Short Communication

In Vitro Hepatotoxicity and Cytochrome P450 Induction and Inhibition Characteristics of Carnosic Acid, a Dietary Supplement with Antiadipogenic Properties

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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 EC_{50} 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 P450 enzymes investigated, although it did inhibit CYP2C9- and CYP3A4-catalyzed reactions with K_{i} 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) (4aR,10aS)-5,6-dihydroxy-1,1-dimethyl-7-propan-2-yl-2,3,4,9,10,10a-hexahydrophenanthrene-4α-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 antiangiogenic 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 publically 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).

ABBREVIATIONS: CA, carnosic acid; DDI, drug-drug interaction; P450, cytochrome P450; LDH, lactate dehydrogenase; PB, phenobarbital; KHB, Krebs-Henseleit buffer; FDA, U.S. Food and Drug Administration; RIF, rifampicin.
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 50 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 Tecan 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 mM 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) of media. The caspase-3/7 activity was measured using the Caspase-Glo 3/7 Assay (Fig. 1). In Assay 2, the P450 activity assay contained selective P450 probes (e.g., phenacetin for CYP1A2), 10 nM NADPH, and 100 mM potassium phosphate buffer, the activity assays were run for 10 min, and samples were prepared for liquid chromatography/tandem mass spectrometry analysis. The control reactions were the 10-s time points from the inactivation assay; therefore, the control reactions were the complete reaction mixture at essentially time 0. The percentage of the control numbers in Table 1 was based off of these controls. For example, if at 10 s the P450 activity was 20 (relative number) and at time 30 min the activity was 10 (relative number), then the percentage of control would be 50%. It is also important to note that control reactions without CA were run to ensure that there was no nonspecific loss of P450 activity (e.g., P450 heme destruction caused by NADPH-dependent lipid peroxidation during a 30-min incubation). If there was greater than 25% loss of activity over 30 min, then the earlier time of 15 min was used for the calculation; however, this was not the case over the course of these experiments in human liver microsomes.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Calculated EC50 values for human hepatocyte cytotoxicity due to treatment with either carnosic acid or tamoxifen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>Carnosic Acid</td>
</tr>
<tr>
<td>082511 (fresh, ATP)</td>
<td>4 h</td>
</tr>
<tr>
<td>083111 (fresh, ATP)</td>
<td>102</td>
</tr>
<tr>
<td>OHO (cryo, ATP)</td>
<td>127</td>
</tr>
<tr>
<td>OHO (cryo, LDH)</td>
<td>56.8</td>
</tr>
<tr>
<td>N.D.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

N.D., not determined. Data could not be fit to an inhibitor vs. dose-response model; N.A., not applicable; cryo, cryopreserved. Data were not obtained for a 24-h time point.

mRNA Analysis. mRNA isolation, quantitation, reverse transcription, real-time polymerase chain reaction, and data analyses have been previously published (Dickmann et al., 2011).

Statistics. Values were expressed as mean ± S.D. and compared using analysis of variance followed by a Bonferroni posttest. For clarity, only comparisons against the appropriate control group are shown. Statistical analyses were performed using Stata/SE, version 10 (StataCorp LP, College Station, TX). A p value < 0.05 was considered statistically significant.

Results

Carnosic Acid Cytotoxicity. Cultured cryopreserved human hepatocytes from three donors were exposed to increasing amounts of carnosic acid for 24 h. Untreated hepatocyte morphology demonstrated good cell-to-cell formation, compact nuclei, and clear cytoplasm. In cells treated with high concentrations of carnosic acid (50–100 μM), nuclei were not observed and the cytoplasm appeared granular and filled with vacuoles (data not shown). ATP measurements (Supplemental Fig. 1) and cell morphology (data not shown) indicated that 100 μM carnosic acid decreased cell viability by less than 10% of untreated or vehicle control-treated cells. As measured by ATP levels, a full dose-response curve revealed a viability EC50 of 95.7 ± 35.6 μM at 4 h and 94.8 ± 36.7 μM at 24 h for carnosic acid compared with 71.4 ± 30.3 μM at 4 h and 34.4 ± 19.2 μM at 24 h for tamoxifen (Table 1; Fig. 1; Supplemental Fig. 3). An LDH assay was also performed in one donor at 4 h to assess membrane integrity (Fig. 1; Table 1). Carnosic acid did not cause an appreciable increase in LDH such that a cytotoxicity EC50 value could not be estimated, whereas the EC50 of tamoxifen was 67.6 μM. The caspase-3/7 assay performed after 4 h of treatment indicated a dose-dependent increase in caspase-3/7 signal from tamoxifen; however, no increase was observed with carnosic acid at all concentrations tested (Fig. 2).

Cytochrome P450 Induction. Human cryopreserved hepatocytes exposed to 0.1, 1.0, and 10 μM carnosic acid for 48 h were screened for induction of selected phase I and II drug-metabolizing enzymes and drug transporters (Fig. 3). Carnosic acid (10 μM) resulted in a 13.9-fold increase in CYP3A4 mRNA, a 4.5-fold increase in CYP2B6 mRNA, and a 2.4-fold increase in SULT2A1 mRNA. Induction of
CYP2B6 and CYP3A4 mRNA levels. The reason for this is unknown, both control inducers and carnosic acid compared with increases in

Increases in CYP2B6 and CYP3A activity in donor 083111 were low for

mM phenobarbital (Fig. 4A). CYP3A activity was increased by 14.6-

activity, respectively, compared with a 7.5- and 2.9-fold increase by 1

carnosic acid resulted in a 5- and 1.9-fold increase in CYP2B6

hydroxytestosterone (CYP3A activity) were also increased after treat-

CYP2B6 and CYP3A4 mRNA and activity was further investigated in

two additional hepatocyte donors with carnosic acid, and the U.S. Food and Drug Administration (FDA) recommended CYP2B6 and CYP3A4 positive control inducers, PB and rifampicin (RIF). In donor 082511, 1 mM PB and 10 μM RIF resulted in a 19.3-fold increase in CYP2B6 mRNA and a 55.9-fold increase in CYP3A4 mRNA, whereas 10 μM carnosic acid resulted in a 4.2- and 33.2-fold increase in CYP2B6 and CYP3A4 mRNA, respectively (Supplemental Fig. 4A). In a second donor (083111), 1 mM PB and 10 μM RIF resulted in a 29.1-fold increase in CYP2B6 mRNA and a 12.3-fold increase in CYP3A4 mRNA, whereas 10 μM carnosic acid resulted in a 3.6- and 5.6-fold increase in CYP2B6 and CYP3A4 mRNA, respectively (Supplemental Fig. 4B).

Formation of hydroxybupropion (CYP2B6 activity) and 6β-hydroxytestosterone (CYP3A activity) were also increased after treatment with carnosic acid. In donors 082511 and 083111, 10 μM carnosic acid resulted in a 5- and 1.9-fold increase in CYP2B6 activity, respectively, compared with a 7.5- and 2.9-fold increase by 1 mM phenobarbital (Fig. 4A). CYP3A activity was increased by 14.6- and 2.4-fold in donors 082511 and 083111, respectively, by 10 μM carnosic acid compared with 17.5- and 2.0-fold by 10 μM RIF (Fig. 4C). Increases in CYP2B6 and CYP3A activity in donor 083111 were low for both control inducers and carnosic acid compared with increases in CYP2B6 and CYP3A4 mRNA levels. The reason for this is unknown, although data pertaining to this donor were used in the current analysis because the overall response of CYP2B6 and CYP3A activity induction by phenobarbital and RIF was greater than 2-fold, respectively. The percentage of CYP2B6 activity for 10 μM carnosic acid based on the phenobarbital positive control was 61.6 and 49.3% for donors 082511 and 083111, respectively, and the percentage of CYP3A activity based on the rifampicin control was 82.6 and 142% (Fig. 4, B and D).

Carnosic acid did not inhibit cytochrome P450 enzyme activity to any appreciable extent for the isoforms surveyed (Supplemental Table 1). Time-dependent inhibition of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A by carnosic acid was less than 11% after a 30-min incubation, although carnosic acid did show an inhibitory effect toward CYP2C9- and CYP3A4-catalyzed reactions with \( K_i \) values of 9.2 and 4.3 μM, respectively.

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.

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). The reduction of
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 EC₅₀ 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 according to manufacturers (Sanchez et al., 2006).

According to the European Food Safety Authority (Aguilar et al., 2008), the predicted maximal exposure to carnosic acid as a food additive is approximately 0.2 mg/kg body weight/day. Based on average body weight, total plasma volume, and 65% bioavailability, maximal plasma concentrations are calculated at approximately 3 nM. Therefore, at concentrations used as a food additive, it is assumed that carnosic acid levels would not cause significant hepatotoxicity or DDI. However, at doses needed to elicit statistically significant weight loss and antiadipogenic effects in ob/ob mice (Wang et al., 2011), it is calculated that plasma concentrations in these animals would exceed 100 μM based on the published pharmacokinetic parameters of carnosic acid in the rat (Yan et al., 2009; Doolaege et al., 2011). This concentration is equal to or greater than the carnosic acid concentrations used in the current human hepatocyte studies.

Carnosic acid clearly exhibits a drug interaction potential with CYP3A4 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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Authorship Contributions

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.
References


Greenhill C (2011) Liver: carnosic acid could be a new treatment option for patients with NAFLD or the metabolic syndrome. Nat Rev Gastroenterol Hepatol 8:122.


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TOXICITY AND P450 INDUCTION AND INHIBITION BY CARNOSIC ACID

1267


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.
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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*Drug Metabolism and Disposition*

**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>
<thead>
<tr>
<th>Reversible Inhibition*: P450 Isoform</th>
<th>IC₅₀/Kᵢ (μM)</th>
<th>Time-Dependent Inhibition: P450 Isoform</th>
<th>%Control (30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>&gt;30/nd</td>
<td>CYP1A2</td>
<td>89.2%</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>&gt;30/nd</td>
<td>CYP2B6</td>
<td>89.3%</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>&gt;30/nd</td>
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<tr>
<td>CYP2C9</td>
<td>16.4 ± 1.3/ 9.2 ± 0.8</td>
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<td>97.0%</td>
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<tr>
<td>CYP2C19</td>
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<td>106.9%</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>&gt;30/nd</td>
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<td>94.5%</td>
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<td>CYP2E1</td>
<td>&gt;30/nd</td>
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<tr>
<td>CYP3A</td>
<td>7.1 ± 0.9/4.3 ± 1.0</td>
<td>CYP3A</td>
<td>94.3%</td>
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*nd = not determined