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Impact of isomalathion on malathion cytotoxicity and genotoxicity in human HepaRG cells

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ABSTRACT

Isomalathion is a major impurity of technical grade malathion, one of the most abundantly applied insecticides; however little is known about its hepatotoxicity. In the present study, cytotoxicity and genotoxicity of malathion and isomalathion either individually or in combination, were assessed using the metabolically competent human liver HepaRG cell line. Isomalathion reduced cell viability starting at a 100 μM concentration after a 24 h exposure. It also significantly induced caspase-3 activity in a dose-dependent manner starting at 5 μM. On the contrary, even at concentrations as high as 500 μM malathion affected neither cell viability nor caspase-3 activity. Moreover, co-exposure of both compounds resulted in decreased toxicity of isomalathion. By contrast, malathion and isomalathion either separately or in combination, slightly induced micronuclei formation at low concentrations and had additive genotoxic effects when combined at 25 μM. Individually or combined isomalathion directly inhibited activity of carboxyesterases which are involved in detoxication of malathion. In addition, transcripts of CYP2B6 and CYP3A4, two CYPs responsible for malathion phase I metabolism, were strongly induced by the mixture while isomalathion alone only moderately decreased CYP1A2 and increased CYP2B6 transcripts. However, these CYPs were not altered at the protein or activity levels. Taken altogether, our results showed that isomalathion was much more cytotoxic than malathion while both compounds had comparable genotoxic effects in HepaRG hepatocytes at low concentrations and brought further support to the importance of considering impurities and interactions during evaluation of health risks of pesticides.

1. Introduction

Extensive use of pesticides has caused both environmental and public health concerns. Because of its low mammalian toxicity and high selectivity toward insects [1], malathion has become one of the most commonly used organophosphate insecticides for both domestic and commercial agricultural purposes; it has been employed in malaria eradication programs in Africa and Central America and in wide-scale pest-control in the United States. Exposure of workers who applied the insecticide or harvested the crops was estimated to be 1–3 mg/kg/day and 1–270 μg/kg/day, respectively which might give rise to a range of plasma concentrations between 0.03 and 80 μM [2]. Its rapid degradation by carboxylesterases (CEs) competes with the cytochrome P450 (CYP)-catalyzed formation of the toxic metabolite, malaoxon [3]. In human liver, malaoxon formation is mainly catalyzed by CYP1A2 and, to a lesser extent, CYP2B6 at low malathion concentrations (<50 μM), whereas at high levels the role of CYP3A4 becomes relevant [4]. Alterations or individual variations in both CYP and CE activities could result in increased malaoxon formation, enhancing malathion toxicity. Technical grade malathion (90–95%) contains several impurities, such as isomalathion and various trimethyl phosphorothioate esters, formed during production and/or storage, that can potentiate malathion-induced toxicity up to 10-fold [5,6]. The presence of isomalathion, a toxic degradation product, in commercial formulations was implicated in the 1976 epidemic malathion poisoning of 2800 spraymen (including five deaths) among 7500 field workers, during the Pakistan malaria control program. The greatest toxicity was observed with the formulation containing the highest level of isomalathion [7]. Isomalathion has been found to inhibit CEs in a non-competitive manner, shifting the metabolic pathway toward the bioactivation reaction [8–10]. However, little is known about isomalathion toxicity.

Malathion has been found to be genotoxic in several *in vivo* and *in vitro* studies. However, data were usually obtained with the technical grade and high doses of the compound [11,12]. No increase in the frequency of micronucleated cells and no inhibition of proliferation in lymphocyte cultures from two cohorts of

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applicators intermittently exposed to relatively low doses of malathion were observed [13]. On occupational settings, pesticide applicators exposed to technical grade malathion and other insecticides were reported to exhibit higher levels of chromosome aberrations and sister chromatid exchanges [14]. Weak or negative results have been reported with pure-grade malathion [15]. Noticeably, most genotoxic studies have been performed in the absence of a bioactivation system.

In the present study, mixtures containing both malathion and its major impurity, isomalathion, were used to better understand their toxic and genotoxic effects in the metabolically competent human liver HepaRG cell line [16]. Since *in vivo* hepatic concentrations are usually higher than circulating ones, malathion concentrations around 50 μM could be easily achieved in exposed individuals, and therefore, a 50 μM maximal concentration was used *in vitro* to be representative of the *in vivo* situation. Moreover, the presence of isomalathion levels as high as 0.2% is documented in actual commercial formulations, and it is known that additional amounts can be formed during storage [6]. This study showed for the first time, the potent toxic and genotoxic effects of isomalathion in an *in vitro* metabolically competent cell model and highlighted the necessity to take into consideration the impurities present in technical pesticide grade.

2. Materials and methods

2.1. Chemicals

Malathion, isomalathion, methylthiazoletetrazolium (MTT), 6β-hydroxytestosterone, 16β-hydroxytestosterone and *p*-nitrophenyl acetate (PNPA) were purchased from Sigma (St. Quentin Fallavier, France). 2′,7′-dichlorodihydrofluorescein (H2DCFDA) was from Invitrogen-Molecular Probe (Cergy-Pontoise, France).

2.2. Cell cultures

HepaRG cells are derived from a human cholangiohepatocarcinoma; they are untransformed cells and were used before passage 18. Briefly, they were seeded at a density of 2.6×10^4 cells/cm^2 as described previously [17,18]. They were first incubated in the Williams' E medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 2 mM glutamine, and 5×10^{-7} M hydrocortisone hemisuccinate for 2 weeks. Then, HepaRG cells were shifted to the same medium supplemented with 2% dimethylsulfoxide (DMSO) for two further weeks in order to obtain confluent differentiated cultures with maximum functional activities. At this time, these cultures contained hepatocyte-like and progenitors/primitive biliary-like cells at around 50% each [19]. The medium was renewed every 2 or 3 days. During treatment, the cells were exposed to the compounds in a medium supplemented by 2% FCS and 1% DMSO.

2.3. Preparation of microsomal fractions

Human liver tissue samples were homogenized in 50 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA. Microsomal fractions were the sediment and supernatant, respectively, from the last of three successive centrifugations at 4 °C (3000*g*, 10 min; 8000*g*, 20 min; and 30,000*g*, 60 min).

2.4. Cell viability

Cytotoxicity of malathion and isomalathion, was evaluated by the MTT spectrometry assay [17]. Briefly, after treatment, medium was removed and serum-free medium containing MTT (0.5 mg/ml) was added to each well and incubated for 2 h at 37 °C. After removal of the incubation solution, water-insoluble formazan was dissolved in DMSO and absorbance was measured at 550 nm. Data were expressed as the mean of three independent experiments.

2.5. Caspase-3 activity

After treatment with pesticides, differentiated HepaRG cells were harvested and stored as pellets at −80 °C. After cell lysis, 40 μg of protein was incubated with 80 μM Ac-DEVD-AMC in caspase-3 activity buffer (20 mM PIPES pH 7.2, 100 mM NaCl, 10 mM dithiotreitol, 1 mM EDTA, 0.1% CHAPS and 10% sucrose) at 37 °C for 1 h. Caspase 3-mediated cleavage of Ac-DEVD-AMC peptide was continuously measured by spectrofluorimetry using excitation/emission wavelengths of 380/440 nm [20].

2.6. Evaluation of oxidative stress

Cells were incubated for 2 h at 37 °C with 5 μM H2DCFDA; then they were washed with cold phosphate buffered saline (PBS), and scraped in a solution containing an equal volume of potassium buffer (10 mM, pH 7.4) and methanol (v/v), supplemented with 0.1% Triton X-100. Fluorescence intensity of cell extracts was determined by spectrofluorimetry using excitation/emission wavelengths of 498/520 nm.

2.7. Real time – quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from HepaRG cells with the SV total RNA isolation system (Promega, Madison, WI). Five hundred nanograms of total RNA were reversed–transcribed into cDNA using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). RT-qPCR was performed by the fluorescent dye SYBR green methodology using the SYBR green PCR master mix and the ABI Prism 7000 (Applied Biosystems). Primers pairs for each transcript are described in Table 1. Amplification curves were read with the ABI Prism 7000 SDS Software using the comparative cycle threshold method and the relative quantification of the steady-state mRNA levels was normalized against 18S RNA.

Table 1
List of primer sequences used in RT-qPCR.

Gene	Name	Forward primer	Reverse primer
CE1	Carboxylesterase 1	AGGTCCTGGGGAAGTATGCC	TGCATCTTGGGAGCACATAGG
CE2	Carboxylesterase 2	GGAGTGGTGTGAGAGATGCG	CAGGTTAGAGCCCTCACGG
CYP1A2	Cytochrome P450, family 1, subfamily A, polypeptide 2	TGGAGACCTTCCGACACTCCT	CGTTGTGTCCTTTGTTGTGC
CYP2B6	Cytochrome P450, family 2, subfamily B, polypeptide 6	TTCCTACTGCTTCCGTCTATCAAA	GTGCAGATTCCCACAGCTCA
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4	CTTCATCCAATGGACTGCATA	TCCCAAGTATAACAGCACTCTACACAGAC
HO1	Hemeoxygenase 1	ACTTTCAGAAGGGCCAGGT	TTGTTGCGCTCAATCTCCT
MnSOD	Manganese superoxide dismutase	GGGTTGGCTTGGTTTCAATA	CTGATTTGGACAAGCAGCAA
Nrf2	NF-E2-related factor 2	TCAGCATGCTACGTGATGAAG	TTTGCTGCAGGGAGTATTCA