ABSTRACT:

Carnosic acid is a phenolic diterpene isolated from rosemary (Rosmarinus officinalis), which may have anticancer, antiadipogenic, and anti-inflammatory properties. Recently, carnosic acid was shown to prevent weight gain and hepatic steatosis in a mouse model of obesity and type II diabetes. Based on these results, carnosic acid has been suggested as a potential treatment for obesity and nonalcoholic fatty liver disease; however, little is known about the safety of carnosic acid at doses needed to elicit a pharmacological effect. For this reason, hepatotoxicity and cytochrome P450 inhibition and induction studies were performed in primary human hepatocytes and microsomes. Measuring cellular ATP, carnosic acid showed a dose-dependent increase in hepatotoxicity with an EC50 value of 94.8 ± 36.7 μM in three human hepatocyte donors without a concurrent increase in the apoptosis markers caspase-3/7. In human liver microsomes, carnosic acid did not exhibit significant time-dependent inhibition for any of the cytochrome P450 enzymes investigated, although it did inhibit CYP2C9- and CYP3A4-catalyzed reactions with Ki values of 9.2 and 4.3 μM, respectively. Carnosic acid also induced CYP2B6 and CYP3A4 mRNA and enzyme activity in a dose-dependent manner. At 10 μM, carnosic acid increased CYP2B6 enzyme activity 61.6 and 49.3% in two donors compared with phenobarbital, and it increased CYP3A enzyme activity 82.6 and 142% compared with rifampicin. These results indicate the potential for drug interactions with carnosic acid and illustrate the need for an appropriate safety assessment before being used as a weight loss supplement.

Introduction

Carnosic acid [(CA) (4αR,10αS)-5,6-dihydroxy-1,1-dimethyl-7-propan-2-yl-2,3,4,9,10,10a-hexahydrophenanthrene-4a-carboxylic acid; Supplemental Fig. 1] is a phenolic diterpene found in the leaves of the rosemary plant (Rosmarinus officinalis) and is used routinely as a food and cosmetic additive due to its antioxidant and antimicrobial properties (Opdyke, 1974; Aguilar et al., 2008). In addition, CA has demonstrated anti-inflammatory, anticancer, photoprotective, and antiangiogenetic activities in vitro (Reuter et al., 2007; Russo et al., 2009; Takahashi et al., 2009; Yesil-Celiktas et al., 2010; Tsai et al., 2011). Recently, CA was shown to induce significant weight loss and reduced visceral adiposity in ob/ob mice fed a diet supplemented with carnosic acid (Wang et al., 2011). A significant reduction in serum triglyceride and cholesterol and improved glucose tolerance were also observed. These observations led to an opinion article suggesting that carnosic acid may be a new treatment option for patients with nonalcoholic fatty liver disease or the metabolic syndrome (Greenhill, 2011).

Carnosic acid as a food additive has a good safety profile and does not pose a health concern (Aguilar et al., 2008). However, little is known about the safety of carnosic acid at doses that are needed to achieve therapeutic effect for weight loss. At concentrations shown to elicit weight reduction and antiadipogenesis in mice and rats, no adverse effects were reported (Ibarra et al., 2011; Wang et al., 2011). However, in a separate study, rats treated with a rosemary extract showed hepatomegaly, anemia, and reduction in fetus body weight (de Oliveira Guerra et al., 2009). Whether these effects are due to carnosic acid or another constituent is currently unknown.

Carnosic acid as a therapy for human disease and weight loss, although attractive, poses many challenges. To date, there are no publicly available, adequate, and well controlled data on safety and efficacy of carnosic acid to treat any human diseases. Because it is categorized as a dietary supplement, relevant safety and efficacy studies at therapeutic doses probably will not be performed as would be for new drug entities developed by the pharmaceutical industry. Carnosic acid is also readily available without prescription and will probably not be administered under the supervision of a healthcare provider. Therefore, individuals are free to self-dose with little guidance. For these reasons, the current in vitro safety study was carried out to determine the extent of hepatotoxicity and drug interactions with carnosic acid. Based on our observations, we predict that carnosic acid has the potential to cause drug-drug interactions (DDIs) due to cytochrome P450 (P450) induction.

Materials and Methods

Reagents and Chemicals. With the exception of cytotoxicity assays listed below, the source of all reagents and chemicals used in this study have been previously published (Dickmann et al., 2011; VandenBrink et al., 2011).
Cell Culture. Human hepatocytes were plated and cultured according to the methods described in Dickmann et al. (2011). Cells were treated the day after plating. Media plus appropriate concentrations of carnosic acid or controls were replaced daily for cytochrome P450 induction studies.

Cytotoxicity Assays. Lactate dehydrogenase (LDH) levels were measured using the CytoTox-ONE Homogeneous Membrane Integrity Assay from Promega (Madison, WI) according to the manufacturer’s protocol using 30 μl of media from treated cells at 4 h. Values were compared with 50 μl of media from cells lysed using the supplied lysis buffer, which represents maximal LDH release. ATP levels were measured using the CellTiter-Glo Luminescence Assay from Promega at 4 and 24 h of treatment using adhered cells and 100 μl of media. An ATP standard curve (0.01–10 μM) was performed, and all samples fell within the linear range. Caspase-3 and caspase-7 levels were measured using the Caspase-Glo 3/7 Assay, which measures activities of both caspases, according to manufacturer’s protocol using 100 μl of media plus adhered cells. A positive control standard curve was run with purified caspase-3 purchased from Enzo Life Sciences (Farmingdale, NY). Tamoxifen was used as a positive control for all experiments with staurosporine as an added positive control for the caspase-3/7 experiments. Fluorescence and luminescence were measured on a Tecxan Safire (San Jose, CA).

Cytochrome P450 Activity Assays in Hepatocyte Culture. After a 48-h incubation with drug [carnosic acid, rifampin, or phenobarbital (PB)], cells were washed with 100 μl of Krebs-Henseleit buffer (KHB). Cells were then incubated with KHB containing either 250 μM bupropion for 30 min or 150 μM testosterone for 20 min. KHB containing probe substrate and metabolites was removed and frozen at −70°C until analysis.

Inhibition Assays and Liquid Chromatography/Tandem Mass Spectral Analysis. All conditions for cytochrome P450 inhibition assays, metabolite quantitation, and calculations of remaining enzyme activity and inhibition constants have been previously published (VandenBrink et al., 2011). In brief, the time-dependent inhibition experiment was performed as a two-step transfer assay. In Assay 1, the inactivation assay contained 1.0 mg/ml microsomes, 10 μM CA, and 100 mM potassium phosphate buffer and is initiated with NADPH; after given time periods (10 s, 1 min, 2 min, 5 min, 10 min, 15 min, and 30 min), 10 μl of the inactivation assay (minimizing competitive inhibition) was transfer to an P450 activity assay (total volume, 250 μl). The per-Downloaded from dm...
CYP2B6 and CYP3A4 mRNA levels. The reason for this is unknown, as both control inducers and carnosic acid caused increased CYP2B6 and CYP3A activity in donor 083111. Significant differences were indicated by the following: *, p < 0.05 or ***, p < 0.001.

Carnosic acid caused toxicity in cultured human hepatocytes with EC_{50} values similar to tamoxifen after 4 h of treatment. After 24 h of treatment, EC_{50} values for carnosic acid toxicity generally remained constant, whereas those for tamoxifen were decreased approximately 3-fold. This result could be due to different mechanisms of toxicity. Tamoxifen has been shown to induce toxicity through apoptosis followed by secondary necrosis (Mandlekar and Kong, 2001).

Discussion

There is increasing awareness among physicians and researchers that dietary supplements or "nutraceuticals" may pose safety risks to individuals due to liver toxicity and drug interactions. For instance, usnic acid, a dietary supplement promoted for weight loss, was linked to 21 reported cases of hepatotoxicity, seven of which resulted in complete liver failure (Favreau et al., 2002; Frankos, 2005). As such, the FDA issued a warning against using supplements containing usnic acid. Many dietary supplements also cause significant drug interactions resulting in either inhibition or induction of cytochrome P450 enzymes. Hyperforin, a major component of St. John’s wort, is a potent CYP3A4 inducer and can cause clinically significant drug interactions with many CYP3A4 substrates (Piscitelli et al., 2000; Hebert et al., 2004; Morimoto et al., 2004), whereas constituents isolated from black cohosh and licorice have been shown to inhibit CYP3A metabolism (Tsukamoto et al., 2005a,b). Due to the widespread and unregulated use of dietary supplements to treat human illness, more research around their potential to cause hepatotoxicity and drug interactions is needed.

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FIG. 2. Dose response for caspase-3/7 activity in human hepatocytes (donor OHO) treated with either carnosic acid or tamoxifen for 4 h. Caspase-3/7 activity was measured as described under Materials and Methods. Error bars represent the S.D. of three individual wells. The EC_{50} for caspase-3/7 production by tamoxifen was 87.9 \mu M. An EC_{50} for caspase-3/7 production by carnosic acid could not be determined. Significant differences are indicated by the following: *, p < 0.05 or ***, p < 0.001.

FIG. 3. mRNA expression of select phase I and II drug-metabolizing enzymes and drug transporters from human hepatocytes (donor Hu4199) treated with 0.1, 1.0, and 10 \mu M CA for 48 h. RNA was processed and analyzed according to the protocols described under Materials and Methods using ribosomal 18s RNA as an endogenous control. Values are expressed relative to vehicle-treated cells, and the error bars represent the S.D. of three individual wells. Significant differences are indicated by the following: *, p < 0.05; **, p < 0.01; or ***, p < 0.001, compared with vehicle treatment.
ATP in combination with the relative lack of caspase-3/7 activity or LDH release after 4 h of treatment suggests that carnosic acid acts as a mitochondrial toxin, although more work is needed to establish the exact mechanism of carnosic acid toxicity in hepatocytes. Our data are in contrast to a recent report suggesting that carnosic acid induces apoptosis in a neuroblastoma cell line (Tsai et al., 2011), although cell type and length of exposure are possible explanations for the discrepancy. Although the EC$_{50}$ of cellular toxicity is in the 50 to 100 µM range, it could be asserted that concentrations this high are viable in patients due to the lack of professional dosing guidance for dietary supplements. In patients presenting with hepatotoxicity and acute liver failure from usnic acid, 1350 mg/day were being ingested, which was the recommended dose based on average body weight, total plasma volume, and 65% bioavailability, maximal plasma levels could likely achieve those concentrations shown to elicit weight loss and antiadipogenic concentrations in these animals would exceed 100 µM CA for 48 h. Values are expressed relative to vehicle-treated cells (A and C) or as the percentage of positive control (B and D) with error bars representing the S.D. of three individual wells. A and B, hydroxybupropion formation. C and D, 6β-hydroxytestosterone formation. The dashed line in B and D indicates the 40% of positive control demarcation. Metabolite quantitation is described under Materials and Methods. Significant differences are indicated by the following: **, p < 0.01 or ***, p < 0.001, compared with vehicle treatment.

Carnosic acid clearly exhibits a drug interaction potential with CYP2A4 substrates and to a lesser extent with CYP2B6 substrates due to up-regulation of these enzymes. Following the FDA draft guidance recommendations, if carnosic acid were a new drug entity, then the in vitro data would likely trigger a clinical DDI study given that circulating plasma levels could likely achieve those concentrations shown to elicit cytochrome P450 induction in vitro (Huang, 2006). The mechanism of CYP3A4 and CYP2B6 induction is currently unknown, although it likely occurs via the pregnane X receptor, constitutive androstane receptor, or both. There is also potential for carnosic acid to cause drug interactions based on CYP3A4 and, to a lesser extent, CYP2C9 inhibition based on carnosic acid inhibition constants for these enzyme-mediated reactions. It cannot be ruled out at this time that a carnosic acid metabolite is responsible for cytochrome P450 induction, inhibition, or cellular toxicity because metabolite identification and rates of formation have not been established, and of course the probability for carnosic acid to cause clinical drug interactions or hepatotoxicity is dependent on dose and human metabolism and pharmacokinetic characteristics of the drug, which at this time are unknown.

Although carnosic acid may have some desirable pharmacodynamic characteristics, caution is warranted when taking this compound with CYP2B6 or CYP3A4 substrates. Appropriate safety margins also need to be established before this compound could be a viable weight loss supplement or treatment for nonalcoholic fatty liver disease.

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Participated in research design: Dickmann, Lin, and VandenBrink.
Conducted experiments: Dickmann and VandenBrink.
Performed data analysis: Dickmann, Lin, and VandenBrink.
Wrote or contributed to the writing of the manuscript: Dickmann, Lin, and VandenBrink.
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liquid chromatography method for determination of carnosic acid in rat plasma and its

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**In Vitro** Hepatotoxicity and Cytochrome P450 Induction and Inhibition Characteristics of Carnosic Acid, a Dietary Supplement with Anti-adipogenic Properties

Leslie J Dickmann, Brooke M. VandenBrink, and Yvonne S. Lin

*Drug Metabolism and Disposition*

**Supplemental Figure 1.** Chemical structure of carnosic acid ((4aR,10aS)-5,6-dihydroxy-1,1-dimethyl-7-propan-2-yl-2,3,4,9,10,10a-hexahydrophenanthrene-4a-carboxylic acid).
**Supplemental Figure 2.** Cytotoxicity in three cryopreserved human hepatocyte donors (BD228, Hu4199, and Hu4237) exposed to carnosic acid for 24 hours. Cells were cultured and total cellular ATP was measured as described in the Materials and Methods section.

DMSO = Dimethylsulfoxide; CA = Carnosic Acid. Significant differences are indicated by: *p<0.05, **p<0.01, or ***p<0.001 compared to vehicle treated cells.
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Drug Metabolism and Disposition

Supplemental Figure 3. Dose response curves for (A) Donors 082531 and (B) 083111. ATP measurements were performed and data fit as described in the Materials and Methods section. Significant differences are indicated by: *p<0.05, **p<0.01, or ***p<0.001.
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Drug Metabolism and Disposition

Supplemental Figure 4. mRNA levels measured in human hepatocytes exposed to either 1 mM phenobarbital (PB), 10 µM rifampicin (RIF), or 0.1, 1.0, or 10 µM carnosic acid (CA) for 48 hours. Values are expressed relative to vehicle treated cells, and the error bars represent the standard deviation of three individual wells. RNA was processed and analyzed according to protocols described in the Materials and Methods section using ribosomal 18s RNA as an endogenous control. (A) Donor 082511. (B) Donor 083111. Significant differences are indicated by: *p<0.05, **p<0.01, or ***p<0.001 compared to vehicle treated cells.
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Supplemental Table 1. Inhibition of cytochrome P450 activity by carnosic acid and selective cytochrome P450 inhibitors in pooled human microsomes. The concentration of carnosic acid used in the TDI assays was 10 μM. Full assay details can be found in the Materials and Methods section.

<table>
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<tr>
<th>Reversible Inhibition*</th>
<th>Time-Dependent Inhibition:</th>
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<tr>
<td><strong>P450 Isoform</strong></td>
<td><strong>IC₅₀/Kᵢ (μM)</strong></td>
</tr>
<tr>
<td>CYP1A2</td>
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</tr>
<tr>
<td>CYP2B6</td>
<td>&gt;30/nd</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>&gt;30/nd</td>
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<tr>
<td>CYP2D6</td>
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<tr>
<td>CYP2E1</td>
<td>&gt;30/nd</td>
</tr>
<tr>
<td>CYP3A</td>
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*nd = not determined