Teratological Interaction Between the Bis-Triazole Antifungal Agent Fluconazole and the Anticonvulsant Drug Phenytoin

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ABSTRACT

Previous studies implicated the cytochrome P450 (CYP) system as critical in the teratogenic bioactivation of phenytoin (PHT). Fluconazole (FCZ) is an antifungal bis-triazole with potent inhibitory effect on the principal CYP-dependent metabolic pathway of PHT. In this study an in vivo experimental model was used to evaluate the potential ability of FCZ (2, 10, or 50 mg/kg intraperitoneally) to modulate PHT (65 mg/kg intraperitoneally) teratogenesis on day 12 (plug day = day 1) Swiss mice. PHT alone elicited embryocidal and malformative effects, with cleft palate as the major malformation. Pretreatment with the non-embryotoxic dosage of 10 mg FCZ/kg potentiated PHT-induced teratogenesis, as indicated by a twofold (from 6.2% to 13.3%) increment of cleft palate incidence (P < 0.05). Combined treatment with 50 mg FCZ/kg plus PHT resulted in a statistically significant (P < 0.05) increment of the resorption incidence recorded after PHT-alone exposure, but possibly as a consequence of the increased embryolethality, in the loss of the potentiative effect on PHT teratogenesis. Although the mechanistic nature of teratological interaction between FCZ and PHT remains to be established, these results may not support CYP system-mediated metabolic conversion as the mechanistic component of PHT teratogenesis. Teratology 59:81–87, 1999.

The anticonvulsant drug phenytoin (PHT; diphenylhydantoin sodium) has been significantly implicated as causative factor in human teratogenesis (Hanson and Smith, ’75; Strickler et al., ’85; Buehler et al., ’90; Scolnik et al., ’94; Nulman et al., ’97), and is a proven animal teratogen (Massey, ’66; Harbison and Becker, ’70; Finnell et al., ’92, ’93, ’94).

Fluconazole (FCZ) is a bis-triazole antifungal agent with a potent inhibitory effect on the principal CYP-dependent pathway responsible for PHT metabolism (Levy, ’95). In this light, we reasoned that FCZ could represent a useful probe to further investigate the potential role of the CYP system in the teratogenic bioactivation of PHT.

MATERIALS AND METHODS

Drugs

PHT (purity approximately 99%) was purchased from Sigma Chemical Co. (St. Louis, MO). Dosing solutions

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were prepared shortly before administration by dissolving PHT in 0.9% (w/v) NaCl containing 0.002 M NaOH at a final pH of 10.7–10.9. The FCZ used in the study was the compound commercially available for intravenous infusion (Diflucan®, Pfizer), formulated as an iso-osmotic solution, and containing 2 mg of FCZ and 9 mg of NaCl per ml.

### Animals and breeding procedure

The animals used in the study were sexually mature Swiss outbred mice, obtained from the animal quarters of the Catholic University of the Sacred Heart (Rome, Italy). Animals were housed in solid-bottom polycarbonate cages with stainless steel tops, containing heat-treated hardwood chips. Standard rodent laboratory food and tap water were provided ad libitum throughout the study. Animals room was maintained at 22°C ± 6°C with a relative humidity of 55% ± 5%. A 12-hr light/dark cycle was used. For breeding, one male was housed with 4–5 nulliparous females throughout the dark cycle. At the end of this period, the presence of a vaginal plug was taken as an index of copulation. The dark cycle. At the end of this period, the presence of a vaginal plug was taken as an index of copulation. The

### Table 1. Effect of FCZ-PHT interaction on maternal parameters in the Swiss mouse

<table>
<thead>
<tr>
<th></th>
<th>Vehicle + vehicle</th>
<th>FCZ* (10 mg/kg) + vehicle</th>
<th>FCZ* (50 mg/kg) + vehicle</th>
<th>Vehicle + PHT**</th>
<th>FCZ (2 mg/kg) + PHT</th>
<th>FCZ (10 mg/kg) + PHT</th>
<th>FCZ (50 mg/kg) + PHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of pregnant females</td>
<td>14</td>
<td>14</td>
<td>17</td>
<td>28</td>
<td>20</td>
<td>24</td>
<td>19</td>
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<tr>
<td>Percent maternal lethality (no.)</td>
<td></td>
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<tr>
<td>Maternal body weight (g ± SEM) at term</td>
<td>65.80 ± 3.03</td>
<td>59.39 ± 2.07</td>
<td>69.32 ± 2.44</td>
<td>63.64 ± 1.58</td>
<td>60.17 ± 1.46</td>
<td>60.50 ± 1.67</td>
<td>59.84 ± 1.36</td>
</tr>
<tr>
<td>Maternal corrected body weight (g ± SEM)**</td>
<td></td>
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<tr>
<td>Maternal weight gain (g ± SEM) (days 12-19)</td>
<td>45.46 ± 1.75</td>
<td>40.64 ± 1.60</td>
<td>48.24 ± 1.38</td>
<td>46.47 ± 0.95</td>
<td>42.52 ± 0.74</td>
<td>45.22 ± 1.06</td>
<td>45.13 ± 0.61</td>
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<tr>
<td>Maternal corrected body weight (g ± SEM)**</td>
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<tr>
<td>Maternal weight gain (g ± SEM) (days 12-19)</td>
<td>19.45 ± 1.53</td>
<td>18.46 ± 1.39</td>
<td>21.99 ± 1.41</td>
<td>17.04 ± 1.13</td>
<td>16.49 ± 1.20</td>
<td>14.26 ± 1.03</td>
<td>14.28 ± 1.30</td>
</tr>
</tbody>
</table>

* Administered intraperitoneally on gestational day 12, 3 hr before PHT administration.

** 65 mg/kg administered intraperitoneally.


system in the mouse (La Delfa et al., '89; Morita et al., '92). PHT was administered at 65 mg/kg, since this dosage was previously found to be teratogenic in the mouse (Wells et al., '89). On the morning of gestational day 19, females were weighed and then sacrificed. After hysterectomy, the uterus was weighed, and uterine contents were evaluated for number of implantations, resorptions, and dead and live fetuses. Viable fetuses were weighed, sexed, and carefully examined for gross malformations, including girth palate. Approximately half of the fetuses from each litter were double-stained with alizarin red and alcian blue, according to the method proposed by Inouye ('76) and Kimmel and Trammel ('81) as modified by Kuczuk and Scott ('84), and examined for skeletal abnormalities. The remaining fetuses were fixed in Bouin's solution and evaluated for soft-tissue abnormalities, using the free-hand razor blade section technique devised by Wilson (65). All morphological evaluations were conducted with the aid of a stereo microscope (Stemi SV11, Zeiss, Oberkochen, Germany).

### Statistical analysis

Continuous data were compared by one-way analysis of variance (ANOVA) followed, if significant, by a Student-Newman-Keuls multiple range test. Binomial data were compared using the chi-square test with Yates correction. All numerical data reported are expressed as the mean ± SEM. The null hypothesis was rejected at the 5% significance level (P < 0.05).

### RESULTS

#### FCZ alone

Administration of FCZ at 10 or 50 mg/kg neither induced appreciable effects on maternal well-being, nor affected the maternal body weight parameters shown in Table 1. Maternal treatment with FCZ at 10 mg/kg did not reveal embryocidal effects (Fig. 1). Conversely, FCZ
at 50 mg/kg significantly reduced embryo viability, as evidenced by the increase of resorption incidence from 5.98% (vehicle group) to 12.55% (P < 0.05) (Fig. 1). The mean fetal weights of FCZ-exposed litters (either 10 and 50 mg/kg) were comparable to the control value (Table 2). Morphological evaluation revealed that fetuses exposed at 50 mg/kg of FCZ had an increased incidence (P < 0.05) of rib abnormalities (full supernumerary ribs and short supernumerary ribs) in comparison to control fetuses (Fig. 2). The highest dose of FCZ also caused a low but measurable incidence of dilated renal pelvis (Fig. 3). No other morphological anomalies were noted in FCZ-exposed fetuses.

### PHT alone

Intraperitoneal administration of PHT (65 mg/kg) rapidly induced signs of maternal nervous system impairment, including sedation, ataxia, and in some instances, opisthotonus. These signs persisted for several hours. Despite distress, maternal weight gain was not affected by PHT in comparison to the control group (Fig. 1). PHT treatment nearly doubled the embryolethality of the control group (Fig. 1), but this increment reached only borderline statistical significance (P = 0.058). Mean fetal body weight was not lessened by PHT (Table 2). As shown in Figure 4, maternal PHT administration significantly (P < 0.05) increased the low cleft-palate incidence observed in vehicle-exposed fetuses, from 1.28% to 6.20%. Additional morphological anomalies were significantly (P < 0.05) increased by PHT with respect to controls, including rib abnormalities (Fig. 2) and dilated renal pelvis (Fig. 3). Without statistical relevance were the remaining defects observed, including exencephaly (1.45%), ectrodactyly (0.36%), and tail defect (0.36%) (data not shown).

### FCZ + PHT

Pregnant animals pretreated with FCZ (at any dose level) did not show appreciable modifications of clinical sequelae (maternal nervous system impairment) induced by PHT administration. For unknown reasons, one dam of the group given FCZ 50 mg/kg plus PHT died during the night of the treatment day (Table 1). Maternal and fetal weight values of groups pretreated with FCZ (2, 10, or 50 mg/kg) were comparable to values recorded after treatment with PHT alone (Tables 1 and 2, respectively). In terms of embryolethality, the combined administration of 50 mg FCZ/kg plus PHT resulted in a 37% increase (P < 0.05) of embryonic loss in comparison with the PHT-alone group (Fig. 1). A similar resorption rate increase (33.4%) was noted between groups treated with 50 mg/kg of FCZ alone and 50 mg/kg FCZ plus PHT, but in this case statistical analysis did not reveal significance (Fig. 1). In terms of malformations, a significant interaction between agents was found in response to a lower dose of FCZ. In fact, as shown in Figure 3, FCZ at 10 mg/kg induced a twofold (from 6.2% to 13.3%) increment of cleft palate incidence (P < 0.05). In contrast with expectations, pretreatment with FCZ at the maximal dose did not significantly enhance PHT-induced cleft palates (Fig. 4). FCZ pretreatment also caused an approximately twofold increment of PHT-induced dilated renal pelvis (when given at 10 or 50 mg/kg; Fig. 3), and a decreased frequency of fetuses with rib anomalies (when given at 2, 10, or 50 mg/kg; Fig. 2). But these changes were not significant statistically. Sporadic malformations found (data not shown) included cases of exencephaly (0.99%; in the group pretreated with 5 mg FCZ/kg), and gastroschisis and open eyelids (0.49%; in the group pretreated with 50 mg FCZ/kg).

### DISCUSSION

In the present study it was found that FCZ, at the nonembryotoxic level of 10 mg/kg, significantly potentiated PHT-induced teratogenesis, as evidenced by a twofold increase in cleft palate incidence. In treatment of fungal infections, FCZ is prescribed at a maximum recommended daily dose of 1,600 mg (Debruyne, '97). Assuming a human patient of 60 kg, this dosage corresponds to 26 mg/kg/day. Thus, the FCZ dosage that we found effective in enhancing the teratogenic effect of PHT (10 mg/kg) is within the human therapeutic range. This idea is also supported by major differences in the pharmacokinetic profiles existing between humans and mice. Peak plasma concentrations of FCZ, normalized to a 1 mg/kg dose level following oral administration, were found to be twofold higher in humans (1.4 µg/ml) than in mice (0.7 µg/ml) (Humprey et al., '85). Differences in half-lives were more than fourfold (22 hr in humans vs. 4.8 hr in mice) (Humprey et al., '85). On the other hand, it should be considered that a single dose given all at once is likely to result in plasma concentrations higher than those achieved with several doses spread over a day. With
respect to PHT, assuming again a patient of 60 kg, the dosage administered to Swiss mice (65 mg/kg) was approximately four times the maximal human dosage (1,000 mg/day) (McNamara, ’95), although it has been reported that an oral administration of 75 mg/kg of PHT in the mouse results in plasma drug concentrations in the human therapeutic range (Sulik et al., ’79).

Interestingly, FCZ increased cleft palate incidence without altering the incidence of remaining PHT-induced developmental disorders. This may suggest that FCZ promotes selective modifications of molecular imbalances that compound PHT embryotoxicity. Alternatively, it is possible that a single dose of PHT at a single gestational time may have missed the critical times of susceptibility for the other developing primordia.

In contrast with a dose-response model, increment of FCZ exposure to 50 mg/kg failed to maintain cleft palate incidence at a significantly higher level in comparison to the PHT-alone-treated group. This inverted teratogenic dose-response relationship may be a consequence of embryonic loss increment that followed 50 mg FCZ/kg-PHT coadministration, with a resulting reduced survival of affected conceptuses. In this regard it is worth remembering that embryolethality precluding the recognition of other developmental disorders is a well-characterized phenomenon in teratogenesis (Wilson, ’77). The finding of an increased embryo mortality after the combined treatment 50 mg/kg of FCZ plus PHT appears explainable in terms of a nearly additive interaction between agents, considering that both exhibited a 12% incidence of embryolethality when separately administered, and a 19% incidence of embryolethality when given in combination. The evidence that FCZ is per se capable of eliciting embryotoxicity is not surprising considering that the embryopathic effects of this triazole have been shown by in vivo (Tachibana et al., ’87; data on file at the Roerig Division, U.S. Pharmaceuticals Group, Pfizer, New York, NY) and in vitro
(Tiboni, ‘93) studies. Possible developmental effects of FCZ appear to deserve attention in view of a recently postulated fetal fluconazole embryopathy (Lee et al., ‘92; Pursley et al., ‘95; Aleck and Bartley, ‘97).

The primary molecular mechanism of action of FCZ is inhibition of ergosterol biosynthesis, leading to inhibition of fungal cell growth and division (Vanden Bossche, ‘85; Saag and Dismukes, ‘88; Como and Dismukes, ‘94). FCZ mediates this effect via competition for oxygen at the catalytic heme iron atom of CYP enzymes, thereby decreasing C-14 demethylation of lanosterol, the precursor intermediate of ergosterol (Vanden Bossche, ‘85; Saag and Dismukes, ‘88; Como and Dismukes, ‘94). In contrast to imidazoles, FCZ has shown a high specificity for fungal CYP enzymes while binding only weakly to the mammalian CYP enzymes (Como and Dismukes, ‘94). On the other hand, FCZ has been recently recognized as a potent inhibitor of the mammalian CYP system. Morita et al. (‘92), using the ratio of 6β-hydroxycortisol to cortisol in urine as an indicator of hepatic oxidative drug-metabolizing capacity in humans, found that therapeutic doses of FCZ (200 mg/day) decreased up to 50% of the original level. In the same study, FCZ (1–10 mg/kg given intraperitoneally) significantly prolonged pentobarbital sleeping time in mice in a dose-dependent manner. La Delfa et al. (‘89), employing the [14C] antipyrine breath test, provided evidence supporting FCZ (1 or 10 mg/kg administered by gavage) as a potent, partially selective, and reversible inhibitor of the CYP-dependent enzyme system in mice. Of particular relevance to the present study, FCZ was found to be a potent inhibitor of CYP 2C9 (Levy, ‘95; Kunze et al., ‘96; Black et al., ‘96), the CYP isoform that catalyzes parahydroxylation of PHT to p-hydroxyphenytoin, accounts for 70–90% of total PHT clearance (Levy, ‘95). Consistent with this notion, symptomatic PHT toxicity has resulted from concomitant administration of FCZ (Howitt and Oziemsky, ‘89; Mitchell and Holland, ‘89; Cadle et al., ‘94). Moreover, coadministration of FCZ and PHT in healthy volunteers induced a marked increase in the mean PHT area under the concentration–time curve (Lazar and Wilner, ‘90; Blumm et al., ‘91; Touchette et al., ‘92) without affecting FCZ levels (Lazar and Wilner, ‘90).

From this perusal of the literature, it is tempting to infer a causal link between the inhibitory properties of FCZ on the CYP enzyme system and the FCZ-induced potentiation of PHT teratogenesis found in the present study. If so, then our findings would argue against a role for CYP system-catalyzed bioactivation in the mechanism of PHT teratogenesis. A possible explanation of the enhancement of PHT teratogenesis by FCZ is that inhibition of maternal PHT hydroxylation resulted in an enhanced maternal plasma concentration of PHT and a transfer to the developing conceptus. Since FCZ has been shown to cross rodent placenta with fetal tissue concentrations approximately equal to maternal plasma concentration (Diflucan, Drug Information Full-text, American Society of Health-System Pharmacist Inc., Bethesda, MD), it is also possible that inhibition of the low activities of embryonic CYP enzymes contributed to the potentiation of PHT teratogenesis. Our study appears congruent with data provided by Harbison and Becker (‘70). These investigators found that a pharmacological stimulus apparently effective in enhancing PHT metabolism (pretreatment with phenobarbital) was associated with a decrement of PHT embryotoxicity and that, on the other hand, a pharmacological exposure (pretreatment with SKF 525A) consistent with inhibition of PHT metabolism resulted in enhancement of PHT embryotoxicity. Conversely, Finnell et al. (‘93, ‘94) found that preadministration of stiripentol, an anticonvulsant with broad inhibiting effects on CYP enzymes, significantly reduced the overall incidence of PHT-induced fetal defects in three strains of mice. These two studies differed in route of drug administration, duration of administration, and strains of mice used, partly accounting for resultant discrepancies. It also appears possible that the protective effect elicited by stiripentol on PHT dysmorphogenesis was of a pharmacological (more than toxicokinetic) nature, as indicated by the fact that stiripentol coadministration to PHT toxicity and that, on the other hand, a pharmacological stimulus apparently effective in enhancing PHT metabolism (pretreatment with phenobarbital) was associated with a decrement of PHT embryotoxicity and that, on the other hand, a pharmacological exposure (pretreatment with SKF 525A) consistent with inhibition of PHT metabolism resulted in enhancement of PHT embryotoxicity. Conversely, Finnell et al. (‘93, ‘94) found that preadministration of stiripentol, an anticonvulsant with broad inhibiting effects on CYP enzymes, significantly reduced the overall incidence of PHT-induced fetal defects in three strains of mice. 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It has been postulated that azole compounds may also affect the fungal cell by inhibition of cytochrome c oxidase and peroxidative enzymes, with a resultant increase in intracellular peroxide generation (Vanden Bossche, '85; Saag and Dismukes, '88). This pathway, if not specific for fungal cells, may possibly be involved in the potentiation elicited by FCZ on PHT teratogenicity. Of note, this study represents part of the growing evidence implicating free radicals, generated via lipoxigenases or prostaglandin synthetase activities, as the ultimate embryotoxic intermediates of PHT (reviewed by Wells and Winn, '96).

**LITERATURE CITED**


