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 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 CYP3A4 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.
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 (Farmdingale, 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 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 transferred to an P450 activity assay (total volume, 250 µl). In Assay 2, the P450 activity assay contained selective P450 probes (e.g., phenacetin for CYP1A2), 10 mM 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 time 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>Donor</th>
<th>Carnosic Acid</th>
<th>Tamoxifen</th>
<th>EC_{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>082511 (fres)</td>
<td>102</td>
<td>114</td>
<td>72.7</td>
</tr>
<tr>
<td>083111 (fres)</td>
<td>127</td>
<td>118</td>
<td>101</td>
</tr>
<tr>
<td>OHO (cry, ATP)</td>
<td>56.8</td>
<td>52.5</td>
<td>40.5</td>
</tr>
<tr>
<td>OHO (cry, LDH)</td>
<td>N.D.</td>
<td>N.A.</td>
<td>67.6</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. 2) and cell morphology (data not shown) indicated that 100 µM carnosic acid decreased cell viability to less than 10% of untreated or vehicle control-treated cells. As measured by ATP levels, a full dose-response curve revealed a viability EC_{50} of 95.7 ± 35.6 µM at 4 h and 94.5 ± 36.3 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 EC_{50} value could not be estimated, whereas the EC_{50} of tamoxifen was 67.6 µM. The caspase-3/7 assays 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 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 a 7.5- and 2.9-fold increase by 1 mM RIF (Fig. 4C). Increases in CYP2B6 and CYP3A3 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 enzymes to any appreciable extent for the isozymes 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 Ki 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 EC50 values similar to tamoxifen after 4 h of treatment. After 24 h of treatment, EC50 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

**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 EC50 for caspase-3/7 production by tamoxifen was 87.9 μM. An EC50 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 μM 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.

TOXICITY AND P450 INDUCTION AND INHIBITION BY CARNOUSIC ACID
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, lack of professional dosing guidance for dietary supplements, in patients presenting with hepatotoxicity and acute liver failure from usnic acid, where at this time are unknown.

However, at doses needed to elicit statistically significant weight loss and antidiogenic 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 and ADME characteristics, caution is warranted when taking this compound with nonalcoholic fatty liver disease.

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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.