Melatonin enhanced bexarotene efficacy in experimental mammary carcinogenesis

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The aim of this paper was to test lower, safe bexarotene dose administered alone and in combination with melatonin to improve its efficacy. Mammary carcinogenesis was induced by N-methyl-N-nitrosourea in female Sprague-Dawley rats, administered in two doses intraperitoneally between 42.-54. postnatal days and chemoprevention was initiated 7 days prior to first N-methyl-N-nitrosourea injection and lasted 15 weeks. Bexarotene, particularly in combination with melatonin decreased mammary tumor incidence and frequency with a shift from poorly to well differentiated carcinomas. Bexarotene alleviated glycaemia and liver/heart muscle glycogen concentration decreased as well as liver/thymus malondialdehyde increased in comparison with control group.

The combination of bexarotene and melatonin is therefore beneficial in preventive-curative model of experimental mammary carcinogenesis and may be applied in oncological practice as such.

Key words: mammary carcinogenesis, female rats, bexarotene, melatonin, prevention

Retinoids act through two intracellular receptors of steroid receptor superfamily – retinoic acid receptors (RAR) and retinoid X receptors (RXR). RAR homodimers or RAR-RXR heterodimers are transcription factors controlling cell proliferation and differentiation. Furthermore, RAR-RXR heterodimers are ligands for numerous nuclear hormone receptors.

RAR ligands are known to suppress carcinogenesis: retinylacetate decreased tumor incidence in 7,12-dimethylbenz(a)anthracene (DMBA) – induced mammary carcinogenesis in female rats [1, 2], but had little efficacy in N-methyl-N-nitrosourea (NMU) model which, however, was improved with simultaneous melatonin (MEL) administration [3]. Another retinoid fenretinide (N-4-hydroxyphenylretinamide, 4-HPR) was effective in numerous experimental tumor model prevention, but Moon et al. [4] reported high toxicity of 4-HPR (frequent liver damage, high triacylglycerolemia and skin lesions). RXR agonists – rexinoids show similar efficacy in tumor prevention [5] and treatment [6]. Selective RXR agonist bexarotene (BEXA) is the most frequently used rexinoid in clinical practice [7]. Bexarotene exerts preventive and treatment effects through cell cycle inhibition, apoptosis and differentiation induction [8]. Its administration, both preclinical and clinical, is not without side effects, metabolic disturbances, particularly hypertriacylglycerolemia were reported [6].

Metabolic changes in tumor host do not reflect just metabolic alterations in tumor tissue; in experimental carcinogenesis metabolic alterations after carcinogen administration were recorded both in tumor-bearing and non-tumor bearing animals. Decrease of insulinemia (and glycaemia) is the hallmark of carbohydrate metabolism in cancer disease. The liver glycogen decreases and serum concentration of contrainsular hormones and IGF-1 increases. In addition carcinogen administration predominantly inhibits fatty acid oxidation and lipogenesis while lipolysis alterations are negligible [9,10]. Cancer progression is also associated with protein degradation

and cachexia [11]. Typically carcinogenesis is also accompanied by increase in reactive oxygen products formation with concomitant tissue antioxidative capacity decrease [14].

MEL is one of the oldest and most uniform molecules in living organisms with pleiotropic functions. MEL acts as potent antioxidant, regulates circadian rhythms and reproduction and contributes to general fitness. Reactive oxygen species (ROS) – scavenging abilities, antiinflammatory, proapoptotic, and antiangiogenic activities predestine MEL as "natural" oncostatic substance. MEL is effective particularly in colorectal and mammary carcinogenesis prevention. MEL inhibits experimental mammary carcinogenesis through various mechanisms including estrogen receptor modulation, prolactin antagonism and liver fatty acid metabolism inhibition [12,13]. In female rats, MEL inhibits mammary tumor growth alone and in combination with other substances in vivo [14]. MEL has also been used in adjuvant chemotherapy in some human neoplasms [15].

The aim of our work was to analyse the effect of relatively low BEXA dose administered alone and in combination with MEL in preventive-curative mammary carcinogenesis model in Sprague-Dawley rats. We decided to evaluate tumor growth and selected serum and tissue metabolic parameters.

Material and methods

Female rats of Sprague-Dawley strain obtained from Germany (distribution by An-Lab, Prague, Czech Republic) aged 33-35 days were adapted to standard vivarium conditions with temperature 23±2°C, relative humidity 60-70%, artificial regimen light:dark = 12:12h, with lights on from 7:00 h (light intensity 150 lux per cage).

The animals were fed Ssniff diet (Soest, Germany) and drank tap water *ad libitum*. Mammary carcinogenesis was induced by NMU (Sigma, Deisenhofen, Germany). NMU was administered intraperitoneally in two doses, each per 50mg/kg b.w., between 42th-54th postnatal days (with a seven-day interval between doses). NMU was freshly prepared by dissolving in isotonic saline solution (the average volume per animal was 0.5 ml). BEXA (Eisai Ltd., London, Great Britain) was administered per os, starting 7 days before and ending 15 weeks after the first NMU dose in a concentration of 26.7 mg/kg b.w. 3 days a week (Monday, Wednesday, Friday). MEL (Sigma, Deisenhofen, Germany) was applied daily, starting 7 days before and ending 15 weeks after the first NMU dose, in drinking water (20 μg/ml) from 15:00 to 8:00 (from 8:00 to 15:00 animals drank tap water without melatonin). The solution was freshly prepared three times a week. Twenty mg of the substance were diluted in 0.4 ml of 30 % ethanol and mixed up with tap water to the desired volume. The animals were divided into four groups: 1. CONT – control group, 17 animals without chemoprevention but with carcinogen administration, 2. BEXA – 17 animals with carcinogen administration and BEXA chemoprevention, 3. BEXA+MEL – 17 animals with carcinogen administration and BEXA and MEL chemopreven-

tion, 4. INT – 19 intact animals without chemoprevention and carcinogen administration. The animals were weekly weighed and investigated in order to record the incidence, number, location, and size of tumors. Food and water intake was recorded in 2nd, 4th, 7th, 9th, 11th, and 14th week of the experiment. In the last week of the experiment all animals were sacrificed by quick decapitation and final investigation was performed: inspection of body organs, mammary tumor excision and size measurement (length, width). Tumor samples were preserved in 10% formalin solution for histopathological investigation. The tumors were classified according to the criteria for the classification of rat mammary tumors [16] with an additional parameter – carcinoma differentiation grade. Malignant tumor samples were divided into low-grade (LG) and high-grade (HG) carcinomas. The criteria for categorization were chosen according to the standard diagnostic patterns and include: solidization, cell atypia, mitotic activity index, and necrosis.

As HG carcinomas were considered tumors with ≥2 positive criteria, LG carcinomas were tumors with ≤1 positive criterium. Positive solidization was considered if >30% of tumor sample displayed solid growth, high mitotic activity index was recorded when > 10 mitoses were observed in 10 high power fields and necrosis was determined by the presence of comedo (not infarct). Selected parameters of lipid and carbohydrate metabolism were analysed: serum concentration of triacylglycerols (TAG), cholesterol (CH), phospholipids (PL), corticosterone (CST), and glucose (GLU); liver concentration/content of TAG, CH, malondialdehyde (MDA, a lipid peroxidation marker), PL, and glycogen (GLY); heart muscle concentration/content of GLY, thymus concentration/content of MDA. The concentration of PL was determined from the lipid phosphorus by Bartlett's method [17], total CH according to Zlatkis et al. [18], GLY according to Roe and Dailey [19], MDA was determined using a reaction with thiobarbituric acid [20], TG and GLU concentrations were determined by commercial sets (Pliva-Lachema), CST was measured using fluorimetry according to Guillemin et al. [21]. Statistical evaluation of selected mammary carcinogenesis parameters, selected parameters of lipid and carbohydrate metabolism and body mass, food, and water intake were evaluated by a combination of non-parametric and parametric tests. Tumor incidence was evaluated by Mann-Whitney U–test, other parameters by one-way analysis of variance or Kruskal-Wallis test. Tumor volume was calculated according to the formula: V (mm³) = π x S_1^2 x S_2 / 12; (S_1<S_2), S1 and S2 are tumor diameters (S1 < S2).

Experiment was approved by the State Veterinary and Food Administration of Slovak Republic by accreditation No. Ro-2819/09-221.

Results

BEXA administration decreased mammary tumor incidence in preventive-curative experimental mammary carcinogenesis model but this was significant only in combination with melatonin (BEXA+MEL). BEXA alone and

in combination with MEL considerably decreased tumor frequency per group when compared to the CONT group (Tab 1). Total tumor number in the CONT group was 36 and decreased to 13 in the BEXA and to 8 in the BEXA+MEL groups. We observed mainly malignant tumors, particularly the cribriform and the cribriform-papillary type. In contrast, only two benign tumors were found (in the BEXA group). Markedly more heterogeneous spectrum of carcinomas (with occurrence of comedo type) in the control group compared to treated groups was observed. The presence of more homogeneous tumor types with dominat pure cribriform lesions in the group BEXA+MEL was found (Tab 2). Carcinomas in the control group were characterized by lower degree of differentiation (predominant high grade tumors) with the signs of solid growth, higher cellular atypia and mitotic activity. Detailed cytologic assessment of invasive lesions (poorly differentiated HG tumor or well-differentiated LG tumor) revealed favourable HG:LG ratio in the BEXA (4:7) and in the BEXA+MEL (3:5) groups in comparison with the CONT group (21:15). No animals died during the experiment and no macroscopic tissue lesions were found by autopsy.

Food intake between 2nd-9th week in the CONT group was decreased in comparison with the INT rats. BEXA administration (alone and in combination with MEL) only decreased food intake in 14th week and water intake only in 2nd week of experiment as compared to the CONT group. The body mass gain in the CONT group was lower in comparison with the INT group and was not altered by BEXA or BEXA+MEL administration (not shown).

While glycaemia was lower in the CONT group when compared to the INT, serum lipid parameters remained unaltered. Liver TAG and CH content in the CONT group was decreased with no effect of BEXA and BEXA+MEL administration. Liver PL content in the CONT group was lower and rose in groups with chemoprevention. Similarly heart muscle glycogen content in the CONT group was decreased and recovered with the use of chemopreventives. Lipid peroxidation in liver and thymus (measured by concentration of MDA) increased in the CONT group, BEXA administration decreased it which was more prominent in BEXA+MEL group where the MDA concentration/content did not differ from the values of the INT group (Tab 3-5).

Table 1. Effects of BEXA and BEXA+MEL on mammary carcinogenesis parameters.

Group	CONT	BEXA	BEXA+ MEL
	n=16	n=17	n=17
Tumor incidence (%)	75.0 ± 11.2	58.8±12.3 (-21.6)	**23.6±10.6** [aa](-68.5), [b][-59.9]
Tumor frequency per group	2.44 ±0.55	**0.76 ±0.18** [aa](-68.9)	**0.47 ±0.26** [aa](-80.7), [-38.2]
Tumor frequency per animal	3.25 ±0.55	**1.30 ±0.15** [aa](-60.0)	2.00 ±0.71 (-38.5), [+53.8]
Tumor volume (cm³)	1.20 ±0.35	1.52 ±0.61 (+26.7)	1.35 ±0.70 (+12.5), [-11.2]
Tumor latency (days)	86.32 ±2.94	91.54 ±3.91 (+6.1)	86.0 ±6.28 (-0.4), [-6.1]

Data are expressed as means±S.E.M
Significant difference between groups is designated as: [a] $P<0.05$ vs CONT; [aa] $P<0.01$ vs CONT , values in round brackets are calculated as %-ual deviation from the 100% of CONT group ; [b] $P< 0.05$ vs BEXA; [bb] $P< 0.01$ vs BEXA , values in square brackets are calculated as %-ual deviation from the 100% of BEXA group.
CONT – control group with NMU, **BEXA** – BEXA (26.7 ug/g b.w), **BEXA+MEL** – BEXA (26.7 ug/g b.w) with MEL (20 ug/ml drinking water), n=number of individuals

Table 2. Histopathological classification of mammary tumors

Malignant lesions – types	CONT	BEXA	BEXA+MEL	n
cribriform	11	2	5	(18)
cribriform and papillary carcinoma	10	5	1	(16)
papillary and cribriform carcinoma	6	3	1	(10)
papillary carcinoma	3	1	1	(5)
cribriform and comedo carcinoma	5	-	-	(5)
cribriform, papillary carcinoma and comedo	1	-	-	(1)
Number of malignant lesions	**36**	**11**	**8**	**(55)**
Forms: high to low grade ratio	21/15	4/7	3/5	
Benign tumors				
tubular- and fibroadenoma	-	1	-	(1)
fibrosarcoma	-	1	-	(1)
Number of benign tumors	-	**2**	-	**(2)**
Total number	**36**	**13**	**8**	**(57)**

The columns indicate the number of tumors in groups. The lines are the numbers of tumor types. Other details: see Tab. 1

Table 3. Effects of BEXA and BEXA+MEL on selected metabolic parameters in serum.

	INT	CONT	BEXA	BEXA+MEL
Serum				
GLU (mmol/l)	5.01±0.10	**3.09±0.13** aaa{-38.3}	3.43±0.14 (+11.0)	**4.33±0.14** ccc (+40.1), ddd [+26.2]
TAG (mmol/l)	0.56±0.04	0.51±0.03{-8.9}	0.54±0.043 (+5.9)	0.48±0.03 (-5.9), [-11.1]
CH (mmol/l)	3.34±0.19	3.49±0.25{+4.5}	3.65±0.17 (+4.6)	3.72±0.15 (+6.6), [+1.9]
PL (mmol/l)	1.94±0.12	1.70±0.22{-12.4}	1.72 ±0.11(+2.4)	1.84±0.11 (+8.2), [+5.7]
CTS (ng/ml)	182.05±34.36	193.21±21.70{+6.1}	246.40±25.50 (+27.5)	166.23±33.06 (-14.0), [-32.5]

Data are expressed as means±S.E.M. Significant difference between groups is designated as: **aaa** p < 0.001 (CONT vs INT), **ccc** p < 0.001 (BEXA + MEL vs CONT), **ddd** p < 0.001 (BEXA + MEL vs BEXA); values in curly brackets are calculated as %-ual deviation from the 100% of INT group; values in round brackets are calculated as %-ual deviation from the 100% of CONT group, values in square brackets are calculated as %-ual deviation from the 100% of BEXA group, GLU – glucose, TAG – triacylglycerols, CH – cholesterol, PL – phospholipids, CTS – corticosterone.

Table 4. Effects of BEXA and BEXA+MEL on selected metabolic parameters in liver.

	INT	CONT	BEXA	BEXA+MEL
Liver				
TAG (µmol/g)	18.47±0.91	15.51±1.50{-16.0}	**8.83±0.56** bbb (-43.1)	**10.07±0.84** cc (-35.1), [+14.0]
TAG (µmol)	134.73±7.46	**97.41±10.36** aa{-27.7}	**59.45±3.17** bbb (-39.0)	**62.53±5.25** cc (-35.8), [+5.2]
CH (µmol/g)	22.66±0.74	22.40±1.59{-1.1}	18.05±0.59 (-19.4)	18.42±0.56 (-17.8), [+2.0]
CH (µmol)	159.59±5.88	148.33±9.36{-7.1}	**121.89±4.26** b (-17.8)	**122.73±4.53** c (-17.3), [+0.7]
MDA (nmol/g)	20.22±2.30	**43.24±3.99** aaa{+113.8}	**26.90±1.76** bb (-37.8)	**19.17±2.42** ccc (-55.7), d [-28.7]
MDA (nmol)	144.05±18.15	**291.74±33.41** aaa{+102.5}	**190.77±13.33** b (-34.6)	**125.29±15.44** ccc (-57.1), dd [-34.3]
PL (µmol/g)	41.78±1.38	**37.59±1.46** a{-10.0}	40.40±0.86 (+7.5)	**41.69±0.77** c (+10.9), [+3.2]
PL (µmol)	294.03±14.92	257.38±10.34{-12.5}	282.92±14.07 (+9.9)	281.53±11.37 (+9.4), [-0.5]
GLY (µmol/g)	14.28±1.28	**9.82±0.71** a{-31.2}	13.75±1.93 (+40.0)	**15.41±1.28** cc (+56.9), [+12.1]
GLY (µmol)	107.76±11.22	**70.75±5.87** a{-34.3}	104.80±17.33 (+48.1)	**102.19±9.68** cc (+44.4), [-2.5]
Liver weight (g)	7.07±0.19	6.77±0.28{-4.24}	6.79±0.19 (+0.3)	6.68±0.18 (-1.3), [-1.6]

Data are expressed as means±S.E.M. Significant difference between groups is designated as: a - CONT vs INT (**a** p < 0.05; **aa** p < 0.01; **aaa** p < 0.001); b - BEXA vs CONT (**b** p < 0.05; **bb** p < 0.01; **bbb** p < 0.001); c - BEXA + MEL vs CONT (**c** p < 0.05; **cc** p < 0.01; **ccc** p < 0.001); d - BEXA + MEL vs BEXA (**d** p < 0.05; **dd** p < 0.01); GLY – glycogen, TAG – triacylglycerols, CH – cholesterol, PL – phospholipids, MDA – malondialdehyde . Other details: see Tab. 3

Discussion

Inhibitory effect of BEXA in experimental mammary carcinogenesis up to total tumor regression was reported by Gottardis et al. [22] and Bischoff et al. [23]. BEXA was effective also in estrogen receptor-negative tumor prevention in transgenic mice bearing mammary tumors[24]. Lubet et al. [25] and Grubbs et al. [7] focused on dose-dependent BEXA efficacy in NMU-induced rat mammary carcinogenesis. Daily BEXA administration at different concentrations during 120 days decreased mammary tumor frequency per animal by 78-96% (administration by gavage) or by 38-92% (administration in the diet), respectively. High BEXA doses prominently reduced proliferation and induced apoptosis in tumor cells with simultaneous serum IGF-1 decrease [25]. Grubbs et al. [7] tested new UAB retinoids and BEXA using the same long-term experimental model but chemoprevention was administered only for 7 days. High doses of BEXA and 4-methyl-UAB30 decreased mammary tumor frequency by 75-80% with concomitant proliferation decrease and apoptosis induction. As expected, retinoid administration induced dose-proportional triacylglycerol increase [7].

The aim of this study was to use lower BEXA dose in combination with other substance to enhance rexinoid effect on NMU treated rats. A similar approach was used by Bischoff et al. [23] with tamoxifen and Suh et al. [26] with arzoxifen. We also evaluated selected parameters of BEXA metabolic impact. Among other results we proved that the chosen BEXA dose did not increase triacylglycerolemia. NMU administration without chemoprevention (CONT group) significantly decreased glycaemia, TAG and GLY liver content and decreased (nonsignificantly) liver PL and CH content and heart muscle GLY concentration while liver and thymus MDA content was higher in comparison with INT. Preventive-curative BEXA administration decreased liver and thymus MDA accumulation and decreased heart muscle GLY, but reinforced the liver TAG and CH content decrease. BEXA+MEL combination normalised (to INT level) glycaemia, liver PL, liver and heart muscle GLY, and liver and thymus MDA, liver TAG and CH content remained decreased. Similar changes following NMU administration, particularly liver TAG content decrease in tumor-non-bearing and tumor-bearing female rats in the same experimental mammary carcinogenesis protocol were reported by Chamilová et al. [9, 10]. They used raloxifen and

Table 5. Effects of BEXA and BEXA+MEL on glycogen and malondialdehyde in heart muscle and thymus.

	INT	CONT	BEXA	BEXA+MEL
Heart muscle				
GLY (µmol/g)	7.13±0.67	5.53±0.56{-22.4}	7.03±0.54 (+27.1)	**7.69±0.66** [c] (+39.1), [+9.4]
GLY (µmol)	5.44±0.52	4.16±0.37{-23.5}	5.14±0.48 (+23.6)	5.85±0.66 (+40.6), [+13.8]
Weight (g)	766.63±18.43	717.00±28.30{-6.5}	725.41±22.25 (+1.2)	717.77±15.21 (+0.1), [-1.1]
Thymus				
MDA (nmol/g)	15.25±0.60	**20.36±1.49** [a]{+33.5}	18.63±0.92 (-8.5)	**16.27±0.60** [c] (-20.1), [d] [-12.7]
MDA (nmol)	2.91±0.12	3.58±0.27{+23.0}	3.23±0.24 (-9.8)	**2.85±0.20** [c] (-20.4), [-11.8]
Weight (g)	198.11±8.77	174.25±12.54{-12.0}	168.72±11.67 (-3.2)	177.29±12.38 (+1.7), [+5.1]

Data are expressed as means±S.E.M. Significant difference between groups is designated as: **a** $p < 0.05$ (CONT vs INT), **c** $p < 0.05$ (BEXA + MEL vs CONT), **d** $p < 0.05$ (BEXA + MEL vs BEXA); GLY – glycogen, MDA – malondialdehyde . Other details: see Tab. 3

tamoxifen, in combination with MEL as chemopreventives and, similarly to our results, chemoprevention did not alleviate lipid metabolism disturbances. We assume this is a result of decreased lipogenesis following carcinogen administration and cancer progression. No macroscopic/microscopic liver lesions were found. We confirmed MEL efficacy as "co-chemopreventive" substance, possibly as ROS scavenger [27] which was demonstrated by attenuated tissue MDA formation.

To summarize, lower dose of the rexinoid BEXA alleviated the carcinogen-induced metabolic changes and the combination with other substance MEL enhanced its preventive – curative action in experimental mammary carcinogenesis. Since MEL has been shown to enhance the chemopreventive efficacy of celecoxib in the same model previously [28], the combination of BEXA plus MEL may be considered in oncological practice too.

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