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Experimental evaluation of the effects of anticancer modulation therapy on MAPK/PI3K/AKT/mTOR/NF-κB signaling with non-toxic drugs

Експериментална евалуација ефеката антиканцерске модулационе терапије на *MAPK/PI3K/AKT/mTOR/NF-кВ* сигнализацију нетоксичним лековима

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SUMMARY

Introduction/Objective Large diversity in molecular mechanisms of cancer regulation allows some marketed pleiotropic non-oncological non-toxic pharmaceuticals to be used in oncology, which reduces duration and cost of novel anticancer treatment research. Up to date, there are no published results *in vivo* on anticancer effects of certain combinations of non-oncological pleiotropic drugs (disulfiram, metformin, deoxycholic acid, mebendazole) that influence MAPK/PI3K/AKT/mTOR/NF-κB signaling.

Methods The anticancer effects of certain aforementioned repurposed drugs combinations, <50 % LD₅₀ (equivalent to usual human dose) were assessed by fibrosarcoma growth kinetics (measured daily in vivo by calipers) and tumor proliferation (Ki-67, PCNA), neoangiogenesis (CD34, CD31), glucose metabolism (GLUT1), NO metabolism (iNOS) and apoptosis (COX4, Cytochrome C) in hamsters, randomly allocated to control and experimental groups (6 animals per group). Animals were sacrificed 19 days after BHK-21/C13 tumor inoculation. Tumors were excised, measured and Biophysical, blood collected. pathohistological, toxicological, hematological and biochemical analyses were performed.

Results Disulfiram with metformin, disulfiram with deoxycholic acid and deoxycholic acid with metformin are the combinations that have shown significant antitumor effects on the fibrosarcoma growth kinetics and tumor immunohistochemical markers in hamsters (p<0.05). All used drugs in efficacious combinations can inhibit MAPK/PI3K/AKT/mTOR/NF- κ B signaling. Addition of NF- κ B stimulator mebendazole to effective two-drug combinations rescued cancer growth, indicating that these pathways may be responsible for antitumor action.

Conclusion Combinations of non-oncological drugs: disulfiram with metformin, disulfiram with deoxycholic acid and deoxycholic acid with metformin have the potential to be used as effective non-toxic adjuvant anticancer therapy in oncology.

Keywords: disulfiram; deoxycholic acid; metformin; hamsters; BHK-21/C13; fibrosarcoma

Сажетак

Увод/Циљ Велика разноликост у молекуларним механизмима регулације канцера омогућава да се неки плејотропни неонколошки нетоксични лекови, који су већ на тржишту, користе у онкологији, што смањује трајање и цену нових истраживања антиканцерских третмана. До данас, не постоје објављени резултати *in vivo* о антиканцерским ефектима одређених комбинација неонколошких плејотропних лекова (дисулфирам, метформин, деоксихолна киселина, мебендазол) који утичу на *МАРК/РІЗК/АКТ/тОR/NF-кВ* сигнализацију.

Антиканцерски ефекти Методе одређених комбинација наведених пренамењених лекова, дозе < 50% LD₅₀ (еквивалентно уобичајеној дози за људе), процењени су кинетиком раста фибросаркома (мерено свакодневно *in vivo* помоћу калипера) и маркерима туморске пролиферације (Ki-67, PCNA), неоангиогенезе (CD34, CD31), метаболизма глукозе (GLUT1), метаболизма NO (iNOS) и апоптозе (COX4, *Citohrom C*) код хрчака, који су насумично распоређени у контролне и експерименталне групе (шест животиња по групи). Животиње су жртвоване 19 дана након инокулације тумора ВНК-21/С13. Тумори су изрезани, измерени и прикупљена је крв. патохистолошке, Урађене биофизичке, cy токсиколошке, хематолошке и биохемијске анализе. Резултати Дисулфирам ca метформином, дисулфирам са деоксихолном киселином И метформином деоксихолна киселина са cv комбинације које су показале значајне антитуморске ефекте на кинетику раста фибросаркома и имунохистохемијске маркере тумора код хрчака (р < 0,05). Сви коришћени лекови у ефикасним комбинацијама инхибирати могу MAPK/PI3K/AKT/mTOR/NF-ĸB туморску

сигнализацију. Додавање *NF-кВ* стимулатора мебендазола ефикасним комбинацијама два лека сачувало је раст канцера, што указује да ови путеви могу бити одговорни за антитуморско деловање.

Закључак Комбинације неонколошких лекова: дисулфирам са метформином, дисулфирам са деоксихолном киселином и деоксихолна киселина са метформином имају потенцијал да се користе као ефикасна нетоксична помоћна антиканцерска терапија у онкологији.

Кључне речи: дисулфирам; деоксихолна киселина; метформин; хрчци; *BHK-21/C13*; фибросарком

INTRODUCTION

Activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling has been found in many types of tumors, including breast, colon, prostate, skin, lymphoid tumors [1]. NF- κ B is antiapoptotic factor responsible for cancer occurrence, development and resistance to chemo- and radio-therapy. Hence, therapeutic blockade of NF-B or upstream signals of the cascade MAPK/PI3K/AKT/mTOR/NF- κ B (mitogen-activated protein kinase / phosphatidylinositol 3-kinase / protein kinase B – PKB / mammalian target of rapamycin / NF- κ B) in cancer cells provides an attractive strategy for the development of anticancer drugs.

For our *in vivo* analysis we selected: registered, non-oncological, low-toxic, pleiotropic drugs with common anticancer mechanisms already established *in vitro*, e.g. via NF- κ B modulation.

Antialcoholic drug disulfiram inhibits NF-κB signaling pathway, and hence inhibits proliferation and induces apoptosis of various cancer cell lines [2, 3].

Antidiabetic drug metformin inhibits MAPK, AKT, mTOR and NF- κ B in various cancer cells, resulting in inhibition of proliferation and stimulation of apoptosis *in vitro* and in mouse xenograft models *in vivo* [4]. Metformin inhibited proliferation by suppression of NF- κ B in: lung, ovarian, gastric and prostate human cancer cells [5].

Deoxycholic acid, used for liver cirrhosis and for serum cholesterol lowering, can produce oxidative stress [6]. The early phase of oxidative stress is associated with temporary activation of the NF- κ B pathway, but sustained oxidative stress decreases NF- κ B activity [7]. Deoxycholic acid induces programmed cell death via MAPK/PI3K/AKT/mTOR/NF- κ B signaling [8]. It has been shown that deoxycholic acid inhibits NF- κ B activity, limits cancer cell proliferation, invasion and induces apoptosis *in vitro* in human: pancreatic, gastric, lung, prostate, breast, colon and hepatic carcinoma cells [9, 10].

In this study we have applied pleiotropic non-toxic drugs, modulators of NF- κ B, with *in vitro* approved anticancer characteristics: disulfiram, metformin, deoxycholic acid, mebendazole and their combinations.

If stimulation of the NF- κ B can block or eliminate the anticancer effect, i.e. can "rescue" the cancer, than anticancer treatment targets NF- κ B. To test whether NF- κ B inhibition underlies the anticancer mechanism of examined drug therapy, we co-medicated a NF- κ B stimulator mebendazole for tumor rescue.

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

METHODS

Single and combined anticancer treatments with repurposed drugs: **I.** disulfiram and metformin; **II.** disulfiram and deoxycholic acid; **III.** deoxycholic acid and metformin were analysed simultaneously in three separate independent experiments with three different control groups.

For our rescue treatments we have used antihelmintic drug mebendazole, which strongly depolymerizes microtubules and thus activates NF-κB [11, 12].

Animal model

We have conducted experiments on Syrian golden hamsters (6 males per group; weight, ~70 g; age, ~13 weeks).

Our study followed internationally recognized guidelines on animal welfare, as well as local and national regulations (ARRIVE guidelines; Law on animal welfare of the Republic of Serbia; University of Novi Sad Rules for work with experimental animals).

All animals were subjected to protocols approved by the University of Novi Sad Animal Ethics Committee (Novi Sad, Serbia): Doc. No. EK: II-E-2020-07; Doc. No. EK: I-2022-01; No. 04-150/15; Doc. No. EK: I-2022-02; and approved by the Ministry of Agriculture, Forestry and Water Management – Veterinary Directorate (Belgrade, Serbia): No. 323-07-03995/2022-05; No. 323-07-03996/2022-05; No. 323-07-03997/2022-05.

Treatments with disulfiram, metformin, deoxycholic acid, mebendazole (all Galenika a.d.) and their combinations in experimental groups were initiated after the subcutaneous inoculation of 1 ml of BHK-21/C13 cell suspension ($2x10^6$ cells/ml) into the animals' back for fibrosarcoma development. BHK-21/C13 cells were produced at Department of Histology and Embryology, Faculty of Medicine, University of Novi Sad, Serbia. The humane endpoints were: 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. General condition, behavior, general clinical

signs (diarrhea, breathing disorders, neurological signs), tumor location and ulceration, appearance of multiple tumors were monitored on daily basis. Tumor diameter and body weight were measured daily.

Each treatment was administered via a gastric probe daily after cancer cell inoculation.

I. Disulfiram and metformin experiment: peroral treatment with 1) water (control group with inoculated tumor); 2) disulfiram 50 mg/kg; 3) metformin 500 mg/kg; 4) combination of disulfiram 50 mg/kg and metformin 500 mg/kg; 5) disulfiram double dose 100 mg/kg (for validation); 6) metformin double dose 1000 mg/kg (for validation); 7) combination of disulfiram 50 mg/kg, metformin 500 mg/kg and mebendazole 460 mg/kg (for tumor rescue); 8) mebendazole 460 mg/kg. Two single drug treatments were applied with doubled doses of disulfiram alone and metformin alone (maximal tolerable < 50% LD₅₀) on two groups of animals for validation of synergistic combinatory two-drug effect.

II. Disulfiram and deoxycholic acid experiment: peroral treatment with 1) water (control group with inoculated tumor); 2) disulfiram 50 mg/kg; 3) deoxycholic acid 100 mg/kg; 4) combination of disulfiram 50 mg/kg and deoxycholic acid 100 mg/kg; 5) combination of disulfiram 50 mg/kg, deoxycholic acid 100 mg/kg and mebendazole 460 mg/kg (for tumor rescue); 6) mebendazole 460 mg/kg.

III. Deoxycholic acid and metformin experiment: peroral treatment with 1) water (control group with inoculated tumor); 2) deoxycholic acid 100 mg/kg; 3) metformin 500 mg/kg; 4) combination of deoxycholic acid 100 mg/kg and metformin 500 mg/kg; 5) combination of deoxycholic acid 100 mg/kg, metformin 500 mg/kg and mebendazole 460 mg/kg (for tumor rescue); 6)mebendazole 460 mg/kg.

The animals were sacrificed 19 days post-inoculation. 90 mg/kg of pentobarbital was administered intraperitoneally to each animal before sacrification. For animal blood biochemical and hematological analyses 2-3 ml of the total collected blood was used. After exsanguination, vital organs were removed for pathological, histological and toxicological examinations. Toxicity was analyzed based on the gross and microscopic standard pathological examination of main organs, tissues and whole bodies, influence on body weight, determination of organ-weight to body-weight ratios of brain, heart, lungs, stomach, liver intestine and kidneys in treated hamsters versus control. Biochemical and hematological blood tests were also performed. Weights of the animals were measured before the sacrification. All animals were in a good condition during the study. Humane endpoints were not reached and none of

the hamsters were euthanized prior to the end of the experiment. The tumor diameters and the tumor burdens were evaluated daily using calipers and the following ellipsoid volume formula: volume = $4\pi abc/3$, where a, b and c are half-diameters. After sacrifice, the tumors were excised and weighed, their diameters were exactly measured using calipers, and the exact tumor volume was determined using the standard water volume displacement method. The relative tumor weight (tumor burden) was determined as the weight ratio of tumor/animal.

In all experiments, drugs were dissolved in water and administered to hamsters daily in 1 ml/100 g animal weight. Doses were < 50% of oral median lethal LD₅₀ for hamsters and equivalent to human doses (by normalization to surface area).

The tumor density was calculated as density=mass/volume; the tumor surface area (S) was calculated using the standard ellipsoid surface 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.

For the verification of tumor growth, tissue penetration, expansion of necrosis and hemorrhagic areas, proliferation, angiogenesis, apoptosis, glucose and NO-metabolism, tumor slices (4 µm) were assessed pathohistologically and immunohistochemically.

Immunohistochemistry

In addition to the principal hematoxylin and eosin (HE) staining, immunohistochemical staining was performed to assess tumor proliferation (Ki-67, PCNA), neoangiogenesis (CD34, CD31), glucose metabolism (GLUT1), NO metabolism (iNOS) and apoptosis (COX4, Cytochrome C) (Table 1).

Tissue sections preparation, staining, and analyzing

Tumor sections (4 μ m) were deparaffinized in xylene (100%) and rehydrated in descending ethanol series (100% twice for three min.: 95% for three min, and 70% for three 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 five 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 two hours at room temperature. Primary antibodies dissolved in TBS with 1% BSA were incubated at 4°C overnight. The sections were washed twice for five 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; Abeam) dissolved in TBS with 1% BSA was added to the sections for two hours at room temperature. The sections were washed three times for five min. with TBS. For visualization, the chromogen 3,3-diaminobenzidine tetrahydrochloride (cat. no. K3468; Liquid DAB + Substrate – Chromogen System; Dako; Agilent Technologies, Inc.) was added and incubated for 10 min. at room temperature. The sections were washed with water for five min. and were stained with Mayer's hematoxylin for five min. at room temperature.

The stained tumor slices were assessed using Leica DMLB 100T (Leica Microsystems GmbH) microscope at 400× magnification. Images were captured using a Leica MC190 HD camera (Leica Microsystems GmbH). Immunoexpression was evaluated based on the positive cells counts (stained/total number of cells) or on the stained portions of surface area (stained surface / whole surface) in the tumor sections (mean of 10 measurements) using UTHSCSA Image Tools for Windows version 3.00.

Blood biochemical tests and hematological analyses

Standard laboratory blood analyses were performed: glucose, serum proteins, albumins hemoglobin, sedimentation, erythrocytes, leucocytes, lymphocytes, monocytes, granulocytes, platelets, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration, in all experiments.

Statistical analysis

Means, standard deviations (SD) or standard errors (SEM) were calculated for the experimental data. The differences among the groups in all measured parameters were determined using one-way ANOVA followed by a Student-Newman-Keuls post hoc test. P<0.05 indicated a statistically significant difference. The two-sided Mann-Whitney U tests were additionally performed to check significances obtained by parametric testing. Data analysis was conducted using TIBCO Statistica 13.3.1 software (TIBCO Software, Inc.) in all experiments.

RESULTS

After inoculation of BHK-21/C13 cells, fibrosarcoma was developed in all hamsters. Animals had isolated, well-demarcated solid tumors without adverse effects on general health and well-being. The maximal tumor diameters after sacrifice were <3.5 cm in all experiments. The maximal tumor burdens after sacrifice were much below 10% of the animal body weight in all experiments. Pathological, histopathological and toxicological analysis following autopsy revealed no signs of toxicity on main organs (heart, lungs, stomach, intestine, liver, kidneys and brain), nor metastases or ascites.

The experimental and control groups were statistically compared for glucose levels, hemoglobin levels, hematocrit levels, serum proteins, sedimentation, red and white blood cell counts, platelet number, but no significant differences were observed among the groups in all three experiments (p > 0.05).

In all experiments, peroral co-treatment with examined dual drug combination significantly inhibited tumor growth as indicated by significant decreases in tumor weight, volume, max. diameter, density, tumor surface area, relative tumor weight, tumor surface area/volume ratio, tumor surface/max. diameter ratio, tumor surface area/weight ratio, tumor surface area/density ratio, tumor max. diameter/density ratio, compared with control, as shown for the I. disulfiram and metformin experiment in Table 2, Figure 1, Figure 2; for the II. disulfiram and deoxycholic acid experiment in Table 3, Figure 3, Figure 4, and for the III. deoxycholic acid and metformin experiment in Table 4, Figure 5, Figure 6.

In all experiments, the pathohistological and immunohistochemical evaluation revealed a decrease in tissue penetration, an expansion of necrosis and hemorrhagic areas, significantly decreased proliferation status of tumor cells, as demonstrated by Ki-67 (and additionally by PCNA for the I. disulfiram and metformin combination), significant inhibition of glucose metabolism, as demonstrated by GLUT1, significant inhibition of NO metabolism, as demonstrated by iNOS staining, significant inhibition of tumor vasculature, as demonstrated by CD31 (and additionally by CD34 for the I. disulfiram and metformin combination), and significant difference in apoptosis intensity, as demonstrated by COX4 and Cytochrome C, in all analyzed slices of tumors from animals treated with the examined dual drug combination, compared with the control group and the single treatment groups, as shown for the I. disulfiram and metformin experiment in Figure 7; for the II. disulfiram and deoxycholic acid experiment in Figure 8. and for the III. deoxycholic acid and metformin experiment in Figure 9. Results gained with HE staining and different antibodies are illustrated by Figure 10.

In all experiments, only the examined dual drug combination resulted in a statistically significant (p < 0.05) antitumor effects compared with control (Tables 2, 3, and 4, Figures 1–9). In the I. disulfiram and metformin experiment, neither disulfiram, nor metformin single treatments, even in double doses, exhibited significant anticancer effect in comparison to control (Table 2, Figure 1, Figure 2, Figure 3).

In all three experiments (I. disulfiram and metformin; II. disulfiram and deoxycholic acid; III. deoxycholic acid and metformin), co-treatment with NF- κ B stimulator mebendazole inhibited anticancer activity of the examined dual drug combination. Mebendazole rescued tumor progression suppressed by each examined dual drug combination of the two NF- κ B inhibitors. This indicates that synergistic antitumor effects of each examined dual drug combination (I. disulfiram and metformin; II. disulfiram and deoxycholic acid; III. deoxycholic acid and metformin) may be caused by NF- κ B suppression.

During the course of all experiments, the treatments had no significant effect on the body weight of the hamsters (compared with the control), as shown for the I. disulfiram and metformin experiment in Table 2; for the II. disulfiram and deoxycholic acid experiment in Table 3 and for the III. deoxycholic acid and metformin experiment in Table 4.

The results of all three experiments confirmed the significant synergistic anticancer effects of each examined dual drug co-treatment (I. disulfiram and metformin; II. disulfiram and deoxycholic acid; III. deoxycholic acid and metformin) on hamster fibrosarcoma, without toxicity.

DISCUSSION

In our experiments, disulfiram doses were 50 and 100 mg/kg, i.e. \sim 10% and \sim 20% of hamster oral LD₅₀ respectively (oral LD₅₀ rat: 500 mg/kg, oral LD₅₀ mouse: 1013 mg/kg). Dose of 50 mg/kg corresponds to an usual human dose of 4 mg/kg by normalization to body surface.

Metformin doses of ~25% and ~50% of the oral LD_{50} for hamsters were selected in our experiments. Since oral metformin LD_{50} is about 2,000 mg/kg (2,400 mg/kg in mice, 1,770 mg/kg in rats), 500 mg/kg and 1000 mg/kg were used in this study. The daily dose of 500 mg/kg metformin in hamsters corresponds to the maximum daily dose of 40 mg/kg in patients with diabetes normalized to body surface.

Deoxycholic acid lowered the serum cholesterol after the administration of 750 mg/day for 3-4 weeks. The effect of deoxycholic acid ingestion 750 mg/day on bile acid kinetics was studied in healthy volunteers [13]. Equivalent dose for hamsters based on body surface for human dose 750 mg/day is 100 mg/kg (750 mg/day = 12 mg/kg/day x 7.4 \approx 100 mg/kg/day, where 7.4 is biometric conversion factor for hamsters based on body surface) [14]. This dose for hamsters (100 mg/kg/day), equivalent to oral human dose, is significantly below 25% hamster LD₅₀ (oral LD₅₀ in mouse 1000 mg/kg, oral LD₅₀ in rat 1370 mg/kg). This is the underlying rationale for using deoxycholic acid dose of 100 mg/kg for hamsters in our study.

In our experiments, mebendazole was administered to hamsters orally at a dose of 460 mg/kg daily (~50% of oral LD_{50} for hamsters), equivalent to an oral human dose of 62 mg/kg/day normalized to body surface area (biometric conversion factor 7.4 [14]), which is comparable to human daily dose of 50 mg/kg/day for the 1-6 months treatment of echinococcosis.

Since in our study doses for disulfiram, metformin, deoxycholic acid and mebendazole for hamsters were equivalent to used human doses (based on surface body area biometric conversion factor 7.4 [14]), it follows that anticancer effects seen in our study can be achievable with usual human doses in oncology.

Previous research has found that disulfiram suppressed proliferation of various malignant cell types also via ROS activation, as well as simultaneous NF- κ B inhibition. Simultaneous induction of ROS and inhibition of NF- κ B by disulfiram induces cell cycle arrest and apoptosis [2, 3].

Studies published so far show that metformin inhibits complex I of the mitochondrial electron transport chain, which leads to membrane depolarization and the release of ROS with the chemical damage of cell components and apoptosis [5]. ROS, elevated by metformin, activates AMPK, inhibits mTOR signaling and expression of NF- κ B [4, 5], activates tumor proliferation suppressor p53, arresting the cell cycle.

Deoxycholic acid has been shown to cause damage to mitochondrial membrane. Also, it was shown that induction of oxidative stress by deoxycholic acid leads to impaired NF- κ B transcriptional activity, which facilitates apoptosis [6–10].

As can be seen from cited publications, efficacious anticancer combinations of repositioned drugs in our experiments (I. disulfiram and metformin, II. disulfiram and deoxycholic acid, III. deoxycholic acid and metformin) encompass agents with NF-κB

inhibitory effect. Since results of our study show that well known NF- κ B stimulator mebendazole annulled anticancer effects of examined two-drug combinations (I. disulfiram and metformin, II. disulfiram and deoxycholic acid, III. deoxycholic acid and metformin) and rescued tumor growth in all three experiments, it can be supposed that NF- κ B inhibition is important underlying mechanism of observed anticancer effects.

Our findings confirmed the significant anticancer effects of non-oncological drug combinations: I. disulfiram with metformin, II. disulfiram with deoxycholic acid and III. deoxycholic acid with metformin on hamster fibrosarcoma, without toxicity. The single treatments did not exhibit significant antisarcoma effects. Rescue treatments with co-medicated mebendazole to combined two-drug therapies in all three experiments indicate important underlying role of NF- κ B in observed anticancer effects.

CONCLUSION

The anticancer properties of the three examined two-drug combinations (I. disulfiram and metformin, II. disulfiram and deoxycholic acid, III. deoxycholic acid and metformin) in hamsters, with used doses equivalent to standard human doses, suggest that effective nontoxic oncological therapies in humans and prevention of cancer relapse using these drug combinations may be achievable and that their administration may be an effective and safe approach in novel nontoxic adjuvant anticancer treatment.

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Table 1. Antibody information

Antibodies	Manufacturer	Cat. No.	Dilution ratio
Ki-67	Thermo Fisher Scientific, Inc.	RB-9043-P0	1:300
PCNA	Thermo Fisher Scientific, Inc.	RB-9055-P	1:300
CD34	Abcam	ab81289	1:200
CD31	Abcam	ab28364	1:200
GLUT1	Thermo Fisher Scientific, Inc.	RB-9052-P0	1:200
iNOS	Thermo Fisher Scientific, Inc.	RB-9242-P0	1:100
Cytochrome C	Abeam	ab133504	1:200

Table 2. Characteristics of animals and quantitative pathological characteristics of tumors in

	Ham	ster		Tumor		
	Weight at	Weight at	Weight	D _{max}	Volume	
	start (g)	end (g)	(g)	(cm)	(cm ³)	
C - Cont	rol group					
Mean	67.5	94.5	4.42	3.37	3.61	
\pm SD	5.55	5.11	0.48	0.32	0.55	
D - Grou	p treated with dist	ulfiram (50 mg/kg	g/day)			
Mean	68.3	90.2	4.30	3.22	3.55	
\pm SD	6.23	6.88	0.79	0.56	0.82	
M - Grou	up treated with me	tformin (500 mg/	kg/day)			
Mean	69.1	91.0	4.12	3.30	3.33	
\pm SD	4.32	5.73	0.68	0.64	0.57	
*DM - C	Broup treated with	the combination of	of disulfiram	(50 mg/l	(g/day) and	
metform	in (500 mg/kg/day	<i>i</i>)				
Mean	68.5	96.2	0.63	1.25	0.59	
\pm SD	5.72	4.92	0.25	0.43	0.25	
2D - Group treated with disulfiram double dose (100 mg/kg/day)						
Mean	70.2	97.2	4.13	3.11	3.40	
\pm SD	5.72	5.76	0.66	0.40	0.65	
2M - Group treated with metformin double dose (1000 mg/kg/day)						
Mean	69.5	91.5	3.92	2.70	3.27	
\pm SD	5.21	5.41	0.60	0.53	0.72	
DMMb - Group treated with the combination of disulfiram (50 mg/kg/day),						
metformin (500 mg/kg/day) and mebendazole (460 mg/kg/day)						
Mean	70.0	94.9	4.41	3.34	3.63	
\pm SD	6.22	6.26	0.39	0.26	0.45	
Mb - Group treated with mebendazole (460 mg/kg/day)						
Mean	68.8	94.8	4.22	3.05	3.35	
± SD	4.29	4.39	0.81	0.61	0.75	

control and treated groups (the disulfiram and metformin experiment)

D_{max} = longest tumor diameter (cm);

*p < 0.05

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Table 3. Characteristics of animals and quantitative pathological characteristics of tumors in

control and treated groups (the disulfiram and deoxycholic acid experiment)

	Han	nster		Tumor			
	Weight at start (g)	Weight at end (g)	Weight (g)	D _{max} (cm)	Volume (cm ³)		
Control gr	oup						
Mean	68.1	95.1	3.22	2.92	2.62		
±SD	1.85	2.01	0.2	0.1	0.19		
Group trea	Group treated with disulfiram (50 mg/kg/day)						
Mean	67.5	92.3	3.06	2.88	2.55		
±SD	2.44	2.49	0.19	0.16	0.17		
Group treated with deoxycholic acid (100 mg/kg/day)							
Mean	70.1	93.4	1.83	2.74	1.49		
±SD	2.09	2.14	0.11	0.18	0.11		
*Group tre	*Group treated with the combination of						
disulfiram	(50 mg/kg/	day) and de	oxycholic a	cid (100 mg	/kg/day)		
Mean	69.3	95.2	0.25	0.94	0.23		
±SD	2.18	1.96	0.07	0.09	0.06		
Group treated with the combination of disulfiram (50 mg/kg/day), deoxycholic acid (100 mg/kg/day) and mebendazole (460 mg/kg/day)							
Mean	69.9	93.8	4.16	3.25	3.4		
±SD	2.42	2.43	0.17	0.13	0.15		
Group treated with mebendazole (460 mg/kg/day)							
Mean	68.3	93.9	3.21	2.85	2.69		
+SD	1 99	2.09	0.18	0.19	0.16		

D_{max} = longest tumor diameter (cm);

Table 4. Characteristics of animals and quantitative pathological characteristics of tumors in

control and treated groups (the deoxycholic acid and metformin experiment)

	Hamster		Tumor		
	Weight at	Weight at	Weight	Dmax	Volume
	start (g)	end (g)	(g)	(cm)	(cm ³)
Control group					
Mean	69.1	96.2	3.1	2.97	2.5
±SD	1.77	2.07	0.1	0.21	0.09
Group treated	with deoxycholi	ic acid (100 mg/	(kg)		
Mean	70.3	94.3	2.2	2.96	1.76
±SD	2.19	2.11	0.05	0.11	0.05
Group treated with metformin (500 mg/kg/day)					
Mean	70.1	92.2	2.65	2.71	2.10
±SD	1.75	2.27	0.09	0.15	0.08
*Group treated	d with the comb	ination of deoxy	cholic acid (100) mg/kg/day) and	d metformin
(500 mg/kg/da	iy)				
Mean	68.9	95.2	1.45	0.98	1.4
±SD	2.32	2.04	0.03	0.07	0.03
Group treated	with the combin	ation of deoxyc	holic acid (100	mg/kg/day), me	tformin (500
mg/kg/day)					
and mebendaz	ole (460 mg/kg/	day)			
Mean	70.9	94.7	2.71	2.96	2.2
±SD	2.39	2.41	0.12	0.11	0.10
Group treated	with mebendaze	ole (460 mg/kg/	lay)		
Mean	69.5	95.7	2.29	2.88	1.83
±SD	2.01	1.99	0.07	0.12	0.06

D_{max} = longest tumor diameter (cm);



Figure 1. Tumor volume growth during course of the I. disulfiram and metformin experiment: interpolated line chart between average values and SD values;





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;

DM Mb – group treated with the combination of disulfiram, metformin and mebendazole; Mb – group treated with mebendazole;



Figure 3. Tumor volume growth during course of the II. disulfiram and deoxycholic acid experiment: interpolated line chart between average values and SEM values;



Figure 4. Means and standard errors (SEM) of quantitative pathological and physicochemical characteristics of the excised tumors in the **II.** disulfiram and deoxycholic acid experiment; C – control group; DS – group treated with disulfiram; DA – group treated with deoxycholic acid; DD – group treated with the combination of disulfiram and deoxycholic acid; DDMb – group treated with the combination of disulfiram, deoxycholic acid and mebendazole; Mb – group treated with mebendazole;



Figure 5. Tumor volume growth during course of the III. deoxycholic acid and metformin experiment: interpolated line chart between average values and SEM values;



Figure 6. Means and standard errors (SEM) of quantitative pathological and physicochemical characteristics of the excised tumors in the III. deoxycholic acid and metformin experiment.

C – control group; DA – group treated with deoxycholic acid; M – group treated with metformin; DM – group treated with the combination of deoxycholic acid and metformin; DM Mb – group treated with the combination of deoxycholic acid, metformin and mebendazole; Mb – Group treated with mebendazole;





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; DM Mb – group treated with the combination of disulfiram, metformin and mebendazole; Mb – group treated with mebendazole;



Figure 8. Means and standard errors (SEM) of histopathological-immunohistochemical characteristics of the excised tumors in the II. disulfiram and deoxycholic acid experiment; C – control group; DS – group treated with disulfiram; DA – group treated with deoxycholic acid; DD – group treated with the combination of disulfiram and deoxycholic acid; DDMb – group treated with the combination of disulfiram, deoxycholic acid and mebendazole; Mb – group treated with mebendazole;



Figure 9. Means and standard errors (SEM) of histopathological-immunohistochemical characteristics of the excised tumors in the III. deoxycholic acid and metformin experiment; C – control group; DA – group treated with deoxycholic acid; M – group treated with metformin; DM – group treated with the combination of deoxycholic acid and metformin; DM Mb – group treated with the combination of deoxycholic acid, metformin and mebendazole; Mb – group treated with mebendazole;



Figure 10. Illustration of results obtained by used staining methods; HE staining images of BHK fibrosarcoma sections (the first row): A – numerous mitotic figures within the tissue of an experimental BHK sarcoma (arrow); B – tumor angiogenesis; C – multiple areas of tumor necrosis (*); D – frequent areas of fresh bleeding in the tumor tissue (arrow); immunohistochemical staining images of hamster fibrosarcoma sections: Ki-67, PCNA, GLUT1, iNOS, CD34, CD31, COX4, and Cytochrome C, examples from the control group and the group treated with the examined combination