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Dysmorphogenic effects of some fungicides derived from the imidazole on rat embryos cultured in vitro

Elena Menegola*, Maria L. Broccia, Francesca Di Renzo, Erminio Giavini

Department of Biology, University of Milan, Via Celoria 26, 20133 Milan, Italy

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Abstract

Like triazole-derivatives, imidazole-derivatives exert their antifungal and toxicological properties by inhibiting P450 enzymes (Cyps). At the embryonic level, Cyp enzymes are involved also in the catabolism of the retinoic acid. Specific effects of triazole-derivatives have been reported on developing rodent embryos, and were correlated to an imbalance of the retinoid homeostasis. The aim of this work was to investigate if imidazole-derivatives are able to induce specific malformations similar to those observed after triazole-derivative exposure. Post implantation rat embryos were exposed in vitro to 1000 μM Imidazole and to 5–100 μM of the imidazole-derivatives Ketoconazole and Enilconazole. After 48 h in culture, the embryos exposed to the imidazole-derivatives showed specific malformations, quite similar to those observed after triazole-derivative exposure. The common dysmorphogenic effects of the azole-derivatives of the two classes could be due to the inhibition of retinoid catabolism. From this point of view, the contemporaneous exposure to these substances or their therapeutic use could be considered as potentially dangerous for human conceptuses.

Keywords: Fungicides; Cyp; Retinoic acid catabolism; Branchial apparatus; Whole embryo culture; Ketoconazole; Enilconazole; Imazalil

1. Introduction

Imidazole-derivatives are anti-mycotic compounds used as anti-fungals in agriculture and clinically against fungal infections of the skin, hair, nails as well as against vaginal and systemic mycosis. Like other azole-derivatives, the triazole-derivatives, their anti-mycotic properties are due to the inhibition of a specific cyt P450 enzyme (Cyp51) involved in fungine cell wall synthesis.

For both of these compound classes (imidazole- and triazole-derivatives) the inhibition of some mammalian cyt P450 enzymes has been demonstrated in vitro and in vivo. In particular, the triazole-derivative Fluconazole, and the imidazole-derivative Ketoconazole, have been related to the inhibition of the enzyme involved in the retinoic acid catabolism (Cyp 26) in vitro, and in vivo to the maintenance of the therapeutic plasmatic level of retinoic acid, by inhibiting its degradation [1–5].

As far as their teratological properties are concerned, the imidazole-derivatives used in therapy, Miconazole, Clotrimazole, Econazole, Terconazole and Ketoconazole were classified by the U.S. Food and Drug Administration, according to risk in pregnancy, as category C (teratogenic or embryotoxic in animal studies without available reports or studies on humans) [6]. For this reason, the imidazole-derivatives used for systemic therapy against deep-seated infections and extensive cutaneous diseases and/or hair or nail mycosis are not recommended during the first trimester or the whole pregnancy period. As far as intravaginal preparations are concerned, these agents are widely used during pregnancy, considering their low systemic absorption (1.4% for Miconazole; 3–10% for Clotrimazole; 5–16% for Terconazole). Ketoconazole is available as shampoo (2% in an aqueous suspension), cream (2% in an aqueous cream vehicle), tablets (200 mg, used orally at doses of 200 and 400 mg/day in treatment of oral and chronic mucocutaneous candidiasis

* Corresponding author. Tel.: +39 02 5031 4756; fax: +39 02 5031 4802.
E-mail address: elena.menegola@unimi.it (E. Menegola).

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and of 600–800 mg/day in patients not responding to regular doses) [7]. The dose of 80 mg/kg has been indicated to be teratogenic or embryotoxic in rats by the manufacturer [7] and by Van Cauteren and Marsboom [8], even if Nishikawa et al. [9] reported (after treatment regimen orally before fertilization and during the first 7 days of pregnancy, during days 7–17 or during the perinatal period) reduced fertility in the female rats at 20 mg/kg and above, induction of cleft palate and patent incisive foramina at 40 mg/kg and above, and increased neonatal loss at 40 mg/kg and above. Finally, pregnant rats treated with 10 or 25 mg/kg from day 6 to 21 of gestation delivered pups with feeding and drinking difficulties from the age of 8 to 9 weeks, probably due to malocclusion of incisors, even if there were no overt teratogenic effects following maternal exposure of up to 50 mg/kg Ketoconazole, level at which extensive fetal toxicity was observed [10].

As far the imidazole-derivatives used in plant protection are concerned, Enilconazole (Imazalil) is used as a seed dressing in cereals (4–30 g/100 kg seed). The admissible daily intake has been calculated from the European Commission Peer Review Program as 0.03 mg/kg bw/day [11]. As far as its teratological properties are concerned, the European review of its toxicological properties indicates that there is no clear evidence of teratogenicity [11]. Tihienpont et al. [12] reported, however, that Imazalil is not embryotoxic or teratogenic in experimental species. Enilcolazole is also used as a veterinary pharmaceutical, for the treatment of dermatomycoses or ringworm induced by pathogenetic fungi and for the treatment of nasal infections. After dermal application, its bioavailability is 5–10% of that observed after intravenous dosing. Teratological studies in rats and rabbits showed that Enilconazole is embryotoxic at maternotoxic dose levels. In particular, several multigeneration studies in the rat showed an increase of gestation duration, a decrease of the number of live pups and an increase of the number of stillborn pups at the dose level of 80 mg/kg. In rabbits, the dosage of 20 mg/kg Enilconazole during the whole organogenetic period elicited a decreased maternal body weight gain and food consumption, a decreased litter size and an increase in embryonal resorption [13].

Recently, the other azole-derivatives, the triazole-derivatives, were related to high dysmorphogenic properties both after exposure in vitro [14–17] and after treatment of pregnant mice [18]. For embryos cultured in vitro the observed abnormalities were specifically related to abnormal branchial arch morphogenesis, due to the abnormal organization of branchial ectomesenchyme derived from rhombencephalic neural crest cells migrating from the abnormally segmented hindbrain [15–17]. After in vivo exposure with a single treatment regimen during the time corresponding to the embryonic stage of 5–13 somites, severe abnormalities occurred to the cranio-facial elements derived from the oral (first) and iomandibular (second) branchial arches [18]. The abnormalities observed both after in vitro and in vivo exposure to the studied triazole-derivatives are quite similar to those reported after retinoic acid exposure in vitro and in vivo (abnormal branchial arch morphogenesis, cranio-facial defects) [19–21]. Moreover, the contemporaneous exposure in vitro to subteratogenic concentrations of the triazole-derivative Fluconazole and of retinoic acid were able to produce synergistic effects [22]. Taking together these data, the current hypothesis on the dysmorphogenic mechanism of triazole-derivatives considers the possibility of a severe inhibition of the embryonic Cyp enzyme activity, producing a consequent increase of the endogenous embryonic retinoic acid content.

As the inhibition of Cyp enzymes is the common mechanism producing antifungal and toxic properties both for imidazole- and for triazole-derivatives, and considering that Cyp 26 isoforms (an enzyme family which control the catabolism of the morphogen retinoic acid) are expressed in the developing embryo [23,24], the aim of the present work was to test the hypothesis that some imidazole-derivatives could lead in vitro to dismorphogenic effects similar to those obtained in the previous published studies after triazole-derivative exposure. To determine the dysmorphogenic potency of some imidazole-derivatives the post implantation rodent whole embryo culture method has been used. The agrochemical imidazole-derivative Enilconazole (Imazalil) and the clinically used imidazole-derivative Ketoconazole were chosen as representatives of this class of molecules.

In contrast to the dysmorphogenic effects obtained after triazole-derivative exposure, the parental molecule Triazole was unable to elicit in vitro abnormalities at the branchial apparatus level. After the in vitro exposure to the limit concentration of 5000 μM Triazole, in fact, the only observed embryotoxic effect was a diffuse blood discoloration [16]. However, literature data do not report that Triazole and Imidazole themselves, used for the synthesis of the antimycotic-derivatives, exert any antifungal nor Cyt P450 inhibition activity. For this reason, to test the hypothesis that the Cyt P450 inhibition activity is crucially related to the dysmorphogenic properties of these molecules, the parental compound Imidazole was included in the chosen substances to test in the present work.

2. Materials and methods

Pregnant Crl:CD rats (Charles River, Italy), maintained in an air-conditioned room (T: $20 \pm 2\,^{\circ}C$, humidity: $55 \pm 5\%$, light cycle: hours 06:00–18:00) with food (Mucedola, Italy) and tap water ad libitum, were sacrificed in the afternoon of day 9 of gestation (9.5-day, considering day 0 the morning when sperm was found in the vaginal smear) and embryos of 1–3 somites were explanted, randomly distributed to experimental groups and cultured according to the method proposed by New [25] into 20-ml glass culture bottles (4–5 embryos/bottle) containing heat-inactivated sterile rat serum (1 ml/embryo), and antibiotics (Penicillin 100 IU/ml;