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Metformin in chemically-induced mammary carcinogenesis in rats

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In this paper the chemopreventive effect of peroral antidiabetic metformin in mammary carcinogenesis in female Sprague-Dawley rats was evaluated. Mammary carcinogenesis was induced by N-methyl-N-nitrosourea (NMU) administered in two intraperitoneal doses each per 50 mg/kg b.w. between 43.-55. postnatal days. Metformin was administered in drinking water (at a concentration of 50 μg/ml and 500 μg/ml) 13 days before the first NMU dose until the termination of the experiment. During the experiment the animals were weekly weighed and palpated for the presence of mammary tumors, the incidence, latency, tumor frequency, and tumor volume were recorded. The experiment was terminated 18 weeks after the first NMU dose, basic tumor growth parameters and metabolic and hormonal variables were evaluated. Metformin did not significantly alter the tumor growth although a delay in tumor onset was recorded after higher metformin dose. Metformin altered metabolic and hormonal variables. Insulinemia decreased after both metformin doses in comparison with intact rats without changes in glycemia, triacylglycerols concentration was decreased in liver and increased in serum when compared to intacts. Higher metformin dose attenuated lipoperoxidation in liver.

Key words: metformin, mammary carcinogenesis, rat, metabolism

Breast cancer is the most prevalent cancer disease in women all over the world. The prevention of this neoplasia represents a challenge for oncology. Nowadays there is a rising evidence that substances primarily used in other diseases´ therapy such as coxibs, statins, and antidiabetics (biguanides and thiazolidinediones) may also be useful in prevention of breast cancer as well as other neoplasms.

Biguanides inhibit fatty acid oxidation, suppress liver gluconeogenesis, increase insulin receptors' availability, inhibit monoamine oxidase activity [1]. First relevant reports on their oncostatic activity come from the end of 70-ies – phenformin inhibited dimethylbenz(a)anthracene-induced mammary carcinogenesis in female rats [2], prolonged survival and inhibited spontaneous mammary carcinogenesis in female C3H/Sn mice [3], another biguanide analogue buformin had the same effect in female rats [4]. Phenformin inhibited radiation carcinogenesis [5], 1,2-dimethylhydrazine-induced colon carcinogenesis [6], and NMU-induced mammary tumor growth in rats [7]. However, phenformin and buformin were withdrawn from the market due to lactic acidosis risk. From this point of view another biguanide compound metformin is more suitable which is nowadays widely used in type 2 diabetes treatment. Metformin exerts its effects through AMP-activated protein kinase (AMPK). AMPK is a regulator of various processes including cell growth and proliferation, fatty acid synthesis, and mRNA translation [8]. Activation of AMPK by AICAR (5-aminoimidazole-4-carboxamide ribonucleoside) inhibited breast, glioma, and prostate cell proliferation [9]. Metformin had a suppressive effect on tumor growth *in vitro*. Isakovic et al. [10] reported proliferation inhibition and apoptosis induction in glioma cell, similarly, the proliferation of prostate cancer cell lines [11] and ovarian cancer cell lines was inhibited after metformin treatment [12]. Breast cancer cell lines growth was also inhibited by metformin – Phoenix et al. [13] reported growth inhibition of both estrogen receptor α negative (MDA-MB-231, MDA-MD-435) and positive (MCF-7, T47D) cell lines after metformin treatment. Metformin inhibited translation initiation in MCF-7 breast cancer cells, resulting in global protein synthesis decrease [14]. Inhibition of AMPK by compound C decreased antiproliferative properties of metformin on ovarian cancer cells [12] and glioma cells [10]. On the other hand, AMPK

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pathway inhibition (using siRNA against the two catalytic subunits of AMPK) did not prevent the antiproliferative effect of metformin in prostate cancer cell lines [11]. Thus the antiproliferative effect of metformin may not entirely depend on AMPK activation and there could be the other mechanism which remains to be revealed.

The *in vivo* reports on metformin oncostatic activity, however, are scarce. Metformin treatment resulted in tumor growth reduction in mice bearing LNCaP xenografts [11] and p53 deficient colon cancer HCT116 xenografts [15], however, the growth of HCT116 $p53^{+/+}$ cells was not affected [15]. Metformin inhibited pancreatic tumor growth in hamsters [16] and increased mammary tumor latency and overall surviving in HER-2/neu transgenic mice [17]. On the other hand, a study carried out in athymic nude mice suggested metformin may stimulate angiogenesis [13], therefore the metformin effect *in vivo* should be further analysed. Human studies suggest metformin may lower neoplastic diseases´ incidence in patients with diabetes mellitus type 2 – the cancer incidence in patients treated with metformin was lower than in those treated with other hypoglycaemic drugs [18]. Cancer-related mortality in diabetic patients using metformin in comparison to those using sulfonylureas or insulin was lower too [19].

Materials and methods

Female rats of Sprague-Dawley strain (AnLab, Prague, Czech Republic) aged 30–35 days were used in the experiment. The animals were adapted to standard vivarium conditions with temperature 23±2°C, relative humidity 60-70%, artificial regimen light:dark 12:12 (lights on from 7 a.m., light intensity 150 lux per cage). During the experiment the animals (4 per cage) were fed the MP diet (Top-Dovo, Dobrá Voda, Slovak Republic) and drank tap water ad libitum.

Mammary carcinogenesis was induced by N-methyl-N-nitrosourea (NMU) (Sigma, Deisenhofen, Germany) administered in two intraperitoneal doses (50 mg/kg b.w.) between 43.-55. postnatal days (with a week interval between doses). NMU solution was freshly prepared prior to carcinogen administration by dissolving NMU in physiological solution (the volume dose per rat was 0.5 ml).

Chemoprevention with metformin (Zentiva N.V., Slovak Republic) began 13 days before the first carcinogen administration and lasted until the end of experiment – 18 weeks after the first NMU application. Metformin was administered in tap water at two concentrations – 50 μg/ml (corresponding to 5 mg/kg/day) and 500 μg/ml (corresponding to 50 mg/kg/day). Metformin solution was freshly prepared 3 times a week by dissolving metformin in a tap water.

Animals were randomly assigned to one of four experimental groups: (1) NMU, control group without chemoprevention; (2) NMU+MF5, chemoprevention with metformin at a dose of 5 mg/kg/day; (3) NMU+MF50, chemoprevention with metformin at a dose of 50 mg/kg/day; (4) INT, intact group. Each group except the intact group consisted of 16 animals, the intact group consisted of 12 animals. Animals were weekly weighed and palpated in order to register the presence, number, location, and size of each palpable tumor. Food and water intake of animals during 24 hours was monitored in 9^{th} and 16^{th} week of experiment (dated from the first NMU injection), overall in 6 measurements (3 times in a given week). Daily intake of metformin ranged from 1.08-1.51 mg/rat/day in NMU+MF5 and 11.2-14.7 mg/rat/day in NMU+MF50, respectively. In the last – 18^{th} week of experiment the animals were quickly decapitated, mammary tumors were excised and weighed and tumor size was recorded. Macroscopic changes in selected organs (liver, kidney, stomach, intestine, and lung) were evaluated at autopsy. Selected organs (heart muscle, thymus, liver, spleen, adrenals, and periovarial fat tissue) were removed and weighed. Basic metabolic and hormonal parameters were determined in serum and selected organs: serum concentration of glucose (GLU); serum and liver concentration of triacylglycerols (TAG), cholesterol (CH), and phospholipids (PL); liver and heart muscle glycogen (GLY) concentration; liver and thymus malondialdehyde (MDA) concentration; serum corticosterone (CTS), insulin (INS), and IGF-1 concentration. GLU and TG were measured using commercial sets (Lachema, Brno, Czech Republic), INS and IGF-1 were determined using commercial RIA sets (Linco Research, St Charles, MO, USA and DRG Instruments GmbH, Germany, respectively), PL were measured from lipid phosphorus according to Bartlett et al. [20], CH according to Zlatkis et al. [21], GLY according to Roe and Dailey [22], MDA was measured in reaction with thiobarbituric acid according to Satch [23], CTS was measured using fluorimetry according to Guillemin et al. [24]. The following basic parameters of mammary carcinogenesis were evaluated in each group: tumor incidence (as the percentage of tumor-bearing animals per group), tumor frequency (as the average number of tumors per group), tumor volume, and latency (the period from carcinogen administration to the appearance of first tumor).

Tumor incidence was evaluated by Mann-Whitney U-test, other parameters by one-way analysis of variance or Kruskal-Wallis test, respectively, the criterion for the choice of the relevant test was the Bartlett´s number value. Tumor volume was calculated according to: $V = \pi \cdot (S_1)^2 \cdot S_2 / 12$; S_1 and S_2 are tumor diameters; $S_1 < S_2$. The experiment was carried out from July to November.

Results

The body mass gain was not changed after metformin administration in comparison with control group but was significantly lower in all three groups with carcinogen administration in comparison with the intact group (Figure 1). Similarly, the periovarial white fat weight in groups with administered carcinogen was decreased when compared to

intacts (data not shown). The food and water intake was not changed in groups with chemoprevention in comparison with control group. When compared to intact group, the food intake in 9th week in groups with chemoprevention was decreased and water intake was decreased in all groups with carcinogen administration. These changes did not persist as in 16th week no differences in food and water intake were recorded (Table 1). No significant changes in tumor incidence were recorded although the tumor onset was delayed in groups with chemoprevention. Tumor frequency was decreased in 8th week and latency was increased until the 12th week in NMU+MF50 in comparison with NMU+MF5 (data during experiment are not shown). Tumor growth parameters recorded after the experiment termination were not significantly changed (Table 2). Systemic IGF-1 levels were lower in all three experimental goups when compared to intacts, significantly higher IGF-1 level was recorded in NMU+MF50 when compared to NMU+MF5 (Table 3).

Metformin in higher dose decreased liver and heart muscle GLY concentration when compared to lower dose. The heart muscle GLY concentration in NMU+MF5 was increased when compared to NMU. Serum TAG concentration in all groups administered with carcinogen was increased when compared to intacts, due to large interindividual differences this increase was significant only in NMU+MF50. In liver, however, TAG concentration decreased (significantly after both metformin doses) when compared to intacts. Liver CH concentration in NMU+MF5 was decreased in comparison with NMU. No changes in PL concentration were recorded either in serum or liver. Liver MDA concentration in NMU and NMU+MF5 was higher when compared to intacts, metformin in higher dose decreased it to the level of intacts. Both metformin doses decreased serum CTS level when compared to NMU. Serum INS level was decreased after both metformin doses in comparison with intacts, the glycemia, however, was not changed (Table 3).

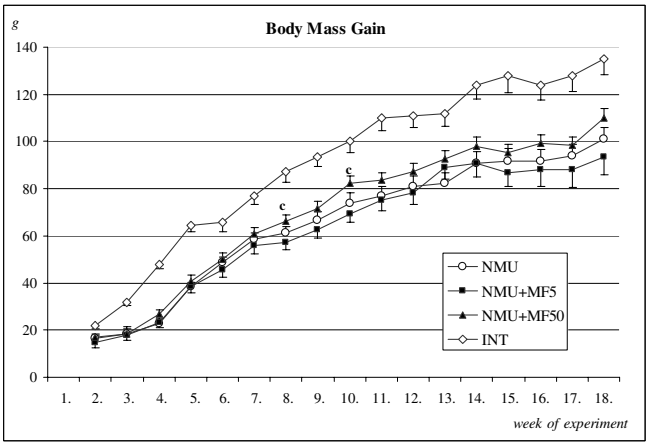

Figure 1 Chemoprevention of NMU-induced mammary carcinogenesis in Sprague-Dawley rats by metformin: body mass gain
Data are expressed as means ± S.E.M. Significant differences between NMU+MF5 and NMU+MF50 are designated as c for $p \leq 0.05$.
Abbreviations: NMU – control group without chemoprevention; NMU+MF5 – chemoprevention with metformin (5 mg/kg/day), NMU+MF50 – chemoprevention with metformin (50 mg/kg/day), INT – intact group.

Table 1. Chemoprevention of NMU-induced mammary carcinogenesis in Sprague-Dawley rats by metformin: food and water intake

	9th week		16th week	
Group	**Food intake (g/rat/day)**	**Water intake (ml/rat/day)**	**Food intake (g/rat/day)**	**Water intake (ml/rat/day)**
NMU	18.3 ± 0.639	25.3 ± 1.45 **a**	18.5 ± 0.987	28.3 ± 1.71
NMU+MF5	17.1 ± 0.443 **aa**	21.6 ± 1.98 **a**	18.6 ± 0.876	30.2 ± 2.62
NMU+MF50	17.7 ± 0.296 **aa**	22.4 ± 1.40 **a**	19.1 ± 0.815	29.4 ± 1.75
INT	19.3 ± 0.411	34.4 ± 4.35	20.3 ± 0.901	27.3 ± 0.847

Data are expressed as means ± S.E.M. Significant differences in comparison with intact goup are designated as **a** for $p \leq 0.05$, **aa** for $p \leq 0.01$.
Abbreviations: NMU – control group without chemoprevention; NMU+MF5 – chemoprevention with metformin (5 mg/kg/day), NMU+MF50 – chemoprevention with metformin (50 mg/kg/day), INT – intact group

Table 2. Chemoprevention of NMU-induced mammary carcinogenesis in Sprague-Dawley rats by metformin

Experimental group	**Incidence (%)**	**Latency (days)**	**Frequency**	**Tumor volume (cm^3)**
NMU (n=16)	88	68.7 ± 5.09	3.81 ± 0.737	0.852 ± 0.227
NMU+MF5 (n=12)	88	66.2 ± 5.34	3.88 ± 0.894	1.17 ± 0.516
NMU+MF50 (n=15)	94	75.3 ± 3.87	4.88 ± 0.758	1.04 ± 0.224

Data are expressed as means ± S.E.M.
Abbreviations: NMU – control group without chemoprevention; NMU+MF5 – chemoprevention with metformin (5 mg/kg/day), NMU+MF50 – chemoprevention with metformin (50 mg/kg/day), INT – intact group, n – number of animals per group

Table 3. Chemoprevention of NMU-induced mammary carcinogenesis in Sprague-Dawley rats by metformin: metabolic and hormonal alterations

	NMU n=16	NMU+MF5 n=12	NMU+MF50 n=15	INT n=12
Serum				
GLU (mmol/l)	5.03 ± 0.150	5.14 ± 0.107	5.01 ± 0.092	5.25 ± 0.087
TAG (mmol/l)	1.10 ± 0.151	1.17 ± 0.220	1.18 ± 0.084 **fff**	0.700 ± 0.076
CH (mmol/l)	2.19 ± 0.228	2.04 ± 0.070	2.06 ± 0.089	2.20 ± 0.084
PL (mmol/l)	1.73 ± 0.133	1.76 ± 0.110	1.64 ± 0.070	1.54 ± 0.225
CTS (ng/ml)	519 ± 57.2	360 ± 41.3 **a**	365 ± 20.3 **b**	437± 62.8
INS (ng/ml)	0.144 ± 0.023	0.134 ± 0.015 **e**	0.111 ± 0.015 **ff**	0.190 ± 0.021
IGF-1 (ng/ml)	583 ± 52.7 **dd**	512 ± 36.4 **eee**	642 ± 34.0 **c ff**	850 ± 45.7
Liver				
GLY (μmol/g)	7.15 ± 1.12	9.16 ± 1.09	6.33 ± 0.650 **c**	8.98 ± 1.37
TAG (μmol/g)	20.0 ± 2.46	18.0 ± 2.78 **e**	13.5 ± 1.20 **fff**	26.3 ± 2.48
CH (μmol/g)	12.9 ± 0.438	11.6 ± 0.359 **a**	11.3 ± 0.653	12.7 ± 0.558
PL (μmol/g)	46.9 ± 1.69	46.7 ± 2.36	45.4 ± 1.63	46.3 ± 1.52
MDA (nmol/g)	50.4 ± 5.50 **dd**	43.2 ± 3.07 **ee**	30.0 ± 1.31 **bb ccc**	30.5 ± 1.67
Heart Muscle				
GLY (μmol/g)	5.73 ± 0.679	8.59 ± 0.889 **a**	5.12 ± 0.770 **cc**	7.02 ± 0.965
Thymus				
MDA (nmol/g)	17.6 ± 1.16	18.6 ± 1.25	17.6 ± 1.09	19.4 ± 2.39

Data are expressed as means ± S.E.M. Significant differences between groups are designated as follows: NMU vs NMU+MF5: **a** for $p \leq 0.05$; NMU vs NMU+MF50: **b** for $p \leq 0.05$; **bb** for $p \leq 0.01$; NMU+MF5 vs NMU+MF50: **c** for $p \leq 0.05$, **cc** for $p \leq 0.01$, **ccc** for $p \leq 0.001$; NMU vs INT: **dd** for $p \leq 0.01$; NMU+MF5 vs INT: **e** for $p \leq 0.05$, **ee** for $p \leq 0.01$, **eee** for $p \leq 0.001$; NMU+MF50 vs INT: **ff** for $p \leq 0.01$, **fff** for $p \leq 0.001$.

Abbreviations: NMU – control group without chemoprevention; NMU+MF5 – chemoprevention with metformin (5 mg/kg/day), NMU+MF50 – chemoprevention with metformin (50 mg/kg/day), INT – intact group, n – number of animals per group, GLU – glucose, TAG – triacylglycerols, PL – phospholipids, CH – cholesterol, CTS – corticosterone, INS – insulin, IGF-1 – insulin-like growth factor 1, GLY – glycogen, MDA – malondialdehyde

Discussion

This study is the first report on metformin in chemically-induced mammary carcinogensis in rats. Metformin administration had no significant effect on mammary tumor growth parameters. During the first weeks of experiment, however, the latency was significantly increased in NMU+MF50 and the tumour frequency was lower in the 8th week of experiment in NMU+MF50 when compared to NMU+MF5 (data not shown). No changes in tumor incidence and frequency in HER-2/neu transgenic mice after metformin treatment (100 mg/kg b.w. in drinking water) were found by Anisimov et al. [17] either; however, the latency of spontaneous mammary tumors was increased as well as overall surviving. Increased life span and spontaneous mammary carcinogenesis inhibition (as seen by 4-fold tumor incidence reduction) was recorded in female C3H/Sn mice after phenformin given in a dose of 2 mg/mouse five times a week orally, the calculated daily dose was in the ranges 60-80 kg/b.w. [3]. Our dose, however, was lower (5 mg/kg/day and 50 mg/kg/day), therefore it seems higher dose may be required for significant oncostatic activity.

The relation between circulating IGF-1 levels and the risk of commom cancers have been studied extensively. IGF-1 is a potent cell survival factor that suppresses apoptosis. Increased IGF-1 levels are associated with increased risk of colorectal, prostate, and premenopausal breast cancer. As for postmenopausal breast cancer, previous studies did not confirm this association [25, 26], however, some recent studies did [27]. In animal studies, the IGF-1 is downregulated in cancer cachexia [28] and similarly, in cancer patients the serum IGF-1 levels decrease as their illness aggravates [29]. In our experiment, the IGF-1 levels decreased in all three experimental groups in comparison with intact group; the lowest level was recorded in NMU+MF5 (which was significantly lower in comparison with NMU+MF50 too). The body mass gain in all three experimental groups was lower in comparison with intacts, the decreased food intake was recorded in 9th week of experiment but did not persist, therefore we relate the body mass gain decrease to energy metabolism disturbances resulting from tumor burden. The serum IGF-1 decrease was accompanied with insulin decrease in all experimental groups (non-significant in the control group), glycemia, however, was not changed. Metformin and other biguanides impact on insulin/IGF-1 signaling has been suggested as a mechanism involved both in ageing and carcinogenesis by Anisimov group [30, 17, 31], the effective dose, however, has to be found by further research.

Tumor burden is connected with metabolic disturbances. The total lipid body content decreases due to enhanced lipid mobilisation and oxidation in host tissues. As the disease progresses, cancer cachexia develops as a result of food intake reduction, increased energy expenditure, or a combination of the two. Patients with cancer cachexia often exhibit a relative glucose intolerance and insulin resistance with increased Cori cycle activity. The solid tumors mostly gain

energy from the anaerobic glucose metabolism. Fat oxidation rates elevate leading to adipose tissue loss, this appears to be the result of reduced lipogenesis rather than increased lipolysis due to decreased lipoprotein lipase enzyme level [32]. In our experiment, a decrease in body weight gain and periovarial fat weight was recorded in all groups with carcinogen administration when compared to intact group as a result of cancer cachexia.

In diabetes, the free radical production increases. In patients with diabetes, metformin reduced platelet superoxide anion production [33] and prevented impairment of the antioxidant properties of serum albumin [34]. In our experiment liver lipid peroxidation measured by MDA production increased in NMU and NMU+MF5, higher metformin dose decreased it to the level of intacts. As increase in free radicals production is often related to aging and even carcinogenesis promotion, this effect of metformin may be regarded as beneficial.

Generally serum cortisol increases in patients with malignant tumors [35]. In patients with breast cancer and weight loss serum cortisol was elevated in comparison with those without weight loss [36]. In our experiment serum CTS level in the control group was higher when compared to intacts (non-significantly due to large individual variations), metformin administration decreased it (non-significantly when compared to intacts). The same beneficial effect can be expected in humans receiving metformin, however, this has to be verified.

As mentioned above glycemia was not changed. In NMU+MF5 heart muscle GLY concentration was increased in comparison with both NMU and NMU+MF50 as well as in liver when compared to NMU+MF50.

Lipid metabolism alterations in cancer include higher serum TAG and lower total and HDL cholesterol concentrations [36]. This was seen also in our study – lipomobilisation resulted in liver TAG concentration decrease in comparison with intacts (significant after both metformin doses) and subsequent serum TAG levels increase (although due to individual variations this was significant only in NMU+MF50). Similarly, TAG concentration increase in serum and decrease in liver was found in our previous work [37] in female rats with NMU-induced mammary carcinogenesis. Serum and liver CH concentrations were not changed (only in NMU+MF5 the liver CH concentration was decreased in comparison with control group). Serum and liver PL concentrations were not changed either.

Although in our experiment metformin failed to act as an oncostatic substance, it should be taken into consideration the experimental animals are administered with high carcinogen dose (either once or twice) to induce carcinogenesis unlike the humans that are permanently exposed to relative small doses of carcinogens during lifetime. The other cause may be a low dose of metformin used in our experiment in comparison with other authors and that one used in humans for diabetes treatment. When calculated to body surface area, the doses used in our experiment were approximately 50 and 500 mg/m^2, respectively, whereas in humans (with daily dose 1.0 – 2.5 g) the doses can be 2-3 fold higher. The cancer incidence decrease recorded in diabetic patients may also be a result of so-called metabolic rehabilitation – biguanides treatment in breast and colon cancer patients improved the survival and slightly decreased contralateral breast tumor incidence [38, 39, 40]. Nevertheless clinical data support the idea of metformin use in cancer prevention at least in diabetic patients and so the possible chemopreventive metformin activity should be further analysed.

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