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Metformin and melatonin improve histopathological outcome of NMU-induced mammary tumors in rats

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ABSTRACT

Objective: Numerous reports showed inhibition of carcinogenesis after metformin (MF) and melatonin (MEL) administration. However, most *in vivo* studies used standard diet type, with relatively low fat content. As increase in fat intake may have a considerable impact on malignant transformation, we evaluated the effects of these two substances in a model of mammary carcinogenesis in rats fed a high-fat diet (10%).
Methods: Mammary tumors were induced by *N*-methyl-*N*-nitrosourea (NMU) in female rats of sensitive Sprague-Dawley strain. MF was administered in a diet (0.2%), MEL was administered in drinking water (20 mg/L). The chemoprevention was initiated 12 days prior to tumor initiation, both substances were administered through the termination of the experiment on 16th week after carcinogen application. Analysis of basic parameters of tumor growth, histopathological profile, and serum IGF-1 level were performed together with immunohistochemical detection of Ki67 (proliferation marker) and caspase-3 and BCL-2 (apoptosis markers) in mammary cancer cells.
Results: Although neither tumor incidence nor frequency were changed after MF and/or MEL administration, MF and MEL decreased high-grade/low-grade (HG/LG) tumor ratio. MEL decreased proliferation in mammary cancer cells; positive correlations between histological grade and Ki67 expressions were found after single administration of both MF and MEL. Serum IGF-1 levels were reduced to the level of intact rats in all groups receiving chemoprevention.
Conclusions: MF and MEL administration did not inhibit growth of NMU-induced mammary tumors in rats in a significant manner but both substances ameliorated tumor histopathological profile. Surprisingly, combined treatment had no such effect.

1. Introduction

Cancer may be prevented or delayed by many substances, both natural and synthetic. In recent years, attention has been drawn to drugs with useful “side effects” which are primarily used for the therapy of other diseases. This group includes antidiabetic drugs glitazones (or thiazolidinediones) and MF (1,1-dimethylbiguanide) which is currently the only available biguanide for diabetes treatment. Since the first reported use in 1957 [1], MF has become a drug of first choice to treat type 2 diabetes. Apart from antidiabetic properties, MF also exerts oncostatic effects. Antiproliferative properties of MF are mainly contributed to AMPK-mediated inhibition of mTOR [2] but MF also inhibits the expression and function of estrogen receptors (ER) [3] and can target cancer stem cells and induce epigenetic changes [4]. Numerous preclinical studies confirmed growth suppression of various types of neoplasia including mammary cancer, both *in vitro* and *in vivo* after treatment with MF [5,6]. Some reports, however, did not support oncostatic efficacy of MF [7,8]. As *in vivo* studies differ in many aspects of experimental protocol including different dosage of MF, unambiguous conclusion cannot be drawn. Human reports are encouraging: an

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extensive meta-analysis by Gandini et al. [9] showed lower cancer risk and overall cancer-related mortality (including breast cancer patients) in MF users.

Data regarding MF effects in nondiabetic populations are, unluckily, much less available. Short-time administration of MF between biopsy and surgery decreased proliferation [10–15] and stimulated apoptosis [14,16] in breast cancer tumor samples, however, the effect of MF seems to depend on insulin resistance status [15,16]. Addition of MF to hormonal therapy decreased the numbers of metastatic cases in nondiabetic breast cancer women [17]. Further research is needed to evaluate the effect and benefits of MF in nondiabetic cancer patients.

MEL (N-acetyl-5-methoxytryptamine), an indolamine which is produced not only in pineal gland but in other tissues too [18], has been established as a molecule with pleiotropic effects. Apart from chronobiotic function, MEL acts as a potent antioxidant [19], immunomodulator [20], has anti-angiogenic properties [21] and is involved in regulation of cell cycle, apoptosis [22] and metabolism [23]. In addition, MEL modulates ER activity and inhibits the production of estrogens via aromatase inhibition [24], thus may modulate the growth of hormone-dependent cancers [25]. These multiple actions contribute to oncostatic ability of MEL [26–28]. MEL is useful when administered as an adjuvant: many relevant studies showed improved life expectancy and amelioration of adverse side effects of chemotherapy in patients with solid tumors [29]. MEL is well-tolerated even at high doses [30], the ideal dose, however, is not standardized yet. The time factor plays an important role too. Preclinical data showed that anti-cancer actions of the circadian MEL signal heavily involve MT1 receptor-mediated mechanisms [26] and as the receptor density and sensitivity fluctuate throughout the 24 h cycle, timing of MEL dose may have a crucial impact on its oncostatic effects [31]. Clinical studies with sufficient sample sizes must be conducted to find the link between the effects and underlying mechanism of action before the combination of MEL and chemotherapeutic agents is recommended as a novel anticancer treatment. Similarly, further research is needed before MEL can be established as a preventive agent against human cancers.

To prevent and/or treat cancer more effectively, combination of agents is used. In this work, we evaluated the preventive/curative effect of MF and MEL administered alone and in combination, in a rat model of mammary cancer induced by a direct alkylating agent, NMU. This experimental system is widely used for the study of mammary tumorigenesis as it closely mimics human breast cancer disease including ER positivity [32]. Unlike other *in vivo* chemoprevention studies, we used a diet with intermediary high-fat content (10% of total fat) in attempt to reflect the situation in human population more precisely. Both substances were administered in a preventive-curative manner, almost two weeks before tumor initiation through the termination of the experiment, to cover both the initiation and promotion/progression stage of carcinogenesis. This experimental basis for the prevention of breast cancer was suggested by Mehta [33]. The effect of combined treatment with MF and MEL using this experimental protocol has not been reported before. MF dose corresponded to recommended daily dose for treatment of type 2 diabetes (1700 mg/day), MEL dose was chosen according to our previous experience from similar experiments. This study is a follow-up to our previous work where we confirmed proapoptotic effects of MF and MEL combination in a rat model of breast cancer using a procarcinogen 7,12-dimethylbenz(a)anthracene (DMBA) [34].

2. Material and methods

2.1. Model of mammary carcinogenesis

Female rats of Sprague-Dawley strain (Velaz, Prague, Czech Republic) aged 30 days were used in the experiment (n = 82). After arrival, the animals were adapted to standard conditions of animal facility (23 ± 2 °C, a relative air humidity of 60–70%, 12:12 h light-dark cycle). The animals (5–6/cage) were fed a high-fat diet (10% of total fat, 7.5% from palm olein, 2.5% from lard; Biofer, Slovak Republic) and drank tap water or MEL solution, *ad libitum*. Mammary carcinogenesis was induced by NMU (Cat. No. N1517 Sigma-Aldrich, Deisenhofen, Germany) administered intraperitoneally (50 mg/kg) on the 42nd postnatal day (according to Thompson and Adlakha [35]). NMU solution was prepared by dissolving NMU in 0.9% NaCl (average volume dose/rat: 0.5 ml).

2.2. Experimental design

Administration of MF (Siofor, Berlin-Chemie AG, Berlin, Germany) and MEL (Cat. No. M5250, Sigma-Aldrich, Deisenhofen, Germany) started 12 days before carcinogen application and lasted for 16 weeks until the end of experiment. MF was administered in a diet at a concentration of 2 g/kg (0.2%). MEL was administered in tap water at a concentration of 20 mg/L from 3 p.m. to 8 a.m. (the rats were drinking tap water between 8 a.m. to 3 p.m.). The animals were assigned randomly to one of the five experimental groups: (1) CONT, control group, no chemoprevention; (2) MF, chemoprevention with metformin; (3) MEL, chemoprevention with melatonin; (4) MF + MEL, chemoprevention with combination of metformin and melatonin; and (5) INT, intact group, no intervention. The number of animals in each experimental group was 18, except for intact group with 10 animals. The animals were weighed and palpated each week, and the presence, number, location, and size of palpable tumors were registered. Basic parameters of tumor growth, tumor incidence (the percentage of tumor-bearing animals per group), latency period (the period from carcinogen administration to the appearance of the first palpable tumor), tumor frequency (the average tumor number per group) and tumor volume (average and cumulative), were evaluated in each group. Intake of food and water over the 24-h period was monitored at weeks 4, 9, and 14 of the experiment (dated from carcinogen administration). The average daily intake of MF ranged from 27 to 29 mg/rat. The average daily intake of MEL ranged from 0.43 to 0.49 mg rat. After the termination of the experiment, the rats were sacrificed by quick decapitation, mammary tumors were excised, and tumor samples were taken for further analysis. Blood was collected and centrifuged and serum samples were stored at −80 °C until evaluation. IGF-1 concentration was determined in serum using a commercial ELISA (Cat. No. CSB-E04582 r; Cusabio, Wuhan, Hubei Province, P.R. China).

2.3. Histopathological and immunohistochemical analysis of mammary tumors

A sample of each mammary tumor was fixed in 10% formalin, paraffin-embedded, and stained by hematoxylin-eosin. Histopathological classification of mammary tumors was performed according to Russo and Russo [36]. In invasive carcinomas, the grade was determined according to our modifications of the grading system described previously [37,38]. Briefly, the resulting grade was determined as assessment of 4 following parameters: solid growth pattern (if more than 30% of the tumor showed this pattern), high mitotic activity index (10 or more mitoses per 10 high-power field), occurrence of necrosis (comedonecrosis, not infarct necrosis), and the presence of cellular atypia. Tumors with two and more positive criteria were classified as HG carcinomas. LG carcinomas were tumors with one or no positive criterion. Typing and grading of all tumors were performed by two independent experienced pathologists (assoc. prof. Karol Kajo, prof. Marián Adamkov). Paraffin blocks with the most representative tumor area were selected for immunohistochemical analysis. A total of 16–18 specimens from each group chosen according to findings in the HG/LG ratio (and having the largest representation of vital tumor epithelial component, i.e. without regressive changes such as extensive necrosis) were analyzed. Ki67 (proliferation marker), cleaved caspase-3 and BCL-2 (apoptosis markers) expressions were evaluated. An analysis of

correlation between tumor grade and Ki67 expression was performed. Semiquantitative immunohistochemical analysis was performed, as described previously [38–40]. For detection, a monoclonal Ki67 antibody (Cat. No. M7248; Dako, Glostrup, Denmark), polyclonal caspase-3 antibody (Cat. No. ab2302; Abcam, Cambridge, MA, USA) and BCL-2 antibody (Cat. No. sc-492; Santa Cruz Biotechnology, Paso Robles, CA, USA) were used. All steps of the immunohistochemical staining were processed according to the manufacturers' recommendations as we described previously [37,38]. The primary antibodies were visualized by a secondary staining system (EnVision, Dual Link System-HRP, cat. no. K060911, Dako North America, Carpinteria, CA, USA) using diaminobenzidine tetrahydrochloride (DAB) as a substrate. Negative controls included omission of primary antibody. A morphometric method was used for evaluation of antigens detected by immunohistochemistry. The sections were screened and digital images (400x magnification) were taken with Olympus Evolt E-420 installed in an Olympus BX41 N microscope. Expression of Ki67 was detected in the nucleus, caspase-3 was analyzed in the cytoplasm and BCL-2 was detected as membrane-associated cytoplasmic protein. Protein expression was quantified as the average percentage of antigen positive area in standard fields (0.5655 mm^2) of tumor hotspot areas. Digital images were analyzed using QuickPhoto Micro software, version 2.3 (Promicra, Prague, Czech Republic). Phase analysis of positive immunoreactivity was performed to determine the antigen positive area. The values were compared between treated and nontreated (control) tumor cells of female rats. Seventy-five up to eighty images for one protein were analyzed. Morphometric analyses were performed by two independent investigators (prof. Marián Adamkov, assoc. prof. Peter Kubatka).

2.4. Statistical analysis

Total tumor incidence and HG tumor incidence were evaluated by the Mann-Whitney *U* test. Other parameters were evaluated using the Kruskal-Wallis test or one-way analysis of variance, respectively. Statistical analysis was performed using GraphPad Prism, version 5.01 (GraphPad Software, Inc., CA, USA). The results were considered statistically significant at $P < 0.05$. Tumor volume was calculated according to the formula: $V = \pi.S_1^2.S_2/12$; S_1 and S_2 are tumor diameters; $S_1 < S_2$ [41].

3. Results

During the experiment, the body weight gains in all groups with induced tumors were lower compared to the intact group, lower body weight gain was also recorded in groups with MEL administration compared to the CONT group between weeks 3–10 after the tumor induction. Water intake was slightly higher in the MEL group compared to the MF group in the second half of the experiment, but no other differences were recorded, and food intake did not differ among groups (data not shown).

Chemopreventives did not significantly decrease mammary tumor incidence and frequency although lower incidence was recorded in the MEL group during the whole experiment (Fig. 1, Table 1). A slight non-significant delay in tumor latency (8 or 9%, respectively) was detected in all groups with chemoprevention. MF prominently reduced tumor burden (by 54 and 51%, respectively, as cumulative and average tumor volume/rat) although due to sporadic large tumors it did not reach the statistical significance. Only slight decrease in cumulative and average tumor volume was recorded in the MEL group (by 10% and 11%, respectively) and in the MF + MEL group (by 9% and 34%, respectively). Tumor induction increased serum IGF-1 level in the CONT group compared to the INT group, chemopreventives decreased serum IGF-1 to the level of intact rats (Table 1).

Histopathological evaluation showed that cribriform and papillary carcinomas prevailed in all groups except of MF group, where papillary and cribriform carcinomas were the most abundant (prevailing tumor characteristic is the first in order) (Table 2, Fig. 2). MF and MEL administered alone increased proportion of LG tumors versus HG tumors as HG/LG tumor ratio decreased significantly (by 58% and 64%, respectively), but combination of both substances had no such effect (Table 2). Improvement of histopathological profile in the MF and MEL group was probably related to the decreased proliferation: significant positive correlations between histological grade and Ki67 expression in the MF group ($r = 0.313$; $P = 0.0491$) and MEL group ($r = 0.318$; $P = 0.0457$) were observed although Ki67 expression significantly decreased only in the MEL group (Fig.3). Immunohistochemical analysis did not prove changes in expressions of caspase-3 and BCL-2 (Figs. 3, 4). Altogether, MEL showed slightly better effect than MF in terms of histopathological outcome improvement.

At necropsy, one animal from the MEL group showed hepatomegaly, splenomegaly was observed in three animals from the CONT and MEL group, in one animal from the MF group and in two animals from the MF + MEL group. Other macroscopic organ changes were not found.

4. Discussion

A controversy regarding MF ability to inhibit breast cancer and other cancers in nondiabetic subjects persists. An extensive research done particularly by Anisimov group showed that MF inhibits growth of spontaneous and transplantable mammary tumors in mice [42–45] but not all authors confirmed this [8]. Results of studies carried out in models of chemically-induced mammary carcinogenesis are not consistent either.

In the present work, we evaluated the effect of MF in the NMU model of rat mammary cancer. MF was administered in a preventive-curative manner, in a dose within the range of human therapeutic dose (approximately 860 mg/m^2 in a 200 g rat with a body surface of 0.0325m^2). MF did not significantly change tumor growth parameters but reduced proportion of aggressive, fast growing HG tumors. Although histological grade positively correlated with Ki67 expression, staining for Ki67 and also for apoptotic markers (caspase-3, BCL-2) did not prove any changes. Similarly, in our recent study, using the same experimental protocol of chemoprevention in the DMBA model, MF had no significant effect on proliferation and apoptosis despite decrease in cumulative tumor volume [34].

It is well known that insulin-like signaling systems control cell proliferation and survival [46]. High serum concentrations of IGF1 are associated with an increased risk of breast and other cancers [47]. Reduction in circulating levels of insulin/IGF1 in animal models by MF may contribute to its ability to decrease cancer incidence [48]. Serum IGF1 concentration was increased in control rats, MF reduced it to the level of intact rats. Reduction of circulating levels of IGF-1 in non-diabetic breast cancer women receiving hormonal therapy decreased the numbers of metastatic cases [17]. Reduction in circulating level of IGF1 may be relevant not only for mammary cancer but also for a variety of other forms of cancer [46].

Tumor enhancing properties of higher fat content may have interfered with MF effects in this study, but MF was not effective in the NMU model with a standard diet type in our previous work [49] and in studies of other authors [8,50] too. Zhu et al. [51] confirmed inhibitory effect of MF on tumor progression only at higher doses. The dose of MF that we used before [49] was probably too low (50 mg/kg corresponding to cca 400 mg/m^2), below the recommended human therapeutic dose (1700 mg corresponding to cca 940 mg/m^2) and, likewise, in above mentioned reports it did not exceed the maximum level of human therapeutic dose (2.5 g/day corresponding to cca 1500 mg/m^2). In the report by Checkley et al. [52], MF in a slightly higher dose than a maximal human therapeutic dose (2 mg/ml in drinking water) suppressed growth of NMU-induced tumors in rats fed a high-fat diet even when its administration was delayed until total tumor volume per rat reached ≥ 1.0 cm^3. The same group reported that MF in a rat model of postmenopausal breast cancer decreased the size of existing mammary

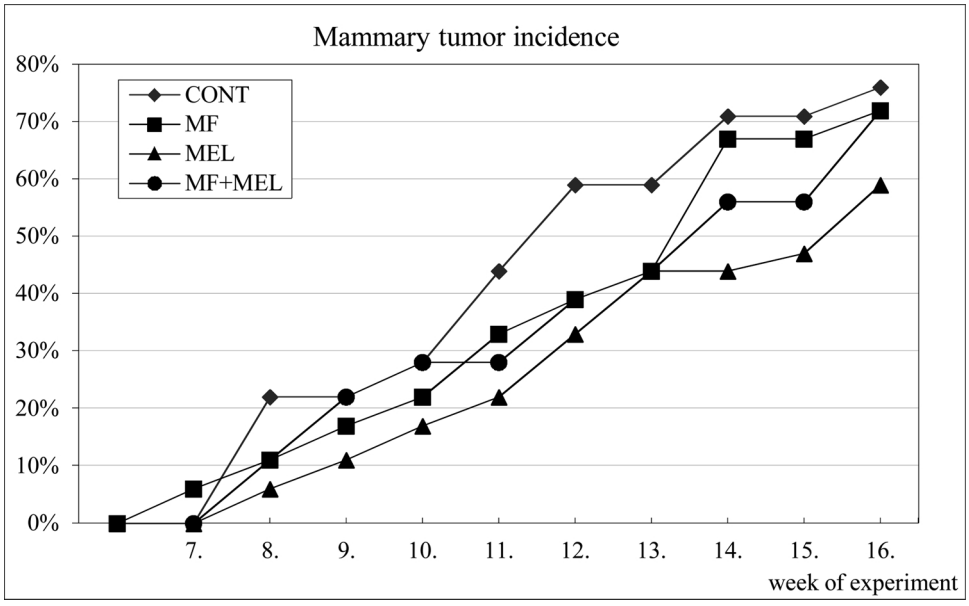

Fig. 1. Mammary tumor incidence.
CONT, control group with no chemoprevention; MF, chemoprevention with metformin; MEL, chemoprevention with melatonin; MF + MEL, chemoprevention with metformin and melatonin.

tumors and prevented the formation of new ones and had a positive effect on tumor microenvironment [53]. Immunohistochemical analysis did not show changes in proliferation and apoptosis after MF treatment in this and our previous experiment [34] either. Likewise, Ki67 staining was not changed in the study by Thompson et al. [8] but was decreased in the study by Checkley et al. [52].

There may be several reasons for weak effect of MF in carcinogenesis. Modulation of tumor progression may require activation of AMPK, a key mechanism that regulates cell proliferation. In a work of Zhu et al. [51], inhibition of mammary tumor growth was recorded only with concomitant activation of AMPK by higher doses of MF. The other factor may be the availability of organic cation transporters (OCT) in tumor cells [51,52] which are responsible for cellular uptake of MF. More precise analysis of tumor samples is needed to clarify how MF modulates tumor growth.

Considering numerous reports, and numerous ways how MEL may modulate carcinogenesis, its role in cancer prevention/inhibition seems more established. In this work we used the dose we found efficient in our previous experiments (0.43-0.49 mg/rat/day), corresponding roughly to 14 mg/m^2 which is about 5-times higher than a dose used for the treatment of disruptions of biological rhythms and sleep in humans (5 mg). MEL did not affect tumor growth parameters in a significant manner, but it decreased proliferation of cancer cells and reduced proportion of HG tumors. Serum IGF-1 dropped to the level of intact rats. MEL had similar effects in the DMBA model using a high-fat diet, it did not significantly alter mammary tumor growth parameters but it stimulated apoptosis in cancer cells [34].

Previously, we showed that MEL administered in the same experimental protocol using a standard diet type decreased both incidence and frequency in the DMBA [54] and NMU model [55–58] but not in all experiments [58–60]. Similarly, contradictory results were reported by other authors. MEL administered in the very similar dose (0.5 mg/rat/day) for the period of 9–22 weeks after tumor initiation, decreased both incidence and frequency of mammary tumors in the NMU model [61,62] and the tumor size in the DMBA model [63] but not in the report by Teplitzky et al. [64].

An ideal dose of MEL for tumor inhibition remains unknown. Early *in vitro* results indicated that only physiological doses are efficient [65] but in some mammary cancer lines only pharmacological concentrations suppressed the growth and induced the apoptosis of cancer cells [22]. Most *in vivo* studies used doses that can be regarded as supraphysiological. In a study by Anisimov et al. [66] MEL at a dose of 2 mg/L (in drinking water, cca 0.3 mg/kg/day) decreased the incidence of spontaneous mammary tumors in female SHR mice, higher dose (20 mg/L, cca 3 mg/kg/day) was ineffective and even increased the rate of spontaneous tumors in female CBA mice [67]. MEL (200 mg/L corresponding to 10 mg/kg) did not suppress the growth of transplantable HER2-positive breast tumors in female FVB/N mice either [43]. On the other hand, MEL at 50–200 mg/kg inhibited growth of mammary tumors in TG.NK transgenic mice with a dose-related statistically

Table 1
Mammary tumor growth parameters. Data are expressed as means ± S.E.M or total numbers. Values in parentheses are calculated as percentual deviation from the 100% of the noninfluenced control group. CONT, control group with no chemoprevention; MF, chemoprevention with metformin; MEL, chemoprevention with melatonin; MF + MEL, chemoprevention with metformin and melatonin; INT, intact group. Significant group difference versus INT: * ($P < 0.05$).

Experimental group	CONT	MF	MEL	MF + MEL
Incidence (%)	76%	72% (-5 %)	59% (-22 %)	72% (-5 %)
Non-tumor bearing rats	4 (out of 17)	5 (out of 18)	7 (out of 17)	5 (out of 18)
Latency period (days)	71.2 ± 4.98	77.0 ± 5.34 (+8 %)	77.9 ± 5.82 (+9 %)	77.6 ± 5.47 (+9 %)
Frequency	1.94 ± 0.489	1.83 ± 0.556 (-6 %)	1.94 ± 0.481	2.67 ± 0.577 (+38 %)
Cumulative tumor volume/rat (cm^3)	2.22 ± 0.741	1.03 ± 0.389 (-54%)	1.99 ± 0.882 (-10%)	2.01 ± 0.631 (-9%)
Average tumor volume/rat (cm^3)	1.14 ± 0.345	0.564 ± 0.163 (-51 %)	1.02 ± 0.388 (-11 %)	0.752 ± 0.217 (-34 %)
Serum IGF-1 (INT: 210 ± 14.7) (ng/ml)	305 ± 18.2 *	254 ± 17.9	281 ± 24.2	219 ± 22.3

Table 2
Histopathological profile of mammary tumors. Data are expressed as total numbers. Values in parentheses are calculated as percentual deviation from the 100% of the noninfluenced control group. CONT, control group with no chemoprevention; MF, chemoprevention with metformin; MEL, chemoprevention with melatonin; MF + MEL, chemoprevention with metformin and melatonin; HG, high-grade carcinoma; LG, low-grade carcinoma. Significant group differences are designated as follows: versus CONT: [a] ($P < 0.05$); versus MF: [b] ($P < 0.05$); versus MEL: [c] ($P < 0.05$).

	CONT		MF		MEL		MF + MEL	
Malignant tumors	HG	LG	HG	LG	HG	LG	HG	LG
Cribriform carcinoma	6	6		1	1	5	8	3
Papillary carcinoma	1		1					
Cribriform and papillary carcinoma	5	7		2	2	12	6	13
Cribriform and comedo carcinoma					1			
Cribriform, papillary and comedo carcinoma	1				1			
Papillary and cribriform carcinoma	1	4	6	21		7	3	8
Papillary, cribriform and comedo carcinoma					1			
Papillary and tubular carcinoma		2						
Tubular carcinoma		1						1
HG/LG ratio	14/20		7/24 [a] (-58%)		6/24 [a] (-64%)		17/25 [b, c] (-3%)	
Other lesions								
Intraductal proliferations			1					

significant negative trend for the incidence [68]. A positive dose-dependent effect of long-term MEL administration (1, 3 and 10 mg/kg) on DMBA-induced mammary tumors was reported by De Jonage-Canonico et al. [69]. In the DMBA model, higher dose of MEL (5 mg/day/rat) administered in a preventive-curative manner increased tumor latency and survival [70]. Similarly, MEL (10 mg/kg daily) inhibited incidence and frequency of rat mammary adenocarcinomas induced by DMBA in both short-term preventive administration and long-term curative administration [71]. In our previous research in the same animal model, a high dose of MEL (100 mg/L in drinking water) decreased frequency of mammary tumors [72]. Altogether, animal studies differ greatly in experimental protocol and the results are not unambiguous. Thus, no clear conclusion regarding the optimal dose of MEL can be made so far.

Most unexpectedly, the combination of both substances failed to exert a positive effect on tumor growth, histopathological profile or markers of proliferation and apoptosis, unlike our previous work where MF and MEL combination inhibited tumor progression in the DMBA model by apoptosis stimulation. NMU-induced carcinomas, though, show higher aggressiveness when compared with the DMBA-induced ones, and consequently a worse response to the therapy [73]. This was also seen in the present and our previous study after evaluation of grading as an additional parameter of tumor growth and morphology [34]. Correlation analysis may elucidate these differences in the future, but so far, the samples are relatively too small to make a solid conclusion. Differences in pharmacokinetics between these two models should be considered too.

The combination of MF (0.5 g/L) and MEL (2 mg/L) inhibited Ehrlich tumor growth in mice but had no effect on spontaneous mammary tumors [74]. Higher dose of MF (2 g/L) and MEL (200 mg/L) increased the cytotoxicity of paclitaxel on transplantable mammary

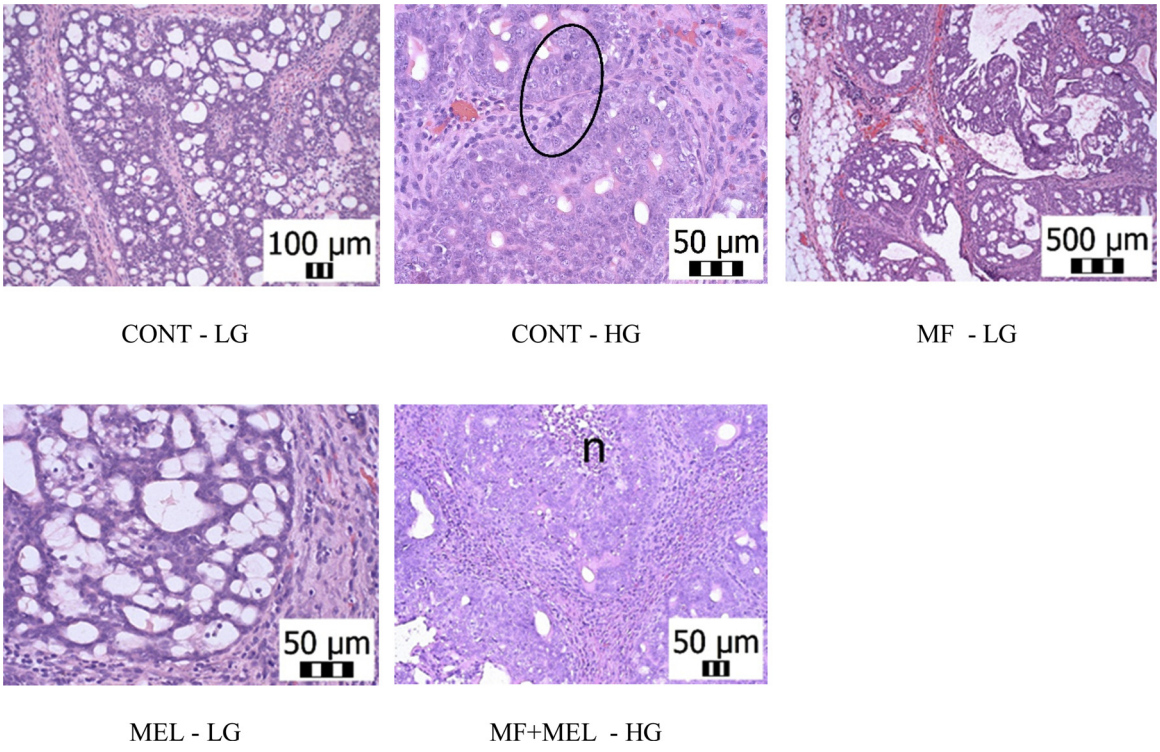

Fig. 2. Representative pictures of mammary tumors.
In the CONT group, a typical LG carcinoma shows retained glandularity with cribriform formations (CONT - LG), HG carcinoma shows reduction of glandular structures, with tendency to solid growth and increased mitotic activity (indicated within elipse, CONT - HG). In the MF group, LG carcinoma with predominant papillary and cribriform growth pattern is shown (MF - LG). Predominant cribriform LG carcinoma from the MEL group is shown (MEL - LG). Cribriform HG carcinoma from the MF + MEL group shows solidization and comedo necrosis indicated as n (MF + MEL - LG). The specimens were stained with hematoxylin and eosin.
CONT, control group with no chemoprevention; MF, chemoprevention with metformin; MEL, chemoprevention with melatonin; MF + MEL, chemoprevention with metformin and melatonin; HG, high-grade carcinoma; LG, low-grade carcinoma; n, necrosis.

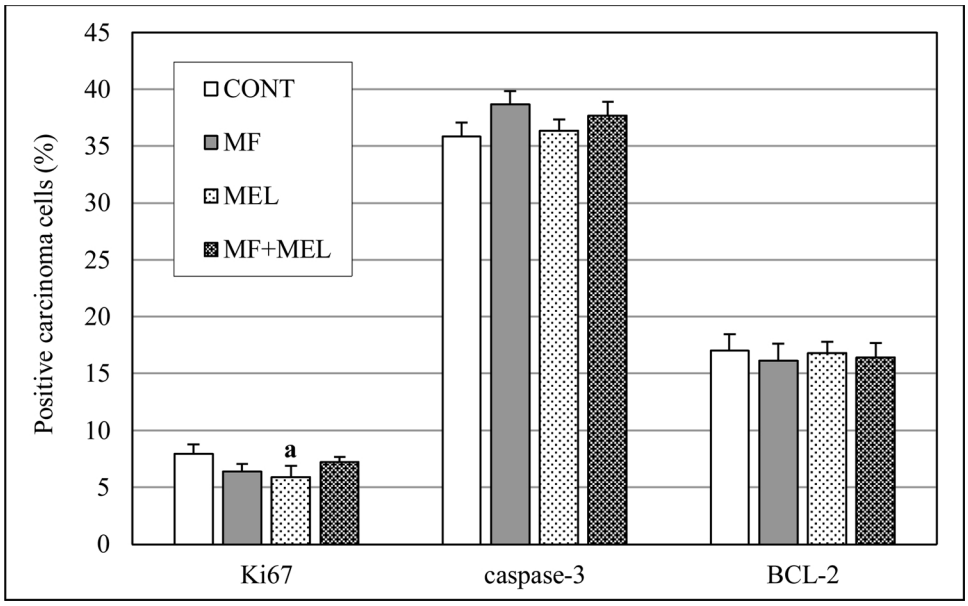

Fig. 3. Immunohistochemical analysis of Ki67, caspase-3 and BCL-2 expressions in mammary carcinoma cells.
Data are expressed as means ± S.E.M. CONT, control group with no chemoprevention; MF, chemoprevention with metformin; MEL, chemoprevention with melatonin; MF + MEL, chemoprevention with metformin and melatonin.
Significant group difference versus CONT is designated as **a** ($P < 0.05$).

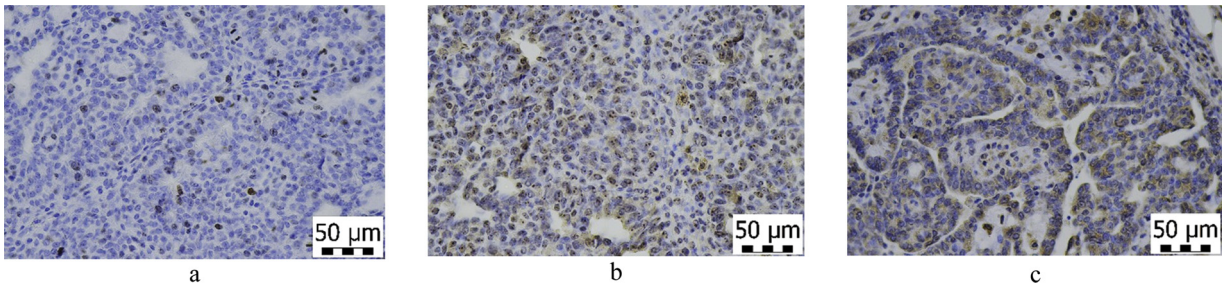

Fig. 4. Expression patterns of Ki67 (a), caspase-3 (b) and BCL-2 (c) in mammary tumor cells.
Representative images of IHC sections are shown (400x magnification).

tumors in mice [43]. We found no other reports on MF and MEL combination on mammary cancer progression. Further studies are needed to clarify the combined potential of these two drugs.

Nevertheless, there is solid evidence that MEL potentiates the effects of chemopreventives/chemotherapeutics. In animal models of breast cancer, MEL enhanced the effects of retinoids [55,64,75,76], non-steroidal anti-inflammatory drugs [54], statins [77,78], natural substances [60,68] and paclitaxel [43]. Clinical results are limited, but an extensive research of Lissoni group showed that MEL decreased toxicity and increased efficacy of chemotherapeutics in patients with solid tumors and improved overall life quality [79–81]. Preliminary results in limited number of patients with breast cancer showed that low doses of cyclophosphamide combined with biological multimodal treatment which included MEL (known as Di Bella Method), showed positive results in terms of efficacy and survival, with a favorable profile of tolerability [82].

5. Conclusions

Our results did not confirm significant inhibitory effects of MF and MEL on parameters of mammary tumor growth. However, single administration of these substances had a positive impact on histopathological profile in terms of reducing proportion of aggressive, fast growing tumors. Surprisingly, this effect was not seen after combined treatment. Data from animal studies indicate that inhibition of malignant transformation requires higher doses of MF than those used for diabetes treatment in humans, and similarly, supraphysiological doses of MEL seem to be efficient [43,74]. Further, well-controlled studies are needed to find the optimal dose of both substances in cancer prevention/treatment.

Ethical approval

Animals were treated in accordance with the principles established in the Law No. 377/2012 and 436/2012 of Slovak Republic for the Care and Use of Laboratory Animals. The experiment was approved by the State Veterinary and Food Administration of the Slovak Republic (accreditation No. Ro. 2054/13-221, 2368/12-221 and 2765/11-221/3).

Conflicts of interest

The authors declare that they have no conflict of interests.

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