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EFFICACY OF NATURAL L-ASPARAGINE IN THE COMPLEX THERAPY FOR MALIGNANT TUMORS IN EXPERIMENTAL STUDIES

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Aim: **To study the influence of natural L-asparagine on the efficacy of cytostatic therapy for malignant tumors in experimental investigations.** ***Materials and Methods:*** **Female C57B1/6 mice weighing 18–20 g were selected for the experiments. Lewis' lung carcinoma (LLC) and melanoma B16 cells were used in the study. Animals were inoculated with tumor cells intramuscularly. Solution of L-asparagine in a volume of 0.2 ml per mouse (in appropriate doses) was administered to the animals using gastric probe, daily, for 14 days. Cyclophosphane was administered intraperitoneally in total doses of 180 mg/kg and 90 mg/kg on days 3 and 7 after tumor implantation. The percentage of tumor growth inhibition was calculated and inhibition index and frequency of metastasis were assessed.** ***Results:*** **It has been shown that despite low activity of L-asparagine with regard to primary tumor, the level of metastasis inhibition is rather high (up to 91% depending on experimental model, therapy regimen and follow-up period). The analysis of previously obtained data and our studies indicate that L-asparagine derived from burdock (*Arctium lappa*) root has not only its own antimetastatic activity but it is also able to increase antimetastatic activity of cyclophosphane partially reducing toxic effect of cyclophosphane on the organism without decreasing its antitumor and antimetastatic activities.** ***Conclusion:*** **L-asparagine derived from burdock (*Arctium lappa*) root can be effective in the complex anticancer therapy with the use of appropriate chemotherapy doses and regimens.**

Key Words: **Lewis lung carcinoma, melanoma B-16, L-asparagine, cyclophosphane.**

Despite many advances in cancer treatment, traditional standard methods (surgery, radiation therapy and chemotherapy) do not result in a complete cure of cancer patients [1, 2]. Up to XVIII century, tumor diseases were treated mainly with herbal extracts and many empirically obtained data were confirmed by up-to-date medicine [3, 4]. Herbs are "soft" drugs, which combine a high biological activity with minimal side effects [5]. Many studies have shown that many herbal extracts, having moderate inhibitory effect on tumor development, reduce toxic effect of cytostatic agents on blood cells and increase the functional activity of cells-effectors of immunity system as well as increase chemotherapy efficacy and protect against progression of metastases during surgery [6–9].

Studies of foreign authors and folk medicine experience in the use of extracts from burdock (*Arctium lappa*) for cancer treatment are of great interest. Burdock seeds contain arctiin and arctigenin, which have anticancer effects. Cytotoxicity of these compounds has been proven on cell cultures and inoculated tumors [10–12]. Arctigenin shows cytotoxicity in 100% of cases during oxygen-glucose deprivation in tumor cells. The antiproliferative activity of glycosides is caused by apoptosis induction [13, 14]. Extracts from burdock leaves and roots are used in medicine as agents having diuretic, diaphoretic, blood purifying and anti-inflammatory effects. Antineoplastic effects of ethanol and dichloromethane extracts of burdock roots have been proved in experimental studies [15]. Apoptosis is an important component in the mechanism of some antitumor agents. It is known that asparagine can be inductor of apoptosis in nervous tissue [16]. We have found that the concentrated juice obtained from fresh burdock roots has not only cytostatic but also apoptosis-induced activity. The burdock root contains up to 10.1% of free amino acids, maximum amount of asparagine (6.4% in terms of raw material and up to 50.0% in terms of the amount of amino acids) [17].

Thus, it seems rather logical and interesting to study antitumor activity of L-asparagine as one of the main components included into the composition of the studied agents derived from the burdock root.

MATERIALS AND METHODS

Female C57B1/6 mice weighing 18–20 g were obtained from the Pharmacology Research Institute in Tomsk, Russia. Research was conducted in accordance with the principles set out in the Guide for the Care and Use of Laboratory Animals (Moscow Breeding Nursery, Russian Acad. Med. Sci.).

Tumor strains. Lewis lung carcinoma (LLC) and melanoma B16 cells were used in the study. Strains obtained from the Russian Cancer Research Center (Russia) are highly adequate models for studying anti-tumor effect of biologic reaction modifiers of plant origin. Animals were inoculated with tumor cells intramuscularly at the doses of 1×10^6 (LLC) and 5×10^6 (B16) tumor cells per mouse.

Agents. *Natural L-asparagine* (Tomsk). The agent was dissolved in warm distilled water. The obtained solution in a volume of 0.2 ml per mouse (at doses of 100, 250 and 500 mg/kg was administered to the animals

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Abbreviations used: LLC – Lewis's lung carcinoma; D-16 – melanoma B-16; CPH – cyclophosphane; IMI – index of metastasis inhibition; TGI – percentage of tumor growth inhibition.

using gastric probe in therapeutic regimen (48 h after tumor implantation) daily, for 14 days.

Cyclophosphane. Cyclophosphane (Ministry of Medical Industry, Russia) was used in mono-and combined therapy for experimental tumors. Cyclophosphane was administered intraperitoneally at the total doses of 180 mg/kg and 90 mg/kg on days 3 and 7 after tumor implantation.

Changes in tumor growth were assessed by measuring the tumor volume according to the Shrek formula: $V = A \times B \times C$, where A, B, and C are orthogonally related tumor sizes.

Percentage of tumor growth inhibition was calculated by the formula: TGI% = [(Vc-Ve/Vc] × 100%, where TGI% is a coefficient of tumor growth inhibition; Vc is the average tumor volume in the control group of animals; Ve is the average tumor volume in experimental group of animals.

Index of metastasis inhibition. Metastatic spread into lungs was evaluated on days 10 and 20 after tumor inoculation. Mice were killed by cervical dislocation, and their lungs examined. Index of metastatic inhibition characterized the extent of metastatic involvement. It was calculated by the formula: IMI = (Ac × Bc)-(A × B)/(Ac × Bc) × 100%, where Ac and A are the numbers of animals with metastases in the control and experimental groups; Bc and B are the average numbers of metastases in animals of the control and experimental groups.

Frequency of tumor metastasis is the percent of animals with metastases with respect to the overall number of animals in the group.

Statistical analysis of results was carried out using STATISTICA 6.0 software. Comparison between the groups was made by nonparametric Mann — Whitney test.

RESULTS AND DISCUSSION

Influence of the combination therapy with L-asparagine and cyclophosphane on the growth of Lewis lung carcinoma. The ability of amino acid of L-asparagine to inhibit tumor growth and modulate cytostatic therapy for experimental malignant tumors was studied on mice with hematogenous metastasis of Lewis's lung carcinoma. This model is suitable for studying anti-tumor and antimetastatic activities of agents. Double injection of cyclophospane with a 96-h interval is one of the standard chemotherapy regimens in therapy of experimental tumors, in particular, LLC [18]. We injected cyclophosphane intraperitoneally in total doses of 180 mg/kg or 90 mg/kg on days 3 and 7 after tumor implantation. Therapy for LLC with L-asparagine at a dose of 250 mg/kg administered alone resulted in insignificant antineoplastic activity of this agent at late stages of oncogenesis (up to 21.5% on day 20). Cyclophosphane at a dose of 180 mg/kg led to statistically significant inhibition of tumor growth ($p < 0.05$) only at early stages of the experiment (on day 7 and 10 with a maximum rate of up to 61.8% on day 10 (Table 1).) Combination of L-asparagine and CPH at a dose of 180 mg/kg demonstrated insignificant inhibition of primary tumor growth as compared to monotherapy with CPH. Thus, it has been shown that L-asparagine has not only insignificant activity in monotherapy regimen, but it also has insignificant ability to enhance therapeutic effect of CPH with regard to primary tumor. The attempt to reduce the CPH dose to 90 mg/kg resulted in ineffective therapy. Antitumor effect of the combination therapy (TGI up to 21.9% on day 20) was compared to that of using asparagine in monotherapy regimen. CPH administered in the reduced dose demonstrated insignificant antitumor effect (on day 20, TGI was up to 14.9% at a dose of 90 mg/kg versus 44.7% at a dose of 180 mg/kg).

Therapy with CPH at the doses of 180 mg/kg and 90 mg/kg resulted in respectively 26.7 and 10.1-fold reductions in the number of metastases on day 20 in animals with LLC, which received the combination therapy with L-asparagine and CPH compared to animals receiving no this combination therapy (Table 2). Mice treated with CPH alone at the doses of 180 mg/kg and 90 mg/kg demonstrated 18.8 and 2.7-fold reductions in the number of metastases, respectively. It should be noted that IMI in animals receiving therapy with asparagine in combination with CPH at the doses of 180 mg/kg and 90 mg/kg was rather high (98.5% and 90.1%, respectively). Metastases in animals treated with asparagine and CPH at a dose of 180 mg/kg were observed 2.6 times more frequently than in animals receiving the reduced dose of cyclophosphane. LLC therapy with L-asparagine in combination with 180 mg/kg CPH led to insignificant weight loss of the liver and statistically significant weight loss of the spleen (Table 3). The data obtained can be indirect confirmation of reduction in toxic effect of CPH given in the combined therapy. We previously showed the reduction in mice spleen cellularity after CPH injection that was partially decreased after

Table 1. Influence of L-asparagine (dose of 250 mg/kg) on the growth of Lewis lung carcinoma

Days	Groups of animals															
	Non treted n=15	Asparagine n=15			CPH (I) n=15			Asparagine +CPH (I) n=15			CPH (II) n=15			Asparagine+CPH (II) n=15		
	V	V	TGI %	p	V	TGI %	p	V	TGI %	p	V	TGI %	p	V	TGI %	p
7	1.2±0.2	1.5±0.1	-	0.24	0.6±0.1	49.58	0.03	0.5±0.1	55,46	0.02	1.2±0.2	1.68	0.73	1.3±0.2	-	0.88
10	2.9±0.1	3.0±0.1	-	0.19	1.13±0.1	60.35	0.0008	1.09±0.1	61.75	0.0006	3.0±0.2	-	0.6	2.6±0.2	7.72	0.37
13	4.0±0.9	4.4±0.3	-	0.67	1.4±0.2	64.4	0.01	2.5±0.2	37,37	0.18	4.7±0.5	-	0.56	3.8±0.3	4.04	0.81
17	5.7±1.2	4.7±0.5	18.71	0.39	3.2±0.3	43.36	0.042	3.8±0.2	34,27	0.2	5.9±0.7	-	0.81	5.1±0.3	11.36	0.83
20	7.0±0.9	5.5±0.8	21.45	0.25	3.9±0.	44.74	0.021	4.3±0.3	39.06	0.027	6.0±0.6	14.91	0.39	5.5±0.2	21.88	0.22

Notes: CPH (I) – dose of 180 mg/kg; CPH (II) – dose of 90 mg/kg, V – tumor volume (cm^3), TGI – tumor growth inhibition, n – number of mice.

administration of asparagine, i.e. toxic effect of CPH on the organism was reduced.

Table 2. Influence of L-asparagine (dose of 250 mg/kg) on metastatic spread of Lewis lung carcinoma

Groups of animals	Average number of metastases, IMI					
	10 day			20 day		
	M ± SE	IMI %	p	M ± SE	IMI %	p
Non-treated, n=15	1±0,58	-	-	101.3±8.7	-	-
Asparagine, n=15	1±1	50.7	0.83	66.5±4.4	34.4	0.034
CPH (I) , n=15	0±0	100.0	0.19	5.4±2.2	94.7	0.024
Asparagine + CPH (I), n=15	0.3±0.3	83.5	0.38	3.8±1.8	98.5	0.024
CPH (II), n=15	0±0	100.0	0.19	37.6±12.0	62.9	0.024
Asparagine + CPH (II), n=15	0±0	100.0	0.19	10±2.0	90.1	0.025

Notes: CPH (I) – dose of 180 mg/kg; CPH (II) – dose of 90 mg/kg, V – tumor volume (cm^3), TGI – tumor growth inhibition, n – number of mice; IMI – index of metastasis inhibition.

Since the combined administration of CPH and L-asparagine more markedly inhibits metastatic spread nearly not influencing on the primary tumor, it can be suggested that in this case the immune-mediated inhibition of metastases but not the direct effect of CPH on tumor cells takes place.

Influence of monotherapy with L-asparagine on the growth of melanoma B-16. A statistically significant inhibition of melanoma-16 growth on days 10 and 15 of the experiment was found in animals, which received L-asparagine at a dose of 500 mg/kg (Table 4). Similar results were obtained on days 8 and 15 in animals, which received L-asparagine at a dose of 250 mg/kg. It should be noted that on day 13 after tumor inoculation the rate of tumor growth inhibition reduced to 4–7% depending on the dose, then, on the day 15 the rate of tumor growth inhibition slightly increased to 8–11%. Tumor volumes of the control group animals were significantly greater than those of the examined animals (see Table 4).

The study of the influence of natural L-asparagine derived from burdock roots on metastatic spread of melanoma B-16 showed a high anti-metastatic effect of this agent (Table 5). A statistically significant inhibition of metastasis was found on days 13 and 15 of the experiment (up to 80% and 91%, respectively). The phenomenon of inverse relationship between the dose of the agent and antimetastatic effect clearly detectable on day 15 is of great interest. The metastatic frequency at all stages of the tumor process was 100% in all groups of animals.

Studies of activity of L-asparagine derived from the burdock root extract using the model of LLC showed insignificant antitumor and antimetastatic activities of this agent administered alone. However, the combination of asparagine and CPH at a dose of 180 mg/kg demonstrated statistically significant increase in metastasis inhibition (up to 99% on day 20 of the experiment). Similar tendency was observed using another experimental model (melanoma B-16). It was shown that asparagine used alone had insignificant antitumor activity, however, the rate of metastasis inhibition was extremely high (up to 91% depending on the therapy regimen and follow-up period). Thus, the analysis of previous and our study results indicate that natural L-asparagine derived from burdock roots has not only its own anti-tumor activity but it is also able to increase anti-tumor activity of CPH (at a total therapeutic dose of 180 mg/kg) partially reducing its toxic effect on the organism.

Table 4. Influence of L-asparagine on the growth of melanoma B-16

Days	Groups of animals						
	Non-treated n=15	L-asparagine (500 mg/kg), n=15			L-asparagine (250 mg/kg), n=15		
	V	V	TGI %	p	V	TGI %	p
8	0.5±0.1	0.4±0.1	33.3	0.1	0.4±0.07	35.2	0.031
10	2.0±0.2	1.5±0.1	17.2	0.05	1.7±0.1	14.2	0.14
13	3.5±0.2	3.0±0.1	6.6	0.07	3.3±0.1	4.3	0.5
15	4.9±0.1	4.3±0.2	11.2	0.03	4.5±0.1	7.9	0.02

Notes: V – tumor volume (cm^3); ITG – inhibition of tumor growth.

Table 5. Influence of L-asparagine on metastatic spread of melanoma 16

Groups of animals	Average number of metastases and IMI					
	13 day			15 day		
	M ± SE	IMI %	*p*	M ± SE	IMI %	*p*
Non-treated, n=15	2.9±1.1			29.5±4.5		
L-asparagine (500 mg/kg), n=15	0.6±0.3	75.0	0.02	8.5±3.4	42.0	0.03
L-asparagine (250 mg/kg), n=15	0.4±0.2	80.0	0.004	1.7±0.7	91.0	0.05

Notes: IMI – index of metastasis inhibition.

Considering previously conducted studies, it can be concluded that the agents derived from the burdock root have detectable antitumor and antimetastatic activities. Asparagine as one of the active components of these agents, therefore, can be promising for cancer therapy. Since these agents are used as a rule in the complex therapy for malignant tumors, we consider that further experimental studies on the influence of L-asparagine on chemotherapy efficacy and surgery are required.

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Table 3. Weight coefficients of the organs of LLC-bearing mice treated with L-asparagine (dose of 250 mg/kg) and CPH

Organ	Groups of experimental animals, weight of organs (g)						
	Intact mice, n=8	LLC, n=8	Asparagine, n=8	CPH(I), n=8	Asparagine + CPH(I), n=8	CPH(II), n=8	Asparagine + CPH(II), n=8
Spleen	0.1±0.01	0.41±0.05	0.34±0.04 p=0.22	0.31±0.03 p=0.22	0.21±0.0 p=0.016	0.4±0.02 p=0.17	0.43±0.03 p=0.17
Liver	1.01±0.02	1.3±0.01	1.16±0.16 *p*=0.62	1.24±0.11 *p*=0.6	1.00±0.14 *p*=0.35	1.28±0.06 *p*=0.05	1.32±0.06 *p*=0.6

Notes: CPH (I) – dose of 180 mg/kg; CPH (II) – dose of 90 mg/kg, V – tumor volume (cm^3), TGI – tumor growth inhibition, n- number of mice.

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