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ONCOLOGY

O₂·- AND NO-ASSOCIATED MECHANISMS OF SELECTIVE ACTION OF REDOX-ACTIVE COBALT COMPLEXES ON TUMOR TISSUE

I.I. Ganusevich[1], A.P. Burlaka[1], E.P. Sidorik[1], I.Ya. Levitin[2], A.L. Sigan[2], S.P. Osinsky[1], *
[1]R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NASU, Kiev, Ukraine
[2]A.N. Nesmeyanov Institute of Organo-Element Compounds, RAS, Moscow, Russia

Aim: To study the influence of redox-active cobalt(III) complex with tetradentate Schiff base and nicotinamide as an axial ligand on the rate of superoxide radical-anions generation and levels of NO in tumor and normal tissues of Lewis lung carcinoma bearing mice as well as activity of matrix metalloproteinases 2 and 9 (MMPs) in tumor. *Methods:* The superoxide radical-anions formation and NO level in tissues were assessed by EPR method with the use of 1-hydroxy-2,2,6,6-tetramethyl-4-oxopiperidin and diethyldithiocarbomate spin traps, respectively. MMPs activities were determined by zymography in polyacrylamide gel. *Results:* It was observed that the rate of superoxide radical-anions generation was selectively increased in tumor tissue (by a factor of 6−7) accompanied with the decrease of NO level (by a factor of 2) due to tested complex administration. Activities of MMPs in tumor were significantly decreased. *Conclusion:* It is supposed that the one of mechanisms of detected earlier antimetastatic effect of complex is based on its ability to induce the formation of high level of superoxide radical-anions selectively in the tumor tissue that results in the damage of its regulatory functions, in particular alteration in the regulation of NO-synthase, decrease of NO generation as well as activities of MMPs.
Key Words: redox-active cobalt complex, radical oxygen species, nitric oxide, matrix metalloproteinases, antimetastatic action.

It is well known the crucial role of oxidative stress in the development of variety of pathological disorders, including cancer [1–3]. Reactive oxygen species (ROS) and nitric oxide (NO) at physiological concentrations are the key signaling molecules that mediates many processes in the organism. However, the elevated levels of ROS and NO induce disfunction of the electron transfer chain and cellular oxidation that form cellular hypoxia accompanied with modification of signaling pathways and development of pathological processes [4–6]. It was shown that ROS and NO can rearrange of the extracellular matrix by activation of latent forms of matrix metalloproteinases resulting in the stimulation of invasion and metastasis [7].

The active search of means for the regulation of the redox-dependent processes in cell in order to influence the oxidative stress is actual till now [8]. Two groups of substances may be considered for this role: substances with antioxidative features that are able to scavenge the radicals and stop oxidative damages of biomolecules, and those that can act as direct or indirect donors of O_2^- and/or NO^- enhancing the oxidative disturbances in tissues, at first in tumor ones. Transition metals are assumed very promising for the realization of last goal because of their variable valency that allows to induce the chain reaction through Fenton chemistry.

In recent years the anti-tumor activities of a number of metal complexes, e. g. of ruthenium, gallium, vanadium and other metals have been demonstrated [9–13]. Among compounds of the transition and non-transition metals which have been investigated (e. g. platinum, rhodium, ruthenium, iron, copper, cobalt), cobalt(III) complexes are worthy of particular atten-

tion because of high electron affinity of this metal in the trivalent state, its facile one-electron reducibility ($Co^{III/II}$), aptitude to bind to DNA and pronounced radiosensitizing activity [14, 15].

Our attention was drawn to cobalt(III) complexes with the symbolic formula $[Co(acac_2en)(NH_3)_2]Cl$ where $acac_2en$ is the chelating tetradentate ligand with two oxygen and two nitrogen donor-atoms each, i.e. the aliphatic residue of Schiff bases composed from acetylacetone and ethylenediamine in a ratio of 1 : 2 [16]. It was shown that this complex (which has anti-inflammatory -bacterial activities) showed also anti-tumor effects on the Ehrlich ascites carcinoma. We prepared complexes with the substitution of ammonium as an extra axial ligand in complexes with biogenous or synthetic nitrogenous bases [17].

It was shown earlier that the newly-synesized compounds, in particular cobalt complex with nicotinamide as axial ligand (AC-30 complex) display the significant antitumor and especially antimetastatic effects in experiments *in vivo* with rodent transplanted tumors [18]. It was also demonstrated that the selectivity of biological effects of complexes, in particular activation of lipid peroxidation, reduction of bioenergetic status, and DNA damage, were notably greater in tumor compared to normal tissues in tumor-bearing animals [19].

The present study was aimed to study the influence of cobalt complex AC-30 that has demonstrated the most high antitumor activity on the O_2^- generation and NO^- level in tumor and normal tissues to consider the possible ROS- and NO-dependent mechanisms of the AC-30 antitumor effect.

MATERIALS AND METHODS

43 female C57Bl/6 mice (IEPOR, NAS of Ukraine) with a body weight of 20–24 g bearing Lewis lung carcinoma (3LL) were used. The principles and methods of transplantation were conventional. Single-cell

suspension of 3LL (5×10^5 cells in 200 μl of buffered saline) was injected intramuscularly into the leg of mice. Animals were kept in Makrolon cages bedded with dust-free wood granules, and had free access to a standard diet and water *ad libitum*. All animal experiments in this study were approved by the regional animal ethics committee.

Octahedral cobalt(III) complex with tetradentate Schiff bases of general formula [Co(acac$_2$en)L$_2$]Cl, containing ligand (L = nicotinamide, complex AC-30) was tested. Compound was dissolved in *aqua pro injectionibus* immediately before use. It was given intraperitoneally (*i. p.*) at a dose of 12 mg/kg of body weight. This dose was chosen in accordance with the earlier experiments that have shown the therapeutic efficacy of complex at an above mentioned dose [18].

AC-30 was injected in a two regimens: 1) In experiments aimed to detect the action of AC-30 on the formation of superoxide radical-anions formation and NO generation by tumor as well as normal tissues of tumor-bearing mice (tumor volume 0,9–1,2 см^3) complex was injected i. p. at a single dose of 12 mg/kg. The animals were killed after 40 min after complex administration. 2) In experiments aimed to determine the influence of AC-30 on matrix metalloproteinase activity complex was injected at a dose of 12 mg/kg ten times with a one-day break between each administration. The animals were killed on the 21st days after the first injection.

Tumor, liver and kidney were snap frozen in liquid nitrogen immediately after excision (mice were anesthetized by pentobarbital that did not influence on indicating parameters) and stored at −70 °C. The superoxide radical-anions formation was assessed by the method of EPR with the use of 1-hydroxy-2,2,6,6-tetramethyl-4-oxopiperidin (Russia) and Spin Traps technology [20]. The rates of superoxide radical-anions generation were calculated in nmol/g fresh tissue per min. The level of NO was determined using diethyldithiocarbomate spin trap (Sigma, USA) and EPR technology [20]. The generation of NO was registered during 5 min followed by cessation of NO generation by liquid nitrogen (77 °K). The levels of NO were calculated in nmol/g fresh tissue.

Activities of MMP-2 and MMP-9 were determined by zymography in 12% polyacrylamide gel with the addition of 0.1% of gelatin as substrate and using MMP-2 and MMP-9 standards (Sigma, USA) [21]. The level of activity was calculated in accordance with standard programme TotalLab 1.01.

The obtained results were statistically evaluated by computer programs Statistics 6.0 and Exel 2003 using parametric and correlation analysis.

RESULTS AND DISCUSSION

It was observed that the level of O$^{-}_2$ generation in tumor tissue of untreated tumor-bearing animals is higher than in normal ones (Table 1). It may indicate the increase of oxidative stress in malignant tissue which is accompanied with significant damages of mitochondrial respiration and development of cellular hypoxia [7]. Fig. 1 clearly demonstrates the dramatic increase of the level of superoxide radical-anions generation in tumor tissue (by a factor of 6.8)

Table 1. The rate of superoxide radical-anions generation in the tumor and normal tissues of mice bearing Lewis lung carcinoma treated with AC-30 complex

Group	The rate of superoxide radical-anions generation (nmol/g of fresh tissue per min)		
	Tumor	Liver	Kidney
Control (n = 8)	1.76 ± 0.21	1.45 ± 0.12	1.23 ± 0.16
AC-30 (n = 8)	12.04 ± 2.05*	1.92 ± 0.5*	2.37 ± 0.72*

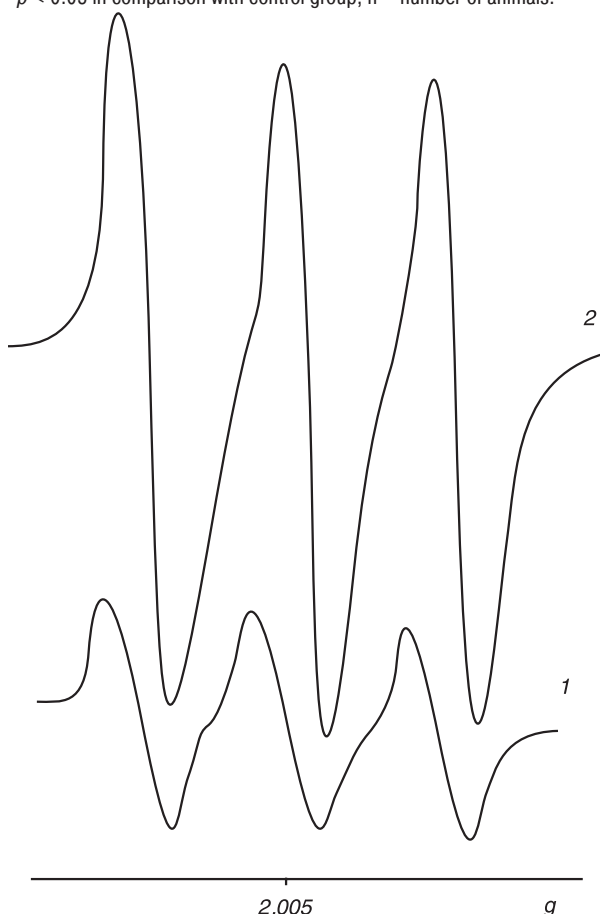*$p < 0.05$ in comparison with control group; n – number of animals.

Fig. 1. EPR spectra characterize the level of superoxide radical-anions generation in Lewis lung carcinoma: 1) untreated mice, 2) after injection of AC-30 complex (spectrum was decreased by a factor of 5 to be presented here)

It was shown also that AC-30 complex displays minimal influence on the level of NO$^{\cdot}$ synthesized by inducible NO-synthase (iNOS) in normal tissue (Table 2). At the same time, the level of NO in tumor was significantly decreased due to administration of AC-30 (by a factor of 2.3). It may be supposed that it results from overhigh level of O$^{-}_2$ generation. It is known that superoxide radical-anions at the high concentration are capable to modulate the NO-synthase that results in the start of O$^{-}_2$ formation instead of NO$^{\cdot}$. Moreover, O$^{-}_2$ through interaction with NO$^{\cdot}$ can generate peroxynitrite (ONOO$^{\cdot}$), which is known as a strong oxidant with wide spectrum of deleterious effect on cellular structures [22–24].

Table 2. The levels of NO in the tumor and normal tissues in Lewis lung carcinoma-bearing animals treated with AC-30 complex

Group	The levels of NO in the tumor and normal tissues (nmol/g of fresh tissue)		
	Tumor	Liver	Kidney
Control (n = 8)	3.2 ± 0.7	4.9 ± 1.2	3.9 ± 0.15
AC-30 (n = 8)	1.4 ± 0.5*	4.6 ± 1.8	4.2 ± 0.2

*$p < 0.05$ in comparison with control group; n – number of animals.

In our previous experiments the activation of latent forms of matrix metalloproteinases, in particular MMP-2 and MMP-9 by $O_2^{\cdot-}$ and $NO^{\cdot}$ was shown [7]. It was well known the crucial role of MMPs in metastasis by destruction of extracellular matrix [25, 26].

Fig. 2 demonstrates the significant decrease of activity of MMP-2 and MMP-9 in tumor tissue after administration of AC-30 complex in comparison with untreated control. It has to be noted that these results are corresponded with the data that significant inhibition of metastasis was observed under influence of AC-30 complex: the number of lung metastases was decreased by 56%, and the volume of of metastases — by 90% [18].
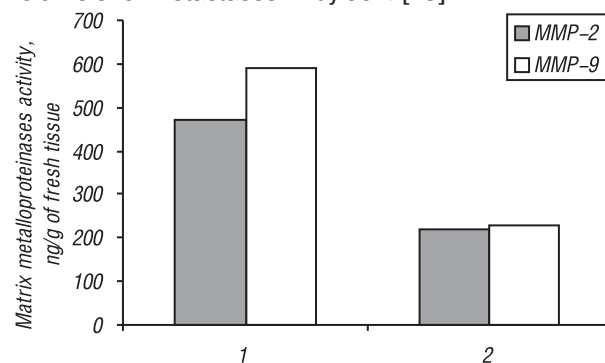

Fig. 2. Activity of matrix metalloproteinase-2 and -9 in Lewis lung carcinoma: 1 — untreated control (n = 10), 2 — treated with AC-30 complex (n = 17)

In the context of the biological activity of the complexes it is interesting to note their much more effective inhibition of metastasis than their effects on the growth of a primary tumor. In this connection it is notable that ruthenium complexes also reveal greater inhibition of metastases then on the growth of primary tumors [27]. It is very important that ruthenium complex was reported to act as a scavenger of nitric oxide, which is known to stimulate angiogenesis crucial to tumor growth and development of metastases [28].

Thus, the current study has allowed to propose the following mechanisms of antimetastatic action of AC-30 complex: 1) generation of the overhigh levels of $O_2^{\cdot-}$ in tumor tissue that induces oxidative damage in cancer cells; 2) inhibition of MMP-2 and -9 activities due to decrease of NO level in tumor tissue that results in the inhibition of metastases formation; 3) scavenging of nitric oxide that results in the inhibition of neo-angiogenesis in forming metastatic foci.

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O^{-}_2 И NO-АССОЦИИРОВАННЫЕ МЕХАНИЗМЫ СЕЛЕКТИВНОГО ДЕЙСТВИЯ РЕДОКС-АКТИВНЫХ КОМПЛЕКСОВ КОБАЛЬТА НА ОПУХОЛЕВУЮ ТКАНЬ

Цель: исследовать влияние редокс-активного комплекса кобальта (III) с тетрадентатным основанием Шиффа и никотинамидом в качестве аксиального лиганда на уровень образования супероксидного радикал-аниона и оксида азота в опухолевой и нормальных тканях мышей с карциномой Льюис и установить взаимосвязь этих показателей с активностью ММП-2 и -9 в опухоли. *Методы:* электронный парамагнитный резонанс при комнатной температуре и температуре жидкого азота (77 °К), технология Spin Traps, зимография в полиакриламидном геле. *Результаты:* установлено, что под действием комплекса в опухолевой ткани мышей с карциномой Льюис селективно повышается (в 6–7 раз) скорость образования супероксидных радикал-анионов и снижается (в 2 раза) уровень образования радикальных форм оксида азота; при этом значительно снижается активность ММП-2 и -9. *Выводы:* высказывается предположение, что в основе механизма установленного ранее антиметастатического действия изученного комплекса кобальта лежит его способность селективно создавать сверхвысокие уровни супероксидного радикал-аниона в опухолевой ткани, что приводит к нарушению его регуляторных функций, в частности к изменению регуляции NO-синтаз, снижению продукции NO и активности желатиназ.
Ключевые слова: редокс-активный комплекс кобальта, радикальные формы кислорода, оксид азота, матриксные металлопротеиназы, антиметастатическое действие.