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Effect of metformin on fibrosarcoma in hamsters

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Abstract. – OBJECTIVE: We investigated the effect of metformin on an *in vivo* solid tumor model of fibrosarcoma in hamsters.

MATERIALS AND METHODS: 33 Syrian golden hamsters of both sexes, weighing approximately 100 g, were randomly allocated to 3 experimental and 2 control groups. 2 x 10^6 BHK-21/C13 cells in 1 ml were injected subcutaneously into the animals' back in 4 groups. The first experimental group (7 animals) started peroral treatment with metformin 500 mg/kg daily via a gastric probe 7 days before tumor inoculation, the second (8 animals) 3 days before inoculation and the third (6 animals) immediately after inoculation. After 2 weeks, when the tumors were approximately 2-3 cm in the control group with tumors (6 hamsters), all animals were sacrificed. The blood was collected for glucose and other analyses. The tumors were excised and weighed and their diameters were measured. The tumor samples were histologically assessed and the main organs toxicologically analyzed, including 6 control animals that had received metformin without tumor inoculation. Tumor volume was determined using the formula Lx $S^2/2$, where L was the longest and S the shortest diameter. Ki-67-positive cells in the tumor samples were quantified; images were taken and processed by software UTHSCSA Image Tools for Windows Version 3.00. Statistical significances of differences in tumor weight, volume, number of Ki-67-positive cells and other parameters were determined by the Student´s *t*-test.

RESULTS: Metformin inhibited fibrosarcoma growth in hamsters without toxicity. The seven-day pretreatment was important for the statistically significant effect.

CONCLUSIONS: Administration of metformin as an anti-tumor drug might be an effective and safe therapeutic approach in novel non-toxic therapies for human sarcomas.

Key Words:
Metformin, Hamsters, BHK-21/C13, Fibrosarcoma.

Introduction

Generally, anticancer candidate metformin activates liver kinase LKB1-mediated AMPK (5′AMP-activated protein kinase), which reduces mTOR complex 1 signaling and S6K1 phosphorylation implicated in protein synthesis and cancer cells proliferation[1,2]. LKB1 is a major upstream kinase of AMPK. AMPK also directly inhibits the raptor (regulatory associated protein) of mTOR[1]. On osteosarcoma MG-63 cells *in vitro* metformin upregulated the phosphorylation of AMPKa at Tyrl72 site and downregulated the phosphorylation of mTOR and its downstream effectors of ribosomal protein S6 kinases[3]. It also inhibited the proliferation, metastasis and cancer stem-like sphere formation[3].

Metformin does not necessarily act via the AMPK to induce MG-63 osteosarcoma cell line growth inhibition and apoptosis[3]. In addition, metformin directly inhibits mTOR C1 signaling, increases REDD1, a negative regulator of mTOR, in a p53-dependent manner and inhibits the expression of cyclin D1 and retinoblastoma protein (key regulators of the cell cycle) in different cell lines independent on AMPK[4].

The participation of mTOR in the genesis of sarcoma is related to the primordial role of the IGF (insulin-like growth factor) system in these tumors. Insulin/IGF-1 is involved not only in the regulation of glucose uptake, but also in carcinogenesis through the upregulation of insulin/IGF-1

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receptor signaling pathway[5]. Furthermore, the inhibition of mTOR and mTOR C1-mediated signal pathway results in a direct antiangiogenic effect[6]. Thus, mTOR inhibitors were a natural choice to test *in vivo* and clinically in sarcomas[6].

Metformin also inhibits the matrix metalloproteinase-9 (MMP-9) activity in human fibrosarcoma HT-1080 cells *in vitro* independently of AMPK[7]. The inhibition of protein synthesis, mRNA and transcriptional activity of MMP-9 gene takes place through the Ca-dependent suppression of the phorbol-12-myristate-13-acetate (PMA) induced phosphorylation of extra cellular signal-regulated kinase activator protein 1 by metformin. MMP-9 is highly expressed in human sarcoma and other cancer cells. Metformin inhibited the PMA-induced invasion and migration of human fibrosarcoma cells *in vitro* via the Ca-dependent signaling pathways, and therefore, has the potential to be an antisarcoma drug[7].

Metformin affects the glycolytic capacity and decreases mitochondrial respiration, lowering the energetic efficacy in lymphocytic leukemia cells *in vitro*[8]. The inhibition of mitochondrial respiratory chain complex I results in the ATP depletion, accumulation of AMP and phosphorylation of AMPK in lymphocytic leukemia cells *in vitro*[8]. AMPK is a negative regulator of the Warburg effect[8]. The activation of AMPK and reduction of glucose metabolism (inhibition of oxidative phosphorylation) opposes tumor progression. Glucose-starvation or glycolysis inhibition was shown to potentiate metformin's cytotoxicity in lymphocytic leukemia cells *in vitro*[8].

The combination of metformin and 2-deoxyglucose, a competitive inhibitor of glycolysis (widely used in PET/CT scan), is effective against a broad spectrum of *in vitro* and *in vivo* human carcinoma (gastric, esophageal, breast) preclinical models[9]. The dual inhibition of the tumor energy pathway is suggested[9]. The combination of metformin and 2-deoxyglucose was more effective than either compound alone in eight-sarcoma cell lines[10]. Sarcoma cells in cultures were 2-5-fold more sensitive to the combination than normal cells[10]. The combination of metformin and 2-deoxyglucose blocked *in vitro* prostate cancer cell cycle in G_2-M and induced p53 dependent apoptosis[11].

The disturbance of the one-carbon pool by metformin's antifolate activity on breast cancer cells *in vitro* is a new recognized mechanism of AMPK induction[12,13]. Metformin *in vitro* on breast cancer cells can act similarly to an antifolate chemotherapeutic agent inhibiting DNA replication and cell proliferation[12]. The concept that metformin acts as a mitochondrial complex I poison, which leads to AMP/ATP imbalance[8-11], has been significantly amended after findings that metformin can function as an antifolate that induces the ataxia teleangiectasia mutated (ATM) kinase and secondary downstream AMPK, following the alteration of the carbon flow through the folate-related one-carbon metabolic pathways in breast cancer cells *in vitro*. Although metformin causes the alteration of carbon flow through the folate-dependent one-carbon metabolism *in vitro*, this does not imply direct inhibition of the folic acid cycle enzymes targeted by methotrexate and thereby, induces significantly fewer side effects[12].

Metformin treatment exhibits an effect similar to vitamin B12 deficiency in breast cancer cells[12] that likely causes defects in *de novo* purine/pyrimidine biosynthesis, homocysteine accumulation and blockade of glutathione biosynthesis. Furthermore, a few studies have addressed vitamin B12 metabolism alteration behind metformin´s antifolate anticancer activity[12,14].

The induction of lymphocytic leukemic cell death by metformin *in vitro*[8] is also associated with the inhibition of Mcl-1 a Bcl-2 family survival protein[8]. Lymphocytic leukemic cells *in vitro* are more addicted to the protective activity of Bcl-2 antiapoptotic proteins than normal cells. Metformin in lymphocytic leukemic cells *in vitro* inhibited the activation of transcription factors lying on leukemic pro-survival and pro-activation pathways, particularly PI3/AKT and NF-κB/STAT3[8].

Metformin produced the following changes in the apoptotic-related and cell cycle-related proteins of myeloma *in vitro*[15]: it activated caspase-3, caspase-9, PARP-1, Bak and p21, inactivated Mcl-1, Ki-67, HIAP-1, cyclin D1, CDK4 and CDK6 and inhibited insulin growth factor 1 receptor (IGF-IR), phosphatidyl inositol 3-kinase (PI3K), protein kinase B (PKB/AKT) and mTOR.

Several independent pathways RAS (pancreatic cancer), AKT (breast cancer cells) and HIF-1α (rat diabetes model) may contribute to the anticancer effect of the drug *in vitro*[16]. Certain gens (CDKN1A, ESR1, MAX, MYC, PPARGC1A, SP1, STK11, CHOP, CAV-1, HO-1, SGK-1, Par-4), through which metformin possibly elicits its anticancer effect *in vitro*, are proposed[16,17]. Some *in vitro* findings on breast cancer cells indicate that metformin´s primary effect is the inhibition of the mRNAs subset[17]. Metformin *in vitro* caused

transcriptional regulation of unfolded protein response on breast cancer cells[17].

Additionally, metformin activates the PERK-ATF4 pathway in breast cancer cells resulting in endoplasmic reticulum stress signaling and upregulates the CHOP mRNA level[17]. Metformin inhibits the PLCε gene expression and Notch1/Hes and androgen receptor signaling pathways in prostate cancer cells[18]. Moreover, metformin has shown influence on autophagy, cell migration (metastatic state), senescence, cancer stem cells and cell immunity in different cancer cell lines, which was explained by different mechanisms[19,20].

However, despite the significant *in vitro* antiproliferative effect on cell cultures, it has not yet been investigated and fully explained if and how exactly metformin inhibits cancer cell proliferation *in vivo* in experimental animals and what its therapeutic antitumor value is. Only a few contradictory studies exist about metformin's anticancer effect *in vivo* on solid tumors, including sarcomas, in experimental animals[9,15,21-23]. Host metabolism may be indirectly anticancer prepared with metformin by the reduction of gluconeogenesis and circulating insulin[1]. Metformin also improves glucose and lipid metabolism by decreasing the concentration of total serum bile acids[24]. Therefore, the aim of this study is to answer whether metformin can in any way suppress solid tumor growth in an experimental animal model, such as fibrosarcoma in hamsters.

Materials and Methods

Animal Model

The study was carried out on 33 12-20 week-old Syrian golden hamsters of both sexes (weighing approximately 100 g), after obtaining the approval of the Ethics Committee of the University of Novi Sad, Faculty of Medicine, Republic of Serbia, under approved institutional Experimental Animal Care and Use of Protocols.

The animals were randomized into 2 control groups (6 hamsters per group) and 3 experimental groups (with 6, 7 and 8 hamsters). The treatment in two experimental groups, with 7 and 8 animals, was initiated 7 or 3 days respectively before subcutaneous inoculation of BHK-21/C13 cells[25] (2 x 10^6 in 1 ml) into the animals' back, for the production of the s.c. tumor (BHK fibrosarcoma[26]) and continued for 14 days after inoculation (when the tumors were approximately 2-3 cm in the untreated control group). At the end of the experiment all animals were sacrificed, including those treated for 14 days in the control group without tumor inoculation.

For the treated groups, metformin (Galenika, Belgrade, Serbia) was dissolved in physiological saline and administered once daily via a gastric probe in a dose of 500 mg/kg (in 1 ml per 100 g weight), equivalent to a human dose of 40 mg/kg (by normalization to surface area), which is the maximum dose used in diabetic patients. The control group with tumors received isovolemic vehicle only (1 ml/100 g animal).

At sacrifice, the tumors were excided, tumor weight and size were measured and tumor volume was calculated as $L \times S^2/2$ (where L was the longest, and S the shortest diameter). Tumor slices were assessed immunohistochemically (Ki-67) for verification of tumor growth and blood samples were collected for glucose and other conventional blood tests (erythrocytes, leucocytes, lymphocytes, monocytes, granulocytes, platelets, hemoglobin, hematocrit, MCV, MCH, MCHC, serum proteins, albumins, sedimentation, partial thromboplastin time). Because the dosage of metformin used in our study (500 mg/kg/d) was higher than 850 mg/d usually used in diabetic patients, the weight of hamsters was measured to evaluate possible side effects caused by metformin. The main animal organs were toxicologically analyzed.

Ki-67 staining

Tumor slices were fixed in cold methanol, blocked with 4% bovine serum albumin/phosphate buffered saline (BSA/PBS) for 1 hour at room temperature and incubated with anti-Ki-67 pAb (1:50, Sigma-Aldrich, St. Louis, MO, USA). Polyclonal anti-rabbit FITC (1:80, Sigma-Aldrich, St. Louis, MO, USA) was used as the secondary antibody. Nuclei were counterstained with Hoechst 33258 fluorochrome stain (Sigma-Aldrich, St. Louis, MO, USA). Images were taken using Leica MC190HD camera (Leica Camera AG, Wetzlar, Germany) and processed by software UTHSCSA Image Tools for Windows Version 3.00[27]. In each sample image the number of Ki-67-positive cells was obtained. The mean numbers of Ki-67-positive cells (20 tumor images of each animal) were compared between the groups.

Statistical Analysis

The differences between the groups in tumor weight, volume, mean number of Ki-67-positive

cells marked on images and other measured parameters were determined using the Student´s *t*-test. The results were considered to be statistically significant at $p < 0.05$.

Results

Subcutaneous inoculation of BHK-21/C13 cells into hamsters resulted in fibrosarcoma formation at the site of injection in all inoculated animals. Peroral treatment with metformin significantly inhibited tumor growth. This was verified in the 7-day pretreated group by significantly decreased tumor weight and volume and also by a reduced proliferation status of tumor cells as shown by Ki-67 staining on hamster tumor sections (Table I). Note that in our experimental model only the 7- day pretreatment was related to statistically significant antitumor effects.

Pathological evaluation revealed a similar scale of necrotic areas in tumors with or without metformin pretreatment and treatment, even though the tumors in the case of metformin pretreatment and treatment were smaller.

Metformin pretreatment and treatment had no significant effect on the body weight of the animals during the course of the study (Table I).

Metformin caused a slight decrease in the fasting blood glucose levels in the hamsters, as shown in Table I. The experimental and control groups were also statistically compared in terms of red and white blood cells, platelet number, hemoglobin levels, hematocrit, serum proteins, sedimentation, activated partial thromboplastin time and other examined blood laboratory values, but no significant difference was observed between the groups.

Examination of the main organs revealed no pathological or toxicological changes in the control and experimental groups.

Discussion

The effects of metformin treatment of various types of cancer cell cultures are defined by various direct molecular, energetic and genetic mechanisms of influence on cell growth, proliferation, migration, senescence, autophagy and apoptosis. Despite remarkable *in vitro* anti-cancer and chemosensitizing effects, the mechanisms of metformin´s effect on tumor growth *in vivo* against solid tumors, such as sarcomas, have not yet been fully understood. In particular, its efficacy has not been sufficiently investigated and confirmed.

The combination of metformin 250 mg/kg/d administered intraperitoneally and 2-deoxyglucose 500 mg/kg/d administered intraperitoneally significantly inhibited subcutaneous tumor growth in mouse xenograft models after carcinoma (breast, esophagogastric) inoculation, which was not the case with either component alone[9].

In the human myeloma xenograft mouse model, like in our study, the animals that received intraperitoneal metformin treatment of 200 mg/kg/d had a lower tumor burden than the control group[15]. Furthermore, metformin treatment remarkably prolonged the survival of tumor-bearing mice[15]. Immunohistochemically, metformin activated caspase-3, caspase-9, p21 and repressed Mcl-1, Ki-67, cyclin D1, AKT, mTOR and 4E-BP1 in this xenograft model[15].

In accordance with our study, intraperitoneal metformin treatment 2-4 mg/kg/d significantly inhibited the B- and T-cell lymphoma growth after subcutaneous inoculation into nude mice[21]. In these murine xenograft models, the phosphorylation of AMPK was remarkably increased, with mTOR decreased in the metformin-treated animals.

The efficacy of 200-500 mg/kg/d of metformin administered p.o. or intraperitoneally was not confirmed against Ewing sarcoma xenografts in athymic nude mice[22]. It is assumed that hypoxia, a common feature of solid tumors, changed the activation of AMPK and the inhibition of mTOR signaling.

At a dose of 250 mg/kg/d administered p.o. intragastrically, metformin inhibited tumor growth in an oesophageal squamous cell carcinoma xenograft model[23]. AMPK, p53, $p21^{cip1}$, $p27^{kip1}$ and cyclin D1 were involved in the inhibition of tumor growth in this *in vivo* study. When metformin was administered 7 days before implantation and continued until the end of the study, tumor size during the experiment was significantly smaller when compared with the group treated only after implantation (without pretreatment)[23]. When metformin was given post-implantation, no significant difference in tumor size in the treatment group, compared with the controls, was observed (until 42 days)[23]. The use of metformin prior to implantation in this model[23] significantly delayed tumor development. Rapid cell proliferation and relatively weak angiogenesis in these tumors caused hypoxia and ischemia, leading to necrosis. Metformin could inhibit tumor angiogenesis in

Table I. Characteristics of animals and tumors in control and metformin treated groups.

Hamster No	Weight (g)		Sex	Pre-treatment (days)	Tumor		Ki-67 $\bar{x}$ for 20	Serum glucose mmol/l
	Beginning	End			Weight mg	Volume cm³		
Control group without metformin treatment, with inoculated tumor								
1	91	95	F	0	2060	1.50000	17	4.8
2	105	117	F	0	6880	5.00000	23	3.9
3	93	94	F	0	260	0.18750	14	3.3
4	94	110	F	0	1720	1.00000	20	5.1
5	105	101	M	0	2910	2.11981	18	4.5
6	97	95	M	0	1400	1.25025	19	4.2
$\bar{x}$	97.5	102			2538	1.84293	18.5	4.3
± SD	6.12	9.51			2297	1.67114	3.02	0.65
Control group without tumor inoculation, treated with 500 mg/kg metformin dose								
1	90	92	F	0	0	0	0	2.4
2	102	112	M	0	0	0	0	2.9
3	100	110	M	0	0	0	0	3.8
4	88	90	F	0	0	0	0	8.7
5	91	90	M	0	0	0	0	3.9
6	102	105	F	0	0	0	0	4.1
$\bar{x}$	95.50	99.83						4.3
± SD	6.50	10.32						2.25
Treated (dose 500 mg/kg)								
1	93	103	F	7	500	0.36000	7	3.3
2	96	112	F	7	300	0.21600	11	3.1
3	75	88	F	7	30	0.02250	3	3.5
4	80	80	M	7	1030	0.75000	9	3.9
5	81	73	M	7	140	0.12500	12	3.7
6	136	121	M	7	15	0.00892	8	4.1
7	98	105	F	7	170	0.10035	9	3.4
$\bar{x}$	94.14	97.43			312	0.22611	8.43	3.57
± SD	20.46	17.54			358	0.26049	2.94	0.35
p (t-test)					< 0.02	< 0.02	< 0.01	< 0.05
1	91	89	F	3	10	0.0240	6	6.9
2	90	93	M	3	450	0.01250	14	3.9
3	107	120	F	3	1880	0.7955	16	2.9
4	87	92	M	3	1950	1.680	25	3.7
5	65	72	F	3	1390	0.870	16	4.1
6	88	95	M	3	1410	0.690	15	3.3
7	77	79	F	3	1500	0.710	16	4.6
8	72	75	F	3	190	0.042	7	3.4
$\bar{x}$	84.63	89.37			1097.5	0.6171	14.38	4.1
± SD	13.04	15.15			766.23	0.5561	5.93	1.24
p (t-test)					> 0.05	> 0.05	> 0.05	> 0.05
1	90	105	M	0	2900	2.10109	17	3.2
2	92	110	F	0	1400	1.02050	13	3.5
3	78	90	M	0	1200	0.87055	7	3.7
4	86	81	M	0	2900	2.11060	21	3.1
5	82	75	F	0	410	0.29889	10	5.2
6	126	122	M	0	1700	1.22066	18	10.8
$\bar{x}$	92.33	97.17			1752	1.27038	14.33	4.9
± SD	17.27	18.15			987	0.71619	5.28	2.98
p (t-test)					> 0.05	> 0.05	> 0.05	> 0.05

the aforementioned model[23]. The inhibition of the angiogenesis process in this model may be via the AMPK dependent and independent ways.

In our experimental sarcoma model on hamsters, the 7-day metformin pretreatment before BHK-21/C13 cells inoculation was also important for a significant effect of the drug on tumor weight, volume and number of Ki-67 stained cells in histological preparations. On the basis of previously published results (on 200-500

mg/kg/d metformin treatment of Ewing sarcoma[22] and 250 mg/kg/d oesophageal squamous cell carcinoma[23] xenografts), we can speculate that a sufficiently long metformin pretreatment period in our sarcoma model has minimized the influence of tumor protecting hypoxia and pronounced the influence of the systemic metformin host effects (reduction of gluconeogenesis and circulated insulin). We suppose that in our experimental model a treatment period longer than 14 days and/or a larger number of animals is needed to achieve significant antitumor effects of metformin in other experimental groups.

The various levels of metformin found in the tumors (colorectal cancer cells) of xenograft-bearing mice (9-56 μM) treated perorally (5 mg/ml via drinking water, for 16 days) corresponded to these in the plasma. They indicated consistent delivery of the drug to tumor tissue and were sufficient for direct antitumor action[28]. The 15-day treatment with 125 mg/kg/d i.p. of metformin, 30 min after the last injection, produced mice plasma concentration of 66-215 μM and xenograft concentration of 42-100 μM[28]. We used the same order of magnitude in oral hamster doses. It should be mentioned that in the investigations of molecular mechanisms of metformin actions the majority of *in vitro* treatments use much higher concentrations (1000 ×), between 1 and 50 μM[28]. Such high concentrations cannot be safely achieved in humans. The maximum metformin dose in diabetic patients, up to 3 g/d, is equivalent to 500 mg/kg/d of our hamster doses, normalized to body surface. The anticancer activities of our doses in hamsters and the possibility of achieving comparably high nontoxic metformin levels in humans by increased dosing suggest the prospect of realizing effective nontoxic metformin antitumor therapy in humans. Further clinical trials will elucidate weather metformin has the potential to become an adjuvant to current antitumor, and especially antisarcoma, therapies.

Conclusions

Since non-toxic metformin p.o. doses given for a sufficiently long period prior and post BHK21/C13 inoculation significantly inhibited sarcoma growth in hamsters, metformin may be a safe novel candidate for adjuvant human sarcoma therapy.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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