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Inhibition of Human MDR1 and BCRP Transporter ATPase Activity by Organochlorine and Pyrethroid Insecticides

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Abstract

Despite the growing evidence suggesting that pesticides contribute to chronic diseases, there is a limited understanding of how these chemicals are removed from cells and whether pesticides can alter the disposition of drugs. The present study examined the effects of two classes of insecticides (organochlorine and pyrethroid) on the ATPase activity of the human multidrug resistance protein 1 (MDR1) and breast cancer resistance protein (BCRP) efflux transporters. Using plasma membranes from cells over-expressing MDR1 and BCRP, it was demonstrated that the organochlorine pesticide DDT (*o,p'*-DDT and *p,p'*-DDT isomers), as well as its metabolite (*p,p'*-DDD), inhibit both MDR1 and BCRP ATPase activity. In addition, *p,p'*-DDE, and two pyrethroid pesticides inhibited BCRP ATPase activity between 4 and 7 μ M. Additional research is necessary to further characterize the functional inhibition of MDR1 and BCRP activity and determine whether pesticides alter the transporter-mediated disposition of other chemicals.

Keywords

insecticides; transporter; brain; BCRP; MDR1; pesticides

INTRODUCTION

Each year approximately 5.2 billion pounds of pesticides are used worldwide in agricultural and residential settings [1]. Human exposure to pesticides results from the environmental persistence of some chemicals including organochlorine insecticides such as dichlorodiphenyltrichloroethane (DDT) and the increasingly widespread use of newer agents

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such as synthetic pyrethroids [2,3]. Chronic exposure and elevated body burdens of pesticides have been linked to negative human health effects such as diabetes, cancer, and neurodegenerative diseases including Parkinson's and Alzheimer's diseases [4–7]. Because of the high potential for exposure to pesticides in the environment, it is important to examine the cellular mechanisms that regulate the disposition of pesticides and the potential for target organ toxicity.

The ATP-binding cassette (ABC) transporters are a superfamily of transmembrane proteins that protect vulnerable organs from the accumulation of toxic compounds by actively removing them from the cell (reviewed in [8]). ABC transporters move substrates out of the cell against concentration gradients using energy derived from the hydrolysis of ATP. The multidrug resistance protein 1, or P-glycoprotein (MDR1/P-gp/*ABCB1*), was initially found to be over-expressed in cancer cells, but was later detected in normal human tissues as well [9,10]. MDR1 protein is localized to the apical membrane of proximal tubule cells, placental trophoblasts, hepatocytes, and capillary endothelial cells that constitute the blood-brain barrier (reviewed in [8]). Substrates of MDR1 include several structurally diverse compounds such as colchicine, doxorubicin, digoxin, imatinib, prazosin, and verapamil [11–14]. Similar to MDR1, the breast cancer resistance protein (BCRP/*ABCG2*) is strongly expressed on the apical membranes of cells in the placenta, liver, mammary glands, kidneys, and brain (reviewed in [8]). BCRP transports chemicals such as mitoxantrone, glyburide, cimetidine, and sulfasalazine [15–18]. MDR1 and BCRP also exhibit an overlap in substrate specificity, including the anticancer drug imatinib and the antihypertensive drug prazosin [19,20].

A number of pesticides have been reported to interact with MDR1 as substrates and/or inhibitors. Incubation of human HepG2 hepatoma cells with the organochlorine pesticide *p,p'*-DDT resulted in the cellular retention of the MDR1 substrate, rhodamine 123, suggesting that *p,p'*-DDT inhibits MDR1 activity [21]. Interestingly, the DDT metabolite, *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE) did not alter the retention of rhodamine 123 in HepG2 cells [21]. Inhibition of MDR1 function by micromolar concentrations of another organochlorine pesticide endosulfan as well as the pyrethroid pesticide permethrin has been demonstrated in mouse melanoma cells (B16/F10) over-expressing the human MDR1 transporter [22]. In addition, ATPase activity can be stimulated by endosulfan and another pyrethroid pesticide cypermethrin in plasma membranes from the Chinese hamster ovary cells that contain the MDR1 transporter, indirectly suggesting that these pesticides are substrates for MDR1. Furthermore, in reconstituted proteoliposomes containing the hamster MDR1 protein, endosulfan (50 μ M) and cypermethrin (70 μ M) each inhibited ATPase activation stimulated by the MDR1 substrate, tetramethylrosamine [23]. In comparison to MDR1, little is known about whether pesticides can act as substrates and inhibitors of BCRP.

The purpose of this study was to determine whether organochlorine and pyrethroid pesticides and/or metabolites can activate and/or inhibit human MDR1 and BCRP ATPase activity in plasma membrane fractions (Figure 1). Knowledge of the *in vitro* interactions of pesticides with MDR1 and BCRP will provide guidance on how transporters may contribute to the overall disposition and toxicity of pesticides.

MATERIALS AND METHODS

Chemicals

All pesticides were purchased from Chem Service (West Chester, PA). Unless specified, all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

ATPase Assay

The human MDR1 and BCRP PREDEASY ATPase Kits were purchased from Xenotech (Lenexa, KS) and used to determine the activation and inhibition of transporter-dependent ATPase activity by pesticides. *Activation.* To determine activation, plasma membranes from *Spodoptera frugiperda* (Sf9) cells transfected with human MDR1 or BCRP were incubated (37°C) in 96-well plates with assay medium, 2 mM ATP, and pesticides (0.03–100 µM) in the presence and absence of 1.2 mM sodium orthovanadate for 30 minutes. Sodium orthovanadate inhibits ABC transporter ATPase activity and is used to calculate vanadate-sensitive activity by subtraction from the total ATPase activity. *Inhibition.* Inhibition was analyzed in a similar manner, with the addition of specific MDR1 and BCRP activators (MDR1 40 µM verapamil; BCRP 10 µM sulfasalazine) to the assay medium. Following incubation (37°C) of the membranes with a colorimetric reagent, liberation of inorganic phosphate was detected by absorption at 610 nm using a Spectramax spectrophotometer (Sunnyvale, CA). Concentration-response experiments for the activation and inhibition studies of MDR1 (activation: verapamil, inhibition: cyclosporin A) and BCRP (activation: sulfasalazine, inhibition: Hoechst 33342) were also performed. DMSO was used to prepare pesticide stock solutions (5 mM) and its concentration in the final reaction mixture did not exceed 2%. All incubations were performed in triplicate. The basal (MDR1: 8–16 nmol P_i/min/mg protein; BCRP: 7–20 nmol P_i/min/mg protein) and stimulated (MDR1: 30–60 nmol P_i/min/mg protein; BCRP: 25–50 nmol P_i/min/mg protein) ATPase activity was consistent with the manufacturer data sheets for each ATPase kit. In this study, values for the basal activity were subtracted from the stimulated transporter ATPase activity.

Data Analysis

Data are presented as mean ± standard error of three determinations from one experiment. Non-linear regression analysis was performed for activation and inhibition experiments using the GraphPad Prism V5 software program (GraphPad, La Jolla, CA). Data generated from compounds that activated ATPase activity were fit to a curve using Michaelis-Menten kinetics for the calculation of V_{max} and K_m, the maximum velocity and concentration at half of V_{max}, respectively. Compounds that inhibited ATPase activity were analyzed by fitting the data to a concentration-response-inhibition curve to generate IC₅₀ values, or the concentration of half the maximal inhibition. R² values were calculated to determine the goodness of fit.

RESULTS

Characterization of ATPase assays

Prototypical activators and inhibitors of MDR1 and BCRP ATPase activity were used to confirm specific transporter activity. The MDR1 substrate, verapamil, activated MDR1

ATPase activity at concentrations over 0.5 μM with maximal activation observed at 10 μM (Figure 2A. V_{max} : 17.3 nmol P_i released/min/mg protein, K_m : 1.9 μM). Activation of MDR1 by 40 μM verapamil was inhibited by cyclosporin A at concentrations as low as 0.3 μM (Figure 2C. IC_{50} : 0.7 μM). Likewise, the BCRP substrate, sulfasalazine, stimulated BCRP ATPase activity (Figure 2B. V_{max} : 24.0 nmol P_i released/min/mg protein, K_m : 0.40 μM) which was inhibited by co-incubation with Hoechst 33342 (Figure 2D. IC_{50} : 3.1 μM).

Effect of pesticides on basal and stimulated MDR1 and BCRP ATPase activity

Of the ten pesticides screened for interaction with MDR1 and BCRP, none of the compounds stimulated baseline ATPase activity (data not shown). Four organochlorine pesticides and/or metabolites (*o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDD, and endosulfan) inhibited MDR1 ATPase activity (Figure 3). All four compounds yielded IC_{50} values ranging from 3.8 μM (*p,p'*-DDT) to 33.6 μM (endosulfan) when analyzed by nonlinear regression (Table 1). These same four compounds along with *p,p'*-DDE, and two pyrethroids (permethrin and resmethrin) inhibited BCRP-mediated ATP hydrolysis (Figure 4). Of these seven pesticides, the inhibitory potency, or IC_{50} values, ranged from 3.0 μM to 10.4 μM (Table 1). Cypermethrin, deltamethrin, and dieldrin did not inhibit stimulated MDR1 or BCRP ATPase activity (data not shown).

DISCUSSION

The current study investigated the interactions of ten insecticides and/or metabolites with human MDR1 and BCRP using isolated plasma membrane fractions. Overall, seven of the chemicals inhibited ATPase activity suggesting that they may reduce the transport of MDR1 and/or BCRP substrates. This is important because reduced activity of ABC transporters by chemicals has been found to alter the disposition and subsequently toxicity of substrates *in vivo*. For example, the antiparasitic drug metabolite, trichlabendazole sulfoxide, increased the plasma concentration of BCRP substrate, sulfasalazine, in wild-type mice as compared to BCRP knockout mice [24]. Similarly, a chemical interaction (insecticide Spinosad® and antiparasitic ivermectin) mediated by MDR1 resulted in elevated brain levels of ivermectin in dogs, which has been associated with neurotoxicity [25,26]. Such findings highlight the importance of evaluating chemical interactions mediated by transporters.

Our data support previous research demonstrating *p,p'*-DDT and endosulfan as inhibitors of human MDR1 transport function [21,23,27]. Also in accordance with previous work, we found limited interaction of *p,p'*-DDE with human MDR1 [21,22]. Despite the structural similarities between *p,p'*-DDT and *p,p'*-DDE, differences in the interactions with MDR1 may be due to the double bond present between the two main chain carbons of *p,p'*-DDE. This is supported by the inhibition of MDR1 ATPase activity by two structurally similar *p,p'*-DDT analogues, *o,p'*-DDT and *p,p'*-DDD, that do not contain the double bond. In the present study, neither permethrin nor cypermethrin altered MDR1 ATPase activity stimulated by verapamil. This observation is in contrast to previous reports that suggested both pyrethroids inhibit or stimulate MDR1 function. These divergent results may be due to the use of different substrates (doxorubicin and tetramethylrosamine) and/or species (hamster) of MDR1 in the model system [22,23].

BCRP ATPase activity was inhibited by the same four organochlorine pesticides and metabolites that inhibited MDR1 ATPase activity, along with *p,p'*-DDE and two of the pyrethroid pesticides, permethrin and resmethrin. Despite the overall inhibitory effect of the organochlorine and pyrethroid pesticides on BCRP, the neonicotinoid pesticide imidacloprid did not interact with BCRP, demonstrating specificity of the assay (data not shown). In addition, we tested the ability of *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDD, and endosulfan to inhibit the activation of the human multidrug resistance-associated protein 2 (MRP2) transporter. All four chemicals produced only modest inhibition of MRP2 suggesting that the broad overlap in organochlorine-transporter interaction is specific to MDR1 and BCRP and is not a result of global ATPase inhibition (data not shown).

None of the pesticides activated ATPase activity alone indicating that the compounds are likely not substrates for either transporter. However, our laboratory has observed false negative results in this assay. Specifically, we have observed activation of MDR1 by the antipsychotic drug, risperidone but not its metabolite paliperidone (9-hydroxyrisperidone) (data not shown), despite the fact that both chemicals are MDR1 substrates [28]. Further work is necessary to interpret IC₅₀ values from transporter ATPase assays to concentrations achievable from occupational and environmental exposures to pesticides. Considering the shortcomings of subcellular assays, the ATPase assay should be used as a screening method to investigate potential interactions of chemicals with ABC transporters. Quantification of substrate accumulation in cell over-expression systems and *in vivo* pharmacokinetic studies should be conducted following screening with the ATPase assay to directly identify substrates of ABC transporters and the *in vivo* implications of chemical interactions mediated by transporters. Future attention should also be placed on how combinations of pesticides can additively or synergistically inhibit transporter activity.

In conclusion, a number of the insecticides tested inhibited MDR1 and BCRP ATPase activity revealing the potential role of pesticides in altering the disposition and accumulation of other chemicals. Future research should further characterize the interaction of the seven pesticides of interest with MDR1 and BCRP to better understand the influence of pesticides on xenobiotic disposition.

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Non-Standard Abbreviations

ABC	ATP-binding cassette
BCRP	breast cancer resistance protein
DDD	dichlorodiphenyldichloroethane

DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
MDR	multidrug resistance protein
MRP	multidrug resistance-associated protein
P-gp	P-glycoprotein

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Figure 1. Structure of organochlorine and pyrethroid insecticides or metabolites

Figure 2. Prototypical activation and inhibition of MDR1 and BCRP ATPase activity

(A, B) Membranes were incubated with ATP and varying concentrations of activator (MDR1: verapamil, BCRP: sulfasalazine) in the presence and absence of sodium orthovanadate for 30 minutes. (C, D) Membranes were incubated with ATP, activator (verapamil 40 μ M; sulfasalazine 10 μ M), and varying concentrations of inhibitor (MDR1: cyclosporin A, BCRP: Hoechst 33342) in the presence and absence of sodium orthovanadate for 30 minutes. The amount of inorganic phosphate released was determined by spectrophotometry following addition of a colorimetric reagent. Data are presented as the vanadate-sensitive ATPase activity of three replicates (mean $\pm$ SE) from one experiment. R^2 values demonstrate the goodness of fit of the curves generated by non-linear regression analysis.

MDR1

Figure 3. Inhibition of MDR1 ATPase activity by insecticides

MDR1 membranes were incubated with ATP, verapamil (40 μM), and varying concentrations of test insecticide, in the presence and absence of sodium orthovanadate for 30 minutes. The amount of inorganic phosphate released was determined by spectrophotometry following addition of a colorimetric reagent. Data are presented as the vanadate-sensitive ATPase activity of three replicates (mean $\pm$ SE) from one experiment. R^2 values demonstrate the goodness of fit of the curves generated by non-linear regression analysis.

BCRP

Figure 4. Inhibition of BCRP ATPase activity by insecticides

BCRP membranes were incubated with ATP, sulfasalazine (10 μM), and varying concentrations of test organochlorine (A) and pyrethroid (B) insecticides, in the presence and absence of sodium orthovanadate for 30 minutes. The amount of inorganic phosphate released was determined by spectrophotometry following addition of a colorimetric reagent. Data are presented as the vanadate-sensitive ATPase activity of three replicates (mean $\pm$ SE) from one experiment. R^2 values demonstrate the goodness of fit of the curves generated by non-linear regression analysis.

Table 1Inhibition of MDR1 and BCRP ATPase activity by insecticides^a.

Pesticide	IC ₅₀ (μM)	
	MDR1	BCRP
<i>o,p'</i> -DDT	7.8	10.4
<i>p,p'</i> -DDT	3.8	5.0
<i>p,p'</i> -DDD	10.7	3.0
<i>p,p'</i> -DDE	-	4.0
Dieldrin	-	-
Endosulfan	33.6	6.9
Cypermethrin	-	-
Deltamethrin	-	-
Permethrin	-	7.0
Resmethrin	-	5.6

^aData are presented as IC₅₀ values calculated by non-linear regression analysis (n=3) from one experiment. Dashes (–) indicate that an IC₅₀ value was unable to be generated for the insecticide-transporter interaction.