Azole Fungicides Disturb Intracellular Ca\textsuperscript{2+} in an Additive Manner in Dopaminergic PC12 Cells

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Humans are exposed to complex mixtures of pesticides and other compounds, mainly via food. Azole fungicides are broad spectrum antifungal compounds used in agriculture and in human and veterinary medicine. The mechanism of antifungal action relies on inhibition of CYP51, resulting in inhibition of fungal cell growth. Known adverse health effects of azole fungicides are mainly linked to CYP inhibition. Additionally, azole fungicide-induced neurotoxicity has been reported, though the underlying mechanism(s) are largely unknown. We therefore investigated the effects of a group of six azole fungicides (imidazol, flusilazole, fluconazole, tebuconazole, triadimefon, and cyproconazole) on cell viability using a combined alamar Blue/CFDA-AM assay and on oxidative stress using a H\textsubscript{2}-DCFDA fluorescent assay. As calcium plays a pivotal role in neuronal survival and functioning, effects of these six azole fungicides and binary and quaternary mixtures of azole fungicides on the intracellular calcium concentration ([Ca\textsuperscript{2+}]) were investigated using single-cell fluorescence microscopy in dopaminergic PC12 cells loaded with the calcium-sensitive fluorescent dye Fura-2. Only modest changes in cell viability and ROS production were observed. However, five out of six azole fungicides induced a nonspecific inhibition of voltage-gated calcium channels (VGCCs), though with varying potency. Experiments using binary IC\textsubscript{50} and quaternary IC\textsubscript{90} mixtures indicated that the inhibitory effects on VGCCs are additive. The combined findings demonstrate modulation of intracellular Ca\textsuperscript{2+} via inhibition of VGCCs as a novel mode of action of azole fungicides. Furthermore, mixtures of azole fungicides display additivity, illustrating the need to take mixture effects into account in human risk assessment.

Key Words: in vitro neurotoxicology; Fura-2 Ca\textsuperscript{2+}-imaging; calcium homeostasis; voltage-gated calcium channels; dose addition; triazole/imidazole fungicides.

Azole fungicides constitute around 40 broad-spectrum fungicides that contain at least one triazole or imidazole moiety in their structure and are used for agricultural, horticultural, and pharmaceutical applications. The primary antifungal mechanism of action of these fungicides relies on inhibition of CYP51 (lanosterol 14 alpha-demethylase), a key enzyme for sterol biosynthesis in fungi (for review, see Zarn et al., 2003). Inhibition of this enzyme leads to a depletion of ergosterol, a vital ingredient of the fungal cell wall, resulting in growth inhibition and death of fungi. Besides inhibition of CYP51, which is also present in humans, azole fungicides are known to exert a range of adverse health effects in mammals, including disturbance of mammalian steroidogenesis, induction of craniofacial malformations, and birth defects (Crofton, 1996; Giavini and Menegola, 2010; Menegola et al., 2005; Zarn et al., 2003). Adverse effects of azole fungicides on mammalian (sex-specific) steroidogenesis are mainly linked to disruption of cholesterol synthesis (Zarn et al., 2003) or interaction with CYP 17/19 (Roelofs et al., 2013; Sanderson et al., 2002), whereas the mechanisms underlying other side effects are largely unknown.

A limited number of azole fungicides is known to exert neurotoxic effects in vitro and in vivo, including effects on uptake and release of monoamines in rat synaptosomes and neurobehavioral effects (Crofton, 1996; Moser et al., 2001). Calcium plays a pivotal role in many inter- and intraneuronal processes, including (dopaminergic) neurotransmission (for review, see Westerink, 2006), gene transcription (Carrasci and Hidalgo, 2006), neurodegeneration (Mattson, 2012), and neurodevelopment (Pravettoni et al., 2000). Neuronal cells, therefore, rely heavily on strict regulation of their intracellular calcium concentration ([Ca\textsuperscript{2+}]). For this reason, we investigated the effect of acute exposure to six selected azole fungicides on basal and depolarization-evoked intracellular calcium levels in PC12 cells, which are a widely used and extensively characterized model for mature dopaminergic neurons (Westerink and Ewing, 2008).

The selection of azole fungicides consisted of fungicides used as veterinary (imidazol) or human (fluconazole) pharmaceutical or as agricultural fungicide (triadimefon, flusilazole, tebuconazole, and cyproconazole) (Table 1). For humans, exposure to fungicides is primarily via food and occupational routes
### Table 1

Molecular Structures and Chemical-Physical Properties of the Six Selected Azole Fungicides

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(For review, see Oates and Cohen, 2011). Though current risk assessment approaches are predominantly based on individual azole fungicides, human exposure to these chemicals occurs predominantly to a complex mixture. We, therefore, included several binary mixtures and a quaternary mixture of fungicides to more closely resemble the human exposure situation in a qualitative way and to reveal whether additivity applies.

### Materials and Methods

**Chemicals.** Fura-2-AM, CFDA-AM, and 2,7-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) were obtained from Molecular Probes (Invitrogen, Breda, The Netherlands); all other chemicals were obtained from Sigma-Aldrich (Swijndrecht, The Netherlands), unless otherwise noted. Flusilazole, triadimefon, imazalil, tebuconazole, and cyproconazole were of 99.8% purity (Pestanal grade, Riedel de Haën, Seelze, Germany). Fluconazole was obtained using the fluorescent dye H$_2$DCFDA as described previously (Etienne et al., 2009).

**Cell culture.** Rat dopaminergic pheochromocytoma (PC12 cells; Greene and Tischler, 1976) were grown for 10 passages in RPMI 1640 (Invitrogen) supplemented with 5% fetal calf serum and 10% horse serum (ICN Biomedicals, Zoetermeer, The Netherlands) in a humidified incubator at 37°C and 5% CO$_2$ as described previously (Dingemans et al., 2009, Heusinkveld and Westerink, 2012). For Ca$^{2+}$ imaging experiments, cells were cultured in poly-l-lysine-coated glass-bottom dishes (MatTek, Ashland, MA) as described previously (Dingemans et al., 2009, Heusinkveld and Westerink, 2012). For cell viability experiments, cells were cultured in poly-l-lysine-coated 24-well plates (Greiner Bio-one, Solingen, Germany) at a density of $5 \times 10^{4}$ cells/well. For experiments assessing the production of reactive oxygen species (ROS), which is indicative for oxidative stress, cells were seeded in poly-l-lysine-coated 48-well plates at a density of $2.5 \times 10^{5}$ cells/well.

**Cell viability assay.** To assess the effects of the compounds on cell viability, a combined alamar Blue/CFDA-AM (aB/CFDA) assay was used (protocol adapted from Bopp and Lettieri, 2007) to determine respectively mitochondrial activity and membrane integrity. Cells were exposed in serum- and phenol red-free medium to concentrations up to 100µM for up to 24 h. Mitochondrial activity of the cells was recorded as a measure of cell viability with the aB assay, which is based on the ability of the cells to reduce resazurin to resorufin. In the same experiment, membrane integrity was assessed indirectly using a CFDA-AM assay, which is based on nonspecific cytoplasmic-esterase activity. Briefly, cells were incubated for 30 min with 12.5 µM aB and 4 µM CFDA-AM. Resorufin was measured spectrophotometrically at 540/590 nm (Infinite M200 microplate; Tecan Trading AG, Mannedorf, Switzerland), whereas hydrolyzed CFDA was measured spectrophotometrically at 493/541 nm.

**Measurement of ROS production using H$_2$DCFDA.** The involvement of oxidative stress in the observed reduction in cell viability was investigated using the fluorescent dye H$_2$DCFDA as described previously (Heusinkveld et al., 2010). Briefly, cells were loaded with 1.5µM H$_2$DCFDA for 30 min at 37°C. Subsequently, cells were exposed for up to 24 h to 0.1–100µM compound, and fluorescence was measured spectrophotometrically at 488/520 nm (Infinite M200 microplate; Tecan Trading AG). Rotenone (100µM) was included as positive control for oxidative stress (Radad et al., 2006).

**Ca$^{2+}$ imaging microscopy.** Changes in the [Ca$^{2+}$]$_i$ were measured on a single-cell level using the Ca$^{2+}$-sensitive fluorescent ratio dye Fura-2 AM as described previously (Hendriks et al., 2012; Heusinkveld and Westerink, 2012). Briefly, cells were loaded with 5µM Fura-2 AM (Molecular Probes; Invitrogen) for 20 min at room temperature, followed by 15 min de-esterification. After de-esterification, the cells were placed on the stage of an Axiovert 35M inverted microscope (Zeiss, Göttingen, Germany) equipped with a TILL Photonics Polychrome IV (TILL Photonics GmbH, Gräfelfing, Germany). Fluorescence, evoked by 340 and 380 nm excitation wavelengths (F340 and F380), was collected every 6 s at 510 nm with an Image SensiCam digital camera (TILL Photonics GmbH). After 5-min baseline recording, cells were depolarized by high K$^+$-containing saline (100mM K$^+$) for 18 s. Following a 10-min recovery period, cells were exposed for 20 min (Fig. 3A) to DMSO (0.1%), methylhistamine (1 or 10µM), a single fungicide (0.1–100µM), or a mixture of equitoxic concentrations of azole fungicides. Subsequently, cells were depolarized a second time to evaluate effects of fungicide exposure on depolarization-evoked Ca$^{2+}$ influx. Minimum and maximum ratios ($R_{min}$ and $R_{max}$) were determined at the end of the recording by addition of ionomycin (5µM) and EDTA (17mM).
In a separate set of experiments, exposure to azole fungicides prior to the second depolarization was reduced from 20 min to 12 s to assess whether lipophilicity, and thus intracellular accumulation, plays a role.

Changes in the F340/F380 ratio (R), reflecting changes in $[\text{Ca}^{2+}]_i$, were further analyzed using custom-made MS-Excel macros calculating F340/F380 ratios and applying a correction for background fluorescence. Free cytosolic $[\text{Ca}^{2+}]$, was calculated using Grynkiewicz’s equation $[\text{Ca}^{2+}] = K_i \times (R - R_{\text{min}}) / (R_{\text{max}} - R)$, where $K_i$ is the dissociation constant of Fura-2 determined in the experimental set-up (Deitmer and Schild, 2000). The mean basal $[\text{Ca}^{2+}]$, during exposure was determined to quantify effects on basal $[\text{Ca}^{2+}]$. The maximum amplitude of $[\text{Ca}^{2+}]$, observed during depolarization was used to investigate possible effects on the depolarization-evoked increase of $[\text{Ca}^{2+}]$, (Hendriks et al., 2012, Heusinkveld and Westerink, 2011).

**Data analysis and statistics.** Cell viability data are presented as % viability ± SD compared with control from at least three independent experiments consisting of four replicates per experiment. Data on production of ROS are presented as % increase in ROS ± SD compared with time-matched controls from three independent experiments consisting of four replicates per experiment.

Data from fluorescence microscopy are presented as mean $[\text{Ca}^{2+}]_i$ ± SD from the number of cells ($n$) indicated, obtained from 3–9 independent experiments ($N$). Treatment ratio (TR; see also Fig. 3A) represents the second depolarization-evoked increase in $[\text{Ca}^{2+}]_i$ in treated cells as a percentage of the response to the first depolarization, expressed relative to the TR in control cells.

Statistical analyses were performed using GraphPad Prism v6.01 (GraphPad Software, San Diego, California). Continuous data were compared using one-way ANOVA with post hoc Bonferroni test where applicable. Concentration-response curves for inhibition of depolarization-evoked Ca$^{2+}$ influx were measured at concentrations of 0.1–100µM with intermediate concentrations where needed. Concentration-response curves were fit using a nonlinear sigmoidal fit using GraphPad Prism v6.01. Calculated IC$_{50}$ and IC$_{90}$ concentrations are presented ± 95% confidence interval of the effect size. A $p$ value ≤ 0.05 is considered statistically significant.

MDL ISIS/Draw version 2.5 was used to draw chemical structures.

**RESULTS**

**Effects of Azole Fungicides on Cell Viability**

To exclude that effects on calcium homeostasis are confounded by cytotoxicity, cell viability was assessed using a combined alamar Blue/CFDA-AM assay. PC12 cells were exposed to different concentrations of azole fungicides (0.1–100µM) for 24h. The results from the alamar Blue assay demonstrate that exposure to 0.1 or 1µM imazalil does not result in an increase in mitochondrial activity (112 ± 5%; $N = 3$; 120 ± 10%; $N = 3$; Fig. 1A), whereas exposure to 10 or 100µM does induce an increase in mitochondrial activity (179 ± 21%; $N = 3$, $p \leq 0.001$; 183 ± 36%; $N = 3$, $p \leq 0.001$; Fig. 1A) suggestive for oxidative stress. At these concentrations, none of the other tested fungicides induces a change in mitochondrial activity (results not shown).

The results from the CFDA-AM demonstrate that exposure to 0.1, 1, or 10µM imazalil does not induce a significant change in membrane integrity (97 ± 5%; $N = 3$; 95 ± 3%; $N = 3$; 96 ± 3%; $N = 3$; Fig. 1B), whereas exposure to 100µM decreases membrane integrity to 74 ± 12% ($N = 3$, $p \leq 0.001$; Fig. 1B). None of the other compounds induces a change in membrane integrity (results not shown).

**Effects of Azole Fungicides on $[\text{Ca}^{2+}]_i$**

Baseline $[\text{Ca}^{2+}]_i$ in PC12 cells is typically low (=100nM; Fig. 3A) with only minor fluctuations. Upon depolarization with 100mM K$^+$, $[\text{Ca}^{2+}]_i$ displays a strong, transient increase...
(2.0 ± 0.7 μM;  n = 80; K1: Fig. 3A) that decreases to near basal [Ca²⁺] within minutes. During a subsequent 20-min exposure to DMSO-containing saline (1 μl/ml), basal calcium levels remain low. Similarly, 20-min exposure to fluconazole, tebuconazole, fluconazole, cyproconazole, and triadimefon (0.1–100 μM) does not affect basal [Ca²⁺], (results not shown). However, compared with DMSO, exposure to 100 μM imazalil induced a significant increase in basal [Ca²⁺] amounting to 316 ± 49nM ( n = 47;  p ≤ 0.001; data not shown), whereas lower concentrations (0.1–30 μM) did not induce a change in basal [Ca²⁺].

When control cells are challenged with a second depolarization following 20-min exposure to DMSO, [Ca²⁺] increases to 1.9 ± 0.8 μM ( n = 80; K2: Fig. 3A), (TR: 95 ± 1%;  n = 98; Fig. 3A; set at 100% for controls). Cells exposed to fluconazole (0.1–100 μM) for 20 min have a TR that is not different from control cells (Fig. 3B). However, in cells exposed for 20 min to fluconazole, imazalil, tebuconazole, cyproconazole, and triadimefon, the TR is concentration-dependently reduced (Fig. 3B; Table 2), demonstrating that these fungicides inhibit VGCC-mediated Ca²⁺ influx with clearly different potencies. To assess whether lipophilicity, and thus accumulation in the cell or membrane, plays a role in the observed effects on VGCCs, cells were exposed for 12 s instead of 20 min to 30 μM cyproconazole. The results reveal a comparable effect of cyproconazole on VGCC-mediated Ca²⁺ influx (TR12s: 79 ± 15%,  n = 43 vs. TR20min: 83 ± 17%,  n = 55; data not shown).

As human exposure is in general to complex mixtures of pesticides, a number of mixture experiments were performed. To discriminate between additivity, antagonism, and synergism, mixtures were composed based on (calculated) IC₅₀ and IC₂₀ concentrations. Mixtures were composed of either two compounds (imazalil and fluconazole; tebuconazole and cyproconazole) at their relative IC₂₀ concentrations or of these four compounds at their relative IC₁₀ concentrations. The results from the mixture experiments of IC₂₀ concentrations imazalil (2.5 μM; IC₂₀ ± 3.5%; Table 2) and fluconazole (1.3 μM; IC₂₀ ± 3.1%; Table 2) display additive inhibition of depolarization-evoked [Ca²⁺], amounting to 34 ± 13% ( n = 40; Fig. 4B). Exposure to the calculated IC₂₀ concentrations of cyproconazole (32.7 μM; IC₂₀ ± 5.6%; Table 2) and tebuconazole (5.2 μM; IC₂₀ ± 3.9%; Table 2) also displays additive inhibition of depolarization-evoked [Ca²⁺], amounting to 41 ± 14% ( n = 55; Fig. 4B).

To assess whether additivity applies for more complex mixtures at IC₁₀ levels (a nonsignificant decrease in TR), mixture experiments were performed with the calculated IC₁₀ concentrations of four fungicides (imazalil: 1.3 μM ± 2.7%; fluconazole: 0.6 μM ± 2.5%; tebuconazole: 3.4 μM ± 3.0%; cyproconazole: 23 μM ± 4.3%; Table 2). Inhibition of depolarization-evoked [Ca²⁺], upon exposure to this IC₁₀ mixture amounted to 45 ± 15% ( n = 46; Fig. 4B), again indicating that dose addition applies.

A recognized pathway of inhibition of VGCCs is via activation of neuronal histamine H3 receptors (H3R) leading to cAMP-dependent downregulation of VGCCs and as a result inhibition of depolarization-evoked Ca²⁺ influx and neurotransmission (Ferrada et al., 2008; Threlfell et al., 2004). To assess whether binding of azole fungicides to the H3R could have caused the observed reduction in the depolarization-evoked [Ca²⁺], cells, we investigated the presence of functional H3Rs in PC12 cells by 20-min incubation with the natural agonist methylhistamine prior to evoking depolarization. No effect was observed on the degree of calcium influx in histamine-exposed cells compared with the solvent control, indicating that our PC12 cells do not contain functional H3Rs. We, therefore, conclude that fungicide-based activation of H3Rs plays no role in the observed inhibition of VGCCs.

**DISCUSSION**

The present results demonstrate that the azole fungicides imazalil, flusilazole, tebuconazole, and cyproconazole concentration-dependently inhibit depolarization-evoked calcium influx. Fluconazole does not induce an inhibition of depolarization-evoked calcium influx with exposures up to 100 μM. Though PC12 cells contain multiple subtypes of VGCCs, including L-, N-, and P/Q-type VGCCs (Dingemans et al., 2009; Heusinkveld and Westerink, 2012), all five compounds induce a (near) complete inhibition at the highest concentrations, indicative of a nonspecific inhibition of VGCCs. IC₅₀ values (Table 2) range from 5 μM (flusilazole) to 65 μM (cyproconazole), revealing a one order of magnitude difference in potency. Exposure of cells to binary IC₂₀ or quaternary IC₁₀ mixtures provides clear indications for additivity with respect to inhibition of depolarization-evoked calcium influx. The results from the cytotoxicity assays indicate that imazalil (≥ 10 μM, 24 h) is linked to an increase in mitochondrial activity (aB assay), which is only at the highest concentration (> 10 μM) related to a minor loss of viability (CFDA-AM
The results of the oxidative stress assay indicate only an increase in oxidative stress for exposure to imazalil and flusilazole (100 µM). None of the other four fungicides induced an effect on oxidative stress. Although the observed increase in basal [Ca\textsuperscript{2+}] upon exposure to 100µM imazalil could be related to acute cell stress, we conclude that the observed effects on depolarization-evoked calcium influx are not related to acute cytotoxicity or the occurrence of excess oxidative stress. Furthermore, it is unlikely that interactions with (steroidogenic) CYP enzymes or formation of (reactive) metabolites plays a role in the observed effects as (our) PC12 cells have poor metabolic capacity and cytochrome activity. In addition, the duration of calcium imaging experiments appears too short for considerable formation of metabolites rendering an effect of metabolites unlikely. However, in the in vivo situation, interaction with (steroidogenic) CYP enzymes is likely to play a role as (in vitro) effect concentrations for interaction of azole fungicides with (steroidogenic) CYP enzymes are in the same range (see Roelofs et al., 2013; Sergent et al., 2009).

In a study on neurobehavioral effects of a range of azole compounds, it has been reported that a strict structure-activity relationship is apparent for neurobehavioral effects caused by azole fungicides concentration-dependently inhibit depolarization-evoked [Ca\textsuperscript{2+}] in PC12 cells. (A) Example traces of cytosolic [Ca\textsuperscript{2+}] illustrating the inhibition of the second depolarization-evoked increase in [Ca\textsuperscript{2+}] (K2) in PC12 cells exposed to DMSO (Control; upper trace), imazalil (30µM; middle trace), and tebuconazole (100µM; lower trace). (B) Concentration-response curves of all six azole fungicides on the TR. Data points display average data (± SD) from at least 28–80 individual cells (four to nine experiments per concentration). Difference from control (C): *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.
table 2

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<th>NOEL</th>
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**FIG. 4.** Benchmark dose approach for mixture composition. (A) Fitted concentration-response curves for flusilazole (Fi), imazalil (Im), tebuconazole (Tb), triadimefon (Tf), and cyproconazole (Cp). Dashed lines alongside the curves represent the 95% confidence interval of the fitted curves. (B) Bargraph displays that the binary combinations of (near) IC⁵₀ and the quaternary combination of (near) IC⁵₀ exert an additive inhibition of TR. Bars display average data (± SD) from the number of cells indicated in the bars (≥four experiments per concentration). Difference from control: ***p ≤ 0.001.

noticeable differences. First, fluconazole contains two triazole moieties, whereas the other fungicides contain only one, pointing toward the single triazole or imidazole moiety as effective group. The presence of two triazole moieties could cause steric hindrance in binding of the molecules to receptors or channels. Second and more likely, the log K₁₀₀ value of fluconazole (0.25) indicates a low lipophilicity, whereas the other fungicides are more lipophilic (Table 1). In this data set, lipophilicity of the test compounds appears inversely correlated to the potency (IC₅₀) of VGCC inhibition because a higher lipophilicity appears related to a higher potency. This indicates that the target of azole fungicides could be located in the lipid membrane or even intracellularly. However, results from experiments with short exposure (12-s exposure; data not shown) demonstrate that effects develop immediately upon exposure, suggesting that azole fungicides act on an extracellular target such as the VGCC itself or a receptor-activated pathway that results in a specific inhibition of VGCCs. Activation of the neuronal histamine H3 receptor (H3R) is linked to inhibition of VGCCs via G-protein-coupled pathways acting on cAMP. As a result, H3R activation causes negative regulation of striatal dopaminergic and serotoninergic neurotransmission (Ferrada et al., 2008; Threlfell et al., 2004).

Considering the structure analogy between azole fungicides and the natural H3R agonist (R)-(-)-α-methyl-histamine, we hypothesized that binding of azole fungicides to the H3R could underlie the observed inhibition of VGCCs. However, experiments with the natural agonist revealed that these receptors are not functional in PC12 cells, and therefore, another mechanism must be involved. Consequently, it cannot be excluded that binding to the H3R plays a role in the in vivo situation.

Maintenance of intracellular calcium homeostasis is pivotal for proper development and functioning of the nervous system. Identification of inhibition of VGCCs as a novel mode of action for azole fungicides is therefore of importance. Notably, inhibition of VGCCs is a well-known common feature of several man-made (persistent) chemicals, such as brominated flame retardants (Dingemans et al., 2010; Hendriks et al., 2012), organochlorine insecticides (Heusinkveld and Westerink, 2012), and PCBs (Langeveld et al., 2012) and thus appears a common denominator for various groups of structurally different environmental contaminants and pesticides in risk assessment (Westerink, 2013). In that way, it can be expected that a complex mixture of compounds—all below their individual effects levels—may evoke adverse effects because additivity may apply.

Human exposure data or data on pharmacokinetics of singleazole fungicides are largely lacking. In addition, data on human exposure to mixtures of azole fungicides are limited to data on technical mixtures of fungicides applied in agriculture, residue levels in food, and (limited) data on animal-based kinetics. According to a recently published study on pesticide residue levels on food in France, residue levels of among others imazalil, tebuconazole, and cyproconazole are often > 10 µg/kg (Nougadère et al., 2012), indicating that human exposure to (mixtures of) azole fungicides is very likely. An animal-based

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**TABLE 2**

NOEL Concentrations and (Calculated) Inhibitory Concentrations of Azole Fungicides on Depolarization-Evoked [Ca²⁺] (µM)

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PBPK study in rats on triadimefon indicated that upon single exposure (50 mg/kg, iv) a rapid increase in triadimefon throughout the body, including the brain, with tissue concentrations peaking between 10 and 100 μM (Crowell et al., 2011). Using the PBPK modelling, the authors calculated a human equivalent dose to the rat NOAEL for neurotoxicity of 0.86 mg/kg/day, which is only 25-fold higher than the human oral reference dose (RfD; 0.034 mg/kg/day; Crowell et al., 2011). This indicates that current safety margins may not be sufficient, in particular, when additivity applies because exposure to multiple no effect concentrations may thus result in adverse effects (Kortenkamp, 2008).

In conclusion, azole fungicides inhibit depolarization-evoked calcium influx via a specific inhibition of VGCC, which likely reduces (dopaminergic) neurotransmission. Furthermore, exposure to complex mixtures reveals additive inhibition of VGCC-mediated calcium influx. The combined findings thus illustrate the need for inclusion of (complex) mixtures and the use of common assessment groups based on neurotoxicological endpoints in human risk assessment studies.

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