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Influence of EMAP II, IFN-α2b and its medicinal preparations on the MGMT protein amount in human cells *in vitro*

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Aim. *To study the effect of EMAP II, IFN-α2b and its medicinal preparations on the amount of O⁶-methylguanine-DNA methyltransferase (MGMT) protein in human cells in vitro.* ***Methods***. *The human cells of 4BL and Hep-2 lines were treated with the purified recombinant proteins EMAP II, IFN-α2b and its commercial me dicinal preparations. Changes in the MGMT gene expression were studied at a protein level by Western blot analysis.* ***Results***. *Treatment of Hep-2 and 4BL cells with EMAP II at the concentrations of 0.02 μg/ml and 2 μg/ml respectively led to induction of the MGMT gene expression. EMAP II at the concentrations of 0.2–20 μg/ml caused decrease of the MGMT protein amount in Hep-2 cells. The regulating activity of EMAP II was also observed for MARP (anti-Methyltransferase Antibody Recognizable Protein). IFN-α2b and Laferon-PharmBiotek with the activity of 200 and 2000 IU/ml were shown to cause an increase of the MGMT protein amount in Hep-2 cells.* ***Conclusions***. *The purified recombinant proteins EMAP II and IFN-α2b which are substrates for the medicinal preparations influenced on the amount of MGMT protein in the human cell cultures in a concentration-dependent manner. At the same time the effect of medicinal preparations differs from that of the purified protein IFN-α2b. Possibly it depends on the presence of stabilizing components in their compositions.*

Keywords: MGMT, MARP, IFN-α2b, EMAP II, human cell cultures.

Introduction. The repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) eliminates O^6-methylguanin adducts in DNA and protects normal cells from damaging effects of alkylating agents. At the same time MGMT makes tumor cells resistant to alkylating drugs such as temozolomide [1, 2].

Therefore, in medicine MGMT is considered as a target which needs to be regulated. Various regulators of the MGMT enzyme activity and expression are known but they are often toxic not only for tumor but also for normal human cells [3]. Cytokines are natural factors and some of them were shown to be promising in the *MGMT* gene expression regulation. For example, IFN-β down-regulated the *MGMT* transcription and sensitized the resistant glioma and neuroblastoma cells to temozolomide *in vitro* [4, 5]. One of the members of interleukin family – IL-24 down-regulated the *MGMT* gene expression via activation of p53 and therefore helped to overcome the melanoma cells resistance to temozolomide [6].

Interferons elicit pleiotropic biological effects, so they are widely used either alone or in combination with other antitumor agents, in particular with nitrosoureas. Preadministration of interferons to patients might be a part of a novel biochemotherapy approach that may help to overcome the resistance to alkylating drugs. The combined therapy with IFN-β and temozolomide was shown to provide better clinical outcomes in the patients whose tumor cells had an unmethylated *MGMT* promoter [7]. For the patients with progressive malignant glioma, the complex therapy with BCNU and IFN-α2b (Intron

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A, «Schering Corporation», USA) appeared to be a feasible and promising treatment strategy. The effect of IFN-α2b on the cell sensitivity to BCNU was proposed to be implemented by inhibition of the *MGMT* gene expression [8]. However, this suggestion needs to be confirmed.

EMAP II (endothelial monocyte-activating polypeptide II) is a multifunctional cytokine, which is formed in malignant tumors of mammals due to the alternative splicing and posttranslational processing of its precursor – the p43 protein [9]. EMAP II suppresses the endothelial cell migration; stimulates their apoptosis and influences the activity of monocytes, neutrophils and macrophages, facilitating inflammatory processes in tumors [10]. The antitumor activity of this cytokine was evidenced in the experimental models of glioma, sarcoma, stomach and pancreas cancer [11].

In our previous works different exogenous cytokines, growth factors and plant components (extracts, lectins) were shown to be able to change the level of the *MGMT* gene expression in some human cell cultures [12, 13]. To our opinion IFN-α2b and EMAP II which possess antitumor activity were the most promising agents for this purpose. One of the important questions in this study was determination of the dependence of IFN-α2b and EMAP II action on their concentration. Moreover the another problem has arrived: does the effect of cytokine medicinal preparation differ from that of their purified substances?

Additionally it should be noted that the human MGMT protein has a molecular weight of 22–24 kDa [14]. However, in our previous works the Western blot analysis with monoclonal anti-MGMT antibodies, clone 23.2, revealed two highly specific immunoreactive bands: 24 kDa (classic MGMT protein) and 48 kDa (anti-Methyltransferase Antibody Recognizable Protein or MARP) [12, 15]. In those works the MARP nature and its induction by exogenous cytokines in human cells *in vitro* have been discussed.

The aim of the present work was to compare the effect of different concentrations of purified IFN-α2b and EMAP II and medicinal preparations of IFN-α2b on the MGMT and MARP protein amount in human cell cultures.

Materials and methods. The following human cell lines were used: a standard line Hep-2 (laryngeal cancer) and 4BL line (fibroblast-like cells) derived in our laboratory [16]. Cells were cultivated in standard DMEM (PAA) with 10 % FBS («Sigma») and antibiotics penicillin (0.02 %) and streptomycin (0.02 %) at 37 °C with 4 % CO_2.

The following interferon agents were used: human recombinant protein IFN-α2b («Interpharmbiotek», Ukraine) in 0.1 M NaCl; Laferon-PharmBiotek («Interpharmbiotek») containing the human recombinant protein IFN- α2b and additional components: NaCl, Dextran 70, KH_2PO_4, Na_2HPO_4; Laferobion («Biofarma», Ukraine) containing the human recombinant protein IFN-α2b and additional components: NaCl, Dextran 70, KH_2PO_4, $Na_3PO_4 \times 12H_2O$.

Recombinant EMAP II protein was expressed in *E. coli*, purified and studied as described previously [17–20].

For cytokine treatments, $8 \cdot 10^5$ cells were plated and allowed attaching during 24 h. The next day, the cells were treated with cytokines and cytokine-containing preparations in the serum-free DMEM growth medium. After 8-h exposure the medium was removed, the cells were rewashed with PBS buffer and harvested in DMEM with 10 % of serum during 16 h. The cells were then trypsinized, washed, centrifuged, and stored at –20 °C. Control intact cells were subjected to the similar procedure but without adding cytokines.

Protein extracts were obtained from cell pellets in lysis buffer (50 mM Tris HCl; 0.1 mM EDTA; 5 mM DTT; pH 7.5). The suspensions were incubated on ice, exposed to three 10-s pulses of sonication with 30-s intervals and 50 mM of PMSF in ethanol were added. Sonicates were then centrifuged at 13,000 *g* for 30 min at 4 °C. The supernatants were collected and frozen at –80 °C for later use. SDS-PAGE (12 % gel) was performed by Laemmli method [21].

The concentration of total protein in cell lysates was measured colorimetrically according to Bradford method [22].

The following antibodies were used: anti-MGMT monoclonal antibodies, clone 23.2, isotype IgG2b («Novus Biologicals», USA), secondary antibodies conjugated with horseradish peroxidase («Sigma», USA). The procedure of MGMT identification in the samples was performed by Western blot analysis according to the methodological instructions of the manufacturer of mAbs [23]. Densitometry of stained membranes was used for

loading control according to [24] by ScionImage 4.0.2 and Origin 8.1 programs.

Results and discussion. It was shown by Western blot analysis that cytokine EMAP II at a concentration of 0.02 μg/ml induced the MGMT gene expression (Fig. 1, lane *2*) in a clone of Hep-2 cells which did not express the *MGMT* gene under normal conditions (Fig. 1, lane *1*). However, an increase of EMAP II doses to 0.2–20 μg/ml did not lead to any changes of the MGMT protein amount in these cells (Fig. 1, lanes *3–5*). After the treatment of Hep-2 cells, that normally expressed the *MGMT* gene, with cytokine EMAP II at a concentration of 2 μg/ml we observed decrease of the MGMT protein amount (Fig. 1, lanes *6* and *7*). Thus, the EMAP II effect on the MGMT protein amount in Hep-2 cells differs depending on concentrations.

In our previous work, 4BL cells were shown to lose the possibility to express the *MGMT* gene during more than 130 passages cultivation and cell line stabilization [26]. However, the treatment of these cells (137 passage) with cytokine EMAP II led to induction of the *MGMT* gene expression (Fig. 2), which allowed us to suggest reversibility of the *MGMT* gene silencing. Induction of the *MGMT* gene expression in 4BL cells was detected after 8-h incubation with EMAP II at a concentration 2 μg/ml. However this effect almost disappeared during longer incubation time – 16 and 32 h (Fig. 2, lanes *3* and *4*). A reduction of the MGMT protein amount may be the result of increasing time of serum-free conditions.

The regulating activity of EMAP II was observed not only for the MGMT protein but also for MARP. The MARP protein is stably expressed in both Hep-2 and 4BL cells in contrast to MGMT. An inverse concentration dependence was observed after treatment of Hep-2 cells with recombinant protein EMAP II: at concentrations of 0.02 and 0.2 μg/ml it induced the MARP expression, but at higher concentrations (2 and 20 μg/ml) it inhibited the MARP expression (Fig. 3, lanes *2–5*). EMAP II caused a slight increase of the MARP amount in 4BL cells (Fig. 3, lanes *7–9*).

In our earlier work IFN-α2b-containing preparation Laferobion («Biofarma») at concentrations of 200 and 2000 IU/ml was shown to cause a dramatic decrease in the MGMT protein amount in Hep-2 cells [12]. However, in our current work it was shown that IFN-α2b at concentrations of 200 and 2000 IU/ml increases the

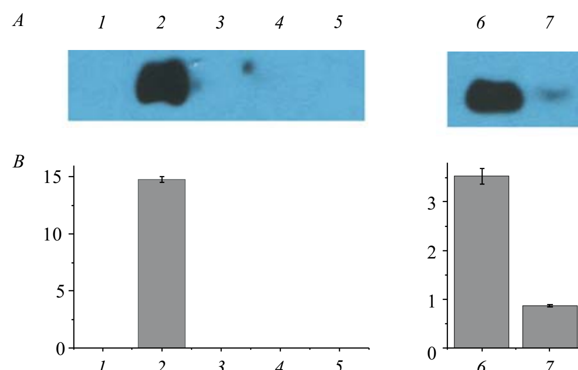

Fig. 1. Effect of cytokine EMAP II at different concentrations on the amount of MGMT protein in MGMT non-expressing (*A*) and MGMT-expressing (*B*) Hep-2 cells: *A* – Western blot analysis (*1* – 0 μg/ml; *2* – 0.02 μg/ml; *3* – 0.2 μg/ml; *4* – 2 μg/ml; *5* – 20 μg/ml); *B* – Western blot analysis (*1* – 0 μg/ml; *2* – 2 μg/ml); *C*, *D* – results of densitometry (the vertical bar represents the level of MGMT protein amount, conventional densitometry units)

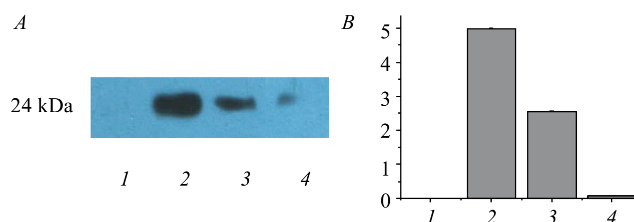

Fig. 2. Time-dependent effect of cytokine EMAP II on the amount of MGMT protein in 4BL cells: *A* – Western blot analysis; *1–4* – 4BL, 137 passages + EMAP II (*1* – 0 μg/ml, 8 h; *2* – 2 μg/ml, 8 h; *3* – 2 μg/ml, 16 h; *4* – 2 μg/ml, 32 h); *B* – results of densitometry (the vertical bar represents the level of MGMT protein amount, conventional densitometry units)

MGMT protein amount in Hep-2 cells (Fig. 4, lanes *2* and *3*). The similar tendency was observed after treatment of Hep-2 cells with Laferon-PharmBiotek preparation («Interpharmbiotech») (Fig. 4, lanes *6* and *7*).

At the same time no changes in the MGMT protein amount were observed after treatment of 4BL cells with IFN-α2b and Laferon (data not shown).

Laferon-PharmBiotek and Laferobion preparations have similar composition (recombinant human IFN-α2b, salts, dext ran) but they showed an opposite effect on the MGMT protein amount. According to the literature data, a preparation Intron A (recombinant IFN-α2b, EDTA, NaCl, m-Kresol, Polysorbate 80, Na_2HPO_4, NaH_2PO_4) decreased the *MGMT* gene expression in patients, who had progressive malignant glioma [8]. Thus Laferobion and Intron A showed similar regu lating effects although they have different composition of stabilizing components [8, 12].

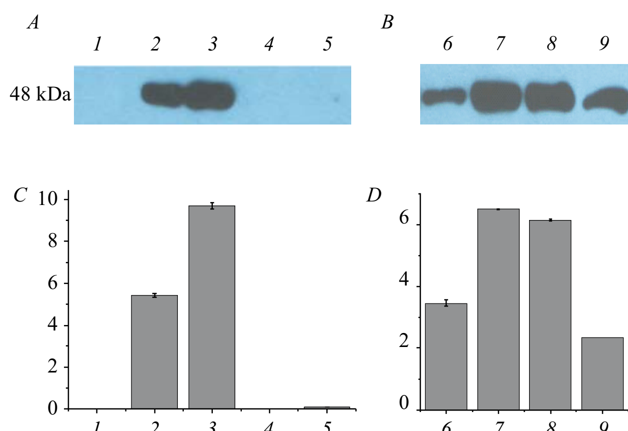

Fig. 3. Effect of recombinant protein EMAP II on the amount of MARP in human cell cultures: *A* – *1–5* – Hep-2 + EMAP II (*1* – 0 μg/ml; *2* – 0.02 μg/ml; *3* – 0.2 μg/ ml; *4* – 2 μg/ml; *5* – 20 μg/ml); 6–9 – 4BL, 137 p. + EMAP II (*6* – 0 μg/ml, 8 h; *7* – 2 μg/ml, 8 h; *8* – 2 μg/ml, 16 h; *9* – 2 μg/ml, 32 h); *B* – results of densitometry (the vertical bar represents the level of MARP amount, conventional densitometry units)

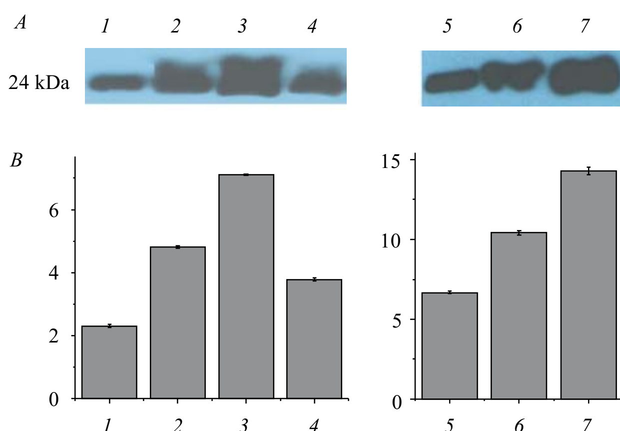

Fig. 4. Effect of IFN-α2b and Laferon-PharmBiotek on the MGMT protein amount in Hep-2 cells: *A* – Western blot analysis (*1* – IFN-α2b, 0 IU/ml; *2* – IFN-α2b, 2000 IU/ml; *3* – IFN-α2b, 200 IU/ml; *4* – IFN-α2b, 2 IU/ml; *5* – Laferon, 0 IU/ml; *6* – Laferon, 2000 IU/ml; *7* – Laferon-PharmBiotek, 200 IU /ml); *B* – results of densitometry (the vertical bar represents the level of MGMT protein amount, conventional densitometry units)

According to these data we may suggest that regulating effect of the IFN-α2b-containing preparations depends not only on the composition but also on their purification degree. Therefore these results could be useful for planning the experiments with the IFN-α2b-containing preparations and for IFN-α2b therapy.

There are several hypotheses about the mechanisms of the *MGMT* gene expression regulation under the influence of cytokines. According to one of them IFN-β regulates the MGMT transcription via p53 protein and

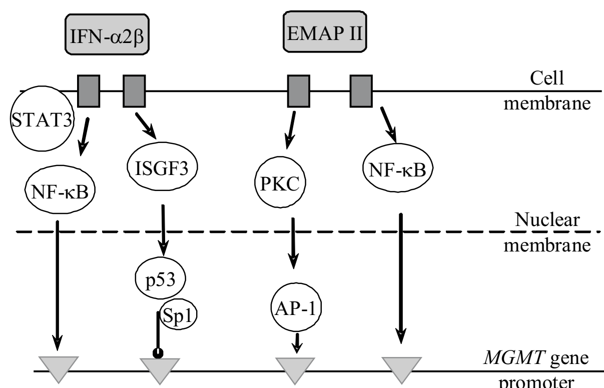

Fig. 5. Possible cell signaling pathways involved in regulation of the *MGMT* gene expression under the influence of IFN-α2b and EMAP II; STAT3 – Signal transducer and activator of transcription 3; ISGF3 – Interferon-stimulated gene factor 3

specificity protein 1 (Sp1) [4, 5]. Another one suggests that IFN-β activates transcriptional factor NF-κB and thus affects the transcription of *MGMT* gene, which belongs to the NF-κB target genes [26]. According to the literature data, all IFNs of type I (IFN-α, IFN-β, IFN-ε, IFN-ω) bind to the specific cell surface receptor complex known as the IFN-β receptor (IFNAR) [27]. Therefore we suppose that regulation of the *MGMT* gene expression by IFN-β and IFN-α2b may involve the similar pathways (Fig. 5).

The way of EMAP II impact on the *MGMT* gene expression remains unclear. In some works it was shown that EMAP II at low and high concentrations activates various signaling pathways in cells [28, 29]. For instance, in a blood–tumor barrier (BTB) model, EMAP II at low concentration (0.05 nM) induced three isoforms of protein kinase C (PKC): PKC-α, β, and ζ, and, through them, caused functional, biochemical, and morphological alterations in BTB [29]. PKC is known to regulate the *MGMT* gene expression [30]. Its regulating effect is mediated by binding to an activator protein 1 (AP-1) and further binding to the AP-1 site in the *MGMT* gene promotor, affecting the *MGMT* gene transcription. Moreover, EMAP II can also activate the NF-κB transcription factor [31]. Therefore we suppose that regulation of the *MGMT* gene expression by IFN-α2b and EMAP II occurs with participation of transcription factors NF-κB, Sp1 and AP-1. A scheme of possible cell signaling pathways involved in these processes is shown in Fig. 5.

We plan further study of the mechanisms of the *MGMT* gene expression regulation under the influence of cytokines to establish a connection between the action of cytokines and the repair processes in human cells.

Conclusions. The EMAP II influence on the MGMT protein amount depends on the concentration. The treatments with cytokine IFN-α2b at concentrations of 200 and 2000 IU/ml lead to the increase of MGMT protein amount in Hep-2 cells. The effect of the purified protein IFN-α2b on the MGMT and MARP protein amounts differs from that of medicinal preparations. Possibly it depends on the presence of stabilizing components in their compositions.

Вплив EMAP II, IFN-α2b та його медичних препаратів на кількість білка MGMT у клітинах людини *in vitro*

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Резюме

Мета. *Дослідити вплив EMAP II, IFN-α2b та його медичних препаратів на кількість білка MGMT у клітинах людини in vitro.* ***Методи***. *Клітини людини 4BL і Hep-2 обробляли EMAP II, IFN-α2b і його комерційними препаратами. Зміни в експресії гена MGMT на рівні білка досліджували за використання Вестерн-блот аналізу.* ***Результати***. *Обробка клітин Hep-2 і 4BL цитокіном EMAP II в концентрації 0,02 і 2 мкг/мл відповідно призводить до індукції експресії гена MGMT. EMAP II в концентраціях 0,2–20 мкг/мл знижує кількість білка MGMT у клітинах Hep-2. Регулювальну активність EMAP II спостерігали також і відносно MARP (білка, який розпізнається моноклональними анти-MGMT антитілами). Показано, що IFN-α2b і Лаферон-ФармБіотек з активністю 200 і 2000 МО/мл підвищують кількість білка MGMT у клітинах Hep-2.* ***Висновки***. *Очищені рекомбінантні білки EMAP II і IFN-α2b, які є субстратами для медичних препаратів, впливають на кількість білка MGMT у клітинах людини in vitro залежно від концентрації. У той же час дія медичних препаратів відрізняється від ефекту очищеного білка IFN-α2b, що, можливо, пов'язано з присутністю стабілізувальних компонентів у його складі.*

Ключові слова: MGMT, MARP, IFN-α2b, EMAP II, культури клітин людини.

Влияние EMAP II, IFN-α2b и его медицинских препаратов на количество белка MGMT в клетках человека *in vitro*

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Резюме

Цель. *Исследовать влияние EMAP II, IFN-α2b и его медицинских препаратов на количество белка MGMT в клетках человека in vitro.* ***Методы***. *Клетки человека 4BL и Hep-2 обрабатывали EMAP II, IFN-α2b и его коммерческими препаратами. Изменения в экспрессии гена MGMT исследовали с использованием Вестерн-блот анализа.* ***Результаты***. *Обработка клеток Hep-2 и 4BL цитокином EMAP II в концентрации 0,02 и 2 мкг/мл соответственно приводит к индукции экспрессии гена MGMT. EMAP II в концентрациях 0,2–20 мкг/мл снижает уровень экспрессии гена MGMT в клетках Hep-2. Регулирующую активность EMAP II наблюдали также и относительно MARP (белка, распознаваемого моноклональными анти-MGMT антителами). Показано, что IFN-α2b и Лаферон-ФармБиотек с активностью 200 и 2000 МЕ/мл повышают количество белка MGMT в клетках Hep-2.* ***Выводы***. *Очищенные рекомбинантные белки EMAP II и IFN-α2b, являющиеся субстратами для медицинских препаратов, влияют на количество белка MGMT в клетках человека in vitro зависимым от концентрации образом. В то же время действие медицинских препаратов отличается от влияния очищенного белка IFN-α2b, что, возможно, связано с присутствием стабилизирующих компонентов в его составе.*

Ключевые слова: MGMT, MARP, IFN-α2b, EMAP II, культуры клеток человека.

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