

A New Aromatase Inhibitor, FR901537

II. Pharmacological and Antitumor Effects

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The pharmacological and antitumor effects of FR901537, a new aromatase inhibitor, isolated from *Bacillus* sp. No. 3072, were studied. Treatment for four consecutive days with FR901537 inhibited the androstenedione-induced increase in the uterus weight in immature rats. FR901537 had no effect on the uterus, adrenal glands, ovary or pituitary weights in mature rats following 14 days of treatment. The antitumor activity of FR901537 on 7,12-dimethylbenz(a)anthracene-induced mammary tumors was studied in ovariectomized, testosterone propionate (TP)-treated rats as a postmenopausal tumor model. Ovariectomy caused the regression of the mammary tumors and the growth of tumors was remarkably stimulated following TP treatment. Further, in the rats treated with FR901537 and TP, the TP-induced tumor growth was significantly inhibited by FR901537. These results suggest that FR901537 is a promising drug in the treatment of estrogen-dependent mammary tumors in postmenopausal women.

The antiestrogen tamoxifen, which competes with estrogen for its receptor, is being used as a first line drug in endocrine therapy for estrogen-dependent breast cancers¹). However, a strategy using aromatase inhibitors has recently aroused considerable interest^{2,3}). Aromatase is a cytochrome P-450 enzyme responsible for the conversion of androgens to estrogens and believed to be a key enzyme in the biosynthesis of estrogens⁴). Therefore, inhibitors of this enzyme should reduce the supply of estrogen to breast cancer cells and may be of value in the treatment of estrogen-dependent breast cancers^{5,6}).

From our screening program for aromatase inhibitors from microbial products, we found that *Bacillus* sp. No. 3072 produced a new aromatase inhibitor, FR901537, which has a unique chemical structure as shown in Fig. 1, and that it has a potent and specific inhibitory activity against human placental and rat ovarian aromatases⁷).

In this study we examined the pharmacological actions of FR901537 *in vivo* and the antitumor activity of FR901537 on 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat mammary tumors, and compared them with those of aminoglutethimide (AG), the first aromatase inhibitor developed⁸).

Materials and Methods

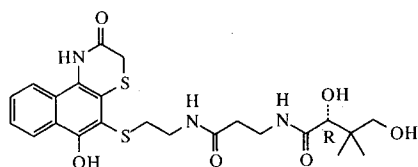
Chemicals

FR901537 was prepared in our Research Laboratories. AG and androstenedione were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Testosterone propionate (TP) was purchased from Nacalai Tesque, Kyoto, Japan and DMBA from Tokyo Kasei Kogyo Co., Tokyo, Japan. FR901537 and AG were suspended in 0.5% methylcellulose (MC) in water and given orally at a volume of 5 ml/kg body weight. 0.5% MC in water was given to the control rats. Androstenedione was suspended in sesame oil and given subcutaneously at a volume of 2 ml/kg body weight. TP was dissolved in 5% benzyl alcohol-95% sesame oil and given subcutaneously at a volume of 2 ml/kg body weight.

Animals

Female Sprague-Dawley rats were purchased from Japan SLC, Inc., Shizuoka, Japan. The rats were kept in conditions of constant temperature and humidity and fed a standard diet and water *ad libitum*.

Fig. 1. Structure of FR901537.



Evaluation in Immature Rats

Drug and androstenedione (32 mg/kg) were given orally and subcutaneously, respectively to immature rats (21 days old) once a day for 4 consecutive days. Four hours after the final drug treatment, rats were killed and their uteri were removed. The uteri were weighed and calculated as mg/100 g of body weight.

Evaluation in Mature Rats

Drug was given orally to mature rats (7 weeks old) once a day for 14 consecutive days and on the day following the last drug treatment the body weight and weights of uteri, ovary, adrenal glands and pituitary were measured. The weight of each organ was calculated as mg/100 g of body weight. The body weight gain was calculated as the difference between the body weight on the first and final days of drug treatment.

Antitumor Activity against DMBA-induced Mammary Tumors in Ovariectomized Rats

DMBA (20 mg) was given orally to rats (7 weeks old) to induce the mammary tumors. When the tumors were induced and deemed of sufficient size to start the experiment, 77 days after DMBA treatment, the rats were ovariectomized under pentobarbital anesthesia. TP (20 mg/kg) was given subcutaneously three times a week for 3 weeks starting 1 day after ovariectomy to maintain the tumor growth. Drug was given orally twice a day for 3 weeks at a dose of 32 mg/kg per each treatment starting 1 day after ovariectomy.

The two perpendicular axes of tumors were measured once a week and the tumor area was calculated as the product of the two axes. The body weights of rats were checked once a week and the appearance of the new tumors was also examined. Three hours after the last drug treatment, blood was collected under ether anesthesia. The serum 17β -estradiol (E_2) level was measured after ether extraction, using E_2 radioimmunoassay (RIA) kits purchased from Diagnostic Products Corporation, Los Angeles, CA, U.S.A. Blood was removed from the non-ovariectomized normal group, the same batch of rats as used in this DMBA experiment.

At the end of the treatment period, the response of individual tumors to the drug was classified as follows; CR (complete remission, disappearance of the tumor), PR (partial remission, reduction of $> 50\%$ in the initial tumor area), NC (no change, increase or decrease of $< 50\%$ in the initial tumor area) or PD (Progressive disease, increase of $> 50\%$ in the initial tumor area). The tumor regression rate was expressed as the sum of CR% and PR%.

Statistical Analysis

Analysis of variance was performed and the Dunnett test was used to determine the significance of differences.

Results

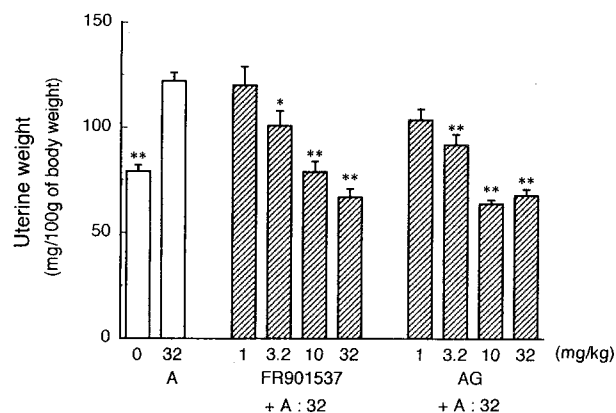
Effect in Immature Rats

The growth of uteri is modulated by estrogen in rats⁹ and when androstenedione is administered to immature rats, estrogen is produced from the administered androstenedione by aromatase, leading to a stimulation of the uterine growth. Therefore, to evaluate the inhibitory activity of FR901537 or AG on aromatase *in vivo*, drug was given to immature rats with androstenedione and the effect on the androstenedione-induced uterine growth was examined. As shown in Fig. 2, androstenedione induced a significant increase in the uterine weight which was dose dependently inhibited by FR901537, being completely counteracted at doses higher than 10 mg/kg. AG in doses of 10 and 32 mg/kg also inhibited this increase in uterine weight. The ED_{50} of FR901537 and AG was 3.1 mg/kg and 1.4 mg/kg, respectively.

Effect in Mature Rats

Inhibition of estrogen synthesis *in vivo* causes a decrease in the uterine weight of mature rats, since the uterine growth is maintained by estrogens⁹. Inhibition of steroidogenic enzymes including side chain cleaving enzyme *in vivo*, by drug such as AG, results in the induction of hypertrophy of endocrine organs such as adrenal glands in rats¹⁰⁻¹²). Therefore, to evaluate the specificity of inhibitory activity of FR901537 or AG on aromatase or other steroidogenic enzymes *in vivo*, drug was given to mature rats for 14 days and the effect on

Fig. 2. Effects of FR901537 and AG on androstenedione-induced uterine growth in immature rats.



Drug was given orally to immature rats with androstenedione (A), and their uteri were removed and weighed. Each group comprised of 10 rats. Each column represents the mean \pm SE.

***: $P < 0.05$, $P < 0.01$, compared with the androstenedione-treated group (Dunnett test).

Table 1. Effects on endocrine organs in mature rats.

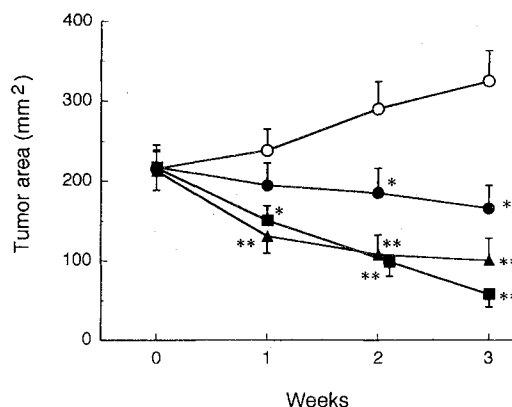
Drug	Dose (mg/kg)	Body weight gain(g)	mg/100g of body weight			
			Uterus	Ovaries	Adrenals	Pituitary
Control		47 ± 3 a)	189 ± 18	45 ± 1	29 ± 1	5.0 ± 0.2
FR901537	3.2	45 ± 2	168 ± 14	50 ± 2	29 ± 1	NT b)
	10	43 ± 2	198 ± 27	50 ± 2	29 ± 0	NT
	32	45 ± 2	195 ± 31	50 ± 2	32 ± 2	5.3 ± 0.2
AG	3.2	47 ± 2	163 ± 16	49 ± 1	34 ± 1*	NT
	10	48 ± 2	136 ± 6	54 ± 2**	41 ± 2**	5.8 ± 0.5
	32	53 ± 2	131 ± 10	61 ± 2**	48 ± 1**	4.4 ± 0.1

Drug was given orally to female mature rats once a day for 14 days and the endocrine organs were removed and weighed. Each group comprised of 10 rats.

***: $P < 0.05$, $P < 0.01$, compared with the control group (Dunnett test).

a), Mean ± SE. b), NT: Not tested.

Fig. 3. Antitumor effects of FR901537 and AG on DMBA-induced rat mammary tumors.



○: Ovariectomy (OVX) + TP, ●: OVX + TP + FR901537, ▲: OVX + TP + AG, ■: OVX alone. Rats with DMBA-induced mammary tumors were ovariectomized 77 days after DMBA treatment. TP was given subcutaneously three times a week for 3 weeks and drug was given orally twice a day for 3 weeks at a dose of 32 mg/kg per each treatment. The two perpendicular axes of the tumors were measured once a week and the tumor area was calculated as the product of the two axes. No. of rats used in each group is shown in Table 2. Each point represents the mean ± SE.

***: $P < 0.05$, $P < 0.01$, compared with the TP-treated, ovariectomized group (Dunnett test).

the weight of endocrine organs was examined. Treatment with AG caused a significant increase in the adrenal and ovarian weights in doses higher than 3.2 and 10 mg/kg, respectively, and a decrease in uterine weight, but did not affect the pituitary weight of mature rats. On the other hand, FR901537 in all doses tested had no effect on weights of these endocrine organs (Table 1).

Antitumor Effect in DMBA-induced Mammary Tumors

The antitumor effects of FR901537 and AG (32 mg/kg

twice a day) on the DMBA-induced mammary tumors were studied in ovariectomized, TP-treated rats as a postmenopausal tumor model. The results are shown in Fig. 3. In ovariectomized rats, the tumor area calculated from the length of two axes was progressively reduced and 3 weeks after ovariectomy it was decreased to about 25% of the initial tumor area. When TP was given to ovariectomized rats, the tumor area increased remarkably and 3 weeks after the starting of treatment it reached about 150% of the initial tumor area. The increase in the tumor area following TP treatment was significantly, although not completely, inhibited by FR901537. With AG treated-rats, a rapid and complete inhibition in the increase of the tumor area was observed. The effect of AG was almost the same as ovariectomy.

The response of the tumors to each drug at the end of the treatment period was investigated and shown in Table 2. The ovariectomized rats showed 78% (50% CR + 28% PR) tumor regression. TP prevented the tumor regression induced by ovariectomy and in this group only 6% (3% CR + 3% PR) tumor regression was observed. When FR901537 was given with TP to ovariectomized rats, the effect of TP was partially inhibited and the tumor regression rate in this group was 41% (23% CR + 18% PR). Treatment with AG abolished the TP-induced tumor growth, and the tumor regression rate as a result of AG treatment was 79% (36% CR + 43% PR).

The number of tumors that newly appeared during the drug treatment period was examined and are shown in Table 2. The number of new tumors in the TP-treated, ovariectomized group was higher than in the other three groups. There was no difference between the FR901537- and AG-treated groups.

Table 2. Antitumor effects on DMBA-induced mammary tumors.

TP	Drug	Body weight gain (g)	No. of rats	No. of tumors	No. of responders (%)				New tumors/rat
					CR	PR	NC	PD	
-	0.5%MC	41 ± 3	9	32	16 (50%)	9 (28%)	6 (19%)	1 (3%)	0.2
+	0.5%MC	29 ± 6	11	40	1 (3%)	1 (3%)	19 (48%)	19 (48%)	1.3
+	FR901537	41 ± 4	12	44	10 (23%)	8 (18%)	23 (52%)	3 (7%)	0.3
+	AG	41 ± 6	12	44	16 (36%)	19 (43%)	6 (14%)	3 (7%)	0.2

Rats with DMBA-induced mammary tumors were ovariectomized, and treated with drug and TP, as described in the legend of Fig. 3. At the end of the treatment, the response of individual tumors to the drug was classified as follows; CR (complete remission, disappearance of the tumor), PR (partial remission, reduction of >50% in the initial tumor area), NC (no change, increase or decrease of <50%) or PD (progressive disease, increase of >50%).

Table 3. Serum E_2 levels in rats with DMBA-induced mammary tumors.

Treatment	No. of rats	Serum E_2 levels (pg/ml)
Normal	5	16.1 ± 5.6*, a)
OVX b)	5	0.3 ± 0.1
OVX+TP	5	4.6 ± 0.7
OVX+TP+FR901537	5	5.5 ± 1.2
OVX+TP+AG	5	3.0 ± 0.7

Three hours after the last drug treatment, the blood was collected from the animals, 5 per each group, and the serum E_2 level was measured after ether extraction, using RIA kits.

*: $P < 0.05$, compared with the OVX+TP group (Dunnett test).

a), Mean ± SE. b), OVX: ovariectomy.

The body weight of the drug treated and control animals were checked once a week and compared (Table 2). Treatment with TP slightly reduced the gain in body weight, while treatment with FR901537 and AG did not affect the body weight gain.

The serum E_2 level was analyzed by RIA after ether extraction. As shown in Table 3, the serum E_2 level was low in the ovariectomized group. Treatment with TP caused an increase in the serum E_2 level, but the level was not restored to that of normal control rats. This increase in the serum E_2 level following TP treatment was not affected by treatment with FR901537 or AG.

Discussion

The *in vivo* endocrine efficiency of FR901537, which was isolated from the fermentation broth of a bacterium, *Bacillus* sp. No. 3072, was evaluated in immature and mature rats. Growth of uteri in immature rats was stimulated by androstenedione. FR901537 and AG counteracted the androstenedione-induced stimulation

with similar potency (Fig. 2). These results suggest that administered androstenedione was converted into estrone (E_1) by the aromatase enzyme, leading to the increase in uterine weight and that the inhibition of this conversion by FR901537 or AG resulted in the decrease in uterine weight. However, FR901537 did not affect the uterine weight of mature rats following 14 days treatment (Table 1). The discrepancy in the effects of FR901537 on the uterine weight in immature and mature rats might possibly be related to a feedback regulatory mechanism in the intact mature rats^{13,14}). Although FR901537 reduced the ovarian estrogen synthesis by aromatase inhibition, in intact mature rats this inhibition of estrogen synthesis lead to an increase in luteinizing hormone and follicle-stimulating hormone levels *via* a feedback mechanism. This increase may in turn stimulate the synthesis of aromatase, counteracting the inhibitory effects of FR901537. However in the case of immature rats, it is possible that all the functions of the ovary are not fully developed and the feedback linkage was inactive. On the other hand, AG was active in both immature and mature rats. As AG is reported to inhibit another cytochrome P-450 enzyme such as the side cleaving enzyme^{10~12}) or to stimulate the estrone sulphate (E_1S) metabolism^{15,16}) as mentioned below, a mechanism other than aromatase inhibition may be involved in the decrease in the uterus weight.

It is well-known that AG is not selective in its inhibition of aromatase and that it also inhibits other steroidogenic enzymes including 11 β -hydroxylase, resulting in the inhibition of adrenal steroidogenesis^{10~12}). In fact patients receiving AG also require glucocorticoid replacement therapy¹⁷). As shown in Table 1, in this study AG induced adrenal and ovarian hypertrophy, but FR901537 did not, suggesting that FR901537, unlike AG, shows inhibitory activity specific for aromatase. This result is consistent with our previous findings that FR901537 did not inhibit bovine adrenal gland 11 β -hydroxylase *in vitro*⁷).

Finally the antitumor effect of FR901537 against DMBA-induced mammary tumors was studied in

ovariectomized, TP-treated rats as a postmenopausal tumor model. Ovariectomy caused regression of the tumors and treatment with TP prevented this tumor regression. FR901537 and AG counteracted the TP-induced growth of the mammary tumors. AG was more active than FR901537 and the effect of TP was almost completely inhibited by AG (Fig. 3, Table 2). However, the blood E_2 level after the final treatment with FR901537 or AG was almost the same as that in TP-treated rats. The blood E_2 level was higher in TP-treated rats than in ovariectomized-rats (Table 3). Thus, we found that the potency of the antitumor effects of FR901537 and AG was different while the serum E_2 level was equal. We have no evidence to explain this difference, but it is speculated that the antitumor effects of AG may be due to other factor(s) in addition to its aromatase inhibition. Recent investigations have suggested that besides aromatase inhibition, the stimulation of E_1S metabolism may be a possible mechanism of AG action^{15,16}. E_1S is a biologically inactive sulphate conjugate of E_1 but may be an important estrogen source for tumor cells¹⁸. It is reported that E_1S may be taken up by breast cancer cells, hydrolyzed to E_1 and reduced to E_2 inside the tumor cells^{18,19}. If AG stimulates the metabolism of E_1S and promotes its clearance, treatment with AG resulted in a decrease in the estrogen supply to tumor cells, leading to the inhibition of the tumor growth. Therefore, the antitumor effect of FR901537 is suggested to be attributed to its aromatase inhibition alone, while the effect of AG due to aromatase inhibition and additional stimulation of E_1S metabolism.

In summary we found that FR901537 is a potent and specific aromatase inhibitor, and shows antitumor effects against DMBA-induced, estrogen-dependent mammary tumors in the postmenopausal tumor model. These results suggest that FR901537 is a promising drug in the treatment of estrogen-dependent mammary tumors in postmenopausal women.

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