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Immunohistochemical evidences of anticancer actions of metformin with other repurposed drug combinations and correlation with hamster fibrosarcoma tumor size

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Abstract: The aim was to detect and correlate anticancer effects of metformin in combinations with other repurposed drugs, already registered for other indications, which may be immediately applied and clinically investigated in oncology, reducing the time and cost of research for new cancer treatments. Immunohistochemistry was performed for tumors treated by dual drug combinations containing metformin with deoxycholic acid, caffeine, itraconazole, nitroglycerin, disulfiram or diclofenac. The drugs were applied in Syrian golden hamsters (6 animals per group) with the inoculated BHK21/C13 fibrosarcoma in doses equivalent to usual human doses, <50 % LD_{50}. The anticancer effects were assessed by: p53 (mutational status); Ki-67 and PCNA (tumor proliferation); CD34 and CD31 (neoangiogenesis); GLUT1 (glucose metabolism); iNOS (NO metabolism); COX4, Cytochrome C and caspase 3 (apoptosis); immunohistochemical markers. Also, biophysical characteristics of fibrosarcoma, animal blood samples and the toxicity on main organs were analyzed. Treatments significantly ($P < 0.05$) reduced mutational status, tumor proliferation, neoangiogenesis, glucose metabolism, NO metabolism and modulated apoptosis, in correlation with tumor size, without toxicity and influence on biochemical blood and hematological tests. The administration of metformin in two-drug combination with deoxycholic acid, caffeine, itraconazole, nitroglycerin, disulfiram or diclofenac may be recommended for further clinical investigations in oncology.

Keywords: tumour markers; fibrosarcoma; metformin; repurposed drugs; hamsters.

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INTRODUCTION

Anticancer effects of some pleiotropic low cost non-oncological, marketed drugs, such as metformin, deoxycholic acid, caffeine, itraconazole, nitroglycerin, disulfiram or diclofenac are reported *in vitro* (cell lines), but investigations *in vivo* (experimental animals) and clinical investigations, especially of their certain combinations, are not yet conducted.

The activation of NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) signaling has been found in many types of tumors, including breast, colon, prostate, skin and lymphoid tumors.[1] NF-κB, as the master stimulator of cancer development and maintenance, stimulates proliferation, angiogenesis and modulates apoptosis (inhibits apoptosis in cancer cell lines).

Metformin inhibited proliferation and stimulated apoptosis of various cancer cell lines by suppression of NF-κB in lung,[2] human ovarian,[3] human gastric adenocarcinoma[4] and prostate cancer cells.[5]

It has been shown that deoxycholic acid inhibited NF-κB activity, limited cancer cell proliferation, invasion and induced apoptosis *in vitro*: in human pancreatic cancer cells,[6] gastric carcinoma cells,[7] lung cancer cells,[8] prostate cancer cells,[8] breast cancer cells,[8] colon cancer cells,[9,10] gastric carcinoma cells[11] and hepatic carcinoma cells.[12]

Caffeine inhibits NF-κB and induces apoptosis in many human cancer cell lines (lung, pancreatic, leukemia) *in vitro*.[13,14]

Itraconazole exhibits significant anticancer effects in various cancer cell lines *in vitro via* inhibition of NF-κB, angiogenesis, folate, autophagy and cholesterol transportation.[15,16] The same as metformin, itraconazole inhibits protein synthesis, cell growth, proliferation and stimulates apoptosis.[17]

Nitroglycerin liberates nitric oxide (NO) in the tissues, which modifies tumor cell metabolism by modulating the Warburg effect in cancer therapy.[18] Similarly to metformin,[19] NO from nitroglycerin causes folate and B_{12} deficiency.[20,21] Nitroglycerin inhibits NF-κB, downstream related proteins and stimulates apoptosis *in vitro*.[22]

Disulfiram inhibits proliferation and stimulated apoptosis of various cell lines *in vitro* by NF-κB suppression: leukemia,[23] endometriotic,[24] lymphoid,[25] glioblastoma,[26] colorectal,[27] non-small cell lung[28] and gastric cancer cells.[29]

Diclofenac inhibits NF-κB in human and mouse hepatoma cells *in vitro*.[30,31] Diclofenac also inhibits NF-κB activation, inhibited proliferation and stimulated apoptosis in various tumour cells *in vitro*.[32]

The opposite functional crosstalk between NF-κB and p53 has a key role in the pathogenesis of most tumours. p53, a tumour suppressor protein, nuclear transcription factor described as “the guardian of the genom”, stops the formation of tumours and prevents mutation. p53 is associated with cell cycle arrest, angio-

genesis inhibition and apoptosis modulation (induces apoptosis in cancer cell lines).

We screened non-oncological pleiotropic drugs (inhibitors of NF-κB) with approved anticancer characteristics *in vitro*, and their combinations on experimental BHK-21/C13 fibrosarcoma in hamsters and correlated indicators of tumour growth (tumour weight and immunohistochemical parameters expression).

Frequently used mouse models offer an attractive system for cancer therapy research. However, there are important limitations: firstly, concerning the immune mediated rejection of cancer xenograft and secondly, models that use the immune deficient mice (in order to avoid the rejection) can produce metastases.[33] Cell lines were the main component of any experimental model. BHK-21/C13 cells are able to undergo malignant transformation when they are subcutaneously injected into hamsters.[33]

A model of BHK-21/C13 cell culture induced sarcoma in Syrian hamsters is easy reproducible, tumour is solitary, big enough in short time, never produced metastases and without influence of the host immune mechanisms. Immunologically, hamsters do not recognize BHK-21/C13 cells as tumorigenic and tumour is enormously growing. The tumour is locally infiltrative. BHK-21/C13 cells are tumorigenic only for hamsters (except nude mice). Only whole live cells are tumorigenic, not DNA, or cell extracts. This model is excellent for the pharmacological examination of antitumor agents, because the tumour never produces metastases and is not influenced by immune rejection, in contrast with some other animal tumour models (Fig. 1).

EXPERIMENTAL

Animal model

The study was performed on Syrian golden hamsters (6 hamsters per group; males only; age, 12–15 weeks; weight, ~80 g). The animals were obtained from the Pasteur Institute and were maintained under standard animal housing conditions at 25±2 °C and 60±2 % humidity under a 12-h light/dark cycle. The animals had ad libitum access to food and water.

BHK-21/C13 cells were subcutaneously inoculated (1ml, 2×10^6 cells/ml) into the back of all hamsters by the same researcher (Fig. 1). After the tumour inoculation, the following characteristics were monitored: general condition; general clinical signs (diarrhoea, breathing disorders, neurological signs); behaviour; body weight (measured daily); tumour diameter, location and ulceration; appearance of multiple tumours.

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: П-Е-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. 323-07-09359/2020-05; No. 323-07-03995/2022-05; No. 323-07-03996/2022-05; No. 323-07-03997/2022-05.

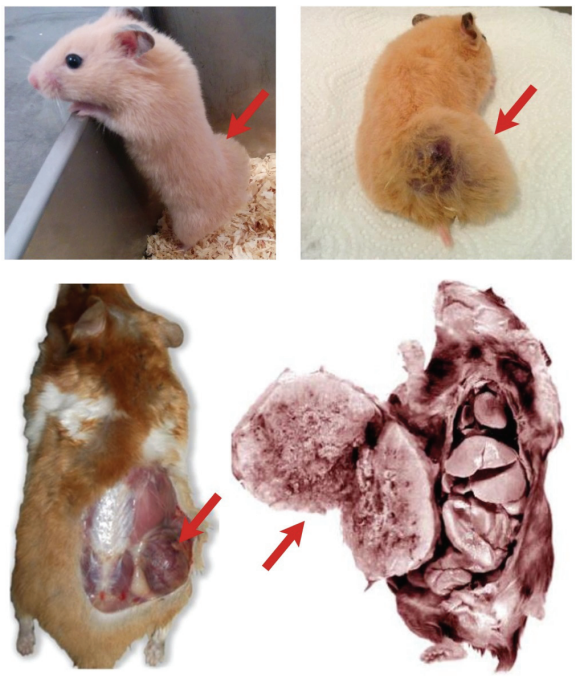

Fig. 1. Syrian hamster with BHK-21/C13 cell culture inoculated subcutaneously. Large tumor on the inoculation site, without spreading on internal organs.

The treatments were initiated after the subcutaneous inoculation of 1 ml of BHK-21/C13 cell suspension (2×10^6 cells/ml) into the back for the development of a subcutaneous fibrosarcoma tumour. Single metformin, deoxycholic acid, caffeine, itraconazole, nitroglycerin, disulfiram or diclofenac and dual combinations with metformin (all from Galenika a. d.) were applied perorally in 1–2 ml of fluid (saline), according hamster body mass, *via* a gastric probe, in daily doses equivalent to usual human doses by normalization to body surface area, < 50 % of hamster oral median lethal dose LD_{50} (Table I).

BHK-21/C13 cells were produced in DMEM substrate with 4.5 g/L glucose, with 10 % foetal bovine serum (FBS), 2 mM glutamine and 1 % penicillin/streptomycin (all from Capricorn Scientific), at 37 °C, in a 5 % CO_2 humidified atmosphere. BHK-21/C13 cells were subcultured twice a week once they reached a confluency of 70–80 %.

Immunohistochemistry

Immunohistochemical p53, Ki-67, PCNA, CD34, CD31, GLUT1, iNOS, COX4, cytochrome C and caspase 3 staining was performed to assess tumour mutational status (p53), proliferation (Ki-67, PCNA), neoangiogenesis (CD34, CD31), glucose metabolism (GLUT1), NO metabolism (iNOS) and apoptosis (COX4, Cytochrome C, caspase 3). COX4 is a mitochondrial cytochrome C oxidase marker of apoptosis (overexpressed in cancer cells), cytochrome C is a useful marker of mitochondrial and cellular damage and apoptosis in tumours and caspase 3 is a crucial mediator of apoptosis (frequently activated death protease, detects endogenous levels of cleaved caspase 3). In immunohistochemical staining, the following primary antibodies were used: p53 (Thermo Fisher Scientific, Inc.; DO-7; Product # MA5-12557;

1:100), Ki-67 (Thermo Fisher Scientific, Inc.; cat. no. RB-9043-P0, 1:300), PCNA (Thermo Fisher Scientific, Inc.; cat. no. RB-9055-P, 1:300), CD34 (Abcam; cat. no. ab81289; 1:200), CD31 (Abcam; cat. no. ab28364; 1:200), GLUT1 (Thermo Fisher Scientific, Inc.; cat. no. RB-9052-P0; 1:200), iNOS (Thermo Fisher Scientific, Inc.; RB-9242-P0; 1:100), COX4 (Abcam; cat. no. ab185056; 1:1,000), Cytochrome C (Abcam; cat. no. ab133504; 1:200) and caspase 3 (Abcam; cat. no. ab13847; 1:200). Briefly, sections (5 μm) were deparaffinized in xylene (100 %) and rehydrated in descending ethanol series (100 % twice for 3 min; 95 % for 3 min and 70 % for 3 min). For antigen retrieval, the sections were microwaved (850 W; ~98 °C) for 20 min in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA solution, 0.05 % Tween 20, pH 9.0), washed twice for 5 min with TBS plus 0.025 % Triton X-100 (with agitation) and blocked by immersion in 10 % goat serum (cat. no. G6767; Sigma–Aldrich; Merck KGaA) in TBS with 1 % BSA (cat. no. T6789; Sigma–Aldrich; Merck KGaA) for 2 h at room temperature. Primary antibodies dissolved in TBS with 1 % BSA were incubated at 4 °C overnight. The sections were washed twice for 5 min with TBS plus 0.025 % Triton X-100 (with agitation) and incubated with 0.3 % H_2O_2 in TBS for 15 min at room temperature. Horseradish peroxidase-conjugated goat polyclonal rabbit immunoglobulin G secondary antibody (cat. no. ab6721; Abcam) dissolved in TBS with 1 % BSA was added to the sections for 2 h at room temperature. The sections were washed three times for 5 min with TBS. For visualization, the chromogen 3,3-diaminobenzidine tetrahydrochloride (cat. no. K3468; Liquid DAB + Substrat-Chromogen System; Dako; Agilent Technologies, Inc.) was added and incubated for 10 min at room temperature. The sections were washed with water for 5 min and were stained with Mayer's hematoxylin for 5 min at room temperature. The stained tumour 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 tumour sections (mean of 10 measurements) using UTHSCSA Image Tools for Windows version 3.00.

Blood biochemical tests and haematological analyses

2–3 ml of the total collected blood was used for standard laboratory analyses: glucose, serum proteins, albumins haemoglobin, sedimentation, erythrocytes, leucocytes, lymphocytes, monocytes, granulocytes, platelets, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration, in all three experiments. The serum, obtained from the blood samples by centrifugation at 2,000×*g* for 10 min, was analysed using an auto chemistry analyser (Rayto Life and Analytical Sciences Co., Ltd.). Commercial tests for the determination of glucose concentration (cat. no. 21503), serum proteins (cat. no. 11500) and albumins (cat. no. 11547; BioSystems S.A.) were used. An auto haematology analyser (Abacus Junior Vet; Diatron; Stratec SE) was used for enumeration of blood cells.

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.). 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.

RESULTS AND DISCUSSION

After inoculation of BHK-21/C13 cells, fibrosarcoma was developed in all hamsters (Fig. 2). Animals had isolated, well-demarcated solid tumours without any adverse effects on general health and well-being. The maximal tumour diameters after sacrifice, were < 3.5 cm in all experiments. The maximal tumour 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.

TABLE I. Weights ± *SD* (g) and control/treatment difference *P*-values of extirpated hamster fibrosarcomas treated with single drug and various combinations of metformin 500 mg/day with another repurposed drug

Drugs/dose, mg/kg	Control	Single 1.	Single 2.	Combination	Control/ /Single 1. *P*-values	Control/ /Single 2. *P*-values	Control/ /Combin. *P*-values
1. Metformin/500	2.90	2.45	3.06	1.36	0.61375	0.89427	0.04501
2. Deoxycholic acid/100	±1.39	±1.28	±2.11	±0.61			
1. Metformin/500	2.54	1.10	2.32	0.42	0.30021	0.78122	0.04110
2. Caffeine/100	±2.30	±0.77	±1.31	±0.32			
1. Metformin/500	4.85	3.20	3.99	1.13	0.39604	0.55283	0.02921
2. Itraconazole/250	±3.35	±1.65	±0.98	±0.41			
1. Metformin/500	2.67	2.42	1.97	1.44	0.59372	0.13005	0.01542
2. Nitroglycerin/25	±1.05	±0.98	±1.02	±0.75			
1. Metformin/500	4.38	4.01	4.22	0.65	0.39856	0.60277	0.00013
2. Disulfiram/50	±0.50	±0.69	±0.99	±0.27			
1. Metformin/500	3.80	3.10	2.80	0.19	0.30005	0.16483	0.00080
2. Diclofenac/60	±0.77	±0.89	±1.16	±0.15			

The experimental and control groups were statistically compared for glucose levels, haemoglobin levels, haematocrit levels, serum proteins, sedimentation, red and white blood cell counts, platelet number, but no significant differences were observed among the groups in all experiments ($P < 0.05$).

Peroral co-treatment with dual drug combinations containing metformin with deoxycholic acid, caffeine, itraconazole, nitroglycerin, disulfiram or diclofenac significantly inhibited tumour growth as indicated by significant decreases in tumour weight, compared with the control group and the single treatment groups (Table I).

The immunohistochemical evaluation (Fig. 3) revealed significantly ($P < 0.05$) decreased tumour mutational status, as demonstrated by p53; significantly ($P < < 0.05$) decreased proliferation status of tumour cells, as demonstrated by Ki-67 and PCNA; significant ($P < 0.05$) inhibition of tumour vasculature, as demonstrated by CD31 and CD34; significant ($P < 0.05$) inhibition of glucose metabolism, as demonstrated by GLUT1; significant ($P < 0.05$) inhibition of NO meta-

bolism, as demonstrated by iNOS staining; and significant ($P < 0.05$) decrease in apoptosis intensity, as demonstrated by COX4, cytochrom C and caspase 3, in all analysed slices of tumours from animals treated with the combinations, compared with the control group and the single treatment groups. Furthermore, captivating correlation were obtained between tumour weight (g) and all analysed immune-histochemical parameters expression (Table II).

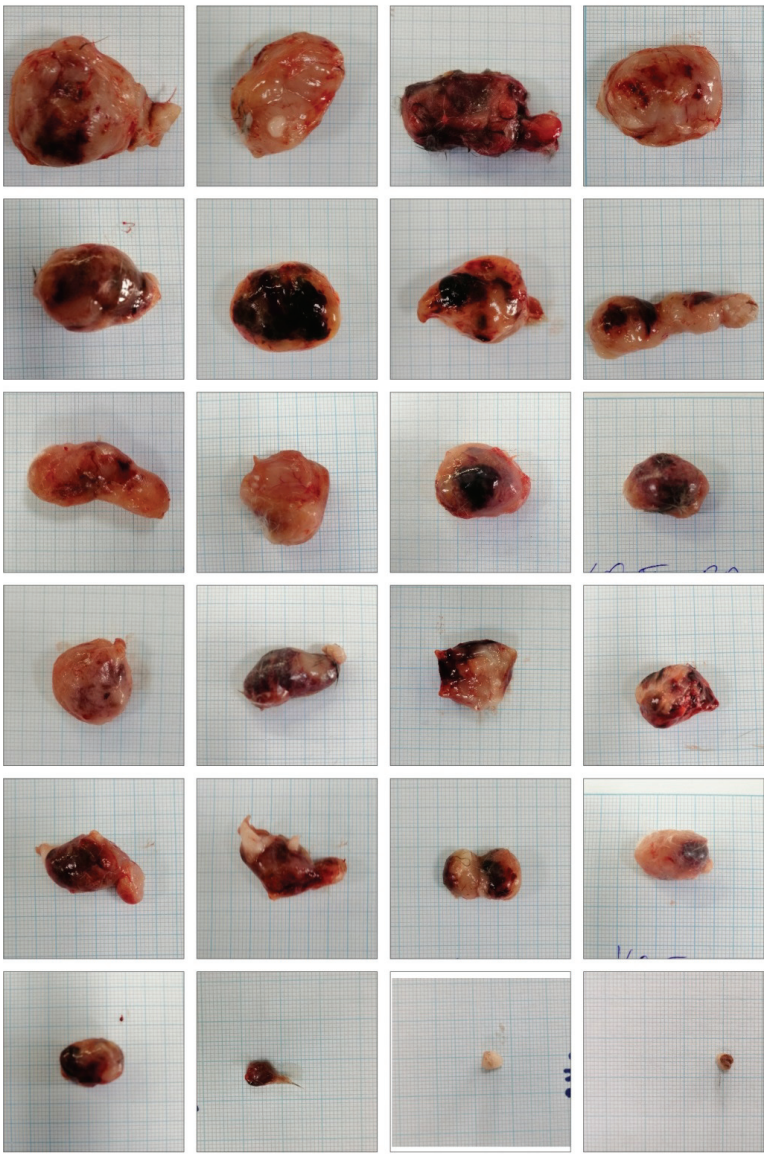

Fig. 2. Extirpated hamster fibrosarcomas placed on one-millimeter grid paper.

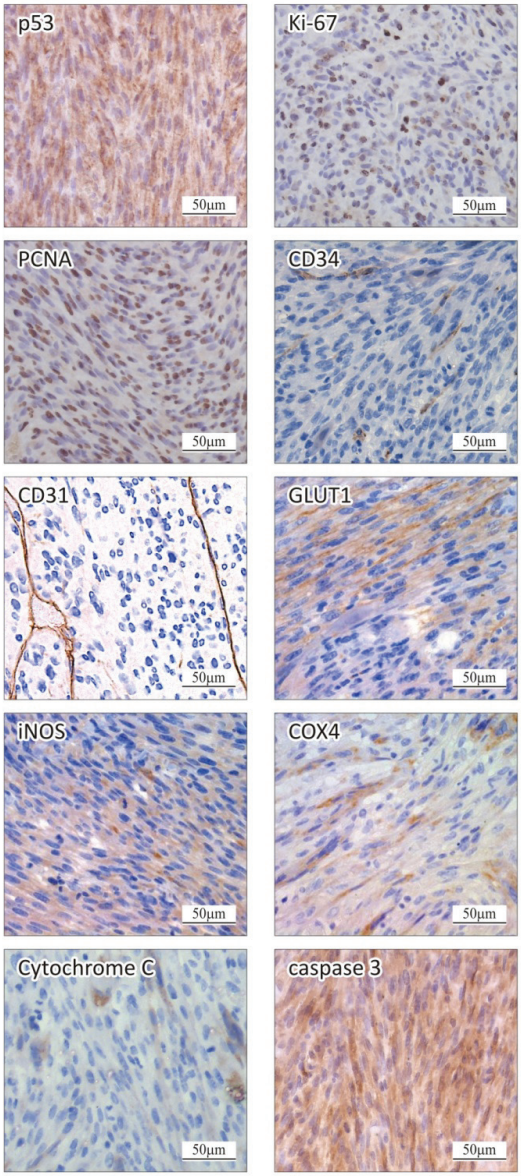

Fig 3. Extirpated hamster fibrosarcoma immunohistochemical staining images (examples from the control groups); illustration for used immunehistochemical stainings (p53, Ki-67, PCNA, CD34, CD31, GLUT1, iNOS, COX4, cytochrome C and caspase 3).

The results (Table I) confirmed the significant ($P < 0.05$) synergistic anticancer effects of all examined drug combinations on hamster fibrosarcoma, with significant correlation ($P < 0.05$) between decrease in tumour weight and immunehistochemical staining grade (Table II), without toxicity on main organs (based on the gross and microscopic standard pathological examination of organs, tis-

sues and whole bodies), influence on body weight, biochemical and haematological blood tests.

TABLE II. Correlation of tumour weight (g) and immunohistochemical expression (intensity)

Immunohistochemical parameter	Line fit plot	Pearson correlation coefficient (r)	P-value×10^7
p53	$y = 8.095x + 1.597$	0.98511	2.1600
Ki-67	$y = 8.630x + 1.522$	0.98501	2.1672
PCNA	$y = 8.367x + 1.664$	0.98506	2.1622
CD34	$y = 1.002x + 0.576$	0.96396	0.70657
CD31	$y = 1.326x + 0.328$	0.95803	0.012902
GLUT1	$y = 0.091x + 0.050$	0.95784	0.013131
iNOS	$y = 0.080x + 0.031$	0.98646	1.4477
COX4	$y = 0.940x + 0.278$	0.96552	0.59297
Cytochrome C	$y = 0.925x - 0.221$	0.97395	0.19512
Caspase 3	$y = 7.791x + 1.782$	0.94972	0.021730

Findings of significant correlation between the significantly reduced tumour weight and immunohistochemical markers expression validate the anticancer effects of the investigated repurposed drug combinations.

Our explanation of the positive correlation between the tumour weight and the used immunohistochemical staining markers intensity, or more precisely, the explanation of the treated tumour weight decrease and the correlated p53 ("the guardian of the genom") staining decrease (what seems contrary to the usual expectation); the reduced expression of proliferation indicators Ki-67 and PCNA (as expected); the reduced expression of neoangiogenesis indicators CD34 and CD31 (as expected); reduced expression of glucose metabolism indicator GLUT1 (as expected); the reduced expression of NO metabolism indicator iNOS (as expected); and the reduced expression of apoptosis indicators COX4, cytochrome C, caspase 3 (what seems contrary to usual expectation), based on recent literature, is as follows.

The tumour suppressor protein p53 is overexpressed in a large fraction of human tumours, including sarcomas.[34] It has been found that: 1) p53 abnormalities may be an early event that contributes to the neoplastic transformation; 2) p53 overexpression may be related to the progression toward more aggressive tumour forms.[34] The p53 protein is frequently mutated in many human cancers.[34] The mutant p53 protein has a much longer half-life than the wild-type protein and accumulates in large amounts in the nuclei of transformed cells. In contrast, the levels of the wild-type p53 protein are normally so low that they cannot be detected by immunohistochemistry.[34] Immunohistochemical detection of p53: 1) strong homogeneous staining pattern (aggressive tumor); 2) rare scattered positive nuclei (treated tumour). p53 protein overexpression (mutated) was significantly associated with parameters of biological aggressiveness. Mutant p53 pro-

tein may be unable to perform the normal p53 function of suppressing cell proliferation.[34] p53 is the most commonly mutated gene in cancer, including soft tissue sarcoma in humans.[35]

Ki-67 and PCNA proteins – cellular markers for proliferation, CD34 – a marker of endothelial cells and vascular differentiation, CD31 – a useful specific adjunctive marker for tumour vasculature endothelial differentiation (more specific and sensitive than CD34), GLUT1 – associated with glucose transport across membranes (overexpressed in cancer), iNOS – marker of NO metabolism in the cytoplasm, associated with the tumour progression, proliferation and angiogenesis (overexpressed in various neoplastic processes) were in our experiments positively correlated to tumour weight. This could be expected, since the tumour cell proliferation, neoangiogenesis and glucose or NO metabolism intensity are involved in the tumour biological outcomes, such as tumour weight.[1]

COX4, Cytochrome C and caspase 3 – markers of apoptosis, were in our study also directly correlated with tumour weight. Disregulation of apoptosis is a hallmark of cancer, enabling tumour cells to evade cell death and promote uncontrolled growth. In cancer cells increased levels of cleaved caspase 3 have been observed, indicating ongoing apoptotic events that might be ineffective or stopped by pro-survival pathways.[36] Rate of apoptosis, as measured by both caspase 3 activation or by other methods and nucleosome release are higher in breast cancer than in non-malignant breast tissue.[37] This finding would appear to conflict with the widely held belief that apoptosis is reduced in malignancy. The proliferation/apoptosis ratio, however, may be higher in carcinomas than in the corresponding normal tissue.[37] Tumour growth is the result of cell proliferation and cell loss by apoptosis.[38] By the comparison with non-neoplastic breast tissue, caspase 3 appeared to be upregulated in malignant breast tissue (> 75 % of the specimens).[38] The caspase-3 protein overexpression appears to be involved in the apoptotic pathways influenced by wild-type p53 (mutated p53).[38] The caspase 3 expression correlates with poor prognostic parameters such as higher histologic grade and high proliferation in breast carcinoma patients.[39] The caspase 3 expression could be linked with malignancy progression and correlates with the malignancy of head and neck cancer in humans.[40]

Repurposing approved non-oncology drugs can significantly expedite the process of identifying effective anticancer treatments overcoming drug resistance in cancer and improve patient outcomes in a cost-effective manner.[41] A good illustration of relevance and importance of drug repurposing for cancer treatment are clinical investigations available up to 2024 from multiple resources (PubMed, Google Scholar, ClinicalTrials.gov, Drug Bank database, ReDo database and the National Institutes of Health).[42] The antidiabetic agents contribute to 52 clinical trials: metformin (37 trials/Phase I– III), pioglitazone (10 trials/Phase I and II), desmopressin (2 trial Phase I and II), dapaglifozin (1 trial/Phase I), epalrestat (1

trial/Phase II), acarbose (1 trial/Phase II) against different cancer types (*e.g.*, in clinical trial Phase III: breast, colorectal, endometrial, prostate and non-small cell lung cancer).[42]

CONCLUSION

The results of our experiments confirmed the significant anticancer effects of the metformin co-treatments with deoxycholic acid, caffeine, itraconazole, nitroglycerin, disulfiram or diclofenac on hamster fibrosarcoma, without toxicity. The anticancer properties of the examined two-drug combinations in hamsters, documented by significant decrease in tumour weight and immunohistochemical staining grade, further supported by the significant correlation between the two, with used doses equivalent to standard human doses, suggest that the effective nontoxic oncological therapies in humans and prevention of cancer relapse using these drug combinations may be achievable. The administration of metformin in combination with deoxycholic acid, caffeine, itraconazole, nitroglycerin, disulfiram or diclofenac might be an effective and safe approach in novel nontoxic adjuvant anticancer treatment and can be recommended for further clinical investigations.

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ИЗВОД

ИМУНОХИСТОХЕМИЈСКИ ПОКАЗАТЕЉИ АНТИКАНЦЕРСКОГ ДЕЛОВАЊА КОМБИНАЦИЈА МЕТФОРМИНА СА ДРУГИМ ПРЕНАМЕЊЕНИМ ЛЕКОМ И КОРЕЛАЦИЈА СА ВЕЛИЧИНОМ ТУМОРА НА ФИБРОСАРКОМУ ХРЧКА

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Циљ је био откривање и корелација антиканцерских ефеката метформина у комбинацији са другим пренамењеним лековима, већ регистрованим за друге индикације, који се могу одмах применити и клинички испитати у онкологији, што смањује време и трошкове истраживања нових терапија канцера. Имунохистохемија је урађена за туморе лечене комбинацијама два лека, које садрже метформин са деоксихолном киселином, кофеином, итраконазолом, нитроглицерином, дисулфирамом или диклофенаком. Лекови су примењени код сиријских златних хрчака

(6 животиња по групи) са инокулисаним BHK21/C13 фибросаркомом, у дозама еквивалентним уобичајеним дозама за људе, <50 % LD_{50}. Антиканцерска дејства су процењена помоћу имунохистохемијских маркера: p53 (мутациони статус); Ki-67 и PCNA (пролиферација тумора); CD34 и CD31 (неоангиогенеза); GLUT1 (метаболизам глукозе); iNOS (NO метаболизам); COX4, cytochrome C и caspase 3 (апоптоза). Такође, анализиране су биофизичке карактеристике фибросаркома, узорци крви животиња и токсичност за главне органе. Третмани су значајно (P < 0,05) смањили мутациони статус, пролиферацију тумора, неоангиогенезу, метаболизам глукозе, метаболизам *NO* и модулирали апоптозу, у корелацији са величином тумора, без токсичности и утицаја на биохемијске крвне и хематолошке тестове. Примена комбинација два лека – метформина са: деоксихолном киселином, кофеином, итраконазолом, нитроглицерином, дисулфирамом или диклофенаком, може се препоручити за даља клиничка испитивања у онкологији.

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REFERENCES

1. D. Verzella, A. Pescatore, D. Capece, D. Vecchiotti, M. V. Ursini, G. Franzoso, E. Alesse, F. Zazzeroni, *Cell Death Dis.* **11** (2020) 210 (https://doi.org/10.1038/s41419-020-2399-y)
2. L. Li, T. Wang, M. Hu, Y. Zhang, H. Chen, L. Xu, *Front. Oncol.* **10** (2020) 1605 (https://doi.org/10.3389/fonc.2020.01605)
3. L. Ma, J. Wei, J. Wan, W. Wang, L. Wang, Y. Yuan, Z. Yang, X. Liu, L. Ming, *J. Exp. Clin. Cancer. Res.* **38** (2019) 77 (https://doi.org/10.1186/s13046-019-1090-6)
4. C. C. Lu, J. H. Chiang, F. J. Tsai, Y. M. Hsu, Y. N. Juan, J. S. Yang, H. Y. Chiu, *Int. J. Oncol.* **54** (2019) 1271 (https://doi.org/10.3892/ijo.2019.4704)
5. J. Yang, J. Wei, Y. Wu, Z. Wang, Y. Guo, P. Lee, X. Li, *Oncogenesis* **4** (2015) e158 (https://doi.org/10.1038/oncsis.2015.18)
6. Z. Wu, Y. Lü, B. Wang, C. Liu, Z. R.Wang, *World J Gastroenterol.* **9** (2003) 2759 (http://dx.doi.org/10.3748/wjg.v9.i12.2759)
7. J. S. Pyo, Y. S. Ko, G. Kang, D. H. Kim, W. H. Kim, B. L. Lee, J. H. Sohn. *J. Cancer Res. Clin. Oncol.* **141** (2015) 1181 (https://doi.org/10.1007/s00432-014-1890-1)
8. J. P. Phelan, F. J. Reen, N. Dunphy, R. O'Connor, F. O'Gara, *BMC Cancer* **16** (2016) 476 (https://doi.org/10.1186/s12885-016-2528-2)
9. S. Yui, R. Kanamoto, T. Saeki, *Nutr. Cancer* **60** (2008) 91 (https://doi.org/10.1080/01635580701525893)
10. J. Ignacio Barrasa, N. Olmo, P. Pérez-Ramos, A. Santiago-Gómez, E. Lecona, J. Turnay, M. Antonia Lizarbe, *Apoptosis* **16** (2011) 1054 (https://doi.org/10.1007/s10495-011-0633-x)
11. H. B. Yang, W. Song, M. D. Cheng, H. F. Fan, X. Gu, Y. Qiao, X. Lu, R. H. Yu, L. Y. Chen, *Mol. Med. Rep.* **11** (2015) 2749 (https://doi.org/10.3892/mmr.2014.3004)
12. P. M. Rodrigues, M. B. Afonso, A. L. Simão, P. M. Borralho, C. M. P. Rodrigues, R. E. Castro, *Sci. Rep.* **5** (2015) 17528 (https://doi.org/10.1038/srep17528); *Erratum* in: *Sci. Rep.* **6** (2016) 27828 (https://doi.org/10.1038/srep27828)
13. M. Hałas, M. Izdebska, A. Klimaszewska-Wiśniewska, M. Gagat, D. Radciniewska, A. Glińska, K. Gizler, E. Bielińska, A. Grzanka, *Open Life Sci.* **9** (2014) 727 (https://doi.org/10.2478/s11535-014-0315-0)

14. E. D. Osarieme, D. T. Modupe, O. P.Oluchukwu, *Arch. Clin. Biomed. Res.* **3** (2019) 326 (https://doi.org/10.26502/acbr.50170077)
15. H. Tsubamoto, T. Ueda, K. Inoue, K. Sakata, H. Shibahara, T. Sonoda, *Oncol. Lett.* **14** (2017) 1240 (https://doi.org/10.3892/ol.2017.6325)
16. M. D. Navarro-Martínez, J. Cabezas-Herrera, J. N. Rodríguez-López, *Int. J. Antimicrob. Agents* **28** (2006) 560 (https://doi.org/10.1016/j.ijantimicag.2006.07.012)
17. C. L. Li, Z. X. Fang, Z. Wu, Y. Y. Hou, H. T. Wu, J. Liu, *Biomed. Pharmacother.* **154** (2022) 113616 (https://doi.org/10.1016/j.biopha.2022.113616)
18. J. Mintz, A. Vedenko, O. Rosete, K. Shah, G. Goldstein, J. M. Hare, R. Ramasamy, H. Arora, *Vaccines* **9** (2021) 94 (https://doi.org/10.3390/vaccines9020094)
19. A. Garcia, G.Tisman, *J. Clin. Oncol.* **28** (2010) e19 (https://doi.org/10.1200/jco.2009.25.7857)
20. M. A. Erkurt, İ. Aydoğdu, N. Bayraktar, İ. Kuku, E. Kaya, *Turk. J. Hematol.* **26** (2009) 197 (https://pubmed.ncbi.nlm.nih.gov/27265632/)
21. P. Pradhan, *J. Vector. Borne. Dis.* **46** (2009) 100 (https://pubmed.ncbi.nlm.nih.gov/19502689/)
22. V. Sukhatme, G. Bouche, L. Meheus, V. P. Sukhatme, P. Pantziarka, *Ecancer* **9** (2015) 568 (https://doi.org/10.3332/ecancer.2015.568)
23. B. Xu, S. Wang, R. Li, K. Chen, L. He, M. Deng, V. Kannappan, J. Zha, H. Dong, W. Wang, *Cell. Death. Dis.* **8** (2017) e2797 (https://doi.org/10.1038/cddis.2017.176)
24. O. Celik, A. Ersahin, M. Acet, N. Celik, Y. Baykus, R. Deniz, E. Ozerol, I. Ozerol, *Eur. Rev. Med. Pharmacol. Sci.* **20** (2016) 4380 (https://pubmed.ncbi.nlm.nih.gov/27831632/)
25. J. Zha, F. Chen, H. Dong, P. Shi, Y. Yao, Y. Zhang, R. Li, S. Wang, P. Li, W. Wang, B Xu, *J. Transl. Med.* **12** (2014) 163 (https://doi.org/10.1186/1479-5876-12-163)
26. M. A. Westhoff, S. Zhou, L. Nonnenmacher, G. Karpel-Massler, C. Jennewein, M. Schneider, M. E. Halatsch, N. O. Carragher, B. Baumann, A. Krause, T. Simmet, M. G. Bachem, C. R. Wirtz, K. M Debatin, *Mol. Cancer. Res.* **11** (2013) 1611 (https://doi.org/10.1158/1541-7786.mcr-13-0435-t)
27. W. Wang, H. L. McLeod, J. Cassidy, *Int. J. Cancer.* **104** (2003) 504 (https://doi.org/10.1002/ijc.10972)
28. K. Butcher, V. Kannappan, R. S. Kilari, M. R. Morris, C. McConville, A. L. Armesilla, W. Wang, *BMC Cancer* **18** (2018) 753 (https://doi.org/10.1186/s12885-018-4617-x)
29. J. Zhang, K. Pu, S. Bai, Y. Peng, F. Li, R. Ji, Q. Guo, W. Sun, Y. Wang, *J. Int. Med. Res.* **48** (2020) 300060520925996 (https://doi.org/10.1177/0300060520925996)
30. B. Herpers, S. Wink, L. Fredriksson, Z. Di, G. Hendriks, H. Vrieling, H. de Bont, B. van de Water, *Arch. Toxicol.* **90** (2016) 1163 (https://doi.org/10.1007/s00204-015-1536-3)
31. L. Fredriksson, B. Herpers, G. Benedetti, Q. Matadin, J. C. Puigvert, H. de Bont, S. Dragovic, N. P. Vermeulen, J. N. Commandeur, E. Danen, M. de Graauw, B. van de Water, *Hepatology* **53** (2011) 2027 (https://doi.org/10.1002/hep.24314)
32. Y. Takada, A. Bhardwaj, P. D. Potdar, B. Aggarwal, *Oncogene* **23** (2004) 9247 (https://doi.org/10.1038/sj.onc.1208169)
33. A. M. Cimpean, D. Lalošević, V. Lalošević, P. Banović, M. Raica, O. A. Mederle, *In Vivo* **32** (2018): 791 (https://doi.org/10.21873/invivo.11309)
34. G. Toffoli, C. Doglioni, , C. Cernigoi, S. Frustaci, T. Perin, B. Canal, M. Boiocchi, *Ann. Oncol.* **5** (1994) 167 (https://doi.org/10.1093/oxfordjournals.annonc.a058771)

35. P. Das, D. Kotilingam, B. Korchin, J. Liu, D. Yu, A. J. Lazar, R. E. Pollock, D. Lev, *Cancer* **109** (2007) 2323 (https://doi.org/10.1002/cncr.22680)
36. L. Flanagan, M. Meyer, J. Fay, S. Curry, O. Bacon, H. Duessmann, K. John, K. C. Boland, D. A. McNamara, E. W. Kay, H. Bantel, H. Schulze-Bergkamen, J. H. Prehn, *Cell Death Dis.* **7** (2016) e2087 (https://doi.org/10.1038/cddis.2016.7)
37. N. O'Donovan, J. Crown, H. Stunell, A. D. Hill, E. McDermott, N. O'Higgins, M. J. Duffy, *Clin. Cancer Res.* **9** (2003) 738 (https://pubmed.ncbi.nlm.nih.gov/12576443/)
38. L. Nakopoulou, P. Alexandrou, K. Stefanaki, E. Panayotopoulou, A. C. Lazaris, P. S. Davaris, *Pathobiology* **69** (2001) 266 (https://doi.org/10.1159/000064337)
39. A. Nassar, D. Lawson, G. Cotsonis, C. Cohen, *Appl. Immunohistochem. Mol. Morphol.* (2008) **16** 113 (https://doi.org/10.1097/PAI.0b013e318032ea73)
40. F. F. V. E. Silva, M. E. Padín-Iruegas, V. C. A. Caponio, A. I. Lorenzo-Pouso, P. Saavedra-Nieves, C. M. Chamorro-Petronacci, J. Suaréz-Peñaranda, M. Pérez-Sayáns, *Int. J. Mol. Sci.* **23** (2022) 11937 (https://doi.org/10.3390/ijms231911937)
41. R. Mohi-ud-din, A. Chawla, P. Sharma, Prince A. Mir, F. H. Potoo, Ž. Reiner, I. Reiner, D. A. Ateşşahin, J. Sharifi-Rad, R. H. Mir, D. Calina, *Eur. J. Med. Res.* **28** (2023) 345 (https://doi.org/10.1186/s40001-023-01275-4)
42. M. A. Hijazi, A. Gessner, N. El-Najjar, *Cancers* **15** (2023) 3199 (https://doi.org/10.3390/cancers15123199).