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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Effects of metformin and its combinations with other repurposed drugs on fibrosarcoma in hamsters

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SUMMARY

Introduction/Objective Many drugs registered for various other indications can act selectively on tumor receptors, signaling pathways, metabolic processes, bioenergetic factors, enzymes, proteins and genes that regulate tumor proliferation, apoptosis, and neoangiogenesis without affecting these activities in healthy cells. Introduction of new drugs is a very long, complex, and expensive process of research. Detecting an anticancer effect in drugs already registered for other indications and forming their combinations may directly reduce the time and cost of such research.

Methods Anticancer efficacy of metformin and its combinations with caffeine, itraconazole and nitroglycerin was tested on fibrosarcoma experimentally induced by BHK21/C13 cells in Syrian golden hamsters (six animals per group, randomly allocated to control and experimental groups, doses equivalent to usual human doses). After animal sacrifice, tumors were excised and their size, biophysical characteristics, histology, and immunohistochemistry were assessed. Blood samples were collected for hematological and biochemical analyses and the main organs were toxicologically analyzed. Statistical significance was determined by one-way ANOVA followed by the Student–Newman–Keuls post hoc test.

Results Two-drug combinations of metformin with caffeine or itraconazole or nitroglycerin showed significant antitumor effects on hamster fibrosarcoma compared to control, regarding all tested tumor parameters ($p < 0.05$) without toxicity.

Conclusion Administration of metformin in combination with caffeine or itraconazole or nitroglycerin might be an effective and safe approach in novel nontoxic adjuvant anticancer treatment.

Keywords: metformin; caffeine; itraconazole; nitroglycerin; hamsters; fibrosarcoma

INTRODUCTION

Metformin activates 5´AMP-activated protein kinase (AMPK), which reduces mammalian target of rapamycin (mTOR) complex 1 signaling, inhibits nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), protein synthesis, and cancer cell proliferation [1]. Metformin inhibits glycolytic capacity and mitochondrial respiration in lymphocytic leukemia cells *in vitro* [2]. AMPK activation and glucose metabolism reduction negatively regulate Warburg effect (aerobic glycolysis – tumor cells preferentially use glucose rather than oxidation for energy production) and inhibit tumor progression [2]. Also, suppression of the Warburg effect in cancer cells by metformin decreases aerobic glycolysis and promotes oxidative phosphorylation, making cancer cells vulnerable to chemotherapy. Metformin interacts with respiratory electron transport chain in mitochondria to cause reactive oxygen species (ROS) production and oxidative stress [1]. Metformin therapy is also connected with both cyanocobalamin and folic acid deficiencies in patients with diabetes [3].

Caffeine induces apoptosis in many human tumor cells (lung, pancreatic, leukemia) *in vitro* [4]. Caffeine enhances tumor cells susceptibility to antineoplastic drugs and radiotherapy [5]. An important finding was that caffeine increased antifolate activity of pemetrexed in the various mesothelioma cell lines [6].

Itraconazole exhibits significant anticancer effects in different cancer tissues *in vitro* via suppression of the following: AMPK/mTOR pathway, neoangiogenesis, folic acid activity and autophagy [7, 8], Hedgehog signaling [9], P-glycoprotein (P-gp), and cholesterol transportation [7]. Itraconazole also induces chemosensitization [7]. Itraconazole, as ergosterol biosynthesis inhibitor, showed synergy with antifolates [8]. In addition to antifolate activity, the published studies have shown that itraconazole, as metformin, activates AMPK and thus downstream inhibits mTOR, protein synthesis, cell growth, proliferation, and stimulates apoptosis [9].

Nitroglycerin acts through the liberation of nitric oxide (NO) in the tissues. NO may modify cancer tissue metabolism by modulating the Warburg effect in oncological treatment [10]. NO donors are especially useful as chemotherapeutic and radio-therapeutic sensitizing preparations and increase cancer hemodynamics, amplifying the effects of cancer treatment [10]. NO can produce nitrosative stress, showing effects likewise to oxidative stress (elevation

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of ROS) [10]. As NO from nitroglycerin results in cyanocobalamin and folic acid deficiency [11, 12] similar to metformin [3], it can be expected that the mixture of nitroglycerin and metformin has synergistic antitumor effects via cyanocobalamin and folic acid deficiency. Nitroglycerin inhibits NF-κB and downstream P-gp [13].

In order to contribute to anticancer treatments, we conducted this study aiming to define the new efficacious, non-toxic and low-cost pleiotropic drug combinations, that can be immediately used in oncology.

METHODS

Three two-drug combinations – metformin and caffeine, metformin and itraconazole, metformin and nitroglycerin – were investigated in three separate independent experiments with three different control groups and simultaneously with appropriate investigated single and combined drug treatments.

Hamster model

Experiments were performed on *Mesocricetus auratus* (six male Syrian golden hamsters per group; 12–15 weeks old; body mass ~90 g). The hamsters were kept under default housing conditions: diurnal light cycle 12 hours of light / 12 hours of dark, at temperatures 25°C ± 2°C and humidity 60% ± 2%. The hamsters had access to food and water *ad libitum*.

The experiments for this study were performed in accordance with national regulations for the handling of laboratory animals: Law on Animal Welfare of the Republic of Serbia dated June 10, 2009 and the University of Novi Sad Rules for Work with Experimental Animals, dated June 11, 2020. All the animals were met with protocols approved by the University of Novi Sad Animal Ethics Committee (Novi Sad, Serbia): No. 04-81/25-5 dated July 22, 2020, Doc. No. EK: П-Е-2020-07; No. 04-150/15 dated March 14, 2022, Doc. No. EK: I-2022-01; No. 04-150/15 dated March 14, 2022, Doc. No. EK: I-2022-02; and approved by the Ministry of Agriculture, Forestry and Water Management – Veterinary Directorate (Belgrade, Serbia): No. 323-07-09359/2020-05 dated September 2, 2020; No. 323-07-03995/2022-05 dated March 28, 2022; No. 323-07-03996/2022-05 dated March 28, 2022; No. 323-07-03997/2022-05 dated March 28, 2022.

Treatment with metformin, caffeine, itraconazole, and nitroglycerin (all Galenika a.d., Belgrade, Serbia) and their co-administration to animals started after the hypodermic injection of 1 ml of BHK-21/C13 cell suspension (2×10^6 cells/ml) into the backside for the hypodermal fibrosarcoma growth. The following criteria for the humane termination of an animal's life were defined: serious body mass loss (20%), diminished activity/responsiveness with loss of body mass, poor posture, incapability to eat, urinate, or defecate, largest cancer dimension > 3.5 cm, cancer burden > 10% body mass, or cancer ulceration. The following characteristics were observed: general condition; general clinical characteristics (breathing disorders, diarrhea, neurological signs); behavior; body weight (measured daily); tumor diameter, location and ulceration; appearance of multiple tumors.

Each of three experiments included four groups of hamsters which received different daily therapies via a gastric probe after fibrosarcoma cell inoculation.

The first experiment: peroral application of 1) water (control group with inoculated tumor); 2) 500 mg/kg metformin; 3) 100 mg/kg caffeine; or 4) combination of 500 mg/kg metformin and 100 mg/kg caffeine.

The second experiment: peroral application of 1) water (control group with inoculated tumor); 2) 250 mg/kg metformin; 3) 250 mg/kg itraconazole; or 4) combination of 250 mg/kg metformin and 250 mg/kg itraconazole.

The third experiment: peroral application of 1) water (control group with inoculated tumor); 2) 1000 mg/kg metformin; 3) 50 mg/kg nitroglycerin; or 4) combination of 500 mg/kg metformin and 25 mg/kg nitroglycerin.

The hamsters were sacrificed 19 days after fibrosarcoma cell inoculation. Before animal sacrification, intraperitoneal dose of 90 mg/kg pentobarbital was applied. The hamsters were evaluated for sleep into coma at 5 minutes by combined methods, such as a toe pinch, lack of respiration and lack of reaction on palpation. Immediately after confirmation of loss of consciousness, total cardiac exsanguination was performed. Depending on animal weight, the volume of blood extracted was 3–5.5 ml. Two to three milliliters of the blood obtained was subjected to biochemical and hematological analyses. After exsanguination and life deprivation, main organs (brain, heart, lungs, kidneys, liver, stomach, intestine) were excised for pathological, histological, and toxicological examination. At the time of sacrifice, the weights of the animals were documented. All hamsters were in a good state during experiments, and none of the animals were euthanized before the end of the examination. During the experiment, the fibrosarcoma diameters and the tumor burdens were measured daily using calipers. The next formula for ellipsoid volume was used: volume = $4\pi abc/3$, where a, b, and c are ellipsoid half-diameters. After animal life deprivation, the cancers were excised, weighed and tumor diameters were exactly determined. The exact cancer volume was obtained by determination of the water level in a graduated cylinder before and after the submergence of the tumor (commonly used water volume displacement method).

In all the experiments, the drugs were dissolved in water and daily administered to animals in 1 ml/100 g body mass doses. The doses were < 50% of oral median lethal LD_{50} for hamsters and equivalent to human doses (by normalization to surface area).

The relative tumor weight (tumor burden) was calculated as tumor mass and animal body weight ratio. The tumor density was determined as density=mass/volume. The tumor surface area (S) was determined using the formula from three ellipsoid half diameters (a, b, and c): $S = 4\pi\{[(ab)^{1.6} + (ac)^{1.6} + (bc)^{1.6}]/3\}^{1/1.6}$.

Tumor slices (4 μm) were analyzed pathohistologically and immunohistochemically for the determination

of tumor development, tissue infiltration, necrosis and hemorrhagic zones expansion, proliferation, angiogenesis, apoptosis, glucose, and NO-metabolism.

Immunohistochemical examinations

In addition to the primary hematoxylin and eosin staining, immunohistochemical Ki-67, PCNA, CD34, CD31, COX4, cytochrome C, GLUT1 and iNOS staining (Thermo Fisher Scientific, Inc., Waltham, MA, USA; Abcam, Cambridge, UK) was performed according to already published methodology [14], to analyze cancer cell mitosis (Ki-67, PCNA), angiogenesis (CD34, CD31), apoptotic activity (COX4, cytochrome C), glucose turnover intensity (GLUT1), and nitric oxide expression (iNOS). The stained fibrosarcoma slices were analyzed under microscope (Leica DMLB 100T, Leica Microsystems GmbH, Wetzlar, Germany) with 400× magnification. Images were taken by a Leica MC190 HD camera (Leica Microsystems GmbH). The Ki-67 and PCNA staining images were analyzed using the UTHSCSA Image Tool for Windows Version 3.00. Individual Ki-67 or PCNA-positive cells were counted in each sample image. The mean numbers of Ki-67 and PCNA-positive cells in 20 cancer images from each hamster were compared among the experimental groups. Immunoexpression level was assessed by measuring part of stained surface area (stained/whole surface ratio) in the fibrosarcoma slices (mean of 20 measurements) by software UTHSCSA Image Tools for Windows Version 3.00.

Blood biochemical tests and hematological analyses

Blood was collected for standard laboratory analyses: glucose, serum proteins, albumins hemoglobin, sedimentation, leucocytes, granulocytes, lymphocytes, monocytes, platelets, erythrocytes, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration, hematocrit, in all three experiments.

Statistical evaluation

Means and standard deviations were determined for the experimental data. The differences among the groups in all measured parameters were calculated using one-way ANOVA followed by a Student–Newman–Keuls *post hoc* test. A probability p-value less than 0.05 was considered to be statistically significant. Data analysis was performed using TIBCO Statistica 13.3.1 software (TIBCO Software Inc., Palo Alto, CA, USA) in all experiments.

RESULTS

The subcutaneous application of BHK-21/C13 cell culture caused fibrosarcoma production in all animals. Experimental animals had separated, well-delimited solid tumors without side effects on the overall state and welfare. The largest tumor diameters after animal life deprivation, were < 3.5 cm in all experiments. The maximal tumor burdens after animal life deprivation were much below 10% of the hamster body weight in all experiments. Pathological, histopathological and toxicological analysis following autopsy revealed no marks of toxic influence on main organs (brain, heart, lungs, kidneys, liver, stomach, and intestine), nor ascites or metastases.

The experimental and control groups were parametrically and nonparametrically tested for sedimentation, red and white blood cell counts, platelet number, glucose levels, hematocrit levels, hemoglobin levels, serum proteins, but no statistically important inequalities were detected among the groups in all three experiments ($p > 0.05$).

Treatment with metformin and caffeine

Treatment with combination of metformin and caffeine significantly suppressed cancer development as demonstrated by statistically important reduction of fibrosarcoma weight, volume, and Ki-67 (mean for 20), compared with control (Table 1).

Only the comedication of metformin with caffeine produced statistically important ($p < 0.05$) anticancer effects in comparison to the control. Neither metformin, nor caffeine given alone showed significant antitumor effects compared to the control. The treatments had no statistically important effects on the body weight of the animals during the experiment, in comparison to the control (Table 1).

The results proved the statistically important antitumor influence of the metformin and caffeine combination on experimental fibrosarcoma, without toxic effect.

Treatment with metformin and itraconazole

Treatment with the combination of metformin and itraconazole significantly suppressed cancer development as demonstrated by statistically important reduction of fibrosarcoma weight, length, volume, surface area, relative weight, density, ratio of tumor surface area to volume, compared with the control and single treatments (Table 2, Table 3, Figure 1, Figure 2).

The pathohistological and immunohistochemical analysis showed a decrease in tissue insertion, an extension of necrosis and hemorrhagic areas, statistically important reduction in cancer cell proliferation, as shown by Ki-67, statistically important reduction of the following: glucose metabolism, as demonstrated by GLUT1; NO metabolism, as demonstrated by iNOS staining; tumor vasculature, as demonstrated by CD34; and apoptosis intensity, as demonstrated by COX IV in all examined cancer slices from hamsters treated with the combination of metformin and itraconazole, in comparison with the control group and the single-treatment groups (Table 3, Figure 1, Figure 2).

Only the combined treatment with metformin and itraconazole produced statistically important ($p < 0.05$) anticancer effects in comparison to the control. Neither metformin, nor itraconazole given alone showed significant antitumor effects compared to control (Table 2, Table 3, Figure 1, Figure 2). The treatments had no statistically

Table 1. Characteristics of animals and tumors in control and groups treated with metformin and caffeine, with significance (p-values)

	Hamster			Tumor				
	Weight at start (g)	Weight at end (g)	Serum glucose (mM/l)	Weight (g)	D_{max} (cm)[a]	Volume (cm^3)	Density (mg/mm^3)	Mean[c] No Ki-67-positive cells
Control group with inoculated tumor, without treatment								
Mean	88.7	99	4.4	2.54	1.71	1.84	1.38	19.1
± SD	6.05	8.75	0.75	2.3	0.29	1.67	0.194	2.99
Group treated with metformin (500 mg/kg) daily								
Mean	86.36	88.72	4.2	1.1	1.58	0.82	1.34	14.45
± SD	12.9	13.99	0.99	0.81	0.42	0.56	0.123	6.03
p			> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
Group treated with caffeine (100 mg/kg) daily								
Mean	95.11	100.1	5	2.32	1.85	1.96	1.18	16.28
± SD	10.95	12.55	3.02	1.31	0.5	1.11	0.177	4.86
p			> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
Group treated with metformin (500 mg/kg) and caffeine (100 mg/kg) daily								
Mean	91.47	98	3.95	0.42	1.12	0.36	1.17	13.27
± SD	12.05	13.75	2.31	0.32	0.22	0.11	0.087	3.39
p			> 0.05	< 0.05[b]	< 0.05[b]	< 0.05[b]	< 0.05[b]	< 0.05[b]

[a]Largest tumor diameter (cm);
[b]p < 0.05 significant difference between treated groups compared to control;
[c]mean of 20 analyses of each tumor

Table 2. Characteristics of animals and tumors in control and groups treated with metformin and itraconazole

	Hamster			Tumor				
	Weight at start (g)	Weight at end (g)	Serum glucose (mM/l)	Weight (g)	D_{max} (cm)[a]	Volume (cm^3)	Density (mg/mm^3)	Mean[b] No Ki-67-positive cells
Control group with inoculated tumor, without treatment								
Mean	115	133.6	5.77	7.99	3.1	7.41	1.09	22
± SD	15	23.2	2.39	5.76	0.7	5.45	0.031	7
Group treated with metformin (250 mg/kg) daily								
Mean	88.7	95.4	4.95	4.59	2.5	4.33	1.075	18.2
± SD	14.3	9.8	3.11	2.14	0.45	1.95	0.04	4.8
Group treated with itraconazole (250 mg/kg) daily								
Mean	102.7	107.2	4.82	6.57	3.12	6.32	1.074	19
± SD	19.9	9.3	3.74	0.98	0.11	0.98	0.042	4.1
Group treated with metformin (250 mg/kg) and itraconazole (250 mg/kg) daily								
Mean	97.2	102.5	5.57	1.97	1.93	1.89	1.032	11.2
± SD	7.3	8.1	2.81	0.71	0.44	0.64	0.012	5.9

[a]Largest tumor diameter (cm);
[b]mean of 20 analyses of each tumor

Table 3. Statistical evaluation of tumor characteristics following treatment with metformin and itraconazole

Group comparison	Tumor (p-values)							
	Weight	Relative weight	Volume	Length	Surface area	Density	Surface/ volume	Mean Ki-67
C/M	0.200	0.840	0.122	0.137	0.349	0.490	0.045[a]	0.615
C/I	0.609	0.891	0.553	0.675	0.648	0.470	0.047[a]	0.769
C/M+I	0.034[a]	0.047[a]	0.037[a]	0.043[a]	0.045[a]	0.002[a]	0.048[a]	0.040[a]
M/I	0.052	0.217	0.047[a]	0.011[a]	0.021[a]	0.967	0.721	0.763
M/M+I	0.019[a]	0.014[a]	0.017[a]	0.061	0.009[a]	0.030[a]	0.457	0.048[a]
I/M+I	0.003[a]	0.003[a]	0.004[a]	0.006[a]	0.001[a]	0.040[a]	0.561	0.024[a]

C – control group; M – group treated with metformin (250 mg/kg); I – group treated with itraconazole (250 mg/kg);
M+I – group treated with the combination of metformin (250 mg/kg) and itraconazole (250 mg/kg);
[a]p < 0.05

important effects on the body weight of the animals during the experiment, in comparison to the control (Table 2).

The results proved the statistically important antitumor influence of the metformin and itraconazole combination on experimental fibrosarcoma, without toxic effect.

Treatment with metformin and nitroglycerin

Treatment with the combination of metformin and nitroglycerin significantly suppressed cancer development as demonstrated by statistically important reduction of fibrosarcoma weight, length, volume, density, compared with the control (Table 4).

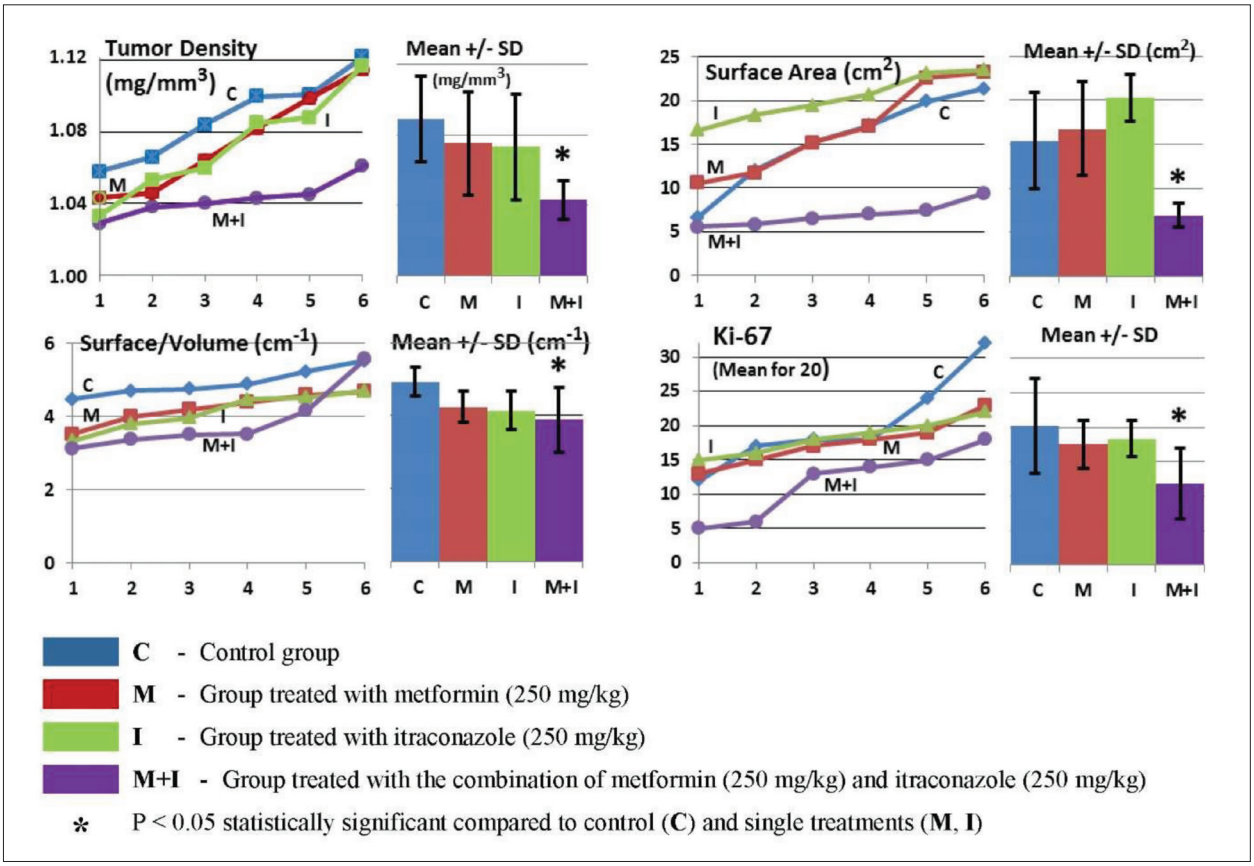

Figure 1. Biophysical and immunohistochemical characteristics of the excised tumors: tumor density, surface area, surface/volume ratio and Ki-67 positivity among the groups of animals treated with metformin and itraconazole;

*p < 0.05, as indicated

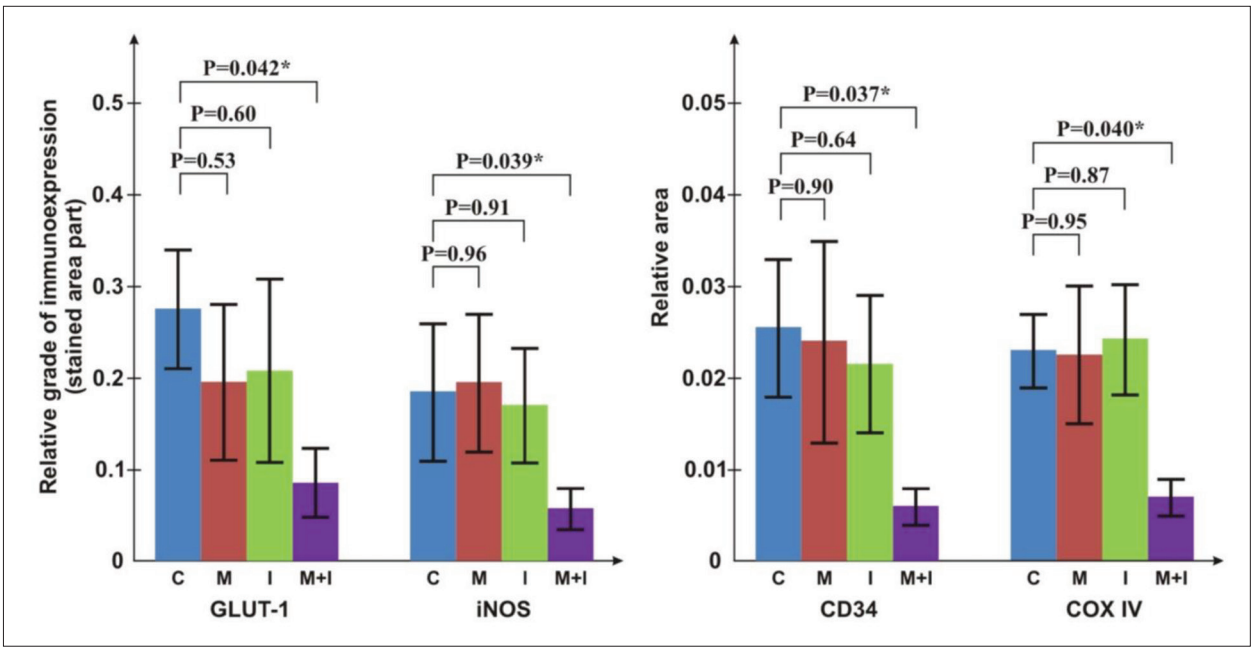

Figure 2. Immunohistochemical characteristics of the excised tumors (Mean ± SD);

GLUT-1, iNOS, CD 34, COX IV, in the second experiment;
C – control group; M – group treated with metformin (250 mg/kg); I – group treated with itraconazole (250 mg/kg); M+I – group treated with the combination of metformin (250 mg/kg) and itraconazole (250 mg/kg);
*p < 0.05, as indicated

Table 4. Comparison of fibrosarcoma growth between hamsters treated with metformin and nitroglycerin, with significance (p-values)

	Hamster			Tumor				
	Weight at start (g)	Weight at end (g)	Serum glucose (mM/l)	Weight (g)	D_{max} (cm)[a]	Volume (cm^3)	Density (mg/mm^3)	Mean[c] No Ki-67 positive cells
Control group (C)								
Mean	65.89	87.2	6.77	3.7	3.13	3.11	1.21	19.5
± SD	8.32	6.44	2.41	0.89	0.39	0.95	0.09	5.8
Group treated with metformin (1000 mg/kg) daily (M)								
Mean	62.73	82.83	5.55	3.63	3.01	3.07	1.24	19
± SD	7.34	7.02	1.31	0.88	0.51	0.87	0.095	7.06
p (MN/M)				0.011[b]	0.010[b]	0.020[b]	0.020[b]	0.010[b]
Group treated with nitroglycerin (50 mg/kg) daily (N)								
Mean	67.35	85.74	6.21	3.38	2.93	2.83	1.22	18.5
± SD	4.21	4.03	1.91	0.87	0.58	0.81	0.03	7.72
P(MN/N)				0.029[b]	0.037[b]	0.048[b]	0.010[b]	0.017[b]
Group co-treated with metformin (500 mg/kg) and nitroglycerin (25 mg/kg) daily (MN)								
Mean	70.04	87.72	6.27	2.33	2.32	2.11	1.12	10.2
± SD	6.03	6.32	1.92	0.69	0.43	0.50	0.09	3.79
P(MN/C)				0.010[b]	0.002[b]	0.030[b]	0.037[b]	0.003[b]

C – control group; M – metformin; N – nitroglycerin; MN – combination of metformin and nitroglycerin;
[a]largest tumor diameter (cm).
[b]$p < 0.05$ significant difference between treatments;
[c]mean of 20 analyses of each tumor

Table 5. Statistical evaluation of immunohistochemical tumor characteristics (p-values)

Group comparison	Tumor (p-values)							
	Ki-67	PCNA	CD 34	CD 31	GLUT-1	iNOS	COX 4	Cytochr. C
C/M	0.8895	0.9705	0.5870	0.4110	0.4610	0.4190	0.1126	0.9870
C/N	0.6790	0.6404	0.4130	0.2910	0.2590	0.3090	0.2370	0.4019
C/M + N	0.0032[a]	0.0082[a]	0.0153[a]	0.0157[a]	0.0091[a]	0.0081[a]	0.0085[a]	0.0137[a]
M/N	0.9670	0.7105	0.9120	0.6062	0.6010	0.7890	0.2470	0.0461[a]
M/M + N	0.0097[a]	0.0089[a]	0.0487[a]	0.0909	0.0077[a]	0.0087[a]	0.0094[a]	0.0179[a]
N/M + N	0.0167[a]	0.0091[a]	0.0801	0.2029	0.0074[a]	0.0085[a]	0.0413[a]	0.0109[a]

C – control group; M – group treated with metformin; N – group treated with nitroglycerin;
M + N – group treated with combination of metformin and nitroglycerin;
[a]$p < 0.05$

The pathohistological and immunohistochemical analysis showed a decrease in tissue insertion, an extension of necrosis and hemorrhagic areas, statistically important reduction in cancer cell proliferation, as shown by Ki-67 and PCNA, statistically important reduction of: glucose metabolism, as demonstrated by GLUT1; NO metabolism, as demonstrated by iNOS staining; tumor vasculature, as demonstrated by CD34 and CD31; and statistically important reduction in apoptosis intensity, as demonstrated by COX IV and cytochrome C, in all examined cancer slices from hamsters treated with the combination of metformin and nitroglycerin, in comparison with the control group and the single-treatment groups (Table 4, Figure 3, Table 5).

Only the combined treatment with metformin and nitroglycerin produced statistically important ($p < 0.05$) anticancer effects in comparison with the control. Neither metformin, nor nitroglycerin given alone showed significant antitumor effects compared to the control (Table 4, Table 5, Figure 3). The treatments had no statistically important effects on the body weight of the animals during the experiment, in comparison to the control (Table 4).

The results proved the statistically important antitumor influence of the metformin and nitroglycerin combination on experimental fibrosarcoma, without toxic effect.

DISCUSSION

Our three experiments suggested that dual therapy by using two repositioned drugs with anticancer and NF-κB activity (such as metformin combinations with caffeine, itraconazole, and nitroglycerin) shows effectiveness against fibrosarcoma in hamsters, contrary to ineffective monotherapy with each of them.

In the Introduction, many pathways are listed and examples of literature for several relevant *in vitro* experiments are given, yet none of this is shown for BHK21/C13 cancer cell line used in our *in vivo* experiments. For proposing a feasible synergistic influence free from the toxic effects, the reasonable approach would be a double-hit one targeting distinct pathways or different targets within the same pathway. In agreement with our effective combined treatment experiments with two NF-κB inhibitors, it can be supposed that combined treatment affects different targets within NF-κB pathway and that NF-κB is one of signaling pathways underlying anticancer mechanism of our three effective two-drug combinations with metformin.

Three weeks peroral administration of metformin, efavirenz and fluoxetine combination resulted in drastic decrease of cancer weight and volume in human colon cancer

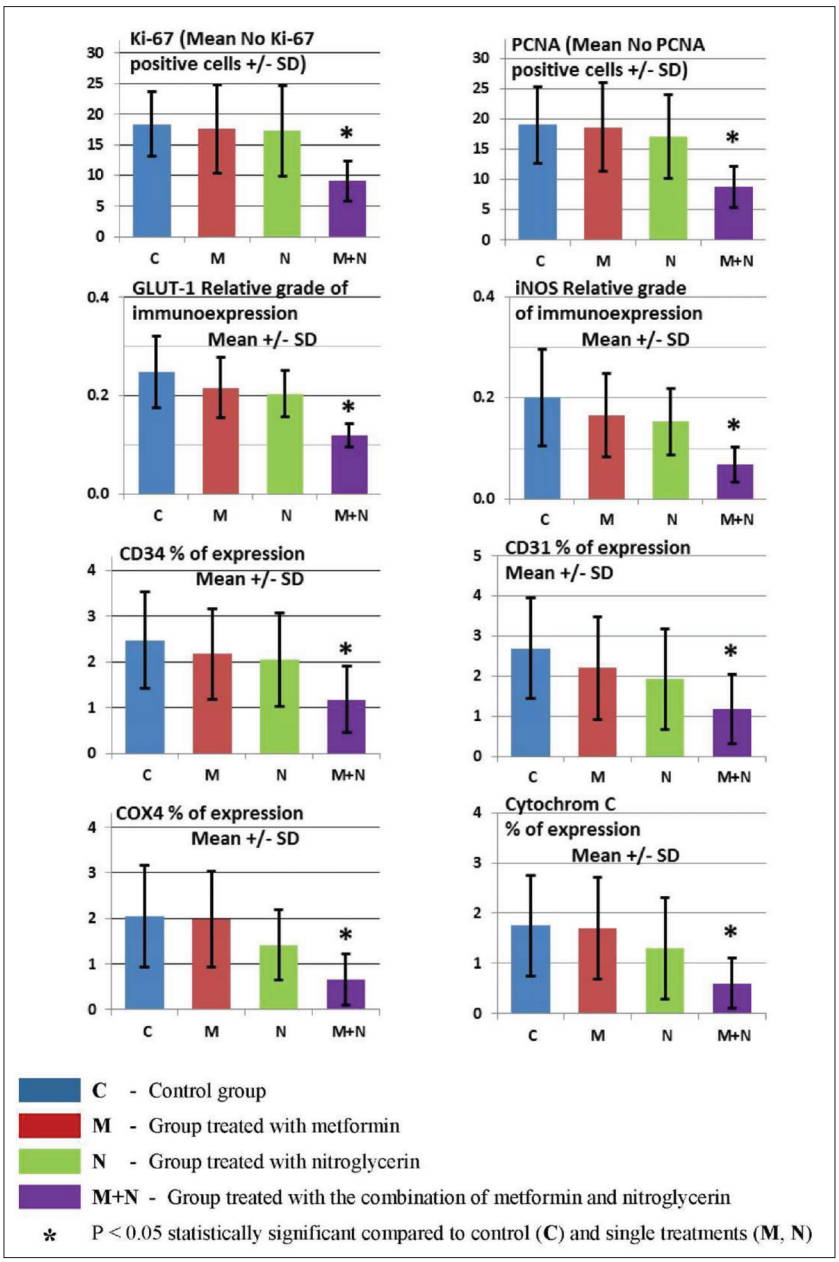

Figure 3. Means and standard deviations (SD) of immunohistochemical-histopathological characteristics of the excised tumors in the third experiment: Ki-67, PCNA, GLUT-1, iNOS, CD34, CD31, COX4, cytochrom C;

*p < 0.05, as indicated

xenografts of mice compared with untreated controls [15]. Profound anticancer activities *in vitro* and *in vivo* of the drug combination used in that study were explained by ROS amplification, which caused DNA damage, apoptosis, autophagy, and necroptosis [15].

The results of a recent meta-analysis showed favorable clinical signal on the response rate after adding metformin to chemotherapy in the breast cancer treatment [16]. Breast cancer patients receiving chemotherapy have been shown to experience better therapeutic responses with the use of metformin [16].

Comedication of metformin with various oncological drugs showed significant synergism in different cancer types [17]. Metformin activated AMPK, causing inhibition of mTOR and also reduced protein kinase B (PKB = Akt), causing inhibition of the phosphatidylinositol 3 (PI3)/Akt/mTOR and various pathways (RAS/RAF/MAPK/ERK), reducing transcription, protein synthesis, and proliferation [17].

Disclosure of anticancer effects in the tested non-oncological marketed drug combinations may be the first step to finding effective, cheap and immediately applicable treatment for tumors.

CONCLUSION

The results of our three experiments proved significant anticancer effects of the metformin co-treatments with caffeine, or itraconazole, or nitroglycerin on hamster fibrosarcoma, without toxicity. Opposite to the examined drug combinations, monotherapies did not show anticancer effects. Anticancer properties of the three examined two-drug combinations (metformin and caffeine, metformin and itraconazole, metformin and nitroglycerin) in hamsters, with used doses equivalent to standard human doses, suggest that effective nontoxic oncological therapies in humans and cancer relapse prevention using these drug combinations may be attainable. Treatment with metformin in combination with caffeine, or itraconazole, or nitroglycerin may be a promising efficacious nontoxic new adjuvant anticancer therapy and invites further clinical investigation.

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Ефекти метформина и његових комбинација са другим пренамењеним лековима на фибросарком код хрчака

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САЖЕТАК

Увод/Циљ Многи лекови регистровани за различите друге индикације могу селективно деловати на туморске рецепторе, сигналне путеве, метаболичке процесе, биоенергетске факторе, ензиме, протеине и гене који регулишу пролиферацију, апоптозу и неоангиогенезу тумора без утицаја на ове активности у здравим ћелијама. Увођење нових лекова је веома дуг, сложен и скуп процес истраживања. Откривање антиканцерског ефекта код лекова који су већ регистровани за друге индикације и формирање њихових комбинација могу директно смањити време и цену таквог истраживања.

Методе Антиканцерска ефикасност метформина и његових комбинација са кофеином, итраконазолом и нитроглицерином тестирана је на фибросаркому експериментално изазваном ћелијама *BHK21/C13* код сиријских златних хрчака (шест животиња по групи, насумично распоређених у контролне и експерименталне групе, дозе једнаке уобичајеним дозама за људе). После жртвовања животиња, тумори су ексцидирани и одређене су њихове величине, биофизичке карактеристике, хистологија и имунохистохемија. Узети су узорци крви за хематолошке и биохемијске анализе, а главни органи су токсиколошки анализирани. Статистичка значајност је одређена једносмерним *ANOVA* тестом, који је пратио *Student–Newman–Keuls post hoc* тест.

Резултати Комбинације два лека, метформина са кофеином, или итраконазолом, или нитроглицерином, показале су значајне антитуморске ефекте на фибросарком хрчка у поређењу са контролом, у односу на све тестиране параметре тумора ($p < 0,05$), без токсичности.

Закључак Примена метформина у комбинацији са кофеином, или итраконазолом, или нитроглицерином може бити ефикасан и безбедан приступ новој нетоксичној адјувантној антиканцерској терапији.

Кључне речи: метформин; кофеин; итраконазол; нитроглицерин; хрчци; фибросарком