A combination of resveratrol and melatonin exerts chemopreventive effects in *N*-methyl-*N*-nitrosourea-induced rat mammary carcinogenesis

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The neurohormone melatonin is primarily involved in the regulation of circadian rhythms, but also acts as an antioxidant and anticarcinogenic agent, especially in breast cancer. Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a widely known polyphenolic agent from red wine, which has been shown to exert antioxidant, anti-inflammatory and anticarcinogenic effects. The objective of this study was therefore to investigate the effects of melatonin in combination with resveratrol in a rat model of experimental mammary carcinogenesis. Female Sprague–Dawley rats aged 31 days were used in the experiment. Mammary carcinogenesis was induced by *N*-methyl-*N*-nitrosourea (NMU), which was administered in two intraperitoneal doses (50 mg/kg of body weight). Chemoprevention with resveratrol and melatonin started 2 weeks before the first dose of NMU and lasted until the end of the experiment. The basic parameters evaluated were: tumour incidence, latency period, tumour frequency per group and tumour volume. In addition, oestrogen receptors ERα and ERß, melatonin receptor MT1, proliferating cell nuclear antigen and vascular endothelial growth factor were determined by immunohistochemical staining. The combination of resveratrol and melatonin reduced tumour incidence by approximately 17% and significantly decreased the quantity of invasive and in-situ carcinomas. Food intake declined in the second and seventh weeks after the administration of carcinogen. Resveratrol in combination with melatonin returned food intake to the level of intact controls. Resveratrol in combination with melatonin has some protective effects on NMU-induced rodent breast cancer. Further studies are necessary to confirm these effects of this promising combination. *European Journal of Cancer Prevention* 21:163–170 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Breast cancer is the most frequently diagnosed neoplastic disease in women at the beginning of the 21st century. The majority of breast tumours, at least those at an early stage, express oestrogen receptors, where oestrogen stimulates cell proliferation and promotes tumour growth (Rice and Whitehead, 2006; Williams, 2010). Two oestrogen receptors (ERs), namely ERα and ERβ, encoded by different genes, have been identified. ERs belong to a family of proteins that function as ligand-activated transcription factors. The activation of ERα, for example, is known to promote the growth of breast tumours, whereas the specific function of ERβ is less understood, although in cell lines such as MDA-MB-231 (Lazennec *et al.*, 2001) or MCF-7 (Paruthiyil *et al.*, 2004), activation of ERβ inhibits cell proliferation. Furthermore, normal proliferating rat mammary epithelial cells rarely express ERα; whereas in 30–47% of proliferating epithelial cells, ERβ can be detected (Ali and Coombes, 2000). The differences in the terminal regions of ERs contribute to the cell and promoter differences in the transcriptional activity and their ability to respond to different ligands (Katzenellenbogen *et al.*, 2000).

The indolic hormone melatonin, produced mainly by the pineal gland, was shown to have an oncostatic role in breast cancer (Sanchez-Barcelo *et al.*, 2003; Hoang *et al.*, 2007). By suppression of ERα expression and behaving as a selective oestrogen receptor modulator (SERM), melatonin was shown to block ER-mediated cancer cell proliferation (Sanchez-Barcelo *et al.*, 2003; Hill *et al.*, 2009). Melatonin also exerts permissive effects on the oestrogen synthesis in the gonads and downregulates peripheral oestrogen-synthesizing enzymes, for example aromatase. Therefore it decreases the levels of circulating and locally produced oestrogens (Sanchez-Barcelo *et al.*, 2005).

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In studies of a rat model of experimental carcinogenesis using chemocarcinogens such as *N*-methyl-*N*-nitrosourea (NMU) or 7,12-dimethylbenz(a)anthracene (DMBA), melatonin has been shown to have preventive and curative effects on DMBA-induced mammary adenocarcinomas (Lenoir *et al.*, 2005) and application of the hormone increases the survival time of the animals (Saez *et al.*, 2005). Although the effects of melatonin at physiological levels are mediated through activation of G-protein-coupled melatonin membrane receptors, for example MT_1 found in breast cancer (Ekmekcioglu, 2006; Hill *et al.*, 2009; Rogelsperger *et al.*, 2009), at pharmacological doses, direct antioxidative effects of melatonin may block breast cancer cell growth (Reiter *et al.*, 2009).

Another important antioxidative agent effective in inhibiting the growth of experimental mammary tumours is resveratrol, a naturally occurring polyphenolic compound present in grapes, berries, peanuts and red wine. In addition, resveratrol sensitizes cancer cell lines to different chemotherapeutic drugs as well as radiotherapy (Hsieh, 2009; Niu *et al.*, 2011). The growth-inhibitory effects of resveratrol are mediated through cell-cycle arrest, caused by the upregulation of cell cycle determinants including p21Cip1/WAF1 and p53 and downregulation of survivin, cyclins D1 and E, respectively. Furthermore, resveratrol was shown to exert antiangiogenic effects through the inhibition of vascular endothelial growth factor (VEGF) synthesis and secretion (Trapp *et al.*, 2010).

In a rat model of experimental carcinogenesis, it has been shown that resveratrol at a concentration of 100 μg/kg suppressed DMBA-induced mammary carcinogenesis presumably through its antiproliferative effects (Banerjee *et al.*, 2002). Furthermore, resveratrol was shown to activate the transcription of oestrogen-regulated genes by binding to ERα and also to ERβ, but with comparable or slightly higher affinity than 17β-oestradiol (Bowers *et al.*, 2000; Garvin *et al.*, 2006).

Furthermore, studies in breast cancer cell lines provided evidence that resveratrol inhibits the growth of both ER-positive (MCF-7; Goswami and Das, 2009) and ER-negative (MCF-10; Lu *et al.*, 2008) breast cancer cell lines through an induction of apoptosis dose dependently. At concentrations in the lower μmol/l range, resveratrol stimulates the growth of MDA-MB-435 human cancer cells (Fukui *et al.*, 2010) and ERα-positive breast cancer lines (Bhat *et al.*, 2001) in the absence of 17β-oestradiol (E2), whereas in the presence of E2, resveratrol has been shown to inhibit oestradiol stimulated growth (Garvin *et al.*, 2006). At higher concentrations, it inhibited the growth of both ERα-positive and ERα-negative cell lines.

As melatonin and resveratrol have been shown separately to exert anticarcinogenic effects, especially in breast cancer, the objective of this study was specifically to investigate whether a combination of both agents may be beneficial for mammary carcinogenesis.

Materials and methods

Animals

Ninety-six female Sprague–Dawley rats were obtained from AnLab (Prag, Czech Republic). Rats aged 30 days and weighing 100–130 g were used in the experiment. The animals were adapted to standard vivarium conditions with a temperature of 22–24°C, relative humidity of 50–60%, and artificial regimen light:dark equal to 12 : 12 h, with lights on from 7 : 00 h (light intensity 150 lux per cage). During the experiment the rats were fed the Ssniff food in pellets (Soest, Germany). Resveratrol (gift from Department of Clinical Pharmacy, University of Vienna) was compressed into pellets in a concentration of 100 mg/kg and was administered *ad libitum*. Melatonin (Sigma, Diesenhofen, Germany) was administered in drinking water from 15 : 00 h to 8 : 00 h at a concentration of 20 mg/l. For the remainder of the day, the rats drank only tap water *ad libitum*. The experiment was conducted according to the principles provided in Law No. 23/2009 of the Slovak Republic for the Care and Use of Laboratory Animals.

Induction of mammary carcinogenesis with *N*-methyl-*N*-nitrosourea

Mammary carcinogenesis was initiated with two intraperitoneal doses (50 mg/kg of body weight each) of NMU (Sigma, Deisenhofen, Germany; Bojkova *et al.*, 2010). The first dose was injected on the 43rd postnatal day and the second one on the 50th postnatal day (Fig. 1).

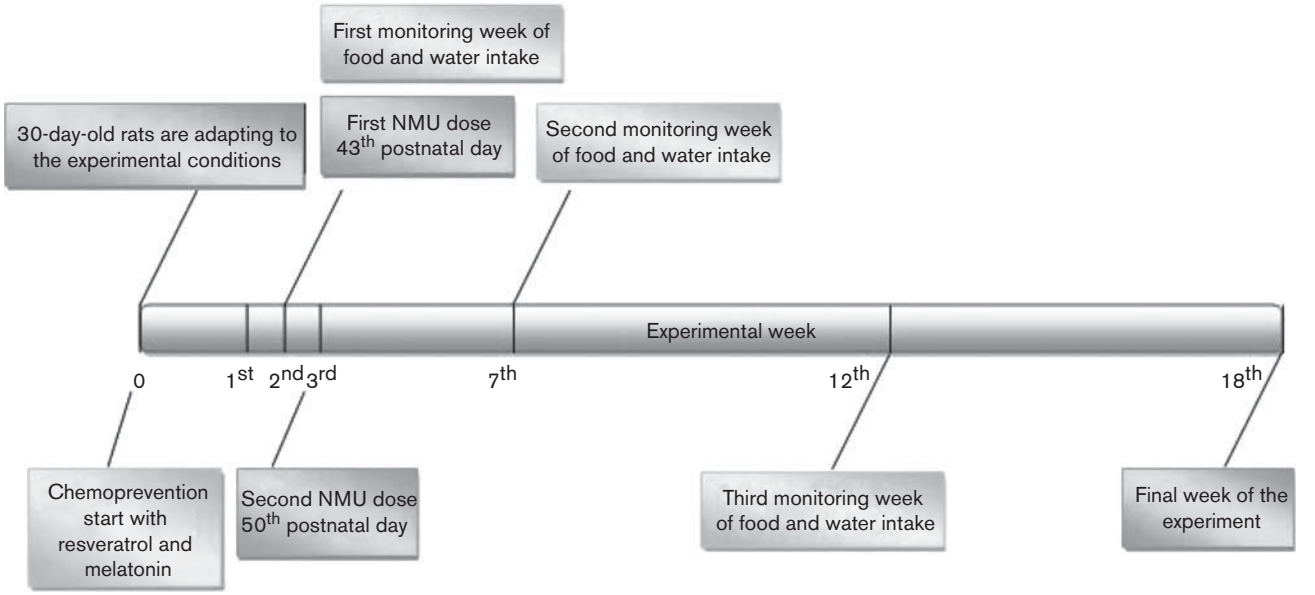
Experimental design

Chemoprevention with resveratrol and melatonin started 2 weeks before the first dose of NMU and lasted until the end of the experiment (18 weeks). Animals were divided into five groups: (a) chemoprevention only with resveratrol, (b) chemoprevention with melatonin, (c) combined chemoprevention with resveratrol and melatonin, (d) control group without chemoprevention (NMU) and (e) intact group composed of rats without carcinogenesis initiation and treatment.

Once a week, rats were weighed and palpated to register the presence, number, location and size of each palpable tumour; in the second, seventh and 12th weeks of the experiment, the food and water intake were also monitored.

At the end of the experiment, after overnight fasting, animals were killed by quick decapitation between 08 : 00 and 11 : 00 h, mammary tumours were excised and tumour size was recorded. The basic parameters evaluated were: tumour incidence (% of tumour-bearing animals in every group), latency period (day when the first tumour appeared), tumour frequency per group (the average tumour number per group) and tumour volume [calculated according to the formula $V = \pi \times (S_1)^2 \times S_2/12$; $S_1 < S_2$, where S_1 and S_2 are tumour diameters].

Fig. 1



Experimental design. The experiment lasted 18 weeks. Thirty-day-old rats were adapted to the standard vivarium conditions for a week. At the same time, 2 weeks before the first chemocarcinogen dose, the chemoprevention started, and lasted until the end of the experiment. In the second, seventh and 12th weeks, the food and water intake were monitored.

Enzyme-linked immunosorbent assay

Melatonin concentrations in blood plasma were measured using the competitive immunoassay with a capture antibody technique (Bühlmann, Allschwil, Switzerland). Frozen plasma samples were thawed and diluted 1 : 1 with the bidestilled water. After an overnight incubation with an antimelatonin antibody (polyclonal Kennaway G280, component of the ELISA Kit), the biotin conjugate, was added. After washing, the enzyme label streptavidin conjugated to horseradish peroxidase (HRP) was added. After 1 h incubation, the samples were washed and tetramethylbenzidine substrate was mixed in. Thirty minutes of incubation was followed by the addition of the Stop solution and measurement of the absorbence at the 450 nm.

Detection of ERα, ERβ, MT1, proliferating cell nuclear antigen and vascular endothelial growth factor proteins by immunohistochemistry

From tissue blocks with breast carcinoma and the surrounding nonmalignant resection margin, 4-μm sections were prepared. Sections were deparaffinized in xylene; and for immunohistochemistry, the endogenous enzyme activity was blocked with 3% hydrogen peroxide in methanol. To detect intracellular antigen, a permeabilization step with 0.1% Tween-20 in phosphate-buffered serum was carried out before antigen retrieval was performed using the microwave technique in citrate buffer, followed by blocking with 10% bovine serum albumin in phosphate-buffered serum. Thereafter, sections were incubated with the primary antibodies [ERα ab2746; ERβ H-150: sc-8974; Mel-1A-R (R-18): sc-13186; VEGF: sc-507; Santa Cruz Biotechnology Inc. (Heidelberg, Germany, Europe), PCNA: clone PC10, DakoCytomation)] overnight in a humidified chamber. After washing, secondary antibodies (HRP Rabbit/Mouse, HRP Goat, Dako Envision) were labelled with peroxidase. Antigens were visualized using 3,3-diaminobenzidine chromogen. Cell nuclei were counterstained with haematoxylin. Sections were viewed in an Axioplan 2 microscope (Carl Zeiss, Jena, Germany).

The evaluation of tumour slides

The evaluation of immunohistological and immunofluorescence slides was carried out according to the four classes of the Breslow scale. Counting of at least 500 cells using high-power fields (40 × objective lens) was conducted after scanning of the whole section at medium magnification. The positivity of the sections was evaluated as the percentage of the positive brown-stained cells. Results were evaluated using the semiquantitative immunoreactive score (IRS) technique of Remmele and Stegner (1987) (Table 1).

Statistical analysis

All data in tables are presented as mean ± standard error of the mean. The data were evaluated using the GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, California, USA) analysis of variance. Differences among groups were considered significantly different at a P value of less than 0.05, respectively.

Table 1 Semiquantitative immunoreactive score scale according to Remmele and Stegner (1987)

A	
	0: no cells with positive reaction
	1: up to 10% cells with positive reaction
	2: 11–50% cells with positive reaction
	3: 51–80% cells with positive reaction
	4: >80% cells with positive reaction
B	
	0: no colour reaction
	1: low intensity of colour reaction
	2: average intensity of colour reaction
	3: intense colour reaction

No reaction: score 0 points; weak reaction: score 1–2 points; average intensity of the reaction: score 3–4 points; intense reaction: score 6–12 points.

Results

Food and water intake in the study groups

Female Sprague–Dawley rats were exposed to NMU and the effect of a treatment on food and water intake was monitored in the second and seventh weeks and in the 12th experimental week (Table 2). The chemocarcinogen NMU significantly reduced food intake from 20.54 ± 0.38 g to 18.8 ± 0.31 g and 18.80 ± 0.31–$17.05 + 0.37$ g per rat in the second and seventh weeks ($P = 0.01$ and 0.05), respectively, whereas no difference was seen in week 12 (18.52 ± 0.44 g and 17.28 ± 0.39 g, respectively).

In NMU-treated rats, in comparing the effect of melatonin (1.5–2 mg/rat/day), resveratrol (7 mg/kg of birth weight/day) and the addition of resveratrol to melatonin, we found that melatonin and resveratrol alone had no effect on the amount of ingested food, but resveratrol in combination with melatonin significantly increased food intake (Table 2). In the resveratrol/melatonin treated group, the food intake in weeks 2 and 7 was significantly higher than in the NMU group and reached values similar to that in non-treated control rats (intact group).

Water intake was not influenced by NMU treatment compared with the control during the period investigated. Daily water intake in the second week after NMU administration ranged from 29.19 to 34.13 ml and was not influenced by chemocarcinogen, although in the following weeks, water intake decreased in the resveratrol and resveratrol/melatonin groups. Rats treated with melatonin alone after NMU injection had the same water intake as untreated intact rats until the end of the experiment.

Melatonin levels

As melatonin was applied through the drinking water and to correct for the endogenous melatonin in blood, we analyzed melatonin levels in the serum. We found that either melatonin alone or the addition of resveratrol to melatonin significantly increased the concentration of melatonin in blood plasma compared with the NMU or resveratrol groups, respectively (Table 3).

Effects of melatonin, resveratrol and their combination on rat mammary tumorigenesis

At the end of the experiment, after 18 weeks, the NMU group had a cancer incidence of 94%. Although melatonin increased the tumour incidence by approximately 0.3% and resveratrol application did not influence the tumour incidence significantly, the addition of melatonin to the resveratrol-containing diet lowered tumour incidence by approximately 17–77.8%, but it did not reduce the average tumour volume in the different groups (mean, $2.99 \pm 0.71\,cm^3$ per rat). The latency period (ranging between 70 and 126 days, median 91 days) was approximately 7 days longer in the resveratrol group compared with the other groups (NMU = 84 days, resveratrol = 91 days).

Histopathology of breast tumours

All tumours observed were subjected to a histological examination of the tumour sections (Fig. 2). Most of the rats had more than one tumour in the mammary glands. On average, in the control group 3.11 ± 0.31 tumours per rat were observed, whereas in the melatonin, resveratrol and resveratrol/melatonin groups, the average was 3.57 ± 0.62, 2.42 ± 0.38 and 2.72 ± 0.64 tumours, respectively. In total, in the NMU group, 53 tumours; in the melatonin group, 62 tumours; in the resveratrol group, 45 tumours and in the combined resveratrol and melatonin group, 40 tumours were observed.

Histopathological inspection showed that in the NMU group, 36 tumours were invasive and 17 were in-situ (IS) carcinomas (Fig. 2). Importantly, in the resveratrol/melatonin group, we observed a significant decrease in the number of invasive carcinomas compared with the NMU group, whereas resveratrol alone had no effect and melatonin even showed a slightly higher number of invasive tumours.

In addition, the number of IS carcinomas was significantly reduced in the resveratrol/melatonin and resveratrol groups compared with the NMU group. Again, melatonin alone had no effect.

Evaluation of ERα, ER, MT1, proliferating cell nuclear antigen and vascular endothelial growth factor expression in breast cancer tissues

Immunohistochemical analysis for ERα and ERβ in seven tissue samples from each group showed a strong positivity for both receptors with an IRS between 9 and 12. The staining pattern presented in Figs 3 and 4 revealed an intensive immunoreactivity in the nuclei of tumour cells for ERα and ERβ, respectively. No differences in the IRS were seen with regard to the expression of both receptors in different tumours and the dietary supplements.

We also examined immunolocalization of the melatonin receptor MT1 in the tumour section (IRS 3–6). In the melatonin and resveratrol/melatonin groups, the staining

Table 2 Food and water intake in *N*-methyl-*N*-nitrosourea-treated rats during the melatonin, resveratrol and resveratrol plus melatonin-containing diet

	Week after *N*-methyl-*N*-nitrosourea administration	Resveratrol	Resveratrol and melatonin	Melatonin	*N*-methyl-*N*-nitrosourea	Intact
Food intake	2.0	18.93 ± 0.40*	20.63 ± 0.43‡	18.45 ± 0.22***	18.80 ± 0.31**	20.54 ± 0.38
	7.0	17.09 ± 0.39*	19.01 ± 0.39‡	16.68 ± 0.27**	17.05 ± 0.37*	18.48 ± 0.31
	12.0	17.18 ± 0.37	17.86 ± 0.41	17.19 ± 0.41	17.28 ± 0.39	18.52 ± 0.44
Water intake	2.0	31.33 ± 0.77	29.19 ± 1.35	33.56 ± 1.09	34.13 ± 1.35	33.56 ± 1.66
	7.0	28.75 ± 1.00*	28.00 ± 1.51**,†	31.75 ± 1.58	33.38 ± 1.24	34.63 ± 1.31
	12.0	27.06 ± 2.00§	30.19 ± 2.15†	31.13 ± 1.00	37.88 ± 2.13	32.38 ± 0.96

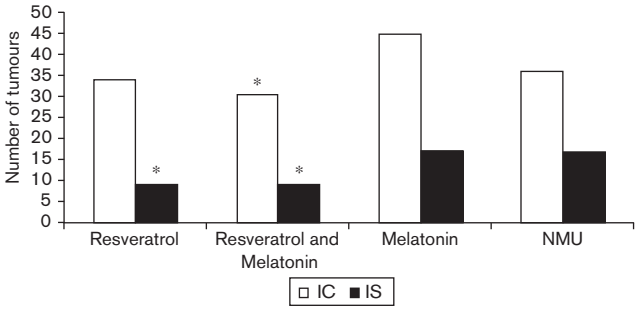
Data are expressed as g or ml/per rat (mean ± standard error of the mean).
Significance versus *N*-methyl-*N*-nitrosourea group (†$P<0.05$; ‡$P<0.01$ and §$P<0.001$). Significance versus untreated control group ('intact') is given by asterisks (*$P<0.05$; **$P<0.01$ and ***$P<0.001$).

Table 3 Melatonin concentration in blood plasma

N-methyl-*N*-nitrosourea	Melatonin
8.62 ± 0.50	12.88 ± 0.72***,†
Resveratrol	Resveratrol and melatonin
6.9 ± 1.08	14.58 ± 0.68***,‡

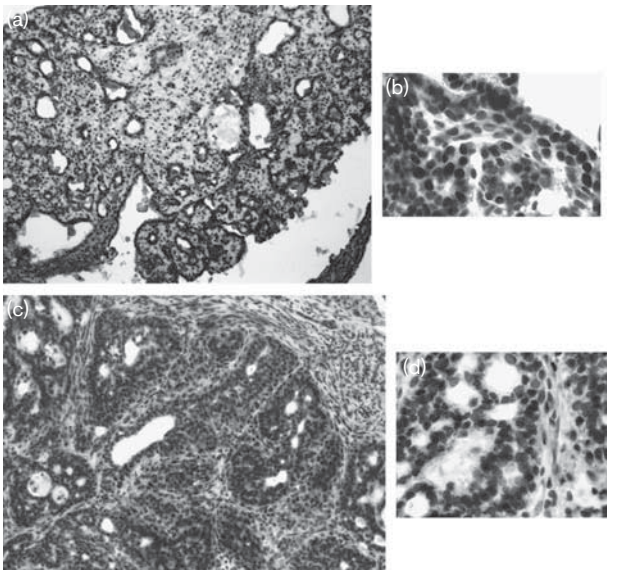
Melatonin (pg/ml) was measured in blood plasma at the end of the experiment using the ELISA method. Data are expressed as mean ± standard error of the mean.
Significance versus resveratrol (***$P<0.001$), versus *N*-methyl-*N*-nitrosourea group (†$P<0.01$ and ‡$P<0.001$).

Fig. 2



Histological analysis of mammary tumours. *Values are statistically different ($P<0.05$) compared with the *N*-methyl-*N*-nitrosourea (NMU) group. IC, invasive carcinomas; IS, in-situ carcinomas.

Fig. 3



Expression of oestrogen receptors, ERα and ERβ, in invasive cancerous tissue. Paraffin-embedded sections from breast tissue were stained for ERα (a,b) and ERβ (c,d) by immunohistology in the *N*-methyl-*N*-nitrosourea group. Brown ERs staining of milk ducts is shown. Magnification in (a and c): 10 ×, in (b and d): 40 ×.

intensity and number of stained cells was reduced by 10–20% revealing a lower IRS score of less than 5.

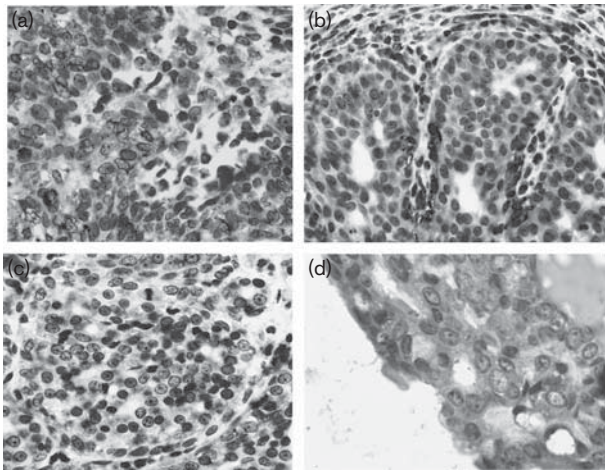
To investigate whether the dietary supplements would influence tumour cell proliferation, the PCNA, a cancer-associated marker of poor prognosis, was measured. However, our analysis showed a high IRS of 9–12 in all tumours, indicating tumours with a high rate of proliferation. This was also observed for VEGF, a marker for tumour angiogenesis (data not shown).

Discussion

We studied the chemopreventive effects of the pineal hormone melatonin and the phytoagent resveratrol, both showing potential anticancer therapeutic effects, in a rat model of chemical-induced breast cancer. Long-term administration of resveratrol and melatonin significantly decreased the number of invasive and IS carcinomas, slightly decreased the tumour incidence and prevented the decline in food intake after carcinogen application.

Resveratrol and melatonin are known as antioxidants that could exert growth-inhibitory and antimutagenic effects in breast cancer. Previous studies showed, for example, that resveratrol inhibits the growth of cancer cell lines *in vitro* and tumours *in vivo* when administered at relative high concentrations (Goldberg *et al.*, 2003; Whitsett *et al.*, 2006). However, lower concentrations could stimulate the growth of the tumours (Fukui *et al.*, 2010). In these cases, resveratrol shows weak oestrogenic activity and can, for example, stimulate the growth of ERα-positive MCF-7 human breast cancer cells *in vitro* (Levenson *et al.*, 2003). But the question is: Which dose is high enough to exert antioestrogenic effects? For example, concentrations of up to 40 mg/kg of body weight in rats seem to be

Fig. 4



Melatonin receptor (MT1) expression in breast tumours containing >10% MT1 receptor. Immunohistological staining in (a) *N*-methyl-*N*-nitrosourea, (b) resveratrol, (c) melatonin in combination with resveratrol and (d) melatonin groups is shown in specimens from invasive tumours of rats. Magnification in (a–d): 40 ×.

breast cancer promoting (Bove *et al.*, 2002; Fukui *et al.*, 2010), but when studying lung cancer, resveratrol in concentrations of between 5 and 25 mg/kg of birth weight suppressed the metastatic activity of the tumours (Busquets *et al.*, 2007) or reduced the tumour volume, tumour weight and metastasis occurrence in mice bearing Lewis lung carcinoma tumours at the concentrations of 2.5–10 mg/kg of birth weight [reviewed in Shankar *et al.* (2007)]. Some in-vitro studies suggest that concentrations less than 10 µmol/l are not high enough to suppress the growth of the cancer cell lines (Levenson *et al.*, 2003; Fukui *et al.*, 2010). However, Bhat *et al.* (2001) determined that resveratrol in concentrations of 1–2.5 µmol/l promoted the growth of the ER-positive breast cancer cell lines and that levels of 5–10 µmol/l suppressed the survival of the colonies.

Bowers *et al.* (2000) were able to observe the difference between the effectiveness of resveratrol at different concentrations in hormone-dependent and independent cell lines and also in *in vivo* experiments. Some studies indicate that ER-positive breast cancer cell lines are more sensitive to resveratrol than those that are ER negative. For example, by using the ERα-positive MCF-7 and ERα-negative MDA-MB-231 cell lines, it has been shown that resveratrol induced apoptotic cell death in MCF-7 but not in MDA-MB-231 at concentrations of 100–150 µmol/l (Pozo-Guisado *et al.*, 2004). Bowers *et al.* (2000) postulated that, depending on the tissue, resveratrol has mixed agonist/antagonist effects for ERα and ERβ. Resveratrol (500 µmol/l) was reported to induce higher oestrogen response element (ERE)-driven reporter activity by ERβ than any concentration of E_2 (0.01–1 mmol/l) examined in COS-1 cells. Resveratrol-liganded ERβ has higher transcriptional activity than E_2-liganded ERβ at a single palindromic ERE. This indicates that tissues expressing specifically ERβ could be more sensitive to the oestrogen agonist activity of resveratrol. Our findings indicate that even if the dosage of resveratrol was probably not high enough to influence the tumour incidence or tumour volume in the resveratrol-treated rat group, it may influence the ratio between invasive and IS carcinomas. Resveratrol slightly decreased the tumour frequency and nonsignificantly prolonged the latency period. The breast cancer tissue showed high expression of ERα and ERβ and the previously described mechanism probably influenced the breast tissue according to the agonistic activity of resveratrol.

Melatonin is an indolic hormone produced by a variety of plants and animals, especially mammals. The effects of melatonin in the carcinogenesis of breast cancer have been investigated in several studies (Saez *et al.*, 2005; Treeck *et al.*, 2006). Melatonin could, for example, increase the survival time of tumour-bearing animals, by bringing them to their optimal physiological status (Saez *et al.*, 2005). As reviewed in Sanchez-Barcelo *et al.* (2003), melatonin increased tumour latency, lowered tumour incidence, reduced the number and size of tumours and lowered the rate of tumour growth. However, in some studies, melatonin has only a very weak influence, no effect or even a cancer-promoting impact on tumour frequency or tumour volume in breast cancer (Bojkova *et al.*, 2000; Mocikova-Kalicka *et al.*, 2001). It could increase the tumour incidence when administered alone (Orendas *et al.*, 2009). No inhibitory effect was observed with 1 and 100 nmol/l of melatonin on the growth of breast and ovarian cancer cells in culture medium supplemented with 1 nmol/l 17-β oestradiol, irrespective of their ERα receptor status (Treeck *et al.*, 2006).

Physiological levels of melatonin mainly act after binding to G-protein-coupled melatonin receptors (Ekmekcioglu, 2006; Rogelsperger *et al.*, 2009). The subtype MT_1 is downregulated after treatment with SERM, 4-OH tamoxifen, in ERα-positive MCF-7 human breast cancer cell lines (Treeck *et al.*, 2006). The downregulation of MT1 receptors was also observed after estradiol treatment in ovarian membranes in Sprague–Dawley rats (Clemens *et al.*, 2001). Melatonin treated hormone-dependent tissues, such as breast or ovaries, seem to be influenced by the presence of oestradiol. Melatonin downregulates the synthesis of oestradiol and thereby reduces the transcription activity of the cells. It could inhibit the binding of oestradiol–ERα complex on the ERE domain in the DNA, and this is why it is called SERM (Sanchez-Barcelo *et al.*, 2005).

Although melatonin inhibits E2-ERα-induced transcription at several ERE-driven promoters, it does not inhibit (or even enhance) ERβ activation by treatment with oestradiol (Del Rio *et al.*, 2004).

We have investigated the effects of melatonin administered alone and in combination with resveratrol. Melatonin increased the tumour incidence by approximately 0.3% and tumour frequency by approximately 15%, and affected tumour volume. However, in combination with resveratrol, the tumour frequency was decreased. The most interesting result was a lowered tumour incidence of approximately 17%. The reason for the superior effect of resveratrol/melatonin is unknown. Homeostatic mechanisms, where positive effects of one substance are potentiated and negative effects suppressed by the other compound, may play a role. For example, in a recent study it was shown that melatonin synergistically increases resveratrol-induced heme oxygenase-1 expression, leading to higher neuroprotection in neurons (Kwon *et al.*, 2011). Furthermore, melatonin was found to be highly effective in reversing pro-oxidant DNA damage of low-dose resveratrol in purified calf thymus DNA (Lopez-Burillo *et al.*, 2003). Combination therapy also has the advantage that compounds acting through different mechanisms may be used in lower doses, and thus potential side effects can be reduced.

The relationship between melatonin doses used in our rats and those applied in humans seems to be correlative. The doses of melatonin administered in rats, ranging from 20 to 500 µg/day (i.e. 0.5–2.5 mg/kg of birth weight per day), correspond, for example, to the 10–40 mg daily dosage (i.e. 0.15–0.6 mg/kg of birth weight per day) of melatonin in patients with various advanced cancers shown to reduce the risk of death 1 year later (Mills *et al.*, 2005). This dose is almost 5-fold to 10-fold higher than that used for the treatment of insomnia or jet lag.

The effective dose of resveratrol for animals as well as humans has not yet been precisely established. As mentioned before, relatively high doses seem to have antitumorigenic effects, whereas small amounts could exert stimulatory effects on breast cancer cell lines. Therefore, the question as to the slender border between the doses has not yet been answered.

The method of administration of resveratrol and melatonin in our experiment essentially resembles the method of administration of both substances for humans. Resveratrol is currently found in every pharmacy in the form of tablets or capsules, injections or even natural creams. Melatonin is also available in the form of tablets or capsules, which is the most practical form of administration for humans.

In summary, the reported data demonstrate for the first time the effects of the resveratrol–melatonin combination in breast cancer *in vivo*. Administered alone, melatonin had no inhibitory effect on tumour incidence or tumour growth. However, long-term administration of resveratrol and melatonin significantly decreased the number of invasive and IS carcinomas, slightly decreased the tumour incidence and prevented food intake reduction after carcinogen application. Further studies are necessary to confirm these effects of this promising combination.

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Conflicts of interest

There are no conflicts of interest.

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