In vivo mutagenicity of conazole fungicides correlates with tumorigenicity

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Triadimefon, propiconazole and myclobutanil are conazoles, an important class of agricultural and therapeutic fungicides. Triadimefon and propiconazole are mouse liver tumorigens, while myclobutanil is not. All three conazoles are generally inactive in short-term genotoxicity tests. We studied the in vivo mutagenicity of these three conazoles using the Big Blue® mouse assay system. Groups of mice were fed either control diet or diet containing 1800 p.p.m. triadimefon, 2500 p.p.m. propiconazole or 2000 p.p.m. myclobutanil. After 4 days of feeding, mice were immediately euthanized, livers were removed, DNA isolated and lacI genes recovered into infectious bacteriophage lambda particles by in vitro packaging. Bacteriophage with mutations in the lacI gene was detected by infecting into Escherichia coli, and mutant frequencies were determined using a colorimetric plaque assay. Propiconazole induced a 1.97-fold increase in mutant frequency compared to concurrent controls (P = 0.018) and triadimefon induced a 1.94-fold increase compared to concurrent controls (P = 0.009). Myclobutanil did not induce any change in mutant frequency (P = 0.548). These results provide the first evidence that the hepatotumorigenic conazoles are capable of inducing mutations in liver in vivo while the non-tumorigenic myclobutanil is not, suggesting that mutagenicity may represent a key event in conazoles tumorigenic mode of action.

Introduction

Conazoles are a diverse group of commercially important fungicides with clinical and agricultural applications. They exert their fungicidal activity by inhibiting the synthesis of ergosterol, primarily by inhibiting the activity of lanosterol 14α-demethylase. Some conazoles, including fenbucounazole, triadimefon and propiconazole, have been shown to induce hepatoadenomas in the livers of mice receiving the chemical in feed (1–3). Other conazoles, such as myclobutanil, did not induce tumours in mouse liver under biasay conditions. All these conazoles, regardless of tumorigenicity, have been found to be generally inactive in a variety of short-term tests of genotoxicity including Ames test, Saccharomyces cerevisiae mutation assay, mouse lymphoma mutation assay, DNA repair assays in rat and human cells, mouse fibroblast transformation assays, dominant lethal test in mouse and chromosomal aberration tests (1,2,4–8). Triadimefon has been reported to induce chromosomal aberrations and micronuclei in one study in rat bone marrow (9), although it did not affect general bone marrow activity and failed to increase micronuclei in mice, compared to controls. The observed tumorigenicity of fenbucounazole has been proposed to occur via a tumour promotion effect in a manner similar to phenobarbital (3), which has been demonstrated to be mediated by activation of nuclear receptor superfamily members constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (10–12). There is ample evidence that many of the toxicological effects of conazoles are also mediated through activation of CAR/PXR-mediated pathways alone are not sufficient to differentiate between tumorigenic and non-tumorigenic conazoles (15,16).

The present studies were undertaken to assess the in vivo mutagenicity of triadimefon, propiconazole and myclobutanil in mouse liver. We used the Big Blue® transgenic rodent mutagenesis assay to determine the mutant frequency in mouse liver of two tumour-producing conazoles and one non-tumorigenic conazole. The basis of this assay is the presence of a lacI reporter gene that is stably integrated into the mouse genome. This gene serves as a recoverable mutation target that can be evaluated to assess in vivo mutagenicity following systemic administration of a chemical. This assay allows for detection and quantitation of mutagenic effects of the chemical in the whole animal and has been successfully used to demonstrate target tissue mutagenicity by a wide range of chemical carcinogens (17).

Materials and methods

Animal dosing

Two separate studies, conducted at different times with independent populations of animals, were undertaken to assess the in vivo mutagenicities of the conazoles. In Study 1, we assessed the effect of administration of 1800 p.p.m. triadimefon in feed. The effects of either 2500 p.p.m. propiconazole or 2000 p.p.m. myclobutanil were assessed in Study 2. Each study included an independent control group which received diet containing no conazoles. Male C57BL/6 Big Blue® mice were obtained from Stratagene (La Jolla, CA). Animals were housed two per cage in a room under a 12:12 h light:dark cycle with controlled temperature and humidity in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee. Animals were ear tagged with a unique identifying number and randomly assigned to treatment groups. The animals were ~8 weeks old at time of treatment.

Animals were either fed a diet of regular feed (Purina Mills Rodent Diet 5002) or the same diet containing 1800 p.p.m. triadimefon, 2500 p.p.m. propiconazole or 2000 p.p.m. myclobutanil for 4 days ad libitum. These doses were selected to be the same as those used in previous in vivo toxicology and transcriptomic studies (15,16).

The feed containing triadimefon was prepared by Bayer CropScience. Propiconazole (Orbit; 94.2% active ingredient) was obtained from Syngenta Crop Protection (Greensboro, NC) and myclobutanil (Eagle; 95.8% active ingredient) was obtained from Dow AgroSciences LLC (Midland, MI). Feeds containing propiconazole and myclobutanil were prepared on-site as previously described (15).

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lacI mutation assay

After 4 days of treatment, animals were immediately euthanized by carbon dioxide asphyxiation and cervical dislocation. Livers were removed, flash frozen in liquid nitrogen and stored at −80°C. DNA was extracted using the RecoverEase™ DNA isolation kit (Stratagene). The lacI-λ shuttle vector was recovered as phage particles by exposing the genomic DNA to λ packaging extract in vitro (Transpack™, Stratagene). The phage particles were assayed for lacI mutations by infecting a repair-deficient Escherichia coli strain (recA- uvrC-), Tet-R, pKM101) developed by James Fuscoe (National Center for Toxicological Research, Jefferson, AR) and plated on XGAL medium as previously described (18). Wild-type viral plaques appear white, while plaques formed by lacI virus are blue. The mutant frequency for each animal was determined from the ratio of blue mutant plaques to white wild-type plaques. A minimum of 1.75 × 104 plaques was analysed for each animal. Sectored and pinpoint plaques were not included in the analysis of mutant frequencies as mutants of this type are considered to arise ex vivo in the plating bacteria and are not representative of in vivo mutagenesis (19). Analyses of the statistical significance of increases in mutant frequency were performed using the generalized Cochran–Armitage test (20,21).

Results

The mutant frequencies observed in the control and conazole-treated animals are summarized in Table I. A 1.94-fold increase in mutant frequency was observed in liver tissues from mice administered 1800 p.p.m. triadimefon (P = 0.009) relative to concurrent controls. Mice receiving 2500 p.p.m. of propiconazole for 4 days had a 1.97-fold increase in mutant frequency compared to concurrent controls (P = 0.018). In contrast, myclobutanil did not induce any change in mutant frequency (P = 0.548). These analyses were conducted in two independent studies conducted at different times, each study having its own group of untreated control animals. Triadimefon was assessed in Study 1, while propiconazole and myclobutanil were assessed in Study 2. Because of variability in absolute mutant frequencies in control groups between these two studies, it is inappropriate to directly compare in vivo mutant frequencies among conazoles. We therefore calculated relative mutant frequencies by dividing the observed mutant frequency in each animal by the mean mutant frequency of the concurrent control group. The relative mutant frequencies in the control and treated mouse livers are shown in Figure 1. The mutant frequencies in each control group are within historical ranges.

Although sectored plaques and pinpoint plaques were not included in the scoring for mutants, there was not a large treatment-related difference in the numbers of these plaques detected relative to concurrent controls, although there was a difference between the levels observed in the two independent studies. In the first study, eight sectored plaques and seven pinpoint plaques were observed among the control animals, while six sectored plaques and seven pinpoint plaques were detected among the triadimefon-treated animals. Thus, the frequencies of sectored and pinpoint plaques were slightly lower in the triadimefon-treated group than in the control group in the first study. The levels of sectored and pinpoint plaques in the second study were much lower, with no sectored plaques observed in any treatment group, one pinpoint plaque observed in the control group, no pinpoint plaques in the myclobutanil-treated group and three pinpoint plaques observed in a single animal among the propiconazole-treated animals. Interestingly, the one propiconazole-treated animal yielding pinpoint plaques was animal 57, the animal with the lowest individual mutant frequency.

Discussion

Previous studies of myclobutanil, propiconazole and triadimefon in a variety of short-term in vitro and in vivo tests have generally failed to demonstrate genotoxicity (1,2,5–8), with the exception of a single positive report of genotoxic activity for triadimefon in rat bone marrow (9). Propiconazole and triadimefon have therefore been considered to be non-genotoxic tumorigens. However, a variety of chemicals have been identified which are tumorigenic and which demonstrate in vivo mutagenicity but are negative in short-term genotoxicity assays for a variety of reasons (22). These include dichloroacetic acid (23), oxazepam (24), p-dichlorobenzene, hexachlorobenzene, hexachloroethane, ochratoxin A and nitroacetic acid (22) and dicyclanil (25).

Two prevailing hypotheses for mechanisms by which ‘non-genotoxic’ hepatocarcinogens induce tumours are the induction of hepatocyte proliferation and the induction of liver enzymes (26). The specific increases in mutant frequencies in the livers

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Animal ID</th>
<th>Total plaques</th>
<th>Mutant plaques</th>
<th>Mutant frequency (×10⁻⁵)</th>
<th>P value relative to concurrent control</th>
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<tbody>
<tr>
<td>Study 1</td>
<td>Control feed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1800 p.p.m.</td>
<td>Triadimefon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>234 091 124</td>
<td>7</td>
<td>1.73 0.0006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>234 091 124</td>
<td>7</td>
<td>1.73 0.0006</td>
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<td>23</td>
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<td>1.73 0.0006</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>1800 p.p.m.</td>
<td>1 389 320 78</td>
<td>5.61 0.23</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table I. Mutant frequencies in the lacI gene recovered from liver

*One-tailed Cochran–Armitage P-value relative to control feed, Study 1.
**One-tailed Cochran–Armitage P-value relative to control feed, Study 2.
—Two-tailed Cochran–Armitage P-value relative to control feed, Study 2.
of mice treated with hepatotumorigenic conazoles observed in the present study cannot be explained by increased cell proliferation. Previous measurements of cell proliferation in mouse liver following administration of the same concentrations of these conazoles showed that only propiconazole and the non-tumorigen, myclobutanil, significantly induced increased hepatic cell proliferation after 4 days of treatment (15). Further, studies have shown that cell proliferation per se does not increase mutant frequencies in mouse liver (27,28), nor does treatment with carcinogens generally considered to be non-genotoxic, including di(2-ethylhexyl)phthalate, heptachlor or sodium phenobarbital (29,30). The mutational data for phenobarbital provide an interesting comparison to the present study. Administration of phenobarbital in the feed at 2500 p.p.m. for 180 days failed to yield a significant increase in mutation frequency, although some statistically significant differences were observed between the mutant spectra in phenobarbital-treated animals compared to historical controls (30). In another study using the LacZ transgenic MutatiMouse assay, administration of phenobarbital in feed at 1500 p.p.m. for 5 days also failed to induce any increase in mutant frequency (31). Propiconazole and triadimefon, in contrast, induced highly significant increases in mutant frequency after only 4 days of administration, clearly differentiating the mutagenic activities of these conazoles from phenobarbital.

The E.coli strain used in this study was designed to be capable of fixing DNA damage into mutations, increasing the ability of the assay to detect the induction of promutagenic damage in the transgene. However, neither triadimefon-treated nor propiconazole-treated animals showed any increase in the frequencies of sectored or pinpoint plaques recovered relative to concurrent untreated controls. Thus, to the extent that sectored and pinpoint plaques may represent \textit{ex vivo} mutation fixation (19), our data do not provide any indication that \textit{ex vivo} mutagenesis accounts for the increases in mutant frequencies induced by triadimefon and propiconazole. While we cannot entirely rule out the possibility that some of the normal morphology plaques recovered from triadimefon- and propiconazole-treated animals may have resulted from \textit{ex vivo} mutation fixation, our data show that these tumorigenic conazoles are capable of inducing promutagenic DNA damage \textit{in vivo}.

The abilities of these three conazoles to induce changes in hepatophysiology \textit{in vivo} have also been studied and found to show many similarities (15). Observed conazole-induced changes include increased liver hypertrophy, increased pentoxy-resorufin-O-dealkylase activity and decreased serum cholesterol and high-density lipoprotein levels. Transcriptional profiling has demonstrated additional similarities and some differences in gene expression patterns among these conazoles (16). Each of these three conazoles induces hepatic hypertrophy, induces cell proliferation and induces CYP2B family expression, protein and enzymatic activity, presumably through CAR activation (not measured). Therefore, nuclear receptor activation alone is unable to explain why triadimefon and propiconazole are mouse liver tumorigens while myclobutanil is not. Propiconazole and triadimefon both induced expression alterations that indicated changes in cell cycle regulation, apoptosis, calcium signalling and many signalling pathways. However, triadimefon and propiconazole also each induced changes in the expression of unique gene pathways, with triadimefon inducing more alterations in biosynthesis pathways and retinoid acid metabolism genes, while propiconazole induced greater effects on the expression of genes responding to oxidative stress and on the insulin-like growth factor/P13K/AKT/PTEN/mTor and Wnt-beta-catenin pathways (16). Three gene pathways were identified which were altered following 4, 30 and 90 days of conazole administration for the tumorigenic conazoles triadimefon and propiconazole, but not the non-tumorigen myclobutanil. These pathways involve the degradation of aliphatic amino acids, butanoate metabolism and nuclear receptors involved in lipid metabolism and toxicity. After 90 days of treatment, both triadimefon and propiconazole induced significant changes in the expression of genes and pathways that control the adherens junctions and actin cytoskeleton (16). Examination of these gene expression data reveals no genes related to DNA repair or DNA damage response whose expression at 4 days was significantly different between tumorigenic and non-tumorigenic conazoles.

Increases in mutant frequency have been observed in DNA isolated from chemically induced liver tumours in \textit{lacI} transgenic mice (32). Inflammatory responses or other processes within the tumour microenvironment may lead to increased mutation. Although many tumours are themselves genetically unstable and promutagenic (33), the 4-day time point used in the present study is considered too brief to allow for the formation of even preneoplastic lesions and precludes the possibility that the presence of small tumours in the liver contributes to the observed increases in mutant frequency.

The increased \textit{in vivo} mutation induction in the target tissues at the tumorigenic doses of triadimefon and propiconazole and the failure of myclobutanil to induce mutations represent a useful biomarker that clearly differentiates between these two tumorigenic and one non-tumorigenic conazoles, suggesting that mutagenesis may represent a key event in hepatotumorigenesis in the mouse induced by treatment with triadimefon and propiconazole. The specific promutagenic damage induced by these conazoles remains to be elucidated; thus, the relevance of the mutagenicity observed in this study to the potential of these two conazoles to act as human mutagens is uncertain. Sequence analysis of the mutants induced by these conazoles is underway and may provide additional insight into the nature of the promutagenic damage and the pathways contributing to its formation.
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References


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