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HIGHLY SELECTIVE INHIBITION OF ESTROGEN BIOSYNTHESIS BY CGS 20267, A NEW NON-STEROIDAL AROMATASE INHIBITOR

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Summary—CGS 20267 is a new non-steroidal compound which potently inhibits aromatase *in vitro* (IC_{50} of 11.5 nM) and *in vivo* (ED_{50} of 1–3 μg/kg p.o.). CGS 20267 maximally inhibits estradiol production *in vitro* in LH-stimulated hamster ovarian tissue at 0.1 μM with an IC_{50} of 0.02 μM and does not significantly affect progesterone production up to 350 μM. In ACTH-stimulated rat adrenal tissue *in vitro*, aldosterone production was inhibited with an IC_{50} of 210 μM (10,000 times higher than the IC_{50} for estradiol production); no significant effect on corticosterone production was seen at 350 μM. *In vivo*, in ACTH-treated rats, CGS 20267 does not affect plasma levels of corticosterone or aldosterone at a dose of 4 mg/kg p.o. (1000 times higher than the ED_{50} for aromatase inhibition *in vivo*). In adult female rats, a 14-day treatment with 1 mg/kg p.o. daily, completely interrupts ovarian cyclicity and suppresses uterine weight to that seen 14 days after ovariectomy. In adult female rats bearing estrogen-dependent DMBA-induced mammary tumors, 0.1 mg/kg p.o. given daily for 42 days caused almost complete regression of tumors present at the start of treatment. Thus compared to each other, CGS 16949A and CGS 20267 are both highly potent in inhibiting estrogen biosynthesis *in vitro* and *in vivo*. The striking difference between them is that unlike CGS 16949A, CGS 20267 does not affect adrenal steroidogenesis *in vitro* or *in vivo*, at concentrations and doses several orders of magnitude higher than those required to inhibit estrogen biosynthesis.

INTRODUCTION

Potent and selective inhibition of the aromatase enzyme should lead *in vivo* to an inhibition of estrogen biosynthesis without attenuation of steroid hormone biosynthesis in other steroidogenic tissues like the adrenal cortex. Aminoglutethimide (AG), was the first non-steroidal aromatase inhibitor to be reported in the literature [1, 2]. Since then, AG has been effectively used in the treatment of estrogen-dependent breast cancer in postmenopausal women and its anti-tumor efficacy has been well-documented (for reviews see Refs [3, 4]). However, AG is a relatively non-selective aromatase inhibitor in that it also inhibits enzymes involved in adrenal steroidogenesis at concentrations similar to those at which it inhibits aromatase [5, 6]. Thus, the search was initiated for non-steroidal aromatase inhibitors which were primarily much more selective in their action as inhibitors of estrogen biosynthesis and which were also more potent as aromatase inhibitors than AG.

We have reported extensively on the endocrine and anti-tumor effects *in vitro* and *in vivo* in rats of a non-steroidal aromatase inhibitor, CGS 16949A {4-(5,6,7,8-tetrahydro-imidazo-[1,5a]-pyridin-5-yl)benzonitrile monohydrochloride} [7–12]. Others have reported on the endocrine effects of CGS 16949A in postmenopausal women with breast cancer [13–15]. These reports profile CGS 16949A as being a potent and efficacious inhibitor of estrogen biosynthesis *in vitro*, *in vivo* in animals and in postmenopausal women. However, at high concentrations *in vitro* and high doses *in vivo* in rats and in women with breast cancer, CGS 16949A also inhibits aldosterone biosynthesis [6,15–17].

Recently, we reported on another non-steroidal aromatase inhibitor, CGS 18320B {bis-(*p*-cyanophenyl)-imidazo-1-yl-methane hemisuccinate} [18, 19]. The pharmacological profile of CGS 18320B is very similar to that of CGS 16949A, except that it is an even weaker inhibitor of aldosterone production *in vitro* [19] and it is more efficacious in non-tumor- and tumor-

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bearing animals, probably due to its longer duration of action [18].

We now report on a third non-steroidal aromatase inhibitor, CGS 20267 [4,4′-(1H-1,2,4-triazol-1-yl-methylene)-bis-benzonitrile]. The chemical structures of these three aromatase inhibitors along with the reference compound AG are shown in Fig. 1.

AROMATASE INHIBITION

In vitro

The IC_{50}s (concentration of inhibitor which inhibits enzyme activity by 50%) with which AG, CGS 16949A, CGS 18320B and CGS 20267 inhibit microsomal aromatase obtained from human placenta are shown in Table 1. The methodology for this *in vitro* assay has been described in detail previously [7]. All of the three new non-steroidal aromatase inhibitors inhibit aromatase at low nanomolar concentrations. Thus, they are all over two orders of magnitude more potent than AG. Further, all three new inhibitors show competitive inhibition of the aromatase enzyme as evidenced from the generation of Lineweaver–Burk plots.

In vivo

We have reported previously on the details of a method developed by us which we use as a standard method for the assessment of aromatase inhibition *in vivo* [8, 12]. The method measures inhibition of the uterine weight increase resulting after treatment of immature female rats with a standard dose of androstenedione. This uterine hypertrophy is caused by the estrogen produced when androstenedione is aromatized in the ovary. Thus, inhibition of this androstenedione-induced uterine hypertrophy is an index of aromatase inhibition *in vivo*.

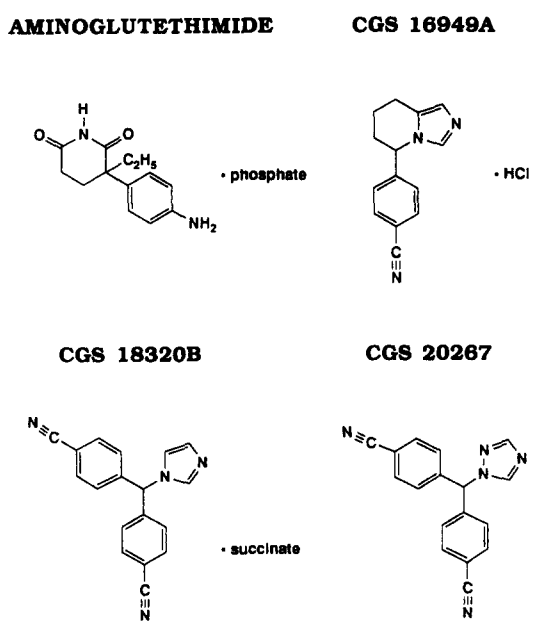

Fig. 1. Chemical structures of AG and three new non-steroidal aromatase inhibitors.

Table 1. Inhibition of human placental aromatase *in vitro*[a]

Compound	IC_{50} (nM)	Relative potency
AG	1900	1
CGS 16949A	5	400
CGS 18320B	3.5	550
CGS 20267	11.5	160

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

The results obtained when the three non-steroidal aromatase inhibitors, and AG, are administered orally in this model are shown in Fig. 2. The estimated ED_{50}s for the inhibition of the androstenedione-induced uterine hypertrophy in this assay system are 30 mg/kg and 30, 10 and 1-3 μg/kg for AG, CGS 16949A, CGS 18320B and CGS 20267, respectively. Thus, the three new inhibitors are over three orders of magnitude more potent than AG *in vivo*, and CGS 20267 is more potent than either CGS 16949A or CGS 18320B.

SELECTIVITY OF AROMATASE INHIBITION

In vitro

We have recently reported on two methods developed by us to assess selectivity of aromatase inhibition [5, 6]. These *in vitro* methods measure the luteinizing-hormone(LH)-stimulated estrogen and progesterone production in hamster ovarian tissue [5] and adrenocorticotrophic-hormone(ACTH)-stimulated production of corticosterone and aldosterone in rat adrenal tissue [6]. Inhibition curves are generated using various concentrations of each inhibitor. Figure 3 shows such curves obtained in hamster ovarian tissue and rat adrenal tissue for CGS 16949A and CGS 20267. The IC_{50}s with which the three inhibitors and AG inhibit estrogen, progesterone, corticosterone and aldosterone production are shown in Table 2.

Of the four inhibitors, AG is by far the least selective and the least potent inhibitor of aromatase. AG inhibits the production of estrogen at concentrations which are only one order of magnitude lower than those for progesterone, corticosterone or aldosterone. The progression

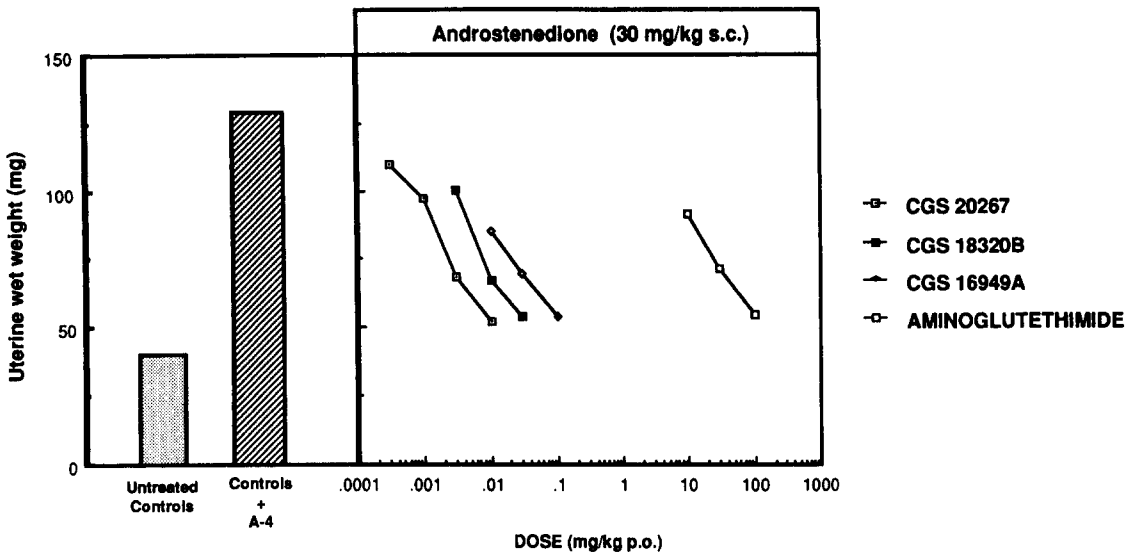

Fig. 2. Inhibition of the androstenedione-induced uterine hypertrophy by AG, CGS 16949A, CGS 18320B and CGS 20267: an assay for the inhibition of aromatase *in vivo*.

of increased selectivity in going from CGS 16949A to CGS 18320B to CGS 20267 is most evident when one looks at the IC_{50}s for inhibition of aldosterone production. This, coupled with the lack of inhibition of either progesterone or corticosterone at the highest tested concentration of 350 μm, makes CGS 20267 the most selective of the three aromatase inhibitors. As with the inhibition of placental aromatase *in vitro*, all three of the new inhibitors show about

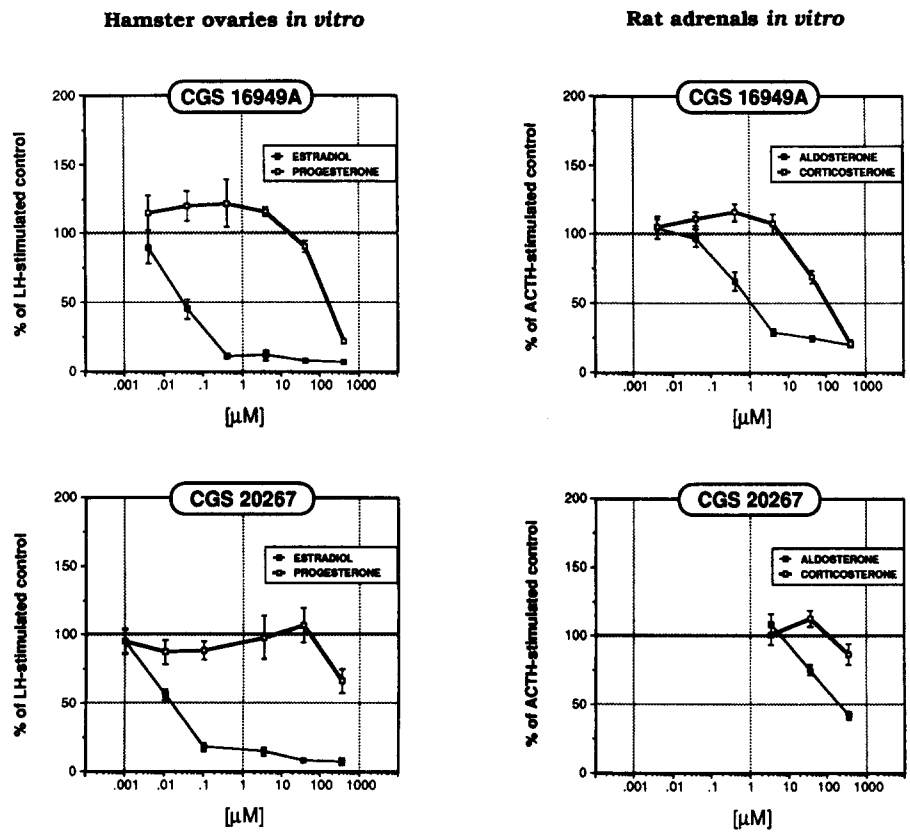

Fig. 3. Inhibition of the LH-stimulated production of estradiol and progesterone in hamster ovaries and the ACTH-stimulated production of corticosterone and aldosterone in rat adrenals by CGS 16949A and CGS 20267 *in vitro*. Results are reported as mean ± SEM.

Table 2. Selectivity of aromatase inhibition *in vitro*: inhibition of estrogen and progesterone production in hamster ovarian tissue and corticosterone and aldosterone production in rat adrenal tissue[a]

Compound	IC_{50} (μM) for the inhibition of the production of:			
	Estrogen	Progesterone	Corticosterone	Aldosterone
AG	13	60	50	110
CGS 16949A	0.03	160	100	1
CGS 18320B	0.06	>300	110	6
CGS 20267	0.02	>350	>350	210

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

the same potency in the inhibition of estrogen production.

In vivo

We have recently reported on a method to assess inhibition of adrenal steroidogenesis *in vivo* [6], which complements the methodology used to assess selectivity *in vitro*. The design of this *in vivo* method is very similar to the *in vitro* method which uses rat adrenal tissue. Male rats are treated with a single dose of ACTH 18 h prior to the administration of either vehicle or a single oral dose of the inhibitor being tested; 2 h after the administration of the inhibitor, the animals are sacrificed and the plasma concentrations of corticosterone and aldosterone are measured. The results of such an experiment with CGS 16949A and CGS 20267 are shown in Fig. 4. In the upper panel, one sees that these *in vivo* results mirror those obtained *in vitro*

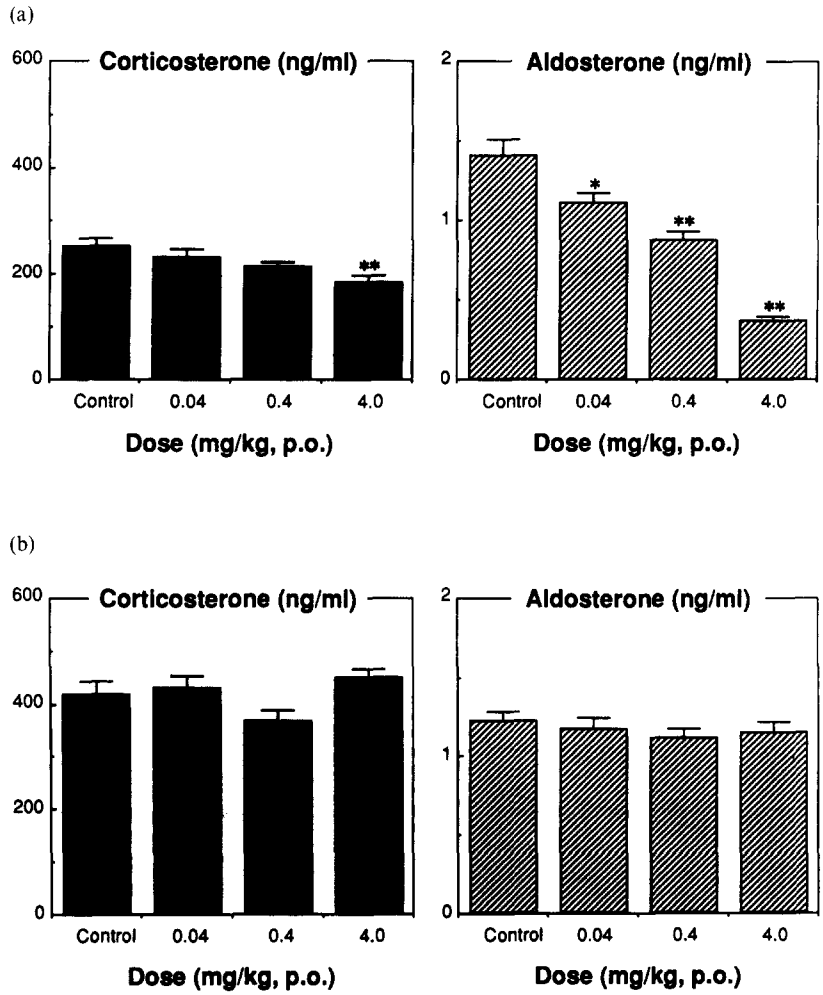

Fig. 4. Effects of single oral doses of (a) CGS 16949A and (b) CGS 20267 on plasma concentrations of corticosterone and aldosterone in adult male rats pretreated with ACTH. Results are reported as mean ± SEM ($N = 5$ per treatment group; *$2P < 0.05$, **$2P < 0.01$, Dunnett's t-test).

for CGS 16949A. Plasma aldosterone concentrations are more effectively and dose-dependently suppressed as compared to corticosterone concentrations. When the same doses of CGS 20267 were administered, there was no significant effect on the plasma concentrations of either aldosterone or corticosterone. The highest dose of CGS 20267 used here (4 mg/kg) is over 1000 times higher than the ED_{50} for inhibition of aromatase *in vivo* (1–3 μg/kg). Thus, these *in vivo* results for both CGS 16949A and CGS 20267 agree well with the *in vitro* results described above.

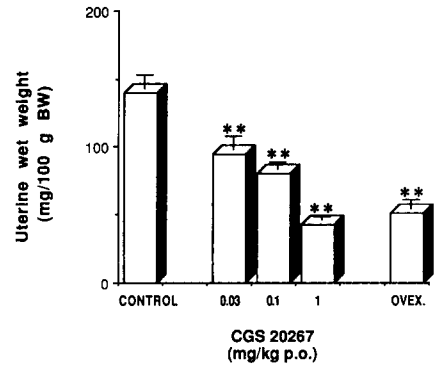

Fig. 5. Effect of CGS 20267 (administered orally, once daily for 14 days) and ovariectomy on uterine wet weight in adult cyclic female rats. Results are reported as mean ± SEM ($N = 8$ per treatment group; $^{**}2P < 0.01$, Dunnett's t-test).

ENDOCRINE EFFICACY *IN VIVO*

Administration of aromatase inhibitors to adult, cyclic female animals should result in attenuation of estrogen biosynthesis and should mimic the sequelae of surgical castration in terms of weight reductions and changes in estrogen-dependent target tissues. We have reported previously on the effects elicited by CGS 16949A, when administered orally once daily for 14 days. At the maximum daily oral dose of 3 mg/kg used in these experiments, ovarian cyclicity was disrupted such that after the fourth day of treatment, all 8 animals in this dose group showed diestrus smears till the end of treatment. Although uterine weight was significantly suppressed at this dose (3 mg/kg), it was only suppressed by about 20% of control values [8].

Using the same experimental protocol as was used in the studies with CGS 16949A [8], CGS 20267 was administered to adult cyclic female animals at doses of 0.03, 0.1 and 1 mg/kg. In addition to the normal control group of animals, a reference control group was included where the animals were ovariectomized on Day 1 of the experiment. The results of this treatment with CGS 20267 and of ovariectomy on uterine weight are shown in Fig. 5. There is a very effective and dose-dependent reduction in uterine weight with increasing doses of CGS 20267. The highest dose used (1 mg/kg) reduced uterine weight to that seen 14 days after ovariectomy in the reference control group. Thus, this dose of CGS 20267 (1 mg/kg) can be considered as one which effects medical castration in these animals in terms of reduction in uterine weight. In terms of ovarian cyclicity, all animals in the ovariectomized control group were in continuous diestrus after Day 4 (of the 14-day treatment period); in the 0.1 mg/kg-treated group all animals but one were in continuous diestrus after Day 4 and in the 1 mg/kg group all animals after Day 3 were in continuous diestrus. Thus, the effects of CGS 20267 on ovarian cyclicity were also equivalent to those resulting from surgical castration.

ANTI-TUMOR EFFICACY *IN VIVO*

The tumor model used for these studies was the estrogen-dependent DMBA-induced mammary carcinoma in adult female rats. Details of the experimental methodology have been reported elsewhere [9].

We have previously reported on the results obtained when animals bearing DMBA-induced mammary tumors were treated with either CGS 16949A or AG [9]. CGS 16949A markedly and dose-dependently reduced the mean tumor volume of palpable mammary tumors and also almost totally suppressed the appearance of new tumors during the treatment period. The esti-

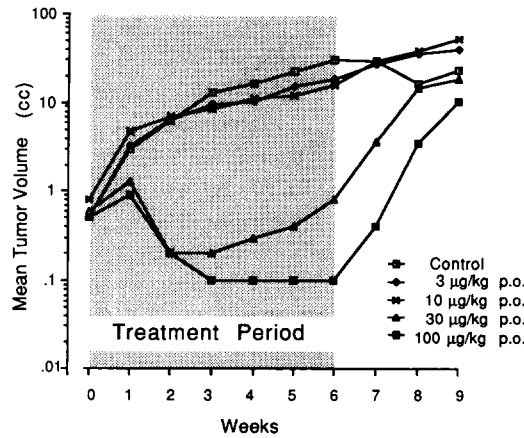

Fig. 6. Effect of once-daily oral doses of CGS 20267 on mean tumor volume of DMBA-induced mammary tumors in adult cyclic female rats ($N = 15$ per treatment group).

mated ED_{50} of this anti-tumor effect of CGS 16949A was between 0.1–0.5 mg/kg/day. AG, under the same conditions, is only marginally effective in reducing tumor growth at its maximally tolerated dose of 100 mg/kg/day.

The results obtained with CGS 20267, using the same experimental conditions as those reported for CGS 16949A [9], are shown in Fig. 6. Just as with CGS 16949A, CGS 20267 also causes marked and dose-dependent regression of DMBA-induced mammary tumors, in terms of mean tumor volume. The daily doses of 30 and 100 μg/kg caused a marked reduction in mean tumor volume, whereas the 3 and 10 μg/kg p.o. were ineffective. From these results one could estimate that the ED_{50} for this anti-tumor effect lies at about 30 μg/kg/day.

We have also previously reported on the effects of ovariectomy on the regression of DMBA-induced mammary tumors [10]. It was shown that at a dose of 4 mg/kg/day, CGS 16949A was as effective as ovariectomy in causing regression of tumors. The extent of the reduction in mean tumor volume with the 100 μg/kg/day dose of CGS 20267 shown in Fig. 6 is also similar to that seen previously after ovariectomy. However, detailed studies to directly compare the effects of ovariectomy and CGS 20267 on tumor growth are in progress and will be reported subsequently.

Thus, in the DMBA-induced mammary carcinoma model, both CGS 16949A and CGS 20267 are highly effective in causing tumor regression. At all doses tested, both CGS 16949A and CGS 20267 were well-tolerated over the duration of the treatment period.

CONCLUSIONS

The endocrine and anti-tumor effects of CGS 20267 have been reported and compared in terms of potency, selectivity and efficacy of aromatase inhibition with those of CGS 16949A and the reference compound AG. Both CGS 16949A and CGS 20267 are orders of magnitude more potent, selective and efficacious than AG. When compared to each other, CGS 20267 and CGS 16949A are both very potent inhibitors of estrogen biosynthesis *in vitro* and *in vivo* with CGS 20267 being the more potent of the two *in vivo*. Thus, with CGS 20267, we report for the first time on a pharmacological agent which inhibits estrogen biosynthesis so effectively that uterine weight is reduced to that seen after surgical castration. However, CGS 20267 does differ significantly from CGS 16949A in terms of selectivity. Unlike CGS 16949A, CGS 20267 does not affect corticosterone production *in vitro* and plasma corticosterone *in vivo*, at very high concentrations and doses. *In vitro*, CGS 20267 is 200 times weaker than CGS 16949A in inhibiting aldosterone production and *in vivo* it does not affect plasma aldosterone concentrations at high doses, making CGS 20267 one of the most selective aromatase inhibitors reported to date.

Thus, the two new aromatase inhibitors CGS 16949A and CGS 20267 are compounds whose pharmacological profiles in terms of their endocrine and anti-tumor effects in animals represent an important advance in the search for aromatase inhibitors which are more potent, selective and efficacious than AG.

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