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

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Abstract

Carnosic acid is a phenolic diterpene isolated from rosemary (*Rosmarinus officinalis*) which may have anti-cancer, anti-adipogenic, 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 non-alcoholic 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 cytochrome 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 to phenobarbital, and increased CYP3A enzyme activity 82.6% and 142% compared to 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 ((4aR,10aS)-5,6-dihydroxy-1,1-dimethyl-7-propan-2-yl-2,3,4,9,10,10a-hexahydrophenanthrene-4a-carboxylic acid, Supplemental Figure 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 anti-oxidant and anti-microbial properties (Aguilar et al, 2008; Opdyke, 1974). In addition, carnosic acid has demonstrated anti-inflammatory, anti-cancer, photoprotective, and anti-adipogenic activities *in vitro* (Reuter et al, 2007; Russo et al, 2009; Takahashi et al, 2009; Tsai et al, 2011; Yesil-Celiktas et al, 2010). Recently, carnosic acid 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 non-alcoholic fatty liver disease (NAFLD) 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 anti-adipogenesis 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 likely 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 likely not be administered under the supervision of a health care 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 upon our observations, we predict that carnosic acid has the potential to cause drug-drug interactions due to cytochrome 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 50 μL of media from treated cells at 4 hours. Values were compared to 50 μL media from cells lysed using the supplied lysis buffer, which represents maximum LDH release. ATP levels were measured using the CellTiter-Glo Luminescence Assay™ from Progemga at 4 and 24 hours 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 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 hour incubation with drug (carnosic acid, rifampin or phenobarbital), cells were washed with 100 μL of Krebs-Hanseleit buffer (KHB). Cells were then incubated with KHB containing either 250 μM bupropion for 30 minutes or 150 μM testosterone for 20 minutes. 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). Briefly, the time-dependent inhibition experiment was performed as a two-step transfer assay. Assay 1: the inactivation assay contained 1.0 mg/mL of microsomes, 10 µM of CA and 100 mM KPi buffer and is initiated with NADPH; after given time periods (10 sec, 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). Assay 2: the P450 activity assay contained selective P450 probes (e.g. phenacetin for P450 1A2), 10 mM NADPH and 100 mM KPi buffer, the activity assays were run for 10 min and samples were prepared for LC/MS/MS analysis. The control reactions were the 10 sec time points from the inactivation assay; therefore, the control reactions were the complete reaction mixture at essentially time zero. The percent of the control numbers in Table 1 were based off of these controls. For example, if at time 10 seconds the P450 activity was 20 (relative #) and at time 30 minutes the activity was 10 (relative #), the percent of control would be 50%. It is also important to note that control reactions without CA were run to ensure that there was no non-specific 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 minutes, the earlier time of 15 minutes was used for the calculation; however this was not the case over the course of these experiments in human liver microsomes.

**mRNA analysis.** mRNA isolation, quantitation, reverse transcription, real-time PCR, and data analysis have been previously published (Dickmann et al, 2011).

**Statistics.** Values were expressed as mean ± SD and compared using ANOVA followed by a Bonferroni post-test. For clarity, only comparisons against the appropriate control group are shown. Statistical analyses were performed using StataSE, version 10 (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 hours. 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 Figure 2) and cell morphology (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 hours and 94.8 ± 36.7 µM at 24 hours for carnosic acid compared to 71.4 ± 30.3 µM at 4 hours and 34.4 µM ± 19.2 at 24 hours for tamoxifen (Table 1, Figure 1, and Supplemental Figure 3). A lactate dehydrogenase (LDH) assay was also performed in one donor at 4 hours to assess membrane integrity (Figure 1 and 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 hours 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 (Figure 2).

**Cytochrome P450 induction.** Human cryopreserved hepatocytes exposed to 0.1, 1.0, and 10 µM carnosic acid for 48 hours were screened for induction of selected Phase I and II drug metabolizing enzymes and drug transporters (Figure 3). 10 µM carnosic acid 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 FDA recommended CYP2B6 and CYP3A4 positive control inducers, phenobarbital and rifampicin. In donor 082511, 1 mM phenobarbital and 10 µM rifampicin resulted in a 19.3-fold increase in CYP2B6 mRNA and a 55.9-fold increase in CYP3A4 mRNA, while 10 µM carnosic acid resulted in a 4.2-fold and 33.2-fold increase in CYP2B6 and CYP3A4 mRNA, respectively (Supplemental Figure 4A). In a second donor (083111), 1 mM phenobarbital and 10 µM rifampicin resulted in a 29.1-fold increase in CYP2B6 mRNA and a 12.3-fold increase in CYP3A4 mRNA,
while 10 µM carnosic acid resulted in a 3.6-fold and 5.6-fold increase in CYP2B6 and CYP3A4 mRNA, respectively (Supplemental Figure 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-fold and 1.9-fold increase in CYP2B6 activity, respectively, compared to a 7.5-fold and 2.9-fold increase by 1 mM phenobarbital (Figure 4A). CYP3A activity was increased by 14.6-fold and 2.4-fold in donors 082511 and 083111, respectively, by 10 µM carnosic acid compared to 17.5-fold and 2.0-fold by 10 µM rifampicin (Figure 4C). Increases in CYP2B6 and CYP3A activity in donor 083111 was low for both control inducers and carnosic acid compared to 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 since the overall response of CYP2B6 and CYP3A activity induction by phenobarbital and rifampicin was greater than 2-fold, respectively. The percent 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 percent CYP3A activity based on the rifampicin control was 82.6% and 142% (Figures 4B and 4D).

**Cytochrome P450 inhibition.** 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 minute incubation, although carnosic acid did show an inhibitory effect towards 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 USFDA 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 (Hebert et al, 2004; Morimoto et al, 2004; Piscitelli et al, 2000), while constituents isolated from black cohosh and licorice have been shown to inhibit CYP3A metabolism (Tsukamoto et al, 2005a; Tsukamoto et al, 2005b). 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 hours of treatment. After 24 hours of treatment, EC_{50} values for carnosic acid toxicity generally remained constant while those for tamoxifen were decreased approximately 3-fold. This could be due to different mechanisms of toxicity. Tamoxifen has been shown to induce toxicity through apoptosis followed by secondary necrosis (Mandlekar & Kong, 2001). The reduction of ATP in combination with the relative lack of caspase-3/7 activity or LDH release after 4 hours 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 is 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 50-100 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 the manufacturers (Sanchez et al, 2006).
According to the European Food Safety Authority (Aguilar et al, 2008), the predicted maximum exposure to carnosic acid as a food additive is approximately 0.2 mg/kg bw/day. Based on average body weight, total plasma volume and 65% bioavailability, maximum 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 anti-adipogenic 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 (Doolaege et al, 2011; Yan et al, 2009). 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 USFDA draft guidance recommendations, if carnosic acid were a new drug entity, the in vitro data would likely trigger a clinical drug-drug interaction 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 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 as 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 non-alcoholic fatty liver disease.
Acknowledgments

The authors would like to thank Amgen Inc. for laboratory resources.

Authorship Contributions

Participated in research design: Dickmann, Lin, VandenBrink

Conducted experiments: Dickmann and VandenBrink

Performed data analysis: Dickmann, Lin, VandenBrink

Wrote or contributed to the writing of the manuscript: Dickmann, Lin, VandenBrink

Contributed new reagents: Not applicable
References


Greenhill C (2011) Liver: Carnosic acid could be a new treatment option for patients with NAFLD or the metabolic syndrome. *Nature Reviews Gastroenterology and Hepatology* **8**: 122


Figure Legend

Figure 1. Dose response curves for human hepatocytes (Donor OHO) treated with either carnosic acid or tamoxifen for 4 hours. Open circles (carnosic acid) and open squares (tamoxifen) represent ATP levels expressed as the percent of vehicle treated cells. Closed circles (tamoxifen) and closed squares (carnosic acid) represent LDH levels expressed as the percent maximal LDH release from cells treated with a membrane lysis buffer. Error bars represent the standard deviation of 3 individual wells. Assays were performed according to the protocols described in the Materials and Methods section. Significant differences are indicated by: * p<0.05, ** p<0.01, or *** p<0.001.

Figure 2. Dose response for caspase-3/7 activity in human hepatocytes (Donor OHO) treated with either carnosic acid or tamoxifen for 4 hours. Caspase-3/7 activity was measured as described in the Materials and Methods section. Error bars represent the standard deviation of 3 individual wells. The EC$_{50}$ for caspase-3/7 production by tamoxifen was 87.9 µM. An EC$_{50}$ for caspase-3/7 production by carnosic acid could not be determined. Significant differences are indicated by: * p<0.05, ** p<0.01, or *** p<0.001.

Figure 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 carnosic acid (CA) for 48 hours. RNA was processed and analyzed according to protocols described in the Materials and Methods section using ribosomal 18s RNA as an endogenous control. Values are expressed relative to vehicle treated cells, and the error bars represent the standard deviation of three individual wells. Significant differences are indicated by: * p<0.05, ** p<0.01, or *** p<0.001, compared to vehicle treatment.

Figure 4. Hydroxybupropion (CYP2B6 activity) and 6β-hydroxytestosterone (CYP3A activity) formation 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 (A and C) or as the percent of positive control (B and D) with error bars representing the standard deviation 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.
quantitation is described in the Materials and Methods section. Significant differences are indicated by: * p<0.05, ** p<0.01, or *** p<0.001, compared to vehicle treatment.
Table 1. Calculated EC\textsubscript{50} values for human hepatocyte cytotoxicity due to treatment with either carnosic acid or tamoxifen. Individual assay parameters and data fitting are described in the Materials and Methods section. Data plots for Donors 082511 and 083111 are shown in Supplemental Figure 3.

<table>
<thead>
<tr>
<th>Donor</th>
<th>EC\textsubscript{50} (\mu M)</th>
<th>Carnosic Acid</th>
<th>Tamoxifen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 hr</td>
<td>24 hr</td>
</tr>
<tr>
<td>082511 (fresh, ATP)</td>
<td>102</td>
<td>114</td>
<td>72.7</td>
</tr>
<tr>
<td>083111 (fresh, ATP)</td>
<td>127</td>
<td>118</td>
<td>101</td>
</tr>
<tr>
<td>OHO (cryo, ATP)</td>
<td>56.8</td>
<td>52.5</td>
<td>40.5</td>
</tr>
<tr>
<td>OHO (cryo, LDH)</td>
<td>ND\textsuperscript{a}</td>
<td>NA\textsuperscript{b}</td>
<td>67.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a}ND = Not determined. Data could not be fit to an inhibitor vs. dose response model.

\textsuperscript{b}NA = Not applicable. Data was not obtained for a 24 hr time point.
Figure 1

- Tamoxifen (LDH)
- Carnosic Acid (LDH)
- Tamoxifen (ATP)
- Carnosic Acid (ATP)
Figure 2

![Graph showing Caspase-3/7 activity (% of vehicle treated) vs. Log [Drug] (μM). The graph compares Tamoxifen (●) and Carnosic acid (△). The y-axis represents Caspase-3/7 activity (% of vehicle treated), ranging from 0 to 250. The x-axis represents Log [Drug] (μM), ranging from 0.0 to 3.0. The graph includes error bars indicating variability.](image-url)
Figure 3

The figure shows mRNA expression (relative quantitation) for various genes under different conditions.

- **Untreated**
- **0.1% DMSO**
- **0.1 μM CA**
- **1.0 μM CA**
- **10 μM CA**

The genes tested include CYP1A2, CYP2B6, CYP2C39, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP4A11/22, SULT1A, SULT2A1, UGT1A, UGT2B7, MDR1, BSEP, MRP2, BCRP, and GAPDH.
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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).
**In Vitro** Hepatotoxicity and Cytochrome P450 Induction and Inhibition Characteristics of Carnosic Acid, a Dietary Supplement with Anti-adipogenic Properties

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*Drug Metabolism and Disposition*

**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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*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*</th>
<th>Time-Dependent Inhibition:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>P450 Isoform</td>
<td>IC₅₀/Kᵢ (μM)</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>&gt;30/nd</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>&gt;30/nd</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>&gt;30/nd</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>16.4 ± 1.3/ 9.2 ± 0.8</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>&gt;30/nd</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>&gt;30/nd</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>&gt;30/nd</td>
</tr>
<tr>
<td>CYP3A</td>
<td>7.1 ± 0.9/4.3 ± 1.0</td>
</tr>
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</table>

*nd = not determined