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PRISTINE C_{60} FULLERENES INHIBIT THE RATE OF TUMOR GROWTH AND METASTASIS

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Aim: **To estimate the impact of C_{60} fullerene aqueous solution (C_{60}FAS) on the rate of transplanted malignant tumor growth and metastasis.** ***Methods:*** **Lewis lung carcinoma was transplanted into C57Bl/6J male mice. Conventional methods for the evaluation of antitumor and antimetastatic effects have been used.** ***Results:*** **The C_{60}FAS at low single therapeutic dose of 5 mg/kg inhibited the growth of transplanted malignant tumor (antitumor effect) and metastasis (antimetastatic effect): the maximum therapeutic effect was found to be of 76.5% for the tumor growth inhibition; the increase of animal life span by 22% was found; the metastasis inhibition index was estimated as 48%.** ***Conclusion*****: It was found that water-soluble pristine C_{60} fullerenes efficiently inhibit the transplanted malignant tumor growth and metastasis.**
Key Words**: Water-soluble pristine C_{60} fullerenes, Lewis lung carcinoma, tumor growth.**

Current status of anticancer therapy indicates the necessity for active search of new agents that will be effective against primary tumor and metastases but demonstrate the minimal level of side effects. As promising antitumor agents could be proposed C_{60} fullerenes — a unique class of carbon allotropes, which exhibits the biological activity both *in vitro* and *in vivo* [1–3].

The water-soluble pristine (unmodified) C_{60} fullerenes are nontoxic at low physiological concentrations [4–7], they can penetrate through the membrane of cells [8–10] and have strong antioxidant properties [11]. Murugesan et al. [12] 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. It is known that an imbalance in the levels of these factors causes many serious diseases including malignant growth [13].

Thus, the purpose of this study was to evaluate the impact of water-soluble pristine C_{60} fullerenes on the transplanted tumor growth and metastasis.

The samples of C_{60} fullerene aqueous solution (C_{60}FAS) were prepared as follows [14]. We used a saturated solution of pure C_{60} fullerenes (purity 99.5%) in toluene and the same amount of distilled water in an open beaker. Two phases are formed. Then we applied an ultrasonic bath as long as the toluene needs to evaporate completely. Meanwhile the water phase became yellow colored, indicating that the aqueous fullerene solution has been formed. Thereafter we filtered the aqueous solution from undissolved C_{60} fullerenes. As a result we prepared the C_{60}FAS sample with maximum concentration of C_{60} fullerenes in water 1.0 mg/ml. The C_{60}FAS sample is stable during 18 months at 4 °C.

Theoretical calculations [15–16] showed that C_{60}FAS contains both the single C_{60} molecules and their clusters and solids (with sizes of ~0.7–4 nm in dependence of C_{60} fullerene concentration in water) in the hydrated state. Moreover, C_{60} fullerenes structure the water, absorbed by DNA molecules [17], and thus they can affect the DNA functioning in the biological systems.

State of C_{60} fullerenes in water was monitored using STM technique (NT-MDT, Russia). Samples were deposited on Au(111) surface by precipitation from aqueous solution droplet.

It is important to note that used C_{60}FAS in our experiments 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 of b.w.) were kept in a vivarium on a standard diet. The average temperature in a vivarium was 20±1 °C. All experiments were performed in accordance with 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 ~$5 \cdot 10^5$, antitumor effect) or to the pad of animal's limb (initial number of tumor cells ~$1 \cdot 10^6$, antimetastatic effect). It is well known that this tumor is characterized by a high degree of metastasis into the lung.

The C_{60}FAS in the volume of 0.1 ml (initial concentration of C_{60} fullerenes in water was 1.0 mg/ml) was injected intraperitoneally to the animals with transplanted tumor (group 1). Injection of C_{60}FAS was started in a day after transplantation of tumor, which visually appeared on the 10^{th} day. The schedule of C_{60}FAS administration was based on the data obtained by [18].

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Abbreviations used: C_{60}FAS – C_{60} fullerene aqueous solution

Finally, group 0 (mice with transplanted tumor without C_{60}FAS injection) was used as a control. Initial number of animals in each group was 5 in Experiment 1 (antitumor effect), 7 in Experiment 2 (antitumor effect) and 7 in Experiment 3 (antimetastatic effect). On the 20th day (Experiment 3) all animals were sacrificed and autopsied to calculate the number of metastases in the lung by conventional method.

It is also important to note that these experiments were performed in different time: Experiment 1 — end of spring — summer 2010; Experiment 2 — end of winter — spring 2011; Experiment 3 — end of spring — summer 2011.

Antitumor effectiveness of the applied technology was estimated by following quantitative indicators:

$k_1^{TGI} = [(V_0 - V_1)/V_0] \cdot 100\%$ — tumor growth inhibition (*TGI*, %): , where V_0 and V_1 are the average values of tumor volume in animals of group *0* (control) and experimental group *1*, respectively; $V = (a + b)^3/16$, where *a* and *b* are the length and width (in mm) of the tumor site;

$k_1^{IAL} = [(t_1 - t_0)/t_0] \cdot 100\%$ — increasing of animal life (*IAL*, %): , where t_0 and t_1 are average life span of animals (in days) in group *0* (control) and experimental group *1*, respectively;

$k_1^{MII} = [(A_0 \cdot B_0 - A_1 \cdot B_1)/A_0 \cdot B_0] \cdot 100\%$ — metastasis inhibition index (*MII*, %): , where A_0 and A_1 are frequency of metastasis in the group 0 (control) and experimental group 1, respectively; B_0 and B_1 are average number of metastases in certain organ of animals in group 0 (control) and experimental group 1, respectively.

Statistical analysis of results was performed using STATISTICA software package. On significance of differences was analysed using the parametric (Student's t-test) method [19]. The differences were considered as valid at $p<0.05$.

The STM images of submonolayer C_{60} fullerene film deposited from aqueous solution (C_{60} fullerene concentration in water was 1.0 mg/ml) on Au(111) surface are shown on Figure. They revealed almost random arrangement of C_{60} fullerene clusters with sizes up to ~2.8 nm (the first stable sphere-like cluster consisting of 13 hydrated C_{60} fullerenes [15–16]) (Figure, *a*). Despite of the mobility of C_{60} molecules on Au(111) at room temperature we were able to image single C_{60} molecules (Figure, *b*).

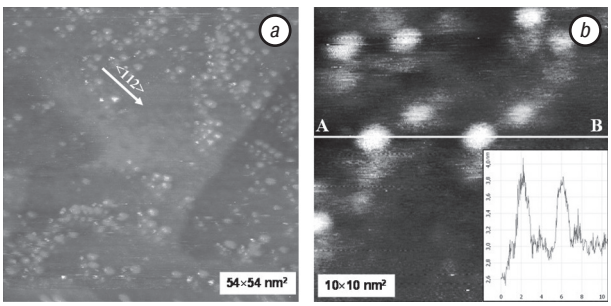

Figure. *a*) Large scale STM image of submonolayer C_{60} fullerene film deposited from aqueous solution (C_{60} fullerene concentration in water was 1 mg/ml) on Au(111) surface. Some C_{60} fullerene clusters are aligned along preferential direction <112>; *b*) Single C_{60} fullerenes. Lateral size is increased because of shape of the tip. Inset: cross-section along line AB. Scanning parameters: I_t=40 pA, U_t=0.7 V.

Results of antitumor effect of C_{60}FAS, obtained in Experiment 1 and Experiment 2, are presented in Table 1 and 2, respectively.

Table 1. Experiment 1 (antitumor effect): start of tumor transplantation – 25.05.2010. Start of C_{60}FAS injection after tumor transplantation – 27.05.2010

Days after tumor transplantation	Group 0 (control) n	Group 1 (injection of C60FAS after tumor transplantation) n	Tumor growth inhibition, k_1^{TGI}, %*
11	5	5	54.5
14	5	5	71.9
17	5	5	74.0
21	3	5	74.4
23	2	5	76.5
25	2	5	73.1
28	2	5	
30	1	5	
36	0	5	
39		5	
43		3	
66		1	
77		0	

Note: n – number of mice; *the differences are statistically significant compared with the control ($p < 0.05$).

Table 2. Experiment 2 (antitumor effect): start of tumor transplantation – 17.02.2011. Start of C_{60}FAS injection after tumor transplantation – 19.02.2011. The increase of animal life span (k_1^{IAL}, %) is given in parentheses

Days after tumor transplantation	Group 0 (control) N	Group 1 (injection of C60FAS after tumor transplantation) n	Tumor growth inhibition, k_1^{TGI}, %*
13	7	7	4.0
16	7	7	25.1
21	7	7	5.4
23	4	7	17.0
27	2	6	5.0
29	2	6	
30	0	6	
33		4	
34		2	
37		0	
		(k_1^{IAL}=21.8%)*	

Note: n – number of mice; *the differences are statistically significant compared with the control ($p < 0.05$).

The inhibition effect of C_{60} fullerenes on the tumor growth was observed on the 11th day (Experiment 1) and the 13th day (Experiment 2) of the experiment. The maximum value of inhibition of the tumor growth was found to be of 76.5% on the 23th day (Experiment 1) and of 25.1% on the 16th day (Experiment 2) of the experiment. Last animal in the control group 0 died on the 36th day (Experiment 1) and the 30th day (Experiment 2) of the experiment. It should be noted that all 5 mice, injected by C_{60} fullerenes (group 1; Experiment 1), lived 39 days. Last animal of the experimental group 1 died on the 77th day (Experiment 1) and the 37th day (Experiment 2) of the experiment. The increase of animal life was found to be of 21.8% in Experiment 2. Moreover, it was determined that the average tumor volume in the control group exceeded this parameter in the group 1 by ~3 fold on the 28th day after tumor transplantation in Experiment 2.

The metastasis inhibition index (k_1^{MII}) was obtained as 48% in the study of antimetastatic effect of C_{60}FAS (Experiment 3).

In conclusion, the C_{60}FAS containing hydrated single C_{60} molecules and C_{60} clusters with size up to ~2.8 nm without showing direct cytotoxicity at low sin-

gle therapeutic dose of 5 mg/kg [6] demonstrates the inhibition of growth of transplanted tumor (antitumor effect): the maximum therapeutic effect was determined as 76.5% in Experiment 1 and 25.1% in Experiment 2; the increase of animal life span by 21.8% was found in Experiment 2. The metastasis inhibition index was obtained as 48% (antimetastatic effect, Experiment 3). Finally, the anticancer affect of C_{60}FAS was confirmed by histological data for the animal tumors of control and investigated group in Experiment 3 [20]. The obtained results can be explained as a result of the high antioxidant activity of C_{60} fullerenes [11], neutralizing excess reactive oxygen species in the cell [20], and possibly blocking the specific cell receptors, for example, endothelial growth factor receptors [12]. The proposed method of C_{60}FAS application for the inhibition of tumor growth is promising for experimental oncology and may be tested in the future studies [21].

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