

Molecular Mechanisms for the Inflammation-Resolving Actions of *Lenabasum*

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Abbreviations: AA, Arachidonic acid; CBD, cannabidiol; COX, cyclooxygenase; CYPGs, cyclopentenone prostaglandins; cPLA2, cytosolic PLA2; FMLP, N-Formylmethionine-leucyl-phenylalanine; GPCR, G-protein coupled receptor; LXA₄, lipoxin A₄; LOX, lipoxygenases; PGJ, 15-deoxy- $\Delta^{12,14}$ -prostaglandin-J₂; PLA2, Phospholipase A2; SSC, systemic sclerosis; SPMs, specialized pro-resolving mediators; PTGDS, Prostaglandin-H2 D-isomerase; THC tetrahydrocannabinol.

Abstract

A first-in-class cannabinoid analog called lenabasum that is a CB2 agonist is being developed as an inflammation-resolving drug candidate. Thus far specific therapeutic targets include scleroderma, cystic fibrosis, dermatomyositis and lupus all of which represent unmet medical needs. Two somewhat independent molecular mechanisms for this type of action are here proposed. Both pathways initially involve the release of free arachidonic acid following activation of the CB2 receptor and PLA₂ by lenabasum. The pathways then diverge into a COX-2 mediated and a lipoxygenase mediated route. The former leads to increased levels of the cyclopentenone prostaglandin 15-deoxy- $\Delta^{12,14}$ -prostaglandin-J₂ that can activate the NLRP3 inflammasome, which, in turn releases caspase-3 leading to apoptosis and the resolution of chronic inflammation. The lipoxygenase mediated pathway stimulates the production of lipoxin A₄ as well as other signaling molecules called specialized pro-resolving mediators (SPMs). These also have inflammation-resolving actions. It is not well understood under which conditions each of these mechanisms operate and if there is cross-talk between them. Thus, much remains to be learned about the mechanisms describing the actions of lenabasum.

Significance Statement.

The resolution of chronic inflammation is a major unmet medical need. The synthetic non-psychoactive cannabinoid lenabasum could provide a safe and effective drug for this purpose. Two putative molecular mechanisms are suggested to better understand how lenabasum produces this action. In both different metabolites of arachidonic acid act as mediators.

I. Introduction

The effects of cannabinoids on the eicosanoid family of signaling molecules have been under investigation for some time (Burstein, 2019). Among the compounds studied were the phytocannabinoids tetrahydrocannabinol (THC) and cannabidiol (CBD). In addition, synthetic agents such as ajulemic acid (also called anabasum or lenabasum), CP55,940, JWH-133 and WIN-55,212-2 were reported on. The actions studied were; alteration of behavioral responses, reduction of pain sensation, resolution of inflammation, hypotensive and vasorelaxant responses, anti-cancer activity and reduction of intraocular pressure. Thus, there appear to be a number of cannabinoid and eicosanoid interactions that could be exploited for therapeutic purposes (Burstein, 2019). In the example of lenabasum, these will be discussed below and a brief history of its development now follows.

The events leading to the discovery and development of lenabasum began with investigations into the metabolism of the cannabinoids (Burstein et al., 1972). It was concluded from these studies that the terminal metabolite of THC is THC-11-oic acid. Interestingly, it was observed that this metabolite, unlike THC, was not psychoactive, however, it appeared to have anti-inflammatory activity (Burstein et al., unpublished). This observation was exploited for the purpose of finding the drug candidate ajulemic acid (Fig.1), that was subsequently reported (Burstein et al., 1992) and later named lenabasum. Lenabasum is the 9-carbon 1,1-dimethylheptyl side-chain analog of THC-11-oic acid. In this study, it was shown that lenabasum had potent activity in the mouse paw edema assay (est. ED-50; 0.025 mg/kg) and in the mouse hot plate assay(est. ED-50; 0.10 mg/kg). In the hot plate assay, a “bell-shaped” curve was seen suggesting a different mechanism than in the paw edema assay. In a later preclinical report, this idea was confirmed and it was concluded that “Available evidence indicates that CT-3 (lenabasum) exhibits two distinct pharmacological properties: an anti-inflammatory property occurring at a very low dose and an analgesic property occurring at a higher dose” (Dajani et al., 1999).

Since 1992 several studies have confirmed and expanded the therapeutic properties of lenabasum. Thus, adjuvant-induced polyarthritis in rats was used as a model of chronic inflammation and joint tissue injury (Zurier et al., 1998). Histopathologic studies of tibiotarsal joints showed that lenabasum treatment (0.1 mg/kg 3 times/week) reduced pannus formation and joint tissue injury by about 80% when compared to vehicle treated rats. A subsequent study also in rats further

confirmed the analgesic actions of lenabasum and, additionally, showed that it did not alter motor function in the rotarod procedure at 4.64 mg/kg i.v.; the latter being a desirable property for a drug candidate (Burstein et al., 1998).

Because of its potent effects on cell adhesion (Burstein et al., 1992), it was decided to test lenabasum in a cancer model for possible inhibition of metastatic disease. Lenabasum administered p.o. to nude mice at a dosage several orders of magnitude below that which produces toxicity, inhibited the growth of subcutaneously implanted U87 human glioma cells (Recht et al., 2001). It was shown that lenabasum can produce significant antitumor activity and its effects are mediated by the action of CB2 receptors. A dramatic decrease in ascites in treated animals suggested concurrent anti-inflammatory and anti-metastatic action (unpublished findings).

Interleukin-1 beta (IL-1b) and tumor necrosis factor-alpha (TNF-a) are critical factors in the progression of inflammation and joint tissue injury in patients with rheumatoid arthritis. Thus, the effects of lenabasum in peripheral blood and synovial fluid monocytes on these factors in samples from both normal and rheumatoid arthritis subjects was examined (Zurier et al., 2003). Cells were treated with lenabasum (0-30 microM) *in vitro*, and then stimulated with lipopolysaccharide. Drug treatment suppression was maximal (50.4%) at 10 microM ($p < 0.05$ vs untreated controls, $N=7$).

Studies were done to provide some insight into the mechanism of the anti-inflammatory action of lenabasum (Bidinger et al., 2003). Thus, apoptosis resulting from lenabasum treatment of human T cells *in vitro* was determined by measuring annexin V expression, caspase-3 activity, DNA fragmentation, and microscopy. Lenabasum induced apoptosis of T cells in a dose- and time-dependent manner. Incubation of cells with 10 microM lenabasum resulted in significant increases (2.5- to 8.4-fold) in Annexin-V-positive cells. Apoptosis preceded loss of cell viability as shown by trypan blue dye exclusion, confirming that cell loss was due to programmed cell death rather than necrosis.

A pharmacokinetic analysis showed that lenabasum has limited brain penetration, with a brain/plasma ratio of 0.4 following oral administration (Dyson et al., 2005). They suggested that this is the basis for the lack of psychoactivity rather than a higher affinity for CB2 vs CB1. In contrast to other reports, they found that lenabasum binds to human cannabinoid *receptors in vitro* with high affinity at hCB1

($K_i = 6$ nM) and hCB2 ($K_i = 56$ nM). The sample of compound used was prepared by them and no purity data were provided raising the possibility of impurities with high affinity for CB1 receptors. The likelihood of such a possibility has been demonstrated more recently (Tepper et al., 2014).

Analgesic actions of lenabasum in animal models of neuropathic and inflammatory pain have been reported (Mitchell et al., 2005). It was compared to HU-210 and both were found to be effective. However, lenabasum, unlike HU-210, did not exhibit any of the motor side effects associated with THC and other non-selective cannabinoid receptor agonists. This again suggests a lack of CNS activity by lenabasum.

The reduction by lenabasum of urinary frequency induced by nociceptive stimuli in the bladder has been observed in rats (Hiragata et al., 2007). It was effective when given i.v. at a dose 10 mg/kg. Evidence was presented supporting a mechanism involving CB1 rather than CB2 which is proposed for inflammation-resolving action. It was suggested that lenabasum could be effective in the treatment of patients with painful bladder syndrome or interstitial cystitis.

Unlike the traditional anti-inflammatory agents (e.g, NSAIDs) that inhibit the production of eicosanoids, lenabasum stimulates their production through the arachidonic acid cascade (Stebulis et al., 2008). However, the difference is in the specific eicosanoids involved. Lenabasum treatment results in the elevation of the inflammation-resolving molecule PGD₂ and the resulting PGJ series that promote the resolution of inflammation. The NSAIDs generally act by inhibiting the production of the pro-inflammatory eicosanoid PGE₂. In the study cited here, the effect of lenabasum on cyclooxygenase 2 (COX2) expression and eicosanoid production in human fibroblast-like synovial (FLS) cells was measured. Treatment of FLS cells with lenabasum increased 15d-PGJ₂ production in a concentration dependent manner but did not affect PGE₂ production significantly.

Eicosanoids produced by the sequential actions of 5- and 15-lipoxygenases (LOX), promote the resolution of inflammation. The levels of one of these, lipoxin A₄, is elevated 2-5 fold in FLS treated with lenabasum (Zurier et al., 2009). Administration of lenabasum to mice with peritonitis resulted in a 25-75% decrease in cells invading the peritoneum and caused a 7-fold increase in LXA₄ measured by mass spectrometry.

The issue of lenabasum purity was addressed (Tepper et al., 2014). In its synthesis, the use of purified starting material and careful purification of the final product, resulted in a preparation that was 99.88% pure. Not taking these precautions can result in the production of byproducts with high affinity for CB1. The receptor affinity ratio of Ki CB1/Ki CB2 was 12.3 for this material. PGJ production in HL-60 cells (est. ED-50 = 5 microM) was blocked by the CB2 antagonist SR144528 but not by the CB1 antagonist. SR141716. *In vivo* functional responses in mice such as catalepsy and hypothermia with lenabasum that are CB1 mediated were absent at doses up to 30 mg/kg. Thus, it was observed that “conclusions from previous reports on certain actions of AJA (lenabasum) should now be reconsidered, in particular those claiming psychotropic activity”.

Just such a report was made where it was stated that lenabasum was not suited for use as a treatment for inflammation in humans because of its CB1 mediated activity (Sumariwalla et al., 2004). No information was given about the source or the purity of the lenabasum used to obtain the data reported. It seems possible that their material contained a significant amount of impurities with high affinity for CB1 making their conclusions about lenabasum not relevant. A detailed response to Sumariwalla *et al* was subsequently published (Burstein and Zurier, 2004). It is worth noting that in none of the human trials with lenabasum, involving a total of more than 900 subjects up to now, were cannabimimetic actions observed.

A detailed study on the pharmacology of lenabasum in rodents also claimed CB1 mediation for the observed actions (Vann et al., 2007). These included antinociception, catalepsy, hypothermia, and hypomobility in mice as well as a rat model of inflammatory pain. Unfortunately, no information on the purity of the lenabasum was reported raising serious questions on the significance of their conclusions. The study authors concluded that lenabasum, like THC, exhibits psychoactive and therapeutic effects at nearly equal doses in these preclinical models. However when ultra-pure preparations of lenabasum were used in these same tests, none of these effects were observed except at doses over 100-fold higher than doses with anti-inflammatory activity (Tepper and Burstein, 2014). Likewise, the clinical trial findings do not support this assumption (Karst et al., 2003) (Salim et al., 2005) (Karst, 2007) (Spiera et al., 2020).

Lenabasum was found to be effective in a novel model of acute inflammation where UV-killed *E. coli* were injected intra dermally into healthy humans (Motwani et

al., 2018). Following a 5 mg b.i.d. dose of lenabasum, inhibition of the neutrophil chemoattractant LTB_4 was observed; the inhibition of anti-phagocytic eicosanoids (PGE_2 , TxB_2 , and $PGF2a$) was also reported. At a dose of 20 mg, the biosynthesis of the specialized pro-resolving lipid mediators including LXA_4 , LXB_4 , $RvD1$, and $RvD3$ was stimulated. It was concluded that “for the first time a striking anti-inflammatory and pro-resolution effects of a synthetic analog of THC in healthy humans” has been demonstrated.

Several publications reported on the effects of lenabasum on $PPAR\gamma$ (Gonzalez et al., 2012, Lucattelli et al., 2016 al., Liu et al., 2003, Ambrosio et al., 2007). It is important to note that concentrations of lenabasum ranging from 10 to 40 micromolar were required to bind and activate $PPAR\gamma$ whereas its binding affinity to CB2 is 51 nM (Tepper et al., 2014). Thus, it seems not likely that $PPAR\gamma$ has an important role in the therapeutic actions of lenabasum.

As a continuation of its development program, Corbus Pharmaceuticals recently completed a Phase 3 multicenter, double-blind, randomized, placebo-controlled study assessing the efficacy and safety of lenabasum for the treatment of diffuse cutaneous systemic sclerosis (SSc). Approximately 354 subjects were enrolled in this study at about 60 sites in North America, Europe, Australia, and Asia. The duration of treatment with the study drug was 52 weeks (ClinicalTrials.gov Identifier: NCT03398837). Ongoing clinical trials by Corbus also include cystic fibrosis, dermatomyositis and lupus.

Following this history of lenabasum, a stepwise presentation of two proposed mechanisms for the inflammation-resolving action of lenabasum is now given.

II. COX mediated Mechanism for the Resolution of Inflammation

a. Cannabinoid receptor CB2. CB2 is a G protein coupled seven transmembrane receptor (GPCR) that shows binding affinity for several cannabinoids and cannabinoid analogs including lenabasum (Figure 1). It is expressed mainly in the periphery, primarily in cells of the immune system (Felder and Glass, 1998). By contrast the other primary cannabinoid receptor subtype, CB1, is found mainly in the CNS, where it mediates the psychotropic action of THC. Activation of CB2 can initiate a series of events eventually resulting in the resolution of chronic inflammation (Figure 2).

Lenabasum has greater affinity for CB2 over CB1 (Tepper et al., 2014) increasing its usefulness as a clinical agent for humans. Among the various preparations reported, highly purified lenabasum (called JBT-101 in Tepper et al.) had the weakest affinity for CB1 while the original preparation (HU-239) showed the strongest affinity for CB1. The CB1/CB2 ratio of affinities was 12.3 for JBT-101 whereas that for HU-239 was 0.19, a 65-fold difference. Similarly, Dyson et al, reported a greater affinity for CB1 vs CB2 for a preparation of lenabasum of unstated purity (Dyson et al., 2005). However, it was recently discovered that impurities can be present in most preparations that account for the additional CB1 activities (Tepper et al., 2014). This is in agreement with the observed CB1 mediated functional responses that were significantly decreased for purified lenabasum compared with HU-239.

b. Phospholipase A2 (PLA2). Cannabinoid-activated PLA2 promotes the release of free arachidonic acid (AA) from membrane glycerophospholipids (Burstein et al., 1994; Burstein, 2019). AA is a major precursor of eicosanoids such as the prostaglandins (PGs), leukotrienes (LTs) and resolvins. Thus far, at least 19 enzymes that possess PLA2 activity have been found in mammals (Dennis, 1994). The secretory PLA2 (sPLA2) family, in which 10 isozymes have been identified, consists of low-molecular-weight, Ca^{++} -requiring, secretory enzymes that have a role in a number of biological processes, such as inflammation, host defense, and atherosclerosis. The cytosolic PLA2 (cPLA2) family consists of 3 enzymes, among which cPLA2 α plays an essential role in the initiation of AA metabolism. Intracellular activation of cPLA2 α is tightly regulated by Ca^{++} and phosphorylation.

Cannabinoids have been reported to modulate the turnover and metabolism of membrane phospholipids in mouse brain preparations through the activation of phospholipases (Hunter et al., 1986). There was a dose related increase in PLA₂ activity leading to an elevation of free AA from phosphatidylcholine. Among the cannabinoids studied was THC-11-oic acid, which was the template molecule used to design lenabasum.

Interestingly, the endocannabinoid anandamide also promotes the release of free AA from phospholipids by activation of PLA₂ (Wartmann et al., 1995). Moreover, it was shown that the MAP kinase signal transduction pathway is involved in this response. WI-38 fibroblasts treated with anandamide showed increased MAP kinase

activity and increased phosphorylation of the arachidonate-specific cytoplasmic phospholipase A2 (cPLA2). Anandamide is a ligand for both CB1 and CB2.

c. Arachidonic acid. Arachidonic acid (AA) is a key precursor in the synthesis of a number and variety of members of the eicosanoid super family of messenger molecules. These include the prostaglandins, the leukotrienes, the lipoxins, etc. and require free AA for their synthesis. Almost all of the AA in the body is found as a component of various phospholipids and needs to be freed by phospholipase action to be converted to any of the eicosanoids.

One example is seen in the treatment of human fibroblast-like synovial (FLS) cells with lenabasum that caused a robust release of free AA (Stebulis et al., 2008). FLS were isolated from synovial tissue or synovial fluid of patients with inflammatory arthritis (4 rheumatoid arthritis, 1 psoriatic arthritis). They reported a 1.5-2 fold increase in AA release from FLS cells treated with lenabasum in comparison to vehicle treated controls.

d. COX-2. Cyclooxygenase (COX), whose full name is prostaglandin-endoperoxide synthase, is an enzyme that is responsible for the formation of prostanoids from AA. The specific reaction catalyzed is the conversion of AA to PGH_2 , via a transient PGG_2 intermediate. There are two types of COX enzymes, COX-1 and COX-2. Both enzymes produce prostaglandins that regulate inflammation, pain, and fever; however, only COX-1 produces prostaglandins that activate platelets and protect the gastrointestinal tract. COX-2 can promote the production of anti-inflammatory prostaglandins such as the cyclopentenone prostaglandins (CYPGs). NSAIDs inhibit both COX enzymes and reduce synthesis of prostaglandins.

Lenabasum elevated the steady state levels of COX-2 mRNA in FLS (Stebulis et al., 2008). This occurred in a concentration dependent manner in both unstimulated and $\text{TNF}\alpha$ -stimulated cells. A 2-fold induction of COX-2 was seen 4 hours after stimulation of cells with $\text{TNF}\alpha$. However, this was small when compared with the 5–8-fold induction of COX-2 in cells exposed to lenabasum (Stebulis et al. 2008).

e. PGD Synthase. Prostaglandin-H2 D-isomerase (PTGDS) is an enzyme that in humans is encoded by the *PTGDS* gene. The molecule encoded by this gene is a glutathione-independent PGD synthase that catalyzes the conversion of PGH_2 to PGD_2 . PGD_2 plays an important role in inflammatory reactions. It is

produced by mast cells, T helper 2 (Th2) lymphocytes and dendritic cells among others (Shimizu et al., 1979).

f. PGD₂ The most important prostaglandins found *in vivo* are: prostaglandin E₂ (PGE₂), prostacyclin (PGI₂), prostaglandin D₂ (PGD₂) and prostaglandin F_{2α} (PGF_{2α}). They are produced in many tissues and each cell type generates one or two dominant products that can function as autocrine or paracrine lipid mediators to maintain local homeostasis. Prostaglandin production is usually low in uninflamed tissues but increases in acute inflammation resulting in the recruitment of leukocytes and the infiltration of immune cells.

Through non-enzymic pathways PGD₂ is converted to PGJ₂, Δ¹²-PGJ₂, Δ¹²-PGD₂, 15-deoxy-Δ^{12,14}-PGJ₂, or 15-deoxy-Δ^{12,14}-PGD₂ (Fukushima, 1990). Due to this instability, PGD₂ or its metabolites must act in close proximity to the site of their synthesis. PGD₂ activates two distinct seven transmembrane, G-protein coupled receptors: the D-type prostanoid receptor (DP₁) and the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2 or DP₂). Although DP₁ and CRTH2/DP₂ share the same ligand, they have little sequence homology.

15-d-Δ^{12,14}-PGJ₂ (PGJ₂) (see Figure 3A) is a member of the cyclopentenone prostaglandins (CYPGs) (Burstein, 2020). This small group of AA metabolites is characterized by an alpha-beta unsaturated keto group that readily undergoes a Michael addition reaction with proteins containing a free SH group. Thus, they may show a variety of biological activities in various models including anti-inflammatory and ant-viral effects. No clinical applications for the CYPGs have so far been reported despite their discovery more than three decades ago. This likely is due to their instability when administered *in vivo*; a stable analog suitable for drug development is apparently not yet available. Thus, an agent that can generate a CYPG *in situ* close to the site of action may provide a viable approach to this problem in therapeutics. Lenabasum shows promise in doing just that.

15-d-Δ^{12,14}-PGJ₂ is produced from PGD₂ that undergoes a non-enzymic unregulated dehydration reaction to form a new 9,10 double bond to yield a product possessing a cyclopentenone ring. 15-d-Δ^{12,14}-PGJ₂ has two electrophilic sites at carbons 9 and 13, however, 9 is more electrophilic than 13 and therefore is more active than carbon 9 in forming covalent bonds in transformations involving the Michael addition reaction (Burstein and Zurier, 2009). This reaction changes the

activity of various functionally important target proteins and is a possible mechanism whereby CYPGs may influence cell function. As an example, treatment of FLS cells with lenabasum elevated PGJ₂ production in a concentration dependent manner, however, PGE₂ production was not changed significantly (Stebulis et al., 2008)..

Regarding mechanism, IκB serves to concentrate NFκB in the cell cytoplasm, thus preventing it from entering the nucleus and acting as a factor to induce the transcription of genes, many of which are able to regulate inflammatory responses (Scher and Pillinger, 2009). 15-d-Δ^{12,14}-PGJ₂ forms a covalently bonded adduct with the IKK-β subunit of IκB kinase thereby inhibiting the kinases activity and promoting the entry of NFκB into the nucleus. This can stimulate the transcription of more than 150 proteins many of which regulate inflammatory responses. The ultimate effect of this inhibition is to resolve inflammation.

Direct administration of 15-deoxy-Δ^{12,14}-PGJ₂ into mice with bleomycin-induced lung disease reduced the lung injury, suggesting mediation by this CYPG that is produced by prostaglandin D synthase in this effect (Ando et al., 2003). Intravenous injection of PGDS cDNA-expressing fibroblasts reduced lung edema, leukocyte infiltration in bronchoalveolar lavage fluid, and pulmonary collagen content at 4 weeks after instillation of bleomycin. This suggests resolution of inflammation. Also, the survival rate in mice injected with PGDS-expressing fibroblasts was higher than that in control mice.

g. The NLRP3 Inflammasome. Intracellular multiprotein signaling complexes called inflammasomes can initiate a response to pathologic insults in various tissues (Spel and Martinon, 2020). One of these, the NLRP3 inflammasome is a vital component of the innate immune system, which promotes caspase-1 activation and the secretion of proinflammatory cytokines IL-1β/IL-18 (Kelley et al., 2019). Activation of the NLRP3 inflammasome has been connected to several inflammatory disorders that include cryopyrin-associated periodic syndromes, Alzheimer's disease, diabetes, and atherosclerosis. The eicosanoid 15d-PGJ₂ and related cyclopentenone prostaglandins (CYPGs) inhibit caspase-1 activation by the NLRP1 and NLRP3 inflammasomes. In a mouse peritonitis model of gout. Using monosodium urate crystals to activate NLRP3, PGJ₂ caused a significant inhibition of cell recruitment and associated IL-1β release (Maier et al., 2015). Moreover, inflammasome inhibition is independent of PPARγ. For example, an earlier study

also demonstrated that 15d-PGJ₂ has anti-inflammatory effects on macrophages that are deficient in PPAR γ (Chawla et al., 2001).

In vivo levels of PGJ₂ are believed to be in the nanomolar range. So, one may question the physiological relevance of the micromolar concentrations required for inflammasome inhibition *in vitro*. However, it has been demonstrated that upon dilution into culture media containing FBS a majority (97–99%) of the PGJ₂ is inactivated by a Michael 1-4 addition reaction. Therefore, the levels actually available for the cells *in vitro* are likely at physiological concentrations. Additionally, it can be argued that systemic measurements of free CYPG *in vivo* are lower than the actual levels found within localized pockets of inflammation or in the cell vicinity, given the high reactivity of these eicosanoids with serum and intracellular proteins (Burstein, 2020).

In a Phase 3 trial, one of the unmet needs currently being studied for lenabasum treatment is systemic sclerosis (SSC). To provide mechanism data, levels of IL-1 β and IL-18 were measured in primary human macrophages that were derived from monocytes (Chintalacharuvu et al. 2020)¹. Lenabasum significantly inhibited IL-1 β and IL-18 secretion by monocyte-derived macrophages, with IC₅₀ = 66.73 \pm 3.92 nM and 349.23 \pm 21.27 nM, respectively. MCC950, an inflammasome activation inhibitor, was included as a positive control. The authors concluded that “Lenabasum inhibits inflammasome activation, which could contribute to potential therapeutic efficacy in SSC and other autoimmune diseases.”

h. Caspase. The caspase family of cysteine proteases can function as primary effectors during apoptosis. They do this by dismantling cellular structures such as the cytoskeleton, cell junctions, mitochondria, endoplasmic reticulum, Golgi, and the nucleus (Taylor et al., 2008). There are two types of caspases: initiator caspases, such as caspase 2,8,9,10,11,12, and effector caspases, such as caspase 3,6,7. The effector caspases proteolytically degrade many intracellular proteins to carry out a cell death program. There are caspases that can promote both pyroptotic and apoptotic cell death and are activated by inflammasomes (Sagulenko et al., 2018).

Previously it was reported that lenabasum stimulated caspase-3 activity in T lymphocytes (Bidinger et al. 2003). At a concentration of 10 microM, it caused a 9.6 fold increase in enzyme activity. The cells used were human peripheral blood T

lymphocytes that were isolated from healthy volunteers. Positive controls included measurement of caspase-3 activity in cells treated with TNF α and camptothecin, known inducers of apoptosis. Lenabasum yielded activity comparable to those of the controls.

i. Apoptosis. The action called apoptosis is found in multicellular organisms and is a type of programmed cell death. Unlike necrosis, that is a form of traumatic cell death resulting from acute cellular injury, apoptosis is a highly regulated and controlled process. It produces cell fragments called apoptotic bodies that are removed by phagocytic cells. Apoptosis has a role in biological processes ranging from embryogenesis to ageing and is involved in normal tissue homeostasis as well as many human diseases. Apoptosis is characterized by a series of changes to the cellular architecture that contribute to cell death and also prepare cells for removal by phagocytes thereby preventing adverse immune responses. Several widely used drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) can alter the levels of apoptosis.

Lenabasum-induced apoptosis was demonstrated in T cells in a dose- and time-dependent manner using several methods (Bidinger et al. 2003). These were, measurement of annexin V expression, caspase-3 activity, DNA fragmentation, and microscopy. Incubation of cells with 10 microM lenabasum caused significant increases of 2.5 to 8.4 fold in Annexin-V-positive cells. Caspase-3 activity was also elevated significantly as was DNA fragmentation. In addition, the cells showed shrinkage and blebbing that is a characteristic of apoptosis.

In a separate study, it was reported that apoptosis was induced in RAW264.7 monocytes that were incubated for 3 days in the presence of the NF- κ B ligand (RANKL) (George et al., 2008). Measurement of apoptosis was done using FLOW cytometry (annexin V and propidium iodide) and measurement of caspases 3 and 8. Cells were incubated in the presence of RANKL before 0–30 microM lenabasum was added to the media for another 24 hours. Measurement of caspase 3 and 8 activities showed induction of apoptosis by lenabasum when added to differentiated multinucleated cells.

III. Lipxygenase (LOX) mediated Mechanism for the Resolution of Inflammation.

The CB₂ and PLA₂ steps of the LOX mediated mechanism for the inflammation-resolving action of lenabasum are the same as in the COX mediated mechanism in the citation, however, at this point the released AA takes a different pathway in some systems for reasons that are not clear (Figure 2). An entire new family of eicosanoid signaling molecules is generated, some of which contribute to the resolution of inflammation (Dalli and Serhan, 2016; Serhan and Samuelsson, 1988). These include the lipoxins, resolvins, protectins and maresins, together called specialized pro-resolving mediators (SPM).

a. Lipoxygenases. Lipoxygenases are enzymes that catalyze the formation of dioxygenated hydroperoxides from polyunsaturated fatty acids (PUFAs) such as linolenic acid and arachidonic acid (Mashima and Okuyama, 2015). They are expressed in immune, epithelial, and tumor cells and perform a variety of physiological actions, which include inflammation, skin disorder, and tumorigenesis. Six LOX isoforms have been identified and characterized; 15-LOX, 15-LOX-2, 12-LOX, 12R-LOX, 5-LOX and eLOX-3. 5-LOX activity is tightly controlled by 5-LOX activating protein (FLAP) by means of the distribution of 5-LOX in the nucleus. Lipoxygenase action results in the production of lipoxins that can promote resolution of inflammation (Parkinson, 2006). Of particular relevance to the mechanism of action of lenabasum is lipoxin A₄ (LXA₄).

Lipoxin A₄ (Figure 3B) is a pro resolving mediator that is formed by the sequential actions of 5- and 15-lipoxygenases, which can promote the resolution of inflammation. For example, in mice with peritonitis, dosing with lenabasum resulted in a 69% decrease in the number of cells invading the peritoneum, and a 7-fold increase in LXA₄ that was identified by mass spectrometry (Zurier et al., 2009). When added to whole human blood cells, the 12/15 LOX inhibitor baicalein reduced the lenabasum-induced increase in LXA₄. LXA₄ stimulates its anti-inflammatory pro resolving responses *via* a G protein-coupled receptor (GPCR), termed ALXR that was identified in human and animal tissues (Serhan and Chiang, 2002). In another system, RAW 264.7 cells, lenabasum also promoted a rise in LXA₄ levels (Table 2).

LXA₄ is a high affinity ligand and activator of the FPR2 receptor. This receptor that is also termed the ALX, ALX/FPR, or ALX/FPR2 receptor, is a GPCR initially identified as a receptor for the leukocyte chemotactic factor, N-Formylmethionine-leucyl-phenylalanine (FMLP), based on its amino acid sequence similarity to the known FMLP receptor, FPR1. There are no experimental data to support the

suggestion that lenabasum-induced LXA₄ binds to the FPLR2 receptor. However, this can be considered as a putative event in the inflammation resolving MOA of lenabasum.

IV. Resolution of Inflammation.

The events described above and depicted in Figure 2 suggest putative mechanisms for the inflammation-resolving action of lenabasum. There are several reports that lenabasum can resolve chronic inflammation. For example, oral administration of lenabasum reduces joint tissue damage in rats with adjuvant-induced arthritis (Zurier et al., 2003). In another animal model of inflammation, bleomycin-induced scleroderma, it again showed a decrease in pulmonary fibrosis (Gonzalez et al., 2012; Lucattelli et al., 2016).

The results from a Phase 2 trial in subjects with scleroderma are in agreement with the preclinical studies (Spiera et al., 2020a). The authors reported that “Gene expression in inflammation and fibrosis pathways was reduced and inflammation and fibrosis (was) improved by histological evaluation of skin biopsies from the lenabasum group compared to the placebo group (all $p < 0.05$)”.

Further evidence for lenabasum-induced resolution in a novel model of acute inflammation driven by intra dermal UV-killed *E. coli* in healthy humans has been reported (Motwani et al., 2018). Lenabasum at a dose of 20 mg stimulated the biosynthesis of specialized pro-resolving lipid mediators including LXA₄, LXB₄, RvD1, and RvD3. It was concluded that lenabasum reduces the onset and speeds up the resolution phase of inflammation in this human model.

Suppression of cytokine release during inflammation can result in the resolution of chronic inflammation. Thus, lenabasum has been shown to inhibit the release of the cytokines IL-1 β (Zurier et al., 2003) and IL-6 (Parker et al., 2008), promoters of inflammation. IL-1 β release appears to be inhibited by the action of lenabasum more than the other cytokines tested. This is supported by experiments done in human peripheral blood monocytes that were stimulated to release IL-1 β by the addition of lipopolysaccharide (Table 2). Also, IL-1 β release was significantly reduced in cells incubated with lenabasum (10^{-12} to 10^{-7} M) *in vitro* or in cells from donors given lenabasum (3 to 10 mg) orally. Interestingly, TNF- α secretion was not affected

under these conditions. IL-1 β is a member of the interleukin 1 family of cytokines. The synthesis of the IL-1 β precursor is a result of stimulation of innate immune cells by Toll-like receptors (TLRs) or RIG-like receptors (RLRs). However, to bind to the IL-1 receptor, the IL-1 β precursor has to be cleaved by a cysteine protease called caspase-1 that needs to be activated. It is an important mediator of the inflammatory response, and is involved in a variety of activities, including cell proliferation, differentiation, and apoptosis.

V. Summary. (Table 3)

The resolution of inflammation is needed to restore host cells and tissues to their normal status. In one mechanism, the resolution of acute inflammation is promoted by molecular families called specialized pro-resolving mediators (SPMs) that include lipoxins, resolvins, protectins and maresins (Serhan, 2014). The preclinical data reported suggested novel mechanisms involving SPMs, including LXA₄, to explain the resolution of inflammation. What occurs is a switch of arachidonic acid-derived prostaglandins and leukotrienes to lipoxins that suppress neutrophil recruitment and induce programmed death by apoptosis (Serhan and Savill, 2005). In a second mechanism, COX-2 action and its products PGD₂ and 15-d-PGJ₂ mediate inflammation-resolving action (Gilroy and Colville-Nash, 2000; Stebulis et al., 2008). The inflammatory response must be actively terminated when no longer needed to prevent damage to healthy tissues. Failure to do so results in chronic inflammation, fibrosis and cellular destruction. CB2 agonists like Lenabasum target both major pathways leading to resolution of inflammation, providing a uniquely effective way to treat chronic inflammatory diseases. In addition, based on these mechanisms it is worth speculating that lenabasum would reduce the so-called “cytokine storms” that occur in other pathological events that are characterized by severe inflammation.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript. Burstein

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Footnotes.

1. S. Chintalacharuvu , P. Zang , B. White , S. Atamas, Lenabasum, A Cb2 Agonist, Inhibits Inflammasome Activation, *Ann Rheum Dis*, **79**, supplement 1, (2020), 1372

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Figure 1. Chemical structure of lenabasum (also called ajulemic acid).

Systematic name: (6a,10a)-3-(1,1-Dimethylheptyl)-1-Hydroxy-6,6-Dimethyl-6a,7,10,10a Tetrahydro-6h-Benzo[c]chromene-9-Carboxylic Acid. MW = 400.5 Da.

Color code: carbon, grey; hydrogen, white; oxygen; red.

Figure 2. Mechanisms proposed for the anti-inflammatory actions of lenabasum. Lenabasum binds to and activates the CB2 cannabinoid receptor. Phospholipase activity (PLA-2) is then stimulated to promote the release of free

arachidonic acid from its phospholipid storage sites. Two pathways, cyclooxygenase (COX-2) or lipoxygenase (LOX) mediated, can then occur ultimately resulting in the resolution of chronic inflammation. COX-2 action elevates PGH_2 that is then followed by PGD synthase action that causes the elevation of PGD_2 . In turn, this is then rapidly converted in an unregulated process to the cyclopentenone prostaglandin 15-d-PGJ_2 . This signaling molecule modulates the activity of the inflammasome NLRP3. Caspases are then released that go on to promote apoptosis and resolution of inflammation. The second independent pathway, initiated by the actions of 5, 12 and 15 lipoxygenases, stimulates the synthesis of the lipoxin LXA_4 as well as other eicosanoids. LXA_4 reduces IL-1 β -induced production of the inflammatory cytokines IL-6 and IL-8, and of matrix metalloproteinases *in vitro* and *in vivo*. The eventual result is the resolution of inflammation.

Figure 3. Chemical structures. A; $15\text{-d-}\Delta^{12,14}\text{-PGJ}_2$ (11-oxo-prosta-5Z,9,12E,14E-tetraen-1-oic acid) MW 316.4 and B; lipoxin A_4 (5S,14R,15S-Trihydroxy-6E,8Z,10E,12E -eicosatetraenoic acid) MW 352.4

Table 1. Reduction of IL-1b secretion by lenabasum* Adapted from (Zurier and Burstein, 2016).

<u>Dose (mg p.o.)</u>	<u>% Reduction IL-1b **</u>
3	14.6
6	35.6
10	47.4

*Cells were stimulated peripheral blood monocytes from healthy volunteers administered oral lenabasum.

**Mean percent reduction in 3 subjects 5 hrs after lenabasum administration.

Table 2. Lenabasum-induced LXA₄ Synthesis in RAW Cells (Burstein et al, unpublished). Culture plates were seeded with 20,000 RAW cells / 500ml media and 50 ml of TNF/well and incubated for 18 hrs at 37°C. After washing 2x, the cells were treated as shown in the table. The media were then assayed by an LXA₄ enzyme Immunoassay.

Report	Inflammation resolving	Inflammation promoting
Zurier et al., 1998	-	iPGE down
Zurier et al., 2003	-	IL-1 β down
Parker et al., 2008	-	IL-6 down
Stebulis et al. 2008	COX-2 mRNA up	-
Stebulis et al. 2008	elevated 15d-PGJ ₂	-
Bidinger et al. 2003	stimulated caspase-3	-
Bidinger et al. 2003	annexin V expression	-
Bidinger et al. 2003	DNA fragmentation	-
Zurier et al., 2009	elevated LXA ₄	-
Motwani et al., 2018	LXA ₄ , LXB ₄ , RvD1, and RvD3 increased	-

Table 3. Effects of lenabasum on mediators of inflammation. A

summary of the changes induced by the actions of lenabasum are shown.

They were observed in diverse model systems under a variety of conditions.

Treatment (n=3)	Conc (microM)	LXA ₄ (ng/ml)
Lenabasum	1.8	244
Lenabasum	5.3	266
Lenabasum	16	430
Lenabasum	48	498
Vehicle	-	187

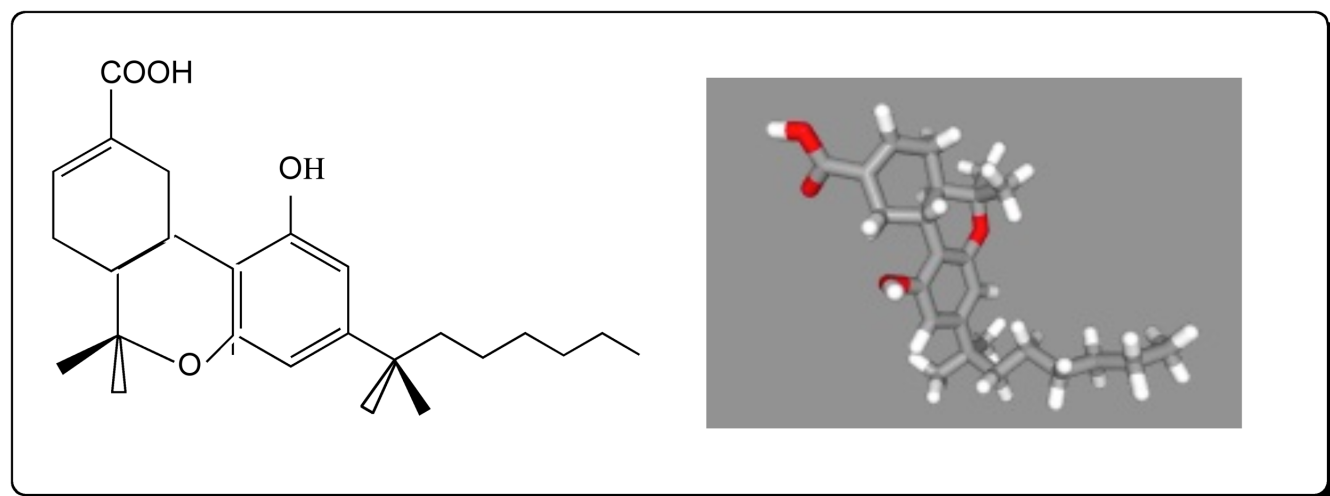


Figure 1

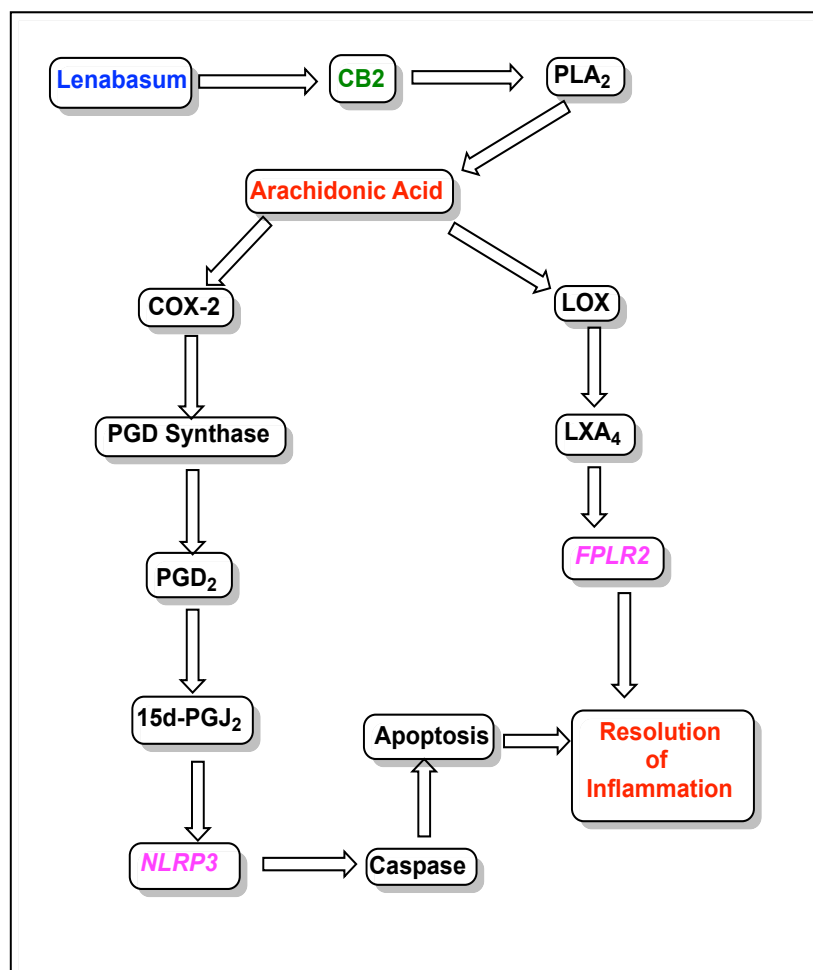


Figure 2

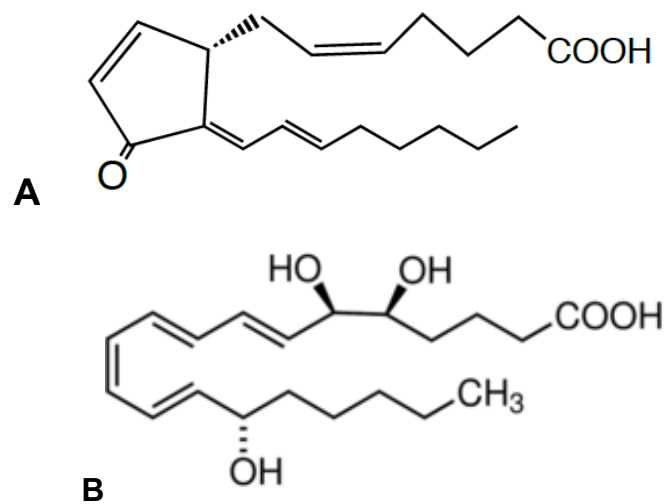


Figure 3