In vitro screening of azole fungicides for antiandrogenic effects – comparison with in vivo effects

Camilla Taxvig, M. Sc.
Danish Institute for Food and Veterinary Research
Dept. of Toxicology and Risk Assessment

eSi Meeting, September 29-30 2006, Alcante, Spain
Disposition

- Background
- Aim of the study
- Study design
- The In Vitro assays
- Results
- Summery
- Conclusion
Background

- Growing concern of permanent damage to the endocrine and nervous systems after exposure to even low levels of pesticides under development.

- Azole fungicides are used in large amounts in the control of fungi in grain crops and to a lesser extent in vegetable and fruit production.

- The fungicides are relatively fat-soluble, and readily absorbed across the gastrointestinal cannel. Therefore, the public is exposed to the fungicides if residues exist in food products.

- In general, the azole fungicides have a low acute toxicity but little is known about their potential health risks at low chronic exposures.
Prochloraz has multiple mechanisms of action *in vitro*:

- **Aryl hydrocarbon (AhR) agonism**  \( EC_{50} \approx 1 \, \mu M \) (Long *et al.*, 2003)
- **Aromatase inhibition**  \( IC_{50} = 0.3 \, \mu M \) (Vinggaard *et al.*, 2000)
- **ER antagonism**  \( IC_{50} \approx 25 \, \mu M \) (Andersen *et al.*, 2002)
- **AR antagonism**  \( IC_{50} = 4 \, \mu M \) (Andersen *et al.*, 2002)
Aim of the study

The results are part of a larger project, which main object is to investigate the effects of some frequently used azole fungicides on the endocrine system, including *in vitro* and *in vivo* examinations, and to assess whether *in vitro* assays can be used to predict *in vivo* effect.

Epoxiconazole    Tebuconazole
**Study design**

**In Vitro Screens**
- AR reporter gene assay and steroid synthesis testing in H295R cells
- CHO or human adrenocortical carcinoma cells

**In Vivo Screens**
- *In utero* and perinatal exposure

**Dosing GD 7-PND 16:**
- Control, Epoxiconazole, Tebuconazole

**GD 7–21** → **PND 1-16**

Fetuses were analyzed for effects on hormone levels

Number of nipples and AGD recorded

(Typical endpoints to test for feminization of male offspring and masculinization of the female offspring)
Assay procedure for AR reporter gene assay

1st day
- Seeding cell
- 24h at 37°C and 5% CO₂

2nd day
- Add plasmids and sample
- Transfected 5h with: expression vector pSVAR0 and the MMTV-LUC reporter plasmid in a ratio of 1:100
- 20h

3rd day
- Measure luminescence

CHO-K1

Androgen Receptor
Receptor plasmid
ARE
Luciferase
Reporter plasmid
H295R cell assay - Test Design

**Seeding**
2x10^5 cells/well in 24-well culture plates

**Acclimatization**
24h at 37°C and 5% CO₂

**Exposure**
48h at 37°C and 5% CO₂

**Hormone Determination**
Delfia time-resolved fluorescence kit

---

**H295R Cell Basal Hormone Production**
*Time Series*

- **Progesterone**
- **Testosterone**
- **Estradiol**

![Graph showing time series of hormone production](image)
Results

Steroid synthesis assay – H295R cells
Results – *In Vitro*

The AR reporter gene assay

Androgen receptor antagonism in vitro
Results – *In Vivo*

**Effects on offspring after perinatal exposure**

**Hormone data GD 21 foetus**

<table>
<thead>
<tr>
<th></th>
<th>17α-hydroxyprogesterone (pg/testis)</th>
<th>Testosterone (ng/testis)</th>
<th>Progesterone (ng/testis)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>1.95±0.54 (4)</td>
<td>1.75±0.71 (5)</td>
<td>0.037±0.025 (5)</td>
</tr>
<tr>
<td><strong>Tebuconazole 50 mg/kg</strong></td>
<td>8.39±2.59* (7)</td>
<td>1.25±0.40 (7)</td>
<td>0.103±0.035* (7)</td>
</tr>
<tr>
<td><strong>Tebuconazole 100 mg/kg</strong></td>
<td>6.59±3.88* (9)</td>
<td>0.88±0.46* (9)</td>
<td>0.084±0.063 (9)</td>
</tr>
<tr>
<td><strong>Epoxyconazole 15 mg/kg</strong></td>
<td>1.76±1.36 (6)</td>
<td>1.62±0.59 (8)</td>
<td>0.029±0.019 (8)</td>
</tr>
<tr>
<td><strong>Epoxyconazole 50 mg/kg</strong></td>
<td>0.94±0.48 (13)</td>
<td>1.11±0.56 (20)</td>
<td>0.027±0.019 (20)</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD
() = n; * significance level P < 0.05
Results – In Vivo

### Anogenital distance PND 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>T-50</th>
<th>T-100</th>
<th>E-15</th>
<th>E-50</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGD (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cubic root of body weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* # only one pup
T-50: Tebuconazole 50 mg/kg, T-100: Tebuconazole 100 mg/kg, E-15: Epoxiconazole 15 mg/kg, E-50: Epoxiconazole 50 mg/kg

### Nipple retention PND 13

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>T-50</th>
<th>T-100</th>
<th>E-15</th>
<th>E-50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of nipples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* #
**In Vitro**
- Both tebuconazole and epoxiconazole inhibited testosterone and estradiol production, and increased progesterone production.
- Tebuconazole and epoxiconazole proved to be antagonists of the androgen receptor.

**In vivo**
- Tebuconazole caused an increase in testicular progesterone levels and a decrease in the testosterone levels.
- Tebuconazole increased the number of nipples in the male pups and increased AGD in female pups.

---

<table>
<thead>
<tr>
<th>P: Prochloraz, T: Tebuconazole, E: Epoxiconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro</strong></td>
</tr>
<tr>
<td>AR effects</td>
</tr>
<tr>
<td>Steroid synthesis</td>
</tr>
<tr>
<td>Testosterone</td>
</tr>
<tr>
<td>Estradiol</td>
</tr>
<tr>
<td>Progesterone</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
</tr>
<tr>
<td>AGD/cubic root of bw</td>
</tr>
<tr>
<td>AGD/cubic root of bw male</td>
</tr>
<tr>
<td>Nippels male</td>
</tr>
<tr>
<td>Testosterone GD 21</td>
</tr>
<tr>
<td>Progesterone GD 21</td>
</tr>
</tbody>
</table>
Conclusions

- The results obtained *in vitro* are in good agreement with the effects observed *in vivo*.

- Tebuconazole and prochloraz showed antiandrogenic effects both *in vitro* and *in vivo*.

- Antiandrogenic effects were also seen for epoxiconazole *in vitro*, however the observed effects *in vivo* was not quite what might be predicted from the *in vitro* experiments, which can be due to the fact, that the *in vitro* screen may be more sensitive than *in vivo*. 
Thank you for your attention!