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THE PEPTIDOGLYCAN FRACTION ENRICHED WITH MURAMYL PENTAPEPTIDE FROM *Lactobacillus bulgaricus* INHIBITS GLIOBLASTOMA U373MG CELL MIGRATION CAPABILITY AND UPREGULATES PARP1 AND NF-κB LEVELS

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Peptidoglycan is a universal component of bacterial walls that exerts various biological activities, including tumoricidal effect. Anti-cancer effect of various peptidoglycan fractions and their derivates is different. Muramyl pentapeptide (MPP) is the most complete building block of peptidoglycan. MPP can stimulate cell reactivity as well as other muropeptides. In the present study, we evaluated inhibitory MPP effect on viability and migration of glioblastoma cells U373MG. As markers of cell reactivity we determined the amounts of proteins PARP1 and NF-κB. MPP exposure induced decrease in viability and migration activity of glioblastoma cells. Besides, MPP treatment increased the amounts of PARP1 and NF-κB in a dose-dependent manner. Furthermore, NADH level in exposed glioblastoma cells was depleted as compared to control. Thus, MPP exhibits tumoricidal effect in glioblastoma cells U373MG via depletion NADH content and consequently metabolic energy level. Moreover, upregulation of the amounts of PARP1 and NF-κB in glioblastoma cells could be an important mechanism of the inhibition of cell migrative capability and the progress of the tumor.

The obtained results evidenced that muramyl pentapeptide could initiate lack of migration via metabolic energy expenditure as a result of gliotypic reactivity. Further studies are actual and extremely required to clarify tumoricidal effect of this muropeptide in glia-derived tumors.

Key words: peptidoglycan, muramyl pentapeptide, PARP1, NF-κB, glioblastoma U373MG, cell reactivity.

Many probiotic ingredients are recognized to be beneficial to human health. Furthermore, several microbial compounds exert the anti-tumor activity [1–3]. Lactobacillus species are the most studied microorganisms among all probiotics [4]. During last decades, anticancer effects of cytoplasm fractions, cell wall components from lactobacilli, live whole and heat-killed cells have been confirmed as inhibiting tumor agents [5–9]. Furthermore, peptidoglycan is confirmed as one of important source of bioactive compounds which are potent to stimulate both innate immunity and exhibit anti-cancer activity in various cell types. Anti-tumor effects of different peptidoglycan fractions were reported with respect to various tumor types including sarcoma, leukemia, melanoma and lung cancer [10–13]. Other toxic effects of muramyl peptides include pyrogenicity, acute polyarthritis [14] and serum amyloid A protein increase were reported [15, 16]. Recently antiproliferative and proapoptotic effects of whole peptidoglycan fraction from *Lactobacillus paracasei* was demonstrated in human colon cancer HT-29 cell line [11]. The various cytotoxic effects of certain peptidoglycan fractions were reported with respect to human and animals cancer types in compare to non-transformed cells [1, 11]. The enzymatic cleaving of bacterial peptidoglycan generates the biology active fragments containing N-acetylmuramyl peptides named muropeptides. Most studies of muropeptides tumoricidal effect have been focused on both synthetic and natural derivatives which

exhibit meaningful level of adjuvant activity [17]. However, several muropeptides were confirmed to be effective tool to modulate cellular response, but not only to enhance proinflammatory cytokines production [18]. Despite of progress in the study of the mechanisms to enhance innate immunity with peptidoglycan derivates, direct muropeptides inhibitory effect on cancer cells remains unknown. Recently there was demonstrated anti-cancer effect of both whole peptidoglycan extracts and separated peptidoglycan fractions from *Lactobacillus* [1, 11]. However, the mechanisms of anticancer effect individual muropeptides isolated from probiotic bacteria walls are unexplored. Muramyl pentapeptide (MPP) is one of peptidoglycan cleaved fragment which confirmed as bioactive muropeptide with promising immunomodulatory property [19]. In spite of well-studied biology activity of muramyl dipeptide (MDP), anticancer activity of muramyl pentapeptide has no reported in literature. Previously, MPP isolated from L. delbrueckii has been confirmed to stimulate leukocytes in a course of early postnatal development as well as to enhance innate immunity [19]. However, cytotoxicity of MPP with respect to tumor cells has no determined. Prevailing number of tumoricidal activity of muropeptides had shown antiproliferative and cytotoxic effect which associated with the modulation cytokines and chemokines production [1, 12, 18]. Elevated cytokines production can initiate either cell surviving or cell death in depend on the power and the duration of stimuli. The inhibition of the glial cell viability by stimulation with proinflammatory factors is well known phenomenon [20–22]. Glial cells, as well as the other cell types, produce inflammatory factors as a result of various stimulations. Nuclear factor kappa B (nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB) is one universal adaptor of cellular response by cytokines production [23]. Besides, NF-κB is critic molecular target of muropeptides effects in respect with the initiation cell reactivity by translational control including in glial cells [24]. Other widespread regulator of cell reactivity is the enzyme poly-(ADP-ribose)-polymerase (PARP — E.C. 2.4.2.30). PARP is a member of small family which provides the regulation of cell function via ADP-ribosilation of target proteins [25]. Multiple regulatory roles of PARP in various cell types were described last years including the regulation of transcription, the cellular stress response, mRNA stability, cell division, and protein degradation [26, 27]. Furthermore, PARP can serve as a coactivator of NF-κB and modulate its transcriptional activity as a result of cell stimulation [28]. Thus, unique muropeptide stimulus to reactivate the cells can induce functional cooperation of PARP and NF-κB in course of cell response. In addition, cellular response which accompanied by extensive activation of both NF-κB and PARP can initiate cell death via dysregulation in cytokines production and extended metabolic energy expenses [29, 30]. The cytokines involved in fundamental pathways regulation including differentiation and proliferation in both normal and cancer cells. There was reported that several muramyl peptides can decrease mitochondrial bioenergetics ratio [31]. Thus, tumor-associated abnormality in energy metabolism could be a prospective target to inhibit cancer progress including invasion. Moreover, non-malignant cells possesses more power mechanisms to adapt to various stimuli in compare to cancer cells [32]. Therefore, muropeptide could initiate in cancer cells metabolic abnormality which abrogate energetic expenditure in proliferation and migration. MDP and its derivates remain the most studied as anti-cancer agents among all muropeptides. However, several reports have showed that the structure of mupopeptides is critic to exhibit mitochondria-targeted toxicity and to induce inefficiency in ATP synthesis. Besides, anti-cancer impact of any muropeptides on the energy metabolism in brain tumors remains undisclosed. Muropeptide-initiated reactivity was determined in various cell types including macrophages, lymphocytes, dendrite monocytes, epithelial and glial cells [9, 10, 33–36]. Despite of number study the biology activity of muropeptides in various mesenchymal end epithelial cells, there are no any reports on the impact of muropeptides or its derivates on glial-derived cancer suppression.

Recently there was demonstrated that peptidoglycan and its derivates can affect critic metabolic pathways in both glial cells and neurons [37, 38]. Astrocytes are responsible for vital processes in a brain. However, they serve a source of the most widespread and mortal tumor type known as glioma. Glioma is a prevailing brain cancer type of astrocyte-derived tumors [39]. Glioblastoma is most aggressive grade of the gliomas among brain tumors [40]. Glioblastoma cells have a high rate of migration and extremely potent to invasion [41]. Unique structure of muropeptides

allows to affect the most cell types via specific extracellular and intracellular receptors [42–44]. The transport of muropeptides into the cells mediated with peptide transporter PepT1 which expressed in many cell types including glial cells [37]. Tens mutations recognized in glioma cells which responsible for high metabolic rate and resistance to apoptosis [41]. Therefore, muropeptide exposure could initiate in glioma cells abnormal cellular response that can suppress aggressive tumor phenotype. Cancer cells exposure to muropeptide could initiate signaling pathways which responsible for defence-directed cell reactivity similarly to adaptive response in normal non-malignated cells. But similar initiation in tumor cells could induce abnormal dysregulation in cellular response machinery and switch to programmed cell death as it was demonstrated in several reports [45–47]. Taking into account that muropeptides were confirmed as anticancer agent with respect to various tumor types, we can presume cytotoxic effect of muramyl pentapeptide in glioma cells. Nevertheless, the impact of MPP on glioma cell reactivity has never been addressed. In this study, the in vitro effects of muramyl pentapeptide on PARP and migration capability were examined.

The objectives of presented study were to elucidate the role of PARP1 and NF-κB in anticancer effect of muramyl pentapeptide from *Lactobacillus delbrueckii* strain in glioma U373MG cells. In addition, we examined the migration capability U373MG cells exposed to various doses of muramyl pentapeptide.

Materials and Methods

Chemicals and Antibodies

Acrylamide, N,N′-Methylenebis (acrylamide), TEMED, β-mercaptoetanol, Sodium dodecyl sulfate (SDS), Tris base, Bovine serum albumin (BSA), Phenylmethylsulfonyl fluoride (PMSF), Benzamidine, Leupeptine, DL-Dithiothreitol (DTT), Ponceau S, P-cumaric acid, Luminol, Methanol, Glycerol, Dimethyl sulphoxide (DMSO), was obtained from Sigma Aldrich (St. Louis, USA). Dulbecco's Modified Eagle Medium (DMEM), Penicillin/Streptomycin, Fetal Bovine Serum (FBS), Trypsin-EDTA and DPBS were obtained from Gibco Life Technologies (Paisley, UK). H_2O_2, NaOH, HCl, glycine, Tween-20, bromophenol blue, β-mercaptoethanol, NP-40, EDTA, EGTA, β-glycerophosphate was purchased from Merck (Darmstadt, Germany). The primary antibodies against PARP and NF-κB were obtained from Abcam (Cambridge, UK). Anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Dallas, USA). Anti-mouse and anti-rabbit secondary antibodies conjugated with HRP were obtained from Advansta (CA, USA).

MPP (MPP: MurNAc-l-Ala-d-Glu-l-Lys-d-Ala-d-Ala) was isolated and purified from *Lactobacillus delbrueckii subsp. Bulgaricus* accordingly protocol of Central laboratory of ENZIM Group Biotechnology company ("Enzim" Ltd, Ukraine). Briefly, isolation of soluble peptidoglycan fragments carried out with limited enzymatic lysis. *L. delbrueckii subsp. Bulgaricus* cells were grown in the exponential phase in MRS broth at 32 °C. The cells were separated from culture medium by centrifugation at 5,000 g for 20 min at room temperature. The obtained pellet was resuspended in sterile PBS and washed by centrifugation at 5,000g for 10 min. The washing procedure was repeated three times to remove any trace of the medium. After washing, the last pellet was resuspended in PBS containing D-glucose and lysozyme. Lysozyme's lyses was performed with circle agitation at 32 °C for 30 min. Soluble peptidoglycan fragments were then separated with centrifugation at 10,000 g for 30 min at 4 °C. Obtained pellet was resuspended and heated at 60 °C for 15 min. Extracted peptidoglycan solution was concentrated at 4 °C by flash evaporation. Concentrated peptidoglycan solution was divided for the fractions with column chromatography in Sephadex G-25. The fraction with the range of molecular weight 800–1 500 Da was collected and concentrated to 1/7 of the obtained volume and lyophilized.

Amino acid composition analysis of muropeptide fraction

Amino acid composition of aforementioned muropeptide fraction was analyzed with using acid hydrolysis of chromatography purified fraction. Briefly, the sample muropeptide fraction with concentration 1mg/ml was mixed with equal volume 6M HCl in glass container and sealed. The hydrolysis was performed in nitrogen atmosphere at 110 °C for 24 h. The resulting product was mixed for neutralization with 6 M NaOH and then diluted with 0.02 M HCl. Amino acid composition was measured with using amino acid analyzer Hitachi L-8800 (Hitachi Corp., Japan).

Cell culture

U373MG (RRID: CVCL 2219), human glioblastoma cells were purchased from the

American Type Culture Collection (ATCC, USA). Glioblastoma cells was cultured and maintained according to the recommendations made by the ATCC. Cell line were maintained in DMEM, containing 10% fetal bovine serum (FBS) 1% penicillin/ streptomycin and 1% L-glutamine at 37 °C CO_2 humidified incubator. All treatments were performed when cell growth entrances in the logarithmic phase. The glioma U-373MG cells were seeded and cultured in DMEM for 2–3 days to mount to 70% confluence. Then the glioma cells were exposed to MPP in 25, 50, 100 and 200 µg/ml concentrations for 48 h. The cells were collected by scratching without trypsinization and lysed with RIPA buffer containing protease and phosphatase inhibitors cocktail.

Cell viability (MTT assay)

The effect of MPP on the viability of U373MG cells was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Bahuguna et al., 2017). Briefly, U373MG cells (1×10^4 cells/well) were seeded in 96-well plates with DMEM, then incubated for 24 hours at 37 °C, 95% humidity and 5% CO_2 conditions. After that, the cells were treated with various (25, 50, 100 and 200 µg/ml) MPP doses for 48 h. Subsequently, the medium was replaced with fresh medium containing 10 µl of MTT-labeled reagent for 4h. Then dimethyl sulfoxide (DMSO, 100 µl) was added into wells and incubated overnight. The values of optical density were measured at 570 nm using an ELISA plate reader (SpectraMax 384 Plus, Molecular Devices, USA).

NADPH determination

The cellular NADPH was assessed using NADPH estimation kit (ab65349, Abcam, UK). Briefly, 1×10^6 cells were plated in 6-cm dishes; on the next day, after treatment, the cells were trypsinized and subjected to extraction by using 300 µl of the Extraction Buffer. The NADPH level were measured according to the manufacturers' protocols and the NADPH levels were calculated as (OD vehicle — OD treatment), where ODs were measured using a SpectraMax plus 384 microplate reader at (460 nm).

Cellular migration scratch-assay

Glioblastoma U373MG cells were seeded in 24-well tissue culture plates with medium containing FBS for 16 h to achieve monolayer formation. A mechanical scratch was made with the help of a 200 µl pipette tip to create the constant-dimension strips, and the plates were then washed with PBS. The cells treated with serum-free DMEM/ medium containing different concentrations 25, 50, 100 and 200 µg/ml of the MPP. The wound areas were photographed immediately after treatment and at time intervals of 24, 48 and 84 hours after with using an invert microscope (Olympus CKX41, Jupon). The percentage of every gap distance was recorded.

Western blotting

Glioblastoma U373MG control and exposed to MPP cells were harvested by scratching without trypsinization. Subsequently, cells were lysed in the lysis buffer containing a proteinase and phosphatase inhibitor cocktail, and proteins were extracted and then the protein concentration was determined using the Bradford protein assay. The content of PARP, NF-κB and β-actin was detected with immunoblotting method. Briefly, the equal amount of protein samples was separated in gradient 5–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a polyvinylidene difluoride membrane (PVDF) (Millipore, USA). The immunoblot membranes were incubated with blocking solution (5% skim milk) at room temperature for 1 h, followed by incubation overnight with corresponding antibody, anti-PARP1 (Abcam, ab194586, 1:2 500), anti-NF-κB P65 (Abcam, ab88940, 1:2 500), and mouse monoclonal anti-β-actin (Santa Cruz, sc-47778, 1:3 000) at 4 °C. The membrane was washed with TBS-T and incubated with a 1:5 000 dilution of HRP-conjugated secondary antibody for 1 h at room temperature. After washing, the blots were visualized by an enhanced chemiluminescence method using X-ray films and quantitated by densitometry using Image J software (USA).

Statistical analysis

All experiments were performed in triplicate and standard deviation values were calculated. Statistical analysis was performed by t-test and One-Way ANOVA test using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA).

Results and Discussion

The results of amino acid composition in peptidoglycan fragments isolated with Sephadex-25 chromatography have shown the present Ala, Asp, Lys and Glu in equimolar ratios into MPP fraction in

respect with pentapeptide structure. Gly is amino acid residue which forms the cross-links between individual pentapeptides in intact peptidoglycan of bacterial walls. Gly is confirmed as a component co-isolated with muropeptides (Table) [1, 48].

Cytotoxic effect of MPP was characterized in vitro with cell viability and migration scratch-assay. The results of cell viability measuring in control and treated with MPP glioblastoma U373MG cells showed dose-dependent cytotoxic effect (Fig. 1).

The results on the impact of MPP onto U373MG cells migration test in a course of scratch-assay have shown dose-dependent effect in a range concentration from 25 to 200 μg/ml. (Fig. 2). U373MG cells were exposed to muramyl pentapeptide in medium without FBS as well as untreated control group to avoid the effect of growth factors on migration activity. The final time of cell migration into gap was determined as full closed gap in untreated control cells. In present study this time was found equal 84 hours.

The metabolic energy deficit in glioblastoma cells initiated by the exposure to MPP was determined via measuring of NADPH content. The results of NADPH content measured in control and treated with MPP glioblastoma cells have showed the suppression of NADH production in exposed to MPP cells. Clear inhibitory dose-dependent effect was determined in a range MPP concentration 25 μg/ml — 200 μg/ml (Fig. 3).

Amino acid content in isolated peptidoglycan fragments

Amino acid	μg/mg material
Ala	12.35
Asp	9.83
Lys	10.07
Glu	9.04
Gly	5.12

The PARP1 expression in control and exposed to MPP glioblastoma U373MG cells was upregulated except the lowest dose exposure (Fig. 4, *a*). The results of NF-κB expression in control and exposed to MPP glioblastoma U373MG cells have showed an increase in almost all treated cell groups (Fig. 4, *b*).

Despite of well-studied adjuvant effect of various muropeptides, the muropeptide-induced reactivity in nonimmune cells remains uncovered. Muropeptides exhibit the stimulating effect in immune cells through leukocytes reactivity including differentiation, proliferation and cytokine production [49–51]. However, muropeptides exhibit not only immunostimulation effect. Several findings evidence multiple effects of muropeptides including anti-inflammatory and cytotoxic activity. For instance, the damaging of intestinal epithelial cells reported in the study of MDP infusion in rat small intestine [35].

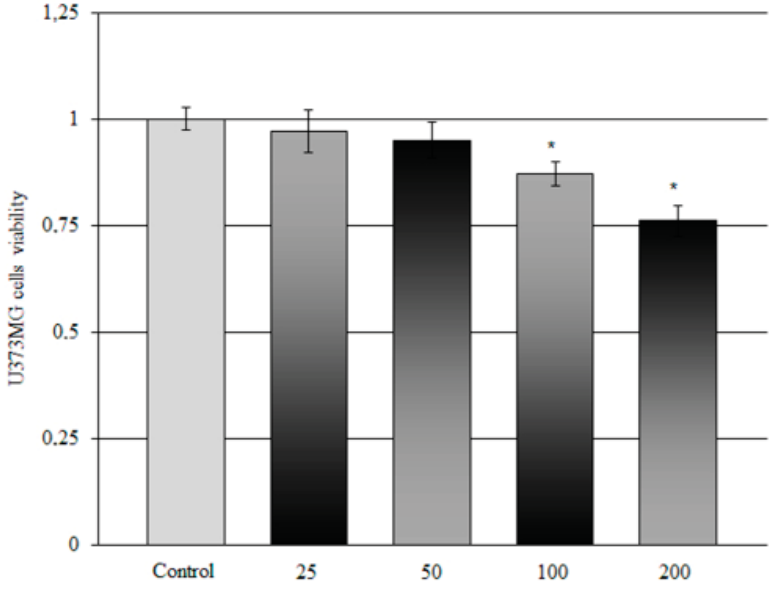

Fig. 1. **Effects of MPP exposure on U373MG cells viability**
Data are expressed as the Mean ± S. E. M.; $n = 3$;
* — $P < 0.05$ significance of differences compared with control group

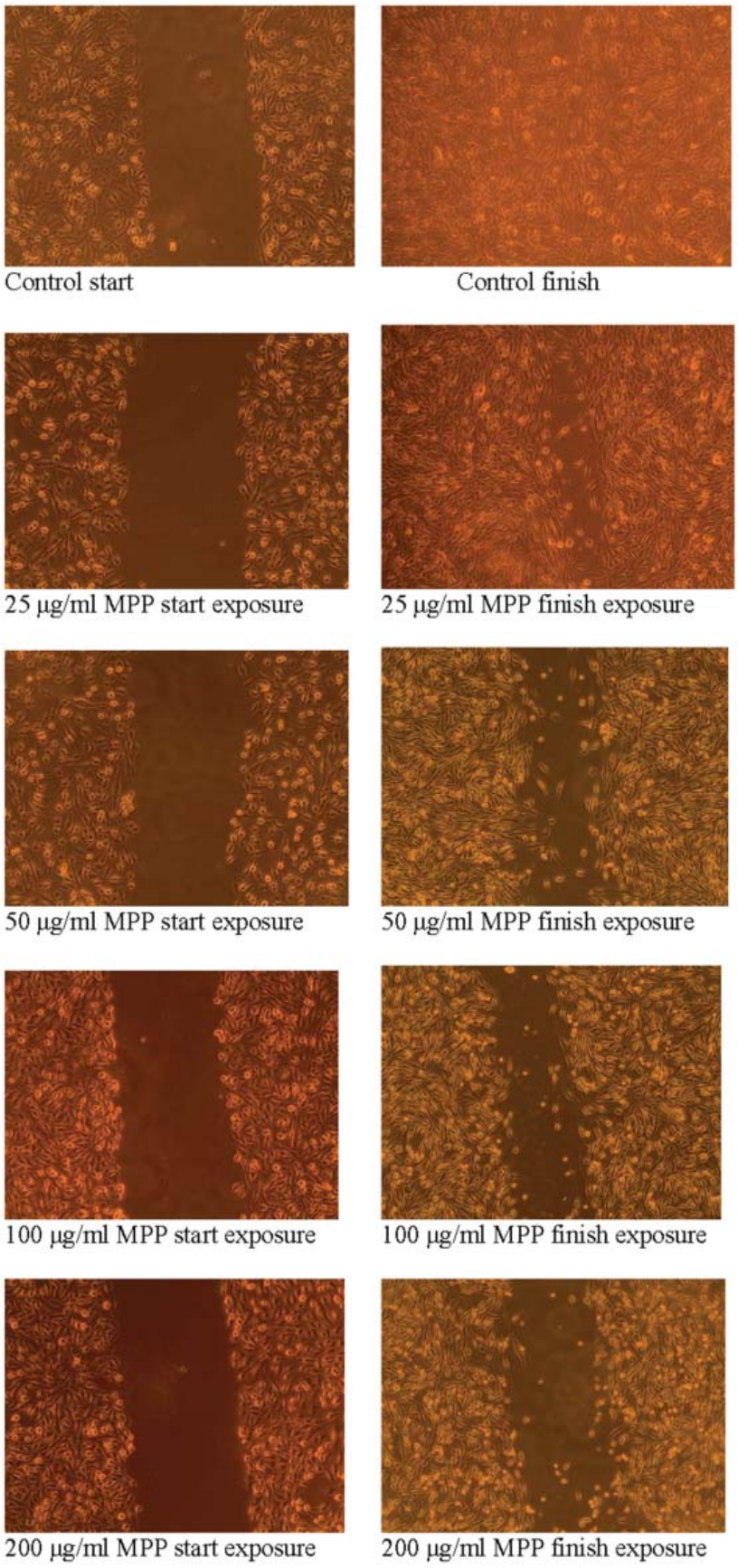

Fig. 2. **The results of migration scratch-assay in control and exposed to various MPP doses U373MG cells**

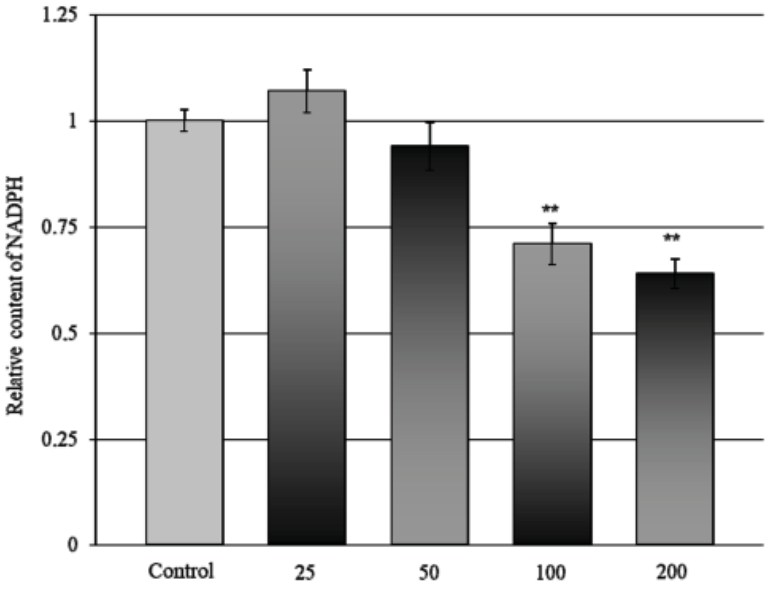

Fig. 3. **Effects of the MPP on NADPH level in control and exposed to various MPP doses U373MG cells**
Data are expressed as the Mean ± S. E. M.; $n = 3$;
** — $P < 0.01$ significance of differences compared with control group

Peptidoglycan structure is strong evolutionary conserved and is recognized by eukaryotic cells as a danger signaling [44]. Unique structure of peptidoglycan is constructed with N-acetylmuramic acid and N-acetylyglucosamine disaccharide chains which crosslinked to each other by short amino acid chains. Enzymatic lyses of bacterial wall generates several biology active compounds containing N-acetylmuramyl peptides which called muropeptides. MDP is most studied and smallest biologically potent product of bacterial walls hydrolysis. MDP as a rule consists two amino acid residues D-Ala and D-isoGln (or D-Glu). On other hand, muramyl pentapeptide is the main structural subunit of peptidoglycan which is synthesized in cytoplasm as disaccharide-pentapeptide building block [52]. However, the isolation MPP requires more complex procedure in compare to muramyl dipeptide purification because MPP fraction could contaminated with three- and four-muropeptides [1, 53]. Isolated in our study MPP agreed with respect to amino acid content to those peptidoglycan fractions reported other authors [1, 48, 54]. In present study the cell viability test and migration scratch-assay were applied to determine cytotoxic effect of MPP on astrocyte-derived human glioblastoma cells. The exposure to 25–200 μg/ml MPP induced in glioblastoma cells dose-dependent decrease in cell viability (Fig. 1).

Obtained in our study results accord with reported by Wang and coauthors cytotoxic effect on different tumor cell lines exposure to whole peptidoglycan extract [11]. It deserves to be mentioned that most effective fraction of peptidoglycan extract was found in a range molecular weight 600–1000 Da that is close to molecular mass of MPP fraction applied in our study. Furthermore, Fichera and coauthors have cleaved bacterial walls with penicillin G treatment that could resulted in the generation various number of amino acid residue in the muropeptide chains [1]. As it was mentioned in Materials and methods we have used lysozyme to cleave peptidoglycan of *Lactobacillus* that generates predominantly muramyl pentapeptide fragments [1, 48, 54]. Presented in our study tumoricidal effect of MPP evidences the direct cytotoxic effect of this muropeptide type in glia-derived tumor cells.

Glioblastoma is characterized by extensive metabolic rate and extremely high capability to the proliferation and metastases [39, 41]. Glial-derived cancer have multiple mutations including p53 protein which responsible for the limiting of cancer progression. On other hand, p53 controls TP53-induced glycolysis regulatory phosphatase (TIGAR) functioning which regulate the pathways of glucose utilization [55]. TIGAR is important apoptosis regulator which protects the cells against oxidative-induced apoptosis. Furthermore,

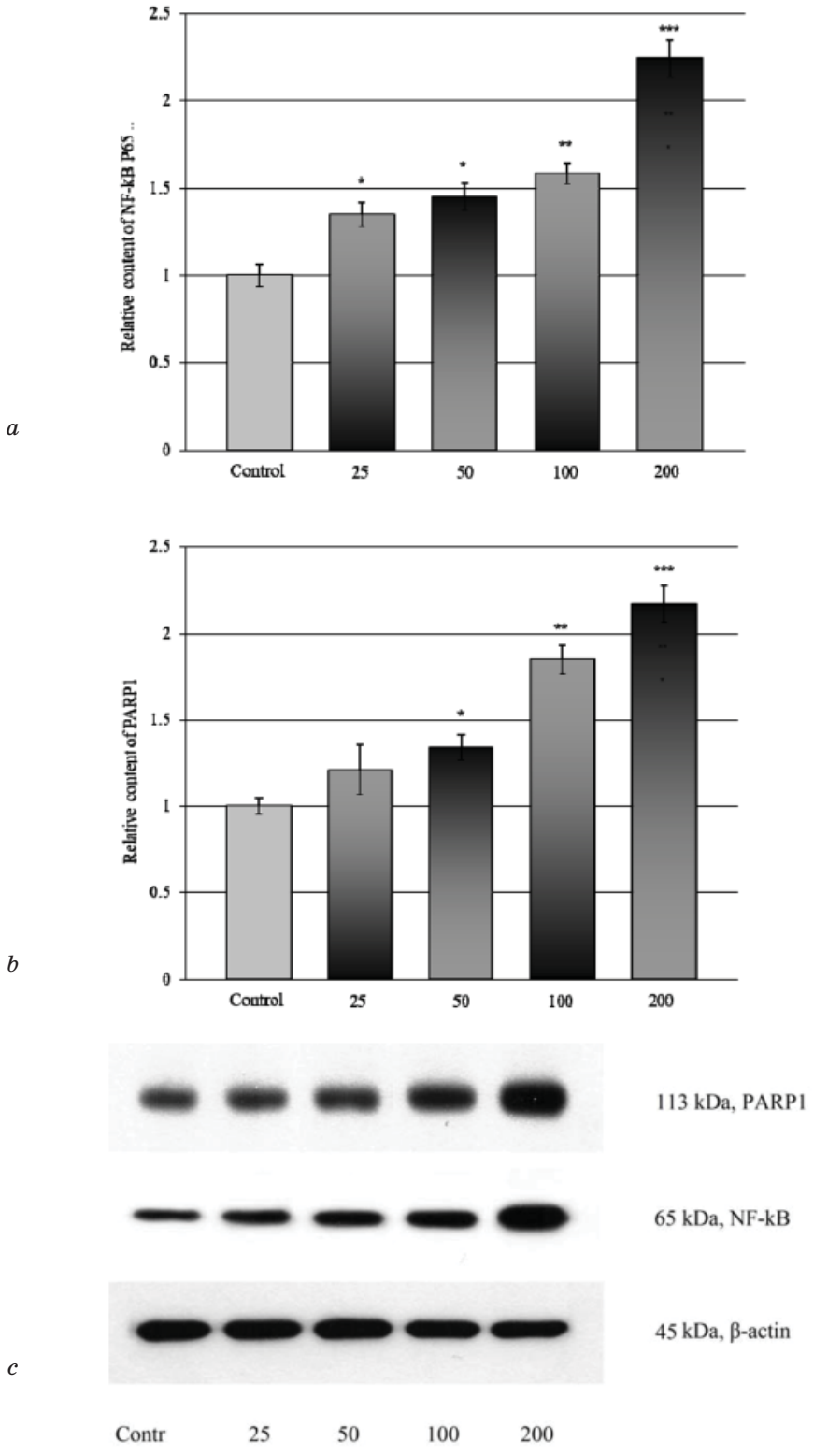

Fig. 4. **Effects of the MPP on PARP1 and NF-κB expression in control and exposed to various MPP doses U373MG cells:**

a — relative PARP1 content; *b* — relative NF-κB content; *c* — western blot results

Data are expressed as the Mean ± S. E. M.; $n = 3$;

* — $P < 0.05$, ** — $P < 0.01$; *** — $P < 0.001$ significance of differences compared with control group

the switching of glucose utilization pathways in glioma could tightly relate to the initiation either apoptosis or autophagy [56]. Thus, the initiation of metabolic energy depletion can limit cancer growth. Taking into account that gliomas prevailingly generate metabolic energy via glycolysis, the inducing an abnormality in energy expenses could be a prospective target to suppress tumor progress. Obtained migration test results have showed dose-dependent effect of MPP in glioblastoma cells exposed to various MPP doses. Accordingly, literature present data is first report in respect with muropeptide inhibition of glioblastoma cells motility. Glioblastoma U373MG cells are one of most potent to invasion brain tumor that accompanied by high metastasis rate [39].

Observed in our study results have showed that MPP in a dose 200 μg/ml induce significant upregulation both PARP1 and NF-κB expression in glioblastoma cells. Recently, Yamaguchi and coauthors have reported that MDP can stimulate NF-κB expression [57]. In addition, muropeptides as a initiators of innate immune response exert multiple effects on cytokine production, cell survival and programmed cell death [44]. According to our results, MPP exposure in range 25–200 μg/ml doses induced both PARP1-dependent and NF-κB-dependent cellular response in glioblastoma U373MG cells. Moreover, observed glioblastoma cells reactivity had accompanied by the suppression of migration capability and NADH content depletion. Unfortunately, experimental data on the impact of muropeptides onto cell migration extremely limited and contradictory. There were reported inhibitory impact of bacterial wall compounds on macrophage migration [58]. Contrary, the study of cell walls isolated from different bacterial species has showed the definite increase in monocyte migration [59]. To the best of our knowledge, the results on the inhibitory effect of MPP on glioblastoma migration capability are presented for the first time.

NADH depletion in MPP exposed glioblastoma cells is tightly relates to PARP activation. Activated PARP decreases NADH content in course of the poly-ADP-ribosylation, protein targets ADP-ribosylation and DNA remodeling [32]. Long term or extensive PARP involving in cellular response can lead to cell damage. For instance, PARP activation is able to trigger astroglia to cell death that reported in reactive astrocytes located in post ischemic injury areas [60]. The activation astrocytes with bacterial walls exposure reported in Phulwani and Kielian study agreed with our results [61]. However, the distinct role PARP in both normal and malignant cells is unknown. PARP can serve as a coactivator of NF-κB and provoke cellular reactivity [28]. The abundant PARP expression is under strict control in normal glial cells. In glial-derived tumors multiple mutations mediate a lack of native mechanisms to limit metabolic energy consumption. Thus, MPP-induced glioblastoma reactivity could be effective cause to trigger metabolic energy expenses from proliferative activity to cell death.

Cell migration is energy consuming process. The results of migration test evidence that MPP exposure in dose-dependent manner suppress glioblastoma migration. Therefore, dose-dependent upregulation of PARP expression could be one of key mechanism in this MPP inhibitory effect. On other side, observed in our study anti-migration effect of MPP exposure could be mediated complex cell response initiated with both extra- and intra-cellular receptors of muropeptides. Mammalian cells can recognize peptidoglycan and its derivates with specific pathogen recognition receptors [62]. First group of them is extracellular receptors including peptidoglycan recognition proteins (PGRPs) and C-type lectin-like receptors (CTLRs) [63]. Furthermore, muropeptides as cleaved peptidoglycan fragments possess stimulate intracellular receptors including NOD2 and hexokinase [42, 43]. Absorbed muropeptides are recognized by intracellular molecule NOD2 in various cell types. Pathogen recognition receptor NOD2 plays important role not only in innate immune systems it can modulate adaptive cellular response through the production of chemokines and toxic peptides [43, 44].

Despite of well-studied effect of muropeptides on NOD family receptors, the precise mechanism underlying how NOD receptors regulate cellular response in non-immune cells is not yet fully understood. Moreover, the structure of muropeptides is critic to initiate specific cell response. For instance, Nagao and coauthors have reported that various muropeptides can induce macrophage migration with different efficacy dependent on the amino acid residues content [58]. There are limited data on the NOD-dependent stimulation of NF-κB transcriptional activity and involving other factors which responsible for cell reactivity in non-immune cells [37]. The stimulation of NOD2 with muropeptides initiates RIP2

kinase polyubiquitination and activates cellular reactivity via NF-κB pathway of transcriptional regulation that ultimately upregulates proinflammatory cytokines production including tumor necrosis factor alpha (TNF-α) [64, 65]. Furthermore, PARP1 phosphorylation in a course of cellular response can promote NF-κB activity through its poly-ADP-ribosylation [66, 67]. Taking into account, that long term PARP activation can induce cellular energetic disturbances, the initiation of PARP-dependent cell response may lead to cell dysfunction or death through NF-κB involving [68]. In this context, observed in our study upregulation of both PARP and NF-κB could serve an important source of the exponential growth in glial cell reactivity induced with MPP exposure [30].

Extensive glial cell reactivity accordingly requires metabolic energy expenses. Tumoricidal effect of PARP1 activation can be developed through multiple features of this enzyme which require ATP for both ribosylation of proteins and PAR synthesis. Especially, PARP1 can modulate the differentiation of several cell types in a course of cellular response [69, 70]. Thus, PARP1-induced events of differentiation in glioblastoma cells which exhibit phenotype of non-differentiated astrocytes could limit glioma aggressiveness and potency in proliferation and invasion. The various isotypes of muropeptides can stimulate NOD2 with different affinity as well as induce variable cellular response. Besides, NOD2 agonists are recognized to induce the resistance to apoptosis that could promote the surviving of infected with bacteria macrophages via suppression of FasL-induced apoptosis [71, 72]. However, the stimulatory mechanisms of muropeptides including MPP remains unknown.

It deserves to be mentioned that glioblastoma treatment with low (25 μg/ml) dose MPP induces mild increase in NADPH level, NF-κB and PARP expression. Observed changes could characterize cellular response directed to cell surviving and involving both NF-κB and PARP in programmed cell death regulation. Thus, obtained results support hypothesis that low doses of muropeptides can exert cytoprotective effect via anti-apoptotic pathways [12, 16].

The study of the functional interaction between the regulation of programmed cell death and immune signaling pathway brings unexpectable results on the applying regulatory mechanisms to inhibit tumor growth. There was demonstrated that both NOD2 and RIPK2 are involved in the regulation of cell death and inflammation through the caspase-1-dependent pathway [43]. Thus, one of important consequence of MPP effect in glioma could be depletion of apoptosis resistance. MPP stimulation can initiate extensive energy consumption directly on the first step of pathogen receptors initiation. There was reported that NOD2 active form requires ATP bindings in equimolar concentration. Besides, only active NOD2 form can bind muropeptide with following dimerization. The dimerization of NOD2 initiates the polymerization of receptor-interacting serine/threonine-protein kinase 2 (RIPK2). RIPK2 is a member of the RIP kinase family and central adaptor kinase in the NOD pathway [43]. RIP kinases are involved in regulation cell death and pathogenesis of chronic diseases [73]. Furthermore, RIPK2 is critic indispensable to activate both NF-κB and MAPK pathways in a course of NOD-mediated cellular response. RIPK2 can bind the tumor necrosis receptor (TNFR) that can switch NF-κB function from surviving to cell death [74].

Despite of unknown role of NOD2 in glial cells reactivity, there is an evidence of glial response to muropeptides signaling. Ribes and coauthors demonstrated that MDP can cause a release of NO and stimulate bacterial phagocytosis in microglial cells [34]. However, in microglia there no observed upregulation of pro-inflammatory cytokines accompanied by this glial response induced with muropeptide. Chauhan and coauthors demonstrated that muropeptides could elevate cytokine production in astrocytes and initiate astrogliosis [37]. Taking into account that extensive astrogliosis can induce irreversible damages and consequently cell death, we can expect the metabolic abnormality in glia-derived tumors as a result of MPP-induced gliosis. Aforementioned data evidence the biology activity of muropeptides with respect to potent effect on the regulation both energy metabolism and glial cells reactivity.

Numerous studies of tumoricidal effects probiotics, bacterial walls fractions, peptidoglycan fragments confirmed that the effect of muropeptides on cellular response of various cell types tightly relate to its anticancer activity. On other hand, muropeptides could affect vital processes in tumor cells directly through complex mechanism of cellular response regulation. Taking into account that there is a principal difference in energy metabolism between non-malignant and tumor

cells, we can presume reciprocal cellular response to muramyl pentapeptide in glial-derived tumor cells compared to normal glia response. In any event, further comparative study of MPP effect on normal glial cells and glioblastoma cells is required to clarify this hypothesis. The results of present study agreed with those presented of Kim and coauthors that bacterial peptidoglycans exhibit significant antiproliferative activity [4]. Moreover, our results accord with the data reported by Fichera and Gunter that peptidoglycan from *L. casei* decrease various tumor cell types viability [18]. Therefore, observed in our study NADH depletion and the suppression of migration could be a result of extensive cellular response to MPP signaling in glioblastoma cells.

Conclusion

Muramyl pentapeptide exposure induces disturbances in NADH content, inhibits migrative capability and upregulate PARP1 and NF-κB expression in glioblastoma U373MG cells. Obtained results evidence that muramyl pentapeptide could initiate a lack of migration via metabolic energy expenditure as a result of gliotypic reactivity. The future studies are actual and extremely required to clarify tumoricidal effect of MPP with respect to glia-derived tumors.

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Research involving Human Participants and/or Animals

The manuscript does not contain studies with human participants or animals.

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ФРАКЦІЯ ПЕПТИДОГЛІКАНУ, ЗБАГАЧЕНА МУРАМІЛ ПЕНТАПЕПТИДОМ З *Lactobacillus bulgaricus*, ПРИГНІЧУЄ МІГРАЦІЙНУ ЗДАТНІСТЬ КЛІТИН ГЛІОБЛАСТОМИ U373MG І ПІДВИЩУЄ РІВНІ PARP1 ТА NF-κB

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Пептидоглікан є універсальним компонентом бактеріальних стінок, що виявляє різну біологічну активність, включаючи антипухлинний ефект. Протипухлинний ефект різних фракцій пептидогліканів та його похідних є варіабельним залежно від їхньої структури. Мураміл пентапептид (MPP) є найбільш повною складовою частиною пептидоглікану. MPP може стимулювати клітинну реактивність так само, як й інші муропептиди. У цій роботі ми оцінювали інгібіторну дію MPP на життєздатність та міграцію клітин гліобластоми U373MG. Як маркери клітинної реакційної здатності визначали кількість протеїнів PARP1 та NF-κB. Вплив MPP, індукований у клітинах гліобластоми, знижує життєздатність клітин та міграційну активність. Крім того, дія MPP спричиняє підвищення кількості PARP1 і NF-κB у дозозалежний спосіб. Рівень НАДФН в стимульованих клітинах гліобластоми був знижений порівняно з контролем. Таким чином, MPP виявляє протипухлинну дію в клітинах гліобластоми U373MG через виснаження вмісту НАДН і, як наслідок, рівня метаболічної енергії. Крім того, регуляція кількості PARP1 та NF-κB в клітинах гліобластоми може бути важливим механізмом пригнічення міграційної здатності клітин і прогресії пухлини.

Отримані результати вказують на те, що мураміл пентапептид може ініціювати зниження міграції шляхом підвищення витрат метаболічної енергії в результаті гліальної реактивації. Подальші дослідження є актуальними і вкрай необхідними для уточнення протипухлинної дії цього муропептиду в гліальних пухлинах.

Ключові слова: пептидоглікан, мураміл пентапептид, PARP1, NF-κB, гліобластома U373MG, реакційна здатність клітин.

ФРАКЦИЯ ПЕПТИДОГЛИКАНА, ОБОГАЩЕННАЯ МУРАМИЛ ПЕНТАПЕПТИДОМ ИЗ *Lactobacillus bulgaricus*, ИНГИБИРУЕТ МИГРАЦИОННУЮ СПОСОБНОСТЬ КЛЕТОК ГЛИОБЛАСТОМЫ U373MG И УВЕЛИЧИВАЕТ УРОВНИ PARP1 И NF-κB

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Пептидогликан является универсальным компонентом бактериальных стенок и проявляет разную биологическую активность, включая антиканцерогенное действие. Противораковое действие различных фракций пептидогликана и его производных вариабельно в зависимости от их структуры. Мурамил пентапептид (MPP) является наиболее полной составной частью пептидогликана. MPP может стимулировать клеточную реактивность, также как и другие муропептиды. В настоящей работе мы оценивали ингибирующее действие MPP на жизнеспособность и миграцию клеток глиобластомы U373MG. Экспрессию PARP1 и NF-κB определяли в качестве маркера клеточной реактивности.

Воздействие MPP индуцировало в клетках глиобластомы снижение жизнеспособности клеток и миграционную активность. Кроме того, воздействие MPP дозозависимо увеличивало PRAP1 и NF-κB экспрессию. Уровень NADH в стимулированных клетках глиобластомы был снижен по сравнению с контролем. Таким образом, МПП проявляет противоопухолевое действие в клетках глиобластомы U373MG путем истощения содержания NADH и, следовательно, уровня метаболической энергии. Кроме того, усиление экспрессии PARP1 и NF-κB в глиобластоме может быть важным механизмом в ингибировании развития опухолевого фенотипа в клетках глиобластомы.

Полученные результаты указывают на то, что мурамил пентапептид может инициировать снижение миграции путем увеличения затрат метаболической энергии в результате глиальной реактивации. Дальнейшие исследования актуальны и крайне необходимы для понимания антиканцерогенного действия этого муропептида на опухоли глиального происхождения.

Ключевые слова: пептидогликан, мурамилпентапептид, PARP1, NF-κB, глиобластома U373MG, клеточная реактивность.