DETECTION OF TUMOR RESPONSE TO Co(III) AND Fe(III) COMPLEXES BY ^{31}P–NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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РЕГИСТРАЦИЯ ОТВЕТА ОПУХОЛЕВЫХ КЛЕТОК НА ВОЗДЕЙСТВИЕ КОМПЛЕКСАМИ Co(III) И Fe(III) МЕТОДОМ ^{31}P–ЯМР–СПЕКТРОСКОПИИ

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A comparative analysis of the effects of several Co(III) and one Fe(III) complexes, as well as that of $CoCl_2$ on the "bioenergetic status" as well as tumor hypoxia in Guerin carcinoma has been carried out using ^{31}P NMR. Existence of the inverse correlation between the changes in the "bioenergetic status" of tumor tissue after complexes administration and their antitumor efficacy has been shown in experiments *in vivo*. The hypoxia levels in tumor that were registered under these conditions have been corresponded to antitumor activities of the complexes. Complexes were shown to exhibit a time-dependent influence on tissue energy metabolism. It has been also demonstrated that ^{31}P NMR spectroscopy may be used to characterize the physiological state of tissues and applied on optimization of drug design and prediction of antitumor efficacy of newly synthesized substances.

***Key Words*: Co(III) complexes, Fe(III) complex, ^{31}P NMR-spectroscopy, Guerin carcinoma.**

С помощью метода ^{31}P-ЯМР проведен сравнительный анализ влияния нескольких комплексных соединений Co(III), комплексных соединений Fe(III), а также $CoCl_2$ на "биоэнергетический статус" и гипоксию клеток карциномы Герена. В экспериментах *in vivo* продемонстрировано наличие обратной корреляции между изменением "биоэнергетического статуса" опухолевой ткани после введения комплексов и их противоопухолевой эффективностью. Степень гипоксии опухоли, определенная в таких условиях, соответствовала противоопухолевой активности комплексов. Установлено, что указанные комплексные соединения влияют на энергетический метаболизм тканей в зависимости от продолжительности периода воздействия. Выявлено, что метод ^{31}P-ЯМР можно применять для характеристики физиологического состояния тканей, оптимизации производства эффективных противоопухолевых препаратов и прогнозирования противоопухолевой активности впервые синтезированных соединений.

***Ключевые слова:* комплексные соединения Co(III), комплексные соединения Fe(III), ^{31}P-ЯМР-спектроскопия, карцинома Герена.**

There is an increasing interest to understanding the interactions between metal complexes and biological systems. Initial studies investigating the biological activity of metal containing compounds have demonstrated inhibition of the growth of tumor cells both *in vitro* and *in vivo* [1, 2]. In this connection cobalt compounds may be of particular interest because of the coordinative capacity of metal center and their ability to catalyze various processes with involvment of biosubstrates, in particular generation and consumption of reactive oxygen species which can attack cellular biomolecules. These data suggest that further investigations with cobalt–containing complexes are warranted [2, 3].

Studies of experimental tumors have suggested that high–resolution ^{31}P–nuclear magnetic resonance (^{31}P NMR) spectroscopy (MRS) may become a useful tool in prediction and assessment of tumor treatment response [4, 5]. ^{31}P NMR spectroscopy has been used to study tumor metabolism during unperturbed tumor growth [6] and to monitor tumor response to radiation therapy [7], hyperthermia [8], chemotherapy [9] and photodynamic therapy [10]. These studies have shown that one of the critically important area being researched by MRS is the "bioenergetic status" of the tissues that give the possibility to provide information on physiological state

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Abbreviations used: GC — Guerin carcinoma; PCA – perchloric acid; MRS – ^{31}P–nuclear magnetic resonance spectroscopy; Pcr – phosphocreatin; ^{31}P NMR – ^{31}P–nuclear magnetic resonance.

of the tissue and any changes of which may evidence the activity of the agents [11]. Moreover, it has been concluded that MRS could be useful to detect changes in tumor energy status induced by alterations in tumor oxygenation [12, 13] and tumor hypoxia considered as important parameter of tumor metabolic status [14].

This study was carried out as comparative analysis to determine whether the ^{31}P NMR spectral changes are reflecting the changes in "bioenergetic status" and hypoxia level of Guerin carcinoma upon administration of Co(III) complexes, Fe(III) complex and $CoCl_2$ *in vivo* and, next, to estimate and predict the antitumor efficacy of the newly synthetized complexes in the aim to find out the most active one.

MATERIALS AND METHODS

Experimental animals. All studies were conducted with strain IEPOR bred rats (Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv, Ukraine) weighting 150–200 g and bearing Guerin carcinoma (GC) transplanted subcutaneously into the right flank. The principles and methods of transplantation were conventional. Animals were kept in Makrolon cages bedded with dust–free wood granulate, and had free access to a standard diet and tap water.

Animals were taken into experiment 8–10 days after transplantation (tumor volume < 1.5 cm^3), anaesthetized with pentobarbital sodium (45 mg/kg, i.p.) and were kept at normal body core temperature (37–38 °C). It was shown that it was absolutely necessary to keep the body core temperature at the normal level to obtain reproducible spectra. When the body core temperature was controlled the anesthesia itself was found to have insignificant effects, if any at all, on the NMR spectra [15].

All animals were housed as a control group (untreated animals) and the groups treated with respective complexes. Tumor and muscle tissues from each GC–bearing animal have been investigated.

All experiments had been approved by the regional animal ethics committee.

Materials. Co(III)complexes: AC–30, AC–40, AC–100 of general formula $[Co(acac_2en)\ L_2]X$ differing in axial ligands (L), the related Fe(III) complex (AC–80) lacking extra ligand, and $CoCl_2$ (a referent Co–containing substance) were tested *in vivo*. All compounds have been dissolved in aqua pro injectionibus immediately before use.

Co(III) complexes (AC–30 and AC–40) were administered i.v. at the dose 15 mg/kg of body weight; complex (AC–100) – at the dose 12 mg/kg (i.v.); $CoCl_2$ – at the dose 15 mg/kg (i.p.); Fe (III) complex – at the dose 5 mg/kg (i.p.). Doses have been chosen as therapeutic ones in accordance with LD_{50} of the substances. The ways of administration were chosen according to the toxicity of the substances.

Each complex and $CoCl_2$ have been tested on a group of 6–11 animals. Then perchloric acid (PCA) extractions both of Guerin carcinoma and hip muscle tissues from individual animal have been analysed for the levels of all phosphocontaining substances and compared with respective samples from control group.

Perchloric acid extraction. For PCA tissue extraction, both tumor (1.0–1.5 cm^3) and hip muscle were surgically resected and immediately frozen in liquid N_2. The frozen samples were pulverized, ground to a fine powder in liquid N_2, and mixed with 1.2 N PCA. The mixture was slowly thawed and then incubated on ice with continuous stirring.

Since the PCA extraction method may induce the hydrolysis of certain acid–labile cell constituents such as NADPH, NADH, inositol phosphates, fructose 1,6–diphosphate, phosphocreatin (PCr), etc. PCA extraction has being performed in the cold [16]. The chilled distilled deionized water was added prior to centrifugation to remove cellular debris. pH of the PCA extracts was adjusted to 7.6–8.0 with 5 N KOH; then the samples were centrifuged to remove the $KClO_4$ precipitate. Divalent ions were removed by addition of Chelex (10 mg/5 ml) (Sigma, USA). Samples were clarified by filtration, lyophilized and kept at –20 °C. Before the NMR recording the lyophilized samples were dissolved in 1.0 ml of D_2O, centrifuged again and transferred to the NMR tubes.

Measurements by ^{31}P NMR. ^{31}P NMR spectra of PCA extracts were recorded with a Mercury–300 BB Spectrometer (Varian, USA) equipped with Sparcs station 4, using a probe of 5 mm inner diameter. ^{31}P NMR spectra were measured at 121.5 MHz. Spectra were usually obtained up to 1,000 transients with a spectral window of 12,000 Hz, 20 K data points, 45° pulse angle, repetition rate 12 s and a line broadening of 10 Hz. Acquisition time was 2 s; relaxation delay was 10 s.

As a standard, methylenediphosphonic acid, trisodium salt (Sigma, USA) was used. All ^{31}P chemical shifts in the spectra were set relative to PCr by setting the PCr signal to 0.00 ppm. Areas of the spectral signals were measured by the integration mode of the spectrometer.

The results are presented as mean ± s.d. Statistical significance was determined using Students *t*–test.

RESULTS AND DISCUSSION

^{31}P NMR spectra of muscle of untreated animals represented the "classic» ^{31}P NMR spectra (Fig. 1) and also served as a control for the procedure of PCA extraction for each tumor tissue sample.

The water soluble phosphate metabolites of PCA extracts of the Guerin carcinoma were examined by nuclear magnetic spectrometry (Fig. 2). ^{31}P NMR spectra of the Guerin carcinoma showed a similar profile with variations in the relative content of metabolites.

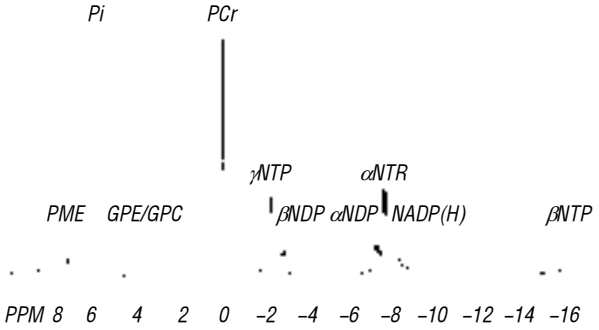

Fig. 1. ^{31}P NMR spectrum of PCA extract of muscle tissue of Guerin carcinoma bearing rat

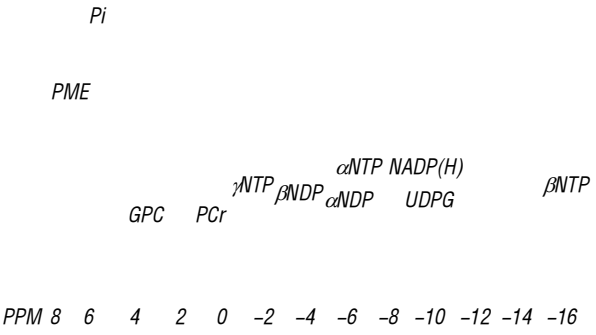

Fig. 2. ^{31}P NMR spectrum of PCA extract of Guerin carcinoma of untreated rat

The spectra of untreated rats revealed the presence of PME (mainly phosphocholine and phosphoethanolamine, and including AMP as well as phosphorylated sugars such as glucose–6–phosphate, fructose–6–phosphate, and fructose–1,6–diphosphate), Pi, PDE (mainly glycerophosphocholine and glycerophosphoethanolamine), PCr, γ–NTP and β–NDP, α–NTP and α–NDP (together with other compounds such as NADPH/NADP$^+$ and NADH/NAD$^+$), DPDE (possibly including UDPG), and β–NTP that were assigned in accordance with literature data [8]. Only the β–phosphate resonance of NTP was used to monitor NTP levels since the α– and γ–peaks overlap other resonances.

PCr was almost undetectable in the majority of GC–derived sample spectra because the tumor size is the main criterion for PCr content. All experiments presented in this paper have been carried out with approximately the same range of tumor volume (by 1.5 cm^3) to avoid differences in volume–dependent biochemical properties such as "bioenergetic status", pH, blood supply per viable tumor and level of tumor oxygenation and each spectrum has been recorded for each tissue sample of separate individual rat. As a rule, PCr may be identified only in tumor which volume is not more than 400 mm^2 [13]. That is the reason why PCr was not used in our study as a representative parameter of the tumor.

The "bioenergetic status" has been derived from MRS measurements in slightly alternative ways, the most common and probably most reliable of which (for tumors) is the ratio βNTP/Pi. The ratio of βNTP to total signal in the ^{31}P spectrum (βNTP/ΣP) and Pi/βNTP have been also used [13, 17]. In this study the Pi/βNTP has been applied to characterize the "bioenergetic status" as the more convenient parameter. Moreover, it has been shown that the ^{31}P spectrum reflects the degree of hypoxia of viable cells and the metabolic alterations required to sustain ATP synthesis as the oxygen supply diminishes and the changes in Pi/βNTP ratio correlates with tumor oxygenation [18].

The PME/β–NTP ratio that was used additionally is the most frequently applied ^{31}P MRS parameter for investigation of the "bioenergetic status" because the information about phosphorylated intermediates in the glycolytic pathway might be available from the phosphomonoester region of the ^{31}P spectrum and analysis of the PME/βNTP changes may confirm more completely the changes in "bioenergetic status" obtained with Pi/βNTP.

The Pi/βNTP and the PME/β–NTP ratios were used for estimation of the changes in the bioenergetics and level of hypoxia of Guerin carcinoma tissue induced *in vivo* by Co(III) complexes (AC–30, AC–40, AC–100), Fe (III) complex, and $CoCl_2$ (Fig. 4).

Energy status of Guerin carcinoma upon AC–40 Co(III) complex administration. The levels of β–NTP, Pi and PME in tumor tissue decreased (2.2, 1.5 and 1.8–fold, respectively) in 2 h after injection of

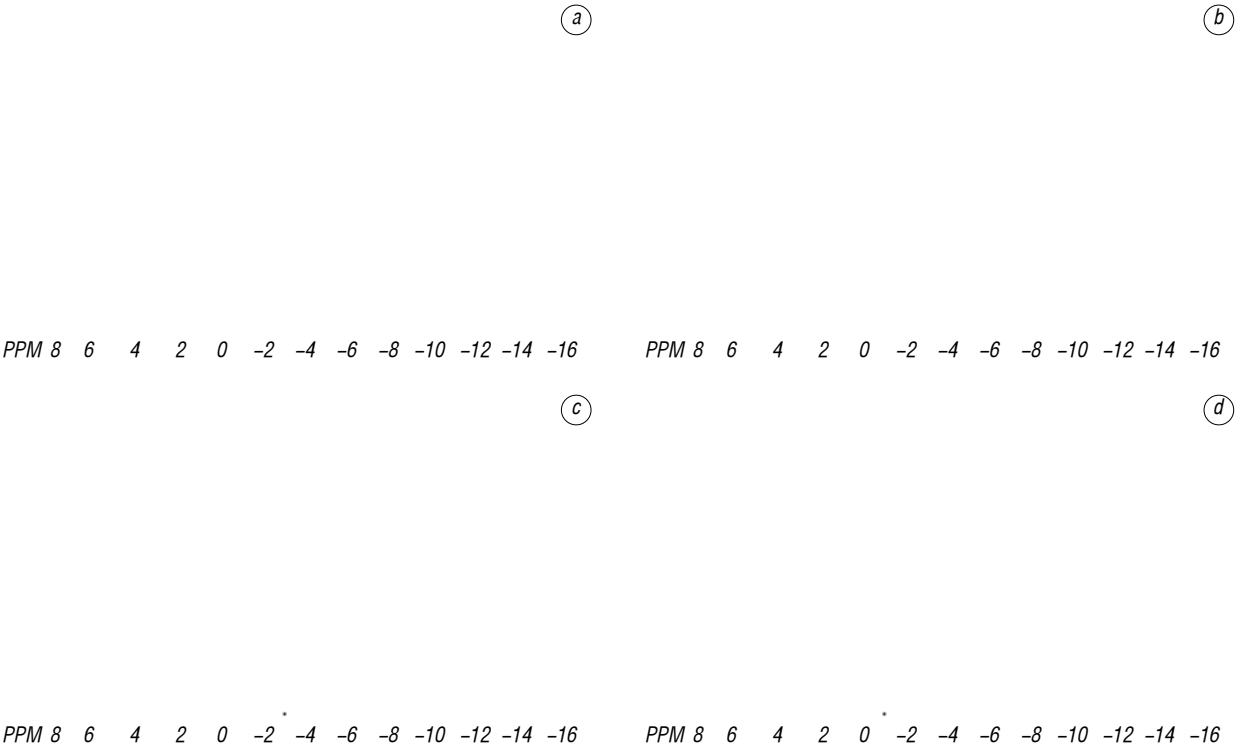

Fig. 3. ^{31}P NMR spectra of PCA extracts of Guerin carcinoma before (a) and in 2 h (b), 6 h (c) and 24 h (d) after administration of complex AC–30

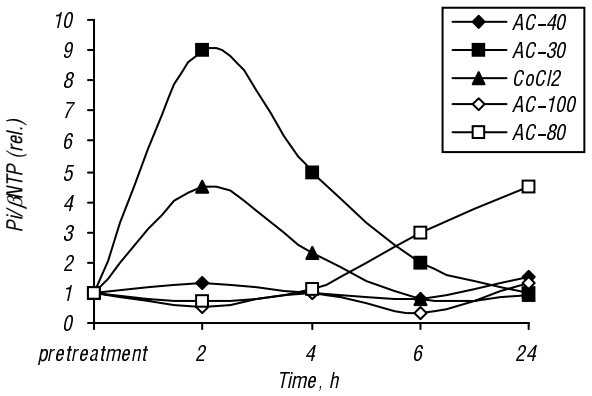

Fig. 4. Time–course of relative changes of metabolite ratios Pi/βNTP after administration of complexes AC–40, AC–30, AC–100, Fe(III) complex and $CoCl_2$

AC–40 complex and increased in 6 h after injection (2.6, 1.4 and 1.45–fold respectively) in comparison with their pre–treatment levels; 24 h after injection, peak resonances for PME and Pi returned almost to the pre–treatment levels whilst β–NTP level returned to the level reached at the 2 h point.

After injection of AC–40 complex, the Pi/βNTP and PME/βNTP ratios increased, reaching maximum values in 2 h after injection by a factor of 1.5 and 1.2, respectively. 6 h after i.v. injection of AC–40 complex, there was approximately a 1.9–fold decrease with respect to the base line both for the Pi/βNTP and PME/βNTP ratios and then in 24 h the Pi/βNTP and the PME/βNTP ratios increased by a factor of 1.7 and 2.3 in comparison with pre–treatment levels.

In muscle tissue the P_i/β–NTP and PME/βNTP ratios remained stable within the observation period with exception for the P_i/β–NTP ratio that decreased (–37% in 6 h), ($p < 0.05$) and almost normalized to the baseline value 24 h after injection.

Those data demonstrated two waves of the decrease in tumor energy status (in 2 h and 24 h after AC–40 injection) and insubstantial increase of the tumor hypoxia fraction.

Energy status of Guerin carcinoma upon AC–30 Co(III) complex administration. Representative ^{31}P MRS spectra of tumor tissues in different intervals after AC–30 injection are shown on Fig. 3.

Upon AC–30 administration, the levels of high–energy phosphates in tumor tissues declined significantly and peak resonances for β–NTP were absent in seven from ten spectra. In the rest of spectra, there was maximal 5.7–fold decrease of the βNTP resonance in 2 h after injection. Concurrent with the initial drop of the β–NTP, there was an increase in the Pi and PME resonances by a factor of 1.6 and 1.3, respectively, in 2 h after injection. 6 h after i.v. injection of AC–30, the β–NTP level returned to 60% of its pre–treatment level (in two of ten spectra peak resonances for β–NTP were absent) and almost returned to the initial level in 24 h. In 6 h after injection 2.0–fold decrease of the PME level was registered; the last one gradually returned to pretreatment level in 24 h (–30%). Pi resonance tended to reach its pre–treatment level within 6 to 24 h.

It should be noted that within the observation period the relative intensity of the peak resonances for PDE compounds involved in biosynthesis and degradation of membrane phospholipids changed similarly to PME.

For DPDE (possibly including UDPG) the 2.7–fold decrease in the relative intensity of the peak resonance in 2 h after injection and then the return to pretreatment levels in 6 h after injection were recorded.

After AC–30 injection, the Pi/βNTP and the PME/βNTP ratios both increased drastically and reached maximum value (9.0–fold and 7.6–fold, respectively) in 2 h after injection; then in 6 h after injection the Pi/βNTP ratio declined close to the pre–treatment levels (+20% higher), whilst PME/βNTP ratio became 28% lower than baseline data; up to 24 h the Pi/βNTP and the PME/βNTP ratios remained stable.

In 2 h after i.v. injection of AC–30 the levels of high–energy phosphates in muscle tissue of Guerin bearing rats changed only marginally. In 6 h the relative intensity of the peak resonances for PME and β–NTP remained at the same levels with the exception for P_i showing 1.9–fold increase.

In 24 h after injection of AC–30, the increase in relative intensity of the peak resonances for PME, P_i and for β–NTP was observed in comparison with pretreatment levels (1.3–, 2.4– and 1.4–fold respectively).

A 40% drop in PCr level in muscle tissue in 6 h after injection is noteworthy, too. Then it remained on the same level for 24 h.

In 2 h after AC–30 injection, the Pi/βNTP ratio did not changed, but it reached maximum value in 6 h (1.8–fold increase). In 24 h after the complex administration, there was no return to pre–treatment value. The PME/βNTP ratio remained stable with marginal changes only.

It is noteworthy that complex AC–30 injection caused significant reduction of energy status of tumor tissue and increase of tumor hypoxia fraction in 2 h after administration. This effect was prolonged and was observed in 24 h with almost full recovery to the pre–treatment levels. At the same time, this phenomenon was also observed in muscle tissue, but in a considerably smaller extent, and was recorded only in 6 h after complex AC–30 injection. Those data allowed to propose that tumor tissue is more sensitive to AC–30 because of its higher hypoxia level and the ability of this complex to enhance the inherent tumor hypoxia. Possibly, the antitumor efficacy of the cobalt complexes results from the reduction of energy status of tumor tissue as well as the increase in tumor hypoxia.

Energy status of Guerin carcinoma upon Fe(III) complex administration. In 2 h after injection of Fe(III) complex the level of β–NTP increased (+62%), the Pi resonance fall (–72%) and PME resonance was almost unchanged in tumor tissue in comparison with pre–treatment level. In 24 h after injection in peak resonances for β–NTP and Pi 2.7– fold decrease and 1.7–fold increase were recorded, respectively, whilst that for PME remained unchanged (+16%).

In 2 h after injection of Fe (III) complex, the Pi/βNTP ratio decreased by a factor of 2.2 and the PME/βNTP

ratio by a factor of 1.8, but in the end of observation period those ratios increased 4.5–fold and 3.0–fold for the P_i/β–NTP and for the PME/βNTP, respectively.

After injection of Fe(III) complex the level of β–NTP remained stable in muscle tissue samples during the observation period; in 24 h the 1.7–fold decrease in Pi resonance was recorded.

During the observation period the P_i/β–NTP and PME/βNTP ratios remained stable changing marginally around the baseline; only the PME/βNTP ratio decreased by a factor of 2.0 in 24 h after injection.

The presented data have shown that the energy status and hypoxia level both of tumor and muscle tissues didn't decrease in 24 h after Fe(III) complex injection. Therefore, according to the ^{31}P NMR spectroscopy data, one may conclude that Fe(III) complex would have no antitumor effect; that statement has been confirmed in experiments *in vivo*.

Energy status of Guerin carcinoma upon Co(III) complex AC–100 administration. In 2 h after AC–100 injection, the levels of β–NTP, Pi and PME in tumor tissue increased by a factor of 3.5, 1.5 and 1.3 respectively, in comparison with pretreatment level and in 6 h remained unaltered. Therefore, in 2 h after AC–100 injection the Pi/βNTP and PME/βNTP ratios decreased by a factor of 2.3 and 2.7, respectively, in comparison with pretreatment levels and then remained nearly constant.

In muscle tissue in 2 h after AC–100 injection the 2.7, 2.1 and 1.5–fold increase in the relative intensity of β–NTP, Pi and PME resonances, respectively, was recorded; the 1.8 and 1.3–fold decrease in the Pi/βNTP and the PME/βNTP ratios, respectively, was recorded.

In 24 h after injection, the P_i/β–NTP and the PME/βNTP ratios remained almost stable and slightly changed similarly to the values shown for tumor tissue without recovery to baseline.

The data obtained indicate that after the AC–100 injection, there was no decrease of the energy status both in tumor or muscle tissues. Moreover, hypoxia levels also were not affected by complex AC–100 administration in those tissues as it was demonstrated by means of the P_i/β–NTP ratio increase. As it was shown by the ^{31}P NMR spectroscopy measurements, complex AC–100 was found to be uneffective for tumor treatment, which was demonstrated independently in experiments *in vivo*.

Energy status of Guerin carcinoma upon $CoCl_2$ solution. For evaluation of the possible effect of Co(II) aquation which may be formed due to metabolism of Co complexes, the effect of a plain Co(II) salt on the energy status both in tumor and muscle tissues was examined, also.

2 h after of $CoCl_2$ injection the level of β–NTP decreased by a factor of 2.0; concurrent with the initial decrease in β–NTP, there was a 2.2–fold increase in both the Pi and PME resonances in tumor tissue in comparison with pre–treatment level. 6 h after i.v. injection of the $CoCl_2$ the level of β–NTP exceeded the baseline by a factor of 2.0 whereas decline in the Pi and PME resonances was registered; the last ones almost returned to the pretreatment levels and remained unchanged to the end of observation. The β–NTP resonance also normalized in 24 h after i.v. injection.

Following injection of the $CoCl_2$ solution the Pi/βNTP and the PME/βNTP ratios increased both by a factor of 4.0, reaching maximum values at 2 h after injection; in 6 h these resonances both declined below the baseline pronounced (–50 and 42% respectively, $p < 0.05$) and at the end of observation they attained steady states close to the baseline.

In muscle tissue the relative intensity of the peak resonances for β–NTP, Pi and PME continuously decreased after injection of the $CoCl_2$ during all observation period, reaching minimum levels at 6 h after $CoCl_2$ administration (3.4–fold for the β–NTP, and 4.0–fold both for Pi and PME decrease).

Both the P_i/βNTP and the PME/βNTP ratios also changed insignificantly declining transiently since 2 h to the end of the observation; in 24 h they remained stable within the reduced values (–20 and 15% respectively), and there were no return to pretreatment values.

The data obtained has indicated that there was the definite depression of tumor energy status. NMR data point also on the increase of tumor hypoxia fraction in 2 h after injection of the $CoCl_2$ solution but it was of very short duration. There results have shown the definite role of Coion in realization of biological effects of coordination cobalt(III) compounds such as complexes AC–30 and AC–40. Taking into account the literature data, such effect could be anticipated.

The significance of ^{31}P NMR as an indicator of tumor oxygenation. Among tumor microenvironmental factors (i.e. metabolic and energetic status and pH distribution) tumor hypoxia is considered as one of the multifactorial causes of tumor treatment resistance. Experimental and clinical evidence suggests that the hypoxic fraction in solid tumors may influence their growth, increase malignant progression, enhance metastatic potential, and reduce sensitivity to conventional treatment modalities [19].

Previous studies have indicated a relation between tumor oxygenation and metabolic status. Okunieff et al. [6] noted that the increase in the hypoxic fraction occurs in parallel to the decrease in high–energy phosphates. Rofstad et al. [15] noted a linear correlation between bioenergetic status and hemoglobin oxygen saturation in three tumor models, indicating a relationship between high energy phosphates and tumor oxygen supply. For the different tumor models this relation was not identical, indicating that intrinsic cellular properties of different tumor models influence tumor hypoxia and energetic status. Vaupel et al**.** [13] noted a significant correlation between the partial pressure of oxygen (measured by oxygen electrodes and NMR–measured metabolite ratios) and concluded that NMR could be used for detection of the changes in tumor energetics induced by changes in tumor oxygenation. In the study of EMT–6 murine tumor, Fu et al. [17] noted significant correlations between hypoxic fraction and metabolic ratios measured by ^{31}P NMR spectroscopy.

It has been found that signal intensity ratios such as Pi/PCr, Pi/вNTP, Pi/ (PCr + вNTP), or related forms are sensitive to acute alterations in tumor oxygen tension produced by regional occlusion [20] and the alterations in the ratios correlate with tumor oxygenation [12, 12].

Such changes in ^{31}P MRS signal ratios have been reported in numerous studies suggesting that spectral examination of tumors may provide a method of measurement of the chronic oxygenation state of tumors [18–21]. Since the hypoxic fraction of a tumor is also influenced by oxygenation status one would expect a correlation between ^{31}P MRS measurements and hypoxic fraction of the tumor. Taking to account the tumor volume, in the present study the Pi/βNTP ratio has been chosen as the most reliable parameter for tumor bioenergetic status as well as for tumor hypoxia level.

In this study ^{31}P NMR spectroscopy was used to examine the state of Guerin carcinoma oxygenation after administration of Co(III) complexes: AC–40, AC–30, AC–100 as well as Fe(III) complex and $CoCl_2$ applied *in vivo* (see Fig. 4).

Examination of the ^{31}P NMR spectra of the PCA extraction of tumor tissue have shown the ability of some complexes to elevate the tumor hypoxia level.

Administration of some Co(III) complexes (AC–40, AC–100) and Fe (III) complex didn't cause any substantial increase in the Pi/βNTP ratio neither in tumor nor in muscle tissue. The 4.5–fold increase in this ratio in tumor tissue was registered after $CoCl_2$ and Fe(III) complex injections (in 2 and in 24 h respectively). The administration of AC–30 complex induced 9.0–fold increase of the Pi/βNTP ratio in 2 h.

It may serve as the convincing evidence of the elevated hypoxia level in tumor tissue. At the same time in muscle tissue the maximum increase of the Pi/bNTP ratio was observed in 6 h and it was much lower than that in tumor tissue. Those data pointed to the assumption that these complexes possess some selectivity with respect to tumor tissue and have time–dependent effect on tumor and muscle tissues. This assumption was confirmed in experiments *in vivo* with complex AC–30 which manifested the highest antitumor activities in experiments *in vivo* [22, 23].

It is noteworthy that neither complexes AC–40, AC–100 nor Fe(III) complex could affect the tumor oxygenation and they are much less effective as anticancer agents.

In this study the investigation has been carried out on the tumors with the definite size. It was demonstrated by means of ^{31}P NMR spectroscopy that in Guerin carcinoma of the studied volume the hypoxic fraction is present and is significantly increased after treatment of experimental animals with complex AC–30. Our data indicated that under the hypoxic condition complexes have toxic activity and are able to enhance such condition by themselves.

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