Antitumor Activity of New Copper Complexes on Ehrlich Ascites Carcinoma *In Vivo*

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Abstract. Despite certain progresses, cancer remains a major cause of death for both humans and companion animals, world wide. Chemotherapy is the treatment of choice for many cancer types, but it has important limitations because of lack of specificity and numerous side effects, therefore finding new cytostatic compounds represent a very active research field. Nowadays, a number of cooper complexes have shown an anticancer effect but the pharmacokinetics and mechanisms are not fully elucidated yet. In the present study we evaluated the anticancer potential of one new synthesized copper complex using a transplantable tumor model on laboratory mice. The tumor model of choice was Ehrlich Ascites Carcinoma inoculated intraperitoneally in female Swiss mice. The investigation was focused not only on antiproliferative parameters as body weight gain, volume and cytological characteristics of ascitic fluid, tumor cell concentration and viability but also on the assessment of side effect of tumor growth on general health status. The cooper complex showed an acceptable degree of toxicity in preliminary studies. It was responsible for an inhibition up to 80% of body weight gain, and prevents the accumulation of the ascitic fluid up to ten fold. The tumor cell concentration was consequently decreased by 18-fold, but the cooper complex did not seem to influence the cell viability. The hematological parameters and peritoneal cytology revealed also significant changes in response to therapy. The new synthesized cooper complex showed promising results; it was able to prevent the tumor growth and consequently it seems to improve the body health status. This represents a promising starting point for further studies dedicated to create new anticancer molecules. .

Keywords: Cooper complexes, anticancer properties, Ehrlich Ascites Carcinoma

INTRODUCTION

The synthesis and the chemical investigation of metallodrugs are of significant importance because their medicinal application. These applications include use in cancer therapy; copper (II) complexes being in focus for decades. The interest for copper complexes is justified because they have biologically accessible redox potentials and a significant affinity for the nuclear bases (Beckford et al, 2012). The concentration of copper levels are tightly regulated, although elevated copper levels are found in many cancer types. Copper seems to play a critical role in angiogenesis, which is a critical event in tumor biology (Buac, et al. 2012).

The cellular targets are not fully elucidated yet, but DNA molecule represents the most likely action site (Garcia-Gimenez et al, 2013). Despite numerous studies, the metal containing drugs are relatively rare, a platinum containing compound, cisplatin is one of the most effective antitumor drug. owever, cisplatin therapy is associated with severe side effects, which limit the clinical use (Lopez et al, 2013). Therefore, finding new potential metal based anticancer drugs to reduce toxicity is a very active research domain world wide (Zuo, et al, 2012).

MATERIALS AND METHODS

Synthesis of cooper complex: The synthesis occurs in the Department of Inorganic Chemistry, the molecule has the formula $Cu(L)_2(py)_2(H_2O)$, in which the ligand (HL) is (N-(5-(4-metilphenyl)-[1,3,4]-tiadiazol-2-il)-benzene sulfonamide). Briefly, the synthesis occurs in several steps. A solution of $CuSO_4 \cdot 5H_2O$ (4mmols) in 20 ml of pyridine : H_2O [v : v = 1 : 1] was added dropwise, under continuous stirring to a solution of HL ligand (1 mmol) dissolved in 25 ml pyridine : H_2O [v: v = 2 : 3]. The resulting solution was stirred at room temperature for one hour and left to stand at room temperature. After two months by the slow evaporation of the solvent, green-blue crystals suitable for X-ray diffraction were obtained. The crystals were purified by filtration, washing and drying several times, until they kept a constant mass.

The injectable form was obtained by dissolving the copper complex (CuL_2) crystals in a mixture of glycerol formal and 1,2- propanediol (Sigma-Aldrich) in rapport 2:3 (adapted accordingly the patent no. A01251/30.11.2010). The final concentration of CuL_2 was 100mg/ml. Before injection, the mixture was further diluted in sodium chloride 0.9% sterile solution, while the control groups were injected with excipient dissolved in sodium chloride only.

The animals were caged in groups of 8 per cage, at controlled temperature of 21-22ºC, humidity (40-60%) and reversed 12/12h light/dark cycle (light off at 10 a.m.). Standard lab chow, provided by National Institute for Research and Development "Cantacuzino" Bucharest (Batch no. 2 / 26.03.2010), and water were freely available. The animal tests and experiments were allowed by the Bioethical Board of the Faculty of Veterinary Medicine Cluj-Napoca.

The experiment was carried out on 24 white Swiss female mice, 35.65 ±0.54 g body weight. The animals were divided in six equal experimental groups. The first group was the Control receiving placebo therapy only and no tumor cells. The other two groups were inoculated with Ehrlich Ascitic Carcinoma (EAC); EAC groups received 10^6 ascitic cells/animal intra peritoneal, in the day 0, and the experiment spanned for 14 days. The second group received in addition to EAC a placebo therapy similar to those described for control, while the third group benefit from CuL2 treatment, i.p., prepared as described above, in the day 1 and 6 - 15 mg./kg bw. In the end, the blood was harvested from the retro orbitary sinus under deep narcosis, latter the euthanasia was made by prolonged narcosis. Total amount of ascitic fluid was measured, and viable tumor cell concentration was counted in a Burker camera (liquid diluted 1:100). Cell viability was assessed by tripan blue staining.

Blood hematology was investigated with Abacus Junior Vet, automatic analyzer Diatron Messtechnik, Budapest, Hungary.

Statistics - the data were expressed as the mean and standard error of the mean (SEM). T Student multiple range test was used to assess the differences between the two groups when appreciate the variation of ascitic fluid and cell concentration. Additionally one-way analysis of variance ANOVA, followed by post hoc Dunnett's range test procedure was done for pair-wise comparisons among the hematological parameters while two-way ANOVA followed by Bonferroni post test was the choice for variation of the body weight.

Statistical significance was at $p<0.05$ (95% confidence interval). Statistical values and figures were obtained using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA.

RESULTS AND DISCUSSIONS

Expectedly, the development of EAC induced anemia in tumor bearing mice (Tab.1). The therapy seems to provide no protective effect; the values were very similar those

untreated. The anemia was responsible for increased anysocytosis but the RBC indices remained within normal range (Tab.2).

The influence of copper complex therapy of EAC inoculated mice on the values
of the red blood cells (RBCs), hemoglobin (HGB) and hematocrit (HCT) (mean ± S.E.M)

	RBC 10^{12}/l	HGB g/l	HCT %
Control	8.00±0.47	128.83±7.95	38.58±1.98
EAC	5.39±0.53	87.8±9.56	26.79±2.73
EAC + Cu(L)$_2$	5.09±0.55	82.8±9.14	26.75±2.47

EAC – Ehrlich ascites carcinoma inoculated group, **EAC + Cu(L)$_2$** - Ehrlich ascites carcinoma inoculated group treated with copper complex Normal values: RBC 6.5-10.1 10^6/µl, Hb 101-161 g/l, Ht 22.8-48.0 % (Jain, 1993)

Tabel 2

The influence of copper complex therapy of EAC inoculated mice on the values of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red blood cells distribution width (RDW) (mean ± S.E.M)

	MCV fl	MCH pg	MCHC g/l	RDWs fl
Control	48.33±0.49	16.08±0.19	332.00±5.07	17.08±0.16
EAC	49.8±0.19	16.23±0.59	327.8±6.11	36.1±0.83
EAC + Cu(L)$_2$	53±1.30	16.28±0.33	307.6±7.50	39.06±2.68

EAC – Ehrlich ascites carcinoma inoculated group, EAC + Cu(L)$_2$ - Ehrlich ascites carcinoma inoculated group treated with copper complex Normal values: MCV 42.3-55.9 fl MCH 13.7-18.1 pg MCHC 29.5-35.1 g/dl (Jain, 1993)

Tabel 3

The influence of copper complex therapy of EAC inoculated mice on the values of total platelet count (PLT), platelet hematocrit (PCT), mean platelet volume (MPV), platelet distribution width (PDW) (mean ± S.E.M.)

	PLT 10^3cells/µl	PCT %	MPV fl	PDWs fl
Control	558.00±35.53	0.39±0.03	7.01±0.08	7.4±0.23
EAC	1309.8±222.35	0.882±0.16	6.66±0.21	6.94±0.40
EAC + Cu(L)$_2$	696.6±121.88*	0.476±0.08	6.72±0.12	6.9±0.21

EAC – Ehrlich ascites carcinoma inoculated group, **EAC + Cu(L)$_2$**
Ehrlich ascites carcinoma inoculated group treated with copper complex , *= $p<0.05$ as compared to EAC group, Normal values: PLT 780-1540 10^3cells/µl (Jain, 1993)

Tabel 4

The influence of copper complex therapy of EAC inoculated mice
on the WBC count and differential count (mean ± S.E.M.)

	WBC 10^9/l	LYM 10^9/l	MID 10^9/l	GRA 10^9/l
Control	6.85±0.74	5.08±0.62	0.18±0.02	1.59±0.17
EAC	71.94±6.29	13.19±2.15	6.74±1.70	51.99±5.30
EAC + Cu(L)$_2$	27.27±6.54**	5.88±1.14*	1.89±0.74*	19.48±5.18**

EAC – Ehrlich ascites carcinoma inoculated group, EAC + Cu(L)$_2$
Ehrlich ascites carcinoma inoculated group treated with copper complex
*= $p<0.05$ as compared to EAC group, **= $p<0.01$ as compared to EAC group

Normal values: WBC 2.61-10.05 10^3cells/µl, N 0.4-2.0 10^3cells/µl, L 1.27-8.44 10^9/l, M 0-0.29 10^3cells/µl, E 0-0.17 10^3cells/µl, B 0-0.02 10^3cells/µl (Jain, 1993)

The EAC inoculation was associated with significant systemic inflammatory syndrome reflected hematological by leukocytosis and increased platelets count.

The granulocytes were in a very high number (neutrophilia), and middle cells were also increased. The copper complex therapy reduces the platelets total count and prevents the leukocytosis throughout down regulation of granulocytes synthesis.

The EAC development was responsible for body weight gain, and remarkably, copper complex therapy provided a strong protective effect, the animals subject to therapy maintain the body mass almost unchanged during entire experiment long (Fig 1.).
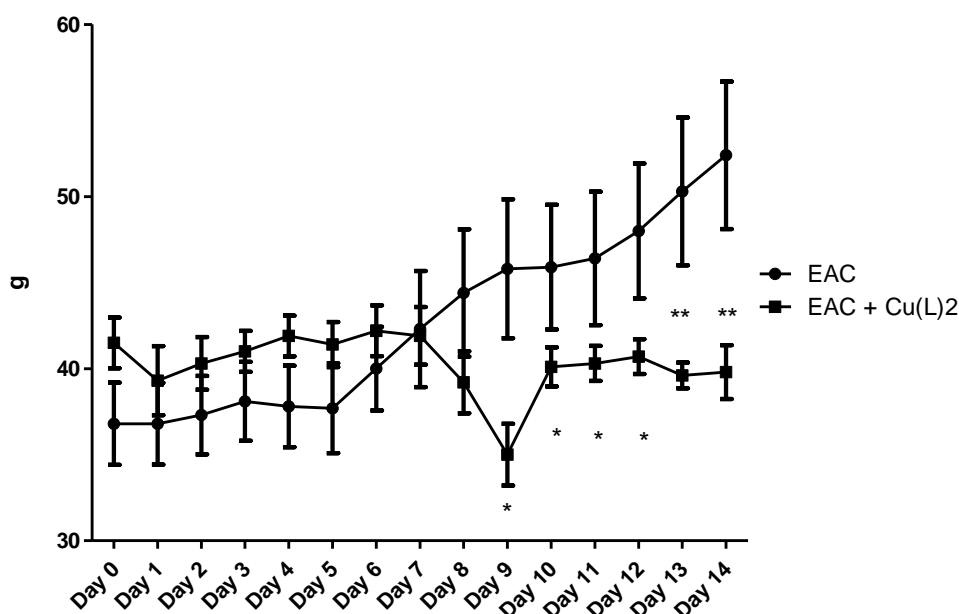

Fig. 1 The influence of copper complex therapy of EAC inoculated mice on the body weight gain (mean ± S.E.M).

EAC – Ehrlich ascites carcinoma inoculated group, EAC + Cu(L)$_2$
- Ehrlich ascites carcinoma inoculated group treated with copper complex ,
*= p<0.05 as compared to EAC group, **= p<0.01 as compared to EAC group

The ascitic volume followed the trend of the body weight variation, therefore the cooper complex treated group shown very few amount of ascitic fluid (Fig 2a).

The antiproliferative effect of copper complex was obvious also in the cellular concentration in the ascitic fluid (Fig.2b), but it has no influence on viable cells concentration (Fig.3c). The antitumor effects of copper complexes are intricate and not fully understood. Many copper complexes do not even penetrate the cell membrane and they require conjugation with specific peptides (Kanemaru et al. 2011). The most widely accepted target of copper complexes is genetic material, in support of this hypothesis; well-documented studies proved that the copper complexes are able to bind the DNA molecule (Beckford, et al. 2012). Furthermore, ternary copper complexes can interact with the DNA molecule and they induce the DNA cleavage in presence of UV light, by a photo-redox pathway with generation of hydroxyl radicals (Garcia-Gimenez et al. 2013). Other studies showed that neutral and cationic copper bis(thiosemicarbazone) complexes inhibit DNA synthesis (Palanimuthu, et al. 2013).

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The interaction with the DNA molecule leads to an antiproliferative effect, which was already proved on model on human cancer cells in vitro (Caco-2 cells) (Garcia-Gimenez *et al.* 2013), and in nude mice xenografts (HCT116 cells) (Palanimuthu, *et al*. 2013).
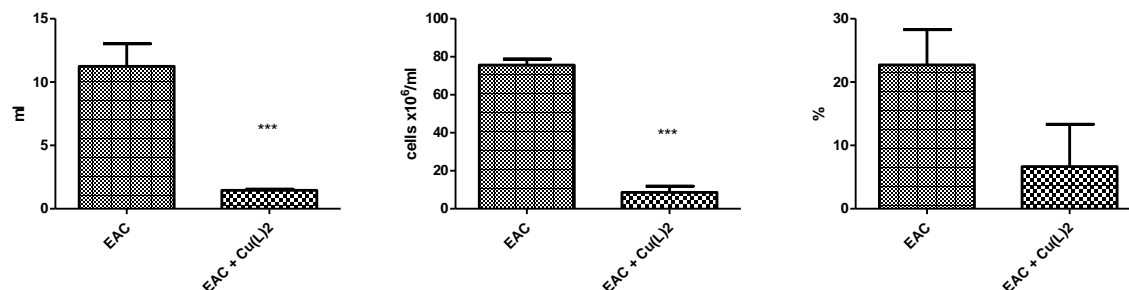

Fig. 2 The influence of copper complex therapy of EAC inoculated mice on the variation of **a)** the volume of the ascitic fluid (ml), **b)** the viable cellular concentration within ascitic fluid, (10^6/ml), **c)** the percentage of non viable cells (%)(mean ± S.E.M.)
EAC – Ehrlich ascites carcinoma inoculated group, **EAC + Cu(L)$_2$** - Ehrlich ascites carcinoma inoculated group treated with copper complex ; ***= $p < 0.001$ as compared to EAC group

However, the anti-mitotic mechanism is not the only one proposed for the copper complexes anticancer activity. Chatterjee et al. 2009, proved that copper chelate modulate the regulatory cytokine production pattern of tumor associated macrophages, it alter the immunosuppressive phenotype of tumor associated macrophages by reprogramming its proinflammatory against immunosuppressive pattern, by elevating the production of IL 12 and down regulating the synthesis of IL 10 and TGF β. Furthermore, Cu^{2+} and several copper complexes were proved to inhibit the NF-kB pathway (Kanemaru et al. 2011).

On the other hand, several studies showed that some copper complexes can trigger the proteasome inhibition (Zhai, et al. 2010) and apoptosis in human breast (MCF-7) and prostate cancer cells (PC-3) (Zuo, et al. 2012). Copper complexes seem to induce a global apoptotic mechanism. Cu complex may interact with mitochondria impairing the energetic metabolism of the cell, the nucleus resulting in DNA fragmentation, and additionally they generate reactive oxygen species (Lopez, et al. 2013). Other studies consider the DNA cleavage as a consequence of oxidative stress (Zhensheng, et al., 2012). The toxicity of copper complexes seems to be lower than classic cancer therapy, additionally copper –doxorubicine complex preserved the anticancer activity or doxorubicine reduced toxicity and allowed a multi dose therapeutic protocol on experimental models (Kheirolomoom, et al. 2010).

CONCLUSIONS

In conclusion, in preliminary study, the administration of copper complex has a significant antiproliferative effect on mouse transplantable tumor model; the therapy prevents the body weight gain, reduces the volume of the ascitic fluid and reduces the tumor cell concentration. In our point of view, those findings justify further pharmacological and chemical studies.

ACKNOWLEDGMENTS

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