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# INHIBITION OF AROMATASE *IN VITRO* AND *IN VIVO* BY AROMATASE INHIBITORS

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## INTRODUCTION

The aromatase enzyme complex effects the conversion of androgens to estrogens (see Figure 1) and is found in several mammalian tissues. In the female, the most important of these are the ovary, adipose tissue, breast, brain and liver. In female rats, aromatase is found almost entirely in the ovaries which are the site of estrogen

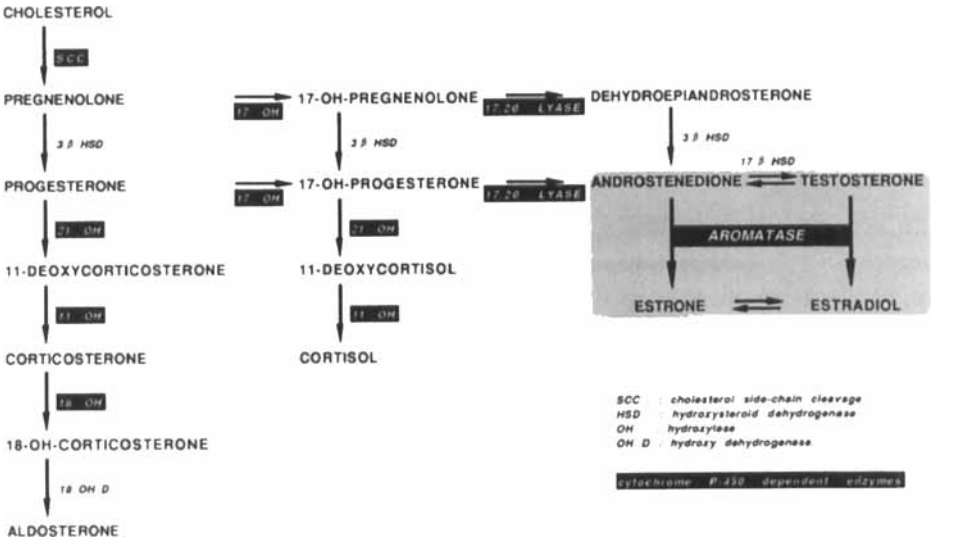


FIGURE 1 A composite of the main pathways of mammalian steroidogenesis from the steroidal precursor cholesterol.

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biosynthesis. In the adult premenopausal woman, the ovary is the predominant source of circulating estrogens and thus is the tissue with the highest amount of aromatase. In the postmenopausal woman, however, the major source of circulating estrogens has been shown to be from the conversion of adrenal androgens to estrogens by aromatase in peripheral tissues like adipose tissue (including the breast).[1] Thus aromatase is a key enzyme in the biosynthesis of estrogens. Since inhibition of aromatase could be a highly effective way of reducing the concentrations of circulating estrogens, it would also be effective therapy in the treatment of estrogen-dependent disease, the most important of which is estrogen-dependent breast cancer.

Like several other steroidogenic enzymes, aromatase is a cytochrome P-450-dependent enzyme. Many nonsteroidal inhibitors of aromatase are compounds which bind and thus inhibit this component of the aromatase enzyme system. Since different cytochrome P-450s are also subunits of other steroidogenic enzymes (marked in Figure 1), the selectivity with which inhibitors inhibit aromatase becomes an important factor. Lack of selectivity could lead to inhibition of the biosynthesis of other important steroid hormones which would then contribute during therapy to the appearance of undesirable side-effects. Thus the past two decades have seen the synthesis and development of pharmacological agents which potently inhibit aromatase with high selectivity for aromatase inhibition.

## CHEMISTRY OF AROMATASE INHIBITORS

Inhibitors of the aromatase enzyme are basically of two chemical types. They are either derivatives of steroids which act as competitive inhibitors of the natural aromatase enzyme substrate androstenedione or they are nonsteroidal in nature and bind to the cytochrome P-450 of the aromatase enzyme complex. Although aminoglutethimide (a nonsteroidal compound) and testolactone (a steroidal compound) had been developed as an inhibitor of adrenal steroidogenesis and an androgen antagonist, respectively and were already in use as therapeutic agents in the treatment of metastatic breast cancer, it was not until the mid 1970s that their aromatase inhibiting properties were first identified.[2,3] The first steroids designed to be aromatase inhibitors were reported by Schwarzel *et al.*,[4] and Brodie *et al.*[5] identified 4-hydroxy-androstenedione (CGP 32349) amongst the early series of steroidal aromatase inhibitors reported. Since then there has been a large number of compounds synthesized based on the structure of aminoglutethimide[6,7] and also a large number of steroidal compounds[8,9] which have been synthesized as aromatase inhibitors. The first steroidal compound which was structurally designed to be an aromatase inhibitor and developed as a treatment for breast cancer was 4-hydroxy-androstenedione[10] (CGP 32349). Recently, new nonsteroidal imidazole derivatives, CGS 16949 A (5,6,7,8-tetrahydroimidazo-[1,5a]pyridin-5-yl}-benzonitrile hydrochloride and CGS 18320B [bis-(p-cyanophenyl)-imidazo-1-yl-methane hemisuccinate], have been synthesized which show extremely high potency as aromatase inhibitors.[11,12,13]

The goal of any development program for aromatase inhibitors is the synthesis of a compound which potently, selectively and efficaciously inhibits the aromatase enzyme both in *in vitro* and *in vivo* systems. For the purposes of this review primary focus will be placed on nonsteroidal inhibitors. They are aminoglutethimide, as our starting point of reference, and the new imidazole derivatives CGS 16949A and CGS 18320B.

TABLE I

Inhibition of human placental aromatase *in vitro* using a product isolation method with $^{14}$C-androstenedione as the radiolabeled substrate. Results are expressed as the concentration at which each inhibitor inhibits aromatase by 50% ($IC_{50}$) and the inhibition constant ($K_i$) determined from a Lineweaver-Burk plot. For experimental details see Steele *et al.*[9]

| Inhibitor | $IC_{50}$ nM | $K_i$ nM |
|---|---|---|
| Aminoglutethimide | 1900 | 530 |
| Testolactone | 8000 | 4600 |
| CGP 32349 | 62 | 20 |
| CGS 16949A | 5 | 1.4 |
| CGS 18320B | 3 | 0.5 |

## POTENCY

Potency of aromatase inhibitors is usually assessed in an *in vitro* system first described by Thompson & Siiteri.[2] These authors used the human placenta as the source of their aromatase enzyme preparation and then measured the inhibition of the aromatization of the natural substrate androstenedione to the estrogen estrone. This aromatization reaction can either be followed by measuring the amount of tritiated water released when (1$\beta$,2$\beta$-$^3$H)-androstenedione, is aromatized to estrone, as was done by Thompson and Siiteri,[2] or by isolating the $^{14}$C-labeled estrogens which result when $^{14}$C-androstenedione is aromatized as reported by Steele *et al.*[11] Using the latter product isolation method, a variety of aromatase inhibitors were assessed for their ability to inhibit aromatase. The results are expressed as the concentrations at which they inhibited the conversion of androstenedione to estrogens by 50% ($IC_{50}$) and as the classic inhibition constant ($K_i$) obtained from a Lineweaver-Burk plot. The results obtained with both steroidal and nonsteroidal aromatase inhibitors are shown in Table 1.

The nonsteroidal aromatase inhibitors CGS 16949A and CGS 18320B inhibit aromatase at nanomolar concentrations and are at least 10 times to over 600 times as potent as any of the other inhibitors.

## SELECTIVITY

Aminoglutethimide, which is a potent aromatase inhibitor, also inhibits other cytochrome P-450-dependent enzymes. Before the aromatase inhibiting properties of aminoglutethimide were recognized, it was used as a pharmacologic agent to effect a so-called medical adrenalectomy. Thus although aminoglutethimide is a potent inhibitor of aromatase it was not selective in its action. We have recently reported a method which assesses the degree of selectivity of an aromatase inhibitor.[14] Briefly, in this *in vitro* method, steroidogenesis is stimulated by incubating hamster ovarian slices with the gonadotrophin luteinizing hormone (LH) either in the presence or absence of the aromatase inhibitor being tested. The release of LH-stimulated progesterone and estradiol into the medium is then measured. A selective inhibitor of aromatase will inhibit the production of estradiol without inhibiting the production of progesterone.

The results of this selectivity assay are presented in Table II and are expressed as the concentration at which each of the listed inhibitors inhibits the production of

TABLE II

Selectivity of aromatase inhibition in the LH-stimulated hamster ovarian tissue assay *in vitro*. Results are expressed as the concentration at which each inhibitor inhibits estrogen (E) and progesterone (P) production by 50% ($IC_{50}$). The Selectivity Index (P/E) is defined as the ratio, $IC_{50}$ P production: $IC_{50}$ E production. For experimental details see Häusler *et al.*[12]

| Inhibitor | E Production $IC_{50}\,\mu M$ | P Production $IC_{50}\,\mu M$ | Selectivity Index P/E |
|---|---|---|---|
| Aminoglutethimide | 13 | 60 | 5 |
| CGP 32349 | 0.88 | $\geqslant 330$ | $\geqslant 375$ |
| CGS 16949A | 0.03 | 160 | 5000 |
| CGS 18320B | 0.06 | $> 300$ | $> 5000$ |

either progesterone or estradiol by 50% ($IC_{50}$). As can be seen, aminoglutethimide inhibits estradiol production with a slightly higher potency than that for progesterone production. On the other hand, CGS 16949A, CGS 18320B and CGP 32349 inhibit estradiol production at concentrations which are orders of magnitude lower than those for the inhibition of progesterone production. So as to be able to compare the selectivity of these inhibitors, a simple selectivity index has been devised which is the ratio of the $IC_{50}$s for the inhibition of progesterone and estradiol production (P/E). The larger the magnitude of the selectivity index, the more selective the inhibitor in this system. Thus it can be seen from Table II that CGS 18320B and CGS 16949A with a selectivity index of over 5,000 and CGP 32349 with an index of over 375 are much more selective than aminoglutethimide which has an index of about 5.

## INHIBITION OF AROMATASE *IN VIVO*

### *Standard Assay for Aromatase Inhibitors:*

To be able to compare the efficacy with which putative inhibitors of aromatase inhibit the enzyme *in vivo*, we have developed a standard assay to measure this parameter. The assay is based on a finding which was reported as early as 1975 by Knudsen and Mahesh.[15] They found that androgens could cause ovulation and an increase in uterine weight in immature female rats and that the increase in uterine weight was abolished by ovariectomy. They reasoned that the ovary was able to convert the exogenous androgen to an estrogen and that it was this estrogen which was responsible for the uterine hypertrophy.

We treated intact and ovariectomized immature female rats with varying doses of androstenedione for 4 days after which the animals were sacrificed, their uteri removed and weighed. The results are shown in Table III. As had been reported by Knudsen & Mahesh,[15] the dose-dependent uterine hypertrophy seen in the intact animals was greatly reduced in the ovariectomized animals. There was, however, an increase in uterine weight in these animals as compared to ovariectomized controls a finding which has also been previously reported.[16,17] These authors ascribed this to a direct effect of androgens on the uterus, since in the same reports, they also documented measurable amounts of androgen receptor in the immature rat uterus. The presence of a weak uterotrophic effect in the absence of ovaries could, however, also be explained by the peripheral or extra-glandular conversion of androstenedione to estrogens by aromatase as is the case in postmenopausal women. To test whether this peripheral aromatization of androgens could be responsible for the uterotrophic

TABLE III
Uterine wet weight in immature intact and ovariectomized female rats administered androstenedione with or without an aromatase inhibitor (CGS 16949A). For experimental details, see legend to Figure 2.

| Treatment | Uterine wet weight (mg) (Mean ± S.E.M.) | |
|---|---|---|
| | Intact animals | Ovariectomized animals |
| Control | 36.4 ± 2.7 | 17.0 ± 0.5 |
| Androstenedione | | |
| 10 mg/kg s.c. | 101.6 ± 8.1** | 36.4 ± 1.5** |
| 30 mg/kg s.c. | 137.6 ± 6.8** | 54.8 ± 2.5** |
| 100 mg/kg s.c. | 161.7 ± 5.0** | 55.0 ± 7.3** |
| Androstenedione 30 mg/kg s.c. + CGS 16949A 100 μg/kg p.o. | 54.2 ± 2.8** | 56.6 ± 0.9** |

** $2p < 0.01$ Dunnett's t-test

effect, we treated immature ovariectomized rats with a combination of androstenedione and a maximally effective dose of CGS 16949A (100 μg/kg p.o.). As is shown in Table III, CGS 16949A was unable to antagonize the weak uterotrophic effect of androstenedione in ovariectomized animals. Thus a direct effect of androstenedione on the uterus is probably the more plausible explanation.

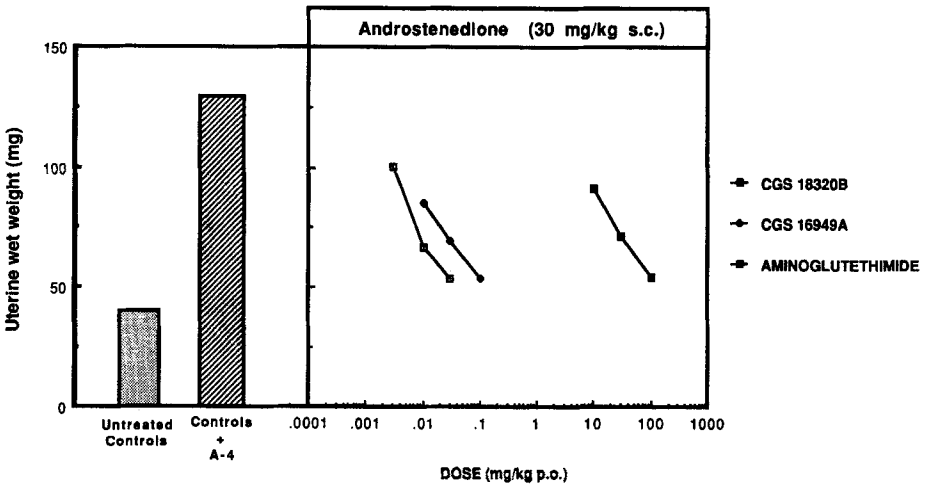


FIGURE 2 Dose-response relationship for the 3 nonsteroidal aromatase inhibitors in the *in vivo* assay for inhibition of aromatase. In a typical experiment, immature female rats (50 gm bodyweight) are divided into 5 groups of 5 animals each: a control group which receives only vehicle; a group which only receives androstenedione (A4) (30 mg/kg s.c.) and 3 groups which each receive a different dose (p.o.) of the aromatase inhibitor being tested + androstenedione (30 mg/kg s.c.). All animals are treated once daily for 4 days. Four hours after the last dose, the animals are sacrificed, their uteri removed, freed of adhering fat and connective tissue and weighed (uterine wet weight).

In our standard assay for aromatase inhibition *in vivo*, we treat intact immature female rats with a fixed dose of androstenedione (30 mg/kg/day s.c.) for four days with or without the concommitant administration of the aromatase inhibitor to be tested. Figure 2 shows the dose-dependent decrease in uterine hypertrophy caused by the reference compound aminoglutethimide and the two nonsteroidal aromatase inhibitors CGS 16949A and CGS 18320B. In this assay, CGS 16949A is about 1000 times and CGS 18320B 3000 times as potent as aminoglutethimide.

Since the basis of the androstenedione-induced uterine hypertrophy in this assay for aromatase inhibition is the estrogenic effect exerted by the estrogen produced *in situ*, compounds which exert anti-estrogenic effects would also antagonize the uterine hypertrophy and behave as aromatase inhibitors. Thus the two limitations of this assay are that the aromatase inhibitors being tested exert nether anti-estrogenic nor androgenic effects. For this reason CGP 32349, which has inherent weak androgenic properties cannot be appropriately tested in this assay. Further, in this assay, tamoxifen (an anti-estrogen) does inhibit the uterine hypertrophy although the effect is not dose-dependent (unpublished data). Aminoglutethimide, CGS 16949A and CGS 18320B do not show any androgenic or anti-estrogenic properties in standard bio-assays for these effects.

### *Anti-tumor Effects of Aromatase Inhibitors in Tumor-bearing Rats*

The adult female Sprague-Dawley rat bearing DMBA-induced mammary tumors is a standard model for the study of anti-tumor agents which affect the growth of these hormone-dependent mammary tumors. These DMBA-induced tumors are particularly sensitive, in terms of growth inhibition, to agents which have the ability to lower circulating estrogens. Thus they represent an animal model in which the efficacy of aromatase inhibitors can be assessed. The use of this model in the study of the anti-tumor efficacy of the aromatase inhibitor CGS 16949A has been reported by Schieweck *et al.*[18] and the anti-tumor efficacy of CGS 18320B has been reported by Bhatnagar *et al.*[19] Both studies show CGS 16949A and CGS 18320B to be very potent and efficacious in causing almost complete regression of DMBA-induced mammary tumors at maximally effective doses of 4 mg/kg/day and 1 mg/kg/day respectively. Furthermore, at these doses, both aromatase inhibitors almost completely suppress the appearance of new tumors during the course of treatment.

To demonstrate that this potent anti-tumor efficacy was directly dependent on the inhibition of aromatase, and thus the suppression of circulating estrogens, tumor-bearing animals were treated with a combination of a maximally effective dose of the aromatase inhibitor and estradiol. The dose of estradiol used was one which served as a replacement dose of estrogen in ovariectomized adult animals and thus was a physiologic and not a pharmacologic dose of estradiol. The results of the studies with CGS 16949A + estradiol were reported by Schieweck *et al.*[20] and those with CGS 18320B were reported by Bhatnagar *et al.*[13] They were able to show that, in both instances, a replacement dose of estradiol completely antagonizes the anti-tumor effect of maximally effective doses of both CGS 16949A and CGS 18320B in terms of regression of growth and suppression of the appearance of new tumors during treatment.

## CLINICAL RESULTS

Of the consequences of treatment with aromatase inhibitors *in vivo*, reduction in circulating estrogens is an important parameter of endocrine efficacy. Thus Dowsett *et al.*[21] have shown that treatment with CGP 32349 leads to a consistent suppression of circulating levels of estradiol and Santen *et al.*[22] have shown similar effects with CGS 16949A. Another parameter which is slightly more difficult to assess is the inhibition of aromatization *in vivo*. Very recently, Reed *et al.*[23] have shown that CGP 32349 (500 mg i.m.) almost completely inhibited the conversion of androstenedione to estrone in postmenopausal women with breast cancer. Dowsett *et al.*[24] have also demonstrated this inhibition with CGS 16949A (1 mg p.o. bd) in the same patient population. These results very strongly suggest that the suppression of circulating estrogens in response to treatment with aromatase inhibitors like CGP 32349 and CGS 16949A is a consequence of the inhibition of aromatase *in vivo*.

Several reports have appeared on the anti-tumor effects which accompany the endocrine effects of CGP 32349 in patients with advanced metastatic breast cancer. Coombes *et al.*,[25] in a review of these data, have shown that response rates in patients with advanced metastatic breast cancer treated with CGP 32349 have been similar to those reported with other forms of endocrine therapy. These results have recently been further corroborated by Höffken *et al.*[26] Although clinical studies on the anti-tumor efficacy of CGS 16949A are in progress, no published reports have appeared as yet.

## CONCLUSIONS

We have reviewed here the advances made in the development of nonsteroidal aromatase inhibitors using aminoglutethimide as a reference compound. Considerable progress has been achieved in terms of potency, selectivity and efficacy since the advent of aminoglutethimide (Orimeten®/Cytadren®) for the treatment of postmenopausal breast cancer. An animal model which effectively demonstrates the inhibition of aromatase *in vivo* by these new aromatase inhibitors has been developed. Further, inhibition of aromatase *in vivo* has been shown to be the predominant mechanism by which these inhibitors exert their anti-tumor effects in the DMBA-induced rat mammary tumor model.

Clinically, the new aromatase inhibitors suppress circulating estrogens and inhibit aromatase *in vivo*. Anti-tumor response rates achieved with one these inhibitors, CGP 32349, compare very favourably with those seen with other endocrine therapies for advanced breast cancer in postmenopausal women.

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