Chemopreventive Effects of Anastrozole in a Premenopausal Breast Cancer Model

PETER KUBATKA[1], VLADIMÍRA SADLOŇOVÁ[1], KAROL KAJO[2], GABRIELA NOSÁĽOVÁ[1], DANIELA OSTATNÍKOVÁ[3] and KATARÍNA ADAMICOVÁ[2]

[1]*Department of Pharmacology, Jessenius Faculty of Medicine, Comenius University, Sklabinská 26, 037 53 Martin;*
[2]*Department of Pathological Anatomy, Jessenius Faculty of Medicine, Comenius University, Kollárová 2, 036 59 Martin;*
[3]*Institute of Physiology, Medical Faculty, Comenius University, Bratislava, Sasinkova 2, Slovak Republic*

Abstract. *Background: Monotherapy with aromatase inhibitors has no established role in premenopausal breast cancer in women and is an area of future exploration. Materials and Methods: In this study, chemopreventive effects of anastrozole in the model of N-methyl-N-nitrosourea–induced premenopausal mammary carcinogenesis in female rats were evaluated. Anastrozole was dietarily administered at two concentrations: 0.05 mg/kg (ANA 0.05) and 0.5 mg/kg (ANA 0.5). Basic parameters of experimental carcinogenesis and side-effects on selected organs after anastrozole treatment in animals were assessed. Results: In the ANA 0.5 group, anastrozole suppressed tumor incidence by 40% ($p<0.05$) and tumor frequency by 57% ($p<0.01$), as well as lengthening the latency period by 10 days ($p=0.084$) compared to control animals. Adverse effects of anastrozole on the genital system (uterus and vagina) and lipid and bone metabolism in rats were not found. Anastrozole did not alter serum concentrations of estradiol, testosterone or dehydroepiandrosterone in animals. An increase in the body weight gain of rats in the ANA 0.5 group compared with the controls ($p<0.01$) was observed. Conclusion: This study is the first about the antineoplastic effects of anastrozole in a model of premenopausal mammary carcinogenesis in female rats.*

Approximately two thirds of all breast cancers are estrogen-receptor-positive. In these cases, estrogens and their metabolites have been implicated in both initiation and progression of disease (1). The enzyme aromatase, which catalyzes the last step of estrogen synthesis, is primarily found in adipose tissue, muscle and skin, as well as in breast tissue. Aromatase inhibitors are effective in the suppression of aromatase enzyme activity and decrease circulating levels of estrogens to nearly undetectable levels in postmenopausal women. For this reason, aromatase inhibitors are primarily used in the breast cancer treatment of postmenopausal women. Recently, potent third-generation aromatase inhibitors have been introduced for breast cancer treatment: the steroidal exemestane and the non-steroidals letrozole and anastrozole. Based on results of clinical trials, exemestane (2), letrozole (3) and anastrozole (4) were approved for the treatment of metastatic breast cancer in the postmenopausal population. Recent adjuvant trials have established the value of the aforementioned drugs in the treatment of early-stage breast cancer in postmenopausal women (5-7). The tolerability of aromatase inhibitors in these trials appeared to be as good as that of tamoxifen, moreover, some serious adverse events associated with tamoxifen use were avoided.

The research on the use of aromatase inhibitors in premenopausal women with estrogen-receptor positive breast cancer is in its infancy. Treatment and prevention of premenopausal breast cancer in women by aromatase inhibitors depends upon the primary source of estradiol in breast tissue. Several experimental methods suggested the importance of *in situ* estrogen production in the mammary gland. The concept of the key role of *in situ* estradiol synthesis rather than uptake of plasma estradiol by breast tissue exists (8-12). Based on this concept, aromatase inhibitors can lower breast tissue estradiol levels without causing a reduction in plasma estradiol levels. This would protect a premenopausal patient treated with aromatase inhibitors from developing of osteoporosis, urogenital atrophy and vasomotor instability, while still reducing the

Correspondence to: Peter Kubatka, RNDr., Ph.D., Department of Medical Biology, Jessenius Faculty of Medicine, Comenius University, Malá Hora 4, 037 54 Martin, Slovak Republic. Tel: +421 434132535, Fax: +421 434134807, e-mail: kubatka@jfmed.uniba.sk

Key Words: Premenopausal, mammary carcinogenesis, rat, chemoprevention, anastrozole, aromatase inhibitors.

Table I. *Effects of anastrozole in mammary carcinogenesis in female rats at the end of the experiment.*

Group	CONT	ANA 0.05	ANA 0.5
All animals/tumor bearing animals	20/20	20/15	20/12
Tumor parameters			
Incidence (%)	100.0	75.0 (–25%)	60.0[a] (–40%)
Frequency per group*	2.45±0.34	2.45±0.54 (0%)	1.05±0.35[b,c] (–57%)
Latency* (days)	73.15±3.92	73.27±4.20	83.42±3.50 (+10 days)
Volume* (cm^3)	0.74±0.23	1.11±0.21 (+50%)	1.02±0.36 (+38%)

CONT, control group; ANA 0.05, group with anastrozole administered at a concentration of 0.05 mg/kg in food; ANA 0.05, group with anastrozole administered at a concentration of 0.5 mg/kg in food. *Data are expressed as means±SEM. Values in brackets are calculated as % deviation from the values of the control group (with the exception of latency). Significantly different: [a]$p<0.05$ *vs.* CONT, [b]$p<0.01$ *vs.* CONT, [c]$p<0.05$ *vs.* ANA 0.05.

incidence of breast cancer (13). Several clinical trials pointed to a significant association between local estrogen production and tumor growth in the breast (14-16). For these reasons, intratumoral aromatase could be an important therapeutic target against breast cancer. The effectiveness of aromatase inhibitors in premenopausal women with breast cancer and their long-term side-effects and safety profile is intensively discussed among oncologists (13, 17, 18).

In our previous experiments, the significant chemopreventive effect of letrozole (19) and apparent neoplastic effects of exemestane (submitted for publication by Kubatka *et al*., 2008) in a premenopausal model of mammary carcinogenesis in female rats were found. The main goal of this experiment that mimics the situation in high-risk premenopausal women for breast cancer is to evaluate the preventive effects of anastrozole in mammary carcinogenesis as well as the adverse effects of the drug on several organs in female rats.

Materials and Methods

Female Sprague-Dawley rats obtained from AnLab (Prague, Czech Republic) aged 30-34 days were adapted to a vivarium controlled for temperature (23±2˚C), relative humidity (50-60%) and photoperiod (12 h light: 12 h dark). During the experiment, animals drank tap water *ad libitum*. The chow containing anastrozole (Arimidex) synthesized by Astra Zeneca UK was prepared at SSNIFF Spezialdiäten GmbH (Soest, Germany). Anastrozole was administered at two concentrations in the chow: 0.05 mg/kg (0.000005%) and 0.5 mg/kg (0.00005%). Mammary carcinogenesis was induced by *N*-methyl-*N*-nitrosourea (MNU; Sigma, Deisenhofen, Germany) administered intraperitoneally in one dose of 50 mg/kg body weight on average the 42nd postnatal day. MNU was freshly prepared and dissolved in isotonic saline solution. Chemoprevention with anastrozole began 7 days before carcinogen administration and lasted until the end of the experiment, 14 weeks after MNU application. Animals were randomly assigned to one of three experimental groups: (i) control group without chemoprevention; (ii) chemoprevention with anastrozole at 0.05 mg/kg of chow (ANA 0.05); (iii) chemoprevention with anastrozole at 0.5 mg/kg of chow (ANA 0.5). Each group consisted of 20 animals. The animals were weighed and palpated weekly and the occurrence, number, location and size of each palpable tumor were registered.

In the 14th week of the experiment (dated from the MNU injection) animals were sacrificed by quick decapitation and mammary tumors, uteri, vaginas and left femori were excised. Subsequently, uteri and vaginas were weighed and the tumor size was registered. Specimens of mammary tumors, uteri and vaginas were fixed in 10% buffered formalin; 3-mm long specimens of femori taken from the middle of the diaphysis were decalcified. Specimens were embedded in paraffin using conventional automated systems. The blocks were cut to obtain 4 to 5 μm-thick sections and were stained with hematoxylin-eosin. Histopathological examination and measurement of compact bone thickness were performed by light microscopy.

At sacrifice, the blood was also 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 rat mammary carcinogenesis were evaluated: incidence, frequency, volume and latency of tumors. The effect of anastrozole on food and water intake of animals was recorded.

The tumor incidence was evaluated by Mann-Whitney test, other parameters by one-way analysis of variance or Kruskal-Wallis test. The tumor volume (V) was calculated according to:

$V=\pi (S_1)^2 S_2/12$, where S_1 and S_2 are tumor diameters ($S_1<S_2$).

The experiment was approved by the State Veterinary and Food Administration of the Slovak Republic by accreditation No. Ro-247/221-06.

Results

The significant tumor-suppressive effects of dietary administered anastrozole in a model of premenopausal mammary carcinogenesis in female rats are outlined in Table I. The antineoplastic activity of anastrozole in the group administered the higher concentration of drug (ANA 0.5) was characterized by suppression of tumor incidence and tumor frequency, as well as by lengthening of tumor latency

Table II. *Histopathological classification and number of mammary tumors.*

Mammary tumors	CRI	CRI-PAP	PAP-CRI	PAP	CRI-CYS	CRI-COM	TUB	SOLID	CRI-SOLID	SOLID-CRI-TUB	CRI-SOLID-PAP
CONT	30	10	3	3	-	1	1	1	-	-	-
ANA 0.05	32	10	4	-	1	-	-	-	-	1	1
ANA 0.5	14	5	-	-	1	-	-	-	1	-	-

CRI, cribriform; PAP, papillary; CYS, cystic; COM, comedo; TUB, tubular. Dominant type in mixed tumors is the first in order.

in rats. Tumor-suppressive effects of anastrozole administered at the lower dose (ANA 0.05) in this study were not found with the exception of a non-significant decrease of tumor incidence by 25% (p=0.074) compared to control rats. In anastrozole-treated groups, the average tumor volume was higher; the evaluation of this parameter was biased as a consequence of the higher frequency of new tumors with small volumes presented in the control group. The histopathological classification of mammary tumors is summarized in Table II.

In the groups with anastrozole, no changes in weights or histology of uteri and vaginas were found at the end of experiment. Anastrozole altered neither serum concentrations of estradiol, testosterone, dehydroepiandrosterone (Table III) nor parameters of lipid metabolism (triacylglycerols, total cholesterol and LDL- and HDL-cholesterol) in rats (data not shown). Anastrozole increased femoral compact bone thickness in animals of both groups treated with anastrozole (p<0.001). An increase in body weight gain (p<0.01) and final body weight (p<0.05) in rats were found in the ANA 0.5 group compared to controls.

The actual anastrozole doses were calculated in accordance with the amount of chow consumed, measured in the 7th and 11th week of the experiment. An average daily dose of anastrozole per rat was 0.73 μg and 7.32 μg in the ANA 0.05 and ANA 0.5 groups respectively.

Table III. *Serum levels of estradiol, testosterone and dehydroepiandrosterone after anastrozole treatment in female rats.*

Group	CONT	ANA 0.05	ANA 0.5
Estradiol (pg/ml)	22.296±0.609	25.468±2.235	24.626±2.180
Testosterone (ng/ml)	0.267±0.026	0.307±0.041	0.253±0.032
Dehydroepiandrosterone (ng/ml)	0.390±0.074	0.549±0.067	0.351±0.074

Data are expressed as means±SEM.

Discussion

This is the first study reporting the antineoplastic effects of anastrozole in a conventional (premenopausal) model of mammary gland cancer in female rats. The effective dose of anastrozole of 7.32 μg per rat used in the ANA 0.5 group of our experiment was equivalent to the daily clinical dose of Arimidex administered to postmenopausal women with breast cancer.

If local aromatization for breast tissue is crucial, aromatase inhibitors would block this *in situ* estradiol biosynthesis. Several experimental methods have been used to determine the biological importance of *in situ* estrogen production by breast tissue. Using immunohistochemical techniques, high focal levels of aromatase in breast tumors were found, supporting the concept that aromatase might act in an autocrine or paracrine fashion in breast tissue (8-10). Other data also supported the biological importance of aromatase in breast cancer tissue (11). In another experiment, administration of the aromatase substrate androstenedione stimulated growth of aromatase-positive MCF-7 cells implanted in ovariectomized nude mice, but did not cause growth stimulation in aromatase-negative MCF-7 cells (12). The same authors designed an additional experiment where the relative importance of *in situ* production *versus* uptake of plasma estradiol was found using silastic implants that produced plasma estradiol in castrated rats (12). These results led to the hypothesis that an important determinant of tissue estradiol levels (and consequently estradiol-induced carcinogenesis) is its local production in the mammary gland. This concept provides a rationale for the use of aromatase inhibitors in the treatment and prevention of breast cancer in women with functional ovaries. The design of our experiment was based on this concept.

Anastrozole used in our premenopausal breast cancer model demonstrated significant antineoplastic effects in female rats. Moreover, anastrozole did not cause any changes in serum concentrations of estradiol or its precursors in comparison with untreated animals in our study. Based on the suppression of mammary gland carcinogenesis without changes in plasma estradiol levels in rats after anastrozole treatment, our results distinctly confirm the hypothesis of the crucial role of *in situ* estradiol biosynthesis in breast tissue. In our similarly designed experiment, the remarkable tumor-suppressive effect of dietary-administered letrozole in

concentrations equivalent to clinical doses (1 mg/kg) and 10-fold higher doses (10 mg/kg) was recorded (19). In this experiment, the atrophy of the genital system in rats observed after letrozole treatment was dose dependent. Contrary to the antineoplastic effects of anastrozole and letrozole, the results of our study with the dietary-administered steroidal aromatase inhibitor exemestane in concentrations equivalent to clinical doses (10 mg/kg) and in concentrations 10 times lower (1 mg/kg) demonstrated its significant tumor-promoting activity in rat mammary carcinogenesis (submitted for publication by Kubatka *et al*., 2008). It is postulated that the effects of exemestane and its metabolite 17-hydroexemestane, with a chemical structure related to the natural estrogen precursor androstenedione, may be a result of their androgenic action through the androgen receptor (21). This may have implications for several end organ effects of exemestane, including its mitotic activity on rat mammary gland observed in our study. After oral administration of exemestane in rats, the main plasma metabolite of the drug is 17-hydroexemestane. This ligand is bound to androgen receptor, which is consequently converted into transcriptional factor that probably triggers expression of mitotic factors in mammary gland cells. This hypothesis regarding 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 or other steroids in rats. The remarkable difference in the effects of exemestane observed in postmenopausal women with breast cancer and female rats in our experiment are probably caused by species-dependent dissimilarities in the structure of gene regulation areas (promotors) of responsible genes.

Aromatase inhibitors have been proven to provide the most effective endocrine therapy in the metastatic and adjuvant setting for postmenopausal women with breast cancer. Questions remain about the long-term side-effects and safety profile of aromatase inhibitors. Results of ongoing studies may indicate a role of aromatase inhibitors in the prevention of breast cancer. The effectiveness and safety of therapy with aromatase inhibitors in premenopausal patients with breast cancer is unknown; this is an area for future exploration. Anastrozole in our model of premenopausal breast cancer demonstrated a significant antineoplastic effect without adverse effects on the genital system, or bone and lipid metabolism in female rats. Our experiments provided a strong rationale for the use of the non-steroidal aromatase inhibitors anastrozole and letrozole in premenopausal patients with breast cancer.

Acknowledgements

The experiment was supported by Comenius University grants no. 67/2007 and no. 66/2007. We thank Dr. Marko Kapalla, Head of Alpha Medical Ltd. (Institute of Laboratory Diagnostics), Ružomberok, for lipid metabolism evaluation.

References

1 Kelsey JL and Bernstein L: Epidemiology and prevention of breast cancer. Annu Rev Public Health *17*: 47-67, 1996.

2 Paridaens R, Therasse P, Dirix L, Beex L, Piccart M, Cameron D, Cufer T, Roozendaal K, Nooij M and Mattiacci MR: First-line hormonal treatment for metastatic breast cancer with exemestane or tamoxifen in postmenopausal patients – A randomized phase III trial of the EORTC Breast Group. Proc Am Soc Clin Oncol *22*: 6S, 2004.

3 Mouridsen H, Sun Y, Gershanovich M, Perez-Carrion R, Becquart D, Chaudri-Ross HA and Lang R: 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 *9*: 489-496, 2004.

4 Thürlimann B, Hess D, Koberle D, Senn I, Ballabeni P, Pagani O, Perey L, Aebi S, Rochlitz C and Goldhirsch A: Anastrozole ('Arimidex') *versus* tamoxifen as first-line therapy in postmenopausal women with advanced breast cancer: Results of the double-blind cross-over SAKK trial 21/95 – a sub-study of the TARGET (Tamoxifen or 'Arimidex' Randomized Group Efficacy and Tolerability) trial. Breast Cancer Res Treat *85*: 247-254, 2004.

5 Coombes RC, Hall E, Gibson LJ, Paridaens R, Jassem J, Delozier T, Jones SE, Alvarez I, Bertelli G, Ortmann O, Coates AS, Bajetta E, Dodwell D, Coleman E, Fallowfield LJ, Mickiewicz E, Andersen J, Lonning PE, Cocconi G, Stewart A, Stuart N, Snowdon CF, Carpentieri M, Massimini G, Bliss JM and van de Velde C: A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. N Engl J Med *350*: 1081-1092, 2004.

6 Thürlimann BJ, Keshaviah A, Coates AS, Mouridsen H, Mauriac L, Forbes JF, Paridaens R, Castiglione-Gertsch M, Gelber RD, Rabaglio M, Smith I, Wardley A, Price KN and Goldhirsch A: A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. N Engl J Med *353*: 2747-2757, 2005.

7 Baum M, Buzdar A, Cuzick J, Forbes J, Houghton J, Howell A and Sahmoud T: 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 *98*: 1802-1810, 2003.

8 Esteban JM, Warsi Z, Haniu M, Hall P, Shively JE and Chen S: Detection of intratumoral aromatase in breast carcinomas. An immunohistochemical study with clinicopathologic correlation. Am J Pathol *140*: 337-343, 1992.

9 Santen RJ, Martel J, Hoagland F, Naftolin F, Roa L, Harada N, Hafer L, Zaino R and Santner SJ: Stromal spindle cells contain aromatase in human breast tumors. J Clin Endocrinol Metab *79*: 627-632, 1994.

10 Bernstein LM, Larionov AA, Kyshtoobaeva AS, Pozharinsski KM, Semiglazov VF and Ivanova OA: Aromatase in breast cancer tissue localization and relationship with reproductive status of patients. J Cancer Res Clin Oncol *122*: 495-498, 1996.

11 Bulun SE, Sharda G, Rink J, Sharma S and Simpson ER: Distribution of aromatase P450 transcripts and adipose fibroblasts in the human breast. J Clin Endocrinol Metabol *91*: 1273-1277, 1996.

12 Yue W, Wang JP, Hamilton CJ, Demers LM and Santen RJ: *In situ* aromatization enhances breast tumor estradiol levels and cellular proliferation. Cancer Res *58*: 927-932, 1998.
13 Santen RJ, Yue W, Naftolin F, Mor G and Berstein L: The potential of aromatase inhibitors in breast cancer prevention. Endocr Rel Cancer *6*: 235-243, 1999.
14 Wysowski DK, Comstock GW, Helsing KJ and Lau HL: Sex hormone levels in serum in relation to the development of breast cancer. Am J Epidemiol *125*: 791-799, 1987.
15 Toniolo PG, Levitz M, Zeleniuch-Jacquotte A, Banerjee S, Koenig KL, Shore RE, Strax P and Pasternack BS: A prospective study of endogenous estrogens and breast cancer in postmenopausal women. J Natl Cancer Inst *87*: 190-197, 1995.
16 Dorgan JF, Longcope C, Stephenson Jr HE, Falk RT, Miller R, Franz C, Kahle L, Campbell WS, Tangrea JA and Schatzkin A: Serum sex hormone levels are related to breast cancer risk in postmenopausal women. Environ Health Perspect *105*: 583-585, 1997.
17 Goss PE and Strasser K: Aromatase inhibitors in the treatment and prevention of breast cancer. J Clin Oncol *19*: 881-894, 2001.
18 Freedman OC, Verma S and Clemons MJ: Pre-menopusal breast cancer and aromatase inhibitors: treating a new generation of women. Breast Cancer Res Treat *99*: 241-247, 2006.
19 Kubatka P, Sadloňová V, Kajo K, Nosáľová G and Fetisovová Ž: Preventive effects of letrozole in the model of premenopausal mammary carcinogenesis. Neoplasma *55*: 42-46, 2008.
20 Russo J, Russo IH, Rogers AE, van Zwieten MJ and Gusterson B: Pathology of Tumours in Laboratory Animals. Tumours of the Rat. Tumours of the Mammary Gland. IARC Sci Publ *99*: 47-78, 1990.
21 Campos SM: Aromatase inhibitors for breast cancer in post-menopausal women. Oncologist *9*: 126-136, 2004.

Received April 10, 2008
Revised June 10, 2008
Accepted June 24, 2008