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ORIGINAL CONTRIBUTIONS

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INFLUENCE OF ACONITINE-CONTAINING HERBAL EXTRACT BC1 ON PROLIFERATIVE AND ELECTROKINETIC CHARACTERISTICS OF ENDOTHELIAL CELLS

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Aim: **To evaluate the influence of new antitumor preparation BC1 produced on the base of *Aconitum* species on viability and electrokinetic properties of endothelial cells for estimation of mechanisms of its antitumor and antimetastatic activity.** ***Materials and Methods:*** **Cytotoxic/cytostatic action of BC1 toward murine aorta endothelial cells (MAEC) and tumor LLC/R9 cells was studied using MTT-test. Apoptotic rate of MAEC was performed by flow cytometry. Electrokinetic properties of MAEC were determined by linear rate of their migration in electric field with the voltage of 20 V/cm.** ***Results:*** **After 24 h of incubation with BC1, IC_{50} for actively proliferating MAEC was 0.95 ± 0.06 μg/ml and was 9-fold and 14-fold lower ($p < 0.05$) than that index for confluent endotheliocytes and LLC/R9 cells respectively. The ability of BC1 to alter electrokinetic characteristics of MAEC and to induce apoptosis has been demonstrated. At the concentration of $IC_{50}/10$, BC1 caused 2-fold decrease of ζ-potential and surface density of charge of MAEC compared to the control ($p < 0.05$) whilst at the concentration of $IC_{50}/20$ — inversion of surface charge of the majority (80%) of the cells in association with BC1-induced apoptosis.** ***Conclusion:*** **High sensitivity of actively proliferating MAEC to the action of BC1 was revealed as well as the ability of that preparation to cause apoptosis and inversion of surface charge of endothelial cells.**

Key Words: **aconitine containing herbal extract BC1, endothelial cells, apoptosis, ζ-potential, surface density of charge.**

As we have shown earlier [1], BC1, an aconitine-containing herbal extract possessed the pronounced antitumor and antimetastatic effects toward transplantable tumor LLC/R9 and had low efficacy toward parental Lewis lung carcinoma (LLC) cells. These two variants of LLC differ by basic parameters of growth and progression, namely by the rates of growth of primary tumor, sensitivity to *in vivo* therapy with cisplatin and by metastatic and angiogenic potentials [1–3]. The latest as well as the data of literature on the mechanism of action of aconitine [3–10] form the base for the suggestion on the possible angiogenesis-mediated antitumor action of aconitine-containing plant extract BC1. Voltage-gated sodium channels (VGSC) are recognized as the main molecular target of the action of aconitine [6–10]; that's why the influence BC1 may lead to the alteration of charge of cell's surface [11–13], and, consequently, of the main functional patterns of the cells [14–16]. Proliferating endothelial cells form the main chain of tumor angiogenesis [12, 17–22]; therefore, the aim of present study was the evaluation of the direct cytotoxic/cytostatic action of BC1 on endothelial cells and its influence at low concentrations on electrokinetic characteristics of endotheliocytes.

MATERIALS AND METHODS

Cell lines. Endothelial cells of MAEC line isolated from murine aorta and spontaneously immortalized in culture [23, 24] were used. MAEC preserve the main biological properties of normal endotheliocytes, including the ability to differentiation and formation of procapillary structures that allow to use them as an adequate model for the study of pro- and anti-angiogenic agents *in vitro*. MAEC were incubated in DMEM medium (Sigma, USA) supplemented with 10% FBS (Sigma, USA), 2 mM L-glutamine and 40 μg/ml gentamycin. LLC/R9 is a LLC subline obtained by 9 sequential treatments with cisplatin *in vivo* [1–3]. LLC/R9 cells were incubated in RPMI 1640 medium (Sigma, USA) supplemented with 10% FBS (Sigma, USA), 2 mM L-glutamine (Sigma, USA) and 40 μg/ml gentamycin. All cell lines were cultured at the standard conditions at 37 °C in humidified atmosphere with 5% CO_2.

BC1 is a new antitumor preparation produced on the base of extract of *Aconitum species* [1]. The calculation of BC1 concentrations was based on the content of aconitine as one of the main active substance.

Test for cytotoxic/cytostatic action. The test for cytotoxic/cytostatic action of BC1 on endotheliocytes of MAEC line and LLC-R9 tumor cells has been performed after 24 and 48 h of incubation of the cells with BC1 at the standard conditions. MAEC cells were seeded in 96-well plates in 0.1 ml of complete medium (5 x 10^4 cells/ml, exponential growth for 48 h) and 2.5 x 10^5 cells/ml (confluent growth). LLC/R9 cells (2.5 x 10^5/ml) were seeded at the same volume. 2 h later, BC1 was added in 10-fold sequential dilutions (to final concentration of 6 to 0.002 μg by aconitine/ml). In 24 and 48 h, the number of cells per well was determined using MTT-test [25]. To evaluate cytotoxic/cytostatic action of BC1 on the cells, IC_{50} index was counted using standard function on the base of the optimal approximation of the model to the data of experimental study by the method of nonlinear regression.

Determination of apoptosis rate in endothelial cells cultured with BC1 at low concentrations. MAEC cells (5 x 10^4/ml) were seeded in 60 mm Petri dishes in a volume of 6 ml of complete culture medium and incubated with BC1 at the concentrations of 0.08 and 0.04 μg/ml ($IC_{50}/10$ and $IC_{50}/20$ respectively) for 2 days at the standard conditions. Then the cell nuclei were isolated, stained with propidium iodide and DNA content was analyzed on flow cytofluorimeter (Becton Dickinson, USA) equipped with argon laser, at the exitation wave's length of 488 nm [26]. The number of apoptotic cells per 10^4 cells was counted using standard program.

Determination of electrophoretic mobility of MAEC cells. The linear speed of migration of MAEC

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Abbreviations used: LLC – Lewis lung carcinoma; MAEC – murine aorta endothelial cells; VGSC – voltage-gated sodium channels.

cells in electric field after 48 h-incubation with BC1 at concentrations of $IC_{50}/10$ and $IC_{50}/20$ was measured using an original device containing special glass capillary with plain walls, allowing to use small volumes of studied cell suspensions and minimizing external influence on electrophoretic mobility of cells at the standard conditions in 0.1 M phosphate buffer at pH 7.15 and 20 °C. The distance between platinum electrodes was 5 cm, and voltage was 100 V. The value of electrokinetic potential (EKP or ζ) was counted, according to the equation of M. Smolukhovsky [27] by the formula ζ- = 14 x U, where U is electrophoretic mobility expressed in ex-systemic units, as the relation of the linear speed of cell migration *v* in electric field determined experimentally (μm/s) to the voltage of the field (V/cm), i.e.

$$\xi = \frac{14 \times v}{E} \text{ (mV)}$$

The surface density of the charge was counted via value of ζ-potential using equation of Quinke — Helmholtz according to which $\zeta = q^{sdc} \cdot \delta/\varepsilon_a$, where q^{sdc} is the surface density of the charge, C/m²; δ (width of double electric layer (DEL)) is a constant equal to 10^{-10} m; ε_a is an absolute dielectric permeability that is equal to the product of electric constant, and dielectric permeability of water $\varepsilon_0 \cdot \varepsilon_{H20}$ at 37 °C.

Statistical analysis of the results was performed using descriptive methods, by Student's *t*-criterium and by the method of nonlinear regression.

RESULTS

The study of cytotoxic/cytostatic action of BC1 on endothelial cells of MAEC line has demonstrated that the inhibition of growth of endotheliocytes strongly depends on the density of the cells: the sensitivity to BC1 of the cells at low density allowing their exponential growth was nearly 10-fold higher than that at confluency (Fig. 1). At the same time the cytotoxic/cytostatic effect of BC1 toward LLC/R9 cells was lower than that toward MAEC cells: IC_{50} for tumor cells was by 50% higher than the index for endothelial cells at confluency ($p < 0.05$), and 13 times higher than that for actively proliferating endotheliocytes ($p < 0.05$) (see Table 1).

Table 1. IC_{50} indexes for BC1 determined on LLC/R9 and MAEC cells

Cell lines	IC_{50} (24 h of incubation)	IC_{50} (48 h of incubation)
LLC/R9	13.3 ± 0.9	ND
MAEC (exp)	0.95 ± 0.06	0.8 ± 0.004
MAEC (confluent)	8.7 ± 2.1	ND

Note: ND – not determined.

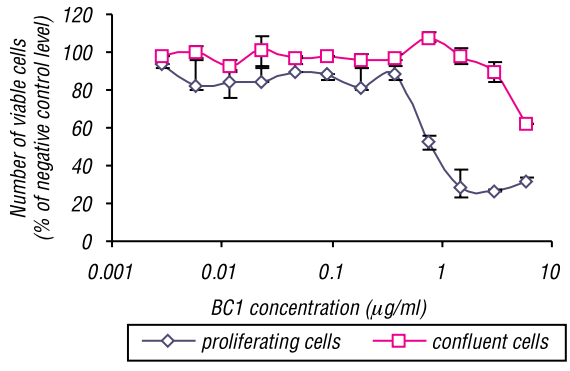

Fig. 1. Survival (%) of endothelial cells incubated for 24 h with BC1 at wide range of concentrations

The prolongation of incubation of actively proliferating endothelial cells with BC1 up to 2 days strengthens the efficacy of the inhibiting effect of this agent (Fig. 2) and results in statistically significant decrease of IC_{50} by 19% ($p < 0.05$, see Table 1); if the concentration of BC1 is lower than 0.1 μg/ml, the cytostatic effect is nearly absent. This fact is supported by the data of MTT-test (see Fig. 2), and by the results on cell cycle phase distribution of endotheliocytes (Fig. 3). As one may see from Fig. 3, no statistically significant alterations of cell cycle distribution were registered if endotheliocytes were incubated with BC1 at the concentrations of 0.08 μg/ml ($IC_{50}/10$) and 0.04 μg/ml ($IC_{50}/20$).

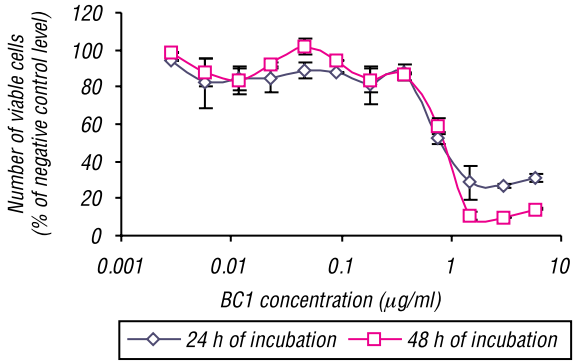

Fig. 2. Influence of BC1 on viability of exponentially growing MAEC cells

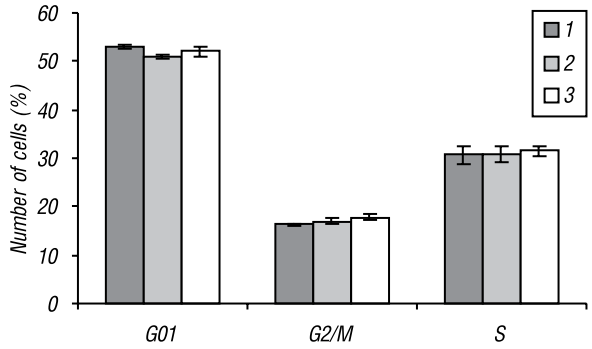

Fig. 3. Influence of BC1 on cell cycle distribution patterns of MAEC: 1 — control; 2 — 0.04 μg/ml BC1 ($IC_{50}/20$); 3 — 0.08 μg/ml BC1 ($IC_{50}/10$)

The evaluation of the ability of BC1 to induce apoptosis in endothelial cells has revealed that BC1 at the concentration of 0.04 μg/ml significantly increases the number of apoptotic cells by 20% compared to the control ($p < 0.05$) (Fig. 4), but at higher concentration ($IC_{50}/10$) didn't influence this index; in both cases no necrotic cells were detected.

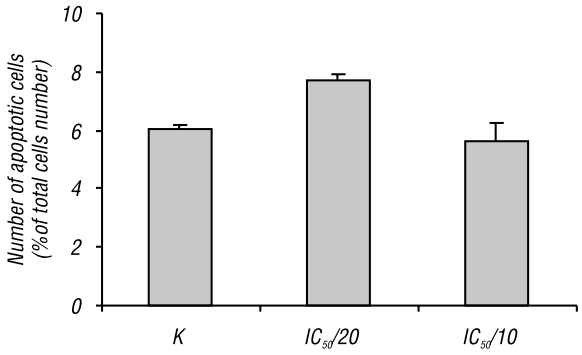

Fig. 4. Influence of BC1 on apoptosis rate of exponentially growing MAEC cells (48 h of incubation): 1 — control; 2 — 0.04 μg/ml BC1 ($IC_{50}/20$); 3 — 0.08 μg/ml BC1 ($IC_{50}/10$)

The study of the influence of BC1 on electrophoretic mobility of MAEC has shown that at lower concentration the agent causes more notable alterations of electrokinetic parameters of endothelial cells than these at higher concentration. In the absence of BC1, nearly 20% of the cells possess positive surface charge and migrate to cathode in electric field with the voltage of 20 V/cm (Table 2), and their distribution by the value of electrokinetic potential possesses unimodal character with the median of 9.56 mV (Fig. 5). At the concentration of $IC_{50}/10$, BC1 decreased electrophoretic mobility of the cells causing nearly 2-fold decrease ($p < 0.05$) of ζ-potential and absolute value of surface charge (Table 3), but the number of migrating cells and the direction of migration didn't alter compared to the control cells, and the cell distribution by the value of electrokinetic potential preserved unimodal character — ζ-potential of more than 90% cells was in the range of 3.5–5.0 mV.

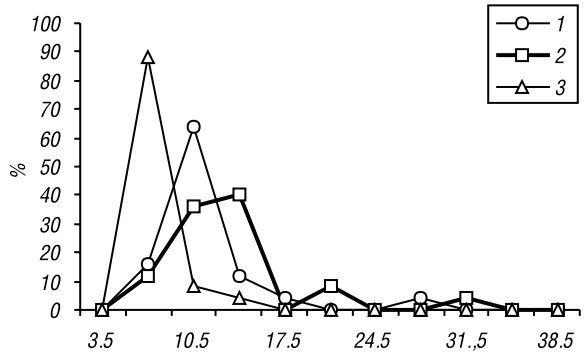

Fig. 5. Distribution of MAEC cells (%) by the value of electrokinetic potential (ζ, mV): 1 — control; 2 — 0.04 μg/ml BC1 ($IC_{50}/20$); 3 — 0.08 μg/ml BC1 ($IC_{50}/10$).

Table 2. Electrokinetic characteristics of endotheliocytes in electric field (20 V/cm) after 48 h action of BC1

Parameters	Control	BC1 (μg/ml)	
		0.04	0.08
% mobile cells	22.5%	82%	20%
Charge of cells and direction of migration in electric field	(+)/cathode	(-)/anode	(+)/cathode

Table 3. Influence of BC1 on electrokinetic characteristics of endothelial cells of MAEC line

Action	ζ-potential (mV)	Surface density of charge (q x 10^{-2} C/m^2)	Surface density of charge (q x 10^{-2} C/m^2) (% of mobile cells)
Control	9.56 ± 1.12	+6.85 ± 0.80	+6.11 ± 0.16 (64%)
BC1 – $IC_{50}/20$ (0.04 μg/ml)	11.84 ± 1.14	-8.49 ± 0.82	-7.83 ± 0.20 (76%)
BC1 – $IC_{50}/10$ (0.08 μg/ml)	4.76 ± 0.46	+3.41 ± 0.33	+2.89 ± 0.06 (88%)

Note: determined in the limits of main mode of cell's distribution.

At the concentration of $IC_{50}/20$, the influence of BC1 on electrophoretic properties of endothelial cells significantly differs from that at concentration of $IC_{50}/10$: BC1 induced negative surface charge in the majority of cells resulting in the increased number of migrating cells in total, their migration toward anode, and also to heterogenization of the population of the cells by the value of ζ-potential. The latest as well as an absolute value of surface charge in the main mode of distribution (composed from 64% of cells) didn't differ from these in control cells, whilst the rest of the cells (approximately 36%) were characterized by the higher values of ζ-potential and respectively by higher surface charge (see Fig. 5, Table 3).

DISCUSSION

In pharmacological studies *in vivo* using LLC/R9 model [1], the significant antitumor effect of aconitine-containing herbal extract BC1 has been revealed, and the elevation of its efficacy upon decreasing total dose of BC1 has been detected [1]. However, in the study of the direct cytotoxic/cytostatic action of this preparation toward tumor cells of LLC/R9 subline *in vitro* it has been shown that IC_{50} index was multifold higher than effective antitumor dose of BC1 that is equal to 0.03 ± 0.01 μg per 1 g of body weight, thus pointing on other mechanism of antitumor action of BC1. We suppose that the latest may be an angiogenesis-dependent one, because LLC/R9 tumor model sensitive to BC1 differs from the parental LLC (insensitive to the agent) by its angiogenic potential [3]. Our research has demonstrated nearly 10-fold difference ($p < 0.05$) in IC_{50} indexes for confluent and exponentially growing endotheliocytes, thus evidencing on the marked selective influence of BC1 on proliferating cells as well as its more potent effect upon prolongated incubation with these cells (see Fig. 1, 2). It's necessary to note that the range of non-cytotoxic concentrations of BC1 toward proliferating MAEC cells upon 48 h of incubation was more wide (see Fig. 2), but at BC1 concentrations > 1 μg/ml more pronounced cytotoxic action was detected that in the case of 24 h incubation.

As far as BC1 contains aconitine as an active substance, that agent may play a role as a proapoptotic factor toward endothelial cells or alter their surface charge [5–7]. That's why the next experiments were directed on the study of the influence of BC1 on the rate of apoptosis and electrokinetic characteristics of endothelial cells at two doses that correlate with antitumor ones in pharmacological studies *in vivo* on LLC/R9 model, are in non-cytotoxic range and respond to IC_{50} index for proliferating MAEC (see Table 1) — $IC_{50}/10$ (0.08 μg/ml) and $IC_{50}/20$ (0.04 μg/ml). It was revealed that whilst at the lower dose BC1 induced apoptosis of the cells (see Fig. 4) and alteration of their main electrokinetic characteristics influencing the value and sign of the surface charge, the decrease of adhesive properties of the cells, at higher dose BC1 didn't induce of apoptosis, nor inversion of the charge, but caused a significant ($p < 0.05$), more than 2-fold decrease of ζ-potential and surface density of charge (see Table 3). Such oppositely directed effects of BC1 on endothelial cells may be explained by the mechanism of action of aconitine. As it was reported [4–10], aconitine modifies 2 types of membrane voltage-gated sodium channels in cells. The first type is stable, and the range of voltage where the activation occurs, is shifted toward negative potentials; such channels are unable to be completely inactivated and their selectivity is strongly altered, that's why they may pass the potassium ions as well as sodium ions [4–8, 10]. The second type of modified sodium channels is unstable because upon the action of aconitine the flow of ions through them is blocked [4, 9]. Our data are supporting such hypothesis: if at concentration of BC1 of 0.08 μg/ml there are significant differences between the values of ζ-potential and surface charge compared with these in the control, the mobility of endothelial cells in electric field is the same as in the control. And, *vise versa*, the

low concentration didn't influence the absolute value of ζ-potential and surface charge, but the character of distribution by ζ-potential is bimodal, and the surface charge of the cells is changed to negative one.

So, BC1 at the concentration of 0.04 μg/ml causes the induction of apoptosis and inversion of charge of endothelial cells. It is known that the surface charge of the cells is being formed by a number of membrane-associated molecules (positively and negatively charged); at physiologic pH migrating cells possess certain charge that determine their galvanotaxis in electric field or toward anode (corneal endothelium, fibroblasts, peritoneal macrophages, osteoclasts, a number of cancer cells of epithelial origin [14, 15, 28–30]), or toward cathode (epidermal and corneal keratinocytes, pigmental retinal epithelium, embryonic fibroblasts and osteoblasts [11, 15, 16, 28–30]). Possibly, the inversion of cell's charge upon the action of 0.04 μg/ml of BC1 may be linked to the altered flow of monovalent ions through VGSC [8, 9, 31–34]. It has been shown [12] that the stimulation of endothelial cells with electric field leads to elevated secretion of VEGF and reorientation of endothelium, where the single cells move to anode, whilst confluent cells — to cathode. It was reported that in the place of injury and in tumor the negative charge is induced [11–13, 16, 28, 35– 37], promoting migration of endothelial cells to the place of injury or to tumor cells. Aconitine activates VGSC that similarly to stimulation with weak electric field, may result in reorganization of endothelium [12, 13], elevation of VEGF secretion, change in cell orientation (perpendicularly to the direction of electric field) and migration — a key event in angiogenesis [12, 28]. The disturbancy of VGSC selectivity upon the action of BC1 may lead to the diffusion of potassium ions in the cells through sodium channels [10, 33–35] influencing in turn the level of intracellular Ca^{2+} [31, 35]. Consequently, it may lead to the suppression of endothelial cell proliferation via inhibition of kinases of receptors of proangiogenic growth factors [10, 32–34, 36], to secretion of proapoptotic factors [38–42] or to inhibition of endothelial cell migration and differentiation [13, 14]. Upon tumor angiogenesis *in vivo,* the charge of endothelial cells and smooth muscle cells or pericytes supports cell-to-cell contacts and formation of neovasculature [12–14, 16, 20], but the inversion of the charge of endotheliocytes due to the action of BC1 may inhibit that process. Maybe, this fact may explain the antitumor and antimetastatic effect of BC1 *in vivo* using experimental model LLC/R9 and its strengthened action upon lowered dose.

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ВЛИЯНИЕ АКОНИТИНСОДЕРЖАЩЕГО РАСТИТЕЛЬНОГО ЭКСТРАКТА BC1 НА ПРОЛИФЕРАЦИЮ И ЭЛЕКТРОКИНЕТИЧЕСКИЕ ХАРАКТЕРИСТИКИ ЭНДОТЕЛИАЛЬНЫХ КЛЕТОК

Цель: **для выяснения механизмов противоопухолевой и антиметастатической активности нового противоопухолевого агента BC1 с возможным ангиогенез-опосредованным действием оценивали его влияние на пролиферацию и электрокинетические характеристики эндотелиальных клеток.** ***Материалы и методы:*** **цитотоксическое/цитостатическое действие BC1 в отношении эндотелиальных клеток аорты мыши (MAEC) и опухолевых клеток LLC/R9 определяли с помощью MTT-теста. Уровень апоптоза оценивали методом проточной цитофлуориметрии. Электрокинетические характеристики MAEC определяли по линейной скорости их движения в электрическом поле с напряженностью 20 В/см.** ***Результаты:*** **установлено, что IC_{50} для активно пролиферирующих MAEC к действию BC1 через 24 ч инкубации с агентом равнялся 0,95 ± 0,06 мкг/мл и был в 9 ($p < 0.05$) и в 14 раз ($p < 0.05$) ниже, чем аналогичный показатель для конфлюентных эндотелиоцитов (8,7 ± 2,1 мкг/мл) и LLC/R9 (13,3 ± 0,9 мкг/мл) соответственно. Обнаружена способность BC1 существенно изменять электрокинетические характеристики MAEC, а также индуцировать их апоптоз. В концентрации $IC_{50}/10$ BC1 приводил к двукратному ($p < 0,05$) по сравнению с контролем снижению ζ-потенциала и поверхностной плотности заряда MAEC; в концентрации $IC_{50}/20$ — к инверсии поверхностного заряда большинства (около 80%) клеток, что ассоциировалось с повышением уровня BC1-индуцированного апоптоза.** ***Выводы:*** **выявлена высокая чувствительность активно пролиферирующих MAEC к действию BC1, а также способность последнего индуцировать апоптоз и приводить к инверсии их поверхностного заряда, что позволяет рассматривать эндотелиальные клетки в качестве одной из мишеней действия BC1 как противоопухолевого и антиметастатического агента.**

Ключевые слова: **аконитинсодержащий растительный экстракт BC1, эндотелиальные клетки, апоптоз, ζ-потенциал, поверхностная плотность заряда.**