STUDIES OF THE TOXICOLOGICAL POTENTIAL OF CAPSINOIDs
XIII: Inhibitory Effects of Capsaicin and Capsinoids on Cytochrome P450 3A4 in Human Liver Microsomes

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ABSTRACT

This study evaluated the potential effects of capsinoids (ie, capsiate, dihydrocapsiate, nordihydrocapsiate) and a single capsaicinoid (ie, capsaicin) on liver microsomal cytochrome P450 3A4-mediated midazolam 1’-hydroxylase activity. Where possible, an inhibition curve was prepared, and the concentration at which enzyme activity dropped to 50% was calculated. Capsaicin clearly inhibited cytochrome P450 3A4 activity; losing 50% of the activity at 21.5 µmol/L. No enzyme inhibition was observed in the presence of capsiate, dihydrocapsiate or nordihydrocapsiate (<100 µmol/L). Preincubation increased the capsaicin inhibitory activity against cytochrome P450 3A4 in a time-dependent manner. Enzyme activity was slightly reduced by capsiate, dihydrocapsiate and nordihydrocapsiate to the same level as that attained with tolbutamide, the negative control compound.

Capsaicin was shown to inhibit cytochrome P450 3A4, probably through a mechanism-based inhibition. In contrast, capsiate, dihydrocapsiate and nordihydrocapsiate did not inhibit cytochrome P450 3A4 activity and were unlikely to be mechanism-based inhibitors of CYP3A4.
INTRODUCTION

Capsaicin, a member of the capsaicinoids family, is a major component of the fruit of pungent chili peppers.\textsuperscript{6} By contrast, capsiate (CAS No. 205687-01-0), dihydrocapsiate (DHC, CAS No. 205687-03-2) and nordihydrocapsiate (CAS No. 220012-53-3), members of the capsinoid family, are found in the fruit of CH-19 Sweet, a cultivar of \textit{Capsicum annuum} L. whose pungency is weak.\textsuperscript{15,16} Studies employing rats as test subjects demonstrate that capsaicin elevates body temperature and suppresses accumulation of body fat,\textsuperscript{7,13} while the administration of capsiate to mice, produces the same effects (ie, promotes energy metabolism and suppresses body fat accumulation).\textsuperscript{23} A clinical study reported similar effects (ie, elevation of body temperature and increased oxygen consumption) when humans received an oral dose of CH-19 Sweet extract.\textsuperscript{24} Recently, multiple safety evaluations of CH-19 Sweet extract and DHC, the main capsinoid in CH-19 Sweet extract, have been published. A variety of toxicity studies have demonstrated that CH-19 Sweet extract\textsuperscript{1,3,18,19,29} and DHC,\textsuperscript{4,5,17,20,30,31} have a low potential for toxicity. Metabolism and pharmacokinetic studies\textsuperscript{2,27} have demonstrated rapid and apparently complete metabolism of capsinoids, most likely in the GI tract and/or alimentary canal, as these compounds have not been detected in the portal vein. However, the current data cannot determine if some metabolism occurred very rapidly following absorption. The data suggested almost exclusive absorption of VOH, its major metabolite, into the portal vein. In the portal compartment, VOH undergoes nearly complete metabolism to sulfuric and glucuronic acid conjugates; VOH was almost non-detectable in the systemic plasma. As would be anticipated, based on these findings, neither capsinoids nor VOH are found in tissues. It is their metabolites, the glucuronide of VOH, the sulphate of VOH, and the sulphate of vanillic acid, which were identified in the plasma and
tissues (the kidney and liver attaining the highest concentrations). Elimination from the body is rapid and mainly through the urine, although lesser and significant amounts were detected in the feces.

Attempts to employ capsaicin in food and drug preparations have been hindered by its strong pungent taste. The less pungent nature of capsinoids (compared with capsaicin) may offer the possibility of increasing their usefulness in food and medicine.

It has been reported that several cytochrome P450 (CYP) isozymes participate in the metabolism of capsaicin. CYPs are oxidative enzymes that take part in the electron transfer system in the endoplasmic reticulum, and are involved in the oxidative metabolism of numerous xenobiotics. Some CYP isozymes play important roles in the metabolism of medicinal compounds and unanticipated adverse reactions caused by drug-drug interactions mediated by CYPs have been reported. Hence, when capsaicin and/or capsinoids are consumed, their potential effect on CYPs (and the metabolism of drugs being concurrently consumed) should be considered.

In this study, we examined the possible inhibitory effects of capsaicin and capsinoids on the most important CYP for the metabolism of drugs, CYP3A4. In addition, we investigated the potential for these compounds (capsinoids and capsaicin) to induce mechanism-based inhibitory (MBI) effects (also known as suicide inhibition). Substances which affect CYP via MBI have the potential to cause more serious toxicity than those that act via reversible inhibition. This is because for MBI, the inhibitory effect caused by the MBI continues after the compound has been eliminated from the blood or tissues.
MATERIALS AND METHODS

Materials

Capsiate, DHC and nordihydrocapsiate were synthesized in the laboratories of Ajinomoto Co., Inc. (Tokyo, Japan). Capsaicin, tolbutamide, midazolam, imipramine, K₂HPO₄, KH₂PO₄ and MgCl₂ hydrate were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan) and 1’-hydroxymidazolam was obtained from Ultrafine Chemicals (Manchester, UK). Human liver microsomes, pooled from 50 individuals, were purchased from Xeno Tech, LLC (Kansas City, KS). Reduced β-nicotinamide-adenine dinucleotide phosphate (β-NADPH), D-glucose 6-phosphate disodium salt (G-6-P) and glucose-6-phosphate dehydrogenase derived from yeast (G-6-P DH) were obtained from Oriental Yeast Co., Ltd (Tokyo, Japan). Acetonitrile and methanol were purchased from Junsei Chemical Co., Ltd (Tokyo, Japan). Ethylenediaminetetraacetate dipotassium was purchased from Dojindo Laboratories Co., Ltd (Kumamoto, Japan).

Methods

Preparation of the enzyme inhibition curve

The inhibitory effects of the individual compounds (capsiate, DHC, nordihydrocapsiate and capsaicin) on CYP3A4-mediated midazolam 1’-hydroxylase activity were examined.

The reaction mixture (450 μL), prepared on ice, contained the following compounds (their concentrations in the final mixture are provided in parentheses): EDTA (0.1 mmol/L), potassium phosphate buffer at pH 7.4 (0.1 mol/L), human liver microsomes (0.2 mg protein/mL), the test compounds dissolved in methanol (at various concentrations), and midazolam (3 μmol/L) dissolved in methanol. The concentration of midazolam was based on the Kₘ value of the reaction.³²
The reaction mixture was equilibrated at 37°C for 5 min. The reaction was initiated by adding a β-NADPH generating system (50 µL) to the mixture (final volume of the reaction mixture: 500 µL). The components of the β-NADPH generating system consisted of β-NADPH (2 mmol/L), G-6-P (19 mmol/L), magnesium chloride hexahydrate (10 mmol/L), G-6-P DH (2 units). The volume of methanol was set at ≤1%, a level which did not inhibit the enzymatic reaction. After four minutes, the reaction was terminated by the addition of acetonitrile solution (3 times the reaction mixture volume) containing imipramine (1.5 ng/mL, as an LC-MS/MS internal standard).

The mixture was centrifuged at 1500 g for 10 min and the supernatant employed for HPLC analysis. CYP3A4-mediated midazolam 1’-hydroxylase activity in human liver microsomes was measured by determining the 1’-hydroxymidazolam concentrations. The inhibitory effects and residual activities (percent) were calculated by comparing the enzyme activities in the presence and absence of the compounds. The inhibition curves were prepared by plotting the compound concentrations versus residual activity.⁹ When ≥50% inhibition was observed, the inhibitory concentration (IC₅₀) was calculated based on the following equation using the least square method.¹⁴

\[
\text{Residual Activity (\%)} = \frac{\text{IC}_{50}}{\text{IC}_{50} + I} \times 100
\]

(I = the concentration of the inhibitor).

Investigation of mechanism-based inhibition (MBI)

A reaction mixture (447.5 µL), prepared on ice, contained the following compounds (their concentrations in the final mixture are provided in parentheses): EDTA (0.1 mmol/L), potassium phosphate buffer at pH 7.4 (0.1 mol/L), human liver microsomes (containing 0.2 mg protein/mL), and the test compounds (100 µmol/L) dissolved in methanol.
The reaction mixture was equilibrated at 37°C for 5 min (in the absence of β-NADPH). A preincubation was initiated by adding the β-NADPH generating system (50 µL). At 0, 5, 10 and 30 min after the preincubation, a quantity of midazolam is added; sufficient to achieve a final concentration of 3 µmol/L (approximately 2.5 µL). The final concentration of the methanol added to the reaction mixture was set at ≤1 %. The reaction was terminated after 4 min. and specimens were prepared for analysis as described above. The ratio (percentage) of enzyme activity at each time point during preincubation to that at 0 min was calculated. The preincubation time versus the ratios of enzyme activity were plotted to examine the possibility of MBI.

Analysis

The quantity of 1'-hydroxymidazolam (a metabolite of midazolam) in the reacted mixture was determined by LC-MS/MS using imipramine as the internal standard. An Agilent 1100 LC system (Agilent Technologies, Wilmington, DE) was employed together with a Develosil ODS-UG-3 column (2.0 mm i.d. × 50 mm, Nomura Chemicals Co., Ltd., Aichi, Japan). The mobile phases consisted of a 0.1% formic acid aqueous solution (mobile phase A) and a 0.1% formic acid acetonitrile solution (mobile phase B). The column temperature: was maintained at 55°C, the injection volume was 8 µL, and flow rate: 0.3 mL/min. The 1'-hydroxymidazolam was eluted in the LC through the systematic alteration of the mobile phase over a period of 12 minutes. The process was initiated using a mixture of mobile phase A and B in a ratio of 95:5 and gradually over a five minute period changing the ratio (A to B) to 40:60. The 40:60 ratio was then maintained for a period of 1 minute. At the end of the one minute eluting period, the mobile phase was switched back to a mobile phase ratio of 95:5 over the next two minutes. With the mobile phase flow rate at
0.5 mL/min and the ratio of A to B at 95:5, elution continued for another 4 minutes to wash the column. The eluate sample that was produced during the 3.5 to 8 minute period of the 12 minute elution time was injected into an MS/MS. The autosampler temperature was 4 °C.

For the MS/MS analyses, an API3000 system (Applied Biosystems, Foster City, CA) was employed. Turboionspray ionization (positive) and multiple reaction monitoring (MRM) were adopted as the ionization method and measurement mode, respectively. Monitoring of 1’-hydroxymidazolam was performed using a Q1 of 342.02 and Q3 of 202.94; imipramine was monitored using Q1 and Q3 of 281.17 and 86.20, respectively. The collision energies of 1’-hydroxymidazolam and imipramine were 37 eV and 29 eV, respectively.

Statistical Evaluation

The data generated in this study were expressed as the mean (and standard deviation) of three trials (ie, three independent values). Statistical analyses were conducted using Dunnett’s test (SAS for windows, version 8.2).

RESULTS

The relationship between the concentration of each compound and residual activity of CYP3A4-mediated midazolam 1’-hydroxylase activity in human liver microsomes is shown in Figure 1. The residual activity was almost 100% in the presence of capsiate, DHC and nordihydrocapsiate at the maximum concentration of 100 µmol/L. However in the case of capsaicin, the residual activity decreased in a concentration-dependent manner and the IC₅₀ was 21.5 µmol/L (Table 1).

Figure 2 shows the preincubation time dependence of midazolam 1’-hydroxylase activity (where preincubation time 0 = 100%). The slight inhibition of CYP3A4-mediated
midazolam 1’-hydroxylase activity by capsiate, DHC and nordihydrocapsiate was equal to that observed with the negative control, tolbutamide; the inhibition being approximately 11% - 13% and 8%, in the three compounds and tolbutamide, respectively. The inhibition of enzyme by capsaicin was approximately 27% and 41% at 5 and 30 min. respectively, and their inhibition were statistically-significant compared to that by tolbutamide (p<0.01).

**DISCUSSION**

It has been reported that capsaicin is metabolized by various types of CYPs (ie, CYP1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4)25, and that capsaicin inhibits CYP3A1, 2A2, 2B1, 2B2, 2C6 and 2C11 found in rodent (rat and hamster) liver microsomes.33 The findings suggest the possibility of a drug-herb interaction when capsaicin is concomitantly administered with drugs that are metabolized by CYPs. On the other hand, there is no information about the metabolism and enzyme inhibitory activity of capsiate, DHC and nordihydrocapsiate.

MBI refers to inhibition in which the metabolic intermediate generated from an inhibition by CYP covalently binds to CYP and thereby inactivates CYP. MBIs are characterized by inhibition of the targeted enzyme in a preincubation-time dependent manner.22 There is a report which suggests that capsaicin acts as a suicidal or mechanism-based inhibitor of CYP2E1.28 Compared with reversible inhibition, there is a higher possibility that MBI might cause a serious drug interaction, especially in the case of CYP3A4, which is important in the metabolism of medicinal drugs.

The inhibitory effect (IC₅₀ value) of capsiate, DHC, and nordihydrocapsiate on CYP3A4 was >100 µmol/L indicating there was no inhibition observed. In contrast, capsaicin demonstrated an obvious inhibitory effect (IC₅₀ = 21.5 µmol/L). The structure of
Capsaicin is markedly similar to that of capsiate; the only difference is the fact that the ester bond in the structure of capsiate is replaced by an amide bond in capsaicin (Fig. 3). The ester bond is vulnerable to enzymatic hydrolysis by esterase(s). One of the reasons for the differences in inhibitory effects of these compounds on CYP3A4 is likely the lability of capsinoids in human liver microsomes. Capsiate, DHC and nordihydrocapsiate inhibited CYP3A4 activity slightly, but not significantly, during preincubation, equivalent to the effect observed with tolbutamide. Tolbutamide is a typical CYP2C9 substrate and was used as the negative control. This slight decrease was ascribed to the probable inactivation of the enzyme due to incubation at 37°C, but not to inhibition of the enzyme. Based on the present results, we conclude that capsiate, DHC and nordihydrocapsiate do not exert MBI effects on CYP3A4. Capsaicin, however, does inhibit the enzyme activity in a time-dependent manner (to a maximum of 41%) indicating that capsaicin is most likely a mechanism-based inhibitor of CYP3A4.

These data may be complementary to the results of previously reported pre-clinical toxicological investigations. In one of those studies, it was suggested that toxicity decreased in the progression from capsaicin to CH-19 Sweet extract to DHC. This relationship is consistent for acute toxicity, mutagenicity and chronic toxicity, while no distinction could be made for teratology due to the absence of observable affects for all these compounds. Whether the differences in toxicity are related to the differences in metabolism, specifically to the relative extent of inhibition on cytochrome P450 3A4, will require additional investigation.

Conclusion
In conclusion, capsaicin markedly inhibited CYP3A4 activity (IC$_{50}$ = 21.5 μmol/L) and is probably a mechanism based inhibitor of CYP3A4. On the other hand, capsiate, DHC and nordihydrocapsiate did not inhibit the activity of CYP3A4 and are unlikely to exert an MBI effect on CYP3A4.
REFERENCES


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Table 1.

IC$_{50}$ of the test compounds for CYP3A4 activity (midazolam 1'-hydroxylase activity) in human liver microsomes.

<table>
<thead>
<tr>
<th>test compounds</th>
<th>IC$_{50}$ (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>capsaicin</td>
<td>21.5</td>
</tr>
<tr>
<td>capsiate</td>
<td>&gt;100</td>
</tr>
<tr>
<td>dihydrocapsiate</td>
<td>&gt;100</td>
</tr>
<tr>
<td>nordihydrocapsiate</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*Values are the mean of three experiments*
Figure 1.

The inhibitory effects of the individual compounds (capsiate, DHC, nordihydrocapsiate and capsaicin) on CYP3A4-mediated midazolam 1’-hydroxylase activity in human liver microsomes.

Data are the mean of three experiments. Results are expressed as means ± standard deviation.
Figure 2.

Preincubation (Mechanism Based Inhibition or Suicide Inhibition) time dependence of CYP3A4 (midazolam 1’-hydroxylase) activity in human liver microsomes.

The concentration of each compound was 100 µmol/L. Data points represent the mean of three experiments. Results are expressed as means ± standard deviation.

* * p < 0.01 : Significant difference compared to tolubutamide (negative control).

Statistical analyses were conducted with Dunnett's test. (SAS for windows version 8.2)
Figure 3.
Chemical structures of capsaicin and capsinoids.

General Capsinoids Structure

R₁: Nordihydrocapsiate
R₂: Capsiate
R₃: Dihydrocapsiate

Capsaicin