Physicochemical and pathohistological changes in experimental fibrosarcoma tumors of hamsters treated with metformin and itraconazole

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Abstract. The anticancer effects of metformin (an antihyperglycaemic agent) and itraconazole (an antifungal agent), which are established non-oncologic drugs, were investigated in the present study. The weight, diameter, volume, density, surface, surface to volume ratio and immunohistochemistry of experimental fibrosarcoma tumors were investigated in hamsters treated with metformin and itraconazole. Briefly, the hamsters were injected with BHK-21/C13 cells in order to induce fibrosarcoma, and the animals were treated daily with metformin, itraconazole or a combination of the two drugs. Subsequently, blood samples were obtained for biochemical analyses and the tumors were excised, weighed and measured. The tumor samples were pathohistologically and immunohistochemically assessed for proliferation marker protein Ki-67, hematopoietic progenitor cell antigen CD34, cytochrome c oxidase subunit 4 (COX4), glucose transporter 1 (GLUT1) and inducible nitric oxide synthase (iNOS), and vital organs were toxicologically tested. Ki-67-positivity and cytoplasmic marker (CD34, COX4, GLUT1, iNOS) immunoexpression in the tumor samples were quantified. The results revealed that the combination of metformin and itraconazole significantly altered the physicochemical and pathohistological characteristics of the hamster fibrosarcoma tumors, including absolute and relative weight, volume, density, length, surface area, surface to volume ratio, Ki-67-positivity and the immunoexpression of cytoplasmic markers, without indications of toxicity. Furthermore, metformin with itraconazole demonstrated antiproliferative functions in cervical carcinoma HeLa, colon carcinoma HT-29, lung carcinoma A549 and fibrosarcoma BHK-21/C13 cells, with markedly lower cytotoxicity in the normal fetal lung MRC-5 cells. In conclusion, the administration of metformin in combination with itraconazole may inhibit the growth of fibrosarcoma tumors *in vivo* and the proliferation of various malignant cell lines *in vitro*, suggesting that this may be an effective and safe approach as a nontoxic anticancer adjuvant and relapse prevention therapy.

Introduction

Metformin, a standard clinical drug, used for the treatment of type 2 diabetes mellitus and polycystic ovary syndrome, reduces cellular energy production and inhibits growth of various cultured cancer cell lines (1,2). Metformin has certain anticancer effects on BHK-21/C13-induced fibrosarcoma in hamsters (3). Notably, metformin exerts anticancer effects *in vitro* via the following main mechanisms: Inhibition of AMP-activated protein kinase (AMPK)/serine/threonine-protein kinase mTOR signaling, anti-angiogenesis, or folate and autophagy inhibition (3). The drug also exhibits anticancer effects by altering host response mechanisms, decreasing gluconeogenesis and regulating circulating insulin, lipid metabolism and serum bile acids (4). In addition, other possible anticancer mechanisms of action have been identified for metformin, including transcriptional regulation of certain genes (5), cobalamin deficiency (6), inhibition of neurogenic locus notch homolog protein 1/transcription factor HES and androgen receptor signaling pathways (7), and regulation of tight junctions via the myosin light-chain kinase-MLC signaling pathway (8).

In patients with diabetes, metformin is usually administered in combination with the most common and safe antimycotic agent itraconazole, since fungal infections occur frequently in diabetes (9). This combination is nontoxic in humans (10).

Similar to metformin, itraconazole also possesses anticancer properties. The *in vitro* anticancer functions of itraconazole in cancer cell cultures include the following: Inhibition of AMPK/mTOR signaling (11), anti-angiogenesis (11), antilymphangiogenesis (11), folate and autophagy inhibition (11,12), inhibition of Hedgehog signaling (13,14),

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inhibition of P-glycoprotein (P-gp), chemosensitization (reversed multiple drug resistance, particularly to cytotoxic antitumor drugs), inhibition of the transportation and pumping of cholesterol, and inhibition of the Wnt/β-catenin signaling pathway (11). There is notable synergy between antifolates and itraconazole, which inhibits ergosterol biosynthesis (12).

The pharmacokinetic interaction between metformin and itraconazole by mutual competitive inhibition of metabolism through hepatic and intestinal cytochrome P450 3A (CYP3A)1/2 leads to a significant increase in the areas under the serum concentration/time curves of metformin and itraconazole following oral or intravenous (IV) administration in rats, implying an improved effect of the drugs (15,16). Metformin and itraconazole are metabolized by the CYP3A subfamily in rats and humans; the two drugs have been demonstrated to significantly inhibit the metabolism of each other by CYP3A4 in human microsomes (15). In humans, metformin is predominantly excreted in urine unchanged, with <20% of the IV dose being metabolized (15). In contrast, itraconazole is eliminated exclusively by hepatic metabolism, where CYP3A4 in humans or CYP3A1/2 in rats is involved to produce several metabolites, including 7-hydroxyitraconazole, the major metabolite in both species (15).

Itraconazole inhibits P-gp, decreasing the elimination of organic cation drugs and increasing their absorption (11). Metformin is present as a cation at physiological pH (17). Organic cation transporters 1 and 3 are active transporters of metformin (18); therefore, as a potent P-gp and CYP3A4 inhibitor, itraconazole can increase the plasma concentrations of P-gp and CYP3A4 substrates, including metformin, and enhance their effects (19).

The aim of the present study was to investigate the anticancer effects of combining nontoxic drugs on experimental tumors. Preclinical and limited clinical studies have proposed the use of metformin (3) or itraconazole (11) as promising nontoxic anticancer agents. To the best of our knowledge, no published results of the anticancer effect of the combination of these drugs exist to date. The possibilities of synergistic anticancer metformin-itraconazole interactions (15-18) and safe multitargeting therapy (9,10), based on previous separate preclinical studies (3,11,12), were the main reasons for testing this drug combination on an experimental hamster fibrosarcoma model.

Sarcoma models are of fundamental importance in cancer treatment research due to the multiple pathological and clinical entities, resistance to current therapies and high mortality attributed to these malignancies (20). Sarcomas are a large family of diverse mesenchymal malignant tumors derived from connective and soft tissues, such as bone, muscle, cartilage, fat, vascular tissue, skin or hematopoietic tissue (21). Sarcomas affect ~200,000 individuals worldwide each year and represent a higher percentage of overall cancer morbidity and mortality in children and adolescents compared with adults (22,23). Sarcomas account for >20% of all pediatric solid malignant cancers (24). Notably, osteosarcoma is the most common primary bone tumor and mainly affects adolescents and young adults (25), and rhabdomyosarcoma is the most common soft tissue sarcoma of childhood (26). There are >100 different histological subtypes of soft tissue sarcomas, which develop in a number of different locations and tissues (23). Fibrosarcoma,

which is a common soft tissue sarcoma, accounts for ~10% of all sarcomas and occurs at all ages; in children, the majority of tumors are diagnosed in the first year and tend to occur in the lower extremities, whereas head and neck lesions account for ≤20% of cases. Despite treatment protocols that combine chemotherapy, including methotrexate, cisplatin, doxorubicin and ifosfamide, surgery and radiotherapy, the 5-year survival rate for patients diagnosed with sarcoma is ~60%, whereas for patients with metastatic disease the survival rate is ~30%. Current treatment strategies for sarcomas, including fibrosarcoma, are associated with significant side effects, and there have been no significant improvements in prognosis in the last decade (23). In addition, chemotherapy dose increase has not improved the outcome (22,23). Therefore, there is a need for improved nontoxic therapies for the treatment of sarcoma and fibrosarcoma (20,22,23). The main rationale for the use of metformin and itraconazole for separate or combined antisarcoma therapy in an animal fibrosarcoma model was the antifolate anticancer effect of the two drugs on malignant cells *in vitro* (3,11,12). It was hypothesized that an interaction between the folic acid cycle and these drugs, which is different from the effects of the antifolate drug methotrexate, may produce synergistic anticancer effects similar to this antifolate drug, without toxicity.

To validate the results on tumor progression obtained by the measurements of fibrosarcoma dimensions, markers of cancer mitosis (proliferation marker protein Ki-67), vasculature (hematopoietic progenitor cell antigen CD34), apoptosis [cytochrome *c* oxidase subunit 4 (COX4)], glucose metabolism [glucose transporter 1 (GLUT1)] and nitric oxide (NO) production [inducible NO synthase (iNOS)] were immunohistochemically assessed in fibrosarcoma specimens (3,11,14). Ki-67 immunoexpression was evaluated by stained cell counting (3). The relative immunoexpression of GLUT1, iNOS, CD34 and COX4 markers was graded according to the portion of stained sample areas. For further validation of the results of co-treatment with metformin and itraconazole, the *in vitro* antiproliferative functions of the combination treatment in cervical carcinoma HeLa, colon carcinoma HT-29, lung carcinoma A549, fibrosarcoma BHK-21/C13 and normal fetal lung MRC-5 cells were tested.

Materials and methods

Animal model. The present study was performed on 48 Syrian golden hamsters (22 males and 26 females; age, 12-20 weeks; weight, ~100 g). The animals were obtained from the Pasteur Institute and were maintained under standard animal housing conditions at 25±2˚C and 60±2% humidity under a 12-h light/dark cycle. The animals had *ad libitum* access to food and water.

The study protocol was conducted in accordance with the requirements of the national regulations for the handling of laboratory animals and the study was approved by the University of Novi Sad Animal Ethics Committee (approval no. 01-78/18-5; dated April 26, 2016).

The hamsters were randomized into eight groups (n=6 hamsters/group) with equal numbers of males and females due to the potential role of sex hormones on the response to the treatment. Treatment with metformin, itraconazole

Table I. Characteristics of animals in the control and treated groups.

| | 2-week treatment | | | | | | 3-week treatment | | | | | |
| | Hamster | | | Tumor | | | Hamster | | | Tumor | | |
No.	Weight at start (g)	Weight at end (g)	Sex	Weight (g)	Dmax (cm)[a]	Volume (cm³)	Weight at start (g)	Weight at end (g)	Sex	Weight (g)	Dmax (cm)[a]	Volume (cm³)
A, Control group												
1	91	102	F	2.370	2.4	2.20	104	160	F	7.700	3.5	7.00
2	87	98	F	2.580	3.4	2.24	138	176	F	16.890	3.5	16.00
3	82	93	M	0.550	1.8	0.35	112	123	F	11.380	3.5	10.50
4	94	99	M	3.040	2.4	2.80	115	118	M	2.580	2.5	2.30
5	80	91	M	1.600	2.3	1.50	134	141	M	1.270	2.0	1.20
6	84	92	M	0.670	2.1	0.60	105	112	M	7.930	3.4	7.20
Mean	86.3	95.8	-	1.802	2.4	1.67	118.0	138.3	-	7.960	3.07	7.37
±SD	5.4	4.4	-	1.034	0.54	0.96	14.6	25.4	-	5.750	0.65	5.44
B, Group treated with metformin												
1	124	138	F	2.470	2.5	2.40	79	91	F	7.690	2.8	7.00
2	146	155	F	1.270	2.2	1.20	78	84	F	6.690	3.0	6.00
3	118	133	F	3.960	2.6	3.80	75	87	F	4.110	2.2	3.80
4	87	112	M	1.640	2.0	1.50	112	108	M	2.660	2.2	2.50
5	78	101	M	0.800	1.6	0.75	84	86	M	3.860	2.8	3.70
6	91	73	M	0.710	1.8	0.70	87	92	M	2.510	1.9	2.40
Mean	107.3	118.7	-	1.808	2.12	1.56	85.8	91.3	-	4.587	2.48	4.23
±SD	26.2	29.5	-	1.234	0.39	1.15	13.5	8.7	-	2.137	0.44	1.88
C, Group treated with itraconazole (250 mg/kg)												
1	92	107	F	3.100	2.0	3.00	89	98	F	7.230	3.0	7.00
2	132	121	F	1.160	2.1	1.10	99	102	F	7.900	3.2	7.50
3	112	110	F	0.800	1.9	0.75	140	107	F	6.700	3.2	6.50
4	149	129	F	0.630	1.2	0.60	112	100	F	5.970	3.2	5.50
5	125	98	M	0.780	1.9	0.70	95	110	M	5.090	3.0	4.80
6	51	71	M	2.260	2.4	2.00	82	85	M	6.530	3.2	6.00
Mean	110.2	106.0	-	1.455	1.92	1.36	102.8	100.3	-	6.570	3.13	6.22
±SD	34.7	20.3	-	1.001	0.397	0.95	20.8	8.7	-	0.977	0.10	0.99

Table I. Continued.

D, Group co-treated with metformin and itraconazole (250 mg/kg)

No.	2-week treatment[b] Hamster Weight at start (g)	Weight at end (g)	Sex	Tumor Weight (g)	Dmax (cm)[a]	Volume (cm³)	3-week treatment[c] Hamster Weight at start (g)	Weight at end (g)	Sex	Tumor Weight (g)	Dmax (cm)[a]	Volume (cm³)
1	93	93	F	0.350	1.2	0.34	94	112	F	2.970	1.8	2.80
2	101	104	F	0.120	0.9	0.12	98	99	F	2.090	1.9	2.00
3	109	113	F	0.400	1.6	0.40	96	94	F	1.440	1.7	1.40
4	84	85	F	0.170	1.1	0.16	105	98	M	2.450	2.7	2.35
5	118	117	M	0.460	1.4	0.45	89	104	M	1.920	2.2	1.85
6	106	114	M	0.250	1.0	0.24	88	90	M	1.040	1.4	1.00
Mean	101.8	104.3	-	0.292	1.20	0.28	95.0	99.5	-	1.973	1.95	1.90
±SD	12.1	12.9	-	0.134	0.26	0.13	6.3	7.7	-	0.690	0.45	0.65

[a]Longest tumor diameter (cm); [b]Treated with 500 mg/kg metformin; [c]Treated with 250 mg/kg metformin. F, female; M, male.

(both Galenika a.d.) and their combination in experimental groups was initiated 3 days prior to the subcutaneous inoculation of 1 ml BHK-21/C13 cell suspension (27) ($2x10^6$ cells/ml) into the back for the development of a subcutaneous fibrosarcoma tumor (28). BHK-21/C13 cells were cultured in DMEM media with 4.5 g/l glucose (Capricorn Scientific), supplemented with 10% fetal bovine serum (FBS; Capricorn Scientific), 2 mM glutamine (Capricorn Scientific) and 1% penicilin/streptomicin (Capricorn Scientific), at 37˚C, in a 5% CO_2 humidified atmosphere. Cells were subcultured twice a week once they reached confluency of 70-80%. Tumor inoculation and drug administration were performed by the same researcher to maintain consistency. The following humane endpoints were established: Significant body weight loss (20%), decreased activity/responsiveness with loss of body weight, impaired posture, inability to eat, urinate or defecate, tumor diameter >10% body weight, or tumor ulceration. The following aspects were monitored: Behavior, general condition, body weight (measured daily), general clinical signs (diarrhea, neurological signs, breathing disorders), tumor diameter, anatomical location, incidence of multiple tumors and tumor ulceration.

Two sets of experiments were performed. The first set involved four groups of animals administered different treatments via a gastric probe daily for 3 days prior to cancer cell inoculation: Perioral treatment with i) physiological saline; ii) 500 mg/kg metformin; iii) 250 mg/kg itraconazole; or iv) combination of 500 mg/kg metformin and 250 mg/kg itraconazole. The animals were sacrificed 2 weeks post-inoculation. The second set of experiments followed the same protocol as the first set, with the exception that the daily dose of metformin was 250 mg/kg and the animals were sacrificed at 3 weeks. To sacrifice the animals, a dose of 60 mg/kg pentobarbital was administered intraperitoneally. The animals were assessed for loss of consciousness at 5 min by a combination of methods, including a toe pinch, lack of visible respiration and lack of reaction on digital palpation. An additional dose of 30 mg/kg pentobarbital was administered if loss of consciousness was not observed. Total cardiac exsanguination was performed immediately following confirmation of loss of consciousness. The volume of blood extracted by total cardiac exsanguination was 3-5.5 ml, depending on animal weight and/or sex. Only 2-3 ml of the total collected blood was used for blood biochemical analysis. Following exsanguination and animal death, vital organs (heart, lungs, liver, kidneys and brain) were removed. The weight of the animals at the time of sacrifice is presented in Table I. In a previous study, subcutaneous inoculation of $1x10^7$ BHK-21/C13 cells produced fibrosarcoma, which killed the animals in 40 days (28). Therefore, 21 days was the maximum duration of the experiments in the present study. All animals were in a good condition during the study, and none of the hamsters were euthanized prior to the end of the experiment. In the course of experiment, the tumor burdens were evaluated daily using calipers and the following ellipsoid volume formula: Volume=4πabc/3, where a, b and c are half-diameters (29,30). The maximal tumor burden in the present study, 1 day prior to sacrifice, was <3.2% of body weight for the 2-week treatment groups and <10% of body weight for the 3-week treatment groups. After sacrifice, the tumors were excised, weighed, their diameters were measured

Table II. Statistical evaluation of tumor characteristics following 2-week treatment.

Group comparison	Tumor (P-values)							
	Weight	Relative weight	Volume	Density	Length	Surface area	Surface/volume	Mean Ki-67
C/M	0.999	0.850	0.936	0.025[a]	0.311	0.592	0.0499[a]	0.250
C/I	0.610	0.862	0.600	0.450	0.170	0.251	0.110	0.170
M/I	0.615	0.950	0.803	0.410	0.472	0.590	0.999	0.850
C/M+I	0.009[a]	0.01[a]	0.0095[a]	0.009[a]	0.006[a]	0.0085[a]	0.049[a]	0.0065[a]
M/M+I	0.019[a]	0.0088[a]	0.021[a]	0.150	0.005[a]	0.0078[a]	0.601	0.031[a]
I/M+I	0.029[a]	0.037[a]	0.0205[a]	0.022[a]	0.0085[a]	0.0095[a]	0.650	0.042[a]

[a]P<0.05. C, control group; M, group treated with metformin (500 mg/kg); I, group treated with itraconazole (250 mg/kg); M+I, group treated with the combination of metformin (500 mg/kg) and itraconazole (250 mg/kg).

Table III. Statistical evaluation of tumor characteristics following 3-week treatment.

Group comparison	Tumor (P-values)							
	Weight	Relative weight	Volume	Density	Length	Surface area	Surface/volume	Mean Ki-67
C/M	0.201	0.833	0.120	0.480	0.133	0.351	0.049[a]	0.479
C/I	0.610	0.888	0.551	0.401	0.655	0.656	0.048[a]	0.535
M/I	0.051	0.213	0.049[a]	0.900	0.0095[a]	0.019[a]	0.713	0.803
C/M+I	0.035[a]	0.049[a]	0.036[a]	0.005[a]	0.045[a]	0.047[a]	0.046[a]	0.044[a]
M/M+I	0.018[a]	0.015[a]	0.015[a]	0.035[a]	0.062	0.008[a]	0.452	0.053
I/M+I	0.002[a]	0.002[a]	0.003[a]	0.045[a]	0.007[a]	0.0001[a]	0.552	0.021[a]

[a]P<0.05. 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).

and the exact tumor volume was determined using the standard water volume displacement method.

Metformin was dissolved in physiological saline and administered at a dose of 250 or 500 mg/kg (in 1 ml/100 g animal weight), equivalent to a human dose of 20 or 40 mg/kg (by normalization to surface area), which are the daily doses taken by patients with diabetes (31). The dose of itraconazole (250 mg/kg) was equivalent to the maximum human dose of 20 mg/kg, and was prepared and administered in the same manner as metformin. The control groups received physiological saline (1 ml/100 g animal weight). The relative tumor weight was determined as the weight ratio of tumor/animal. The tumor volume was determined using the standard water volume displacement method, by measuring the water level in a graduated cylinder prior to and following the submergence of the tumor. The tumor density was calculated as density=mass/volume. The tumor surface area (S) was calculated using the ellipsoid formula from three half diameters (a, b and c): $S=4\pi\{[(ab)^{1.6} + (ac)^{1.6} + (bc)^{1.6}]/3\}^{1/1.6}$. The ratio of tumor surface area to volume (S/V) was also calculated.

Tumor slices (4 μm) were assessed pathohistologically and immunohistochemically for the verification of tumor growth and angiogenesis. The weight of the hamsters was recorded in order to evaluate possible side effects of metformin and/or itraconazole. The vital animal organs (brain, heart, lungs, liver and kidneys) were analyzed histopathologically and no

obvious toxicological effects of drugs used in this experiment were detected.

Immunohistochemistry. In addition to the principal hematoxylin and eosin staining, immunohistochemical Ki-67, CD34, COX4, GLUT1 and iNOS staining was performed to assess tumor cell proliferation. Tissue sections were stained at room temperature with hematoxylin for 5 min and with Eosin G for 30 sec. For the Ki-67 staining, tumor slices were fixed in 10% neutral buffered formalin for 48 h at room temperature, blocked with 4% bovine serum albumin (Capricorn Scientific) in PBS for 1 h at room temperature, and incubated overnight at 4˚C with anti-Ki-67 primary antibody (Thermo Fisher Scientific, Inc.; cat. no. RB-9043-P0, 1:300). Sections were incubated with fluorescein isothiocyanate-conjugated polyclonal anti-rabbit secondary antibody (cat. no. F7512; 1:80; Sigma-Aldrich; Merck KGaA) for 2 h at room temperature. The nuclei were counterstained with Hoechst 33256 (Sigma-Aldrich; Merck KGaA). In further immunohistochemical staining, the following primary antibodies were used: CD34 (Abcam; cat. no. ab81289; 1:200), COX4 (Abcam; cat. no. ab185056; 1:1,000), GLUT1 (Thermo Fisher Scientific, Inc.; cat. no. RB-9052-P0; 1:200) and iNOS (Thermo Fisher Scientific, Inc.; RB-9242-P0; 1:100). Briefly, sections (5 μm) were deparaffinized in xylene (100%) and rehydrated in descending ethanol series (100% twice for 3 min; 95% for
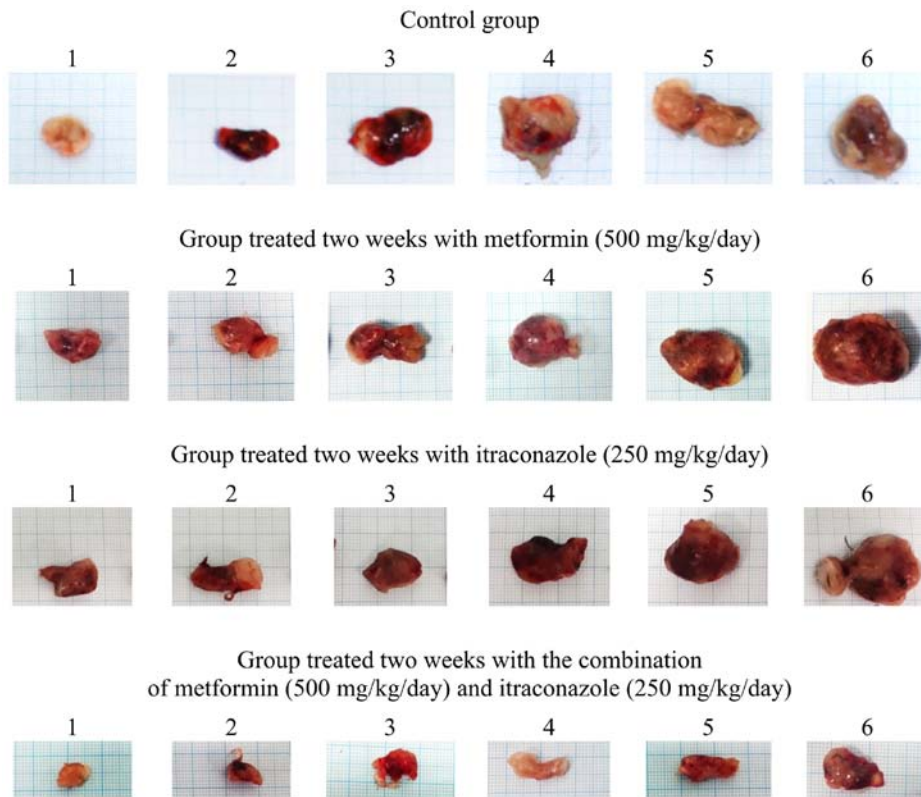
Figure 1. Excised fibrosarcoma tumors placed on 1-mm grid paper in ascending weight order for visual dimension comparison following 2-week treatment.
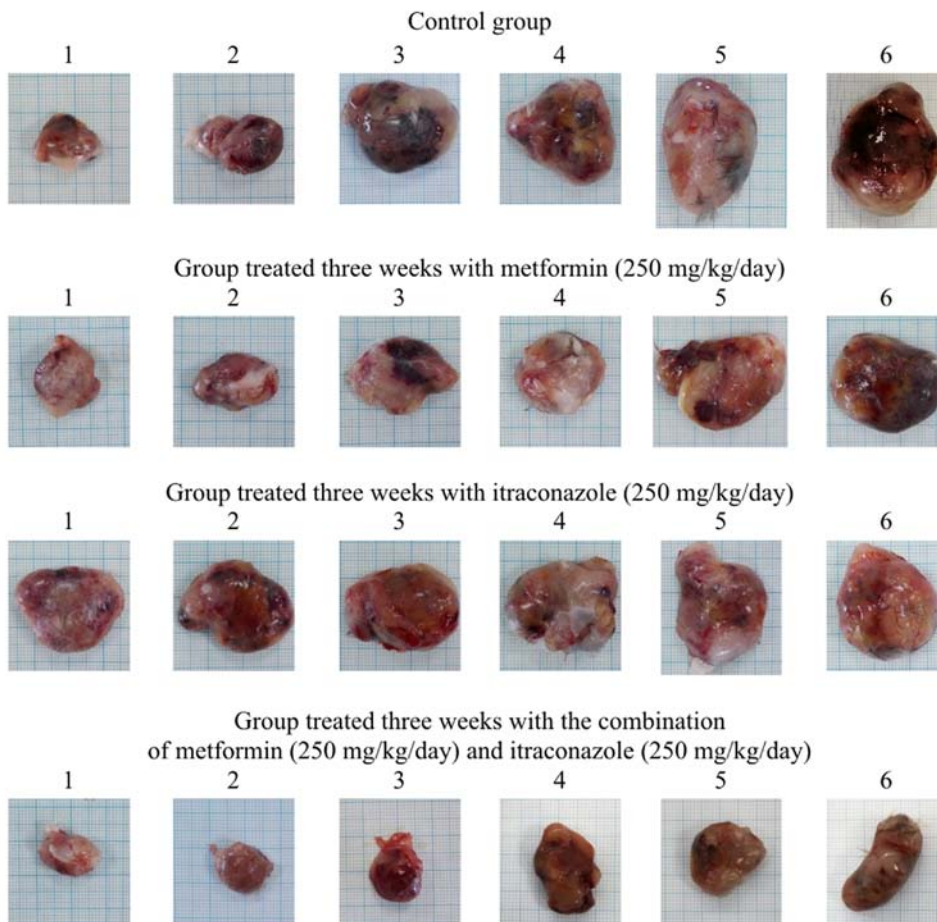

Figure 2. Excised fibrosarcoma tumors placed on 1-mm grid paper in ascending weight order for visual dimension comparison after 3-week treatment.
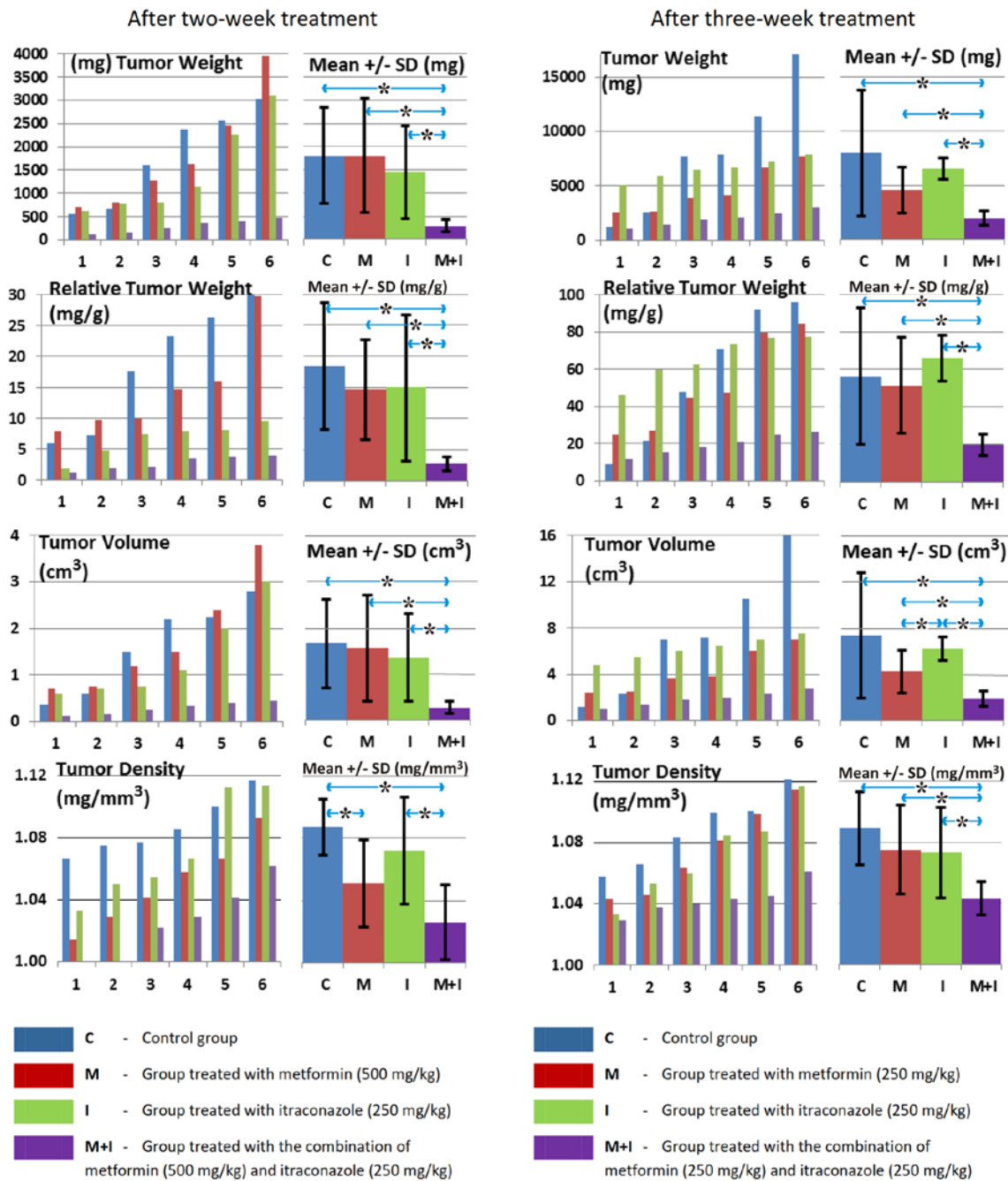
Figure 3. Characteristics of the excised tumors. Tumor weight, relative weight, volume and density, among the treated groups of animals. *P<0.05, as indicated.

3 min and 70% for 3 min). For antigen retrieval, the sections were microwaved (850 W; ~98°C) for 20 min in Tris-EDTA buffer [10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20 (pH 9.0)], washed twice for 5 min with TBS plus 0.025% Triton X-100 (with agitation) and blocked by immersion in 10% goat serum (cat. no. G6767; Sigma-Aldrich; Merck KGaA) in TBS with 1% BSA (cat. no. T6789; Sigma-Aldrich; Merck KGaA) for 2 h at room temperature. Primary antibodies dissolved in TBS with 1% BSA were incubated at 4°C overnight. The sections were washed twice for 5 min with TBS plus 0.025% Triton X-100 (with agitation) and incubated with 0.3% H_2O_2 in TBS for 15 min at room temperature. Horseradish peroxidase-conjugated goat polyclonal rabbit immunoglobulin G secondary antibody (cat. no. ab6721; Abcam)

dissolved in TBS with 1% BSA was added to the sections for 2 h at room temperature. The sections were washed three times for 5 min with TBS. For visualization, the chromogen 3,3-diaminobenzidine tetrahydrochloride (cat. no. K3468; Liquid DAB + SubstratChromogen System; Dako; Agilent Technologies, Inc.) was added and incubated for 10 min at room temperature. The sections were washed with water for 5 min and stained with Mayer's hematoxylin for 5 min at room temperature. The stained tumor slices were assessed using Leica DMLB 100T (Leica Microsystems GmbH) microscope at x400 magnification. Images were captured using a Leica MC190 HD camera (Leica Microsystems GmbH). The images of Ki-67 staining were processed using the UTHSCSA Image Tool for Windows version 3.00 (32). In each sample image,
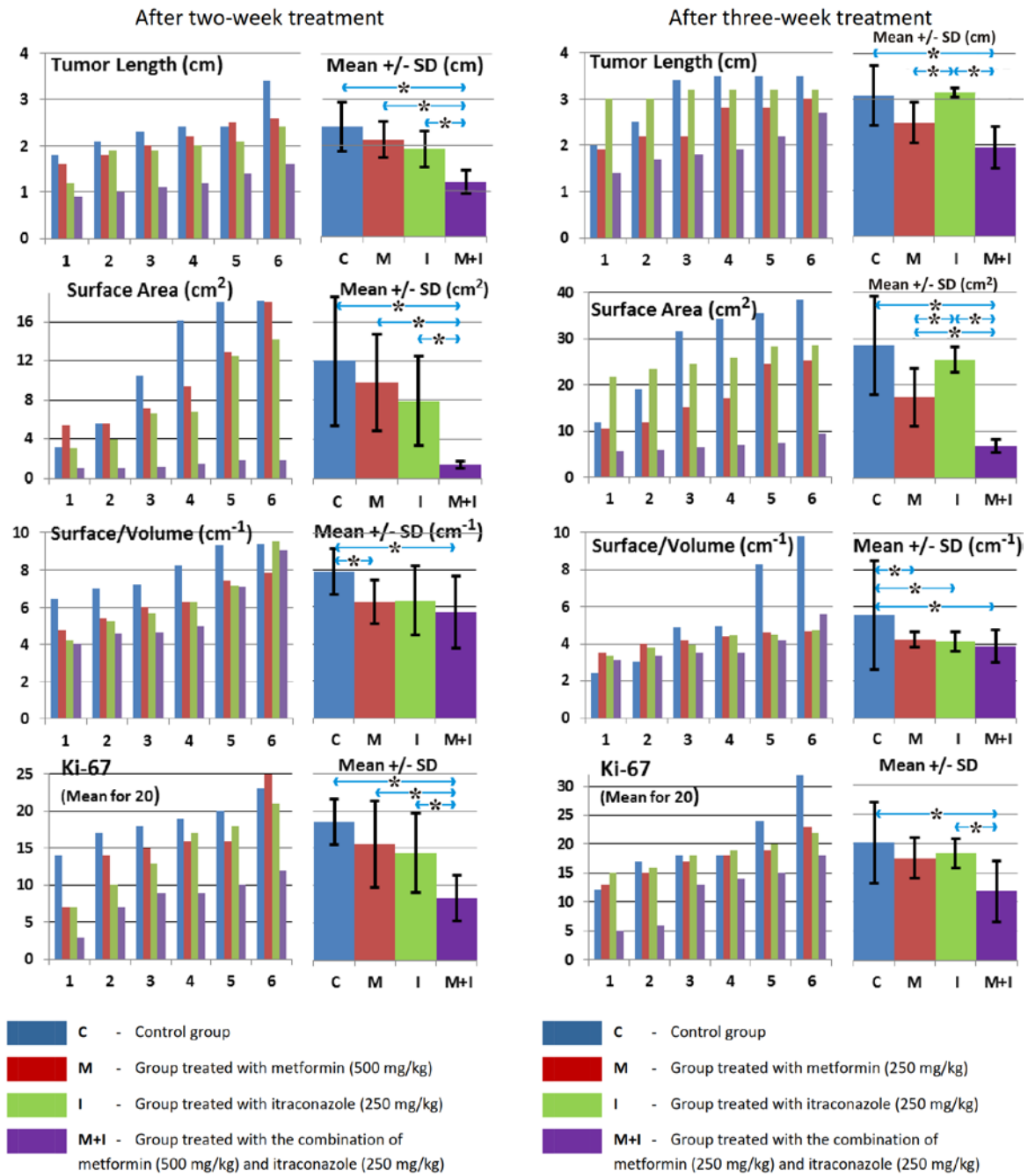
Figure 4. Characteristics of the excised tumors. Tumor length, surface area, surface/volume ratio and Ki-67 positivity, among the treated groups of animals. *P<0.05, as indicated.

the number of Ki-67-positive cells was counted. The mean numbers of Ki-67-positive cells in 20 tumor images from each animal were compared among the groups.

CD34, COX4, GLUT1 and iNOS staining was used to evaluate the vasculature, apoptosis, glucose metabolism and NO production in the tumor specimens, respectively. Immunoexpression was evaluated based on the measured portion of stained surface area (stained surface/whole surface) in the tumor sections (mean of 10 measurements) using UTHSCSA Image Tools for Windows version 3.00.

In vitro antiproliferative assay. The tested treatments were evaluated for their *in vitro* antiproliferative effects in hamster fibrosarcoma BHK-21/C13, human cervical carcinoma HeLa (CCL-2), colon carcinoma HT-29 (HTB-38), lung carcinoma A549 (CCL-185) and normal fetal lung MRC-5 (CCL-171) cells. All cell lines were obtained from the American Type Culture Collection. The cell lines were authenticated and mycoplasma testing was conducted. The cell lines were cultured in DMEM with 4.5 g/l glucose containing 10% FBS and 1% penicillin-streptomycin in an incubator at 37°C with 5% CO_2. A standard MTT assay (33) was performed to evaluate the cytotoxic effects of the treatments following exposure to doses of 50, 100, 250, 350, 500 μM and 1, 2, 3, 4, 5, 10, 20, 50 mM at 37°C for 48 h. The antiproliferative effect was expressed as the half maximal inhibitory concentration (IC_{50}).
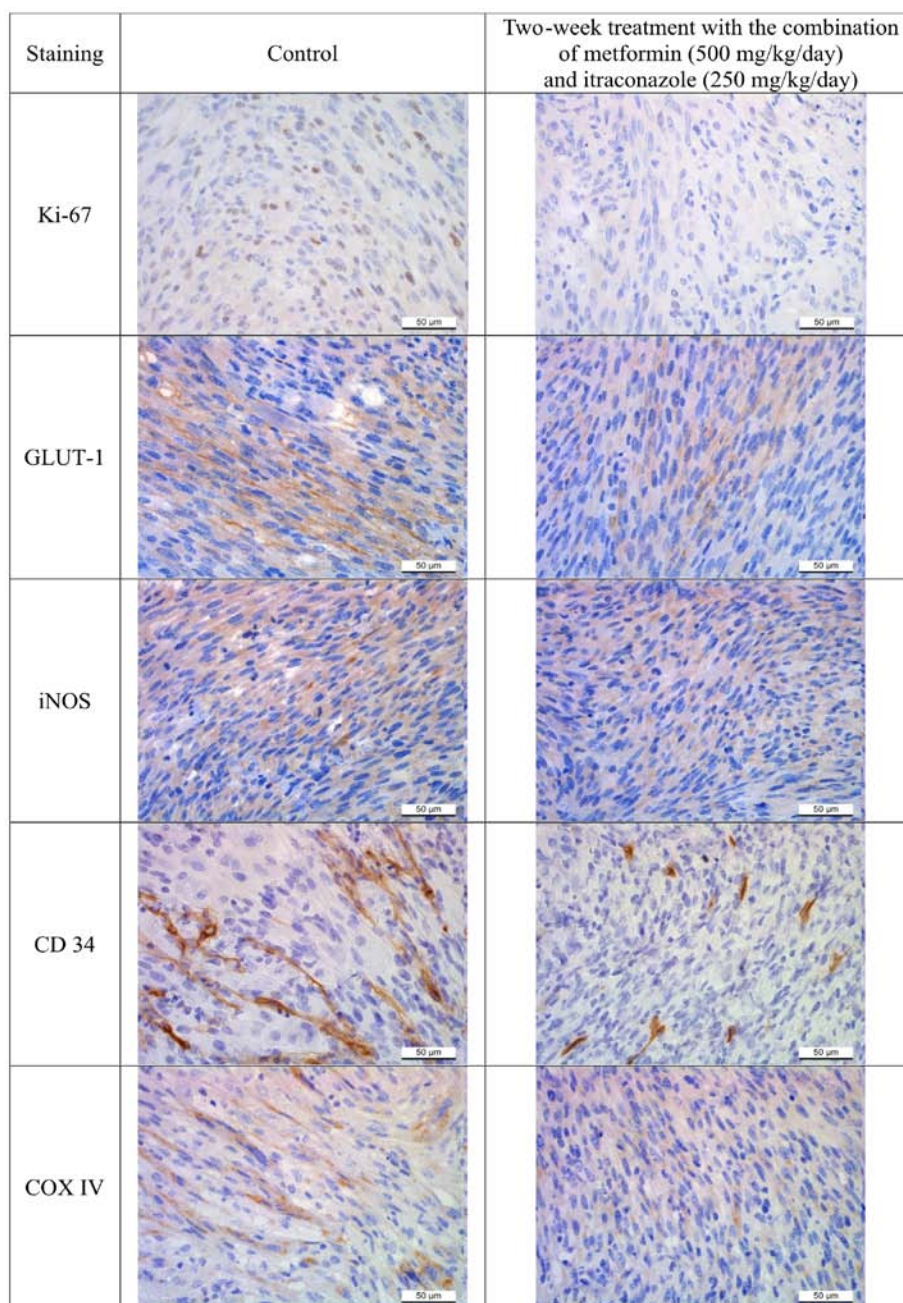
Figure 5. Inhibition of tumor growth and vasculature by metformin and itraconazole. Marked decreases were observed in the immunohistochemical staining of Ki-67, GLUT1, iNOS, CD34 and COX4 in the group treated with the combination of metformin and itraconazole compared with the control group. COX4, cytochrome *c* oxidase subunit 4; GLUT1, glucose transporter 1; iNOS, inducible nitric oxide synthase.

Blood biochemical tests. Blood samples were collected from the hamsters for blood measurements, including glucose, erythrocytes, leucocytes, lymphocytes, monocytes, granulocytes, platelets, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, serum proteins, albumins and sedimentation. Full blood cell counts were obtained using an auto hematology analyzer (Abacus Junior Vet; Diatron; Stratec SE). The serum, extracted by centrifugation at 2,000 x g for 10 min from the blood samples, was analyzed using an auto chemistry analyzer (Rayto Life and Analytical Sciences Co., Ltd.) and by commercial tests for concentration determination of glucose (cat. no. 21503), serum proteins (cat. no. 11500) and albumins (cat. no. 11547; BioSystems S.A.).

Statistical analysis. The data are presented as means ± standard deviations. The differences among the groups in tumor weight, volume, density, length, surface area, S/V ratio, mean number of Ki-67-positive cells and other measured parameters were determined using one-way ANOVA followed by a Student-Newman-Keuls post hoc test. Data analysis was conducted using TIBCO Statistica 13.3.1 software (TIBCO Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Co-treatment with metformin and itraconazole has antitumor effects in a hamster fibrosarcoma model. The subcutaneous
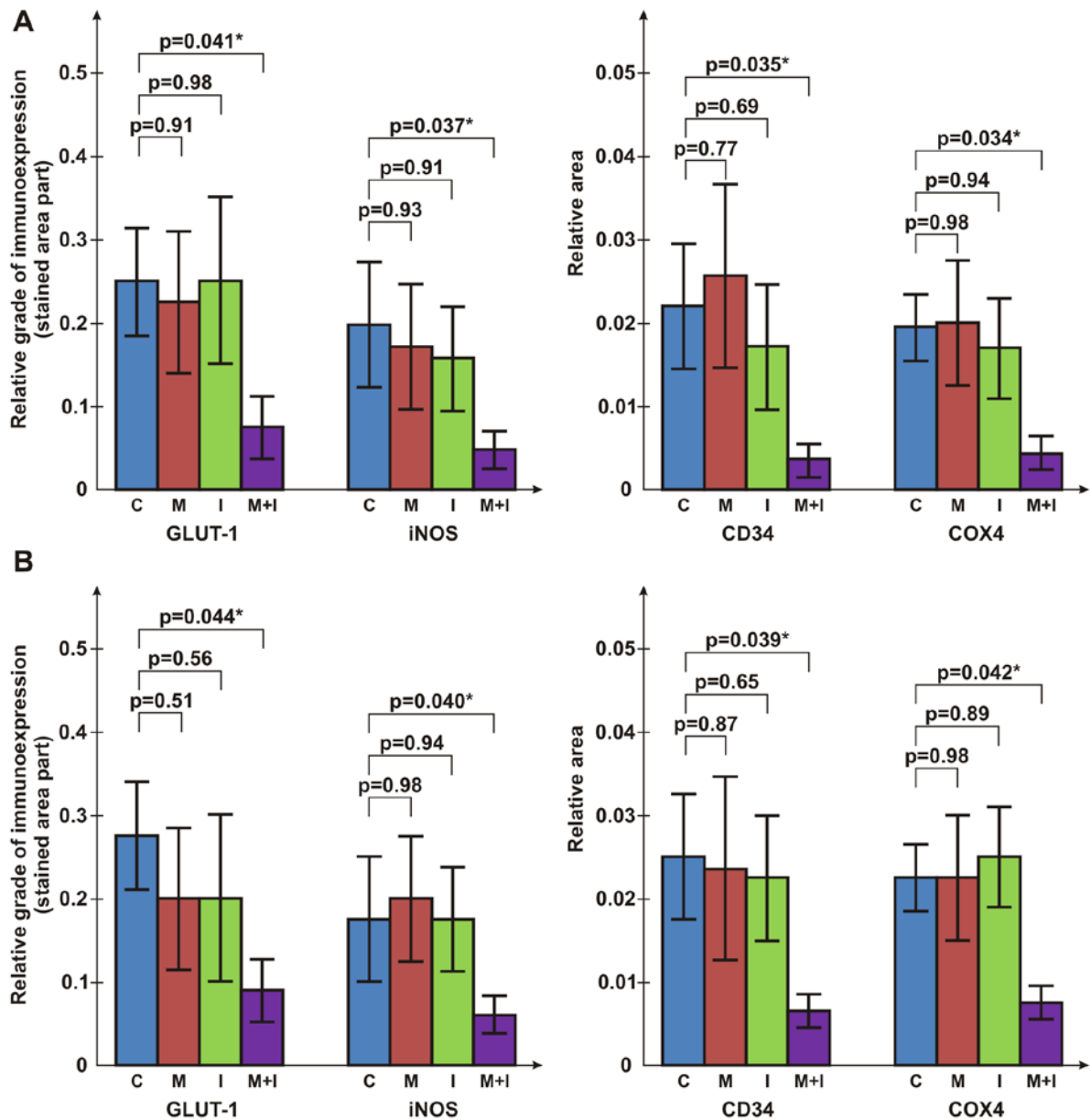
Figure 6. Extent of immunoexpression of GLUT1, iNOS, CD34 and COX4. Mean stained area in the groups treated for (A) 2 and (B) 3 weeks. *P<0.05. (C) control group; COX4, cytochrome *c* oxidase subunit 4; GLUT1, glucose transporter 1; I, group treated with itraconazole; iNOS, inducible nitric oxide synthase; M, group treated with metformin; M+I, group treated with the metformin/itraconazole combination.

inoculation of BHK-21/C13 cells into hamsters (Table I) resulted in fibrosarcoma formation at the site of injection in all inoculated animals. All experimental animals had isolated, well-demarcated solid tumors without adverse effects on general health and well-being. Pathological and histopathological analysis following autopsy revealed no signs of metastases or ascites. Perioral co-treatment with metformin and itraconazole significantly inhibited tumor growth at 2 and 3 weeks, as indicated by significant decreases in tumor weight, relative weight, volume, density, length, surface area and S/V ratio, as well as by the decreased proliferation status of tumor cells, as demonstrated by Ki-67 staining of the hamster tumor sections at 2 and 3 weeks (Tables II and III; Figs. 1-4). Notably, following a 3-day pre-treatment, only the combination of metformin and itraconazole resulted in a statistically significant antitumor effect compared with control (Tables II and III).

The pathohistological and immunohistochemical evaluation revealed a decrease in tissue penetration, an expansion of necrosis and hemorrhagic areas, inhibition of tumor growth and vasculature in all analyzed slices of tumors from animals treated with the combination of metformin and itraconazole compared with the control group (Figs. 5 and 6). The combined metformin and itraconazole therapy exhibited tumor cytotoxicity between the early and late tumor treatment stages (days 14-21 post tumor inoculation; Tables II and III; Figs. 3, 4 and 6).

The treatments had no significant effect on the body weight of the hamsters during the course of the study (Table I).

Treatment with metformin alone resulted in a nonsignificant (P>0.05) decrease in the fasting blood glucose levels in the hamsters (Tables IV and V). Additionally, the experimental and control groups were statistically compared for

Table IV. Blood analysis following 2-week treatment.

A, Control group

Hamster no.	Leucocytes (10⁹/l)	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)	Erythrocytes (10¹²/l)	Hemoglobin (g/l)	Hematocrit (%)	MCV	MCH	MCHC (g/l)	Platelets (10⁹/l)	Sedimentation	Glucose (mM/l)	Serum proteins (g/l)	Albumins (g/l)
1	9.60	86.1	1.8	12.0	4.68	112	34.63	74	23.9	323	512	1/2	5.1	57.79	27.49
2	27.41	44.0	0.8	55.2	5.29	136	35.36	67	25.8	386	201	1/2	4.2	52.80	43.00
3	18.14	53.6	8.0	45.6	6.14	134	37.01	60	21.6	361	265	1/2	4.8	54.72	33.84
4	13.38	82.3	4.4	13.3	5.47	114	34.74	64	20.9	329	413	1/2	4.5	50.68	29.32
5	18.69	73.6	6.3	20.1	4.30	92	27.66	64	21.4	332	564	1/2	3.3	89.80	41.70
6	29.14	78.5	7.2	14.3	4.98	114	32.08	64	23.0	356	484	1/2	3.9	53.10	45.30
Mean	19.39	69.68	4.75	26.75	5.14	117.00	33.58	65.50	22.77	347.83	406.50	-	4.30	59.82	36.78
± SD	7.66	16.97	2.95	18.77	0.64	16.24	3.31	4.72	1.86	24.16	144.39	-	0.65	14.88	7.56

B, Group treated with metformin (500 mg/kg)

Hamster no.	Leucocytes (10⁹/l)	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)	Erythrocytes (10¹²/l)	Hemoglobin (g/l)	Hematocrit (%)	MCV	MCH	MCHC (g/l)	Platelets (10⁹/l)	Sedimentation	Glucose (mM/l)	Serum proteins (g/l)	Albumins (g/l)
1	11.49	43.8	3.6	52.6	5.52	124	36.82	67	22.4	336	538	1/2	3.7	58.94	32.62
2	16.06	73.4	13.7	12.7	6.20	126	38.38	62	20.4	329	228	1/2	4.1	52.60	41.40
3	31.09	41.7	5.1	53.1	6.37	145	37.76	59	22.5	379	330	1/2	4.6	52.41	44.10
4	16.34	90.0	0.7	9.3	7.49	151	43.72	58	20.2	346	146	1/2	3.9	43.50	21.90
5	6.75	81.2	0.8	17.9	4.81	113	34.34	71	23.5	329	312	1/2	3.3	58.56	33.35
6	17.29	77.8	3.3	19.0	6.27	130	40.24	64	20.7	322	544	1/2	4.1	58.94	36.77
Mean	16.50	67.98	4.53	27.43	6.11	131.50	38.54	63.50	21.62	340.17	349.67	-	3.95	54.16	35.02
± SD	8.17	20.30	4.80	20.00	0.90	14.10	3.19	4.93	1.36	20.68	162.06	-	0.44	6.07	7.83

C, Group treated with itraconazole (250 mg/kg)

Hamster no.	Leucocytes (10⁹/l)	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)	Erythrocytes (10¹²/l)	Hemoglobin (g/l)	Hematocrit (%)	MCV	MCH	MCHC (g/l)	Platelets (10⁹/l)	Sedimentation	Glucose (mM/l)	Serum proteins (g/l)	Albumins (g/l)
1	15.94	77.5	0.8	21.7	4.87	117	32.64	67	23.9	357	679	1/2	3.5	55.48	29.93
2	15.23	78.5	0.7	20.8	5.69	126	36.74	65	22.2	343	295	1/2	3.7	57.34	25.12
3	5.96	84.7	5.2	10.1	5.09	114	34.01	67	22.5	336	434	1/2	3.2	43.10	20.10
4	36.38	35.0	11.7	53.3	4.83	123	34.79	72	25.5	354	265	1/2	5.2	57.21	36.04
5	12.10	84.1	5.8	10.2	5.68	124	37.40	66	21.9	332	464	1/2	3.1	61.63	40.70
6	17.32	78.7	5.2	16.1	4.45	101	29.82	67	22.7	340	479	1/2	9.9	53.56	42.27
Mean	17.16	73.08	4.90	22.03	5.10	117.50	34.23	67.33	23.12	343.67	436.00	-	4.77	54.72	32.36
± SD	10.25	18.91	4.04	16.11	0.50	9.27	2.78	2.42	1.35	9.93	148.87	-	2.63	6.29	8.83

Table IV. Continued.

D, Group co-treated with metformin (500 mg/kg) and itraconazole (250 mg/kg)

Hamster no.	Leucocytes (10⁹/l)	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)	Erythrocytes (10¹²/l)	Hemoglobin (g/l)	Hematocrit (%)	MCV	MCH	MCHC (g/l)	Platelets (10⁹/l)	Sedimentation	Glucose (mM/l)	Serum proteins (g/l)	Albumins (g/l)
1	14.16	58.8	0.7	40.5	3.39	85	24.51	72	25.0	345	406	1/2	3.5	59.90	26.60
2	29.18	86.8	3.8	9.4	5.48	118	38.21	70	21.5	308	542	1/2	3.3	61.63	40.70
3	20.66	34.5	13.9	51.6	6.13	129	35.94	59	21.1	360	421	1/2	3.9	58.10	43.20
4	10.82	88.6	1.7	9.7	5.99	141	39.17	65	23.6	361	416	1/2	4.1	48.80	24.20
5	16.49	65.7	8.0	26.3	5.95	132	38.44	65	22.2	344	475	1/2	3.1	57.98	40.19
6	9.82	73.5	6.5	21.0	4.78	109	32.96	69	23.9	332	551	1/2	3.2	58.97	37.99
Mean	16.86	67.98	5.77	26.42	5.29	119.00	34.87	66.67	22.88	341.67	468.50	-	3.52	57.56	35.48
± SD	7.21	20.10	4.85	16.92	1.05	20.05	5.56	4.68	1.52	19.77	65.07	-	0.40	4.50	8.02

Normal ranges: leucocytes 3-16·10⁹/l; lymphocytes 50-81%; monocytes 0-3%; granulocytes 17-30%; erythrocytes 4-7·10¹²/l; hemoglobin 100-180 g/l; hematocrit 35-50%; MCV 67-77; MCH 23-27; MCHC 200-360 g/l; platelets 200-500·10⁹/l; sedimentation 1/2; glucose 3-11 mM/l; serum proteins 45-75 g/l; albumins 23-43 g/l.

red and white blood cell counts, platelet number, hemoglobin levels, hematocrit levels, serum proteins and sedimentation, but no significant differences were observed among the groups ($P>0.05$). In addition, examination of the vital organs revealed no pathological or toxicological differences between the experimental and control groups.

Co-treatment with metformin and itraconazole has antiproliferative effects in cancer cell lines. The antiproliferative effects in fibrosarcoma, carcinoma and normal cell lines, expressed as IC_{50}, for all treatments are presented in Table VI. Co-treatment with metformin and itraconazole exhibited selective cytotoxicity against the malignant cell lines (HeLa, HT-29, A549 and BHK-21/C13). The combination demonstrated favorable antiproliferative effects in the normal fetal lung MRC-5 cells, suggesting that this treatment may be safe and efficient.

Discussion

A number of common or separate anticancer mechanisms of metformin (3) and itraconazole (11) have been identified. Among the potential anticancer targets of these drugs, AMPK/mTOR inhibition, anti-angiogenesis, and folate and autophagy inhibition are notable common functions. The chemosensitization properties of itraconazole (34) are also important, since they enhance the therapeutic efficacy of anticancer drugs through the inhibition of P-gp.

The combination of metformin and itraconazole in the present study resulted in reductions in fibrosarcoma tumor weight, relative weight, volume, density, length, surface area, S/V ratio, proliferation, vasculature and tissue penetration. These effects, as well as increased tumor necrosis and hemorrhage observed in the immunohistochemical and histological evaluation, may be due to the cytotoxicity of the treatment, which may alter the expression of proteins that regulate the proliferation and apoptosis of tumor cells, and vascular growth (35). The radiologically determined (4-dimension computed tomography) density of non-small cell lung cancer tumors has been reported to vary between 0.236 and 1.010 g/cm³ (36). Denser tumors are more likely to have poorer outcomes and result in shorter disease-free survival times (36). The Ki-67 protein is a cellular marker for proliferation, present during all active phases of the cell cycle (G_1, S, G_2 and M), and absent in the resting phase. The Ki-67 antigen can be exclusively detected within the cell nucleus. GLUT1 facilitates the transport of glucose across membranes and is overexpressed in cancer (3). Increased glycolysis, with increased expression of GLUT1, is associated with poor cancer prognosis. Increased iNOS immunostaining in the cytoplasm denotes tumor progression by increasing proliferation and angiogenesis (11), which is associated with poor outcome in various types of cancer. CD34 is a marker of endothelial cells and vascular differentiation (11). COX4 is a mitochondrial cytochrome *c* oxidase marker overexpressed in cancer cells (14).

In a previous study, the metformin levels in the colorectal cancer cells of xenograft-bearing mice that were successfully treated orally and intraperitoneally corresponded to the plasma concentrations (37). This indicates a consistent delivery of the drug to the tumor tissue. In the present study, the same orders

Table V. Blood analysis following three-week treatment.

A, Control group

Hamster no.	Leucocytes (10⁹/l)	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)	Erythrocytes (10¹²/l)	Hemoglobin (g/l)	Hematocrit (%)	MCV	MCH	MCHC (g/l)	Platelets (10⁹/l)	Sedimentation	Glucose (mM/l)	Serum proteins (g/l)	Albumins (g/l)
1	23.25	62.2	12.5	25.3	4.03	111	28.41	64	24.1	375	519	1/2	2.9	52.03	35.18
2	20.20	60.2	8.1	31.7	5.72	118	36.26	72	25.2	350	490	1/2	4.9	55.10	40.12
3	16.61	81.7	1.8	16.5	3.47	90	23.46	68	25.8	383	192	1/2	2.6	50.60	34.40
4	11.77	80.2	3.6	16.2	5.71	12.6	36.79	64	22.1	343	428	1/2	2.5	57.20	37.00
5	10.44	78.6	4.6	16.8	6.95	149	44.06	63	21.5	339	448	1/2	7.9	61.24	35.55
6	13.78	66.6	4.0	29.4	5.67	129	37.98	67	22.8	340	445	-	6.7	55.68	29.56
Mean	16.01	71.58	5.77	22.65	5.26	101.60	34.49	66.33	23.27	355.00	420.33	-	4.58	55.31	35.30
± SD	4.99	9.68	3.89	7.04	1.28	47.77	7.36	3.39	1.54	19.15	116.75	-	2.31	3.79	3.46

B, Group treated with metformin (250 mg/kg)

Hamster no.	Leucocytes (10⁹/l)	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)	Erythrocytes (10¹²/l)	Hemoglobin (g/l)	Hematocrit (%)	MCV	MCH	MCHC (g/l)	Platelets (10⁹/l)	Sedimentation	Glucose (mM/l)	Serum proteins (g/l)	Albumins (g/l)
1	14.89	76.5	6.2	15.9	5.80	136	35.67	61	23.4	381	484	1/2	4.5	59.50	44.00
2	25.13	71.31	6.9	21.9	3.94	95	27.21	69	24.1	349	445	1/2	4.5	48.30	31.40
3	18.33	54.5	0.2	15.3	6.73	145	42.81	64	21.5	339	400	1/2	5.1	53.95	29.93
4	9.93	62.3	7.1	30.6	7.22	145	45.13	63	20.0	320	544	1/2	6.7	41.60	31.78
5	10.19	83.1	0.3	16.7	7.00	152	48.83	63	21.7	347	418	1/2	3.4	42.40	25.30
6	14.30	80.4	2.7	16.9	6.72	146	43.09	64	21.9	339	357	1/2	3.1	44.90	23.40
Mean	15.46	71.35	3.90	19.55	6.24	136.50	40.46	64.00	22.10	345.83	441.33	-	4.55	48.44	30.97
± SD	5.69	11.07	3.24	5.90	1.22	20.96	7.78	2.68	1.46	20.04	65.94	-	1.29	7.05	7.23

C, Group treated with itraconazole (250 mg/kg)

Hamster no.	Leucocytes (10⁹/l)	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)	Erythrocytes (10¹²/l)	Hemoglobin (g/l)	Hematocrit (%)	MCV	MCH	MCHC (g/l)	Platelets (10⁹/l)	Sedimentation	Glucose (mM/l)	Serum proteins (g/l)	Albumins (g/l)
1	10.24	85.8	2.0	12.2	6.65	148	44.04	66	22.3	336	365	1/2	3.7	46.70	22.90
2	29.03	72.4	3.7	21.9	4.63	105	38.33	65	22.7	346	417	1/2	3.2	49.30	37.20
3	18.87	81.2	4.6	14.1	6.29	134	39.98	64	21.3	335	220	1/2	5.6	40.00	20.00
4	16.27	83.9	1.3	14.9	6.74	143	42.73	63	21.4	336	347	1/2	7.3	45.60	22.60
5	15.77	82.6	2.0	16.4	6.75	141	40.55	61	20.9	348	436	1/2	5.1	57.40	27.00
6	7.64	84.2	0.8	15.0	6.89	143	41.44	60.	20.7	344	332	1/2	6.1	49.90	24.80
Mean	16.30	81.68	2.40	15.75	6.33	135.67	41.18	63.17	21.55	340.83	352.83	-	5.17	48.15	25.75
± SD	7.49	4.81	1.46	3.31	0.85	15.69	2.03	2.32	0.79	5.81	76.54	-	1.53	5.74	6.08

Table V. Continued.

D, Group co-treated with metformin (250 mg/kg) and itraconazole (250 mg/kg)

Hamster no.	Leucocytes (10⁹/l)	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)	Erythrocytes (10¹²/l)	Hemoglobin (g/l)	Hematocrit (%)	MCV	MCH	MCHC (g/l)	Platelets (10⁹/l)	Sedimentation	Glucose (mM/l)	Serum proteins (g/l)	Albumins (g/l)
1	6.78	86.1	5.4	8.5	5.91	132	37.69	64	22.4	351	37.7	1/2	4.1	52.39	22.79
2	5.83	84.4	0.8	14.8	7.56	150	43.01	57	19.9	349	266	1/2	5.4	51.68	25.15
3	27.97	78.6	2.1	19.3	5.17	119	34.78	67	23.0	341	189	1/2	7.2	44.70	19.20
4	18.50	65.6	5.7	28.7	6.19	135	38.43	62	21.9	352	557	1/2	3.4	45.70	27.10
5	18.97	77.0	4.1	18.9	6.11	129	38.92	63	20.9	332	485	1/2	4.6	45.60	23.60
6	32.62	81.8	2.5	15.7	5.70	126	36.46	64	22.1	345	271	1/2	6.0	43.00	35.80
Mean	18.45	78.92	3.43	17.65	6.11	131.83	38.22	62.83	21.70	345.00	300.95		5.12	47.18	25.61
± SD	10.84	7.36	1.95	6.66	0.80	10.46	2.78	3.31	1.12	7.56	191.51		1.37	3.89	5.65

Normal ranges: leucocytes 3-16·10⁹/l; lymphocytes 50-81%; monocytes 0-3%; granulocytes 17-30%; erythrocytes 4-7·10¹²/l; hemoglobin 100-180 g/l; hematocrit 35-50%; MCV 67-77; MCH 23-27; MCHC 200-360 g/l; platelets 200-500·10⁹/l; sedimentation 1/2; glucose 3-11 mM/l; serum proteins 45-75 g/l; albumins 23-43 g/l.

Table VI. Experimentally obtained half maximal inhibitory concentration values of analyzed drugs on various cell lines after 48 h exposure.

Drug	Normal fetal lung MRC-5	Cervix carcinoma HeLa	Colon carcinoma HT-29	Lung carcinoma A549	Hamster fibrosarcoma BHK-21/C13
Metformin (mM)	37	0.42	0.56	2.2	5.8
Itraconazole (mM)	50	0.70	2.70	4.7	11.0
Metformin + itraconazole (1:1; mM each)	33	0.29	0.42	1.5	3.5

of magnitude of oral metformin doses were administered to the hamsters, compared with the doses administered in a previous study (37). For the 2-week treatment, metformin and itraconazole doses of ~30% of the median lethal dose (LD_{50}) for this species were selected; as metformin LD_{50} is 1,450, 500 mg/kg was used. The daily dose of 500 mg/kg metformin in hamsters is equivalent to the maximum daily dose of 40 mg/kg in patients with diabetes normalized to body surface (38). The dosage of 500 mg/kg/day metformin used in 2-week treatment was twice higher compared with the equivalent of the 20 mg/kg/day typically administered to patients with diabetes. Therefore, a lower dose of metformin (250 mg/kg) was selected for the 3-week treatment to prevent possible toxicity induced by prolonged application of the maximal dose.

During the 2- and 3-week treatments, tumor diameters reached ~2.5 and 3.5 cm in the untreated control groups, respectively. Since the metformin doses in the 2- and 3-week treatment groups were different, the effects of the treatments were evaluated separately for each treatment period. The 3-week treatment results confirmed and validated the results of the 2-week treatment, which demonstrated the significant anticancer effects of the co-treatment on hamster fibrosarcoma.

Itraconazole treatment has been reported to suppress the growth of medulloblastoma and basal cell carcinoma in a mouse allograft model at serum concentrations comparable to those in patients undergoing antifungal therapy (39). The itraconazole levels achieved in the tumors were similar to those in serum, indicating that itraconazole exhibits good tumor tissue penetration (39). The human daily dose of 20 mg/kg itraconazole is equivalent to a daily dose of 250 mg/kg in hamsters normalized to body surface (38). In previous studies, the anti-angiogenic effect of itraconazole was reported to function synergistically in combination with the effects of classical toxic chemotherapeutic drugs, such as paclitaxel, in epithelial ovarian (40) and non-small cell lung carcinoma (41) xenograft mouse models.

The anticancer properties of the present metformin and itraconazole co-treatment doses in hamsters, and the possibility of achieving comparably high nontoxic levels in humans, suggest that the prospect of effective nontoxic oncological therapy and prevention of cancer relapse using this drug combination may be achievable. The present *in vitro* results from normal and malignant cell lines (MRC-5, BHK-21/C13, HeLa, HT-29 and A549) supported the *in vivo* findings from the hamster fibrosarcoma tumors. In addition, in other studies, metformin (42) and itraconazole (11) separately inhibit tumor cell migration and invasion *in vitro*. Furthermore, metformin alone inhibits invasion and migration of human fibrosarcoma cells (42). Therefore, metformin may have the potential to prevent fibrosarcoma metastasis. Itraconazole alone inhibits the proliferation of cancer-associated fibroblasts, serving a key role in cell proliferation, invasion and metastasis in cancer (11). Therefore, the effects of co-treatment with metformin and itraconazole on the migration and invasion of fibrosarcoma cells need to be studied further. Clinical trials are required to elucidate whether the combination of metformin and itraconazole has the potential to become an adjuvant or relapse prevention treatment in current anticancer and particularly antisarcoma therapies.

In conclusion, nontoxic oral doses of metformin combined with nontoxic doses of itraconazole significantly inhibited sarcoma growth in hamsters and exhibited anticancer properties in various cancer cell lines; this combination may be a candidate for novel safe anticancer adjuvant or relapse prevention therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KJP made substantial contributions to the conception and design of the investigation, and performed physical biometrical analyses of the hamster tumors. DJP was a major contributor in writing the manuscript and in performing the blood analyses. DM performed the randomization, tumor inoculation and treatment of hamsters. DL performed the histological and toxicological analyses. IČ performed the immunohistochemical analyses. JKP made substantial contribution to the statistical analysis and interpretation of all experimental data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the University of Novi Sad (approval no. 01-78/18-5; dated April 26, 2016).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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