Food and Chemical Toxicology 59 (2013) 564–571

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

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

Inhibition of methyleugenol bioactivation by the herb-based constituent nevadensin and prediction of possible *in vivo* consequences using physiologically based kinetic modeling

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ARTICLE INFO

Article history:
Received 8 January 2013
Accepted 21 June 2013
Available online 4 July 2013

Keywords:
Methyleugenol
PBK
Nevadensin
Inhibition
Bioactivation
In vivo

ABSTRACT

Methyleugenol (ME) occurs naturally in a variety of spices, herbs, including basil, and their essential oils. ME induces hepatomas in rodent bioassays following its conversion to a DNA reactive metabolite. In the present study, the basil constituent nevadensin was shown to be able to inhibit SULT-mediated DNA adduct formation in HepG2 cells exposed to the proximate carcinogen 1′-hydroxymethyleugenol in the presence of nevadensin. To investigate possible *in vivo* implications of SULT inhibition by nevadensin on ME bioactivation, the rat physiologically based kinetic (PBK) model developed in our previous work to describe the dose-dependent bioactivation and detoxification of ME in male rat was combined with the recently developed PBK model describing the dose-dependent kinetics of nevadensin in male rat. The resulting binary ME–nevadensin PBK model was used to predict the possible nevadensin mediated reduction in ME DNA adduct formation and resulting carcinogenicity at the doses of ME used by the NTP carcinogenicity study. Using these data an updated risk assessment using the Margin of Exposure (MOE) approach was performed. The results obtained point at a potential reduction of the cancer risk when rodents are orally exposed to ME within a relevant food matrix containing SULT inhibitors compared to exposure to pure ME.

1. Introduction

Methyleugenol (ME) is a compound naturally occurring in a variety of spices and herbs, including clove, allspice, cinnamon leaves, walnuts, basil, nutmeg, anise, pimento, citronella, laurel fruits leaves and others, as well as in their essential oils (Smith et al., 2002). The general population is primarily exposed to ME via ingestion of food stuffs flavored with ME containing essential oils (Smith et al., 2010), including for instance, candy, cookies such as gingersnaps, ice cream, tomato ketchup and relish (Burdock, 1995; Leung, 1998). Realistic daily intake levels of ME have been estimated to range from 0.014 mg/kg bw/day (Smith et al., 2002) to 0.217 mg/kg bw/day (SCF, 2001). Given that at present addition of ME as a pure compound to individual food categories is no longer allowed within the European Union (European Commission (EC), 2008), the value of 0.014 mg/kg bw/day, resulting mainly from herbs and spices and their essential oils (Smith et al., 2002), is taken in the present study as the value for current levels of dietary human intake.

In 2000, the carcinogenicity of ME was investigated in mice and rats (NTP, 2000). The NTP reported that ME is carcinogenic in both rats and mice when dosed at high dose levels as pure compound suspended in 0.5% methylcellulose (NTP, 2000). ME is unreactive by itself but undergoes metabolic activation to produce an

Abbreviations: NTP, National Toxicology Program; SCF, Scientific Committee on Food; FEMA, Flavor and Extract Manufacturers Association; PBK, physiologically based kinetic; NEV, nevadensin; DMSO, dimethylsulfoxide; ME, methyleugenol; 1′ACME, 1′-acetoxymethyleugenol; 1′HME, 1′-hydroxymethyleugenol; 1′HMEG, 1′-hydroxymethyleugenol glucuronide; 1′HMES, 1′-sulfooxymethyleugenol; 1′OME, 1′-oxomethyleugenol; 7HC, 7-hydroxycoumarin; 7HCS, 7-hydroxycoumarin sulphate; 2′dG, 2′-deoxyguanosine; $^{15}N_5$-2′dG, $^{15}N_5$-2′-deoxyguanosine; ME-3′-N^2-dG, N^2-(*trans*-isomethyleugenol-3′-yl)-2′-deoxyguanosine; ($^{15}N_5$) ME-3′-N^2-dG, $^{15}N_5$-labeled N^2-(*trans*-isomethyleugenol-3′-yl)-2′-deoxyguanosine; E-3′-N^2-dG, N^2-(*trans*-isoestragol-3′-yl)-2′-deoxyguanosine; PCP, pentachlorophenol; SULT, sulfotransferase; NMWL, nominal molecular weight limit; L, liver; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; RT, retention time; hr, hour; min, minute; bw, body weight; K_i, inhibition constant.

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http://dx.doi.org/10.1016/j.fct.2013.06.043

electrophilic metabolite that acts as the DNA reactive intermediate (Miller et al., 1983; Randerath et al., 1984; Gardner et al., 1997; Smith et al., 2002). Fig. 1 displays the bioactivation pathway of ME which starts with hydroxylation of the 1′-position of ME to produce the proximate carcinogenic metabolite 1′-hydroxymethyleugenol (Miller et al., 1983; Gardner et al., 1997; Smith et al., 2002). In a next step, 1′-hydroxymethyleugenol can be sulfonated by SULT to form 1′-sulfoxymethyleugenol that can form DNA adducts (Smith et al., 2002). Recently, studies using *Salmonella typhimurium* TA100 strains expressing different human SULTs revealed that human SULT1A1 and SULT1C2 are specifically able to activate 1′-hydroxymethyleugenol to DNA reactive metabolites (Herrmann et al., 2012).

It is important to note that the carcinogenicity observed in studies in which animals are exposed to high dose levels of the pure compound by gavage may not be representative for the situation where humans are exposed to ME at low levels via dietary intake with a food matrix being present. For the related alkenylbenzene estragole it was actually demonstrated that other ingredients present in alkenylbenzene containing herbs or food items may inhibit the SULT mediated bioactivation of estragole to its ultimate DNA reactive 1′-sulfooxy metabolite (Jeurissen et al., 2008; Alhusainy et al., 2010). For instance a methanolic basil extract was shown to cause a dose dependent inhibition of DNA adduct formation in 1′-hydroxyestragole exposed human HepG2 cells (Jeurissen et al., 2008). In a subsequent study, the flavonoid nevadensin (Fig. 2) was identified as the potent SULT inhibitor present in these methanolic basil extracts (Alhusainy et al., 2010). So far the existence of such matrix dependent interactions with the bioactivation of alkenylbenzenes was only demonstrated for estragole (Alhusainy et al., 2010, 2012). Therefore the objective of the present study was to study the potential of nevadensin to inhibit the SULT mediated bioactivation and subsequent DNA adduct formation of ME using human HepG2 cells as an *in vitro* model. To obtain some insight in the *in vivo* relevance of these observations our physiologically based kinetic (PBK) model for bioactivation and detoxification of ME in male rat (Al-Subeihi et al., 2011) was combined with the recently developed PBK model for nevadensin in male rat (Alhusainy et al., 2013). This combined PBK model was previously validated using *in vivo* data on liver adduct formation in male Sprague–Dawley rats orally dosed with the structurally related alkenylbenzene estragole and nevadensin (Alhusainy et al., 2013).

Using this binary PBK model the effects of combined *in vivo* exposure to ME and nevadensin could be quantified as well given that the SULT inhibition by nevadensin is non-competitive in nature and therefore by definition independent of the nature of the SULT substrate.

2. Materials and methods

2.1. Materials

2′-Deoxyguanosine was purchased from Sigma (Basel, Switzerland), and 1,2,3,7,9-$^{15}N_5$-2′-deoxyguanosine was obtained from Cambridge Isotope Laboratories (Cambridge, MA). Fetal bovine serum (FBS), DMEM/F12 (L-glutamine, 15 mM HEPES) medium and phosphate buffered saline (PBS) (pH 7.4) were purchased from Gibco (UK). Dimethyl sulfoxide (DMSO), methanol, zinc sulphate (heptahydrate), phosphodiesterase I from *Crotalus adamanteus* (venom phosphodiesterase), phosphodiesterase II from bovine spleen (spleen phosphodiesterase), nuclease P1 and alkaline phosphatase were purchased from Sigma (Schnelldorf, Germany). Acetonitrile was purchased from Biosolve BV (Valkenswaard, The Netherlands). Formic acid and ethanol were obtained from VWR Merck (Darmstadt, Germany). Nevadensin was purchased from Apin Chemicals (Milton, UK). Pentachlorophenol (PCP; 98%) was obtained from Sigma-Riedel de Haen (Seelze, Germany). 1′-Hydroxymethyleugenol and 1′-acetoxymethyleugenol were synthesized as described previously (Al-Subeihi et al., 2011). Human HepG2 cells were purchased from the American type culture collection (Manassas, Virginia).

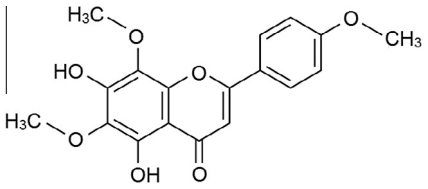

Fig. 2. Structural formula of nevadensin.

2.2. Synthesis of N^2-(trans-isomethyleugenol-3′-yl)-2′-deoxyguanosine (ME-3′-N^2-dG) and $^{15}N_5$-labeled N^2-(trans-isomethyleugenol-3′-yl)-2′-deoxyguanosine (($^{15}N_5$) ME-3′-N^2-dG)

ME-3′-N^2-dG was synthesized via a reaction between 1′-acetoxymethyleugenol and 2′-deoxyguanosine following the protocol of Punt et al. (2007). In brief, 250 μL of a 0.01 g/mL solution of 1′-acetoxymethyleugenol in DMSO were added to 2250 μL of 2.5 mM 2′-deoxyguanosine in 2.5 mM ammonium carbonate (pH 7.4). The same reaction was performed for the synthesis of ($^{15}N_5$) ME-3′-N^2-dG in which 1′-acetoxymethyleugenol was allowed to react with $^{15}N_5$-labeled 2′-deoxyguanosine. The incubations were stirred for 48 h at 37 °C. Both ME-3′-N^2-dG and ($^{15}N_5$) ME-3′-N^2-dG were purified by HPLC–UV on a M600 liquid chromatography system (Waters, Milford, MA) equipped with an Alltima C18 5 μm column, 150 × 4.6 mm (Alltech, Breda, The Netherlands). The gradient was made with ultrapure water and acetonitrile. The flow rate was 1 mL/min. A linear gradient was applied from 10% to 15% acetonitrile over 30 min after which the percentage of acetonitrile was increased to 100% over 2 min, kept at 100% for 1 min, lowered to 10% in 2 min, and kept at these initial conditions for 10 min for equilibration of the system. Detection was carried out using a Waters 966 photodiode array detector (Waters, Milford, MA) at 260 nm. Both ME-3′-N^2-dG and ($^{15}N_5$) ME-3′-N^2-dG were collected at a retention time of 11.4 min. The purity of both ME-3′-N^2-dG and ($^{15}N_5$) ME-3′-N^2-dG was more than 98%, according to LC-MS/MS and HPLC analyses.

2.3. Cytotoxicity test

The cytotoxicity of nevadensin, 1′-hydroxymethyleugenol, pentachlorophenol, and DMSO was evaluated using the MTT test (Mosmann, 1983; Hussain et al., 1993). HepG2 cells were plated in a 96-well plate at a density of 2×10^4 cells per well 24 h prior to exposure. Just before exposure the medium was removed and cells were washed with 100 μL HBSS. Cells were exposed to 0.5% DMSO (control) or the test compounds in exposure medium (DMEM/F12 + glutamax + 50 μg/mL gentamicin) at concentrations of nevadensin, 1′-hydroxymethyleugenol, and/or pentachlorophenol as indicated and added from respectively 5000, 1000, and 400 times concentrated stock solutions in DMSO for 4 h in a humidified atmosphere at 37 °C. Then, 5 μL of a 5 mg/mL MTT solution in PBS were added and the cells were incubated for another hour. Thereafter, the medium was removed and 100 μL of DMSO were added to all wells to dissolve the formazan crystals. The absorbance was measured at 562 nm and cell viability was defined as the ratio between the absorbance measured for nevadensin, 1′-hydroxymethyleugenol, pentachlorophenol or DMSO treated cell samples and the absorption measured for untreated cell samples.

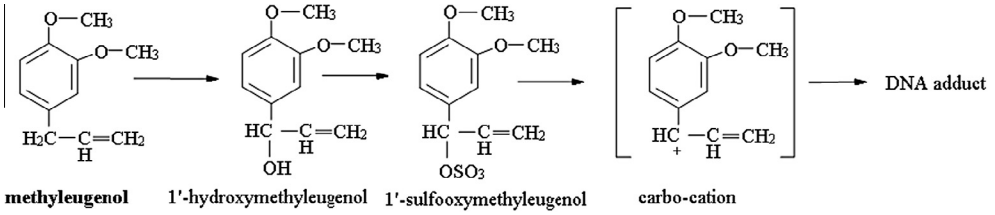

Fig. 1. Structural representation of the pathway for bioactivation of ME.