Preventive effects of letrozole in the model of premenopausal mammary carcinogenesis

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Single – agent therapy with aromatase inhibitors has no established role in premenopausal women with breast cancer. In this study, tumor suppressive effects of letrozole in the prevention of N-methyl-N-nitrosourea – induced mammary carcinogenesis in female Sprague-Dawley rats were evaluated. Letrozole was dietary administered in two concentrations – 1 mg/1 kg (LETRO 1), and 10 mg/1 kg (LETRO 10). Letrozole suppressed incidence of mammary gland cancer by 93 % ($P<0.00002$) in the group LETRO 1 in comparison with control animals. Total suppression of mammary carcinogenesis was observed in the group LETRO 10. In the groups with letrozole, uterine and vaginal atrophy was found at the end of experiment. In letrozole – treated animals in comparison with untreated animals, increased plasmatic triacylglycerol concentrations ($P<0.0001$) were observed, but total cholesterol and cholesterol of low- and high- density lipoprotein fractions were not significantly changed. An increase in body weight gain and food intake was found in the groups LETRO 1 and LETRO 10 compared with the control group ($P<0.0001$).

The present study points to high tumor suppressive effects of letrozole in premenopausal model of mammary carcinogenesis in female rats.

Keywords: mammary carcinogenesis, rat, chemoprevention, letrozole, aromatase inhibitors.

Aromatase inhibitors have revolutionized the treatment of postmenopausal women with hormone receptor – positive breast cancer. Aromatase inhibitors are now the first choice endocrine therapy in the metastatic breast cancer of postmenopausal women. These endocrine agents also seem likely to become soon the standard adjuvant therapy for postmenopausal patients with hormone – responsive breast cancer, either alone or in sequence with tamoxifen. There is evidence, that third generation aromatase inhibitor letrozole, is superior to tamoxifen in adjuvant treatment [1, 2] and in treatment of metastatic disease [3] for postmenopausal women with estrogen receptor – positive breast cancer. Some trials showed that aromatase inhibitors reduced the incidence of contralateral breast cancer [4, 1, 5]. The data of these trials point out to fact that aromatase inhibitors could be useful in the prevention of breast cancer in women with increased risk of developing the disease. In direct comparisons with tamoxifen in adjuvant therapy, aromatase inhibitors have a better toxicity profile with fewer patients stopping therapy because of drug – related adverse effects. These data have prompted breast cancer chemoprevention trials with aromatase inhibitors. Results of ongoing trials using exemestane and anastrozole may indicate a role for aromatase inhibitors in the prevention of breast cancer.

Based on mechanism of action, aromatase inhibitors are primarily used in the postmenopausal population and single – agent therapy with aromatase inhibitors has no established role in premenopausal breast cancer patients. It is known, that premenopausal ovaries are relatively resistant to blockade with first generation aromatase inhibitors [6]. Several experimental methods have been used to determine the biological importance of breast *in situ* estrogen production versus uptake of estradiol from plasma by breast tissue [7, 8, 9, 10]. Results of above cited experiments suggested the importance of *in situ* estrogen production in the breast and led to the hypothesis that an important determinant of tissue estradiol levels

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is local production in the mammary gland. There is also clinical evidence suggesting that local production of estrogens may contribute to breast tumor growth [11, 12, 13]. Based on this hypothesis, the estradiol levels in breast tissue itself would be an important predictor of later carcinogenesis in the mammary gland and therefore intratumoral aromatase could be a potential therapeutic target. The efficacy and toxicity of aromatase inhibitors in the treatment of premenopausal breast cancer patients are discussed among oncologists [14, 15, 16]. For reasons mentioned above, research on the use of aromatase inhibitors in premenopausal population with estrogen receptor – positive breast cancer in adjuvant or preventive settings is required.

Our model of mammary carcinogenesis in female rats mimics situation in high risk premenopausal women (e.g. with positive family history of breast cancer). The aim of this experiment is to evaluate the oncostatic activity of aromatase inhibitor letrozole in the chemoprevention of mammary gland cancer in female Sprague-Dawley rats. The adverse effects of letrozole in rats will be evaluated in this experiment.

Materials and methods

Female rats of Sprague-Dawley strain obtained from AnLab (Prague, Czech Republic) aged 33 – 37 days were used in the experiment. The animals were adapted to standard vivarium conditions. During the experiment, animals drank tap water ad libitum. The chow containing letrozole (Femara) synthesized by Novartis was prepared at Kocanda Mill (Prague, Czech Republic). Letrozole was administered in the chow in two concentrations – 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 in two intraperitoneal doses (50 mg/kg b.w.) on average postnatal days 44 and 51. Chemoprevention with letrozole began 7 days before carcinogen administration and lasted until the end of the experiment – 17 weeks after NMU application. Animals were randomly assigned to one of three experimental groups: (1) control group without chemoprevention; (2) chemoprevention with letrozole in concentration of 1 mg/1 kg of chow (LETRO 1); (3) chemoprevention with letrozole in concentration of 10 mg/1 kg of chow (LETRO 10). Each group consisted of 20 animals. The animals were weekly weighed and palpated in order to register the presence, number, location and size of each palpable tumor.

In the last – 17th week of the experiment (dated from the first NMU injection), the animals were quickly decapitated. The mammary tumors, the uterus as well as the vagina of each animal were excised and the tumor size was recorded. The effect of letrozole on uterine and vaginal weights (absolute and relative) was observed. 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 mm thick sections and were stained with hematoxylin-eosin. Histopathologic examination was performed by light microscopy. Blood was collected, at sacrifice, from each animal. In the serum, the concentrations of triacylglycerols, total cholesterol, and cholesterol values of the low-density lipoprotein (LDL), and high-density lipoprotein (HDL) fractions were measured. Lipid metabolism changes were measured by automatic biochemical analyser AU 640 (Olympus).

The tumors were classified according to the criteria for the classification of rat mammary tumors [17]. The following parameters of mammary carcinogenesis were evaluated in each group: tumor incidence as the percentage representation of tumor bearing animals, tumor frequency as the number of tumors per group, cumulative tumor volume as the sum of tumor volumes in the group and latency period determined by the period from carcinogen administration to the appearance of first tumor in an animal.

The individual body weight gain of animals was recorded from prevention initiation till the end of the experiment. Food intake of animals during 24 h in 8th and 16th week after carcinogen administration was observed.

Tumor incidence was evaluated by Mann-Whitney U-test, other parameters by one-way analysis of variance (ANOVA) or Kruskal-Wallis test. 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

In generally remarkable tumor suppressive effect of letrozole in the prevention of mammary carcinogenesis in fe-

Table 1. Chemopreventive effects of letrozole in NMU-induced mammary carcinogenesis in female Sprague-Dawley rats at the end of experiment.

Group	CONT	LETRO 1	LETRO 10
all animals / tumor bearing animals	20 / 15	19 / 1	19 / 0
number of tumors	32	1	0
tumor incidence (%)	75.0	5.2 [a] (- 93 %)	0
tumor frequency per group*	1.60±0.29	0.05±0.05 [a] (- 97 %)	0
tumor latency* (days)	98.00±2.82	112.00±0 (+ 14.5 %)	–
cumulative tumor volume** (cm^3)	14.14	4.15 (- 70.5 %)	0

CONT – control group, LETRO 1 – group with administered letrozole in concentration of 1 mg/kg in food, LETRO 10 – group with administered letrozole in concentration of 10 mg/kg in food. *Data are expressed as means±SEM, **data are expressed as a sum of volumes. Values in brackets are calculated as %-ual deviation from the 100% of non-influenced control group. Significantly different, [a] $P<0.00002$ vs CONT

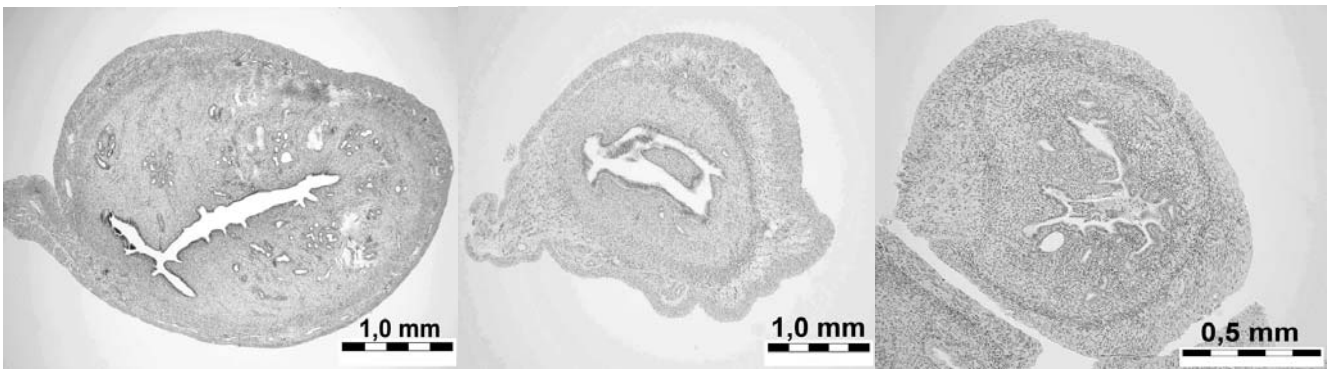


Figure 1. Uterine histology after 18 weeks of letrozole administration. Uterine atrophy (B, C) is characterized by a decrease of endometrial thickness in letrozole – treated rats in comparison with control animals (A).
A – control group, B – group LETRO 1, C – group LETRO 10. Magnification: 40x (A, B) and 100x (C).

male Sprague-Dawley rats were observed (Table 1). Histopathology of mammary tumors: in the control group, 32 mammary tumors were evaluated, 29 malignant and 3 benign (91: 9 %). Adenocarcinomas were cribriform (20), tubular (3), tubulo-alveolar (2), papillary (2), alveolar (1) and comedo (1). Benign lesions were 2 adenomas and 1 fibroadenoma. Only one tumor – fibroadenoma in the group LETRO 1 was observed. The diffuse high-grade malignant lymphoma infiltrations in the liver and spleen were observed in one animal of the group LETRO 1 and two animals of the group LETRO 10.

After 18 weeks of letrozole administration, reduced absolute and relative uterine and vaginal weights ($P < 0.0001$) in both treated groups in comparison with the control group were observed (Table 2). Decreased endometrial thickness and endometrial atrophy after letrozole administration were noted (Figure 1). This atrophy was characterized by the reduction of number and presence of smaller endometrial glands. Histopathologic examination of vaginal epithelium showed also atrophic changes. While in untreated animals the vaginal epithelium was composed of stratified squamous epithelial cells, in letrozole – treated animals was composed of epithelium with 2 – 6 layers in group LETRO 1, or only 2 layers in group LETRO 10, respectively (Figure 2).

With regard to lipid metabolism, letrozole increased the plasma levels of triacylglycerols in both letrozole – treated groups in comparison with control animals ($P<0.0001$). Significant changes in total cholesterol, LDL- and HDL-cholesterol between treated and untreated animals were not exhibited (Table 3).

Evaluation of body weight gain (Table 2) and food intake revealed significant increase in letrozole – treated groups ($P<0.0001$). The increase in body weight was reflected mainly in an increased of body fat content. An increase in the body

Table 2. Effects of letrozole on uterine, vaginal and body weights.

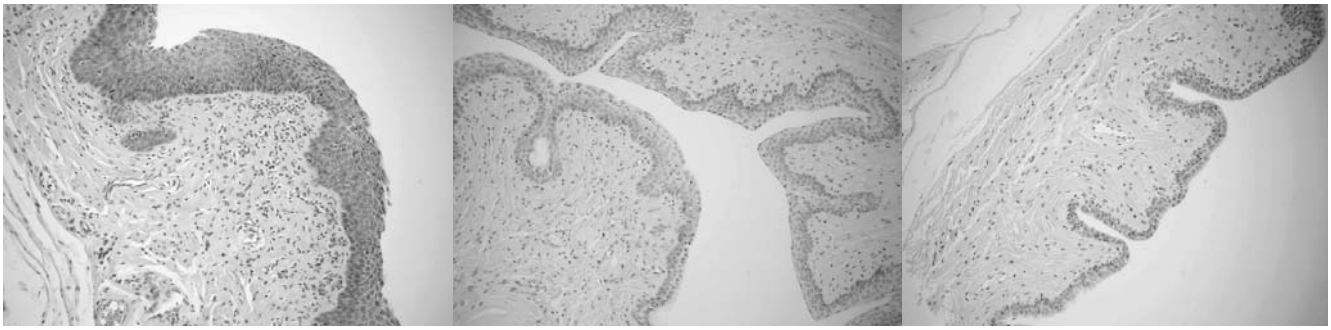
Group	uterine wet weight		vaginal wet weight		body weight gain (g)
	absolute (g)	relative* (%)	absolute (g)	relative* (%)	
CONT	0.541±0.031	0.205±0.013	0.176±0.005	0.066±0.002	129.15±5.40
LETRO 1	0.238±0.019 [a]	0.067±0.006 [a]	0.159±0.011	0.043±0.003 [a]	235.37±12.13 [a]
LETRO 10	0.122±0.004 [a,b]	0.032±0.001 [a,b]	0.143±0.010 [c]	0.038±0.003 [a]	237.63±10.56 [a]

Data are expressed as means±SEM. *Relative organ weight (%) = [absolute organ weight (g) / body weight (g)] x 100. Significantly different, [a] $P<0.0001$ vs CONT, [b] $P<0.0001$ vs LETRO 1, [c] $P<0.01$ vs CONT

Table 3. Effects of letrozole on plasma lipid metabolism.

Group	Total cholesterol (mmol/l)	HDL- cholesterol (mmol/l)	LDL- cholesterol (mmol/l)	Triacylglycerols (mmol/l)
CONT	1.35±0.05	0.52±0.02	0.11±0.01	0.67±0.04
LETRO 1	1.35±0.07	0.50±0.02	0.14±0.01	1.33±0.15 [a]
LETRO 10	1.52±0.12	0.48±0.03	0.23±0.07	1.36±0.11 [a]

Data are expressed as means±SEM. Significantly different, [a] $P<0.0001$ vs CONT



A B C

Figure 2. Effects of 18 weeks of letrozole treatment on vaginal histology. Atrophic vaginal epithelium (B, C) in letrozole – treated rats is characterized by a reduction of epithelium cell layers compared with control animals (A).
A – control group, B – group LETRO 1, C – group LETRO 10.
Magnification: 200x (A, B, C).

weight markedly correlated with increase in food intake in rats from groups LETRO 1 and LETRO 10 ($r=0.80$, $P<0.0001$ or $r=0.81$, $P<0.0001$, respectively). An average daily food intake per rat was: 15.63±0.39 g (control group), 19.77±0.57 g (LETRO 1) and 20.24±0.42 g (LETRO 10). The letrozole doses were calculated in accordance with the amount of chow consumed, measured in 8th and 16th week of the experiment. An average daily dose of drug per rat was 19.8 μg in group LETRO 1 and 202.4 μg in group LETRO 10.

Discussion

This study is the first report on tumor suppressive effects of letrozole in model of premenopausal mammary carcinogenesis in female Sprague-Dawley rats. Antitumor effect of letrozole was recorded in all evaluated parameters of mammary carcinogenesis. The daily average dose of letrozole – 19.8 μg per rat (group LETRO 1) used in our experiment is equivalent to daily clinical dose of Femara administered in postmenopausal breast cancer patients. This experiment provided a rationale for clinical trials of letrozole in premenopausal breast cancer patients.

Estrogens play an important role in many physiological processes and systems, like the urogenital or the cardiovascular. Several adverse effects of letrozole in our experiment were observed. Histological examination showed atrophic changes of the uterus and vagina in letrozole – treated animals, what points out to drug's antiestrogenic effects on rat genital system in our preclinical model of premenopausal breast cancer. These histopathological changes were dose dependent. Similar effects of letrozole on the rat uterus were noted also in other experimental studies in rats [18, 19]. The effects of chronic aromatase inhibitor administration are being assessed in adjuvant therapy and prevention trials and will be key factor in determining the usefulness of these agents in long – term therapy settings.

The plasma estradiol deprivation observed after treatment with aromatase inhibitors is the risk factor for serum lipid metabolism changes in patients. Letrozole in MA-17 study of the National Cancer Institute of Canada's Clinical Trial Group did not significantly alter serum cholesterol, HDL cholesterol, LDL cholesterol or triglycerides in postmenopausal women treated up to 36 months following at least 5 years of adjuvant tamoxifen therapy [20]. Also incidence of cardiovascular events in letrozole group of MA-17 study did not reach statistical significance in comparison with control group. In our study a significant increase in serum triacylglycerols and no significant changes in total cholesterol and cholesterol in high- and low- density lipoprotein fractions were found. In the study of Goss et al. [21] letrozole given to ovariectomized rats had no effects of serum cholesterol and low-density lipoprotein cholesterol in comparison with control animals. Potential adverse effects on the cardiovascular system – specifically on lipid metabolism in clinical trials have not been conclusively demonstrated. Therefore a new generation aromatase inhibitors, including letrozole, are now being introduced for the adjuvant breast cancer treatment of postmenopausal women.

Actions of estradiol on many aspects of physiological and behavioural regulation of energy balance are well – know [22, 23]. A hypothesis about the role of estradiol in mediating leptin's effects on body weight exists. Significant increase of food intake in letrozole – treated rats characterized by increase in body weight and body fat content in our experiment was observed. This result points out to antiestrogenic effect of letrozole on food intake regulations in rats.

Aromatase inhibitors are now the first choice endocrine therapy in the metastatic setting for postmenopausal women. These endocrine agents also seem likely to soon become the standard adjuvant therapy for postmenopausal patients with hormone – responsive breast cancer, either alone or in sequence with tamoxifen, but monitoring and management of

bone decalcification associated with their application are essential and are being addressed in ongoing trials. Further studies with longer follow – up are required to clarify the effects of aromatase inhibitors on lipid metabolism and cardiovascular health. The results from the aromatase inhibitor prevention trials with the identification of breast cancer risk reduction are awaited with interest. The role of aromatase inhibitors in premenopausal breast cancer patients is area of next exploration. Premenopausal administration of letrozole in humans will be limited by adverse effects of the drug and risk/benefit ratio will be considered.

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References

[1] GOSS PE, INGLE JN, MARTINO S, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. N Engl J Med 2003; 349: 1793–1802.

[2] THÜRLIMANN BJ, KESHAVIAH A, COATES AS, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. N Engl J Med 2005; 353: 2747–2757.

[3] MOURIDSEN H, SUN Y, GERSHANOVICH M, et al. Superiority of letrozole to tamoxifen in the first-line treatment of advanced breast cancer: evidence from metastatic subgroups and a test of functional ability. Oncologist 2004; 9: 489–496.

[4] BAUM M, BUZDAR A, CUZICK J, et al. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early-stage breast cancer: results of the ATAC (Arimidex, Tamoxifen Alone or in Combination) trial efficacy and safety update analysis. Cancer 2003; 98: 1802–1810.

[5] COOMBES RC, HALL E, GIBSON LJ, et al. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. N Engl J Med 2004; 350: 1081–1092.

[6] SANTEN RJ, SAMOJLIK E, WELLS SA. Resistance of the ovary to blockade of aromatization with aminoglutethimide. J Clin Endocrinol Metab 1980; 51: 473–477.

[7] SANTEN RJ, MARTEL J, HOAGLAND F, et al. Stromal spindle cells contain aromatase in human breast tumors. J Clin Endocrinol Metab 1994; 79: 627–632.

[8] BERNSTEIN LM, LARIONOV AA, KYSHTOOBAEVA AS, et al. Aromatase in breast cancer tissue localization and relationship with reproductive status of patients. J Cancer Res Clin Oncol 1996; 122: 495–498.

[9] BULUN SE, SHARDA G, RINK J, et al. Distribution of aromatase P450 transcripts and adipose fibroblasts in the human breast. J Clin Endocrinol Metabol 1996; 91: 1273–1277.

[10] YUE W, WANG JP, HAMILTON CJ, et al. In situ aromatization enhances breast tumor estradiol levels and cellular proliferation. Cancer Res 1998; 58: 927–932.

[11] WYSOWSKI DK, COMSTOCK GW, HELSING KJ, et al. Sex hormone levels in serum in relation to the development of breast cancer. Am J Epidemiol 1987; 125: 791–799.

[12] TONIOLO PG, LEVITZ M, ZELENIUCH-JACQUOTTE A, et al. A prospective study of endogenous estrogens and breast cancer in postmenopausal women. J Natl Cancer Inst 1995; 87: 190–197.

[13] DORGAN JF, LONGCOPE C, STEPHENSON Jr HE, et al. Serum sex hormone levels are related to breast cancer risk in postmenopausal women. Environ Health Perspect 1997; 105: 583–585.

[14] SANTEN RJ, YUE W, NAFTOLIN F, et al. The potential of aromatase inhibitors in breast cancer prevention. Endocr Rel Cancer 1999; 6: 235–243.

[15] GOSS PE, STRASSER K. Aromatase inhibitors in the treatment and prevention of breast cancer. J Clin Oncol 2001; 19: 2767.

[16] FREEDMAN OC, VERMA S, CLEMONS MJ. Pre-menopusal breast cancer and aromatase inhibitors: treating a new generation of women. Breast Cancer Res Treat 2006; 99: 241–247.

[17] RUSSO J, RUSSO IH, ROGERS AE, et al. Pathology of tumors in laboratory animals. Tumors of the rat. Tumors of the mammary gland. IARC Sci Publ 1990; 99: 47–78.

[18] BHATNAGAR AS, BATZL C, HAUSLER A, et al. The role of estrogen in the feedback-regulation of follicle-stimulating-hormone secretion in the female rat. J Steroid Biochem Mol Biol 1993; 47: 161–166.

[19] SINHA S, KASETA J, SANTNER SJ, et al. Effect of CGS 20267 on ovarian aromatase and gonadotropin levels in the rat. Breast Cancer Res Treat 1998; 48: 45–51.

[20] WASAN KM, GOSS PE, PRITCHARD PH, et al. The influence of letrozole on serum lipid concentrations in postmenopausal women with primary breast cancer who have completed 5 years of adjuvant tamoxifen (NCIC CTG MA.17L). Ann Oncol 2005; 16: 707–715.

[21] GOSS PE, QI S, JOSSE RG, PRITZKER KPM, et al. The steroidal aromatase inhibitor exemestane prevents bone loss in ovariectomized rats. Bone 2004; 34: 384–392.

[22] VARMA M, CHAI JK, MEGUID MM, et al. Effect of estradiol and progesterone on daily rhythm in food intake and feeding patterns in Fischer rats. Physiol Behav 1999; 68: 99–107.

[23] PELLEYMOUNTER MA, BAKER MB, McCALEB M. Does estradiol mediate leptin's effects on adiposity and body weight? Am J Physiol 1999; 276: E955-E963.