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The Cannabinoid Acids, Analogs and Endogenous Counterparts

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Abstract

The cannabinoid acids are a structurally heterogeneous group of compounds some of which are endogenous molecules and others that are metabolites of phytocannabinoids. The prototypic endogenous substance is N-arachidonoyl glycine (NAGly) that is closely related in structure to the cannabinoid agonist anandamide. The most studied phytocannabinoid is Δ^9 -THC-11-oic acid, the principal metabolite of Δ^9 -THC. Both types of acids have in common several biological actions such as low affinity for CB1, anti-inflammatory activity and analgesic properties. This suggests that there may be similarities in their mechanism of action, a point that is discussed in this review. Also presented are reports on analogs of the acids that provide opportunities for the development of novel therapeutic agents, such as ajulemic acid.

Keywords

cannabinoid; Δ^9 -THC-11-oic acid; ajulemic acid; NAGly; lipoamino acid; prostaglandin; lipoxin

1. Introduction

Since an earlier review on this topic published in 1999¹, there have been a considerable number of new research findings. This paper represents an update of the field and also gives a more detailed and comprehensive treatment of this subject. The present review includes not only phytocannabinoid-derived acids, but also their synthetic analogs and lipoamino acid counterparts, sometimes called elmiric acids. In contrast to earlier beliefs, the cannabinoid acids have a number of biological activities that are of potential therapeutic interest and will be discussed here.

2. Cannabinoid acids as plant constituents

The penultimate intermediates in the biosynthesis of Δ^9 -THC are two molecules, tetrahydrocannabinolic acid A and tetrahydrocannabinolic acid B, containing a carboxyl group at either the 2 or 4 positions on the aromatic ring (Fig. 1)²⁻⁴. These compounds are

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readily decarboxylated when heated to give Δ^9 -THC especially when the plant material is consumed by smoking. It is possible that some of the oral preparations of *Cannabis* such as *Bhang* and *Majun* may contain these acids. Little is known about their pharmacology, however, several reports suggest that these compounds may have biological activities ⁵⁻⁸. Similar acids of the other phytocannabinoid acids such as Cannabidiolic acid, Cannabigerolic acid, Cannabidivarinic acid, Cannabichromenic acid, (5aS,6S,9R,9aR) - Cannabielsoic acid A, (5aS,6S,9R,9aR) - Cannabielsoic acid B, (1aS,3aR,8bR,8cR) - Cannabicyclic acid, and Cannabinolic acid have all been isolated from plant extracts ³.

3. Acid metabolites of the phytocannabinoids

The earliest report suggesting the existence of acid metabolites of Δ^9 -THC was made by a Swedish group ⁹. They observed that a large proportion of the urinary metabolites in the rabbit were acidic in nature. However, no structural assignments were made. Subsequently, in a scaled up repetition of this study, large enough samples of two metabolites were isolated to allow identifications by proton NMR and low-resolution mass spectrometry ¹⁰. The structures were shown to be the 1' hydroxy and 2' hydroxy derivatives of Δ^9 -THC-11-oic acid (Fig. 2, D). The occurrence of Δ^9 -THC-11-oic acid itself as a urinary metabolite was reported in a subsequent study that also described its synthesis and lack of psychotropic activity ¹¹. Apparently, the possibility of other activities was not investigated by them or any other researchers until some years later (see section 3.3). Figure 3 shows examples of metabolites of Δ^9 -THC.

3.1. Acid metabolites from *in vivo* metabolism

The most widely studied cannabinoid acid is Δ^9 -THC-11-oic acid (Fig. 2, D). It is the terminal metabolite of Δ^9 -THC (Fig. 2, A) that is generated in a three-stage process going through the hydroxyl (Fig. 2, B) and aldehyde (Fig. 2, C) intermediates. This route occurs in humans and in every other species thus far examined ¹¹⁻¹². In contrast to its precursors, it is devoid of psychotropic activity, however, it does have biological actions described in section 3.3 that may contribute to the pharmacological profile of Δ^9 -THC. Two other cannabinoids CBD and CBN are also metabolized similarly to give a carboxyl-containing product (Fig. 4). There is also much interest in Δ^9 -THC-11-oic acid as the principle analyte in the forensic assays for marijuana use (see section 3.4). A more recent review of cannabinoid pharmacokinetics contains detailed information on the production of Δ^9 -THC-11-oic acid in humans ¹³.

3.2. Acid metabolites from *in vitro* metabolism

A cytochrome P450 (P450 MUT-2) catalyzes the *in vitro* oxidation of 11-oxo- Δ^8 -THC to Δ^8 -THC-11-oic acid as shown in Figure 2 ^{17, 18}. This isozyme, a member of the P450 2C gene subfamily was isolated from hepatic microsomes of untreated male mice. 11-oxo- Δ^9 -THC and 11-oxo-cannabinol (11-oxo-CBN) are also substrates for this enzyme and produce the corresponding carboxylic acid. The data indicate that P450 MUT-2 is a major enzyme for metabolizing cannabinoids by mouse hepatic microsomes.

3.3. Actions of acid metabolites

3.3.1. Psychotropic activity—⁹-THC-11-oic acid was synthesized and tested for behavioral responses in Rhesus monkeys ¹¹. It was found to be inactive and was not studied for any other actions. More than a decade passed before it was discovered that, in fact, it did have potentially important biological activities. For example, THC-induced catalepsy in mice can be substantially inhibited by the prior administration of ⁹-THC-11-oic acid, the major metabolite of THC in most species including humans ⁵. This suggests that the intensity and duration of psychotropic action of THC may depend to a degree on the levels of this metabolite at its sites of action. Evidence was also reported that it has activity as an anti-inflammatory agent (see Section 3.3.2).

3.3.2 Anti-inflammatory effects—Several reports contained data showing that THC and other cannabinoids, in particular, CBD, can stimulate the production of pro inflammatory eicosanoids such as PGE₂ ¹⁹⁻²⁵. Subsequently, it was reported that its metabolite, ⁹-THC-11-oic acid, could inhibit this process, suggesting that the acid is an anti-inflammatory agent, however, the mechanism for this action was not well characterized ⁸. In any case, the findings suggested the possibility, that this effect may influence the *in vivo* actions of THC by inhibiting its stimulatory action on cellular prostaglandin synthesis.

⁸-THC-11-oic acid was active in the mouse ear edema test where it was about as efficacious as phenidone; however, its potency was less than either phenidone or indomethacin ²⁶. It also showed activity as an antagonist to platelet activating factor (PAF), which may help explain the known properties of THC as a bronchodilator, antipyretic and anti-rheumatic agent. Its activity in preventing PAF-induced mortality was comparable to naproxen.

⁹-THC-11-oic acid produced a 20% stimulation of phospholipase A₂ (PLA₂) activity in mouse brain synaptosomes resulting in the increased release of free arachidonic acid from exogenously added [1-¹⁴C]-phosphatidylcholine ²⁷. This effect could provide precursor material for the synthesis of anti-inflammatory eicosanoids such as PGJ₂ and LXA₄. A more detailed study of this process is discussed for the analog AJA in Section 4.1.2.

3.3.3 Analgesic activity—Both delta ⁹-THC and ⁹-THC-11-oic acid produce analgesic action in the mouse hot plate test done at 50°C ²⁸. Of special interest is the time course in the hot plate test when each substance is administered orally at a dose of 20 mg/kg. At ten minutes, ⁹-THC is actually hyperalgesic whereas the acid is mildly analgesic. The latter action for both reaches a maximum at 30 minutes where the acid shows an effect twice as potent as ⁹-THC. Prior administration of either indomethacin or ⁹-THC-11-oic acid inhibits the hyperalgesic response. In the ring test, the metabolite does not produce a cataleptic state in the mouse, which eliminates catalepsy as a cause for the hot plate response.

The anti-nociceptive effect of ⁸-THC-11-oic acid activity was investigated further with particular regard to the influence of certain experimental parameters in the hot plate test ²⁹. These included the intensity of the thermal stimulus, the composition of the vehicle and a possible role for Cu⁺⁺. A temperature effect similar to that seen with nonsteroidal anti-

inflammatory drugs (NSAIDs) was observed; at 55°C observable anti-nociception was produced, however, at a surface temperature of 58°C no drug effect was seen. This suggests that opioid receptors are not involved. Non-aqueous vehicles such as peanut oil increased the potency of ⁸-THC-11-oic acid possibly due to increased bioavailability. Finally, the substitution of purified drinking water for tap water reduced the drug response that could be partially restored by adding Cu⁺⁺ to the purified drinking water. An increase in the inhibitory effect of ⁸-THC-11-oic when Cu⁺⁺ was added to the media was seen in a cell culture model where the acid reduced THC-induced prostaglandin synthesis. It has been shown that many anti-inflammatory agents form chelate complexes with Cu⁺⁺ that are more efficacious than the unchelated drug ³⁰.

3.4. Forensic aspects of acid metabolites

⁹-THC-11-oic acid and its glucuronides are the principal constituents in urine samples following the use of marijuana. An improved assay that is applicable for routine urine cannabinoid testing has recently been reported ^{31, 32}. A sensitive and specific method for the extraction and quantification of ⁹-THC, 11-OH-⁹-THC, ⁹-THC-11-oic acid, cannabidiol, cannabinol, ⁹-THC-glucuronide and ⁹-THC-11-oic acid-glucuronide in human urine was developed and validated. This method was tested on urine specimens collected from individuals participating in studies of controlled administration of *Cannabis*, and was reported to be a useful procedure for determining *Cannabis* use in forensic toxicology applications.

3.5. Chemical stability of acid metabolites

Data have been reported demonstrating the reactivity of ⁹-THC-11-oic acid in water containing free chlorine ³³. It was rapidly degraded following pseudo-first order kinetics to yield seven chlorine-containing products. Chlorinations involving the aromatic ring and the double bond were identified by mass spectrometry. The extent of the reaction was dependent on the level of organic matter content in the aqueous milieu; the greater the organic matter content, the lower the amount of chlorination. These results indicate a need for caution when handling cannabinoids in media such as chlorinated drinking water, for example, in forensic assays.

A comprehensive study of ⁹-THC, 11-hydroxy-⁹-THC, ⁹-THC-11-oic acid, cannabidiol, cannabinol, and ⁹-THC-11-oic acid glucuronide stability in urine was reported ³⁴. They concluded that analysis should be conducted in frozen urine within 3 months if non-hydrolyzed ⁹-THC-11-oic acid, or ⁹-THC-11-oic acid glucuronide quantification is required.

4. Synthetic analogs of acid metabolites

4.1. Ajulemic acid (AJA, HU-239, CT-3, IP-751, JBT-101)

Ajulemic acid is a totally synthetic compound that, over the years, has been prepared in several laboratories using different procedures. This has given rise to slight variations in the composition and amounts of the accompanying impurities some of which seem to have potent binding affinity for CB1. Each organization has assigned a code that will be indicated

in parentheses after the name as follows. They are: HU-239, The Hebrew University; CT-3, Atlantic Pharmaceuticals; IP-751, Indevus Pharmaceuticals and JBT-101, JB Therapeutics.

AJA (Fig. 5A) is a ⁸-dimethylheptyl side-chain analog of ⁹-THC-11-oic acid, the major metabolite of the active ingredient of marijuana, ⁹-THC. AJA was designed to have increased anti-inflammatory properties and reduced psychotropic activity, compared to the ⁹-THC parent. AJA (HU-239) was found to be effective at reducing paw edema in mice induced by arachidonic acid or platelet activating factor, at diminishing leukocyte adhesion in peritoneal cells from AJA (HU-239) dosed mice, and at producing analgesia³⁵. Subsequent studies have substantiated and expanded these findings and have demonstrated that it can minimize the adverse effects of adjuvant-induced arthritis in rats³⁶. AJA (CT-3) was well tolerated in nonclinical toxicity studies in mice, rats, and dogs (Atlantic Pharmaceuticals, unpublished data). A Phase 1 clinical trial designed to measure the safety and pharmacokinetics of AJA (CT-3) resulted in no clinically relevant adverse events and no evidence of marijuana-like psychoactivity (Atlantic Pharmaceuticals, unpublished data). In a Phase 2a trial, AJA (CT-3) showed efficacy in patients with chronic neuropathic pain³⁷. Thus, AJA may have significant potential for therapeutic benefit in the treatment of pain and chronic inflammation with a low potential for abuse and other adverse events. The actions of AJA are summarized in Table 1. The enantiomer of AJA shown in Figure 5B showed greatly reduced activities suggesting receptor mediation. A chiral analysis of the enantiomer revealed the presence of 10-20% of AJA that was probably the source of its activities (Atlantic Pharma, unpublished).

4.1.1. Analgesic activity—Potent anti-nociceptive effects were reported for AJA (HU-239) in the mouse hot plate assay³⁵. When tested over the range of 0.025-1.0 mg/kg, a bell-shaped dose-response relationship with a peak effect at 0.05 mg/kg was observed when the assay was done at 55°C. It was also reported that, at a dose of 0.1 mg/kg, no response in the ring test for catalepsy was seen suggesting a separation of psychotropic from analgesic activities. In a subsequent study of AJA (CT-3), positive effects were reported in several pain assays³⁸. These included the mouse hot plate assay at 48°C, the mouse p-phenylquinone writhing test and the mouse formalin anti-nociception test. In addition, it was observed that it did not alter motor function in the rota rod procedure at 4.64mg/kg i.v.; again suggesting a separation of activities. Further studies using the hot-plate (55°C) and the tail clip tests in mice and in the tail clip test in rats showed potency similar to morphine sulfate after i.g. and i.p. administration³⁹. In the rat, oral administration of AJA (Novartis) (0.1-1 mg/kg) produced up to 60% reversal of mechanical hyperalgesia⁴⁰. The anti-hyperalgesic activity was prevented by the CB1 antagonist SR141716A but not the CB2 antagonist SR144528 suggesting mediation by the CB1 receptor. They concluded that while “it shows significant cannabinoid-like CNS activity, it exhibits a superior therapeutic index compared to other cannabinoid compounds, which may reflect a relatively reduced CNS penetration”. A study comparing AJA (IP-751) with HU-210 again suggested a separation of psychoactive properties from analgesic effects⁴¹. Using a nerve-injury induced model of neuropathic pain and the CFA-induced model of inflammatory pain, efficacy was shown for both compounds, however, only HU-210 was effective in the rota rod test. They concluded ‘that ajulemic acid reduces abnormal pain sensations associated with chronic pain without

producing the motor side effects associated with Δ^9 -THC and other non-selective cannabinoid receptor agonists". A model of tonic inflammatory pain involving the injection of platelet activating factor (PAF) into the hind paw of rats was carried out (Walker et al., unpublished). The PAF injection control led to a drop in the withdrawal threshold to mechanical pressure (from 83g to 40g). A series of doses of AJA(CT-3), 3.0, 4.5, 6.8, 10.1 and 15.2 mg/kg, i.p. were administered and the paw withdrawal thresholds at 40-60min post-drug were measured. An approximate ED-50 of 4.2mg/kg was estimated for the reversal of PAF-induced allodynia. A complete reversal was seen at around 6.8mg/kg, above which anti-nociception was observed. Tepper et al. have prepared highly purified AJA (JBT-101) and compared its cannabinoid receptor binding constants with those obtained from other preparations^{*}. CB2 binding did not vary greatly among the various samples tested, however, CB1 binding showed a wide range of affinities (K_i =5.7-628nM). The CB1 binding activity is substantially reduced in JBT-101. This finding may explain the conclusion reached by Vann et al.⁴² that "AJA, like Δ^9 -THC, exhibits psychoactive and therapeutic effects at nearly equal doses in preclinical models, suggesting similar limitations in their putative therapeutic profiles". This conclusion is inconsistent with the results obtained in humans where another preparation of AJA (CT-3) that has low CB1 binding was used and showed a good therapeutic profile^{37, 43}.

In humans, a Phase 1 trial using healthy subjects did not reveal any adverse events, including psychotropic effects, in an acute, high dose treatment with AJA (CT-3) (Atlantic Pharmaceuticals, unpublished data). In a Phase 2a randomized, placebo-controlled, double blind crossover trial in patients with refractory neuropathic pain, it was effective in 30% of the subjects (Karst *et al.*, 2003). The scores obtained on the ARCI-M questionnaire indicated an absence of cannabimimetic activity under these conditions. These data raise a question about the applicability to AJA of preclinical tests for cannabinoids such as those employed by Vann, et al., e.g. drug discrimination⁶⁵. This issue has been discussed in the literature⁴³⁻⁴⁶.

4.1.2. Anti-inflammatory effects—The *in vivo* anti-inflammatory effects of AJA have been demonstrated in six rodent models. These are: 1) the adjuvant-induced chronic arthritis model³⁶, 2) cytokine-induced acute inflammation³⁶, 3) arachidonic acid-induced paw edema³⁵, 4) PAF-induced paw edema³⁵, 5) leukocyte adhesion of mouse peritoneal cells³⁵ and 6) interstitial cystitis^{47, 48}.

Chronic poly-arthritis was induced in rats by intradermal injection of Freund's complete adjuvant³⁶. AJA (CT-3) treatment (0.1 mg/kg/day in oil, p.o.) began 3 days after adjuvant injection and was administered on Mondays, Wednesdays, and Fridays for 5 weeks. Arthritis was scored in all four paws where it reduced the numbers of severely inflamed paws, improved weight gain, and minimized arthritic damage in joints observed histologically and compared to untreated controls. The degree of protection afforded by AJA (CT-3) treatment was greater in the histological measures than in the clinical measures. In a second study,

^{*}Mark A. Tepper, Robert B. Zurier and Sumner H. Burststein, Highly Purified Ajulemic Acid is a CB2 Agonist with Reduced CB1 Activity (2013) 23rd Annual Symposium on the Cannabinoids, International Cannabinoid Research Society, Research Triangle Park, NC, USA, P1-20.

acute inflammation was induced by injecting IL-1 β and TNF α into air pouches formed on the backs of mice. It reduced the numbers of leukocytes in exudates of these pouches by 42.3% (0.1 mg/kg/day) and by 65.5% (0.2 mg/kg/day).

Figure 7 shows the inhibitory action of AJA (HU-239) in the mouse paw edema model of inflammation. Arachidonic acid (1.0 mg) was injected into a mouse hind paw and the volume of the paw was measured by fluid displacement³⁵. Edema induced by either arachidonic acid or PAF was completely inhibited at a dose of 0.2 mg/kg, p.o. in oil. The maximum inhibition occurred at 90 minutes after AJA (HU-239) administration, although significant reductions were noted at all time points tested (30, 60, 90, 120 and 180 minutes). In a second experiment³⁵, reductions in the numbers of adhering peritoneal leukocytes resulted from oral administration of AJA (HU-239) in oil at doses of 0.01 to 1.0 mg/kg. Cells were collected from mice 6 hours after dosing and were cultured for 18-20 hours before adhesion was measured. Only 24% as many cells adhered after *in vivo* treatment with 1.0 mg/kg than with vehicle.

MMPs (matrix metalloproteinases) are important targets for agents designed to treat inflammatory arthritis. They promote cartilage degradation and bone erosion, and lead to joint deformities and crippling. The effect of AJA (CT-3) on MMP production in human fibroblast-like synovial cells (FLS) and a possible role for PPAR- γ was investigated⁴⁹. MMP-3 (stromelysin-1) release in FLS cells that were stimulated by TNF α was reduced by 88% following AJA (10 μ M) treatment. The effect appeared to be PPAR- γ independent, however the mechanism of action was not determined. Several other MMPs were also inhibited, but less so than MMP-3.

AJA (CT-3) was shown to induce apoptosis of T cells in a dose- and time-dependent manner and preceded the loss of cell viability, showing that cell loss was due to programmed cell death rather than necrosis⁵⁰. T lymphocytes in the synovium of rheumatoid arthritis patients are resistant to apoptosis and this is thought to be a factor in joint tissue injury in patients. Annexin V expression, caspase-3 activity, DNA fragmentation, and microscopy were used to demonstrate AJA-induced apoptosis. Thus, its joint sparing effect previously reported³⁶ may be explained in part by this effect.

Addition of AJA (CT-3) to human peripheral blood and synovial fluid monocytes *in vitro* reduced both the steady-state levels of IL-1 β mRNA and the secretion of IL-1 β in a concentration-dependent manner⁵¹. However, it had no effect on TNF α gene expression in, or secretion from, peripheral blood monocytes. Interleukin-1beta and tumor necrosis factor-alpha are needed for the progression of inflammation and joint tissue injury in patients with rheumatoid arthritis. Therefore, inhibition of the former supports the suggestion that AJA would be effective in the treatment of this disease.

The anti-inflammatory effects of AJA can best be understood at the molecular level by its actions at several points of the arachidonic acid cascade that regulate the expression of the eicosanoid family. The initial event in the cascade is the release of free arachidonic acid from cellular phospholipid storage pools where AJA (CT-3) stimulates a robust response (Fig. 8). Treatment of human synovial fibroblasts with AJA (CT-3) results in a concentration

dependent increase in COX-2 expression⁵². A consequence of this is an increase in anti-inflammatory eicosanoid production. Pretreatment of synovial cells with the CB2 antagonist SR144528 partially inhibits release suggesting a role for this receptor. Figure 9 shows a similar effect in the HL-60 cell line where AJA (JBT-101) is compared with THC that is less active and CBD that is inactive (Burststein et al., unpublished data).

AJA (IP-751) also stimulates a product of the lipoxygenase branch of the cascade lipoxin A₄ (LXA₄). Addition of AJA (0-30 μ M) *in vitro* to human blood or synovial cells increased production of LXA₄ 2- to 5-fold⁵³. Administration of AJA (IP-751) to mice with peritonitis resulted in a 75% reduction of cells invading the peritoneum, and a 7-fold increase in LXA₄ identified by mass spectrometry. Blockade of the 12/15 LOX enzyme, which mediates LXA₄ synthesis *via* 15-HETE production, reduced by >90% the ability of AJA (IP-751) to enhance production of LXA₄ *in vitro*. These results further suggest that AJA may be useful for conditions characterized by chronic inflammation and tissue injury.

4.1.3. Actions on bladder irritation and hyperactivity—Effects of AJA (IP-751) on bladder over activity induced by bladder irritation in rats have been reported^{47, 48}. It can suppress normal bladder activity and urinary frequency induced by bladder nociceptive stimuli, probably by suppression of bladder afferent activity. These inhibitory effects of AJA (IP-751) are at least in part mediated by the CB1 receptor. These findings identify a potential new approach for the treatment of the painful bladder syndrome, interstitial cystitis.

4.1.4. In vitro and in vivo anti-neoplastic activity—AJA (CT-3) was reported to be moderately active in inhibiting growth *in vitro* against a variety of neoplastic cell lines⁵⁴. It was more effective than its enantiomer, HU-235, suggesting receptor mediation. Moreover, CB2 but not CB1 receptor antagonists blocked its effects. At a dose of 0.1 mg/kg, it inhibited modestly but significantly the growth of tumors in nude mice that were subcutaneously implanted with U87 human glioma cells. Interestingly, the *in vitro* effects on cells were accompanied by the development of lipid droplets consisting of di and tri glycerides. The significance of these lipids is not immediately apparent.

A subsequent experiment using a SCID-NOD mouse flank tumor model again showed anti-neoplastic efficacy for AJA (CT-3) at a dose of 0.1 mg/kg (Recht and Salmonsén, unpublished data). On day 28 post inoculation, the vehicle treated group had a 1/5 unhealthy survivor, whereas treated mice had 3/5 tumor bearing healthier looking survivors. More importantly, the vehicle treated control group all showed evidence of malignant ascites suggesting metastatic disease while the AJA treated group did not develop ascites and generally appeared healthier. A major problem in oncology is the lack of effective ways to control metastatic disease. These preliminary findings suggest that AJA should be further investigated for possible use in this type of treatment.

4.1.5. Anti-fibrotic effects—It has been recently reported that AJA (JBT-101) prevents progression of fibrosis *in vivo* and inhibits fibrogenesis *in vitro* by stimulating PPAR- γ signaling⁵⁵. Its efficacy in pre-established fibrosis was studied in a modified model of bleomycin-induced dermal fibrosis and in mice over expressing a constitutively active

transforming growth factor beta (TGF β) receptor. A reduction in skin thickness and development of skin fibrosis following oral administration of AJA (1 mg/kg) was compared to control levels ($p < 0.001$) with mock treated mice. They also observed that AJA (JBT-101) stimulated the expression of PPAR- γ in dcSSc fibroblasts (diffuse cutaneous systemic sclerosis) from patients, increasing the production of an endogenous anti-inflammatory ligand PGJ₂. It also significantly decreased collagen neosynthesis by scleroderma fibroblasts *in vitro*, an effect that was reversed by co-treatment with the selective PPAR- γ antagonist GW9662. Effective treatment of fibrosis in scleroderma patients remains a major unmet medical need.

4.1.6. Multiple sclerosis—A major problem for patients with the autoimmune disease multiple sclerosis is the occurrence of spasticity. In an animal model of this effect, AJA (CT-3) treatment at 0.1 mg/kg i.v. resulted in a robust, visibly apparent reduction of spastic activity⁵⁶. Its activity at this dose was absent when the experiment was done in CB1 receptor knockout mice. The authors concluded, “This provides further evidence for the peripheral nerve mode of action via CB1 receptors”. They also studied the extent to which AJA crosses the blood brain barrier (BBB). AJA (CT-3) is a hydrophobic molecule with a calculated log BBB brain/blood coefficient of 0.46, which is greater than would predict CNS penetration. Cyclosporine A is a widely used immunosuppressant agent that also reduces the action of the BBB. Significant cannabimimetic effects were induced by AJA (CT-3) following pretreatment with cyclosporine A (50 mg/kg i.v.) that included hypothermia and visible sedation. AJA (CT-3) induced hypothermia was studied in a series of mouse strains. Based on genetic considerations, it was concluded that AJA is a “CNS-excluded cannabinoid CB1 receptor agonist” and is a member of a novel class of agents that are safe in humans.

4.1.7. Suppression of osteoclastogenesis—Osteoclasts are large multinucleated cells formed by the fusion of hematopoietic precursors of the monocyte/macrophage lineage. In many inflammatory diseases such as periodontal disease, rheumatoid arthritis, and metastatic cancers, excess osteoclast activity leads to bone resorption. Addition of AJA (IP-751) to stimulated precursor mouse macrophages and bone marrow cells in culture suppresses development of multinucleated osteoclasts (osteoclastogenesis), and prevents further osteoclast formation in cultures in which osteoclastogenesis had already begun⁵⁷. Thus, reduction of osteoclastogenesis may be an additional mechanism whereby AJA prevents bone erosion in joints of rats with adjuvant arthritis.

4.1.8. In vitro metabolism and metabolic effects—Plasma samples obtained from a clinical study on 21 patients suffering from neuropathic pain with hyperalgesia and allodynia³⁷ were analyzed for AJA and its glucuronide. For example, plasma levels of 599 ± 37.2 ng/ml (mean $\pm$ R.S.D., $n=9$) AJA were obtained for samples taken two hours after the administration of an oral dose of 20 mg AJA (CT-3). The mean AJA glucuronide concentration at two hours was 63.8 ± 128 ng/ml⁵⁸. No other metabolites were reported.

The *in vitro* metabolism of AJA (IP-751) by cryopreserved hepatocytes from rats, dogs, cynomolgus monkeys and humans was studied in which it was shown that only minor amounts of biotransformation products were observed⁵⁹. These were primarily side chain

hydroxylation metabolites and glucuronides. Unchanged amounts of AJA detected after a two hour incubation were 103%, 90%, 86% and 83% for rat, dog, monkey and human hepatocytes, respectively. AJA (IP-751) also showed low inhibitory activity against several human cytochrome P450 isozymes, CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5, using human hepatic microsomes as the test system. These data suggest that AJA would display a high safety profile when administered to human subjects.

4.2. HU-235: a putative metabolite of dexanabinol

The S,S enantiomer of AJA, HU-235, was tested for analgesic and anti-inflammatory activity³⁵. This compound is a putative metabolite of dexanabinol so that its activities may have significance for the actions of dexanabinol *in vivo*. It was observed to have moderate efficacy in the mouse hot plate and paw edema assays, however, these findings are questionable. A subsequent chiral analysis of this sample of HU-235 revealed that it was contaminated with approximately 20-30% of AJA (Atlantic Pharmaceuticals, unpublished data), suggesting that the AJA was responsible for the observed activities.

4.3. (-)-CBD-11-oic acids and their dimethylheptyl (DMH) homologs

A major, non-psychotropic constituent of *Cannabis* is cannabidiol (CBD) that is similarly metabolized to give CBD-11-oic acid (Figure 4A). This compound, its dimethylheptyl analog (HU-320, Figure 5C) and their enantiomeric isomers were synthesized and their cannabinoid receptor binding affinities measured⁶⁰. The results from the binding experiment were surprising in that, as expected, the isomers with the R, R “natural” stereochemistry were inactive however, unexpectedly, the S, S “unnatural” isomers displayed significant affinity for CB1. No explanation for this unusual observation was offered. HU-320 was studied for its anti-arthritis potential in mice at doses of 1 and 2 mg/kg⁶¹. The findings were similar to those reported earlier for AJA (Section 4.1.2) further supporting the hypothesis that carboxylic acid derivatives of cannabinoids are biologically active substances with potential therapeutic applications (Section 3.3). A comparison between HU-320, AJA and ⁹-THC in the mouse tetrad assay⁴² was done with all compounds a single dose of 20 mg/kg in which AJA responded in similar fashion to ⁹-THC. This dose is 200 times that needed for a potent anti-arthritis effect as previously reported³⁶. Thus, the results of the tetrad assay in this study are probably not of any consequence for possible therapeutic applications contrary to the conclusion reached by the authors.

4.4. Comparison of AJA with arachidonic acid

A comparison of the structure of AJA (Fig. 10, B) with the long chain fatty acid arachidonic acid (Fig. 10, A) gives rise to some interesting speculations. If the two structures are superimposed in a two dimensional representation, a considerable overlap can result (Fig. 10, C). This suggests that AJA may serve as a surrogate for arachidonic acid in a variety of biological events. For example, AJA has a profound effect on the activity of COX-2 where arachidonic acid is the principle substrate. Another example is the action of PPAR- γ where it was shown that AJA binds to⁶² and activates⁶³ this important member of the nuclear receptor super family. AJA may also substitute for some of the metabolites of arachidonic

acid such as the prostaglandins and the lipoxins. For example, LXA₄ (Fig. 10, D) has been shown to be a potent mediator in the resolution of chronic inflammation^{53, 64}. A second important example is PGJ₂ (Fig. 10, E).

5. Endogenous counterparts

An endogenous subfamily of eicosanoids, the lipoamino acids, represents possible counterparts to the cannabinoid acids. These substances are conjugates of long chain fatty acids and α -amino acids joined by an amide linkage. They produce a variety of biological actions including the resolution of inflammation and analgesic responses in preclinical models. The most widely studied member of this family is N-arachidonoyl glycine (NAGly, Fig. 11), which is similar in structure to anandamide (arachidonoyl ethanolamide), a CB1/CB2 agonist. In fact, the two are metabolically interrelated; oxidation of the hydroxyl group of anandamide leads to NAGly^{65, 66}. This “endogenous counterpart” hypothesis is further supported by structural overlay considerations between AJA and arachidonic acid (Fig. 10, C). In addition, a number of activities of the two are shared, for example, low affinity for the CB1 receptor. Several aspects of this topic have been previously reviewed^{67, 68}.

5.1. Lipoamino acids (Elmiric acids, EMA)

The lipoamino acids, which we have termed elmiric acids⁶⁷, are emerging as an important family of endogenous signaling molecules⁶⁹ that may act as physiological regulators of pain and inflammation⁷⁰. The existence of these endogenous substances was first predicted more than 16 years ago⁶⁵ when several examples were synthesized and shown to exhibit anti-inflammatory and analgesic activity in mice. Subsequent studies identified several natural EMAs in rat brain extracts⁷¹. They may be thought of as a branch of the eicosanoid super family with whom they share several features.

5.2. Occurrence, metabolism and tissue distribution of the lipoamino acids

The prototypic EMA, NAGly, shown in Figure 11, is found in rat brain, spinal cord, and other tissues where it occurs in amounts greater than the closely related endocannabinoid, anandamide⁷¹. The rank order of dry weight tissue content in the rat is: spinal cord > small intestine >> kidney > glabrous skin >> brain > testes > lung > liver > blood > spleen > heart. The values were obtained by LC/MS selected ion monitoring using lyophilized samples of fresh tissue samples.

Tan et al. used a targeted lipidomics approach with specific enrichment steps, nano-LC/MS/MS and high-throughput screening of the data sets to measure the endogenous levels of lipoamino acids in rat brain^{72, 73}. They identified 50 different lipoamino acids that were present at 0.2 to 69 pmol/g of wet rat brain. They observed that in compounds with the same amino acid group, those with palmitoyl, stearoyl, and oleoyl groups were more abundant than those with arachidonoyl or docosahexaenoyl groups. This matches the abundance of these fatty acids either as free acids or as phospholipids, suggesting that coupling of fatty acids with amino acids is a general route for lipoamino acid biosynthesis.

Evidence has been published showing that cytochrome c catalyzes the synthesis of NAgly from arachidonoyl coenzyme A and glycine in the presence of hydrogen peroxide⁷⁴. Other heme-containing proteins, hemoglobin and myoglobin, were significantly less effective in promoting the synthesis of NAgly compared to cytochrome c. The formation of N-arachidonoyl serine, N-arachidonoyl alanine, and N-arachidonoyl- γ -aminobutyric acid from arachidonoyl CoA and the respective amino acids mediated by cytochrome c was reported⁷⁵. Interestingly, arachidonoyl CoA and ethanolamine were found to react spontaneously to form anandamide, independent of cytochrome c and hydrogen peroxide.

COX-2 selectively metabolizes NAgly to N-PGH₂gly and N-hydroxyeicosatetraenoic-gly⁷⁶. Site-directed mutagenesis identified the side pocket residues of COX-2, especially Arg-513, as critical determinants of the COX-2 selectivity towards NAgly. These findings suggest a possible role for COX-2 in the regulation of NAgly synthesis and demonstrate the formation of a novel class of eicosanoids from NAgly metabolism. The lipoamino acids were efficiently oxygenated by 12S- and 15S-lipoxygenases⁷⁷. This suggests that fatty acid oxygenases may play an important role in the metabolic inactivation of lipoamino acids or may convert them to bioactive derivatives.

5.3. Actions

The widespread occurrence of NAgly both in species and tissue sites within a given species suggests that it may have a variety of physiological and pharmacological effects. A summary of the actions of NAgly is shown in Table 2. Some of the actions shown in the table are discussed in more detail further on as are other actions not shown in Table 2. There is some evidence that there are several proposed mechanisms that can help explain this multiplicity of actions. One of these relating to anti-inflammatory effects is discussed in Sections 5.4 and 6.

5.3.1. Analgesic effects of NAgly—A preliminary report on the actions of NAgly was made in 1997⁶⁵. At that time, an increase in withdrawal time in the mouse hot plate assay at 55°C and a lack of effect in the mouse ring test for catalepsy was observed. The latter indicated a major difference when compared with the closely related endocannabinoid anandamide and suggested that CB1 was not a target for NAgly. A more complete study was subsequently published supporting the earlier findings and further suggesting that NAgly was an oxidative metabolite of anandamide⁷⁸.

Peripherally administered NAgly at 275 nmol was 50% effective in suppressing phase 2 (tonic pain phase) of formalin-induced pain behavior⁷¹. This suggests that NAgly can suppress formalin-induced hyperactivity in nociceptive afferents either directly on the nerve, or indirectly by modulating their immediate interstitial environment. It was concluded that suppression of formalin-induced pain by NAgly might have relevance to postoperative and chronic pain states. A study of NAgly in a rat model of neuropathic pain, namely, intrathecal administration of NAgly (700 nmol) was reported to reduce the mechanical allodynia induced by partial ligation of the sciatic nerve⁷⁹. They also observed that HU-210, but not NAgly, produced a reduction in rotarod latency. It was suggested that NAgly might provide a novel approach to alleviate neuropathic pain. The actions of NAgly on neurons within the

superficial dorsal horn, a key site for the actions of many analgesic agents were examined⁸⁰. The study suggested that NAgly enhanced inhibitory glycinergic synaptic transmission within the superficial dorsal horn by blocking glycine uptake *via* GLYT2. NAgly also decreased excitatory NMDA-mediated synaptic transmission. They suggested that the study provided a cellular explanation for the spinal analgesic actions of NAgly.

5.3.2. Anti-inflammatory actions of NAgly—Reports from our laboratory suggest that NAgly reduces responses to inflammatory stimuli in both *in vitro* and *in vivo* models^{67, 70, 81}. A mini library of lipoamino acids (elmirc acids) was synthesized and evaluated for activity as potential anti-inflammatory agents in which prostaglandin production was compared with effects on an *in vivo* model of inflammation (Table 3). LPS stimulated RAW 267.4 mouse macrophage cells were the *in vitro* model and phorbol ester-induced mouse ear edema served as the *in vivo* model. The prostaglandin responses were found to be strongly dependent on the nature of the fatty acid part of the molecule. Polyunsaturated acid conjugates produced a marked increase in media levels of PGJ₂ with minimal effects on PGE production. It is reported in the literature that prostaglandin ratios in which the J series predominates over the E series promote the resolution of inflammatory conditions⁸². Several of the elmirc acids tested produced such favorable ratios suggesting their potential anti-inflammatory activity. The ear edema assay results were generally in agreement with the prostaglandin assay findings indicating a connection between them (Table 3). The second study was in a mouse peritonitis model involving migration of pro inflammatory cell types into the peritoneal cavity following a challenge with thioglycollate⁸¹. NAgly reduced migration by 60% at a dose of 0.3 mg/kg administered orally. Evidence was presented suggesting that GPR18 played a significant role in this therapeutic response.

The nature of the fatty acid is a dominant factor in anti-inflammatory activities both *in vitro* and *in vivo* since increasing unsaturation results in greater effects in both models (Table 3). Interestingly, the steric volume of the amino acid, in going from glycine to 1,1-dimethylglycine does not seem to appreciably change the responses in either model.

5.3.3. Inhibition of FAAH activity—NAgly is a potent inhibitor of the fatty acid amide hydrolase (FAAH), the enzyme primarily responsible for the degradation of the endocannabinoid N-arachidonylethanolamine (anandamide)⁸³. A mini library of several N-arachidonoyl-amino acids was synthesized including the D and L isomers of N-arachidonoyl alanine. These were tested for inhibitory activity on FAAH preparations from mouse, rat, and human cell lines, and from mouse or rat brain. NAgly was the most potent compound on the rat and mouse enzymes whereas N-arachidonoyl isoleucine was active only on human FAAH. The data suggest that an increase in anandamide levels may, in part, account for the analgesic effects of NAgly in rodents. Data from another study suggested that it may serve as an endogenous regulator of tissue anandamide concentrations⁸⁴.

5.3.4. Other actions of NAgly—Palmitoyl glycine (PALgly); Arachidonoyl glycine (NAgly); Palmitoyl l-alanine (PAL-l-ala); Arachidonoyl l-alanine (NA-l-ala); Palmitoyl γ -aminobutyric acid (PALgaba); Arachidonoyl γ -aminobutyric acid (NAgaba) were tested *in vitro* for their effects on cell proliferation (Fig. 12)⁸⁵. The results demonstrated a wide range

of responses from 10-100% inhibition of proliferation. Both the fatty acid and the amino acid regions of the molecule influenced the activity of the structures that were tested. No data on mechanism were reported.

The occurrence of lipoamino acids in mouse bone was reported ⁸⁶. N-oleoyl-L-serine (OS) had the highest activity in an osteoblast proliferation assay and it was found that it triggers a G_i-protein-coupled receptor and Erk1/2. It also promotes osteoclast apoptosis through the inhibition of Erk1/2 phosphorylation and the receptor activator of nuclear-κ-B ligand (RANKL) expression in bone marrow stromal cells and osteoblasts. In intact mice, OS moderately increases bone volume density mainly by inhibiting bone resorption. Moreover, in a mouse ovariectomy model for osteoporosis, OS efficiently rescues bone loss by increasing bone formation and markedly limiting bone resorption.

A primary beta-cell-based functional assay to monitor intracellular Ca⁺⁺ flux ([Ca⁺⁺]_i) was used to screen an assortment of compounds, leading to the discovery of three novel insulin secretagogues: NAgly, 3β-(2-diethylamino-ethoxy) androstene hydrochloride (U18666A) and 4-androstene-3,17-dione ⁸⁷. NAgly increased [Ca⁺⁺]_i through stimulation of the voltage-dependent Ca⁺⁺ channels that was dependent on the extracellular glucose level. It was suggested that NAgly might be useful for the development of insulinotropic factors and drugs for treating type 2 diabetes.

NAgly inhibits store-operated Ca⁺⁺ entry (SOCE), a ubiquitous Ca⁺⁺ entry pathway regulating multiple cellular functions, in a time- and concentration-dependent manner ⁸⁸. The inhibitory action on SOCE was found in the human endothelial cell line EA.hy926, the rat pancreatic beta-cell line INS-1 832/13, and the rat basophilic leukemia cell line RBL-2H3. These findings demonstrated the STIM1/Orai1-mediated SOCE machinery as a molecular target of NAgly that might have multiple implications in cell physiology.

The levels of the signaling lipids anandamide, 2-arachidonoyl glycerol, N-arachidonoyl glycine, N-arachidonoyl-γ-amino butyric acid, and N-arachidonoyl dopamine in seven different brain areas (pituitary, hypothalamus, thalamus, striatum, midbrain, hippocampus, and cerebellum) in male rats, and in female rats at five different points in the estrous cycle were measured ⁸⁹. The report provides a basis for an understanding into how differences in gender and hormonal status may influence mechanisms regulating endocannabinoid production.

NAgly produces vasorelaxation in rat small mesenteric arteries predominantly *via* activation of BK (Ca⁺⁺) channels ⁹⁰. The data obtained in this study suggest that NAgly activates an unknown G(i/o)-coupled receptor that stimulates endothelial release of nitric oxide, which then activates BK (Ca⁺⁺) channels in smooth muscle. It was also concluded that NAgly might activate BK (Ca⁺⁺) channels through G(i/o)- and nitric oxide-independent mechanisms.

A complex response relationship between the amino acid and fatty acid groups in the inhibition of cancer cell proliferation was observed ⁸⁵. Thus, a robust inhibition was seen when arachidonoyl glycine or gaba derivatives were tested; however, the palmitoyl analogs were completely inactive (Figure 12). This is similar to the effects seen in the anti-

inflammatory studies (Table 3). In sharp contrast, the L-alanine derivatives gave a reverse order of activity i.e. palmitoyl-L-ala was very active whereas arachidonoyl-L-ala was only moderately active. The reason for this anomaly is not immediately apparent.

The actions of NAgly on endothelial electrical signaling in combination with vascular reactivity were recently reported⁹¹. The data identified NCX ($\text{Na}^+/\text{Ca}^{++}$ exchanger) as a Ca^{++} entry pathway in endothelial cells and NAgly as a potent G-protein-independent modulator of endothelial electrical signaling. The study also revealed a dual effect of NAgly on endothelial electrical responses. It was concluded that in agonist pre stimulated cells, NAgly opposes hyperpolarization and relaxation via inhibition of NCX-mediated Ca^{++} entry, while in unstimulated cells it promotes hyperpolarization *via* receptor-independent activation of BKCa channels.

Lipoxin A₄ has been proposed to be an important mediator in the resolution of chronic inflammation⁹³. Figure 13 shows a robust stimulation of LXA₄ resulting from exposure to NAgly. An analog with no anti-inflammatory activity PALgly had no effect on LXA₄ synthesis. In Section 4.1.2., a similar effect is described for AJA where blockade of the 12/15 LOX enzyme was also reported to reduce LXA₄ levels.

5.3.5. Other lipoamino acids—N-Palmitoylglycine (PALgly) was partially purified from rat lipid extracts and it occurs in high levels in rat skin and spinal cord⁹⁴. PALgly levels were increased in fatty acid amide hydrolase (FAAH) knockout mice, suggesting enzymic regulation. PALgly has no anti-inflammatory actions, however, it potently inhibited heat-evoked firing of nociceptive neurons in rat dorsal horn and also induced transient Ca^{++} influx in native adult dorsal root ganglion (DRG) cells and a DRG-like cell line (F-11). The nonselective calcium channel blockers ruthenium red, SK&F96365, and La^{+++} blocked PALgly-induced Ca^{++} influx. It also stimulated the production of nitric oxide through calcium-sensitive nitric-oxide synthase enzymes present in F-11 cells and was inhibited by the nitric-oxide synthase inhibitor 7-nitroindazole.

N-linoleoyl glycine (LINGly) was tested in the mouse peritonitis assay where it showed activity in reducing leukocyte migration at doses as low as 0.3mg/kg⁹². Peritoneal cells from analog treated mice produced elevated levels of 15-deoxy-^{13,14}-PGJ₂ that seemed to correlate with a decrease in peritoneal cell number. A small group of N-linoleoyl analogs was studied for their ability to stimulate 15-deoxy-^{13,14}-PGJ₂ production in mouse macrophage RAW cells (Table 4). The D-alanine derivative was the most active while the D-phenylalanine showed almost no response. A high degree of stereo specificity was observed when comparing the D and L alanine isomers; the latter being the less active suggesting that the response is receptor mediated. LINGly is a more stable molecule than NAgly so that it might be a good substitute for the latter in future studies.

5.4. Mediation of NAgly actions by GPR-18

NAgly possesses analgesic properties but lacks the psychotropic activity of cannabinoids such as THC or anandamide. It has been shown that NAgly has low affinity for the cannabinoid CB1 receptor⁹⁵, however, it appears to activate the orphan G-protein coupled receptor (GPCR), GPR18^{81,96}. GPR18, a member of the GPCR super family, was

discovered and its sequence reported in 1997⁹⁷. In a subsequent report, Kohno et al. de-orphanized GPR18 with the identification of NAgly as a putative ligand based on functional responses⁹⁶. It was the only “hit” out of a group of 200 bioactive lipids screened suggesting a high degree of structural requirements for functional activity. Saturable, high affinity binding has been observed (Fig. 14) that is consistent with the designation of NAgly as a ligand for GPR18 (Burststein et al., unpublished). There are additional functional data supporting the ligand nature of NAgly and, possibly, a second molecule, called abnormal CBD⁹⁸.

McHugh et al. found that NAgly is an extremely effective recruiter of BV-2 microglia and its effects can result in anti-inflammatory actions in the brain⁹⁸⁻¹⁰¹. They reported that NAgly potently acts on GPR18 to produce directed migration, cell proliferation and perhaps other MAPK-dependent actions. These results advance our understanding of lipid-based signaling mechanisms employed by the CNS to actively recruit microglia to sites of injury. The NAgly-GPR18 signaling pathway offers a novel approach for developing therapeutic agents to elicit a population of regenerative microglia, or alternatively, to prevent the accumulation of misdirected, pro-inflammatory microglia that contributes to and intensifies neurodegenerative disease.

NAgly may also have an important role in peripheral sites. In a subsequent study from the same group, evidence was presented that NAgly, is effective in driving the migration of the human endometrial cell line, HEC-1B¹⁰². They concluded that these data potentially explain the unusual results from previous studies in the field in which the effects of CB1 and CB2 were unable to account for the observed phenomena in endometrial cells.

A recent study examined the occurrence and role of GPR-18 in the anterior murine eye¹⁰³. GPR18 was expressed in the ciliary epithelium, the corneal epithelium and the trabecular meshwork. NAgly, was detected in the mouse eye at a level comparable to that seen in the brain. It significantly reduced intraocular pressure in all of the mice tested following topical administration. Evidence was obtained for a functional GPR18-based signaling system in the murine anterior eye, including receptors and ligands. The findings suggested that GPR18 might serve as a target for the development of novel ocular hypotensive medications.

Further support for the role of GPR18 in the *in vitro* actions of NAgly was obtained by the use of polyclonal antibodies as recently reported⁸¹. It was found that treatment of the cells with 3 μ M NAgly caused a 100% rise in PGJ concentrations in the media. The addition of a polyclonal antiGPR18 reduced this response by about 50% suggesting that GPR18 is a mediator of this action. The further addition of a GPR18 blocking peptide completely abolished the antibody action showing that this is a specific effect. These observations also raise the possibility that antiGPR18 can be used to implicate this receptor in other actions of the elmiric acids.

Using the β -arrestin PathHunter™ assay system, a newly developed, generic GPCR assay format that measures β -arrestin binding to GPCRs, an evaluation of receptor and ligand pairing for NAgly and GPR-18 was reported¹⁰⁴. They found no interaction between the two in contradiction to all of the above reports showing the opposite. Another study showing

GPR18 coupling in a native neuronal system with endogenous signaling pathways and effectors was recently published¹⁰⁵. The addition of NAgly to GPR18-expressing neurons did not inhibit calcium currents but instead potentiated currents in a voltage-dependent manner. These results suggested to the authors that NAgly is not an agonist for GPR18. They concluded, “GPR18 signaling involves non canonical pathways not examined in these studies” and suggested that there may be actions of NAgly that are mediated by it through such pathways. However, most of the experiments they reported were carried out only at 10 μ M NAgly casting doubt on the significance of the findings. For example, several earlier reports showed inverted dose response relationships for NAgly^{102,81; 68} suggesting that rather different findings might have been obtained at lower concentrations.

In summary, the majority of evidence thus far reported supports the existence of a NAgly-GPR18 mediated mechanism of action. However, when all of the reports are considered, the possibility of more than one mechanism has been raised. Additional studies are therefore needed for a better understanding of the molecular pharmacology of the lipoamino acids.

6.0. Putative mechanisms for the anti inflammatory actions of AJA and NAgly

Although not complete, there are sufficient data to allow a proposed mechanism to be made for the anti-inflammatory actions of AJA and NAgly (Figure 15). In keeping with structural similarities (Section 4.4), there are also striking similarities in their mechanisms. The initial step would be the activation of one of three likely GPCR receptors: CB1, CB2 or GPR18 (steps 1 and 2). There are data showing that AJA does not bind significantly to CB1 but is a ligand for CB2*; there is no evidence for AJA binding to GPR18. However, in the case of NAgly, there is ample evidence for high affinity, saturable binding to GPR18 (Figure 14), but, little is known about its effects on CB2. It was reported to have no meaningful affinity for CB1⁹⁵. There may be a role for PPAR- γ in some of the actions of NAgly¹⁰⁶ through a positive feedback step to PPAR- γ ¹⁰⁷. This possibility is suggested by the data shown in Figure 16.

NAgly induced an increase in intracellular Ca^{++} concentration in GPR18-transfected cells that was significantly greater than that in mock-transfected cells⁹⁶ supporting step 2 of the proposed mechanism. No such data are available for AJA. Activation of phospholipases is a well-known effect of increased intracellular Ca^{++} ion concentration (step 3)¹⁰⁸. With both AJA and NAgly this results in the mobilization and release of free fatty acids (Figure 8 and Table 5) from their phospholipid storage sites; in particular free arachidonic acid (step 4), which is a substrate for a number of eicosanoid metabolites. Step 5 indicates a selective transformation of arachidonic acid to PGD_2 that is mediated by COX-2¹⁰⁹ and PGD synthetase⁵². In an unregulated process¹⁰⁹, PGD_2 is rapidly converted to PGJ_2 (Figure 9 and Table 4) a potent agent for the resolution of chronic inflammation¹¹⁰ as shown in steps 7 and 8. An additional metabolic pathway for free arachidonic acid is mediated by lipoxygenases resulting in the production LXA_4 ⁵³ that can also achieve a resolution of chronic inflammation as indicated in steps 6 and 9. These mechanistic similarities indeed support the suggestion that the lipoamino acids are endogenous counterparts of the cannabinoid acids.

7.0 Conclusions

Following their discovery¹⁰, the cannabinoid acids were determined to be biologically inactive¹¹ and for some time little was reported to dispute this claim. However, subsequent studies showed they produce a number of physiological and pharmacological actions¹. These include anti-inflammatory activity, analgesia and inhibition of THC-induced catalepsy. The synthetic analogs showed additional actions that include anti-neoplastic, anti-cystic, anti-fibrotic, suppression of osteoclastogenesis and reduction of spasticity in multiple sclerosis¹¹. More recent reports describe a family of endogenous counterparts of the acids that are structurally different but share many of the actions of the acids⁶⁷. Studies on mechanism(s) for these actions thus far show a lack of involvement of CB1, thereby, explaining the absence of psychotropic activity for all of these substances. Data have been obtained for the involvement of CB2 and the orphan receptor GPR18. A role for the anti-inflammatory eicosanoids PGJ₂ and LXA₂ has been observed that may include a feedback step involving PPAR- γ . There is some evidence for inhibition of FAAH activity, which results in increased levels of anandamide. All of these findings indicate that potent and stable analogs would make good drug candidates for several indications. Ajulemic acid is such a substance that is currently undergoing Phase 2 trials for the treatment of lupus, scleroderma and cystic fibrosis. Further exploitation of the therapeutic potential of the cannabinoid acids is likely to result in the discovery other candidates and indications.

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Biography

Born: Boston, MA, 1932

BS, Massachusetts Institute of Technology, 1953

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My research activities have centered on the structure and function of natural and synthetic cannabinoids and their mechanism of action. This research led to the discovery of the principal route of metabolism of THC, from which it was demonstrated that the terminal carboxylic acid metabolites of THC are non-psychoactive, yet retain their activity as analgesic and anti-inflammatory agents. This resulted in the design and discovery of ajulemic acid (JBT-101) a novel non-psychotropic cannabinoid drug under development for the treatment of pain, inflammation and fibrotic disease. Recent studies led to the discovery of a novel lipid signaling pathway involving NAgly. At The Worcester Foundation for Experimental Biology where, in addition to studies on cannabinoids, work was done in the areas of steroid and prostaglandin biochemistry.

Abbreviations

NAgly	N-arachidonoyl glycine
AJA	ajulemic acid
THC	tetrahydrocannabinol
CBD	cannabidiol
CBN	cannabinol

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Figure 1.
Cannabinoid acids as plant constituents. A. tetrahydrocannabinolic acid A. B. tetrahydrocannabinolic acid B

Figure 2. Principal route of metabolism for ⁹-THC in most species. Compounds A (⁹-THC), B and C all showed similar biological activities. While devoid of psychotropic activity in mice and humans, compound D, the terminal carboxy metabolite showed NSAID-like action in several animal models, albeit at low potency. This prompted the synthesis of a more potent analog, ajulemic acid (*vide infra*).

Figure 3.

Examples of metabolites of 9 -THC that have been detected and identified. A. 9 -THC-11-oic acid itself. B. Monohydroxy 9 -THC-11-oic acid; arrows indicate positions of hydroxyl groups. C-F. Side-chain degradation products.

Figure 4. Metabolites of other phytocannabinoids, ¹⁻³. A. CBD-11-oic acid. B. CBN-11-oic acid

Figure 5.

Structures of synthetic analogs of cannabinoid acids. A. R,R'-1,1'-dimethylheptyl-⁸-THC-11-oic acid (AJA). B. S,S'-1,1'-dimethylheptyl-⁸-THC-11-oic acid. C. S,S'-1,1'-dimethylheptyl-⁹-CBD-11-oic acid (HU-320); D. R,R'-1,1'-dimethylheptyl-⁹-CBD-11-oic acid.

Figure 6.

Dose--response curve of AJA (CT-3) on PAF--induced mechanical allodynia. Platelet--activating factor (PAF) injected in the hind paw of rats was used as a model of tonic inflammatory pain. PAF injection led to a drop in the withdrawal threshold to mechanical pressure (83g to 40g). Varying doses of AJA (3, 4.5, 6.8, 10.1, 15.2 mg/kg, i.p.) were administered and the paw withdrawal thresholds at 40--60min post--drug were plotted. An approximate ED-50 of 4.2mg/kg was estimated for reversal of PAF-induced allodynia. Complete reversal was seen at around 6.8 mg/kg, above which anti nociception was observed (Walker et al., unpublished).

Figure 7. Inhibition by Ajulemic Acid of Arachidonic Acid-Induced Mouse Paw Edema. Adapted from Burststein, et al. (1992). Conditions as reported.³⁵

Figure 8.

Release of free arachidonic acid is promoted by ajulemic acid (IP-751). Human synovial cells were labeled and treated as reported.⁵² Partial inhibition resulted from pre treatment with the CB2 receptor antagonist SR144528 (unpublished data).

Figure 9. Stimulation of PGJ₂ synthesis by AJA (JBT-101), THC and CBD in HL-60 cells. Conditions used were as previously reported.⁸¹

A. Arachidonic Acid **B. Ajulemic Acid** **C. Overlay Structure: red is an arachidonate moiety** **D. Lipoxin A₄** **E. Prostaglandin J₂****Figure 10.**

A comparison of the structure of AJA with the structure of arachidonic acid. A-C depicts the similarities in structure between arachidonic acid and AJA. The structures of lipoxin A₄ and PGJ₂ are shown for comparison purposes.

Figure 11.

The structure of NAgly. There are three regions of the molecule that are of pharmacological interest. Region 1 confers a high degree of specificity of action. Polyunsaturated residues produce molecules with analgesic and anti-inflammatory action whereas saturated structures, in this action, are inactive. Region 2 is related to metabolic stability since NAgly is degraded by FAAH (fatty acid amide hydrolase) activity. Region 3, the amino acid residue, can have an effect on the analgesic and anti-inflammatory activities depending on steric factors and the chiral nature of the amino acid ⁴.

Figure 12.

Structural effects of lipoamino acids on rat basophilic leukemia (RBL-2H3) cell proliferation (Adapted from Burststein et al., 2008). Cells were treated with the lipoamino acids (10 μ M) followed by treatment with LPS (10 ng/ml). Cell numbers were obtained using the CellTiter-Glo assay. Compounds studied were: Palmitoyl glycine (PALgly); Arachidonoyl glycine (NAgly); Palmitoyl l-alanine (PAL-l-ala); Arachidonoyl l-alanine (NA-l-ala); Palmitoyl γ -aminobutyric acid (PALgaba); Arachidonoyl γ -aminobutyric acid (NAgaba).

Figure 13. Stimulation of lipoxin A₄ levels in mouse macrophage RAW 264.7 cells (Burstein et al., unpublished). Cell culture and treatment conditions were as previously reported.⁸¹ Media were assayed for LXA₄ using an enzyme Immunoassay kit. PALgly is included as a control.

Figure 14.

Binding isotherm for NAGly using (A) GPR18 transfected HEK-293 cell membranes compared with (B) wild type HEK-293 membranes (Burststein et al., unpublished). 25 mg of membranes were incubated in binding buffer (50 mM Tris, 0.5 mM EDTA, 0.1% BSA, pH 7.4) in the presence of increasing amounts of ^3H -NAGly (specific activity 200 Ci/mmol). Membranes were filtered with a 96 well GF/B filter plate and washed with 500 μl wash buffer (25 mM HEPES, 1 mM CaCl_2 , 5 mM MgCl_2 , 0.25 mM NaCl) using a Filtermate 196 Harvester. Filter plates were treated with 1% BSA prior to use to reduce non-specific binding. TB, total binding; NSB, non-specific binding.

Figure 15.

A proposed unified mechanism for the anti-inflammatory actions of AJA and NAGly.

Figure 16.

The PPAR- γ inhibitor GW9662 reduces the stimulation of PGJ₂ levels by NAgly in RAW cells (Burstein et al., unpublished data). Cells were cultured and treated as previously reported.⁹²

Table 1

Reported actions of ajulemic acid

Observed Response	Potency	Model	References
Reduces paw edema in mice	0.1 mg/kg	Arachidonic acid or PAF induction	Burstein et al., 1992
Reduces leukocyte adhesion	0.5 mg/kg	Mouse peritoneal cells	Burstein et al., 1992
Produces analgesia	0.05 mg/kg	Mouse hot plate at 55°C	Burstein et al., 1992
Adjuvant-induced chronic arthritis	0.1 mg/kg	Male rats	Zurier et al., 1998
Reduces leukocyte migration	0.2 mg/kg	Subcutaneous air pouch	Zurier et al., 1998
Decreases chronic neuropathic pain	20 mg b.i.d.	Humans with refractory pain	Karst et al., 2003
Reduction of writhing	1.2 mg/kg i.v.	p-phenylquinone writhing test	Burstein et al., 1998
Reduction of pain	4.6 mg/kg i.v.	Formalin anti-nociception test	Burstein et al., 1998
Analgesia	4.4 mg/kg i.g.	Mouse tail clip test	Dajani et al., 1999
Reversal of hyperalgesia	0.1-1 mg/kg	Inflammatory pain in the rat	Dyson et al., 2005
Increased latency	3.3 mg/kg	Tail flick assay	Dyson et al., 2005
Hypothermia	10 mg/kg	Core temperature	Dyson et al., 2005
Reduced mechanical allodynia	10 mg/kg	Nerve-injury induced model	Mitchell et al., 2005
Reduced mechanical allodynia	10 mg/kg	CFA-induced inflammatory pain	Mitchell et al., 2005
Inhibits metalloproteinases	10 μ M	Human synovial cells	Johnson et al., 2007
Induction of apoptosis	1 μ M	T lymphocytes	Bidinger et al., 2003
Decreases secretion of IL-1 β	5 μ M	Synovial fluid monocytes	Zurier et al., 2003
Increased COX-2 expression	20 μ M	Human synovial cells	Stebulis et al., 2008
Increased production of LXA ₄	0-30 μ M	Human blood or synovial cells	Zurier et al., 2009
Suppression of bladder activity	1.44 μ M	Rats	Hayn et al., 2008
Inhibition of cell proliferation	5.8-16 μ M	Cancer cells in vitro	Recht et al., 2001
Anti metastatic activity	0.1 mg/kg	SCID-NOD mouse flank tumor	Recht & Salmonsens, unpub
Prevents progression of fibrosis	1 mg/kg	Bleomycin-induced dermal fibrosis	Gonzalez et al., 2012
Reduction of spastic activity	0.1 mg/kg i.v.	Rat multiple sclerosis model	Pryce et al., 2013
Suppresses osteoclastogenesis	15 μ M	Precursor mouse macrophages	George et al., 2008

Table 2

Reported actions of NAgly

Observed Response	Potency	Model	Source
Formalin-induced pain suppression	275 nmol	Rat formalin test	Huang et al., 2001
Inhibits cAMP production	10 nM	GPR18-transfected CHO cells	Kohno et al., 2006
Resolution of inflammation	3 μ M	RAW 264.7 cells	Burstein et al., 2011
Cell migration	100 nM	BV-2 microglia	McHugh et al., 2010
Mouse peritonitis inhibition	0.3 mg/kg	Leukocyte migration	Burstein et al., 2011
Inhibition of glycine transport	3.4 μ M	Endogenous inhibitor of GLYT2	Edington et al., 2009
Induction of cell migration.	44 nM	Human endometrial HEC-1B cells	McHugh et al., 2012a
Macrophage apoptosis	10 μ M	RAW 264.7 cells	Takenouchi et al., 2012
Modulates synaptic transmission	30 μ M	Rat spinal cord slices	Jeong et al., 2010
Relaxant in mesenteric arteries	30 μ M	Activation of BK (Ca^{++}) channels	Parmar et al., 2010
Inhibition of T-type Ca channels	1 μ M	TsA-201 cells	Barbara et al., 2009
Neuropathic pain model	700 nM	Paw withdrawal threshold	Vuong et al., 2008
Regulation of anandamide levels in vitro & in vivo	10 mg/kg & 10 μ M	RAW 264.7 cells and rat blood	Burstein et al., 2002
Stimulate PGJ production	3 μ M	RAW 264.7 cells	Burstein et al., 2007
Inhibition of FAAH activity	8.5-50 μ M	FAAH preparations	Grazia Cascio et al., 2004
Increased $[\text{Ca}^{++}]_i$	10 μ M	Rat primary β -cells	Ikedo et al., 2005
Inhibits store-operated Ca^{++}	10 μ M	EA.hy926, INS-1 832/13, RBL-2H3 cell lines	Deak et al., 2012
Endothelial electrical signaling	3 μ M	EA.hy926 cells	Bondarenko et al., 2013
Reduces intraocular pressure	1% topical	Mouse eye	Caldwell et al., 2013

Table 3*In vivo vs In vitro* anti-Inflammatory effects (Burstein et al., unpublished)

Test Molecule	PGJ ₂ Activity * (% Control)	Activity in Ear Edema Assay **
Palmitoyl glycine (PALgly)	100	0
Oleoyl 1,1-dimethyl glycine	118	+
γ -Linolenoyl glycine (LINGly)	203	++
Arachidonoyl glycine (NAGly)	213	++
Arachidonoyl l-alanine	212	++
Arachidonoyl 1,1-dimethyl glycine	220	+++

* Stimulation of PGJ₂ synthesis by 10 μ M compound in mouse macrophage RAW cells.

** Relative potency in inhibiting phorbol ester-induced ear swelling in mice. Compounds applied topically in safflower oil at 1 mg/ml.

Table 4Stimulation of Prostaglandin J₂ levels in mouse macrophage RAW 264.7 cells

Treatment	Dose (μM)	PGJ ₂ (pg/ml)	SD
N-linoleoylglycine	1	<200	-
N-linoleoylglycine	2.5	1843	431
N-linoleoylglycine	5	3907	293
N-linoleoylglycine	10	7315	1444
N-arachidonoyl glycine	10	6847	625
DMSO	-	<200	-

Table 5

Stimulation of arachidonic acid release from RAW cells by NAgly^a (Adapted from Burstein et al., 2008)

NAgly (μM)	Arachidonic Acid (Treated/Control)	SD
1.5	1.0	0.21
3.0	1.2	0.13
6.0	1.4	0.32
12.0	1.85	0.31
24.0	2.95	0.25

^aFollowing a 2 hr labeling period with ¹⁴C-arachidonic acid, the media (RPMI+0.1% BSA) were changed and the cells treated for 1 hr with NAgly in 10 μl of DMSO. The control was 10 μl of DMSO. Release was measured by LSC on a 0.1 ml aliquot of medium. Values shown are the means ± SD. The cells were treated with non-radioactive NAgly.