Food and Chemical Toxicology 109 (2017) 130–142

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

# Steviol, an aglycone of steviol glycoside sweeteners, interacts with the pregnane X (PXR) and aryl hydrocarbon (AHR) receptors in detoxification regulation

Jan Dusek [a, c], Alejandro Carazo [a, c], Frantisek Trejtnar [a, c], Lucie Hyrsova [a], Ondřej Holas [b], Tomas Smutny [a, c], Stanislav Micuda [d], Petr Pavek [a, c, *]

[a] Department of Pharmacology and Toxicology, Charles University, Faculty of Pharmacy in Hradec Kralove, Heyrovskeho 1203, Hradec Kralove, Czech Republic
[b] Department of Pharmaceutical Technology, Charles University, Faculty of Pharmacy in Hradec Kralove, Heyrovskeho 1203, Hradec Kralove, Czech Republic
[c] Centre of Drug-Dietary Supplements Interactions and Nutrigenetics, Charles University, Faculty of Pharmacy, Heyrovskeho 1203, Hradec Kralove, Czech Republic
[d] Department of Pharmacology, Charles University, Faculty of Medicine, Simkova, Hradec Kralove, Czech Republic

ARTICLE INFO

*Article history:*
Received 26 April 2017
Received in revised form
20 August 2017
Accepted 1 September 2017
Available online 5 September 2017

*Keywords:*
Food-drug interactions
Drug metabolism
Nuclear receptors
Steviol
Stevioside

ABSTRACT

*Stevia rebaudiana* Bertoni is a herb known for the high content of natural sweeteners in its leaves. Its main secondary metabolite stevioside is used as non-caloric sweetener.

No information, however, is available on whether stevioside or steviol interact with drug-metabolizing enzymes and pose the potential risk of food-drug interactions. Similarly, data are lacking on the interactions of steviol and stevioside with key nuclear receptors controlling the expression of the main drug metabolizing enzymes.

We studied the interactions of steviol and stevioside with the pregnane X (PXR), vitamin D (VDR), constitutive androstane (CAR), farnesoid X (FXR), glucocorticoid (GR) and aryl hydrocarbon (AHR) receptors, which control expression of genes of xenobiotic metabolism. In addition, the inhibitory activities of steviol and stevioside towards the major cytochrome P450 enzymes CYP3A4, CYP2C9, CYP2D6, CYP1A2 and CYP2B6 were evaluated *in vitro*.

We found that steviol moderately activated the PXR and AHR, resulting in the induction of their target genes including CYP3A4 and CYP1A2 in primary human hepatocytes. A weak inhibition of CYP3A4 and CYP2C9 with steviol was also found.

Our results provide mechanistic data indicating that stevioside and stevia sweeteners may have the potential to induce food-drug interactions, a finding that warrants future prospective clinical investigation.



## 1. Introduction

Pharmacokinetic drug-drug interactions (DDIs) can lead to undesired drug exposure resulting in insufficient efficacy or even toxicity. Similarly, different foodstuffs can affect the body's response to drugs and cause food-drug interactions (FDIs). The most important FDIs occur at the level of metabolism, due to either the induction or inhibition of drug metabolizing enzymes, mainly those of the cytochrome P450 superfamily (CYPs).

*Stevia rebaudiana* Bertoni is a perennial herb of significant economic importance due to the high content of natural, dietetically valuable sweeteners in its leaves (Ramesh et al., 2006; Wölwer-

*Abbreviations:* ADI, accepted daily intake; AHR, arylhydrocarbon receptor; CAR, constitutive androstane receptor; CYP, cytochrome P450; DDIs, drug-drug interactions; FDIs, food-drug interactions; FXR, farnesoid X receptor; GR, glucocorticoid receptor; GRAS, Generally Recognized As Safe; 3-MC, 3-methylcholanthrene; NR, nuclear receptor; PXR, pregnane X receptor; VDR, Vitamin D receptor.

* Corresponding author. Department of Pharmacology and Toxicology, and Centre of Drug-Dietary Supplements Interactions and Nutrigenetics, Charles University, Faculty of Pharmacy in Hradec Kralove, Heyrovskeho 1203, Hradec Kralove, Czech Republic.
*E-mail address:* pavek@faf.cuni.cz (P. Pavek).

http://dx.doi.org/10.1016/j.fct.2017.09.007
0278-6915/

Rieck, 2012). The most abundant steviol glycosides are stevioside and rebaudioside A, both of which have the highest content in the plant (5–10% and 2–4%, respectively) (Chatsudthipong and Muanprasat, 2009; Wölwer-Rieck, 2012). Stevioside, a diterpenoid glycoside, is a non-caloric sweetener, and is considered about 250–300 times sweeter than sucrose (Duke and deCellier, 1993). In many countries, stevioside and other stevioside glycosides are used as food additives (code E 960) in sweetened foodstuffs, candies, toothpaste, ice cream, chewing gum, soy sauce and beverages; they are also used as a sweetener in pharmaceutical products (Gardana et al., 2010; Jeppesen et al., 2002). Stevioside is hydrolyzed into steviol following ingestion by bacteria in the distal gastrointestinal tract. The aglycone steviol, but not stevioside itself, is then absorbed into the body. Other steviol glycosides are similarly hydrolyzed and absorbed as steviol in humans (EFSA., 2015; Chatsudthipong and Muanprasat, 2009).

Due to its wide and ever-increasing consumption, the toxicology of stevioside has been extensively studied (see the latest dossier report from the 69th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2009). Highly purified steviol glycosides have repeatedly received Generally Recognized As Safe (GRAS) status from the US Food and Drug Administration in the past (FDA). JECFA has proposed the accepted daily intake (ADI) of stevioside at up to 4 mg/kg body weight expressed as steviol, which was recently accepted by the European Commission (Authority, 2011; JECFA, 2009) although the latest animal and clinical data suggest that a higher ADI of between 6 and 16 mg/kg could be justified (Roberts et al., 2016).

Pharmacokinetic interactions can occur as changes in the activity of the metabolic enzymes via nuclear receptor mediated-induction or due to cytochrome P450 enzyme inhibition (Isoherranen et al., 2012; Pavek and Smutny, 2014; Sinz, 2013). The pregnane X receptor (PXR) is a member of the nuclear receptor (NR) superfamily of ligand-activated transcription factors, with PXR having been found to bind a huge variety of endobiotics and xenobiotics including many clinical drugs, herbal components and dietary compounds (Pavek and Smutny, 2014; Smutny et al., 2013). Cytochrome P450 3A4 (CYP3A4) and several other CYP isoforms, such as CYP2B6 and CYP2Cs, phase II enzymes (e.g. UGT1A1) and transporters (e.g. ABCB1/MDR1), are induced through PXR activation. Other "xenobiotic sensors" activated by drugs, xenobiotics and food ingredients such as the constitutive androstane receptor (CAR, NR1I3) and the aryl hydrocarbon receptor (AHR) control mainly CYP2B6 and CYP1 family enzymes, respectively (see reviews (Pavek and Smutny, 2014; Stejskalova et al., 2011a,b)).

Recently, the artificial sweeteners aspartame, acesulfame, cyclamate and saccharin have been tested for interactions with the AHR and GR. These sweeteners have been shown to be safe in this regard, i.e. with no interactions with the tested receptors (Kamenickova et al., 2013). Nevertheless, data have never been reported on interactions of stevioside and its principal metabolite steviol with the PXR, CAR, FXR and VDR. Similarly, interactions of stevioside and steviol with dominant cytochrome P450 enzymes have so far never been addressed in the scientific literature.

## 2. Materials and methods

### 2.1. Chemicals

Stevioside hydrate (sc-272502, Lot NoA1411) and steviol (sc-473800) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Steviol hydrate (Cat. No. H8664, purity ≥98%) was also obtained from Sigma-Aldrich. Rifampicin (a prototypical ligand of the human PXR), 6-ethylchenodeoxycholic acid (6-ECDCA, a prototypical ligand of the human FXR), 6-(4-chlorophenyl)imidazo [2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime, CITCO, a prototypical ligand of the human CAR), dexamethasone (a prototypical ligand of GR), 1α,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2$vit $D_3$, a prototypical ligand of VDR), 3-methylcholanthrene (3-MC, a prototypical ligand of AHR), α-naphthoflavone (a prototypical inhibitor of CYP1A enzymes), ketoconazole (a prototypical inhibitor of CYP3A4), fluconazole (a prototypical inhibitor of CYP2C9), tiklopidine (a prototypical inhibitor of CYP2B6), quinidine (a prototypical inhibitor of CYP2D6), dimethyl sulfoxide (DMSO), 7-ethoxyresorufin, dicumarol, and human recombinant insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM media and 0.5% trypsin solution were purchased from ThermoFisher Scientific/Invitrogen/Gibco (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from PAA (Pasching, Austria) or from GE Healthcare Life Sciences (Hyclone™ FBS, Logan, UT). Phenol red-free OPTI-MEM® medium and penicillin/streptomycin were purchased from Invitrogen/ThermoLife Technologies (Waltham, MA, USA). All other chemicals were of the highest quality that was commercially available. Stock solutions (1,000x) were prepared in DMSO.

### 2.2. Cell lines

The human Caucasian hepatocellular carcinoma (HepG2), human hepatocellular carcinoma (Huh7), Caucasian colon adenocarcinoma (CACO2), human choriocarcinoma (JEG3) and cholangiocarcinoma hepatic (HepaRG) cell lines were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). HepG2 cells were maintained in antibiotic-free Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% non-essential amino acids (NEAA). Huh7 were maintained in antibiotic-free DMEM with 10% FBS. JEG3 cells were maintained in antibiotic-free DMEM with 10% FBS, 1% L-glutamine and 1% NEAA. CACO2 cells were maintained in antibiotic-free Minimum essential medium (MEM) with 20% FBS at 37 °C. HepaRG cells were maintained in William's medium E with 10% Hyclone™ FBS, 1% L-glutamine, 100 U/100 μg per mL of penicillin/streptomycin, 5 μg/mL human recombinant insulin and $5 \times 10^{-5}$ M hydrocortisone, at 37 °C in a humidified atmosphere containing 5% $CO_2$. To differentiate HepaRG cells into hepatocyte-like cells, they were cultured for at least four weeks without trypsination and in the last two weeks in medium also containing 1.5% DMSO to stimulate hepatocyte-phenotype differentiation (Hyrsova et al., 2016). The stably transfected gene reporter cell line AZ-AHR generated from HepG2 cells and using the DRE-luc construct is from here on referred to by the term DRE-luc cells (Novotna et al., 2011). DRE-luc cells have been cultivated in DMEM with 10% FBS and 1% NEAA medium.

MTS-based cell proliferation assay has been performed according to manufacture's protocol described in Supplementary Information (CellTiter 96 $AQ_{ueous}$ Non-Radioactive Cell Proliferation Assay, Promega, Hercules, CA).

### 2.3. Primary cultures of human hepatocytes

Human long-term hepatocytes in monolayer (Batch HEP220913 and HEP220944, Biopredic International, Saint Grégoire, France) were prepared from livers of a 69-year-old Caucasian male and a 72-year-old female. The medium was replaced with serum-free medium the day after delivery of primary human hepatocytes, and the cultures were allowed to stabilize for an additional 48 h prior to treatment. The cultures were maintained at 37 °C in 5% $CO_2$ in a humidified incubator and treated with stevioside and steviol at indicated concentrations together with prototype PXR and AHR ligands.