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ANTICANCER ACTIVITY OF ACONITINE–CONTAINING HERBAL EXTRACT BC1

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Aim: **The objective of the study was the investigation of anticancer activity of aconitine-containing herbal extract BC1 against two tumor strains with different metastatic potency: strongly metastatic Lewis lung carcinoma (LLC) and its weakly metastatic counterpart (LLC-R9).** ***Results:*** **It was shown that low proliferative activity and high metastatic potential of LLC correlated with high refractoriness of this tumor to BC1 action, while significant inhibition of tumor growth and metastasis by BC1 were observed against actively proliferating and weakly metastatic LLC-R9. Maximal antitumor activity of BC1 (> 70% of inhibition of primary tumor growth) and antimetastatic action (88% of metastatic inhibition) were observed at the dose of MTD/20. High efficacy of BC1 against LLC-R9 was shown to be accompanied by 2-fold increase of apoptosis rate predominantly in diploid cells.** ***Conclusions:*** **The obtained results showed the ability of aconitine-containing herbal extract BC1 to inhibit growth of primary tumor and metastases of actively proliferating and weakly metastatic variant of Lewis lung carcinoma.**

Key Words: **aconitine-containing herbal extract, anticancer activity, Lewis lung carcinoma.**

Nowadays, great attention is given to search of new anticancer agents because traditional cytotoxic therapy has shown limitation of its possibilities and low efficiency in the treatment of locally–advanced malignancies. Modern strategy for development of new effective anticancer drugs includes search of the substances expressing rather antiinvasive and antiangiogenic properties than direct cytotoxic effect on tumor cells. Despite the trends of molecular biology and chemistry providing fast escalation of synthesized *de novo* drugs, plants still remain a traditional source of medicinal compounds; up to 40% of modern drugs may directly or indirectly be related to natural compounds. Several plant–derived compounds have been approved as anticancer drugs. Vinblastine, vincristine, etoposide, teniposide, taxol, taxotere, topotecan and irinotecan, just to name a few. At present, great attention is attracted to diterpenoid alkaloids, for some of which high antineoplastic activity and significant antiangiogenic effect have been demonstrated [1–5]. Striking example of diterpenoid alkaloids is akonitine — the toxic substance of plant origin belonging to the class of neurotoxins. Alkaloids of *Aconitum* species are considered to be of anti–inflammatory, analgetic and cardiotropic effects [6]. Main mechanism of pharmacological action of aconitine is connected with its effect on voltage–gated sodium channels (VGSC).

It is well known that ion channels can exert a profound influence on cellular homeostasis. Ion channel activity was shown to be involved in a variety of cellular activities, including proliferation/apoptosis, cell adhesion, cell movement, secretion and gene expression [7, 8].

VGSCs are mainly known for mediating representative cell membrane depolarization and conduction of electrical signalling in nerves and muscles. However, VGSCs were shown also to be expressed in traditionally non–excitable cell types, particularly in cancer cells of epithelial origin [9–10].

Using whole–cell patch clamp recording technique it was shown that VGSCs occur only in strongly metastatic prostate cancer cells of both rat and human [11–14].

Due to the important role of VGSCs in the vital activity of cells as well as increased expression of these channels on malignant cells, VGSCs are considered to be a challenging molecular target for anticancer and antimetastatic therapy. Modifiers of Na^+ channels to which aconitine belongs, represent a new arsenal for search and creation of effective anticancer drugs [15].

Thereupon, the objective of this study was the investigation of anticancer activity of aconitine–containing herbal extract BC1 against two tumor strains with different metastatic potency.

MATERIALS AND METHODS

Experimental animals and tumor strains. The studies were carried out in female C57Bl/6 mice weighting 18–22 g aged 2 to 2.5 months from the vivarium of R.E.Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of National Academy of Sciences of Ukraine (Kiev, Ukraine). All investigations with animals had been approved by the regional animal ethics committee.

Two variants of Lewis lung carcinoma (LLC) cell lines possessed markedly different metastatic ability — the

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Abbreviations used: CDDP — cis–diamminedichloroplatinum; LLC and LLC–R9 — variants of Lewis lung carcinoma; VGSCs — voltage–gated sodium channels.

strongly metastatic parental LLC and its weakly metastatic LLC–R9 counterpart — were used in the study (National Bank of Cell Lines and Tumor Strains, IEPOR).

Both cell lines LLC and LLC–R9 were expanded *in vitro* in RPMI 1640 (Sigma, USA), supplemented with 2 mM glutamine, 10% FBS and 40 μg/ml gentamicine and were incubated at 37 °C in incubator with humidified atmosphere containing 5% CO_2. The cells mechanically removed from the plates were inoculated intramuscularly into the mice (10^6cells in 0.1 ml of Hanks' solution).

Cell line LCC–R9 was derived from parental LLC after the selection procedure *in vivo*. For that LLC cells were expanded *in vitro* (as it was mentioned above) and were inoculated into syngenic mice. The treatment by cis–diamminedichloroplatinum (CDDP) (5 injection of 1.2 mg/kg CDDP every other day, total dose 6.0 mg/kg) was initiated as tumor reached 2 mm in diameter. The tumors relapsed after CDDP treatment were passaged into mice and selection was continued over 9 cycles of passage and retreatment.

Treatment of animals. Herbal extract BC1 containing aconitine as an active component was dissolved in sterile water and 0.4 ml of solution was administered *per os* to mice daily beginning in a day after cancer cell inoculation. The duration of treatment was 3 weeks (5 administrations per week). Dosing of BC1 was estimated with respect to MTD (maximum tolerated dose). Preliminary study showed that MTD of BC1 for female C57Bl/6 mice was 0.18 ± 0.06 μg of aconitine/g of body weight. Two dosing schedules for each cell line were used in the study: total doses of BC1 for LLC–bearing mice were MTD/3 and MTD/6 while total doses for LLC–R9–bearing mice were MTD/6 and MTD/20.

CDDP (Sigma, USA) was used as a reference agent. 0.3 ml of CDDP water solution was administered to mice intraperitoneally (i.p.) twice a week during 3 weeks (total dose was 6 μg/g of body weight).

0.4 ml of water was administered *per os* (in time regime corresponding to that of BC1 input) to each mouse which was not treated with BC1 while 0.3 ml of sterile water was injected i.p. twice a week during 3 weeks to each animal which was not treated with CDDP.

Study of anticancer activity. For study purposes, the animals were assigned to 8 groups: groups 1–4 consisted of LLC–bearing mice while groups 5–8 included LLC–R9–bearing mice. The variability of body weight in each group and between different groups didn't exceed 2% and 5% correspondingly.

The animals of the groups 1 and 5 receiving water *per os* and i.p. were used as corresponding negative controls. The mice of the groups 2 and 6 treated by CDDP were considered as positive controls. Total dose of BC1 input to each mouse was MTD/3 (3rd group), MTD/6 (groups 4 and 7) and MTD/20 (8th group).

The body weight as well as tumor volume were measured twice a week during the experiment.

For estimation of antitumor activity of BC1 and CDDP tumor growth inhibition (*TGI*) was calculated on 19th and 26th days (after cancer cell inoculation) using the formula:

$$TGI = \frac{(V - V_k)}{V_k} \cdot 100\% \quad (1)$$

where V is a mean tumor volume calculated for groups treated by anticancer agents; V_k is average tumor volume per corresponding negative control group.

For estimation of antimetastatic activity of anticancer agents all animals in experiments were sacrificed on 26th day after cancer cell inoculation under either anaesthetic and number and volume distribution of lung metastases were evaluated by routine methods. The number of lung metastases (as a portion of their total number per mouse) in avascular phase (when the volume of metastases is smaller than 1.75 mm^3) was analyzed in comparison with the number of metastases in vascular phase (metastases volume is greater than 1.75 mm^3).

Analysis of LLC and LLC–R9 growth kinetics was carried out for the animals of the negative control groups (1st and 5th) by Weibull's mathematical model

$$V = V_0 \exp(\lambda(t - t_{lag})^{\beta}) \quad (2)$$

where V is the volume of the tumors averaged per group, V_0 is the volume of primary tumor cell inoculum (10^6 cells/0.1 ml) mice. Parameter λ reflects the rate of cell division in exponentially growing tumor. Parameter β characterizes high cell density growth inhibition of the tumor: the lesser the value of β the more considerably tumor growth is inhibited. t_{lag} — time after cancer cell inoculation that is necessary for cells to form cellular aggregate with growth ability in animal organism.

The values of growth kinetic parameters (model parameters) were determined from the best fit of mathematical model (2) to experimentally measured growth kinetics of the tumor using nonlinear regression analysis (Microcal Origin v. 6.0).

BC1 induced apoptosis in LLC and LLC–R9 tumors. Fresh single cell suspensions were prepared from LLC and LLC–R9 by mechanically disaggregation of tumor tissues on 26th day after cancer cell inoculation. Propidium iodide was used to stain the DNA and the sub–diploid population of cells from a cell cycle profile was analyzed by flow cytometry (Becton Dickinson equipped by argon laser) [16]. The portion of apoptotic cells was counted on 10^4 cells.

Statistical analysis of the results was performed using descriptive methods, *t*–test and nonlinear regression analysis.

RESULTS

The comparative characteristics of LLC and LLC–R9 cell lines. One of essential differences between LLC and LLC–R9 variants was the difference in rates of their growth and metastasic potential. Kinetics of LLC growth is characterized by lower rate of cell division ($p < 0.01$) as compared to kinetics of LLC–R9 growth (Fig. 1, Table 1). Such differences of kinetic parameters conditioned that the volume of primary tumor in LLC–bearing mice on 26th day after tumor cell inoculation was 2.5–fold less ($p < 0.05$) than that in LLC–R9–bearing mice within the same period of observation. Perhaps, the lower rate of LLC growth was

caused by more prolonged cell cycle and/or lower mitotic activity of tumor cells of this type. The latter is confirmed by over twice greater ($p < 0.05$) number of apoptotic cells in LLC on 26th day after tumor cell inoculation than that in LLC–R9 (54.5% ± 7.2% and 27.8% ± 2,1%, respectively).

In spite of lower primary tumor growth rate, LLC is characterized by higher level of metastasis. On 26th day after tumor cell inoculation the level of metastasis (taking into account the amount of metastases and volume of metastatic lesion) in LLC–bearing mice was 3–fold higher ($p < 0.05$), than that in LLC–R9–bearing mice (Table 2, 3). Difference in metastatic potential between two tumors correlated with statistically reliable ($p < 0.05$) difference in the amount of aneuploid cells (Table 4). About 3–fold prevalence of aneuploid cells observed in LLC pointed to lower differentiation rate of this tumor in comparison with LLC–R9 [17, 18]. Less expressed ability of these cells to inhibit their proliferation due to high cell density indicated lower degree of LLC cell differentiation as compared with LLC–R9. Thus, kinetic parameter of tumor growth β (see Table 1) for LLC was significantly higher ($p < 0.05$), than that of LLC–R9.

Antitumor and antimetastatic activity of BC1 against LLC. It was found that BC1 at doses of MTD/3 and MTD/6 didn't show anticancer activity against LCC. No statistically reliable difference was detected in the volume of a primary tumor between treated and untreated by BC1 LLC–bearing mice (see Table 2). It should be noted that administration of CDDP to the animals with LLC didn't result in inhibition of primary tumor growth: on 26th day the volume of tumor in this group was similar to that in control.

It was also revealed that BC1 (at both doses) as well as CDDP didn't inhibit metastasis of LLC. There were no differences in the level of metastasis between all four groups of LLC–bearing mice. Thus, the number of lung metastases as well as total volume of lung metastatic injury in mice treated by anticancer agents varied at a narrow range of values and didn't significantly differ from that in control group.

Antitumor and antimetastatic activity of BC1 against LLC–R9. In contrast to LLC, BC1 exerted significant anticancer effect on LLC–R9 (see Table 3). Thus, it was found, that BC1 at doses MTD/6 and MTD/20 strongly ($p < 0.05$) suppressed the growth of a primary tumor by over 70% in comparison with the control animals. Meanwhile there was no statistically significant difference in efficiency of inhibition of primary tumor growth between LLC–R9–bearing mice administered by BC1 and CDDP.

Antimetastatic effect of BC1 was dose–dependent with a tendency to increase antimetastatic effect with decrease of drug dose (see Table 3). Essential antimetastatic effect estimated by both the number of metastases (inhibition by 88%, $p < 0.05$) and the volume of metastatic lesion in lung (inhibition by 93%, $p < 0.05$), was registered for a dose of BC1 equal to MTD/20.

It should be mentioned, that the amount of metastases in avascular phase was about 3 times less ($p < 0.05$) in the group of animals, receiving BC1 at a dose of MTD/6 than that in the mice of control group (see Table 4). However, absence of both increase of the number of metastases and total volume of metastatic lesion in lung after administration of BC1 at a dose of MTD/6 evidenced that shift observed in volume distribution of lung metastases to the vascular phase is connected more likely with cumulative inhibiting effect of BC1 on the metastatic process, than with the stimulation of tumor angiogenesis.

Unlike LLC, which was shown to be extremely refractory against CDDP, this anticancer drug revealed different action on LLC–R9 growth and metastasis. Despite significant inhibition of LLC–R9 growth, CDDP didn't display antimetastatic effect. Moreover in the group of animals treated by CDDP, some tendency towards the stimulation of metastatic process in comparison with control group was observed. In contrast to CDDP, antimetastatic effect of BC1 at a dose of MTD/20 was markedly expressed and statistically reliable ($p < 0.01$): the number and volume of lung metastases were 14 and 40 times correspondingly lesser after BC1 therapy than that after administration of CDDP.

Comparative analysis of anticancer effect of BC1 against two different variants of LLC. An analysis of anticancer effect of BC1 revealed significant dependence of its efficacy upon the attributes of tumor model. In case of LLC BC1 didn't exert inhibiting effect on primary tumor growth. Meanwhile, BC1 at the same

Table 1. Model parameters of growth kinetics of LLC and LLC–R9

Tumor strain	Model parameters		
	λ, days $^{-\beta}$	β	t_{lag}, days
LLC	1.23 ± 0.01	0.28 ± 0.01	10.5 ± 0.0
LLC–R9	1.78 ± 0.1	0.22 ± 0.02	12.0 ± 0.2

Table 2. Influence of BC1 on the growth and metastasis of LLC

Number of mice groups	Tumor volume, mm^3		Number of lung metastases	Volume of lung metastases, mm^3
	19th day	26th day		
1st group	63.6 ± 17.0	245.2 ± 86.1	20.1 ± 8.7	74.4 ± 40.7
2nd group	60.0 ± 15.9	192.4 ± 53.1	20.2 ± 6.5	79.8 ± 38.1
3rd group	134.0 ± 30.4	244.1 ± 957.2	26.0 ± 11.9	81.1 ± 45.3
4th group	79.1 ± 20.5	338.2 ± 65.0	19.6± 7.7	72.1± 27.3

Table 3. Influence of BC1 on the growth and metastasis of LLC–R9

Number of mice groups	Tumor volume, mm^3		Number of lung metastases	Volume of lung metastases, mm^3
	19th day	26th day		
5th group	508.5 ± 180.6	663.3 ± 198.5	6.2 ± 1.9	12.2 ± 5.7
6th group	158.4 ± 71.5	98.1 ± 45.0	10.0 ± 3.1	33.9 ± 13.1
7th group	180.1 ± 81.5	155.4 ± 98.1	4.0 ± 3.5	9.6 ± 8.7
8th group	114.5 ± 32.7	194.1 ± 50.1	0.7 ± 0.3	0.8 ± 0.5

Table 4. Number of diploid cells (%) in LLC and LLC–R9 after BC1 treatment

Tumor strain	Total dose of BC1			
	—	MTD/3	MTD/6	MTD/20
LLC	27.5 ± 3.8	36.2 ± 18.6	24.9 ± 2.4	—
LLC–R9	77.0 ± 23.0	—	25.7 ± 2.7	22.6 ± 4.9

Table 5. Relative number of lung metastases as a function of metastatic volume on 26th day after LLC–R9 cell inoculation

Volume of lung metastases, mm^3	Relative average number of metastasis per group			
	5th group	6th group	7th group	8th group
? 1.75	0.68 ± 0.13	0.67 ± 0.08	0.23 ± 0.17	0.5 ± 0.4
> 1.75	0.32 ± 0.07	0.33 ± 0.05	0.77 ± 0.18	0.5 ± 0.4

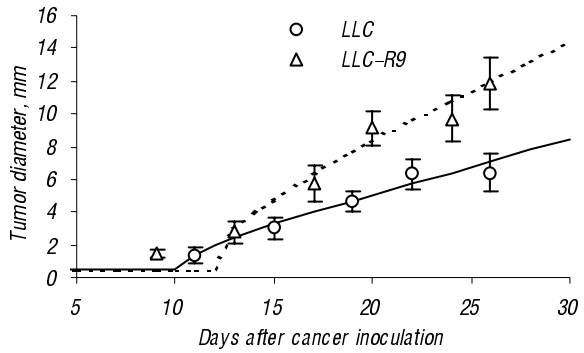

Fig.1. Growth kinetics of LLC and LLC–R9. Solid and dotted lines — best fit of experimental model to LLC and LLC–R9 growth kinetics correspondingly; symbols — experimental data

dose possessed significant ($p < 0.05$) inhibition of LLC–R9 growth by over 75%. It should be noted that such difference of anticancer activity of BC1 between two variants of LLC was manifested at early stages of tumor growth and remained in a week after the end of BC1 treatment (Fig. 2). The obtained results of direct anticancer effect are confirmed by findings of cytofluorimetric study of the level of apoptosis in LLC and LLC–R9 after metronomic administration of various doses of BC1 to the animals. As shown at Fig. 3, in case of LLC BC1 at no one of the examined doses rendered the effect on the amount of cells being in apoptosis. On the contrary, BC1 at both doses (MTD/6 and MTD/20) statistically reliable ($p < 0.05$) twice increased the number of apoptotic cells that was in a good agreement with high antitumor efficacy of BC1 against LLC–R9. Analysis of cell ploidity of various types

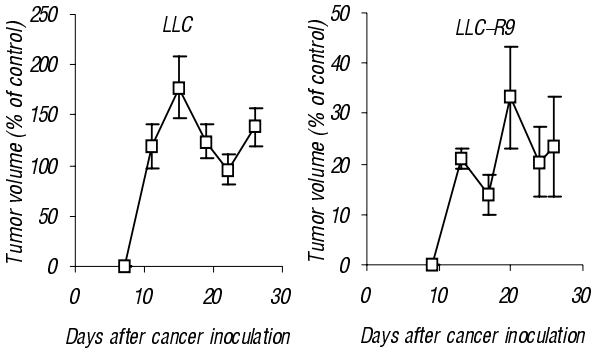

Fig. 2. Influence of BC1 (total dose — MTD/6) on the growth kinetics of LLC and LLC–R9

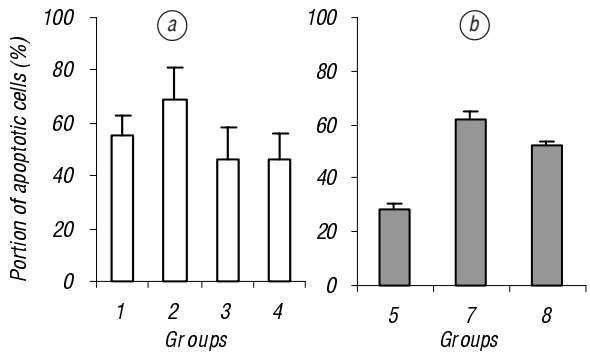

Fig. 3. Portion of apoptotic cells in LLC (*a*) and LLC–R9 (*b*) after BC1 treatment *in vivo* at day 26

of LLC after the impact of BC1 showed, that administration of BC1 to the animals with LLC didn't change ratio of diploid and aneuploid cells in this tumor. Meanwhile, BC1 at both doses statistically reliable ($p < 0.05$) decreased portion of diploid cells in LLC–R9 by three times (see Table 4), evidencing that BC1 induced apoptosis mainly in LLC–R9 cells with normal diploid set of DNA and practically didn't effect aneuploid cancer cells.

DISCUSSION

Ion channel subtypes and membrane potential have been implicated in many aspects of cell biology, including the cell cycle, apoptosis, cell adhesion, cell motility, exocytosis, and multidrug resistance, all of which are relevant to the neoplastic process. Among many known voltage–gated channels, fast sodium channels have been of special importance as a starter of activation of other voltage–gated channels like potassium and calcium channels. The activation of VGSC, results in increased permeability for sodium ions. Short abundant influx of Na(+) ions creates a cascade of events resulting in redistribution of extracellular and intracellular concentrations of ions.

Ion pumps, which regulate ion flow across cells, act as selective targets for drugs used to treat different diseases. Aconitine is considered to target voltage–gated Na(+) channels via promotion of Na(+) channel opening and induction of depolarization of the resting membrane potential, and thus drastically affect the electrophysiological property of cells and tissues. Such properties of aconitine may result in antitumor and/or antimetastatic action.

The results obtained in this study clearly indicated the ability of aconitine–containing herbal extract BC1 to inhibit tumor growth and metastasis. Efficacy of anticancer and antimetastatic action of BC1 depends at a great extent on physiological (both tumor and cellular) peculiarities of the different variants of LLC. Low proliferative activity and high metastatic potential of LLC correlated with high refractoriness of this tumor to CDDP and BC1 action. Significant inhibition of tumor growth of actively proliferating LLC–R9 may be related to direct cytotoxicity of BC1 with dominant cyclospecific mechanism of action. It was also confirmed by significant antitumor activity of cyclospecific drug CDDP against LLC–R9. However, differences observed between BC1 and CDDP in their ability to inhibit metastasis of LLC–R9, pointed out an existence of other possible mechanisms of anticancer and antimetastatic action of BC1 in addition to direct cytostatic action of BC1. The analysis of the available data concerning the role of VGSCs in vital activity of cells showed that BC1 could exhibit antiinvasive and/or antiangiogenic action [19, 20]. Finally, anticancer effect of BC1 independently upon mechanism(s) of its action, should be realized through the induction of apoptosis in tumor cells. Such form of cell death can occur as the result of direct effect of BC1 on apoptosis–associated systems in tumor cells and/or indirectly through possible activation of immune system and/or inhibition of tumor angiogenesis.

Further research presumes specification of the malignant tumors with high sensitivity to BC1 and investigation of the mechanisms of its anticancer action.

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ПРОТИВООПУХОЛЕВОЕ И АНТИМЕТАСТАТИЧЕСКОЕ ДЕЙСТВИЕ АКОНИТИНСОДЕРЖАЩЕГО РАСТИТЕЛЬНОГО ЭКСТРАКТА BC1

Цель: **изучить противоопухолевую активность аконитинсодержащего растительного экстракта BC1 в отношении двух опухолевых моделей с различным метастатическим потенциалом: высоко метастатической карциномы легкого Льюис (LLC) и ее низко метастатического варианта (LLC-R9).** ***Результаты:*** **показано, что низкая пролиферативная активность и высокий метастатический потенциал LLC коррелировал с высокой рефрактерностью этой опухоли к действию BC1. В то же время BC1 проявлял противоопухолевое и антиметастатическое действие в отношении активно пролиферирующей и низко метастазирующей LLC-R9. Максимальное противоопухолевое действие (ингибирование первичной опухоли > 70%) и антиметастатический эффект (ингибирование метастазирования на 88%) наблюдались при суммарной дозе BC1 МПД/20.** ***Выводы:*** **полученные результаты свидетельствуют о высокой противоопухолевой и антиметастатической активности акинитинсодержащего растительного экстракта BC1 в отношении активно пролиферирующего и низко метастатического варианта карциномы легкого Льюис.**

Ключевые слова: **аконитинсодержащий растительный экстракт, противоопухолевая активность, карцинома легких Льюис.**