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COMPARATIVE STUDY OF ANTITUMOR EFFECT OF PRISTINE C_{60} FULLERENES AND DOXORUBICIN

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The purpose of this study was mainly focused on the technology of application of C_{60} fullerenes in the combined therapy with doxorubicin for treatment of malignant tumors. Growth experiments of transplanted malignant tumors in the presence of pristine C_{60} fullerenes, doxorubicin and C_{60} in combination with doxorubicin were performed on groups of mice. It was found that C_{60} in combination with doxorubicin injection efficiently inhibits the growth of transplanted malignant tumor as well as metastases of Lewis lung carcinoma. The findings demonstrate the possibility of using C_{60} in combination with doxorubicin in anticancer therapy.

Key words: C_{60} fullerene; doxorubicin; Lewis lung carcinoma; tumor growth inhibition; increasing of animal life span; metastasis inhibition index.

Cancer remains a major cause of premature mortality [1]. This stimulates development of new effective medications capable of selectively inhibit growth and proliferation of tumor cells. One of such drugs is an anthracycline antibiotic, doxorubicin (Dox) [2, 3]. Its molecule is a complex heterocyclic compound, poorly soluble in water and weakly polar organic solvents. However, Dox is able to form inclusion compounds with the DNA molecules and this determines the mechanism of its impact on cancer cells. Intercalation of the DNA molecules with Dox (insertion between adjacent pairs of complementary nucleotides) interferes with the ability of cancer cells to reproduce the genetic information. On the other hand, Dox is capable of producing pathological side effects, primarily a negative impact on the functioning of heart [2, 4]. In this regard, there is a need to develop alternative methods of tumors treatment and to find new substances, which act locally in tumor, causing its destruction. One of the mechanisms of such destruction is the stimulation of tumor cells death by necrosis or apoptosis with preserving of normal cells viability. Biologically active nontoxic C_{60} fullerenes [5–7], which are able to penetrate through the membrane of cells [8, 9] and have strong antioxidant and antiviral properties [10, 11], could be used for preventing the growth of malignant neoplasms. In fact, some fullerene derivatives have shown promising anticancer activity [12]. Namely, Murugesan et al. [13] have demonstrated the substantial antiangiogenic activity of C_{60} fullerenes against either basic fibroblast growth factor- or vascular endothelial growth factor-induced angiogenesis in the chick chlorioallantoic membrane model. Meng et al. [14] have reported that fullerene derivatives are capable to regulate in low levels simultaneously more than 10 angiogenic factors in the mRNA level that is further confirmed at the protein level. This result indicates that fullerene-containing materials serve as a potent antiangiogenesis inhibitor that can simultaneously target multiple angiogenic factors. Yin et al. [15, 16] found that metallofullerene nanoparticles penetrate plasma membrane of tumor cells, effectively inhibit their proliferation and decrease the activities of those enzymes which are responsible for catalyzing the production of reactive oxygen species *in vivo*. In addition, the effect of amino-acid derivatives of C_{60} fullerene in combination with anticancer drug adriamicin as modifiers of biological reactions in the treatment of metastatic tumors was studied *in vivo* [17]. It was found that amino-acid derivatives of C_{60} fullerene penetrate the lipid bilayer of biological membrane and are the effective inhibitors of lipid peroxidation. The maximum

therapeutic effect of the amino-acid derivatives of C_{60} fullerene use (dose 50 mg/kg) in combination with adriamicin (in dose 1 mg/kg) was 40% for the index of metastases inhibition. Within the chemically-induced model of breast cancer the authors [18] investigated the possible protective role of fullerenol to the action of antitumor drug Dox, which caused pulmonary toxicity in rats. These results clearly indicate that the using only Dox (in dose 8 mg/kg) significantly impairs lung function, but preliminary injection of fullerenol (in dose 100 mg/kg; 30 min before Dox) prevents its toxic effect through the inhibition of oxidative stress.

Considering the importance of using C_{60} fullerenes for the cancer treatment [19], the purpose of this work was mainly focused on comparative analysis of antitumor effect of pristine (unmodified) water-soluble C_{60} fullerenes and Dox. Moreover, we propose the technology of application of C_{60} fullerenes in the combined chemotherapy with Dox for treatment of malignant tumors.

Materials and Methods

The C_{60} fullerene aqueous solution (C_{60}FAS) with maximum concentration of C_{60} 1.0 mg/ml used for the experiments was prepared as follows [20]. Theoretical calculations [21] showed that this C_{60}FAS contains both single C_{60} molecules and their clusters (with sizes of about 0.7–4 nm depending of C_{60} fullerene concentration) in the hydrated state. The STM images of submonolayer C_{60} fullerene film deposited from aqueous solution on Au(111) surface demonstrate both C_{60} fullerene clusters with sizes up to ~2.8 nm (the first stable sphere-like cluster consisting of 13 hydrated C_{60} fullerenes [21]) and single C_{60} molecules [22]. Moreover, C_{60} fullerenes structure the water, absorbed by DNA molecules [23], and thus they can affect the DNA functioning in the biosystem. Finally, it is important to note that used C_{60}FAS does not show a cytotoxic effect with respect to both normal and transformed cells at concentrations below 1.0 mg/ml [6].

The male mice of C57Bl/6J line (20–21 g weight) were kept in a vivarium on a standard diet. The average temperature in a vivarium was 25±1 °C. We have to mention that in all experiments performed in the present work we followed the international principles of European Convention for protection of vertebrate animals.

Tumor transplantation (Lewis lung carcinoma) was performed by intramuscular injection to the animal's limb (initial number of tumor cells was equal to ~$5 \cdot 10^5$; antitumor effect, Experiment 2) or to the pad of animal's limb (initial number of tumor cells was equal to ~$1 \cdot 10^6$; antimetastatic effect, Experiment 1). As is known, the strain of this tumor is characterized by a high degree of lung metastases damage.

The C_{60}FAS in the volume of 0.1 ml (the initial concentration of C_{60} fullerenes in water was 1.0 mg/ml) was injected intraperitoneal to the animals after the transplantation of tumor (group 1) 5 times with interval through a day given the fact that C_{60} fullerenes, introduced intraperitoneal to animals (dose 500 mg/kg), excreted from the body within 2–4 days [24]. Dox («Ebewe», Austria; the initial concentration was 0.1 mg/ml) was injected intraperitoneal to the animals after the transplantation of tumor (group 2) in the volume of 0.1 ml (dose 0.5 mg/kg) one-time for 5 days given the fact that Dox excreted in the urine from the body within 5 days. The C_{60}FAS in the volume of 0.1 ml (the initial concentration was 1.0 mg/ml) was injected intraperitoneal to the animals after the transplantation of tumor one-time for 5 days 30 min prior to the introduction of Dox (group 3). Introduction of Dox (group 2) and C_{60}FAS in combination with Dox (C_{60}+Dox) (group 3) started on the 10^{th} day after the transplantation of tumor, when it appeared visually. Finally, group 0 (mice with transplanted tumor without C_{60}FAS or Dox injection) was used as control. The initial number of animals in each group was 7. On the 20^{th} day of Experiment 1 (antimetastatic effect) all animals were put to death for the purpose of calculating the number of metastases in the lung of each animal.

The antitumor effectiveness of the applied technique was estimated using such quantitative indicators as the metastasis inhibition index, tumor growth inhibition and increasing of animal life span [19].

The statistical analysis of obtained results was performed by use the STATISTICA software package including the Student's t-test [25].

Results and Discussion

The results obtained from Experiment 1 (antimetastatic effect) are presented in Table 1. They clearly demonstrate that the metastasis inhibition index for group 2 (Dox injection) is almost twice the value of this index for group 1 (C_{60}FAS injection) and different in ~1.2 times from the metastasis inhibition index for group 3 (injection of C_{60}+Dox) on 20^{th} day after the tumor transplantation.

Table 1. **Experiment 1 (antimetastatic effect)**

Experimental groups	Metastasis inhibition index, %*
1	48±3
2	94±4
3	79±4

Notes: start of tumor transplantation — 20.05.2011. Start of C_{60}FAS injection after the tumor transplantation — 22.05.2011 (group 1). Start of Dox (group 2) and C_{60}FAS in combination with Dox (group 3) injection after the tumor transplantation — 30.05.2011. Initial number of animals in experimental groups was n = 7;
* the differences are statistically valid compared with the control ($P < 0.05$).

The results extracted from Experiment 2 (antitumor effect) are presented in Table 2. Antitumor effect of drugs was recorded on the 10th day after the transplantation of tumor in all experimental groups. Tumor growth inhibition in group 1 (C_{60}FAS injection) and group 3 (injection of C_{60}+Dox) shows a tendency to decrease from 45 to 22% (21th day after the tumor transplantation) and from 34 to 21%, respectively. On the contrary, tumor growth inhibition in group 2 (Dox injection) increased from 20%, reached a maximum of 28% (14th day after the tumor transplantation), and then stopped altogether. Finally, the increasing of animal life span in group 1 and group 3 are close in value, but above this parameter in ~1.5 times for group 2.

For visual comparison of tumor growth, Fig. presents the animals in group 0 (control; Fig., *a*), group 1 (C_{60}FAS injection; Fig., *b*), group 2 (Dox injection; Fig., *c*) and group 3 (injection of C_{60}+Dox; Fig., *d*) on 20th day after the tumor transplantation in Experiment 2. Estimations demonstrate that the average tumor volume in control over this parameter in the experimental groups 1, 2 and 3 in ~1.5, ~3 and ~4.5 times, respectively.

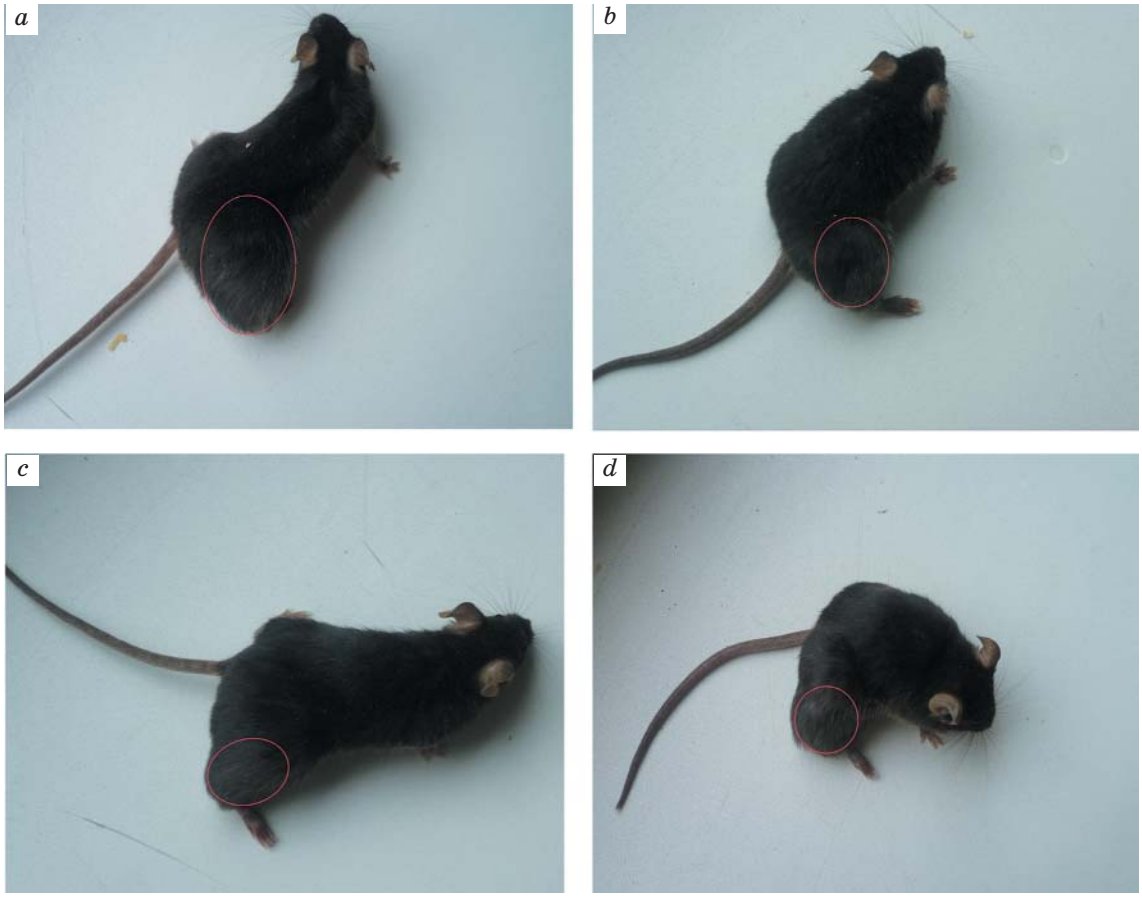
It is also important to note that the visual observation of experimental animals revealed that animals in group 1 (C_{60}FAS injection) and group 3 (injection of C_{60}+Dox) compared to the animals in experimental group 2 have greater mobility and that the color of their hair was more explicit shade. Finally, the life span duration for last animal in group 1 and group 3 (42 days after the tumor transplantation) over this parameter in a control group 0 in ~1.5 times.

This study clearly demonstrates that such drugs as C_{60} fullerenes, Dox and C_{60}+Dox cause an antitumor response at low therapeutic doses. Specifically, it was observed that a treatment with C_{60}+Dox enhances the antitumor activity, namely contributes to the probable inhibition of tumor growth as well as metastases of Lewis lung carcinoma in male mice C57Bl/6J line: the maximum therapeutic effect reached 34% for the tumor growth inhibition and 79% for the metastasis inhibition index; the increasing of animal life reached 24.4%. These results can be explained as a result of the high antioxidant activity of C_{60} fullerenes [10], neutralizing excess reactive oxygen species in the cell, and possibly blocking the specific cell receptors, for example, endothelial growth factor [13, 14]. In addition, we can assume that the injection of pristine C_{60} fullerenes in combination with Dox effectively prevents/reduces its toxic affect

Table 2. **Experiment 2 (antitumor effect)**

Number of days after tumor transplantation	Group 0 (control)	Group 1 (injection of C_{60}FAS)	Tumor growth inhibition*, %	Group 2 (injection of Dox)	Tumor growth inhibition*, %	Group 3 (injection of C_{60}FAS +Dox)	Tumor growth inhibition*, %
10	n = 7	n =7	45±4	n = 7	20±1	n = 7	34±3
14	n = 7	n = 7	36±3	n = 7	28±2	n = 7	34±3
19	n = 5	n = 5	25±2	n = 7		n = 7	33±3
21	n = 4	n = 5	22±2	n = 4		n = 6	21±1
29	n = 0	n = 3		n = 2		n = 4	
37		n = 1		n = 0 (15±1)%*		n = 1	
42		n = 0 (21.4±1.4)%*				n = 0 (24.4±1.6)%*	

Notes: start of tumor transplantation — 11.06.2011. Start of C_{60}FAS injection after the tumor transplantation — 13.06.2011 (group 1). Start of Dox (group 2) and C_{60}FAS in combination with Dox (group 3) injection after the tumor transplantation — 21.06.2011. Initial number of animals in experimental groups was n=7. Increasing of animal life is given in parentheses. * the differences are statistically valid compared with the control ($P < 0.05$).



Visual comparison of tumor size for animals of group 0 (control: *a*), group 1 (C_{60}FAS injection: *b*), group 2 (Dox injection: *c*) and group 3 (injection of C_{60}FAS in combination with Dox: *d*) on 20th day after the tumor transplantation

on the organism (like a fullerenol through the inhibition of oxidative stress [18]), especially cardiotoxic effect, that requires further using histological and biochemical approaches (in particular, identifying indicators of apoptosis and necrosis of tumors, level of oxygen radicals in the mitochondria of tumor cells). The proposed technology of treatment with C_{60}+Dox can be very promising in clinical oncology for the inhibition of tumor growth [26].

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ПОРІВНЯЛЬНЕ ДОСЛІДЖЕННЯ ПРОТИПУХЛИННОГО ЕФЕКТУ НЕМОДИФІКОВАНИХ ФУЛЕРЕНІВ C_{60} І ДОКСОРУБІЦИНУ

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Метою дослідження було розроблення технології застосування фулеренів C_{60} у комбінованій терапії з доксорубіцином для лікування злоякісних пухлин. Серію експериментів у присутності немодифікованих фулеренів C_{60}, доксорубіцину і C_{60} у комбінації з доксорубіцином проводили на групі мишей з трансплантованими злоякісними пухлинами. Встановлено, що введення C_{60} у комбінації з доксорубіцином ефективно пригнічує як ріст пухлини, так і метастазування карциноми легень Льюїса. Результати демонструють можливість використання C_{60} у комбінації з доксорубіцином у протипухлинній терапії.

Ключові слова: фулерен C_{60}, доксорубіцин, карцинома легень Льюїса, інгібітор росту пухлини, подовження життя тварин, коефіцієнт інгібування метастазів.

СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ ПРОТИВООПУХОЛЕВОГО ЭФФЕКТА НЕМОДИФИЦИРОВАННЫХ ФУЛЛЕРЕНОВ C_{60} И ДОКСОРУБИЦИНА

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Целью исследования была разработка технологии применения фуллеренов C_{60} в комбинированной терапии с доксорубицином для лечения злокачественных опухолей. Серию экспериментов в присутствии немодифицированных фуллеренов C_{60}, доксорубицина и C_{60} в сочетании с доксорубицином проводили на группе мышей с трансплантированными злокачественными опухолями. Установлено, что введение C_{60} в сочетании с доксорубицином эффективно подавляет как рост опухоли, так и метастазирование карциномы легких Льюиса. Результаты демонстрируют возможность использования C_{60} в сочетании с доксорубицином в противоопухолевой терапии.

Ключевые слова: фуллерен C_{60}, доксорубицин, карцинома легких Льюиса, ингибитор роста опухоли, продление жизни животных, коэффициент ингибирования метастазов.