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IN VITRO POTENCY AND SELECTIVITY OF THE NON-STEROIDAL ANDROGEN AROMATASE INHIBITOR CGS 16949A COMPARED TO STEROIDAL INHIBITORS IN THE BRAIN

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Summary—A sensitive *in vitro* ^3H_2O microassay for aromatase activity was used to evaluate the potency and selectivity of three aromatase inhibitors in mammalian (gerbil) and avian (ring dove) hypothalamus. The steroidal inhibitors, 1,4,6-androstatrien-3,17-dione (ATD) and 4-hydroxy-androstenedione (4-OH-A) were compared with a new non-steroidal imidazole inhibitor, CGS 16949A [4-(5,6,7,8-tetrahydroimidazo-[1,5-a]-pyridin-5-yl)benzonitrile HCl]. Adult male dove hypothalamic aromatase is highly active [V_{max} = 5.3 pmol testosterone (T) converted/h/mg protein], has high substrate binding affinity (K_m = 4.0 nM), and direct involvement in control of sexual behaviour. With [1β-^{3}H]T or [1β-^{3}H]A as substrate, male dove preoptic aromatase activity was inhibited more effectively and selectively by CGS 16949A. Thus, K_is and IC_{50}s for aromatization were ~50 times lower for the non-steroidal inhibitor, and inhibition of the other major androgen-metabolizing enzymes (5α/β-reductase) occurred at concentrations at least one order of magnitude greater than for ATD and 4-OH-A. Neonatal male gerbil hypothalamic aromatase activity (V_{max} = 1.3 pmol T converted/h/mg protein) was lower than in the dove. Aromatase inhibition by CGS 16949A is more potent in the neonatal gerbil than in the dove (K_is of 0.03 and 0.60 nM, respectively, with A as substrate). We conclude that the imidazole is an effective aromatase inhibitor in both the adult and developing brain.

INTRODUCTION

Conversion of circulating androgens to active and inactive metabolites is a regulatory step in their action on the male brain [1]. An enzyme complex consisting of a specific form of cytochrome *P*-450, aromatase-system cytochrome *P*-450 (*P*-450$_{AROM}$), and the flavoprotein NADPH–cytochrome *P*-450 reductase [2], is localized in brain areas such as the preoptic area (POA) associated with male sexual behaviour [1]. The aromatase converts testosterone (T) to its active metabolite, estradiol-17β (E_2) which has specific effects on behaviour [3] and the sexual differentiation of the brain during development [4]. Evidence for this is suggested by the direct action of estrogen on male sexual behaviour, the effectiveness of aromatase inhibitors in eliminating behaviour, and the detection of aromatase in brain areas associated with behaviour [1, 5]. Results obtained with steroidal inhibitors such as 4-hydroxy-androstenedione (4-OH-A) and 1,4,6-androstatrien-3,17-dione (ATD) are difficult to interpret, because they affect other pathways of T metabolism [6] and bind to steroid receptors in the brain [7, 8]. To find out more about the functional role of aromatase and E_2 formation in brain cells, it is essential to obtain selective aromatase inhibitors that do not affect other enzymes or steroid receptor systems in the brain. The problem has been to find an active, regulated brain aromatase system with a known physiological function to test the inhibitors. The intracellular aromatase system localized in the POA of the male dove (*Streptopelia risoria*) is highly active, regulated by T [9, 10], and is a well known model for the study of brain aromatase. The enzyme is influenced by photoperiod [11] and the behavioural environment of the male [12]. Estrogen is also involved in the sexual differentiation of the brain and behaviour of the male gerbil (*Meriones unguiculatus*) [13]. Therefore, in both the adult male dove and neonatal

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male gerbil, studied in this paper, the brain estrogen has a specific functional role. The present study compares the new non-steroidal aromatase inhibitor CGS 16949A, known to be effective for ovarian, placental and adrenal aromatase [14, 15], with the two steroidal aromatase inhibitors 4-OH-A and ATD (Fig. 1) by measuring their inhibitory action on formation of ^3H_2O from [1β-^{3}H]T or [1β-^{3}H]A *in vitro*. The imidazole inhibitor presents advantages as a tool for investigating the physiology and biochemistry of the brain aromatase in view of its non-steroidal structure which should prevent binding to steroid receptors and interference with steroid receptor-dependent processes. The effects of CGS 16949A on brain aromatase have not yet been studied, and the inhibitory mechanism has not been evaluated previously using a specific ^3H_2O microassay.

EXPERIMENTAL

Substrates and inhibitors

[1β,2β-^{3}H]T and [1β-^{3}H]A were purchased from New England Nuclear-Dupont (Boston, MA). [1β-^{3}H]T was prepared from [1β,2β-^{3}H]T by removal of the labile 2β-tritium atom under mild alkaline treatment [16]. All substrates were purified by TLC prior to use. 4-OH-A and ATD were obtained from Sigma Chemical Co. (St Louis, MO) and Steraloids Inc. (Wilton, NH), respectively. CGS 16949A was provided by Ciba-Geigy (Basel, Switzerland).

Animals and tissue preparation

Male doves were in breeding condition in the laboratory. Neonatal male Mongolian gerbils were obtained from breeding pairs on the day of birth [17]. The brains were frozen rapidly (within 2 min of sacrifice) on dry ice. Samples of the preoptic-anterior hypothalamic area ($\sim$1–2 mg/brain), known to contain aromatase activity from pilot studies, were obtained using the microdissection procedure for frozen brain sections described previously [10]. Since there are technical difficulties in dissecting samples consisting only of POA from the neonatal gerbil brain, both POA and anterior hypothalamic tissue were dissected as a single sample (POA–AH) using procedures for neonatal tissue sampling similar to those described previously [18]. Kinetic studies were carried out on pooled preoptic tissue obtained from 6 sexually active male doves and 12 neonatal (day 1) male gerbils. Studies of the aromatase inhibitors were carried out on pooled male dove POA ($N = 4$) and neonatal (day 1) male gerbil POA–AH ($N = 6$).

Enzyme assays

The effects of aromatase inhibitors on the activity of the aromatase and other steroid metabolizing enzymes were measured *in vitro* by a modified micro-assay based on the release

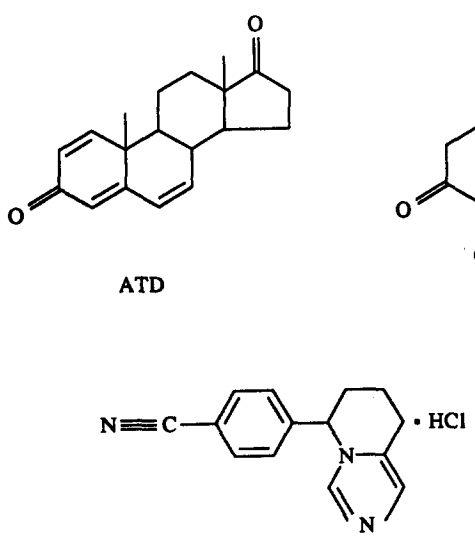

Fig. 1. Chemical structure of three aromatase inhibitors: 1,4,6-androstatriene-3,17-dione (ATD); 4-hydroxy-androstene-3,17-dione (4-OH-A); 4-(5,6,7,8-tetrahydroimidazo-[1,5-a]-pyridin-5-yl)benzonitrile monohydrochloride (CGS 16949A).

(and liquid scintillation counting) of ^3H_2O from the aromatase stereospecific substrates [1β-^{3}H]T or [1β-^{3}H]A, using solid phase extraction of steroids onto C-18 (reverse phase) chromatographic sorbent [19, 20]. Because E_2 can be further metabolized or bound to receptors, the ^3H_2O aromatase assay has advantages over the product formation assay for the evaluation of inhibitors. The ^3H_2O assay has been validated by direct comparison with the [^{3}H] E_2 formation assay using the same batch of tissue [19]. All enzymatic studies were performed in 100 mM Tris buffer, pH 7.4, containing 50 mM KCl and 1 mM EDTA. Brain samples were homogenized manually with a glass homogenizer on ice. Incubations were performed in a microplate with 30 μl homogenate in the presence of 0.2 mM NADPH and saturating substrate concentration (300 nM), for 30 min at 37°C. A range of substrate concentrations was employed if kinetic determinations of K_m, V_{max} or K_i were required. Reactions were stopped by freezing samples on dry ice, and adding 10 μg each of 9 "cold" "carrier" steroids in 160 μl ethanol–water (1:15). Other ^{3}H steroid metabolites were resolved by silica-gel TLC in 85:15 dichloromethane–ether followed by liquid scintillation counting. All metabolites have been previously identified using re-crystallization to constant specific activity, and derivative formation [21]. Duplicate determinations of each kinetic point were performed, with a coefficient of variation <10%. Interassay variation (<1.0%) was checked by including internal controls of active brain aromatase samples in all experiments. All experiments were repeated, with comparable results.

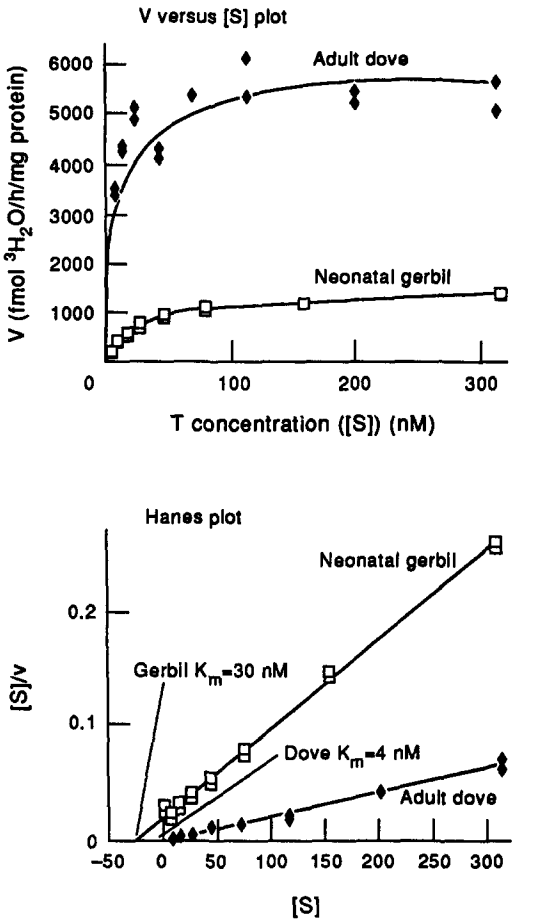

Fig. 2. Characterization of aromatase kinetics from both adult dove POA and neonatal (day 1) gerbil POH–AH. ^3H_2O formation from [1β-^{3}H]T is shown as a function of substrate concentration, including Hanes plot derivation. The standard incubation conditions were as described in the Experimental section. Points represent means of duplicate determinations.

RESULTS

Enzyme kinetics

With either T or A as substrate, adult male dove POA aromatase yielded linear activity plots with time and sample dilution, and linear Michaelis–Menten kinetics (Fig. 2). Dove POA aromatase had a mean K_m of 4.0 ± 1.2 nM, V_{max} of 5.3 pmol T converted/h/mg protein, and pH preference between 7.0–8.0 with T as substrate. When [^{3}H]A was used as the aromatase substrate in the ^3H_2O assay, the enzyme kinetics were similar to those determined using T. We also examined gerbil brain aromatase which is most active neonatally. Neonatal gerbil POA–AH aromatase was less active (mean V_{max} = 1.3 pmol T converted/h/mg protein), had a weaker T substrate binding affinity (mean K_m = 30 nM), and had a similar pH preference to the dove enzyme. The strong substrate binding affinity and activity of the dove aromatase relative to the adult rodent brain enzyme [22] indicates that the male dove POA is a good *in vitro* system to study aromatase inhibitors and their mechanism of action in the adult brain.

Potency of inhibition

The study of inhibitory kinetics with both T and A provided a double confirmation of aromatase inhibitor potencies. Using [^{3}H]T as substrate, CGS 16949A inhibited dove POA aromatase activity at much lower concentrations than the two other aromatase inhibi-

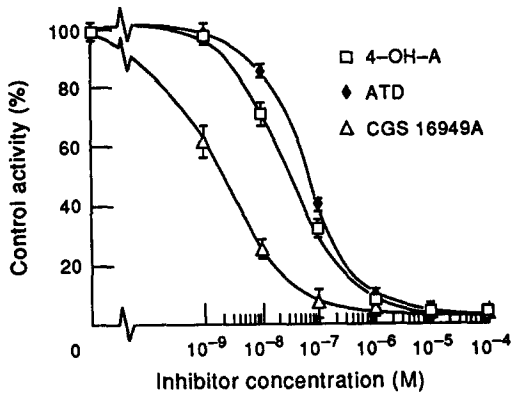

Fig. 3. Dose–response curves for the inhibition of adult male dove POA aromatase by ATD, 4-OH-A and CGS 16949A. Incubations were performed at a saturating substrate concentration (300 nM T). Values are expressed as the mean of duplicate determinations.

tors, 4-OH-A and ATD (Fig. 3). Similar inhibition profiles comparing CGS 16949A to ATD (mean IC_{50}s = 3.7 and 270 nM, respectively, with A as substrate: other posterior hypothalamic data not included) were obtained using posterior hypothalamic samples. K_i determinations with dove POA aromatase reinforced the finding that CGS 16949A is more potent ($\geq$50 times) than ATD. Inhibition constants obtained with dove POA aromatase using both T and A as substrate are summarized in Table 1. Neonatal gerbil POA aromatase had a lower K_i with CGS 16949A than in dove (mean K_is = 0.03 and 0.60 nM, respectively, with [^{3}H]A as substrate), suggesting that this inhibitor is more effective for the neonatal gerbil enzyme. In the determinations of K_is, increasing CGS 16949A inhibitor concentration raised the gerbil aromatase mean K_m from 30 to >300 nM, and similarly in dove.

The similar pattern of results when comparing substrates for determination of inhibition constants reinforces the rank order of potency of the aromatase inhibitors as being ATD < 4-OH-A < CGS 16949A. The non-steroidal CGS 16949A was by far the most potent aromatase inhibitor, with the steroidal ATD and 4-OH-A being less potent.

Selectivity of inhibition

The selectivity of aromatase inhibition was examined using dove POA, because this tissue is known to have 2 other major pathways of T metabolism, 5α- and 5β-reduction. With T as substrate, in addition to aromatization, 5β-reduction is the major pathway of androgen metabolism involving both the 5β-reductase catalysed formation of 5β-dihydrotestosterone (5β-DHT) and its subsequent 3α-reduction to form 5β-androstane-3α,17β-diol. All the inhibitors reduced the levels of these two metabolites, but at concentrations well above (at least one order of magnitude) those required to inhibit the aromatase (Table 2). The difference between the IC_{50}s for CGS 16949A inhibition of aromatase and 5β-reductase is approximately 4 orders of magnitude. CGS 16949A is, therefore, highly selective for aromatase. The IC_{50}s for inhibition of both 5β-DHT and 5β-androstane-3α,17β-diol with T as substrate (Table 2) were very similar for a given aromatase inhibitor, indicating that the inhibition step by all three inhibitors is of the 5β-reductase, which results in low levels of both its primary product (5β-DHT), and subsequent metabolites such as the 3α-diol. Other steroid metabolic pathways (such as 5α-reductase) are also inhibited as shown in Table 2, with CGS 16949A being consistently the least inhibitory at all of the concentrations examined. Figure 4(a) shows the effect of one concentration (10^{-4} M) of ATD on the production of various metabolites, with T as substrate for the determination of the IC_{50}s (Table 2). With A as substrate, the major metabolites formed, in addition to estrogens, are 5α-androstane-3β,17β-diol and 5β-androstanedione. As before, CGS 16949A is highly selective for aromatase, with on average 4 orders of magnitude difference between the IC_{50}s for aromatization, and 5α/β-reduction. ATD is less selective, with less than 1 order of magnitude difference between IC_{50}s for inhibition of aromatization and 5α/β-reduction (Table 2). Other steroid metabolites, such as 5α-androstanedione are also inhibited

Table 1. Inhibition of adult male dove and neonatal male gerbil preoptic aromatase activity

	Substrate	Testosterone			Androstenedione	
	Inhibitor:	CGS 16949A	ATD	4-OH-A	CGS 16949A	ATD
Adult dove	K_i (nM)	1.0	50	ND	0.6	25
	IC_{50} (nM)	1.8	71	46	1.0	150
Neonatal gerbil	K_i (nM)	0.08	ND	ND	0.03	ND
	IC_{50} (nM)	0.30	ND	ND	0.30	ND

K_i inhibitor constants were obtained graphically from secondary replots (slope vs [I]), of Lineweaver–Burk plots (1/v vs 1/(S)] performed at several [I]. ND = not determined.

Table 2. IC_{50}s of androgen metabolites inhibited by three aromatase inhibitors in dove brain POA homogenate, with either T or A as substrate. [The inhibitor concentrations ranged from $0–10^{-3}$ M.]

Substrate	Metabolite production	Relative metabolite production	Relevant enzymes	IC_{50} (μM) for inhibition by		
				CGS 16949A	4-OH-A	ATD
T	^3H_2O	1.0	Aromatase	0.0018	0.046	0.071
	5β-DHT	3.0*	5β-Reductase	20.0	4.0	0.40
	5β-Androstane-3α,17β-diol	1.5*	5β-Reductase,(5β)-3α-HSD	10.0	4.4	0.23
	5α-DHT	0.5*	5α-Reductase	—	>1000.0	100.0
	5α-Androstane-3α,17β-diol	0.4*	5α-Reductase,(5α)-3α-HSD	—	—	70.0
A	^3H_2O	1.0	Aromatase	0.001	ND	0.15
	5β-Androstane-3,17-dione	4.0*	5β-Reductase	18.0	ND	0.50
	5α-Androstane-3β,17β-diol	2.1*	5α-Reductase,(5α)-3β-HSD	9.0	ND	0.50
	5α-Androstane-3,17-dione	0.4*	5α-Reductase	—	ND	50.0

HSD, hydroxysteroid dehydrogenase; —, not inhibited; *, minimum estimate since at the substrate concentration employed (300 nM) aromatase is saturated but most other enzyme activities are below their V_{max}s. Aromatase relative activity = 1. ND, not determined.

by ATD (not by CGS 16949A) when A is used as substrate (Table 2). Figure 4(b) shows the effect of 10^{-4} M ATD on formation of various steroid metabolites, with A as substrate, used in the determination of the IC_{50}s for ATD listed in Table 2. An unexpected result was that the 17β-hydroxysteroid dehydrogenase catalysed formation of A, with T as substrate was increased to 140% by 10^{-4} M 4-OH-A.

DISCUSSION

The potency of aromatase inhibitors is usually assessed in an *in vitro* system using human placental microsomes or rat ovarian tissue as the source of aromatase [23]. We show here that a new non-steroidal imidazole inhibitor is highly effective in another steroidogenic tissue, namely brain. Although there is probably a single gene for the aromatase [2], there is no evidence yet that transcripts in the brain, placenta or ovary are identical. Adult dove POA is a useful model for investigating androgen metabolism, because the aromatase is very active relative to mammals [22]. In addition, the enzyme has a functional role, since changes in sexual behaviour in the dove are influenced by brain aromatization [24].

Previous studies of aromatase from human placenta and rat ovary [25, 26] show that the steroid aromatase inhibitors 4-OH-A and ATD bind irreversibly to the enzyme, acting as "suicide substrates" [27]. Newly characterized aromatase inhibitors include the steroidal, irreversibly binding SH 489 [28] and non-steroidal R76713 [8]. A disadvantage of steroidal inhibitors is that, at higher concentrations than those required for aromatase inhibition, they affect other androgen metabolizing pathways and may bind to steroid receptors. Our results using adult and neonatal brain tissue containing active aromatase systems show that the non-steroidal inhibitor CGS 16949A is more potent than either of the steroid aromatase inhibitors 4-OH-A or ATD (1.4 and 1.6 orders of magnitude difference in IC_{50}s, respectively with T as substrate). Similar data confirming the potency of CGS 16949A were obtained using a second dove brain area with high aromatase activity, the posterior hypothalamus. When human placental microsomes were used as the source of aromatase, with A as substrate, Bhatnagar *et al.* [29] found a corresponding difference in IC_{50}s between these inhibitors.

The production of mainly 5β-DHT and 5β-androstane-3α,17β-diol (i.e. 5β-reduction) by adult male dove POA in addition to aromatization when T is provided as substrate, agrees with previous observations on the hypothalamus [33]. Our study revealed a greater inhibition of 5β-reductase than of 5α-reductase (2–3 orders of magnitude difference) by the steroid aromatase inhibitors 4-OH-A and ATD, with some inhibition of 5β-reductase by high CGS 16949A concentrations ($IC_{50} \sim 10^{-5}$ M). Thus, inhibition of 5β-reductase by CGS 16949A only occurs at a concentration 50 times (T as substrate) or 36 times (A as substrate) higher than that required for equivalent inhibition by ATD. Therefore, the non-steroidal imidazole is far more selective than either ATD or 4-OH-A. There is also partial inhibition of 5α-reductase at high concentrations of 4-OH-A and ATD, but no inhibition by CGS 16949A. The influence of 4-OH-A on 5α-reductase activity is also minimal in human foreskin fibroblasts [31]. Inhibition of aromatase by 4-OH-A increases production of A with T as substrate. This shift in metabolism is consistent with results from genital skin fibroblasts [31] and probably arises from an increased T substrate availability for the 17β-hydroxysteroid dehydrogenase due to inhibition of other steroid metabolizing enzymes. The selectivity of the aromatase inhibitor CGS

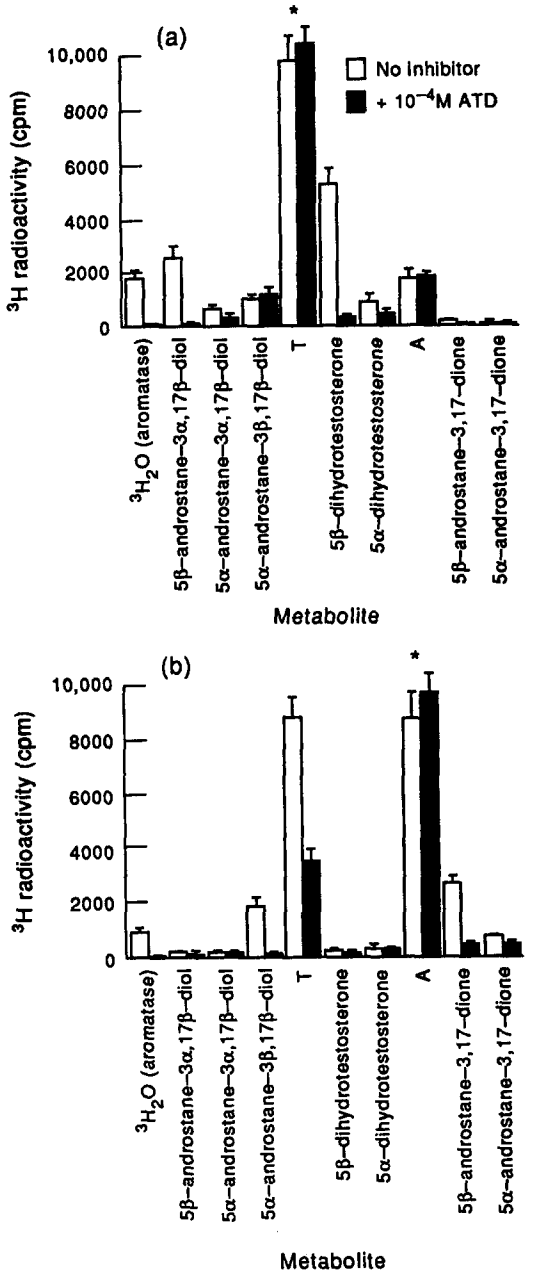

Fig. 4. Relative metabolite levels (mean ± SEM) in dove POA homogenates incubated with either 300 nM [1β-^{3}H]T (a) or [1β-^{3}H]A (b), in the presence or absence of 10^{-4} M ATD. Standard incubation conditions are as described in the Experimental section. *Substrate peak (T or A) divided by 20 for graphic purposes.

16949A has been established for other enzyme systems involved in rat ovarian progesterone biosynthesis [30], and formation of corticosterone and aldosterone [15]. The efficacy of CGS 16949A has also been demonstrated *in vivo* [14]. At a low oral dose CGS 16949A causes almost complete regression of 7,12-dimethylbenz-(α)-anthracene- (DMBA)- induced mammary carcinoma in adult female rats [29] and inhibits ovarian estrogen synthesis in the rat.

This study is the first demonstration of inhibition of neonatal brain aromatase by a non-steroidal imidazole. Brain aromatase of the neonatal gerbil has a weaker substrate binding affinity and more potent inhibition by CGS 16949A than the dove aromatase, indicating that the catalytic sites of the aromatase may differ between species. The results imply that higher substrate binding affinity is directly related to weaker inhibition by non-steroidal aromatase inhibitors. The exact inhibitor mechanism of CGS 16949A on the brain aromatase is still unknown. However, the increase in brain aromatase K_m in both dove and gerbil POA observed with increasing inhibitor (ATD or CGS 16949A) concentration agrees with the finding of Hsiang *et al.* [31] who investigated 4-OH-A inhibition of human foreskin fibroblast aromatase, and is indicative of competitive inhibition. Studies of rat ovarian and placental aromatase also show that the non-steroidal inhibitor acts competitively [14, 32]. There may also be more than one aromatase substrate binding site [34], suggesting that the action of the inhibitor differs depending on the substrate. Further work, including the nature of substrate–inhibitor binding sites, is in progress to demonstrate the mechanism of aromatase inhibition by non-steroidal inhibitors such as CGS 16949A in brain cells.

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