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Disulfiram and metformin combination anticancer effect reversible partly by antioxidant nitroglycerin and completely by NF-κB activator mebendazole in hamster fibrosarcoma

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ABSTRACT

We investigated the anticancer effect of disulfiram and metformin combination on fibrosarcoma in hamsters. Hamsters of both sexes (~ 70 g) were randomly allocated to control and experimental groups (8 animals per group). In all 10 groups, 2×10^6 BHK-21/C13 cells in 1 ml were injected subcutaneously into the animals' backs. Peroral treatments were carried out with disulfiram 50 mg/kg daily, or with metformin 500 mg/kg daily, or with their combination. Validation and rescue grups were treated by double doses of the single therapy and by the combination with addition of rescue daily doses of ROS inhibitor nitroglycerin 25 mg/kg or NF-κB stimulator mebendazole 460 mg/kg, via a gastric probe after tumor inoculation. After 19 days all animals were sacrificed. Blood samples were collected for hematological and biochemical analyses, the tumors were excised and weighed, and their diameters and volumes were measured. The tumor samples were pathohistologically and immunohistochemically assessed (Ki-67, PCNA, CD34, CD31, COX4, Cytochrome C, GLUT1, iNOS), and the main organs were toxicologically tested. The combination of disulfiram and metformin significantly inhibited fibrosarcoma growth in hamsters without toxicity, compared to monotherapy or control. The single treatments did not show significant antisarcoma effect. Co-treatment with nitroglycerin partly rescued tumor progression, probably by ROS inhibition, while mebendazole completely blocked anticancer activity of the disulfiram and metformin combination, most likely by NF-κB stimulation. Combination of disulfiram with metformin may be used as an effective and safe candidate for novel nontoxic adjuvant and relapse prevention anticancer therapy.

1. Introduction

Drugs that promote increase of reactive oxygen species (ROS) may act as cancer inhibitors, causing oxidative stress, deleterious, genotoxic and proapoptotic effect in cancer cells [1]. Drugs that inhibit inducible transcription nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) may suppress expansion, proliferation, diversification, angiogenesis, adhesion, extravasation, matrix degradation, apoptosis, necrosis and therapeutic resistance of canceras [2]. Although high complexity characterizes the cross-talk of ROS and NF-κB, elevated ROS can inhibit NF-κB and promote cell death [3]. At a contrary, NF-κB activation may attenuate ROS and promote survival [3].

Disulfiram in alcoholism acts by inhibiting aldehyde dehydrogenases. Aldehyde accumulation can induce lipid peroxidation, generation of highly reactive free radicals, ROS and oxidative stress, leading to protein and DNA crosslinking and apoptosis [4]. Disulfiram, as a strong divalent metal ion chelator, can chelate Cu^{++}, what improves the transport of Cu into cancer cells and additionaly induces ROS generation and apoptosis [4]. Chelates of disulfiram with Au^{+++}, Cu^{++}, Zn^{++}, Ag^{+}, Ga^{+++} or Fe^{+++} facilitated intracellular metal ions transport, increased intracellular concentration of metal ions, enhanced ROS generation, NF-κB inhibition, decreased human melanoma cells proliferation in vitro, inhibited growth and angiogenesis in melanomas transplanted in immunodeficient mice [5]. Disulfiram exerted simultaneous induction of ROS and inhibition of NF-κB in breast cancer cells [6]. Antiapoptotic factor NF-κB, responsible for cancer initiation,

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progression, chemo- and radio-resistance, inhibits ROS and dampens ROS-induced cytotoxic, apoptotic effects of many conventional anti-cancer drugs. Disulfiram induces ROS, but inhibits NF-κB in several types of cancer [4]. Therefore, disulfiram could be an unique example of simultaneously ROS increasing with NF-κB decreasing drug [4].

Generation of ROS is one of core steps in disulfiram induced apoptosis in non-Hodgkin lymphoma malignant cell lines in vitro and in vivo [7]. Moreover, ROS-related activation of c-Jun N-terminal kinase (JNK) pathway and inhibition of NF-κB may also contribute to the disulfiram induced apoptosis [7]. In rescue experiment with a ROS scavenger N-acetyl-cysteine (NAC), the activation of JNK, the inhibition of NF-κB and apoptosis by disulfiram were abolished in lymphoid malignant cell lines [7].

Disulfiram was shown to induce oxidative stress, increase ROS production, enhances cancer cytotoxicity of pro-oxidants (H_2O_2), disrupts the NF-κB pathway, activates JNK pathway, forms chelate complexes with metal ions, inhibits cancer stem cells, suppresses cancer cell metastasis, targets epigenetic cancer growth mechanisms, suppresses DNA mutilation, inhibits proteasome activity, enhances the cytotoxicity of anticancer drugs and sensitizes tumor cells to radiotherapy [8].

Functional thiol groups and the thiuram structure were responsible for extracellular and intracellular ROS generation and cytotoxicity of disulfiram in non-small lung cancer cell culture [9]. The induced cell death and apoptosis were reversed by addition of ROS-antagonist NAC into the culture medium [9].

Disulfiram exerted its cytotoxicity by irreversible inhibition of aldehyde dehydrogenase (ALDH) activity, inducing ROS accumulation in a dose-dependent and time-dependent manner, producing ROS accumulation in a very short time, causing instant killing of cells via ROS, inhibited spheroid formation, inhibited stemness-related gene expression, sensitized cancer cells for cisplatin, acted synergistically with cisplatin and overcomed cisplatin resistance in breast cancer cell lines in vitro [10].

Disulfiram reduced proliferation, induced apoptosis and necrosis through ROS stimulation by an ALDH independent mechanism in vitro and in vivo (mouse model), on nasopharyngeal cancer cells and cancer-associated fibroblasts [11]. The ROS scavenger NAC reversed the cellular and lipid ROS levels and rescued cancer activities [11].

Inhibitory effects of disulfiram on gastric cancer cells in vitro were associated with decrease of NF-κB, Wnt (wingless-type) ligand and β-catenin [12]. The anti-apoptotic factor NF-κB enhances the transcription of DNA, responses to cellular noxes and additionally decreases the apoptosis by stimulating the anti-apoptotic gene TRAF (tumor necrosis factor receptor-associated factor) [12]. β-catenin activates Wnt target genes (*Drosophila* gene "wingless" and vertebrate homolog "integrated" or "int-1″) that facilitate cell proliferation and metastasis [12].

It was reported that disulfiram suppresses the proteasome and NF-κB pathways by suppressing ubiquitin E3 ligase activity and also affects epigenetic pathways [13].

Metformin induces endoplasmic reticulum (ER) stress and apoptosis in prostate cancer cell lines [14]. ER stress associates ROS increase [15]. A number of reports prove that metformin activates ROS and induces ER-dependent apoptosis [16]. The recent study of anticancer effect of metformin in ovarian cancer cells in vivo [17] shows that metformin activates apoptosis signal-regulating kinase 1 (ASK1), what is associated with accumulation of ROS that triggers ER stress and apoptosis. Also, the ASK1 activation, associated with ROS accumulation, further activates downstream signaling including JNK, contributing to the apoptosis [17]. Thus, metformin exhibited anticancer effect through apoptosis mediated by ASK1/ROS/ER-stress and ASK1/ROS/JNK pathways [16,17]. Furthermore, the pretreatment of ovarian cancer cells with the ROS scavenger NAC reduced several anticancer effects of metformin, suggesting that ROS accumulation plays significant role in metformin anticancer mechanism [17].

Based on different analyses how metformin induces apoptosis [16–18], significant role belongs to an increase in ROS. For example, metformin induces apoptosis of human gastric adenocarcinoma cells in vitro by contribution (activation) of AMPK (adenosine monophosphate activated protein kinase), suppression of mTOR/AKT (mammalian target of rapamycin/protein kinase B) signaling, inhibition of mitogen-activated protein kinase (MAPK) and by stimulation of ROS production [18].

Metformin induced AMPK (the AMP-activated protein kinase promotes catabolic, inhibits anabolic pathways, growth, proliferation) activation leading to downstream ERK (the extracellular-signal-regulated kinase pathway - one of mayor signaling cassettes of MARK – the mitogen activated protein kinase signaling pathway) and NF-κB inhibition, resulting in proliferation inhibition, apoptosis stimulation and cancer stemness inhibition in lung cancer cells, in vitro and in mouse xenograft models in vivo [19].

Nitroglycerin, donor of nitric oxide (NO) increases activity of antioxidant system in rabbits [20], other mammalians [21] and plants [22], can abate the oxidation chemistry mediated by ROS, such as H_2O_2, O^-, with beneficial effects against oxidative stress.

Antioxidative effect of nitroglycerin is detected also in many other studies [23–26]. NO, which is produced from nitroglycerin, acts as an ROS scavenger [26,27], similarly to NAC. Therefore, nitroglycerin can be also used instead of NAC for ROS antagonization in rescue experiments. Nitroglycerin can participate in antioxidant reactions in doses which are without any hypotensive effects [24,26]. In animals treated with nitroglycerin, glutathione levels are higher compared to controls [26]. It is especially important that nitroglycerin administered alone to rats has not caused reduction of NF-κB activation [26]. Nitroglycerin (and ROS) can cause NF-κB inhibition [3], stimulation [28] or may have no effect on NF-κB [26], depending on cell type [29,30]. It is clear that activity of NF-κB is under the influence of ROS (cross-talks) [3] and that the effects of anti-oxidants, as nitroglycerin, on NF-κB activation are stimulus- and cell-specific [29,30].

Benzimidazoles, especially mebendazole, act as NF-κB stimulators in neuroblast cell culture and in vivo in mice [31] and rats [32]. Mebendazole strongly depolymerizes microtubules [33–35]. Microtubule-depolymerizing agents, especially benzimidazole nocodazole and mebendazole, powerfully activate NF-κB [36–42]. As other benzimidazoles, mebendazole stimulates (overexpresses) NF-κB through 2 steps. The first step: mebendazole causes depolymerization, destruction, degradation of microtubules [33,34]. The second step: depolymerization of microtubules stimulates NF-κB [36–42].

Earlier studies have implied the use of disulfiram [4–13] or metformin [43–48] as promising nontoxic anticancer agents. Capabilities of disulfiram and metforin to triger ROS stimulatory and NF-κB inhibitory anticancer effects, based on previous separate single drug preclinical studies [4–19], were the reasons for testing this drug combination in an experimental hamster fibrosarcoma model. Also, there is a need for improved nontoxic therapies for the treatment of human sarcoma [49–53].

From presented cancer treatment studies with disulfiram, metformin, nitroglycerin and mebendazole we can assuredly conclude that disulfiram acts as ROS stimulator and NF-κB inhibitor, metformin also acts as ROS stimulator and NF-κB inhibitor, nitroglycerin acts as ROS inhibitor and mebendazole acts as NF-κB stimulator. We combined disulfiram with metformin in order to increase ROS, decrease NF-κB and increase anticancer effect of the each single drug. Also, we tried to find whether potential anticancer effect can be abolished by ROS inhibition with nitroglycerin or by NF-κB stimulation with mebendazole. If inhibition of the ROS (by nitroglycerin) and/or stimulation of the NF-κB can block or eliminate the anticancer effect, i.e. can "rescue" the cancer, than disulfiram and metformin anticancer combination targets ROS and/or NF-κB.

As the treatment with either drug alone may cause anticancer effects, the hypothesized combinatory effect could be only the addition of anticancer effects from both alone. To resolve this question, a validation was performed with double doses of single treatments.

2. Materials and methods

2.1. Animal model

The study was performed on Syrian golden hamsters (equal number of males and females; age, ~12 weeks; weight, ~70 g). The hamsters 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 is in accordance with the requirements of the national regulations for the handling of laboratory animals and is approved by the University of Novi Sad Animal Ethics Committee (Novi Sad, Serbia), No. 04–81/25–5 dated 22nd July 2020, Doc. No. ЕК: ΠΙ-Е-2020–07 and also by Ministry of Agriculture, Forestry and Water Management - Veterinary Directorate (Belgrade, Serbia), No. 323–07–09359/2020–05 dated 2nd September 2020. (Supplementary data).

Animal randomization was performed making groups (n = 8 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 disulfiram, metformin, nitroglycerin, mebendazole (all Galenika a.d.) and their combination in experimental groups was initiated after the subcutaneous inoculation of 1 ml of BHK-21/C13 cell suspension [54] (2×10^6 cells/ml) into the back for the development of a subcutaneous fibrosarcoma tumor [55]. BHK-21/C13 cells were cultured as previously reported (Supplementary data). The same researcher performed the tumor inoculation and drug administration. 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 > 3.5 cm, tumor burden > 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.

The experiment involved ten groups of animals administered different treatments via a gastric probe daily after cancer cell inoculation: peroral treatment with physiological saline (group 1 – control); disulfiram 50 mg/kg (group 2); 500 mg/kg metformin (group 3); combination of disulfiram 50 mg/kg and 500 mg/kg metformin (group 4). Validation and rescue groups were treated with double doses of the single therapy (groups 5 and 6) and in addition to the combined therapy rescue daily doses of reactive oxygen species (ROS) inhibitor nitroglycerin 25 mg/kg (group 7) or nuclear factor kappa B (NF-κB) stimulator mebendazole 460 mg/kg (group 8). The last two groups were treated with single doses of nitroglycerin 25 mg/kg (group 9) and mebendazole 460 mg/kg (group 10).

The animals were sacrificed 19 days post-inoculation. Dose of 90 mg/kg pentobarbital was administered intraperitoneally to sacrifice the animals. 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. 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 hematological and biochemical analyses. Vital organs (heart, lungs, liver, kidneys and brain) were removed for detailed pathological and histopathological exmination, after exsanguination and animal death. The weights of the

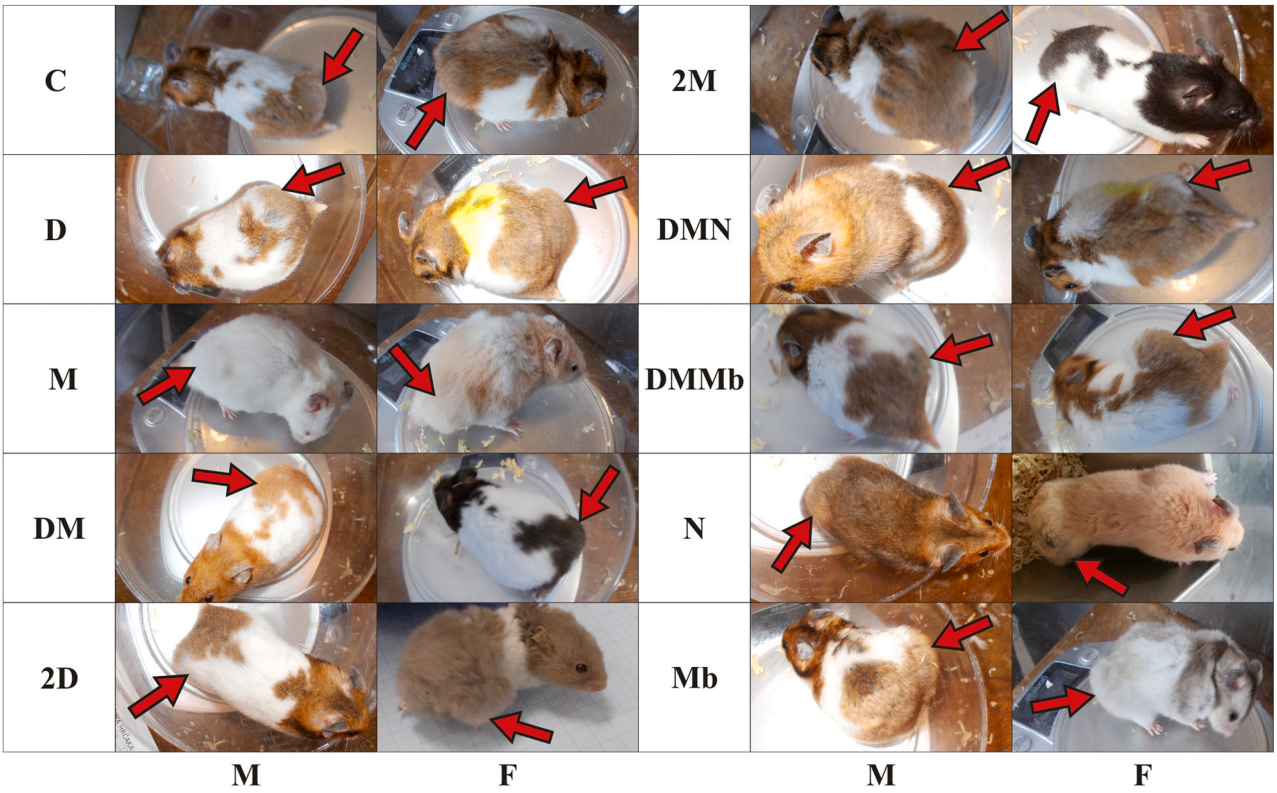

Fig. 1. In the course of experiment, the animal weight, tumor diameter and the tumor burden were evaluated daily. The photos of the first male (M) and female (F) animal in each group before sacrifice are presented: C - Control group; D - Group treated with disulfiram; M - Group treated with metformin; DM - Group treated with the combination of disulfiram and metformin; 2D - Group treated with disulfiram doubled dose; 2M - Group treated with metformin doubled dose; DMN - Group treated with the combination of disulfiram, metformin and nitroglycerin; DMMb - Group treated with the combination of disulfiram, metformin and mebendazole; N - Group treated with nitroglycerin; Mb - Group treated with mebendazole.

animals at the time of sacrifice were recorded. 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 diameters and 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 [56,57] (Fig. 1.). The maximal tumor diameters prior to sacrifice were 3.5 cm. The maximal tumor burdens prior to sacrifice, were 5.82% of the animal body weight. After sacrifice, the tumors were excised and weighed, their diameters were exactly measured, and the exact tumor volume was determined using the standard water volume displacement method.

In our experiment, disulfiram was dissolved (suspended) in physiological saline and administered to hamsters at a dose of 50 mg/kg (~ 10% of oral median lethal dose LD_{50}) daily (in 1 ml/100 g animal weight), equivalent to a usual human dose of 4 mg/kg (by normalization to body surface area) [58–60]. Metformin was dissolved in physiological saline and administered to hamsters at a dose of 500 mg/kg (~ 25% of oral median lethal dose LD_{50}) daily (in 1 ml/100 g animal weight), equivalent to a human dose of 40 mg/kg (by normalization to surface area) [58–60], which are the maximal daily doses taken by patients with diabetes [61]. The dose of nitroglycerin 25 mg/kg (~ 25% of oral LD_{50}) was equivalent to the maximum human dose of 2 mg/kg [58–60], and it was prepared and administered in the same manner as previously. The control group received physiological saline (1 ml/100 g animal weight). In the 2 groups, doses of disulfiram and metformin single drug treatments were doubled. For 2 groups, mebendazole was suspended in pure olive oleum and administered orally at a dose of 460 mg/kg daily (~50% of oral LD_{50} for hamsters), equivalent to an oral human dose of 36,8 mg/kg normalized to body surface area [58–60], which is comparable to human daily dose of 40–50 mg/kg for the 1–6 months treatment of echinococcosis.

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, relative weight, surface/maximal length ratio, surface/tumor weight ratio, surface/density ratio and maximal length/density ratio among the treated groups of animals were also calculated.

Tumor slices (4 μm) were assessed histopathologically for the verification of tumor growth, tissue penetration, expansion of necrosis and hemorrhagic areas, and immunohistochemically for the proliferation, angiogenesis, apoptosis, glucose and NO-metabolism. The weight of the hamsters was recorded to evaluate possible side effects of nitroglycerin and/or metformin. The vital animal organs (brain, heart, lungs, liver and kidneys) were analyzed pathologically and histopathologically, and no obvious changes, nor toxicological effects of drugs used in this study were detected.

2.2. Histological staining procedures

The principal hematoxylin-eosin (HE) staining and immunohistochemical Ki-67, PCNA, CD34, CD31, COX4, Cytochrome C, GLUT1 and iNOS staining (Supplementary data) was performed to assess tumor proliferation (Ki-67, PCNA), neoangiogenesis (CD34, CD31), apoptosis (COX4, Cytochrome C), glucose metabolism (GLUT1) and NO metabolism (iNOS) in all groups in the same manner as previously reported [48].

Primary antibodies (Thermo Fisher Scientific, Abcam), antigen retrieval in sections in goat serum (Sigma-Aldrich), horseradish goat polyclonal rabbit immunoglobulin G secondary antibody (Abcam), visualization with chromogen (Dako; Agilent Technologies, Inc.), staining with Mayer's hematoxylin, Leica microscope with Leica camera (Leica Microsystems GmbH) and UTHSCSA Image Tool for Windows [62] for evaluation of immunoexpression were used as described in our previous report [48].

2.3. Blood hematological analyses and biochemical tests

Samples for standard blood laboratory tests (erythrocytes, leucocytes, lymphocytes, monocytes, granulocytes, platelets, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, glucose, serum proteins, albumins and sedimentation) were collected from animals and processed in the same way as previously reported [48] (Supplementary data).

2.4. Statistical analysis

Mean ± SD are presented. The differences among the groups in all 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 values less than 0.05 were considered statistically significant.

3. Results

BHK-21/C13 inoculation resulted in tumor formation at the site of injection in all animals (Fig. 1.). All hamsters 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.

In the our experiment, *per oral* co-treatment with disulfiram and metformin significantly ($P < 0.05$) inhibited tumor growth as indicated by significant ($P < 0.05$) decreases in tumor weight, length, volume, surface area, relative weight, density, ratio of tumor surface area to volume, surface/maximal length ratio, surface/tumor weight ratio, surface/density ratio and maximal length/density ratio, compared with control, all single treatments and all triple treatments (Table I, Fig. 2.–4., Supplemental Fig. 1.). Precise statistical significances expressed as P values for all mentioned data comparisons are shown in Table II (rows: C/DM, D/DM, M/DM, 2D/DM, 2 M/DM, DMN/DM, DMMb/DM). NF-κB suppression is more effective than ROS suppression ($P < 0.05$) in rescuing tumor growth, by physicochemical comparisons (Table I, Fig. 2.–4., Supplemental Fig. 1.). Exact statistical significances for these data comparisons - P values are shown in Table II (rows: C/DMN, DMN/DM, C/DMMb, DMMb/DM, DMN/DMMb).

In all analyzed slices of tumors from animals treated with the combination of disulfiram and metformin, compared with the control group, all single and all triple treatments, the pathohistological and immunohistochemical evaluation revealed a decrease in tissue penetration, an expansion of necrosis and hemorrhagic areas (Supplemental Fig. 2.), significantly ($P < 0.05$) decreased proliferation status of tumor cells, as demonstrated by Ki-67 and PCNA, significant ($P < 0.05$) inhibition of tumor vasculature, as demonstrated by CD34 and CD31, significant ($P < 0.05$) difference in apoptosis intensity, as demonstrated by COX4 and Cytochrome C, significant ($P < 0.05$) inhibition of glucose metabolism, as demonstrated by GLUT1, and significant ($P < 0.05$) inhibition of NO metabolism, as demonstrated by iNOS staining (Fig. 5., 6.). Precise statistical significances for all these data comparisons as P values are shown in Table III (rows: C/DM, D/DM, M/DM, 2D/DM, 2M/DM, DMN/DM, DMMb/DM). NF-κB is again more effective than ROS suppression in rescuing tumor expansion (Fig. 5., 6.). Exact statistical significances - P values are in Table III (rows: C/DMN, DMN/DM, C/DMMb, DMMb/DM, DMN/DMMb).

Neither disulfiram nor metformin single treatments, regardless the dose, exhibited significant anticancer effect ($P > 0.05$) in comparison to control (Table I, Fig. 2.–6., Supplemental Fig. 1.). Precise statistical significances for all mentioned data comparisons are shown as P values

Table I
Characteristics of animals and quantitative pathological characteristics of tumors in control and treated groups.

No	Hamster			Tumor			Hamster			Tumor		
	Weight at start (g)	Weight at end (g)	Sex	Weight (g)	D_{max} (cm)[a]	Volume (cm^3)	Weight at start (g)	Weight at end (g)	Sex	Weight (g)	D_{max} (cm)[a]	Volume (cm^3)
	Control group (C)						Group treated with metformin double dose (2 M)					
1	69	90	M	4.45	3.4	3.5	71	89	M	3.30	1.9	2.7
2	57	85	M	4.95	3.4	4.0	64	87	M	3.62	2.1	2.9
3	71	96	M	3.90	2.8	2.9	68	95	M	3.50	2.8	2.8
4	66	95	M	4.10	3.0	3.3	66	88	M	4.99	3.5	4.3
5	69	98	F	4.87	3.5	4.2	64	95	F	3.84	2.2	3.0
6	64	86	F	4.98	3.5	4.4	72	90	F	3.67	3.0	2.9
7	67	92	F	4.00	3.5	3.4	62	85	F	3.73	2.0	3.0
8	58	94	F	3.80	2.9	2.9	63	84	F	4.46	2.6	3.7
Mean	65.1	92.0		4.38	3.25	3.58	66.3	89.1		3.89	2.51	3.16
±SD	5.17	4.69		0.50	0.30	0.57	3.73	4.12		0.56	0.56	0.55
	Group treated with disulfiram (D)						Group treated with the combination of disulfiram, metformin and nitroglycerin (DMN)					
1	59	88	M	4.66	3.5	4.0	71	93	M	3.00	2.0	2.7
2	67	94	M	2.97	2.2	2.5	68	88	M	2.65	2.2	2.3
3	70	97	M	4.96	3.5	4.1	73	95	M	3.70	2.5	3.2
4	63	78	M	2.55	3.1	2.0	69	92	M	1.20	1.6	1.0
5	65	89	F	4.94	3.5	4.1	67	89	F	2.30	3.3	2.0
6	62	84	F	3.80	2.5	3.1	70	91	F	1.95	1.7	1.7
7	71	99	F	4.98	3.5	4.2	74	98	F	1.20	1.5	1.0
8	65	86	F	4.91	3.2	4.1	75	94	F	3.73	3.5	3.4
Mean	65.3	89.4		4.22	3.13	3.51	70.9	92.5		2.47	2.29	2.16
±SD	4.03	7.01		0.99	0.51	0.86	2.90	3.25		0.10	0.76	0.91
	Group treated with metformin (M)						Group treated with the combination of disulfiram, metformin and mebendazole (DMMb)					
1	69	92	M	3.44	3.5	2.9	64	97	M	2.25	3.0	3.5
2	71	99	M	3.55	3.3	2.9	67	86	M	4.85	3.5	4.0
3	65	78	M	3.90	3.5	3.1	68	95	M	4.22	3.0	3.4
4	73	92	M	4.60	3.5	3.8	64	98	M	4.20	3.1	3.3
5	75	85	F	4.80	3.3	4.0	65	85	F	4.80	3.5	4.0
6	69	86	F	2.86	2.2	2.3	76	98	F	4.21	3.3	3.3
7	73	91	F	4.20	3.0	3.4	75	91	F	4.55	3.5	3.8
8	70	89	F	4.70	3.5	3.9	71	93	F	4.22	3.2	3.3
Mean	70.6	89.0		4.01	3.22	3.29	68.8	92.9		4.41	3.26	3.58
±SD	3.11	6.19		0.69	0.45	0.59	4.77	5.17		0.28	0.22	0.31
	Group treated with the combination of disulfiram and metformin (DM)						Group treated with nitroglycerin (N)					
1	63	86	M	0.65	1.7	0.6	69	99	M	4.00	3.5	3.2
2	76	95	M	0.40	0.7	0.3	72	96	M	3.40	2.1	2.8
3	66	92	M	0.70	1.7	0.7	68	89	M	3.94	2.5	3.1
4	68	97	M	0.30	1.0	0.3	71	92	M	3.95	2.5	3.2
5	69	96	F	1.00	1.2	0.9	76	98	F	4.60	3.2	3.7
6	70	99	F	0.82	1.7	0.8	75	94	F	2.82	2.5	2.2
7	72	93	F	0.35	0.8	0.3	64	93	F	4.98	3.5	4.3
8	74	95	F	0.95	1.5	0.9	70	95	F	3.20	2.3	2.5
Mean	69.8	94.1		0.65	1.29	0.60	70.6	94.5		3.86	2.76	3.13
±SD	4.23	3.94		0.27	0.42	0.27	3.85	3.25		0.71	0.55	0.66
	Group treated with disulfiram double dose (2D)						Group treated with mebendazole (Mb)					
1	65	90	M	4.80	3.0	3.9	75	95	M	4.97	3.5	4.2
2	73	96	M	3.36	2.8	2.7	71	98	M	2.50	2.0	2.0
3	75	99	M	3.60	2.7	2.8	70	97	M	4.28	3.3	3.5
4	64	88	M	3.90	2.8	3.0	68	89	M	2.40	2.4	2.0
5	68	92	F	4.93	3.5	4.0	67	91	F	4.20	2.7	3.5
6	66	86	F	3.47	3.0	3.0	69	92	F	4.25	2.7	3.6
7	62	85	F	3.45	3.2	2.9	72	88	F	4.28	3.5	3.6
8	65	94	F	4.48	3.5	3.6	66	93	F	4.30	3.5	3.6
Mean	67.3	91.3		4.00	3.06	3.24	69.8	91.6		3.90	2.95	3.25
±SD	4.53	4.92		0.64	0.31	0.51	2.92	3.70		0.93	0.58	0.80

[a] Longest tumor diameter (cm). Sex: F, female; M, male.

in Tables II and III (in both tables rows: C/D, C/M). Between single disulfiram and metformin treatments there are no significant ($P > 0.05$) differences in anticancer activity, regardless the dose (Table I, Fig. 2.–6., Supplemental Fig. 1.). Exact statistical significances for mentioned data comparisons are shown as P values in Tables II and III (in both tables rows: D/M).

The treatments had no significant ($P > 0.05$) effect on the body weight of the hamsters during the course of the experiment, compared with the control (Table I). The experimental and control groups were statistically compared for red and white blood cell counts, platelet number, hemoglobin levels, hematocrit levels, glucose levels, serum proteins and sedimentation (Supplemental Table I), but no significant differences were observed among the groups ($P > 0.05$). In addition, pathological and histopathological examination of the main organs (brain, heart, lungs, liver and kidneys) didn't reveal any pathological, histopathological or toxicological changes, or differences between the experimental and control groups.

During the treatments, tumor diameters reached a maximum of 3.5 cm. The results confirmed the significant anticancer effects of the co-treatment on hamster fibrosarcoma, without toxicity.

Contrary to the combined disulfiram and metformin therapy and combined disulfiram, metformin and nitroglycerin therapy, combined

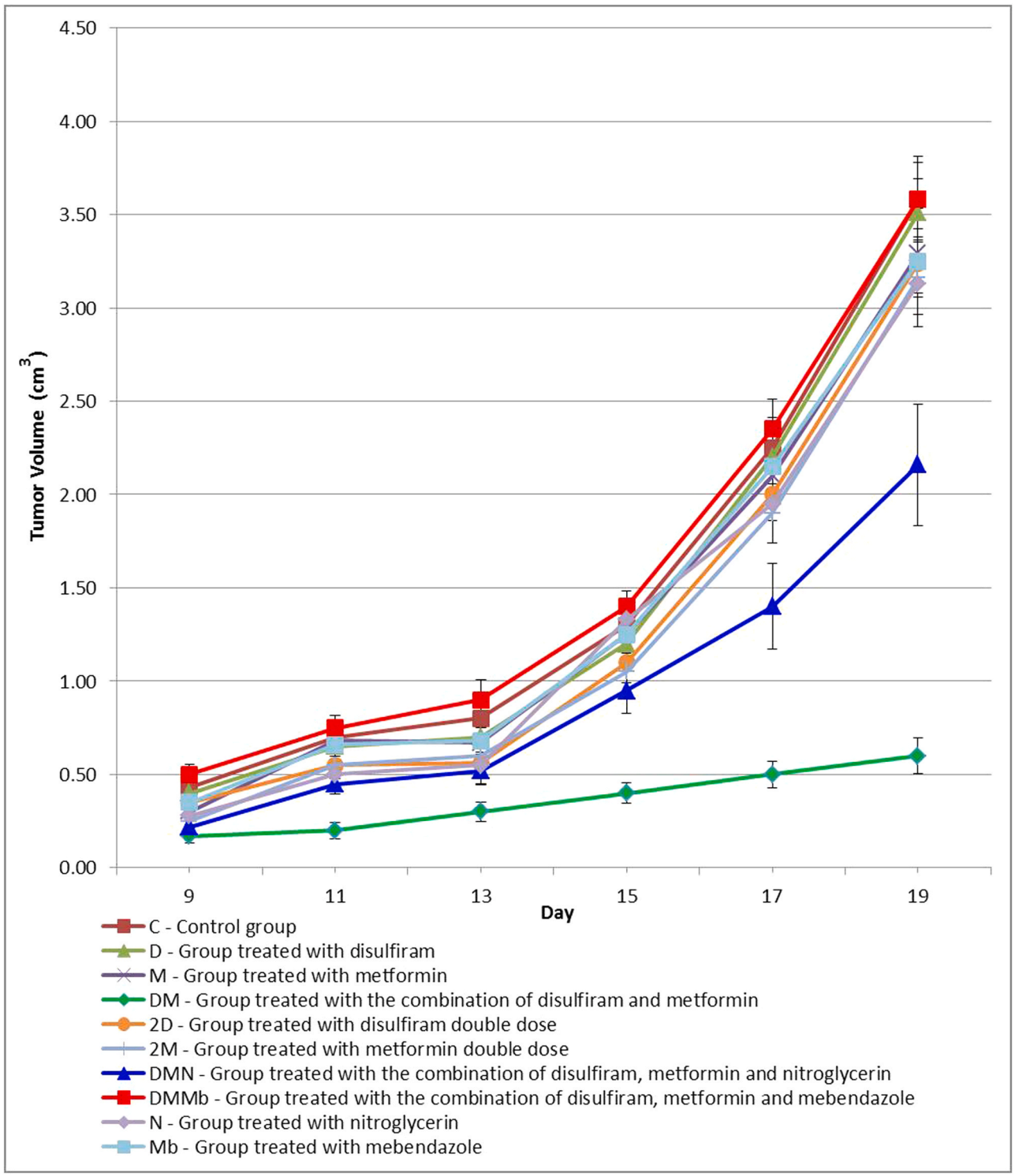

Fig. 2. Tumor volume growth during course of the experiment: interpolated line chart between average values and SEM values.

disulfiram, metformin and mebendazole therapy, single nitroglycerin and single mebendazole therapy have no significant ($P > 0.05$) anticancer effect on fibrosarcoma in hamsters, regarding tumor weight, diameter, volume, density, Ki-67, nor all other quoted physicochemical and immunohistochemical parameters (Table I, Fig. 2.–6., Supplemental Fig. 1.). Precise statistical significances for all mentioned comparisons, as P values, are in Tables II and III (in both tables rows: C/DMMb, C/N, C/Mb). The nitroglycerin given with combined disulfiram and metformin therapy partly inhibited sarcoma growth, in comparison to strong anticancer effect seen with the disulfiram and metformin combination ($P < 0.05$). The mebendazole given with combined disulfiram and metformin therapy, as single mebendazole treatment, do not influence sarcoma growth, in contrast to significant anticancer effect seen with the disulfiram and metformin combination ($P < 0.05$).

Co-treatment with nitroglycerin partly eliminates anticancer effect of the disulfiram and metformin combination. Tumor progression inhibited by disulfiram and metformin combination was partly rescued by nitroglycerin (Table I, Fig. 2.–6., Supplemental Fig. 1.). Precise

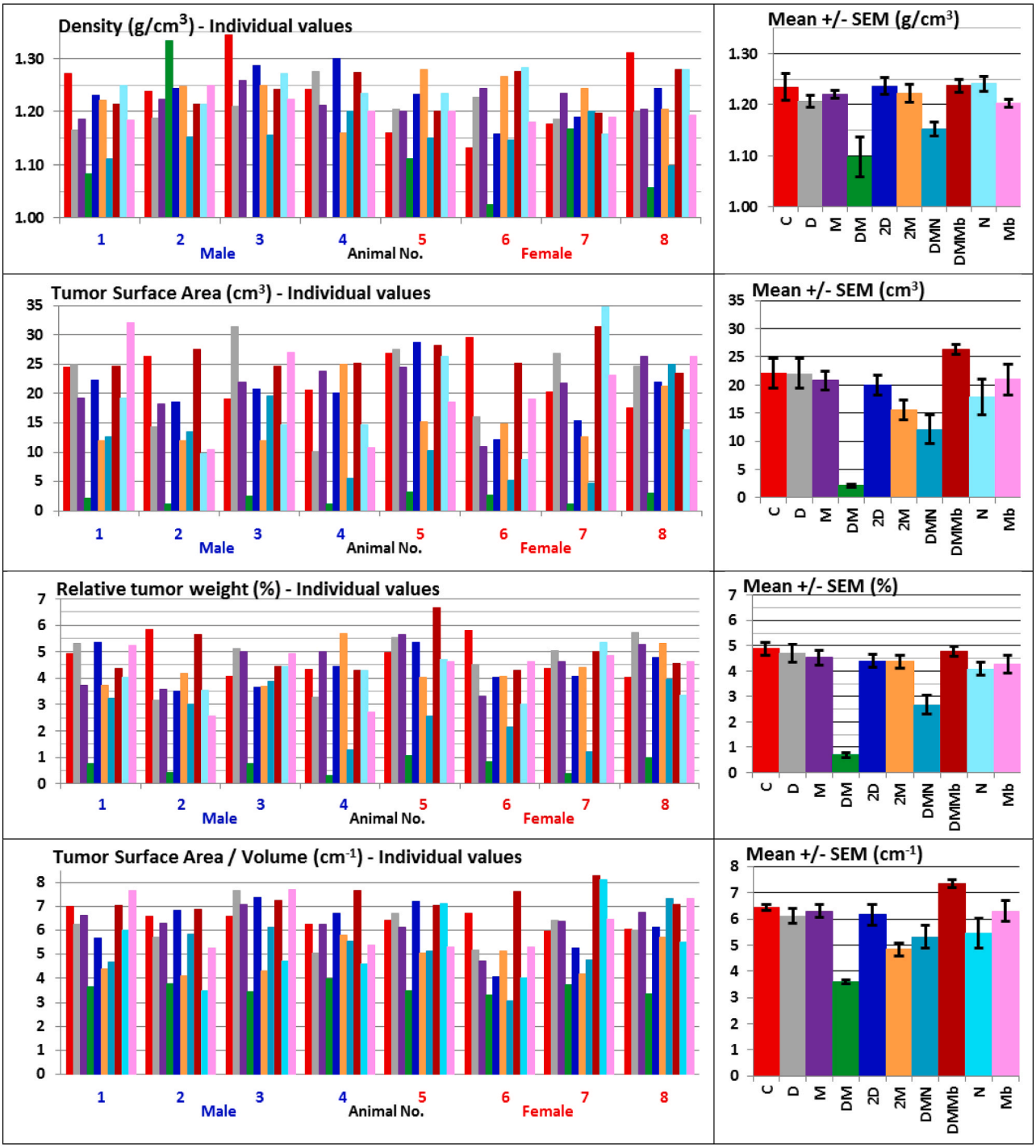

Fig. 3. Individual values with means and standard errors (SEM) of quantitative pathological and physicochemical characteristics of the excised tumors. Density, tumor surface area, relative tumor weight, tumor surface area/volume ratio among the treated groups of animals in the experiment: C - Control group; D - Group treated with disulfiram; M - Group treated with metformin; DM - Group treated with the combination of disulfiram and metformin; 2D - Group treated with disulfiram doubled dose; 2M - Group treated with metformin doubled dose; DMN - Group treated with the combination of disulfiram, metformin and nitroglycerin; DMMb - Group treated with the combination of disulfiram, metformin and mebendazole; N - Group treated with nitroglycerin; Mb - Group treated with mebendazole.

statistical significances for these data as P values are shown in Tables II and III (in both tables rows: C/DMN, DMN/DM). As indicated by CD34 and CD31 markers of angiogenesis, HIF-1α (and downstream target VEGF) activity suppressed by disulfiram and metformin combination, was partly rescued by nitroglycerin (statistical significances in Table III, rows: C/DMN, DMN/DM).

Co-treatment with mebendazole completely eliminates anticancer effect of the disulfiram and metformin combination. Tumor progression inhibited by disulfiram and metformin combination was completely rescued by mebendazole (Table I, Fig. 2.–6., Supplemental Fig. 1.). Precise statistical significances for these data, as P values, are shown in Tables II and III (in both tables rows: C/DMMb, DMMb/DM). As indicated by CD34 and CD31 markers of angiogenesis, HIF-1α (and downstream target VEGF) activity suppressed by disulfiram and metformin combination, was completely rescued by mebendazole (statistical significances in Table III, rows: C/DMMb, DMMb/DM).

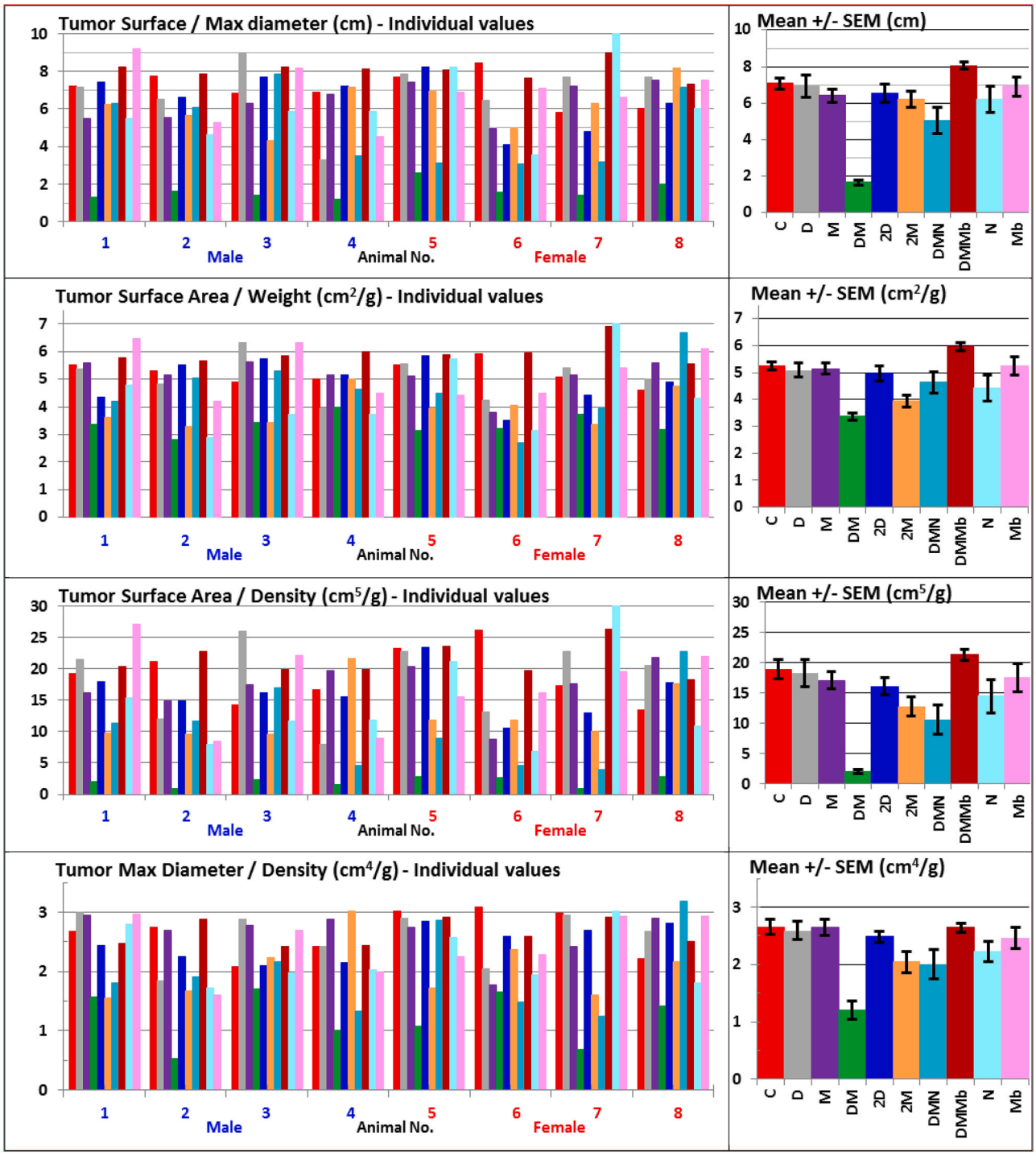

Fig. 4. Individual values with means and standard errors (SEM) of quantitative pathological and physicochemical characteristics of the excised tumors. Tumor surface/max. diameter ratio, tumor surface area/weight ratio, tumor surface area/density ratio, tumor max. diameter/density ratio among the treated groups of animals in the experiment: C - Control group; D - Group treated with disulfiram; M - Group treated with metformin; DM - Group treated with the combination of disulfiram and metformin; 2D - Group treated with disulfiram doubled dose; 2M - Group treated with metformin doubled dose; DMN - Group treated with the combination of disulfiram, metformin and nitroglycerin; DMMb - Group treated with the combination of disulfiram, metformin and mebendazole; N - Group treated with nitroglycerin; Mb - Group treated with mebendazole.

However, although addition of antioxidative NO donor and the ROS scavenger nitroglycerin to the disulfiram and metformin combined treatment partly (statistically significant $P < 0.05$) rescued cancer growth (exact statistical significances in Tables II and III, rows: DMN/DM), it did not completely eliminate the antiproliferative effect of the disulfiram and metformin combination and significantly ($P < 0.05$) differs from control treatment (exact statistical significances in Tables II and III, rows: C/DMN).

At the contrary, the NF-κB stimulator mebendazole added to the combined disulfiram and metformin treatment completely rescued ($P < 0.05$) fibrosarcoma growth (exact statistical significances in Tables II and III, rows: DMMb/DM) without significant ($P > 0.05$) difference from control treatment (exact statistical significances in Tables II and III, rows C/DMMb).

Table II
Statistical significances expressed as *P*-values for comparisons of pathological and physicochemical tumor characteristics.

Group comparison	Weight	Length	Volume	Surface area	Relative weight	Density	Surface/ volume	Surface/ diameter	Surface/ weight	Surface/ density	Diameter / density
C/D	0.597	0.632	0.901	0.699	0.697	0.943	0.420	0.899	0.707	0.893	0.789
C/M	0.283	0.845	0.404	0.391	0.442	0.554	0.539	0.199	0.730	0.461	1.000
[a]C/DM	0.00013[a]	0.00083[a]	0.00055[a]	0.00048[a]	0.00030[a]	0.01109[a]	0.00010[a]	0.00034[a]	0.00081[a]	0.00075[a]	0.00197[a]
D/M	0.671	0.691	0.596	0.687	0.682	0.413	0.601	0.473	0.904	0.716	0.782
[a]D/DM	0.00090[a]	0.00108[a]	0.00096[a]	0.00114[a]	0.00089[a]	0.01601[a]	0.00324[a]	0.00104[a]	0.00206[a]	0.00129[a]	0.00224[a]
[a]M/DM	0.00057[a]	0.00099[a]	0.00063[a]	0.00088[a]	0.00069[a]	0.00910[a]	0.00081[a]	0.00059[a]	0.00123[a]	0.00090[a]	0.00200[a]
C/2D	0.287	0.212	0.283	0.206	0.199	0.999	0.563	0.451	0.462	0.280	0.409
C/2 M	0.086	0.00970[a]	0.261	0.00865[a]	0.198	0.692	0.00101[a]	0.159	0.00487[a]	0.01798[a]	0.01950[a]
2D/2 M	0.697	0.03107[a]	0.721	0.107	0.999	0.537	0.01550[a]	0.693	0.01497[a]	0.154	0.052
[a]2D/DM	0.00051[a]	0.00086[a]	0.00051[a]	0.00098[a]	0.00050[a]	0.00810[a]	0.00102[a]	0.00090[a]	0.00209[a]	0.00092[a]	0.00213[a]
[a]2M/DM	0.00049[a]	0.00491[a]	0.00060[a]	0.00101[a]	0.00053[a]	0.01003[a]	0.00470[a]	0.00079[a]	0.03011[a]	0.00204[a]	0.00849[a]
[a]C/DMN	0.00171[a]	0.00865[a]	0.00721[a]	0.00718[a]	0.00470[a]	0.01414[a]	0.03770[a]	0.02004[a]	0.269	0.01185[a]	0.04771[a]
[a]DMN/DM	0.00150[a]	0.00892[a]	0.00413[a]	0.00688[a]	0.00375[a]	0.202	0.00721[a]	0.00611[a]	0.01643[a]	0.00699[a]	0.01954[a]
C/DMMb	0.922	0.998	1.0	0.115	0.700	0.984	0.00509[a]	0.01787[a]	0.00787[a]	0.279	0.998
[a]DMMb/DM	0.00005[a]	0.00077[a]	0.00009[a]	0.00006[a]	0.00021[a]	0.00782[a]	0.00009[a]	0.00005[a]	0.00052[a]	0.00009[a]	0.00099[a]
[a]DMN/ DMMb	0.00189[a]	0.00894[a]	0.00555[a]	0.00353[a]	0.00414[a]	0.00520[a]	0.00604[a]	0.00616[a]	0.00984[a]	0.00601[a]	0.02976[a]
C/N	0.115	0.04990[a]	0.277	0.249	0.053	0.873	0.111	0.259	0.201	0.270	0.097
C/Mb	0.211	0.273	0.411	0.568	0.273	0.386	0.698	0.790	0.999	0.559	0.400

[a]$P < 0.05$. C - control group; D - group treated with disulfiram; M - group treated with metformin; DM - group treated with the combination of disulfiram and metformin; 2D - group treated with disulfiram double dose; 2M - group treated with metformin double dose; DMN - group treated with the combination of disulfiram, metformin and nitroglycerin; DMMb - group treated with the combination of disulfiram, metformin and mebendazole; N - group treated with nitroglycerin; Mb - group treated with mebendazole.

Furthermore, the addition of the NF-κB stimulator mebendazole to the combined disulfiram and metformin treatment significantly ($P < 0.05$) rescued fibrosarcoma growth (antagonized with the disulfiram and metformin combination) with respect of the addition of the antioxidative NO donor and the ROS scavenger nitroglycerin to the combined disulfiram and metformin treatment (exact statistical significances in Tables II and III, rows DMN/DMMb).

Single treatments with nitroglycerin and mebendazole, for all measured parameters, failed to reduce fibrosarcoma growth compared with the control ($P > 0.05$, exact P values in Tables II and III, rows C/N, C/Mb).

4. Discussion

Previous research has found that metformin at cellular level inhibits complex I of the mitochondrial electron transport chain, which leads to membrane depolarization and the release of ROS [63]. Inhibition of mitochondrial respiratory complex I increases the aberrant flow of electrons to oxygen and creates superoxide within the mitochondrial matrix and can generate the ROS. ROS are involved in the chemical damage of cell components and can promote apoptosis.

When, in vitro, live ovarian cancer cells were treated with metformin, they showed increased ROS [64]. The complex I inhibition is involved in metformin's growth inhibition of ovarian cancer by increasing ROS and sensitizing cancer to additional oxidative stress (possibly produced by other medication) [64]. Metformin elevated intracellular ROS levels and production to 4-fold of control in luminal breast cancer cells [65]. After metformin treatment, it was observed 2.5–3-fold increases in ROS production in the colorectal cancer cells [66].

Furthermore, it has been suggested that elevated ROS by metformin activates AMPK [67], one of main metformin's anticancer mechanisms. Namely, AMPK activation by metformin inhibits mammalian target of rapamycin (mTOR) signaling and activates tumor proliferation suppressor p53, arresting the cell cycle [48]. Elevated ROS production enhance AMPK activation in the endothelium of patients with coronary artery disease and diabetes [67].

Findings that metformin can only transiently inhibit the growth and proliferation of colorectal cancer cells by promoting oxidative stress, by elevating ROS and by activating AMPK, suggest that its use alone is not sufficient for cancer treatment [66]. It was suggested that future studies should consider strategies for synergistic combinations of metformin with other drugs that can inhibit cell growth [66], what is in line with our previous [45–48] and current results.

Metformin inhibited NF-κB in various types of normal and malignant cells [68–71].

Results of NF-κB inhibition by metformin are as follows. Metformin inhibits proinflamatory vascular responses by inhibiting NF-κB [69], regulates inflammation and kidney disease through NF-κB inhibition [72], eliminates susceptibility to common diseases and ageing diseases by inhibiting expression of NF-κB gene [73] and inhibits various tumors growth as a result of inhibiting NF-κB in cancers, e.g. in esophageal squamous carcinoma [71], breast cancer [74] and lung cancer [19] cells.

Metformin (NF-κB inhibitor) has certain anticancer effects on BHK-21/C13 induced fibrosarcoma in hamsters if applied 7 days before tumor inoculation or together with caffeine or itraconazole (both inhibit NF-κB) [45–47], or nitroglycerin [48].

Disulfiram suppressed proliferation of various malignant cell types also via ROS activation [7,75–79], as well as simultaneous NF-κB inhibition [4–6]. Simultaneous induction of ROS and inhibition of NF-κB by disulfiram is detected earlier in breast cancer cells [6].

It is recorded that inhibition of NPL4 (nuclear protein localization protein 4) plays an important role in disulfiram's NF-κB inhibition. The p97 (valosin containing protein - VCP), UFD1L (Ubiquitin fusion degradation protein 1 homolog) and NPL4 complex (p97-UFD1L-NPL4) decreases IκBα (inhibitor of NF-κB) and increases NF-κB activity [80]. The p97-UFD1L-NPL4 complex enhances IκBα proteolysis and causes IκBα reduction, important for NF-κB activation [80]. p97 and UFD1L are important for the activation of NF-κB [80]. Given that NPL4 decreasing – decreases UFD1L, similar results if we decrease NPL4 can be obtained [80]. Based on these data, it can be concluded that the p97-UFD1L-NPL4 protein complex is important for the activation of NF-κB. UFD1L and NPL4 are cofactors of p97 in proteolysis and reduction of IκBα and consequently of NF-κB activation [80]. The NPL4 positively regulates NF-κB [81]. Specific recognition of linear ubiquitin chains by the NPL4 were detected a long time ago [82].

Newer publications [13,83–90] also indicate that disulfiram inhibits NF-κB through inhibition of NPL4.

Targeting NPL4 via disulfiram can be used for the treatment of clear cell renal cell carcinoma, through NPL4, NF-κB, VEGFR and tumor

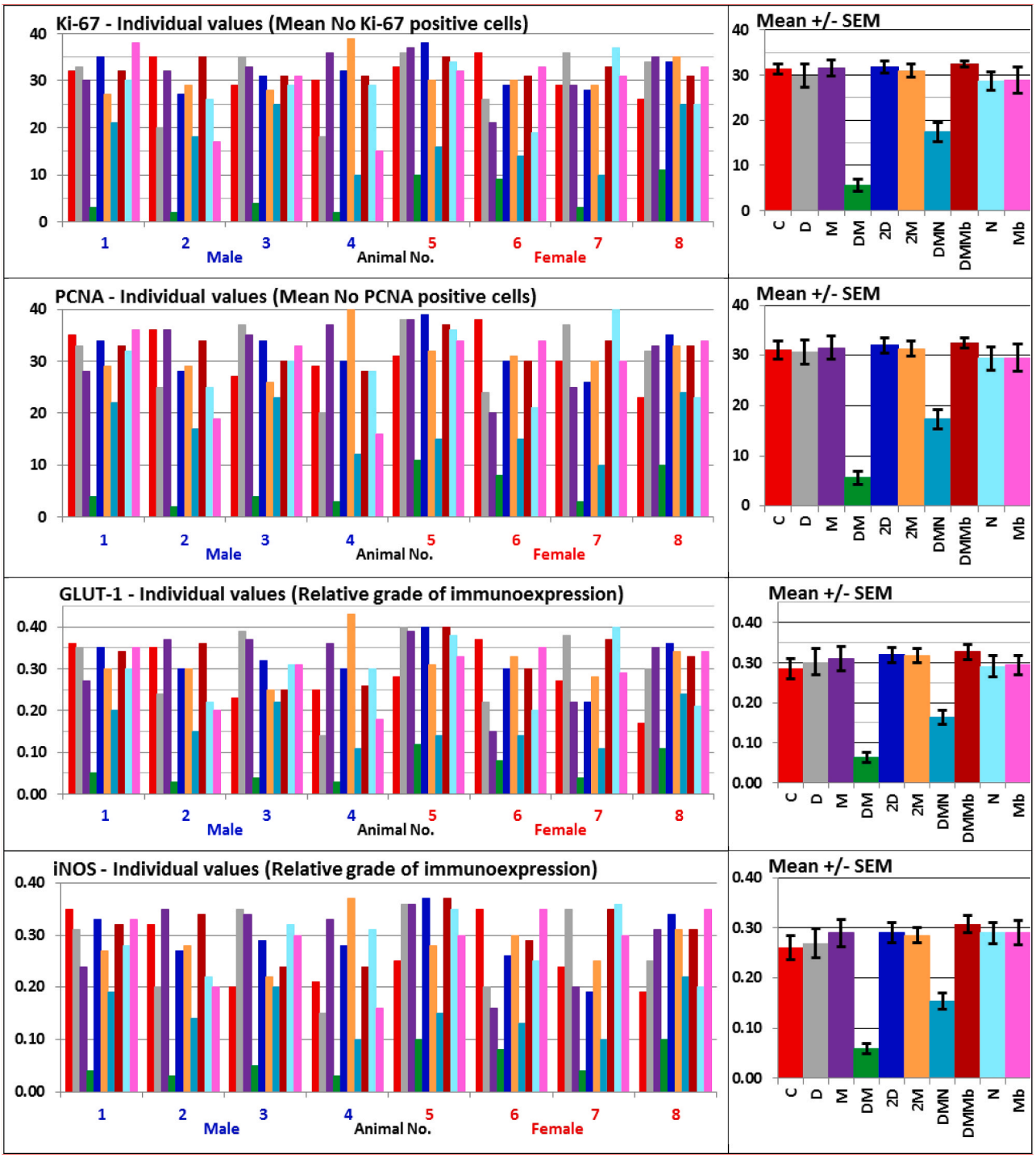

Fig. 5. Individual values with means and standard errors (SEM) of histopathological-immunohistochemical characteristics of the excised tumors in the experiment: Ki-67, PCNA, GLUT-1, iNOS. C - Control group; D - Group treated with disulfiram; M - Group treated with metformin; DM - Group treated with the combination of disulfiram and metformin; 2D - Group treated with disulfiram doubled dose; 2M - Group treated with metformin doubled dose; DMN - Group treated with the combination of disulfiram, metformin and nitroglycerin; DMMb - Group treated with the combination of disulfiram, metformin and mebendazole; N - Group treated with nitroglycerin; Mb - Group treated with mebendazole.

angiogenesis inhibition [83]. NPL4 was increased in renal carcinoma [83]. Greater NPL4 values were connected with poorer survival and lower NPL4 values means better survival in renal cancer [83]. Disulfiram has antitumor effect in renal carcinoma via inhibition of NPL4 [83]. Disulfiram's anticancer activity reflects targeting NPL4 and kills diverse human cancer cell types through aggregation and reduction of NPL4, a subunit of the p97/VCP segregase [84]. Disulfiram as a therapeutic agent for metastatic malignant melanoma increased levels of ROS and DNA damage, immobilized (inactivated) NPL4 in focal clusters in the nucleus and cytoplasm, inhibited NF-κB and favored cancer cell death [85].

The disulfiram metabolite diethyldithiocarbamate (ditiocarb, DTC) cooper complex (CuDTC) bound to NPL4, an adaptor protein of the p97 segregase, induces NPL4 aggregation immobilization and inhibition in breast cancer and myeloma xenograft models [87]. These findings suggest a model in which disulfiram is rapidly converted into CuDTC, which

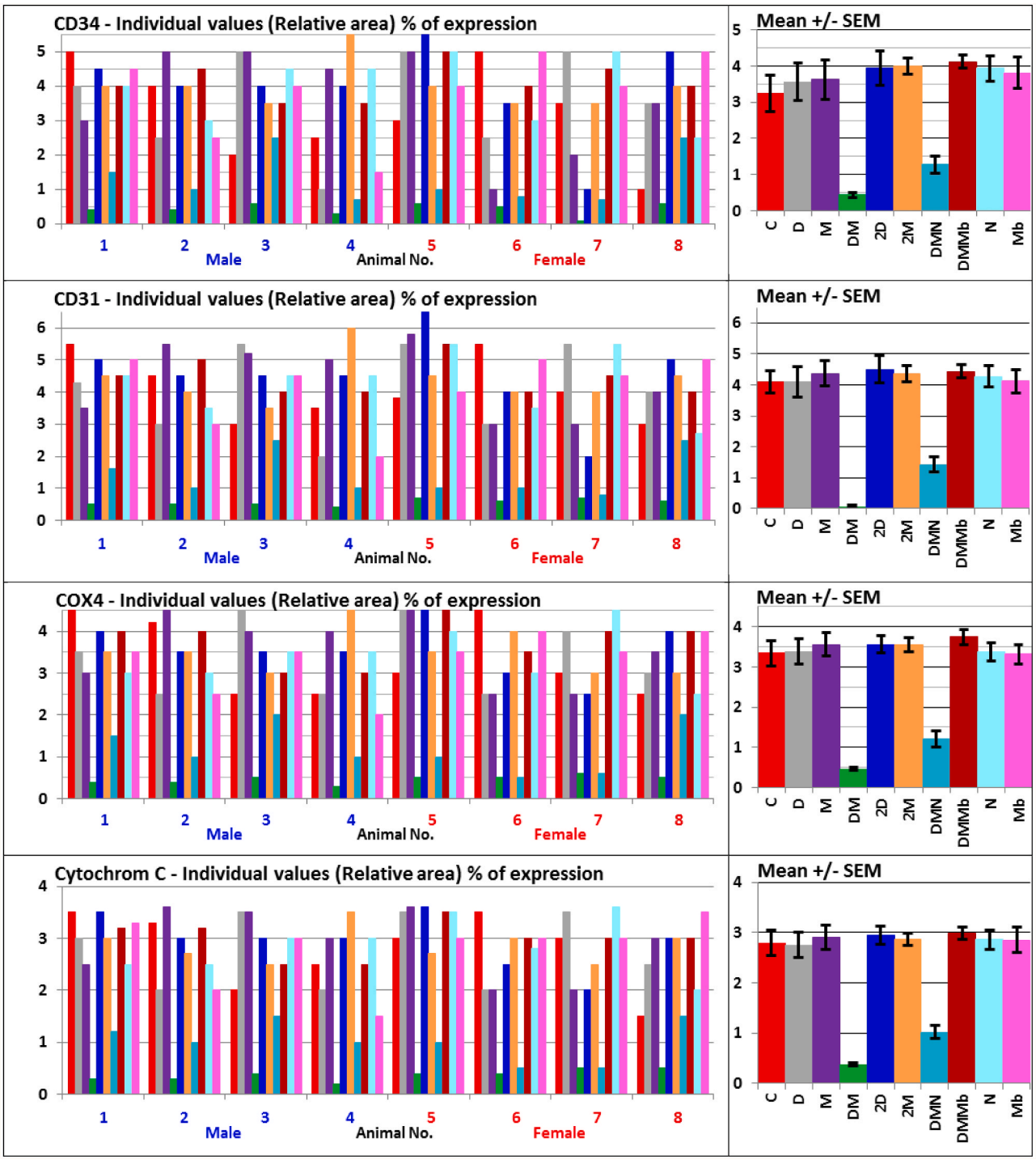

Fig. 6. Individual values with means and standard errors (SEM) of histopathological-immunohistochemical characteristics of the excised tumors in the experiment: CD 34, CD 31, COX 4, Cytochrom C. C - Control group; D - Group treated with disulfiram; M - Group treated with metformin; DM - Group treated with the combination of disulfiram and metformin; 2D - Group treated with disulfiram doubled dose; 2M - Group treated with metformin doubled dose; DMN - Group treated with the combination of disulfiram, metformin and nitroglycerin; DMMb - Group treated with the combination of disulfiram, metformin and mebendazole; N - Group treated with nitroglycerin; Mb - Group treated with mebendazole.

accumulates in tumors and induces NPL4 clustering and inactivation to inhibit p97 segregase and trigger further responses (NF-κB inhibition, stress response, ROS accumulation) that lead to tumor cell death [87].

In the body disulfiram is metabolized to DTC and CuDTC. CuDTC binds to the NPL4, induces NPL4 aggregation, clustering and consequently impairs p97, NF-κB, protein degradation activities [13,86,89,90].

Targeting the NPL4 by disulfiram evokes replication stress (elevation of ROS) and DNA damage while silencing the ATR (serine/threonine-protein kinase or ataxia teleangiectasia and Rad3-related protein; Rad3-DNA helicase for the DNA repair after irradiation) and downstream NF-κB [88] also ARF (alternate reading frame nuclear protein, $p14^{ARF}$ in humans, $p19^{ARF}$ in mice) can activate ATR and then ATR activates NF-κB [91]. In response to different DNA damage, ATR phosphorylates a number of downstream proteins and kinases and two mayor transcription factors: pro apoptotic tumor suppressor p53 and pro survival NF-κB

Table III
Statistical significances expressed as P values for comparisons of histopathological-immunohistochemical tumor characteristics.

Groups (comparison)	Ki-67	PCNA	GLUT-1	iNOS	CD 34	CD 31	COX 4	Cytochrom C
C/D	0.58301	0.90895	0.60303	0.80110	0.59905	1.00000	0.99405	0.98413
C/M	0.90862	0.90859	0.50405	0.41005	0.54100	0.55200	0.55011	0.75341
[a]**C/DM**	0.00011[a]	0.00077[a]	0.00150[a]	0.00179[a]	0.00514[a]	0.00099[a]	0.00103[a]	0.00099[a]
D/M	0.56723	0.79033	0.89340	0.54950	0.99010	0.59904	0.60303	0.59857
[a]**D/DM**	0.00092[a]	0.00089[a]	0.00229[a]	0.00198[a]	0.00499[a]	0.00192[a]	0.00099[a]	0.00097[a]
[a]**M/DM**	0.00079[a]	0.00099[a]	0.00187[a]	0.00172[a]	0.00502[a]	0.00098[a]	0.00090[a]	0.00091[a]
C/2D	0.79852	0.67944	0.32500	0.34909	0.32405	0.47355	0.50250	0.55123
C/2 M	0.80910	0.99605	0.32900	0.39202	0.20710	0.48907	0.58615	0.80700
2D/2 M	0.64200	0.70110	0.99700	0.81105	0.90200	0.57322	1.00000	0.60805
[a]**2D/DM**	0.00048[a]	0.00050[a]	0.00078[a]	0.00093[a]	0.00193[a]	0.00089[a]	0.00047[a]	0.00042[a]
[a]**2M/DM**	0.00055[a]	0.00050[a]	0.00079[a]	0.00069[a]	0.00048[a]	0.00039[a]	0.00029[a]	0.00008[a]
[a]**C/DMN**	0.00569[a]	0.00572[a]	0.00801[a]	0.00811[a]	0.00827[a]	0.00475[a]	0.00587[a]	0.00294[a]
[a]**DMN/DM**	0.00587[a]	0.00579[a]	0.00507[a]	0.00494[a]	0.00831[a]	0.00844[a]	0.00832[a]	0.00244[a]
C/DMMb	0.41400	0.56232	0.28330	0.19850	0.15429	0.41067	0.30147	0.55063
[a]**DMMb/DM**	0.00023[a]	0.00034[a]	0.00075[a]	0.00062[a]	0.00009[a]	0.00176[a]	0.00033[a]	0.00008[a]
[a]**DMN/DMMb**	0.00205[a]	0.00199[a]	0.00217[a]	0.00247[a]	0.00084[a]	0.00086[a]	0.00111[a]	0.00095[a]
C/N	0.28733	0.56954	0.90840	0.36728	0.29906	0.69907	0.98950	0.89205
C/Mb	0.44127	0.59817	0.80210	0.39909	0.42270	0.99959	0.99811	0.90971

[a]$P < 0.05$. C - control group; D - group treated with disulfiram; M - group treated with metformin; DM - group treated with the combination of disulfiram and metformin; 2D - group treated with disulfiram double dose; 2M - group treated with metformin double dose; DMN - group treated with the combination of disulfiram, metformin and nitroglycerin; DMMb - group treated with the combination of disulfiram, metformin and mebendazole; N - group treated with nitroglycerin; Mb - group treated with mebendazole.

[92].

Inherent different strategies for responding to stress lead to a functional antagonism between p53 and NF-κB [93]. A long ago it was found that transcriptional crosstalk between NF-κB and p53 was: NF-κB attenuates p53 and p53 attenuates NF-κB [94]. At a contrary, later was published that p53 induces NF-κB activation by an IκB kinase-independent mechanism involving phosphorylation of p65 (subunit of NF-κB) by ribosomal S6 kinase 1 [95]. Later it was found that complex regulation of NF-κB and p53 crosstalk can be at several steps. These transcription factors can functionally antagonize, cooperate or exhibit independence [96]. In normal cells normal p53 attenuates NF-κB. In tumor cells, mutant p53 promotes NF-κB. Consequently, in normal cells NF-κB inactivates p53. In tumor cells NF-κB activates mutant p53 [96].

p53 is a regulator of NF-κB repression by the glucocorticoid receptor in a mouse model of lipopolysaccharide shock [97]. From previous papers it follows that NPL4 and ATR, which can be suppressed by disulfiram, are important for NF-κB activation and p53 attenuation, i.e. for indirect NF-κB inhibition, p53 activation (in normal cells) and tumor p53 silencing.

A number of common or separate anticancer mechanisms of disulfiram [4–13] and metformin [14–19,45–48] have been identified. Among the potential anticancer targets of these drugs ROS production stimulation and NF-κB inhibition are notable common functions.

The reason for comedication of ROS scavenger nitroglycerin or NF-κB stimulator mebendazole with potentially ROS stimulatory and NF-κB inhibitory disulfiram and metformin combination was: if inhibition of ROS or stimulation of the NF-κB can block or eliminate the anticancer effect, i.e. can "rescue" the cancer, then disulfiram and metformin anticancer combination targets ROS or NF-κB.

Nitroglycerin, which is a NO donor, can as NAC produce antioxidative effect and can also be used as ROS scavenger as NAC [23–27].

However, ROS has dual effects. On the one hand, ROS can trigger biological processes – at low physiological concentrations they induce cell proliferation. On the other hand, at high cancer therapeutic levels, they may damage DNA, proteins and lipids and can cause cell death and trigger necroptosis [98]. ROS are the key factor in the promotion of necroptosis via a positive feedback mechanism, which can be rescued by NAC [99]. ROS are able to trigger programmed cell death and to have the role of Trojan horses, in many metabolic pathways, to eliminate cancer cells [100]. Necroptosis can be prevented by scavenging ROS production in heat stress-induced intestinal injury [101].

Expected NF-κB inhibitory effect of nitroglycerin [48] can be opposite and manifested in NF-κB stimulation, for example in *nucleus trigeminalis caudatus* of migraine animal model [28]. Also, nitroglycerin can be without any effect on NF-κB [26]. Nitroglycerin has not caused NF-κB reduction in the rat model of formalin induced inflammation [26].

Microtubule depolymerization and disruption by mebendazole stimulates NF-κB [33–42], which activates HIF-1α pathway and downstream targets (VEGF, P-gp - MDRP, MRPs). Benzimidazoles, including mebendazole, have no effect on the respiration rates, the membrane potential changes and ROS production of isolated mitochondria [31]. On microtubule depolymerization the cytoplasmic inhibitor of the transcription factor NF-κB, IκB, is degraded, unmasking the nuclear localization sequence of NF-κB and allowing it to translocate to the nucleus, were it can up-regulate a number of genes, including HIF-1α. Increases in HIF-1α transcription could overwhelm the ability of cells to degrade HIF-1α and lead to its stabilization and induction [31]. Mebendazole can abrogate oxidative stress-induced neuronal death, including stroke by microtubule depolymerization and consecutive stimulation of NF-κB, HIF-1α, VEGF, P-gp, MRPs [31,102].

Suppression of NF-κB in cancer leads to the disease regression by: inhibition of mitochondrial respiration, Warburg effect, cell proliferation, angiogenesis, resistance to treatment and metastasis by complex interplay with the ROS and by stimulation of autophagy and necroptosis [103].

Results of the our experiment, realized by giving double doses of either disulfiram or metformin alone against hamster fibrosarcoma growth in vivo, show that there is no simple addition of therapeutic effects that can be caused by doubling the dose of monotherapy.

Possible explanation of our results, based on previously presented studies, may be that disulfiram causes stimulation of ROS production and inhibition of NF-κB identical to the effects of metformin. ROS stimulation additionally inhibits NF-κB [3]. Because if that, dual synergistic effect of the disulfiram and metformin combination on ROS and NF-κB can be partly reversed by ROS inhibitor nitroglycerine (or NAC) and completely reversed by NF-κB stimulator mebendazole. Also, anticancer effect of ROS stimulation depends of NF-κB inhibition. Briefly, the presented interpretation of our results is that dual ROS production stimulation followed by a dual NF-κB inhibition caused by disulfiram and metformin combination, can contribute to the synergistic anticancer properties of the combination therapy through the mentioned underlying complex mechanisms (downstream reduction of HIF-1α, which downstream reduces P-gp, MRPs, VEGF).

Results of recently published in vitro study of effective radiosensitization on glioblastoma cell culture by the combination of metformin and disulfiram-Cu [104] are in accordance with our in vivo results. The combinatory usage of both drugs synergistically decreased the cell growth, induced apoptosis, caused the upregulation of cell death markers BAX, p53, CASPASE-3 and markedly downregulated the expression of the anti-apoptotic protein BCL-2 [104]. It is concluded that the synergistic effect of both metformin and disulfiram, with the support of irradiation can remarkably restrict the growth of the cell line and trigger apoptosis [104]. In this publication, the precise mechanisms, as ROS production stimulation and NF-κB suppression, by which disulfiram with metformin exert upregulation of BAX, p53, CASPASE-3, downregulation BCL-2 and resulting apoptosis were not studied. In our study we find that just ROS production stimulation and NF-κB suppression are underlying precise mechanisms by which the combination affects the regulation of proapoptotic factors.

Also, in accordance with our study, disulfiram with or even without metformin inhibited oesophageal squamous carcinoma in vivo [105]. This effect for disulfiram was independent of copper. In this experiment autophagy was inhibited since increase in total ubiquitinated proteins, LC3B-II, LAMP1 and p62, where detected [105]. Similar results were obtained earlier in vitro on oesophageal squamous carcinoma cell lines by same authors [106]. They found that the combination affects protein degradation/turnover pathways and explained that metformin further enhances the role of disulfiram as a proteasome inhibitor [106].

Several other therapies were combined disulfiram with ROS inducers, for example, with auranofin in hepatocellular carcinoma model in vitro and in vivo (mice xenografts) [107] and with arsenic trioxide (As_2O_3) in melanoma cells [108]. These data strongly support the concept of dependence of disulfiram anticancer effect on the production of ROS.

The disulfiram and metformin combination could target many, not only known, but also unknown mechanisms of cancer growth in which, according our present results, are surely involved ROS and NF-κB. However, the advantage of the non-oncology anticancer drug combination application is in direct, fast implementation of actual recommended multi-target anticancer approach strategy, which may bring the greatest benefit to patients. So, for that, ie. extrapolation of nontoxic drug anticancer efficacy results from experimental animals to humans, interspecies comparisons of bioavailabilities, pharmacokinetic properties and doses must be considered.

Disulfiram is rapidly absorbed from the gastrointestinal tract and more than 80% of an oral dose is generally absorbed in humans and experimental animals [109]. Disulfiram is to some extent converted to its copper complex jet in the stomach [109]. After absorption across the gastrointestinal mucosa into the blood, disulfiram is partly metabolized and disulfiram with its metabolites are uniformly distributed throughout the body in various tissues in animals as well as in man [109]. After oral administration of radiolabeled disulfiram to rats and mice, the intact drug and metabolites were found along the entire length of the gastrointestinal tract and in the blood, liver, kidney, heart, adrenal, thyroid, pancreas, testes, spleen, marrow, muscle and brain [109]. Disulfiram and metabolites are bound to various proteins. In the blood, both disulfiram and metabolites are highly bound principally to albumin, with average binding percentage of 96.1 and 79.5, respectively [109]. The metabolites of disulfiram are mainly excreted via kidney, faeces and the lung [109]. Up to 20% of an oral dose is eliminated as intact drug in the faeces, about 65% via the kidney, mainly as glucuronide (up to 53%) or sulphate (up to 30%) metabolite [109].

Disulfiram doses in our experiments were 50 and 100 mg/kg, i.e. ~10% and ~20% of hamster oral LD_{50} respectively (oral LD_{50} rat: 500 mg/kg, oral LD_{50} mouse: 1013 mg/kg). Dose of 50 mg/kg was equivalent to an usual human dose of 4 mg/kg by normalization to body surface [58–60,110].

Oral bioavailability of metformin (undergoes negligible metabolism) was ~50% in mammals [111]. The metformin levels in the colorectal cancer cells of xenograft-bearing mice that were successfully treated orally and intraperitoneally corresponded to the plasma concentrations [112]. These results indicate a consistent delivery of the drug to the tumor tissue. In the present study, the same orders of magnitude of oral metformin doses were administered to the hamsters as those administered in the mentioned study [112]. For the hamster treatment, metformin doses of ~25% and ~50% of the oral LD_{50} for this species were selected. As oral metformin LD_{50} is about 2000 mg/kg (2400 mg/kg in mice, 1770 mg/kg in rats), 500 mg/kg and 1000 mg/kg were used in our present study. 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 [58–60,110]. The dosage of 500 mg/kg/day metformin was two times higher than the equivalent of the 20 mg/kg/day typically administered to patients with diabetes.

The anticancer properties of the present disulfiram and metformin combination in hamsters and the possibility of achieving comparably high nontoxic levels in humans suggest that the prospect of effective nontoxic oncological treatment using this drug combination may be achievable. Clinical trials are required to determine whether the combination has the potential to become an adjuvant or relapse prevention treatment in current anticancer procedures, and in particular, in antisarcoma therapies. Furthermore, after clinical checking, at the end, it is not impossible to incapsulate both drugs into appropriate nanocarrier for more efficaceous treatment in oncology.

5. Conclusions

In conclusion, combination of nontoxic oral doses of disulfiram and metformin significantly inhibited sarcoma growth in hamsters, compared to nontreatment or single treatments with different doses of each drug. At the contrary to the combined treatment, the single treatments did not exhibit significant antisarcoma effect, nevertheless of the dose. Co-treatment with a ROS scavenger nitroglycerin or NF-κB stimulator mebendazole inhibited anticancer activity of the disulfiram and metformin combination and rescued tumor progression. According to results of nitroglycerin or mebendazole co-treatment, significant components of the disulfiram and metformin combination anticancer activities were ROS production and NF-κB inhibition with downstream inhibition of HIF-1α and subordinate targets (VEGF, P-gp-MDRP, MRPs). Despite of complex, for the most part unknown, crosstalk between ROS and NF-κB, NF-κB can at the contrary of ROS and independently of ROS, affect cancer growth and apoptosis. This follows from our findings that alonely ROS inhibition (by nitroglycerin) only partially rescued cancer treated by the combination (disulfiram and metformin), but that NF-κB stimulation by mebendazole completely rescued cancer treated by the combination and also prevented ROS-induced cell death. The combination of disulfiram and metformin may be a candidate for novel safe anticancer neoadjuvant, adjuvant or risk-reducing and relapse prevention therapies.

Ethics approval and consent to participate

The study was carried out following the approval of the University of Novi Sad Animal Ethics Committee (Novi Sad, Serbia), No. 04–81/25–5 dated 22nd July 2020, Doc. No. ЕК: ИЕ-2020–07 and the approval of the Ministry of Agriculture, Forestry and Water Management - Veterinary Directorate (Belgrade, Serbia), No. 323–07–09359/2020–05 dated 2nd September 2020.

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Patient consent for publication

Not applicable.

Credit authorship contribution statement

KJP made substantial contributions to the conception and design of the investigation and performed the physical and 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. JKP made substantial contribution to the statistical analysis and interpretation of all the experimental data. DL performed the histological and toxicological analyses. MP contributed to the physicochemical analyses of the hamster tumors. IČ performed the immunohistochemical analyses. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests

Data availability

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112168.

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