Neoplastic effects of exemestane in premenopausal breast cancer model

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Aromatase inhibitor exemestane as a single – agent has no established role in the treatment of premenopausal breast cancer women. The aim of this study was to evaluate preventive effects of exemestane in the model of premenopausal N-methyl-N-nitrosourea – induced mammary carcinogenesis in female rats. Exemestane treatment begun 7 days prior to carcinogen administration and continued next 12 weeks – till the end of experiment. Exemestane was dietary administered in two concentrations of 1 mg / 1kg (EXE 1), or 10 mg/1 kg (EXE 10), respectively.

Exemestane increased the tumor frequency by 80.5 % (P=0.034) in the group EXE 1 and by 61.5 % (P=0.045) in the group EXE 10 in comparison with control animals. In the group EXE 10, the incidence of mammary tumors was increased by 11.5 % (P=0.31) and tumor volume by 41.5 % (P=0.23), also the latency was shortened by 8 days (P=0.078) compared with controls. In the groups with exemestane, changes in weights and histology of uterus and vagina were not found at the end of experiment. Exemestane did not alter serum concentrations of estradiol, testosterone and dehydroepiandrosterone. In the group EXE 10 in comparison with untreated animals, exemestane decreased serum concentrations of triacylglycerols by 9 % (P=0.032), total cholesterol by 19.5 % (P=0.0002) and cholesterol of low – density and high – density lipoprotein fractions by 41 % (P<0.0001), or 21.5 % (P=0.0002), respectively. In the group EXE 1, the decrease in cholesterol of low-density lipoprotein fraction by 22.5 % (P=0.0005) was recorded. An increase in food intake (P=0.023) and body weight gain (P=0.036) was found in the group EXE 10 compared with the control group (P<0.05).

The present study points to apparent tumor – promoting effects of dietary administered exemestane in the model of premenopausal mammary carcinogenesis in female rats. Exemestane as a steroidal agent indicated androgenic effects on rat lipid metabolism in this experiment.

Key words: mammary carcinogenesis, rat, chemoprevention, exemestane, aromatase inhibitors.

A link between estrogen exposure and breast cancer initiation and progression was clearly demonstrated by epidemiological studies [1]. Aromatization is the major mechanism of estrogen synthesis in the postmenopausal woman. In these women, aromatase inhibitors block biotransformation of adrenal androgens to estrogens in peripheral tissues (including the breast, muscle, liver, and fat), resulting in undetectable levels of plasma estrogens. For that reason, aromatase inhibitors are primarily used in the breast cancer treatment in postmenopausal population. Recently potent third-generation aromatase inhibitors have been introduced for breast cancer treatment: the steroidal exemestane and two non – steroidal letrozole and anastrozole. Based on results of clinical trials [2, 3, 4] exemestane, letrozole and anastrozole were approved for the treatment of metastatic breast cancer in postmenopausal population. Recent adjuvant trials have established the value of above-mentioned drugs in the treatment of early-stage breast cancer [5, 6, 7].

The premenopausal ovaries are relatively resistant to effects of first generation aromatase inhibitors [8]. This effect is the result of high levels of androstendione in the premenopausal ovary that compete effectively with aromatase inhibitors and do not allow binding of inhibitor to the active site of the aromatase. Also the reflex rising of luteinic hormone and follicle – stimulating hormone after tissue estradiol deprivation is observed. On the other hand, several groups of investigators demonstrated the importance of local aromatization in mammary gland versus uptake of estradiol from plasma by breast tissue [9, 10, 11, 12]. Also some clinical trials pointed out to significant connection of local estrogen production and

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tumor growth in the breast [13, 14, 15]. For reasons mentioned above, intratumoral aromatase could be an important therapeutic target against breast cancer. The effectiveness and safety of aromatase inhibitors in the treatment of premenopausal breast cancer patients are intensively discussed among oncologists [16, 17, 18].

Monotherapy with exemestane has no established role in premenopausal women with breast cancer and is area of future exploration. In our experiments, the significant tumor suppressive effect of non – steroidal letrozole [19] and anastrozole (submitted for publication by Kubatka et al., 2008) in premenopausal model of mammary carcinogenesis in female rats were found. The main goal of this experiment, that also mimics situation in high-risk premenopausal women for breast cancer, is to evaluate the preventive effects of steroidal aromatase inhibitor exemestane in mammary gland carcinogenesis in female Sprague-Dawley rats. The side effects of exemestane in animals will be evaluated in this study.

Materials and methods

Female rats of Sprague-Dawley strain obtained from AnLab (Prague, Czech Republic) aged 30-34 days were adapted to vivarium controlled for temperature (23±2°C), relative humidity (60-70%) and photoperiod (12 h light: 12 h dark). During the experiment, animals drank tap water ad libitum. The chow containing exemestane (Aromasin) synthesized by Pfizer was prepared at SSNIFF Spezialdiäten GmbH (Soest, Germany). Exemestane was administered in two concentrations in the chow – 1 mg/1 kg (0.0001 %), and 10 mg/1 kg (0.001 %). Mammary carcinogenesis was induced by N-methyl-N-nitrosourea (NMU; Sigma, Deisenhofen, Germany) administered intraperitoneally in two doses each per 50 mg/kg b.w. on average postnatal days – 43. and 49. NMU was freshly prepared, dissolved in isotonic saline solution. Chemoprevention with exemestane began 7 days before carcinogen administration and lasted until the end of the experiment – 12 weeks after NMU application. Animals were randomly assigned to one of three experimental groups: (1) control group without chemoprevention; (2) chemoprevention with exemestane in concentration of 1 mg/1 kg of chow (EXE 1); (3) chemoprevention with exemestane in concentration of 10 mg/1 kg of chow (EXE 10). Each group consisted of 20 animals. The animals were weekly weighted and palpated, the occurrence, number, location and size of each palpable tumor were registered.

In the last, 12th week of experiment (dated from the first NMU injection) animals were sacrificed by quick decapitation, mammary tumors, uteri and vaginas were excised, subsequently weighted and the tumor size was registered. Specimens of mammary tumors, uteri and vaginas were fixed in 10% buffered formalin and were embedded in paraffin using conventional automated systems. The blocks were cut to obtain 4 to 5 mcm thick sections and were stained with hematoxylin-eosin. Histopathologic examination was performed by light microscopy. At sacrifice, the blood was collected from each animal. In the serum, the concentrations of estradiol, testosterone, dehydroepiandrosterone, triacylglycerols, total cholesterol, and cholesterol values of the low-density lipoprotein (LDL), and high-density lipoprotein (HDL) fractions were measured. Serum steroids were measured using ELISA kits (DRG Diagnostics, Marburg, Germany) with intra- and interassay coefficients of variations < 5%. Lipid metabolism changes were measured by automatic biochemical analyser Olympus AU 640 (Olympus Optical, Tokyo, Japan).

The tumors were classified according to the criteria for the classification of rat mammary tumors [20]. The following parameters of mammary carcinogenesis were evaluated in each group: tumor incidence as the percentage representation of tumor-bearing animals in the group, tumor frequency per group as the number of all tumors devided by all animals in the group, average tumor volume and latency period determined by the period from carcinogen administration to the appearance of first tumor in an animal. The effect of exemestane on food, water intake and body weight gain (evaluated from prevention initiation until the end of the experiment) of animals was observed. Food and water intake during 24 hours was observed in 7th and 11th week after carcinogen administration, overall in 4 measurements (twice in a given week).

The tumor incidence was evaluated by Mann-Whitney U-test, other parameters by one-way analysis of variance or Kruskal-Wallis test. The tumor volume was calculated according to: $V = \pi \, . \, (S_1)^2 \, . \, S_2 / \, 12$; S_1 and S_2 are tumor diameters ($S_1 < S_2$).

Results

Significant neoplastic effects of dietary administered exemestane in the model of premenopausal mammary carcinogenesis in female Sprague-Dawley rats are outlined in Table 1. Neoplastic activity of exemestane was characterized mainly by increasing of tumor frequency and tumor volume. Histopathological classification of mammary tumors is summarized in Table 2. In both groups with exemestane, the significant changes of serum estradiol, testosterone and dehydroepiandrosterone were not observed (Table 3).

After 13 weeks of exemestane treatment, no changes in absolute and relative uterine and vaginal weights in both exemestane groups in comparison with the control group were observed (Table 4). Also the changes in the thickness of uterine endometrium and myometrium among experimental groups were not found. Histopathologic examination of vaginal epithelium did not exhibit any changes. In animals of both treated and untreated groups, the vaginal epithelium composed of stratified squamous epithelial cells. In some cases, vaginal epithelium was keratinized or/and covered by mucinous cell layer, but these alterations did not depend on exemestane administration.

Table 1. Effects of exemestane in NMU-induced mammary carcinogenesis in female Sprague-Dawley rats at the end of experiment.

Group	CONT	EXE 1	EXE 10
all animals / tumor bearing animals	20 / 17	19 / 16	19 / 18
tumor incidence (%)	85.0	84.21 (- 1 %)	94.74 (+ 11.5%)
tumor frequency per group*	3.00±0.55	5.42±0.97 [a] (+ 80 %)	4.84±0.70 [a] (+ 61.5 %)
tumor latency* (days)	69.59±2.97	66.06±2.21 (- 3.5 days)	61.83±3.06 (- 8 days)
tumor volume* (cm^3)	0.87±0.12	0.89±0.16 (+ 2.5 %)	1.23±0.23 (+ 41.5 %)

CONT – control group, EXE 1 – group with administered exemestane in concentration of 1 mg/kg in food, EXE 10 – group with administered exemestane in concentration of 10 mg/kg in food.
*Data are expressed as means±SEM. Values in brackets are calculated as %-ual deviation from the 100% of non-influenced control group (with exception of latency). Significantly different, [a] $P<0.05$ vs CONT.

Table 2. Histopathological classification of mammary tumors.

Mammary tumors	CONT	EXE 1	EXE 10
Cribriform	37	80	58
Papillary	5	9	9
Cribriform-papillary	4	1	5
Tubulo-alveolar	3	4	2
Cribriform-comedo	3	1	4
Tubular	1	3	-
Papillary-cribriform	1	-	3
Carcinoma in fibroadenoma	1	1	1
Cribriform-cystic	2	-	-
Cystic	1	-	1
Tubulo-alveolar-papillary	-	1	1
Papillary-cystic	-	-	2
Comedo	1	-	-
Tubulo-cribriform	-	1	-
Cribriform-papillary-comedo	-	-	1
Cribriform-tubular	-	-	1
Fibroadenoma	1	2	4
Total number	60	103 (+ 71.5 %)	92 (+ 53.5 %)

Values in brackets are calculated as %-ual deviation from the 100% of non-influenced control group.

Exemestane had beneficial effects on lipid metabolism in rats (Table 5). In the group EXE 10 compared with control animals, the triacylglycerols were decreased by 9 % ($P=0.032$), total cholesterol by 19.5 % ($P=0.0002$) and LDL- and HDL-cholesterol by 41 % ($P<0.0001$), or 21.5 % ($P=0.0002$), respectively. The significant decrease in LDL-cholesterol by 22.5 % ($P=0.0005$) was found in the group EXE 1.

An increase in body weight gain, characterised mainly by an increased body fat content, was found in the group EXE 10 ($P<0.036$) compared with the controls (Table 3). An average daily food intake per rat increased depending on an increasing exemestane dose: 14.90±0.41 g (control group), 15.53±0.85 g (EXE 1) and 16.18±0.34 g (EXE 10). Non – significant correlation between the body weight gain increase and food intake increase ($r=0.643$, $P=0.056$) in animals from the group EXE 10 was found. The exemestane doses were calculated in accordance with the amount of chow consumed, measured in 7^{th} and 11^{th} week of the experiment. An average daily dose of exemestane per rat was 15.5 μg in the group EXE 1 and 161.8 μg in group the EXE 10.

Discussion

Results of this experiment are the first mention about tumor promoting effects of steroidal aromatase inhibitor exemestane in conventional (premenopausal) model of mammary carcinogenesis in female Sprague-Dawley rats. The daily average dose of exemestane – 161.8 μg per rat administered in the group EXE 10 of our experiment was equivalent to daily clinical dose of Aromasin administered in postmenopausal breast cancer patients.

Results of clinical trials showed highly effectiveness of exemestane in endocrine breast cancer therapy in the metastatic, adjuvant and neoadjuvant settings for postmenopausal women. Also experimental study of Di Salle et al. [21] demonstrated antineoplastic effects of intramuscularly administered exemestane in high dose of 100 mg / kg / week in dimethylbenz(a)anthracene induced mammary gland cancer in rats. These facts were main reason to use exemestane in our chemopreventive study. The next argument for our study was to confirm high tumor suppressive effects of non-steroidal letrozole in premenopausal model of mammary

Table 3. Serum levels of estradiol, testosterone and dehydroepiandrosterone after exemestane treatment in female rats.

Group	CONT	EXE 1	EXE 10
Estradiol (pg/ml)	36.965±4.921	44.854±7.454	35.369±2.716
Testosterone (ng/ml)	0.221±0.032	0.172±0.024	0.254±0.032
Dehydroepiandrosterone (ng/ml)	0.350±0.081	0.447±0.050	0.371±0.075

Data are expressed as means±SEM.

Table 4. Effects of exemestane on uterine, vaginal and body weights and on uterine endometrium and myometrium.

Group	uterine wet weight		uterine		vaginal wet weight		body weight gain (g)
	absolute (g)	relative* (%)	endometrium (μm)	myometrium (μm)	absolute (g)	relative*(%)	
CONT	0.578±0.042	0.210±0.014	810.3±106.8	368.8±29.6	0.197±0.012	0.072±0.004	109.60±3.99
EXE 1	0.567±0.029	0.205±0.010	787.0±67.9	362.9±24.5	0.203±0.001	0.074±0.004	113.84±4.58
EXE10	0.599±0.037	0.207±0.014	857.2±51.3	383.6±24.2	0.216±0.010	0.074±0.003	121.37±3.62[a]

Data are expressed as means±SEM. *Relative organ weight (%) = [absolute organ weight (g) / body weight (g)] x 100. Significantly different, [a] P=0.023 vs CONT

Table 5. Effects of exemestane on plasma lipid metabolism.

Group	Triacylglycerols (mmol/l)	Total cholesterol (mmol/l)	LDL- cholesterol (mmol/l)	HDL- cholesterol (mmol/l)
CONT	1.19±0.06	2.23±0.08	0.22±0.01	0.69±0.03
EXE 1	1.21±0.10 (+ 1.5 %)	2.07±0.08 (- 7 %)	0.17±0.01 [b] (- 22.5 %)	0.63±0.02 (- 8.5 %)
EXE 10	1.08±0.12 [a] (- 9 %)	1.79±0.07 [b, c] (- 19.5 %)	0.13±0.01 [b, d] (- 41 %)	0.54±0.02 [b, d] (- 21.5 %)

Data are expressed as means±SEM. Significantly different, [a] P<0.05 vs CONT, [b] P<0.001 vs CONT, [c] P<0.05 vs EXE 1, [d] P<0.01 vs EXE 1. Values in brackets are calculated as %-ual deviation from the 100% of non-influenced control group.

carcinogenesis in female rats observed in our previous experiment [19]. However, the results of this study point out to apparent tumor – promoting activity of dietary administered exemestane in NMU – induced rat mammary carcinogenesis. It is possible, that chemopreventive effect of exemestane in rat mammary carcinogenesis depends on dose and/or way of administration. On the other hand, it is postulated that the effects of exemestane and its metabolite 17-hydroexemestane with the chemical structure related to natural estrogen precursor androstenedione, may be a result of their androgenic action in organism [22]. It was found that affinity of 17-hydroexemestane to androgen receptor is 100 times higher that of the dihydrotestosterone [23]. This fact may have implications for several end organ effects of exemestane including its mitotic activity on rat mammary gland observed in our study. This hypothesis about exemestane and 17-hydroexemestane mitotic activity in rat mammary gland through androgen receptors could be supported by our finding that exemestane did not alter the serum levels of estradiol and its precursors in rats. The remarkably difference in effects of exemestane observed in postmenopausal breast cancer women and female rats in our experiment are probably caused by species dependent dissimilarities in structure of gene regulation areas (promotors) of responsible genes.

Estrogens play a key role in several organs and physiological processes, like the lipid metabolism. Histopathological examination of the uterus and vagina did not reveal changes in exemestane – treated animals, what points out to drug's non-antiestrogenic effects on rat genital system in our preclinical model of premenopausal mammary gland cancer. On the other side, non-steroidal letrozole in our previous experiment caused apparent uterine and vaginal atrophy in rats at the end of experiment [19]. In our last experiment, non-steroidal anastrozole in doses equivalent to clinical doses (0.5 mg / kg of food) did not cause any changes on uteri, vaginas and plasma lipid metabolism of rats (submitted for publication by Kubatka et al., 2008). In this study a significant decrease in serum triacylglycerols, total cholesterol and LDL- and HDL-cholesterol in rats treated with exemestane was found. On the contrary, in letrozole – treated rats of our previous study, increased plasmatic triacylglycerol concentrations (P<0.0001) were observed; total cholesterol and LDL- and HDL-cholesterol were not significantly changed [19]. In the study of Goss et al. [24] exemestane given to ovariectomized rats caused significant reductions of serum cholesterol and LDL-cholesterol in comparison with control animals. A decrease in HDL – cholesterol in our study may seem somewhat surprising, realising that estrogens may elevate this fraction of lipoprotein [25], which is known to have a cardioprotective effect. Similarly, in one clinical study, where the effect of 2 years exemestane treatment in patient at low risk operated for breast cancer was evaluated, a decrease in HDL – cholesterol was observed too [26]. Notably, exemestane with a putative androgenic action had a beneficial action on lipid metabolism by decreasing of serum triacyglycerols, total cholesterol and LDL-cholesterol in this study. However, these data must be confirmed in long – term clinical trials.

The effects of estradiol on some aspects of physiological and behavioural regulation of energy balance are known [27, 28]. In our previous experiment, a significant increase of food intake in letrozole – treated rats characterized by increase in body weight and body fat content was observed [19]. Similarly, exemestane caused an increase in food consumption and body weight gain of rats. An increase in the final body weight of rats after exemestane treatment was also observed in the study of Di Salle et al. [21]. This result points out to

antiestrogenic effect of exemestane on food intake regulations in rats.

The new generation of aromatase inhibitors has become the first choice of the endocrine treatment for advanced breast cancer in postmenopausal patients. These compounds are currently evaluated in adjuvant setting; also the results of several ongoing trials may indicate their role in the chemoprevention of breast cancer. Further studies with longer follow – up are required to clarify the effects of aromatase inhibitors on cardiovascular system and bone metabolism. Our study showed, that dietary administered exemestane in concentrations equivalent to clinical doses and in concentrations 10 times lower demonstrated neoplastic effects on mammary gland in female rats. The markedly reverse effects of steroidal aromatase inhibitor exemestane versus non-steroidal aromatase inhibitors letrozole [19] and anastrozole (submitted for publication by Kubatka et al., 2008) on mammary gland and other end organs in our model of premenopausal mammary carcinogenesis were revealed. Premenopausal administration of exemestane in humans is area of next exploration.

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