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# *In Vivo* and *In Vitro* Effects of the Antifungal Agent Miconazole on Estrogen Biosynthesis in Human Breast Cancer*

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**Abstract**—*This study was undertaken to investigate whether miconazole (MCZ), an antifungal agent, inhibits aromatase activity in human breast cancer tissue preparations* (n = 6). *Aromatase activity in breast cancer tissue was significantly suppressed by an addition of MCZ* (1μ*M*). *The aromatase inhibition rate of MCZ was in the range* 15–58%.

*The change of androgen and estrogen levels was also examined in peripheral blood after administering MCZ* (200 mg) *intravenously to four patients both with breast cancer and two with systemic fungal infection. Serum androstenedione and testosterone levels fluctuated only little. However, serum estrone and estradiol levels tended to decrease after administration of MCZ. These results suggest that MCZ may be useful in the treatment of breast cancer.*

## INTRODUCTION

ESTROGEN BIOSYNTHETASE (aromatase for C-19 steroid) is the most important enzyme for estrogen production. This enzyme is present in gonadal tissue and in various other organs and tissues [1–6]. Aromatase consists of aromatase cytochrome P-450 and NADPH cytochrome P-450 reductase. Recent studies indicate that aromatase activity is significantly higher in estrogen-dependent tumors including breast cancer [7–9], uterine endometrial cancer [10, 11] and uterine leiomyoma tissues [12] than in the corresponding normal tissues. The growth of these tumors seems closely related to endogenous estrogen levels. Estrogens are mainly converted from C-19 steroids by an aromatase action. Thus, clinical use of aromatase inhibitors to decrease endogenous estrogen levels is expected to result in an effective remission of estrogen-dependent tumors.

Much research has been done on aromatase inhibitors in experiment animals both *in vivo* and *in vitro*. However, aminoglutethimide (AG) [13, 14] has been only used clinically in postmenopausal women with advanced breast cancer. AG inhibits aromatase activity but it also suppresses adrenal function: therefore glucocorticoids must be given with AG.

Miconazole (MCZ; 1-{2,4-dichloro-β-[(2,4-dichlorobenzyl)oxo]-phenethyl}imidazole nitrate), one of the antifungal imidazole group, is effective against severe pulmonary fungal disease which occurs frequently in the terminal stage of cancer patients and immunosuppressed patients. The chemical structure of MCZ contains an azole ring. Its antifungal action appears to be due to the suppression of ergosterol biosynthesis [15], and the inhibition of sterol 14α-demethylase activity (especially the binding of nitrogen atoms in the azole ring to cytochrome P-450 14DM) in yeast cells [15].

Recently, Mason *et al.* [16] and ourselves [17] reported that MCZ suppresses aromatase activities in human ovary and placental tissue preparations. Mason *et al.*, moreover, suggested that MCZ binds with the cytochrome P-450 of the human placental aromatase. However, it has been considered unnecessary to give simultaneous glucocorticoids with MCZ [18].

In this study we have investigated whether MCZ inhibits aromatase activity in human breast cancer tissue *in vitro*. We have also examined the dynamics of androgen and estrogen levels in peripheral blood after administering MCZ intravenously to patients with breast cancer.

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## PATIENTS AND METHODS

1. *Steroids and solvents*

[1β-$^3$H]Androstenedione (specific activity 27.8 Ci/mmol) and [6,7-$^3$H]estradiol-17β (specific activity 47.4 Ci/mmol) were purchased from NEN Corporation (Boston, MA, U.S.A.). The solvents used for this experiment were 1st grade and were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Co-enzyme NADPH was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). MCZ was kindly provided by Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan).

2. *The preparation of breast cancer tissue samples and the breast cancer patients*

Human breast cancer tissues used for this experiment were obtained from mastectomy of specimens from patients with breast cancer (Nos. 1–6; aged 38–79 years). The tissues obtained were stored immediately at −80°C until analysis. These patients had not received hormonal therapy prior to surgery. The changes in the serum levels of sex steroid hormones were measured in four patients with untreated breast cancer after an i.v. injection of MCZ (patient No. 7: 40 years old, height 147 cm, weight 47 kg; No. 8: 49 years old, height 158 cm, weight 51 kg; No. 9: 42 years old, height 160 cm, weight 51 kg; and No. 10: 47 years old, height 152 cm, weight 44 kg). They were informed about our research and gave their consent prior to the injection of MCZ.

3. In vitro *assay of aromatase activity in human breast cancer tissue preparation*

The following experiment was done in the cold room. The frozen breast cancer specimen (100–200 mg wet wt) was thawed rapidly; then it was minced and homogenized in 1/15 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 800 ***g*** for 10 min. The supernatant fractions obtained were divided into two samples, and each sample (approx. 0.5 ml) was mixed with or without 1 μM of MCZ. The mixture was incubated with [1β-$^3$H]androstenedione (100 pmol) and NADPH (1 mg) in air at 37°C for 2 h. The incubated sample was then cooled immediately in an ice bath and 10% trichloroacetic acid solution (0.4 ml) added to the cooled sample to terminate the enzyme reaction. Three volumes of chloroform were added to the sample, it was then mixed for 40 s with a Voltex Mixer, and centrifuged at 800 ***g*** for 10 min. The water soluble fraction obtained was eluted by Amberlite XAD-II resin–charcoal minicolumn chromatography. The eluted amount of $^3H_2O$ was measured by a Packard Tri-Carb 460 spectrophotometer, and its value was regarded as the aromatase activity for androstenedione substrate. The protein concentrations of the cancer tissue preparations were measured by a Bio-Rad protein assay kit.

4. In vitro *assay of estrogen receptor in human breast cancer tissue*

The cytosol fraction (105,000 ***g*** supernatant) of breast cancer tissue was incubated with [6,7-$^3$H]estradiol-17β ($E_2$) for estrogen receptor determination. Incubations were carried out with or without excess unlabeled diethylstibestrol. The bound form of [$^3$H]$E_2$ was obtained by dextran-coated charcoal adsorption. Specific binding was expressed as the difference of the binding in each sample with or without unlabeled steroid. A Scatchard analysis was made and the maximum binding sites as a unit of cytosol protein calculated. The detailed procedure for the receptor assay is described elsewhere [19].

5. *The dynamics of serum-free androgens and free estrogens after administration of MCZ to breast cancer patients*

Six patients, four with breast cancer (patient Nos. 7–10) and two with pulmonary candidiasis were given MCZ (Florid-F inj; 200 mg) dissolved in 200 ml of saline solution intravenous infusion over a 1 h period. The dose administered (200 mg) for the cancer patients was fixed to avoid any side-effect caused by miconazole immediately before radical surgery. Blood samples were taken from the patients before and 1, 2, 4 and 8 h after the administration of MCZ. The serum levels of androstenedione ($\Delta^4$A), testosterone (T), estrone ($E_1$) and $E_2$ were measured by radioimmunoassay kit; $\Delta^4$A was measured with an $\Delta^4$A RIA kit (DPC, LA, U.S.A.); T with a T RIA kit (DPC, LA, U.S.A.), with an $E_1$ test set (Travenol, Tokyo, Japan) and $E_2$ with an $E_2$ RIA kit (CEA IRE Sorin, Saclay, France).

## RESULTS

We used six human breast cancer (papillo-tubular adenocarcinomas) tissue samples for this *in vitro* experiment. Aromatase activity (0.19–1.20 pmol/h/100 mg protein) in the breast cancer samples was significantly suppressed by an addition of MCZ (1 μM). The aromatase inhibition rate of MCZ was in the range 15–58%. In the control samples (no incubation sample and no tissue blank sample), levels of aromatase activity were under 10 fmol/h/100 mg protein (Table 1). Aromatase activity was not related to the patient's age. In this study, the cancer tissues available for the experiment were small, so we were unable to examine aromatase activity with MCZ concentrations other than 1 μM. There seemed to be no relationship between aromatase activity in the individual sample, the existence of estrogen receptor and the degree to which the aromatase activity was inhibited by MCZ.

*Table* 1. *The inhibitory rate of MCZ* (1 μ*M*) *on aromatase activity in human breast cancer tissue. The inhibitory rate is a percentage of the value in the control sample which had not received MCZ.*

| Patient (no.) | Age (yr) | Estrogen receptor | Aromatase activity (pmol/h/100 mg protein) | Inhibitory rate of aromatase activity (%) |
|---|---|---|---|---|
| 1 | 38 | − | 0.1 9 | 1 5 |
| 2 | 51 | + | 0.3 2 | 2 8 |
| 3 | 57 | + | 0.4 6 | 2 8 |
| 4 | 49 | + | 1.2 0 | 4 4 |
| 5 | 79 | − | 0.2 3 | 3 6 |
| 6 | 50 | + | 0.1 9 | 5 8 |

Estrogen receptor ···· (+) ; above 10fmol bound/mg protein
(−) ; below 10fmol bound/mg protein

We examined the change of androgen and estrogen levels in the peripheral blood after giving MCZ to four patients with and two without breast cancer. Serum $\Delta^4$A levels and serum T levels fluctuated only minimally (Fig. 1). However, serum estrogen ($E_1$ and $E_2$) levels tended to decrease after the administration of MCZ. Four hours after the administration of MCZ, estrogen levels had almost returned to pre-treatment levels (Fig. 2). Serum $E_1$ levels in two patients without cancer (females aged 56 and 70 years) also decreased significantly 3 h after the administration of MCZ.

## DISCUSSION

This study demonstrated that MCZ significantly inhibits aromatase activity in human breast cancer tissues. However, the degree of inhibitory activity varied between the breast cancer samples. Thus,

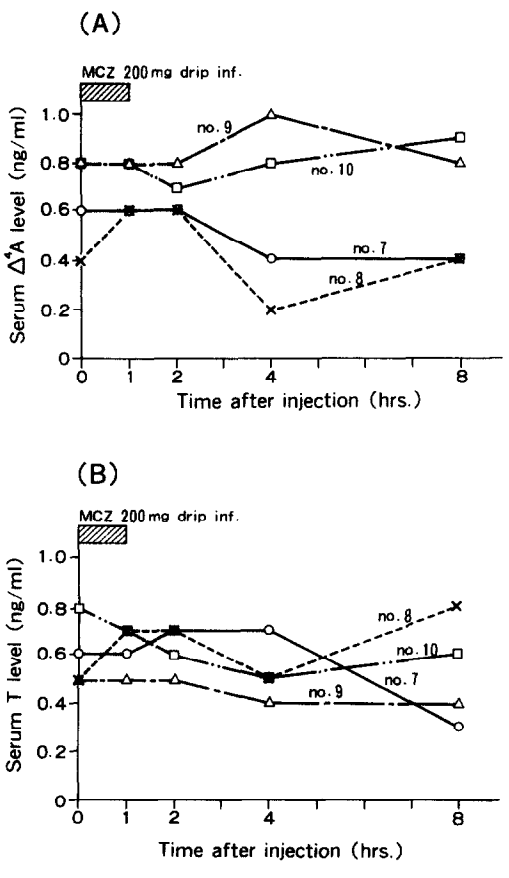


*Fig.* 1. *The change of serum androstenedione and testosterone concentrations after the intravenous infusion of MCZ* (200 mg) *to breast cancer patients.*

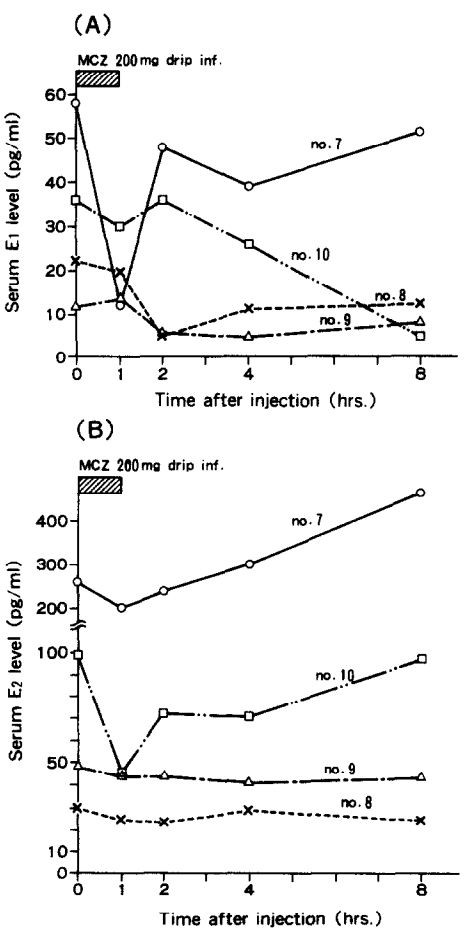


*Fig.* 2. *The change of serum estrone and estradiol concentrations after the intravenous infusion of MCZ* (200 mg) *to breast cancer patients.*

because there was no relationship between the aromatase activity in a sample and its degree of aromatase inhibition by MCZ, the aromatase activity in the individual cancer tissue may not be proportional to the corresponding protein concentration.

MCZ, an antifungal agent, binds various groups of cytochrome P-450 related to steroid hormone biosynthesis in mammals. Sheets *et al.* [20] reported that MCZ binds to cytochrome P-450s in liver microsome and inhibits hydroxylase activities related to steroid hormone. They showed that MCZ, like ketoconazole (KCZ) and clotrimazole (CTZ), inhibits 50% of 6β-, 16α- and 16β-hydroxylase activity at concentrations of 100 nM to 10 μM in rat livers. Mason *et al.* [21] indicated that MCZ and KCZ are potent inhibitors of human fetal adrenal progesterone 16α- and 17α-hydroxylase activities. However, they have less effect on progesterone 21-hydroxylase activity.

These enzyme inhibitory actions of MCZ are due to MCZ's interaction with the various cytochrome P-450s in liver or adrenal tissue, which results in selective inhibition of monoxygenase activity. Also, Mason *et al.* reported, as we [17] did, that MCZ also influences aromatase activity in human placental preparations. MCZ is a potent inhibitor of the aromatase activity of human placental microsomes ($IC_{50}$ value is 0.6 μM) [21] and of ovarian preparations [17]. KCZ, however, is a less effective inhibitor.

MCZ exhibits competitive kinetics with respect to $\Delta^4$A, the aromatase substrate [16]. Moreover, Mason *et al.* [16], using spectrophotometric studies, revealed that MCZ binds to cytochrome P-450 components of the placental microsomal aromatase complex and has negligible effects on NADPH—cytochrome *c* (P-450) reductase activity. Their report states that MCZ is nearly 70 times more effective as an inhibitor than AG, a drug which is in current use for the treatment of estrogen-dependent breast cancer. The apparent inhibitory constant ($K_i$) under assay conditions was 55 nM while the apparent $K_m$ for androstenedione was 220 nM [16]. However, MCZ is a relatively poor inhibitor of the mitochondrial cytochrome P-450-dependent cholesterol side-chain cleavage activity of a placental mitochondria-enriched fraction [16]. The cytochrome P-450 components of steroid hydroxylases are selective sites of interaction with N atoms of the azole ring in MCZ [15].

Clinically, MCZ is given intravenously to severely ill patients with systemic fungal infection. Schurmeyer *et al.* [18] reported that a continuous (24-h) high dose (1 mg/kg/h) MCZ infusion (compared to saline infusion without MCZ) does not have any significant effect on plasma concentrations of cortisol, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, dehydroepiandrosterone and $\Delta^4$A in five male rhesus monkeys. These observations suggest that MCZ may not influence adrenal steroid biosynthesis and may not alter pituitary–adrenal responsiveness to corticotropin-releasing hormone in primates. However, as Lamberts *et al.* [22] showed that MCZ inhibited 21-hydroxylase in primary culture of human adrenals, the lack of effect of MCZ on cortisol production will need to be investigated further. Coster *et al.* [23] gave single oral doses of KCZ (400 mg) as 80 mg/ml suspensions to six healthy male volunteers. Their plasma total T and $E_2$ fell to their respective minimum levels of 18 and 60% of the initial concentrations 6 h after the administration of KCZ. The recovery from both steroids began between 8 and 12 h, and was complete at 24 h [23]. Plasma LH increased 6–24 h after drug intake. The rise was not significant; plasma FSH levels were not modified by KCZ treatment [23]. Thus, it would be demonstrated that MCZ, in its clinical use, may also not influence pituitary gonadotropins. This study is the first report of the effects of MCZ in breast cancer patients.

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