Chemosphere 159 (2016) 256–266

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Combined cytotoxic effects of pesticide mixtures present in the Chinese diet on human hepatocarcinoma cell line

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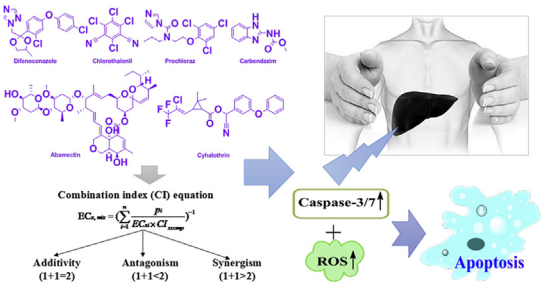
HIGHLIGHTS

- Cytotoxicity of high-risk pesticides was evaluated on human HepG2 cell lines.
- Combined effects of the pesticides were assessed using combination index method.
- Increasing ROS and caspase-3/7 levels were found in some pesticides pairings.
- The mediated activation of caspase-3/7 and ROS level are attributed to apoptosis.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 12 December 2015
Received in revised form
11 April 2016
Accepted 17 May 2016
Available online 11 June 2016

Handling Editor: Frederic Leusch

Keywords:
Pesticide mixtures
Cytotoxicity
HepG2
Combined effects
Cell apoptosis

ABSTRACT

Consumers might be simultaneously exposed to several pesticide residues contained in their food. Based on the results of previous studies, 20 pesticides were selected due to their high exposure levels to which the Chinese population is likely exposed through the diet. The purpose of this study was to measure the cytotoxicity of these pesticides in HepG2 cells *in vitro*, as an alternative approach to assess the toxicity of chemicals. Then, the pesticides and some of the mixtures with comparatively high cell-proliferating inhibitory activities were selected to test the cellular ROS level and apoptosis-related protein Caspase-3/7 content in HepG2 cells. The combined effects of these pesticide mixtures with the prediction was based on a combination index (CI)-isobologram equation and the pesticide combinations exhibited various types of interactions (synergism, antagonism, and additivity). Two individuals, one binary combinations, and three uniform design (UD) mixtures of the pesticides were found to have significant cytotoxic effects, along with significant time- and dose-dependent induction of caspase-3/7 activity *in vitro*, indicating that cytotoxicity caused by these pesticides might be attributed to the pro-oxidative and apoptosis induced potential.

1. Introduction

Pesticides are designed to kill, repel or inhibit the growth of biological organisms (Laetz et al., 2009). Due to the broad spectrum of pesticide usages, human populations are exposed to

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http://dx.doi.org/10.1016/j.chemosphere.2016.05.050
0045-6535/

combination of several different pesticides simultaneously through their food. In light of this, monitoring and risk assessment programs of pesticide were conducted at regional and national levels to identify the main pesticide mixtures to which the local general population is simultaneously and most heavily exposed through their diet (Iñigo-Nuñez et al., 2010; Claeys et al., 2011; Chen et al., 2011; Nougadère et al., 2012; Bakırcı et al., 2014; Lozowicka, 2015). Because China has become one of the largest producers and users of pesticides in the world, the presence of pesticides in food has fairly recently become a major concern for consumers (Zhang et al., 2011). In recent investigations, the risks associated with pesticide residues in fruits and vegetables from China were assessed (Chen et al., 2011; Yuan et al., 2014; Li et al., 2015). In the fruit survey, a total of 310 fruit samples were collected from China's orchards. Although only 8 samples (2.6%) were found to contain pesticide residues above the maximum residue limits (MRLs), 218 samples (70.3% of positive samples) contained more than one pesticide residue, and one sample contained up to 10 compounds (Li et al., 2015). The potential combined effects of pesticide mixtures are likely to occur through dietary exposures.

Traditional risk assessment of pesticides is based on no observed adverse effect levels for individual compounds. In reality, human beings can be simultaneously exposed to several chemicals that might potentially contribute to a cumulative adverse effect in the individuals (Boobis et al., 2008; Müller et al., 2009); therefore, assessing the combined toxicity of pesticides as mixtures has been an ongoing challenge in the research for environmental and human health within the last 20 years (Monosson, 2005). Concentration addition (CA) and independent action (IA) are two traditional concepts that have been widely used to predict mixture toxicities (Altenburger et al., 2003; Backhaus et al., 2003; Jonker et al., 2004, 2005; Loureiro et al., 2009). CA relies on the assumption that mixture components have the same or similar modes of action, while IA is based on the idea that each component acts on a different receptor and together could contribute to a common response as a whole. These two concepts provide a reference structure to which the experimental mixture toxicity can be compared. Recently, the combination index (CI) equation method has successfully been used to study pollutant interactions (Rodea-Palomares et al., 2010, 2012; Rosal et al., 2010; Boltes et al., 2012; González-Pleiter et al., 2013; Chen et al., 2015; Wang et al., 2015).

A series of animal tests, cell functions and their associated endpoints were evaluated following pesticides exposures. Conventional toxicity screening has involved the use of animal models. However, it lacks the ability to generate accurate, sensitive, and cost-effective safety assessments due to interspecies differences (Peters, 2005; Bandele et al., 2012). On the other hand, *in vitro* human cell culture systems using biologically relevant biomarkers has been developed as an alternative approach to assess the toxicity of chemicals that may adversely affect physiological activities (Flynn and Ferguson, 2008). Hepatocytes are the most abundant cell type and primary functional unit of the liver, especially in the process of the metabolism of xenobiotics, therefore HepG2 cell line is often used as a hepatic model. Moreover, induced hepatotoxicity is an essential indicator and frequently used for evaluating the safety of chemicals (Antoine et al., 2009; Bandele et al., 2012).

Based on the results of previous pesticide monitoring and risk assessment, twenty pesticides were selected with high exposure levels in the Chinese diet (Chen et al., 2011; Yuan et al., 2014; Li et al., 2015). The main objective of the present study was to study the cytotoxicity induced by pesticide mixtures using the MTT assay on HepG2 cells; then we assessed the combined effects of the pesticides and their mixtures by comparing the measured effects of the mixture with predictions based on the CA and IA concepts, as well as median-effect/combination index (CI)-isobologram equation method. Further, to evaluate the pathway in the induction of apoptosis, the cellular production of reactive oxygen species (ROS) levels and Caspase-3/7 activity for the pesticides and their mixtures with high cell-proliferating inhibitory activities.

2. Material and methods

2.1. Chemicals

Twelve pesticides (acetamiprid, chlorpyrifos, carbendazim, chlorothalonil, cyhalothrin, cypermethrin, difenoconazole, imidacloprid, iprodione, prochloraz, procymidone, pyrimethanil) and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). American Chemical Society–grade dimethyl sulfoxide (DMSO) and methylthiazolyldiphenyl-tetrazolium bromide (MTT) powder were obtained from AMRESCO (Solon, OH, USA). Eight pesticides (abamectin, carbofuran, isocarbophos, omethoate, parathion, phorate, pyridaben, and triazophos) were purchased from CRM/RM Information Center of China (Putiantongchuang Inc., Beijing, China). All pesticides were purified up to ≥93%. Pesticides stored at 4 °C were dissolved in DMSO at proper concentrations to ensure that the final density of the DMSO exposed to cells was ≤1.0‰. Cells treated with 1.0‰ DMSO were used as the control. Some of the physicochemical properties of the pesticides are listed in Table 1.

2.2. Cell culture

HepG2 (catalogue number TCHu 72) were obtained from the cell bank of the Chinese Academy of Sciences and maintained in a humidified atmosphere of 5.0% CO_2 and 95% air at 37 °C. Minimum Essential Medium (MEM) purchased from Hyclone (Thermo Fisher Scientific, Logan, Utah, USA), was supplemented with 1.0% penicillin/streptomycin and 10% fetal bovine serum (FBS) Gibco (Thermo Fisher Scientific, Logan, Utah, USA). When the cell culture reached 80% confluence, cells were dispersed with 2.5% trypsin-

Table 1
Some physico-chemical properties of the pesticides tested.

Pesticides	IC_{50} (μM)	CAS	Purity (%)	MM (g/mol)
Procymidone	>1000	32809-16-8	99.9	284.1
Iprodione	>1000	36734-19-7	99.5	330.2
Difenoconazole	228.8 ± 1.95	119446-68-3	97.2	406.3
Chlorothalonil	38.96 ± 0.08	1897-45-6	99.3	265.9
Pyrimethanil	>1000	53112-28-0	99.9	199.2
Acetamiprid	>1000	135410-20-7	99.9	222.7
Imidacloprid	>1000	138261-41-3	99.9	255.7
Prochloraz	248.5 ± 0.55	67747-09-5	98.6	376.7
Cypermethrin	>1000	52315-07-8	94.3	416.3
Carbendazim	440.2 ± 42.97	10605-21-7	99.2	191.2
Cyhalothrin	22.12 ± 0.03	68085-85-8	98.3	449.9
Chlorpyrifos	>1000	2921-88-2	99.7	350.6
Carbofuran	>1000	1563-66-2	98.6	221.3
Isocarbophos	>1000	24353-61-5	98.8	289.3
Pyridaben	>1000	96489-71-3	98.5	364.9
Abamectin	35.68 ± 1.29	71751-41-2	93	873.1
Triazophos	>1000	24017-47-8	98.9	313.3
Phorate	>300	298-02-2	1000 μg/mL[a]	260.4
Parathion	>300	56-38-2	1000 μg/mL[a]	291.3
Omethoate	>300	1113-02-6	1000 μg/mL[a]	213.2

[a] With an expanded uncertainty of 7. IC_{50} values are given as means ± S.D. of three independent experiments.