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PHARMACOLOGICAL EFFECT OF AMINOFERROCENE IN MICE WITH L1210 LEUKEMIA

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***Aim*: To study the cytostatic and some biological effects of aminoferrocene using mice with L1210 lymphoid leukemia. *Materials and Methods*: Experiments were performed on BDF1 male mice (DBA/2, female × C57Bl/6, male) with transplantable L1210 lymphoid leukemia. Determination of antitumor activity of Benzyl-Fc Boron (Bn), it was injected intraperitoneally 6 times daily, starting on day 2 after L1210 leukemia cell transplantation. Doses of Bn such as 26; 260 and 2600 μg/kg were used. The determination of intracellular content of cardiolipin, thiols, reactive oxygen species (ROS) and also analysis of Annexin V positivity and mitochondrial transmembrane potential (JC-1 staining) were performed with use of flow cytometry. The levels of "free iron" complexes, transferrin active forms and the rate of NO generation were measured by EPR-specroscopy. *Results*: Six daily injections of Bn at a dose of 26 μg/kg resulted in an increased survival of mice with L1210 leukemia by 28% ($p < 0.05$). Bn led to an increase of apoptotic cells number and ROS amount in leukemia cells. Besides, Bn caused a decrease of cardiolipin and nonprotein thiol compounds content. The membrane electrochemical potential of cell mitochondria was decreased also after Bn administration. Studies using EPR-spectroscopy revealed a significant increase in a level of "free iron", content of transferrin active species and generation rate of NO by inducible NO-synthase in L1210 cells after aminoferrocene administration. *Conclusion*: Our data indicate that Benzyl-Fc Boron can be promising candidate for realizing a new strategy of anticancer therapy with the use of ROS-inducing agents.**

***Key Words*: aminoferrocene, tumor, L1210 leukemia, mitochondria membrane electrochemical potential, cardiolipin, thiols, ROS, transferrin, NO-generation.**

In the last decade, some publications have appeared showing an antitumor activity of ferrocene and its derivatives [1–9]. It has been shown that the representatives of these compounds exhibit cytotoxic activity *in vitro* and lead to retardation of some ascitic tumors growth [9, 10]. According to data of several authors, an antitumor effect of ferrocene and its derivatives is achieved through the production of H_2O_2 and hydroxyl radicals [1, 2, 9]. Other researchers suggest that this effect is realized by the activation of the immune system [1, 10] or due to the influence on estrogen receptors in the case of breast cancer cells [11]. Moreover, the complexes of ferrocene and its derivatives with biologically active molecules [12–16], retinoids [17, 18], hormones and hormone antagonists [19], metals such as Co, Cu, Zn, Pt, Pd, Rh, Ir [20–25], are sometimes used as anticancer agents. For example, aminoferrocene-based prodrugs were found to exhibit significant toxicity towards a range of cancer cell lines (HL-60, JVM-2, BL-2, Raji, HeLa, PANC-1, SK-OV-3, U-373, LNCaP, DU-145, RKO) and primary cancer cells (CLL cells), whereas their effect on normal (non-cancerous) cells including mononuclear cells (MNC's) and fibroblasts was found to be limited [26, 27]. Moreover, it has been demonstrated that aminoferrocenes are activated in the presence of elevated concentrations of reactive oxygen species (ROS), which are conditions characteristic for cancer cells [9]. However, no *in vivo* studies of these promising prodrugs have been reported up to date.

Owing to the above-mentioned, the objective of our work was the study of antitumor activity of the aminoferrocene-based prodrug N-ferrocenyl-N-benzyl-aminocarbonyloxymethyl-phenylboronic acid pinacol ester (briefly Bn) and the study of the mechanism of its action on L1210 murine lymphoid leukemia models. Since Bn is the donor of iron, we have studied some of the indicators that characterize the state of the intracellular ROS/antioxidants balance.

MATERIALS AND METHODS

Synthesis of Bn. N-Ferrocenylaminocarbonyloxymethylphenyl boronic acid pinacol ester (compound 1, 50.0 mg, 108 μmol) was dissolved in anhydrous DMF (2 ml) under a nitrogen atmosphere. The solution was treated with NaH (5.20 mg, 130 μmol, 60% mineral oil suspended) and stirred 30 min at 22 °C. Benzyl bromide (27.8 mg, 19.3 μl, 163 μmol) was added and the reaction was stirred for 2 h at 22 °C. Then, the solvent was removed in vacuum (0.01 mbar) and the product was purified by column chromatography on silica gel using petroleum ether/acetone (4/1, v/v) as eluent. Yield: 55 mg, 100 μmol (92%). TLC (SiO_2, eluent petroleum ether/acetone, 4:1, v/v) $R_f = 0.5$; ^{1}H-NMR (aceton-d_6, 300 MHz) δ (ppm) 7.73 (d, 2 H), 7.39–7.27 (m, 7 H), 5.25 (s, 2 H), 5.02 (s, 2 H), 4.46 (s, 2 H), 4.12 (s, 5 H), 3.97 (s, 2 H), 1.34 (s, 12 H). The spectra are in agreement with those reported for this compound.

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Abbreviations used: Bn – Bensyl-Fc Boron; iNOS – inducible NO-syntase; MTD – maximum tolerated dose; PI – propidium iodide; ROS – reactive oxygen species.

All *in vivo* experiments were performed in compliance with all the requirements of biological ethics on BDF1 male mice (DBA/2, female × C57Bl/6, male) weighing 20–22 g and bred in vivarium of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine. The animals were kept under standard laboratory conditions: in polystyrene cages, coated with galvanized steel mesh, the room temperature was 20–22 °C, air humidity — 60–70%.

Firstly Bn was dissolved in DMSO at 37 °C. Then, a certain amount of PBS was added to obtain the desired concentration of Bn. While determining the acute toxicity indices (MTD and LD50) Bn was injected intraperitoneally at a single dose of 10; 50; 100; 150; 200; 300; 400; 500; 700; 1000; 1500; 3000, and 6000 μg/kg. 6 mice were used to evaluate each dose. Animals were kept under observation for 3 weeks.

While determining the antitumor activity of Bn, it was injected intraperitoneally 6 times daily, starting on day 2 after transplantation of L1210 lymphoid leukemia. L1210 was transplanted intraperitoneally in the amount of $1 \cdot 10^6$ cells per mouse. After the transplantation all of the animals were divided into 4 groups (10 mice in each): I — tumor control (intraperitoneal injection of 0.4 ml of saline); II — injection of Bn at a dose of 26 μg/kg; III — injection of Bn at a dose of 260 μg/kg; IV — injection of Bn at a dose of 2600 μg/kg.

To evaluate the mechanism of Bn action it was injected only in the dose that was effective at determination of average lifetime of animals. There were 5 mice in each group, including the control one. The injection of Bn started 24 hours after L1210 cells transplantation. 6 daily intraperitoneal injections were performed. An ascites was washed out from the abdominal cavity and determination of the number of apoptotic cells, cardiolipin and non-protein thiol compounds content, mitochondria functional activity (JC-1) and the amount of ROS in cells were performed one day after the last injection of Bn.

Measurement of Annexin V and propidium iodide (PI) positivity. Translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane occurs during apoptosis and can be assessed by exploiting the high binding affinity of Annexin V, a Ca^{2+}-dependent phospholipid binding protein to phosphatidylserine. To examine whether cell death occurred via apoptosis or necrosis, PI, a non-permeable stain with affinity towards nucleic acids, which selectively enters necrotic or late apoptotic cells, was used. Therefore, costaining of Annexin V and PI helps discriminate between live cells (PI and Annexin V negative), cells in early apoptosis (Annexin V positive, PI negative), cells undergoing late apoptosis (both Annexin V and PI positive) or necrotic cells (PI positive, Annexin V negative). For detection of apoptotic cells we used apoptosis detection kit (Annexin V-FITC kit, Beckman Coulter, USA). Briefly, L1210 cells ($2.5 \cdot 10^5$/ml) after two washes, cells were resuspended in Annexin V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and Annexin V-FITC was added according to the manufacturers' instructions. The cells were incubated for 10 min in the dark at 37 °C and just 5 min prior to acquisition, PI (0.1 mg/ml) was added and cells were washed and placed then in a flow cytometer (Beckman Coulter Epics XL, USA).

Measurement of cardiolipin content. To determine the cardiolipin content in mitochondria, 10-N-nonyl acridine orange (NAO) was used. Briefly, L1210 cells ($2.5 \cdot 10^5$/ml) were washed with PBS, labeled with NAO (100 nM in methanol, 37 °C, 20 min), acquired at FL1 channel and analyzed in a flow cytometer.

Analysis of mitochondrial transmembrane potential. The mitochondrial transmembrane electrochemical gradient (Dym) was measured using JC-1. JC-1, a cell permeable, cationic, lipophilic dye freely crosses the mitochondrial membrane and forms J-aggregates which fluoresce red; accordingly, viable cells with a normal mitochondrial membrane potential when stained with JC-1 exhibit a pronounced orange fluorescence (FL2). Following an apoptotic stimulus, the resultant decrease in the mitochondrial membrane potential prevents JC-1 from entering the mitochondria and remains as monomers in the cytosol that emits a predominantly green fluorescence (FL1). Therefore, the ratio of J-aggregates/monomers serves as an effective indicator of the cellular mitochondrial transmembrane potential, allowing apoptotic cells to be easily distinguished from their non-apoptotic counterparts. Briefly, L1210 cells ($2.5 \cdot 10^5$/ml) were stained with JC-1 (7.5 mM in PBS, 10 min, 37 °C). Cells were then acquired in a flow cytometer on the basis of quadrant plot to distinguish monomers from J-aggregates. To set the quadrants, cells were treated with H_2O_2 (20 mM, 37 °C, 30 min), representative of cells with depolarized mitochondrial membrane potential.

Flow cytometric determination of intracellular thiols. Non-protein thiols were measured. L1210 cells ($2.5 \cdot 10^5$/ml) were washed, resuspended in PBS and labelled with CMFDA (0.05 mM, 37 °C, 20 min). CMFDA is a cell permeable, non-fluorescent dye that upon entering the cell, rapidly binds with non-protein thiols and becomes non-permeable. The simultaneous cleavage of the diacetate moiety by cellular esterases yields a fluorescent thioether whose fluorescence is acquired in a flow cytometer at FL1 channel.

Measurement of intracellular ROS. CM-H2DCFDA, a lipid soluble membrane permeable dye upon entering cells undergoes deacetylation by intracellular esterases and forms the more hydrophilic, non-fluorescent dye Dichlorodihydrofluorescein (DCFH2). This is subsequently oxidized by ROS with formation of a highly fluorescent oxidation product, Dichlorofluorescein (DCF). The generated fluorescence is directly proportional to the amount of ROS. Fluorescence was analyzed by flow cytometry. The effect of Bn on generation of ROS was measured in L1210 cell ($2.5 \cdot 10^5$ cells). After centrifugation (1500 rpm for 5 min) cells were resuspended in PBS, incubated for 30 min at 37 °C with CM-H2DCFDA (10 mM) for measu-

rement of ROS. Positive control with 25 μM H_2O_2 was also made (data not presented). Fluorescence was acquired in the log mode and expressed as geometrical mean fluorescence channel (GMean). Acquisition was performed on 10,000 gated events.

Determination of "free iron" complexes, the content of active forms of transferrin and the rate of NO generation. Levels of transferrin and "free iron" in L1210 leukemia cells were measured by EPR-spectroscopy method at the temperature of liquid nitrogen (77 K). The speed of generating NO by iNOS (inducible NO-synthase) was also measured by EPR method using a spin trap DETK (Sigma) at a room temperature.

Main blood indices of mice was investigated by the use of a haematological analyzer Particle Counter PCE-210 (Erma Inc., Japan). Statistical processing of the data was performed by the use of the software Statistica v. 7.0 and Student's *t*-criterion. A statistically significant difference was considered to be present at $p < 0.05$.

RESULTS AND DISCUSSION

Synthesis of 4-(N-ferrocenyl-N-benzyl-aminocarbonyloxymethyl)-phenylboronic acid pinacol ester. We previously reported a procedure for preparation of compound Bn under optimized conditions (Fig. 1). In particular, we used compound 1 as a starting material which can be obtained from commercially available starting materials in large quantities with 74% yield [28]. Compound Bn was alkylated in the presence of Cs_2CO_3 using BnCl/(NBu_4)I mixture as an electrophile. This procedure led to clean conversion of 1 into Bn with 71% yield of the analytically pure product. However, due to the low solubility of the (NBu_4)I in the column chromatography eluent it was necessary to adsorb the crude reaction mixture onto silica. Unfortunately, this led to visible decomposition on dry silica which explains the yield of 71%, despite full conversion to the product.

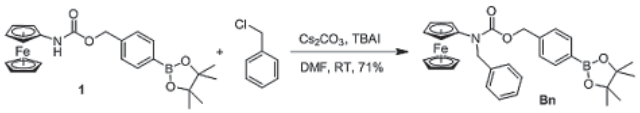

Fig. 1. Outline of the previously reported method of synthesis of compound Bn [28]

For further optimization of the synthesis, an alternative approach to Bn was developed (Fig. 2). It consisted of the alkylation of 1 in the presence of NaH using either benzyl chloride or bromide as electrophiles. This procedure led again to clean conversion of 1 into Bn. However, it was possible to dissolve the crude reaction mixture in the appropriate eluent for column chromatography. Therefore adsorption on silica was not necessary. This led to an isolated yield of analytically pure Bn with a yield of 92%.

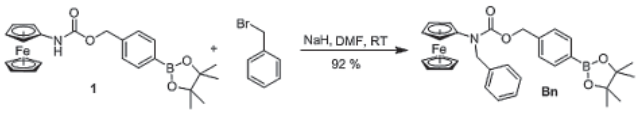

Fig. 2. Outline of the optimized method of synthesis of compound Bn

Biological studies. At the first stage of the investigation an acute toxicity of Bn was studied. That is, it was necessary to determine the maximum tolerated dose (MTD) and the lethal dose which causes the death of animals in 50% (LD50) at a intraperitoneal injection. For this purpose the BDF1 hybrid mice (DBA/2, female × C57Bl/6, male) were used, which were injected intraperitoneally with Bn at a single dose of 10; 50; 100; 150; 200; 300; 400; 500; 700; 1000; 1500; 3000, and 6000 μg/kg. All the above-stated doses of Bn were non-toxic (i.e., all of the mice were alive after Bn injection) (Table 1).

Table 1. Data on the study of acute toxicity of Bn on BDF1 hybrid mice

Dose of Bn, μg/kg	Number of animals per group	Number of alive animals	Number of dead animals
10	6	6	0
50	6	6	0
100	6	6	0
150	6	6	0
200	6	6	0
300	6	6	0
400	6	6	0
500	6	6	0
700	6	6	0
1000	6	6	0
1500	6	6	0
3000	6	6	0
6000	6	6	0

At the second stage of studies an antitumor activity of Bn in relation to the L1210 leukemia was investigated. The studies found that the injection of Bn at a dose of 26 μg/kg resulted in an increase of mice lifetime by 28% (Table 2, Fig. 3). Such doses of Bn as 260 and 2600 μg/kg were ineffective. It should be noted that the increase in the number of past days by 28% for mice with ascitic lymphoid leukemia L1210 should deserve attention, as a minimum percentage of the effect for the positive assessment is the increase of lifetime by 25% [29].

Table 2. Number of past days (days of survival) after L1210 cells transplantation

Group of mice with L1210	Number of past days
I (control), n = 10	13.7 ± 0.6
II (Bn; 26 μg/kg), n = 10	17.5 ± 0.7*
III (Bn; 260 μg/kg), n = 10	14.7 ± 0.5
IV (Bn; 2600 μg/kg), n = 10	13.7 ± 0.6

Note: *p<0.05 compared with control mice.

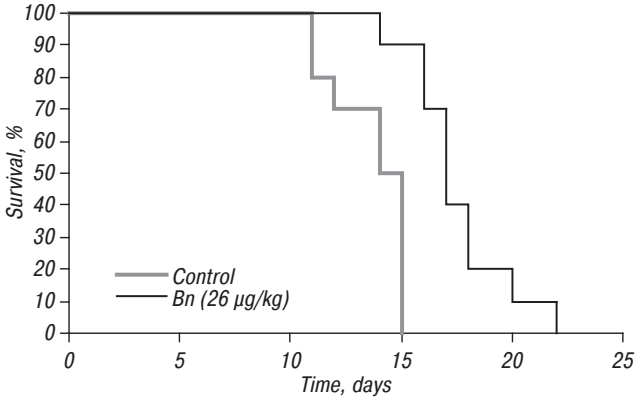

Fig. 3. Mice lifetime after L1210 cells transplantation (Kaplan — Meier survival curves)

In order to identify the mechanism of Bn action in the control mice and animals injected with Bn at a dose of 26 μg/kg, the ascites was washed out from their abdominal cavity. After that, the determination of the number of apoptotic cells, cardiolipin and non-protein thiol

compounds content, mitochondria functional activity (JC-1) and the amount of ROS in cells was performed.

As a result of studies, it was found that 6 daily injections of Bn at a dose of 26 μg/kg led to an increase in the amount of apoptotic cells from 5.24±0.3 to 8.0±0.4% ($p < 0.05$) (Fig. 4). Besides, Bn caused a decrease of cardiolipin content in ascitic cells (Fig. 5). Whereas in the control group a level of fluorescence gave a value of 150.3 ± 10.5 arbitrary units (a.u.), in the group injected with Bn it was 104.1 ± 13.1 a.u. ($p < 0.05$). Cardiolipin is known to be a very important phospholipid of an inner mitochondrial membrane and is involved in ensuring a coupling of oxidative phosphorylation process.

The fluorescence of the dye JC-1 also indicates a deterioration of a mitochondria functional state (Fig. 6). The fluorescence of JC-1 indicates the total value of the membrane electrochemical potential of all mitochondria of the studied cells. The index of green fluorescence (JC-1 monomers) in the control group was 48.2 ± 3.1% and after Bn administration it was 97.0 ± 4.3% ($p < 0.05$).

An important stage in the study was an evaluation of so-called "redox state" of ascitic cells. For this purpose we have determined a content of non-protein thiol compounds (glutathione content practically). Bn led to a decrease of this index in ascitic cells: the level of fluorescence in cells after Bn administration was 132.4 ± 12.5 a.u. and 174.9 ± 15.0 a.u. in the control group ($p < 0.05$) (Fig. 7). This data conformed well with the results of the ROS determination in cells (Fig. 8). In the control group the level of fluorescence was 163.3 ± 20.0 a.u., while after Bn administration it was

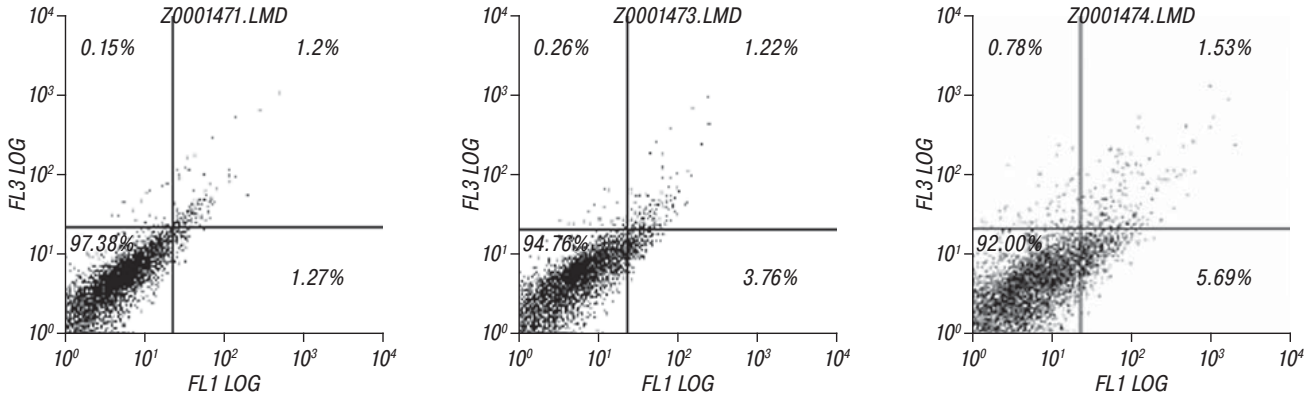

Fig. 4. Alteration of the number of L1210 cells undergoing apoptosis and necrosis after Bn administration. In order from left to right: 1 — control without colour, 2 — cells of the animal from the control group, 3 — cells of the animal administered with Bn

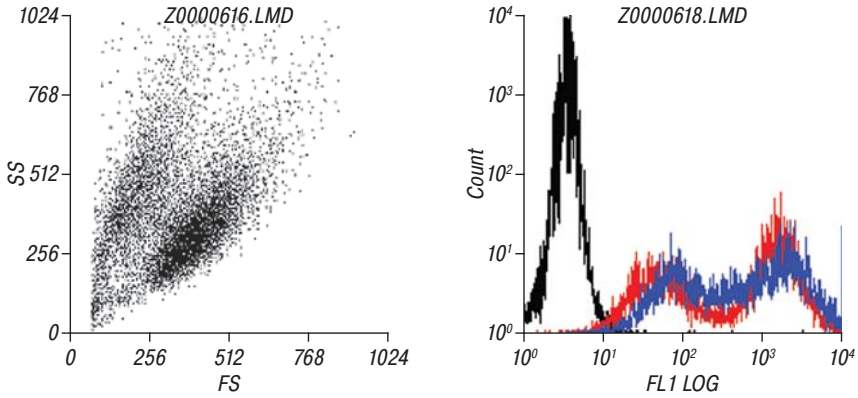

Fig. 5. The content of cardiolipin in L1210 cells after Bn administration (flow cytotometry). On the left — Dot Plot, the cells are distributed by size and granularity. We can see two cell populations. On the right: black — unpainted control, blue — control cells, red — cells from the animal administered with Bn

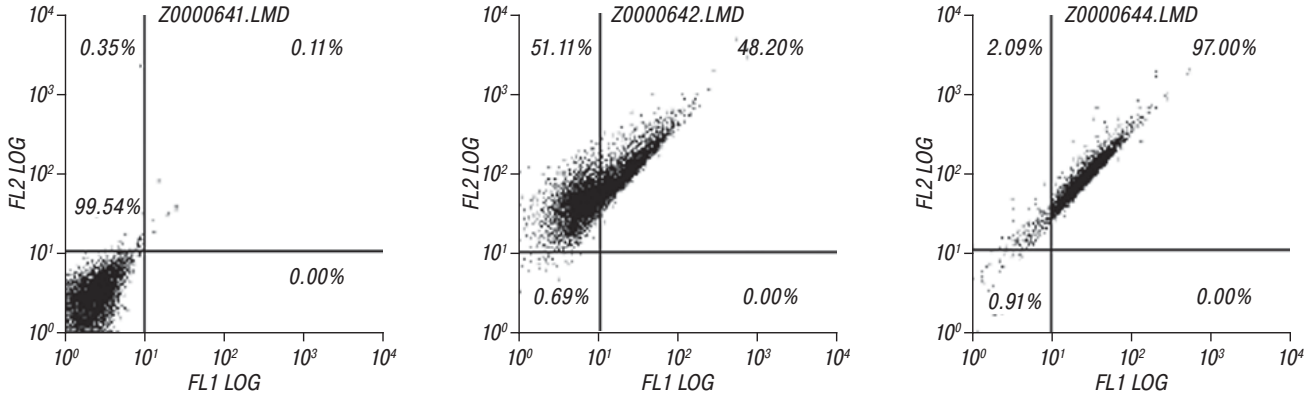

Fig. 6. Alteration in the mitochondria membrane electrochemical potential of L1210 cells after Bn administration (flow cytometry). In order from left to right: 1 — control without colour, 2 — cells of the animal from the control group, 3 — cells of the animal administered with Bn

230.0 ± 18.5 a.u. ($p < 0.05$). In fact, a reduction of non-protein thiol compounds is accompanied by an increase in the amount of ROS.

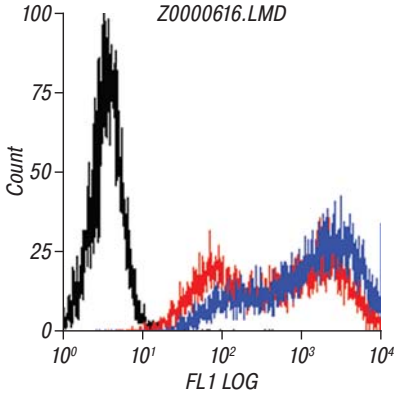

Fig. 7. The content of non-protein thiol compounds in L1210 cells after Bn administration (flow cytometry). Black — unpainted control, blue — control cells, red — cells of the animal administered with Bn

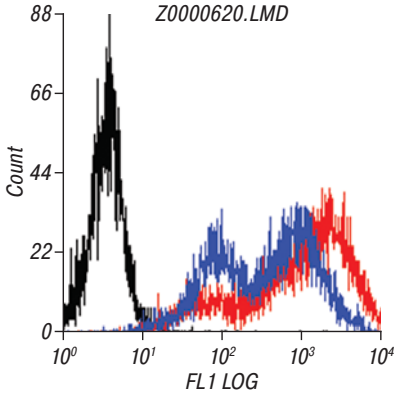

Fig. 8. The content of ROS in L1210 cells after Bn administration (flow cytometry). Black — unpainted control, blue — control cells, red — cells of the animal administered with Bn

Another direction in the investigation of biological effects of Bn was an assessment of its impact on the number of haematological indices. Therefore, main blood indices of the animals with L1210 were determined. It was found that a clear tendency to reduction in the total number of leukocytes in the mice peripheral blood was fixed after a 6 times daily Bn injection (Table 3). Moreover, such decrease occurs due to the reduction in the number of lymphocytes. The total number of erythrocytes, thrombocytes and haemoglobin level in the blood of mice received Bn, did not differ from the blood indices of control animals.

Table 3. Main peripheral blood indices of mice with L1210 after a course of Bn therapy

Indexes	Control (n = 5)	Bn (n = 5)
Number of erythrocytes, $\times 10^9$/ml	10.5 ± 0.4	9.9 ± 0.4
Haemoglobin level, g/dl	15.7 ± 0.6	14.7 ± 0.7
Number of thrombocytes, $\times 10^6$/ml	485.0 ± 67.3	496.0 ± 37.6
Total leukocytes, $\times 10^6$/ml	18.0 ± 2.0	14.4 ± 1.3
Number of lymphocytes, $\times 10^6$/ml	9.8 ± 1.1	5.6 ±0.8*
Number of monocytes, $\times 10^6$/ml	2.7 ± 0.2	2.9 ± 0.3
Number of granulocytes, $\times 10^6$/ml	5.5 ± 0.8	5.9 ± 0.6

Note: *$p < 0.05$ compared with the control group.

Seemingly, an important part in the mechanism of Bn action is its influence on cardiolipin. It is believed that this phospholipid can integrate elements of the mitochondria respiratory chain in an integral complex [30]. It has been found that the peroxide degradation of cardiolipin stimulates a release of cytochrome C from mitochondria into the cytoplasm, and this process is associated with apoptosis [31]. In general, most studies support the idea that cardiolipin directly initiate apoptosis [32, 33]. Even there is a hypothesis that an interaction between cardiolipin and cytochrome C deteriorates before apoptosis start, and this leads to the mitochondrial membrane permeabilization [34]. In addition, cardiolipin supports mitochondrial membrane potential at the required level.

Studies using EPR-spectroscopy revealed a significant increase in a level of "free iron", content of transferrin active species and generation rate of NO by inducible NO-synthase in L1210 cells after aminoferrocene administration, compared with the control group (Table 4). Such alterations in L1210 cells may lead to an increase in generation of ROS and blocking of Fe-S protein centers in mitochondrial electron transport chain, which initiated cell death caused by necrosis or apoptosis.

Table 4. The level of "free iron" and transferrin active species, the rate of NO generation in L1210 cells after aminoferrocene administration

Index	Control (n = 5)	Aminoferrocene (n = 5)
"Free iron", a.u.	0.80 ± 0.10	2.16 ± 0.18*
Content of transferrin active species, a.u.	0.19 ± 0.02	0.37 ± 0.03*
NO generation rate (nmol/2.5 · 10^5 cells)	0.40 ± 0.04	0.77 ± 0.06*

Note: *$p < 0.05$ compared with the control group.

Thereby, the indices which we determined in this study are complementary. Bn leads to a generation of ROS in L1210 cells. And this is accompanied by a decrease of non-protein thiol compounds, which have a leading role in protection of proteins, lipids, nucleic acids in cells from oxidative stress. During the process of activation of free radical oxidation the cardiolipin becomes oxidized releasing cytochrome C and making its transfer into the cytoplasm possible. After release into the cytoplasm, it forms a complex with apoptosis-inducing factor (Apaf-1) and procaspase-9 that activates downstream caspases, which carry out apoptotic cell death. Therefore, our data indicate that aminoferrocenes, particularly Bn, can be promising candidates for realizing a new strategy of anticancer therapy with the use of ROS-inducing agents. Aminoferrocenes are modern direction of redox-biotechnology.

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