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THE INHIBITION OF HUMAN PROSTATIC AROMATASE ACTIVITY BY IMIDAZOLE DRUGS INCLUDING KETOCONAZOLE AND 4-HYDROXYANDROSTENEDIONE

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Abstract—Ketoconazole, an orally active imidazole drug and bifonazole, clotrimazole, econazole, isoconazole, miconazole and tioconazole are known as inhibitors of cytochrome P450 dependent steroidogenic enzymes including human placental aromatase. The aim of the present study was to investigate the effectiveness of these imidazole drugs to inhibit human prostatic aromatase activity compared with the known inhibitor of aromatase 4-hydroxyandrostenedione (4-OHA). The imidazole drugs and 4-OHA inhibited prostatic aromatase activity in a dose-dependent manner. The order of decreasing inhibitory potency determined from IC_{50} values (μmol/L) was: 4-OHA (1.57) > bifonazole (1.6) > tioconazole (1.69) > clotrimazole (1.73) > econazole (1.87) > miconazole (2.0) > isoconazole (2.2) > ketoconazole (4.7). The IC_{50} values for the inhibition of prostatic homogenate aromatase activity are 3–9-fold higher than that for the inhibition of human placental aromatase activity, previously reported, except that of ketoconazole which was 1.5-fold lower than that for the inhibition of placental microsomal aromatase.

The increased ratio of plasma oestrogen/androgen ratio in elderly men [1–4] together with reports of the localization of oestrogen [5, 6] and their receptors in the stromal component of the prostate gland [7, 8] suggests an involvement of oestrogens in the proliferation of the fibromuscular stroma. Further evidence for a role of oestrogens in the regulation of prostatic function comes from the observation of increased androgen receptors in the prostate of rats treated with diethylstilbestrol [9], which could be antagonized, by tamoxifen, and a potentiating effect of oestradiol on the increase in prostate weight induced by androgens [10].

Recently the presence of aromatase activity in benign prostatic hyperplastic (BPH) tissue has been reported [11, 12], this raises the question of the importance of oestrogens in the pathogenesis of benign hyperplasia.

The use of inhibitors of oestrogen biosynthesis for the treatment of BPH has been suggested [13]. Recently ketoconazole, an orally active imidazole drug and related imidazole drugs (bifonazole, clotrimazole, econazole, isoconazole, miconazole, tioconazole) have been reported to inhibit human placental aromatase [14] as well as cytochrome P450 dependent enzymes of the testis [15] and adrenal [16]. The aim of the present study was to determine the effectiveness of ketoconazole and other imidazole drugs to inhibit human prostatic aromatase activity and compared with their effect on other cytochrome P450 dependent enzymes [14–16].

MATERIALS AND METHODS

Materials. The source of materials and imidazole drugs was as previously reported [15]. [1,2,6,7-^{3}H]Androstenedione (sp. act. 90 Ci/mmol) was from Amersham International plc (Amersham, U.K.). 4-Hydroxyandrostenedione was a gift from Ciba-Geigy Pharmaceuticals (Horsham, U.K.).

Preparation of human prostatic homogenate. Hyperplastic prostates were obtained from patients at the time of transurethral resection or retropubic prostatectomy, transported to the laboratory on ice and stored at −70°. Fragments of prostatic tissue were examined histologically and the diagnosis of benign hypertrophy confirmed. For the preparation of the homogenate, prostatic tissue from several patients was pooled.

Frozen tissue was allowed to thaw at room temperature prior to homogenization. All subsequent procedures were carried out at 0–4°. Tissue was washed thoroughly with cold phosphate buffer (50 mmol/L, pH 7.4) to remove blood clots. Charred material was removed and the tissue then finely minced with scissors, suspended in cold phosphate buffer. Homogenization was carried out using 5 × 20 sec bursts of an Ultra-Turrax homogenizer with 2 min cooling periods. Homogenates were divided into portions (80 mg/mL protein) and stored at −70°.

Determination of human prostatic aromatase activity. [^{3}H]Androstenedione (1 μCi/11.1 pmol) was incubated with prostate homogenate (4 mg protein) in the absence or presence of NADPH (500 μmol/L) and in the absence or presence of progesterone (10 μmol/L) for 60 min at 37° in a final incubation volume of 1 mL.

Steroids and drugs were dissolved in 20 μL of ethanol, a similar amount of ethanol was added to control incubations. The presence of this amount of ethanol (2%) in the reaction mixture was without effect on enzyme activity.

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Table 1. Characterization of [^{3}H]oestrone and [^{3}H]oestradiol formed after incubation of [^{3}H]androstenedione with human prostatic homogenate

Metabolite	^{3}H/^{14}C Ratios		
	Starting material	Rechromatography	Acetylation
Oestrone	0.67	0.64	0.63
Oestradiol	0.29	0.31	0.30

In experiments to determine the effect of progesterone on human placental aromatase, the conditions were as described above except that human placental microsomes (200 μg protein), prepared as described previously [14], were incubated in place of prostatic homogenate.

Incubations were terminated by the addition of 1 mL cold chloroform containing: [^{14}C]oestradiol and [^{14}C]oestrone (each 5000 dpm) also non-radioactive androstenedione, testosterone, oestradiol and oestrone (each 100 μmol/L). Incubations were extracted with diethyl ether (3 × 4 mL) and evaporated to dryness [14, 15] and the residue reconstituted in 1 mL of NaOH (1 mol/L) and extracted with toluene (2 × 1 mL). The toluene removes approximately 90% of the androgens. The NaOH was neutralized with HCl to release the oestrogens which were then extracted with diethyl ether (3 × 4 mL).

Extraction efficiency was always greater than 85%. The toluene and ether extracts were separately chromatographed in solvent system chloroform–acetone (185:15, v/v) and chloroform–ethanol (90:10, v/v), respectively. Each experiment was carried out in duplicate and the results are the means of two independent experiments. Prostatic homogenate aromatase activity was quantified from the yields of total [^{3}H]oestrogens formed which had been corrected for manipulative loss using the recovery of [^{14}C]-labelled oestrogens and cross-over of cpm as described previously [15, 16]. The inhibitory effect of the drugs on aromatase activity was calculated as follows:

$$100 - \frac{\text{yield of [}^3\text{H]oestrogens in the absence of drugs}}{\text{yield of [}^3\text{H]oestrogens in the presence of drugs}} \times 100$$

The inter-assay coefficient of variation for aromatase activity was 6.6%.

Radiochemical purity of oestrogens formed. The

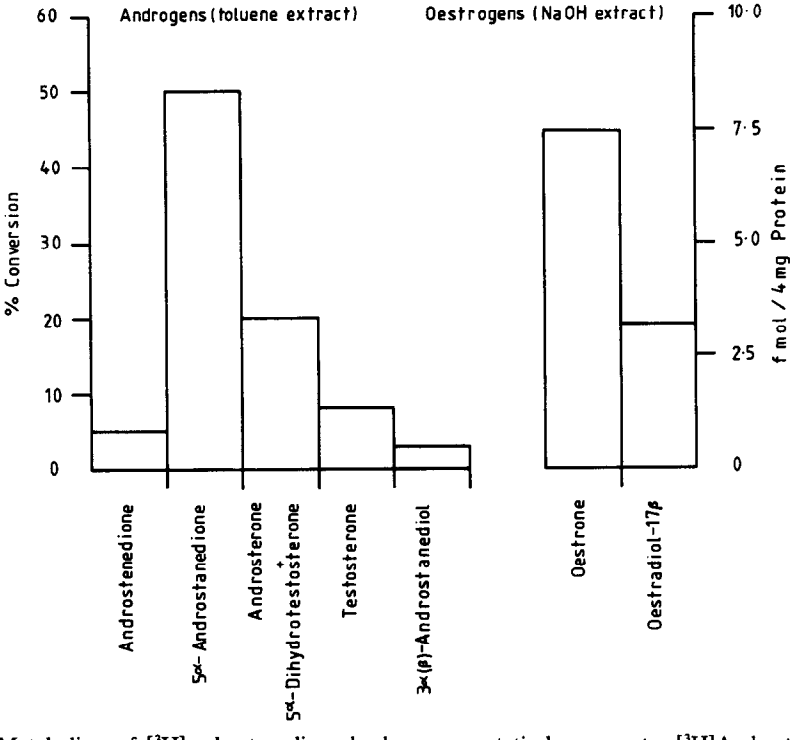

Fig. 1. Metabolism of [^{3}H]androstenedione by human prostatic homogenate. [^{3}H]Androstenedione (1 μCi/11.1 pmol) was incubated with prostatic homogenate (4 mg protein) in the presence of NADPH (500 μmol/L) for 60 min at 37°.

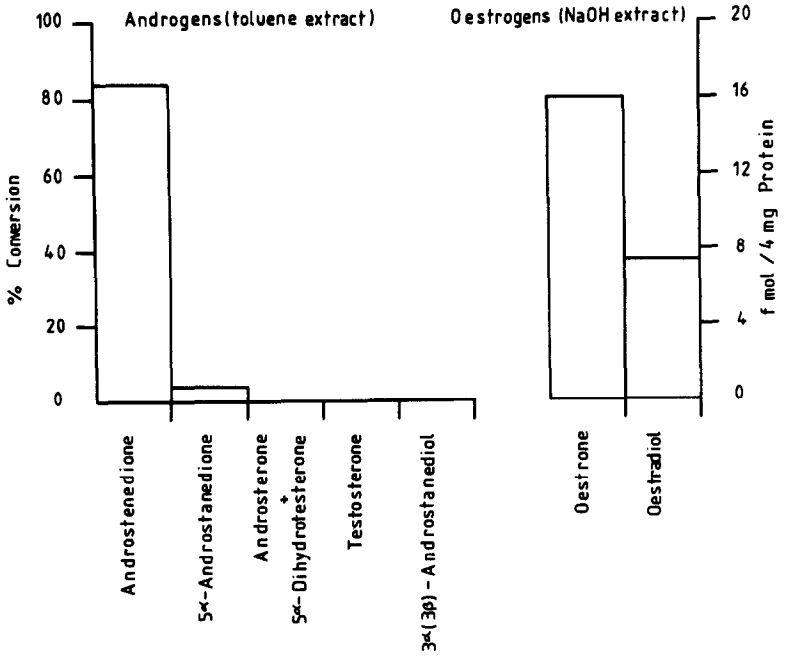

Fig. 2. Metabolism of [^{3}H]androstenedione by human prostatic homogenate in the presence of progesterone. [^{3}H]Androstenedione (1 μCi/11.1 pmol) was incubated with prostatic homogenate (4 mg protein), NADPH (500 μmol/L) and progesterone (10 μmol/L) for 60 min at 37°.

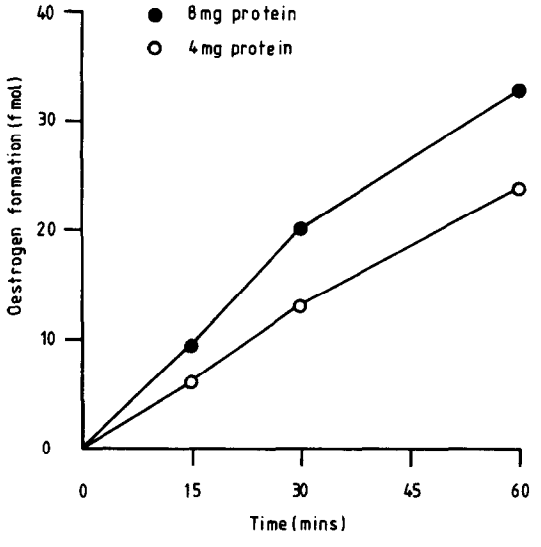

Fig. 3. Effect of incubation time and protein on oestrogen formation by human prostatic homogenate. [^{3}H]Androstenedione (1 μCi/11.1 pmol) was incubated with prostatic homogenate, NADPH (500 μmol/L) and progesterone (10 μmol/L) at 37°.

solvent system used in the chromatography of the oestrogen fraction, chloroform–ethanol (90:10, v/v), separates oestrone and oestradiol-17β from each other, as well as from androstendione and testosterone. Although oestrone and 5α-dihydrotestosterone were not separated there is no conversion of [^{3}H]androstenedine to [^{3}H]5α-dihydrotesterone in the presence of progesterone—which was included in all incubations to inhibit the 5α-reduction of androgens. The radiochemical purity of the oestrone and oestradiol was further checked by scraping and eluting the areas on the silica gel corresponding to each of the oestrogens (pooled from several chromatograms). The ^{3}H:^{14}C ratios of each pool of oestrogen was determined and an aliquot was rechromatographed in chloroform–ethyl acetate (50:50, v/v) which separates oestrone from 5α-dihydrotestosterone. The ^{3}H:^{14}C ratios did not vary by more than 4 and 6% for oestrone and oestradiol, respectively, from the starting material (Table 1). No radioactivity was found associated with the area on the TLC plate corresponding to 5α-dihydrotestosterone but migrated to positions corresponding to oestrone and oestradiol standards.

The ^{3}H:^{14}C ratios of oestrone and oestradiol did not vary by more than 6 and 3%, respectively, at the starting material following acetylation [17]. Moreover, the chromatographic mobilities in ethyl acetate–iso octane (70:30, v/v) of oestrone did not change, confirming the lack of readily acetylatable hydroxyl groups, whereas the mobility of oestradiol increased to that of its acetate.

Boiled tissue blanks were included in each set of experiments and no radioactivity was found associated with the oestrogen region of the chromatograph.

RESULTS

Aromatozation of [^{3}H]*androstenedione by human prostate homogenate*

To investigate the aromatization of [^{3}H]androstenedione by prostate homogenate, preliminary experiments were carried out to determine the effect

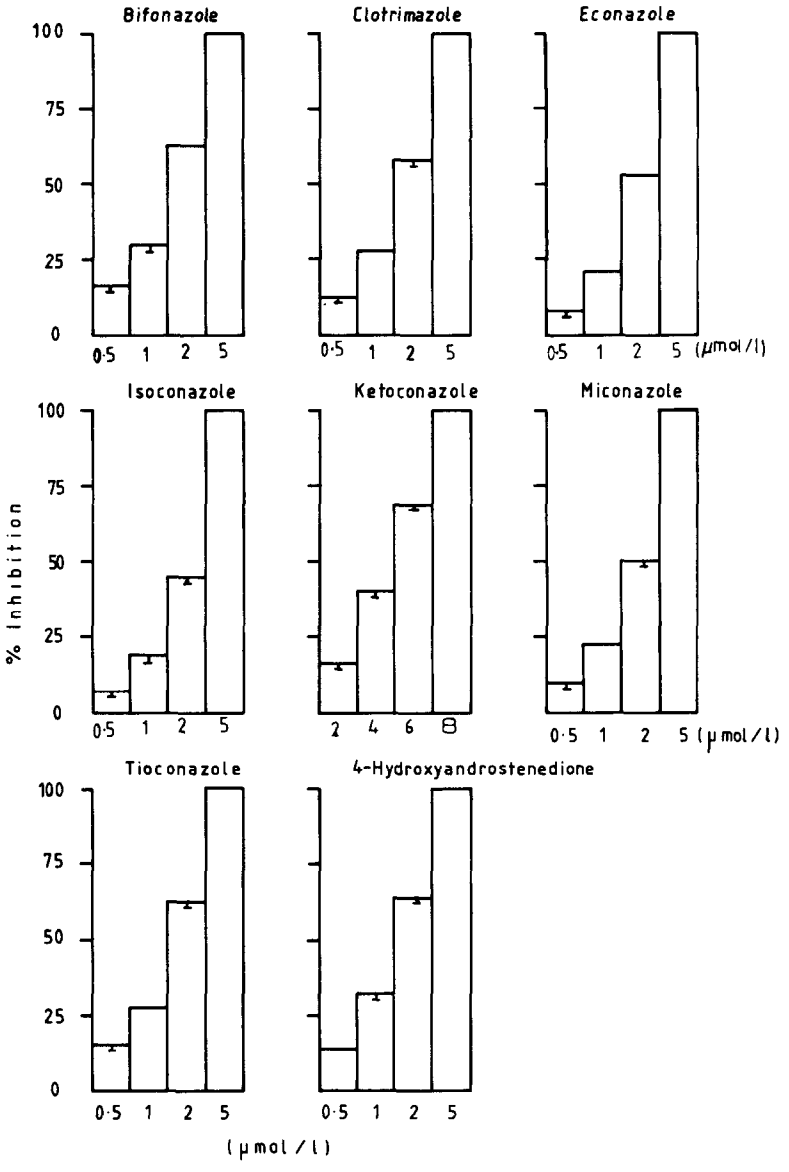

Fig. 4. The effect of increasing concentrations of imidazole drugs and 4-OHA on human prostatic aromatase activity. [^{3}H]Androstenedione (1 μCi/11.1 pmol) was incubated with prostatic homogenate (4 mg protein), NADPH (500 μmol/L), progesterone (10 μmol/L) and increasing concentrations of drugs for 60 min at 37°. Error bars denote the standard deviation around the mean of duplicate incubations of two independent experiments.

of the inclusion of progesterone (10 μmol/L), to inhibit the 5α-reduction of [^{3}H]androstenedione so as to make more substrate available for aromatization. The oestrogen (NaOH extract) and the androgen fraction (toluene extract) were separately chromatographed. In the absence of added progesterone there was conversion to oestrogens and substantial conversion of [^{3}H]androstenedione to 5-reduced androgens, in particular to 5α-androstanedione (Fig. 1). The results of the conversions to androsterone, 5α-dihydrotestosterone and 5α-androstane-3α(β)-diol are shown together since the solvent system used did not separate them. When progesterone was included in the incubation mixture conversion to oestrogens increased by approximately 50% (Fig. 2) whereas the conversion to 5α-reduced androgens was less than 5% of the total radioactivity recovered. The oestrone to oestradiol-17β ratio was approximately 2. There was no effect of progesterone at the concentration studied (10 μmol/L), on human placental aromatase determined under similar incubation conditions. In all subsequent experiments progesterone was included in the incubation mixture to inhibit the 5α-reduction of androgens.

Table 2. IC_{50} values of imidazole drugs and 4-OHA for the inhibition of human prostatic aromatase and human placental aromatase activity

Imidazole drug	IC_{50} (μmol/L) Prostatic homogenate aromatase	Placental* microsomal aromatase
4-Hydroxyandrostenedione	1.57 ± 0.04	0.18
Bifonazole	1.60 ± 0.03	0.31
Tioconazole	1.67 ± 0.01	0.22
Clotrimazole	1.73 ± 0.04	0.43
Econazole	1.87 ± 0.02	0.25
Miconazole	2.00 ± 0.08	0.47
Isoconazole	2.20 ± 0.13	0.67
Ketoconazole	4.70 ± 0.90	7.3

Each value shows the standard deviation around the mean of duplicate incubations of two independent experiments.

* IC_{50} values for human placental microsomal aromatase from Ayub and Levell [14].

The effect of incubation time and protein on oestrogen formation by human prostate homogenate

There was a linear increase of oestrogen formation with incubation time up to 60 min at 4 and 8 mg of protein (Fig. 3). The maximum concentration of oestrogen formation was 33 pmol/L which occurred in the presence of 8 mg of protein and at an incubation time of 60 min.

The effect of imidazole drugs, 4-OHA on human prostatic aromatase activity

The effect of imidazole drugs and 4-OHA on aromatase activity is shown in Fig. 4. There was a dose-dependent inhibition of activity with increasing concentration of drugs. The IC_{50} values (summarized in Table 2) ranged from 1.57 to 4.70 μmol/L; 4-OHA was the most and ketoconazole the least potent inhibitor.

DISCUSSION

In the present study, androstenedione in the presence of NADPH was found to be metabolized by prostate homogenate to oestrogens as well as to testosterone and 5α-reduced products. In agreement with these results Stone *et al.* [11] and Kaburgi *et al.* [12] also reported the presence of aromatase activity in human prostatic tissue, Schweikert [18] reported aromatase activity in fibroblasts from prostatic tissue and Marts *et al.* [19] in rat prostatic tissue. In contrast Smith *et al.* [20] could not detect aromatase activity in prostatic tissue.

Inclusion of progesterone in the incubation mixture resulted in the decrease of 5α-reduced products and an increase of oestrogen formation. This is in agreement with the results of previous studies which report that progesterone prevents the conversion of testosterone into 5α-dihydrotestosterone by acting as a preferential substrate for the 5α-reductase enzyme [21]. Stone *et al.* [11] used 4-MA (*N*,*N*-diethyl-4-methyl-3-oxo 4-aza-5α-androstane 17β-carboxamide) for the same purpose of increasing the yields of oestrogens by inhibiting the 5α-reduction of androstenedione, however they found 4-MA inhibited human prostatic aromatase activity. In the present study, inclusion of progesterone in the incubation mixture resulted in an increase in oestrogen formation, thus indicating that aromatase activity was not inhibited.

In the present study, the imidazole drugs (shown in Fig. 5) and 4-OHA were found to inhibit human prostatic aromatase in a dose-dependent manner.

The IC_{50} values of the imidazole drugs and 4-OHA for the inhibition of prostatic aromatase ranged from 1.57 to 4.70 μmol/L. The order of decreasing inhibitory effect was 4-OHA > bifonazole > tioconazole ± clotrimazole > econazole > miconazole > isoconazole > ketonazole, in comparison the order of decreasing inhibitory effect on human placental aromatase, previously reported [14] and shown in Table 1, was 4-OHA > tioconazole > econazole > bifonazole > clotrimazole > miconazole > isoconazole > ketoconazole.

For both placental and prostatic aromatase, 4-OHA was the most potent inhibitor and ketoconazole the least potent, the other imidazoles being intermediate.

Generally placental aromatase was more sensitive than prostatic aromatase to inhibition, the difference being greatest with the more potent inhibitors. Thus, the IC_{50} values for the inhibition of human prostatic aromatase by 4-OHA, bifonazole, tioconazole, clotrimazole, econazole, miconazole and isoconazole were 9, 5, 8, 4, 7, 4 and 3-fold higher, respectively, than those for the inhibition of human placental aromatase, in contrast the IC_{50} of ketoconazole for prostatic aromatase was 1.5-fold lower than that for placental aromatase.

The results of the present study for 4-OHA are in agreement with those of Stone *et al.* [11] who also reported the inhibition of human prostatic aromatase by 4-OHA.

Of the imidazole drugs studied econazole, miconazole and isoconazole are similar in structure differing only in the number and position of the chlorine

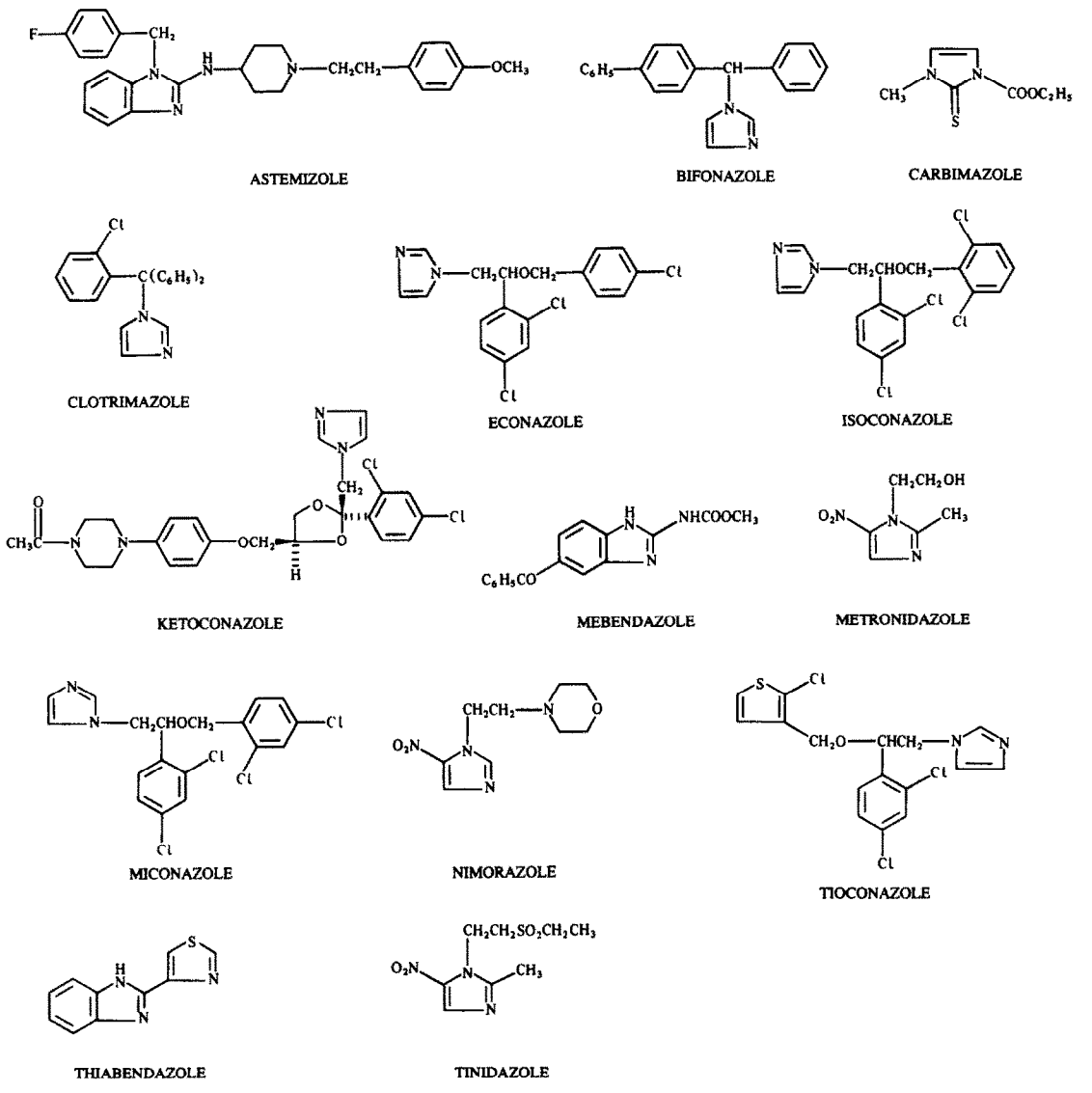

Fig. 5. Structures of imidazole drugs.

atoms on the same aromatic ring (benzyl ether). Their inhibitory effect on prostatic aromatase activity was similar but differed on human placental aromatase; econazole being 2-fold more potent than miconazole and isoconazole [14]. This suggests that the hydrophobic interaction of the N-1 substituent of econazole with the apoprotein near the haem of placental aromatase is stronger than that of miconazole or isoconazole. Previous results of the effect of the imidazole drugs used in the present study on the same enzyme activities (17α-hydroxylase/17,20-lyase) of rat testicular [15] and human adrenal [16] origin showed that the order of the inhibitory potency was similar but the testicular activities were more susceptible to inhibition.

A striking difference of the effect of ketoconazole on prostatic aromatase activity relative to testicular [15] and adrenal [16] 17α-hydroxylase and 17,20-lyase is apparent; it is 29 and 56-fold more potent an inhibitor of testicular and 4 and 8-fold more potent an inhibitor of adrenal 17α-hydroxylase and 17,20-lyase activities.

The demonstration of aromatase activity in human prostatic tissue as reported in the present study and also by others [11, 10, 18, 19] raises the possibility of regulation of intraprostatic levels of oestrogen independently of circulating plasma levels. The localization of higher levels of both oestrogen receptors [22] and 17β-hydroxysteroid oxidoreductase activity converting oestrone to oestradiol in the stromal tissue relative to the epithelial tissue [23] suggest that oestrogens exert their effect mainly on the fibromuscular stromal compartment of the prostate [1–4]. Some evidence for this is provided by the observation that treatment with androstenedione, an aromatizable androgen, caused hyperplasia of the stromal components of canine prostate which could be antagonized by co-administration of 4-OHA [13].

In conclusion, these imidazole drugs in addition to inhibiting the biosynthesis of androgens [15, 16] are potent inhibitors of prostatic aromatase activity. The concentrations at which ketoconazole, has an inhibitory effect on steroidogenic cytochrome P450 dependent enzymes are within the range of the plasma concentrations of ketoconazole (3–20 μmol/L; [24, 25]) achieved during treatment. Ketoconazole has been used for the treatment of androgen dependent disorders such as prostatic cancer [26–28] and could be of potential therapeutic use as an alternative treatment of BPH.

REFERENCES

1. Pirke KM and Doerr P, Age related changes and interrelationship between plasma testosterone, oestradiol and testosterone-binding globulin in normal adult males. *Acta Endocr Copenhagen* **74**: 792–800, 1973.
2. Karr JP and Murphy GP, Cellular growth and hormone receptor. In: *Benign Prostatic Hypertrophy* (Ed. Hinman F Jr), pp. 193–214. Springer, New York, 1983.
3. Nenbaur BL, Endocrine and cellular factors in the development of human benign prostatic hypertrophy. In: *Benign Prostatic Hypertrophy* (Ed. Hinman F Jr), pp. 179–192. Springer, New York, 1983.
4. Seppelt U, Correlation among prostate stroma, plasma estrogen levels and urinary estrogen excretion in patients with benign prostatic hypertrophy. *J Clin Endocr Metab* **47**: 1230–1235, 1978.
5. Ghanadian R and Puah CM, Relationship between oestradiol-17β, testosterone, dihydrotestosterone and 5α-androstane-3α,17β-diol in human benign hypertrophy and carcinoma of the prostate. *J Endocr* **88**: 255–262, 1981.
6. Belis JA, Methodologic basis for the radioimmunoassay of endogenous steroids in human prostatic tissue. *Invest Urol* **17**: 332–336, 1980.
7. Hawkins EF, Nigs M, Brassine C and Tagnon HJ, Steroid receptors in the human prostate 1/oestradiol-17β binding in benign prostatic hypertrophy. *Steroids* **26**: 458–468, 1975.
8. Kreig M, Bartsch W, Thomsen M and Voight KD, Androgens and oestrogens: Their interaction with stroma and epithelium of human prostatic hyperplasia and normal prostate. *J Steroid Biochem* **19**: 155–161, 1985.
9. Bouton MM, Pornin C and Grandadam JA, Estrogen regulation of rat prostate androgen receptor. *J Steroid Biochem* **15**: 403–408, 1981.
10. Walvoord DJ, Resnick MI and Grayhack JT, Effect of testosterone, dihydrotestosterone, estradiol and prolactin on the weight and citric acid content of the lateral lobe of rat prostate. *Invest Urol* **14**: 60–65, 1976.
11. Stone NN, Fair WRI and Fishman J, Estrogen formation in human prostatic tissue from patients with and without benign prostatic hyperplasia. *The Prostate* **9**: 311–318, 1986.
12. Kaburagi Y, Marino MB, Kirdani RY, Greco JP, Karr JP and Sandberg AA: The possibility of aromatisation of androgen in human prostate. *J Steroid Biochem* **26**: 739–742, 1987.
13. Habenicht UF, Schwartz K, Schweikert HU, Neumann F and Etrety MFEl, Development of a model for the induction of estrogen-related prostatic hyperplasia in the dog and its response to the aromatase inhibitor 4-hydroxy-4-androstene-3,17-dione: preliminary results. *The Prostate* **8**: 181–194, 1986.
14. Ayub M and Levell MJ, Structure–activity relationships of the inhibition of human placental aromatase by imidazole drugs including ketoconazole. *J Steroid Biochem* **31**: 65–72, 1988.
15. Ayub M and Levell MJ, Inhibition of testicular 17α-hydroxylase and 17,20-lyase but not 3α(β-hydroxysteroid dehydrogenase-isomerase or 17β-hydroxysteroid oxidoreductase by ketoconazole and other imidazole drugs. *J Steroid Biochem* **28**: 521–531, 1987.
16. Ayub M and Levell MJ, Inhibition of human adrenol steroidogenesis enzymes *in vitro* by imidazole drugs including ketoconazole. *J Steroid Biochem* **32**: 515–524, 1989.
17. Ayub M and Levell MJ, Inhibition of rat testicular 17α-hydroxylase and 17,20-lyase activity by anti-androgens (flutamide, hydroxyflutamide, RU 23908 cyproterone acetate) *in vitro*. *J Steroid Biochem* **28**: 43–47, 1987.
18. Schweikert HU, Conversion of androstenedione to oestrone in human fibroblasts cultured from prostate genital and non-genital skin. *Horm Metab Res* **11**: 635–640, 1979.
19. Marts SA, Padilla GM and Petrow V, Aromatase activity in microsomes from rat ventral prostate and Dunning R3327H rat prostatic adenocarcinoma. *J Steroid Biochem* **26**: 25–29, 1987.
20. Smith T, Chisholm GD and Habib FK, Failure of human benign prostatic hyperplasia to aromatase testosterone. *J Steroid Biochem* **17**: 119–120, 1982.
21. Jenkin JS and McCaffery M, Effects of oestradiol-17β and progesterone on the metabolism of testosterone by human prostatic tissue. *J Endocrinol* **63**: 517–526, 1974.
22. Kreig M, Kloetzl G, Kurfmann J and Voight KD, Stroma of benign prostatic hyperplasia: preferential tissue for androgen metabolism and oestrogen binding. *Acta Endocrol (Kbh)* **96**: 422–432, 1981.
23. Orlowski J and Clark AF, Estrogen metabolism by primary cultures of rat ventral prostate epithelial and stromal cells. *J Steroid Biochem* **29**: 361–368, 1988.
24. Brass C, Galgiani JN, Blaschke TF, De Flice R, O'Reilly RA and Stevens DA, Disposition of ketoconazole, an oral antifungal in humans. *Antimicrob Agents Chemother* **21**: 151–158, 1982.
25. Heyns W, Drochmans A, Van der Schueren E and Verhoeren G, Endocrine effects of high-dose ketoconazole therapy in advanced prostatic cancer. *Acta Endocrol (Kbh)* **110**: 276–285, 1985.
26. Vanuytsel L, Ang KK, Vantomgeren K, Drochmans A, Baert L and Van Der Schueren E, Ketoconazole therapy for advanced prostatic cancer: feasibility and treatment results. *J Urol* **137**: 905–908, 1987.
27. Pont A, Long-term experience with high dose ketoconazole therapy in patients with stage D2 prostatic carcinoma. *J Urol* **137**: 902–904, 1987.
28. Trachtenberg J and Poht A, Ketoconazole therapy for advanced prostate cancer. *Lancet* **2**: 433–435, 1984.