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SODIUM-L-ARGININE SUCCINATE ENHANCES CISPLATIN ANTI-TUMOR ACTIVITY IN P388 LYMPHOCYTAL LEUKEMIA MODEL IN MICE

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Abstract: Low effectiveness of antitumor therapy is one of basic problems in oncology. Besides searching for novel antitumor medicines there is another important vector of this trend for the treatment of oncologic patients namely intensification of existing medicines' effectiveness without changing their safety profile. One of possible approaches to this goal is combining cytostatics with angioprotecting medicines — nitric oxide donors. The present study focuses at plausible change of Cisplatin effectiveness and safety profile on the background of nitric oxide donor — Sodium-L-Arginine Succinate administration. Murine P388 Lymphocytal Leukemia was used as oncogenesis model. The study involved 6 month old male CDF_1 mice (BALB/C female × DBA/2 male). Cisplatin was used as anti-tumor medicine, it was administered once 48 hours after tumor transplantation 8 mg/kg. The animal groups involved with maintaining therapy in addition to cytostatic one also got 12.5 ml/kg of Sodium-L-Arginine Succinate (NAS) solution. The results of the study point out at statistically significant increase of Cisplatin antitumor activity in case of combination with NAS: extended tumor development latent period and lifespan of tumor-carriers. The toxic effect of cytostatic therapy upon vascular endothelium proved to be evened-out by NAS: nitric oxide synthesis increased parallel to endothelin-1 production decrease. The study gives grounds to consider NAS a promising participant in combination with Platinum-containing antitumor drugs in prophylaxis and treatment of their toxic effects upon circulatory system and possibly also as a medicine boosting their effectiveness without increasing negative side-effects' rate.

Key words: Cisplatin, nitric oxide, arginine, vasodilation, P-388 leukemia, Sodium-L-arginine succinate

НАТРИЯ-L-АРГИНИНА СУКЦИНАТ ПОВЫШАЕТ АНТИОПУХОЛЕВУЮ АКТИВНОСТЬ ЦИСПЛАТИНА В МОДЕЛИ МЫШИНОЙ ЛИМФОЛЕЙКОМЫ Р388

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Резюме: Низкая эффективность антиопухолевой терапии — одна из основных проблем в онкологии. Наряду с поиском новых антиопухолевых лекарств имеется еще один важный вектор этого направления, а именно поиск средств для повышения эффективности уже существующих антиопухолевых препаратов без изменения их

профиля безопасности. Один из возможных подходов для достижения этой цели — комбинация цитостатиков и ангиопротекторов, — доноров окисида азота. Настоящее исследование нацелено на возможность изменения эффективности и профиля безопасности цисплатина на фоне донора оксида азота, натрия-L-аргинина сукцината. В качестве модели опухолевой патологии использовали мышиную лимфолейкому Р388. В исследовании использовали 6-месячных мышей-самцов CDF_1 (BALB/C female × DBA/2 male). В качестве антиопухолевого препарата выступал Цисплатин; его вводили однократно в дозе 8 мг/кг через 48 часов после трансплантации опухоли. Основные подопытные группы животных в дополнение к цитостатику также получали 12.5 мл/кг раствора Натрия-L-аргинина сукцината (НАС). Результаты исследования указывают на статистически достоверное увеличение антиопухолевого эффекта Цисплатина в случае комбинации с НАС: зарегистрировано удлинение латентного периода развития опухоли и увеличение продолжительности жизни животных опухоленосителей. НАС уменьшал токсическое действие цитотоксической терапии на сосудистый эндотелий: параллельно с уменьшением продукции эндотелина-1 увеличивался синтез оксида азота. Исследование дает основание для того, чтобы рассматривать НАС в качестве многообещающего участника в комбинации с платина-содержащими лекарствами для профилактики и лечения их токсических эффектов на сосудистую систему, а возможно, также в качестве усилителей их эффективности без повышения величины отрицательных побочных эффектов.

Ключевые слова: Цисплатин, оксид азота, аргинин, вазодилатация, лейкома Р-388, Натрия-L-аргинина сукцинат

INTRODUCTION

Dozens of new anti-tumor agents and treatment routines appear every year in the world. The leading chemotherapy trend is concerned with increased specificity of its influence upon tumor cells and associated stromal elements side by side with minimization of systemic effects upon various organs and tissues that are not directly involved in the pathologic process. However various complications of chemotherapy are still considered to be a wide-spread phenomenon in clinical practice responsible for strict limitations of treatment tactics and worsening the disease prognosis.

Circulatory lesions namely endothelial dysfunctions constitute a considerable part of anti-tumor-therapy complications [1,2,3]. Taking into consideration the role of blood vessels in all physiological and pathological processes elaboration of new medicines for correction of microcirculatory disorders is a very attractive goal for pharmacologists. However, before introducing angioprotectors into clinical practice one should compare the expected benefits of their use with serious risks caused by profound integration of circulatory system into oncogenesis pathogeny.

The important role of blood vessels in the development of malignant tumors is caused by their metabolic and migrational needs. In spite of their ability to survive without adequate oxygen and nutrients supply providing macroergic molecules' synthesis through anaerobic energy-providing mechanisms' activation any tumor cell still needs oxygen and substrates [4]. Invasion of lymphatic and blood vessels with tumor cells and intensive neoangiogenesis create the necessary prerequisites for activation of tumor cells metastatic dissemination with biological fluids flow.

Malignant neoplasms growth inhibition through angiogenesis inducing factors and their receptors block (or direct destruction of microvessels wall) concept was initially proposed by Moses Judah Folkman in 1971 [5]. Nowadays the number of known angiogenesis inhibitors is over one thousand and many of them are used as medicines in combination with traditional cytostatic therapy because of their ability to cope with tumors' multiple drug resistance mechanisms [6,7].

However, neoangiogenesis mechanisms, stages and structural and functional peculiarities of newly-formed blood vessels that provide tumor tissue blood-supply are still under close scrutiny and most of these phenomena aspects and consequences for anti-tumor therapy are yet to be discovered. In spite of considerable scope of studies and detailed pathogenial substantiation the use of antiagiogenic drugs in oncology is still limited and in some cases is empiric in nature. Some researchers also point out target angiogenic therapy with simultaneous increase of chemotherapy toxicity low effectiveness in case of certain tumors [8], reduction of relapse-free period, increase of tumor growth rate and their invasive and metastatic potential [9,10]. Apparently these issues require more profound scrutiny.

Wide introduction of target antiangiogenic therapy in everyday practice is also hindered by possible decrease of basic cytostatic therapy effectiveness through limiting anti-tumor drug access to tumor cells due to impairment or destruction of microcirculatory network in the tumor and peritumorose area. It is this limitation that is the most probable basis for the studies of combined application of cytostatics and maintaining therapy aimed at endothelial dysfunction and hemostasis impairment correction and general angioprotection.

Exogenous nitric oxide (NO) donors namely L-arginine derivates constitute a promising pharmacologic group in this maintaining therapy trend. The researchers' interest towards arginine is mostly explained by its precursor role in nitric oxide synthesis [11]. Nitric oxide is produced by attaching molecular oxygen to

L-arginine guanidine group resulting in NO and L-citrulline formation [12].

NO is one of mediators participating in many a physiologic function realization primarily vascular tone and transcapillary exchange regulation. NO effects are due to its activation effect upon soluble (cytosole) guanilate-cyclase haem group. This enzyme synthesizes cyclic guanosine monophosphate (cGMP) controlling membrane ionic channels, protein phosphorillation and phosphodiesterase activity [13]. In smooth-muscle cells of blood vessels NO-induced cGMP buildup causes miocyte relaxation and consequently vasodilatation, increased blood vessels' permeability and transcapillary exchange [12].

Nowadays a great amount of data is accumulated explaining the role of NO in oncogenesis [14,15,16], direct effect of increased NO concentration upon tumor cells [17,18] and combined effects of NO and traditional cytostatics for overcoming neoplasms' multiple drug-resistance phenomenon [19,20,21]. However contradictory nature of results of the study does not allow a simple conclusion of advisability vs unreasonableness of nitric monoxide donors' inclusion into tumor therapy routines and demands an extension of the studies.

Taking all his into consideration we deemed it intriguing to accomplish studies of Cisplatine, L-arginine and succine acid combination demonstrating pronounced endothelium-protecting effect and contributing to nitric monoxide production in preliminary studies.

2. MATERIALS AND METHODS

2.1. Animals.

All experiments involving animals were carried out in accordance with ethical principles regulated by active Russian legislation and European Convention for Protection of Vertebral Animals Used for Experimental and Other Scientific Purposes. Strasbourg. 18.III.1986. European Treaty Series — № 123.

The study involved 6-month-old male CDF_1 mice (BALB/C female × DBA/2 male). The animals were kept at standard conditions of vivarium, 5–6 in a cage with free access to food and drinking water. The light conditions were 12 hours of light and 12 hours of darkness. During the entire study the animals were examined on a daily basis with assessment of their behavior, appetite, body mass, hair condition, activity. The animals were euthanatized inside a hermetic box by inhalation of diethyl ether.

2.2. Tumor models.

Murine P388 Lymphocytal Leukemia strain obtained from Cancerogenesis and Ageing Laboratory of The Oncologic Institute named after N.N. Petrov was used as neoplasm model. P388 Leukemia is frequently used in studies of malignant lymphoid tumors [22,23,24]. It has a high invasive potential and a tendency to generalization since the first day after transplantation.

Neoplasm model was realized through direct transplantation of malignant cells from tumor-carrying animals. Solid variant of P388 Leukemia was modeled by subcutaneous injection of 10^9 leukemic cells per animal diluted in 0.2 ml of 0.9 % NaCl solution into the right flank of the animal, whereas the ascitic one — by means of intraperitoneal infusion of the same amount of tumor cells.

2.3. Chemicals and Medicines.

Cisplatin (CP) (Cisplatin-Ebeve, Ebeve Pharma, Austria) was used as anti-tumor medicine.

For maintaining therapy (MT) a 14 g/l isotonic solution of Sodium-L-Arginine Succinate ($Na^+[NH=C(NH_2)NH(CH_2)_3CH(NH_2)COOH] + [OOC(CH_2)_2COO]^{2-}$) balanced to blood plasma regarding Sodium ions (147 mmol), Potassium (4 mmol), Chlorides (109 mmol) and Magnesium (1.26 mmol), with physiologic value of hydrogenium index that places no limitations in its application.

2.4. Experimental groups.

120 CDF_1 male mice were randomly divided into 7 groups. The first one (Control, n = 6) was used for yielding endothelial markers physiological parameters. The animals of groups 2 — 4 were used for modeling P388 leukemia solid variant (S), while those from groups 5 — 7 — the ascitic one (A). Groups 2 ($P388_S$, n = 22) and 5 ($P388_A$, n = 16) were used for studying oncogenesis and endothelial markers' dynamics without additional influence. Mice from groups 3 ($P388_S$ + CP, n = 22) and 6 ($P388_A$ + CP, n = 16) 48 hours after tumor transplantation started getting Cisplatin (8 mg/kg). Mice from groups 4 ($P388_S$ + CP + NAS, n = 22) and 7 ($P388_A$ + CP + NAS, n = 16) 48 hours after tumor transplantation started getting Cisplatin (8 mg/kg) and also in one hour after tumor transplantation Sodium-L-Arginine Succinate solution (12.5 ml/kg).

2.5. P388 Leukemia development assessment.

The following parameters of growth and development of P388 Leukemia were studied:

1. Term of solid tumor node emergence (days).
2. Dynamics of solid tumor node growth (three perpendicular sizes; mm^3). Measurements were made on the 10^{th}, 14^{th}, 22^{nd} and 26^{th} day after tumor transplantation.
3. Tumor growth inhibition (TGI): TGI (%) = [Vcontrol - Vexperiment)/Vcontrol]*100%, where V — size of the tumor (mm^3). Clinically substantial level is over 50%.
4. Mean life span (MLS) of experimental animals (days).
5. Life span extension (LSE): LSE = [(MLS(experiment) — MLS(control)]/ MLS(control)*100%.). Clinically substantial level is over 25%.

2.6. Assessment of endothelium condition markers' dynamics.

Using Sodium-L-Arginine Succinate as nitric oxide donor necessitates the assessment of its influence upon metabolism of NO and its antagonist Endothelin-1. These parameters were measured with the aid of "Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit, R&D Systems" and "Mouse Endothelin 1, ET-1 ELISA Kit, Cusabio" commercial kits on the 5^{th} and 15^{th} day in mice with ascitic form of the tumor.

2.7. Statistical analysis.

The results were statistically analyzed using SPSS software. All data obtained directly are presented as M ± SD. The distribution pattern was audited by means of Cholmogorov-Smirnov

criterion. The independent samples' means were compared with the aid of Student's t-criterion (in case of normal distribution) or Mann-Whitney U-criterion (in case of distribution different from normal). The mean values of dependent samplings were compared using Friedman's χ^2-criterion. Correlation analysis was carried out using Pierson's criterion. Precise confidence intervals (CI) were calculated with the aid of Clopper-Pierson method using CONFINT software. Probability over 95% was considered to be a valid level of difference ($p < 0.05$).

3. RESULTS.

3.1. The influence of Cisplatin and Sodium-L-Arginine Succinate combination upon the development of P388 leukemia solid form

Subcutaneous transplantation of P388 leukemia proved to be successful in 100% of the cases. Primary node was revealed on day 9.8 ± 3.62 after transplantation. Cytostatic therapy reliably increased this latent period by 3.9 days ($p = 0.029$). Combined Cisplantin and Sodium-L-Arginine Succinate (NAS) therapy increased it even more — by 4.9 days ($p = 0.011$). However there was no statistically significant difference between groups "$P388_s$ + CP" and "$P388_s$ + CP + NAS" ($p = 0.494$; fig. 1).

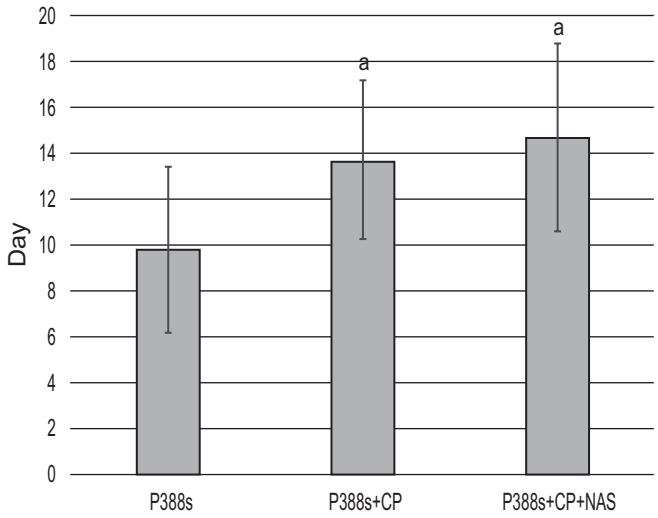

Fig. 1. Term of primary P388 leukemia node debut (days) after transplantation in mice with solid form of neoplasm growth. a — difference from "$P388_s$" group is valid ($p < 0,05$)

Strong correlation was revealed between the term of solid P388 leukemia node revelation and the tempo of the tumor growth ($r = -0.909$, $p < 0.001$). Experimental therapy of the neoplasm with Cisplatin as well as with Cisplatin and NAS combination has been producing a reliable clinically significant effect upon this parameter throughout the entire experiment (table 1).

The increase of the tumor development latent period in mice of groups "$P388_s$ + CP" and "$P388_s$ + CP + NAS" has predictably caused a valid increase of mean lifespan of these animals (fig. 2).

Direct correlation was revealed between the term of the tumor node debut and life span of mice with P388 leukemia ($r = -0.940$; $p < 0.001$). Adding NAS to cytotoxic therapy "$P388_s$ + CP + NAS" resulted in additional 6.2 day increase of this parameter in comparison with "$P388_s$ + CP" group ($p = 0.028$).

Table1

Influence of Sodium-L-Arginine Succinate upon the inhibition of P388 leukemia solid node on the background of cytostatic Cisplatin therapy

Group	Term after P388 leukemia transplantation (days)	TGI, %
$P388_s$	---	---
$P388_s$ + CP	14	64,1*
	18	69,7*
	22	54,2*
$P388_s$ + CP + NAS	14	69,4*
	18	74,7*
	22	54,3*

Commentary: TGI — tumor growth inhibition, * — clinically significant level (> 50%). Assessment of tumor growth inhibition on the 10th and 26th day after tumor transplantation was not made because of insufficient amount of observations on these days.

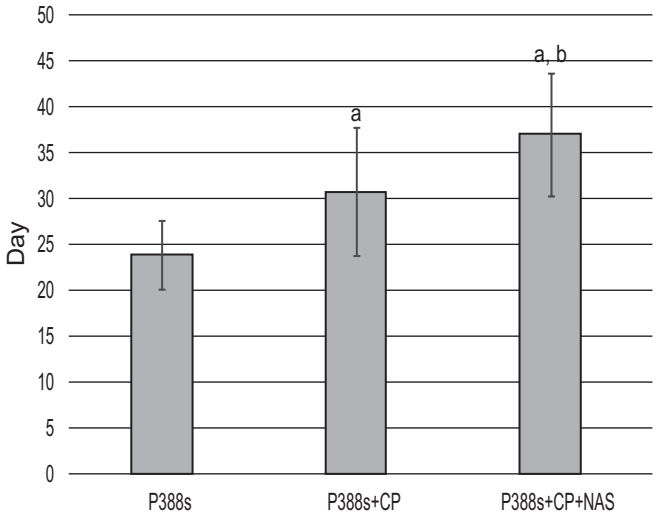

Fig. 2. Mean lifespan of mice with P388 leukemia (solid form of neoplasm growth). a — difference from "$P388_s$" group is valid ($p < 0,05$), b — difference from "$P388_s$ + CP" group is valid ($p < 0,05$)

3.2. The effect of Cisplatin and Sodium-L-Arginine Succinate combination upon the development of P388 leukemia ascitic form

The development of P388 leukemia ascetic form on the background of Cisplatin and Sodium-L-Arginine Succinate combination therapy was similar to that of the tumor solid variant (fig. 3).

Therapy of P388 leukemia with Cisplatin increased mean lifespan of mice with ascetic form by 5.6 days ($p = 0.024$) while combination therapy with Cisplatin and NAS increased this parameter even more — by 12.4 days (225%; $p < 0.001$) in com-

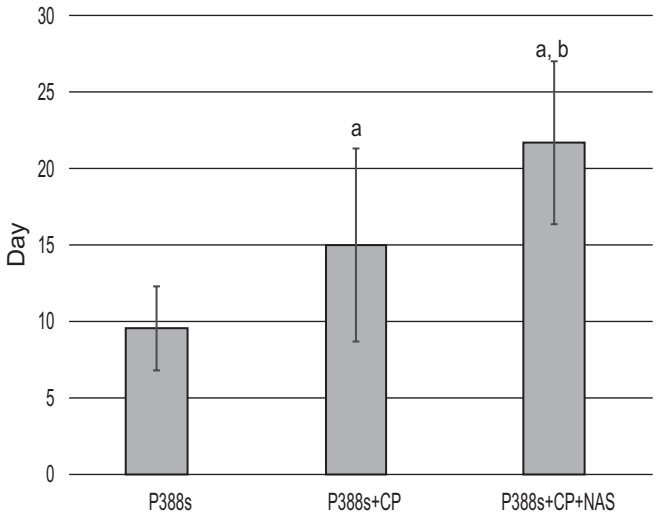

Fig. 3. Mean lifespan of mice with P388 leukemia (ascitic variant of neoplasm growth). a — difference from "$P388_a$" group is valid ($p < 0,05$), b — difference from "$P388_a$ + CP" group is valid ($p < 0,05$)

parison to group "P388a" and by 6.8 days (144%; $p = 0.023$) in comparison to group «P388a + CP».

3.3. The effect of Cisplatin and Sodium-L-Arginine Succinate combination upon the state of blood vessels' endothelium in mice with P388 leukemia

The development of P388 leukemia solid form caused moderate gradual increase of NO blood concentration (fig. 4). This parameter on the 5th and 15th day was found to have increased by 4.4 μmol/l ($p = 0.631$) and 12.5 μmol/l ($p = 0.128$) in comparison to the control values. Therapy with Cisplatin caused a pronounced tendency to NO production inhibition on the 5th day of the experiment (fig. 4).

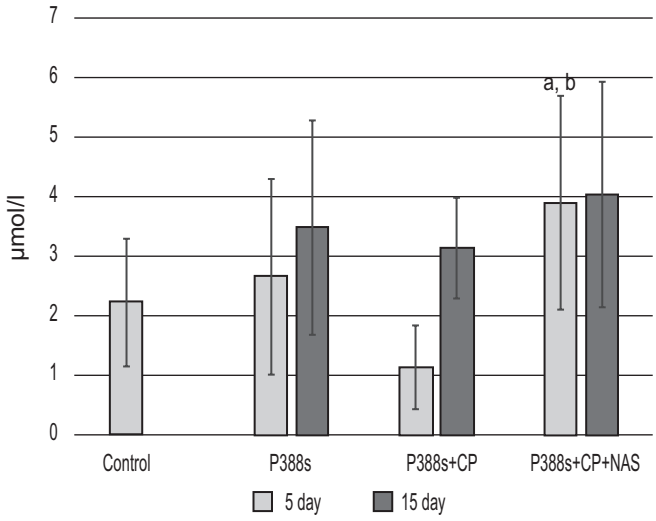

Fig. 4. NO blood concentration changes in mice with P388 leukemia (solid form of neoplasm growth). a — difference from "control" group is valid ($p < 0,05$), b — difference from "$P388_s$ + CP" group is valid ($p < 0,05$)

Adding NAS to cytotoxic therapy reversed this process, i.e. NO level in blood of "$P388_s$ + CP + NAS" group significantly (by 16.4 μmol/l) surpassed that of intact mice and was 27.7 μmol/l higher than in mice of "$P388_s$ + CP" group ($p = 0.010$).

Analogous tendency of NO blood concentration changes has been revealed in case of P388 leukemia ascitic form development (fig. 5).

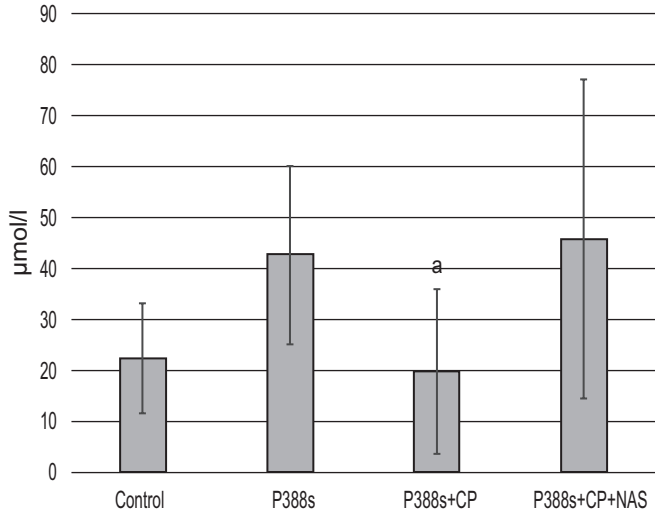

Fig. 5. NO blood concentration changes in mice with P388 leukemia (ascitic form of neoplasm growth). a — difference from "$P388_a$" group is valid ($p < 0,05$)

Increased NO production in mice with transplanted P388 leukemia obviously affected endothelin-1 concentration changes (fig. 6–7).

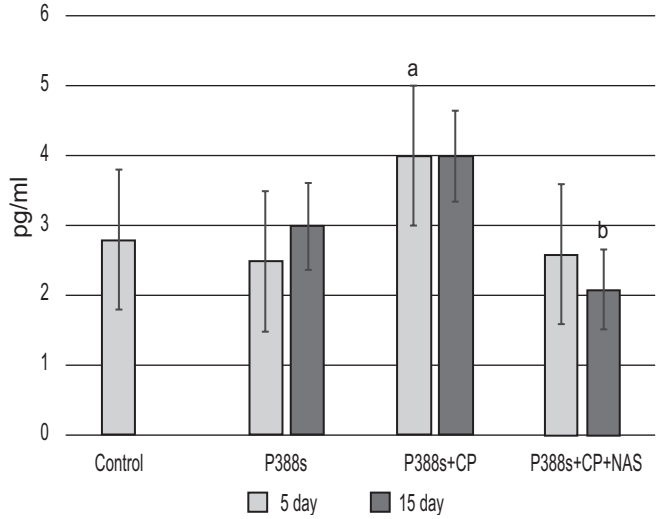

Fig. 6. Endothelin-1 blood concentration changes in mice with P388 leukemia (solid form of neoplasm growth). a — difference from "$P388_s$" group is valid ($p < 0,05$), b — difference from "$P388_s$ + CP" group is valid ($p < 0,05$)

Mice with P388 leukemia solid form demonstrated maximum endothelin-1 concentration in the group treated with Cisplatin. On the 5th day this parameter in group "$P388_s$ + CP" was significantly

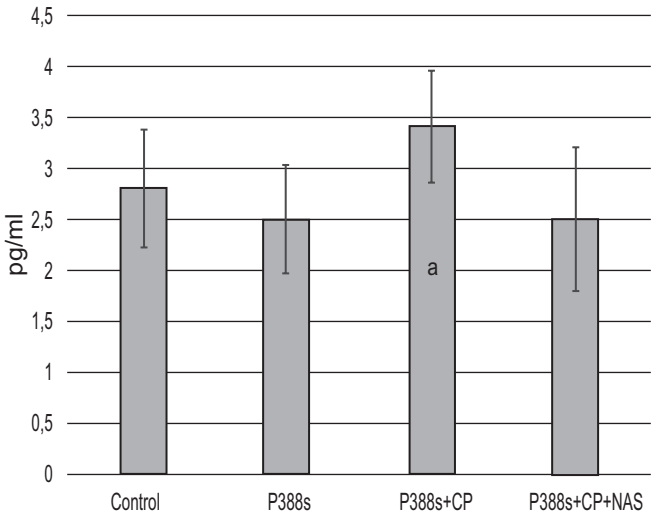

Fig. 7. Endothelin-1 blood concentration changes in mice with P388 leukemia (ascitic form of neoplasm growth).

higher than in mice with tumor not getting cytostatic therapy by 1.2 pg/ml ($p = 0.041$) and it stayed increased during the entire experiment (fig. 6). Endothelin-1 blood concentration in mice with P388 leukemia ascitic variant proved not to change significantly (fig. 7).

Adding maintaining therapy with NAS caused a critical drop of Endothelin-1 production (fig. 6). On the 15th day of the experiment Endothelin-1 level in "$P388_s$ + CP + NAS" group was lower than in group "$P388_s$ + CP" by 1.9 pg/ml ($p = 0.013$).

4. DISCUSSION

The major goal of the study was to ascertain whether it was possible to combine Cysplatin with Sodium-L-Arginine Succinate — nitric oxide donor and a potential instrument of maintaining therapy during the development of vascular pathology in oncologic patients.

Cisplatin was selected for cytostatic therapy because of its sufficient effectiveness against P388 leukemia proved in multiple studies [25,26,27]. Besides Cisplatin is known to produce a distinct toxic effect upon vascular endothelium [28,29], thus making our antitumor therapy model relevant for the assessment of drugs with possible angioprotective effect.

Sodium-L-Arginine Succinate (NAS) is an active combination of arginine and succine acid in isotonic solution. The influence of NAS upon vascular endothelium is of utmost interest. L-arginine is well-known to be a natural substrate for NO production. This reaction is controlled by an NO-synthases family including this enzyme's three major isoforms: the neuronal, macrophagal (inducible) and endothelial ones. The neuronal and endothelial synthases permanently present within the cells are Ca^{2+}-dependant and can synthesize relatively minor amount of NO while the macrophagal NO-synthase activated by specific exposure (e.g. by lipopolisacharides or cytokines) can contribute to comparatively high NO yield [12]. NO as a signaling molecule is known to rapidly diffuse through cytoplasmatic membrane into intercellular space and easily permeate target cells; this process is not receptor-mediated. However high reactivity of NO does limit its half-life period to 1–5 seconds and its possible diffusion distance is very short (25–35 μm) [30,31]. Therefore NO blood concentration is normally permanent enough mostly thanks to endothelial synthase.

In the present study we have revealed a moderate tendency to NO production increase during P388 leukemia development which might be a sign of major endotheliocytes' and paravasal cells' role in the development of this tumor that corresponds to previous studies [32,33]. The treatment of P388 leukemia with Cisplatin was successful: in groups of mice that received cytotoxic therapy the neoplasm-forming latent period increased and the tumor growth slowed down increasing the life-span of tumor carriers. Cisplatin was shown to produce a substantial effect upon blood vessels' endothelium significantly shifting balance of vasodilating and vasoconstrictive influences in favor of vasoconstriction — decreased NO production and increased endothelin-1 synthesis (fig. 4–7).

Adding NAS to experimental anti-tumor therapy resulted in boosting anti-tumor effect of Cisplatin: there was a statistically significant extension of tumor development latent period as well as mean lifespan both in case of P388 leukemia solid form (less active) and quickly developing primarily generalized ascitic variant. The use of NO donating NAS as a tool for maintaining therapy contributed to evening-out of Cisplatin effect upon vascular endothelium. Moreover, NAS was shown to significantly boost NO production not only in comparison to mice that had been getting Cisplatin but also in comparison to intact animals (fig. 4). Administration of NAS also proved to inhibit endothelin-1 synthesis (fig. 6) thus additionally testifying to its favorable effect on vascular endothelium functioning.

We did not initially plan to ascertain precise mechanisms of registered Cisplatin antitumor activity changes in case of its combination with NAS. However we would like to suggest a few of plausible variants in the context of scientific discussion.

Firstly the observed increase of Cisplatin effectiveness in case of combination with NAS may be due to augmented access of the cytostatic to tumor cells. Blood vessels associated with tumor are known to possess considerable morphological and functional peculiarities in comparison to vessels of intact tissue: incomplete coating of the inner surface with endotheliocytes, insufficient basal membrane, varying diameter, chaotic disposition and crimpiness of blood vessels. In many a case blood supply of the tumor may be realized through primitive vessel-like channels lacking endothelium and directly connected to circulatory system — so called "vasculogenic mimicry" of neoplasm phenomenon [34, 35, 36]. In combination with observed hemostasis system activation and consequent tumorigenic trombotic and tromboembolic complications it might upset the access of antitumor drugs to neoplasm and decrease their effectiveness. On this background vasodilation caused by exogenous NO donor administration may increase perfusion of the entire tumor node and augment cytostatic access to target cells.

Another plausible explanation may be in modification of immune system functioning on the background of NAS administra-

tion due to evening-out of Cisplatin immunotoxic effect, modification of tumor cells metabolism producing better conditions for cytostatic influences, direct modifying effect of Cisplatin through chemical bonds with NAS components or a combination of all these mechanisms. All these issues require additional studies and mechanisms of observed antitumor effects of Cisplatin in combination with NAS are not evident at present.

5. CONFLICT OF INTERESTS.

Conflicts of interest: none.

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