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In vitro combined cytotoxic effects of pesticide cocktails simultaneously found in the French diet

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ABSTRACT

Although human populations may be constantly exposed to complex pesticide mixtures through their diet, the human health risk of pesticide exposure is currently assessed on the basis of toxicity data on individual compounds. To investigate the combined toxic effects of pesticide cocktails previously identified in the French diet, we first studied the cytotoxicity induced by seven cocktails composed of two to six pesticides on human hepatic (HepG2) and colon (Caco-2) cell lines using the MTT and neutral red uptake assays. Secondly, we challenged to assess the combined effects of the two most cytotoxic cocktails by comparing the measured effects of the mixtures with the predictions based on additive effects on two concepts—independent action (IA) and concentration addition (CA). For the cocktail composed of dichlorodiphenyltrichloroethane (DDT) and dieldrin, the cytotoxicity of the equimolar cocktail proved greater than the additive effect estimated by the two concepts. Furthermore, apoptosis induction was higher in equimolar cocktail than predicted by summing the effects of DDT and dieldrin. Thus, some supra-additive toxicity was found in the DDT-dieldrin cocktail. Nevertheless, if IA and CA models could reveal combined effects of pesticide cocktails, an accurate evaluation remains challenging.

1. Introduction

A wide range of pesticides are currently used to manage pests in agricultural and household environments. Consequently, food products may simultaneously contain residues of several different pesticides, leading to a constant exposure of human populations to complex pesticide mixtures through their diet. Pesticide residues are mainly present in cereals, vegetables and fruit. They were detected in 46.7% of the 67,887 food samples analysed throughout the European Union in 2008 and several pesticide residues, including organochlorines, organophosphorus, synthetic pyrethroids and phthalimides, were concurrently found in foodstuffs (Crépet and Tressou, 2011; EFSA, 2010; Van Audenhaege et al., 2009).

However, the risk assessment of pesticide residues in food is based on the toxicological evaluation of each individual compound, and there is no internationally-accepted procedure to assess the risk of cumulative exposure to multiple pesticide residues (Reffstrup et al., 2010). Increasing attention has therefore been focused on identifying combined actions, including supra- and infra-additive effects such as synergism, potentiation, antagonism and inhibition. Today, combined actions are commonly predicted on the basis of a concept known as additivity. Two main approaches are generally used for co-exposure scenarios (Goldoni and Johansson, 2007; Groten et al., 2001). One is the Bliss independence criterion, based on the independent action (IA) concept, the other the Loewe additivity model, based on the concentration addition (CA) concept. Both are "non-interaction" models assuming that, in mixtures, chemical effects are simply additive, and are neither infra- nor supra-additive. The IA concept should be used for combinations of chemicals that produce the same toxic effect in the same target organ via dissimilar mechanisms of action (MOA). In contrast, the CA model should be used for mixtures of chemicals that produce the same toxic effect in the same target organ via the same MOA. The United States Environmental Protection Agency (US-EPA) and the European Food Safety Authority (EFSA) have offered guidance on how to perform risk assessment for a mixture of pesticides with a common mechanism (EFSA, 2009; US-EPA, 2002). However, this statement does not address mixtures of pesticides in food, which usually have a different MOA.

Abbreviations: ANOVA, analysis of variance; CA, concentration addition; CI, confidence interval; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DDT, dichlorodiphenyltrichloroethane; DMSO, dimethyl sulphoxide; EFSA, European food safety authority; FCS, foetal calf serum; IA, independent action; MEM, minimum essential medium; MOA, mechanisms of action; MTT, methylthiazolyldiphenyl-tetrazolium bromide; NRU, neutral red uptake; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase d UTP nick end labelling; US-EPA, United States environmental protection agency.

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Furthermore, the mechanisms of toxicity of most pesticides in humans are not well-known.

France has been one of the largest users of pesticides (UIPP, 2009). The presence of pesticides in food has become a major concern for French consumers and food safety authorities fairly recently. In this light, the PERICLES research program coordinated by the French Agency for Food, Environmental and Occupational Health Safety (ANSES), launched in 2009, aims firstly to identify the main pesticide cocktails to which the French general population is simultaneously and most heavily exposed through the diet, and secondly to investigate the possible combined toxic effects of their components in human cells. In the first part of this programme, the dietary exposure of 1898 adults and 1439 children was assessed for 79 pesticide residues in combining individual food quantities from the French national consumption survey INCA2 (Individual and national study on food consumption 2006–2007 (Dubuisson et al., 2010; Lioret et al., 2010) with residue levels in food provided by the French food monitoring programs planned in 2006. Two main criteria were taken into account for cocktails design: (i) identification of groups of consumers with similar patterns of exposure to the 79 pesticides by a statistical approach (Crépet and Tressou, 2011) and (ii) no consideration of the toxicological profiles of the selected compounds. After screening the correlations between the exposure to the 79 pesticides of the most exposed groups of consumers, 25 pesticides with at least one correlation above 0.7 were selected to form seven cocktails composed of two to 6 pesticides (Crépet et al., in press). As the second part of the program, we have investigated the cytotoxic and genotoxic effects of these 7 cocktails on human cell lines and have evaluated their potential combined effects. Recently, we reported that one of these seven cocktails exhibited genotoxicity *in vitro* (Graillot et al., 2012).

In the present study, we first studied the cytotoxicity induced by seven cocktails using the MTT and neutral red uptake (NRU) assays on two human cell lines: the human epithelial colorectal adenocarcinoma Caco-2 cell line as an intestinal model in light of the direct contact of this tissue with food contaminants, and the human hepatocellular carcinoma HepG2 cell line as a hepatic model since the liver is a common target of xenobiotics. Secondly, we assessed the combined effects of the two most cytotoxic cocktails by comparing the measured effects of the mixtures with their predictions based on additive effects.

2. Materials and methods

2.1. Chemicals

Twenty-five pesticides (captan, chlorfenvinphos, chlorpropham, cyprodinil, 4,4′-DDT , dieldrin, diphenylamine, ethion, fenhexamid, fenitrothion, fludioxonil, imazalil, iprodion, lamda-cyhalothrin, linuron, maleic-hydrazide, methidathion, penconazol, phosalone, procymidon, propargit, pyrimethanil, qinoxyfen, tolylfluanid, triadimenol), dimethyl sulphoxide (DMSO), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), neutral red solution (0.33%), methylthiazolyldiphenyl-tetrazolium bromide (MTT) and staurosporine from *Streptomyces* sp. were purchased from Sigma–Aldrich (Saint-Quentin Falavier, France).

Each pesticide was dissolved in DMSO at 50 mM, aliquoted and stored at −20 °C. The final concentration of DMSO in assays was 0.2%.

2.2. Cell culture

Cell culture media and supplements were obtained from invitrogen. The human hepatoma cell line, HepG2 (ATCC HB-8065, passage 15–25), was cultured in Minimum Essential Medium (MEM), a medium supplemented with 10% foetal calf serum (FCS), penicillin (100 IU/ml) and streptomycin (100 µg/ml). The human colonic carcinoma cell line, Caco-2 (ATCC HTB37™, passage 28–40), was cultured in MEM with Glutamax supplemented with 10% FCS, 1% non-essential amino acids, penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cells were maintained at 37 °C under a humidified atmosphere of 5% CO_2.

2.3. Cytotoxicity measurements

Cellular viability was determined by the MTT and NRU assays. The MTT assay was performed according to previously-described procedures (Mosmann, 1983) with minor modifications. HepG2 cells at 3.5×10^4 cells per well and Caco-2 cells at 1×10^4 cells per well were seeded in 96-well plates (Corning, USA) and allowed to adhere for 24 h. The cells were exposed for 24 h to pesticide samples or 0.2 % of DMSO (vehicle control) in a serum-free medium. Treatment media were removed, and 100 µl of MTT solution (0.5 mg/ml in serum-free medium) was added. After a 3-h incubation period, the MTT solution was removed and the formazan crystals were resuspended in 100 µl of acidic isopropanol solution (0.04 N HCl in absolute isopropanol). The absorbance at 570 nm was read using a spectrophotometer (Fluostar Optima, BMG Labtech).

The NRU assay was performed according to the method of Repetto et al. (2008). After the treatment, the medium was removed and 100 µl of 0.003% NR solution was added to each well. After a 3-h incubation period, the NR solution was discarded and 100 µl of 1% acetic acid solution containing 50% ethanol was added to extract the dye from cells. The absorbance at 540 nm was then read using a spectrophotometer (Fluostar Optima, BMG Labtech).

Cell viability was expressed as a percentage of the vehicle control.

2.4. Intracellular reactive oxygen species (ROS) production

Intracellular levels of ROS production were determined spectrophotometrically using the non-fluorescent DCFH-DA probe as previously described (Osseni et al., 1999) with minor modifications. After 24-h subculture, cells were incubated with 20 µM DCFH-DA in PBS for 30 min at 37 °C in the dark. Excess DCFH-DA was removed and cells were rinsed with PBS prior to treatment with cocktails or 0.2% of DMSO (vehicle control) in serum-free medium. Fluorescence intensity was determined at 0-, 2-, 4- and 24-h using a spectrophotometer (Fluostar Optima, BMG Labtech) with a 485 nm excitation wavelength and a 520 nm emission wavelength. Relative ROS production was calculated as a ratio of the fluorescence intensity measurements of the treated sample to the fluorescence intensity measurements of the vehicle control.

2.5. Caspase-3/7 activity

Caspase-3/7 activity was measured by an Apo-ONE Homogeneous caspase-3/7 assay (Promega) according to the manufacturer's instructions. At the end of treatment, the caspase-3/7 substrate Z-DEVD-R110 was added and the reaction prolonged for 1.5 h. Fluorescence was measured with excitation and emission wavelengths of 485 and 520 nm respectively.

2.6. Data analysis of combined cytotoxic effects

To evaluate the combined effect of pesticides, the measured cytotoxic effect of the cocktail was compared with that of the additive prediction based on the concentration–response functions for each cocktail component. Using the concepts of CA and IA (Altenburger et al., 2003), the additivity prediction was calculated according to the mathematical formulations as described by Kortenkamp et al. (2007).

To calculate the additive cytotoxic effect, viability was transformed into cytotoxicity values (Eq. (1)):

$$\text{Cytotoxicity}(\%) = 100 - \text{viability}(\%) \tag{1}$$

The concentration–response relationships of the individual pesticides were determined by the Hill model. To normalise the effects, the bottom and top asymptotes were set to 0% and 100% respectively. The experimental data set was fitted to the Hill function (Eq. (2))

$$E(\%) = \frac{100}{1 + \left(\frac{c}{EC_{50}}\right)^{-p}} \tag{2}$$

where E is the effect in %, c the concentration of the test agent (µM), p the parameter slope and EC_{50} the concentration of the single agent that produces a 50% effect. A nonlinear sigmoid regression analysis for each concentration–response curve was drawn on GraphPad PRISM 5 (GraphPad Software Inc., San Diego, CA, USA).

The total concentration of a cocktail at which a certain effect is generated can be calculated on the basis of the concentration–response curves of individual pesticides using the CA concept according to

$$ECx_{\text{mix}} = \left[\sum_{i=1}^{n} \frac{Pi}{EC_{xi}}\right]^{-1}, \tag{3}$$

In this equation, ECx_{mix} is the total concentration of the cocktail provoking x% effect, EC_{xi} the concentration of component i provoking the x% effect when applied singly, and Pi denotes the relative proportions of the total mixture concentration. The calculation of total mixture concentrations for various effect levels gives a complete interaction overview of an expected concentration–response relationship (Fig. 2).