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 1L-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 amoebacyte 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 (R^2) 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.

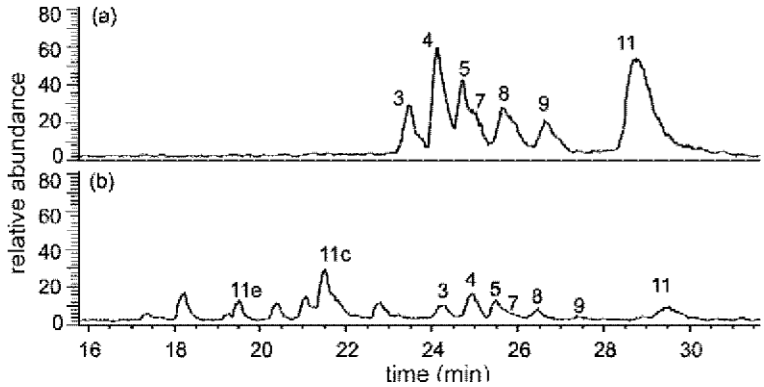


Fig. 1 Base peak chromatograms of an *E. purpurea* extract (a) after incubation with human liver microsomes without NADPH and (b) after incubation with human liver microsomes and NADPH. Because NADPH is a required cofactor for activity of human liver enzymes, metabolism is observed only for the chromatogram in (b). A significant decrease in signal for the relevant alkylamides occurs due to liver enzyme metabolism, and a number of new peaks, which represent metabolites of the alkylamides, are detected in the microsome processed extract.

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.la represents the most abundant alkylamides in. *E. purpurea*, the isomeric mixture of dodeca-2E,4E,8410E/Z-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. la 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.lb).

Three oxidized products were the major metabolites of dodeca2E,4E,8Z,10E/Z-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,10E/Z-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 *a*lkylamide precursor) in negative ion mode LC-MS. Three primary carbons on dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutlyamide could be oxidized to carboxylic acids (Hg.

2), one at the end of the unsaturated alkyl chain (C12), and two on the Nisobutyl 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 **1** Structural assignment of *E. purpurea* alkylamides and their liver enzyme metabolites. Each alkylamide listed was identified in the *E. purpurea* extract using a published method, and the numbers assigned to the alkylamides are consistent with the numbering system used in that publication [13].

ID	*Alkylamides and metabolites identified (mass)*[a]
3	Undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide (229.2)
4	Undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide (229.2)
5	Dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide (243.2)
6	Dodeca-2*E*,4*Z*,10*E*-triene-8-ynoic acid isobutylamide (245.2)
7	Undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide (243.2)
8	Dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide (243.2)
9	Dodeca-2,4-diene-8,10-diynoic acid 2-methylbutylamide (257.2)
10	Dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide (257.2)
11	Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide (247.2)[b]
11c	Carboxylic acid metabolite of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide (277.2)[b,c]
11h	Hydroxylated metabolite of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide (263.2)[b,d]
11e	Epoxide metabolite of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide (263.2)[b,d]
14	Dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide (249.2)
15	Dodeca-2*E*,4*E*-dienoic acid isobutylamide (251.2)

[a] *M* + 1 (MH^+) ions were observed with LC-MS in the positive ion mode for all of the alkylamides and metabolites listed.

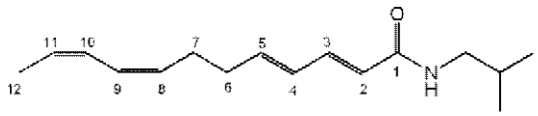
[b] The metabolites shown here for dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide resulted from incubation of both an *E. purpurea* extract and a standard of this compound with human liver microsomes.

[c] The carboxylic acid metabolites were detectable both as *M* + 1 (MH^+) ions in the positive ion mode and as *M* – 1 ($M - H^-$) ions in the negative ion mode.

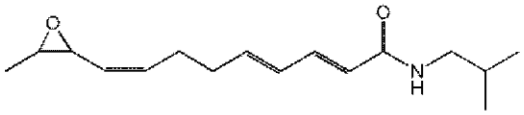
[d] The mass spectra for hydroxylated metabolites demonstrated a significant $MH^+ - 18$ peak (loss of water), which could be used to distinguish these species form isobaric epoxide metabolites, as reported previously [11].

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 immunmodulatory 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,8410Z-tetraenoic add 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.

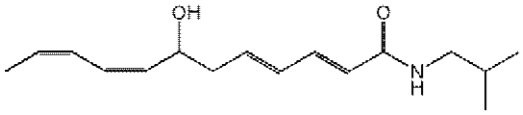
The influence of incubation time on distribution of products formed by enzymatic oxidation of standard dodeca-2E,4E,8Z,10Ztetraenoic add isobutylamide is demonstrated in. Fig. 3. At short incubation times (≤40 min), the most abundant product is the hydroxylated metabolite. Longer incubation (120 min) 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.



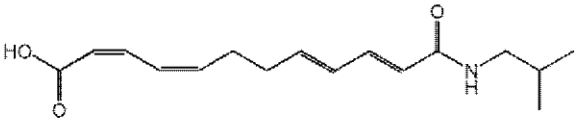
11: dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide



11e: epoxide metabolite of dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide



11h: hydroxylated metabolite of dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide



11c: carboxylic acid metabolite of dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide

Fig. **2** Structures of dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide, the most abundant alkylamide in *Echinacea purpurea*, and several proposed metabolites. The structural assignment of compound **11h** is not certain; the hydroxy group could be located on any of carbons 4–12. However, fragmentation (MS-MS) studies ruled out hydroxylation on the *N*-isobutyl group and carbons 2 and 3. The position of the epoxide on compound **11e** is assigned according to Matthias et al. [11]. The reported structure of compound **11c** (the carboxylic acid metabolite) was verified by mass spectrometry.

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-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide) used for immunomodulatory testing. Concentrations are displayed for the major alkylamide (dodeca-2*E*,4*E*,8*Z*,10*E*/*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

			Concentrations		
Sample ID	***NADPH***	***Microsomes***	***Alkylamide 11 (μg/mL ± SD[a])***	***Alkylamide 11 (μM ± SD)***	***Metabolite 11c (μg eq/mL[b] ± SD)***
E. purpurea extract	–	+	4 ± 1	16 ± 4	0
E. purpurea extract	+	+	0.9 ± 0.08	3.6 ± 0.3	1.2 ± 0.1
alkylamide std.	–	+	1.8 ± 0.04	7.3 ± 0.2	0
alkylamide std.	+	+	0.19 ± 0.05	0.8 ± 0.2	0.8 ± .3
–	–	+	0	0	0
–	+	+	0	0	0

[a] SD = standard deviation of the mean concentration for triplicate samples.

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

In contrast to the observation for the un metabolized 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.

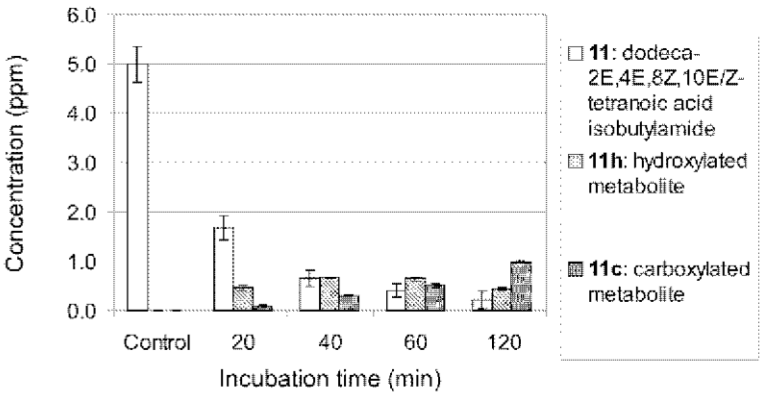


Fig. 3 Time-dependent conversion of dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide to hydroxylated and carboxylic acid metabolites after incubation with human liver microsomes. The error bars represent standard deviations of mean concentrations determined for triplicate reactions.

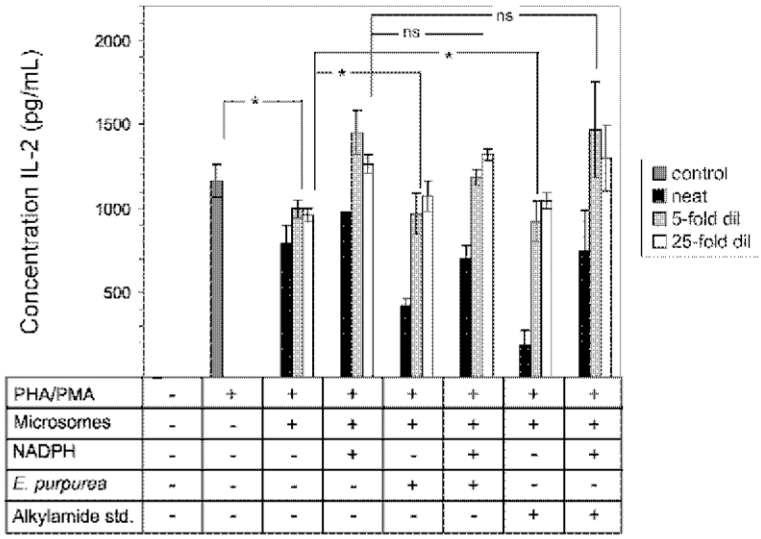


Fig. 4 *Echinacea* alkylamides but not cytochrome P450 metabolites suppress IL-2 production. Human Jurkat T cells were treated with an *E. purpurea* extract and a standard alkylamide (dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide) that had been incubated with human liver microsomes +/- NADPH for 2 h. The assay concentrations of the major alkylamide and its major metabolite in the samples labeled as neat are displayed in Table **2**. The samples without NADPH do not undergo oxidation by liver enzymes, and serve as controls. A statistically significant suppression in IL-2 production is observed due to treatment with the highest concentration of *E. purpurea* extract (containing 4 μg/mL dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide) and the isolated alkylamide (1.8 μg/mL). In contrast, the suppressive effect on IL-2 secretion of the *E. purpurea* extract and the alkylamide after liver microsome + NADPH treatment is no longer statistically significant when compared to the microsomes + NADPH control. The statistical significance of differences among treatments and controls was tested using ANCOVA with alpha = 0.05. A statistically significant difference is indicated by "*"; "ns" signifies no significant difference.

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′-fluoroethyeamide, 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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