Azole Fungicides Affect Mammalian Steroidogenesis by Inhibiting Sterol 14α-Demethylase and Aromatase

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Azole compounds play a key role as antifungals in agriculture and in human mycoses and as nonsteroidal antiestrogens in the treatment of estrogen-responsive breast tumors in postmenopausal women. This broad use of azoles is based on their inhibition of certain pathways of steroidogenesis by high-affinity binding to the enzymes sterol 14α-demethylase and aromatase. Sterol 14α-demethylase is crucial for the production of meiosis-activating sterols, which recently were shown to modulate germ cell development in both sexes of mammals. Aromatase is responsible for the physiologic balance of androgens and estrogens. At high doses, azole fungicides and other azole compounds affect reproductive organs, fertility, and development in several species. These effects may be explained by inhibition of sterol 14α-demethylase and/or aromatase. In fact, several azole compounds were shown to inhibit these enzymes *in vitro*, and there is also strong evidence for inhibiting activity *in vivo*. Furthermore, the specificity of the enzyme inhibition of several of these compounds is poor, both with respect to fungal versus nonfungal sterol 14α-demethylases and versus other P450 enzymes including aromatase. To our knowledge, this is the first review on sterol 14α-demethylase and aromatase as common targets of azole compounds and the consequence for steroidogenesis. We conclude that many azole compounds developed as inhibitors of fungal sterol 14α-demethylase are inhibitors also of mammalian sterol 14α-demethylase and mammalian aromatase with unknown potencies. For human health risk assessment, data on comparative potencies of azole fungicides to fungal and human enzymes are needed. *Key words:* aromatase, CYP19, CYP51, endocrine disruption, follicle fluid meiosis-activating sterol (FF-MAS), lanosterol, meiosis-activating sterols, sterol 14α-demethylase, testis meiosis-activating sterol (T-MAS). *Environ Health Perspect* 111:255–261 (2003). doi:10.1289/ehp.5785 available via *http://dx.doi.org/* [Online 30 October 2002]

Azole fungicides show a broad antifungal activity and are used either to prevent fungal infections or to cure an infection. Therefore, they are important tools in integrated agricultural production. According to their chemical structure, azole compounds are classified into triazoles and imidazoles; however, their antifungal activity is due to the same molecular mechanism. The cell membrane assembly of fungi and yeast is disturbed by blocking the synthesis of the essential membrane component ergosterol. This fundamental biochemical mechanism is the basis for the use of azole fungicides in agriculture and in human and veterinary antimycotic therapies. The enzyme involved is sterol 14α-demethylase, which is found in several phyla. In mammals, it converts lanosterol into the meiosis-activating sterols (MAS). These precursors of cholesterol have been recently discovered to modulate the development of male and female germ cells.

Aromatase is another target enzyme of azole compounds. In steroidogenesis, it converts androgens into the corresponding estrogens. The importance of androgens and estrogens for the development of reproductive organs, for fertility, and in certain sex steroid-dependent diseases is well known. Therefore, azole compounds can be directed against aromatase to treat estrogen-responsive diseases.

The broad use of biologically active compounds in human therapy as well as in nonhuman applications may involve some risks, as exemplified by emerging antibiotic resistance. In agriculture, fungi and yeast are well known to develop resistance to azoles, and some molecular mechanisms of resistance development have been described (Joseph-Horne and Hollomon 1997). The significance of the agricultural azole resistance for human clinical antimycotic therapies has been recently discussed in Europe, but is not clarified yet (Hof 2001).

The antifungal properties of azoles and the issue of azole resistance in agriculture and medicine, however, are not the topics of this review. Our focus is rather on the increasing evidence for adverse effects of high doses of azole fungicides on the mammalian steroid metabolism.

Use of Azoles

Azole fungicides in agriculture. According to the U.S. Environmental Protection Agency (EPA), in 1997 approximately 244,000 and 37,000 tons of fungicides were sold worldwide and in the United States, respectively (U.S. EPA 1999). Unfortunately, precise data on the proportion of azole fungicides are not available. In Switzerland, nearly 40 tons of azoles are sold per year, which is approximately 5% of active ingredients in fungicides.

Maximum residue limits (MRLs) of pesticides approved in Switzerland are listed in the Swiss Ordinance on Foreign Substances and Toxic Components in Foodstuffs (Federal Authorities of the Swiss Confederation 2000). Within the group of the azole fungicides, currently 16 triazoles (bitertanol, cyproconazole, difenoconazole, epoxiconazole, fluquinconazole, flusilazole, flutriafol, hexaconazole, metconazole, myclobutanil, penconazole, propiconazole, tebuconazole, triadimefon, triadimenol, triticonazole) and three imidazoles (imazalil, prochloraz, and triflumizole) are listed. Additionally, there are triazoles with nonfungicidal applications (e.g., azocyclotin is used as an acaricide, paclobutrazole as a growth regulator, carfentrazone as a herbicide, and isazophos as an insecticide). An imidazole moiety is also found in the chloroacetanilid herbicide metazachlor.

Identity, chemical structure and physicochemical and other properties of azole fungicides, as well as their metabolic pathways in animals, plants and soils, are described elsewhere (Roberts and Hutson 1999; Tomlin 1997).

Azoles in treatment of human diseases. In the treatment of systemic and dermal mycoses, azoles play a pivotal role (Bodey 1992; Georgopapadakou 1998). They show significantly fewer side effects in comparison with other antimycotics such as amphotericin B, they can be applied after the emergence of resistance to other antimycotics (Espinel-Ingroff 1997), and they are inexpensive.

Individual azole compounds are found in many different antimycotic formulations. In Switzerland, three triazoles (terconazole, itraconazole, fluconazole) and eight imidazoles (clotrimazole, miconazole, econazole, ketoconazole, tioconazole, isoconazole, oxiconazole, and fenticonazole) are in use (Documed AG 2002).

Another significant application of azoles is the management of advanced estrogen-responsive breast tumors in postmenopausal

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women (Murray 2001; Santen and Harvey 1999). In these women, the estrogen levels dramatically decrease due to the lack of synthesis in the ovaries. At this time point, the adipose tissue becomes the main site of estrogen production. In patients suffering from breast cancer, the tumor and the surrounding tissues express aromatase and hence produce estrogen as well (Santner et al. 1997). This local production can substantially support the growth of estrogen-responsive tumors, and treatment with azole compounds (letrozole, anastrozole, vorozole, or fadrozole) suppresses estrogen production dramatically in these patients. Consequently, tumor growth can be blocked, and sometimes even remission occurs (Bhatnagar et al. 2001; Bisagni et al. 1996; de Jong et al. 1997; Dixon et al. 2000). In Switzerland, currently the two triazoles letrozole and anastrozole are in use at recommended doses of 2.5 mg/day or 1 mg/day, respectively (Documed AG 2002), partially replacing the classical nonsteroidal antiestrogen tamoxifen.

In a clinical trial, anastrozole was also effective in the treatment of estrogen-dependent endometriosis (Takayama et al. 1998). Furthermore, letrozole was used to treat boys with delayed puberty and short stature to induce growth (Wickman et al. 2001). The rationale behind this treatment is that azoles inhibit estrogen-dependent bone maturation. This leads to a delayed closure of the epiphysial growth plates and thereby allows growth.

These examples demonstrate the increasing importance of azole compounds in sex-steroid hormone-dependent diseases.

Target Enzymes of Azoles in Steroidogenesis

In this section we review the molecular characteristics and the involvement in steroidogenesis of both the sterol 14α-demethylase (encoded by the *CYP51* gene) and the aromatase (encoded by the *CYP19* gene), and we summarize the results of inhibition studies with azole compounds.

Sterol 14α-demethylase. Sterol 14α-demethylase is a member of the superfamily of heme-containing cytochrome P450 enzymes involved in metabolism of endogenous and xenobiotic substances. The antifungal effect of azoles is due to inhibition of sterol 14α-demethylase in fungi and yeast, thereby blocking the biosynthesis of ergosterol (Espinel-Ingroff 1997; Georgopapadakou 1998; Joseph-Horne and Hollomon 1997). The subsequent lack of ergosterol is detrimental because ergosterol is an essential sterol component in the membranes of fungi and yeast.

Sterol 14α-demethylase is not only expressed in fungi and yeast but is also found in many other species ranging from bacteria to mammals. The DNA sequences encoding sterol 14α-demethylase of many fungi and yeast are known, as well as the sequences of mice, rats, pigs, and humans (Debeljak et al. 2000; Kojima et al. 2000; Nitahara et al. 1999; Stromstedt et al. 1996). On the protein level, the amino acid sequences are highly conserved along the phylogenetic tree. This fact is considered by many authors as an indication of the pivotal role of sterol 14α-demethylase in all organisms. The homology of the amino acid sequence level between rats and humans is 93% and 40% between fungi and humans (Stromstedt et al. 1996). In humans, the sterol 14α-demethylase is expressed in many different tissues (Raucy et al. 1991).

The common linear precursor squalene is converted to the basic sterol structures in fungi, yeast, plants, and animals by complex reactions, including cyclizations. These sterols are the substrates of the sterol 14α-demethylase (Figure 1). In all species, the sterol 14α-demethylase oxidatively demethylates C-14 of these basic sterols. The reaction has been studied in detail and it was shown to closely resemble the demethylation reaction at C-10 catalyzed by aromatase (Shyadehi et al. 1996). In fungi, the sterol 14α-demethylase reaction leads to an important precursor of ergosterol. In plants, the sterol 14α-demethylase reaction metabolizes obtusifoliol and provides precursors for biosynthesis of phytosterols. In animals, the sterol 14α-demethylase reaction is part of the metabolic pathway leading to biosynthesis of cholesterol. Cholesterol in turn is the substrate for the production of many other sterols (e.g., the sex steroid hormones). In more detail, C-14 demethylation of lanosterol in animals produces the follicle fluid meiosis-activating sterol (FF-MAS), which is further metabolized to the testis meiosis-activating sterol (T-MAS). Otherwise, if lanosterol is reduced by the sterol Δ24-reductase to 24,25-dihydrolanosterol before demethylation, the resulting metabolites are MAS-412 and MAS-414, respectively (Figure 2; Byskov et al. 1995).

Although the specific inhibition of fungal sterol 14α-demethylase by competitive, reversible binding to the heme moiety of the enzyme is the intended effect of azoles in agriculture and clinical antifungal treatments (Ji et al. 2000; Podust et al. 2001; Yoshida and Aoyama 1987), some azole compounds are not very specific in this respect. Clotrimazole, miconazole, sulconazole, tioconazole, and ketoconazole were shown to inhibit many different P450 enzymes (Zhang et al. 2002).

The inhibition of sterol 14α-demethylase by azoles, expressed as the half maximal inhibitory concentration (IC_{50}), depends on the azole derivative and to a lesser extent on the source of sterol 14α-demethylase (Van den Bossche et al. 1987). For example, the IC_{50} values for triadimenol and tebuconazole on the sterol 14α-demethylase prepared from the phytopathogen fungus *Ustilago maydis* are 0.07 and 0.05 nM, respectively, whereas for sterol 14α-demethylase prepared from the

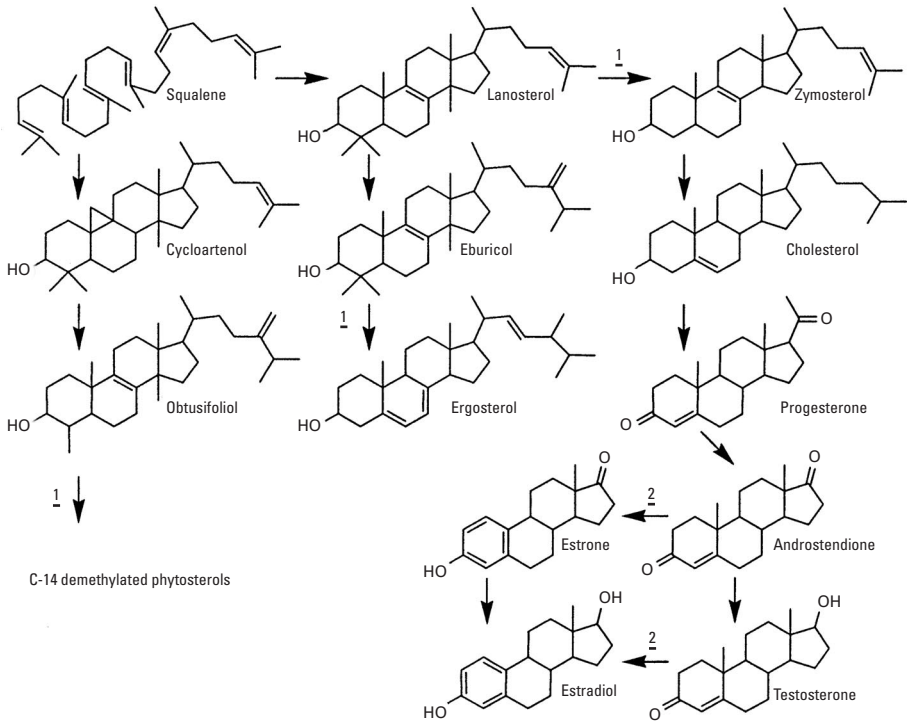

Figure 1. Biosynthesis of phytosterols, ergosterol, and mammalian sex steroid hormones. Arrows indicate one or more enzymatic steps. Reactions catalyzed by sterol 14α-demethylase (1) and aromatase (2) are indicated.

plant *Sorghum bicolor*, the IC_{50} values are only slightly higher (0.24 and 0.16 nM, respectively; Lamb et al. 2001). Ketoconazole and itraconazole, respectively, exhibited IC_{50} values of 60 and 80 nM on *Candida albicans* sterol 14α-demethylase and of 580 and 610 nM on human sterol 14α-demethylases, both enzymes heterologously expressed in yeast (Lamb et al. 1999). For ketoconazole, itraconazole, and fluconazole, IC_{50} values of 180 nM, 330 nM, and 170 μM, respectively, were reported for rat sterol 14α-demethylase expressed in *Escherichia coli* (Nitahara et al. 1999). In crude extracts of yeast, the sterol 14α-demethylase is inhibited by flusilazole approximately 100 times more efficiently than in crude cell-free extracts from rat liver (Trzaskos and Henry 1989). This difference was nearly abolished upon further purification of the enzymes. According to the authors, the differences in the crude extracts are due to different contents in other azole-susceptible P450 enzymes that may scavenge the azoles.

In conclusion, the direct comparison of the IC_{50} values from different studies is hampered by considerable variations in the study designs and test materials used, such as enzyme purity.

Aromatase. Another important P450 enzyme involved in the steroidogenesis is aromatase. Like sterol 14α-demethylase, aromatase catalyzes the oxidative demethylation of sterols. In contrast to sterol 14α-demethylase, which has several substrates in different phyla, aromatase demethylates C10 and specifically converts androstenedione and testosterone, resulting in estrone and estradiol, respectively (Figure 1). However, other nonsteroid substrates of the aromatase, such as 7-ethoxycoumarin are also known (Toma et al. 1996). The aromatase coding DNA sequences of several animals (Hickey et al. 1990; McPhaul et al. 1988; Tanaka et al. 1992; Trant 1994) and humans (Evans et al. 1986; Means et al. 1989; Toda and Shizuta 1993) have been published. On the protein level, the amino acid sequence homology between aromatase from fish and humans is about 50% (Trant 1994) and between rats and humans is about 78% (Hickey et al. 1990). In mammals, aromatase is mainly expressed in the brain and the gonads, but it is also found in placental, adipose, and bone tissue (Conley and Hinshelwood 2001).

The physiologic balance between different sex steroid hormones is crucial for the development, maintenance, and function of the reproductive system as well as for the differentiation of the sexual phenotype during ontogeny. Estrogens (estrone and estradiol) are products of the androgens (androstenedione and testosterone), and the reaction is catalyzed by aromatase. In mammals, differentiation of the male phenotype depends not only on testosterone but also on estradiol generated from testosterone by neuronal aromatase in central nervous system. Therefore, disturbances in aromatase expression and/or changes in its catalytic activity are expected to exhibit negative effects on reproduction parameters. In fact, in transgenic male mice overexpressing aromatase in the testis, the serum estradiol level is increased (Fowler et al. 2000). Half of these animals were infertile and had larger testes and a significantly increased incidence of Leydig cell tumors in testes. In male and female aromatase knockout mice (ArKO), the estradiol levels are below detection limits in both sexes. In males, the testosterone levels are elevated in the first 12–14 weeks only, and female ArKO mice showed a pronounced increase in testosterone levels (Fisher et al. 1998; Robertson et al. 1999).

Aromatase can be inhibited competitively and reversibly by azole compounds, as seen with sterol 14α-demethylase. The IC_{50} values of the azole fungicides prochloraz, imazalil, propiconazole, triadimenol, and triadimefon on microsomal aromatase from human placenta were 0.04, 0.34, 6.5, 21, and 32 μM, respectively (Andersen et al. 2002; Vinggaard et al. 2000). In a rat ovary aromatase assay, the therapeutic drugs anastrozole, fadrozole, and letrozole exhibited IC_{50} of 0.025, 0.007 and 0.007 μM, respectively (Odum and Ashby 2002). Not only azoles but also natural plant constituents such as flavonoids are able to significantly inhibit the aromatase (Jeong et al. 1999).

Biological Effects of Azole Compounds

Based on the inhibitory activity of azoles on key enzymes involved in sex steroid hormone synthesis, it is likely that effects on fertility, sexual behavior, and reproductive organ development will occur depending on dose level and duration of treatment of laboratory animals. Therefore, we reviewed the toxicology monographs of the Joint FAO/WHO (Food and Agriculture Organization/World Health Organization) Meeting on Pesticide Residues (JMPR) on some azole fungicides by focusing on effects that might be induced by disturbed steroidogenesis (Appendix).

Role of sterol 14α-demethylase in germ cell development. As discussed above, in mammals, sterol 14α-demethylase either directly converts lanosterol into FF-MAS or, after preceding reduction of lanosterol by sterol Δ24-reductase into MAS-412. Sterol Δ14-reductase then converts FF-MAS in T-MAS and MAS-412 in MAS-414 (Figure 2). These metabolites of lanosterol have been regarded only as precursors of cholesterol without any biological function in animals. This view dramatically changed recently with the observation that FF-MAS isolated from human follicle fluid and T-MAS isolated from bull testis as well as the MAS-412 and MAS-414 induced resumption of meiosis in cultivated mouse oocytes (Byskov et al. 1995).

In rats, expression of sterol 14α-demethylase in postmeiotic spermatids is significantly higher than in premeiotic spermatids (Stromstedt et al. 1998) and is restricted to

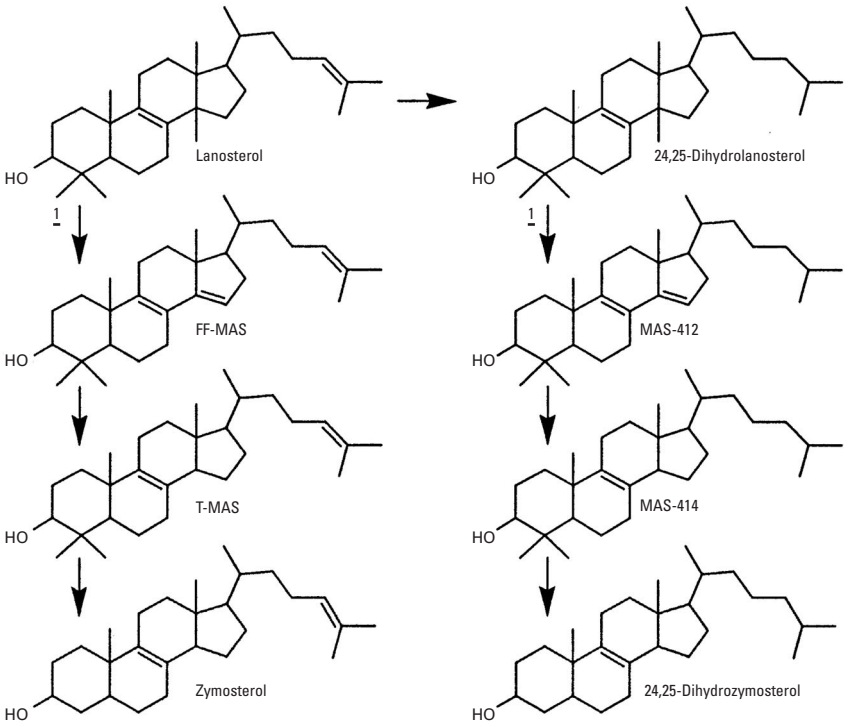

Figure 2. Biosynthesis of MAS. Reactions catalyzed by sterol 14α-demethylase (1) are indicated.

discrete stages of the spermatid development (Majdic et al. 2000). In the developing rat testis, high levels of T-MAS were found. These increased T-MAS levels were due to the selective up-regulated expression of cholestogenic genes, namely the pre-MAS genes such as sterol 14α-demethylase, and down-regulated post-MAS genes (Tacer et al. 2002). In the human hepatoma cell line HepG2 and in rat testes, progestins inhibited the cholesterol biosynthesis, and consequently the levels of FF-MAS and T-MAS were increased (Lindenthal et al. 2001). In mouse oocytes, *in vitro* resumption of meiosis was induced by the specific blocking of the sterol Δ14-reductase, and hence the FF-MAS level was elevated (Leonardsen et al. 2000). In another study, only denuded oocytes responded to higher FF-MAS levels but not cumulus-cell–enclosed oocytes (Downs et al. 2001). Resumption of meiosis *in vitro* by increased FF-MAS levels was also observed in human oocytes (Cavilla et al. 2001). The biological significance of the MAS and their implications on fertilization were recently reviewed (Byskov et al. 2002; Rozman et al. 2002). However, other studies failed to show these pronounced effects of MAS on oocytes (Tsafriri et al. 1998, 2002; Vaknin et al. 2001).

Although the detailed function of the MAS is not fully understood yet, the existing studies indicate an important role of these sterols in the development of testis and spermatids and possibly also an impact on the oocyte development.

Effects induced by aromatase inhibition. Azole fungicides described in the JMPR monographs (see Appendix) and azole compounds used in the management of breast cancer share some common effects *in vitro* and *in vivo*.

The potential of azoles to lower estradiol levels and to delay ovulation in rats has been known for several years as is exemplified by an isomer of flutriafol (Middleton et al. 1986; Milne et al. 1987).

Although on the molecular level (e.g., IC_{50} values) no profound species differences are seen, distinct effects are observed in laboratory animals after azole treatment. In dogs, exposure to letrozole induced histopathologic changes in the testis, whereas in rats no such effects were observed (Walker and Nogues 1994). In female rats, letrozole (Sinha et al. 1998) and vorozole (De Coster et al. 1990) considerably lowered estrogen levels and reduced uterus weights. In a mouse MCF-7 xenograft model, tumor growth was effectively inhibited by letrozole (Lee et al. 1995). The same effect on rat mammary tumor growth was seen with letrozole and a derivative thereof, in addition to a prominent reduction of estradiol levels and uterus weights (Schieweck et al. 1993). In male cynomolgus monkeys, vorozole lowered the estradiol levels (Tuman et al. 1991) and in quails (Foidart et al. 1994) and in female musk shrews (Rissman et al. 1996) it changed sexual behavior. The treatment of growing male rats with vorozole impaired skeletal development and the mineralization of bones (Vanderschueren et al. 1997).

In European pond turtles (Belaid et al. 2001) and in whiptail lizards (Wennstrom and Crews 1995), letrozole affected sex determination, as did fadrozole in chickens and turkeys (Burke and Henry 1999). Juvenile sexually undifferentiated fathead minnows were shown to masculinize when exposed to fadrozole in combination with methyltestosterone (Zerulla et al. 2002). In another study on fathead minnows exposed to fadrozole, reduced brain aromatase activity was accompanied by reduced estradiol and vitellogenin levels, and a decrease in mature oocytes and an increase in sperms in testes was observed (Ankley et al. 2002). In nonhuman primates, it was shown that fadrozole, letrozole, and a derivative of letrozole inhibited the aromatase (Shetty et al. 1997, 1998). Although in female animals the estrogen levels were significantly decreased, the development of follicles seemed normal but the animals did not become pregnant. In the males, the treatment resulted in elevated testosterone levels, and the production of spermatocytes was significantly reduced within the first 30 days of treatment. In contrast, within the first 90 or 120 days of treatment, in ArKO mice the spermatogenesis seemed normal and the spermatozoa *in vitro* were as fertile as the wild-type spermatozoa (Fisher et al. 1998; Robertson et al. 1999). Yet there were fewer litters with these males. The authors suggest alterations in mounting behavior as being the reason for this discrepancy (Robertson et al. 2001). Additionally, elevated prostate weights without malignant changes were observed in ArKO male mice (McPherson et al. 2001).

Many of the effects described can be seen as the result of a disturbed balance of androgens and estrogens, provoked either by chemically blocking aromatase or by the lack of a functional aromatase in the case of knockout animals. In animal studies to investigate the chemical blockage of the aromatase by azoles, it cannot be excluded that the sterol 14α-demethylase also was blocked. Interpretations of these results therefore should include not only changed androgen and estrogen levels but also a disturbed balance of MAS levels.

Summary

Several azole compounds were shown to inhibit the aromatase and to disturb the balance of androgens and estrogens *in vivo*. In fact, the clinical use of azole compounds in estrogen-dependent diseases is based on this effect. Additionally, azole antifungals developed to inhibit the sterol 14α-demethylase of fungi and yeast in agriculture and medicine are also inhibiting aromatase. Therefore, these antifungals may unintentionally disturb the balance of androgens and estrogens. Until now, it is not clear whether this effect is compensated by an increased expression of aromatase or by other unknown mechanisms.

The actual target enzyme of azole antifungals, the fungal sterol 14α-demethylase, is expressed in many species including humans, and it is highly conserved through evolution. Hence, it seems reasonable to assume that most of the azole antifungals used in agriculture and medicine as well as azoles used in management of breast cancer also act as inhibitors on human sterol 14α-demethylase to an unknown extent. Unfortunately, there are few studies on the inhibition of mammalian sterol 14α-demethylases by azole compounds.

The impact of inhibition of the mamalian sterol 14α-demethylase on the cholesterol pool is not clear. Because dietary cholesterol may compensate disturbances in cholesterol levels, the cholesterol pool might be not a very sensitive parameter for sterol 14α-demethylase inhibition. However, spermatogenesis and oogenesis are in part controlled by MAS, and the conversion of lanosterol to MAS exclusively relies on sterol 14α-demethylase activity. The data available provide good evidence that azoles directed against the ergosterol biosynthesis of fungi and yeast have the potential to influence the levels of FF-MAS, T-MAS, androgens, and estrogens in animals and humans.

To our knowledge, structure–activity relationships of azoles with respect to their ability to discriminate between sterol 14α-demethylase and aromatase have hardly been investigated. There is also a lack of data on enzyme regulation of sterol 14α-demethylase and aromatase after azole exposure and on the putative impact of dietary cholesterol. Inhibition of other P450 enzymes by azoles is even less investigated.

The toxicologic profiles of individual azole fungicides, as summarized in the Appendix, provide evidence for endocrine effects. In fact, many of these fungicides have effects on prostate, testis, uterus, and ovaries as well as on fertility, development, and sexual behavior. The current database does not allow us to establish causal relationships of these effects with inhibition of sterol 14α-demethylase and/or aromatase, but the overall view strongly suggests a connection with disturbed steroidogenesis.

Based on the clear effects on steroidogenic enzymes *in vitro*, animal studies should be performed with concomitant analysis of androgen, estrogen and MAS levels and

investigations on inhibition of the fungal sterol 14α-demethylase, mammalian sterol 14α-demethylases, and aromatase are clearly necessary.

The Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) of the U.S. EPA has recommended the aromatase inhibition assay as one of the possible testing system to identify endocrine disrupters (EDSTAC 1998). Since the publication of the EDSTAC report in 1998, recent studies on the impact of MAS on spermatogenesis and oogenesis have been published and, as a consequence, sterol 14α-demethylase should also be considered as a candidate testing system for identifying endocrine disrupters.

Exposure estimates based on residue values from supervised field trials are performed by JMPR. These estimates indicate that the average daily intake of individual azole fungicides is approaching at most a very low percentage of the acceptable daily intake (ADI) established by the JMPR. According to residue data from food random sampling, the MRLs are hardly ever exceeded. Nevertheless, several azole fungicides are found in the candidate list of putative endocrine disrupters within the European Union Community strategy for endocrine disrupters (European Commission DG ENV 2002).

In conclusion, azole fungicides interact with several P450 enzymes in different species and have the potential to affect the endocrine system by interacting with steroidogenesis. Due to the knowledge of the common mechanism on steroidogenesis in target organisms and mammalians, we suggest that azole fungicides should be considered as a group when MRLs are allocated. It will be the challenge to design more specific inhibitors of fungal sterol 14α-demethylases that do not affect animal sterol 14α-demethylases and aromatases.

Appendix

Usually, the hazard assessments of pesticides by the JMPR are based on data of *in vitro* and animal studies providing no observable effect levels (NOEL) for delineation of individual ADIs in humans. Based on the ADI and residue levels in plants after application of recommended use levels, MRLs are allocated to individual foods by the authorities.

In vivo studies of some azole fungicides are summarized below with special focus on effects putatively connected to disturbed steroidogenesis. In the allocation procedure for an ADI by the JMPR, these effects are included.

Bitertanol was evaluated by the JMPR in 1998 (FAO/WHO 1999). An ADI of 0–0.01 mg/kg body weight (bw) was allocated based on a NOEL of 1 mg/kg bw in a three-generation rat study and reduced pup survival rates at 5 mg/kg bw. Histopathologic changes in the adrenal glands of dogs and rats were seen at 1.2 and ≥ 81 mg/kg bw, respectively. In dogs, at ≥ 5 mg/kg bw, cataracts and reduced prostate weight with histopathologic changes were seen. In male rats, at ≥ 300 mg/kg bw, the relative testis weights were increased, and in females, the absolute ovary and adrenal weights were decreased with histopathologic changes. At the maternotoxic level of 100 mg/kg bw, rat fetuses showed effects such as cleft palate and hydrocephalus.

Cyproconazole induced cleft palate, hydrocephalus, and hydronephroses in rat embryos after treatment of the dams with 20 mg/kg bw (the lowest dose tested); ≥ 50 mg/kg bw increased incidences of resorptions and dead fetuses were observed (Machera 1995).

Flusilazole was evaluated by the JMPR in 1995 (FAO/WHO 1996). An ADI of 0–0.001 mg/kg bw was allocated based on a NOEL of 0.14 mg/kg bw in a 1-year dog study. At ≥ 0.7 mg/kg bw, effects on the liver of the dogs were observed. In a 14-day rat study, the levels of testosterone and estradiol were reduced at ≥ 20 mg/kg bw. In isolated Leydig cells, the IC_{50} for testosterone production was 3.5 μM for flusilazole and 1 μM for ketoconazole. In a 2-year rat study, 31 mg/kg bw flusilazole induced Leydig cell tumors of the testis; flusilazole was fetotoxic and embryotoxic at ≥ 9 mg/kg bw in a developmental study. Toxicity to the dams was observed at ≥ 27 mg/kg bw. In *in vitro* teratogenicity assays with rat embryos, changes at the branchial apparatus were seen at 6.25 μM (Menegola et al. 2001).

Hexaconazole was evaluated by the JMPR in 1990 (FAO/WHO 1991). An ADI of 0–0.005 mg/kg bw was allocated based on a NOEL of 0.47 mg/kg bw in a 2-year rat study. At 4.7 ≥ mg/kg bw an increased incidence of Leydig cell tumors in testis was observed. In a 90-day rat study, histopathologic changes were observed in the adrenal glands at ≥ 2.5 mg/kg bw, and reduced testis and adrenal weights were observed at 250 mg/kg bw. In rats, hexaconazole was fetotoxic at ≥ 25 mg/kg bw. Isolated rat Leydig cells showed reduced testosterone and increased progesterone production on exposure to hexaconazole (0.1–30 μM).

In a 29-day mouse study, lack of corpora lutea and smaller ovaries were observed in females and histopathologic changes in the testis and epididymis and enlarged adrenals were observed in males at ≥ 14 mg/kg bw. In a 90-day dog study, weights of ovaries and testes were reduced at ≥ 125 mg/kg bw.

Imazalil was evaluated by the JMPR in 2000 (FAO/WHO 2001). In a 1-year dog study, an ADI of 0–0.03 mg/kg bw was allocated based on a NOEL of 2.5 mg/kg bw and effects on body weight, the liver, and clinical symptoms at 20 mg/kg bw. At a dose range of 5–20 mg/kg bw imazalil was fetotoxic in rats, mice, and rabbits.

Myclobutanil was evaluated by the JMPR in 1992 (FAO/WHO 1993a). An ADI of 0–0.03 mg/kg bw was allocated based on a NOEL of 2.5 mg/kg bw in a 2-year rat study and many effects on the male reproductive system at ≥ 10 mg/kg bw. Reduced testis weight, testis atrophy, reduced or absent spermatid production, necrotic epididymis, and atrophy of the prostate were observed with increasing doses.

Penconazole was evaluated by the JMPR in 1992 (FAO/WHO 1993b). An ADI of 0–0.03 mg/kg bw was allocated based on a NOEL of 3 mg/kg bw and reduced testis weight with atrophic changes at ≥ 17 mg/kg bw in a 1-year dog study. In long-term studies, increased prostate weights were observed in mice at ≥ 9.8 mg/kg bw and in rat testis at 202 mg/kg bw. In a two-generation rat study, relative ovary weights were increased at ≥ 125 mg/kg bw. At the maternotoxic level of 500 mg/kg bw, penconazole was also fetotoxic.

Prochloraz was evaluated by the JMPR in 2001 (FAO/WHO 2002). An ADI of 0–0.01 mg/kg bw was allocated based on a NOEL of 1.3 mg/kg bw and liver effects at ≥ 5 mg/kg bw in a 2-year rat study. In a 90-day rat study, ovary and thyroid weights were increased in females at ≥ 6 mg/kg bw. In a 90-day dog study, reduced testis and prostate weights were observed at ≥ 7 mg/kg bw. In a rat reproduction study, at ≥ 27 mg/kg bw a tendency to prolonged gestation, increased total litter losses, smaller litter sizes, and increased pup mortality was observed. In trout, 25 nM (9.4 μg/L) prochloraz impaired the spermatogenesis (Le Gac et al. 2001).

Propiconazole was evaluated by the JMPR in 1987 (FAO/WHO 1988). An ADI of 0–0.04 mg/kg bw was allocated based on a NOEL of 4 mg/kg bw slight effects on the liver and hematology parameters at ≥ 20 mg/kg bw in a 2-year rat study. In a rat reproduction study, reduced testis and epididymis weights in pups were observed at ≥ 21 mg/kg bw. However, testis weights of rats were increased at 256 mg/kg bw in a short-term study.

Tebuconazole was evaluated by the JMPR in 1994 (FAO/WHO 1995). An ADI of 0–0.03 mg/kg bw was allocated based on a NOEL of 3 mg/kg bw and histopathologic

changes in the adrenal glands and questionable cataracts at 4.5 mg/kg bw in a 1-year dog study. In a 90-day rat study, histopathologic changes in the adrenals were found at ≥ 36 mg/kg bw. In rat reproduction studies, reduced litter sizes and reduced survival indices were observed at ≥ 73 mg/kg bw. In a mouse teratogenicity study, increased incidences of runts and malformations such as cleft palate were observed at ≥ 30 mg/kg bw. In rats and rabbits, malformations and embryotoxicity occurred at ≥ 100 mg/kg bw.

Triadimefon was evaluated by the JMPR in 1981/1985 (FAO/WHO 1982, 1986). An ADI of 0–0.03 mg/kg bw was allocated based on a NOEL of 2.5 mg/kg bw and body weight effects and reduced hematopoesis at ≥ 25 mg/kg bw in a 2-year rat study. In a rat reproduction study, the male/female sex ratio in F_2 was reduced at 77 mg/kg bw and the female fertility index was one-third of control. In a supplementing study, treated males (77 mg/kg bw) were mated with untreated females. The pregnancy rate was significantly lower, but the ratio of pregnant to inseminated females was not affected. It was concluded that triadimefon impaired the sexual behavior of the males. The testosterone level of these male rats was doubled. In *in vitro* teratogenicity studies with rat embryos, changes at the branchial apparatus were seen at 25 µM for both triadimefon and triadimenol (Menegola et al. 2000).

Triadimenol was evaluated by the JMPR in 1989 (FAO/WHO 1990). An ADI of 0–0.05 mg/kg bw was allocated based on a NOEL of 5 mg/kg bw in a rat two-generation study and on retarded development of pups at 20 mg/kg bw. In a mouse short-term study and a dog long-term study, changes in the cholesterol levels were seen at 170 mg/kg bw and 45 mg/kg bw, respectively, and in a 2-year rat study, ovary weights were reduced at 144 mg/kg bw. In rat and rabbit teratogenicity studies, increased incidences of resorptions were observed at ≥ 120 mg/kg bw.

References

Andersen HR, Vinggaard AM, Rasmussen ES, Gjermandsen IM, Bonefeld-Jorgensen EC. 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. Toxicol Appl Pharmacol 179:1–12.

Ankley GT, Kahl MD, Jensen KM, Hornung MW, Korte JJ, Makynen EA, et al. 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). Toxicol Sci 67:121–130.

Belaid B, Richard-Mercier N, Pieau C, Dorizzi M. 2001. Sex reversal and aromatase in the European pond turtle: treatment with letrozole after the thermosensitive period for sex determination. J Exp Zool 290:490–497.

Bhatnagar AS, Brodie AM, Long BJ, Evans DB, Miller WR. 2001. Intracellular aromatase and its relevance to the pharmacological efficacy of aromatase inhibitors. J Steroid Biochem Mol Biol 76:199–202.

Bisagni G, Cocconi G, Scaglione F, Fraschini F, Pfister C, Trunet PF. 1996. Letrozole, a new oral non-steroidal aromastase inhibitor in treating postmenopausal patients with advanced breast cancer. A pilot study. Ann Oncol 7:99–102.

Bodey GP. 1992. Azole antifungal agents. Clin Infect Dis 14(suppl 1):S161–S169.

Burke WH, Henry MH. 1999. Gonadal development and growth of chickens and turkeys hatched from eggs injected with an aromatase inhibitor. Poult Sci 78:1019–1033.

Byskov AG, Andersen CY, Leonardsen L. 2002. Role of meiosis activating sterols, MAS, in induced oocyte maturation. Mol Cell Endocrinol 187:189–196.

Byskov AG, Andersen CY, Nordholm L, Thogersen H, Xia G, Wassmann O, et al. 1995. Chemical structure of sterols that activate oocyte meiosis. Nature 374:559–562.

Cavilla JL, Kennedy CR, Baltsen M, Klentzeris LD, Byskov AG, Hartshorne GM. 2001. The effects of meiosis activating sterol on in-vitro maturation and fertilization of human oocytes from stimulated and unstimulated ovaries. Hum Reprod 16:547–555.

Conley A, Hinshelwood M. 2001. Mammalian aromatases. Reproduction 121:685–695.

De Coster R, Van Ginckel R, Wouters W, Goeminne N, Vanherck W, Byloos M. 1990. Endocrine and antitumoral effects of R76713 in rats. J Enzym Inhib 4:159–167.

de Jong PC, van de Ven J, Nortier HW, Maitimu-Smeele I, Donker TH, Thijssen JH, et al. 1997. Inhibition of breast cancer tissue aromatase activity and estrogen concentrations by the third-generation aromatase inhibitor vorozole. Cancer Res 57:2109–2111.

Debeljak N, Horvat S, Vouk K, Lee M, Rozman D. 2000. Characterization of the mouse lanosterol 14alpha-demethylase (CYP51), a new member of the evolutionarily most conserved cytochrome P450 family. Arch Biochem Biophys 379:37–45.

Dixon JM, Renshaw L, Bellamy C, Stuart M, Hoctin-Boes G, Miller WR. 2000. The effects of neoadjuvant anastrozole (Arimidex) on tumor volume in postmenopausal women with breast cancer: a randomized, double-blind, single-center study. Clin Cancer Res 6:2229–2235.

Documed AG. 2002. Arzneimittel-Kompendium der Schweiz. Available: http://www.kompendium.ch/ [accessed 29 April 2002].

Downs SM, Ruan B, Schroepfer GJ Jr. 2001. Meiosis-activating sterol and the maturation of isolated mouse oocytes. Biol Reprod 64:80–89.

EDSTAC, U.S. Environmental Protection Agency. 1998. Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final Report. Available: http://www.epa.gov/oscpmont/oscpendo/history/finalrpt.htm [accessed 29 April 2002].

Espinel-Ingroff A. 1997. Clinical relevance of antifungal resistance. Infect Dis Clin North Am 11:929–944.

European Commission. 2001. Communication from the Commission to the Council and the European Parliament on the Implementation of the Community Strategy for Endocrine Disrupters – a Range of Substances Suspected of Interfering with the Hormone Systems of Humans and Wildlife. European Community. Available: http://europa.eu.int/comm/environment/docum/01262_en.htm [accessed 29 April 2002].

Evans CT, Ledesma DB, Schulz TZ, Simpson ER, Mendelson CR. 1986. Isolation and characterization of a complementary DNA specific for human aromatase-system cytochrome P-450 mRNA. Proc Natl Acad Sci USA 83:6387–6391.

FAO/WHO (Food and Agriculture Organization/World Health Organization). 1982. Triadimefon. In: Pesticide Residues in Food – 1981 Evaluations. FAO Plant Production and Protection Paper 42. Rome:FAO, 512–547.

———. 1986. Triadimefon. In: Pesticide Residues in Food – 1985 Evaluations. Part II – Toxicology. FAO Plant Production and Protection Paper 72/2. Rome:FAO, 199–202.

———. 1988. Propiconazole. In: Pesticide Residues in Food – 1987 Evaluations. Part II – Toxicology. FAO Plant Production and Protection Paper 86/2. Rome:FAO, 111–129.

———. 1990. Triadimenol. In: Pesticide Residues in Food – 1989 Evaluations. Part II – Toxicology. FAO Plant Production and Protection Paper 100/2. Rome:FAO, 215–235.

———. 1991. Hexaconazole. In: Pesticide Residues in Food – 1990 Evaluations. Toxicology. WHO/PCS/91.47. Geneva:WHO, 81–100.

———. 1993a. Myclobutanil. In: Pesticide Residues in Food – 1992 Evaluations. Part II – Toxicology. WHO/PCS/93.34. Geneva:WHO, 267–289.

———. 1993b. Penconazole. In: Pesticide Residues in Food – 1992 Evaluations. Part II – Toxicology. WHO/PCS/93.34. Geneva:WHO, 291–315.

———. 1995. Tebuconazole. In: Pesticide Residues in Food – 1994 Evaluations. Part II – Toxicology. WHO/PCS/95.2. Geneva:WHO, 137–157.

———. 1996. Flusilazole. In: Pesticide Residues in Food – 1995 Evaluations. Part II – Toxicological and Environmental. WHO/PCS/96.48. Geneva:WHO, 157–179.

———. 1999. Bitertanol. Pesticide Residues in Food – 1998 Evaluations. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 148. Rome:FAO, 54–59.

———. 2001. Imazalil. In: Pesticide Residues in Food – 2000 Evaluations. Part II – Toxicological. Geneva:WHO, WHO/PCS/01.3:195–215.

———. 2002. Prochloraz. In: Pesticide Residues in Food – 2001 Evaluations. Part II – Toxicological. WHO/PCS/02.1. Geneva:WHO, 141–182.

Federal Authorities of the Swiss Confederation. Verordnung vom 26. Juni 1995 über Fremd- und Inhaltsstoffe in Lebensmitteln (Fremd- und Inhaltsstoffverordnung, FIV). Available: http://www.admin.ch/ch/d/sr/817_021_23/index.html [accessed 2 January 2003].

Fisher CR, Graves KH, Parlow AF, Simpson ER. 1998. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. Proc Natl Acad Sci USA 95:6965–6970.

Foidart A, Harada N, Balthazart J. 1994. Effects of steroidal and non steroidal aromatase inhibitors on sexual behavior and aromatase-immunoreactive cells and fibers in the quail brain. Brain Res 657:105–123.

Fowler KA, Gill K, Kirma N, Dillehay DL, Tekmal RR. 2000. Overexpression of aromatase leads to development of testicular Leydig cell tumors: an *in vivo* model for hormone-mediated testicular cancer. Am J Pathol 156:347–353.

Georgopapadakou NH. 1998. Antifungals: mechanism of action and resistance, established and novel drugs. Curr Opin Microbiol 1:547–557.

Hickey GJ, Krasnow JS, Beattie WG, Richards JS. 1990. Aromatase cytochrome P450 in rat ovarian granulosa cells before and after luteinization: adenosine 3′,5′-monophosphate-dependent and independent regulation. Cloning and sequencing of rat aromatase cDNA and 5′ genomic DNA. Mol Endocrinol 4:3–12.

Hof H. 2001. Critical annotations to the use of azole antifungals for plant protection. Antimicrob Agents Chemother 45:2987–2990.

Jeong HJ, Shin YG, Kim IH, Pezzuto JM. 1999. Inhibition of aromatase activity by flavonoids. Arch Pharm Res 22:309–312.

Ji H, Zhang W, Zhou Y, Zhang M, Zhu J, Song Y, et al. 2000. A three-dimensional model of lanosterol 14alpha-demethylase of *Candida albicans* and its interaction with azole antifungals. J Med Chem 43:2493–2505.

Joseph-Horne T, Hollomon DW. 1997. Molecular mechanisms of azole resistance in fungi. FEMS Microbiol Lett 149:141–149.

Kojima M, Morozumi T, Onishi A, Mitsuhashi T. 2000. Structure of the pig sterol 14alpha-demethylase (CYP51) gene and its expression in the testis and other tissues. J Biochem (Tokyo) 127:805–811.

Lamb DC, Cannieux M, Warrilow AG, Bak S, Kahn RA, Manning NJ, et al. 2001. Plant sterol 14 alpha-demethylase affinity for azole fungicides. Biochem Biophys Res Commun 284:845–849.

Lamb DC, Kelly DE, Waterman MR, Stromstedt M, Rozman D, Kelly SL. 1999. Characteristics of the heterologously expressed human lanosterol 14alpha-demethylase (other names: P45014DM, CYP51, P45051) and inhibition of the purified human and Candida albicans CYP51 with azole antifungal agents. Yeast 15:755–763.

Le Gac F, Thomas JL, Mourot B, Loir M. 2001. In vivo and in

vitro effects of prochloraz and nonylphenol ethoxylates on trout spermatogenesis. Aquat Toxicol 53:187–200.

Lee K, Macaulay VM, Nicholls JE, Detre S, Ashworth A, Dowsett M. 1995. An in vivo model of intratumoural aromatase using aromatase-transfected MCF7 human breast cancer cells. Int J Cancer 62:297–302.

Leonardsen L, Stromstedt M, Jacobsen D, Kristensen KS, Baltsen M, Andersen CY, et al. 2000. Effect of inhibition of sterol delta 14-reductase on accumulation of meiosis-activating sterol and meiotic resumption in cumulus-enclosed mouse oocytes in vitro. J Reprod Fertil 118:171–179.

Lindenthal B, Holleran AL, Aldaghlas TA, Ruan B, Schroepfer GJ Jr, Wilson WK, et al. 2001. Progestins block cholesterol synthesis to produce meiosis-activating sterols. FASEB J 15:775–784.

Machera K. 1995. Developmental toxicity of cyproconazole, an inhibitor of fungal ergosterol biosynthesis, in the rat. Bull Environ Contam Toxicol 54:363–369.

Majdic G, Parvinen M, Bellamine A, Harwood HJ Jr, Ku WW, Waterman MR, et al. 2000. Lanosterol 14alpha-demethylase (CYP51), NADPH-cytochrome P450 reductase and squalene synthase in spermatogenesis: late spermatids of the rat express proteins needed to synthesize follicular fluid meiosis activating sterol. J Endocrinol 166:463–474.

McPhaul MJ, Noble JF, Matsumine H, Wilson JD. 1988. Cloning and expression of the chicken ovary aromatase P-450: expression of mRNA in tissues of the Sebright and Leghorn chicken. Trans Assoc Am Physicians 101:219–225.

McPherson SJ, Wang H, Jones ME, Pedersen J, Iismaa TP, Wreford N, et al. 2001. Elevated androgens and prolactin in aromatase-deficient mice cause enlargement, but not malignancy, of the prostate gland. Endocrinology 142:2458–2467.

Means GD, Mahendroo MS, Corbin CJ, Mathis JM, Powell FE, Mendelson CR, et al. 1989. Structural analysis of the gene encoding human aromatase cytochrome P-450, the enzyme responsible for estrogen biosynthesis. J Biol Chem 264:19385–19391.

Menegola E, Broccia ML, Di Renzo F, Giavini E. 2001. Antifungal triazoles induce malformations in vitro. Reprod Toxicol 15:421–427.

Menegola E, Broccia ML, Di Renzo F, Prati M, Giavini E. 2000. In vitro teratogenic potential of two antifungal triazoles: triadimefon and triadimenol. In Vitro Cell Dev Biol Anim 36:88–95.

Middleton MC, Milne CM, Moreland S, Hasmall RL. 1986. Ovulation in rats is delayed by a substituted triazole. Toxicol Appl Pharmacol 83:230–239.

Milne CM, Hasmall RL, Russell A, Watson SC, Vaughan Z, Middleton MC. 1987. Reduced estradiol production by a substituted triazole results in delayed ovulation in rats. Toxicol Appl Pharmacol 90:427–435.

Murray R. 2001. Role of anti-aromatase agents in postmenopausal advanced breast cancer. Cancer Chemoth Pharmacol 48:259–265.

Nitahara Y, Aoyama Y, Horiuchi T, Noshiro M, Yoshida Y. 1999. Purification and characterization of rat sterol 14-demethylase P450 (CYP51) expressed in *Escherichia coli*. J Biochem (Tokyo) 126:927–933.

Odum J, Ashby J. 2002. Detection of aromatase inhibitors in vitro using rat ovary microsomes. Toxicol Lett 129:119–122.

Podust LM, Poulos TL, Waterman MR. 2001. Crystal structure of cytochrome P450 14alpha -sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors. Proc Natl Acad Sci USA 98:3068–3073.

Raucy JL, Carpenter SJ, Trzaskos JM. 1991. Identification of lanosterol 14 alpha-methyl demethylase in human tissues. Biochem Biophys Res Commun 177:497–503.

Rissman EF, Harada N, Roselli CE. 1996. Effect of vorozole, an aromatase enzyme inhibitor, on sexual behavior, aromatase activity and neural immunoreactivity. J Neuroendocrinol 8:199–210.

Roberts T, Hutson D, eds. 1999. Metabolic Pathways of Agrochemicals. Cambridge, UK:The Royal Society of Chemistry.

Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, et al. 1999. Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. Proc Natl Acad Sci USA 96:7986–7991.

Robertson KM, Simpson ER, Lacham-Kaplan O, Jones ME. 2001. Characterization of the fertility of male aromatase knockout mice. J Androl 22:825–830.

Rozman D, Cotman M, Frangez R. 2002. Lanosterol 14alpha-demethylase and MAS sterols in mammalian gametogenesis. Mol Cell Endocrinol 187:179–187.

Santen RJ, Harvey HA. 1999. Use of aromatase inhibitors in breast carcinoma. Endocr Relat Cancer 6:75–92.

Santner SJ, Pauley RJ, Tait L, Kaseta J, Santen RJ. 1997. Aromatase activity and expression in breast cancer and benign breast tissue stromal cells. J Clin Endocrinol Metab 82:200–208.

Schieweck K, Bhatnagar AS, Batzl C, Lang M. 1993. Anti-tumor and endocrine effects of non-steroidal aromatase inhibitors on estrogen-dependent rat mammary tumors. J Steroid Biochem Mol Biol 44:633–636.

Shetty G, Krishnamurthy H, Krishnamurthy HN, Bhatnagar AS, Moudgal NR. 1998. Effect of long-term treatment with aromatase inhibitor on testicular function of adult male bonnet monkeys (*M. radiata*). Steroids 63:414–420.

Shetty G, Krishnamurthy H, Krishnamurthy HN, Bhatnagar S, Moudgal RN. 1997. Effect of estrogen deprivation on the reproductive physiology of male and female primates. J Steroid Biochem Mol Biol 61:157–166.

Shyadehi AZ, Lamb DC, Kelly SL, Kelly DE, Schunck WH, Wright JN, et al. 1996. The mechanism of the acyl-carbon bond cleavage reaction catalyzed by recombinant sterol 14 alpha-demethylase of *Candida albicans* (other names are: lanosterol 14 alpha-demethylase, P-45014DM, and CYP51). J Biol Chem 271:12445–12450.

Sinha S, Kaseta J, Santner SJ, Demers LM, Bremmer WJ, Santen RJ. 1998. Effect of CGS 20267 on ovarian aromatase and gonadotropin levels in the rat. Breast Cancer Res Treat 48:45–51.

Stromstedt M, Rozman D, Waterman MR. 1996. The ubiquitously expressed human CYP51 encodes lanosterol 14 alpha-demethylase, a cytochrome P450 whose expression is regulated by oxysterols. Arch Biochem Biophys 329:73–81.

Stromstedt M, Waterman MR, Haugen TB, Tasken K, Parvinen M, Rozman D. 1998. Elevated expression of lanosterol 14alpha-demethylase (CYP51) and the synthesis of oocyte meiosis-activating sterols in postmeiotic germ cells of male rats. Endocrinology 139:2314–2321.

Tacer KF, Haugen TB, Baltsen M, Debeljak N, Rozman D. 2002. Tissue-specific transcriptional regulation of the cholesterol biosynthetic pathway leads to accumulation of testis meiosis-activating sterol (T-MAS). J Lipid Res 43:82–89.

Takayama K, Zeitoun K, Gunby RT, Sasano H, Carr BR, Bulun SE. 1998. Treatment of severe postmenopausal endometriosis with an aromatase inhibitor. Fertil Steril 69:709–713.

Tanaka M, Telecky TM, Fukada S, Adachi S, Chen S, Nagahama Y. 1992. Cloning and sequence analysis of the cDNA encoding P-450 aromatase (P450arom) from a rainbow trout (*Oncorhynchus mykiss*) ovary; relationship between the amount of P450arom mRNA and the production of oestradiol-17 beta in the ovary. J Mol Endocrinol 8:53–61.

Toda K, Shizuta Y. 1993. Molecular cloning of a cDNA showing alternative splicing of the 5'-untranslated sequence of mRNA for human aromatase P-450. Eur J Biochem 213:383–389.

Toma Y, Higashiyama T, Yarborough C, Osawa Y. 1996. Diverse functions of aromatase: O-deethylation of 7-ethoxycoumarin. Endocrinology 137:3791–3796.

Tomlin CDS, ed. 1997. The Pesticide Manual. 11th ed. Farnham, Surrey, UK:British Crop Protection Council.

Trant JM. 1994. Isolation and characterization of the cDNA encoding the channel catfish (*Ictalurus punctatus*) form of cytochrome P450arom. Gen Comp Endocrinol 95:155–168.

Trzaskos JM, Henry MJ. 1989. Comparative effects of the azole-based fungicide flusilazole on yeast and mammalian lanosterol 14 alpha-methyl demethylase. Antimicrob Agents Chemother 33:1228–1231.

Tsafriri A, Cao X, Vaknin KM, Popliker M. 2002. Is meiosis activating sterol (MAS) an obligatory mediator of meiotic resumption in mammals. Mol Cell Endocrinol 187:197–204.

Tsafriri A, Popliker M, Nahum R, Beyth Y. 1998. Effects of ketoconazole on ovulatory changes in the rat: implications on the role of a meiosis-activating sterol. Mol Hum Reprod 4:483–489.

Tuman RW, Morris DM, Wallace NH, Bowden CR. 1991. Inhibition of peripheral aromatization in the male cynomolgus monkey by a novel nonsteroidal aromatase inhibitor (R 76713). J Clin Endocrinol Metab 72:755–760.

U.S. EPA, Office of Pesticide Programs. 1999. Pesticide Industry Sales and Usage. Available: http://www.epa.gov/oppbead1/pestsales/ [accessed 8 August 2002].

Vaknin KM, Lazar S, Popliker M, Tsafriri A. 2001. Role of meiosis-activating sterols in rat oocyte maturation: effects of specific inhibitors and changes in the expression of lanosterol 14alpha-demethylase during the preovulatory period. Biol Reprod 64:299–309.

Van den Bossche H, Marichal P, Gorrens J, Bellens D, Verhoeven H, Coene MC, et al. 1987. Interaction of azole derivatives with cytochrome p-450 isozymes in yeast, fungi, plants and mammalian cells. In: Symposium on Gibberellin and Sterol Synthesis Inhibitors, 5 March 1987, London, UK. Pestic Sci 21:289–306.

Vanderschueren D, van Herck E, Nijs J, Ederveen AG, De Coster R, Bouillon R. 1997. Aromatase inhibition impairs skeletal modeling and decreases bone mineral density in growing male rats. Endocrinology 138:2301–2307.

Vinggaard AM, Hnida C, Breinholt V, Larsen JC. 2000. Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. Toxicol In Vitro 14:227–234.

Walker UJ, Nogues V. 1994. Changes induced by treatment with aromatase inhibitors in testicular Leydig cells of rats and dogs. Exp Toxicol Pathol 46:211–213.

Wennstrom KL, Crews D. 1995. Making males from females: the effects of aromatase inhibitors on a parthenogenetic species of whiptail lizard. Gen Comp Endocrinol 99:316–322.

Wickman S, Sipila I, Ankarberg-Lindgren C, Norjavaara E, Dunkel L. 2001. A specific aromatase inhibitor and potential increase in adult height in boys with delayed puberty: a randomised controlled trial. Lancet 357:1743–1748.

Yoshida Y, Aoyama Y. 1987. Interaction of azole antifungal agents with cytochrome P-45014DM purified from *Saccharomyces cerevisiae* microsomes. Biochem Pharmacol 36:229–235.

Zerulla M, Lange R, Steger-Hartmann T, Panter G, Hutchinson T, Dietrich DR. 2002. Morphological sex reversal upon short-term exposure to endocrine modulators in juvenile fathead minnow (*Pimephales promelas*). Toxicol Lett 131:51–63.

Zhang W, Ramamoorthy Y, Kilicarslan T, Nolte H, Tyndale RF, Sellers EM. 2002. Inhibition of cytochromes P450 by antifungal imidazole derivatives. Drug Metab Dispos 30:314–318.