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Different DNA damage response of *cis* and *trans* isomers of commonly used UV filter after the exposure on adult human liver stem cells and human lymphoblastoid cells

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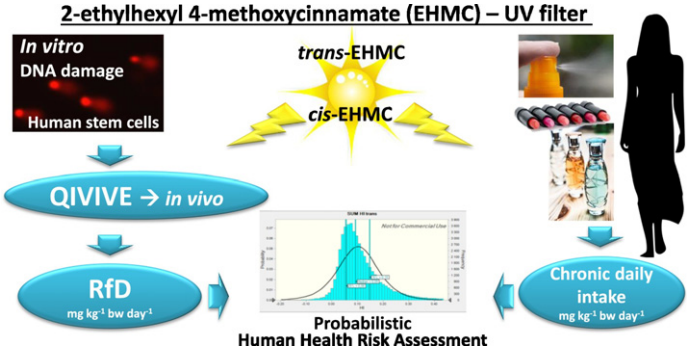
HIGHLIGHTS

- Photoisomerisation product – *cis*-EHMC was used in comet assay for the first time
- Adult human liver stem cells HLh-T1 were used for the first time in comet assay
- In the study, the QIVIVE approach for *in vitro* to *in vivo* extrapolation was used
- *cis*-EHMC may cause seven times higher risks in female population than *trans*-EHMC

GRAPHICAL ABSTRACT



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ABSTRACT

2-ethylhexyl 4-methoxycinnamate (EHMC), used in many categories of personal care products (PCPs), is one of the most discussed ultraviolet filters because of its endocrine-disrupting effects. EHMC is unstable in sunlight and can be transformed from *trans*-EHMC to emergent *cis*-EHMC. Toxicological studies are focusing only on *trans*-EHMC; thus the toxicological data for *cis*-EHMC are missing. In this study, the *in vitro* genotoxic effects of *trans*- and *cis*-EHMC on adult human liver stem cells HL1-hT1 and human-derived lymphoblastoid cells TK-6 using a high-throughput comet assay were studied.

TK-6 cells treated with *cis*-EHMC showed a high level of DNA damage when compared to untreated cells in concentrations 1.56 to 25 μg mL^{-1}. *trans*-EHMC showed genotoxicity after exposure to the two highest concentrations 12.5 and 25 μg mL^{-1}. The increase in DNA damage on HL1-hT1 cells induced by *cis*-EHMC and *trans*-EHMC was detected at the concentration 25 μg mL^{-1}. The No observed adverse effect level (NOAEL, mg kg^{-1} bw day^{-1}) was determined using a Quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) approach: $NOAEL_{trans\text{-}EHMC} = 3.07$, $NOAEL_{cis\text{-}EHMC} = 0.30$ for TK-6 and $NOAEL_{trans\text{-}EHMC} = 26.46$, $NOAEL_{cis\text{-}EHMC} = 20.36$ for HL1-hT1. The hazard index (HI) was evaluated by comparing the reference dose (RfD, mg kg^{-1} bw day^{-1}) obtained from our experimental data with the chronic daily intake (CDI) of the female population. Using

Abbreviations: EHMC, 2-ethylhexyl 4-methoxycinnamate; PCP(s), personal care product(s); UV, ultraviolet; NOAEL, No observed adverse effect level; QIVIVE, Quantitative *in vitro* to *in vivo* extrapolation; RfD, reference dose; CDI, chronic daily intake.

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comet assay experimental data with the more sensitive TK-6 cells, $HI_{cis\text{-}EHMC}$ was 7 times higher than $HI_{trans\text{-}EHMC}$. In terms of CDI, relative contributions were; dermal exposure route > oral > inhalation. According to our results we recommend the $RfD_{trans\text{-}EHMC} = 0.20$ and $RfD_{cis\text{-}EHMC} = 0.02$ for *trans*-EHMC and *cis*-EHMC, respectively, to use for human health risk assessment.
The significant difference in *trans*-EHMC and *cis*-EHMC response points to the need for toxicological reevaluation and application reassessment of both isomers in PCPs.

1. Introduction

Organic ultraviolet (UV) filters are used in sunscreens to protect humans against harmful UV rays. They are also used in many other kinds of personal care products (PCPs) to prevent the photodegradation of polymers and pigments (Ozáez, Martínez-Guitarte, and Morcillo, 2013). One of the most used and discussed substances from the family of organic UVB filters used in PCPs is 2-ethylhexyl 4-methoxycinnamate (EHMC). In Switzerland, EHMC was found in 51% of 116 analyzed PCPs (Manová, Goetz, and Hungerbuehler, 2015; Manová, von Goetz, Hauri, Bogdal, and Hungerbühler, 2013), while in the Danish market it was found in 59 products, including 14 sunscreens, from a total of 291 analyzed PCPs (The Danish Environmental Protection Agency, 2015). In a study from the UK it was found that use of EHMC in PCPs is decreasing (59% in 2005 and 17.8% in 2011; n = 337) (Kerr, 2011), but still EHMC is one of the most used UV-filters with potential endocrine-disrupting (ED) effects, which were confirmed in several studies *in vitro* (Jiménez-Díaz et al., 2013; Kunz and Fent, 2006) and *in vivo* (Axelstad et al., 2011; Carbone et al., 2010; Schreurs, Sonneveld, Jansen, Seinen, and van der Burg, 2005; Szwarcfarb et al., 2008; Tsui, Leung, Lam, and Murphy, 2014). EHMC is included in the priority list of ED active compounds in the European Commission database 2014, and was included also in the SIN database 2014, http://sinlist.chemsec.org/, in EDC DataBank (Montes-Grajales and Olivero-Verbel, 2015), and in the European Union's database of possible endocrine disruptors as a compound with limited knowledge about its health effects and worthy of further investigation based on accumulating evidence (Petersen, Rasmussen, and Gustavson, 2007). Except for endocrine disrupting effect, it was found that EHMC can cause genotoxicity also *in vitro* (Bonin et al., 1982; Nečasová, Bányiová, Literák, and Čupr, 2016).

EHMC can be found in several matrices of the environment all over the world, such as treated wastewater, leachate, marine water, lake sediments, and biota (ECHA, 2014; Thomas et al., 2015; Tsui et al., 2015). High EHMC concentrations were found during recreational peak season in the sea, where EHMC remained present even after the recreational period, which may hypothesize higher persistency in mussels in comparison with other UV-filters (Bachelot et al., 2012). EHMC was found in high concentration also in tap water and drinking water (Díaz-Cruz, Gago-Ferrero, Llorca, and Barceló, 2012; Loraine and Pettigrove, 2006) which suggests that conventional water treatment processes appear to be inefficient at removing the residues of UV filters. These kinds of compounds in drinking water are unregulated and should be routinely monitored. Further studies on the removal efficiency of PCPs should be carried out.

Although EHMC is widely present in aquatic ecosystems, its presence in human tissues, such as urine, blood, and breast milk (Janjua, Kongshoj, Andersson, and Wulf, 2008; Janjua et al., 2004; Markogiannaki, Andrianou, Kalyvas, and Andra, 2014; Schlumpf et al., 2010) correlates with consumer habits rather than with environmental exposure.

In the sun, EHMC can undergo an isomerization which might be connected with a decrease in UV-B filtering efficiency (Durand, Habran, Henschel, and Amighi, 2010; Pattanaargson, Munhapol, Hirunsupachot, and Luangthongaram, 2004). Previous studies reported photodegradation of EHMC in aqueous solution under sunlight irradiation which can lead to partly stable photoproducts (Jentzsch, Olsson, Westphal, Reich, and Leder, 2016). The isomerization causes also the transformation of parental form *trans*-EHMC to emergent *cis*-EHMC. To the best of our knowledge, the toxicological effects of the emergent isomer (*cis*-EHMC) have not been evaluated yet. As the *Cis*-EHMC is not commercially available, the technique to prepare the *cis*-isomer with achieved purity >98% was developed by Nečasová et al. (2016) and *cis*- and *trans*-EHMC were tested for their genotoxic potential using bacterial assays (Nečasová et al., 2016). It was found that *cis*-EHMC may represent a greater risks than *trans*-EHMC in the female population (Nečasová et al., 2016). Related research with a focus on the *cis*-EHMC isomer is necessary and urgent. EHMC, contained in many PCPs, is daily used by people, especially females. They can be exposed to *trans*-EHMC and after sun exposure also to emergent *cis*-EHMC which can cause higher risks than *trans*-EHMC. The research should be focused on human health impacts which might include *in vitro* experiments with human cell lines to predict possible effects at the organismal level.

Under our current state of knowledge, RfD for EHMC was determined only on animal data (Axelstad et al., 2011; ECHA, 2014; Klammer et al., 2007; Schneider et al., 2005). REACH and systems for safety registration of compounds prefer the use of *in vitro* models to determine NOAEL and RfD. The aim is to minimize animal testing and replace it using suitable alternative methods such as *in vitro*, *in silico*. To extrapolate experimental results *in vitro* to *in vivo*, there are 'Quantitative *in vitro* to *in vivo* extrapolation' (QIVIVE) approaches, which are currently an active research area providing an important tool in the light of animal testing restrictions such as in cosmetic area (Grech et al., 2016).

Human stem cells are characterized by their capacity of self-renewal and ability to differentiate into specialized cell types, and thus provide an attractive *in vitro* alternative and a potentially unlimited source of human cells, which could reduce the need for *in vivo* testing (Jennings, 2015). Among different types of stem cells (*e.g.* embryonic stem cells, induced pluripotent stem cells), adult stem cells, also known as somatic or tissue-specific stem cells, are being increasingly recognized as a suitable *in vitro* model to study tissue and organ specific physiological, pathophysiological processes or effects of drugs or toxic chemicals (Kang and Trosko, 2011; Nantasanti, de Bruin, Rothuizen, Penning, and Schotanus, 2016). The *In vitro* models based on normal non-cancerous adult human stem cells may more accurately predict *in vivo* toxicity than traditionally used cancer-derived or viral oncogene-immortalized cell lines, which might have lost or gained key characteristics due to substantial genetic and epigenetic alterations. The self-renewing ability and *in vitro* growth of adult stem cells can be an effective method to extend their proliferative capacity *in vitro* without causing cancer-associated changes or significantly altering phenotypic properties (Lee, Choi, and Ouellette, 2004).

In vitro models based on hTERT immortalized human adult stem cells (HL1-hT1) are a very promising alternative which is easy to standardize, amenable to high-throughput and detailed mechanistic studies (Jennings, 2015). Moreover, there is increasing evidence that adult stem cells represent the key population of cells within a tissue and their alterations by chemical exposures might be involved in the origin or development of various chronic toxicities and diseases (Canovas-Jorda, Louisse, Pistollato, Zagoura, and Bremer, 2014; Persano, Zagoura, Louisse, and Pistollato, 2015). Genotoxic damage and mutations