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RESEARCH

Metformin Effects on Malignant Cells and Healthy PBMC; The Influence of Metformin on the Phenotype of Breast Cancer Cells

Ana Damjanović · Ivana Z. Matić · Marija Đorđić · Marina Nikolić Đurović · Srđan Nikolić · Ksenija Roki · Zorka Milovanović · Jelena Antić-Stanković · Radan Džodić · Svetozar Damjanović · Ksenija Kanjer · Zaki Abu Rabi · Zorica Juranić

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Abstract The aim of research was to determine the effects of maximally therapeutically achievable concentrations of metformin on malignant cells and healthy peripheral blood mononuclear cells (PBMC). Eight patients with T2D or hyperglycemia and nine healthy volunteers were included in the study. For determination of the influence of metformin on the phenotype of breast carcinoma, 1,410 patients with surgically removed tumors were included. From this group 37 breast cancer patients had DM type 2 or hyperglycemia and were pretreated with metformin alone or sometimes in combination with other antidiabetic drugs. Our results proved that metformin at low concentrations induced mild decrease in survival of malignant cells and PBMC stimulated for proliferation, but it didn't affect survival of resting PBMC. The effects of plasma of hyperglycemic patients who were under metformin therapy on autologous PBMC-induced decrease in survival of MDA-MB-361 cells, was noticeable in some patients. Metformin pretreatment for 24 h of HER2+ MDA-MB-361 cells, which were subsequently treated for 48 h with Herceptin, induced additional decline in cell survival. The analysis of influence of metformin on phenotype of breast cancer cells revealed significantly lower number of diabetic cancer patients treated with metformin with overexpressed HER2+ tumors ($p<0.013$), while the number of patients with ER+PR+ tumors was not significantly changed ($p<0.832$). In conclusion, therapeutically used concentrations of metformin exhibit mild cytotoxic action on malignant and dividing normal cells pointing to its preferred role in malignant and autoimmune diseases. The use of metformin was associated with pronounced decrease in HER2 overexpressing tumors.

Keywords Cancer · Herceptin · Metformin · PBMC

A. Damjanović · I. Z. Matić · M. Đorđić · S. Nikolić · K. Roki · Z. Milovanović · R. Džodić · K. Kanjer · Z. Abu Rabi · Z. Juranić (✉)
Institute of Oncology and Radiology of Serbia, Pasterova 14, 11000 Belgrade, Serbia
e-mail: juranicz@ncrc.ac.rs

M. N. Đurović · S. Damjanović
Clinic of Endocrinology, Diabetes and Metabolic Diseases, Clinical Center of Serbia, dr Subotića 13, 11000 Belgrade, Serbia

M. N. Đurović · R. Džodić · S. Damjanović
School of Medicine, University of Belgrade, dr Subotića 8, 11000 Belgrade, Serbia

J. Antić-Stanković
Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11000 Belgrade, Serbia

Introduction

It is well known that some of cancer patients have hyperglycemia due to *diabetes mellitus,* DM. Data from a number of epidemiological studies indicate that disturbances in metabolism characteristic for DM could be associated with increased risk for some forms of cancer like primary cancer of the liver [1], pancreas [2], bladder [3] or colorectal cancer [4]. Possible mechanisms by which DM could be associated with tumor promotion pointed to the high glucose and/or the insulin action. Insulin binds to preassembled receptor heterodimers leading to initiation of signaling through adaptor proteins, the insulin receptor substrates (IRS1-4), which activate the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein-kinase (MAPK) signaling pathways. This results in a cascade of proliferative and anti-apoptotic events [5].

It is established that metformin (1, 1-dimethylbiguanide hydrochloride) in addition to diet and exercise is used as the first line of diabetes therapy. The mechanisms of its action

include a decrease of insulin and glucose in circulation; it was reported that LKB1-mediated activation of adenosine monophosphate protein kinase (AMPK) is required for the glucose lowering effect of metformin [6]. Its antiproliferative action in vivo to various cancers could be mediated through many pathways [7–9]. Special review of overall metformin anticancer action is published by Jalving et al. [10]. It was also reported that metformin has influence on the action of some chemotherapeutics [11–13].

It is well known that cancer is under control of host immune system. Cytotoxicity of peripheral blood mononuclear cells (PBMC) to malignant cells is one of the well known and very important mechanisms of the immune-mediated host anticancer action [14]. It is mainly occurring through the action of natural killer cells (NK) or, in persons with anticancer adaptive immune response through specific T cell tumor toxicity (CTL) and antibody-mediated cell cytotoxicity (ADCC) [15–18]. Furthermore, it is important to note that one external immunodrug Herceptin, synthetic monoclonal antibody to HER2, is very successful antitumor drug which is clinically used for therapy of patients bearing HER2+ tumors [19].

While the evidence of the direct antitumor action of high concentrations of metformin (>2 mM) on some tumor cell lines in vitro, in the presence of fetal bovine serum (FBS), is beyond any doubts, until now there are no data about metformin action on the normal human lung fibroblasts and also there are no records about its action on the human immunocompetent cells (PBMC), especially in the presence of human serum (HS). The data of metformin influence on PBMC cytotoxic action towards malignant cells, and on Herceptin mediated antitumor PBMC action are also absent in recent literature.

Therefore the aim of this work was to determine the cytotoxic action of maximally achievable concentrations of metformin (which could be present in diabetic patients' blood during the therapy) on six human malignant cell lines, on normal human lung fibroblasts, as well as on human PBMC stimulated or non-stimulated for proliferation in the presence of human plasma. Next aim was to determine the effect of plasma of patient with type 2 diabetes (T2D) or hyperglycemia (1 month after the everyday metformin usage) on the decrease in MDA-MB-361 cell survival induced alone or in combination with PBMC in vitro. One of the aims was to determine is there any input of malignant cells pretreatment by metformin on the antiproliferative Herceptin action to HER2+ MDA-MB-361 cells. To get insight whether the therapy of diabetes or hyperglycemia with metformin might induce the changes in phenotype of cancer cells, we determined the prevalence of breast cancer patients with enhanced HER2, estrogen and progesterone receptor expression on tumor cells and compared it with those found in general breast cancer population.

Materials and Methods

Patients

For determination of metformin effect on PBMC survival and function nine healthy control persons and eight patients with hyperglycemia were included in this study. In addition, for determination of the influence of metformin on the phenotype of breast cancer cells 1,410 patients (in the period November 2011–December 2012) with surgically removed tumors were included. From this group 42 patients had DM type 2 or hyperglycemia and were pretreated (not less than 1 month) with metformin alone or sometimes in combination with other antidiabetic drugs. In 37 out of these 42 patients, malignant tumors were found (three of them were with bilateral tumors), while 5 out of 42 patients had benign breast disease. It should be noted that 14 of 37 diabetes patients additionally were treated with some sulfonylurea derivatives, while 3 of 37 used additionally insulin as the antidiabetic therapy.

The study of our project was approved by the Ethics Committee of the Clinical Center of Serbia and the Ethics Committee of the Institute of Oncology and Radiology of Serbia. Written informed consent was obtained from each patient.

Reagents

Stock solution of metformin hydrochloride (Harman Finochem) prepared in a physiological solution was diluted with nutrient medium and applied to target cells to various final concentrations (2 mM, 1 mM, 0.5 mM, 0.25 mM and 0.125 mM). Nutrient medium used in this work was RPMI 1640 supplemented with 3 mM L-glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin, 10 % FBS, (or 10 % human serum or plasma, HS or HP respectively) and 25 mM Hepes; pH was adjusted to 7.2 by bicarbonate solution. Human plasma was obtained from heparinized blood (20 ml of human blood with 0.2 ml of heparin) by centrifugation.

Determination of the Cytotoxic Metformin Action on Malignant Cell Lines

For determination of the cytotoxic metformin action, several malignant cell lines were used: human cervix adenocarcinoma HeLa, human melanoma Fem-x, human breast adenocarcinoma MDA-MB-361, human breast carcinoma MDA-MB-453, human chronic myelogenous leukemia K562 cells and human acute T cell leukemia Jurkat cells. Briefly, 2,000 HeLa cells, 5,000 Fem-x cells, 10,000 MDA-MB-361 cells, 3,000 MDA-MB-453 cells, 5,000 K562 cells and 7,000 or 20,000 Jurkat cells were seeded in 0.1 ml of nutrient medium and incubated at 37 °C in the humidified air atmosphere with 5 % CO_2. Various concentrations of metformin were added 2 h later.

Determination of cell survival was done by MTT test, as it was published earlier, 72 h after the continuous metformin action [17].

Cytotoxic Action of Metformin on Normal Cells

The cytotoxic action of metformin on normal cells was tested on human lung fibroblasts MRC-5, and on immunocompetent peripheral blood mononuclear cells (PBMC). PBMC were obtained from heparinized blood, as it was reported before [17]. Briefly, 5,000 MRC-5 cells were seeded in a nutrient medium, or 150,000 PBMC (isolated from five healthy volunteers) were seeded into 96-well microtiter plates in nutrient medium only, or in a nutrient medium enriched with phytohaemagglutinin (5 μg/ml). After 2 h metformin was added to the wells in five final concentrations. The nutrient medium was added to control wells. Determination of target cell survival was done by MTT test 72 h later.

The Effect of Continuous (24 h) Metformin Action on the Decrease of Malignant Cells Survival Induced by PBMC

Aiming to explain the data obtained testing the direct effect of plasma of hyperglycemic patients who were on metformin therapy on breast cancer cell survival, as well as those immune-mediated, determination of the metformin effect on PBMC and induced decrease in MDA-MB-361 cell survival was done according to previously published test [17].

Target MDA-MB-361 cells (10,000 in nine experiments, or 20,000 in eight experiments) were mixed with 40,000 PBMC for 24 h, in the absence or with presence of 0.5 mM, 1 mM or 2 mM metformin in nutrient medium with 10 % of autologous to PBMC human plasma.

According to the definition of additive action, additive effect was present if the decrease in target cell survival, after the combined action of metformin and PBMC, was equal to the calculated (sum) of decreases in the cell survival after the only metformin, or after the only PBMC action. If this decrease in cell survival was higher than calculated it was defined as the synergistic one, or when it was lower it was defined as the antagonistic action.

The Effect of Plasma of Hyperglycemic Patients Who Were on Metformin Therapy on the Decrease in MDA-MB-361 Cell Survival Induced by PBMC

The effect of plasma of patients with hyperglycemia (who were for 1 month on the therapy with metformin) on the decrease in malignant cell survival induced by PBMC was tested on breast adenocarcinoma MDA-MB-361 cells. Duration of the test was 24 h; 20,000 of target MDA-MB-361 cells per well were incubated with effector 40×10^3 or 80×10^3 PBMC in nutrient medium RPMI 1640 in the presence of 10 % of autologous plasma isolated from heparinized diabetic patients' blood, before and 1 month after the daily metformin usage (500 mg or 1,000 mg metformin was orally taken in two doses, for 1 month). MTT test was used for determination of target cell survival.

Metformin Modulation of Herceptin Antitumor Action

The possibility that metformin could modulate Herceptin antitumor action was tested on HER2+ MDA-MB-361 breast cancer cells. Briefly, 15,000 MDA-MB-361 cells seeded per well were pretreated with 0, 0.5 mM, 1 mM and 2 mM metformin for 24 h, and then Herceptin was added to the target cells in final concentration of 21 μg/ml and 42 μg/ml. The test was finished after next 48 h.

Determination of Target Cell Survival

MTT test was used for determination of target cell survival [17]. Cell survival (S%) is defined as the ratio of the number of target cells present in well with drug(s), and/ or effector cells and number of cells which are present in control well (cells grown only in the presence of nutrient medium) and multiplied by 100. In a few words, after desired incubation time, 10 μl of MTT solution (5 mg/ml in phosphate buffered saline) were added to the samples and 4 h later 100 μl of 10 % sodium dodecyl sulfate. The absorbance at 570 nm of MTT stained cells was read 24 h later. To determine the cell survival, the absorbance at 570 nm of MTT stained target cells was always divided with the absorbance of control cells (Ac) i.e. target cells incubated in nutrient medium with 10 % autologous plasma and multiplied by 100.

$$S(\%) = (A/Ac) \times 100$$

The absorbance of corresponding blank A was always subtracted from A of samples, or from A of controls.

In experiments with PBMC used as effectors, absorbance of sample with PBMC, (A_{PBMC}), was always subtracted from absorbance of corresponding sample with mixture of effector and target cells ($A_{MDA\text{-}MB\text{-}361+PBMC}$) as it was published earlier [17].

Determination of HER2 Expression on MDA-MB-361 Cells

MDA-MB-361 cells were treated with two different concentrations of metformin (0.5 mM and 1 mM) for 72 h. After cytospin of target cells HER2 expression was determined using DACO polyclonal rabbit anti-human c-erbB-2 oncoprotein antibody. Detection system was Lab Vision™ UltraVision™ LP Detection System HRP Polymer/DAB Plus Chromogen.

Determination of the Phenotype of Malignant Cells

Receptor status was assessed analyzing tumor cells obtained at diagnosis by immunohistochemistry. Estrogen and

Table 1 The effect of continuous (72 h) metformin action on the survival of various malignant cell lines, normal human lung fibroblasts MRC-5 and of resting or stimulated to proliferation by PHA (5 μg/ml) healthy human PBMC, incubated in nutrient medium (RPMI 1640 enriched with 10 % FBS or 10 % HP) determined by MTT test. Results are mean from three independent experiments done in triplicate

	2 mM metformin RPMI +10 %FBS S [%]	2 mM metformin RPMI +10 %HP S [%]
HeLa	74.23±2.43	82.09±6.07
Fem-x	68.15±1.67	81.45±12.09
MDA-MB-453	72.50±2.59	86.97±2.71
MDA-MB-361	74.37±9.21	86.33±1.66
K562	74.47±9.27	69.02±5.59
Jurkat	77.54±13.99	82.48±9.06
MRC-5	79.37±5.46	79.99±8.17
PBMC	ND	100.94±6.77
PBMC + PHA	ND	84.57±8.52

progesterone receptor expression was scored from 0 to 8; scores 3 and above were considered positive. Intensities of HER2 expression were graded from 0 to 3+; scores 3+ and 2+ (after confirmation of HER2/neu amplification by additional analysis, chromogenic in situ hybridization) were considered as positive.

Statistical Analysis

Fisher test was used for assessment of the influence of metformin on phenotype of breast tumors; when $p<0.05$ was considered as significantly different.

Results

Metformin Action on Malignant Cell Lines

Data presented in Table 1 and Fig. 1 clearly show that different concentrations of metformin exert similar extent of decrease in the survival of human cervix adenocarcinoma HeLa, human melanoma Fem-x, human breast adenocarcinoma MDA-MB-

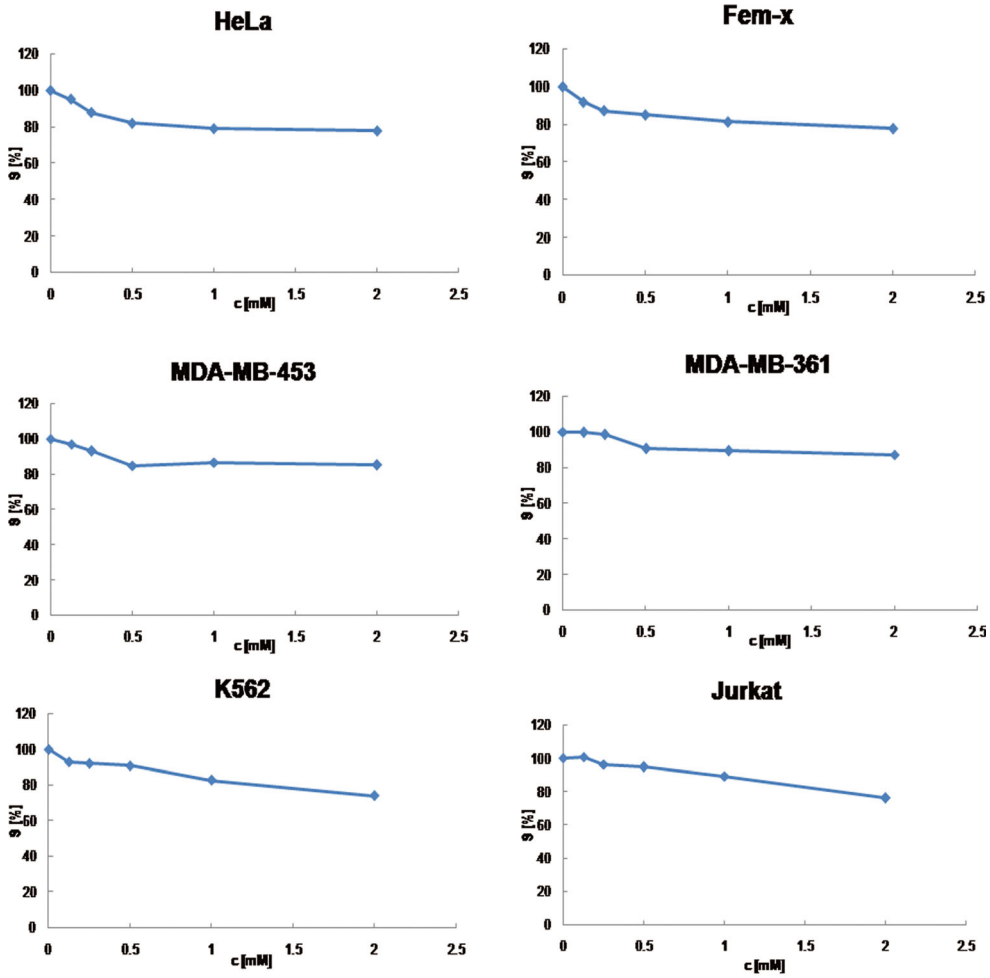


Fig. 1 Survival of various malignant cell lines grown in the presence of different metformin concentrations in nutrient medium (RPMI 1640 enriched with 10 % HP) done in triplicate, determined by MTT test, 72 h after the metformin action. Representative graphs are shown

361, human breast carcinoma MDA-MB-453, human chronic myelogenous leukemia K562 cells and human acute T cell leukemia Jurkat cells, which was not higher than 30 %. The presence of healthy human plasma instead of FBS in nutrient medium did not change the intensity of metformin induced decrease in malignant cells survival.

Metformin Action on Normal Cells

Regarding metformin action on normal cells, obtained data showed that normal human lung fibroblasts MRC-5 and PBMC stimulated for proliferation were sensitive to the anti-proliferative metformin action. On the contrary, resting PBMC were resistant to metformin cytotoxic action, as shown in Table 1 and Fig. 2.

Effect of Metformin on the PBMC Antitumor Action

The effect of exogenously added metformin (in concentrations: 0.5 mM, 1 mM and 2 mM) and of externally added PBMC to autologous plasma of 9 healthy controls and of 8 hyperglycemic patients (before metformin therapy) on malignant MDA-MB-361 cell survival showed that the effect of metformin on PBMC induced antitumor action was additive or synergistic in 11 cases and antagonistic in 6 cases (3 patients and 3 controls), showing that immune-mediated antitumor action of metformin was individually dependent.

The Effect of Plasma of Hyperglycemic Patients Who Were on Metformin Therapy on the Decrease in MDA MB-361 Cell Survival Induced by PBMC

The presence of 10 % plasma (in nutrient medium) of five hyperglycemic patients who were on metformin therapy decreased the mean values of survival of malignant MDA-MB-361 cells. Furthermore, an enhancement of the decrease in malignant MDA-MB-361 cells survival induced by 40,000 of PBMC in the presence of 10 % plasma of these patients in relation to the intensity of the 40,000 PBMC action in the presence of plasma of the same patients before metformin therapy is obtained.

The enhancement of the antitumor action of PBMC to malignant MDA-MB-361 cells is observed in the presence of some patients' plasma who were at least 1 month on the therapy with anti-hyperglycemic drug metformin in relation to PBMC action in the presence of plasma before metformin therapy (Fig. 3a). The suppressive effect of metformin present in plasma on the antitumor action of PBMC could be observed in the other patient (Fig. 3b).

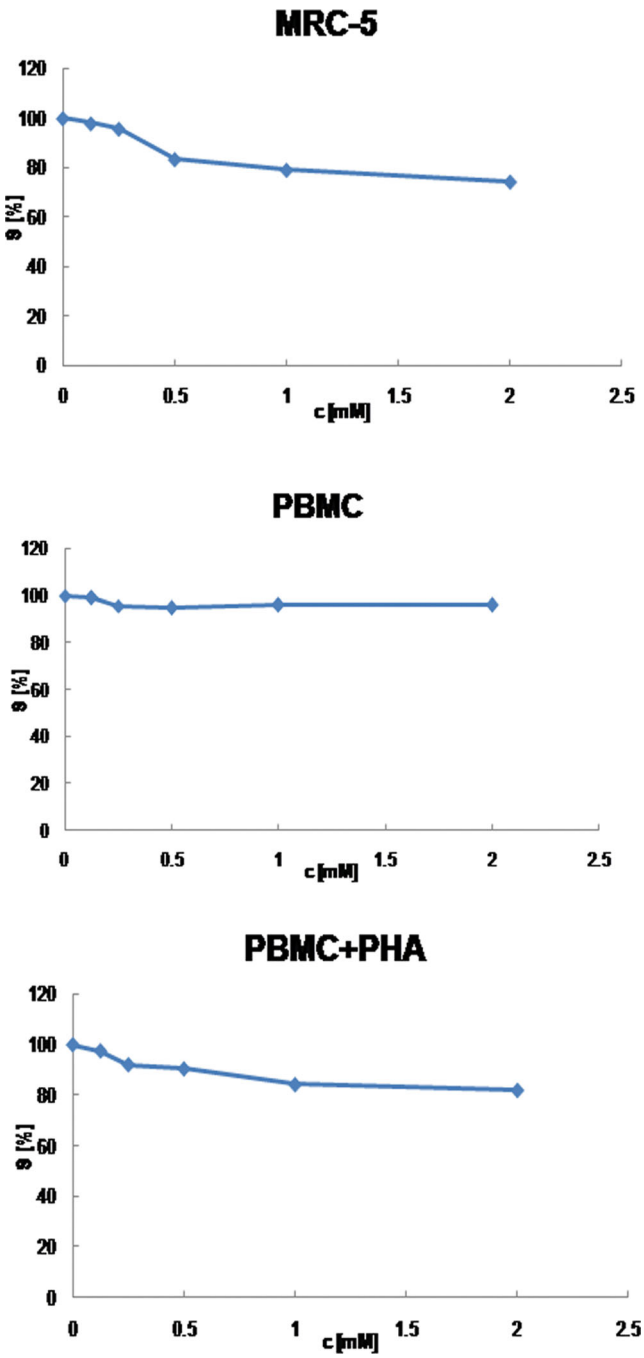
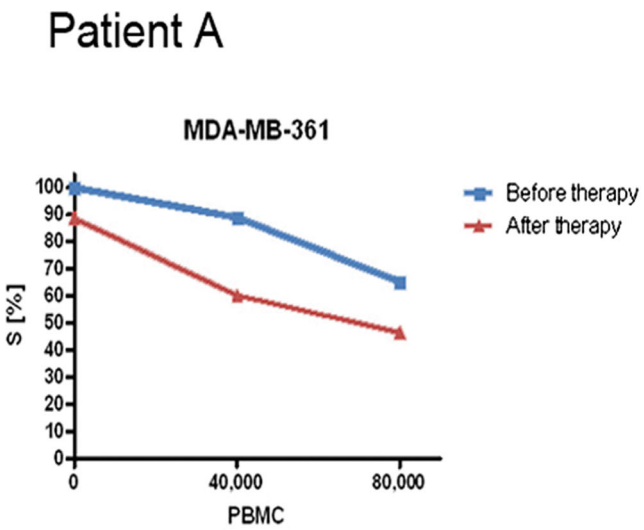


Fig. 2 Survival of normal human lung fibroblasts MRC-5, resting human PBMC, or PBMC stimulated for proliferation with PHA (5 μg/ml) in nutrient medium (RPMI 1640 enriched with 10 % autologous HP) done in triplicate, determined by MTT, 72 h after metformin action. Representative graphs are shown

Metformin Modulation of Herceptin Antitumor Action

Pretreatment (for only 24 h) of MDA-MB-361 cells with various concentrations of metformin, with subsequent addition of Herceptin to these cells, led to mild decrease in target



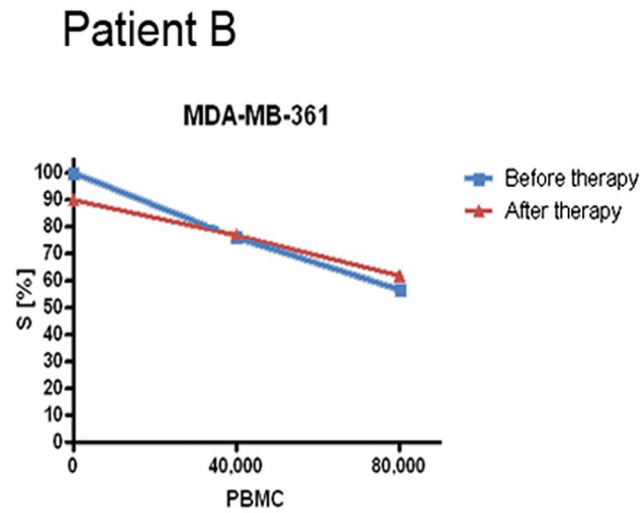


Fig. 3 Survival of target MDA MB-361 cells incubated with PBMC in nutrient medium with 10 % of autologous plasma of two different hyperglycemic patients who were 1 month on metformin therapy, determined by MTT. Representative experiment of the clear synergistic (**a**) and an antagonistic action (**b**) of metformin (especially in the presence of PBMC) could be seen. Duration of the test done in triplicate, was 24 h; 20,000 of target MDA-MB-361 per well were incubated with 40×10^3 and 80×10^3 PBMC in nutrient medium RPMI 1640 in the presence of 10 % of autologous plasma

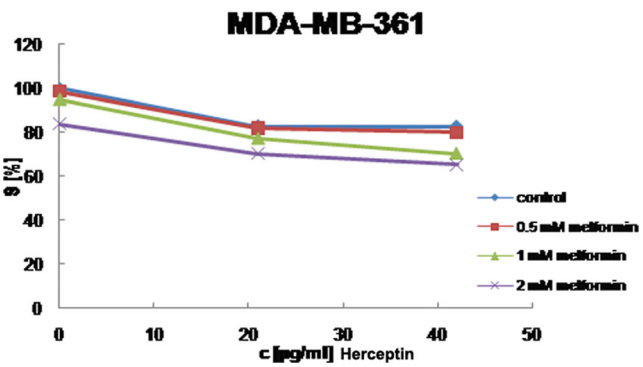


Fig. 4 Survival of malignant human breast cancer MDA-MB-361 cells (S %), pretreated (for only 24 h) with various concentrations of metformin, and then additionally with two different concentrations of Herceptin (c=21 μg/ml and 42 μg/ml)—done in triplicate, was determined by MTT test. Duration of additional Herceptin action was 48 h

cell survival, compared to cells treated only with Herceptin or only with metformin (Fig. 4).

Influence of Metformin on the Phenotype of MDA-MB-361 Cells

Metformin treatment of human breast adenocarcinoma MDA-MB-361 cells did not decrease the levels of HER2 expression on target cells, as it could be seen in Fig. 5.

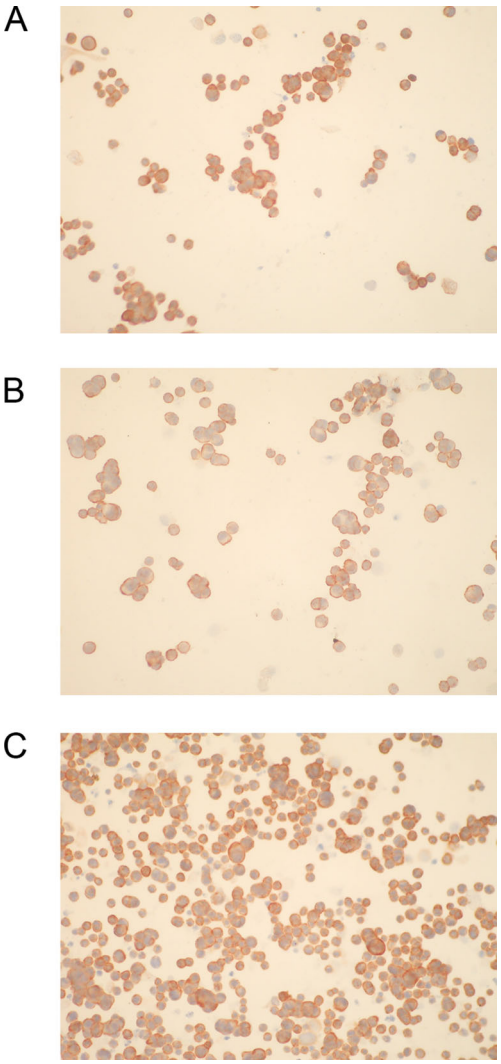


Fig. 5 HER2 expression on MDA-MB-361 cells—control (**a**), cells exposed to 0.5 mM metformin (**b**) and 1 mM metformin (**c**). Representative photomicrographs are shown

Influence of Metformin on the Phenotype of Breast Tumors

Score 3+ of HER2 receptor overexpression was found in 234 out of 1,410 all breast tumor patients surgically treated in the Institute of Oncology and Radiology of Serbia in the period November 2011–December 2012 (16.6 %). Score 3+ of HER2 was not found on tumors of 37 patients treated by metformin: even more tumors with HER2 expression 2+ (found in five patients) were without HER2 amplification. Therefore, significantly lower number of diabetic cancer patients treated with metformin with overexpressed HER2 tumors in relation to whole group of surgically treated patients was found ($p<0.013$).

Percentage of patients with ER+/PR+ tumors in the mentioned general breast cancer population was 85.5 % , while in 30 out of 37 metformin treated patients' tumors ER+/PR+ protein expression was found (81.1 %). Consequently, the number of patients with estrogen and progesterone receptor overexpressing tumors was not significantly changed in the metformin treated group in relation to general patients population.

Obtained data showed that metformin used alone, or in combination with other antidiabetic drugs could modify the phenotype of breast tumor cells.

Discussion

The cytotoxic action of high concentrations of metformin on various malignant cells in nutrient medium with FBS is already reported. The majority of the growth inhibitory effects of metformin on malignant cells are mediated through the inhibition of mTOR signaling pathway which could be preceded via activation of AMPK (LKB1 dependent signaling pathway). This compound can inhibit mTOR signaling through phosphorylation and stabilization of TSC2. It can suppress HER2 protein kinase activation resulting in reduced signaling through downstream pathways. Metformin can block cell cycle at least partially through reduced cyclin D1 expression. Furthermore, metformin can decrease levels of VEGF resulting in the inhibition of angiogenesis; it can induce apoptosis through p53-dependent or independent pathways.

Our results of the effects of applied metformin concentrations on the decrease in malignant cell survival obtained in the presence of FBS in nutrient medium are consistent with previous studies in which extremely high metformin concentrations were tested [9]. The antiproliferative effect of metformin was present even in the presence of human plasma. The small, but notable decrease in survival of stimulated to proliferation PBMC in comparison to the absence of this effect on resting PBMC is published for the first time in this paper and indicate that metformin in these concentrations could have only antiproliferative action on dividing cells, independent of the nature of target cells (malignant or normal proliferative cells). Importantly, these data suggest the possibility of favorable effect of metformin in suppression of autoimmune diseases.

Obtained results indicate that metformin in the plasma of some diabetic patients to whom metformin was given therapeutically has the potential to contribute to the immune-mediated PBMC induced decrease in malignant cell survival. The metformin effect was strictly individually dependent. This observation is especially important for personalized cancer treatment which is the basis for modern oncology. Data of this work also suggest that if natural anti-HER2 IgG antibodies are present in blood of some cancer patients as it was reported earlier [17, 20, 21], they could mediate their own cytotoxic ADCC action contributing to the cytotoxic action of metformin to malignant cells, even without added Herceptin. Our results point out that pretreatment of breast cancer cells with metformin might contribute to the Herceptin-induced or better said, to the natural anti-HER2 IgG mediated decrease in HER2+ breast cancer cell survival expressing its own antiproliferative action on these malignant cells.

Data about metformin influence on the phenotype of breast cancer cells are in agreement with reports in which in different geographic regions (China, Tianjin and Slovenia) significant decrease in number of metformin treated diabetic patients with HER2+ tumors was found [22, 23].

In our preliminary experiments HER2+ immunocytostaining of MDA-MB-361 breast adenocarcinoma cells treated with two different concentrations of metformin (0.5 mM and 1 mM) did not show any downregulation of HER2 expression on the surface of target cells. Apparently, it seems that results of our study are different from recently published data, but experiments in these publications were observed at higher metformin concentrations [24, 25]. Due to this fact decreased presence of HER2+ cancer cells observed in breast tumors might be preferentially caused by specific anti-HER2 immune action.

In conclusion, results of this work show that concentrations of metformin used in these experiments have the potential to decrease HER2+ breast cancer cells survival directly, or sometimes through immune-mediated PBMC antitumor action, and that metformin could contribute to Herceptin-mediated antitumor action.

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