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Can the inhibition of cytochrome P450 in aquatic invertebrates due to azole fungicides be estimated with *in silico* and *in vitro* models and extrapolated between species?

Michele Gottardi[a], Jonathan D. Tyzack[b], Andreas Bender[c], Nina Cedergreen[a,*]

[a] Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark
[b] EMBL-EBI, Wellcome Genome Campus, Hinxton, Cambridgeshire, CB10 1SD, United Kingdom
[c] Centre for Molecular Informatics, Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, United Kingdom

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ABSTRACT

Azole fungicides, designed to halt fungal growth by specific inhibition of fungal cytochrome P450 (CYP51), inhibit cytochrome P450s involved in the metabolism of xenobiotics in several non-target organisms thus raising environmental concern. The present study investigates the degree by which inhibition strengths of azoles toward cytochrome P450 in rat liver, the insect *Chironomus riparius* larvae and the snail *Lymnaea stagnalis* can be extrapolated from estimated *in silico* affinities. Azoles' affinities toward human cytochrome P450 isoforms involved in xenobiotic metabolism (CYP3A4, CYP2C9 and CYP2D6) as well as fungal CYP51 were estimated with a ligand-protein docking model based on the ChemScore scoring function. Estimated affinities toward the selected enzymatic structures correlated strongly with measured inhibition strengths in rat liver (ChemScore *vs.* $\log IC_{50}$ among cytochrome P450 isoforms: $-0.662 < r < -0.891$, $n = 17$ azoles), while weaker correlations were found for *C. riparius* larvae ($-0.167 < r < -0.733$, $n = 9$) and *L. stagnalis* ($-0.084 < r < -0.648$, $n = 8$). Inhibition strengths toward *C. riparius* and rat liver activities were found to be highly correlated to each other (r: 0.857) while no significant relationship was found between either of the species and *L. stagnalis*. The inhibition of cytochrome P450 due to azole fungicides could be estimated *in vitro* and to a lesser extent *in silico* for *C. riparius* but not for *L. stagnalis*, possibly due to different enzymatic susceptibility toward azole inhibition among the species.

1. Introduction

Azole fungicides are a group of plant protection agents extensively use in agriculture, accounting for approximately 25% of the total production of fungicides worldwide (Saxena et al., 2015). Furthermore, they are also used as antifungal drugs in humans as well as in livestock (Kahle et al., 2008). Azole fungicides can enter surface water due to spray drift and run-off after rain events (Riise et al., 2004; Werner et al., 2004), or through effluents of waste water treatment plants (Kahle et al., 2008). Azoles have been measured in surface water in concentrations usually ranging from 0.01 $\mu g/L^{-1}$ to 1 $\mu g/L^{-1}$ with peak concentrations of up to 175 $\mu g/L^{-1}$ (Bjergager et al., 2017).

Azoles were designed to inhibit the fungal cytochrome P450 responsible for ergosterol biosynthesis (CYP51, lanosterol-14 α-demethylase). Ergosterol plays an important role in the function and integrity of fungal membrane, hence, inhibition of its synthesis halts fungal growth (Copping and Hewitt, 1998). The molecular structure of azoles is characterized by the presence of an azole ring, which contains either two (imidazoles) or three (triazoles) nitrogen atoms, resulting in different antifungal selectivity (Maertens, 2004). However, in addition to the target cytochrome P450 of fungi, azoles have also been shown to inhibit cytochrome P450s involved in detoxification in non-target organisms (Pilling et al., 1995; Bach and Snegaroff, 1989; Snegaroff and Bach, 1989). Inhibition of cytochrome P450 dependent detoxification is believed to be the main mechanism responsible for the ability of azoles to synergize the toxicity of other pesticides such as pyrethroid insecticides (Pilling et al., 1995; Cedergreen, 2014). Under certain conditions, cytochrome P450s responsible for detoxification may also be induced thereby synergizing the toxicity of compounds that require activation by cytochrome P450 dependent oxidations to exert their

Abbreviations: ACN, acetonitrile; BSA, bovine serum albumin; DTT, DL-dithiothreitol; ECOD, 7-ethoxycoumarin-*O*-dealkylase; EDTA, ethylenediaminetetraacetic acid disodium salt dihydrate; IC_{50}, half maximal inhibitory concentration; $K_2HPO_43H_2O$, potassium phosphate dibasic trihydrate; KH_2PO_4, potassium dihydrogen phosphate; MFO, Mixed function oxidase; NADPH, ß-nicotinamide adenine dinucleotide-phosphate reduced tetrasodium salt hydrate; PMSF, phenylmethanesulfonyl fluoride

* Corresponding author.
E-mail address: ncf@plen.ku.dk (N. Cedergreen).

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toxic effect, such as certain organophosphate insecticides (Belden and Lydy, 2000). Induction of cytochrome P450s is, however, not specifically related to azoles, but a more general property of several groups of xenobiotics (Cedergreen, 2014), and it will therefore not be discussed further in the present study.

Azoles reversibly inhibit cytochrome P450s by coordination of the azole ring to the heme-iron as well as through molecular interaction with the enzyme's hydrophobic regions (Correia and Ortiz de Montellano, 2005). In general, the inhibitory strength of azole fungicides toward cytochrome P450s is believed to be governed by several factors, namely the affinity of the nitrogen electron lone pair of the azole ring to the heme-iron and the presence of substituents that may sterically modify this affinity, the hydrophobicity of the overall molecule (which is generally favorable for binding, given the hydrophobicity of the enzyme active cavity) and the complementarity between inhibitor geometry and enzyme active site (Correia and Ortiz de Montellano, 2005).

A large suite of cytochrome P450s exists within the same organism, not only catalyzing xenobiotics but also a range of endogenous processes, of which those governing endocrine hormone regulations have received the most attention (James and Boyle, 1998; Rewitz et al., 2006; Snyder, 2007). Cytochrome P450s involved in xenobiotic detoxification are membrane bound proteins primarily present in the endoplasmic reticulum (microsomal fraction) which mainly catalyze oxidation reactions (Koymans et al., 1993; Poulos and Johnson, 2005). Cytochrome P450s involved in detoxification generally lack the substrate specificity of cytochrome P450s involved in the metabolism of endogenous molecules. Different cytochrome P450s involved in detoxification have been shown to metabolize similar substrates and similar cytochrome P450s can metabolize different substrates (James and Boyle, 1998; Rewitz et al., 2006; Snyder, 2007). In the present study, the characterization of cytochrome P450 activity relevant for detoxification and its inhibition in different species is based on the ability of the extracted microsomal fraction to metabolize the same standard substrate, 7-ethoxycoumarin (Aitio, 1978).

Knowledge of inhibition strength of azole fungicides in relevant aquatic species would provide a tool to better understand and assess the ecotoxicological impact of these chemicals. Among the relevant aquatic species, the present study focuses on aquatic invertebrates, which are currently employed as test organisms for ecotoxicological studies and therefore form the basis of aquatic risk assessment of chemicals. Extraction of microsomal material and measurements of cytochrome P450 activities in small aquatic invertebrates used in ecotoxicological studies have been shown to be time consuming and sometimes not possible to carry out with current techniques. This is because organs rich in cytochrome P450s cannot be dissected out of very small organisms, and because homogenization of the whole organisms can release endogenous substances that attenuate *in vitro* enzymatic activity (Gottardi et al., 2015 and references therein). Therefore, the prediction of inhibition strengths, by using computer based approaches and/or by measurements on commercially available enzymatic materials extracted from other species, could provide a quick and cheap screening tool to identify strong cytochrome P450 inhibitors and subsequently potential synergistic substances in environmental chemical mixtures.

Ligand-protein docking is a well-established computer based (*in silico*) method that predicts the orientations of small organic molecules (ligands) in protein binding pockets (de Graaf et al., 2005; Kirchmair et al., 2012) as well as their binding affinities in the form of docking scores (Kirchmair et al., 2012). The method, although not free from limitations (de Graaf et al., 2005; Kirchmair et al., 2012), has been recently used to investigate inhibition of aromatase (cytochrome P450 CYP19 involved in steroidogenesis) by azole fungicides, obtaining good degrees of correlation between docking scores and experimentally measured inhibition strengths of azoles toward aromatase activity in human (r: −0.81), fish brain (r: −0.69) and fish ovaries (r: −0.85) (Saxena et al., 2015). However, the use of this approach for ecotoxicologically relevant aquatic invertebrates presents additional limitations, since the specific types and roles of cytochrome P450 present in these organisms are generally unknown. Therefore, crystallized cytochrome P450 structures are currently not available and since gene sequencing of non-target invertebrates is only in its infancy, also the creation of cytochrome P450 structures from homology models is limited (Gilbert et al., 2005). At present, *in silico* investigations of chemical interactions with cytochrome P450s in aquatic invertebrates must therefore rely on the available (mainly human) cytochrome P450 crystallized structures.

The use of models based on cytochrome P450 structures obtained from different organisms may nonetheless be possible. The overall three-dimensional structure of cytochrome P450s and the site responsible for the catalytic activity (heme-group) are conserved in practically all organisms where cytochrome P450s are found (Poulos and Johnson, 2005; Meunier et al., 2004), whereas the position of structural elements in the active cavity accounts for substrate and/or inhibitor (un)specificity among the different isoforms (Poulos and Johnson, 2005; Meunier et al., 2004). Therefore, inter species extrapolations of inhibition strengths may also be feasible for cytochrome P450s responsible for detoxification, as this group of enzymes is expected to present substrate unspecificity and possibly to be conserved across species potentially exposed to the myriad of natural and artificial xenobiotics present in the environment.

The aim of the present study was to investigate the degree by which an *in silico* ligand-protein docking model, based on available human and fungal cytochrome P450 crystallized structures, could estimate experimentally measured inhibition strengths of selected azole fungicides (Table 1) toward microsomal cytochrome P450 activities of rat liver, insect *Chironomus riparius* larvae and snail *Lymnaea stagnalis*. Rat liver microsomes were chosen as a commercially available source of enzymatic material, widely used for *in vitro* cytochrome P450 activity assays. The non-target invertebrates *C. riparius* and *L. stagnalis* were chosen as model organisms representing insects and molluscs, respectively. Both species are widely present in the environment and are currently used as standard organisms for ecotoxicological studies (OECD, 2016, 2011). In addition, measurable cytochrome P450 activity of *C. riparius* has been shown to be inhibited by a range of azole fungicides in a previous investigation (Gottardi et al., 2015).

The present study tested the following hypotheses: 1) *in silico* estimated affinities (docking scores) between azoles and different cytochrome P450 isoforms are strongly correlated to one another; 2) the estimated affinities, based solely on the azoles' molecular structures and the available crystallized cytochrome P450 structures, can be used to rank the inhibition strength of the azoles equally well in different species and 3) experimentally measured inhibition strengths of azole fungicides toward microsomal cytochrome P450 activities can be extrapolated between species from different phyla.

2. Materials and methods

2.1. Azole fungicides and cytochrome P450 structures

Five imidazoles and 13 triazoles were selected based on their use in agriculture or medicine (Table 1, Fig. S1). Their 3D structures were downloaded from the PubChem (Kim et al., 2016) or ZINC (Irwin et al., 2012) databases in Structure Data File (.sdf) format or Sybyl Mol2 (.mol2) format.

A relatively large number of cytochrome P450 crystallized structures are available in The Protein Data Bank (PDB) (Berman et al., 2000). Among those, the structures of three human cytochrome P450 isoforms involved in xenobiotic detoxification were selected: CYP3A4, CYP2C9 and CYP2D6. Two crystallized structures were selected for each isoform, with or without the presence of a co-crystallized inhibitor. This was done to investigate whether the choice of a specific isoform, or a structure co-crystallized with an inhibitor, had an effect on