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# Combined in silico and in vitro approaches to identify P-glycoprotein-inhibiting pesticides

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## Abstract

The P-glycoprotein (P-gp) efflux pump plays a major role in xenobiotic detoxification. The inhibition of its activity by environmental contaminants remains however rather little characterised. The present study was designed to develop a combination of different approaches to identify P-gp inhibitors among a large number of pesticides using in silico and in vitro models. First, the prediction performance of four web tools was evaluated alone or in combination using a set of recently marketed drugs. The best combination of web tools—AdmetSAR2.0/PgpRules/pkCSM—was next used to predict P-gp activity inhibition by 762 pesticides. Among the 187 pesticides predicted to be P-gp inhibitors, 11 were tested in vitro for their ability to inhibit the efflux of reference substrates (rhodamine 123 and Hoechst 33342) in P-gp overexpressing MCF7R cells and to inhibit the efflux of the reference substrate rhodamine 123 in the Caco-2 cell monolayer. In MCF7R cell assays, ivermectin B1a, emamectin B1 benzoate, spinosad, dimethomorph and tralkoxydim inhibited P-gp activity; ivermectin B1a, emamectin B1 benzoate and spinosad were determined to be stronger inhibitors (half-maximal inhibitory concentration [IC<sub>50</sub>] of 3 ± 1, 5 ± 1 and 7 ± 1 μM, respectively) than dimethomorph and tralkoxydim (IC<sub>50</sub> of 102 ± 7 and 88 ± 7 μM, respectively). Ivermectin B1a, emamectin B1 benzoate, spinosad and dimethomorph also inhibited P-gp activity in Caco-2 cell monolayer assays, with dimethomorph being a weaker P-gp inhibitor. These combined approaches could be used to identify P-gp inhibitors among food contaminants, but need to be optimised and adapted for high-throughput screening.

## KEYWORDS

in silico, in vitro, inhibitor, pesticide, P-glycoprotein

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## 1 | INTRODUCTION

Pesticides include a wide variety of chemicals and are used in many applications such as agriculture and residential settings.<sup>[1]</sup> Thus, the general public is very frequently exposed to pesticides and notably to their residues, present in food.<sup>[2]</sup> However, a link has been established between exposure to pesticides and the incidence of various human diseases.<sup>[1,3]</sup> One of the contributing factors influencing pesticide toxicity is their interactions with plasma membrane transporters.<sup>[4]</sup> Indeed, inhibiting the activities of these transporters could lead to adverse effects not only by impairing the pharmacokinetics of drugs but also by blocking the transport of endogenous substrates.<sup>[5,6]</sup> One of the most important plasma membrane transporters is the ATP-binding cassette (ABC) efflux pump P-glycoprotein (P-gp) (ABCB1/MDR1), which limits the absorption of its substrates at the intestinal level, supports their biliary and renal elimination and protects sensitive tissues.<sup>[7]</sup> Several studies have described the modulation of P-gp activity by pesticides using *in vitro* assays. Indeed, compounds from various chemical pesticide classes (chlordecone, parathion, methylparathion, ivermectin, selamectine, moxidectine and emamectine) have been reported to be P-gp inhibitors.<sup>[8–14]</sup> On the other hand, pesticides lindane, dieldrin, dimethoate, methomyl, aminocarb, chlorpropham and acetamiprid have been described as not inhibiting P-gp.<sup>[15–19]</sup> Finally, endosulfan and diazinon remain ambiguous because studies have reported them either as a P-gp inhibitor or not depending on the *in vitro* assay used.<sup>[13,15,16,20,21]</sup> The diversity of the approaches used, sometimes in association with methodologies of little relevance, could partially explain the discrepancies observed between the results of these various studies. Moreover, many pesticides currently in the market are untested as to their interactions with membrane transporters, as regulatory studies do not include such tests. Therefore, current knowledge about potential P-gp inhibition by pesticides is limited and calls for extensive characterisation.<sup>[4]</sup>

The present study evaluated which pesticides are P-gp inhibitors by combining different approaches. Due to the large number of these chemicals and to prioritise them for their later evaluation *in vitro*, we first used an *in silico* method to predict their potential inhibition of P-gp activity. The selected pesticides were then tested using different *in vitro* models to identify potential P-gp inhibitors. Accumulation and retention assays were performed using the P-gp-overexpressing MCF7R cell subline. The apparent permeability ( $P_{app}$ ) assay was carried out with the Caco-2 cell monolayer, a relevant intestinal model whose functional and morphological characteristics are similar to those of enterocytes.

## 2 | MATERIALS AND METHODS

### 2.1 | Selection of an *in silico* web tool to predict P-gp-inhibiting pesticides

We selected the drugs approved by the Food and Drug Administration (FDA) over the 2010–2020 period from the FDA website

([www.accessdata.fda.gov/scripts/cder/daf/index.cfm](http://www.accessdata.fda.gov/scripts/cder/daf/index.cfm)) and assessed *in vitro* for their P-gp activity inhibition. Data about this P-gp inhibition, including half-maximal inhibitory concentration ( $IC_{50}$ ) values, were collected from drug reviews freely available on the FDA website cited above. In the present study, each drug was classified as a P-gp inhibitor if its  $IC_{50}$  was lower than 100  $\mu$ M, and as a P-gp noninhibitor if its  $IC_{50}$  was higher than 100  $\mu$ M or if no P-gp activity inhibition was observed. Thus, 64 drugs were classified as P-gp inhibitors and 31 as P-gp noninhibitors (Table S1).

Using this set of 95 drugs, we evaluated the prediction performance for P-gp activity inhibition of four web tools: AdmetSAR 2.0 (<http://lmmd.ecust.edu.cn/admet2/>),<sup>[22]</sup> PgpRules (<https://pgprules.cmdm.tw/>),<sup>[23]</sup> pkCSM (<http://biosig.unimelb.edu.au/pkcsml/>)<sup>[24]</sup> and vNN-ADMET (<https://vnnadmet.bhsai.org/vnnadmet/>).<sup>[25]</sup> To predict P-gp activity inhibition, AdmetSAR 2.0, pkCSM and vNN-ADMET require the simplified molecular input line entry specification (SMILES), while PgpRules requires the molecular design limited (MDL) Molfile. Therefore, canonical SMILES of the 95 drugs were collected from the PubChem website (<https://pubchem.ncbi.nlm.nih.gov/>) and then converted into MDL Molfiles using an online converter (<http://www.cheminfo.org/Chemistry/Cheminformatics/FormatConverter/index.html>).

For each individual or combination of web tool(s), the area under the receiver operating characteristics curve (ROC AUC) was determined to be the first prediction performance parameter using GraphPad Prism software (version 5.0; GraphPad Software Inc.) as previously described.<sup>[26]</sup> The Matthews correlation coefficient (MCC) was then calculated as a second prediction performance parameter. The MCC measures the correlation between predictions and real values.<sup>[26]</sup> Thereafter, the prediction of P-gp activity inhibition by 762 pesticides belonging to various chemical classes was evaluated using the most efficient individual web tool or combination of tools.

### 2.2 | Chemicals

Cell culture products (culture medium, nonessential amino acids, penicillin, streptomycin and foetal calf serum [FCS]), Hank's balanced salt solution with  $Ca^{2+}$  and  $Mg^{2+}$  (HBSS) and Hoechst 33342 (H33342) were purchased from Gibco (Invitrogen). 4-Morpholineethanesulphonic acid monohydrate (MES), 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid (HEPES),  $NaHCO_3$ , cyflumetofen, elacridar, rhodamine 123 (Rh123) and verapamil were provided by Merck. Dimethomorph, emamectin B1 benzoate, ivermectin B1a, profoxydim lithium salt (profoxydim), silafluofen, spinosad and tralkoxydim were purchased from Santa Cruz Biotechnology. Oxathiapiprolin and pinoxaden were obtained from HPC Standards GmbH. Fenpicoxamid was provided by LGC standard. Solutions of elacridar, Rh123, verapamil, dimethomorph, emamectin B1 benzoate, ivermectin B1a and tralkoxydim were prepared in dimethyl sulphoxide (DMSO). Solutions of cyflumetofen, profoxydim, silafluofen, spinosad and pinoxaden were prepared in methanol (MeOH). Solutions of oxathiapiprolin and fenpicoxamid were prepared in acetonitrile (ACN).

## 2.3 | Cell culture

Parental human mammary MCF7 cells were used to generate the P-gp-overexpressing MCF7R cell subline by stepwise selection with doxorubicin as previously described.<sup>[27]</sup> MCF7 and MCF7R cells were provided by Dr J. Robert (Bordeaux University). The cells were used at passages 24–40 and grown in a culture medium (Dulbecco's modified Eagle's medium containing 25 mM D-glucose, 4 mM L-glutamine and 1 mM pyruvate), supplemented with 1% nonessential amino acids, 20 IU/mL penicillin, 20 µg/mL streptomycin and 10% FCS, at 37°C in an atmosphere containing 5% CO<sub>2</sub>. For accumulation and retention assays, MCF7 and MCF7R cells were seeded, respectively, at  $2.25 \times 10^4$  and  $1.5 \times 10^4$  cells/cm<sup>2</sup> in 96-well plates. The culture medium was changed three times a week, and cells were used on Day 7 postseeding.

Caco-2 cells were obtained from the American Type Culture Collection (HTB-37) and used in passages 32–48. Cells were grown in a culture medium (Dulbecco's modified Eagle's medium containing 25 mM D-glucose and 4 mM L-glutamine), supplemented with 1% nonessential amino acids, 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% FCS, at 37°C in an atmosphere containing 5% CO<sub>2</sub>. For  $P_{app}$  assays, Caco-2 cells were seeded at  $2.7 \times 10^5$  cells/cm<sup>2</sup> on polyester membrane inserts (0.4 µm pore size, 12 mm diameter) purchased from Corning. The culture medium was changed three times a week, and cells were used on Days 22–24 postseeding.

## 2.4 | Accumulation and retention assays

P-gp activity was evaluated by measuring the cellular accumulation or retention of a reference substrate in the presence or absence of either a pesticide or a reference inhibitor used at a noncytotoxic concentration (Table S2). For accumulation assay, cells are incubated with the reference substrate and the tested pesticides or the reference inhibitor. During the period of accumulation, the substrate enters the cells but is also concomitantly effluxed by P-gp, so inhibition of P-gp by the tested pesticides or the reference inhibitor increases the substrate accumulation. The substrate accumulation is determined at the end of this period. For retention assay, cells are incubated only with the reference substrate (loading period); thereafter, cells are incubated in a substrate-free medium with the tested pesticides or the reference inhibitor (efflux period). During this efflux period, substrate is exclusively effluxed by P-gp (decrease of intracellular concentration of the reference substrate), so inhibition of P-gp by the tested pesticides or the reference inhibitor blocks this efflux and limits the decrease of the intracellular concentration of the reference substrate. The intracellular concentration of the reference substrate is determined at the end of the efflux period. Assays were performed in a transport buffer (NaCl 136 mM, KCl 5.3 mM, KH<sub>2</sub>PO<sub>4</sub> 1.1 mM, MgSO<sub>4</sub> 0.8 mM, CaCl<sub>2</sub> 1.8 mM, HEPES 10 mM, D-glucose 11 mM, pH 7.4). Rh123 and H33342 were used as reference substrates for the accumulation and retention assays, respectively.

Elacridar and verapamil were used as reference inhibitors for the accumulation and retention assays, respectively. Assays were performed as previously described with the following modifications.<sup>[28]</sup> The cell lysate was only used for measuring fluorescence. The retention assay using H33342 was used to measure P-gp activity (rather than breast cancer resistance protein (BCRP/ABCG2) activity). The experiments were performed on both MCF7 and MCF7R cells. The final concentration of solvent (DMSO, MeOH or ACN) for all the solutions was set at 0.3% (vol/vol). Vehicle controls containing DMSO 0.3%, MeOH 0.3% or ACN 0.3% were included for each experiment. The fluorescence of Rh123 (ex: 485 nm; em: 520 nm) and H33342 (ex: 355 nm; em: 460 nm) was measured with a microplate-reading spectrofluorometer (FLUOstar OPTIMA; BMG Labtech). For each independent experiment (biological replicate), the median of three technical replicates was calculated, and then this median value was expressed as a percentage of that of the vehicle control. Three independent experiments were performed.

When the accumulation or retention of the reference substrate was greater than 150% (compared with the vehicle control), the pesticide was considered to be a P-gp inhibitor. Therefore, experiments complementary to the accumulation and retention assays were performed as described above, using various concentrations of the pesticides considered to be P-gp inhibitors to determine IC<sub>50</sub> (Table S3). For each independent experiment (biological replicate), the three technical replicates were expressed as a percentage of the median of three technical replicates of the vehicle control. The IC<sub>50</sub> was determined from a nonlinear regression based on a dose–response stimulation equation (log(agonist) vs. response [three parameters]) using GraphPad Prism software through the following equation:

$$Y = 100 + (Y_{\max} - 100)/(1 + 10^{\log((IC_{50} - X)})}$$

with Y the percentage of reference substrate in the cells (relative to the vehicle control) for a given concentration of pesticide, 100 the percentage of reference substrate in the cells for the vehicle control (arbitrarily set at 100%) and  $Y_{\max}$  the maximal percentage of reference substrate in the cells (relative to the vehicle control) whatever the concentration of pesticide. Three independent experiments were performed.

## 2.5 | Apparent permeability assay

P-gp activity was evaluated by measuring the  $P_{app}$  of a reference substrate (Rh123) in the presence or absence of either a pesticide or a reference inhibitor (verapamil) used at a noncytotoxic concentration. We used two buffers for the  $P_{app}$  assay: an apical transport buffer (ATB) (HBSS, MES 10 mM, NaHCO<sub>3</sub> 4.2 mM, pH 6.5) and a basolateral transport buffer (BTB) (HBSS, HEPES 25 mM, NaHCO<sub>3</sub> 4.2 mM, pH 7.4). The  $P_{app}$  assay was performed bidirectionally from apical (A; donor) to basolateral (B; acceptor) compartments, and from

B (donor) to A (acceptor) compartments to determine  $P_{app\ A-B}$  and  $P_{app\ B-A}$ , respectively. Solutions of dimethomorph 500  $\mu\text{M}$ , emamectin B1 benzoate 20  $\mu\text{M}$ , ivermectin B1a 25  $\mu\text{M}$ , spinosad 50  $\mu\text{M}$ , tralkoxydim 200  $\mu\text{M}$  and verapamil 500  $\mu\text{M}$  were used. The final concentration of solvent (DMSO or MeOH) for all the solutions was set at 1% (vol/vol) (which was previously shown not to alter the integrity of the Caco-2 cell monolayers). Vehicle controls containing DMSO 1%, or MeOH 1% were included for each experiment. Solutions of vehicle control, pesticides and verapamil were prepared both in the ATB and BTB and loaded in the A (500  $\mu\text{L}$ ) and B (1000  $\mu\text{L}$ ) compartments, respectively. The Caco-2 cell monolayers were then equilibrated for 30 min at 37°C. Next, the content of the donor compartment was replaced by Rh123 5.25  $\mu\text{M}$  prepared in the adequate buffer (ATB for the A compartment, BTB for the B compartment) in the presence or absence of either a pesticide or verapamil. The Caco-2 cell monolayers were incubated at 37°C for 2 h. The total volume of the acceptor compartment was then harvested into glass vials and stored at -20°C until analysis. The fluorescence of Rh123 was measured as described above. The  $P_{app}$  value was expressed as  $\text{cm s}^{-1}$  and calculated according to the following equation:

$$P_{app} = (dC \times V)/(C_0 \times dt \times S),$$

with  $dC$  ( $\mu\text{M}$ ) the final concentration of Rh123 in the acceptor compartment,  $V$  ( $\text{cm}^3$ ) the volume of the acceptor compartment,  $C_0$  ( $\mu\text{M}$ ) the initial concentration of Rh123 in the donor compartment,  $dt$  (s) the time of incubation and  $S$  ( $\text{cm}^2$ ) is the surface area of the insert. The efflux ratio (ER) of Rh123 was then calculated according to the following equation:

$$\text{ER} = P_{app\ B-A}/P_{app\ A-B}.$$

The integrity of the Caco-2 cell monolayer was monitored by measuring its transepithelial electrical resistance (TEER) at 37°C both before and after the  $P_{app}$  assay with a Millicell Electrical Resistance System supplied by Merck. For this, ATB was loaded in the A (700  $\mu\text{L}$ ) and B (1500  $\mu\text{L}$ ) compartments. The Caco-2 cell monolayer was then equilibrated for 30 min at 37°C. Thereafter, the TEER was measured twice per insert, and a cell-free insert was included. The TEER value was expressed as  $\Omega\text{ cm}^2$  and calculated according to the following equation:

$$\text{TEER} = (R_M - R_0) \times S,$$

with  $R_M$  ( $\Omega$ ) the resistance mean value of the Caco-2 cell monolayer on insert,  $R_0$  ( $\Omega$ ) the resistance mean value of the cell-free insert and  $S$  ( $\text{cm}^2$ ) the surface area of the insert. Only Caco-2 cell monolayers with a TEER > 250  $\Omega\text{ cm}^2$  before and after the  $P_{app}$  assay were used because this threshold value indicates adequate integrity of the Caco-2 cell monolayer.<sup>[29,30]</sup> Three independent experiments were performed with one technical replicate for each independent experiment.

## 2.6 | Statistical analysis

Statistical analyses were performed using GraphPad Prism software. For accumulation and retention assays with only one applied concentration, data were analysed using the one-sample t test, with '100' as the theoretical mean. Means were considered significantly different from 100 at  $p < 0.05$ . For  $\text{IC}_{50}$ ,  $P_{app}$  and ER values, an analysis of variance was performed. When the experimental condition effect was significant ( $p < 0.05$ ), the values were compared with each other using Bonferroni's test. Differences were considered significant at  $p < 0.05$ . The values presented are mean  $\pm$  SEM.

## 3 | RESULTS

### 3.1 | Selection of an in silico web tool and application to predict P-gp-inhibiting pesticides

We used 64 drugs classified as P-gp inhibitors and 31 drugs classified as P-gp noninhibitors according to FDA drug reports to evaluate the prediction performance of four web tools as to P-gp activity inhibition by determining the ROC AUC and the MCC as parameters. We obtained ROC AUC values of the web tools individually and in combination (Table 1). For individual web tools, values ranged from 0.555 (for vNN-ADMET) to 0.737 (for pkCSM). When the web tools were combined, values ranged from 0.622 (for AdmetSAR 2.0/vNN-ADMET) to 0.791 (for AdmetSAR 2.0/PgpRules/pkCSM). The combination of AdmetSAR 2.0/PgpRules/pkCSM had the best ROC AUC value, and different cut-offs can be applied: a drug can be considered a P-gp inhibitor if one, two, or three of the web tools predicted it to be a P-gp inhibitor. The term cut-off 1, 2 or 3 used hereinafter refers to this classification by one, two or three web tools. Therefore, MCC values of the AdmetSAR 2.0/PgpRules/pkCSM combination were determined for the different cut-offs (Table 2). The MCC value was equivalent between cut-offs 1 and 2, and higher than that obtained with cut-off 3.

Based on the ROC AUC and MCC values, we used the AdmetSAR 2.0/PgpRules/pkCSM combination of web tools with cut-offs of 1 and 2 to evaluate the prediction of P-gp activity inhibition by 762 pesticides. The results indicated that 421 and 187 pesticides were predicted to be P-gp inhibitors with cut-offs 1 or 2, respectively. Therefore, we selected the results obtained with cut-off 2 to limit the number of pesticides to evaluate for the in vitro assays (Table S4). Among these 187 pesticides, we selected nine (cyflumetofen, dimethomorph, fenpicoxamid, ivermectin B1a, oxathiapiprolin, pinoxaden, profoxydim, silafluofen, spinosad) based on their representativeness of the different chemical families, their solvent solubility, their market availability and the availability of P-gp-related data in the literature. We also selected two more pesticides (emamectin B1 benzoate and tralkoxydim) as they inhibited P-gp activity (own laboratory data) yet had been predicted as noninhibitors with the AdmetSAR 2.0/PgpRules/pkCSM combination.

**TABLE 1** ROC AUC values of web tools considered alone or in combination for predicting P-gp inhibiting FDA-approved drugs.

Web tools alone or in combination	ROC AUC
AdmetSAR 2.0	0.625
PgpRules	0.729
pkCSM	0.737
vNN-ADMET	0.555
AdmetSAR 2.0/PgpRules	0.742
AdmetSAR 2.0/pkCSM	0.755
AdmetSAR 2.0/vNN-ADMET	0.622
PgpRules/pkCSM	0.777
PgpRules/vNN-ADMET	0.695
pkCSM/vNN-ADMET	0.712
AdmetSAR 2.0/PgpRules/pkCSM	0.791
AdmetSAR 2.0/PgpRules/vNN-ADMET	0.709
AdmetSAR 2.0/pkCSM/vNN-ADMET	0.725
PgpRules/pkCSM/vNN-ADMET	0.762
AdmetSAR 2.0/PgpRules/pkCSM/vNN-ADMET	0.765

Abbreviations: FDA, Food and Drug Administration; P-gp, P-glycoprotein; ROC AUC, area under the receiver operating characteristics curve.

**TABLE 2** MCC values of the AdmetSAR 2.0/PgpRules/pkCSM combination depend on the cut-off retained for predicting P-gp inhibiting FDA-approved drugs.

Cut-off	MCC
1	0.47
2	0.46
3	0.36

Abbreviations: FDA, Food and Drug Administration; MCC, Matthews correlation coefficient; P-gp, P-glycoprotein.

### 3.2 | P-gp activity inhibition by pesticides based on accumulation and retention assays

There were no morphological changes in the cells after exposure to pesticides (image not shown). Results on MCF7 cells indicated no variation in the intracellular concentration of Rh123 or H33342 in the presence of either the tested pesticides or the reference inhibitor compared with the vehicle control (data not shown). This indicated that there was little or no P-gp activity in MCF7 cells. However, in MCF7R cells, with the reference inhibitor elacridar, we observed a significant increase in Rh123 intracellular concentration ( $270 \pm 2\%$  compared with the vehicle control) (Figure 1A). With a concentration greater than 150%, this result confirmed P-gp activity in MCF7R cells and elacridar's ability to inhibit it. Five of the 11 tested pesticides significantly increased Rh123 intracellular concentration:

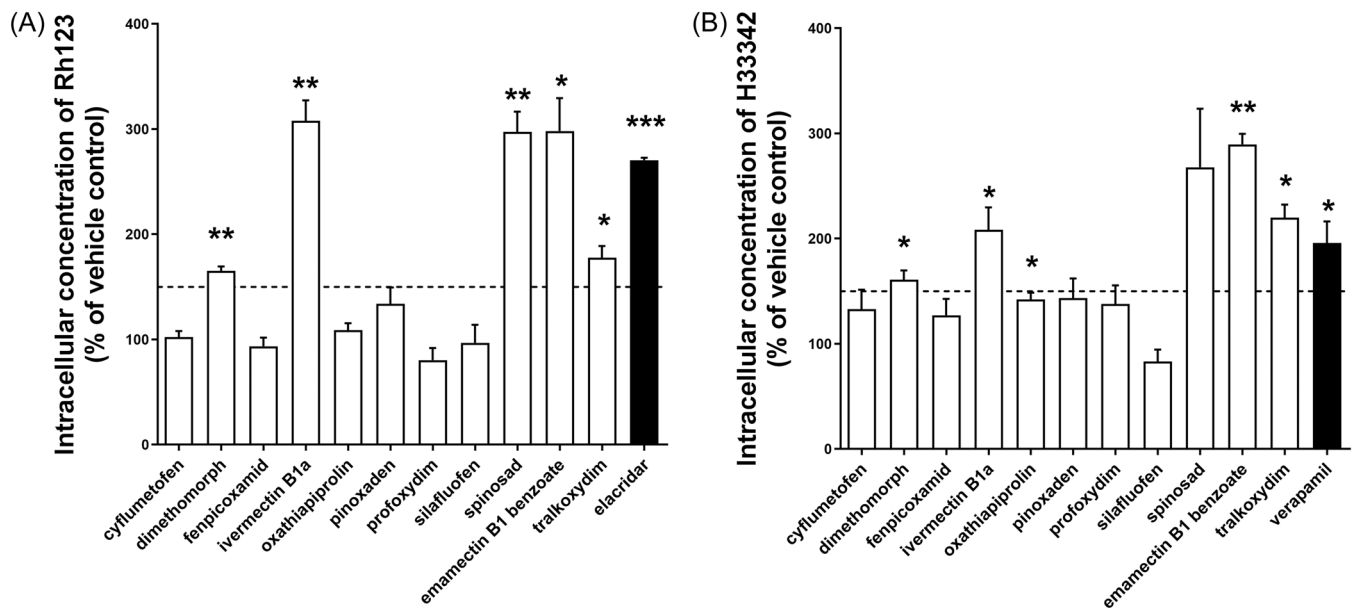
dimethomorph ( $165 \pm 4\%$ ), ivermectin B1a ( $308 \pm 19\%$ ), spinosad ( $297 \pm 19\%$ ), emamectin B1 benzoate ( $298 \pm 31\%$ ) and tralkoxydim ( $178 \pm 11\%$ ).

Reference inhibitor verapamil significantly increased H33342 intracellular concentration ( $196 \pm 20\%$  compared with the vehicle control) in MCF7R cells, thus confirming its ability to inhibit P-gp activity (Figure 1B). For the five pesticides identified above, we also observed an increase in H33342 intracellular concentration beyond 150%: dimethomorph ( $161 \pm 9\%$ ), ivermectin B1a ( $208 \pm 21\%$ ), spinosad ( $268 \pm 56\%$ ), emamectin B1 benzoate ( $290 \pm 10\%$ ) and tralkoxydim ( $220 \pm 13\%$ ). While the difference was significant for dimethomorph, ivermectin B1a, emamectin B1 benzoate and tralkoxydim, only a tendency ( $p < 0.1$ ) was observed for spinosad. In light of the results obtained with the accumulation and retention assays, these five pesticides could be considered as P-gp inhibitors, and their  $IC_{50}$  values were consequently evaluated by complementary experiments.

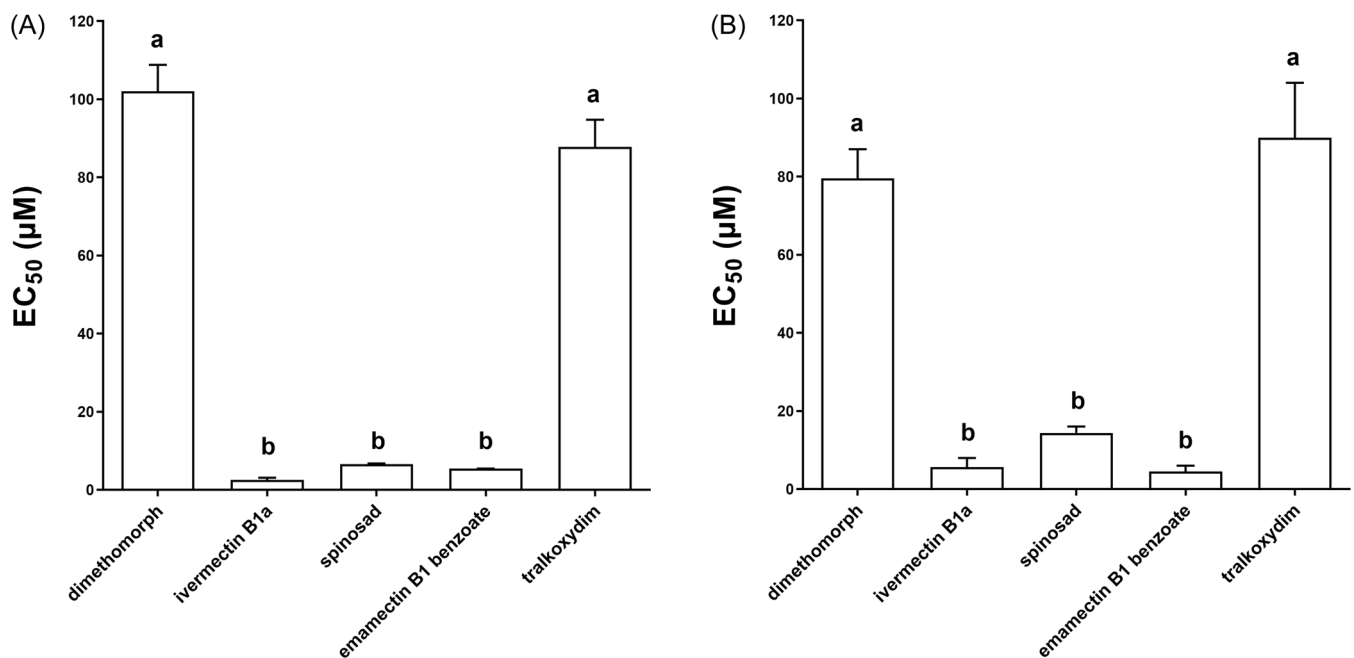
$IC_{50}$  values were determined for the five pesticides being considered based on the Rh123 accumulation assay (Figure 2A).  $IC_{50}$  values were similar for dimethomorph and tralkoxydim ( $102 \pm 7$  and  $88 \pm 7 \mu\text{M}$ , respectively), but were significantly lower for ivermectin B1a ( $3 \pm 1 \mu\text{M}$ ), spinosad ( $7 \pm 1 \mu\text{M}$ ) and emamectin B1 benzoate ( $5 \pm 1 \mu\text{M}$ ). Similar observations were made with the H33342 retention assay (Figure 2B).  $IC_{50}$  values for dimethomorph and tralkoxydim were similar ( $80 \pm 7$  and  $90 \pm 14 \mu\text{M}$ , respectively) and significantly higher than those for ivermectin B1a ( $6 \pm 2 \mu\text{M}$ ), spinosad ( $14 \pm 2 \mu\text{M}$ ) and emamectin B1 benzoate ( $5 \pm 1 \mu\text{M}$ ).

### 3.3 | P-gp activity inhibition by pesticides based on the apparent permeability assay

The pesticides affected the  $P_{app}$  of Rh123 to different degrees (Table 3). The  $P_{app\ A-B}$  of Rh123 was similar for all the tested compounds (ranging from  $7.8 \pm 0.2 \times 10^{-7}$  to  $9.5 \pm 0.4 \times 10^{-7} \text{ cm s}^{-1}$ ). In contrast, the effects on  $P_{app\ B-A}$  of Rh123 were stronger. With verapamil, the  $P_{app\ B-A}$  of Rh123 was significantly lower ( $21.7 \pm 0.5 \times 10^{-7} \text{ cm s}^{-1}$ ;  $-66\%$ ) than the vehicle controls (DMSO and MeOH,  $63.0 \pm 1.2 \times 10^{-7}$  and  $65.8 \pm 3.9 \times 10^{-7} \text{ cm s}^{-1}$ , respectively). Similar significant decreases occurred with spinosad ( $20.8 \pm 1.5 \times 10^{-7} \text{ cm s}^{-1}$ ;  $-68\%$ ), ivermectin B1a ( $19.9 \pm 0.6 \times 10^{-7} \text{ cm s}^{-1}$ ;  $-68\%$ ) and emamectin B1 benzoate ( $19.4 \pm 1.7 \times 10^{-7} \text{ cm s}^{-1}$ ;  $-69\%$ ). The decrease with dimethomorph was smaller ( $33.2 \pm 1.4 \times 10^{-7} \text{ cm s}^{-1}$ ;  $-47\%$ ) but still significant, while no difference was observed with tralkoxydim. The compounds tested affected the ER (Figure 3). In comparison with MeOH ( $8.3 \pm 0.5$ ) and DMSO ( $8.0 \pm 0.3$ ), there was a significant decrease ( $-70\%$ ) with verapamil ( $2.4 \pm 0.1$ ), thus confirming verapamil's ability to inhibit P-gp activity. We also observed a significant decrease in ER with spinosad ( $2.4 \pm 0.1$ ;  $-71\%$ ), dimethomorph ( $3.9 \pm 0.3$ ;  $-51\%$ ), ivermectin B1a ( $2.5 \pm 0.1$ ;  $-68\%$ ) and emamectin B1 benzoate ( $2.4 \pm 0.1$ ;  $-71\%$ ). No modification in the ER was reported with tralkoxydim.



**FIGURE 1** Intracellular concentration of rhodamine 123 (A) and Hoechst 33342 (B) in MCF7R cells in the presence of either pesticide (white bars) or a reference inhibitor (black bars). The pesticides were tested at 100  $\mu\text{M}$  (for cyflumetofen, dimethomorph, fenpicoxamid, oxathiapiprolin, pinoxaden, profoxydim, silafiuofen and tralkoxydim), or 10  $\mu\text{M}$  (for ivermectin B1a, spinosad and emamectin B1 benzoate), while the reference inhibitors elacridar and verapamil were tested at 10 and 100  $\mu\text{M}$ , respectively. Values are shown as mean  $\pm$  SEM and expressed as percentages of the vehicle control. Dotted line: 150%. When the concentration of rhodamine 123 or Hoechst 33342 was higher than 150%, the tested compound was considered to be a P-glycoprotein inhibitor. Three independent experiments were performed. \*, \*\*, \*\*\*: significantly different from the vehicle control ( $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ , respectively).



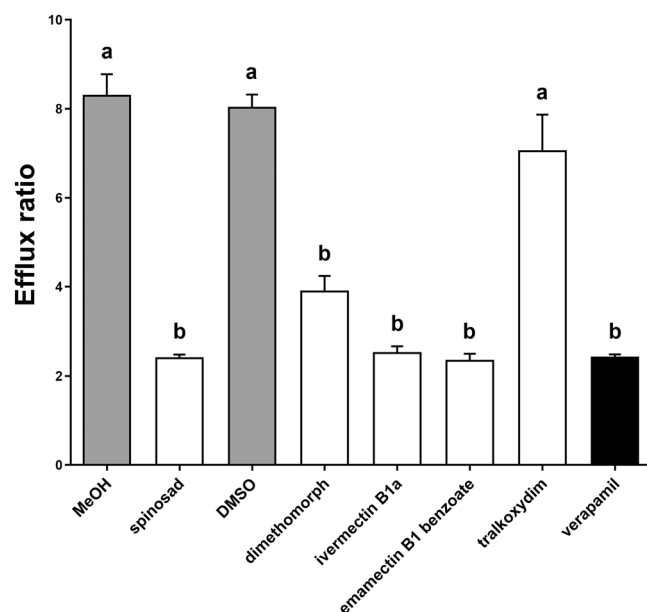
**FIGURE 2** IC<sub>50</sub> of pesticides determined from the intracellular concentration of rhodamine 123 (A) and Hoechst 33342 (B) in MCF7R cells. From various concentrations of pesticides, intracellular concentrations of rhodamine 123 and Hoechst 33342 were expressed as percentages of the vehicle control, and the IC<sub>50</sub> was determined from a nonlinear regression based on a dose-response stimulation equation. Values are shown as means  $\pm$  SEM. Three independent experiments were performed. <sup>a</sup>, <sup>b</sup>: bars without a common letter differ ( $p < 0.001$ ). EC<sub>50</sub>, half-maximal effective concentration; IC<sub>50</sub>, half-maximal inhibitory concentration.

**TABLE 3**  $P_{app}$  of Rh123 in Caco-2 cell monolayers in the presence of pesticides.

Tested compound	$P_{app}$ A-B	$P_{app}$ B-A
MeOH (vehicle control)	7.9 ± 0.4 <sup>ab</sup>	65.8 ± 3.9 <sup>a</sup>
Spinosad	8.6 ± 0.6 <sup>ab</sup>	20.8 ± 1.5 <sup>c</sup>
DMSO (vehicle control)	7.9 ± 0.2 <sup>a</sup>	63.0 ± 1.2 <sup>a</sup>
Dimethomorph	8.6 ± 0.5 <sup>ab</sup>	33.2 ± 1.4 <sup>b</sup>
Ivermectin B1a	7.8 ± 0.2 <sup>ab</sup>	19.9 ± 0.6 <sup>c</sup>
Emamectin B1 benzoate	8.2 ± 0.2 <sup>ab</sup>	19.4 ± 1.7 <sup>c</sup>
Tralkoxydim	9.5 ± 0.4 <sup>ab</sup>	66.5 ± 5.6 <sup>a</sup>
Verapamil	9.0 ± 0.2 <sup>b</sup>	21.7 ± 0.5 <sup>c</sup>

Note:  $P_{app}$  was determined from apical to basolateral compartments ( $P_{app}$  A-B) and from basolateral to apical compartments ( $P_{app}$  B-A). Verapamil was used as a reference inhibitor. Three independent experiments were performed. Values are shown as means ± SEM and expressed in  $10^{-7}$  cm s<sup>-1</sup>. <sup>a, b, c</sup>: within a column, means without a common letter differ ( $p < 0.05$ ).

Abbreviations: DMSO, dimethylsulfoxide; MeOH, methanol;  $P_{app}$ , apparent permeability; Rh123, rhodamine 123.



**FIGURE 3** Efflux ratio of rhodamine 123 in Caco-2 cell monolayers in the presence of pesticides (white bars), a vehicle control (grey bars) or verapamil used as a reference inhibitor (black bar). Values are shown as means ± SEM. Three independent experiments were performed. <sup>a, b</sup>: bars without a common letter differ ( $p < 0.001$ ).

## 4 | DISCUSSION

Using a combination of three web tools, our results indicated that out of 762 pesticides, 187 were predicted to be P-gp inhibitors. Among the 11 pesticides tested in vitro, between four and five (depending on the assay used) were determined to be P-gp inhibitors.

An evaluation of the web tools' prediction performance revealed that the best combination was AdmetSAR 2.0/PgpRules/pkCSM; this combination had ROC AUC and MCC values of, respectively, 0.791, 0.47 or 0.46 (depending on the cut-off applied). These results indicated a correct prediction, as in the literature an ROC AUC value between 0.7 and 0.8 is considered acceptable, and an MCC value of 0.5 corresponded to 75% of cases being correctly predicted.<sup>[31,32]</sup> Indeed, the correct positive and negative predictions represented 78% and 77% of the 95 drugs with either cut-off 1 or 2, respectively. Nevertheless, a nonnegligible number of drugs was not correctly predicted as already reported in a study using web tools to predict P-gp substrates.<sup>[26]</sup> Using cut-off 1, the false positives and false negatives represented 20% and 2% of the drugs, respectively, while they represented 12.5% and 10.5% when using cut-off 2. This relative poor prediction performance by the web tool combination was confirmed in our experiments. Indeed, among the nine pesticides predicted to be P-gp inhibitors and evaluated in vitro, six (cyflumetofen, fenpicoxamid, oxathiapiprolin, pinoxaden, profoxydim and silafluofen) failed to inhibit P-gp activity in MCF7R cells. We were not able to corroborate our results with other studies as these six pesticides had never been evaluated previously. Other pesticides were predicted to be P-gp inhibitors by the web tool combination, but data in the literature reported that they were not able to inhibit P-gp activity in vitro. This was notably the case of numerous pyrethroids such as deltamethrin, tested using a variety of assays.<sup>[16,28]</sup> Moreover, the two pesticides predicted to be P-gp noninhibitors (emamectin B1 benzoate and tralkoxydim) and tested in vitro in our study were in fact able to inhibit P-gp activity in MCF7R cells. Emamectin B1 benzoate also inhibited P-gp activity using the  $P_{app}$  assay with the Caco-2 cell monolayer. Our results confirmed previous experiments describing this pesticide as a P-gp inhibitor in vitro.<sup>[14]</sup> The case of tralkoxydim is more ambiguous, as this pesticide inhibited P-gp activity when using the MCF7R cell model but not when using the Caco-2 cell monolayer model. Moreover, it had not been previously studied in vitro. Another pesticide, phosalone, was predicted to be a P-gp noninhibitor by our combination of web tools, but a previous study had reported that this chemical inhibited P-gp activity in vitro.<sup>[15]</sup> Only three pesticides (ivermectin B1a, dimethomorph and spinosad) predicted *in silico* to be P-gp inhibitors were confirmed by our in vitro assays with both MCF7R cell and Caco-2 cell monolayer models. Nevertheless, predictions by our combination of web tools were correlated for other pesticides such as tebufenozide and methoxyfenozide, described as P-gp inhibitors by a previous study,<sup>[33]</sup> but also imidacloprid and nitenpyram reported in vitro to be P-gp noninhibitors.<sup>[18]</sup>

We report here for the first time that cyflumetofen, fenpicoxamid, oxathiapiprolin, pinoxaden, profoxydim and silafluofen were not able to inhibit P-gp activity in vitro, these pesticides never having been evaluated previously to our knowledge. The logP value (ranging from 3 to 6.5) of these pesticides being equivalent to that of the P-gp inhibitor pesticides, we could exclude a nonentry into the cells to explain this absence of P-gp inhibition. In the present study, the pesticides dimethomorph, ivermectin B1a, spinosad and emamectin



**TABLE 4** Prediction concerning inhibition of intestinal P-gp activity in vivo by luminal gut concentrations of pesticides according to FDA criteria.<sup>a</sup>

Pesticide	ADI <sup>b</sup> (mg/kg body weight)	Oral dose <sup>c</sup> (μmole)	I <sup>d</sup> (μM)	IC <sub>50</sub> <sup>e</sup> (μM)	Ratio I/IC <sub>50</sub>	Potential inhibition of intestinal P-gp activity in vivo
Spinosad	0.024	0.57	2.27	6.59	0.34	No inhibition
Emamectin B1 benzoate	0.0007	0.02	0.10	4.53	0.02	No inhibition
Dimethomorph	0.050	4.51	18.05	79.57	0.23	No inhibition
Ivermectin B1a	0.010	0.40	1.60	2.54	0.63	No inhibition
Tralkoxydim	0.005	0.53	2.13	87.83	0.02	No inhibition

Abbreviations: ADI, admissible daily intake; FDA, Food and Drug Administration; I, intestinal luminal gut concentration; IC<sub>50</sub>, half-maximal inhibitory concentration; P-gp, P-glycoprotein.

<sup>a</sup>The inhibition of intestinal P-gp activity in vivo can be predicted if the ratio  $I_{\text{gut}}/IC_{50} \geq 10$ .

<sup>b</sup>According to the European Union Pesticides database and to the Joint FAO-WHO expert committee on food additives.

<sup>c</sup>Defined for one meal out of two daily meals and a 70 kg body weight.

<sup>d</sup>Calculated as the ratio oral dose/250 mL.

<sup>e</sup>The lowest of the two calculated IC<sub>50</sub> values was retained.

B1 benzoate were found to be P-gp inhibitors using two complementary in vitro models, while tralkoxydim was able to inhibit P-gp activity only in MCF7R cells. For the first time, we report that dimethomorph and tralkoxydim were P-gp inhibitors in vitro, while our results concerning ivermectin B1a, spinosad and emamectin B1 benzoate were in agreement with data in the literature.<sup>[8–10,14,34]</sup> The results obtained with the accumulation and retention assays indicated a stronger inhibition of P-gp activity by ivermectin B1a, spinosad and emamectin B1 benzoate than by dimethomorph and tralkoxydim. These observations were confirmed by the IC<sub>50</sub> values, which ranged from 2.5 to 14.4 μM for ivermectin B1a, spinosad and emamectin B1 benzoate, while IC<sub>50</sub> values for dimethomorph and tralkoxydim ranged from 79.6 to 102.1 μM. Previous experiments have indicated IC<sub>50</sub> values with respect to P-gp activity for ivermectin ranging from 0.25 to 9 μM, and from 0.27 to 3.2 μM for spinosad, while no IC<sub>50</sub> has been reported for emamectin.<sup>[12,21,34–36]</sup> These discrepancies between the studies could be explained by the different assays and reference substrates used. Nevertheless, our results were in accordance with these data. The stronger inhibition of P-gp activity by ivermectin B1a, spinosad and emamectin B1 benzoate was also observed with the  $P_{\text{app}}$  assay. Indeed, these three pesticides had ER values (on average 2.4) close to those of the reference inhibitor, verapamil. The ER value was slightly higher for dimethomorph (3.9), and no different from the negative control for tralkoxydim (7.1). Previous studies have also reported a lower ER with ivermectin and spinosad in Caco-2 and MDCK-MDR1 cell monolayers.<sup>[11,34]</sup> This lower ER value was basically explained by a decrease in  $P_{\text{app B-A}}$  for ivermectin B1a, spinosad and emamectin B1 benzoate, while  $P_{\text{app A-B}}$  remained stable whatever the experimental conditions. Indeed,  $P_{\text{app B-A}}$  decreased for the vehicle control from  $63 \times 10^{-7}$  to on average  $20 \times 10^{-7} \text{ cm s}^{-1}$  for these three pesticides, our results being in agreement with the literature.<sup>[11]</sup> For dimethomorph, the  $P_{\text{app B-A}}$  value was, respectively,  $33 \times 10^{-7} \text{ cm s}^{-1}$ , thus confirming its lower potency in inhibiting the P-gp activity.

In the context of environmental exposure, P-gp activity was unlikely to be inhibited by the tested pesticides according to the FDA criteria for in vivo drug–drug interactions. Indeed, intestinal P-gp activity in vivo is likely to be inhibited if the human luminal gut concentration of the compound exceeds or equals 10 times the IC<sub>50</sub> (Table 4). As IC<sub>50</sub> values are usually over 1 μM, this would require greater pesticide concentrations, which would be unlikely to be reached for the tested pesticides in light of their environmental concentrations. The prediction of a potential inhibition of the activity of intestinal transporters in vivo has also been evaluated for other pesticides, such as allethrin, tetramethrin, malathion, parathion and chlorpropham.<sup>[19,28,37]</sup> These studies also predicted that intestinal transporter activity in vivo would not be inhibited by these pesticides in environmentally exposed humans. Nevertheless, both these previous studies and our own study evaluated each pesticide individually. However, this evaluation needs to be performed for mixtures (not only pesticide combinations but also pesticides with other environmental pollutants, drugs or compounds that naturally interact with P-gp). Pesticide combinations have already been shown to affect P-gp activity.<sup>[15]</sup> Exposure to such mixtures may consequently have to be considered when judging the in vivo relevance of transporter inhibition by pollutants, including pesticides.<sup>[4]</sup> Moreover, the study of P-gp expression regulation upon chronic exposure to pesticides mixtures is worthy of interest and would deserve further experiments.

## 5 | CONCLUSION

In the present study, we applied a combination of in silico and in vitro approaches to identify pesticides that inhibit P-gp activity. The combination of different in silico web tools was helpful in prioritising the pesticides to be tested in vitro. However, the prediction performance of the in silico models needs to be improved by

increasing the number of compounds in the selection and supplementing the model with new informative data. A more robust prediction model could be used to screen a wide range of compounds (like pesticides) before performing complementary *in vitro* experiments. Thus, *in silico* docking studies could improve predictions of the potential inhibition of P-gp activity. In view of the data produced during this research and in agreement with published data, MCF7R cells could be an advantageous model for identifying P-gp-inhibiting compounds *in vitro*, and it therefore needs to be adapted for high-throughput screening.<sup>[38]</sup> A complementary assay focused on the third binding site (M) of the P-gp could reduce false negative results by identifying pesticides which are competitive inhibitors at this third site. Finally, the Caco-2 cell monolayer model could complement this integrative approach as it confirmed 80% of the results obtained with the MCF7R cells and is suitable for representing the intestinal barrier to identify potential P-gp inhibitors among food contaminants.

#### AUTHOR CONTRIBUTIONS

**Nelly Guéniche:** Conceptualisation; data curation; formal analysis; investigation; methodology; writing—original draft. **Zeineb Lakehal:** Data curation; formal analysis; investigation. **Denis Habauzit:** Formal analysis; methodology. **Arnaud Bruyère:** Conceptualisation; supervision; validation. **Olivier Fardel:** Conceptualisation; supervision; validation; writing—original draft; writing—review and editing. **Ludovic Le Hégarat:** Supervision; validation. **Antoine Huguet:** Conceptualisation; data curation; formal analysis; investigation; methodology; supervision; validation; writing—original draft; writing—review and editing.

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#### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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