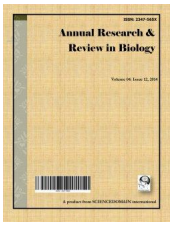

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Anti-cancer Properties of Anphen Sodim and Its Effect on Antiapoptotic Proteins of the Bcl-2 Family

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aims: It's known that apoptosis, necessary for renewal and vital activity of cells, is suppressed in the tumour cell. The strategies of anti-cancer therapy may be a search of the new drugs and development of targeted substances, including the Bcl-2 family proteins, to initiate apoptosis. One of such drugs may be spatially substituted phenol anphen sodium (AS), a derivative of dibunol that can inhibit free radical oxidation and interact with peroxide radicals in the cell, and has also biological activity. The study aims to investigate possible anti-cancer, antioxidant AS action on experimental tumours of the ascitic sarcoma 37 and Lewis carcinoma of F1 (C57Bl × DBA) mice. The influence of AS drug on Bcl-2 family proteins level in blood plasma Lewis carcinoma cells suspension in spleen cells of white mice was determined.
Place and Duration of Investigation: Emanual Institute of Biochemical Physics Russian Academy of Sciences, Moscow, Russia, between October 2013 and March 2018.
Metodology: To examine AS anti-cancer properties, the kinetic curves of tumour development and

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the number of ascitic cells in ascitic fluid were studied; the changes in anti-apoptotic Bcl-2 proteins and Bcl-2 family proteins levels were monitored by immunoblotting.
Results: A significant (100%) anti-tumour effect of the antioxidant sodium antepoxide AS (2-(carboxy) -2- (N-acetylamino) -3- (3 ', 5'-di-t-butyl-4'-hydroxyphenyl) - sodium propionate was seen when it was administered to mice after transplantation of ascites sarcoma cells 37. The reduction of the Bcl-2 protein took place in the blood plasma of F1 mice (C57B1 × DBA), when AS (10^{-4}M) was administered to mice before transplantation by Lewis cells of carcinoma, as shown by immunoblotting. At the same time, this did not change the survival rate of mice. The administration of AS into the Lewis carcinoma cell suspension causes a dramatic decrease in the amount of monomer and homodimer of Bcl-2 protein for 1-3 hours in these cells. AS drug administered during 4 days (10^{-4} M) to white mice caused a change in the ratio of Bcl-2 family proteins in the spleen cells, indicating the onset of the mitochondrial pathway of apoptosis.
Conclusion: The anti-cancer effect of AS can be associated with an effect on the molecular targets of the apoptosis pathway, including the proteins of the Bcl-2 family.

Keywords: Ascites sarcoma 37; Lewis carcinoma; Bcl-2 protein; anti-cancer properties; anphen sodium.

1. INTRODUCTION

Spatially hindered phenols, synthesised in the Institute of Biochemical Physics RAS, antioxidants AS and phenozan potassium, derivatives of dibunol known as anti-inflammatory agents, can inhibit free radical oxidation and interact with peroxide radicals in the cell [1]. They have biological as well as anti-cancer activities [2].

Previously, it was found that antioxidant potassium phenosan [3], prolongs the lifetime of AKR mice, led to an increase in the level of anti-apoptotic Bcl-2 proteins [4], and a decrease in the content of double-stranded DNA breaks in the spleen [5]. Potassium phenosan proved to be effective as an antiepileptic drug, as well as in hypoxia of newborns [6] and was introduced into medical practice, while AS is still under study.

A number of studies have shown that the content of the main apoptosis proteins of protein p53 [7,8] and an antiapoptotic Bcl-2 in tumour cells that allows to characterise the tumour process and its change caused by the action of physicochemical factors [9-14].

Expression of p53 protein and Bcl-2 protein content, that have anti-inflammatory and anti-oxidant functions too [15,16], has been observed in tissues of various tumours including upper respiratory tract and lung tumours [9-11], and in a number of cell lines, for example, human lung carcinoma TW2.6 [11-17].

While tumour growth, the process of apoptosis is usually suppressed, including violation of pro- and anti- apoptotic proteins system balance, as well as the increase of damaging proteins, interfering with the action of caspases, such as XIAP and the newly discovered AVEN protein in tumour cells [18]. Present chemotherapy of tumours is often based on the enhancement of apoptosis in cancer cells, and one of the nowadays directions is the effect on the proteins of the Bcl-2 family. These proteins can be conditionally divided into 3 groups; anti-apoptotic proteins such as Bcl-2, Bcl-XL and others; pro-apoptotic proteins such as BAX, BAK, BOK and others, contain homologous domains (BH1 - BH4, TM), and the third group of apoptosis activators (BAD, NOXA, PUMA and other proteins) comprising BH3-only domain.

It is known that the anti-apoptotic activity of the Bcl-2 protein is based on its ability to inhibit pore formation in the mitochondria and oligomerisation of the BAX apoptosis protein, and also directly bind cytochrome C and displace it from the apoptosome, thereby preventing the activation of caspase. One of the causes of the appearance of pores and the initiation of the apoptosis process in mitochondrial membranes is the imbalance of the apoptotic proteins of the Bcl-2 family. In the process of apoptosis regulation, pro- and antiapoptotic proteins can pair homo- and heterodimers both within their own group (for example, Bcl-2 / Bcl-2; Bcl-2 / Mcl; Bax / Bax; Bax / Bak) and with proteins of the opposite direction (for example, Bcl-2 / Bax) [16,19-22].

The work studied AS anti-cancer effect on ascitic sarcoma and Lewis carcinoma, both in vivo and in vitro experiments. The level of anti-apoptotic Bcl-2 protein was monitored by immunoblotting

methods in the blood plasma of F1 (C57Bl × DBA) mice with Lewis carcinoma, in the suspension of the cells of this tumour and the spleen of healthy white mice.

2. MATERIALS AND METHODS

2.1 Experimental Animals

While studying AS effect on Lewis carcinoma, mouse hybrids of the first generation F1 (C57Bl × DBA) (3-4 months of age, weight 25 g) were used in the work; while studying the ascitic sarcoma 37 in experiments with spleen cells white non-linear mice (cattery of laboratory animals "Stolbovaya") were used. All animal experiments were carried out humanely by trained personnel, following the Animal Care Instructions. Before the extraction of the biological material, the mice were sacrificed under ether anaesthesia.

To study AS anti-cancer activity - 6 x 10^6 tumour cells of ascitic sarcoma 37 strain (0.2 ml of ascitic fluid diluted with saline) were transplanted intraperitoneally in mice (weigh 18-20 g). Tumour growth was assessed by the change in the volume of ascitic fluid and the total number of tumour cells suspended in it according to the standard method [23]. To plot the kinetic curves of tumour volume growth and the number of tumour cells up to the terminal stage, 5 mice were used for each point in the experiment and control.

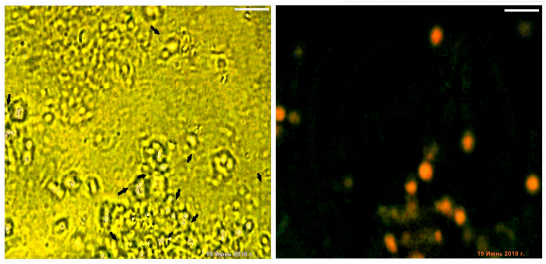
2.2 Lewis' Carcinoma

Lewis carcinoma cells used in the experiments were isolated from the tumour on the 14^{th} day after appropriate transplantation of tumour cells. After grinding, the cell suspension was diluted in physiological saline, injected 0.2 ml (7×10^6 cells/ mouse) into femoral hindlimb muscle of the recipient. To assess the tumour growth, the volume of intramuscular tumour was calculated by using measurements in three planes

2.3 Lewis' Carcinoma Tumour cells-Fluorescence Measurement

The effect of AS on induction of apoptosis was studied in experiments *in vitro*. The suspension of Lewis carcinoma cells (concentration of 5 x 10^8 cells/ml) with medium 199 were incubated with the AS- solution (concentration of 5 mg/ml) at 37°C within 0-3 hours. The control cells contained the same amount of medium 199 instead of AS-solution. Then tumour cells were destroyed by the freezing-thawing cycles and homogenisation, and the resulting suspension was centrifuged. Further, the study of anti-apoptotic proteins was conducted in the supernatant.

To estimate the number of dead tumour cells in non-fixed Lewis carcinoma cells, an ethidium bromide dye was used in a final concentration of 0.1 mg/ml. The fluorescence measurement of tumor cells was performed on a fluorescence microscope upon excitation of 450 nm and a magnification 600 x.

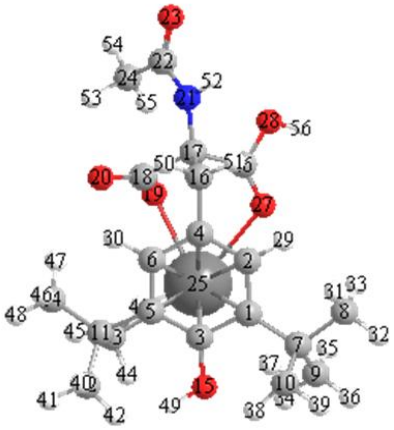


Light microscopic image of these cells is on the left and its fluorescent image - to the right. In the upper right corner – the scale bar equals 30 μm. (The arrows on the left image indicate fluorescent cells.). It can be observed that, only a small number of cells showed fluorescence; red areas indicate the reaction of the dye with a single-chain nucleic acid, and this occurs after the dye penetrates the dead cells. Less than 1% of dead cells were found in the Lewis carcinoma suspension that were used in the experiments.

2.4 Addition of Anphen Sodium

Anphen sodium (2-(carboxy)--2- (N-acetylamino)-3 -(3',5'-*tret.-* butyl-4' - hydroxyphenyl) - sodium propionate) was synthesised at the Institute of Chemical Physics RAS, along with other drugs of the class of spatially hindered phenols class. They have both antioxidant properties so that they may influence many biochemical targets in cells, including superoxide dismutase and protein kinase C proteins. The AS drug was completely non-toxic [1] and stable in aqueous solutions. The drug was administered at a concentration of 10^{-4} M and 10^{-6}M intraperitoneally a day after transplantation of the tumour cells of the sarcoma 37 and 4 days before the transplantation of Lewis carcinoma [24]. When

studying the effect of AS on spleen cells, AS in concentration (10^{-4} M) were administered intraperitoneally to white mice during 4-days. The control mice were injected with an AS-free solution. Five days later the spleen was isolated from mice, protein extracts of spleen cells were prepared and the Bcl-2 family proteins were defined.



The figure presents a 3D-molecular model of AS; red colour- Oxygen, blue colour- Nitrogen, light grey- Hydrogen, dark grey Carbon, in the centre- Sodium.

2.5 Determination of Bcl-2 by Immunoblotting

10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis was carried out in the Laemmli system [25]. Pretreatment of samples for electrophoresis was performed at 90°C for 5 min. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and seen with AEC Staining Kit "Sigma". Bcl-2 proteins in blood serum of mice with Lewis carcinoma was determined by immunoblotting with monoclonal anti-BCL-2 clone10C4 antibodies, and with the second antibody labelled with peroxidase of horseradish immunoglobulin anti- rabbit IgG ("Sigma"). The content of Bcl-2 in Lewis carcinoma cells was determined by monoclonal anti-BCL-2 clone [E17] ab 32124 ("Abcam"), on the synthetic polypeptide site 50-150, including of the BH3 domain of Bcl-2 family proteins. Detection and molecular weight determination by gel electrophoresis and Western blots was conducted with Precision Plus Protein standards family. The estimation of proteins amount in the immunoblotting bands was carried out following the optical density of the bands by using the scanning and "BMP scale" program. In determining the optical density of the bands, the optical density of the background was taken into account. Normalisation of the proteins amount in the immunoblotting band depending on the loading was performed by the optical density of high-molecular protein scans. So the high molecular weight part of the gel after the electrophoresis were cut out and stained with Coomassie Blue R-250. Proteins amount in Western blotting bands was expressed in relative units.

In experiments with the Bcl-2 protein in mouse plasma, during the growth of Lewis carcinoma, and in the immune experiments with the suspension cells of Lewis carcinoma 5 mice were used for each point in the control and after administration of AC.

2.6 Statistics

Statistical processing was performed by STATISTICA 6. Means and 0.95 confidence intervals were determined for n samples not less than 30. The normality of the distribution was estimated from the probability-probability plot for the cumulative functions of the theoretical distribution and the experimental data.

2.7 Reagents

Acrilamid "Panreac" Spain , Twin 80 "Merk", β-mercaptoethanol "Merk",10x Tris-glicin-sds electrophoresis buffer "fermentas" Lithuania, Ecl Western blotting detection reagents reagent 1 "Amercham Pharmacia Biotech", Ecl Western blotting detection reagents reagent 2 "Amercham Pharmacia Biotech", AEC Staining Kit "Sigma", Methanol "SERVA", Albumin Bovine cryst. Lyophil. "SERVA", Potassium chloride "SERVA", SDS Solution 20% "SERVA", Sodium chloride "SERVA",Coomassie Brilliant Blue R 250 "SERVA", Medium 199 (Hanks salts, glutamine) "MERCK".

3. RESULTS

Anticancer effect of AS was studied on the well-known experimental tumour model - ascites sarcoma 37 [24]. Fig. 1 shows the kinetic curve of the changing in the total number of tumour cells in ascitic sarcoma 37 of white mice, as well as a semi-logarithmic anamorphosis of the curve. The curve of the changing in the number of tumour cells in mice

showed the extreme nature, the maximum number of tumour cells in the ascitic fluid was detected at 14-16th day (Fig. 1) the number of cells decreased and the animals entered the terminal phase.

In experiments AS (10^{-4} and 10^{-6}M) was administered 24 hours after the transplantation of cells daily for 11 days (in the logarithmic phase of tumour cells growth). No ascitic cells were detected after AS-administration on the 12 days and in the following days (Fig. 1b), both at a dose of 10^{-4}M and 10^{-6}M, i.e., it was the complete suppress of tumour growth. It should be noted that the experimental mice lived much longer than the control mice, they were observed for three months. After that the experiment was finished.

Further, the study investigated whether AS had an anti-cancer effect after preliminary administration of drug before transplantation of the solid Lewis carcinoma cells. The changes in Bcl-2 proteins level during the tumour growth in blood serum (Fig. 2) was examined by immunoblotting. The control remained stable within a few days after transplantation, but on the terminal stage, a decrease in Bcl-2 proteins level by 15-20% was revealed, and soon the animals died. This is consistent with the literature data on reduction of Bcl-2 level in lymphocytes with age [26] and at certain stages of tumour process.

Besides, it was found that AS had a distant effect on the anti-apoptotic Bcl-2 proteins in the experimental group. On the 18th day AS drug led to a sharp decrease (up to 40-50%) in Bcl-2 proteins level in blood of mice which may be explained as an increase in the apoptosis process.

It was found that the kinetic curves of solid tumour volume growth (exponential dependence) upon AS administration and in control coincided,

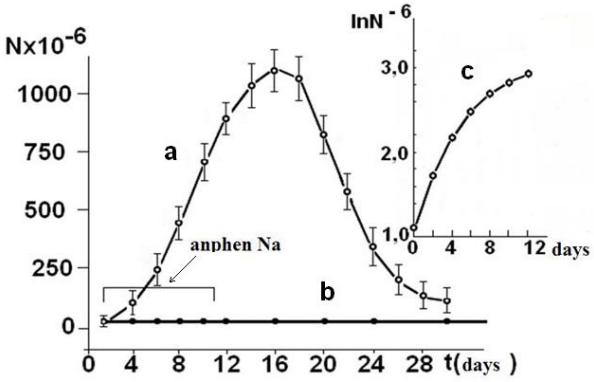


Fig. 1. Kinetic curves of changing the number of ascitic sarcoma 37 cells (a), semi-logarithmic curve of cell growth onset (c) in control, and after daily intraperitoneal injection of AS (10^{-4} and 10^{-6}M), within 11 days, starting 24 hours after transplantation (b)

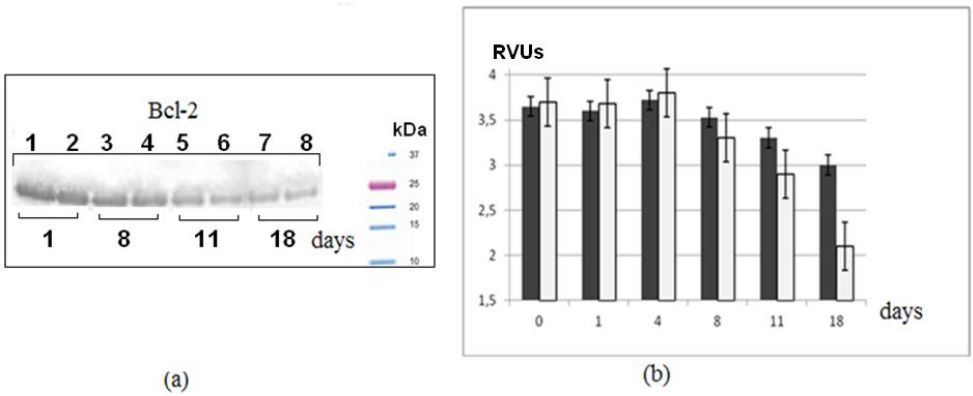


Fig. 2. Change in Bcl-2 protein content in mouse plasma during Lewis carcinoma growth in control and after administration of AS (10^{-4}M). Western blotting (a) and histogram (b)): control (dark columns), AS. (light columns)

the mice in both groups died almost simultaneously on 23rd- 24th day. Haematological toxicity and reduced survival rate of experimental animals make it difficult application of various chemotherapeutic drugs, cytostatics and antimetabolites [27].

Earlier, changes in morphology of erythrocytes were examined by the atomic force microscopy method. It was shown that in comparison with control, the morphological changes of erythrocytes in mice after AS administration was not observed [28]. AS has been characterised as a non-toxic drug [1]. This is of great importance in cancer therapy.

To assess the sensitivity of the tumour to the AS and its ability to influence on the molecular pathways of apoptosis, AS drug (10^{-4}M) was incubated with a suspension of Lewis carcinoma tumour cells at 37°C for 0-3 hours.

Two bands of Bcl-2 proteins were found in the control: monomer at 26 kDa and homodimer at 51 kDa (more intensive-coloured bands) (Fig. 3, Fig. 4). In the control group, the level of anti-apoptotic protein remained fairly stable for 3 hours. It was detected that AS administration led to a regular decrease in the level of homodimer Bcl-2 / Bcl-2 and monomer Bcl-2, which led to a decrease by 80% and 40%, respectively within 1-3 hours. Such a sharp decrease of Bcl-2 proteins content may lead to the programmed cell death- apoptosis.

The anti-apoptotic Bcl-2 family proteins can suppress the onset of apoptosis due to the formation of complexes with pro-apoptotic proteins, such as Bcl-2 / Bax. It was shown that the decrease in Bcl-2 proteins level is correlated in cancer with the onset of apoptosis in liver and spleen cells after administration of drugs in high doses [28].

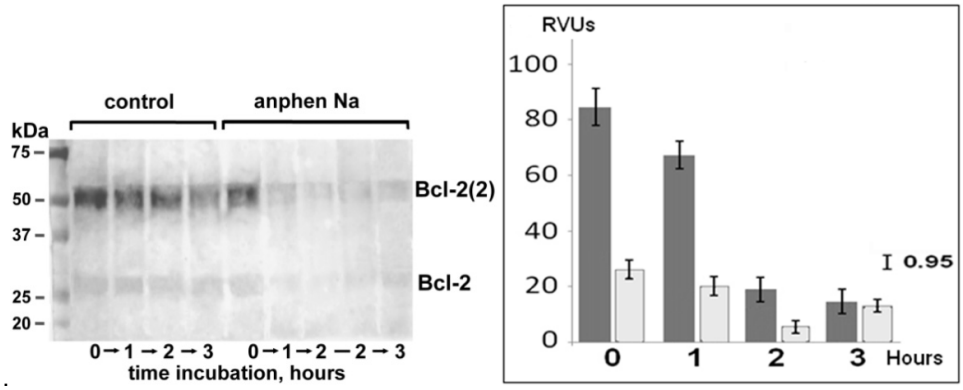


Fig. 3. Change in Bcl-2 protein content of Bcl-2 (monomer) and Bcl-2 (2) (homodimer) proteins in the suspension cells of Lewis carcinoma. in control and in the presence of AS (0-3 hrs). Western blotting (a), and histogram (b): dark column - Bcl-2(2), light column - Bcl-2

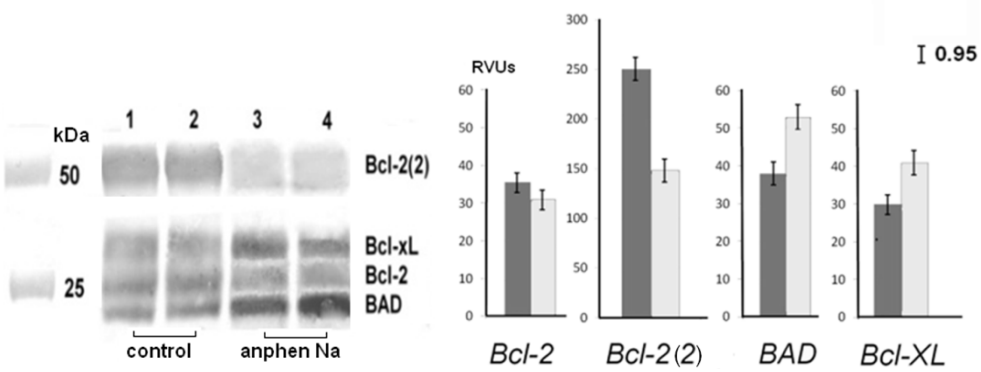


Fig. 4. Western blotting (a) and histogram (b) of Bcl-2 proteins content (monomeric and homodimeric form Bcl-2(2), BAD and Bcl-XL proteins in spleen cells white mice in control (dark) and after AS (10^{-4}M) administration during four days (light)

To check how the drug works in tissue of healthy animals, the following experiment was carried out with the healthy white mice four days later after AS administration.

In this experiment, proteins were seen with the antibody on the synthetic polypeptide of site 50 - 150 of Bcl-2, which contain the homologous BH3 domain, that presents in the Bcl-2 family proteins: Bcl-XL, BAD and others. On the blot, in addition to Bcl-2, protein bands at 23 and 30 kDa were also detected. The correspondence of molecular masses, as well as the antisemitic behaviour of these proteins concerning Bcl-2 under AS action allowed to presume with a high probability, that these were BAD (23 kDa) and Bcl-XL (30 kDa) proteins.

It was found that AS administration into spleen cells resulted (Fig. 3, columns 3, 4) in 25% decreasing of Bcl-2 homodimer (Fig. 3) and monomer contents, but the levels of apoptosis protein activator BAD (30 kDa) and antiapoptotic protein Bcl-XL (23 kDa) were increased (Fig. 4). Increase of anti- apoptotic Bcl-XL proteins level which blocks the formation of the apoptosome, may be due to that Bcl-XL proteins leave the complex with proteins APAF1 and cytochrome C when the apoptosis begins. Increasing of BAD may indicate to the mitochondrial pathway of apoptosis.

4. DISCUSSION

The studies of AS biological properties have shown, that AS on epinephrine model stops the development of heart attack at its initial stage with preservation of the life of animals [29]. It was also established that AS has the protective effect at hypoxia after a single, subcutaneous injection to mice in 5 or 40 mg/kg doses [30]. In the present work, the reliable antitumour effect of AS was revealed at administration drug after transplantation cells of ascitic sarcoma 37 to mice. The antitumour effect of AS may be connected by the fact that both transplantation and administration of AS drug was carried out intraperitonally, and AS could directly affect on the tumour cells, causing their death in apoptosis.

At preliminary administration, AS (10^{-4} M) did not affect the tumour growth rate of Lewis carcinoma and mouse lifespan. But the Bcl-2 content in mice serum decreased faster than in control at the terminal stage of tumour growth and could enhance apoptosis of aging animal blood cells.

It should be noted that action of AS was opposite to phenozan potassium, as phenozan increase the level of Bcl-2 proteins in spleen cells. The reparation effect of phenozan can be associated with activation of protein kinase C [31], which activates Bcl-2 by phosphorylating Ser 70.

It is known that in normal cells anti-apoptotic Bcl-2 protein has antioxidant effects and protects cells from oxygen radicals, necrotic and apoptotic death. In tumour cells, apoptosis is often suppressed, and the anti-cancer therapy aimed to create conditions that enhance apoptosis, including affecting the Bcl-2 family proteins. In the regulation of apoptosis take part a mono proteins, homo and heterodimers between pro, antiapoptotic protein, and proteins BH3 –only, such as Bcl-2/ Bcl-2, Bcl-2/ BAX, Bcl-2/BAD. Bcl-XL/BAD etc. [21]. In this work, the presence of both monomers Bcl-2 and homodimers Bcl-2/ Bcl-2 was observed in Lewis carcinoma cells and spleen cells of white mice. This means that the BH3 region in homodimer remains free and can bind to the monoclonal antibody. It is consistent with the model of dimer “head-to-tail” formation [32]. Where, two binding surfaces are on separate faces of the three-dimensional structure, and they might bind the activated BAX.

Introduction AS into Lewis carcinoma cellular suspension causes a sharp decreasing in mono and homodimers Bcl-2 proteins content during 1-3 hours. This is consistent with the data [30], where changes in the functional state of isolated liver mitochondria were found immediately after AS administration (10^{-4} M). It was shown that the AS dramatically reduces the maximum rate of electron transfer in the respiratory chain of mitochondria in 1-1.5 hours after administration. But a few hours later, these parameters were restored. These data demonstrate that AS, unlike other antioxidants - sterically hindered phenols - besides antioxidant action, effects mitochondria and mitochondrial enzymes.

The mechanism of Bcl-2 proteins action in regulation of apoptosis process is interconnected with p53 protein (PUMA), which suppress transcription of BCL-2 gene. Also PUMA, BAD and other BH3-only proteins could bind to the hydrophobic region of Bcl-2 protein, triggering the apoptosis process. At present, there are a number of natural antitumour compounds such as gossypol, some polyphenols, and a number of targeted anti-cancer drugs which had been synthesised and could also interact with the hydrophobic Bcl-2 pocket [21,22]. It is assumed

that phenolic amphiphilic antioxidant AS is able to form stable compounds with polypeptides, and can interact with the Bcl-2 proteins family, leading to apoptosis of tumour cells.

5. CONCLUSION

In summary, a significant anti-cancer effect of AS antioxidant on sarcoma 37 development was found in mice, but the same effect was not detected in Lewis carcinoma development in AS pre-treated animals. However, it was shown a significant decrease in Bcl-2 protein level in mice blood and mono and homodimers Bcl-2 proteins content in Lewis carcinoma cells suspension. Also, AS administration leads to the change in the balance of pro- and anti-apoptotic Bcl-2 family proteins in the spleen of white mice, that can cause the onset of cell apoptosis. The study considers that non-toxic AS drug,that can stimulate the physiological process of mitochondrial apoptosis, may be of interest for further studies and its possible application as an anti-cancer drug.

ETHICAL DISCLAIMER

As per international standard or university standard was written ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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