Chapter

The Structure and Function of Alkamides in Mammalian Systems

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Abstract

Alkamides, or alkylamides, are fatty acid amides produced by plants from the genera *Echinacea, Acmella, Spilanthes,* and *Heliopsis* among others. Alkamides contain varying head groups, an amide moiety, and a fatty acid tail with varying numbers of carbons and double and triple bonds. Extracts from these plants have been used worldwide by native peoples for the treatment of numerous medical disorders, including bacterial and viral infections, inflammation, liver and kidney disorders, and pain. *In vitro*, these molecules display a variety of different activities depending on the cell type tested. Studies with neurons, macrophages and mast cells have revealed interactions between alkamides and a number of different cells surface receptors and intracellular signaling molecules. Generally, the alkamides have been found to exert suppressive effects, inhibiting cellular activation. In this report we introduce the structure of alkamides and review their effects in a number of different cellular systems. We also describe structure:function studies that have been performed with alkamides. While these studies have not as yet revealed general rules for alkamide activity, interesting insights have been revealed. The stage is set for the development of synthetic, designer alkamides with targeted *in vivo* activities.

Keywords: alkamide, inflammation, immunity, nocioception

1. Introduction

The alkamides, also known as alkylamides, are fatty acid amides which vary in structure and function. Alkamides are found in nature in over 100 plant species, where they are thought to act as a defense against herbivory [1]. Alkamides contain a fatty acid tail, which can vary in the number of carbons and unsaturations, an amide group, and a variable headgroup. The structure of the alkamide dodeca-2E,4E-dienoic acid isobutylamide (A15) from *Echinacea,* which contains an isobutyl headgroup is shown in **Figure 1**. The structures of several other alkamides that have been studied extensively are also shown in **Figure 1**, including spilanthol, pellitorine, sanshool, and capsaicin. Spilanthol, which is found in many plants, including several species in the *Acmella* and *Spilanthes* genera and *Heliopsis longipes*, has 10 carbons and three double bonds in the fatty acid region. Historically, the most common usage for spilanthol has been as an analgesic. Plants containing spilanthol are often called "toothache plants" where the plant matter is chewed, causing a local numbing sensation in the mouth [2]. Pellitorine, found in plants from the *Piper* genus, is similar in structure to A15 from *Echinacea*, with two less carbons and two unsaturations in the fatty acid chain. Sanshool is found in plants in the *Zanthoxylum* genus which includes the Szechuan peppercorn. A number of analogs of sanshool have been identified

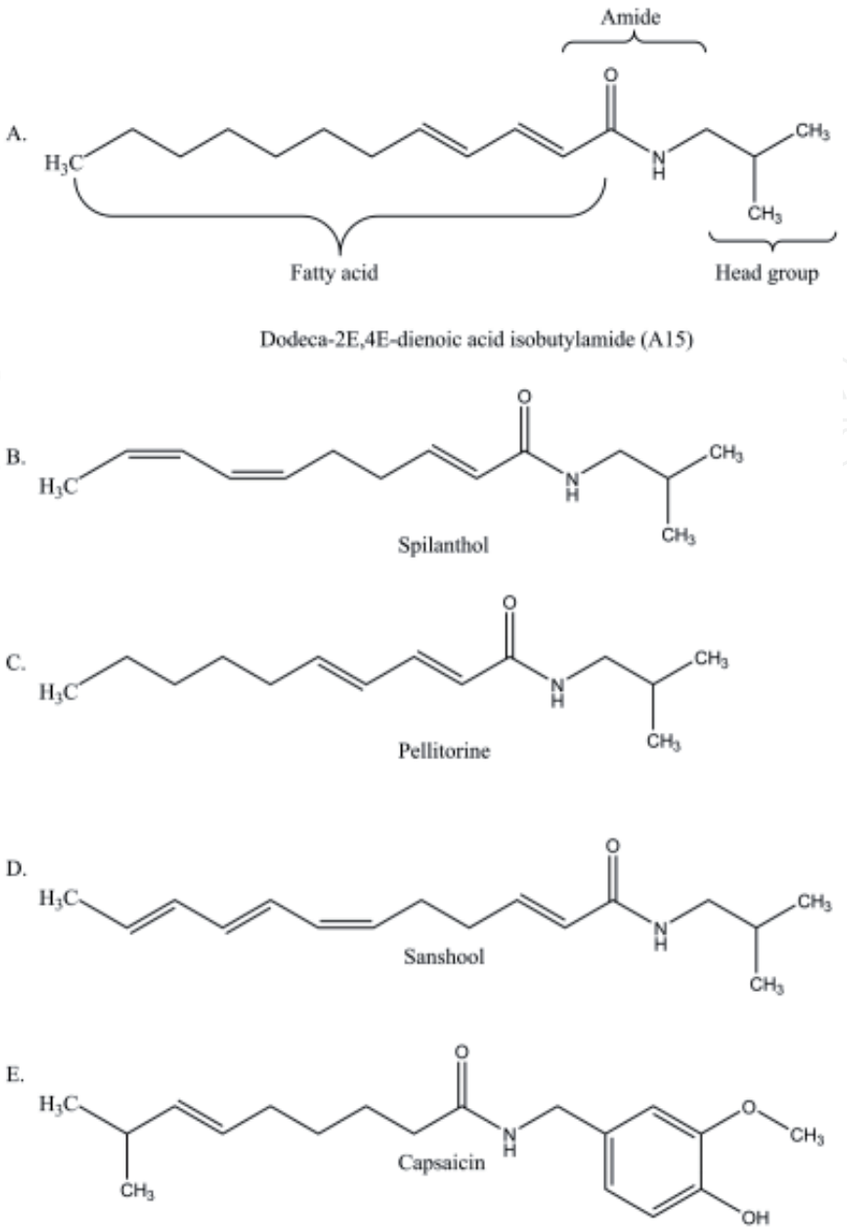

Figure 1.
General structure of alkamides. A. Dodeca-2E,4E-dienoic acid isobutylamide (A15) is shown above as a representation for the general alkamide structure. Other alkamides from Acmella/Spilanthes *(spilanthol) (B),* Piper nigrum *(pellitorine) (C),* Zanthoxylum *(sanshool) (D), and* Capsicum *(capsaicin) (E). Structures B-E are from Boonen et al. [1].*

with hydroxy-α-sanshool believed to be the major bioactive compound in most plant extracts [3]. Hydroxy-α-sanshool contains 12 carbons with multiple double bonds in the fatty acid chain and a hydroxyl group for the headgroup. Capsaicin, which contains a nine-carbon fatty acid, contains methyl groups on the fatty acid chain and an unsaturation at the sixth carbon. Capsaicin also contains an aromatic head group, and through its ability to activate the transient receptor potential (TRP) TRPV1 receptor, is responsible for the painful sensation associated with "hot peppers" [4].

2. Alkamides in plants used in traditional medicine

2.1 Echinacea

Alkamides occur in the flowering plants of the *Echinacea* genus, including the species *purpurea*, *angustifolia*, and *pallida* [5]. Alkamide containing *Echinacea*

extracts have been used historically by a variety of peoples including numerous Native American tribes for a wide range of purposes including treatment of infected wounds, rabies, or painful conditions such as toothaches or snakebites [6]. In 1805, Lewis and Clark learned about the use of this medicinal plant on their famous expedition and mailed seeds and roots to President Jefferson noting it as one of their important finds [6]. Today, *Echinacea* extracts are used to treat a variety of conditions- most often the common cold, but also bronchitis, upper respiratory infections, and more generally as an anti-inflammatory [7, 8]. Recently, the role of the alkamides in the uses for *Echinacea* have been studied by a number of labs.

Alkamides from *Echinacea,* such as A15, have been shown to act on a variety of cell types, including many immune cells such as macrophages, mast cells, and T cells, and also neurons [9–11]. In immune cells, A15 suppresses activation of pro-inflammatory responses such as production of pro-inflammatory cytokines and chemokines, which may account for the reduction of symptoms when *Echinacea* is used to treat respiratory infections [11]. In addition to the modulation of important inflammatory cytokines, alkamides from *Echinacea* are useful in inhibiting activation of mast cells and T cells, which has been linked to the inhibition of calcium-dependent signaling [10]. In neurons, alkamides have been shown to block ion channel activity leading to analgesia, which further reinforces their use to relieve symptoms caused by the common cold or respiratory infections [9]. *Echinacea* extracts have also been tested successfully in clinical trials to treat eczema where a significant reduction in local inflammation was noted [12].

2.2 *Piper longum* and *Piper nigrum*

Plants containing alkamides have not only been used by Native Americans, but they have also been used by people around the globe in China, Mexico, Brazil, Africa, Europe, and India [13]. *Piper* species, such as the long pepper, have been used in traditional medicine to treat a range of conditions such as chronic bronchitis, asthma, viral infections, and diarrhea and their use first appeared in texts by Hippocrates [14]. The plant *Piper longum* L., which contains 16 known alkamides, has been used to treat stomach conditions in ancient Chinese medicine and in traditional Indian medicine to treat abdominal pain and disease, among other diseases and disorders [15, 16]. Modern research has shown that these alkamides can increase melanin content and tyrosinase activity in melanoma cells [15] leading to suggestions that *Piper* extracts might produce an anti-melanoma affect. In addition, pellitorine displayed a strong cytotoxic activity in a study with two tumor-derived cell lines [17]. Alkamides isolated from *Piper longum* have also been shown to suppress NF-κB activation and inhibit the activity of COX-1 and -2 [18]. Inhibition of prostaglandin synthesis was also observed in ionophore stimulated leukocytes treated with pellitorine containing *Piper* extracts [19]. Finally, pellitorine has also been shown to be an effective insecticidal agent against the housefly and *Aedes aegypti* mosquito [20, 21].

2.3 *Phyllanthus*

Traditional healers in India have used the plant *Phyllanthus fraternus* to treat liver disorders, mixing the plant into a paste or using a plant extract [22]. Aqueous extracts from the plant, which are used by Indian healers, possess antioxidant activity and can prevent the oxidation of lipids and proteins [23]. In isolated hepatocyte mitochondria, the extracts are protective against alcohol induced oxidative stress [24]. *Phyllanthus sp.* have also been used in Ghana as an anti-malarial treatment and two alkamides E,E-2,4-octadienamide and E,Z-2,4-decadienamide (both of which lack the alkyl residue on the amine group) are thought to contribute to their anti-malarial activity [25].

2.4 *Spilanthes*

In Mexico, alkamide containing *Spilanthes* plants have been used as insecticides as well as analgesics [26]. In Africa and India, *Spilanthes acmella* is used as a medication to treat malaria [27]. In regions of Brazil, extracts from these plants have also been used as a female aphrodisiac [28]. Spilanthol is the predominant alkamide found in *Spilanthes sp.* with several other alkamides reported in lesser quantities [29]. Commercial preparations of spilanthol are as available for use as oral analgesics and to provide a long-lasting mint flavor in toothpastes [2]. In animal models, analgesia was demonstrated using a *Spilanthes* extract and was found to reduce murine hind paw edema and acetic acid induced tail flick in a dose dependent manner [30]. Spilanthol displays structural similarities to capsaicin, the ligand for the nociceptor channel TRPV1, which may account for its analgesic properties [23]. Isolated spilanthol also displays immunomodulatory properties *in vitro* causing dose dependent reduction in macrophage activation and nitric oxide (NO) production, as well as inhibition of cytokine production and NF-κB activation [31]. Other uses for spilanthol have been investigated including as an antipyretic, antimicrobial, antifungal, diuretic, and vasorelaxant [32].

2.5 *Zanthoxylum clava-herculis*

Zanthoxylum clava-herculis, also known as the toothache tree, Hercules' club, or prickly ash, has been used as a medical plant by Native Americans. In East Asia this plant is used as an analgesic, an antimicrobial, and for the treatment of kidney and liver disorders (Pawlus et al.; [33]). For example, extracts from the bark of *Zanthoxylum* display antimicrobial activity against Gram negative and Gram positive bacteria, and yeast *in vitro* [34]. Several alkamides have been isolated from *Zanthoxylum clava-herculis* including α-sanshool, and the presence of these molecules may explain the activities of this plant [35]. The alkamides in *Zanthoxylum clava-herculis* extracts have been shown to bind cannabinoid receptors, perhaps suggesting the mechanism of analgesic action [36].

2.6 Additional plants

Alkamides have been identified from a variety of other plants representing over 30 different plant families [23] although the role of the alkamides in the activity of the plant extracts has not been thoroughly defined. A few come from the *Solanaceae* family such as *Capsicum annuum* L. which has been used to treat otitis, infections, rheumatism, and headache [1]. Alkamides have also been identified in another plant from the same family, *Nicotiana tabacum* L., which is used in Africa to treat convulsions and as a stimulant [1]. Extracts of *Ricinus communis* L., which is a member of the *Euphorbiaceae* family, contains alkamides and is used by Mediterranean and African cultures to treat respiratory illness, rheumatic pain, and acne [37].

3. Alkamide cellular activities

3.1 Macrophages

Macrophages are important innate immune cells involved in organ homeostasis and defense against microbes [38]. Excess macrophage activation can, however, result in pathophysiological damage [39] and, therefore, it is necessary to identify immunomodulatory compounds which can dampen macrophage responses. Alkamides have been shown to display this activity *in vitro*. For example, alkamides have been shown

to inhibit LPS-induced TNF-α production by human monocytes/macrophages [40]. The authors propose that this effect is mediated by alkamides binding to type 2 cannabinoid receptors (CB2) and altering downstream signaling via cAMP, p38/MAPK, and JNK molecules [40]. CB2 is highly expressed on innate and adaptive immune cells, with the capability to down-regulate cellular activity, and has been proposed as an important therapeutic target [41]. Subsequently, it was shown that the alkamides dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide and dodeca-2E,4E-dienoic acid isobutylamide bind the CB2 receptor directly, with a higher affinity than endogenous cannabinoids, and that binding was associated with increased intracellular calcium level and IL-6 expression. However, contradictory to previous work, it was shown subsequently that the effect on TNF-α, IL-1β, and IL-12p70 expression was independent of CB2 binding [42]. Therefore, there may be multiple cellular targets of alkamides resulting in inhibition of both CB2-dependent and CB2-independent pathways leading to modulation of cytokine production. Taken together, these results demonstrate that alkamides are able to directly bind an important cell surface receptor, with known anti-inflammatory activity, as well as inhibit pro-inflammatory cytokine production through alternative, undefined mechanisms.

Alkamide effects on macrophages have also been studied during viral infection. During infection with influenza A, macrophages are key in elimination of the virus and can also contribute to the symptoms and pathology of influenza A by causing overproduction of inflammatory mediators [43]. It was found that alkamides undeca-2Z,4E-diene-8,10-diynic acid isobutylamide, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide, dodeca-2E,4E-dienoic acid isobutylamide, and undeca-2E-ene-8,10-diynoic acid isobutylamide from *Echinacea* were able to inhibit influenza-induced TNF-α and prostaglandin production, with dodeca-2E,4E-dienoic acid isobutylamide also strongly inhibiting chemokine CCL2, CCL3, and CCL5 production [44]. The inhibition of these mediators may explain the relief from symptoms seen in certain individuals when *Echinacea* extracts are used to treat influenza A.

3.2 T cells

Thymus-derived lymphocytes, or T cells, are a type of lymphocyte whose activity is critical to the immune response to infection, allergic reactions, and cancer [45]. Alkamides have been shown to inhibit IL-2 production in a dose dependent-manner from Jurkat T cells and the effects were independent of cytotoxicity [46]. IL-2 production is an important signaling molecule in T cell function and differentiation and decreasing IL-2 production may limit T cell activation and proliferation reducing the adaptive immune response. On the other hand, reducing IL-2 production in certain situations may have a beneficial effect by decreasing production of pro-inflammatory cytokines [47]. In support of this hypothesis, mitogen-stimulated splenocytes harvested from mice treated with *Echinacea*, produced significantly less IL-1β and TNF-α [48]. These mice also showed enhanced levels of T cell proliferation, both mitogen-induced and in the absence of mitogens. Stimulation of T cell proliferation was also observed using a commercial preparation of *Echinacea augustifolia* in which murine T cells were stimulated with anti-CD3 and the commercial *Echinacea* product [49]. Finally, T cell calcium responses were also found to be inhibited follow ionophore stimulation upon treatment with dodeca-2E,4E-dienoic acid isobutylamide (T. V. [10]).

3.3 Mast cells

Alkamides have been shown to be biologically active against mast cells. Mast cells are myeloid derived immune cells with key roles in regulation of vascular homeostasis, immune responses, and angiogenesis and have important functions in diseases such as

allergy, asthma, cardiovascular disorders, and gastrointestinal diseases [50]. A15 from *Echinacea* was demonstrated to inhibit mast cell degranulation, histamine release, and calcium influx in both primary bone marrow-derived mast cells and the mast cell-like cell line RBL-2H3 [10]. Because A15 was able to block granule release following ionophore stimulation, as well as FCεRI crosslinking, A15 must act on molecular targets regulating both stimulation pathways. Additionally, A15 inhibited TNF-α and prostaglandin E_2 production following ionophore stimulation. In an atopic dermatitis model, mast cell tissue infiltration was diminished following treatment with spilanthol [51]. *In vivo*, oral administration of N-(2-hydroxyethyl) hexadecanamide downregulated mast cell activation and pathology associated with mast cell activation such as edema [52]. In an asthmatic model using OVA-sensitized guinea pigs, *Echinacea* treated animals displayed a significant reduction in exhaled nitric oxide which has been shown to be partially produced by mast cells in asthmatic disease [53].

3.4 Neurons

A popular therapeutic use for alkamides is as pain relievers. Numerous groups have now reported on the analgesic effects of alkamides *in vitro* and *in vivo*. There are multiple types of pain receptors, with different specific receptors mediating mechanical and thermal pain. The neurons bearing these receptors are categorized as C-fibers, which are unmyelinated and small in diameter, and A-fibers, which are myelinated and quick to respond to stimuli mediating "initial fast-onset pain" [54]. Using the alkamide hydroxy-α-sanshool, from the *Zanthoxylum* plant, a selective inhibition of mechanical pain via inhibition of voltage-gated sodium channels on Aδ mechanonociceptors was observed in mice under both naïve and inflammatory conditions, with no influence on thermal pain [55]. Hydroxy-α-sanshool also altered activity levels of cool-sensitive fibers and cold nociceptors in extracellular nerve recording from the lingual nerve in rats [56]. Sanshool was also found to target channels TRPV1 and TRPA1 [57]. Alkamides from *Acmella oleracea* and a synthetic isobutylalkylamide showed long lasting *in vivo* analgesic efficacy when mice were pretreated with the alkamide 15 minutes prior to carrageenan injection to induce pain [58]. Alkamide dodeca-2E,4E-dienoic acid isobutylamide was demonstrated to be biologically active in the central nervous system in mice following intraperitoneal injection and dependent on interaction with the voltage-gated sodium channel, particularly Nav1.8 [59]. TRPV1, a non-specific cation channel that is the receptor for capsaicin and found on neurons, has been shown to be sensitive to isobutylalkylamides [60]. Using *in vitro* dorsal root ganglion cultures, neurons responded to the application of a synthetic isobutylalkylamide with an increase in intracellular calcium in a manner similar to activation by capsaicin [61]. This supports the analgesic effects observed with alkamides due to TRPV1 repeat activation of the channel leading to desensitization and lack of responsiveness [62]. Alkamides, such as pellitorine, also directly inhibit TRPV1 activation by acting as an antagonist which additionally explains the commonly observed analgesic effects [12]. This points to dual actions of alkamides as both TRPV1 agonists and antagonists, which can both lead to channel inactivation and pain relief. Interestingly, low dose synthetic isobutylalkylamide administration was shown the be anti-nociceptive, whereas high doses induced nociceptive behaviors in mice, with the authors suggesting the anti-nociceptive effects arising from blocking of ion channels [63]. Further, lingual application of synthetic isobutylalkylamide activated mechanosensitive neurons through modulation of potassium channels in human testing and caused a tingling sensation, while repeated exposure to the isobutylalkylamide causes desensitization of the channels and lessened tingling [64] supporting the concept of inhibition of neuron activities through desensitization of ion channels.

3.5 Liver and pancreatic cells

Alkamides have be tested as therapeutics for dietary and nutritional disease, particularly in diabetes. For example, daily oral administration of alkamides to diabetic rats was shown to significantly decrease fasting blood glucose level, and total liver cholesterol, and to relieve organ enlargement through activation of the AMPK signaling pathway which reduced fatty acid synthesis [65, 66]. Additionally, alkamides from *Zanthoxylum* were found to cause activation of the mTOR pathway in diabetic rats and ameliorate their protein metabolism disorder [65, 66]. Alkamides from the same plant also increased glucose metabolism preventing hyperglycemia and pancreatic dysfunction through modulation of the main enzymes regulating gluconeogenesis as well as improved amino acid metabolism [67, 68].

3.6 Cancer cells

Alkamides have also been investigated as treatments for cancer. For example, alkamide derivatives of bexarotene were able to induce apoptosis and prevent cell migration and proliferation in triple-negative breast cancer cells, while showing no cytotoxic effects against normal mammary epithelial cells [69]. In addition, a panel of alkamides with varying structure and molecular weights were able to induce differentiation of human leukemia cells to granulocyte-like cells [70].

4. Structure: function studies

4.1 Fatty acid chain saturation

The differences in cellular uptake based on structure could also explain the differences seen in biological activities of alkamides. A number of labs have asked how the structure of various alkamides contributes to their activities. For example, the importance of double bonds in the fatty acid chain was evaluated by measuring inhibition of cytokine production from LPS-stimulated RAW 264.7 macrophage-like cells. Similar levels of inhibition of TNF-α was observed with synthetic versions of dodeca-2E,4E-dienoic acid isobutylamide which all have 12-carbon tails with zero, one, or two double bonds indicating that unsaturated bonds are not required for inhibitory effect [71]. Further, 11–12 carbon isobutylamides containing a double bond at position C2 were found to inhibit chemically induced TNF-α production from human blood, RAW 264.7 macrophage-like cells, and other cell lines [1, 44].

The presence of multiple alkyne groups in the fatty acid chain was also investigated. Alkamides with multiple alkyne groups inhibited the activity COX enzymes, and at higher levels, inhibited prostaglandin E_2 production [44, 72]. Both alkamide A15 and pellitorine, which have highly similar structures, including their fatty acid chain inhibit ionophore stimulated prostaglandin production [19]. Interaction with the endocannabinoid receptor CB2 has been shown to occur with unsaturated alkamides with 11–14 carbons, but there was no affinity observed for *all-trans* tetradeca-2E,4E,8E,10E-tetraenoic acid IBA, indicating specific structural requirements for alkamide receptor interaction [1]. One group showed alkamides required a double bond at the C2 position for interaction with CB2 receptors with a second double bond at C4 increasing affinity, but not required for receptor interaction [1, 59]. Finally, a possible role for double bonds came from studies of the endocannabinoids where it was noted that the alkamide N-benzyl-(9Z,12Z)-octadecadienamide double bonds closely mimic those in endocannabinoid substrates [73].

The number and placement of double bonds has also been shown to impact the ability of alkamides to cross cell barriers. Using a Caco-2 cell monolayer, spilanthol and pellitorine, both 10 carbon alkamides but variable in position and number of double bonds, were tested for their ability to cross the monolayer. Spilanthol transport was significantly better than was the transport of pellitorine, suggesting that the placement and number of double bonds can affect transport [74]. A systematic analysis of bond number and position, and how they affect transport has not been performed.

Positioning of double bonds in spilanthol analogs between carbons two and five altered the physiological activity, with most activity resulting from double bonds at positions two and four [75]. The necessity of double bonds in the fatty acid chain was also evaluated for the activity of α-hydroxysanshool. Unsaturations were found to be required for interaction with TRPA1, but not TRPV1, perhaps indicating that different regions of the alkamide interact with the two receptors [57].

4.2 Fatty acid chain length

Experiments with alkamides using RAW 264.7 macrophages found that the number and placement of double bonds did not affect the activity, however, the length of the fatty acid chain did impact activity with these cells, with shorter fatty acid chains eliminating anti-inflammatory activity [71]. The length of the fatty acid chain was investigated using synthetic variants of dodeca-2E,4E-dienoic acid isobutylamide in an LPS activated RAW 264.7 cell model system. Alkamides with fatty acid tails shorter than 12 carbons did not significantly inhibit LPS-stimulated TNF-α cytokine production, indicating that longer fatty acid chains are required for this activity [71].

Alkamide fatty acid chain length was also evaluated in mast cells with alkamide analogs of varying chain lengths tested for their ability to inhibit intracellular calcium influx and mast cell degranulation. It was found that the shortest (four carbon) and longest (15 carbon) analogs were poor inhibitors of both degranulation and intracellular calcium influx [76]. Interestingly, there seemed to be differences in the optimum chain length and maximum inhibition for calcium influx and degranulation, perhaps suggesting different cellular targets responsible for inhibitory effects. For degranulation, the optimum chain length was eight carbons and for inhibition of calcium influx it was 12 carbons.

4.3 Head group

The head group was also investigated using LPS-stimulated RAW 264.7 cells and results indicated that head group substitutions were well tolerated with biological activity retained with most substitutions [71]. Addition of a carbon into the isobutyl head group did not significantly affect cytokine inhibition, and replacement of the isobutyl group with a benzyl group or six-carbon alkyl chain lessened inhibition, but the molecule was still biologically active [71]. Finally, altering of the amide functional group through addition of a thiazole group rendered the molecule inactive, thus demonstrating the importance of the amide [71]. In other studies using alkamides with benzyl headgroups, some showed affinity for CB2 receptors, which had been previous reported for isobutyl headgroups, with most activity seeming to come from the presence of an alkyl chain with 2 double bonds, rather than the identity of the headgroup [1]. Together, these studies suggest that fatty acid chain length and the amide are the critical determinants of alkamide activity.

5. Summary

Alkamides have been in use in traditional medicine for centuries across cultures worldwide. Alkamides are found in a large number of plant species including those in the genera *Echinacea*, *Piper*, *Phyllanthus*, *Zanthoxylum*, and *Spilanthes*, among others. Some alkamide containing products have made to commercialization in the 21st century such as splinathol in oral products and capsaicin creams. Currently, significant progress has been made into understanding alkamide activity and their use as therapeutics, although many questions regarding the molecular mechanism of alkamide action remain unanswered. At a cellular level, alkamides act on a variety of cell types including mast cells, macrophages, T cells, and neurons and alkamides are able to cross important cellular barriers including the blood–brain-barrier and the gut epithelial barrier. Modulation of cellular activity results in changes in cytokine and chemokine production, as well as cellular activation and signaling. Alkamides may be useful in dietary and nutritional settings with some studies demonstrating efficacy in mitigating effects of diabetes in mice. Additionally, some progress has been made in linking alkamide structure to activity, which could aid in the development of highly targeted drugs. Overall alkamides provide a promising class of plant derived compounds which should be considered when designing and evaluating novel therapeutics.

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