Liver Enzyme-Mediated Oxidation of Echinacea purpurea Alkylamides: Production of Novel Metabolites and Changes in Immunomodulatory Activity

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Abstract:
The medicinal plant Echinacea is widely used to treat upper respiratory infections and is reported to stimulate the human immune system. A major constituent class of Echinacea, the alkyl-amides, has immunomodulatory effects. Recent studies show that alkylamides are oxidized by cytochrome P450 enzymes, but the immunomodulatory activity of these products is unknown. The objectives of this study were to characterize the products formed by incubation of an Echinacea extract and an isolated alkylamide with human liver microsomes, and to evaluate the influence of Echinacea alkylamides and metabolites on cytokine production by Jurkat human T cells. A novel class of carboxylic acid alkylamide metabolites was identified and shown to be the major constituents present after 2-h incubation of alkylamides with human liver microsomes. Echinacea alkylamides suppressed IL-2 secretion by stimulated T cells, and this effect was significantly lessened upon oxidation of the alkylamides to carboxylic acids and hydroxylated metabolites. These findings highlight the importance of considering the influence of liver enzyme metabolism when evaluating the immunomodulatory effects of alkyl-amides.

Key words: Echinacea purpurea Asteraceae, alkylamides, alkamides, cytochrome P450, metabolism, T-lymphocytes, interleukin-2 (IL-2)

Article:

Introduction

Echinacea, a genus native to the plains and prairies of North America, is among the most widely used plant medicines. Echinacea preparations constitute $300 million in annual sales in the USA [1], and physicians in Germany wrote more than three million prescriptions for Echinacea annually in the last decade [2]. Echinacea is reputed to beneficially stimulate the immune system and to aid in the treatment of upper respiratory infections [3]. Clinical trials have reported conflicting results as to the efficacy of Echinacea for this purpose [4], [5]. However, a vast body of research supports the assertion that Echinacea's constituents influence the human immune system [3].

Alkylamides comprise one of the major constituent classes that are associated with Echinacea's activity. In vitro studies have shown Echinacea alkylamides to have immunomodulatory effects, including upregulation of the expression of TNF mRNA by peripheral blood mononuclear cells [6], and suppression of the production of nitric oxide by cultured murine macrophages [7]. Alkylamides were also recently shown to regulate the expression of several cytokines in human whole blood [8]. In vivo, alkylamides have been shown to cause increased phagocytic activity of rat alveolar macrophages after oral administration [9]. Very little is known about the influence of alkylamides on T cells, key mediators in the immune response to upper respiratory infections, but a recent study found that alkylamides from Echinacea suppressed the secretion of the cytokine IL-2 by cultured Jurkat human T-lymphocytes [10].
Recently, Matthias et al. demonstrated that the in vitro metabolism of alkylamides by human liver enzymes produces a number of metabolites [11]. Coker et al. observed degradation of caffeic acid derivatives in Echinacea preparations to result from treatment with microsomal fractions of human livers, and showed that both mutagenic and tumor cell metabolism enhancing effects of specific Echinacea preparations were increased after they were processed by the microsomes [12]. However, the influence of liver enzyme metabolism on the immunomodulatory effects of Echinacea alkylamides has not been reported. The goal of this study was to evaluate how structure and immunomodulatory activity of alkylamide constituents from Echinacea change in response to in vitro oxidation by human liver enzymes. The objectives of this study were to: 1) identify the products formed by enzymatic oxidation of alkylamides from Echinacea purpurea; 2) determine how the distribution of products varies as a function of time; and 3) investigate the influence of both native alkylamides and those oxidized by liver enzymes on cytokine production by cultured leukemic human T-lymphocytic cells.

Materials and Methods

Extract preparation
Extracts were prepared from fresh cultivated roots of E. purpurea (Horizon. Herbs; Williams, OR, USA) within 3 days of harvesting. A voucher specimen was collected and the species verified by Richard Cech (Owner, Horizon Herb). E. purpurea roots (1 g/2 mL solvent) were blended in ethanol (95%) and macerated at 4°C for 7 days. The solvent was removed with a hydraulic press, and the extract was filter sterilized and stored at -70°C. The alkylamides were stable under these storage conditions for at least 12 months. Extracts at the dilution used for cell culture experiments were tested with the limulus amoebocyte lysate (LAL) assay (Cambrex 13ioscience; Rockland, ME, USA) and found to be free of endotoxin above 0.25 endotoxin units per mL. (EU/mL).

Liver enzyme incubations
Dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide (40 μM; Chromadex; Santa Ana, CA, USA) or E. purpurea extract (100 μL) were incubated with human liver microsomes in sterile phosphate buffer (pH 7.4, 0.1 M). Pooled human liver microsomes (40 μL; Moltox; Boone, NC, USA) were used with an assay concentration of 0.2 μM cytochrome P450 s. NADPH (1 mM) or sterile water was added to the appropriate samples or controls just before incubation at 37°C. Reagent grade ethanol (1:2.5) was added to stop the reactions, and the samples were centrifuged to precipitate proteins. Supernatants were filter-sterilized, the solvent was removed under vacuum at 35°C, and the samples were stored at -70°C.

Analysis of alkylamides and metabolites
The extracts were analyzed with a previously published liquid chromatography-mass spectrometry (LC-MS) method [13]. Separation was accomplished using reversed phase HPLC (HP1100 Agilent; Agilent; Palo Alto, CA, USA) with a linear acetonitrile/water gradient and a C18 column. An electrospray ionization mass spectrometer (Thermo Finnigan; San Jose, CA, USA) served as the detector.

Quantitative analysis of dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide was conducted on triplicate supernatants from the microsome reactions. Samples concentrated 20-fold and resuspended in 5% ethanol (aqueous) were also quantified to verify solubility of the alkylamides. Linear regression on a calibration curve of peak area versus concentration of dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide yielded a coefficient of determination (R²) of 0.998. The concentrations of carboxylic acid metabolites in the samples were estimated based on this calibration curve, and were expressed as pg equivalent per mL (μg eq/mL) with an approach used previously for quantification of alkyl-amides in Echinacea [14]. While the resulting metabolite concentrations are only estimates, they can be used to compare the abundance of metabolites among samples.

Cell culture followed by ELISA
The Echinacea alkylamide samples from the microsome reactions were resuspended in 50 μL of 5% ethanol in media by vortexing, vigorously. The effects of the Echinacea alkylamides on IL-2 production by leukemic human T-lymphocytic cells (Jurkat E6.1 clone; ATCC; Manassas, VA, USA) were determined using a method described previously [10]. Briefly, the cells were plated at 10^5 cells/mL and stimulated with phytohemagglutinin
(PHA, 1 μg/mL) and phorbol 12-myristate 13-acetate (PMA, 1 ng/mL). The samples and controls were diluted 10-fold into triplicate wells. Controls included cells with media alone, stimuli alone, and microsome reagents both with and without NADPH. The plates were incubated for 24 h before testing for IL-2 concentration by ELISA.

Statistics
IL-2 concentration data were subjected to one-way analysis of covariance (ANCOVA) using SPSS 14.0 (SPSS Inc.; Chicago IL, USA). Treatment (E. purpurea or alkylamide standard) was used as the main effect and dilutions were used as covariates. Multiple comparisons of main effects were adjusted by Bonferroni with an alpha value of 0.05. Mean values were calculated from triplicate wells, except in two cases where outliers were rejected (as justified by the Q-test) and the mean calculated for the remaining two data points.

Results and Discussion
Incubation with liver microsomes converted alkylamides in the E. purpurea extract to a number of metabolites. This is clearly demonstrated in Fig.1, which shows a chromatogram for an unmetabolized E. purpurea extract (a) and an extract that has been metabolized in vitro by human liver microsomes (b). The largest peak in Fig.1a represents the most abundant alkylamides in E. purpurea, the isomeric mixture of dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide [15]. The MS-MS fragmentation pattern and the HPLC retention time for this peak matched those of standard dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide. The other peaks in Fig.1a represent less abundant alkylamides, as identified in Table 1. A total of 11 different alkylamides were identified in the extracts (Table 1), the structural assignments of which were made as described elsewhere [13]. The concentration of these alkylamides decreased after in vitro metabolism, and new peaks representing alkylamide metabolites were detected (Fig.1b).

Three oxidized products were the major metabolites of dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide (Table 1, Fig. 2). These included hydroxylated and epoxide species, which have previously been reported as products of liver enzyme oxidation of alkylamides [11], and a novel carboxylic acid metabolite. A number of metabolites of the other minor alkylamides in the E. purpurea extract were also detected (Fig. 1), but due to the complexity of the extract, it was not possible to conclusively assign their alkylamide precursors.

The assignments of alkylamide structures displayed in Fig. 2 are based on mass spectrometric fragmentation studies and existing literature. Matthias et al. convincingly demonstrated that the epoxide group on metabolite 11e is located between carbons 8 and 9 [11]. Mass spectral fragmentation studies indicate that hydroxylation of the alkylamide does not occur on the N-isobutyl group, or on carbons 1-4, but the hydroxy group could be located on any of carbons 4—12.

The identity of the new metabolite of dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide as a carboxylic acid derivative was supported by the characteristic increase in mass of 30 Da, and the ability to detect the M — H+ ions of the metabolite (but not its alkylamide precursor) in negative ion mode LC-MS. Three primary carbons on dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide could be oxidized to carboxylic acids (Hg.


2), one at the end of the unsaturated alkyl chain (C12), and two on the N isobutyl group. The location of the carboxylic acid group on C12, as shown in Fig. 2, was supported by the MS-MS for the metabolite, which displayed a fragment at \( m/z = 74 \) consistent with an unmodified isobutylamine [13]. Studies of liver enzyme oxidation of standard isobutylamides further support the terminus of the alkyl chain as the location of the carboxylic acid group. Standard dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide produced both hydroxylated and carboxylic acid products upon incubation with microsomes, but standard undeca-2-ene-8,10-diynoic acid isobutylamide produced only hydroxylated and no carboxylic acid products. This difference is attributed to the terminus of undeca-2-ene-8,10-diynoic acid isobutylamide being blocked by an alkyne group, which prevents oxidation to the carboxylic acid.

<table>
<thead>
<tr>
<th>ID</th>
<th>Alkylamides and metabolites identified (mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide (229.2)</td>
</tr>
<tr>
<td>4</td>
<td>Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide (229.2)</td>
</tr>
<tr>
<td>5</td>
<td>Dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide (243.2)</td>
</tr>
<tr>
<td>6</td>
<td>Dodeca-2E,4Z,8Z,10E-triene-8-ynoic acid isobutylamide (245.2)</td>
</tr>
<tr>
<td>7</td>
<td>Undeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide (243.2)</td>
</tr>
<tr>
<td>8</td>
<td>Dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide (243.2)</td>
</tr>
<tr>
<td>9</td>
<td>Dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide (257.2)</td>
</tr>
<tr>
<td>10</td>
<td>Dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide (257.2)</td>
</tr>
<tr>
<td>11</td>
<td>Dodeca-2E,4Z,10E/Z-tetraenoic acid isobutylamide (247.2)</td>
</tr>
<tr>
<td>11c</td>
<td>Carboxylic acid metabolite of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (277.2)</td>
</tr>
<tr>
<td>11h</td>
<td>Hydroxylated metabolite of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (263.2)</td>
</tr>
<tr>
<td>11e</td>
<td>Epoxide metabolite of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (263.2)</td>
</tr>
<tr>
<td>14</td>
<td>Dodeca-2Z,4E,8Z,10E-triene-8-ynoic acid isobutylamide (249.2)</td>
</tr>
<tr>
<td>15</td>
<td>Dodeca-2E,4E,8Z-diene-8-ynoic acid isobutylamide (251.2)</td>
</tr>
</tbody>
</table>

The concentrations of the major alkylamide (dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide) and its major metabolite (carboxylated dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide) in the samples used for immunomodulatory testing are displayed in Table 2. The conversion of the alkylamide to its products, as measured by the decrease in concentration of dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide, was less complete in the complex *E. purpurea* extract (79% conversion) than for the isolated alkylamide (90% conversion). An alkylamide with a terminal alkyne group was previously shown to inhibit the activity of cytochrome P450 enzymes [11]. The *Echinacea* extract used here contained three such compounds (alkylamides 3, 4 and 7), which were likely responsible for the observed inhibition of enzyme activity observed for the complex extracts.

The influence of incubation time on distribution of products formed by enzymatic oxidation of standard dodeca-2E,4E,8Z,10Z-tetraenoic add isobutylamide is demonstrated in Fig. 3. At short incubation times (≤40 min), the most abundant product is the hydroxylated metabolite. Longer incubation (120 min) favors the carboxylic acid metabolite. After 120 min incubation with the liver microsomes, the majority of the alkylamide is converted to the carboxylic acid and hydroxylated metabolites. Thus, the 120 min incubation samples were utilized for cell culture assays.
Fig. 4 shows the concentration of IL-2 secreted by stimulated cultured T-lymphocytes after concomitant treatment with various alkylamide samples (Table 2) and appropriate controls. The highest concentration of the E. purpurea extract (neat, 4 μg/mL of the major alkylamide dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide) caused 47% suppression in IL-2 secretion as compared to microsome treatment alone. A significant suppressive effect (70% decrease in IL-2 concentration) was also observed for the highest concentration of standard dodeca-2E,4E,8Z,10Z-tetraenoic acid isohutylamide (1.8 μg/mL) as compared to the same control. These IL-2 suppressive effects were statistically significant for the highest concentration of both the E. purpurea extract (p = 0.020) and the alkylamide standard (p < 0.001). The 5-fold dilution of these samples did not induce a statistically significant suppression in IL-2, but a trend toward such an effect is evident.

Table 2 Concentrations of alkylamide constituents in an E. purpurea extract and an alkylamide standard (dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide) used for immunomodulatory testing. Concentrations are displayed for the major alkylamide (dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide) and its carboxylic acid metabolite after 2 h incubation with liver microsomes. Triplicate supernatants from the liver microsome reactions were quantitatively analyzed with LC-MS, and the highest (neat) concentrations used in the cell culture assays (shown below) were calculated based on these analyses. Samples without NADPH do not undergo oxidation by the enzymes in the microsomes and serve as controls.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>NADPH</th>
<th>Microsomes</th>
<th>Alkylamide 11 (μg/mL ± SD)</th>
<th>Concentrations</th>
<th>Metabolite 11c (μg eq/mL ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. purpurea extract</td>
<td>−</td>
<td>+</td>
<td>4 ± 1</td>
<td>16 ± 4</td>
<td>0</td>
</tr>
<tr>
<td>E. purpurea extract</td>
<td>+</td>
<td>+</td>
<td>0.9 ± 0.08</td>
<td>3.6 ± 0.3</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>alkylamide std.</td>
<td>−</td>
<td>+</td>
<td>1.4 ± 0.04</td>
<td>7.3 ± 0.2</td>
<td>0</td>
</tr>
<tr>
<td>alkylamide std.</td>
<td>+</td>
<td>+</td>
<td>0.19 ± 0.05</td>
<td>0.8 ± 0.2</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*SD = standard deviation of the mean concentration for triplicate samples.

*The metabolite concentrations were estimated from the standard curve for dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide, and are reported as μg equivalent to this alkylamide per mL solvent.

In contrast to the observation for the unmetabolized alkylamide samples, a statistically significant suppression in IL-2 production was not observed for the NADPH containing E. purpurea and alkylamide samples, although a trend toward a suppressive effect was still evident (Fig. 4). Thus, the IL-2 suppressive effect of the alkylamides appears to be significantly lessened after liver enzyme metabolism. The remaining suppressive effect on IL-2 secretion observed for the E. purpurea and alkylamide samples + NADPH (Fig.4) could be due to
the residual unoxidized alkylamides still present in the samples (Table 2), or to a slight suppressive effect of the alkylamide metabolites.

The suppressive effect of *E. purpurea* alkylamides on IL-2 secretion by cultured T-lymphocytes observed in these studies (Fig.4) has been reported previously, and it has been shown that, in the micromolar concentration range, this effect is not due to cytotoxicity [9]. While the mechanism of IL-2 suppression is unknown, alkylamides are agonists of the cannabinoid receptor (CB2) [16] which is ubiquitous in immune cells (although at varying expression levels) [17]. Therefore, the IL-2 suppressive effect of unmetabolized alkylamides may be mediated by CB2. Other known CB2 agonists, including the anandamide congener 2-methylarachidonyl-(2'-fluoroethyamide, have been shown to suppress IL-2 expression in activated T cells; however, this activity has been reported to occur by a CB2-independent mechanism [18]. Further investigations are necessary to determine whether the observed IL-2 suppressive effect of alkylamides occurs through CB2 dependent or independent mechanisms.

If CB2 binding were required for alkylamide mediated IL-2 suppression, the loss of this effect upon oxidation of alkylamides would be expected. Ligands of CB2 receptors possess unsaturated lipophilic chains that are necessary for the ligand/receptor interaction [18]. The action of liver enzymes on alkylamides introduces polar functionalities onto the unsaturated alkyl chains of alkylamides (Fig. 2), that likely would decrease their affinity for CB2 receptors.

This study is the first to report a change in immunomodulatory activity of alkylamides in response to metabolism by liver enzymes. In this study, *in vitro* metabolism significantly lessened the IL-2 suppressive effects of alkylamides on T-lymphocytes. However, the alkylamide metabolites may have very different
modulatory effects on other immune parameters, such as monocyte/macrophage activity. Alkylamides have been detected in the serum of patients who ingest Echinacea extracts [14], [20]. Therefore, studies of the immunomodulatory effects of alkylamides in their native form are still quite relevant. However, as demonstrated by the studies reported here, changes in immunomodulatory activity are likely to result as a consequence of liver metabolism, and are an important factor for consideration in future investigations of the mechanism of action of Echinacea and other alkylamide containing medicinal plants.

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