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Induction of human hepatic cytochrome P-450 3A4 expression by antifungal succinate dehydrogenase inhibitors

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ABSTRACT

Succinate dehydrogenase inhibitors (SDHIs) are widely-used fungicides, to which humans are exposed and for which putative health risks are of concern. In order to identify human molecular targets for these agrochemicals, the interactions of 15 SDHIs with expression and activity of human cytochrome P-450 3A4 (CYP3A4), a major hepatic drug metabolizing enzyme, were investigated *in vitro*. 12/15 SDHIs, *i.e.*, bixafen, boscalid, fluopyram, flutolanil, fluxapyroxad, furametpyr, isofetamid, isopyrazam, penflufen, penhiopyrad, pydiflumetofen and sedaxane, were found to enhance CYP3A4 mRNA expression in human hepatic HepaRG cells and primary human hepatocytes exposed for 48 h to 10 μM SDHIs, whereas 3/15 SDHIs, *i.e.*, benzovindiflupyr, carboxin and thi-fluzamide, were without effect. The inducing effects were concentrations-dependent for boscalid (EC₅₀=22.5 μM), fluopyram (EC₅₀=4.8 μM) and flutolanil (EC₅₀=53.6 μM). They were fully prevented by SPA70, an antagonist of the Pregnane X Receptor, thus underlining the implication of this xenobiotic-sensing receptor. Increase in CYP3A4 mRNA in response to SDHIs paralleled enhanced CYP3A4 protein expression for most of SDHIs. With respect to CYP3A4 activity, it was directly inhibited by some SDHIs, including bixafen, fluopyram, fluxapyroxad, isofetamid, isopyrazam, penhiopyrad and sedaxane, which therefore appears as dual regulators of CYP3A4, being both inducer of its expression and inhibitor of its activity. The inducing effect nevertheless predominates for these SDHIs, except for isopyrazam and sedaxane, whereas boscalid and flutolanil were pure inducers of CYP3A4 expression and activity. Most of SDHIs appear therefore as *in vitro* inducers of CYP3A4 expression in cultured hepatic cells, when, however, used at concentrations rather higher than those expected in humans in response to environmental or dietary exposure to these agrochemicals.

1. Introduction

Succinate dehydrogenase (SDH) inhibitors (SDHIs) represent one of the largest families of agrochemical fungicides, with 24 substances listed according to the Fungicide Resistance Action Committee (FRAC) classification, based on mechanism of action (FRAC, 2023). These molecules are increasingly used in agriculture: ten substances are currently authorized at the European level in 2023 (ANSES, 2023; Umetsu and Shirai, 2020). SDHIs are consequently rather widely distributed in the environment and the occupational and general human populations are exposed to them via the respiratory, dermal and dietary routes (Béranger et al., 2020; Ottenbros et al., 2023; Polledri et al., 2019).

SDHIs block mitochondrial respiration in phytopathogenic fungi and human cells by inhibiting the complex II of the mitochondrial respiratory chain, also known as SDH (Bénil et al., 2019; D'hose et al., 2021; Sierotzki and Scalliet, 2013). SDHIs may additionally display SDH-independent effects, which however remain rather poorly identified, despite the fact that these SDH-unrelated effects may contribute to potential toxicity of SDHIs towards human health. In this context, it is noteworthy that some SDHIs such as sedaxane and fluopyram have been shown to activate the nuclear receptor pregnane X receptor (PXR) in rodents (Peffer et al., 2018; Rouquié et al., 2014), whereas the SDHI boscalid activated human PXR (Creusot et al., 2021). A key cellular target of PXR is the hepatic drug metabolizing cytochrome P-450 (CYP)

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3A4 (Willson and Klierer, 2002), induced by various drugs acting as PXR agonists (Zhou, 2008) and involved in the metabolism of 30% of medicines at least (Zhao et al., 2021). As potential activators of PXR, SDHIs may consequently increase CYP3A4 expression and activity in human hepatic cells. The present study was designed to investigate this hypothesis, through analyzing CYP3A4 regulation by 15 SDHIs in cultured human hepatic HepaRG cells and primary human hepatocytes (PHHs), which represent convenient and validated models for studying regulatory pathways of human drug detoxifying proteins (Andersson et al., 2012; Gómez-Lechón et al., 2004). Various SDHIs were found to interact with CYP3A4 expression and/or activity, most of them acting predominantly as CYP3A4 inducers.

2. Material and methods

2.1. Chemicals

SDHI fungicides (benzovindiflupyr, bixafen, boscalid, carboxin, fluopyram, flutolanil, fluxapyroxad, furametpyr, isofetamid, isopyrazam, penflufen, penthiopyrad, pydiflumetofen, sedaxane and thi-fluzamide) were provided by LGC group (Teddington, Middlesex, UK); their acronyms and chemical structures are listed in Figure S1. Rifampicin, omeprazole and the PXR antagonist SPA70 were provided by MedChemTronica (Sollentuna, Sweden). Stock solutions of SDHIs and other chemicals were prepared in dimethyl sulfoxide (DMSO).

$$\% \text{CYP3A4 mRNA expression increase} = \frac{\text{Fold induction}_{\text{SDHI}} - \text{Fold induction}_{\text{Vehicle control}}}{\text{Fold induction}_{\text{Rifampicin}} - \text{Fold induction}_{\text{Vehicle control}}} \times 100 \quad (2)$$

2.2. Cell culture and treatment

Human hepatoma HepaRG cells were cultured in Williams' Eagle medium supplemented with 10% (vol/vol) fetal calf serum (Cytiva, Velizy-Villacoublay, France), 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml bovine insulin (Sigma-Aldrich), 2 mM glutamine (Invitrogen), and 5×10^{-5} M hydrocortisone hemisuccinate (Upjohn, Paris La Défense, France), as previously described (Le Vee et al., 2013), without addition of 2% (vol/vol) DMSO, because this DMSO concentration induces CYP3A4 expression in HepaRG cells (Dubois-Pot-Schneider et al., 2022). PHHs, prepared from human liver fragments from 7 donors, were obtained from Biopredic International (Saint-Grégoire, France), Lonza (Basel, Switzerland), Xenotech (Kansas City, KS, USA) or BioIVT (West Sussex, United Kingdom) (See Table S1 for the list of these PHH batches). PHHs were cultured in Williams' E medium, supplemented with 5 µg/ml bovine insulin, 5×10^{-5} M hydrocortisone hemisuccinate, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine.

For cell treatment, 14 days-cultured HepaRG cells or 2 days-cultured PHHs were exposed for 48 h to 0.1% (vol/vol) DMSO, as chemical vehicle control, to 10 µM SDHIs or to reference CYP inducers (10 µM rifampicin for CYP3A4 and 50 µM omeprazole for CYP1A2). Viability of SDHIs-treated cells was analysed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, as previously described (Carmichael et al., 1987), and through phase-contrast light microscopic examination of the cells.

2.3. RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays

Total RNAs were extracted from cells using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and were quantified

using the NanoDrop 8000 Spectrophotometer™ (Thermo Fisher Scientific). RT-qPCR assays were next performed as previously described (Zerdoug et al., 2023), using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and the fluorescent dye Power SYBR™ Green PCR Master Mix from Applied Biosystems. The used gene primer sequences are listed in Table S2. Amplification curves were analysed with the CFX Maestro software (Bio-Rad, Hercules, CA, USA), using the comparative cycle threshold method. Relative quantification of target mRNA levels was calculated after normalising the total amount of cDNA tested against the internal reference (18 S rRNA), using the $2^{-\Delta\text{Ct}}$ method, giving thus the normalised expression of the target gene. Data were next commonly expressed as fold-change in comparison with 0.1% (vol/vol) DMSO-treated cell control, according to Eq. (1):

$$\text{Fold change} = \frac{\text{Normalised expression}_{\text{Treated sample}}}{\text{Normalised expression}_{\text{Vehicle control}}} \quad (1)$$

with $\text{Normalised expression}_{\text{Treated sample}} = \text{normalised expression of the target gene in cells exposed to SDHIs or to reference CYP inducers (rifampicin or omeprazole)}$ and $\text{Normalised expression}_{\text{Vehicle Control}} = \text{normalised expression of the target gene in control cells exposed to 0.1\% (vol/vol) DMSO}$.

The level of CYP3A4 mRNA expression increase in SDHI-treated HepaRG cells, relative to that found in the positive control, i.e., rifampicin-treated cells, was additionally calculated according to Eq. (2):

The half maximal effect concentration (EC_{50}) values of SDHIs towards CYP3A4 mRNA induction were determined from nonlinear regression of concentration-response data in HepaRG cells, based on the four-parameter logistic function. They were calculated using the Prism 8.3 software (GraphPad, San Diego, CA, USA), through the following Eq. (3):

$$A = \frac{100}{1 + 10^{(\text{Log}(\text{EC}_{50}) - [I]) \times \text{Hill slope}}} \quad (3)$$

with $A = \text{percentage of CYP3A4 mRNA expression in HepaRG cells for a given concentration of SDHI determined as described in Eq. (2)}$, $[I] = \text{the SDHI concentration in the medium}$, and $\text{Hill slope} = \text{a coefficient describing the steepness of the curve}$.

2.4. Western Blot analysis

Cell lysis and protein extraction were performed through exposing cells to RIPA buffer, supplemented with 2 mM dithiothreitol, 1 mM orthovanadate sodium, 1 mM phenylmethanesulfonyl fluoride, 1 mM NaF, and 1X complete Protease Inhibitor Cocktail™ (Roche, Basel, Switzerland), for 30 min. After cell lysate sonication, protein extracts were separated on polyacrylamide gel and electrophoretically transferred onto nitrocellulose membranes. After blocking with Tris-buffered saline containing 3% (vol/vol) bovine serum albumin for 60 min, membranes were incubated overnight at 4 °C with primary monoclonal antibodies against CYP3A4 (clone D9U6N, Cell Signaling Technology, Danvers, MA, USA) or glyceraldehyde phosphate dehydrogenase (GAPDH) (clone 0411, Santa Cruz Biotechnology, Dallas, TX, USA), used here as a loading control. After washing, membranes were next re-incubated with appropriate horseradish peroxidase-conjugated goat secondary antibodies (Agilent-Dako, Santa Clara, CA, USA) for 1 h. Immunolabeled proteins were finally visualized by chemiluminescence,

quantified by densitometry analysis using the Image Lab software (Bio-Rad) and normalized to GAPDH protein expression. CYP3A4 protein levels were finally expressed as fold-change comparatively to CYP3A4 protein expression in control cells.

2.5. CYP3A4 activity assay

CYP3A4 activity was measured by a luminescent method, using the Luciferin-IPA/ P450-Glo™ CYP3A4 assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. For measurement of potential direct inhibition of CYP3A4 by SDHIs, HepaRG cells, pre-treated for 48 h with 10 μM rifampicin in order to enhance their CYP3A4 activity, were exposed to 10 μM SDHIs only during the CYP3A4 assay period. Data were expressed as percentages of CYP3A4 activity found in control cells not exposed to SDHIs, arbitrarily set at 100% or as percentages of CYP3A4 activity inhibition according to the following Eq. (4):

$$\% \text{ CYP3A4 activity inhibition} = 100\% - \% \text{ CYP3A4 activity}_{\text{SDHI}} \quad (4)$$

with $\% \text{ CYP3A4 activity}_{\text{SDHI}}$ = percentage of CYP3A4 activity in the presence of SDHI.

The half maximal inhibitory concentration (IC₅₀) value of isopyrazam towards CYP3A4 activity was determined from nonlinear regression of concentration-response data based on the four-parameter logistic function. It was calculated using the Prism 8.3 software (GraphPad, San Diego, CA, USA), through the following Eq. (5):

$$A = \frac{100}{1 + 10^{(|I| - \text{Log}(\text{IC}_{50})) \times \text{Hill slope}}} \quad (5)$$

with A = percentage of CYP3A4 activity for a given concentration of isopyrazam, [I] = the isopyrazam concentration in the medium, and Hill slope = a coefficient describing the steepness of the curve.

2.6. Molecular descriptor generation

5270 molecular descriptors organized in 30 logical blocks were determined for SDHIs using the Dragon 7.0 software (Kode Cheminformatics, Pisa, Italy) (See https://chm.kode-solutions.net/products_dragon_descriptors.php for a complete list of these descriptors and blocks). SDHIs, initially expressed in SMILES format using the PubChem website (<https://pubchem.ncbi.nlm.nih.gov/>), were converted to 3D format using the MarvinView software (ChemAxon, Budapest, Hungary) before processing by the Dragon 7.0 software to obtain molecular descriptors.

2.7. Prediction of *in vivo* induction of CYP3A4 expression by SDHIs

In vivo predictions of hepatic CYP3A4 expression regulation by SDHIs were performed according to the correlation approach (Fahmi and Ripp, 2010). The ratio maximum unbound plasma concentration of SDHIs ($I_{\text{max,u}}$) versus their EC₅₀ towards CYP3A4 mRNA expression was calculated and considered for the prediction: ratio ≤ 0.001, 0.001 < ratio ≤ 0.01 and ratio > 0.01 would predict an SDHI to be a weak, moderate and strong CYP3A4 inductor, respectively (Fahmi and Ripp, 2010).

As data on the human pharmacokinetics of SDHIs are not currently available, $I_{\text{max,u}}$ values for SDHIs were evaluated using acceptable daily intake (ADI) values for SDHIs provided by the European Food Safety Authority (EFSA, <https://www.efsa.europa.eu/fr>), as described previously (Bénil et al., 2019; D'hose et al., 2021; Spycher et al., 2018). Briefly, ADIs permitted to calculate the maximal theoretical doses of SDHIs ingested at once by a 70 kg-weighting subject for 24 h. These ingested doses were considered to be fully absorbed and distributed into the standard volume (5 L) of blood for the subjects, allowing thus to determine the maximal total blood concentrations of SDHI (I_{max}), as the ratio "ingested dose/blood volume". the $I_{\text{max,u}}$ values, after taking into account the unbound plasma fractions of SDHIs, predicted *in silico* using the online tool Admetlab2.0 (<https://admetmesh.scbdd.com/>) (Xiong et al., 2021).

2.8. Statistical analysis

Data were usually expressed as means ± SD from at least three independent experiments, each being performed in triplicate or quadruplicate. They were statistically analyzed through analysis of variance (ANOVA) followed by Dunnett's post-hoc test. The criterion of significance for statistical tests was $p < 0.05$. Correlations between molecular descriptor indexes and CYP3A4 mRNA induction factors by SDHIs or percentages of direct CYP3A4 activity inhibition by SDHIs were done through Spearman rank correlation. The criterion of significance for statistical tests was $p < 0.05$. Statistical analyses were performed using the Prism 10.2.1 software (GraphPad Software, Boston, MA, USA).

3. Results

3.1. Induction of CYP3A4 mRNA expression by SDHIs

HepaRG cells were exposed for 48 h to 10 μM SDHIs, a concentration that has been previously used for characterizing *in vitro* effect of SDHIs (Bénil et al., 2019) and that appeared to not trigger cytotoxicity, as observed by light microscopic analysis of the cells (data not shown) and MTT viability assay (Fig. S2). As indicated in Fig. 1A, various SDHIs, *i.e.*, bixafen, boscalid, fluopyram, flutolanil, fluxapyroxad, furametpyr, isofetamid, isopyrazam, penthiopyrad and sedaxane, as well as the reference inducer rifampicin, were found to significantly enhance mRNA expression of CYP3A4 in HepaRG cells, with induction factors ranging for SDHIs from 5.1 (for flutolanil) to 13.9 (for isopyrazam). Penflufen and pydiflumetofen also enhanced CYP3A4 mRNA levels in HepaRG cells (by a 3.3- and 3.9-fold factor, respectively), thus reaching the threshold of a 2-fold induction factor for being inducer (Kenny et al., 2018), even if the significance level was not reached (Fig. 1A). Only benzovindiflupyr, carboxin and thifluzamide did not induce CYP3A4 mRNA expression in HepaRG cells (Fig. 1A). In contrast to CYP3A4 mRNA levels, those of CYP1A2 mRNAs were not or very poorly induced by SDHIs in HepaRG cells, with the notable exception of carboxin, which induced CYP1A2 mRNA factor by a 106.7-fold factor (Fig. S3).

CYP3A4 mRNA induction by SDHIs was next studied in PHHs, whose viability was not obviously impaired by a 48-h treatment by SDHIs (Fig. S2). CYP3A4 mRNA data for each of the seven hepatocyte populations considered in the study are reported in Fig. 1B. It is noteworthy that the CYP3A4 induction factors in response to the prototypical inducer rifampicin ranged from 2.6 to 228.0 according to the donor (Fig. 1B), thus illustrating the marked inter-donor variability in CYP3A4 mRNA regulation, as already highlighted (Bulutoglu et al., 2020; Kenny et al., 2018). The regulation of CYP3A4 mRNA expression by most of SDHIs also varied according to donors; for example, the induction factors for CYP3A4 mRNA expression in response to isopyrazam ranged from 1.4 to 373.0 (Fig. 1B). Therefore, to interpret data with PHHs, we applied a criteria previously proposed for classifying a compound as an *in vitro* inducer of CYP3A4 in PHHs (Kenny et al., 2018), *i.e.*, a 2.0-fold induction as a threshold, observed in more than 33.3% of the donors. The SDHIs inducing CYP3A4 mRNA levels by at least a 2-fold factor in at least 3/7 donors can therefore be considered as CYP3A4 inducers. This was the case for bixafen, boscalid, fluopyram, flutolanil, fluxapyroxad, furametpyr, isofetamid, isopyrazam, penflufen, penthiopyrad, pydiflumetofen and sedaxane (Fig. 1B), which also increased CYP3A4 mRNA expression by more than 2-fold in HepaRG cells (Fig. 1A). Benzovindiflupyr, carboxin and thifluzamide, which did not enhance CYP3A4 expression in HepaRG cells (Fig. 1A), also failed to act as CYP3A4 inducers in PHHs (Fig. 1B).

As shown in Fig. 1C, boscalid, fluopyram and flutolanil, corresponding to prototypical SDHIs among the most frequently detected ones in the environment (Manjarres-López et al., 2021; Oh et al., 2007; Polat and Tiryaki, 2023) were shown to induce CYP3A4 mRNA expression in HepaRG cells in a concentration-dependent manner, with EC₅₀ values of 22.5 μM, 4.8 μM and 53.6 μM, respectively.

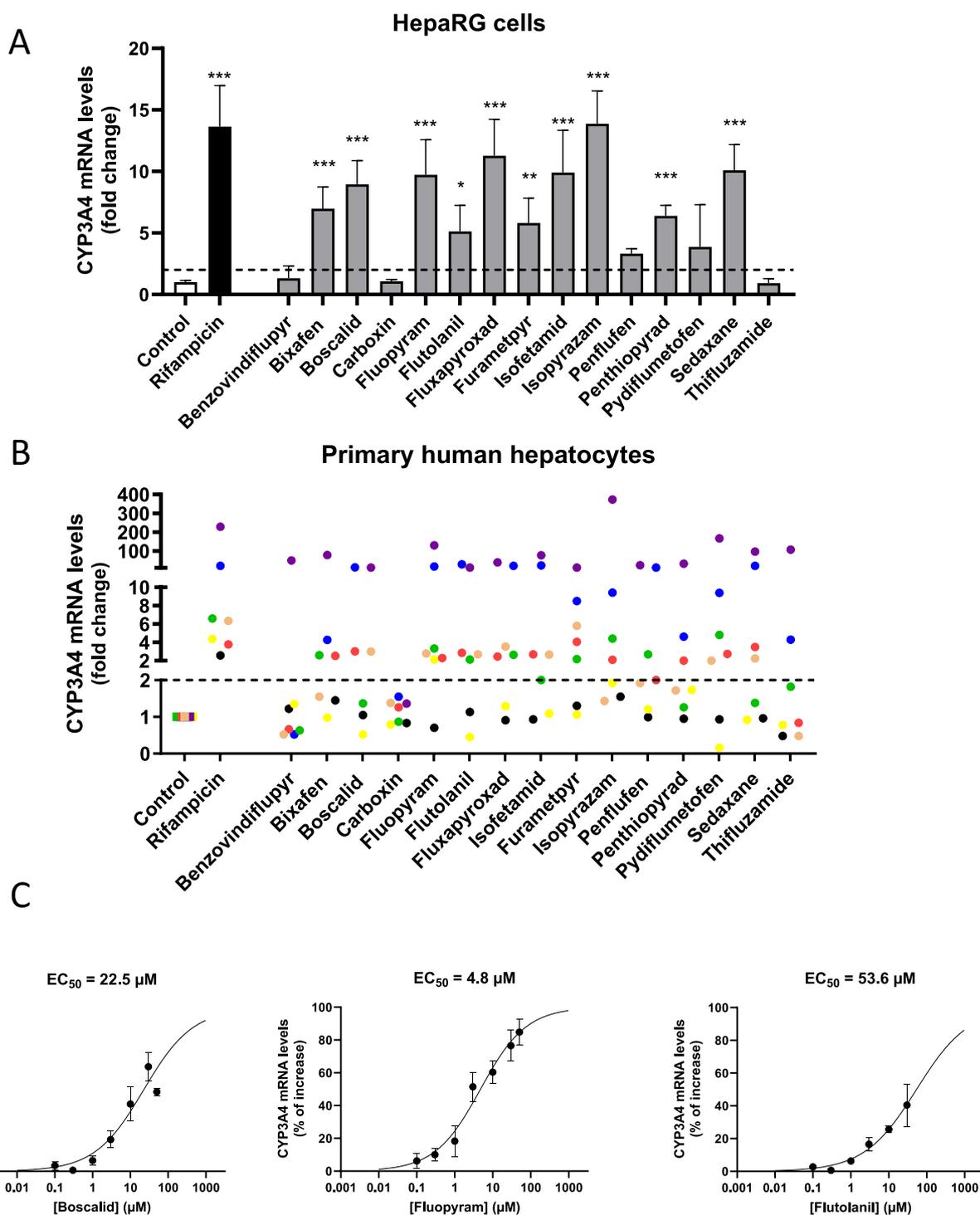


Fig. 1. Effects of SDHIs on CYP3A4 mRNA expression. (A) Human hepatic HepaRG cells or (B) primary human hepatocytes from seven donors were either untreated (control) or treated by 10 μM SDHI or 10 μM rifampicin for 48 h. CYP3A4 mRNA expression was next determined by RT-qPCR. Data are expressed as fold change of CYP3A4 mRNA levels comparatively to control. They are the means ± SD of at least three independent assays for HepaRG cells (A) or correspond to the seven human hepatocyte populations, represented each by a specific colored dot (B). *, p<0.05, **, p<0.01 and ***, p<0.001 when compared to control. The dashed lines represent the threshold for a 2-fold induction. (C) HepaRG cells were exposed to various concentrations of boscalid, fluopyram or flutolanil for 48 h. CYP3A4 mRNA expression was next determined by RT-qPCR. Data are expressed as percentages of CYP3A4 mRNA increase and are the means ± SD of at least three independent assays. Rifampicin (10 μM) was used to determine the 100% induction. The EC₅₀ values are indicated on the top of the graphs.

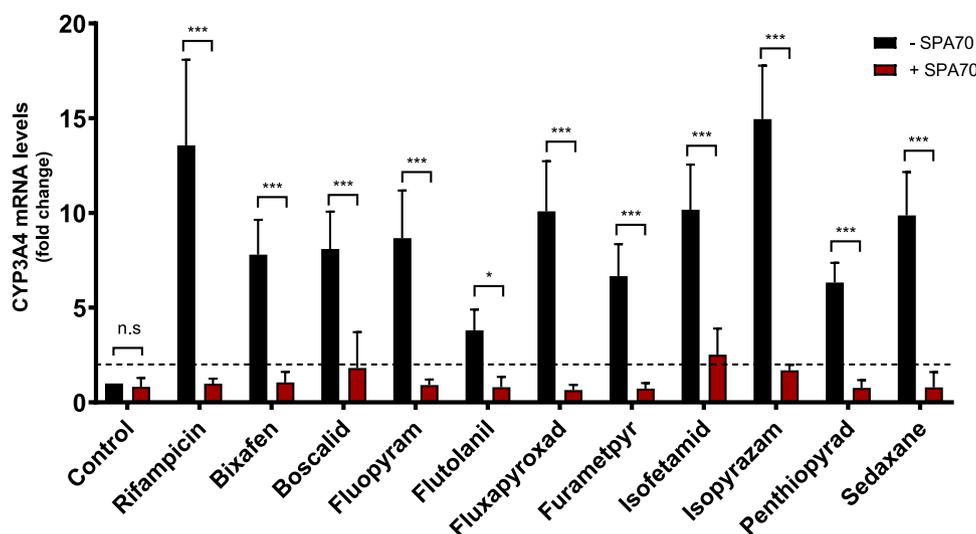


Fig. 2. Effects of the PXR antagonist SPA70 on SDHIs-mediated CYP3A4 mRNA induction. HepaRG cells were either untreated (control) or treated by 10 μ M SDHIs or 10 μ M rifampicin for 48 h, in the absence or presence of 5 μ M SPA70. CYP3A4 mRNA expression was next determined by RT-qPCR. Data are expressed as fold change of CYP3A4 mRNA levels comparatively to control. They are the means \pm SD of at least three independent assays. *, $p < 0.05$, ***, $p < 0.001$ and n.s., not statistically significant.

Table 1

List of SDHI molecular descriptors highly correlated with CYP3A4 mRNA induction ($|r_s| > 0.7$ and $p < 0.005$).

| Molecular descriptor | | Correlation with CYP3A4 induction (fold change) | |
|-----------------------------|-------------------------------------------------------------------------------------------------------------|-------------------------------------------------|------------------------|
| Block | Name | Spearman coefficient (r_s) | Significance (p value) |
| GETAWAY descriptors | R maximal autocorrelation of lag 4 / weighted by polarizability (R4p+) | -0.8357 | 0.0002 |
| 3D matrix-based descriptors | logarithmic coefficient sum of the last eigenvector from reciprocal squared geometrical matrix (VE3sign_RG) | -0.8179 | 0.0004 |
| RDF descriptors | Radial Distribution Function - 050 / weighted by I-state (RDF050s) | 0.8107 | 0.0004 |
| 3D matrix-based descriptors | average coefficient of the last eigenvector from reciprocal squared geometrical matrix (VE2sign_RG) | -0.7878 | 0.0008 |
| GETAWAY descriptors | R maximal autocorrelation of lag 5 / weighted by polarizability (R5p+) | -0.7655 | 0.0013 |
| GETAWAY descriptors | R maximal autocorrelation of lag 4 / weighted by van der Waals volume (R4v+) | -0.7599 | 0.0015 |
| 2D matrix-based descriptors | Hosoya-like index (log function) from Barysz matrix weighted by atomic number (Ho_Dz(Z)) | 0.7357 | 0.0025 |
| 2D matrix-based descriptors | Hosoya-like index (log function) from Barysz matrix weighted by mass (Ho_Dz(m)) | 0.7357 | 0.0025 |
| P_VSA-like descriptors | P_VSA-like on Molar Refractivity. bin 3 (P_VSA_MR_3) | -0.7025 | 0.0043 |

Next, HepaRG cells were co-exposed to SDHI and SPA70. This compound is a potent and specific antagonist of PXR, known to abolish PXR-related induction of CYP3A4 at both mRNA and protein level (Lin et al., 2017). SPA70 consequently fully prevented rifampicin-mediated induction of CYP3A4 mRNA levels in HepaRG cells (Fig. 2). In the

same way, SPA70 hindered CYP3A4 up-regulation caused by the SDHIs bixafen, boscalid, fluopyram, flutolanil, fluxapyroxad, furametpyr, isofetamid, isopyrazam, penthiopyrad and sedaxane (Fig. 2).

Finally, among 5270 molecular descriptors, 9 were found to be rather highly correlated with CYP3A4 mRNA induction ($|r_s| > 0.7$ and $p < 0.005$). These descriptors belong to the blocks “GETAWAY descriptors” (n=3), “2-D matrix descriptors” (n=2), “3-D matrix descriptors” (n=2), “RDF descriptors” (n=1) and P_VSA-like descriptors (n=1) and were either positively or negatively correlated to CYP3A4 mRNA levels (Table 1). Other descriptors were found to be more weakly correlated ($|r_s| < 0.7$ and $p < 0.05$) (n=153) or were not correlated ($p \geq 0.05$) for most of them (n=5108), including “constitutional indices”, such as molecular weight, and “molecular properties”, such as hydrophobicity (LogP) (data not shown).

3.2. Regulation of CYP3A4 protein expression and activity by SDHIs

The SDHIs bixafen, boscalid, fluopyram, flutolanil, fluxapyroxad, furametpyr, isofetamid, isopyrazam, penthiopyrad and sedaxane, and the reference inducer rifampicin, which significantly induced CYP3A4 mRNA levels in HepaRG cells (Fig. 1A), concomitantly enhanced CYP3A4 protein level (Fig. 3A). The fold-induction ranged from 3.6 (for furametpyr, for which significance was not reached, as well as for sedaxane), to 10.1 (for flutolanil) (Fig. 3A).

This repressing effect of isopyrazam towards CYP3A4 activity is rather surprising because this SDHI obviously induced CYP3A4 protein by a 7.4-fold factor, whereas furametpyr and sedaxane are weaker inducer of CYP3A4 protein (Fig. 3A). We hypothesized that isopyrazam may in fact act as a dual regulator of CYP3A4, through behaving as an inducer of its expression and as a direct inhibitor of its activity, as already described for some drugs like ritonavir and meclizine (Dixit et al., 2007; Foo et al., 2015; Wei et al., 2016). We therefore directly measured CYP3A4 activity in HepaRG cells, in the absence or presence of SDHIs, without prior exposure to SDHIs (Fig. 4). Isopyrazam was found to markedly and directly inhibit CYP3A4 activity (by 94.5%), with an IC_{50} value of 3.3 μ M (Fig. S4). Other SDHIs such as isofetamid, penthiopyrad, pydiflumetofen and sedaxane also potentially decreased CYP3A4 activity (inhibition in the 60.5%-85.2% range). Bixafen, fluopyram and fluxapyroxad inhibited CYP3A4 activity in a weaker manner (inhibition by less than 50%), whereas boscalid, flutolanil and furametpyr did not impair CYP3A4 activity (Fig. 4). Other SDHIs such as

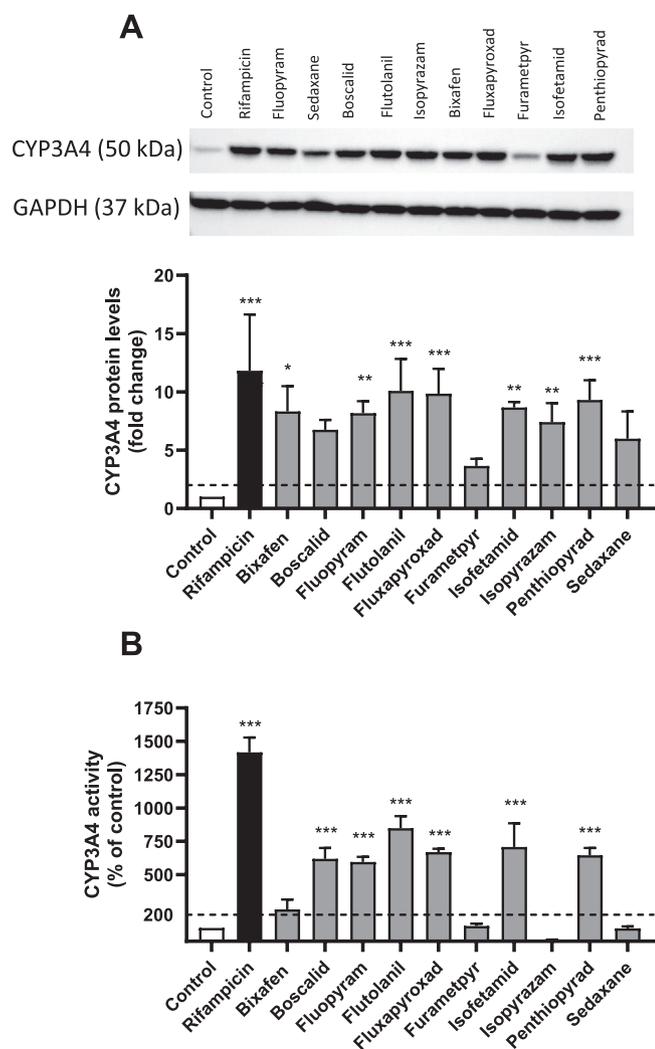


Fig. 3. Effects of a 48-h exposure to SDHIs on CYP3A4 protein expression and activity. Human hepatic HepaRG cells were either untreated (control) or treated by 10 μ M SDHIs or 10 μ M rifampicin for 48 h. (A) CYP3A4 and GAPDH protein expression were determined by Western-blotting and quantified by densitometric analysis. Data shown are a representative blot (upper panel) or as fold change of CYP3A4 protein expression comparatively to control (lower panel). Quantitative data are the means + SD from three independent assays; the dashed line represents the threshold for a 2-fold induction (lower panel). (B) CYP3A4 activity was measured by a luminescent method. Data are expressed as percentages of CYP3A4 activity found in control cells, set at 100%, and are the means \pm SD of three independent assays. The dashed line represents the threshold for a 200% CYP3A4 activity (corresponding to a 2-fold induction). (A, B) *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$ when compared to control.

carboxin, penflufen and thifluzamid also failed to inhibit CYP3A4 activity, whereas benzovindiflupyr weakly reduced it (by 38.2%).

Among 5270 molecular descriptors for SDHIs, 13 were found to be very highly correlated with direct CYP3A4 inhibition activity by the fungicides ($|r_S| > 0.8$ and $p < 0.0005$) (Table S3). These descriptors belong to the blocks “2-D matrix descriptors” ($n=8$), “Information indices” ($n=3$), “Atom-centered fragments” ($n=1$) and “GETAWAY descriptors” ($n=1$) and were positively correlated to percentages of CYP3A4 activity inhibition. Other descriptors were more weakly correlated, with one group of descriptors remaining however rather highly correlated to CYP3A4 activity inhibition ($0.0005 \leq p < 0.005$) ($n=89$) and another group of descriptors being more poorly correlated ($0.005 \leq p < 0.05$) ($n=583$) (data not shown). Most of molecular descriptors were not correlated ($p > 0.05$) ($n=4586$), including “constitutional indices” such as molecular weight, “molecular properties”, such as

topological polar surface area or hydrophobicity (LogP) and “functional group counts”, such as numbers of acceptors or donors for H-bonds (data not shown).

3.3. Prediction of *in vivo* CYP3A4 induction by SDHIs

Prediction of *in vivo* induction of hepatic CYP3A4 in humans exposed to boscalid, fluopyram or flutolanil, which are reference SDHIs commonly present in the environment or the diet (Louca Christodoulou et al., 2018; Spycher et al., 2018) and enhancing *in vitro* CYP3A4 expression and activity (Fig. 3), was performed as previously described (Fahmi and Ripp, 2010; Tastet et al., 2023), using EC_{50} values of these SDHIs towards CYP3A4 mRNA expression and their $I_{max, u}$ calculated from their ADI. As indicated in Table 2, boscalid, fluopyram or flutolanil were predicted to be only weak CYP3A4 inducers in humans.

4. Discussion

The data reported in the present study demonstrate that various SDHIs can interact with human CYP3A4 expression and/or activity, thus adding CYP3A4 as a new potential human off-target of these agrochemicals. The recent demonstration that the SDHI isoflucypram, not analyzed in the present study, as well as boscalid, induced CYP3A4 activity in PHHs (Tinwell and Bars, 2022; Wiemann et al., 2023) fully supports this conclusion. The exact effects towards CYP3A4 observed in human hepatic HepaRG cells depend however of the nature of the SDHIs (See Table 3 for a summary of these effects). Boscalid and flutolanil induced CYP3A4 expression (at mRNA and protein level) and activity, without directly interfering with CYP3A4 activity. Bixafen, fluopyram, fluxapyroxad, isofetamid and penthiopyrad induced CYP3A4 mRNA and protein expression, but concomitantly directly inhibited its activity, thus acting as dual regulators; the resulting effect after 48 h treatment is however an overall induction of CYP3A4 activity, thus suggesting that the inducing effect is likely predominant for these SDHIs, perhaps in relation with a rapid reversibility of the CYP3A4 inhibitory effect, whereas the CYP3A4 protein induction may be a long-lasting action. By contrast, for isopyrazam and sedaxane, which are also dual regulators of CYP3A4, the inhibitory effect towards CYP3A4 most likely predominated, as demonstrated by the lack of significant CYP3A4 activity induction (and even a down-regulation for isopyrazam) after 48 h exposure to these SDHIs.

Furametpyr also did not induce CYP3A4 activity, without however directly inhibiting it; this may be due the relative low and non-significant induction of CYP3A4 protein by this SDHI (Fig. 3A). In the same way, penflufen slightly enhanced CYP3A4 mRNA expression in HepaRG cells, without interfering with the activity. By contrast, pydiflumetofen, which also very moderately increased CYP3A4 mRNA levels in HepaRG cells, markedly inhibited CYP3A4 activity. Benzovindiflupyr, carboxin and thifluzamid, which failed to impair CYP3A4 mRNA expression in HepaRG cells at the tested concentration (10 μ M), did not, or only moderately, reduced CYP3A4 activity. In addition, it is noteworthy that there are some discordances between the levels of CYP3A4 mRNA and protein induction by some SDHIs, especially fluopyram and furametpyr. Thus, both fluopyram and furametpyr induced CYP3A4 mRNA expression in HepaRG cells (Fig. 1A), whereas only fluopyram increased CYP3A4 protein (Fig. 3A). Such discordances have also been reported for drugs; for example, omeprazole and efavirenz similarly induced CYP3A4 mRNA levels in primary human hepatocytes, but only efavirenz concomitantly enhanced CYP3A4 protein level (Järvinen et al., 2023). The reasons for such discordances are unclear, but they may reflect differences in time-courses of CYP3A4 mRNA and protein induction according to inducers. Finally, positive or negative feedback CYP3A4 expression and/or activity regulations may have to be considered for SDHIs, which likely deserve further studies.

The molecular mechanism by which SDHIs can induce human CYP3A4 expression likely involves PXR because the PXR antagonist

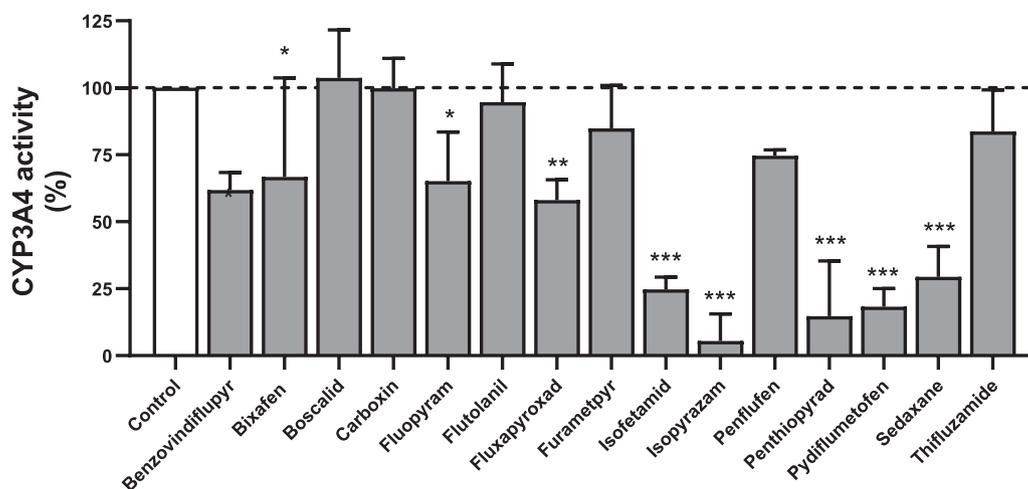


Fig. 4. Direct effect of SDHIs on CYP3A4 activity. CYP3A4 activity was measured using a luminescent method in the absence (control) or presence of 10 μM SDHIs in HepaRG cells pre-treated with 10 μM rifampicin. Data are expressed as percentages of CYP3A4 activity found in control cells, set at 100%, and are the means \pm SD of three independent assays. The dashed line represents 100% CYP3A4 activity. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$ when compared to control.

Table 2

Prediction of *in vivo* CYP3A4 mRNA induction by SDHIs^a.

| SDHI | Concentration data used | F_u ^c | $I_{\text{max}, u}$ ^d | EC_{50} (μM) | Ratio $I_{\text{max}, u}/\text{EC}_{50}$ | Predicted <i>in vivo</i> induction | |
|-------------------|--------------------------------------------------------------------------------------------|--------------------|----------------------------------|------------------------------------|------------------------------------------|------------------------------------|----------------|
| Boscalid | DJA Approximated I_{max}^b (μM) | 1.63 | 0.008 | 0.013 | 22.5 | 0.0006 | Weak induction |
| Fluopyram | | 0.42 | 0.010 | 0.004 | 4.8 | 0.0008 | Weak induction |
| Flutolanil | | 3.90 | 0.012 | 0.047 | 56.3 | 0.0008 | Weak induction |

^a *In vivo* induction of CYP3A4 mRNA expression was predicted according to the ratio $I_{\text{max}, u}/\text{EC}_{50}$ (Fahmi and Ripp, 2010): ratio ≤ 0.001 , 0.001–0.01 would predict an SDHI to be a weak, moderate and strong CYP3A4 inducer, respectively.

^b defined as the maximum plasma concentration.

^c defined as the unbound plasma fraction and predicted *in silico* using ADMETlab 2.0.

^d defined as the maximum unbound plasma concentration.

Table 3

Summary of 10 μM SDHI effects towards CYP3A4 expression and activity in HepaRG cells.

| SDHI | Effect towards human CYP3A4 |
|------------------|----------------------------------------------------------------------------|
| Benzovindiflupyr | Inhibitor |
| Bixafen | Dual regulator (inducer and inhibitor): the inducing effect predominates |
| Boscalid | Inducer |
| Carboxin | No effect |
| Fluopyram | Dual regulator (inducer and inhibitor): the inducing effect predominates |
| Flutolanil | Inducer |
| Fluxapyroxad | Dual regulator (inducer and inhibitor): the inducing effect predominates |
| Furametpyr | Weak inducer |
| Isofetamid | Dual regulator (inducer and inhibitor): the inducing effect predominates |
| Isopyrazam | Dual regulator (inducer and inhibitor): the inhibitory effect predominates |
| Penflufen | No effect |
| Penthiopyrad | Dual regulator (inducer and inhibitor): the inducing effect predominates |
| Pydiflumetofen | Inhibitor |
| Sedaxane | Dual regulator (inducer and inhibitor): the inhibitory effect predominates |
| Thifluzamide | No effect |

SPA70 fully inhibited SDHI-mediated CYP3A4 mRNA level increase. This indicates that these SDHIs enhancing CYP3A4 expression are most likely PXR agonists. The fact that some SDHIs such as sedaxane, fluopyram and boscalid have been previously shown to activate PXR mainly in rodents (Creusot et al., 2021; Peffer et al., 2018; Rouquié et al., 2014)

fully agrees with this conclusion. In this context, it is noteworthy that the diversity of molecules that activate PXR is vast (Bower et al., 2020): PXR activators can be large or small, long or compact, flexible or rigid, aromatic or aliphatic, and acidic, basic, or neutral. This diversity makes it difficult to develop pharmacophores that effectively distinguish PXR activators from nonactivators (Bower et al., 2020) and may explain why only few molecular descriptors ($n=9/5270$) were identified as rather highly correlated with PXR-based CYP3A4 mRNA induction in the present study. Because PXR plays important physiopathological roles beyond drug detoxification processes, notably in inflammation, energy homeostasis, carbohydrate and lipid metabolism and cancer progression (Hukkanen and Hakkola, 2020; Oladimeji and Chen, 2018), its activation by various SDHIs may contribute to potential toxic effects of these agrochemicals, that remain however to be characterized. In contrast to PXR, the aryl hydrocarbon receptor (AhR), implicated in xenobiotic-mediated CYP1A2 up-regulation in hepatic cells, is probably rather poorly activated by most of SDHIs, thus ruling out any unspecific and general activation of drug-sensing receptors by these fungicides. Indeed, among the SDHIs tested in the present study, only carboxin was able to markedly enhance CYP1A2 mRNA levels in HepaRG cells. This SDHI can therefore be considered as a putative AhR agonist, without however concomitantly activating PXR owing to the lack of effect of this SDHI towards CYP3A4 mRNA levels.

Induction of CYP3A4 expression in PHHs in response to SDHIs appears to be variable according to the donors. Applying the criteria previously defined as at least a 2-fold induction of CYP3A4 mRNA expression in more than 33.3% of the donors for being classified as a CYP3A4 inducer (Kenny et al., 2018) nevertheless allows us to similarly discriminate SDHIs as CYP3A4 inducers and non-inducers in both PHHs and HepaRG cells, thus underlining the interest of HepaRG cells as surrogates for PHHs for studying hepatic effects of drugs and pollutants,

as previously reported (Anthérieu et al., 2012; Guillouzo et al., 2007). The cause for the variability of SDHI-related CYP3A4 inducibility among donors remains to be clarified. It would reflect an inherent feature of the PHH model, as a marked inter-donors variation in the level of CYP3A4 induction by the reference CYP3A4 inducer rifampicin was also observed, in agreement with previous studies (Bulutoglu et al., 2020; Kenny et al., 2018; Roymans et al., 2004). Such a variability of CYP3A4 inducibility in PHHs has been claimed to be not obviously dependent of the age of the donor, the level of PXR expression or the cryopreservation status of the hepatocytes (Bulutoglu et al., 2020).

Besides regulating CYP3A4 expression, some SDHIs exerted direct inhibition of CYP3A4 activity, which was even the predominant effect for isopyrazam. Such inhibitory effects towards CYP3A4 have also been reported for other pesticides (Hodgson and Rose, 2008), which can also target activities of additional CYPs, such as CYP1A2, CYP2B6, CYP2C8 and CYP2D6 (Abass et al., 2009). The mechanism by which SDHIs inhibit CYP3A4 activity remains to be determined. Whether SDHIs may act as reversible direct or irreversible time-dependent inhibitors of CYP3A4 activity is therefore likely to deserve further studies. In the same way, it is worthy of interest to investigate if CYP3A4 may metabolize SDHIs, which likely needs additional studies. Interestingly, a limited number of SDHI molecular descriptors ($n=13$) was found to be very highly correlated to CYP3A4 activity inhibition ($p<0.0005$), but these molecular descriptors correspond to rather complex descriptors such as 2D matrix-based descriptors and are not easily interpretable. By contrast, more basic and intelligible descriptors such as molecular weight, lipophilicity, topological surface area, number of H-bond acceptor or number of H-bond donor, previously reported as potentially contributing to discriminate CYP3A4 inhibitors and non-inhibitors (Buchwald and Yamashita, 2014; Gonzalez et al., 2021), did not correlate with direct CYP3A4 activity inhibition among SDHIs, which may be due to the relative small number of SDHIs ($n=15$) analysed in the present study.

The possible *in vivo* relevance of the *in vitro* findings reported in the present study is a key-point to consider, notably because *in vivo* CYP3A4 induction by SDHIs acting as “perpetrators” can lead to enhanced CYP3A4-mediated metabolism of “victims” drugs, thus leading to potential drug-pollutant interactions in humans (Alvares, 1978). In this context, the SDHIs boscalid, fluopyram or flutolanil, which markedly induced *in vitro* CYP3A4 expression and activity, were predicted to act *in vivo* as weak inducers. For inhibition of CYP3A4 activity by isopyrazam, *in vivo* prediction from *in vitro* data was not performed because it requires to know the mechanism of the CYP3A4 inhibition (Vieira et al., 2014), which was not determined in the present study. Nevertheless, isopyrazam $I_{max, u}$ calculated from its ADI (30 $\mu\text{g}/\text{kg}/\text{day}$) is 48 nM and thus more weaker than its IC_{50} value towards CYP3A4 activity (3.3 μM), suggesting that *in vivo* hepatic CYP3A4 inhibition in response to environmental or dietary exposure to isopyrazam is unlikely. Taken together, these predictions would not support major *in vivo* interactions of SDHIs with CYP3A4, either as inducers or inhibitors. This conclusion may however be challenged by the fact that human pharmacokinetics data about SDHIs are currently unknown, for both environmental or occupational exposures; although SDHI $I_{max, u}$ calculated from ADI values are rather low, specific accumulation of SDHIs in the liver cannot be excluded, which may allow to reach hepatic SDHI concentrations active on CYP3A4 expression and activity. The fact that the liver may be a preferential organ target for SDHIs (Ren et al., 2021) agrees with this hypothesis. Moreover, humans are frequently exposed to mixtures of pollutants, including potentially SDHIs, and the inducing or inhibitory effects of these pollutants towards CYP3A4 may be additive or synergistic. The enhanced toxicity of the combined exposure to boscalid and the fungicide pyraclostrobin towards the mitochondrial function of human hepatocytes (Carbone et al., 2023) supports this special attention to SDHI/pollutant mixtures. Finally, the fact that SDHIs would interfere with other detoxifying proteins apart from CYP3A4, such as drug transporters (Kerhoas et al., submitted) or other drug metabolizing enzymes has also to be taken into account for judging the overall possible

effects of these agrochemicals towards the hepatic drug detoxification system, and beyond, the pharmacokinetics features of drugs or environmental chemicals for humans concomitantly exposed to SDHIs.

5. Conclusions

The current work demonstrates that various SDHIs can interact *in vitro* with human hepatic CYP3A4, thus identifying this drug metabolizing enzyme as a human off-target of these fungicides, initially postulated as not or only poorly interacting with human targets. SDHIs can act as inducers of CYP3A4 expression and/or as CYP3A4 inhibitors of CYP3A4 activity, the inducing effects being the predominant ones for most of SDHIs, except for isopyrazam. CYP3A4 induction by SDHIs was fully prevented by the PXR inhibitor SPA70, thus demonstrating the contribution of this xenobiotic-sensing receptor to the SDHI effects. SDHIs can therefore be added to the growing list of pesticides that interfere with human drug metabolizing enzymes, even if the concentrations of SDHIs considered to occur *in vivo* in response to environmental or dietary exposure seems to be too low to act on CYP3A4 expression and/or activity in a notable way, at least when SDHIs are used as single agents. A better characterisation of the SDHI exposure and pharmacokinetics in humans is however likely required in the future, in order to more accurately evaluate the potential consequences of the interactions of SDHIs with CYP3A4 for the human health.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:[10.1016/j.ecoenv.2024.116261](https://doi.org/10.1016/j.ecoenv.2024.116261).

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