A novel aromatase inhibitor, vorozole, shows antitumor activity and a decrease of tissue insulin-like growth factor-I level in 7,12-dimethylbenz[*a*]anthracene-induced rat mammary tumors.

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Abstract. Effects of vorozole, a potent and specific non-steroidal aromatase inhibitor, were evaluated on female Sprague-Dawley (SD) rats with 7,12-dimethylbenz [a] anthracene (DMBA)-induced mammary tumors. Vorozole at a dose of 0.25, 1.0 and 4.0 mg/kg was orally administered once a day for 28 consecutive days. A significant regression in tumor size was observed in each treated group at 1,2,3 and 4 weeks after the start of treatment compared with control group. Tissue insulin-like growth factor I (IGF-I) in the DMBA-induced tumors in each treated group significantly decreased in a dose dependent fashion compared with control group. These results show the mechanism of vorozole in DMBA-induced rat mammary tumors.

Introduction

Breast cancer is frequently estrogen-responsive (1) since this cancer contains estrogen receptors and its growth is stimulated by estrogens. Autocrine and paracrine mechanisms are thought to be involved in the estrogen stimulation of breast cancer cell proliferation (2). Estrogens regulate production of specific growth factors in hormone-dependent human breast cancer (3-7). However the mechanisms of estrogen responsiveness are not fully understood.

The insulin-like growth factors (IGFs) have important roles in normal cellular growth and development. The IGFs have also been implicated in regulation of tumor cell growth (8). Two ligands, IGF-I and IGF-II, have been identified that are homologous to each other and to proinsulin (9,10). They interact with at least two specific cell surface receptors. The type I IGF receptor has a high affinity for IGF-I, a somewhat lower affinity for IGF-II, and a low affinity for insulin. The type II IGF receptor has a high affinity for IGF-II and a moderate affinity for IGF-I (11). The major mitogenic effects of the IGFs are thought to be mediated through the type I IGF receptor (12). Approximately 10-fold higher concentrations of IGF-II than IGF-I were required to stimulate MCF-7 cell proliferation (13). Since IGF-I is a potent mitogen for these cells, local stromal production of IGF-I causes growth of breast cancer by paracrine stimulation (12).

Aromatase is a cytochrome P450 enzyme that catalyzes the conversion of androstendione to estrone and testosterone to estradiol (22-24). The major sites of estrogen biosynthesis are the ovarian granulosa cells in premenopausal women and adipose tissue in postmenopausal women (25). Some authors found aromatase activities in approximately two thirds of human breast cancer (26,27). Inhibition of aromatase has been found to be of therapeutic value in the endocrine treatment of breast cancer in postmenopausal women (28-30). Previous experiments also showed that binding of IGF-I to the type I IGF receptor and type I IGF receptor mRNA levels are stimulated by estradiol (13).

Vorozole (R83842) is a competitive aromatase enzyme that acts specifically to lower production of estrogens and does not compete with either estrogen or androgen receptors (31-33). This new triazole derivative reduces the aromatase activity in rat and human ovarian granulosa cells, and human stromal cells from adipose tissue (34,35).

We have examined whether vorozole alters local production of IGF-I in DMBA-induced mammary cancers of rats as a premenopausal breast cancer model.

Materials and methods

Test compound. Vorozole(+)-6-[(4-chlorophenyl)(1H-1,2,4-triazol-1-yl)methyl]-1- methyl-1H-benzotriazole) (R83842) was disolved in 20% polyethylene glycol for oral administration.

DMBA-induced rat mammary cancers. DMBA-induced rat mammary cancers were obtained as previously described (36). Rats developing solid mammary tumors within 6 - 10 weeks after the DMBA administration were divided into 4 groups

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every week for treatment. If a rat bore several tumors at the start of treatment, only the largest tumor was evaluated. The animals were housed in air-conditioned quaters illuminated from 6 a.m. to 6 p.m. and had free access to pellets (MF, Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water.

Treatment groups of rats. The rats were randomized into four groups with no significant difference of tumor size or average body weight among groups (15 rats per group). Vorozole was given by gavage at a dose of 0.25 (V0.25), 1.0 (V1.0), and 4.0 (V4.0) mg/kg body weight once a day for consecutive 4 weeks. Control group (C) received 1ml of 20% polyethylene glycol daily for the same period. The long and perpendicular axes of tumors were measured conventionally once a week with calipers. The percentage change of tumor size was calculated by product of the long and perpendicular axes. At the end of treatment for 4 weeks all the animals were sacrificed under ether anesthesia. The tumors were excised for assays, and uterine and ovarian wet weights were recorded.

Media and chemicals. IGF-I enzyme immunoassay (EIA) was performed using IGF-I EIA kit, and pre-treatment before IGF-I EIA was performed using pre-treatment reagent (acid-ethanol) from Fujisawa pharmaceutical Co., Ltd. (Tokyo, Japan). ER-EIA was performed using Abbott ER-EIA monoclonal antibodies from Abbott Laboratories, (Abbott Park, IL). The Bio-Rad protein assay dye reagent was purchased from Nippon Bio-Rad (Tokyo, Japan). The other reagents were of the highest grade available.

Sample preparation. Before assay, the specimens were cleared of fat and debris. The specimens had a wet weight of 100-300 mg. The extraction buffer contained 10 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin. Phenylmethylsulfonyl fluoride (PMSF, 1 mM) was added just before use. Samples were homogenized in four volumes of extraction buffer using a micro-homogenizer NS-310E (NICHI-ON, Japan) on ice. All debris and nuclei were removed by centrifugation at 900 × g at 4℃ for 10 min and supernatant was used as whole cell lysate. IGF-binding protein was disposed by acid-ethanol method from this lysate before EIA. The supernatant obtained was centrifuged at 105,000 × g at 4℃ for 60 min and used as the cytosol fraction. ER-EIA was performed using this fraction. ER and IGF-I in the fraction was assayed by ER and IGF-I kit respectively according to the instructions of the manufacturers. Each assay was carried out in duplicate.

Protein assays. Protein concentrations were determined with bovine serum albumin as the reference standard using Bio-Rad protein assay dye reagent (37).

Statistical analysis. Statistical analysis was performed using StatView 4.0 software (Abacus Concepts, Inc.). The data are expressed as mean±SEM. We examined parameters among groups by using analysis of variance and Fisher's PLSD test as Post-Hoc test. A p-value less than 0.05 was considered to be significant.

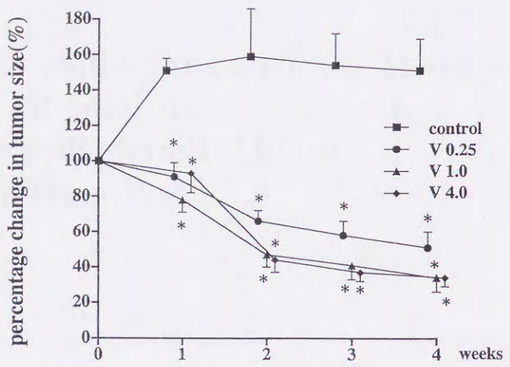

Figure 1. Antitumor effect of vorozole on DMBA-induced rat mammary tumors (15 rats per group). A significant regression in tumor size was observed in each V group 1, 2, 3 and 4 weeks after the start of treatment (*p<0.0001 vs control).

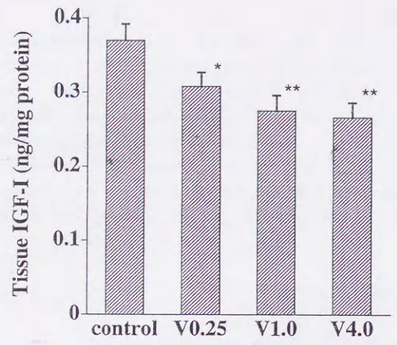

Figure 2. IGF-I in DMBA-induced rat mammary tumors. A significant decrease of tissue IGF-I was observed does-dependently in all groups treated with vorozole(*p<0.05 V0.25 vs control;**p<0.01 V1.0 and V4.0 vs control).

Results

Effects on DMBA-induced rat mammary cancer. A significant suppression of tumor growth was observed in each V group 1, 2, 3 and 4 weeks after the start of treatment (p<0.0001 vs control). There were no significant differeces among vorozole administration groups in the tumor regression (Fig.1).

IGF-I in rat mammary cancer. Tissue IGF-I were 0.370±0.022 ng/mg protein in control, 0.308±0.019 ng/mg protein in V0.25, 0.275±0.021 ng/mg protein in V1.0, and 0.266±0.02 ng/mg protein in V4.0 group, respectively. A significant decrease of tissue IGF-I was observed dose-dependently in the groups treated with vorozole (p<0.05 V0.25 vs control, p<0.01 V1.0 and V4.0 vs control Fig.2).

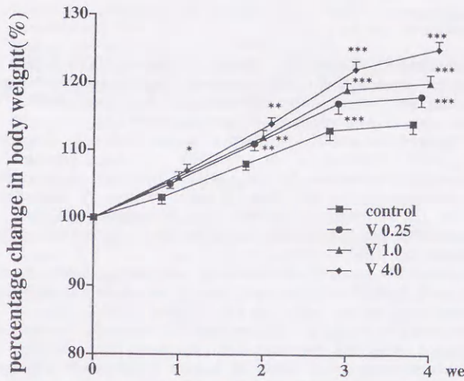

Figure 3. Body weight in female rats treated with or without vorozole. Body weight in all V administration groups increased significantly compared with that in control after 1(V4.0 only,*p=0.0045), 2(**p=0.0003), 3(*** p<0.0001) and 4 weeks(*** p<0.0001). A significant gain in body weight was observed in V4.0 group compared with V0.25 and 1.0 groups at the end of treatment.

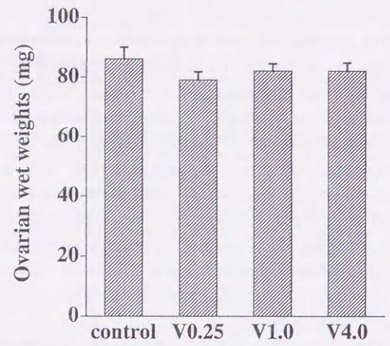

Figure 5. Ovarian wet weight. No significant difference of ovarian wet weight was observed after 4 weeks.

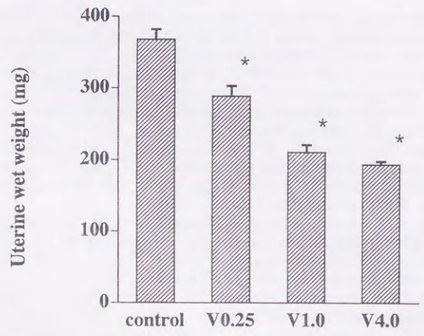

Figure 4. Uterine wet weight. A significant decrease of uterine wet weight was observed in all groups treated with vorozole after 4 weeks(*p<0.0001).

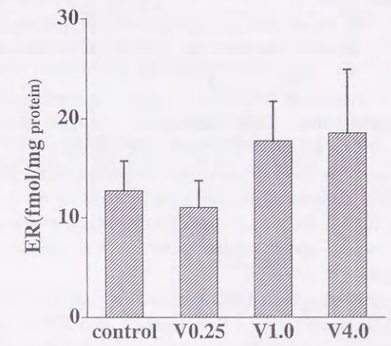

Figure 6. ER levels in DMBA-induced rat mammary tumors. No significant difference in ER levels was observed after 4 weeks. ER levels in V 1.0 and V4.0 groups tended to increase compared with those in control.

Body weight. Vorozole was well tolerated and caused no adverse drug reaction. A significant gain in body weight was observed in V4.0 group compared with control after 1 week (p=0.0045). The body weight in all V administration groups increased significantly compared with that in control after 2 (p=0.0003), 3 (p<0.0001), and 4 weeks (p<0.0001). An increase of body weight in V4.0 group compared with V0.25 and V1.0 groups was significant at the end of treatment (Fig. 3).

Uterine and ovarian wet weights. Uterine wet weights were 368±14 mg in control, 289±14 mg in V0.25, 210±10 mg in V1.0, and 194±4 mg in V4.0 group. A significant decrease in uterine wet weights was shown in each treated group compared with control group (p<0.0001) (Fig.4). There were no significant differences in ovarian wet weights among the groups. (Fig.5).

ER in rat mammary cancer. No significant difference in ER levels DMBA-induced rat mammary cancer was observed after 4 weeks (Fig.6).

Discussion

The DMBA-induced rat mammary cancer model resembles human breast cancer in its histology (38) and response to estrogen ablative therapy (35). It is widely used for evaluation of antitumoral activity of aromatase inhibitors (40,41). We demonstrated clearly the potent effect of vorozole to induce marked regression of existing mammary tumors.

At a dose of 0.25, 1.0 and 4.0 mg/kg of vorozole once a day, this new aromatase inhibitor showed antitumoral effects in this study. At a dose of 0.2 mg/kg twice a day, tumor growth was not signifcantly inhibited whereas serum estradiol levels, measured 6 h after administration, were comparable to those seen in ovariectomized animals (33). The discrepancy

probably resulted to be due to the shorter duration of aromatase inhibition at this dosage (34).

Since other studies have shown that vorozole administration lowers aromatase activities and estrogens in serum or tissue (18,19), we have not measured these activities and estrogens. In this experiment, body weights in all vorozole administration groups increased significantly compared with those in control group. And then V4.0 group showed remarked body weight gain. The body weight gain which was observed in the group treated with aromatase inhibitor can be explained to have occurred by estrogen deprivation (39). Further, doses of 0.25, 1.0, 4.0 mg/kg once a day reduced significantly uterine wet weights. These data inferred that vorozole lowered circulating estrogens in female SD rats.

We showed that tissue IGF-I levels significantly decreased in the rats treated with vorozole. Serum IGF-I level is strongly influenced by hepatic production of IGF-I. Growth hormone is the chief regulator of IGF-I gene transcription in the liver (14,15). On the other hand, in local tissues IGF-I gene is expressed by stromal cells of primary human breast cancers (16). Stromal cells of breast cancers are capable of producing IGF-I not only in response to various physiological signals (17-21), but also in response to pathophysiological signals originating from neighbouring tumor cells (16). DMBA-induced rat mammary tumors have been reported to be type I IGF receptor positive (44). It is logical to propose that IGF-I receptor positive breast cancer cells are influenced by tissue IGF-I bioactivity in their microenvironment rather than by serum IGF-I concentration. So, quantitative measurements of tissue IGF-I levels in regressed DMBA-induced rat mammary cancer may elucidate paracrine mechanism of cell proliferation.

Pekonen *et al.* reported that there was a significant positive correlation between type I IGF receptor and ER in human breast cancer tissue (45). We also observed that ER in DMBA-induced rat mammary tumors treated with vorozole were positive similarily to those in control group. But, ER levels in V1.0 and V4.0 group seemed to be slightly upregulated because estrogen levels were decreased by vorozole. Therefore it is likely that antitumoral effects of vorozole are influenced by both IGF-I and estrogens.

Ovarian wet weights in vorozole groups showed no difference compared with those in control group. This fact is consistent with the report by DeCoster and co-authers that after vorozole treatment the rise in gonadtropins was less pronounced than after ovariectomy (33). On the other hand, after treatment with nonsteroidal aromatase inhibitors (atamestane, CGS 16949A), serum gonadotropin levels and ovarian wet weights increased (42,43). It is likely that vorozole has a different action to gonad.

We conclude that the inhibitory effect on hormone-dependent tumor growth, the body weight gain of rats, the reversible effect on uterine growth, and the inhibitory effect on tissue IGF-I induction observed in the present study following vorozole administration were caused by an aromatase inhibitory action of the drug and that vorozole is an effective agent for premenopausal as well as post-menopausal patients as extrapolated from the data obtained in the present animal model.

References

1. Sutherland RL, Reddel RR, Murphy LC and Taylor IW:Effects of Antiestrogens on Cell Cycle Progression. In: Estrogen/Antiestrogen Action and Breast Cancer Therapy. VC Jordan(ed.) 1986, The university of Wiconsin Press. Wisconsin. pp265-282.
2. Sutherland RL, Reddel RR, Murphy LC and Taylor IW: Estrogens Regulate Production of Specific Growth Factors in Hormone-Dependant Human Breast Cancer. In:Estrogen/ Antiestrogen Action and Breast Cancer Therapy. VC Jordan(ed.) 1986, The university of Wiconsin Press. Wisconsin. pp237-248.
3. Sporn MB. and Roberts AB. Autocrine growth factors and cancer. Nature 313: 745-747, 1985.
4. Salomon DS, Zwiebel JA, Bano M, Losonczy I, Fehnel P, and Kidwell WR. Presence of transforming growth factors in human breast cancer cells. Cancer Res 44: 4069-4077, 1984.
5. Bronzert DA, Pantazis P, Antoniades HN, Kasid A, Davidson N, Dickson RB, and Lippman ME. Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. Proc Natl Acad Sci USA, 84:5763-5767, 1987.
6. Vignon F, Capony F, Chambon M, Freiss G, Garcia M, and Rochefort H. Autocrine growth stimulation of the MCF-7 breast cancer cells by the estrogen regulated 52Kd protein. Endocrinology 118: 1537-1545, 1986.
7. Huff KK, Kaufman D, Gabbay KH, Spencer EM, Lippman ME, and Dickson RB: Secretion of an Insulin-like Growth Factor-I-related Protein by Human Breast Cancer Cells. Cancer Res 46: 4613-4619, 1986.
8. Krywicki RF and Yee D. The insulin-like growth factor family of ligands, receptor and binding proteins. Breast Cancer Res Treat 22: 7-19, 1992.
9. Rinderknecht E, Humbel RE: The amino acid sequence of human insulin-like growth factor-I and its structural homology with proinsulin. J Biol Chem 253: 2769-2776, 1978.
10. Rinderknecht E, Humbel RE: Primary structure of human insulin-like growth factor-II. FEBS Lett 89: 283-286, 1978.
11. Baxter RC: The somatomedins: insulin-like growth factors. Adv Clin Chem 25: 49-115, 1986.
12. De Leon DD, Bakker B, Wilson DM, Hintz RL, Rosenfeld RG. Demonstration of insulin-like growth factor(IGF-I and II) receptors and binding protein in human breast cancer cell lines. Biochem Biophys Res Commun 152: 398-405, 1988.
13. Stewart AJ, Johnson MD, May FEB and Westley BR: Role of insulin-like growth factors and the type I inslin-like growth factor receptor in the estrogen-stimulated proliferation of human breast cancer cells. J Biol Chem 265: 21172-21178, 1990.
14. Roberts CT Jr, Brown AL, Graham DE, Seeling S, Berry S, Gabby KH, Rechler MM. Growth hormone regulates the abundance of insulin-like growth factor I RNA in adult rat liver. J Biol Chem 261: 10025-10028, 1986.
15. Mathews LS, Norstedt G, Palmiter RD. Regulation of insulin-like growth factor I gene expression by growth hormone. Proc Natl Acad Sci USA 83: 9343-9347, 1986.
16. Yee D, Paik S, Lebovic GS, Marcus RR, Favoni RF, Cullen KJ and Lippman ME: Analysis of insulin-like growth factor I gene expression in malignancy: Evidence for a paracrine role in human breast cancer. Mol Endo 3: 509-517, 1989.
17. D'Ercole AJ, Applewhite GT, Underwood LE: Evidence that somatomedin is synthesized by multiple tissues in the fetus. Devel Biol 75: 315-328, 1980.
18. D'Ercole AJ, Stiles AD, Underwood LE: Tissue concentration of somatomedin C: Further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. Proc Natl Acad Sci USA 81: 935-939, 1984.
19. Atkinson PR, Weidman ER, Bhaumick B, Bala RM: Release of somatomedin-like activity by cultured WI-38 human fibroblasts. Endocrinology 106: 2006-2012, 1980.
20. Clemmons DR, Underwood LE, Van Wyk JJ: Hormonal control of immunoreactive somatomedin production by cultured fibroblasts. J Clin Invest 67: 10-19, 1981.
21. Clemmons DR: Multiple hormones stimulate the production of somatomedin by cultured human fibroblasts. J Clin Endocrinol Metab 58: 850-856, 1981.
22. Grodin JM, Siiteri PK, McDonald PC. Source of esrogen production in postmenopausal women. J Clin Endoclinol Metab 36: 207-214, 1973.
23. Poortman J, Thijssen JHH, Schwarz F. Androgen production and conversion to estrogens in normal postmenopausal women and in selected breast cancer patients. J Clin Endoclinol Metab 37: 101-109, 1973.

24. Siiteri PK, Williams JE, Takaki NK. Steroid abnormalities in endometorial and breast carcinoma: a unifying hypothesis. J Steroid Biochem Mol Biol 7: 897-903, 1976.
25. Edman CD, Aiman EJ, Porter JC, McDonald PC. Identification of the estrogen product of extagrandular aromatization of plasma androstendione. Am J Obstet Gynecol 130: 439-447, 1978.
26. Tilson-Mallett N, Santner SJ, Feil PD, Santen RJ. Biological significance of aromatase activity in human breast tumors. J Clin Endoclinol Metab 57: 1125-1128, 1983.
27. Lipton A, Santner SJ, Santen RJ, Harvey HA, Feil PD, White-Hershey D, et al. Aromatase activity in primary and metastatic human breast cancer. Cancer 59: 779-782, 1987.
28. Santen RJ, Demers LM, Adlercreutz H, Harvey H, Santner S, Sanders S, Lipton A. Inhibition of aromatase with CGS16949A in postmenopausal women. J Clin Endoclinol Metab 68: 99-106,1989.
29. Santen RJ, Manni A, Harvey H, Redmond C. Endocrine treatment of breast cancer in women. Endocrine Rev 111: 221-265, 1990.
30. Lipton A, Harvey HA, Demers LM, Hanagan JR, Mulagha MT, Kochak GM, Fitzsimmons S, Sanders SI, Santen RJ. A phase I trial of CGS 16949A. Cancer 65: 1279-1285, 1990.
31. Wouters W, Van Ginckel R, Krekels M, Bowden C, DeCoster R. Pharmacology of vorozole. J Steroid Biochem Molec Biol 44: 617-621, 1993.
32. Wouters W, DeCoster R, Van Dun J, Krekels MDWG, Dillen A, Raeymaekers A, Freyne E, Van Gelder J, Sanz G, Vent M, Jansen M. Comparative effects the aromatase inhibitor R76713 and of its enantiomers R83839 and R83842 on steroid biosynthesis in vitro and in vivo. J Steroid Biochem Molec Biol 37: 1049-1054, 1990.
33. DeCoster R, Van Ginckels RF, Callens MJL, Goeminne NKG, Janssens BLE. Anti-tumoral and endocrine effects of (+)-vorozole in rats bearing dimethylbenzanthracene-induced mammary tumors. Cancer Res 52: 1240-1244, 1992.
34. Wouters W, DeCoster R, Krekels M, Van Dun J, Beerens D, Haelterman C, Raeymaekers A, Freyne E, Van Gelder J, Venet M, Janssen PAJ. R76713, a new specific non-steroidal aromatase inhibitor. J Steroid Biochem 32: 781-788, 1989.
35. Simpson-Herren L and Griswold DP Jr. Studies of the kinetics of growth and regression of 7,12- dimethylbenz(a)anthracene-induced mammary adenocarcinoma in Sprague-Dawley rats. Cancer Res 30: 813-818, 1970.
36. Iino Y, Yoshida M, Sugamata N, Maemura M, Ohwada S, Yokoe T, Ishikita T, Horiuchi R and Morishita Y. 1α-hydroxyvitamin D3, hypercalcemia, and growth suppression of 7,12-dimethylbenz(a) anthracene-induced rat mammary tumors. Breast Cancer Res Treat 22: 133-140, 1992.
37. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254, 1976.
38. Young S and Hallowes RC: Tumors of the mammary gland. In: Pathology of tumors in laboratory animals. VS Turusov (ed.), Vol. 1, Part 1, 1973, International Agency for Research on Cancer, Lyon, France, pp 31-74.
39. McElroy JF and Wade GN. Short- and long-term effects of ovariectomy on food intake, body weight, carcass composition, and brown adipose tissue in rats. Physiol Behav 39: 361-365, 1987.
40. Wing LY, Garrett WM and Brodie AM. Effects of aromatase inhibitors, aminoglutethimide, and 4-hydroxyandrostenedione on cyclic rats and rats with 7,12-dimethylbenz[a]anthracene-induced mammary tumors. Cancer Res 45: 2425-2429, 1985.
41. Schieweck K, Bhatnager AS and Matter A. CGS 16949A, a new nonsteroidal aromatase inhibitor: effects on hormone-dependent and-independent tumors in vivo. Cancer Res 48: 834-838, 1988.
42. Nishino Y, Schneider MR, Michna H and El Etreby MF. Antitumor effect of a specific aromatase inhibitor, 1-methyl-androsta -1, 4-diene-3, 17-dione (atamestane), in female rats bearing DMBA-induced mammary tumors. J Steroid Biochem 34: 435-437, 1989.
43. Tominaga T, Yoshida Y, Shimozuma K, Hayashi K and Kosaki G. Effect of CGS 16949A plus tamoxifen on induced mammary tumors in rats. Eur J Cancer 26: 600-603, 1990.
44. Ruggeri BA, Klurfeld D, Kritchevsky D, Furlanetto R. Growth factor binding to 7,12-dimethylbenz[a] anthracene-induced mammary tumors from rats subject to caloric restriction. Cancer Res 49: 4135-4141, 1989.
45. Pekonen F, Partanen S, Makinen T and Rutanen EM. Receptors for epidermal growth factor and insulin-like growth factor I and their relation to steroid receptors in human breast cancer. Cancer Res 48: 1343-1347, 1988.