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The Potent and Selective Inhibition of Estrogen Production by Non-steroidal Aromatase Inhibitor, YM511

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YM511 inhibited aromatase activities in microsomes from rat ovary and human placenta competitively (IC_{50}s: 0.4 and 0.12 nM, respectively). YM511 was about 3 times more potent than other aromatase inhibitors, such as CGS 16949A, CGS 20267 and R 76713. YM511 decreased the contents of estradiol stimulated by pregnant mare's serum gonadotropin in rat ovary with an ED_{50} of 0.002 mg/kg, indicating that YM511 was equipotent to CGS 20267 and 3 times more potent than the other two inhibitors. Serum estradiol levels in female rats were reduced by YM511 at 0.01 mg/kg into the ovariectomized range. YM511 at 1 mg/kg for 2 weeks decreased rat uterine weight to levels comparable to ovariectomy, showing it was 10 times more potent than other inhibitors. But the maximal inhibitory effect of tamoxifen failed to reach ovariectomized level. YM511 slightly inhibited production of other steroid hormones *in vitro* and *in vivo*. The IC_{50}s of YM511 for aldosterone and cortisol production from adrenal cells were from 5500 to 9800 times higher than that for rat ovarian aromatase and 130,000 times higher for testosterone production, indicating that YM511 is a highly specific aromatase inhibitor. The data suggest that YM511 may be a potent and selective agent for suppressing estrogen-dependent action without affecting serum levels of other steroid hormones.

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INTRODUCTION

Several benign and malignant diseases in women are estrogen-dependent. Among these are breast cancer, cancer of the uterine body, endometriosis and uterine myoma. A variety of endocrine therapies aiming at antiestrogen action have been attempted against these diseases. Tamoxifen, an estrogen receptor antagonist, is effective in preventing breast cancer in postmenopausal women [1]. Furthermore, it is assumed that the potential efficacy of tamoxifen as an antitumor agent in premenopausal women with breast cancer is comparable to that of ovariectomy [2]. However, tamoxifen is reported to increase the risk of cancer of the uterine body because of its estrogenic effect on the endometrium [3]. On the other hand, gonadotropin analogs inhibit estrogen production in the ovary but not in other tissues. Therefore, these analogs are restricted within estrogen-dependent diseases in premenopausal women [4]. Since aromatase is the only enzyme which converts testosterone to estradiol and is present in the ovary, fat, muscle and skin [5, 6], the inhibition of aromatase may have a potential for the treatment of estrogen-dependent diseases in postmenopausal as well as premenopausal women. However, the few clinical experiences of the use of aromatase inhibitors in premenopausal women with breast cancer did not support their use in premenopausal women. Aminoglutethimide [7] and 4-hydroxyandrostendione [8] were ineffective in suppressing estradiol levels in premenopausal women. Therefore, an attempt has been made to find more potent and selective aromatase inhibitors, such as CGS 16949A [9], CGS 20267 [10] and R 76713 [11]. Some of these compounds have been evaluated in postmenopausal women with breast cancer. CGS 16949A was reported to be from 200 to 400 times more potent than aminoglutethimide *in vitro* and was the first non-steroidal selective aromatase inhibitor for which a clinical trial was conducted [12]. The clinical study with this agent showed that this was a potent

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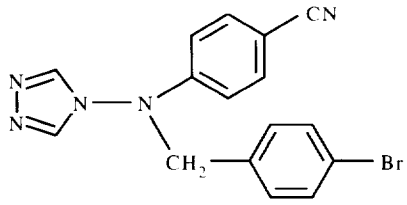

Fig. 1. Chemical structure of YM511.

estrogen suppressant in postmenopausal patients with a concomitant reduction of serum aldosterone levels, indicating that its effect was not totally selective.

In this study, we have evaluated the potency and selectivity of YM511, a novel non-steroidal compound (Fig. 1), in inhibiting aromatase activities *in vitro* and *in vivo* and have compared YM511 with other aromatase inhibitors. The results have shown that YM511 reduced serum estrogen levels and the uterine weight into the ranges induced by ovariectomy without affecting serum levels of other steroid hormones.

MATERIALS AND METHODS

Materials

The materials used in these experiments were obtained from the following sources; pregnant mare's serum gonadotropin (PMSG) and HCG, Seikagaku Co., Tokyo, Japan; [1,2-^{3}H]androstenedione, Dupont-New England Nuclear, Boston, MA, U.S.A.; NADPH, $NADP^+$, glucose-6-phosphate dehydrogenase, 1,4-dithiothreitol and collagenase, Sigma, St Louis, MO, U.S.A.; ACTH, Peptide Institute Inc., Osaka, Japan; and synacthen depot, Ciba-Geigy, Basel, Switzerland. Radioimmunoassays for estradiol, aldosterone, cortisol and testosterone were conducted using assay kits produced by Daiichi Pure Chemicals Co., Dainabot Radioisotope Laboratories Ltd., Baxter Ltd., and CIS diagnostic k.k., Tokyo, Japan, respectively. 4-[*N*-(4-bromobenzyl)-*N*-(4-cyanophenyl) amino]-4H-1,2,4,-triazole (YM511), CGS 16949A, CGS 20267 and R 76713 were synthesized at Yamanouchi Pharmaceutical Co., Ltd., Tsukuba, Japan.

Preparation of rat ovarian and human placental microsomes

Rat ovarian microsomes were prepared according to the method described by Steele *et al.* [9]. Briefly, female Wistar rats at 3 weeks of age, were administered 100 IU of PMSG. Four days later, the rats were sacrificed and ovaries were removed. The ovarian tissues were rinsed with ice-cold 0.15 M KCl and homogenized in 0.25 M sucrose by a polytron homogenizer (Kinematica GMBH, Lucerne, Switzerland). The homogenate was centrifuged at 20,000 *g* for 30 min and the resulting supernatant was centrifuged again at 148,000 *g* for 60 min. The pellet of microsomes was resuspended in 0.05 M potassium phosphate buffer, pH 7.4, and centrifuged again at 148,000 *g* for 60 min. The resulting pellet was resuspended in the above phosphate buffer to prepare the microsome fraction. Human placental microsomes were prepared by the protocol reported by Bullion *et al.* [13] with a minor modification. Briefly, the placenta was freed of membranes and large blood vessels and rinsed with 0.067 M potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose and 0.5 mM 1,4-dithiothreitol and homogenized with a Polytron homogenizer. The homogenate was centrifuged at 12,000 *g* for 20 min and the supernatant was centrifuged again at 105,000 *g* for 60 min. The microsomal pellet was resuspended in 0.067 M potassium phosphate buffer including 20% glycerol, 0.5 mM EDTA and 0.5 mM 1,4-dithiothreitol. The pellet was resuspended with the above same buffer.

Aromatase activity in rat ovarian and human placental microsomes

[1β,2β-^{3}H]androstenedione was incubated with rat ovarian or human placental microsomes in potassium phosphate buffer (pH 7.4). The incubation medium also contained various concentrations of test compounds dissolved in dimethyl sulfoxide (final conc. 0.5%) in the presence of NADPH regenerating system [14] or 5 mM NADPH [13]. The reaction mixture was treated with chloroform and 2.5% activated charcoal to remove residual steroids. The radioactivity in the aliquot of the supernatant was determined by a Packard liquid scintillation spectrometer (model 2500TR). The IC_{50} for aromatase activity was obtained from the linear line drawn by the method of least-squares fitting.

Aldosterone, cortisol and testosterone syntheses from isolated adrenal or testicular cells

Adrenal or testicular cells were prepared from rats or rabbits by collagenase digestion (200 IU/ml) at 37°C. The cells were used after washing twice with 199 medium or Eagle's Minimal Essential Medium (MEM) containing 0.2% BSA (Nissiu Pharmaceuticals, Tokyo) [15].

Aldosterone synthesis in rat adrenal cells. Cells suspended (3×10^5 cells/ml) in 199 medium containing 0.2% bovine serum albumin were preincubated at 37°C for 30 min with various concentrations of test compounds dissolved in dimethyl sulfoxide. This was then followed by a 2 h incubation with 1 ng/ml ACTH to stimulate aldosterone synthesis. The amount of aldosterone released from the cells in the presence or absence of the test compound was measured using RIA.

Cortisol synthesis in rabbit adrenal cells. Cells suspended (5×10^5 cells/ml) in MEM containing 0.2% BSA were preincubated at 37°C for 30 min in the presence of various concentrations of test compounds. Then the cells were incubated for 2 h with 0.1 ng/ml ACTH to stimulate cortisol synthesis. The amount of cortisol released from the cells was measured using RIA.

*Table 1. Inhibitory effects of YM511 on rat ovarian and human placental aromatase activities**

	Inhibition of aromatase activities IC_{50} (nM)	
	Rat ovarian microsomes	Human placental microsomes
YM511	0.4	0.12
CGS 16949A	1.5	0.37
CGS 20267	1.8	0.39
R 76713	2.3	0.33

*Results expressed as the concentration required to inhibit enzyme activity by 50% (IC_{50}).

Testosterone synthesis in rat testicular cells. Cells suspended (1×10^6 cells/ml) in 199 medium containing 0.2% BSA were preincubated at 37°C for 45 min with various concentrations of test compounds. Then the cells were incubated for 2 h with 0.5 IU/ml HCG to stimulate testosterone synthesis. The amount of testosterone released from the cells was measured using RIA.

PMSG-stimulated estradiol syntheses in 3-week-old rats

The *in vivo* inhibition of aromatase activity by the test compounds was evaluated by the methods shown elsewhere [9, 11]. Briefly, 100 IU of PMSG was administered to female Wistar rats. Three days after the PMSG administration, test compounds were administered orally and then estradiol synthesis in the ovary was measured over a period up to 24 h. Estradiol was extracted with chloroform from the homogenate of the ovaries and measured by RIA.

Serum concentration of estrogen and uterine weight in female adults rats

Three hours after YM511 administration or ovariectomy, serum concentrations of estradiol were measured by RIA. The test compound or a vehicle was administered to Wistar rats or ovariectomized rats for 2 weeks. After the last dosing, the uterine weight was measured.

Production of aldosterone and cortisol in vivo

Serum concentrations of aldosterone and cortisol were measured according to the method described by Häusler *et al.* [16]. ACTH (synacthen depot, 1 mg/kg), which stimulated the production of aldosterone and cortisol, was administered subcutaneously to male Wistar rats or guinea pigs at 10 weeks of age. 15 h later, blood samples were collected from the animals which had been treated orally with the test compound 3 h before. The experimental protocols in the present study were approved by the local animal ethics committee for animal studies.

Statistical analyses

Comparisons between experimental groups were made using One-way ANOVA, Newman–Keuls multiple range test. Differences were accepted as significant at the $P < 0.05$ level.

RESULTS

Inhibitory effects of YM511 on rat ovarian and human placental aromatase activities

YM511 inhibited aromatase activity of rat ovarian microsomes with an IC_{50} of 0.4 nM. YM511 also inhibited the activity of aromatase obtained from human placenta and its IC_{50} was 0.12 nM, indicating that YM511 was at least 3 times more potent than other agents examined (Table 1). Kinetic analysis indicated that YM511 was a competitive inhibitor with a K_i of 0.11 nM (Fig. 2).

In vivo inhibitory effect of YM511 on rat ovarian estrogen content stimulated by PMSG

The time-dependent inhibition by YM511 and CGS 16949A on PMSG-stimulated estradiol content in rat ovaries was observed *in vivo*. The maximal inhibition was seen 3 h after the administration of the compounds [Fig. 3(A)]. About a 50% reduction of the estrogen level by YM511 was still seen 24 h after the dosing. YM511 decreased estradiol contents in a dose-dependent manner with the ED_{50} of 0.002 mg/kg, 3 h after the administration [Fig. 3(B)]. YM511 was

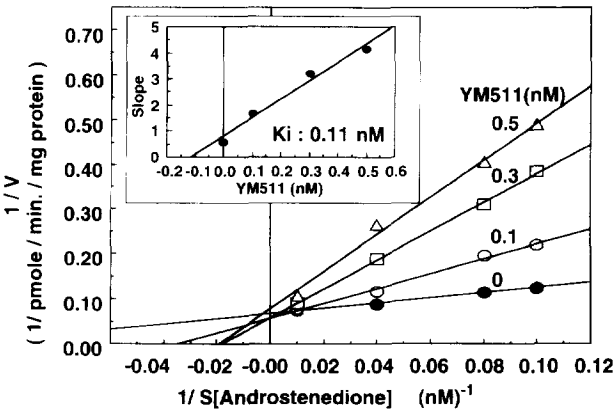

Fig. 2. Lineweaver–Burk plots showing the competitive inhibition of human placental aromatase by YM511. Experimental conditions were as in Materials and Methods.

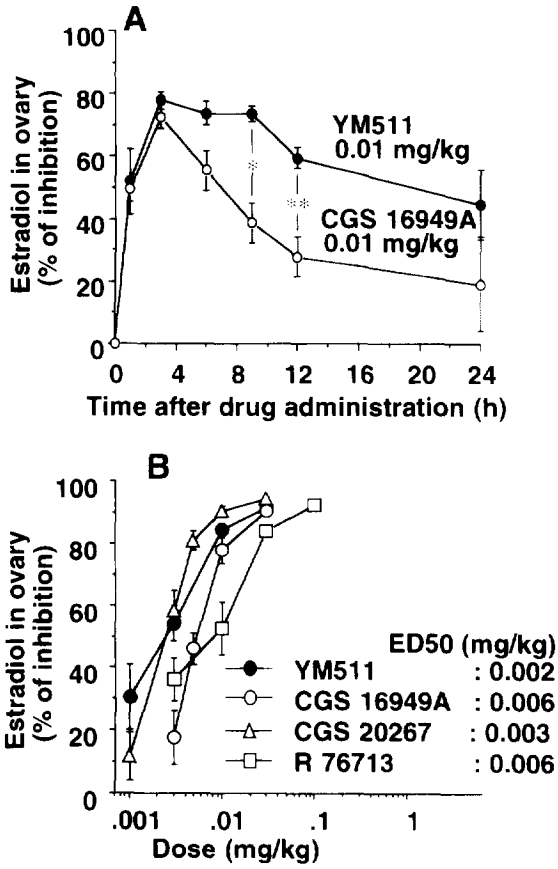

Fig. 3. Time- and dose-dependent inhibition by YM511 on estradiol production stimulated by PMSG in rat ovary. PMSG (100 IU) was administered to female Wistar rats at 3 weeks of age. 3 days later, the test compound was administered orally to the rats. (A) Estradiol content in the ovary was measured over a period up to 24 h. (B) A dose-response relationship between YM511, CGS16949A, CGS20267 and R76713 was observed 3 h after the dosing of the test compounds. Each point with a vertical line shows mean ± SEM from 5–15 rats. $*P < 0.05$, $**P < 0.01$, the effect of YM511 was significantly different from CGS 16949A.

equipotent to CGS 20267 and then three times as potent as CGS 16949A and R 76713.

Reduction of serum concentration of estradiol in female rats

As shown in Fig. 4, YM511 dose-dependently reduced the serum concentration of estradiol in normal adult female rats. The maximum reduction in estradiol level was at a dose of 0.01 mg/kg or more. The decrease in estradiol levels in YM511-treated rats was comparable to that in ovariectomized rats.

Reduction of the uterine weight in female rats

YM511 reduced uterine weight dose-dependently. The inhibitory effect of YM511 at a dose of 1 mg/kg for 2 weeks was comparable to that of ovariectomy (Fig. 5). On the other hand, YM511 was ten times more potent than CGS 16949A, R 76713 and CGS 20267 since 10 times higher doses were required to exert effects comparable to YM511. The maximal inhibitory effect of tamoxifen did not reach ovariectomy level because the saturation of inhibitory action was observed at a dose of 1 mg/kg or more.

Effects of YM511 on aldosterone, cortisol and testosterone syntheses in isolated adrenal and testicular cells from rats and rabbits

In order to confirm that YM511 is a very specific inhibitor of aromatase, effects of YM511 on the syntheses of other steroid hormones were examined *in vitro*. YM511 inhibited aldosterone production from rat adrenal cells and cortisol production from rabbit adrenal cells with the IC_{50}s of 2.19 and 3.9 μM, respectively (Table 2). YM511 showed a very weak inhibitory effect on testosterone production from rat testicular cells (IC_{50}: 52.9 μM). The IC_{50}s of YM511 for aldosterone and cortisol production were from 5500 to 9800 times higher than that for rat ovarian aromatase and 130,000 times more in the case of testosterone production, indicating that YM511 is a highly specific aromatase inhibitor.

Effects of YM511 on serum concentrations of aldosterone and cortisol in vivo

Consistent with the specificity of YM511 to aromatase inhibition *in vitro*, YM511 at the highest dose of 100 mg/kg used in this study, decreased neither aldosterone levels in rats nor cortisol levels in guinea pigs (Table 3). However, CGS 16949A, R 76713 and CGS 20267 significantly reduced serum concentrations of aldosterone in rats (ED_{50}s: 0.26, 4.7 and 32.6 mg/kg, respectively).

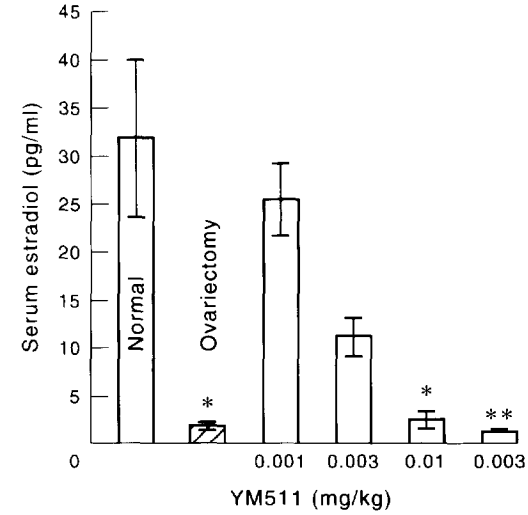

Fig. 4. Effects of YM511 and ovariectomy on serum concentrations of estradiol in adult female rats. 3 h after YM511 administration or ovariectomy, serum concentrations of estradiol were measured by RIA. Each column represents mean ± SEM from 5 rats. $*P < 0.05$, $**P < 0.01$, compared with normal rat.

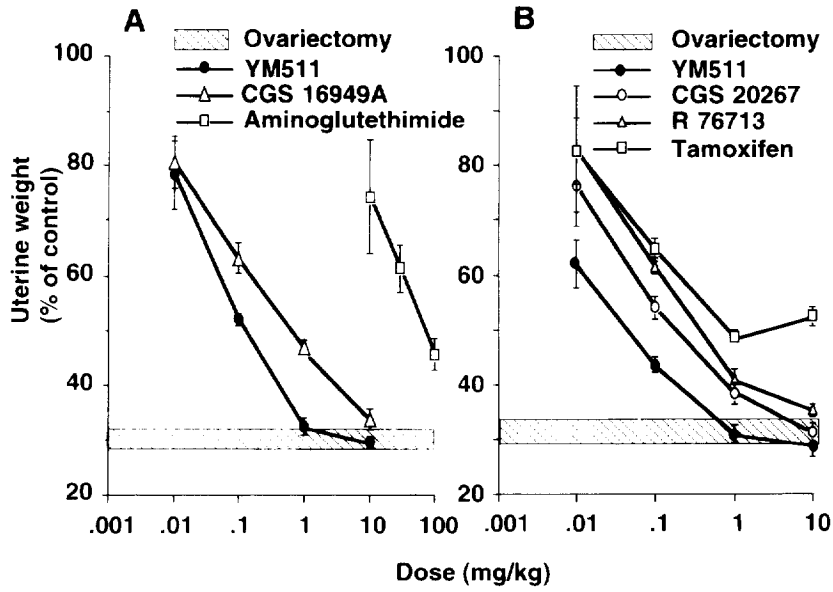

Fig. 5. Effects of YM511 and ovariectomy on the uterine weight in female rats. The test compound or a vehicle was administered to Wistar rats or ovariectomized rats for 2 weeks. After the last dosing, the uterine weight was measured. Each point with a vertical line shows mean ± SEM from 5 rats.

*Table 2. Effects of YM511 on aldosterone, cortisol and testosterone synthesis in adrenal and testicular cells**

	Inhibition of synthesis IC_{50} (μM)		
	Aldosterone (Rat adrenal cells)	Cortisol (Rabbit adrenal cells)	Testosterone (Rat testicular cells)
YM511	2.19	3.9	52.9
CGS 16949A	0.04	2.4	1.8
CGS 20267	2.61	1.6	18.4
R 76713	0.60	3.3	4.7

*Results expressed as the concentration required to inhibit steroid production by 50% (IC_{50}).

*Table 3. Effects of YM511 on ACTH-induced aldosterone and cortisol synthesis in vivo**

	ED_{50} (mg/kg)			
	ACTH-induced aldosterone synthesis in rats		ACTH-induced cortisol synthesis in guinea pigs	
	n†		n	
YM511	5	no effect (100 mg/kg)	5	no effect (100 mg/kg)
CGS 16949A	40	0.26	0	n.d.‡
CGS 20267	65	32.6	0	n.d.
R 76713	15	4.7	0	n.d.

*Results expressed as the dose required to decrease the serum concentration of aldosterone or cortisol by 50% (ED_{50}).
†Number of animals used in each experiment.
‡Not determined.

DISCUSSION

Aminoglutethimide has been shown to inhibit estrogen production almost completely in postmenopausal women with breast cancer [7]. The reduction in serum estrogen levels by this agent was attributed to the inhibition of aromatase activity analyzed by isotope kinetic method in a clinical pharmacological study [17]. However, aminoglutethimide was not potent enough to lower plasma concentration of estradiol below that observed in normal menstruating women [8]. The IC_{50} of CGS 16949A for aromatase activity was 1.7 nM,

indicating that CGS 16949A was 180 times more potent than aminoglutethimide (0.3 μM) [9]. However, CGS 16949A was not a totally specific inhibitor for aromatase in postmenopausal women because it reduced aldosterone concentration at almost the same range of dosing where a decrease in estrogen levels was observed [12]. Recently, it was reported that CGS 20267 and R 76713 were potent and selective aromatase inhibitors [18, 19] but it has not yet examined that these two inhibitors are potent enough to lower estrogen levels in premenopausal women.

The present study clearly showed that YM511 was a potent and selective inhibitor of aromatase based on the following evidence. YM511 inhibited aromatase activities in microsomes from rat ovary and human placenta (Table 1). YM511 decreased ovarian contents of estradiol stimulated by PMSG in rats (Fig. 3). The potency of YM511 with an ED_{50} of 0.002 mg/kg was equipotent to CGS 20267 and three times more potent than CGS 16949A and R 76713. As shown in Fig. 4 using normal female rats, YM511 at a dose of 0.01 mg/kg suppressed serum concentration of estradiol into the range of ovariectomized rats 3 h after the dosing, indicating that the onset of YM511 action was very rapid. YM511 at a dose of 1 mg/kg for 2 weeks decreased uterine weight comparable to ovariectomy but the same levels of reduction of the uterus were not observed until ten times higher doses of CGS 20267, CGS 16949A and R 76713 were given to rats (Fig. 5). These results indicate that the dose of YM511, as well as the other three aromatase inhibitors required for reduction of uterine weight to ovariectomized level, was about 500–3000 times higher than that which suppressed estradiol content in ovary by 50% [calculated from the data in Figs 3(B) and 5]. Such a dose difference with aromatase inhibitors was also reported by others [9, 10].

One of the possibilities which causes the dose difference observed above is the duration of estrogen deprivation induced by ovariectomy and YM511 treatment. As shown in Fig. 3(A), only a 50% reduction of the ovarian level of estrogen by YM511 at a dose of 0.01 mg/kg was observed 24 h after the dosing. This incomplete suppression of estrogen synthesis over 24 h may cause the difference in the regression of the uterus between the two treatments. Therefore, it is likely that the higher dose of YM511 was required to completely suppress the serum concentration of estrogen comparable to ovariectomy. The second possibility is an increase in testosterone level, a substrate of aromatase, which is competing with aromatase inhibitor for aromatase. Testosterone might be increased by gonadotropin, LH, through a feedback mechanism after estrogen deprivation [20]. However, there was no change in serum testosterone level in rats treated with doses of 0.01 and 0.1 mg/kg YM511 although about a 3-fold increase in testosterone level was observed after treatment with 1 mg/kg YM511 (unpublished observations, M. Kudoh). Thus, it was unlikely that an increase in testosterone levels was responsible for the dose difference. The third possibility is the role of progesterone in leading to the development of the endometrium. However, this is not the case because the serum concentration of progesterone was not increased but rather decreased by YM511 (unpublished observations, M. Kudoh). One of the most important findings in this study is that YM511 decreased serum concentrations of estradiol and the uterine weight into the ranges induced by ovariectomy. Tamoxifen, however, did not achieve the same effect. Thus, YM511 could be useful for the treatment of estrogen-dependent diseases.

In accord with the clinical report showing that CGS 16949A significantly reduced serum concentrations of aldosterone [21], this agent inhibited aldosterone production from rat adrenal cells dose-dependently with an IC_{50} of 0.04 μM (Table 2) and also decreased serum concentrations of aldosterone in rats with an ED_{50} of 0.26 mg/kg (Table 3). On the other hand, the IC_{50} of YM511 for aldosterone production from rat adrenal cells was 2.19 μM. This means that inhibition of aromatase activity by YM511 is 5500 times more potent than inhibition of aldosterone production by YM511. YM511 had no inhibitory effect on serum concentrations of aldosterone in rats at a dose of 100 mg/kg, indicating that YM511 was a very weak inhibitor of aldosterone compared to CGS 16949A. Additionally YM511 had a very weak inhibitory effect on cortisol production *in vitro* and *in vivo* (Tables 2 and 3) and also a negligible effect on testosterone production (Table 2). It was reported that CGS 20267 administered for 28 days had no effect on the circulating levels of aldosterone, cortisol, FSH and LH in postmenopausal patients with advanced breast cancer, although serum concentrations of estradiol and estrone were decreased below the limit of detection of the assay [22]. This result suggests that CGS 20267 is a clinically useful aromatase inhibitor which significantly decreases estrogen levels at a dose which lacks significant endocrine side effects. If a ratio of the IC_{50} for rat aromatase inhibition compared to the IC_{50} for aldosterone production from adrenal cells is expressed as an index showing the selectivity of aromatase inhibitor, the ratios of YM511 and CGS 20267 are 5500 and 1500, respectively (see Tables 1 and 2). This index suggests that YM511 is a more than 3 times more specific aromatase inhibitor than CGS 20267. Therefore, it is conceivable that YM511, as well as CGS 20267, is a potent and specific aromatase inhibitor and clinically useful for the treatment of estrogen-dependent diseases, such as breast cancer.

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