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# Synthesis and biological evaluation of a series of fatty acid amides from *Echinacea*



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# ABSTRACT

Alkylamides are lipophilic constituents of *Echinacea* and possess numerous biological activities. Although significant effort has been focused on the study of crude *Echinacea* extracts, very little is known regarding the activities of the individual constituents that make up these herbal treatments. Herein we explore the SAR of simple alkylamides found in *Echinacea* extracts with respect to their ability to decrease the production of the pro-inflammatory mediator TNF- $\alpha$ . Our results have revealed the key structural requirements for activity and provide lead compounds for further investigation of these poorly understood molecules.

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Echinacea extracts are widely used as herbal medicines and are often taken for the treatment of cold-like symptoms due to their proposed immunomodulating capabilities. Fatty acid amides (alkylamides) are the most prevalent lipophilic compounds contained in Echinacea extracts and the only components known to cross the intestinal barrier: however, their constituent makeup varies greatly by species and method of preparation.<sup>1</sup> These variances in crude plant extracts have slowed efforts to utilize such mixtures as medicinal agents and have complicated the analysis of the various components' biological roles.<sup>1c</sup> Although structurally simple, fatty acid amides have diverse biological activities and are involved in signaling pathways relevant to cancer, cardiovascular disease, pain, inflammation, drug addiction, eating disorders, sleep deprivation, anxiety and depression.<sup>2</sup> Significant efforts have been focused on the development of fatty acid amides as analgesic agents due to their inhibition of FAAH, CB-1 and VR-1.<sup>3</sup> Surprisingly, relatively little effort has focused on the systematic study of simple fatty acid amides on pro-inflammatory mediators (such as TNF- $\alpha$ ) even though many members of this family have demonstrated anti-inflammatory effects.<sup>2b</sup> This area has been further complicated by the fact that fatty acid amides have poorly understood and/or multiple mechanisms of action and are known to elicit their anti-inflammatory effects via both

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cannabinoid receptor (CB)-dependent and CB-independent mechanisms.<sup>4</sup> We have previously demonstrated that diene-containing fatty acid amide **1**, a constituent of *Echinacea purpurea*, decreases the production of TNF- $\alpha$  from RAW 264.7 macrophage-like cells in a dose-dependent manner (Fig. 1).<sup>5</sup> Herein we report the chemical synthesis of **1** and a series of analogs to probe the structural requirements for activity and evaluate the general cytotoxicity of these compounds.

We began our synthetic efforts by preparing the lead natural product (1), as this compound was obtained in extremely small quantities from natural sources for the previously reported biological studies. To this end, two-step oxidation of the commercially available diene-containing alcohol **2** to carboxylic acid **3** followed by coupling with isobutyl amine  $(T3P^{\oplus})$  provided fatty acid amide **1** in good yield (Scheme 1).<sup>6</sup> Compound **1** proved identical to the natural product by <sup>1</sup>H and <sup>13</sup>C NMR analysis.

With the natural product in hand, we sought to prepare a series of analogs to explore the role of the double bonds, alkyl chain length in the fatty acid unit and the structure of the amide head group in the observed biological activity of **1**. We prepared compound **4** (Fig. 2) which only bears one unsaturation and a series of chain shortened derivatives (**5–9**, Fig. 2). In all cases the analogs were prepared in one step from the coupling of the corresponding carboxylic acid with isobutyl amine (see SI for details).

To evaluate the impact of these structural changes on the biological activity of **1**, we compared each analog's ability to inhibit TNF- $\alpha$  production by lipopolysaccharide (LPS)-stimulated RAW

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Figure 1. Structure of fatty acid amide 1 from Echinacea.

264.7 cells, a murine, macrophage-like cell line. Each compound was tested at 100  $\mu$ M in combination with 10 ng/mL of LPS and incubated for 18 h. TNF- $\alpha$  concentrations in the culture supernatants were measured after the incubation period using a commercially available ELISA (eBioscience). The effects of each compound on TNF- $\alpha$  production were compared to cells stimulated only with LPS (R595, List Labs). Additionally, RAW 264.7 cells were treated with each compound in the absence of LPS to determine if these molecules themselves could induce the production of TNF- $\alpha$ . None of the compounds were found to induce TNF- $\alpha$  significantly above levels from unstimulated cells (data not shown).

As shown in Figure 3, compounds 4 and 5 both significantly inhibited the production of TNF- $\alpha$ , with levels of suppression similar to that of 1 indicating that the double bonds along the alkyl chain are not critical for inhibition of TNF- $\alpha$  production. Significant inhibition was lost with compounds 6–9 (Fig. 2) indicating that the length of the alkyl chain is critical for determining inhibitory activity. An alkyl chain with at least 11 carbons is apparently required for this activity, in the absence of any unsaturation.

We next sought to explore the impact of the amide head group on the observed activity of **1** and simplified derivatives. Accordingly, **10–17** were prepared (see SI for details) to evaluate the impact of replacing the isobutyl amide with 2-methylbutyl amide (**10–11**), benzyl amide (**12–13**), hexyl amide (**14**), thiazole amide isosteres (**15–16**) and isobutyl amine (**17**) derivatives (Fig. 4). This series of alkyl amide derivatives served to not only evaluate the steric requirements of the amide head group, but also to answer key questions regarding the amide functionality itself.

The addition of a single carbon to the isobutyl amide head group in **10** and **11** did not impair their ability to significantly inhibit TNF- $\alpha$  production (Fig. 5). Replacement of the isobutyl amide group with a benzyl ring in 12 and 13 reduced the overall level of inhibition from  $50.2 \pm 5.1\%$  to  $29.8 \pm 5.5\%$  and  $32.4 \pm 0.8\%$ , respectively, although both still produced significant levels of suppression. Similarly, the addition of a 6-carbon alkyl chain on the amide nitrogen in compound 14 also produced significant suppression. In contrast, the two thiazole-containing compounds 15 and 16 did not display significant TNF- $\alpha$  suppressive activity, highlighting the importance of the amide functionality for the observed activity. Compound 17, which lacks the carbonyl group present in most of these compounds, completely inhibited the production of TNF- $\alpha$  (99.8 ± 0.2%); however, this activity likely arises from cell death, since 17 displayed significant cytotoxic activity (see below). Overall, we found that the isobutyl amide head group is not unique for inhibiting TNF- $\alpha$  production, although there do appear to be structural constraints in this region that limit inhibitory activity.



Scheme 1. Synthesis of fatty acid amide 1.



Figure 2. Analogs prepared to explore the impact of unsaturation and alkyl chain length.



**Figure 3.** TNF- $\alpha$  production in the presence of alkyl chain analogs. Error bars indicate means ± SEM from three independent experiments. One way ANOVA with Tukey's post hoc test was used to determine statistical significance. <sup>\*\*\*</sup> p <0.001. GraphPad Prism software was used for statistical analyses.

The cytotoxic effects of each compound were tested to ensure that inhibition of TNF- $\alpha$  production was not due to cell death. RAW 264.7 cells were treated with each compound at 100  $\mu$ M and incubated for 18 h before supernatants were collected and analyzed for lactate dehydrogenase (LDH) activity (Pierce LDH Cytotoxicity Assay Kit, Thermo Scientific). LDH is a cytosolic enzyme that is released from dead or dying cells. A lysis buffer was used to determine the maximal LDH release while spontaneous background release was determined by treatment with media and ethanol. While most compounds did not display significant cytotoxic effects, **10** and **17** did induce statistically significant increases in cytotoxicity (Fig. 6). As mentioned above for amine **17**, the suppression of TNF- $\alpha$  shown in Figure 5 was likely due to its



Figure 4. Analogs prepared to explore the impact of the amide head group.



**Figure 5.** TNF- $\alpha$  production in the presence of head group analogs. Error bars indicate means ± SEM from three independent experiments. One way ANOVA with Tukey's post hoc test was used to determine statistical significance. <sup>\*\*\*</sup> p < 0.001. <sup>\*\*\*</sup> p < 0.001. GraphPad Prism software was used for statistical analyses.

significant cytotoxic effects. Other factors, including cell permeability, could play a role in the varying activities of the studied compounds and these aspects are under additional study.



**Figure 6.** Cytotoxicity of the alkylamide structural analogs. Error bars indicate means  $\pm$  SD, n = 2. "p < 0.01, ""p < 0.001. V, vehicle; L, lysis buffer. GraphPad Prism software was used for statistical analyses.

In conclusion, the structural requirements of alkylamides to inhibit production of pro-inflammatory cytokines appear to be dependent on the alkyl chain length, and while there is structural flexibility within the amide head group, certain chemical properties within this region appear to be required to avoid reducing inhibitory effects. The double bonds in the alkyl chain of the natural product do not appear to be critical for suppression of TNF- $\alpha$ . This observation is consistent with previous publications in which additional alkylamides produced by Echinacea, as well as other plant genera, which vary in the number and placement of unsaturation along the alkyl chain, are also capable of suppressing cytokine production.<sup>5,7</sup> It is unlikely that these fatty acid amides are exerting their activity selectively on LPS or the LPS-signaling machinery since influenza A-induced production of TNF- $\alpha$  is also blocked. Interestingly, removal of the carbonyl group from the fully saturated version of the natural alkylamide produced a molecule with cytotoxic activity. Further experiments to develop an understanding of this effect and to further probe the SAR of this family of alkylamides are underway and will be reported in due course.

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#### Supplementary data

Supplementary data (experimental details) associated with this article can be found, in the online version, at http://dx.doi.org/10. 1016/j.bmcl.2015.06.024.

## **References and notes**

 (a) Matthias, A.; Penman, K. G.; Matovic, N. J.; Bone, K. M.; De Voss, J. J.; Lehmann, R. P. *Molecules* **2005**, *10*, 1242; (b) Woelkart, K.; Linde, K.; Bauer, R. *Planta Med.* **2008**, 74, 633; (c) Todd, D. A.; Gulledge, T. V.; Britton, E. R.; Oberhofer, M.; Leyte-Lugo, M.; Moody, A. N.; Shymanovich, T.; Grubbs, L. F.; Juzumaite, M.; Graf, T. N.; Oberlies, N. H.; Faeth, S. H.; Laster, S. M.; Cech, N. B. PLoS One 2015, 10, e0124276.

- 2. (a) Ruiu, S.; Anzani, N.; Orru, A.; Floris, C.; Caboni, P.; Maccioni, E.; Distinto, S.; Alcaro, S.; Cottiglia, F. Bioorg. Med. Chem. 2013, 21, 7074; (b) Dang, H. T.; Kang, G. J.; Yoo, E. S.; Hong, J.; Choi, J. S.; Kim, H. S.; Chung, H. Y.; Jung, J. H. *Bioorg. Med.* Chem. 2011, 19, 1520; (c) Ezzili, C.; Otrubova, K.; Boger, D. L. Bioorg. Med. Chem. Lett. 2010, 20, 5959; (d) Matthias, A.; Banbury, L.; Stevenson, L. M.; Bone, K. M.; Leach, D. N.; Lehmann, R. P. Immunol. Invest. 2007, 36, 117.
- (a) Otrubova, K.; Ezzili, C.; Boger, D. L. Bioorg. Med. Chem. Lett. **2011**, 21, 4674; (b) 3. Matovic, N.; Matthias, A.; Gertsch, J.; Raduner, S.; Bone, K. M.; Lehmann, R. P.; Devoss, J. J. Org. Biomol. Chem. 2007, 5, 169.
- 4. Raduner, S.; Majewska, A.; Chen, J. Z.; Xie, X. Q.; Hamon, J.; Faller, B.; Altmann, K. H.; Gertsch, J. J. Biol. Chem. 2006, 281, 14192.
  5. Cech, N. B.; Kandhi, V.; Davis, J. M.; Hamilton, A.; Eads, D.; Laster, S. M. Int.
- Immunopharmacol. 2010, 10, 1268.
- Matovic, N. J.; Hayes, P. Y.; Penman, K.; Lehmann, R. P.; De Voss, J. J. J. Org. Chem. 6. 2011, 76, 4467.
- 7. (a) Sasagawa, M.; Cech, N. B.; Gray, D. E.; Elmer, G. W.; Wenner, C. A. Int. Immunopharmacol. 2006, 6, 1214; (b) Spelman, K.; Iiams-Hauser, K.; Cech, N. B.; Taylor, E. W.; Smirnoff, N.; Wenner, C. A. Int. Immunopharmacol. 2009, 9, 1260; (c) Wu, L. C.; Fan, N. C.; Lin, M. H.; Chu, I. R.; Huang, S. J.; Hu, C. Y.; Han, S. Y. J. Agric. Food Chem. 2008, 56, 2341.