay. The cells were seeded in 96-well plates (Cellstar) at a concentration of 2×10^4 cells/well. At the 24th h cells from monolayers were washed and covered with media modified with different concentrations of the compound tested (each concentration in 6 to 8 repetitions). Samples of cells grown in non-modified medium served as a control. After 24 h and 48 h incubation periods, each plate was examined under inverted microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Alterations in monolayer morphology were registered by an adapted to an inverted microscope digital camera and a computer program EDS-1000E IS.

Neutral red uptake cytotoxicity assay was performed as described by B o r e n f r e u n d and P u e r n e r [13]. Relative cell viability, expressed as a percentage of the untreated control, was calculated for each concentration.

Trypan blue dye exclusion test. The cells were cultured and treated with the complex investigated as described for neutral red uptake cytotoxicity assay. After 24 h and 48 h incubation periods cells were tripsinized and counted after mixing (1:1/ vol:vol) with 0.2% solution of trypan blue (1:1/ vol:vol).

Single cell gel electrophoresis. The alkaline variant of Single cell gel electrophoresis ("Comet Assay"), modified by O l i v e et al. [17]. The comets in the gel were stained with 0.5 μg/ml ethidium bromide and visualized by a fluorescent microscope.

Colony-forming assay. Tumour cells (approximately 10^3 cells/well) were suspended in 0.45% purified agar (Difco) in medium containing different concentrations (ranging from 1 to 200 g/ml) of the mixed ligand copper (II) complex and layered in 24 well microplates (Cellstar). The presence/absence of colonies was registered using an inverted microscope during 20-day period.

Statistical analysis. The data are presented as mean ± standard error of the mean. Statistical differences between control and treated groups were assesses using one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test.

Results

Elemental analysis of the compound. Cu2C38H42N8O2Cl4 (911.71) Found: C 51.02; H 4.71; N 12.33; Cl 15350; Cu 13.82; requires C 50.06; H 4.64; N 12.29; Cl 15.55; Cu 13.94 %. *IR spectrum* (KBr cm^{-1}):— 1620 s (phenyl ring), 1584 s (C=C/C=N), 1520 m (dipy), 1158 w (C-O), 1130 w (C=C/C=N), 1126 s (C-N al) cm^{-1}. Far IR: 635 m (py), 609 w (M-O), 598 w (M-N al), 362 s (M-Cl), 315 s(M-Cl), cm^{-1}. *Magn. Mom.* 1.97 BM.

Spectric and magnetic properties. In the infrared spectra of the complex, bands belonging both to the pirazolonic and dipyridyl ligands can be noticed, some of them were modified as a result of coordination. Thus, the intense band at 1662 cm^{-1} assigned to the $\nu_{C=O}$ mode in the free ligand is missing in the spectrum of the complex and a weak peak in the 1158-1185 cm^{-1} region, attributable to the ν(C-O) appears. These denote the coordination of antipyrine ring through carbonylic oxygen. Additionally, the presence of ν(C=C/C=N) shows the stabilization of antipyrine. The shift of ν(C-N) toward lower frequencies proves the implication of piperazine in coordination.

The value of the magnetical moment is according to the octahedral geometry of the complex.

Neutral red uptake cytotoxicity assay. The data about the effect of $Cu_2(BAMP)(dipy)Cl_4$ on neutral red uptake in LSCC-SF(Mc29) cells are presented in Fig. 1 and Table 1. No uptake of the vital dye was detected when the complex was applied at concentrations of 100 μg/ml and 200 μg/ml for 24 h and 48 h. Administered at lower concentrations the compound was also found to reduce significantly cell viability as compared to the control. Thus, only 8.24% ± 0.69 ($p < 0.01$) and 60.03% ± 4.08 ($p < 0.01$) viable cells were found after 48 h treatment with 10 μg/ml and 1 μg/ml $Cu_2(BAMP)(dipy)Cl_4$, respectively. The IC_{50} (the concentrations producing 50% reduction of neutral red uptake) were calculated to be 3.1 μg/ml ± 0.3 (24 h) and 2.8 μg/ml ± 0.2 (48 h) (Table 1).

Table 1. Inhibitory concentrations (IC_{50}) of $Cu_2(BAMP)(dipy)Cl_4$ for LSCC-SF(Mc29) tumour cell line

Period of treatment	NR	TB	
		Total number of cells	Cell viability
24 h	3.1 ± 0.3	8.6 ± 0.6	5.8 ± 0.4
48 h	2.8 ± 0.2	5.1 ± 0.3	3.6 ± 0.2

NR – the concentrations (μg/ml) of the compound that reduced the neutral red uptake into LSCC-SF(Mc29) cells by 50% as compared to the control;
TB – the concentrations (μg/ml) of the compound established by the trypan blue dye exclusion technique that reduced the total number of cells/cell viability by 50% as compared to the control.

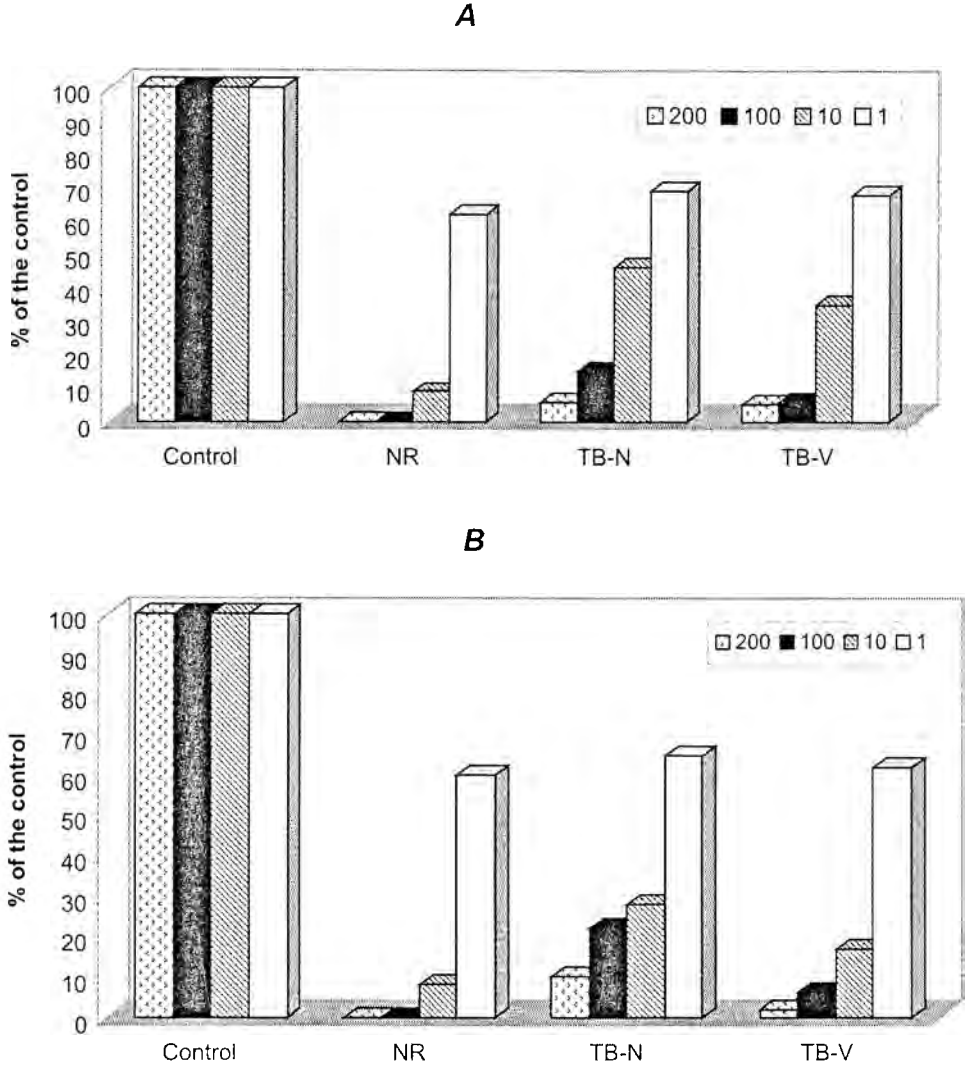

Fig 1. Changes in viability and proliferation of LSCC-SF(Mc29) chicken hepatoma cells treated for 24h (*A*) and 48h (*B*) with different concentrations (1, 10, 100, 200 μg/ml) of $Cu_2(BAMP)(dipy)Cl_4$: effects on neutral red uptake (**NR**) as well as influence on total cell number (**TB-N**) and cell viability (**TB-V**) established by trypan blue dye exclusion test. The figure represents data summarized from three independent experiments

Trypan blue dye exclusion test. The results obtained using trypan blue dye exclusion test are summarized in Fig. 1 and Table 1.

Cytopathological changes. An increase in number of the rounded-up cells, as well as formation of acellular zones were observed after 24 h and 48 h treatment of LSCC-SF(Mc29) cells with 1 or 10 μg/ml $Cu_2(BAMP)(dipy)Cl_4$ (Figs. 2, 3). Applied at higher concentrations (100, 200 μg/ml) the compound was found to induce complete cell destruction.

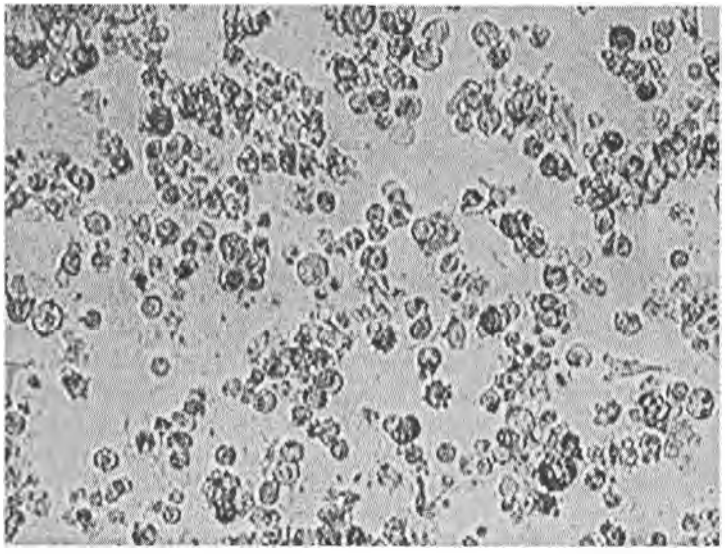

Fig. 2. Monolayer of LSCC-SF(Mc29) tumour cells treated for 24 h with 10 μg/ml $Cu_2(BAMP)(dipy)Cl_4$. Orig. × 6.3

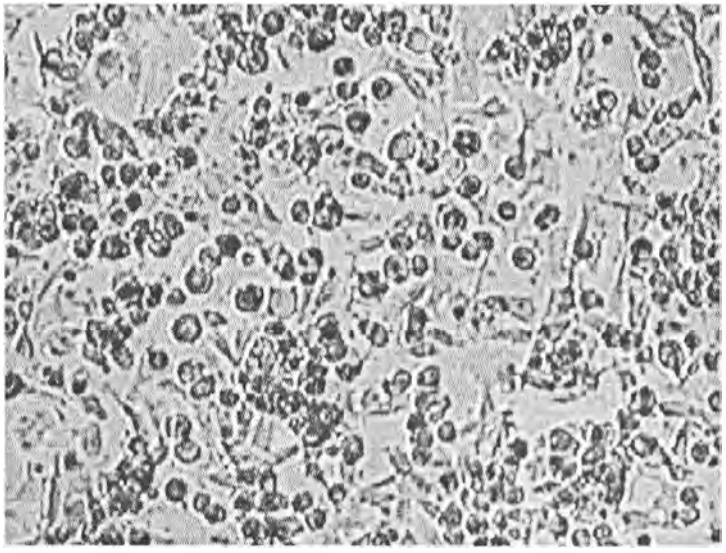

Fig. 3. Monolayer of nontreated LSCC-SF(Mc29) tumour cells. Orig × 6.3

Colony-forming assay. $Cu_2(BAMP)(dipy)Cl_4$ was shown to suppress completely the colony-forming ability of chicken hepatoma cells in semisolid medium when used at concentrations ≥ 10 μg/ml.

DNA damages. DNA damages were observed in about 65% of LSCC-SF(Mc29) tumour cells cultured for 48 h in the presence of 100 μg/ml $Cu_2(BAMP)(dipy)Cl_4$. The total number of the treated cells was found to be reduced by ≈ 84% as compared to the control.

Discussion

The need to find a safe and highly selective cure for neoplastic diseases remains a major challenge for modern science. The discovery of the antitumour efficacy of cisplatin and some related platinum complexes has stimulated the search for other metals with anticancer properties [6, 15]. In the literature there are data that different copper containing compounds possess antineoplastic activity *in vitro* and *in vivo*. Thus, some diphenylphosphinoethane-copper (I) complexes express significant cytotoxic properties against chinese hamster ovary (CHO) and human ovarian carcinoma (PA-1) cell lines as well as on cells obtained from human ovarian carcinomas [11]. Copper (II) complexes of thiosemicarbazones showed encouraging cytotoxic effect against HT-29 (human colon adenocarcinoma) cells [14]. The 2-furfural semicarbazone and thiosemicarbazone copper complexes demonstrate pronounced cytotoxic activity against human lung MB9812, colon SW480, ovary 1-A9 and uterine HeLa-S3 carcinomas [16].

It was found in our previous investigations that some copper (I, II) complexes with Mannich type ligands - N,N′-bis(4-antipyrylmethyl)-piperazine (BAMP) and N.N′-tetra-(antipyryl-1-methyl)-1,2-diaminoethane (TAMEN) exhibited antineoplastic effects *in vitro* on human (8-MG-BA) and animal (LSCC-SF-Mc29, LSR-SF-SR) tumour cell lines [7]. On the basis of their cytotoxic activity these copper (I, II) complexes were graded as follows: $Cu_2(BAMP)(NCS)_4 > Cu_2(BAMP)I_3 > Cu(TAMEN)(NO_3)_2$. In addition, it was also shown that applied at concentrations ranging from 1 to 200 μg/ml both ligands — BAMP and TAMEN, did not reduce significantly the viability and proliferation of tumour cells examined [10]. In order to continue the investigations in this field in the study presented here we report for the first time the data about synthesis, spectral and magnetic properties and antitumour potential *in vitro* of a mixed ligand copper (II) complex $Cu_2(BAMP)(dipy)Cl_4$, containing Mannich base BAMP and 2,2-dipyridyl as a coligand. The decision to start the investigations on biological activity of this compound namely on LSCC-SF(Mc29) chicken hepatoma cells was not occasional because this cell line was proved to be highly sensitive to the cytotoxic and cytostatic effects of different metal complexes [7, 8, 9, 10], alkaloids [1] and photosensitizers [5]. The results obtained revealed that $Cu_2(BAMP)(dipy)Cl_4$ expressed promising antitumour activity *in vitro*. This compound decreased significantly viability and proliferation of LSCC-SF(Mc29) cells in a time and concentration-dependent manner and induced DNA damages in the treated cells. $Cu_2(BAMP)(dipy)Cl_4$ was found to be much more effective than $Cu_2(BAMP)(NCS)_4$ and $Cu_2(BAMP)I_3$. For example, the IC_{50} (48 h) of $Cu_2(BAMP)(NCS)_4$ that reduced the neutral red uptake in chicken hepatoma cells was calculated to be 70 μg/ml ± 2.6 [7], whereas for $Cu_2(BAMP)(NCS)_4$ was 2.8 μg/ml 0.2. Copper complexes examined in the study presented here as well as in our previous experiments differ from each other in ligand (BAMP, TAMEN or BAMP + 2,2dipyridyl) and anion (NCS^-, I^-, NO_3^-, Cl^-). Each of these components (ligands and anions) as well as metal ions influences in different way physico-

chemical and biological properties of the complexes obtained which could explain the differences in their cytotoxic effects. A series of experiments with different copper complexes with Mannich type ligands are underway to study the relationship between the structure and physical and chemical properties of these compouns, and their biological activity. Additional investigations are also planned to clarify the potential antitumour properties of $Cu_2(BAMP)(NCS)_4$ on several tumour and nontumour human and animal cell lines as well as the mechanism(s) of action of this complex.

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