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Diclofenac and metformin synergistic dose dependent inhibition of hamster fibrosarcoma, rescued with mebendazole

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ABSTRACT

We examined whether combinig diclofenac and metformin in doses equivalent to human doses would synergize their anticancer activity on fibrosarcoma inoculated to hamsters and in vitro. Rescue experiment was performed to examine whether the prosurvival NF-κB stimulation by mebendazole can reverse anticancer effects of the treatment. BHK-21/C13 cell culture was subcutaneously inoculated to Syrian golden hamsters randomly divided into groups (6 animals per group): 1) untreated control; treated daily with 2) diclofenac; 3) metformin; 4) combinations of diclofenac and metformin at various doses; 5) combination of diclofenac, metformin and mebendazole; 6) mebendazole. Dose response curves were made for diclofenac and metformin combination. Tumor growth kinetics, biophysical, pathological, histological and immunohistochemical characteristics of excised tumors and hamster organs as well as biochemical and hematological blood tests were compared among the groups. Single treatments had no anticancer effects. Diclofenac (60 mg/kg/day) exhibited significant ($P < 0.05$) synergistic inhibitory effect with metformin (500 mg/kg/day) on all tumor growth parameters, without toxicity and influence on biochemical and hematological blood tests. The same results were obtained with double doses of diclofenac and metformin combination. The addition of mebendazole to the diclofenac and metformin combination rescued tumor expansion. Furthermore, diclofenac with metformin demonstrated antiproliferative effects in hamster fibrosarcoma BHK-21/C13, human lung carcinoma A549 (CCL-185), colon carcinoma HT-29 (HTB-38) and cervical carcinoma HeLa (CCL-2) cell cultures, with markedly lower cytotoxicity in the normal fetal lung MRC-5 cells. In conclusion, diclofenac and metformin combination may be recommended for potential use in oncology, due to synergistic anticancer effect in doses achievable in humans.

1. Introduction

Drug repurposing and drug combinations are major approaches applied to improve cancer therapy by reducing toxicity, increasing efficacy, decreasing dosage (at an equal or increased effect) and also by antagonizing drug resistance in oncology [1]. Diclofenac and metformin can be recognized among potential drug combination candidates for anticancer repurposing, needing approval in following future investigations, as they both target cell proliferative signaling [2–6]. The aim of our investigation is discovery of effective non-toxic anticancer combinations that suppress fibrosarcoma in hamsters and could be immediately applied in oncology, using marketed pleiotropic non-expensive drugs.

Through deeper insight in earlier single drug investigations, it is observed that diclofenac and metformin can separately affect a huge number of similar pathways and molecules, with possibility to treat cancer. These mechanisms include the following: inhibition of NF-κB (diclofenac [7–17], metformin [18–26]); inhibition of glucose metabolism (diclofenac [27–31], metformin [32–34]); folate inhibition (diclofenac [35,36], metformin [37–39]) and increasing of ROS and causing oxidative stress (diclofenac [40,41], metformin [32–34]).

NF-κB is a key regulator of cancer growth, which has been involved

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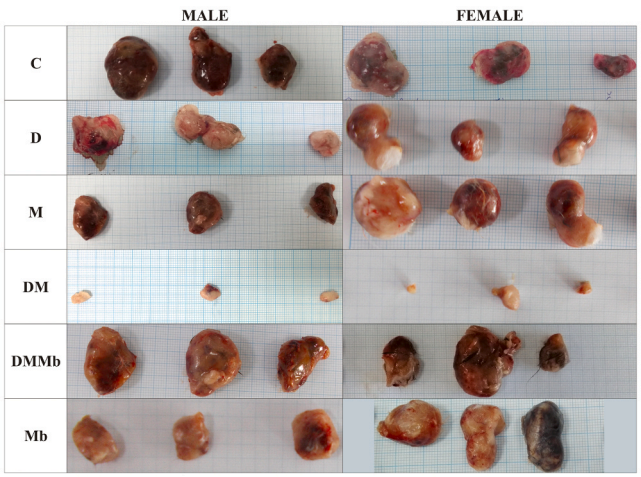

Fig. 1. The first experiment. Extirpated fibrosarcomas placed on one-millimeter grid paper for visual dimension comparison: C - Control group; D - Group treated with diclofenac; M - Group treated with metformin; DM - Group treated with the combination of diclofenac and metformin; DMMb - Group treated with the combination of diclofenac, metformin and mebendazole; Mb - Group treated with mebendazole.

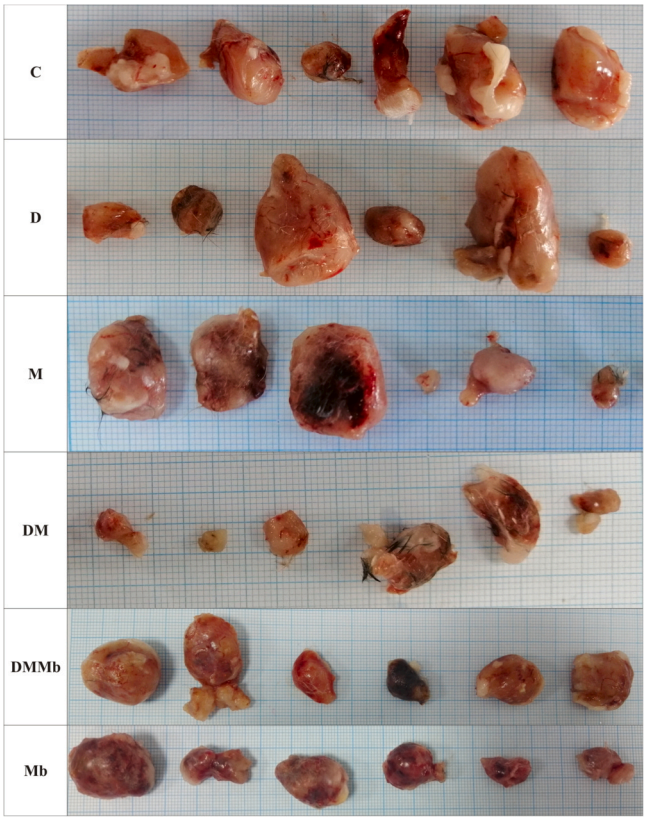

Fig. 2. The second experiment. Extirpated fibrosarcomas placed on one-millimeter grid paper for visual dimension comparison: C - Control group; D - Group treated with diclofenac; M - Group treated with metformin; DM - Group treated with the combination of diclofenac and metformin; DMMb - Group treated with the combination of diclofenac, metformin and mebendazole; Mb - Group treated with mebendazole.

in the pathogenesis of the most malignancies and has a main role in the cellular life/death balance, such as apoptosis, autophagy and necroptosis in cancer development and progression [42]. Mebendazole is shown to activate NF-κB [43–52]. For that reason, which was confirmed in our own research with mebendazole NF-κB stimulation in hamster fibrosarcoma [33,34], we have decided to use this drug for NF-κB activation in our rescue experiment. To connect future work with our previous research with mebendazole [33,34], a triple drug combination is used for experimental fibrosarcoma treatment, and the combination anticancer effect will be analyzed and compared.

Diclofenac and metformin separately exhibit anticancer activities via inhibition of NF-κB and other diverse molecular mechanisms in different cancer cell lines. However, their anticancer capabilities are limited by high doses required for clinical effects with potential adverse effects of such doses, or by limited clinical data regarding efficacy in their separate application. In this study, we tested the combined anticancer effects of diclofenac and metformin on fibrosarcoma, inoculated to hamsters, in doses extrapolated from usual human doses, equivalent for hamsters. We expected that the combined treatment with diclofenac and metformin could target some pathways simultaneously (NF-κB signaling directly or indirectly, affecting NF-κB or upstream signals) and therefore exert synergistic anticancer effects in clinically acceptable doses.

From previous studies it can be seen that NSAID 5-aminosalicylic acid and metformin in combination cooperate to decrease proliferation and induce apoptosis in colorectal cancer cell lines [4]. NF-κB levels showed signicifant decrease in colorectal cancer cell lines upon the addition of metformin to 5-aminosalicylic acid, an anti-inflammatory modulator similar to diclofenac [4]. The combination of the effects of anti-inflammatory drugs and metformin in vitro on cancer cell cultures allows for their potential anti-tumor activity to acumulate, leading to lower drug doses in effective oncological treatments [4].

NF-κB activity is dependent on its phosphorilation and nuclear translocation. Diclofenac inhibits the phosphorilation, activation and nuclear translocation of NF-κB and finally blocks NF-κB activity in human airway epithelial cells [7].

The major anticancer mechanism of NSAIDs according to literature is NF-κB suppression, which subsequently decreases transcription of growth factors, chemokines and proteases, inhibiting angiogenesis, invasion and resistance to apoptosis in ovarian carcinoma cells [8]. Authors are of the opinion that NSAIDs should be investigated in animal models to test likely benefits in ovarian and other cancers [8].

Cancer cells treated with NSAIDs, including diclofenac [9] show suppression and downregulation of NF-κB activity, which may be a mechanism underlying proapoptotic activity and causing a decrease of various NF-κB target expression, including cell addhesion molecules, growth factors and antiapoptotic proteins.

Reduced activation on NF-κB and COX-2 expression was observed after diclofenac application (or diclofenac derivatives with oleanolic acid) in normal hepatocytes (THLE-2), hepatic cancer cells (HepG2) and in mice bearing hepatoma (HepG2) xenografts [10].

Diclofenac has shown the following effects: it has reduced activation of NF-κB in human hepatoma HepG2 cells [11]; it has shown NF-κB inhibition and tumor apoptosis of primary human hepatocytes and liver HepG2 cells in a drug-induced liver injury model [12]; also, diclofenac inhibited tumor necrosis factor-α-induced NF-κB activation, causing human hepatoma HepG2 cells and mouse hepatoma Hepa1c1c7 cells apoptosis in vitro [13], as well as decreased NF-κB in mosquito fish, exhibiting a significant time and/or dose effect relationship under diclofenac exposure [14]. Diclofenac also inhibited NF-κB-driven inflammatory response [15], as well as NF-κB activation and NF-κB-regulated reporter gene expression in various tumor cells [16]; it caused suppression of the nuclear translocation of NF-κB (including phosphorilated NF-κB) and consequently inhibited NF-κB transcription in osteoclasts [17].

Based on citations [7–17], it is obvious that diclofenac inhibits NF-κB, directly or indirectly.

Similarly to diclofenac, metformin inhibits NF-κB [18].

Metformin has suppressed NF-κB activity and obstructed cancer cell proliferation and tubelike formation in colorectal cancer cells [19] and it has negatively regulated NF-κB in mouse macrophage cell line [20].

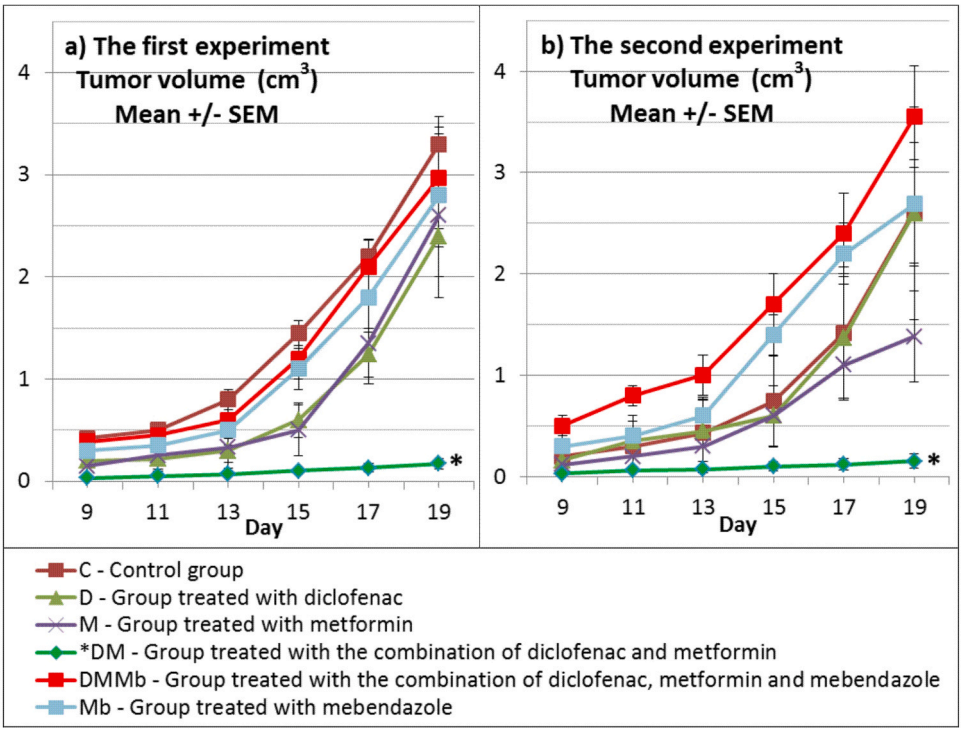

Fig. 3. Tumor volume growth during: a) the first experiment, b) the second experiment; interpolated line between means and SEM values, * statistically significant comparing to control and all other groups $P < 0.05$, as indicated.

The percentage of cell proliferation and levels of NF-κB were considerably reduced in metformin treated breast cancer cell line MCF-7. Metformin may act on the proliferation and the processes of invasion and metastases of MCF-7 cells through blocking NF-κB, which is intensely expressed in breast cancer cells [21].

Metformin prevented the activation of NF-κB, inhibiting cell proliferation and migration of primary breast cancer cells (MBCDF, MBCD3, MBCD4, MBCD17, MBCD23, MBCD25), which were derived from biopsies of mastectomies performed on patients with breast cancer [22].

Pretreatment by metformin attenuated NF-κB in the hippocampus of ishemic rats after transient global cerebral ishemia [23].

Metformin has also inhibited NF-κB in tumor-infiltrating lymphocytes of mice inoculated with cancer [24], and it was found to inactivate the NF-κB signaling pathway in periodontal ligament stem cells [25] as well as inhibit NF-κB in human neuroblastoma cells [26].

To confirm hypothesis that combination of diclofenac and metformin acts synergistically against cancer cells through NF-κB inhibition (direct or indirect – possibly affecting NF-κB expression by targeting upstream signals), rescue experiment with a NF-κB stimulator, such as mebendazole, is needed [43–52]. If NF-κB stimulator mebendazole aborgates expected anticancer effect of the metformin and diclofenac combination, than it can be supposed that the combination acts through NF-κB (directly or indirectly).

During microtubule depolymerization by mebendazole, activation of NF-κB was induced in neuroblast cell culture and in vivo in mice [46] and rats [47].

Depolymerization of microtubule by mebendazole, other benzimidazoles and cold temperatures leads to activation of NF-κB in HeLa S3 cells [48].

Effects of microtubule disrupting agents (colhicine, vinblastine, benzimidazoles) are dependent upon NF-κB activation, since pharmacological inhibition of NF-κB aborgated these effects in human lung cancer cell line [49].

Variety of agents that disrupt microtubules, such as benzimidazoles, e.g. mebendazole, nocodazole etc., stimulated NF-κB in rat myogenic cells during myogenesis [50]. In contrast, taxol, a microtubule stabilizing agent, suppressed NF-κB and inhibited spontaneous differentiation of rat myogenic cells as well [50].

The microtubule depolymerizing agents (colhicine, vinblastine, vincristine, nocodazole, mebendazole) were found to dramatically increase the translocation of NF-κB from the cytoplasm to the nucleus as well as NF-κB activation (14–39 fold increases) in cultured vascular smooth muscle cells [51]. Taxol, which stabilizes microtubules, blocked this effect [51]. Activation of NF-κB allows the active subunit of NF-κB to migrate to the nucleus [51].

Microtubule disrupting agents (colhicine, vinblastine, vincristine), as mebendazole, have been shown to elevate NF-κB production in the murine macrophage cell line [52].

Recently, researchers have found that mebendazole is unique among tubulin-active drugs in activating the MEK-ERK pathway [53]. ERK activates NF-κB by induction of IkBα degradation [54] and mediates cell survival, activation and differentiation. In cancer, MAPK/MEK/ERK/NF-κB signaling cascade activation is mostly associated with tumor growth promotion [53]. Furthermore, activation of NF-κB requires the activity of MEK/ERK [55]. Hyperactivation of NF-κB via the MEK/ERK signaling is indispensable for the inhibition of apoptosis in leukemia cells [55]. The immunological effect of mebendazole, seen in some studies, may be due to monocyte/macrophage activation via ERK activation [56].

According to the our previous reports, combinations which contain metformin with another repurposed non-oncological drug exhibited measurable biometrical, histological and immunohistochemical anticancer effects on subcutaneously inoculated fibrosarcoma (BHK-21/C13 cell line) in hamsters, without toxicity [32–34,57].

Diclofenac, metformin and mebendazole in animals show similar bioavailability and pharmacokinetics to those found in humans [58–61].

The pharmacokinetic parameters of I.V. and P.O. diclofenac show that the drug is comparably absorbed, distributed and eliminated in rats, rabbits, sheep, buffalo calves and humans [58].

Metformin levels in colorectal cancer tissue of xenograft in mice (treated P.O. or I.P.) corresponded to the plasma concentrations (9–215 μM) [59].

Table 1
Characteristics of hamsters and quantitative pathological and biophysical characteristics of extirpated tumors in the first experiment.

	Hamster			Tumor								
No	Weight at start (g)	Weight at end (g)	Sex	Weight (g)	D_{max} (cm)[a]	Volume (cm^3)	Tumor burden (%)	Density (g/cm^3)	Area (cm^2)	D_{max}/ Density (cm^4/g)	Area/ Density (cm^5/g)	Volume/ Density (cm^6/ g)
Control group (C)												
1	75	98	M	4.4	3.4	3.9	4.5	1.113	14.8	3.05	13.3	3.51
2	77	98	M	4.3	3.5	3.7	4.4	1.162	14.1	3.01	12.1	3.18
3	67	95	M	3.9	2.7	3.4	3.6	1.150	12.9	2.35	11.2	2.96
4	72	90	F	4.0	2.6	3.4	4.5	1.176	13.0	2.21	11.1	2.89
5	65	88	F	3.6	2.5	3.1	4.1	1.161	11.0	2.15	9.5	2.67
6	62	83	F	2.3	2.0	2.0	2.8	1.150	8.0	1.74	7.0	1.74
Mean	70	92		3.8	2.8	3.3	4.0	1.152	12.3	2.42	10.7	2.83
±SD	5.9	6.0		0.77	0.57	0.67	0.67	0.021	2.47	0.51	2.2	0.60
Group treated with diclofenac (D)												
1	72	92	M	4.1	2.7	3.5	4.4	1.171	13.4	2.31	11.4	2.99
2	67	95	M	4.0	3.0	3.4	4.2	1.176	12.1	2.55	10.2	2.89
3	61	80	M	1.9	1.8	1.6	2.4	1.188	6.7	1.52	5.6	1.35
4	66	85	F	3.3	2.5	2.8	3.9	1.179	10.0	2.12	8.5	2.38
5	63	84	F	1.3	1.4	1.1	1.5	1.182	5.0	1.18	4.2	0.93
6	65	87	F	2.3	2.2	2.0	2.6	1.150	7.9	1.91	7.0	1.74
Mean	66	87		2.8	2.3	2.4	3.2	1.174	9.2	1.93	7.8	2.05
±SD	3.8	5.5		1.16	0.59	0.99	1.17	0.013	3.23	0.51	2.7	0.84
Group treated with metformin (M)												
1	70	97	M	4.3	2.8	3.7	4.4	1.162	13.3	2.41	11.5	3.18
2	65	99	M	4.0	2.7	3.4	3.4	1.176	12.4	2.30	10.5	2.89
3	68	94	M	3.2	2.6	2.7	3.4	1.185	10.3	2.20	8.7	2.28
4	62	85	F	2.7	1.9	2.3	3.2	1.174	8.7	1.62	7.4	1.96
5	61	82	F	2.0	1.6	1.6	2.4	1.250	6.8	1.28	5.4	1.28
6	65	90	F	2.5	2.3	2.1	2.8	1.190	8.2	1.93	6.9	1.76
Mean	65	91		3.1	2.3	2.6	3.3	1.189	10.0	1.96	8.4	2.23
±SD	3.4	6.7		0.89	0.48	0.80	0.68	0.031	2.53	0.44	2.3	0.71
Group treated with the combination of diclofenac and metformin (DM)												
1	75	92	M	0.17	1	0.15	0.2	1.133	1.51	0.88	1.33	0.13
2	70	85	M	0.42	1.2	0.38	0.5	1.105	2.71	1.08	2.45	0.34
3	67	88	M	0.14	1	0.13	0.2	1.077	1.35	0.92	1.25	0.12
4	63	82	F	0.02	0.4	0.02	0.02	1.000	0.35	0.4	0.35	0.02
5	65	84	F	0.28	1	0.26	0.3	1.077	2.17	0.93	2.01	0.24
6	71	95	F	0.08	0.7	0.07	0.08	1.143	0.88	0.61	0.77	0.06
Mean	69	88		0.185	0.88	0.17	0.22	1.089	1.495	0.80	1.36	0.15
±SD	4.4	5.0		0.145	0.29	0.13	0.17	0.052	0.854	0.25	0.77	0.12
Group treated with the combination of diclofenac, metformin and mebendazole (DMMb)												
1	74	96	M	4.5	2.9	3.8	4.7	1.184	13.2	2.45	11.1	3.21
2	72	99	M	4.6	2.8	4.0	4.6	1.150	13.7	2.43	11.9	3.48
3	68	91	M	3.1	2.5	2.6	3.4	1.192	9.6	2.10	8.1	2.18
4	64	93	F	2.5	2.2	2.1	2.7	1.190	8.2	1.85	6.9	1.76
5	70	98	F	4.7	3.2	4.0	4.8	1.175	14.0	2.72	11.9	3.40
6	69	85	F	1.6	1.7	1.3	2.0	1.231	5.8	1.38	4.7	1.06
Mean	70	94		3.5	2.6	2.97	3.7	1.187	10.8	2.16	9.1	2.52
±SD	3.4	5.2		1.3	0.54	1.14	1.18	0.026	3.39	0.48	3.0	1.00
Group treated with mebendazole (Mb)												
1	65	86	M	1.4	1.5	1.2	1.6	1.170	5.6	1.28	4.8	1.03
2	64	85	M	1.7	1.8	1.4	2.0	1.214	6.4	1.48	5.3	1.15
3	69	90	M	3.0	1.9	2.4	3.3	1.250	8.7	1.52	7.0	1.92
4	70	87	F	3.8	2.4	3.2	4.4	1.190	10.8	2.02	9.1	2.69
5	77	98	F	4.6	2.6	4.2	4.7	1.095	12.4	2.37	11.3	3.84
6	71	99	F	4.6	2.7	4.1	4.6	1.122	12.8	2.41	11.4	3.65
Mean	69	91		3.2	2.2	2.8	3.4	1.174	9.5	1.85	8.2	2.38
±SD	4.7	6.2		1.4	0.48	1.30	1.37	0.058	3.05	0.50	2.9	1.22

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

The estimated tissue distribution parameters for 21 tissues of mice were assumed to be identical in humans in a recent physiologically based metformin pharmacokinetics model of mice for the estimation of concentrations in 21 human tissues [60].

The levels of mebendazole in animals showed similar pharmacokinetics to those found in humans [61].

Since pharmacokinetics of used drugs were not investigated in literature for hamsters with fibrosarcoma, and since drug metabolism/bioavailability can differ between species, we conducted a dose response experiment in order to definitely confirm bioavailability, pharmacokinetics, tumor drug penetration and infiltration, resulting in significant anticancer efficacy, by the same methodology.

2. Materials and methods

2.1. Animal model

Animal experiments were performed according to our previous reports [33,34]. The total of 36 Syrian golden hamsters, randomly divided in 6 groups with equal number of separated males and females were included in the first (pilot) experiment. In the second (explorative) experiment, the 36 male Syrian golden hamsters were randomly divided

Table 2
Characteristics of hamsters and quantitative pathological and biophysical characteristics of extirpated tumors in the second experiment.

	Hamster		Tumor								
No	Weight at start (g)	Weight at end (g)	Weight (g)	D_{max} (cm)[a]	Volume (cm^3)	Tumor burden (%)	Density (g/cm^3)	Area (cm^2)	D_{max}/Density (cm^4/g)	Area/ Density (cm^5/g)	Volume/ Density (cm^6/g)
Control group (C)											
1	72	99	2.5	2.3	2.2	2.6	1.157	8.6	1.99	7.43	1.90
2	68	95	2.8	2.3	2.5	3.0	1.135	9.3	2.03	8.19	2.20
3	61	84	1.2	1.2	1.0	1.4	1.175	4.5	1.02	3.83	0.85
4	63	81	2.1	2.4	1.8	2.6	1.164	7.7	2.06	6.62	1.55
5	70	99	4.8	2.4	4.3	4.8	1.116	12.9	2.15	11.56	3.85
6	66	97	4.5	2.3	3.9	4.6	1.154	12.1	1.99	10.49	3.38
Mean	67	93	2.98	2.15	2.62	3.2	1.150	9.2	1.87	8.02	2.29
±SD	4.2	7.9	1.40	0.48	1.26	1.30	0.021	3.06	0.42	2.78	1.13
Group treated with diclofenac (D)											
1	60	82	1.17	1.4	1.0	1.4	1.166	4.0	1.20	3.43	0.86
2	64	83	1.18	1.2	1.0	1.4	1.178	4.5	1.02	3.82	0.85
3	75	110	6.50	3.0	5.8	5.9	1.121	16.4	2.68	14.63	5.17
4	74	91	1.19	1.3	1.0	1.3	1.191	4.8	1.09	4.03	0.84
5	76	113	6.69	3.3	6.0	5.9	1.115	17.8	2.96	15.96	5.38
6	69	100	1.00	1.2	0.8	1.0	1.250	4.0	0.96	3.20	0.64
Mean	70	97	3.04	1.9	2.6	2.8	1.170	8.6	1.65	7.51	2.29
±SD	6.5	13.3	2.76	0.97	2.56	2.39	0.050	6.62	0.91	6.05	2.31
Group treated with metformin (M)											
1	67	92	2.3	1.9	2.0	2.5	1.153	7.7	1.65	6.68	1.73
2	65	95	2.2	1.9	1.9	2.3	1.161	7.4	1.64	6.37	1.64
3	71	97	3.3	2.2	2.9	3.4	1.146	9.8	1.92	8.55	2.53
4	60	81	0.06	0.5	0.05	0.1	1.188	0.6	0.42	0.81	0.042
5	73	89	1.4	1.5	1.2	1.6	1.166	5.3	1.29	4.55	1.03
6	77	100	0.26	0.8	0.21	0.26	1.238	1.7	0.65	1.37	0.17
Mean	69	92	1.59	1.5	1.38	1.7	1.175	5.4	1.26	4.67	1.19
±SD	6.1	6.7	1.26	0.68	1.11	1.31	0.034	3.62	0.6	3.17	0.97
Group treated with the combination of diclofenac and metformin (DM)											
1	64	82	0.042	0.8	0.04	0.05	1.055	0.8	0.76	0.76	0.038
2	62	85	0.01	0.4	0.01	0.01	1.000	0.3	0.40	0.30	0.01
3	66	92	0.052	0.7	0.05	0.06	1.040	0.9	0.67	0.86	0.048
4	60	91	0.452	1.3	0.43	0.5	1.051	3.25	1.24	3.09	0.409
5	74	103	0.37	1.4	0.35	0.36	1.057	2.64	1.32	2.50	0.33
6	63	90	0.036	0.8	0.034	0.04	1.059	0.64	0.75	0.60	0.032
Mean	65	91	0.160	0.9	0.15	0.17	1.044	1.42	0.86	1.35	0.144
±SD	4.9	7.2	0.196	0.38	0.18	0.21	0.022	1.21	0.35	1.15	0.176
Group treated with the combination of diclofenac, metformin and mebendazole (DMMb)											
1	75	102	5.3	2.6	4.5	5.2	1.178	13.8	2.21	11.71	3.82
2	74	106	5.8	3.5	5.1	5.5	1.137	16.0	3.08	14.07	4.49
3	65	88	2.6	2.0	2.2	3.0	1.182	8.3	1.69	7.02	1.86
4	60	84	2.3	1.7	1.9	2.7	1.210	7.4	1.40	6.12	1.57
5	63	86	3.9	2.4	3.4	4.5	1.147	11.2	2.09	9.76	2.96
6	71	97	4.8	2.4	4.2	4.9	1.143	12.9	2.10	11.29	3.67
Mean	68	94	4.12	2.4	3.55	4.3	1.166	11.6	2.10	9.99	3.06
±SD	6.2	9.1	1.44	0.61	1.288	1.17	0.028	3.30	0.57	3.00	1.15
Group treated with mebendazole (Mb)											
1	78	112	6.0	2.8	5.2	5.3	1.154	15.0	2.43	13.00	4.77
2	70	95	2.4	2.4	2.1	2.5	1.143	8.5	2.10	7.44	1.84
3	67	94	4.0	2.5	3.5	4.2	1.143	11.6	2.19	10.15	3.06
4	69	88	3.1	2.2	2.7	3.5	1.148	9.7	1.92	8.45	2.35
5	73	87	1.3	1.8	1.14	1.5	1.141	5.5	1.58	4.82	1.00
6	77	101	1.8	2.0	1.5	1.8	1.200	6.6	1.67	5.50	1.25
Mean	72	96	3.10	2.8	2.69	3.1	1.155	9.5	1.98	8.23	2.38
±SD	4.5	9.3	1.71	1.37	1.492	1.47	0.023	3.47	0.32	3.04	1.39

[a] Longest tumor diameter (cm).

in 6 groups. For the third (confirmative) experiment, another 36 male Syrian golden hamsters, randomly distributed in 6 equal groups, were used. All animals, in our three experiments, were ~12 weeks age, ~70 g weight, maintained under standard housing conditions and subjected to protocol approved by the University of Novi Sad Animal Ethics Committee (Novi Sad, Serbia): No. 04–81/25–5 dated 22nd July 2020, Doc. No. EK: П-Е-2020–07; No. 04–150/15 dated 14th March 2022, Doc. No. EK: I-2022–01; No. 04–150/15 dated 14th March 2022, Doc. No. EK: I-2022–02; and approved by the Ministry of Agriculture, Forestry and Water Management - Veterinary Directorate (Belgrade, Serbia): No. 323–07–09359/2020–05 dated 2nd September 2020; No. 323–07–03995/2022–05 dated 28th March 2022; No. 323–07–03996/2022–05 dated 28th March 2022; No. 323–07–03997/2022–05 dated 28th March 2022. (Supplementary data). In the first (pilot) experiment, all groups of animals (control and treated) contained equal number of males and females. In the second (explorative) and third (confirmative) experiments, all groups of animals (control and treated) contained equal number of males only, to eliminate the influence of sex on variability of experiment results. Influence of sex and possible different effects of sexual hormones on tumor growth were not investigated during our experiments. Taking the 3 R into account, the number of animals in all groups is reduced to a minimum which can be statistically relevant (6 hamsters per group).

BHK-21/C13 cells, cultured as previously reported (Supplementary data), were subcutaneously inoculated (1 ml, 2 $\times 10^6$ cells/ml) into the back of all hamsters by the same researcher.

Table 3
Statistical significances expressed as *P*-values for comparisons of quantitative pathological and biophysical tumor characteristics in the first experiment.

Groups comparison	Weight	Length (D_{max})	Volume	Tumor burden	Density	Surface area	Length/ density	Surface/ density	Volume/ density
C/D	0.09855	0.20371	0.09999	0.20447	0.05500	0.09999	0.18290	0.05984	0.09988
C/M	0.20964	0.18791	0.18778	0.17504	0.04800[a]	0.23714	0.18129	0.11746	0.23798
[a]**C/DM**	0.00080[a]	0.00146[a]	0.00086[a]	0.00072[a]	0.01997[a]	0.00097[a]	0.00195[a]	0.00103[a]	0.000096[a]
D/M	0.55914	1.00000	0.62817	0.75026	0.29702	0.56699	0.85537	0.58274	0.57005
[a]**D/DM**	0.00395[a]	0.00374[a]	0.00352[a]	0.00294[a]	0.00862[a]	0.00312[a]	0.00496[a]	0.00367[a]	0.00397[a]
[a]**M/DM**	0.00104[a]	0.00248[a]	0.00147[a]	0.00091[a]	0.00751[a]	0.00113[a]	0.00375[a]	0.00132[a]	0.00205[a]
C/DMMb	0.56304	0.50964	0.51047	0.54134	0.04397[a]	0.47697	0.46934	0.44792	0.50980
[a]**DMMb/DM**	0.00246[a]	0.00212[a]	0.00314[a]	0.00187[a]	0.00789[a]	0.00264[a]	0.00297[a]	0.00169[a]	0.00274[a]
C/Mb	0.46005	0.09011	0.47911	0.45891	0.47725	0.18005	0.16995	0.20367	0.48404

[a]$P < 0.05$. C - control group; D - group treated with diclofenac; M - group treated with metformin; DM - group treated with the combination of diclofenac and metformin; DMMb - group treated with the combination of diclofenac, metformin and mebendazole; Mb - group treated with mebendazole.

Table 4
Statistical significances expressed as *P*-values for comparisons of quantitative pathological and biophysical tumor characteristics in the second experiment.

Groups comparison	Weight	Length (D_{max})	Volume	Tumor burden	Density	Surface area	Length/ density	Surface/ density	Volume/ density
C/D	0.93052	0.41237	0.98704	0.65070	0.46926	0.78013	0.51974	0.82493	1.00000
C/M	0.10000	0.06950	0.10326	0.07521	0.23986	0.09247	0.07923	0.06004	0.10042
[a]**C/DM**	0.00699[a]	0.00447[a]	0.00705[a]	0.00294[a]	0.00146[a]	0.00352[a]	0.00749[a]	0.00403[a]	0.00701[a]
D/M	0.29724	0.48024	0.45933	0.47006	0.77924	0.43094	0.47232	0.44275	0.41456
[a]**D/DM**	0.02207[a]	0.04211[a]	0.04450[a]	0.02411[a]	0.00368[a]	0.03572[a]	0.06487	0.04022[a]	0.04911[a]
[a]**M/DM**	0.02012[a]	0.09536	0.02972[a]	0.01905[a]	0.00095[a]	0.03726[a]	0.26934	0.04537[a]	0.03328[a]
C/DMMb	0.25371	0.49272	0.26017	0.21869	0.31072	0.24894	0.47043	0.30065	0.30772
[a]**DMMb/DM**	0.00199[a]	0.00408[a]	0.00214[a]	0.00079[a]	0.00087[a]	0.00158[a]	0.00722[a]	0.00211[a]	0.00283[a]
C/Mb	0.80043	0.45006	0.87935	0.81432	0.59664	0.79453	0.52862	0.86204	0.86795

[a]$P < 0.05$. C - control group; D - group treated with diclofenac; M - group treated with metformin; DM - group treated with the combination of diclofenac and metformin; DMMb - group treated with the combination of diclofenac, metformin and mebendazole; Mb - group treated with mebendazole.

The rationale underlaying the selection of Syrian hamsters as study subjects and context for the choice were as follows. A model of BHK-21/C13 cell culture induced sarcoma in Syrian hamsters is easy reproducible, we regularly induced tumor on the inoculation site of BHK-21/C13 cell culture suspension; tumor is solitary, enormous, and never produced metastases. Tumor cells are very similar as BHK-21/C13 in vitro, and without influence of the host immune mechanisms. Immunologically, hamsters do not recognize BHK-21/C13 cells as tumorigenic. Because of that, transplant is growing to enormous tumor masses. This tumor is localy infiltrative. BHK-21/C13 cells are tumorigenic only for hamsters (except nude mice). Only whole live cells are tumorigenic, not DNA, or cell extracts – it's a transplantation of cells, i.e. BHK tumor is in vivo culture of BHK-21/C13 cells. We believe this model is excellent for pharmacological examination of antitumor agents, because it is not influenced by immune rejection, in contrast with some other animal tumor models.

In the first (pilot) experiment, all drugs were administered to hamsters at 25–50% of oral median lethal doses (LD_{50}) daily. The hamsters were treated orally as follows: in control group 1 with physiological saline; in group 2 with diclofenac 120 mg/kg; in group 3 with metformin 1000 mg/kg; in group 4 with combination of diclofenac 60 mg/kg and metformin 500 mg/kg; in group 5 with combination of diclofenac 60 mg/kg, metformin 500 mg/kg and mebendazole 460 mg/kg; and in group 6 with mebendazole 460 mg/kg.

For extrapolation from human dosing of already registered drugs used in our study, we used the US-FDA Guidance (*Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*) which recommends using a body surface area (BSA) normalization approach based on the following equation [62]:

HED (mg/kg) = Animal dose (mg/kg) × [Animal weight (kg) / Human weight (kg)] $^{(1–EXP)}$,

where HED is the human equivalent dose and EXP is an allometric scaling exponent. The FDA guidance used conversion factors that were derived based on a BSA scaling factor of 0.67, i.e. (1–EXP) = 1 – 0.67 = 0.33.

In the second (explorative) experiment, we used hamster doses which were exactly equivalent to human standard doses, in mg/kg daily (based on body surface area), calculated by the formula: hamster equivalent dose = standard human dose x 7.4 [63–65]. Diclofenac is orally administered in the dose 150 mg/day in humans [58]. Human equivalent dose for hamster is (150 / 60) x 7.4 = 18.5 mg/kg/day (while diclofenac oral LD_{50} for a mouse is 170–389 mg/kg). Metformin standard oral human dose is 35 mg/kg/day [66]. Human equivalent dose for hamster is 35 × 7.4 = 259 mg/kg/day (while metformin oral LD_{50} for a mouse is 1450–3500 mg/kg). Mebendazole (insoluble in water) has poor bioavailability and high oral dosage for systemic use. In cystic echinococcosis treatment, the recommended dose regimen for humans is 40–50 mg/kg/day [67]. Hamster equivalent is 50 × 7.4 = 370 mg/kg/day (while mebendazole oral LD_{50} for a mouse is up to 1280 mg/kg). In our second (explorative) validation experiment, we used the same doses in single and combined treatments of certain drugs: diclofenac 20 mg/kg/day, metformin 250 mg/kg/day and mebendazole 370 mg/kg/day. In the second (explorative) experiment, the hamsters were treated as follows: in control group 1 with physiological saline, in group 2 with diclofenac 20 mg/kg, in group 3 with metformin 250 mg/kg, in group 4 with combination of diclofenac 20 mg/kg and metformin 250 mg/kg, in group 5 with combination of diclofenac 20 mg/kg, metformin 250 mg/kg and mebendazole 370 mg/kg and in group 6 with mebendazole 370 mg/kg.

In the light of the first two experiments, finally we conducted the third (confirmative) dose response experiment. In the dose response experiment the hamsters (6 in each group) were treated as follows: in control group 1 with physiological saline (0% of maximal doses), in group 2 with combination of diclofenac 6 mg/kg and metformin 50 mg/kg (5% of maximal doses), in group 3 with combination of diclofenac 12 mg/kg and metformin 100 mg/kg (10% of maximal doses), in group 4 with combination of diclofenac 30 mg/kg and metformin 250 mg/kg (25% of maximal doses), in group 5 with combination of diclofenac 60 mg/kg and metformin 500 mg/kg (50% of maximal doses) and in group 6 with combination of diclofenac 120 mg/kg and metformin 1000 mg/

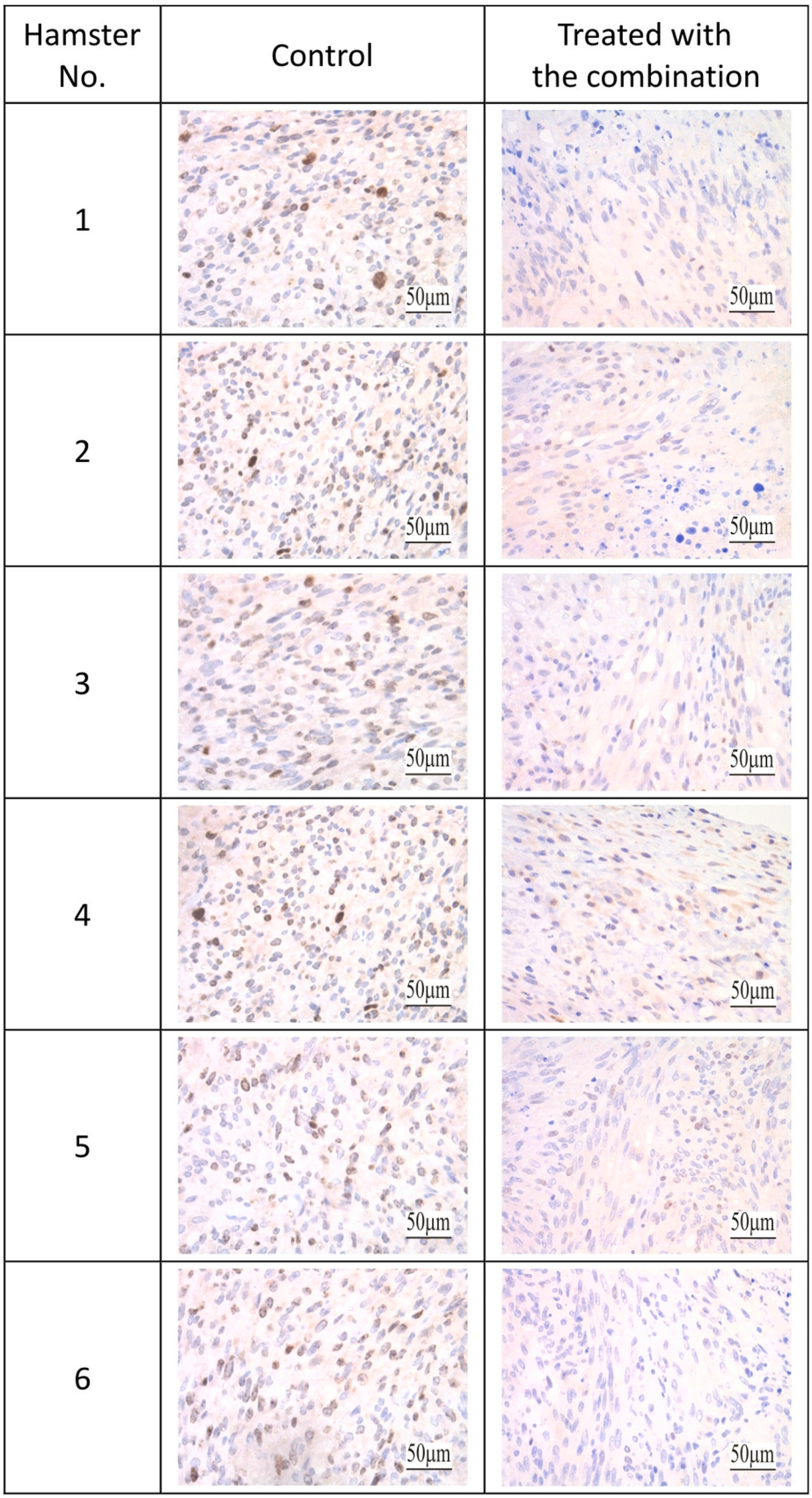

Fig. 4. Extirpated fibrosarcoma PCNA immunohistochemical staining images, illustration for six hamsters (examples for the control and the group treated with the combination of 60 mg/kg/day diclofenac and 500 mg/kg/day metformin).

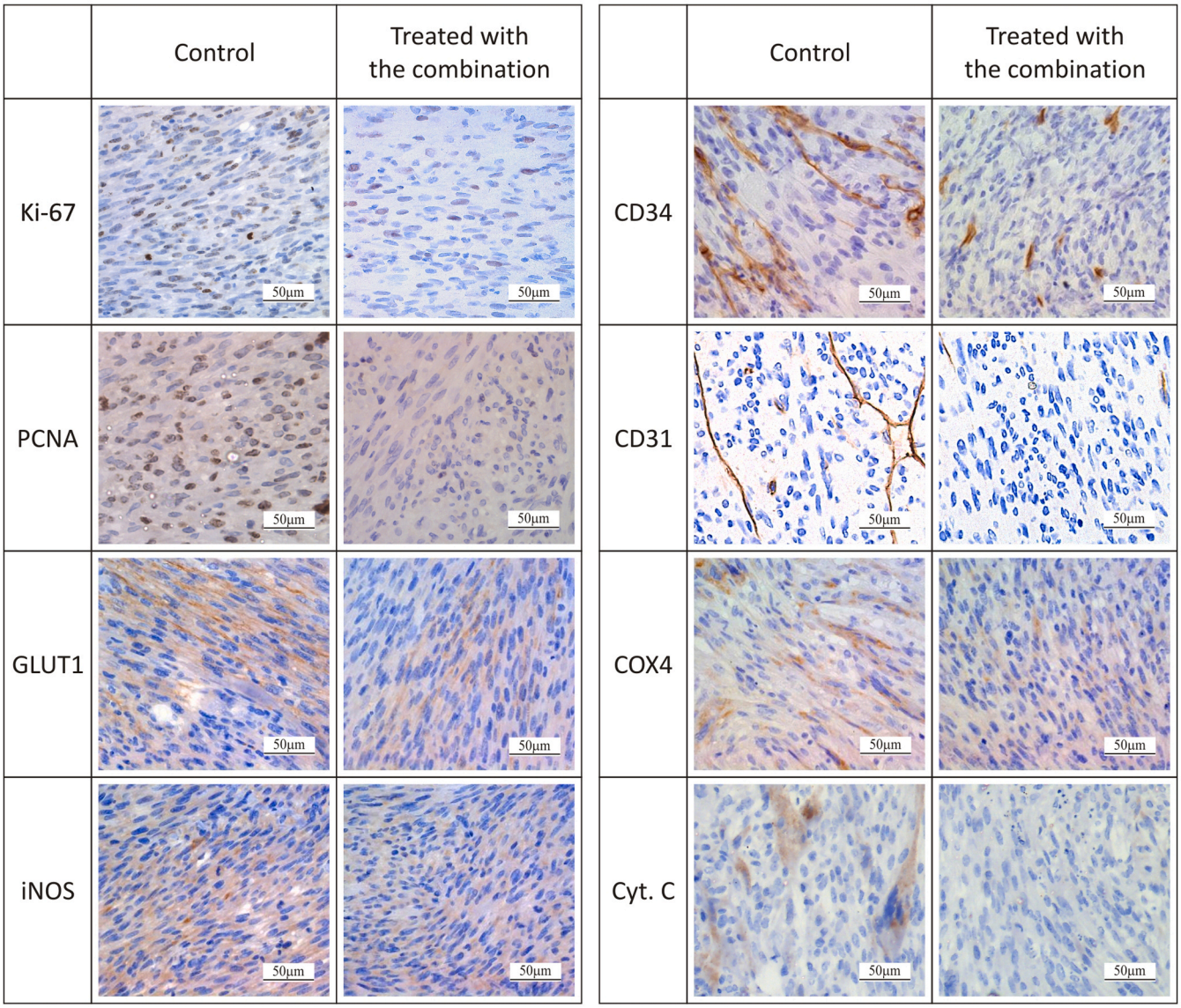

Fig. 5. Hamster fibrosarcoma Ki-67, PCNA, GLUT1, iNOS, CD34, CD31, COX4 and Cytochrome C immunohistochemical staining images, examples from the control group and the group treated with the combination of 60 mg/kg/day diclofenac and 500 mg/kg/day metformin.

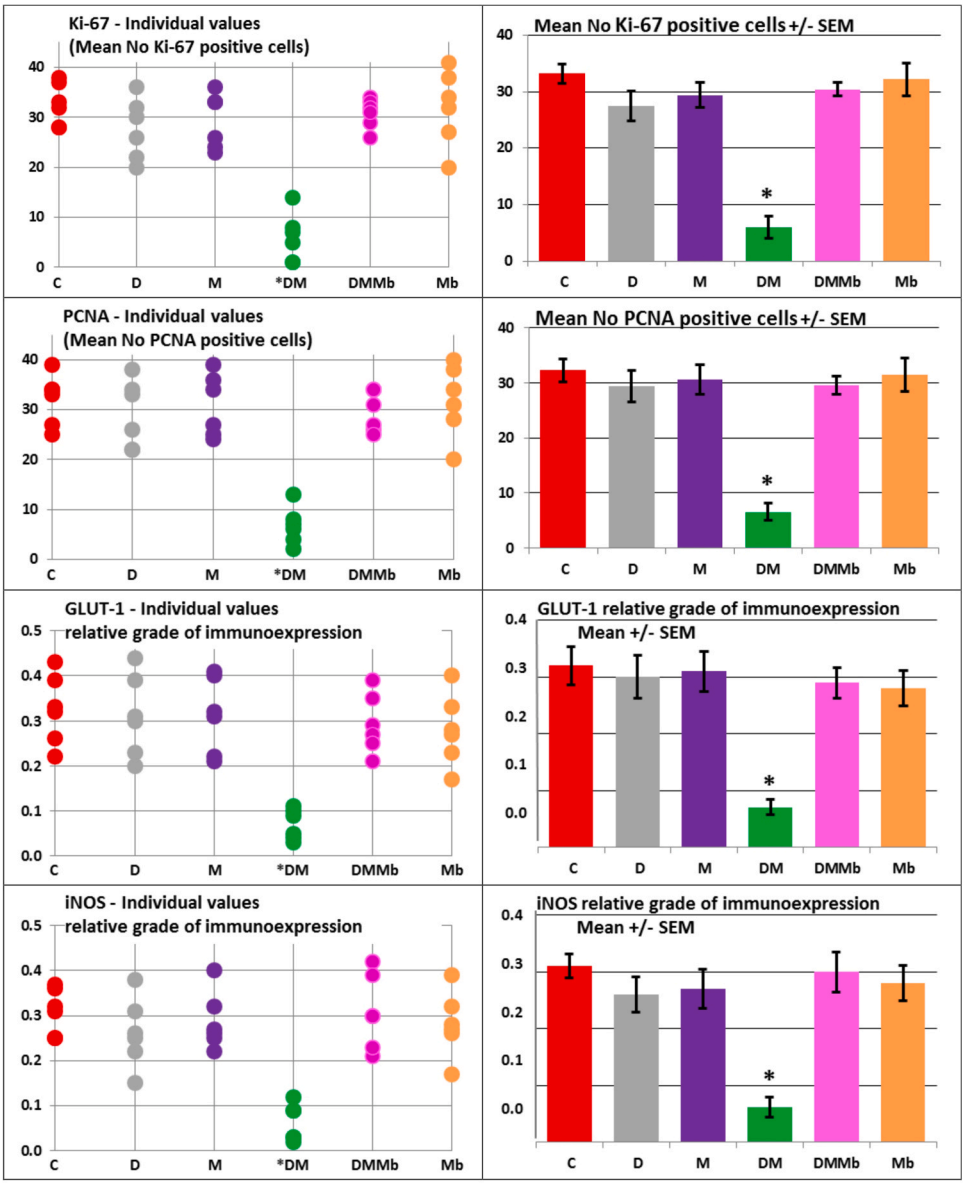

Fig. 6. Individual values, means and standard errors (SEM) of immunohistochemical-histopathological characteristics of the excised tumors in the first experiment: Ki-67, PCNA, GLUT-1, iNOS. C - Control group; D - Group treated with diclofenac; M - Group treated with metformin; DM - Group treated with the combination of diclofenac and metformin; DMMb - Group treated with the combination of diclofenac, metformin and mebendazole; Mb - Group treated with mebendazole. *Statistically significant, $P < 0.05$ as indicated.

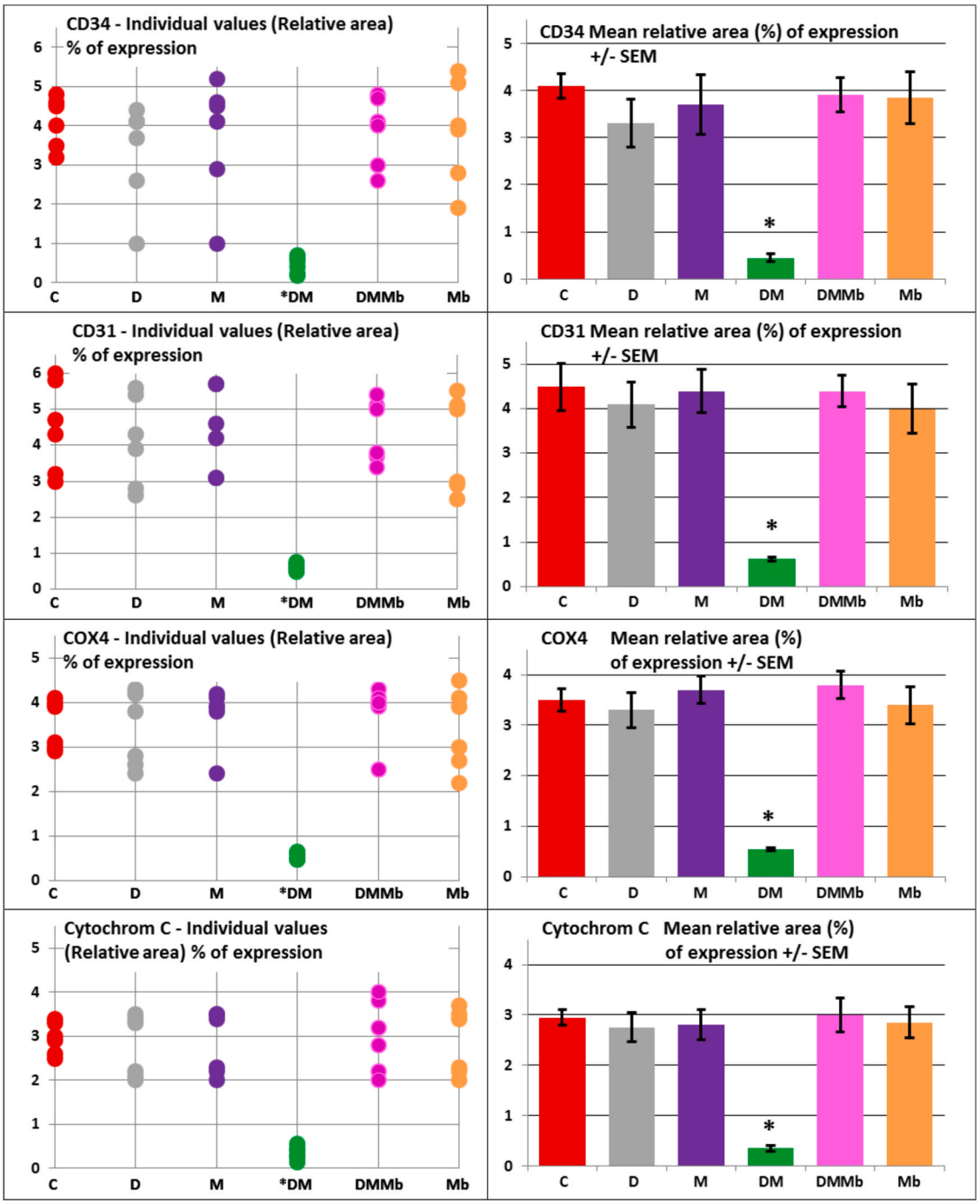

Fig. 7. Individual values, means and standard errors (SEM) of immunohistochemical-histopathological characteristics of the excised tumors in the first experiment: CD34, CD31, COX4, Cytochrom C. C - Control group; D - Group treated with diclofenac; M - Group treated with metformin; DM - Group treated with the combination of diclofenac and metformin; DMMb - Group treated with the combination of diclofenac, metformin and mebendazole; Mb - Group treated with mebendazole. *Statistically significant, $P < 0.05$ as indicated.

kg (100% of maximal doses).

All used drugs (from Galenika a. d.) were applied perorally in 1–2 ml of fluid (saline), according hamster body mass, as solution (metformin) or as suspension (diclofenac, mebendazole), via a gastric probe (daily) after cancer cell inoculation.

Absolute single doses were individually determined based on body mass (same relative dose in mg/kg), but volume of gavage (~1 ml) was based on standard drinking volume (10 ml per 100 g hamster weight daily) and was significantly (~10 times) lower than volume that hamsters need to drink every day.

Humane endpoints were established as previously reported [33,34] (Supplementary data).

All hamsters were assessed for loss of consciousness at 5 min after an intraperitoneal dose of 90 mg/kg pentobarbital (lack of visible respiration, lack of reaction on digital palpation, a toe pinch,) in order to sacrifice the hamsters 19 days post tumor cells inoculation.

After confirmation of loss of consciousness, total cardiac exsanguination (3–5.5 ml) was performed for biochemical and hematological blood analysis and vital organs (heart, lungs, stomach, intestine, liver, kidneys and brain) were removed (after animal death) for pathological,

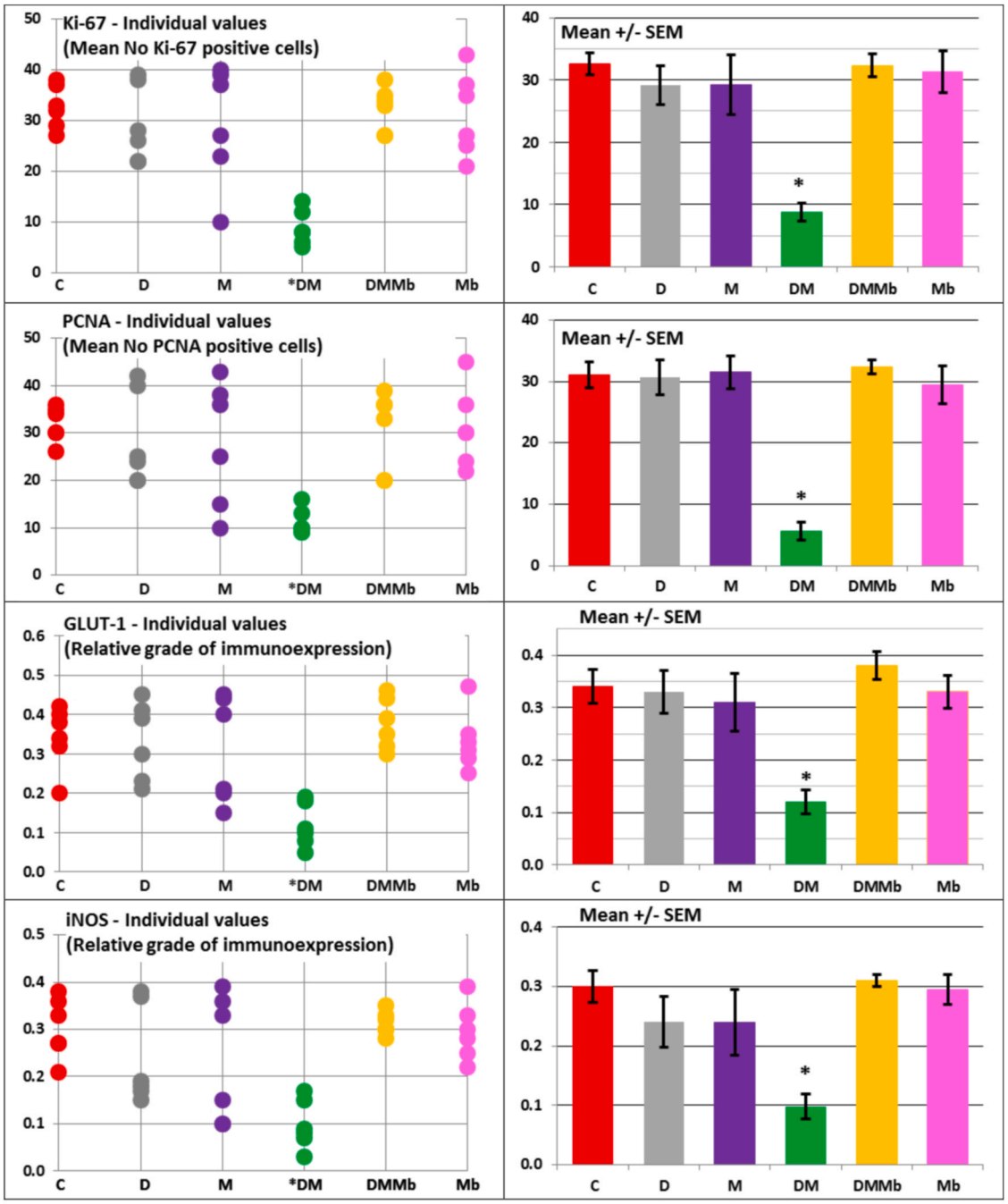

Fig. 8. Individual values, means and standard errors (SEM) of immunohistochemical-histopathological characteristics of the excised tumors in the second experiment: Ki-67, PCNA, GLUT-1, iNOS. C - Control group; D - Group treated with diclofenac; M - Group treated with metformin; DM - Group treated with the combination of diclofenac and metformin; DMMb - Group treated with the combination of diclofenac, metformin and mebendazole; Mb - Group treated with mebendazole. *Statistically significant, $P < 0.05$ as indicated.

histological and toxicological examination.

In the course of the study (during all 3 experiments: pilot, explorative and confirmative) the weights of the hamsters, the tumor diameters and volumes were evaluated daily using calipers and the ellipsoid volume formula. All hamsters were in a good condition during the study, and none were euthanized or died prior to the end of the experiments. In all three experiments, the maximal tumor diameters did not exceed 3.5 cm and the maximal tumor burdens were below 6%, which is in accordance with internationally adopted standards (Supplementary data). After excision tumors were weighed, 3 diameters exactly measured, tumor surface area calculated using ellipsoid formula, exact tumor volumes determined by the standard water volume displacement method [33, 34]. Tumor section up to 5 mm thick (for histological analysis) was taken from the area representing the widest circumference of the tumor nodule. Tumor slices (4 μm) were obtained for further pathohistological and immunohistochemical evaluation.

The combination effect was evaluated with Combination Index (CI) analysis to prove the synergism ($CI < 1$), additive effect ($CI = 1$) or antagonism ($CI > 1$) by using the general formula $CI = C_A/IC_A + C_B/IC_B$, where IC is the concentration (dose) required to produce the given effect, C is the concentration (dose) in combination that provide the same effect for drugs A and B [68].

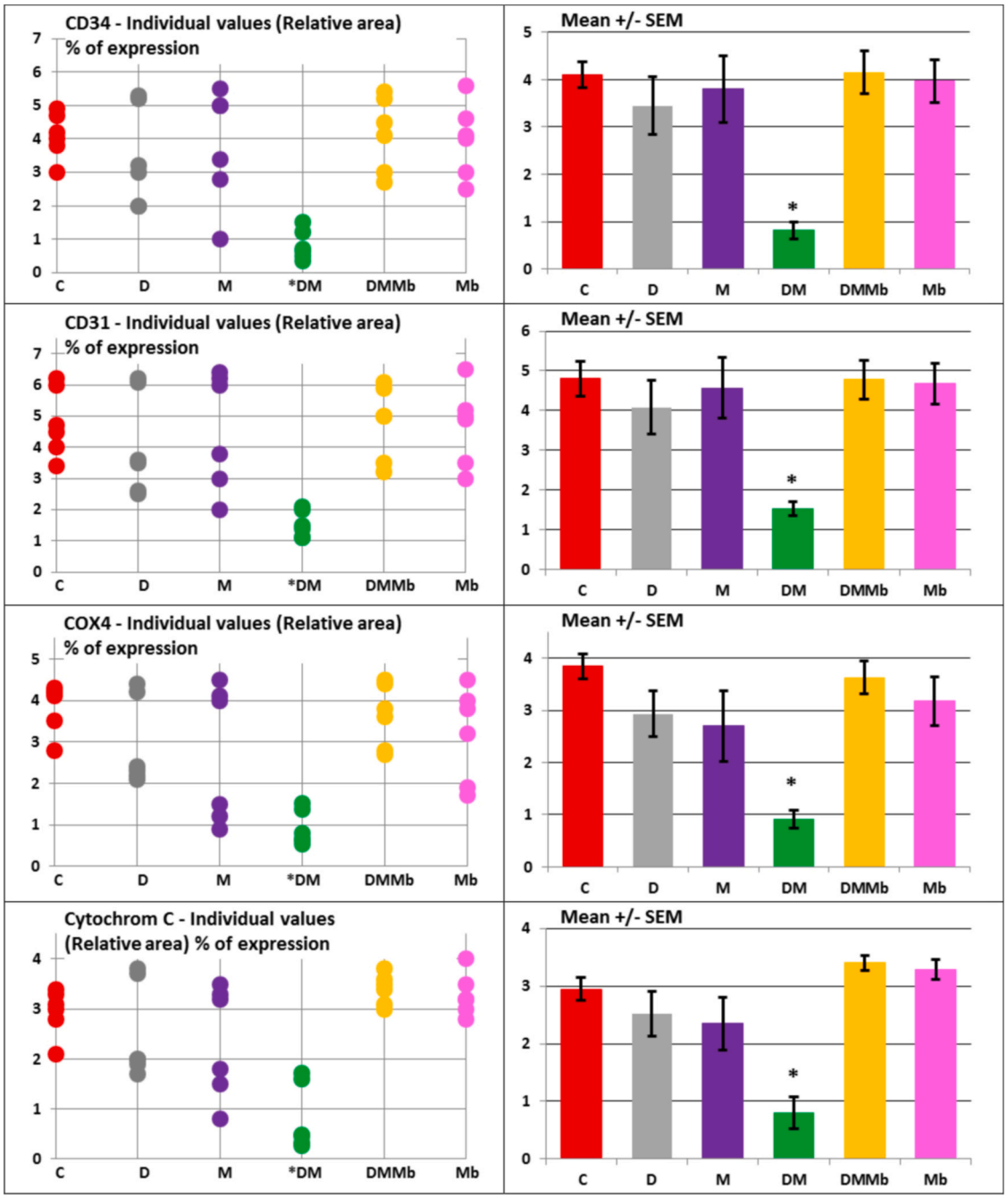

Fig. 9. Individual values, means and standard errors (SEM) of immunohistochemical-histopathological characteristics of the excised tumors in the second experiment: CD34, CD31, COX4, Cytochrom C. C - Control group; D - Group treated with diclofenac; M - Group treated with metformin; DM - Group treated with the combination of diclofenac and metformin; DMMb - Group treated with the combination of diclofenac, metformin and mebendazole; Mb - Group treated with mebendazole. *Statistically significant, $P < 0.05$ as indicated.

2.2. Histological staining procedures

The standard hematoxylin-eosin (HE) staining was performed to assess tumor growth, tissue penetration, expansion of necrosis and hemorrhagic areas. The immunohistochemical staining Ki-67, PCNA (both for tumor proliferation), GLUT1 (glucose metabolism), iNOS (NO metabolism), CD34, CD31 (both for neoangiogenesis), COX4, Cytochrome C (both for apoptosis) was performed as previously reported [33,34] (Supplementary data). Two markers (Ki-67, PCNA for tumor proliferation; CD34, CD31 for tumor angiogenesis; COX4, Cytochrome C for tumor apoptosis) were used to validate and confirm findings for the most important tumor growth processes.

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 [69] for evaluation of immunoexpression were used as described in our previous reports [33,34].

2.3. Blood hematological analyses and biochemical tests

Erythrocytes, leucocytes, lymphocytes, monocytes, granulocytes,

Table 5
Statistical significances expressed as *P*-values for comparisons of immunohistochemical-histopathological tumor characteristics in the first experiment.

Groups (comparison)	Ki-67	PCNA	GLUT-1	iNOS	CD 34	CD 31	COX 4	Cytochrom C
C/D	0.09999	0.48052	0.59343	0.23047	0.22568	0.54216	0.55028	0.53006
C/M	0.22547	0.55097	0.71756	0.45834	0.53972	0.79884	0.54027	0.55526
[a]**C/DM**	0.00098[a]	0.00095[a]	0.00192[a]	0.00104[a]	0.00077[a]	0.00114[a]	0.00075[a]	0.00041[a]
D/M	0.53987	0.64874	0.74873	0.70022	0.55997	0.56944	0.46004	0.80437
[a]**D/DM**	0.00208[a]	0.00155[a]	0.00299[a]	0.00395[a]	0.00402[a]	0.00209[a]	0.00107[a]	0.00081[a]
[a]**M/DM**	0.00108[a]	0.00120[a]	0.00255[a]	0.00405[a]	0.00458[a]	0.00101[a]	0.00079[a]	0.00087[a]
C/DMMb	0.22599	0.30995	0.49999	0.77364	0.56175	0.78544	0.47986	0.79925
[a]**DMMb/DM**	0.00092[a]	0.00097[a]	0.00195[a]	0.00257[a]	0.00110[a]	0.00091[a]	0.00078[a]	0.00090[a]
C/Mb	0.70185	0.71536	0.47045	0.48597	0.56997	0.52875	0.70987	0.65903

[a]$P < 0.05$. C - control group; D - group treated with diclofenac; M - group treated with metformin; DM - group treated with the combination of diclofenac and metformin; DMMb - group treated with the combination of diclofenac, metformin and mebendazole; Mb - group treated with mebendazole.

Table 6
Statistical significances expressed as *P*-values for comparisons of immunohistochemical-histopathological tumor characteristics in the second experiment.

Groups (comparison)	Ki-67	PCNA	GLUT-1	iNOS	CD 34	CD 31	COX 4	Cytochrom C
C/D	0.45838	0.49622	0.80027	0.45855	0.45239	0.46705	0.09999	0.45097
C/M	0.51093	0.49999	0.55234	0.46703	0.64491	0.77292	0.17903	0.45063
[a]**C/DM**	0.00081[a]	0.00090[a]	0.00399[a]	0.00294[a]	0.00099[a]	0.00199[a]	0.00099[a]	0.00247[a]
D/M	0.96974	0.89072	0.71562	1.00000	0.64992	0.72611	0.68136	0.63704
[a]**D/DM**	0.00305[a]	0.00639[a]	0.00522[a]	0.01963[a]	0.00567[a]	0.00894[a]	0.00524[a]	0.00903[a]
[a]**M/DM**	0.00740[a]	0.01950[a]	0.01105[a]	0.02474[a]	0.00589[a]	0.00875[a]	0.01996[a]	0.01485[a]
C/DMMb	0.87946	0.71563	0.45783	0.65704	0.89745	0.96325	0.51996	0.09364
[a]**DMMb/DM**	0.00093[a]	0.00412[a]	0.00105[a]	0.00098[a]	0.00115[a]	0.00272[a]	0.00130[a]	0.00107[a]
C/Mb	0.68901	0.83376	0.69053	0.87045	0.68802	0.86394	0.29038	0.44952

[a]$P < 0.05$. C - control group; D - group treated with diclofenac; M - group treated with metformin; DM - group treated with the combination of diclofenac and metformin; DMMb - group treated with the combination of diclofenac, metformin and mebendazole; Mb - group treated with mebendazole.

platelets, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, glucose, serum proteins, albumins and sedimentation were analysed in the same way as previously reported [33,34] (Supplementary data).

2.4. In vitro antiproliferative assay

The tested treatments (diclofenac, metformin, diclofenac and metformin combination) were evaluated for their in vitro antiproliferative effects in hamster fibrosarcoma BHK-21/C13 and in human cancer cell lines: lung carcinoma A549 (CCL-185), colon carcinoma HT-29 (HTB-38), cervical carcinoma HeLa (CCL-2) and normal human fetal lung MRC-5 (CCL-171) cells. All cell lines were obtained from the American Type Culture Collection. The cell lines were authenticated and mycoplasma testing was conducted. The cell lines were cultured in DMEM with 4.5 g/l glucose containing 10% FBS and 1% penicillin-streptomycin in an incubator at 37 °C with 5% CO_2. A standard MTT assay [70] was performed to evaluate the cytotoxic effects of the treatments following exposure to doses of 50, 100, 250, 350, 500 μM and 1, 2, 3, 4, 5, 10, 20, 50 mM at 37 °C for 48 h. The antiproliferative effect was expressed as the half maximal inhibitory concentration (IC_{50}).

2.5. Statistical analysis

Mean ± SD or ± SE and correlation analysis were determined and one-way ANOVA followed by a Student-Newman-Keuls post hoc test were performed using TIBCO Statistica 13.3.1 software (TIBCO Software, Inc.), as in our previous studies [33,34]. P values less than 0.05 were regarded statistically significant.

To check significances obtained by parametric testing (comparing the means), the two-sided Mann-Whitney U tests (comparing the medians) were additionally performed.

3. Results

All animals (control and treated) survived for the entire duration of the study, in all experiments (the first - pilot, the second - explorative and the third - confirmative experiments).

3.1. Biophysical evaluation of hamster fibrosarcoma characteristics

At the end of the treatments, 19 days after BHK-21/C13 inoculation, all hamsters in all groups had isolated well demarcated solid tumors (Fig. 1., Fig. 2.) at the site of injection, without adverse effects on general health and well-being, and without pathological and histopathological signs of toxicity on main organs (heart, lungs, stomach, intestine, liver, kidneys and brain), metastases or ascites.

In the first two experiments, detectable tumor formation began about 9 days after the inoculation (Fig. 3.) and maximal tumor diameter was 3.5 cm with the tumor burden < 6%, which is in accordance with internationally adopted standards (Supplementary data).

In the first two experiments, only the co-treatment with diclofenac and metformin simultaneously significantly ($P < 0.05$) inhibited tumor growth as manifested by significant decreases in tumor weight, maximal length, volume, burden (relative tumor to hamster weight), density, surface area, maximal length/density ratio, area/density ratio and volume/density ratio, compared with control, all single treatments and the triple treatment (Tables 1–4).

All results obtained by parametric statistical technique were justified by two-sided non-parametric Mann-Whitney U test.

3.2. Immunohistochemical evaluation of hamster fibrosarcoma characteristics

The reason for choosing certain markers for immunohistochemical

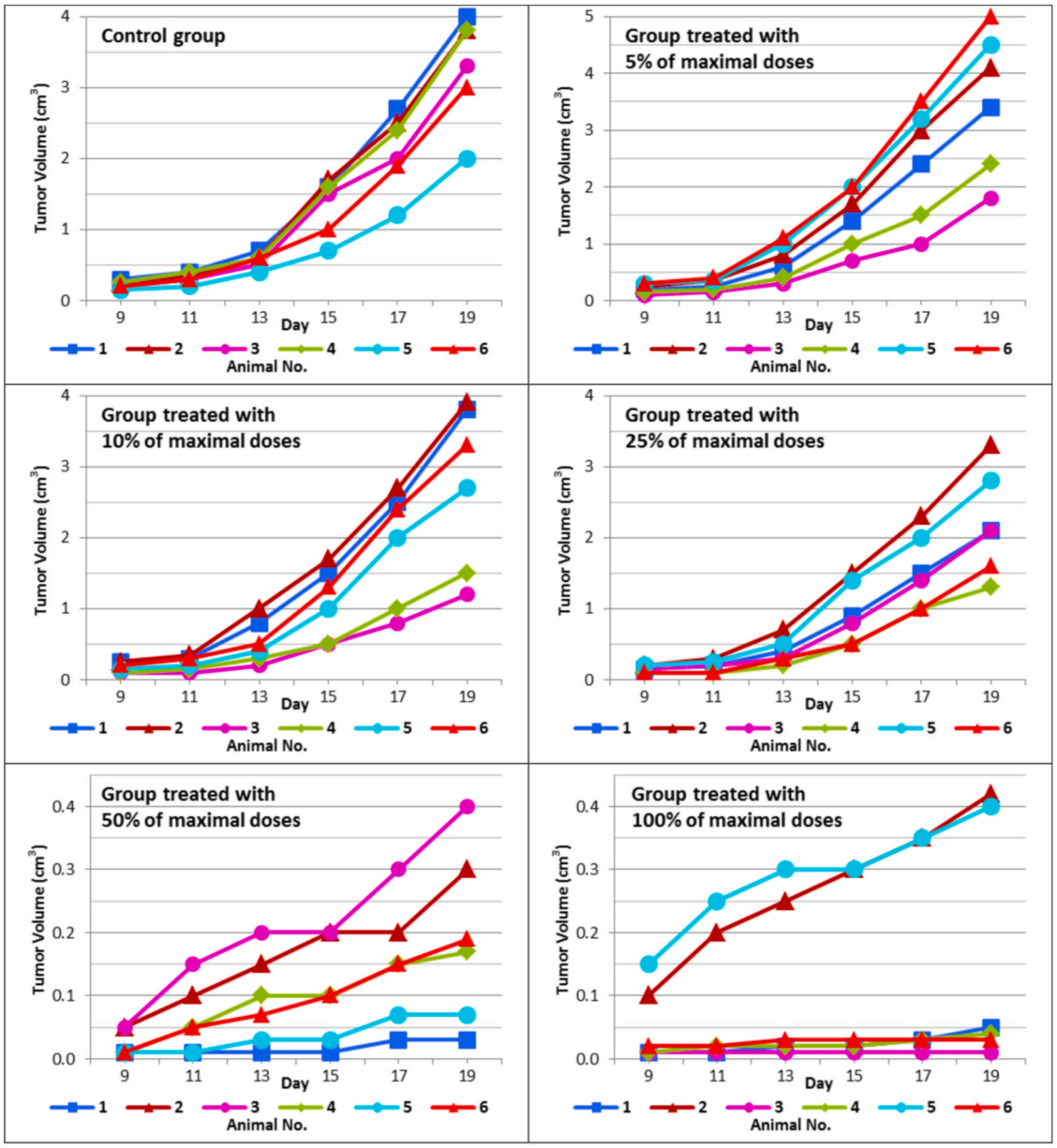

Fig. 10. Tumor volume growth during the third dose response experiment: interpolated lines between individual values.

staining is as follows. The Ki-67 and PCNA proteins are cellular markers for proliferation. CD34, CD31, COX4, Cytochrome C, GLUT1 and iNOS staining are used to evaluate the vasculature, apoptosis, glucose metabolism and NO production in the tumor specimens, respectively. Two markers were used to validate and confirm findings for the most important tumor growth processes (Ki-67, PCNA for tumor proliferation; CD34, CD31 for tumor angiogenesis; COX4, Cytochrome C for tumor apoptosis).

In the experiments, the pathohistological and immunohistochemical evaluation of all analysed slices of tumors confirmed biophysical findings and revealed a decrese in tissue penetration, an expansion of necrosis and hemorrhagic areas, significantly ($P < 0.05$) decreased proliferation of tumor cells, as evidenced by Ki-67 and PCNA, significant ($P < 0.05$) inhibition of glucose metabolism, as denoted by GLUT1, significant ($P < 0.05$) inhibition of NO metabolism, as shown by iNOS staining, significant ($P < 0.05$) inhibition of tumor vasculature, as exhibited by CD34 and CD31 and significant ($P < 0.05$) difference in apoptosis intensity, as evidenced by COX4 and Cytochrome C in animals treated with the diclofenac and metformin combination compared with the control group, all single treatments and the triple treatment (Figs. 4–9., Tables 5 and 6).

In the experiments, neither single diclofenac or metformin treatments exhibited significant anticancer effect ($P > 0.05$) regardless of doubling doses used in the combined treatment (in the first experiment), compared to control, in accordance with all analysed biophysical and immunohistochemical parameters (Figs. 1–9., Tables 1–6). Between single diclofenac and metformin treatments, there are no significant ($P > 0.05$) differences in anticancer activities (Figs. 1–9, Tables 1–6).

In the first two experiments, the combined mebendazole, diclofenac and metformin therapy, as single mebendazole treatment, does not influence sarcoma growth, compared with the control ($P > 0.05$), contrasted by significant anticancer effect seen with the diclofenac and metformin combination ($P < 0.05$), (Figs. 1–3, 6–9., Tables 1–6).

The treatments had no significant ($P > 0.05$) effect on the body

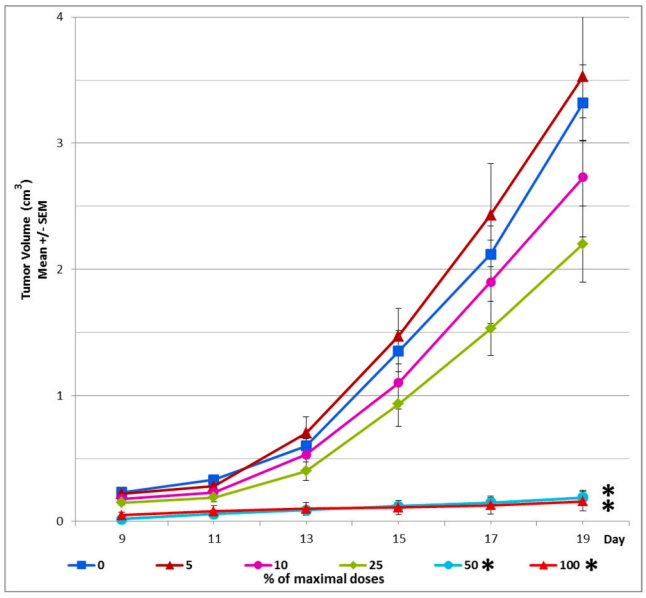

Fig. 11. Tumor volume growth during the third dose response experiment: interpolated line between means and SEM values. *Statistically significant comparing to control and groups treated with lower doses (5%, 10% and 25% of max. doses); $P < 0.05$ as indicated.

weight, red and white blood cell counts, platelet number, hemoglobin levels, hematocrit levels, glucose levels, serum proteins and sedimentation compared with the control in both experiments (Supplemental Table I and Supplemental Table II). We have not registered differences in measured parameters in relation to the sex of experimental animals.

In the experiments, co-treatment with a NF-κB stimulator mebendazole completely eliminates combined antitumor effects of the two NF-κB inhibitors diclofenac and metformin. Cancer progression inhibited by diclofenac and metformin combination was completely rescued by mebendazole (Figs. 1–3, 6–9, Tables 1–6).

These results demonstrate that the significant synergistic anticancer effects induced by co-treatment with two NF-κB inhibitors diclofenac and metformin (in the first experiment with doses 30–50% LD_{50} and in the second experiment with lower doses equivalent to usual human doses) can be attributed at least in part to the synergistic inhibition of NF-κB in hamster fibrosarcoma cells. Furthermore, even doubled doses in single diclofenac and metformin treatments (in the first experiment) had no anticancer effects, which validates our assumption that the combinatory effect could not be only the simple addition of anticancer effects from both drugs given alone.

In the first experiment (same control group, with same methodology and at the same time) we also investigated combination of metformin with mebendazole (6 animals, equal number of males and femails) and the combination of diclofenac with mebendazole (6 animals, equal number of males and femails), but anticancer effects were not found, and fibrosarcoma treatments stayed without significant differences between treated tumors and control.

All results obtained by parametric statistical technique were justified by two-sided non-parametric Mann-Whitney U test.

3.3. Combination index analysis

Since we didn't measure drug concentrations, original form of the Combination Index (CI) calculation [68] is modified in the way that doses were used instead of concentrations, assuming linear kinetics of used drugs (doses linearly correlate to concentrations in the animal body fluids). Because treatments with double doses of single drugs diclofenac and metformin in our first experiment and also treatments with single drugs in the second experiment are stil far from anticancer effectiveness of their combination, CI for all measured effects of the combination are allways CI< 1, indicating synergistic anticancer effect.

In the experiments, synergistic antitumor effect of diclofenac and metformin combination, antagonized by mebendazole, has been observed on hamster fibrosarcoma with CI> 1 for all analysed tumor characteristics.

Diclofenac and mebendazole combination and metformin and mebendazole combination have CI> 1 for all investigated anticancer effects in the first experiment.

3.4. Dose response experiment

As in the first two experiments, in the third experiment detectable tumor formation began about 9 days after the inoculation (Fig. 10., 11.) and maximal measured tumor diameter was 3.5 cm with the tumor burden < 6%, which is in accordance with internationally adopted standards (Supplementary data).

Only combination of diclofenac (D) 60 mg/kg/day and metformin (M) 500 mg/kg/day and combination of 120 mg/kg/day D and 1000 mg/kg/day M exhibited significant anticancer effects ($P < 0.05$) in comparison to control and groups treated with lower doses of diclofenac and metformin components in combination (6 mg/kgD+50 mg/kgM; 12 mg/kgD+100 mg/kgM; 30 mg/kgD+250 mg/kgM) in respect to all analyzed biophysical and immunohistochemical (Ki-67, GLUT1, iNOS, CD31, COX4, Cytochrome C) parameters, without effect on general state, toxicity on main organs, blood hematological and biochemical tests (Figs. 10–14., Table 7 and Supplemental Table III), which was also the case in the first and second experiment. Results of our third dose response experiment show that combination of diclofenac and metformin exhibit anticancer effects in dose dependent manner with significant ($P < 0.05$) positive dose/effect correlation (Fig. 15., 16. and Table 8).

3.5. Co-treatment with diclofenac and metformin antiproliferative effects in cancer cell lines

Co-treatment with diclofenac and metformin has antiproliferative effects in cancer cell lines. The antiproliferative effects in fibrosarcoma, carcinoma and normal cell lines, expressed as IC_{50}, for all treatments are presented in Table 9. Co-treatment with diclofenac and metformin exhibited selective cytotoxicity against the malignant cell lines (BHK-21/C13, A549, HT-29 and HeLa). The combination demonstrated favorable antiproliferative effects in the normal fetal lung MRC-5 cells, suggesting that this treatment may be safe and efficient.

4. Discussion

Our results show statistically significant synergistic anticancer effects of examined combination in daily doses of 60 mg/kg diclofenac and 500 mg/kg metformin and over. Rescue experiment with mebendazole indicates that NF-κB signaling may be an important mechanism underlying observed anticancer effects of the combination. Design of our second experiment was changed in comparison to the first experiment, as follows: gender difference was completely excluded, as the second experiment was conducted only on male hamsters; hamster doses were significantly reduced to be exactly equivalent to human standard doses; the same doses of certain drugs were used in single and combined treatments; stomach and intestine were additionally toxicologically examined, as well as other main organs that were examined in the first experiment (heart, lungs, liver, kidneys and brain).

Results of the second experiment completely confirmed and validated all results obtained in the first experiment, without toxicity and

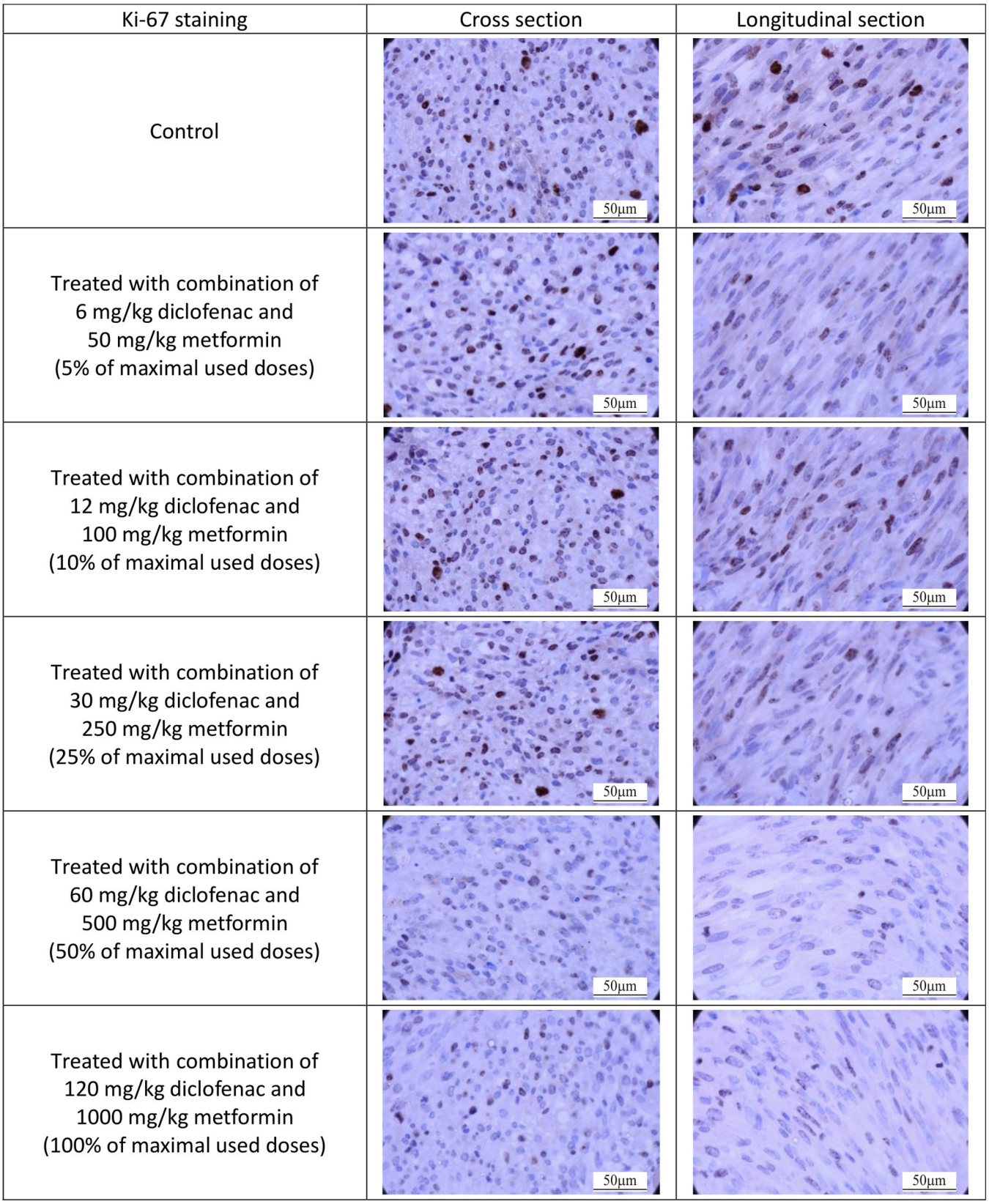

Fig. 12. The third dose response experiment: the extirpated fibrosarcoma Ki-67 immunohistochemical staining images (cross and longitudinal sections), illustration for randomly chosen animals from the control and groups treated with various doses of diclofenac and metformin combination.

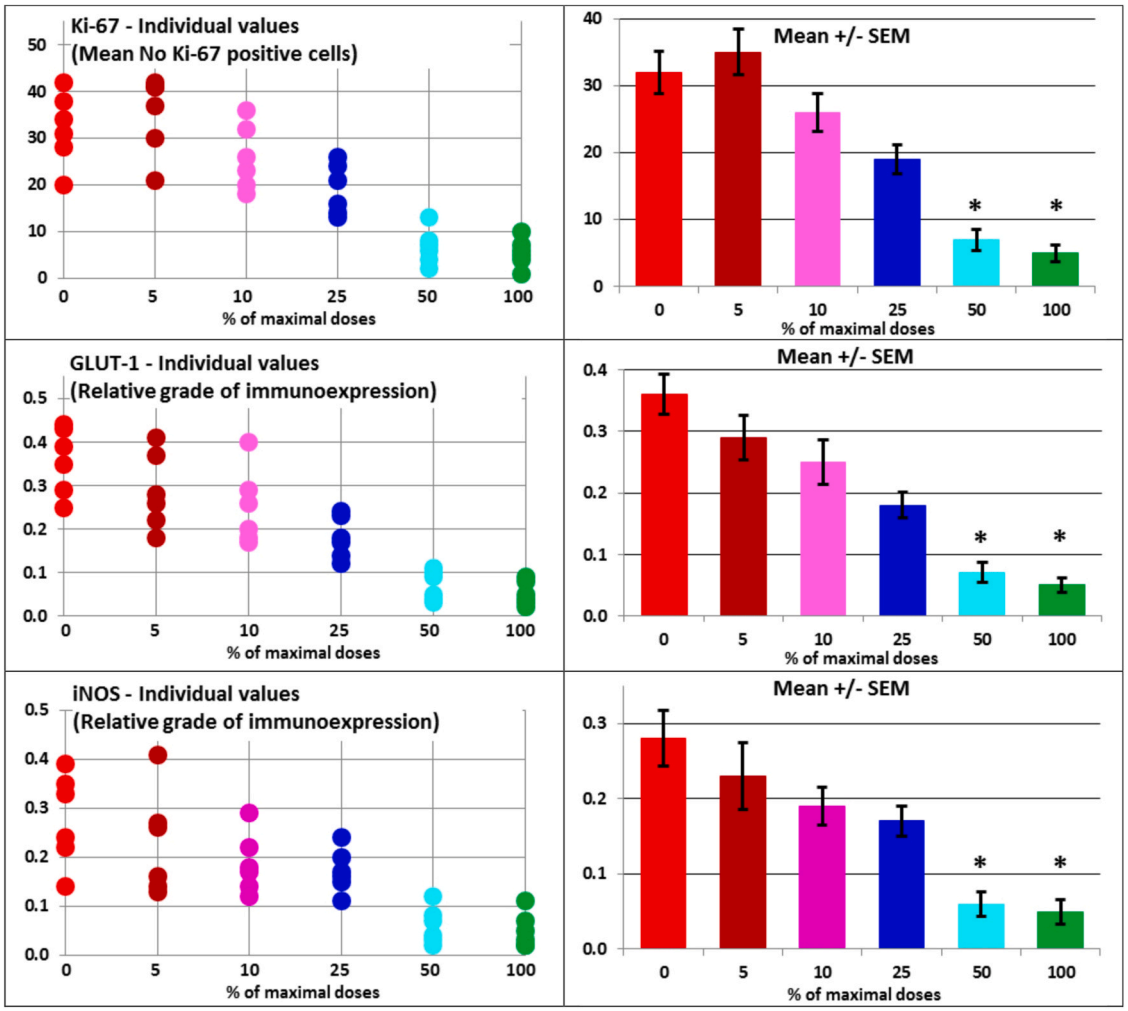

Fig. 13. Individual values, means and standard errors (SEM) of immunohistochemical characteristics of the excised tumors in groups treated with combinations of diclofenac and metformin various doses in the third dose response experiment: Ki-67, GLUT-1, iNOS. 0 - Control group; 5 - group treated with 5% of maximal doses; 10 - group treated with 10% of maximal doses; 25 - group treated with 25% of maximal doses; 50 - group treated with 50% of maximal doses; 100 - group treated with 100% of maximal doses of diclofenac and metformin combination. *Statistically significant comparing to control and groups treated with lower doses (5%, 10% and 25% of max. doses); $P < 0.05$ as indicated.

influence on biochemical blood and hematological tests.

Results of the third, dose response, experiment confirmed the results of the first and the second experiment and confirmed the effectiveness of hamster doses equivalent to standard human doses and larger against cancer progression. By performing the third experiment, dose response study of drug combination (diclofenac and metformin at the same time), paralleled with biological and biochemical analysis, the impact and relevance of the first and second experiment has been highly improved (with so many animals).

Regarding these results, relevant literature is assessed as follows.

In recent work [71] high dose of metformin 25 mg/kg adjusted in 1 ml of drinking water (since a mouse drinks about 3 ml water per day; the metformin dose was ~ 75 mg/kg daily, that is about 6 x lower than human equivalent dose for mouse) and diclofenac 30 mg/kg adjusted in 1 ml of drinking water (~ 90 mg/kg daily, ~ 3 x higher than human equivalent dose for mouse) combination slightly delays xenograft of lung carcinoma growth, inplanted in mice, into the peritoneal cavity. Low-dose of metformin (~ 7.5 mg/kg daily) also delays tumor growth, but not low-dose diclofenac (~ 9 mg/kg daily). Metformin shows a dose-response effect on tumor volume. This is not the case of diclofenac-treated group, where the high-dose diclofenac slightly reduces the tumor volume, but the low-dose group is similar to untreated animals [71]. These findings support our results, despite our use of different doses (25–50% of LD_{50} daily in the first experiment and human equivalent daily doses for hamsters in the second experiment) and different animal and cancer model. In contrast to our work, metformin and diclofenac interaction was explained on the level of enzymes of central carbon metabolism [71]. We proposed another mechanism of synergistic metformin and diclofenac antitumor action (affecting NF-κB and other upstream signals).

Diclofenac and metformin combination can be selected as a synergistic combination based on recently published criteria [72].

Report [73] provides data that suggests that a blockage of glycolysis exerts additive anti-proliferative and anti-migratory effects of metformin and diclofenac combination in some brain tumor and glioma cell lines (in vitro) allowing for reduction of the effective doses of the single agents that may be too high for clinical use and prevention of metabolic rescue mechanisms [73]. As functional effects including proliferation and migration, encompassing oxigen consumption, extracellular lactate levels and effects on the protein level have shown [73], it may be interesting to observe how signal pathways that are involved in proliferation, migration and metastasis formation, i.e., NF-κB are affected by the combined treatment, especially on in vivo models. Our findings on hamster fibrosarcoma in vivo are in accordance with this report in vitro and additionally allow some insight in the role of the NF-κB (or upstream signals).

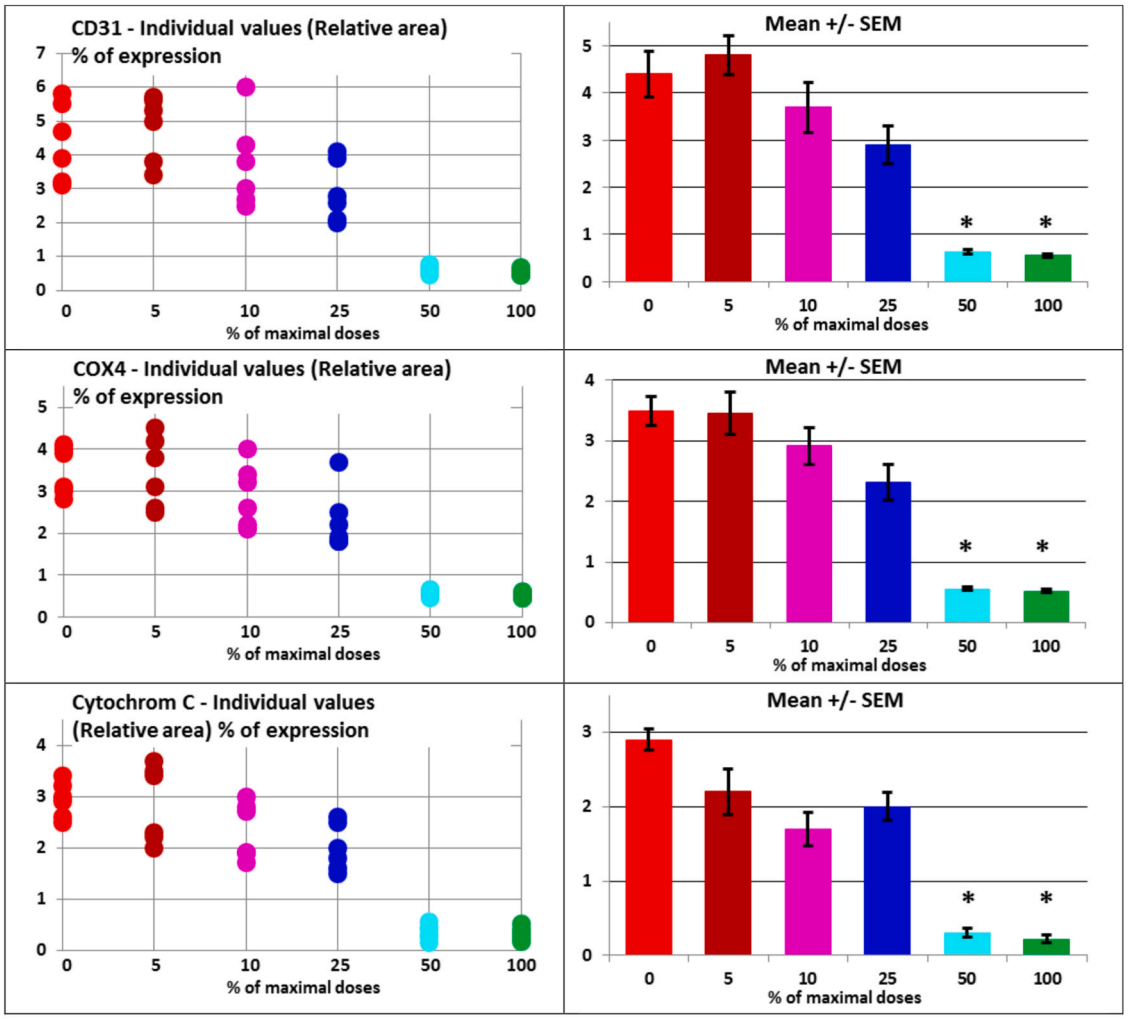

Fig. 14. Individual values, means and standard errors (SEM) of immunohistochemical characteristics of the excised tumors in groups treated with combinations of diclofenac and metformin various doses in the third dose response experiment: CD31, COX4, Cytochrom C. 0 - Control group; 5 - group treated with 5% of maximal doses; 10 - group treated with 10% of maximal doses; 25 - group treated with 25% of maximal doses; 50 - group treated with 50% of maximal doses; 100 - group treated with 100% of maximal doses of diclofenac and metformin combination. *Statistically significant comparing to control and groups treated with lower doses (5%, 10% and 25% of max. doses); $P < 0.05$ as indicated.

Similar study and results, as shown in [73], were obtained with metformin and diclofenac in acute myeloid leukemia cell cultures [74]. Furthermore, authors [74] have shown that low concentrations of metformin and the two NSAIDs, diclofenac and diflunisal, exert a synergistic inhibitory effect in vitro on acute myeloid leukemia cell lines proliferation and induce apoptosis in physiologically achievable concentrations [74].

In accordance with our findings, some authors previously hypothesized that targeting NF-κB could potentiate diclofenac mediated apoptosis [75]. Indeed, combining diclofenac (and other NSAIDs) treatment with NF-κB inhibitors (quinazoline, isohelenin, inhibitor SC-514, wedelolactone) lead to enhanced apoptosis induction in ovarian cancer cells (in vitro) and mice tumors (in vivo). Results indicate that inhibition of NF-κB may lead to a new combinatorial therapy for ovarian cancer [75]. Furthermore, a combination of different NSAIDs (sulindac, sulindac sulfide, diclofenac) results in synergistic anticancer effect in ovarian cancer cells [75].

In our study, rescue experiment with addition of NF-κB stimulator mebendazole to cancer inhibitory treatment (with combination of two NF-κB inhibitors, diclofenac and metformin), which saves cancer growth, clearly indicates NF-κB inhibitory mechanism (direct or indirect, by upstream signals) of the diclofenac and metformin combination synergistic anticancer effects. Furthermore, results of double doses of diclofenac and metformin given alone (single treatments in the first experiment) validate that the combinatory effect was not only the simple addition of anticancer effects from both drugs alone and that the treatment with either drug alone, even with maximal doses, may not cause anticancer effects. For postulating a viable synergistic effect without the toxicities seen in high doses of individual drugs, the rational would be a double hit strategy targeting different pathways or at least different targets within a pathway. According our rescue experiment with NF-κB stimulation by mebendazole, it can be postulated that combined diclofenac and metformin therapy affects different upstream targets within NF-κB pathway.

Results of our present experiments are also in accordance with our previous reports of significant biometrical, histological and immunohistochemical anticancer effects of combinations of two drugs which directly or indirectly inhibit NF-κB (metformin with another repurposed non-oncological non-toxic drug) on subcutaneously inoculated fibrosarcoma in hamsters, without toxicity [32–34,57].

According to our results, the diclofenac and metformin combination can be explored for use as a part of multidrug adjuvant cancer treatments, which use combinations of multiple repurposed drugs in resistant cancer patients [76].

Investigations of sarcoma models are of enormous importance in cancer treatment research due its extremely aggressive patern,

Table 7
Characteristics of hamsters and quantitative pathological and biophysical characteristics of extirpated tumors in the third dose response experiment.

Hamster			Tumor					
No	Weight at start (g)	Weight at end (g)	Weight (g)	D_{max} (cm)[a]	Volume (cm^3)	Tumor burden (%)	Density (g/cm^3)	Area (cm^2)
Control group								
1	69	98	4.8	2.5	4.0	4.9	1.200	13.0
2	65	99	4.6	2.3	3.8	4.6	1.211	12.2
3	66	96	3.8	2.6	3.3	3.9	1.152	12.8
4	71	100	4.6	2.8	3.8	4.6	1.211	13.9
5	64	96	2.3	1.7	2.0	2.4	1.150	7.7
6	62	88	3.7	2.4	3.0	4.2	1.233	11.0
Mean	66	96	3.97	2.38	3.32	4.1	1.193	11.8
±SD	3.3	4.3	0.94	0.38	0.74	0.90	0.034	2.21
Group treated with 6 mg/kg diclofenac and 50 mg/kg metformin (5% of maximal used doses)								
1	65	88	3.9	2.3	3.4	4.4	1.147	11.5
2	61	99	4.9	2.5	4.1	4.9	1.195	13.5
3	62	84	2.2	1.6	1.8	2.6	1.222	6.9
4	70	91	2.8	2.2	2.4	3.1	1.167	9.4
5	77	110	5.4	3.1	4.5	4.9	1.200	14.2
6	85	121	5.9	3.4	5.0	4.9	1.180	17.0
Mean	70	99	4.18	2.52	3.53	4.13	1.185	12.1
±SD	9.4	14.3	1.47	0.65	1.24	1.03	0.026	3.61
Group treated with 12 mg/kg diclofenac and 100 mg/kg metformin (10% of maximal used doses)								
1	72	99	4.6	2.7	3.8	4.6	1.211	12.9
2	69	100	4.7	2.8	3.9	4.7	1.205	13.0
3	60	81	1.3	1.4	1.2	1.6	1.083	4.9
4	65	86	1.6	1.7	1.5	1.9	1.067	6.0
5	70	95	3.1	2.0	2.7	3.3	1.148	9.5
6	72	98	3.9	2.4	3.3	4.0	1.182	11.0
Mean	68	93	3.20	2.17	2.73	3.35	1.149	9.55
±SD	4.7	7.8	1.48	0.56	1.16	1.34	0.062	3.45
Group treated with 30 mg/kg diclofenac and 250 mg/kg metformin (25% of maximal used doses)								
1	74	92	2.4	2.3	2.1	2.6	1.143	8.2
2	70	101	3.7	2.5	3.3	3.7	1.121	12.3
3	66	87	2.2	2.1	2.1	2.5	1.048	7.4
4	65	92	1.4	1.5	1.3	1.5	1.077	5.7
5	67	98	3.2	2.4	2.8	3.3	1.143	9.8
6	69	105	1.8	1.7	1.6	1.7	1.125	6.5
Mean	68.5	96	2.45	2.08	2.2	2.55	1.110	8.32
±SD	3.27	6.68	0.86	0.40	0.74	0.84	0.039	2.41
*Group treated with 60 mg/kg diclofenac and 500 mg/kg metformin (50% of maximal used doses)								
1	62	88	0.03	0.4	0.03	0.00	1.000	0.40
2	70	91	0.31	1.0	0.30	0.34	1.033	2.32
3	64	98	0.43	1.3	0.40	0.44	1.075	2.95
4	62	99	0.18	1.1	0.17	0.18	1.059	1.59
5	71	85	0.07	0.8	0.07	0.08	1.000	0.95
6	74	93	0.20	1.2	0.19	0.22	1.053	1.40
Mean	67	92	0.20	0.97	0.19	0.21	1.037	1.60
±SD	5.15	5.50	0.15	0.33	0.14	0.16	0.031	0.92
*Group treated with 120 mg/kg diclofenac and 1000 mg/kg metformin (100% of maximal used doses)								
1	64	96	0.05	0.8	0.05	0.05	1.000	0.88
2	61	95	0.44	1.2	0.42	0.46	1.048	3.17
3	63	89	0.01	0.5	0.01	0.01	1.000	0.41
4	66	91	0.04	0.9	0.04	0.04	1.000	1.00
5	69	100	0.41	1.5	0.40	0.41	1.025	2.88
6	62	95	0.03	0.7	0.03	0.03	1.000	0.58
Mean	64	94	0.16	0.93	0.16	0.17	1.012	1.49
±SD	2.93	3.88	0.20	0.36	0.20	0.21	0.020	1.21

[a] Longest tumor diameter (cm).
*Statistically significant compared to control ($P < 0.05$)

resistance to current therapies and the high mortality attributed to these malignancies [77]. Sarcomas affect a growing number of ~200,000 individuals worldwide each year and represent a higher percentage of overall cancer morbidity and mortality in children and adolescents compared with adults [78–80]. Sarcomas account for > 20% of all pediatric solid malignant cancers [81].

Generalizing our findings on fibrosarcoma in hamsters to other cancer types or cell lines might be problematic, given the considerable variation in mechanisms and responses to treatment among distinct tumor types. However, our in vitro results with various cancer types: hamster fibrosarcoma BHK-21/C13, human lung carcinoma A549 (CCL-185), colon carcinoma HT-29 (HTB-38) and cervical carcinoma HeLa (CCL-2) are encouraging. Further preclinical investigations of the combination on other cancer types and clinical confirmations on sarcomas and other tumors are needed for implementation in oncology.

5. Conclusions

The results of the present study in vivo indicate that co-treatment with diclofenac and metformin significantly and dose dependently inhibits fibrosarcoma growth in hamsters, in doses equivalent to usual human doses and larger, evidently by mechanisms which can be inhibited by NF-κB stimulator mebendazole. Diclofenac and metformin combinatory dose dependent antisarcoma anticancer effects in hamsters were not only the simple addition of the effects from both drugs alone, since even double doses (maximal doses) of single treatments were

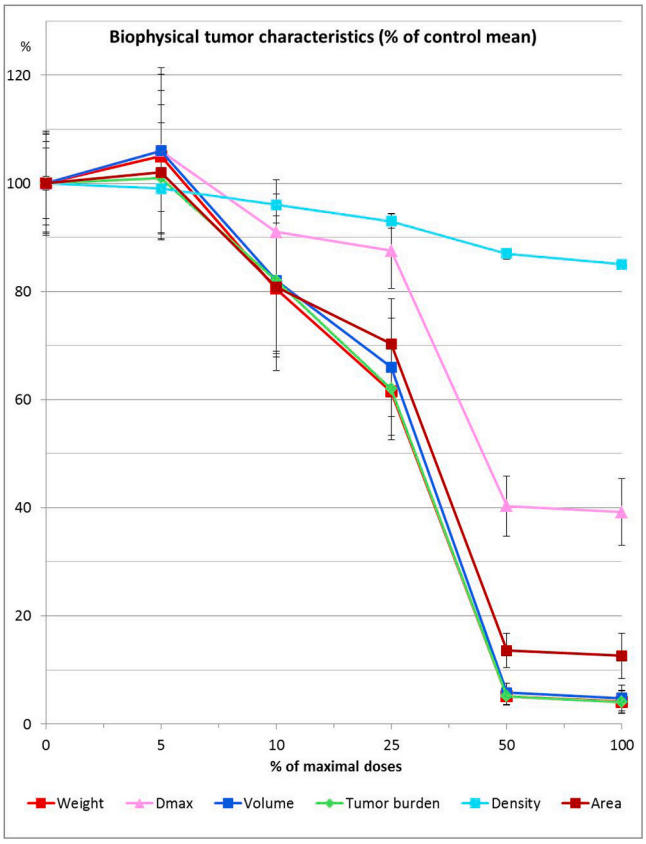

Fig. 15. Dose response curves for biophysical tumor characteristics: weight, maximal diameter, volume, tumor burden, tumor density and surface area for all groups in the third experiment, expressed as % of control mean (mean+/— SE): 0 - Control group; 5 - group treated with 5% of maximal doses; 10 - group treated with 10% of maximal doses; 25 - group treated with 25% of maximal doses; 50 - group treated with 50% of maximal doses; 100 - group treated with 100% of maximal doses of diclofenac and metformin combination.

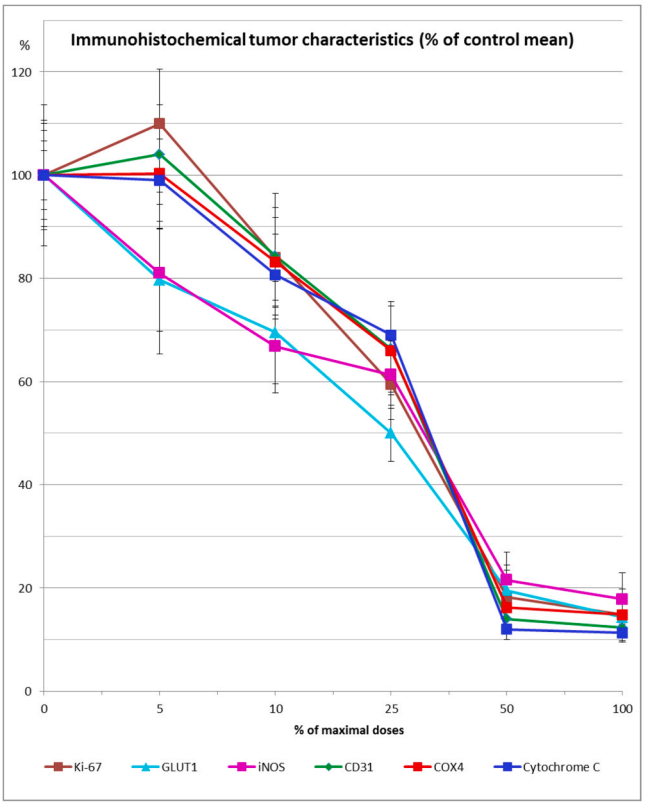

Fig. 16. Dose response curves for immunohistochemical tumor characteristics: Ki-67, GLUT1, iNOS, CD31, COX4, Cytochrom C for all groups in the third experiment, expressed as % of control mean (mean+/— SE): 0 - Control group; 5 - group treated with 5% of maximal doses; 10 - group treated with 10% of maximal doses; 25 - group treated with 25% of maximal doses; 50 - group treated with 50% of maximal doses; 100 - group treated with 100% of maximal doses of diclofenac and metformin combination.

without significant anticancer effects. Antisarcoma anticancer effects of diclofenac and metformin combination in hamsters are potentially due to synergistic interaction on NF-κB inhibition (direct or indirect by upstream signals), since these anticancer effects can be reversed by NF-κB stimulator mebendazole in a rescue experiment.

In vitro, diclofenac and metformin combination also exhibits anticancer antiproliferative effects in various animal and human cancer cell lines: hamster fibrosarcoma BHK-21/C13, human lung carcinoma A549 (CCL-185), human colon carcinoma HT-29 (HTB-38) and human cervical carcinoma HeLa (CCL-2), without effects on normal human fetal lung MRC-5 (CCL-171) cells.

Diclofenac and metformin combination therapy may synergize the anticancer effects of these two drugs in oncology and provide several advantages for fibrosarcoma and probably other cancer chemoprevention and adjuvant therapy, including better efficacy, dose reduction of the individual agents involved to minimize their adverse drug reactions, as well as possible overcoming of drug resistance. Further clinical exploration will be beneficial to fully establish the appropriate place of diclofenac and metformin combination in fibrosarcoma and possibly other cancer patients.

Table 8
Correlation dose % and effect (response).

Tumor	Pearson (r) corr. coeff.	P-value	Line fit plot
W (g)	— 0.9021	0.0139	y = — 0.043 x + 3.715
D_{max} (cm)	— 0.908	0.0123	y = — 0.017 x + 2.371
V (cm^3)	— 0.9042	0.01334	y = — 0.036 x + 3.154
Tumor burden (%)	— 0.9071	0.01255	y = — 0.043 x + 3.793
Density (g/cm^3)	— 0.935	0.006197	y = — $1.868 \cdot 10^{-3}$ x + 1.173
Area (cm^2)	— 0.9053	0.01302	y = — 0.114 x + 11.098
Ki-67	— 0.9022	0.01388	y = — 0.3 x + 30.152
GLUT-1	— 0.8978	0.01514	y = — $2.91 \cdot 10^{-3}$ x + 0.292
iNOS	— 0.9006	0.01433	y = — $2.184 \cdot 10^{-3}$ x + 0.234
CD31	— 0.9015	0.01408	y = — 0.044 x + 4.22
COX4	— 0.9092	0.012	y = — 0.033 x + 3.244
Cytochrome C	— 0.9086	0.01215	y = — 0.028 x + 2.682

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CRediT authorship contribution statement

Dušica J. Popović: Conceptualization, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing - Original Draft, Writing – review & editing. **Kosta J. Popović:** Conceptualization, Formal analysis, Funding acquisition. **Dejan Miljković:** Investigation,

Table 9
Experimentally obtained half maximal inhibitory concentration values of analyzed drugs on various cell lines after 48 h exposure.

Drug	Normal fetal lung MRC-5	Hamster fibrosarcoma BHK-21/C13	Lung carcinoma A549	Colon carcinoma HT-29	Cervix carcinoma HeLa
Diclofenac (mM)	48	9.8	5.1	2.85	0.67
Metformin (mM)	35	6.1	2.4	0.54	0.41
Diclofenac + metformin (1:1; mM each)	34	3.1	1.3	0.40	0.27

Resources. **Jovan K. Popović:** Data curation, Formal analysis, Methodology, Validation, Project administration, Supervision. **Dušan Lalošević:** Investigation, Funding acquisition. **Mihalj Pošа:** Investigation. **Zana Dolićanin:** Investigation. **Ivan Čapo:** Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki. The study was carried out following the approvals of the University of Novi Sad Animal Ethics Committee (Novi Sad, Serbia): No. 04–81/25–5 dated 22nd July 2020, Doc. No. ЕК: П-Е-2020–07; No. 04–150/15 dated 14th March 2022, Doc. No. ЕК: I-2022–01; No. 04–150/15 dated 14th March 2022, Doc. No. ЕК: I-2022–02; and the approvals of the Ministry of Agriculture, Forestry and Water Management - Veterinary Directorate (Belgrade, Serbia): No. 323–07-09359/2020–05 dated 2nd September 2020; No. 323–07-03995/2022–05 dated 28th March 2022; No. 323–07-03996/2022–05 dated 28th March 2022; No. 323–07-03997/2022–05 dated 28th March 2022.

Informed consent statement

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115528.

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