Redox-active cobalt complexes as promising antitumor agents*

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Cobalt(III) complexes with tetradentate aliphatic Schiff´s bases containing also compounds of the vitamin PP series or their analogs as axial ligands were synthesized as potential antitumor agents. The behavior of these redox-active complexes in chemical processes that presumably govern their biological action was studied. These processes include aquation and subsequent decomposition, electrode and homogeneous redox reactions, and catalytic activity in autooxidation of biosubstrates, especially at the stages of generation and consumption of reactive oxygen species (ROS). The antitumor action of these complexes *in vivo* was studied. Changes in the organisms of laboratory animals characteristic of processes involving ROS were followed at the cellular and molecular levels. The tumor-selective action of the complexes is due to specific features of microenvironment of tumor cells. Some of them exhibit a strong antimetastatic effect, which exceeds that for a number of drugs used in clinical practice. A complex with nicotinamide was recommended for preclinical studies. The scope of application of the redox-active transition metal complexes in oncology is discussed.

Key words: cobalt complexes, redox-active complexes, antitumor effect, selectivity of action.

The last reports of the World Health Organization state that at present up to 6 million people in the world die every year of malignant tumors. The experts´ forecasts are not comforting: in the following two decades, this number is expected to reach 20 million and oncological diseases would come out on top among the causes of mortality leaving behind the cardiovascular pathology. The increase in the number of malignant tumor cases is attributed to both the deterioration of the environmental situation in many countries and aging of population. Despite the progress in the oncological sciences, attained, first of all, due to advances in molecular biology, the intense search for new in principle methods for prophylaxis, diagnosis, and therapy of malignant neoplasms does not stop in laboratories throughout the world. In recent years, new antitumor chemotherapeutical preparations, radiation sources, irradiation techniques, and means for the gene therapy and biotherapy of cancer have been developed and proposed for clinical use. An extremely topical task is the development of tumor-selective cytostatic agents, which would strongly injure tumor cells but affect as little as possible the usual organs and tissues and have no serious side effect on the patient´s organism. Certain progress has already been attained along this line, but the problem is still far from being solved. In recent years, especially after decoding of the human genome, the interest in the pathophysiological aspects of the malignant growth has revived and the attempts to relate them to the molecular mechanisms of functioning of cell systems have been resumed. This is a very important turning point in oncol-

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ogy, because, from the standpoint of physical chemistry, a malignant tumor is a complex, highly organized, open (in the thermodynamic sense) system closely connected to the organism where it has appeared and is being developed. Therefore, one should take into account the so-called tumor microphysiology or, in other words, the microenvironment of tumor cells, in particular, microcirculation, oxygenation, acidity, and the bioenergetic status of the tumor tissue.[1—3] They are closely connected to each other and can have a pronounced influence on the properties of the tumor such as invasiveness, metastatic potential, cellular heterogeneity, and tumor progression. It is believed[4] that tumor progression is based on "instability of the genome", which is modulated by the microenvironment of the tumor cells. Experimental and clinical works showed that the direct response of the tumor to irradiation, chemotherapy, local hyperthermia, immunotherapy, and photodynamic therapy and remote results of the therapy and expression of various tumor markers that are of diagnostic value depend appreciably on the state of the tumor blood flow and other microphysiological parameters.[5—7]

The microenvironment of the tumor cells is largely predetermined by the features of the tumor blood flow. It is known that the active angiogenesis, which is typical of a tumor tissue, cannot meet the demands of the growing tumor for the nutrients including oxygen. This results in a pronounced decrease in the oxygenation level of the tumor tissue and, as a consequence, an increase in the hypoxic fraction of tumor cells stable against irradiation and a number of chemotherapeutical preparations. In turn, the increasing tumor hypoxia causes the compensatory activation of glycolysis, giving rise to a large amount of lactic acid, which is retained in the tumor due to the poor blood flow and decreases the extracellular pH. Thus, the tumor tissue is characterized by defective microcirculation and retarded local blood flow, substantial hypoxia, and enhanced acidity. It thus follows that the cell microenvironments in the malignant and normal tissues are substantially different. These specific features of tumor microphysiology, which also largely determine the course of processes in the tumor cells, should be taken into account in the development of new antitumor preparations and treatment of oncological patients.

The elucidation of the specific features of the tumor microphysiology and elaboration of the ways of its modification and control have stimulated the search for, and development of, antitumor preparations that are able to exhibit a cytotoxic effect only in the microenvironment typical of tumor cells. A number of ways for solving this important problem are currently known, namely, (i) the search for hypoxia-dependent substances that exhibit a cytotoxic effect only at high pO_2 values; (ii) the use of inhibitors of angiogenesis; (iii) development of pH-dependent cytostatics whose effect is manifested at low pH values. All these imply the elaborartion of preparations that keenly respond to relatively small (quantitative) changes of the environment.

The discovery[8] of the high anticancer activity of *cis*-$[PtCl_2(NH_3)_2]$ has stimulated the study of the possibility of using compounds of other metals aas well, first of all, transition metals, in the chemotherapy of malignant tumors. The expectancies for their antitumor activity are based on the high and diverse coordination abilities of these metals with respect to bioligands, in particular, proteins and nucleic acids. Preparations based on cisoid platinum(II) complexes, for example, cisplatin, platidiam, carboplatin, and oxaliplatin are widely used in clinical practice, despite their pronounced nephrotoxicity. Although the research has now covered the great part of the Periodic Table and antitumor activities have been found for many complexes of other metals, only iron, cobalt, and ruthenium preparations have reached the first stage of clinical trials.[9—32] It is noteworthy that the most clear-cut and persistent antitumor effects were found for compounds of the Group VIII elements.

The mechanism of the antitumor action of platinum(II) complexes has been described previously.[33] The antitumor effects are found for those complexes where two ligands activated with respect to substitution occupy *cis*-positions relative to each other and the activity is due to the ability of such complexes to suppress DNA replication by forming stable coordination bonds as bridges within a DNA strand.

The principles of action and, correspondingly, of the targeted selection of other metal complexes have not been established as yet. The situation is especially intricate for redox-active complexes, which undergo reversible reduction under conditions similar to the physiological ones. This type of redox systems is known for many transition metals, first of all, Co, Fe, Mn, and Cu and are especially typical of low-spin $Co^{II/III}$ complexes with planar tetradentate macrocyclic and chelating strong- and moderate-field ligands. At least, three possible mechanisms of action have been proposed for these complexes. First, it has been assumed[34] that some of the complexes suppress tumor growth by binding the histidine units of polypeptide chains. In some other cases, release of cytotoxic ligands from the reduced forms of complexes is possible under hypoxic conditions. It is this mechanism that was proposed for the antitumor action observed in *in vitro* experiments[35—37] with mixed-ligand complexes of cobalt(III) with acetylacetone and nitrogenous analogs of yperite. M. E. Vol´pin put forward a hypothesis[38] that redox-active complexes of transition metals able to catalyze the autooxidation of ascorbic acid (AA) can be used in the therapy of cancer. This reaction is known to involve generation of reactive oxygen species (ROS), namely, the superoxide ion, the hydroxyl radical, and hydrogen peroxide. These are potent cytotoxic agents as they can

damage key biomolecules such as DNA.[39] Those of the above-mentioned complexes that would be capable of selective accumulation in malignant tissues may be expected to exhibit the antitumor activity when used together with AA. The fruitfulness of this approach has been demonstrated[40,41] in the cases of the redox-active cobalt phthalocyanine complexes and cobalt complexes of the B_{12} series combined with AA.

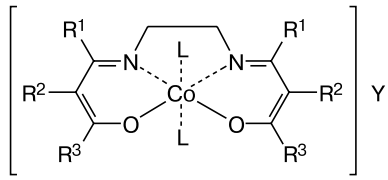
Interestingly, cobalt chelates with aliphatic tetradentate Schiff´s bases, unlike the corresponding complexes with phthalocyanines and B_{12} derivatives exhibit[42] an antitumor action regardless the presence of AA.* Two points are pertinent in this respect. First, AA is by no means the only one bioreductant, vital functions being also borne by other redox systems, for example, those based on nicotinamide adenine dinucleotide or glutathione. Second, redox-active complexes are able to catalyze not only the formation of ROS but also the reactions where ROS are consumed, *i.e.*, they exhibit nonspecific oxidase, peroxidase, and catalase activities.[44] In addition, the complexes themselves can gradually lose the catalytic activity, being destroyed under the action of the ROS formed. These facts suggest that selective attack of malignant neoplasms requires a particular balance between these processes depending on the catalytic activity of the given complex in each of the above-mentioned reactions and on the distribution of oxygen between the normal tissue and the tumor.

From this standpoint, the antitumor action of cobalt complexes with aliphatic tetradentate Schiff´s bases deserves further investigation, in particular, using chemical models, for the following reasons. First, the good prospects of these complexes were pointed out by both publications concerning their biological, in particular antitumor, activities[43] and preliminary results of our investigations.[44] Second, they are convenient objects for studying the dependence of the antitumor effect of redox-active complexes on the structure, reactivity, and physicochemical characteristics, as their structures can be easily varied by introducing additional axial ligands. Of special interest are complexes with biogenic ligands, whose function may be to weaken the side toxic effect caused by the xenobiotic core of the complex, to enhance its antitumor action, and/or to facilitate its transport through cell membranes. Our goal was to study the antitumor effects of cobalt complexes with tetradentate aliphatic Schiff´s bases and biogenic axial ligands; we chose compounds of the vitamin PP series as the axial ligands. Previously, these complexes have been unknown. Encouraging preliminary results of medicobiological trials have already been reported.[45—47]

* For cobalt chelates with aliphatic tetradentate Schiff´s bases, a moderate antitumor activity has been found[43] in addition to the antiinflammatory and bactericide effects.

Chemical studies

Synthesis and characterization of the complexes. Cobalt chelates with tetradentate aliphatic Schiff´s bases derived from ethylenediamine and acetylacetone or its derivatives were prepared. The newly synthesized compounds, [*N*,*N*´-ethylenebis(2-iminopent-3-en-4-olate)]bis(pyridine-3-carboxamide)cobalt(+1) chloride (**1Ac**, Y = Cl), [*N*,*N*´-ethylenebis(2-iminopent-3-en-4-olate)]bis(pyridine-3-carboxamide)cobalt(+1) tetraphenylborate monohydrate (**1Ac**, Y = BPh_4, $\cdot H_2O$), [*N*,*N*´-ethylenebis(3-chloro-2-iminopent-3-en-4-olate)]bis(pyridine-3-carboxamide)cobalt(+1) chloride (**1B**, Y = Cl), [*N*,*N*´-ethylenebis(2-imino-5,5,5-trifluoropent-3-en-4-olate)]bis(pyridine-3-carboxamide)cobalt(+1) chloride (**1C**, Y = Cl), [*N*,*N*´-ethylenebis(2-iminopent-3-en-4-olate)]bis(pyridine-4-carboxamide)cobalt(+1) chloride (**1Ad**, Y = Cl), [*N*,*N*´-ethylenedi(2-iminopent-3-en-4-olate)](pyridine-3-carboxylate)[pyridine-3-carboxylic acid]cobalt (**2**), are Co^{III} complexes. They are stable in air, unlike reduced forms, *i.e.*, Co^{II} complexes, and, therefore, suitable for injections. It is known that axial bases (L, L´) in these oxidized forms are bound much more strongly than in reduced forms, especially if they are strong-field ligands.[48] Therefore, one may hope that these complexes would reach the cell targets being undissociated.

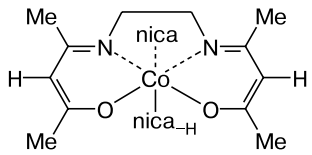


1A*—D

	1Aa	1Ab	1Ac	1Ad	1B	1C	1D
R^1	Me	Me	Me	Me	Me	Me	CF_3
R^2	H	H	H	H	Cl	H	H
R^3	Me	Me	Me	Me	Me	CF_3	CF_3
L	NH_3	im	nic	i-nic	nic	nic	nic

* $[Co(acac_2en)L_2]Y$.

Y = Cl, Br or BPh_4



2

$(Co(acac_2en)(nica)(nica_{-H}))$

Note: im is imidazole, nic is nicotinamide, i-nic is isonicotinamide, nica is nicotinic acid, $nica_{-H}$ is the anionic residue of nicotinic acid

In a study of the antitumor activity of cobalt complexes we showed[24,42] that impurities can have a substantial influence on the pharmacological characteristics of these substances. Therefore, to reduce the content of impurities, we tested and optimized several routes for the synthesis of these complexes. The "half-template" method used previously[49] to prepare Co^{III} complexes with tetradentate Schiff´s bases proved to be the the method of choice. This involves three successive steps: (1) synthesis of a free Schiff´s base, (2) synthesis of a Co^{II} chelate with the Schiff´s base from a Co^{II} salt and this free ligand under anaerobic conditions, and (3) the introduction of the corresponding Lewis base (L), which becomes an axial ligand, followed by oxidation of the reaction mixture with atmospheric oxygen. In all cases, the yields exceeded 70%.

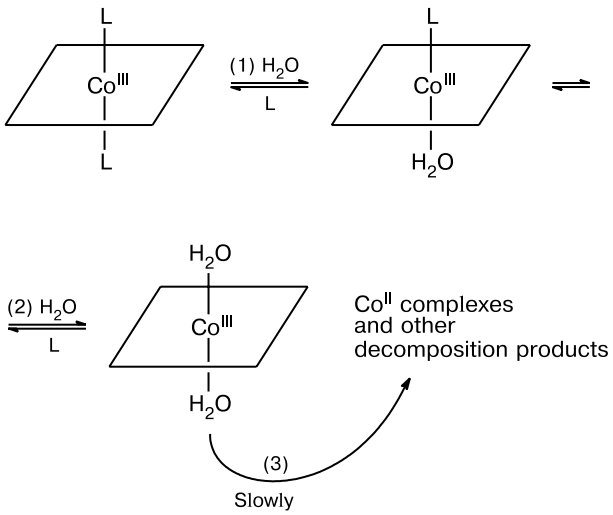
The new complexes **1Ac**, **1Ad**, and **1B—D** and complexes **1Aa** and **1Ab** known previously[50] were identified and characterized by elemental analysis, TLC, and by ^{1}H NMR, UV/Vis, and IR spectroscopy; where appropriate, aquametry was used to determine the water of crystallization. The individuality and the degree of purity of these substances were established by reversed-phase HPLC and capillary electrophoresis with spectrophotometric detection, including that with fast scanning of the spectrum.[51,52] In all cases, the arbitrary purity defined as the ratio of the area of the major peak on the chromatogram or electrophoregram to the sum of all signals at 310 nm was at least 99%.

In addition, the structure of the complex with nicotinamide **1Ac**, which is the most promising antitumor agent, was determined by X-ray diffraction.* Like most of the compounds considered, this is a cationic octahedral Co^{III} complex containing two nicotinamide molecules as monodentate axial ligands, which are coordinated to the central metal ion through the pyridine N atoms (Fig. 1). The neutral complex with nicotinic acid (**2**) is an exception, as it contains both the acid molecule and its anionic residue as the axial ligands.

The physicochemical characteristics of these complexes in the processes assumed to be related to their biological activity have been studied.

Aquation and subsequent decomposition. The behavior of the complexes under pseudo-physiological or related conditions, namely, in aqueous media at pH 5—9 and 20—60 °C, has been studied using mainly spectrophotometric kinetic measurements and ^{1}H NMR spectroscopy. The two axial ligands underwent successive and reversible replacement by water (aquation). This was followed by a slow extensive decomposition of the resulting diaqua complex. In this connection, it should be noted that we were unable to isolate diaqua complex **1A** (L, L´ = H_2O). Apparently, decomposition starts with an electron transfer inside the coordination sphere from the equatorial Schiff´s ligand to the central Co^{III} ion, and the lower electron-donating ability of water compared to nitrogenous bases favors this transfer. Thus, the whole process can be represented by Scheme 1. Selected data on the aquation kinetics and equilibria are presented in Table 1.

Scheme 1



$v_1, v_2 >> v_3$

Both steps of aquation are pseudo-first-order reactions. The pH dependence of the reaction rates indicates the involvement of acid-base catalysis usual for Co^{III} amino complexes.

The aquation of the diammino complex **1Aa** proceeds more easily than that of related complexes with aromatic heterocyclic nitrogenous bases **1Ab—Ad** and **2** to such an extent that kinetic measurements had to be carried out in different temperature ranges, namely, 15—25 and 50—60 °C, respectively.

The first step of the aquation of diammino complex **1Aa** in a neutral medium proceeds approximately 15 times as fast as the second one, which is attributable to the *trans*-labilizing effect of the other (remaining) NH_3 ligand. For other complexes of the series **1A**, the first step of aquation is much slower so that the rates of both steps become comparable. Obviously, this stabilization of the starting complex is due to both the formation of a dative bond by the antibonding MO of the aromatic heterocycles and to the decrease in the *trans*-labilizing effect of the remaining axial ligand, which is a weaker electron donor than ammonia.

Electrode processes. The electrode potentials of redox-active complexes provide important characteristics of these compounds from the standpoint of their possible biological action. Therefore, we studied the behavior of

* Z. A. Starikova, the materials are being prepared for publication.

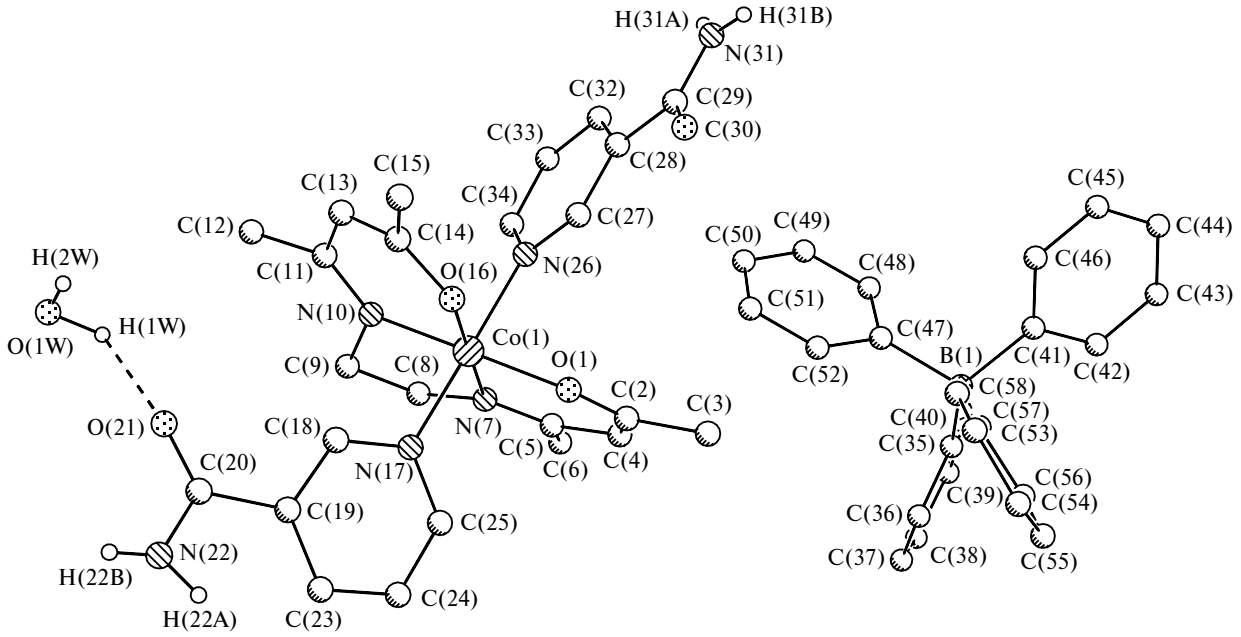


Fig. 1. Molecular structure of $[Co(acac_2en)(nic)_2]BPh_4 \cdot H_2O$ (**1Ac**).

complexes **1** on a glassy carbon electrode in an aprotic medium. The mechanism of their cathodic reduction corresponds to the scheme proposed previously[50] for the electrode reactions of cobalt complexes with tetradentate Schiff´s bases in aprotic solvents (Scheme 2). The electronic effects in the equatorial (polydentate Schiff´s) ligand affect appreciably the potentials of both reduction steps, *i.e.*, $Co^{III/II}$ and $Co^{II/I}$, whereas the variation of the axial ligand (monodentate Lewis base) has a pronounced effect

Table 1. Equilibrium and kinetic constants[a] for the aquation of cobalt(III) complexes with a tetradentate Schiff´s base derived from acetylacetone and ethylenediamine

$$[Co(acac_2en)LL']^+ + H_2O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [Co(acac_2en)L(H_2O)]^+ + L',$$

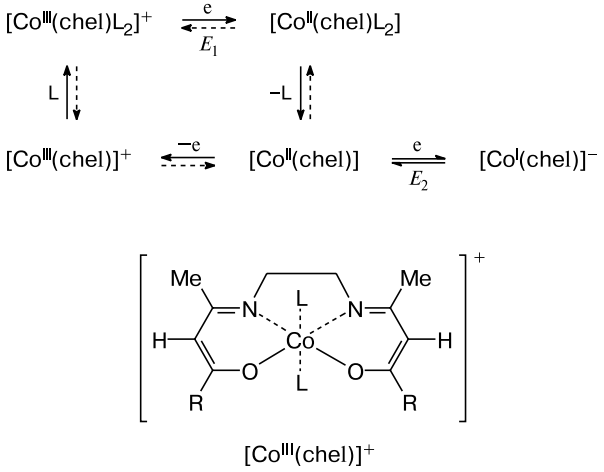
$$[Co(acac_2en)L(H_2O)]^+ + H_2O \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [Co(acac_2en)(H_2O)_2]^+ + L$$

L, L´	$k_1 \cdot 10^2$	$k_2 \cdot 10^2$	$k_{-1} \cdot 10^{-3}$	$k_{-2} \cdot 10^{-2}$	$K_1 \cdot 10^5$	
$K_2 \cdot 10^4$	min^{-1}		L mol^{-1} min^{-1}		mol L^{-1}	
nic	3.31	2.51	1.49	1.83	2.23, 1.77[b]	1.37
i-nic	3.99	3.21	1.93	2.05	2.07, 0.85[b]	1.56
nica, $nica_{-H}$	3.23	1.14	0.80	3.31	4.00, 3.40[b]	0.36

[a] Determination method and conditions: absorption spectroscopy in the visible and UV regions; phosphate buffer with pH 7.4 in H_2O, 60 °C, the sum of concentrations of cobalt(III) complexes was ~10^{-4} mol L^{-1}.

[b] Determined by ^1H NMR spectroscopy in D_2O; 25 °C, the sum of concentrations of cobalt(III) complexes was ~10^{-1} mol L^{-1}.

Scheme 2



Complex	R	L	$-(E_1)_{pc}$	$-(E_2)_{1/2}$
			V	
1Aa	Me	NH_3	1.07	1.80
1Ac	Me	nic	0.62	1.85
1Ad	Me	i-nic	0.60	1.87
1C	CF_3	nic	0.24	1.33

Note. The CV data were obtained on a glassy carbon electrode in a 0.1 *M* solution of Bu_4NBF_4 in MeCN at 25 °C; potential sweep rate 0.1 V s^{-1}; the cathodic peak (E_{pc}) and half-wave ($E_{1/2}$) potentials are referred to SCE.

only on the first step. The reason for this difference has already been discussed.[50]

Homogeneous redox reactions and catalytic processes involving ROS. The reduction of cobalt(III) complexes with tetradentate Schiff´s bases in an aqueous solution has been studied by pulse radiolysis taking **1Ad** as an example. The superoxide, carbon dioxide, and methyl viologen radical anions that were generated upon the pulse served as the test reducing agents. The equilibrium redox potentials of these species are −0.45, −0.65, and −2.15 V (*vs.* SCE). The carbon dioxide and even methyl viologen radical anions but not the superoxide radical anion reduce this complex promptly and efficiently (the second-order rate constants are $2.0 \cdot 10^8$ and $1.8 \cdot 10^8$ L mol^{-1} s^{-1}, respectively). This means that in this case, the $Co^{III/II}$ redox potential falls in the range from −0.45 to −0.65 V (*vs.* SCE), which agrees with CV data.

Two independent methods, namely, CV and the superoxide dismutase (SOD) biosensor, were used to estimate the antioxidant activity of complexes **1Aa**, **1Ac**, and **1Ad**, *i.e.*, the ability of these redox systems to accept the ROS in buffer and physiological solutions compared to that of AA. Both methods gave similar results: in terms of increasing antioxidant activity, these complexes can be arranged in the sequence

$$\mathbf{1Aa} \gg \mathbf{1Ad} > \mathbf{1Ac}. \quad (1)$$

The catalytic autooxidation AA in the presence of complexes in question was studied. To prove the intermediate formation of the ROS and to monitor the reactions involving them, we used enzymological techniques in combination with the same biosensor.

The catalytic activity of complexes **1** and **2** in the autooxidation of AA estimated from the initial rates of the reaction decreases in the sequence

$$\mathbf{1B} \gg \mathbf{1Ad} \gg \mathbf{1Ac} > \mathbf{2} \gg \mathbf{1Aa} > \mathbf{1Ab} > \mathbf{1C}. \quad (2)$$

The apparent catalytic activity of complexes gradually changes as the process develops. The loss of activity per catalytic cycle decreases, while the growth increases in the sequence

$$\mathbf{1Aa} > \mathbf{1Ab} \mid \mathbf{1B} > \mathbf{1Ad} > \mathbf{1Ac} > \mathbf{2} \gg \mathbf{1C}, \quad (3)$$

where the character "|" corresponds to switching of the sign of the effect to the opposite one.

Generally speaking, the change in the apparent catalytic activity of complexes may be due to the following reasons: (1) their destruction under the action of the ROS, (2) gradual change in the ratio (approach to the equilibrium) between the oxidized and reduced forms of the catalyst, and (3) the developing aquation process. The switching of the sign of the effect in the series of the complexes (3) indicates that different situations with predominance of one or another of these effects can occur during the process.

The influence of enzymes that cause either ROS decomposition, namely, SOD and catalase (Cat), or ROS consumption (xanthine oxidase combined with xanthine as the substrate) on the catalytic oxidation of AA has also been followed. In all cases, the processes under interest were inhibited; in the first two cases, this was indicated by a decrease in the degree of conversion of AA, while in the last-mentioned case, by a decrease in the steady-state superoxide concentration. Superoxide dismutase and Cat inhibit the autooxidation of AA to comparable extents, except for the case of the least catalytically active complex **1C** (Table 2). This implies that the extents to which superoxide and the hydroxyl radical (more precisely, hydrogen peroxide) are involved in the process are of the same order of magnitude. A similar situation has also been observed for hydroxocobalamine (B_{12a}),[41] whereas in the case of Fe and Co phthalocyanine complexes, the effect of SOD is much more pronounced.[53]

Table 2. Effect of superoxide dismutase (SOD) and catalase (Cat) on the autooxidation of ascorbic acid (AA) in the presence of cobalt complexes with tetradentate Schiff´s bases[a]

Complex used as catalyst	The ratio complex : AA	Degree of conversion of AA (%) over 1 h			Relative suppression of autooxidation by the enzyme (%)	
		without an enzyme	in the presence of			
			SOD	Cat	SOD	Cat
—[b]	—	26	8	12	72	55
1Aa	1 : 10	28	17	16	39	43
1B	1 : 1000	74	9	18	88	75
1C	1 : 10	52	28	46	46	11
2	1 : 10	35	28	23	21	35
B_{12a}[c]	1 : 10	45	41	37	8	16

[a] Aqueous buffer solutions, pH 7.0, 25 °C, the AA, SOD, and Cat concentrations were $5 \cdot 10^{-5}$ mol L^{-1} and 1 and 2 mg mL^{-1}, respectively.
[b] Blank experiment.
[c] Hydroxocobalamine.

Attempt at matching the reactivity to the antitumor effects. The model approach makes it possible to outline a relationship between the chemical properties and physicochemical characteristics of redox-active complexes, on the one hand, and their antitumor action, on the other hand.

Of the complexes we studied, two complexes, **1Ab** and **1B**, proved to be clearly ineligible due to the high general toxicity of free ligands, imidazole and 3-chloroacetylacetone, respectively.

Complex **1C**, containing a CF_3 group in the equatorial Schiff's ligand, appears to have no prospects either, due to the too high oxidation potential of the $Co^{II/III}$ redox pair (see Scheme 2), *i.e.*, difficulty of oxidation of the Co^{II} reduced form under physiological conditions. This accounts for very low catalytic activity of this complex in the autooxidation of AA (see sequence (2)).

These data in combination with the results of medicobiological tests, which are outlined below, suggest that the stability against aquation and high and persistent catalytic activity in the autooxidation of AA or another bioreductant are favorable for the antitumor action of redox-active cobalt(III) complexes, all other factors being the same.

Medicobiological studies

Complexes **1Aa**, **1Ac**, and **1Ad** were tested in detail. Determination of acute toxicity of **1Ac** (typical example) containing nicotinamide as the axial ligand gave the following results: for F_1 ($C57Bl/DBA_2$) mice, $LD_{50/14}$ = 68.3 mg kg^{-1}; maximum tolerable dose (MTD) = 41 mg kg^{-1}; therapeutic doses, 10—12 mg kg^{-1}; therapeutic index, 4.3; and range of therapeutic effect, 3.4. The decrease in the body weight of animals at doses of up to 60 mg kg^{-1} was not more than 3.8%. These values attest to a moderate acute toxicity of the complex not exceeding that of known antitumor preparations. It is also noteworthy that the complexes under investigation did not exhibit a substantial acute toxicity, in particular, hemo-, hepato-, or nephrotoxicity.

The influence of the complexes on the tumor growth were studied in experiments with transplanted murine tumors, in particular, with mammary adenocarcinoma Ca755, melanoma B16, and Lewis lung carcinoma. All three complexes, first of all, **1Ac** and **1Ad**, exhibited rather high antitumor, especially antimetastatic effects (Figs. 2 and 3, Table 3). It should be emphasized that the antimetastatic effect of the complex with nicotinamide (**1Ac**) proved to be more pronounced than that found for the conventional modern preparations, platidiam and cyclophosphamide (see Fig. 3). On the basis of these results, the complex with nicotinamide, **1Ac**, can be recommended for preclinical studies in order to develop a pharmaceutical form.

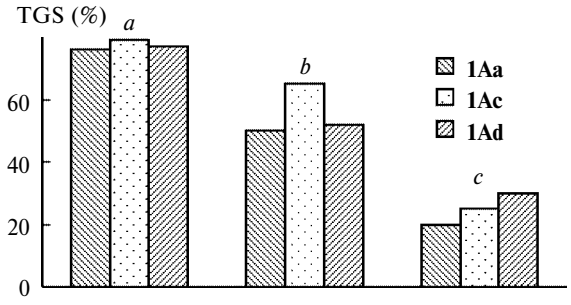


Fig. 2. Antitumor activity of cobalt(III) complexes **1Aa**, **1Ac**, and **1Ad**: tumor growth suppression (TGS) of the primary transplanted tumors in mice having mammary adenocarcinoma (Ca755) (*a*), melanoma B16 (*b*), and Lewis lung carcinoma (*c*).

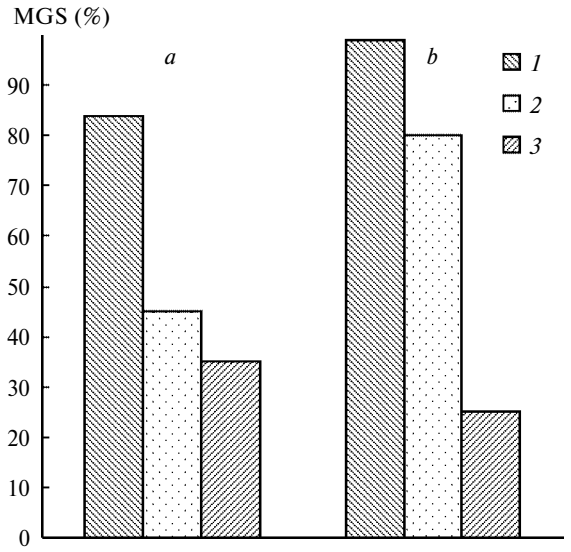


Fig. 3. Antimetastatic activity of complex **1Ac** (*1*), platidiam (*2*), and cyclophosphamide (*3*) in mice with Lewis lung carcinoma (model with amputation): metastasing growth suppression (MGS) as regards the number (*a*) and the bulk of (*b*) metastases.

To evaluate the possible effect of the Co^{2+}_{aq} ions (which are likely to be formed upon complete decomposition of the complexes), we studied the effect of $CoCl_2$ on the growth of transplanted tumors. This cobalt(II) salt exhibited only insignificant antitumor effect (about 8 times less pronounced than that of **1Ac**).

The fact that the most pronounced antitumor activity was found for complex **1Ac** containing nicotinamide as the axial ligand stipulated the necessity of verifying also the effect of free nicotinamide and its combination with complex **1Aa** containing relatively weakly bound ammonia as the axial ligand on the tumor growth. The experiments demonstrated rather low antitumor activity of nicotinamide (below 25%), while the introduction of nicotinamide together with **1Aa** even decreased the antitumor effect of this complex from 76 to 22%. The reason for the last-mentioned fact is obscure. Nevertheless, it was clearly

Table 3. Antimetastatic activity of cobalt(III) complexes

Complex	The number of mice without metastases (%)	Metastasing growth suppression in the lungs (%) determined from	
		the number of metastases	the bulk of metastases
Lewis lung carcinoma — the model without amputation			
1Aa	15	67	70
1Ac	17.5	74	80
1Ad	15	77	78
Lewis lung carcinoma — the model with amputation			
1Aa	27.5	67	90
1Ac	34	84	99
1Ad	35	84	95
Melanoma B16			
1Aa	37.5	75	76
1Ac	40	71	99
1Ad	35	65	85

shown that the pronounced antitumor effect is inherent in complex **1Ac** rather than in the products of its possible transformations.

Study of the mechanisms of the antitumor action of the complexes. We assumed that complexes **1Ac**, **1Ad**, and even (at least partially) **1Aa** can reach the biological targets (tissues, cells) without being decomposed, while in the targets, they are reversibly reduced. This gives rise to the $Co^{II/III}$ redox pairs, which catalyze the autooxidation of biological substrates with the intermediate formation of the ROS. The ROS thus generated attack the biomolecules of the tumor cell (in particular, lipids, proteins, and DNA) to oxidize some of them (lipids) and damage some other (single- and double-strand scissions of DNA). For this to take place, the given $Co^{II/III}$ pair should possess an appropriate redox potential such that the starting Co^{III} complex could be reduced in the hypoxic areas of the tumor tissue selectively with respect to normal tissues and organs.

This assumption was verified in an experiment in mice with Lewis lung carcinoma in which the contents of malondialdehyde (MDA) and reduced glutathione (GSH) were determined and the activity of glutathione-S-transferase (GST) in the tumor tissue and in some normal tissues of the tumor-bearing animal was measured. The obtained results clearly demonstrated a substantial selectivity of the action of the complexes on the tumor. In particular, the content of MDA, which is the final lipid peroxidation product and a marker of its activity, increased 3.5-fold in the tumor 60 min after injection of complex **1Ac**, whereas the content of MDA in the liver and kidneys increased only 1.5- and 2.4-fold, respectively. The content of MDA in the blood did not change. After 24 h, the content of MDA in the tumor remained at the same level, whereas in the normal tissues it has returned to the initial level.

This experiment showed that **1Ac** activates lipid peroxidation in animal tissues, this effect being most pronounced in the tumor. This activation is known to stimulate the protective function of the thiol system, which includes, first of all, GSH and the enzyme GST. Upon the injection of complex **1Ac**, the content of GSH in the tumor halved, whereas its content in the liver remained the same. It was also found that the activity of GST in the tumor decreased as soon as 30 min after the injection. Subsequently, it continued to decrease and the initial level is not restored even after 24 h. Meanwhile, in normal tissues, only a slight decrease in the enzyme activity was observed, the initial activity being restored already after 60 min. These results attest to selective suppression of the protective system of the tumor tissue under the action of **1Ac**. While analyzing the rersults obtained, one can state that the cobalt complexes in question are selectively reduced in the tumor tissue, thus initiating the lipid peroxidation.

The selectivity of action of the complexes was also found in experiments where the bioenergetic status[54] of the tumor and the normal tissues was determined by ^{31}P NMR spectroscopy. The complexes cause a substantial and rather selective decrease in the level of tumor bioenergetics (in normal tissues, only a slight decrease was noted) and an increase in the fraction of hypoxic tumor cells, especially 2 h after the injection. Probably, inhibition of the energetic metabolism in the tumor is a mechanism of action of these complexes.

Damage of DNA in the tumor cells, in particular, an increase in single-strand scissions of DNA is noteworthy, which is indicative of the ability of complexes or their metabolites, including ROS, to interact with this important biomolecule. Experiments *in vivo* showed that administration of the complex in animals with transplanted tumors increased the level of apoptotic cells in the tumor by a factor of almost 3. Note that the level of apoptosis in normal tissues almost was not changed.

Experiments on determination of the influence of **1Ac** on the activity, in the tumor tissue, of type IV matrix metalloproteinases (MMP-2 and MMP-9), which play a key role in the tumor dissemination, were carried out. The activities of MMP-2 and MMP-9 in the tumor were found to decrease markedly after administration of the complex according to the therapeutic regimen, which may imply one more possible mechanism of the antitumor, in particular, antimetastatic action of the complex.

Attention is drawn by the fact that the sequence of the antitumor effects of complexes **1Aa**, **1Ac** and **1Ad** (see sequence (1)) is opposite to the sequence of antioxidant activity. To make this finding general, wider series of complexes are to be studied.

We found that after administration of complex **1Ac** into a tumor-bearing animal, the level of NO formation from L-arginine in the tumor is halved. The fact that the level of NO formation in the normal tissues of the tumor-bearing animal, which is usually 2—3 times higher than in a healthy animal, was not changed after administration of the complex, still lacks an explanation.

The observed decrease in the NO level in the tumor under the action of this cobalt complex is of obvious interest, especially in view of the data that an enhanced level of NO in the tumor is correlated with its growth and metastazing.[55] Note that ruthenium complexes that have a pronounced antitumor effect also exhibit a substantial antioxidant activity.[28,55] These complexes were found[56,57] to "neutralize" NO, due to coordination of the paramagnetic NO molecule to the paramagnetic Ru^{III} complex. This may be followed by transformations of the NO ligand, including catalytic transformations involving biosubstrates, in the metal coordination sphere. It was suggested[58] that these processes are responsible for the antitumor effect of Ru complexes. As a result, the angiogenesis in the tumor, otherwise stimulated by NO, is retarded.

* * *

In conclusion, we would like to emphasize that, irrespective of the mechanism of the antitumor activity of the cobalt complexes we considered, the high selectivity of their effect on the tumor is most important from the clinical oncology standpoint. This selectivity is due, on the one hand, to a set of their chemical properties and, on the other hand, to specific features of the microenvironment of tumor cells. This gives hope for the development of effective antitumor drugs on the basis of these complexes or related structures.

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