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Generation of ROS under the influence of thiazole derivative and its complexes with PEG-based polymeric nanoparticles

M. V. Ilkiv[1], Ya. R. Shalai[1], B. O. Manko[1], Yu. V. Ostapiuk[1], N. E. Mitina[2], A. S. Zaichenko[2], A. M. Babsky[1]

[1] Ivan Franko National University of Lviv
4, Hrushevskoho Str., Lviv, Ukraine, 79005

[2] Lviv Polytechnic National University
12, S. Bandery Str., Lviv, Ukraine, 79013
popovych.marta@gmail.com

Aim. To determine the *in vitro* effect of thiazole derivative and its complex with polyethylene glycol (PEG)-based nanoscale particles on ROS generation in the NK/Ly lymphoma cells and hepatocytes of mice. **Methods.** The effects of BF-1 (N-(5-benzyl-1,3-thiazol-2-yl)-3,5-dimethyl-1-benzofuran-2-carboxamide), PEG-based polymeric nanoparticles (Th1, Th3, Th5) and their complexes (Th2, Th4, Th6) on the ROS production in murine NK/Ly lymphoma cells were studied using fluorescent microscopy. The level of superoxide in both the murine hepatocytes and NK/Ly cells was determined with a spectrophotometric assay. **Results.** BF1, Th2, Th6 and Th5 significantly increased the level of ROS in NK/Ly lymphoma cells by 27.7 %, 28.6 % 22.7 % and 20.1 %, respectively. Meanwhile, Th1, Th3, Th4 did not affect the ROS level. The level of superoxide significantly decreased under the influence of BF1 by 14.7 % and all its complexes with PEG-based polymeric nanoparticles (Th2, Th4, Th6) by 25.5 %, 21.6 % and 13 %, respectively, compared to control. Unlike lymphocytes, in the murine hepatocytes none of the investigated compounds affected the superoxide content. **Conclusions.** Thus, thiazole derivative BF1 may realize its antitumor effect on cancer cells by promoting generation of additional amount of ROS. BF1 and its complexes with PEG-containing polymeric nanoparticles significantly increase the ROS generation in NK/Ly cells. Meanwhile, all investigated compounds did not change the level of superoxide in murine hepatocytes. It can be an evidence of their low toxicity to nontumor cells.

K e y w o r d s: thiazole derivative polyethylene glycol, polymeric nanoparticles, ROS, superoxide radical

Introduction

Reactive oxygen species (ROS) play an essential role in cell metabolism and regulate the cellular signaling of proliferation, inflammation, immune response, autophagy, stress-related response and cell survival [1, 2]. However, the uncontrolled expression of ROS causes an imbalance between the cellular reduction-oxidation conditions, promotes oxidative stress and cytotoxicity of cells, that leads to disorders of cellular functions and the development of different pathologies including oncology diseases [1]. Usually, cancer cells generate elevated ROS levels compared to normal cells.

Interestingly, ROS play a dual role in cancer with both supporting and inhibiting malignant behavior. The DNA damage and genomic instability under the influence of ROS can lead to various oncogenic alterations and promote cancer progression. ROS catalyze many signaling pathways and stimulate cancer cell survival, proliferation, metabolism, angiogenesis and metastasis [1, 2]. Furthermore, ROS can drive metabolic and mitochondrial dysfunctions, activation of oncogenes and contributes to the alteration of some regulatory proteins [2]. On the other hand, intensive oxidative damage and enhanced ROS-dependent death signaling can lead to a tumor-suppressing process. ROS damage the mitochondrial integrity, which promotes a cascade of caspase-related reactions and induces apoptosis of tumor cells [3]. Many chemotherapeutic strategies based on the oxidative damage through the acceleration of ROS content in cancer cells, are successfully applied in clinical practice.

It was established previously, that newly synthesized thiazole derivative N-(5-benzyl-1,3-thiazol-2-yl)-3,5-dimethyl-1-benzofuran-2-carboxamide (BF1) increased the level of ROS in human glioma cells lines and was approximately two times more cytotoxic than doxorubicin [4]. However, the clinical application of the general amount of pro-oxidative agents is limited due to their cytotoxicity toward nontumor cells and their poor solubility in water. PEG-based polymeric nanoparticles (PEG-PNs) may conjugate with many therapeutic anticancer agents increasing solubility and blood-circulation time of antitumor chemicals, improving targeted delivery to tumor tissues, decreasing side effects on healthy cells, and, as a result, these nanoparticles improve the efficacy of drugs [5, 6]. It was established, that combination of BF1 with the polymeric carriers based on polyethylene glycol (PEG) increases the cytotoxicity towards several tumor cell lines [7], causing the apoptotic-like alterations in the mice Nemeth-Kellner lymphoma (NK/Ly) cells [8] and increasing the level of superoxide dismutase (SOD), one of the most important enzyme of the antioxidant defense system [9].

Therefore, this work aimed to evaluate the impact of thiazole derivative free BF1 and conjugated with PEG-based polymeric carriers on the ROS level in the mice NK/Ly lymphoma cells and hepatocytes.

Materials and Methods

Compounds

Thiazole derivative BF1 (Fig. 1) was synthesized at the Department of Organic Chemistry of Ivan Franko National University of Lviv, as described previously [4].

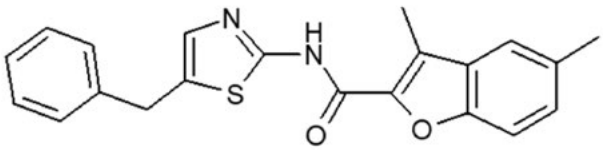

Fig. 1. Structure of N-(5-benzyl-1,3-thiazol-2-yl)-3,5-dimethyl-1-benzofuran2-carboxamide (BF1)

The PEG-based polymeric nanoparticles (Fig. 2), namely, a comb-like copolymer where the backbone is a copolymer of 5-(tert-butylperoxy)-5-methylhexene-3-yne (VEP) and glycidyl methacrylate (GMA) with grafted chains of polyethylene glycol monomethyl ether (mPEG, 550 kDa) (poly(VEP-co-GMA)-graft-mPEG (Th1)); homopolymer of PEG-methacrylate with a molecular weight of the PEG-unit 475 kDa (poly(PEGMA) (Th3)) and its copolymer with dimethyl maleate (DMM) (poly(PEGMA-co-DMM) (Th5)) were synthesized at the Department of Organic Chemistry of the Lviv Polytechnic National University, as described earlier [10].

Water dispersions of polymeric nanoparticles — Th1, Th3 and Th5 and their complexes with the BF1 (Th2, Th4, Th6) were dissolved in dimethyl sulfoxide (DMSO) and the solutions were subsequently transferred in water.

Three experimental groups were prepared: the 1st group — BF1 (10 μM), Th1 (1 g/100 mL) and Th2 (Th1 (1 g/100 mL)) + BF1 (0.03 g/100 mL), the 2nd group — BF1 (10 μM), Th3 (1 g/100 mL) and Th4 (Th3 (1 g/100 mL)) + BF1 (0.03 g/100 mL), and the 3rd group — BF1 (10 μM), Th5 (1 g/100 mL) and Th6 (Th5 (1 g/100 mL)) + BF1 (0.03 g/100 mL). The lymphoma homogenate was incubated for 10 min with each of the compounds (BF1, PN or BF1 + PN). Other experimental explanations are presented in the **Table 1**.

NK/Ly lymphoma model

The experiments were conducted using an experimental model of NK/Ly, which is cultivated in mice. The NK/Ly strain was provided by the Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine, Kyiv, from their collection of tumor cultures. All manipu-

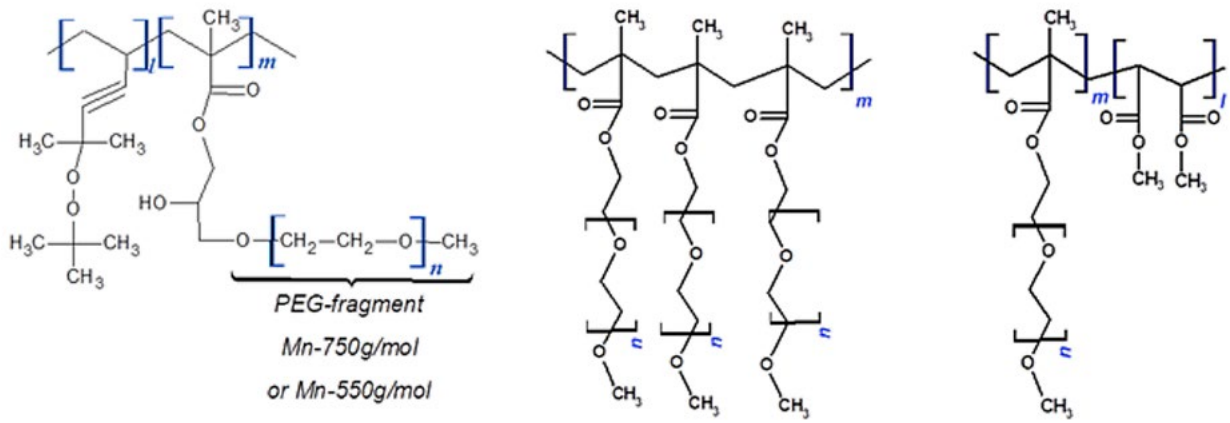

Fig. 2. Structure of PCs: *A* — poly(VEP-*co*-GMA)-*graft*-mPEG (k = 1.4 % mol, l = 98.6 % mol, Mn = 240 kDa); *B* — poly(PEGMA) (k = 99.2 % mol, [IPB-fragment] = 0.8 % mol, Mn = 55 kDa); *C* — poly(PEGMA-*co*-DMM) (k = 87.0 % mol, l = 13.0 % mol, Mn = 47 kDa).

Table1. **Scheme of control and experimental groups used in the study.**

Name of sample	Thiazole derivative	Polymeric nanoparticle
Control	-	-
BF1	+	-
Th1, Th3, Th5	-	+
Th2, Th4, Th6	+	+

lations with animals were carried out in accordance with the European Convention on the Protection of Vertebrate Animals (1986), the article 26 of the Law of Ukraine (No 3447-IV, 2006) "On the Protection of Animals from Cruelty" as well as approved by the Ethics Committee of Ivan Franko National University of Lviv (Protocol of 09.02.2021 No 17-02-2021). Lymphoma was cultivated in non-linear male mice (20–30 g), which were injected intraperitoneally with 200 µl of ascites fluid which contained 100–150 million tumor cells. Tumor growth was monitored by weighing mice daily from the time of lymphoma inoculation. Initially mice were first anesthetized with diethyl ether. Then, to obtain ascites, drainage of the abdominal cavity was performed with a sterile syringe.

Preparation of liver homogenates

To extirpate the liver, the animals were decapitated under ether anesthesia, after which the organ was quickly excised. After removal, the liver was weighed and washed from blood with a cooled solution of the following composition (in mM): NaCl — 140, KCl — 4.7, $MgCl_2$ — 1, glucose — 5, HEPES — 10; pH 7.4. The liver was crushed using a metal press and solution was added in the ratio of 8 ml of solution per 1 g of tissue. Liver tissue was homogenized in a Potter-Evelheim homogenizer (Shalai *et al.*, 2018).

Fluorescence microscopy

The ROS level was recorded using fluorescence microscopy. The method is based on recording differences in the fluorescence of cells treated with a specific dye. An Olympus IX73 inverted microscope and a DP-74 digital camera were used for image acquisition [11].

In order to register the relative values of the amount of ROS, the fluorescent dye dihydroethidium (DHE) was used with the excitation wavelength 540–585 nm and emission > 600 nm. The cell incubation medium had the following composition (in mM): KCl — 90.0, NaCl — 15.0, EGTA — 1, HEPES — 10; pH 7.4. Lymphoma ascites was washed and diluted 10 times. The investigated compounds BF1, PEG-PNs Th1, Th3, Th5 and complexes of BF1 with PEG-PNs (Th2, Th4, Th6) were added to the suspension of cells in final concentrations 10 µl and incubated for 15 min at 37 °C. After incubation, the cells were washed again and DHE (10 µl) was added and incubated again for 15 min (temperature — 37 °C). Rotenone in final concentration 5 % was used as a positive control. Approximately, 5–10 µl were taken from each sample and a drop of solution was placed on a glass slide. It was covered with a cover glass and placed in a microscope (microscope magnification ×12.6). In the field of view, 4–5 different variants of cell images were selected, first in visible light, and then switched to the fluorescent light spectrum.

Fluorescence intensity, which reflected the changes in the amount of ROS, was recorded and evaluated using the ImageJ computer program.

Spectrophotometry

Superoxide anion radical formation in lymphoma cells and hepatocytes was measured using the nitroblue tetrazolium (NTT) test [12]. Solvent was photometered at a wavelength of $\lambda = 540$ nm. Taking into account the dilution, the ratio of the components in the reaction, the extinction of the standard according to the graph, the amount of superoxide radical was calculated:

$$(E \times 11.11)/C \text{ protein} = \text{nmol/g} \times \text{s},$$

where E is the extinction of the sample, 11.11 is the extinction coefficient of the reduced NTT, C — protein concentration, mg/ml.

Statistical analysis

The statistical analysis of the results was made and illustrated using the MS Excel-2013 and Statistica programs. All experiments were repeated five times in each variant. The normality of distribution was assessed with the Shapiro-Wilk test. All data are presented as a mean ± SD. To determine statistically significant differences between the means of independent investigation groups, the one-way analysis of variance (ANOVA) was used. Statistical analyses were performed using t-test. The P values below 0.05 were considered as statistically significant.

Results and Discussion

ROS generation in lymphoma cells

At the initial stage, the effect of rotenone on lymphoma cells was tested to check the validity of the method. This substance causes active ROS generation in all types of cells. Since the studied compounds are dissolved in DMSO, the effect of DMSO on the ROS level in lymphoma cells was also evaluated. In this series of experiments, the control values of the ROS level were 72.4±2.5 relative units (r. u.) of fluorescence intensity. The amount of ROS in lymphoma cells increased by 46 % under the influence of rotenone compared to the control. Noteworthy, DMSO solvent did not affect the content of ROS in lymphoma cells.

At the next stage of the investigation, the effect of thiazole derivative BF1, unconjugated PEG-PN Th1, Th3, Th5 and BF1 complexes with polymers (Th2, Th4, Th6) on the level of ROS in tumor cells was evaluated. Figure 4 shows the fluorescent images of NK/Ly lymphoma cells under the influence of the investigated compounds. Fluorescence was more intense under the action of BF1 (b) compared to the control (a). The most intense fluorescence was observed under the action of rotenone (c). It is noticeable, that the fluorescence of lymphoma cells under the action of the complex Th2 (e) was slightly more intense than the fluorescence under the action of BF1.

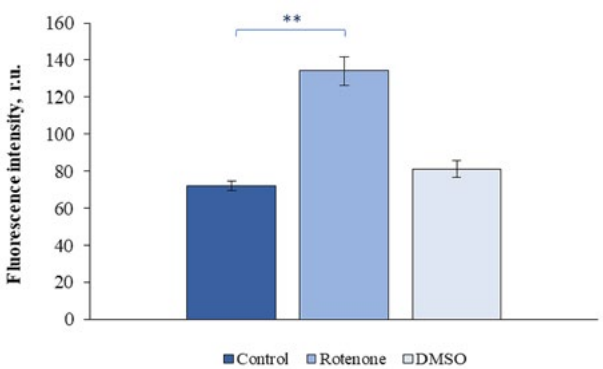

Fig. 3. ROS level in NK/Ly lymphoma cells under the action of rotenone (10 μM) and DMSO (final concentration 5 %). M±m, n = 6. ** — $P < 0.01$

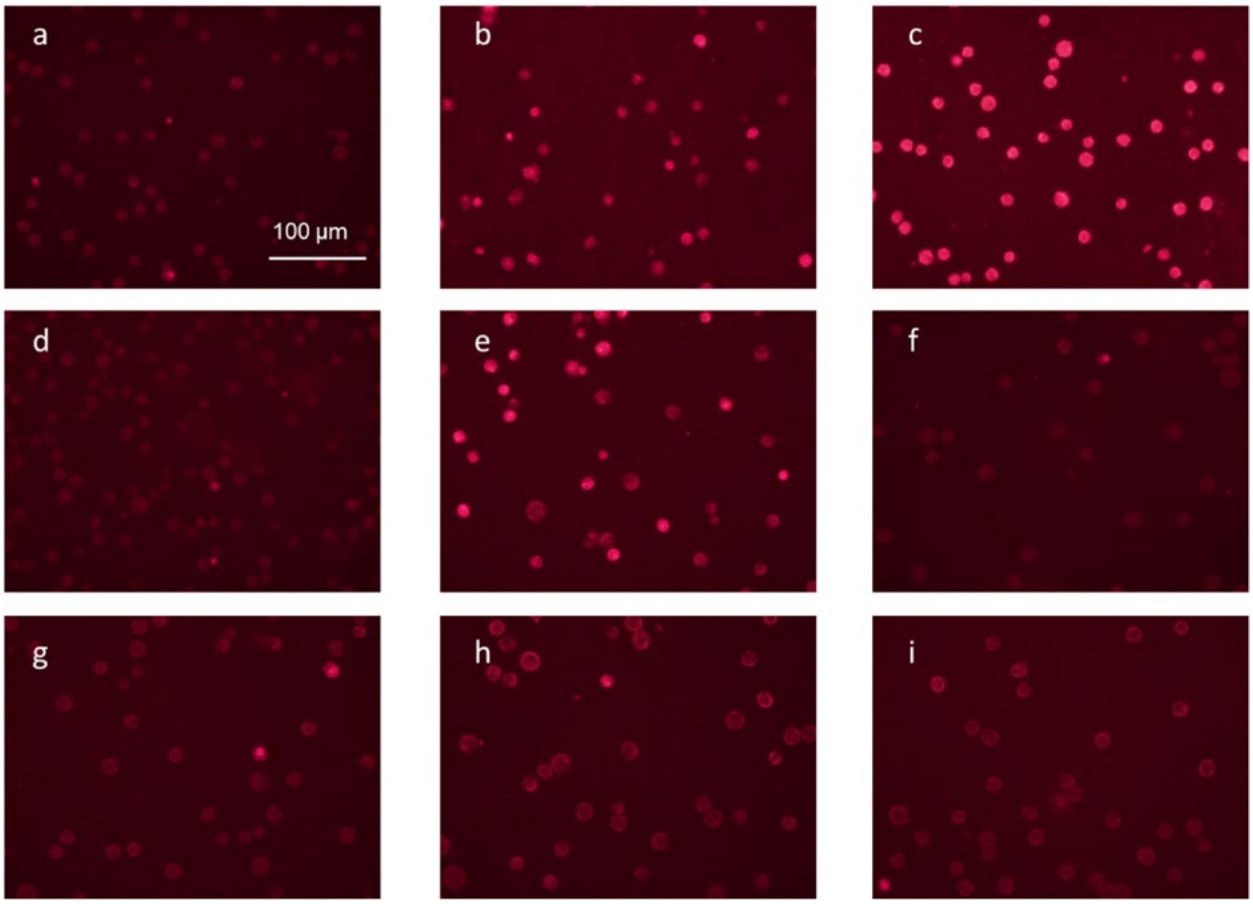

Fig. 4. Fluorescence of lymphoma cells with DHE under the action of thiazole derivative BF1 in complexes with PEG-based polymer nanoparticles: a — control, b — unconjugated thiazole derivative BF1, c — rotenone, d — polymeric nanoparticles based on poly(VEP-*co*-GMA)-graft-mPEG (Th1), e — complex of BF1 with Th1 (Th2), f — polymeric nanoparticles based on poly(PEGMA) (Th3), g — complex of BF1 with Th5 (Th6), h — polymeric nanoparticles based on poly(PEGMA-*co*-DMM) (Th5), i — complex of BF1 with Th3 (Th4).

It is interesting that only under the action of PEG-PN Th5 (h) the fluorescence of lymphoma cells significantly increased.

Statistical analysis of fluorescent images showed that the ROS level in lymphoma cells increased by 27.7 % ($P < 0.001$) under the action of the substance BF1 at a concentration of 10 μM. The ROS level also increased by 28.6 % ($P < 0.001$) and 22.7 % ($P < 0.001$) under the action of the complexes Th2 and Th6. Unconjugated PEG-PNs Th1 and Th3 did not affect the ROS content in lymphoma cells. There was a tendency of decreasing the ROS level under the action of the complex Th4 compared to the unconjugated BF1, but these changes were not statistically confirmed. A significant increase in the ROS level in lymphoma cells by 20.1 % compared to the control was observed under the action of polymeric nanoparticle Th5 ($P < 0.001$) (Fig. 5).

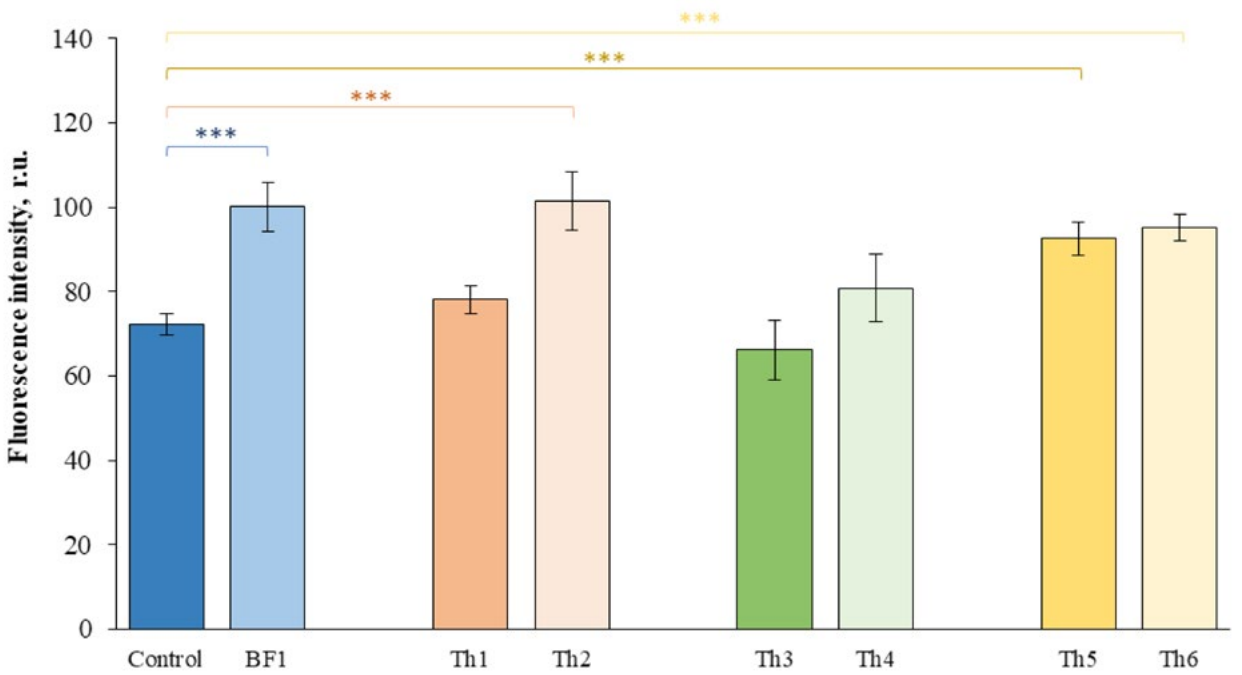

Fig. 5. ROS level in NK/Ly cells under the action of BF1, PEG-based polymeric nanoparticles Th1, Th3, Th5 and complexes of BF1 with PEG-PNs (Th2, Th4, Th6). M±m, n=6. *** — $P < 0.001$

Superoxide radical level in lymphoma cells and hepatocytes

Figure 6 shows the changes in the superoxide radical content under the action of unconjugated BF1, PEG-NPs (Th1, Th3 and Th5) and BF1 in complexes with polymer nanoparticles (complexes Th2, Th4 and Th6). The ontrol values of superoxide radical in this series of experiments were in the range from 0.83±0.03 to 0.95±0.05 nmol/g×s. The level of O_2^- decreased under the action of thiazole derivative BF1 by ~14.7 % ($P < 0.01$) versus control. Complexes Th2 and Th4 significantly decreased the superoxide radical content by 25.5 % ($P < 0.05$), 21.6 % ($P < 0.01$), respectively compared to the control. A slight decrease of this radical was also observed under the action of the complex Th6. Complex Th6 decreased the superoxide radical content by 13.0 % ($P < 0.01$) compared with the control values. Free polymers Th1, Th3 and Th5 did not affect the content of superoxide radical in lymphoma cells.

The content of the superoxide radical in the liver cells of a tumor-bearing mice was also investigated (Fig. 7). The control levels of superoxide radical in the liver ranged from 0.22±0.02 to 0.24±0.00 nmol/g×s. It was found, that BF1 in the studied concentration did not change the level of superoxide radical in the liver cells of tumor-bearing mice. All investigated PEG-PNs or complexes of BF1 with PEG-PNs did not affect the level of superoxide in mice hepatocytes compared to the control.

In our previous study, a pronounced cytotoxic effect of two thiazole derivatives on tumor cells *in vitro* was established [13]. It was

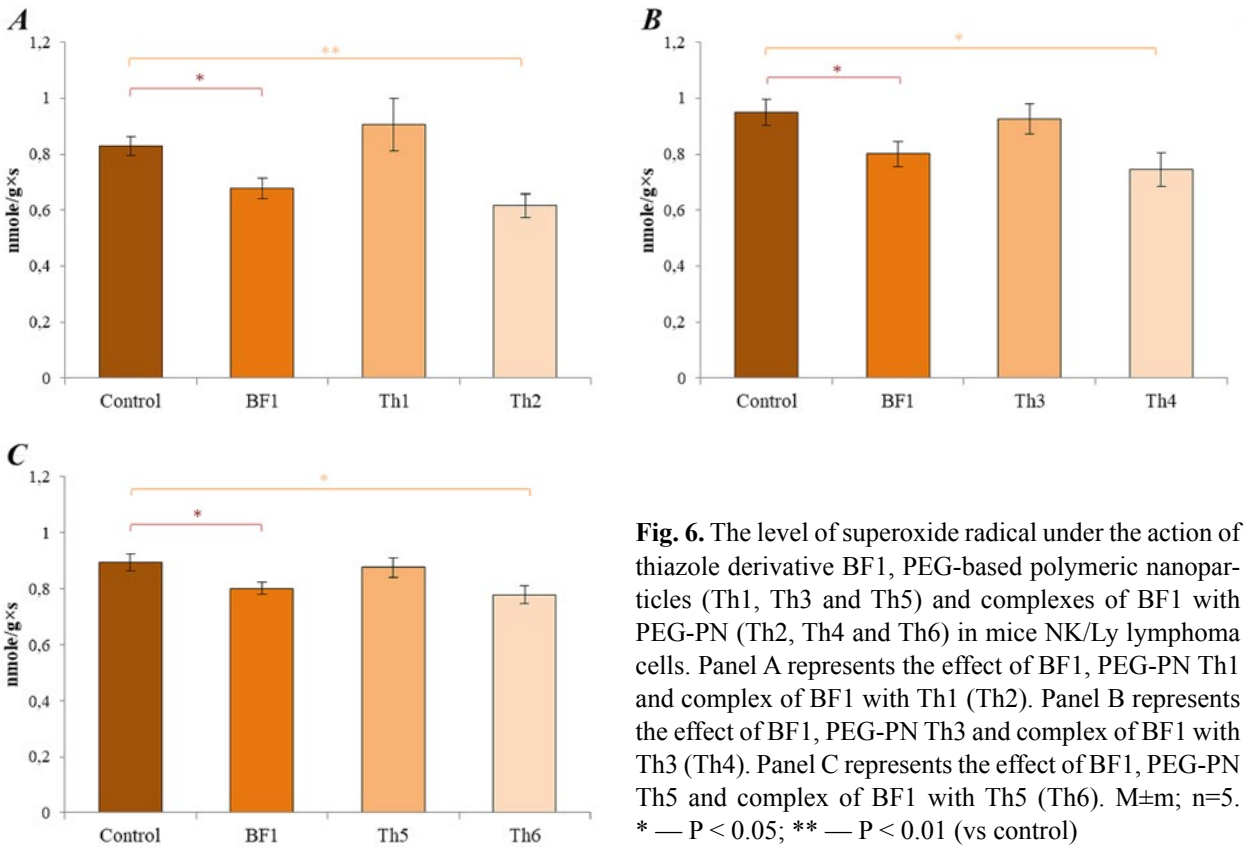

Fig. 6. The level of superoxide radical under the action of thiazole derivative BF1, PEG-based polymeric nanoparticles (Th1, Th3 and Th5) and complexes of BF1 with PEG-PN (Th2, Th4 and Th6) in mice NK/Ly lymphoma cells. Panel A represents the effect of BF1, PEG-PN Th1 and complex of BF1 with Th1 (Th2). Panel B represents the effect of BF1, PEG-PN Th3 and complex of BF1 with Th3 (Th4). Panel C represents the effect of BF1, PEG-PN Th5 and complex of BF1 with Th5 (Th6). M±m; n=5. * — $P < 0.05$; ** — $P < 0.01$ (vs control)

also proved that ROS scavengers significantly reduced the cytotoxicity of BF1 [4]. It was found earlier, that investigated compound BF1 and its complexes with PEG-PNs changed the activity of antioxidant defense enzymes. Under this circumstance, the activity of SOD increased whereas the activity of catalase and glutathione peroxidase decreased [9].

To further clarify the mechanisms of action of investigated compounds, it was important to study the effect of BF1 in complexes with PEG-PNs on the ROS level in cancer cells.

The chemical reactivity of ROS differs from other signaling molecules. At low concentrations, ROS actively participate in the complex mechanisms of controlling cell proliferation and differentiation, and their excessive amount can lead to cell damage [1]. Extra production of ROS and their subsequent accumulation in cells or tissues can contribute to the interaction of these molecules with DNA components, damaging them, and thus leading to the development of carcinogenesis.

Our study confirmed that BF1 increases the general level of ROS in NK/Ly cells compared with the untreated control cells. Though BF1 in complexes with PEG-PNs did not increase the ROS level compared to unconjugated BF1, complex Th2 had a slightly more intensive effect on ROS generation in treated lymphoma

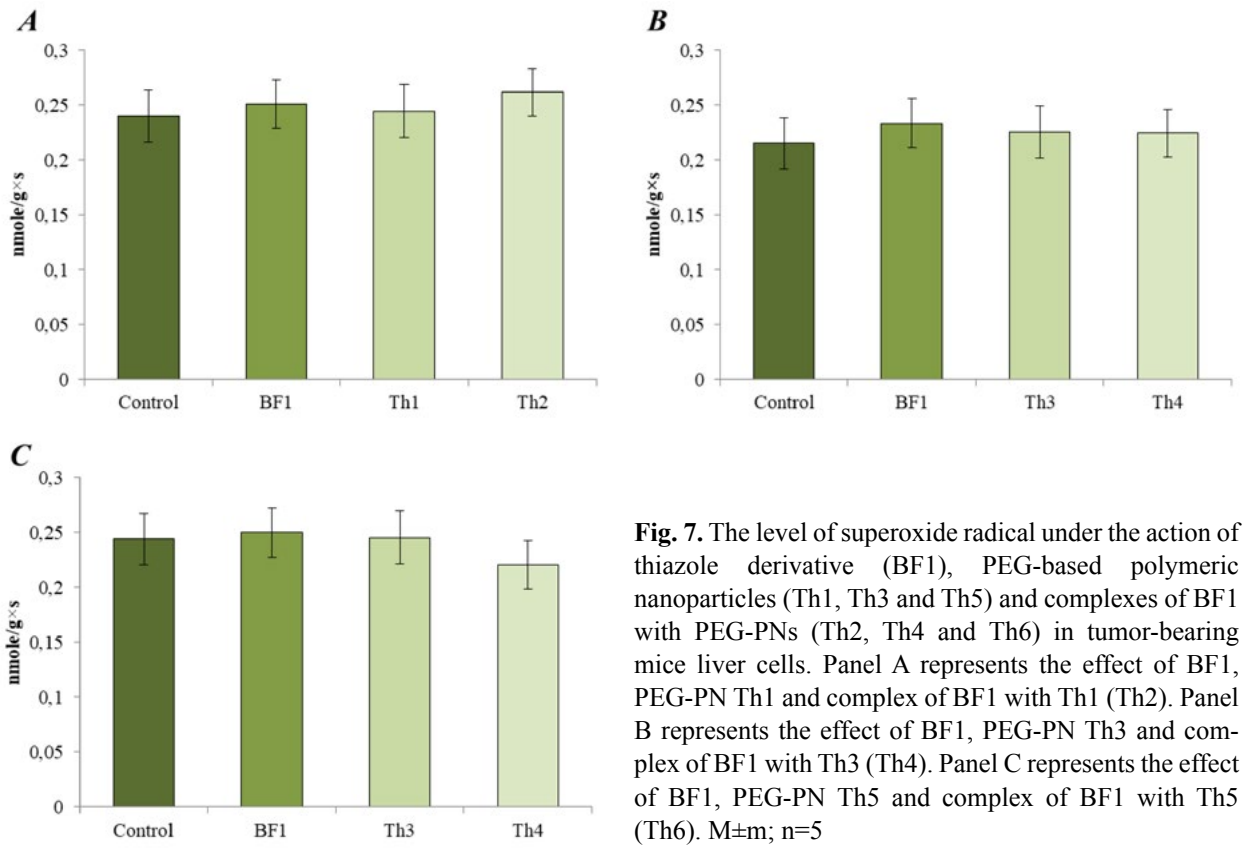

Fig. 7. The level of superoxide radical under the action of thiazole derivative (BF1), PEG-based polymeric nanoparticles (Th1, Th3 and Th5) and complexes of BF1 with PEG-PNs (Th2, Th4 and Th6) in tumor-bearing mice liver cells. Panel A represents the effect of BF1, PEG-PN Th1 and complex of BF1 with Th1 (Th2). Panel B represents the effect of BF1, PEG-PN Th3 and complex of BF1 with Th3 (Th4). Panel C represents the effect of BF1, PEG-PN Th5 and complex of BF1 with Th5 (Th6). M±m; n=5

cells versus BF1 (Fig. 4 and 5). Thus, the cytotoxic effect *in vitro* is the result of the action of the specific compound BF1, but not PNs in lymphoma cells. Our data confirm the previously published results that the investigated compound BF1 and its complexes with PEG-PNs changed the activity of the antioxidant defense enzymes [9]. Thus, disturbing the redox balance by BF1 in lymphoma cells can accelerate the intensive oxidative stress. It is interesting, that free PEG-PNs Th5 significantly increase the ROS level in lymphoma cells even without BF1. It was established, that some polymeric nanoparticles changed the ROS level without any antitumor agents [14, 15].

The free radicals formed under the action of anticancer drugs can cause the oxidative stress in tumor cells and induce cell death [16, 17]. The chemotherapeutic agents that enhance the oxidative stress are toxic to cancer cells because they are involved in biological processes such as cell cycle disruption, DNA damage, and induction of apoptosis.

In our opinion, the main effect that determines the cytotoxicity of the studied substance BF1 is an increase in the level of hydrogen peroxide. It is not a radicalbut in the presence of metal ions, H2O2 decomposes essentially. This agent at low concentrations (<10 μM) increase proliferation, but at higher concentra-

tions can lead to the cell growth arrest and cell death by apoptosis or necrosis [18].

Also, we investigated the level of superoxide radical in lymphoma cells under the action of BF1 and its complexes with PEG-PNs. Expectedly, the investigated substances decreased the generation of superoxide in NK/Ly cells (Fig. 6). These results affirm that BF1 can either directly interact with ROS, especially with superoxide anion, or affect the activity of enzymes like SOD, which forms hydrogen peroxide from $O_2^{\bullet-}$ that released to the cellular cytosol and nucleus, generating oxidative stress.

Many chemotherapeutic drugs have a low selective effect and therefore provoke significant side effects, such as cardiotoxicity, hepatotoxicity, neurotoxicity, nephrotoxicity, and affect the immune system. The action of antitumor substances on the liver could significantly damage the functioning of the organ and the body. However, it was established that the level of superoxide radical in the liver cells of mice did not change under the influence of the studied substances. These results represent the BF1 and its complex with PEG-PNs as potential safety compounds that can be used in clinical trials in the future.

Conclusion

The ROS level in lymphoma cells sufficiently increased under the action of BF1 and its complex with PEG-based polymeric carriers. We believe that ROS generation is a part of the mechanism of thiazole derivatives action. At the same time, unconjugated PNs did not affect the ROS level in lymphoma cells. Thus, the cytotoxic effect *in vitro* is the result of the action of the specific compound BF1, but not PNs in lymphoma cells. The investigated compound and its complexes with PEG-PNs did not change the ROS level in mice hepatocytes, which characterized free BF1 and conjugated BF1 as potentially safe antitumor agents.

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Утворення АФО за впливу похідного тіазолу та його комплексів з ПЕГ-вмісними полімерними наночастинками

М. В. Ільків, Я. Р. Шалай, Б. О. Манько, Ю. В. Остап'юк, Н. Є. Мітіна, О. С. Заіченко, А. М. Бабський

Мета. Дослідити вплив *in vitro* похідного тіазолу N-(5-benzyl-1,3-thiazol-2-yl)-3,5-dimethyl-1-benzofuran-2-carboxamide (BF1) та його комплексів з нанорозмірними частинками на основі поліетиленгліколю (ПЕГ) на утворення АФО в клітинах лімфоми NK/Ly та гепатоцитах мишей. **Методи.** Для оцінки ефекту речовини BF-1, ПЕГ-вмісних полімерних наночастинок (Th1, Th3, Th5) та їхніх комплексів (Th2, Th4, Th6) на рівень АФО в клітинах лімфоми NK/Ly мишей використано метод флюорисцентної мікроскопії. Для визначення рівня супероксидного радикалу в гепатоцитах та лімфоми NK/Ly мишей використано спектрофотометричний аналіз. **Результати.** BF1, Th2, Th6 та Th5 достовірно підвищували рівень АФО на 27,7 %, 28,6 % 22,7 % та 20,1 %, відповідно, позаяк Th1, Th3 і Th4 не змінювали рівень АФО в клітинах лімфоми NK/Ly. Рівень супероксидного радикалу достовірно знижувався за впливу речовини BF1 на 14,7 % і всіх її комплексах з ПЕГ-вмісними полімерними носіями (Th2, Th4 та Th6) на 25,5 %, 21,6 % and 13 %, відповідно, у порівнянні з контролем. На відміну від лімфоми, жодна з досліджуваних сполук не змінювала рівень супероксиду в гепатоцитах миші. **Висновки.** Похідне тіазолу BF-1 може реалізувати свій протипухлинний ефект через посилення генерування АФО в пухлинних клітинах. BF-1 та його комплекси з полімерними носіями достовірно збільшують рівень АФО в лімфомних клітинах, проте не змінюють рівень супероксидного радикалу в гепатоцитах миші. Це можна інтерпретувати як доказ їх низької токсичності щодо непухлинних клітин.

Ключові слова: похідне тіазолу, поліетиленгліколь, полімерні носії, АФО, супероксид радикал.

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