ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Autophagy: An Emerging Target for Developing Effective Analgesics

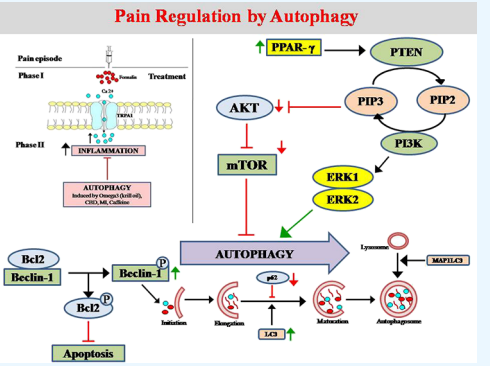
Palaniyandi Manivasakam, Atchayaa Ravi, Janani Ramesh, Divyarathna Bhuvarahamurthy, Kalaiyarasi Kasirajan, Singaravel Vijayapoopathi, Bhuvarahamurthy Venugopal,* and Anton Franz Fliri*

Cite This: *ACS Omega* 2023, 8, 9445−9453

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Inadequate treatment of acute and chronic pain causes depression, anxiety, sleep disturbances, and increased mortality. Abuse and overdose of opioids and the side effects associated with chronic use of NSAID illustrate the need for development of safer and effective pain medication. Working toward this end, an *in silico* tool based on an emergent intelligence analytical platform that examines interactions between protein networks was used to identify molecular mechanisms involved in regulating the body's response to painful stimuli and drug treatments. Examining interactions between protein networks associated with the expression of over 20 different pain types suggests that the regulation of autophagy plays a central role in modulation of pain symptoms (see Materials and Methods). Using the topology of this regulatory scheme as an *in silico* screening tool, we identified that combinations of functions targeted by cannabidiol, myo-inositol, and fish oils with varying ratios of eicosapentaenoic and docosahexaenoic acids are projected to produce superior analgesia. For validating this prediction, we administered combinations of cannabidiol, myo-inositol, and fish oils to rats that received formalin injections in hind paws, prior to substance administration, and showed that analgesic effects produced by these combinations were comparable or superior to known NSAID analgesics, which suggests that these combinations have potential in treatment of pain.



INTRODUCTION

Pain sensation is an idiosyncratic signal that arises from the nervous system when an injury occurs. Understanding the genesis of various acute and chronic pain perceptions has advanced from a one-dimensional to a multidimensional perspective that integrates sensory, cognitive, motivational, and emotional aspects. Acute pain, caused by injuries or inflammations, typically lasts for a short time. This condition is easily diagnosed and treated. However, if despite removal of noxious stimuli and acute pain persists, chronic pain emerges.[1] This condition is highly prevalent in aging populations and the leading cause of illness and declining quality of life in the elderly.[2,3]

Numerous analgesic standalone therapies and combinations have been used to treat pain. Among the most frequent drug classes used are opioid and nonsteroidal anti-inflammatory drug (NSAID) analgesics. However, efficacies of NSAID vary and produce side effects; particularly high chronic doses have been shown to produce hepatic and renal toxicity that can be treatment limiting. The usage of opioids risks emergence of addiction and the loss of efficacy over time due to development of drug tolerance, which may cause dosage variations, requiring drug-tolerant individuals to receive higher doses than others. To overcome these limitations, adjuvant analgesic therapy has been introduced. An adjuvant is a drug that is not primarily intended to be an analgesic but can be used to reduce pain either alone or in combination with other pain medications.[4] Recently, adjuvant analgesics continue to attract both scientific and medical interest as constituents of a multimodal approach to pain management.[5] Considering these limitations of NSAIDs and the abuse liability associated with opioid analgesics, we probed if a common mechanistic underpinning between different types of pain type exists and if this mechanistic link could be used to develop safe and effective products for providing chronic pain relief. Herein, we report, using an EI analytical platform, identification of an effective treatment module generated by exploiting protein network interactions between analgesics that if used alone produce only marginal analgesia.

MATERIALS AND METHODS

Emergent intelligence (EI) is based on system pharmacology and network analysis to identify the cause−effect relationship at biological levels. The methodology has been published

Received: December 22, 2022
Accepted: February 20, 2023
Published: March 3, 2023

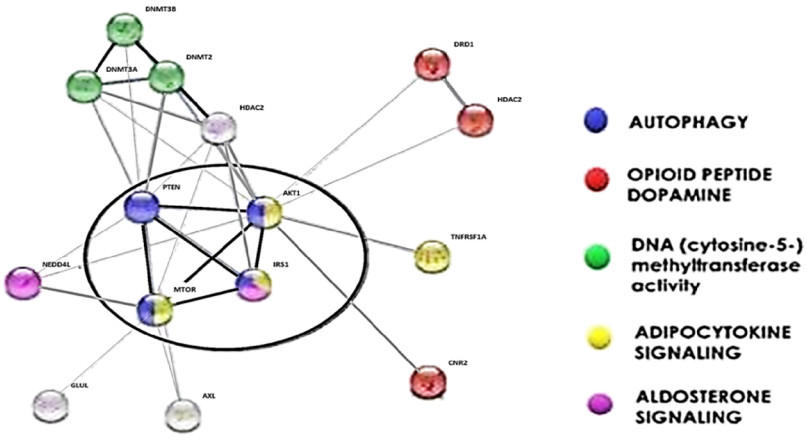


Figure 1. Protein interaction networks of biological processes regulated in pain perceptions. Edges show physical interactions between proteins. Biological processes overlapping with this network fragment regulating pain show that autophagy-related proteins play an essential role and they interact with pain-related pathways such as opioid, cytokine, DNA metabolism, and steroid signaling.

elsewhere (PCT/US2016/06379). This novel methodology identifies, among an infinite number of combinations of protein interactions, network connectivity that is important for regulating information flows through networks of networks controlling the body's response to diseases and drug treatments. The special clustering methodology developed addresses tracking of perturbation-induced information flow through multiple interacting network systems and facilitates determination of the cause−effect relationship.[6] The methodology involves data mining to identify co-occurrence frequencies of different pains with 20,233 proteins in over 15 million Medline abstracts. The proteins identified in this step were entered into the STRING platform to select proteins with high probability of involvement in pain.[7] A network fragment specific to pain was used to identify functions affected by cannabidiol, fish oils, and drugs that complement or synergize with CBD or fish oil, using special clustering analysis.

Animals and Chemicals. All the chemicals used in this study were purchased from Sigma Aldrich, USA, except krill oil and CBD. Krill oil (99% purity) containing 230 mg of omega-3 (128 mg of eicosapentaenoyl-EPA and 65 mg of docosahexaenoyl-DHA) was purchased from RIMFROST Sublime, batch 11,335; Rimfrost AS. We used CBD (≥99%purity) in a powdered form, which was purchased from industrial hemp farms in Colorado, USA. Myo-inositol (≥99% purity) (PubChem substance ID 57654297) was purchased from Sigma Aldrich, USA. All the compounds purchased and used were ≥95% pure by HPLC analysis.

Lewis rats weighing 250−300 g used for the study were obtained from the animal facility of the Establishment, University of Madras, India. The animals were housed in polypropylene cages with sterilized rice husks as bedding material. They were fed with pelleted rat chows, and water was provided *ad libitum*. The experimental protocol and maintenance of the animals were approved by the Institutional Animal Ethical Committee (IAEC), University of Madras, and the ethical registration number was 205/GO/ReBi-S/Re-L/2000/CPSCEA.

Drug Preparations. Drugs such as CBD, acetaminophen, Anacin, and Ibuprofen were solubilized in ethanol, while myo-inositol and caffeine were solubilized in water to prepare 10× stock solutions. The stock was diluted to a 1× working standard solution using saline and prepared freshly on the day of the experiment. For preparing a 1× solution with omega-3, krill oil was added at the end and vortexed for few minutes before every use to avoid solubility issues.

Formalin Pain Test. The formalin pain test was performed in animals that had fasted overnight. Each treatment group included three to five animals. For treatment, 2.5 mL of a 1× drug solution was administered to rats orally. The choice of each drug dosage for oral treatment of rats was based on the human equivalent doses (HED). Saline was used as a negative control. We ensured a one-hour time interval between each oral drug administration and formalin injection. To assess inflammatory pain, one hour after oral drug administration, formalin was injected (5%, 50 μL of formalin diluted in physiological saline, pH 7.4) subplantar, subcutaneously into the dorsal left hind paw using a 30-G needle. Then, the rats were placed immediately in an open plexiglass chamber to count licking and flinches as an index for pain as per the method described by Hunskaar.[8] As these two signs are mutually exclusive, both licking and flinching events were added as total numbers of events per minute.[9] The pain reaction was biphasic, the events up to 6−7 min were considered as an index of nociception, and the events after 10 min were due to inflammatory pain. Each experiment was repeated 2−6 times with three to five animals per treatment group. The formalin control (formalin-injected animal with no oral drugs) was included in each experiment to compare between different experiments. The number of events of licking and flinches in the formalin test was considered as the amount of pain caused by formalin treatment and was expressed as a percent.

The rate of pain inhibition was calculated using the equation below:

$$\text{pain inhibition\%} = (\text{number of events in formalin} - \text{number of events in treatment} / \text{total events in formalin treatment}) \times 100$$

The rate of pain inhibition values was reported as a mean with standard error. The significance was calculated using the analysis of variance (ANOVA) test, and significance between treatments was calculated using the *T*-test from GraphPad Prism software version 9.

https://doi.org/10.1021/acsomega.2c06949
ACS Omega 2023, 8, 9445−9453

RESULTS

The EI analytical platform examines interactions between protein networks and identifies molecular mechanisms involved in regulating the body's response to painful stimuli as well as identifies the right drugs to treat pain. Examining interactions between protein networks associated with the expression of over 20 different pain types suggests that the regulation of autophagy plays a central role in the modulation of different pain symptoms. Using the cocitation frequencies of proteins with over 20 different pain types in Medline and entering results in the STRING platform identified networks of different pain subtypes. Examining proteins mediating information transfers between tissue and molecular process networks and a spectral clustering methodology (described in the patent) identified that the expression of 20 different pain types involves a protein network element (shown in Figure 1) regulating autophagy. This protein network fragment, using an EI-based *in silico* screening tool for identifying substances affecting functions of this circuit, identified that cannabidiol, fish oils, and myo-inositol and its derivatives have functionalities that are expected to produce additive or synergistic effects on reducing pain. To evaluate this prediction, we used rat *in vivo* pain models to analyze the efficacy of the drugs as a single compound (standalone) and in two-way combinations in a formalin-induced pain model.

Examination of functional relationships between biological processes regulated by the protein network fragment shown in Figure 1 implicates that the regulation of autophagy/mitophagy plays a central role in the genesis of various types of pain perceptions. Consistent with observations made by Weng et al. in neuropathic pain,[10] our analysis also indicates that modulation of autophagy-flux mediated interactions between MTOR, PTEN, AKT1, and IRS1 plays a vital role in the expression of pain symptoms.

To evaluate the effects of various pharmacological agents on the protein network circuit shown in Figure 1, we created a protein network using EI analysis, which overlaps with protein interaction networks of over 31,000 pharmacological active substances. Among those substances, EI identified ligands for PIK3 kinases such as eCBS, which includes cannabidiol, omega-3 fatty acids, fish oil (krill oil), and myo-inositol. The projected effects of these substances on proteins of circuit-1 are illustrated in Figure 2. The protein network analysis (Figure 2) suggests that myo-inositol, which is a PIK3 ligand known to activate autophagy flux,[11] can increase analgesic effects of endocannabinoid ligands. Previously reported clinical trials in human and *in vivo* animal models illustrate that autophagy/mitophagy acts as a mechanism contributing to the analgesic effects of eCBS. Protein interaction networks further suggest that combinations of two or more autophagy/mitophagy activating substances may produce superior analgesic products. We focused on omega-3 fatty acids, which are abundant in krill/fish oils, and cannabidiol (CBD) isolated from hemp. In our study, we tested their analgesic effects in combination with autophagy inducers such as myo-inositol[12] and caffeine[13] in formalin-induced inflammatory pain models. Since substances tested in our combination experiments are generally recognized as safe (GRAS), these product combinations may offer an effective and better treatment option for chronic pain.

In order to confirm our *in silico* findings, we established formalin-induced pain models in rats. Generally, formalin injection induces two phases of behaviors. The early rapid and short phase response (lasts up to 10 min) occurs due to a direct chemical activation effect on nociceptors, while the second long phase is caused by inflammatory response (15−60 min).[8] Similar to a previously published study,[9] our formalin controls also showed biphasic response. Hence, this formalin-based pain stimulation test may be best suited to differentiate between inflammatory and noninflammatory pain. Therefore, the drug showing inhibition of pain in the second phase is effective toward reducing inflammatory pain. As the saline control group responses were negligible, we excluded them from our later set of experiments (Figure 3).

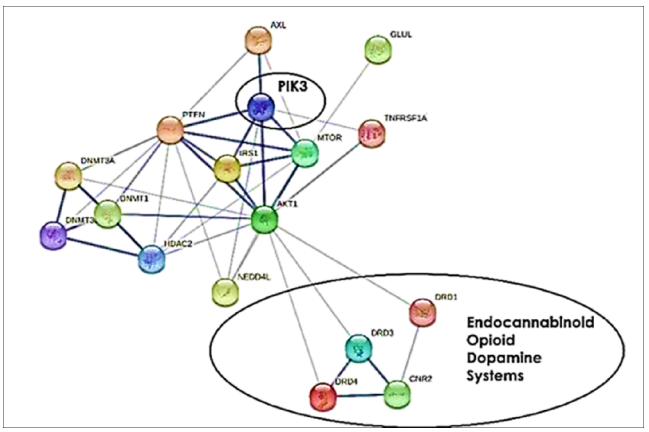


Figure 2. Protein interaction networks of pain perception and 31,000 pharmacological active substances. Overlapping studies of 15 proteins identified (Figure 1) with protein networks of 32,000 pharmacologically active molecules identified that endocannabinoids, opioids, and dopamine interact with proteins involved in autophagy and PI3K serves a pivotal role in regulating pain by these molecules.

First, we tried to verify the formalin-induced pain model of our study by examining the responses for widely used standard pain drugs in the market, namely, Anacin (aspirin 800 mg + caffeine 130 mg), Paracetamol (1000 mg), and Ibuprofen (400 mg). Doses administered to the rats were based on HED. Anacin, an extensively used pain-alleviating drug is a combination of aspirin and caffeine. Our results showed that 85% inhibition of pain was observed with a regular dose of Anacin 800 (aspirin 800 mg with caffeine 65 mg) dose (Figure 4). The reduction of the aspirin dose to 500 mg with 65 mg of caffeine (Anacin 500) shows only partial pain inhibition in phase two. Paracetamol is known to have a wide range of analgesic, antipyretic, and anti-inflammatory effects, which is a reason why it has been extensively used to treat pain.[14] Our results on Paracetamol-treated groups were consistent with the previously published results[14] (Figure 4). When administrated orally, Bonnefont et al.[15] obtained similar results to ours, suggesting that oral administration of Paracetamol might activate serotonergic bulbospinal pathways, which are involved more in nociceptive pain and less efficiently in inflammatory pain. As Paracetamol is weaker than other anti-inflammatory drugs such as NSAIDs and COX-2-selective inhibitors, it does not suppress a higher level of inflammation found in rheumatoid arthritis and acute gout. However, it showed selectivity in inhibiting the prostaglandins (PGs), which were synthesized from arachidonic acid,[15] an activator of different types of pain. We tested the third popular pain drug, Ibuprofen, in the formalin-induced rat model. In those experiments, both the regular dose (400 mg) and the lower dose (200 mg) of Ibuprofen showed similar effects (Figure 4).

https://doi.org/10.1021/acsomega.2c06949
ACS Omega 2023, 8, 9445−9453

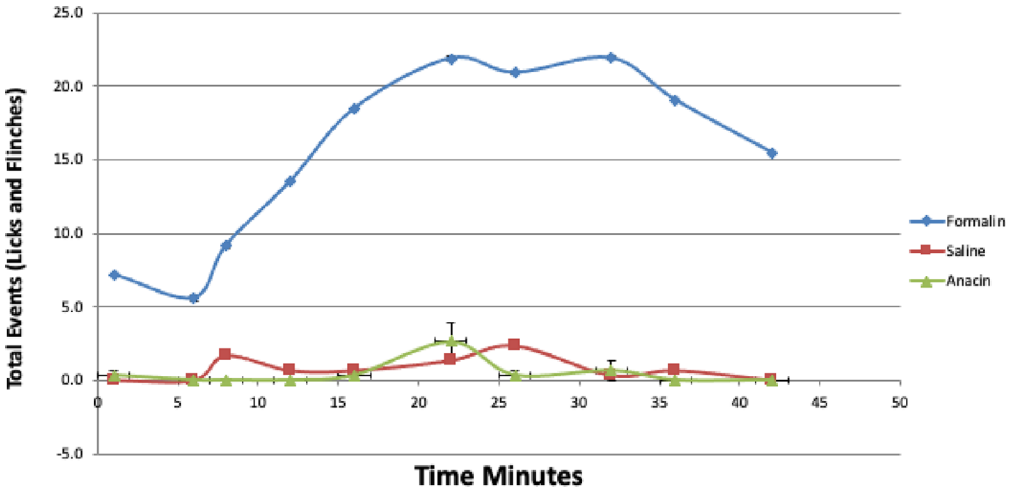


Figure 3. Effect of formalin on inducing pain in rat models. Total events, licking plus flinches, were counted every three minutes, and means ± SE of six different experiments were used to draw the graph.

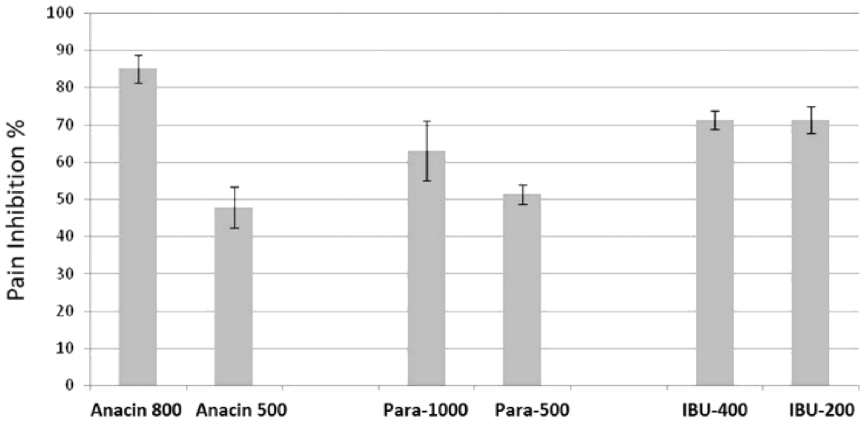


Figure 4. Effect of Anacin, Paracetamol, and Ibuprofen (IBU) on formalin-induced phase 2 pain in rats. All the drug doses are HED. Anacin 800 is a combination of aspirin 800 mg and caffeine 65 mg, Anacin 500 has only 500 mg of aspirin with 65 mg caffeine. Para1000: Paracetamol 1000 mg, Para 500: Paracetamol 500 mg, IBU-400: Ibuprofen 400 mg, and IBU-200: Ibuprofen 200 mg.

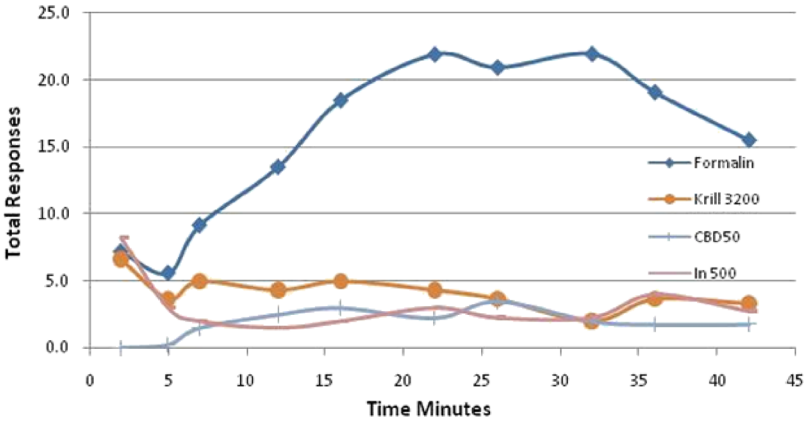


Figure 5. Effect of omega-3 (krill oil), CBD, and myo-inositol on formalin-induced pain in rats. All the doses are human equivalent dose. Krill 3200: krill oil 3200 mg, CBD50: CBD 50 mg, and In500: myo-inositol 500 mg.

After the establishment of the inflammatory pain models in rats, we tested for pain reduction by CBD, omega-3 fatty acid (krill oil), and myo-inositol. We observed that the CBD-treated group showed pain reduction in both phases, while the omega-3 fatty acid and myo-inositol reduced pain only in phase 2 (Figure 5). Later, we tested individual drug molecules in the inflammatory pain model rats, to understand the rats' unique pain response, particularly regarding their inflammatory pain in phase 2. Krill oil (omega-3) showed about 80% pain reduction with 1.6 g of HED (Figure 6a). CBD decreased pain even in lower doses and reached a plateau of 85% pain inhibition in phase 2 with 20 mg of HED (Figure 6b). With

https://doi.org/10.1021/acsomega.2c06949
ACS Omega 2023, 8, 9445−9453

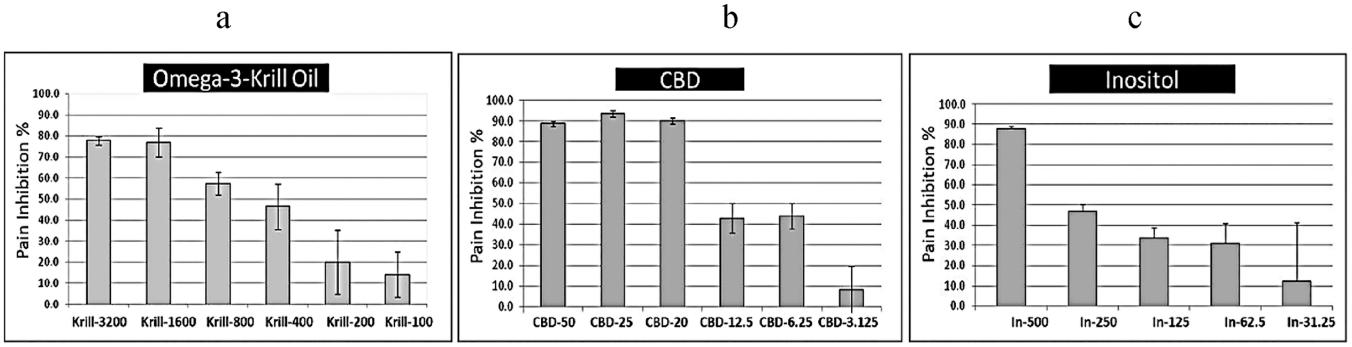


Figure 6. Effect of different doses of omega-3 (krill oil), CBD, and myo-inositol on formalin-induced phase 2 pain in rats. The doses are human equivalent dose (HED) and ranged from 100 to 3200 mg for krill oil, 3.125 to 50 mg for CBD, and 31.25 to 500 mg for myo-inositol.

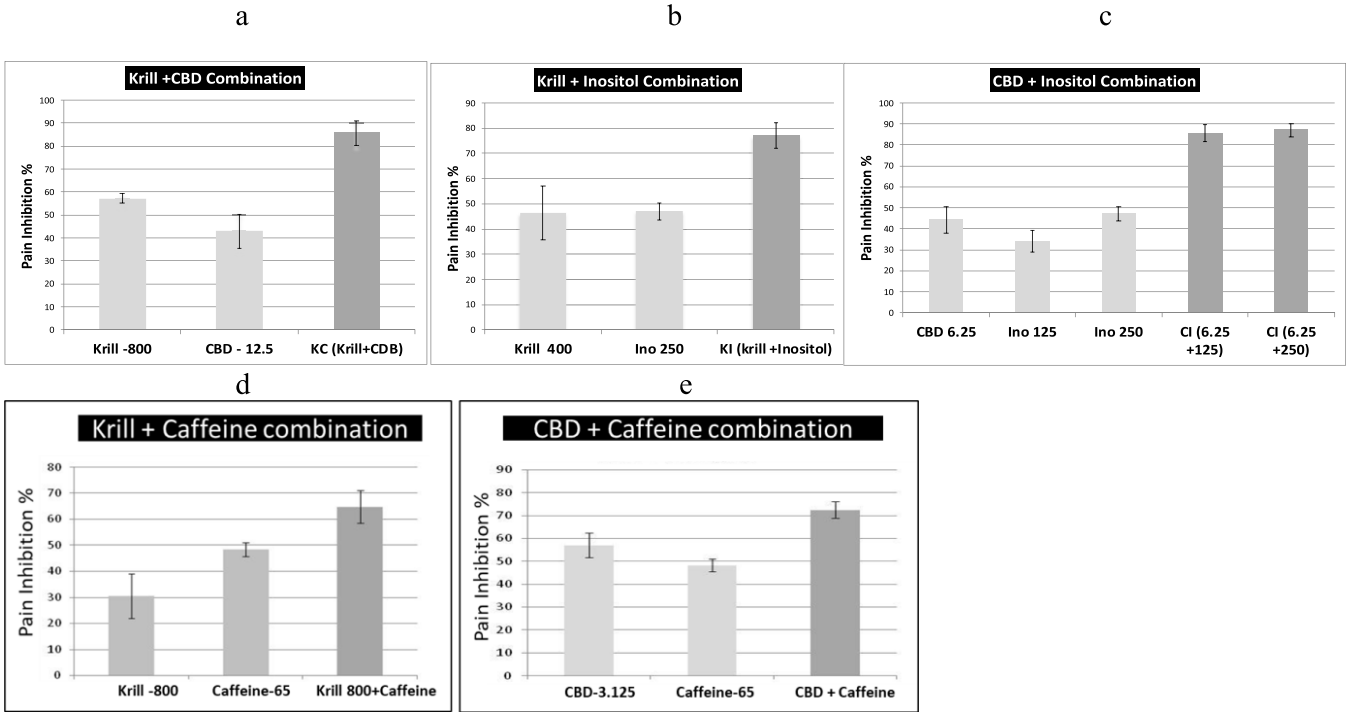


Figure 7. Effect of combinations of cannabinoids and autophagy inducers on formalin-induced pain in rats. The doses are human equivalent dose (HED). 7a, KC: krill oil 800 mg + CBD 12.5 mg; 7b, KI: krill oil 400 mg + inositol 250 mg; 7c, CI: CBD 6.25 mg + inositol 125 or 250 mg; 7d, KC: krill oil 800 mg + caffeine 65 mg; 7e, CBD 3.125 mg + caffeine 65 mg.

500 mg of HED, myo-inositol, an autophagy inducer, supplementation showed a maximum efficacy of 80% pain inhibition (Figure 6c). As caffeine has weak analgesic and anti-inflammatory effects, it showed only 45% inhibition (shown below in combination results).

Based on these results with single compounds in pain inhibition, we tested whether combinations could have an additional effect on pain relief as predicted by EI analysis. Krill oil at an 800 mg HED dose showed 57% pain inhibition, while CBD had 42% pain inhibition with 12.5 mg. Interestingly, this combination was able to produce maximum inhibition (86%), which is comparable to the effect observed by standard analgesics: Anacin, Ibuprofen, and Paracetamol (Figure 7a). Similarly, krill oil's effect was enhanced by adding myo-inositol 250 mg or caffeine 65 mg (Figure 7b,d). For the krill oil 400 mg and myo-inositol 250 mg combination, the individual pain inhibition effects were 46 and 47%, respectively, while the combination effect (77%) was nearly additive (Figure 7b). CBD (6.25 mg) was tested with two doses of myo-inositol (125 and 250 mg). The CBD and myo-inositol combination effect on pain reached a plateau of 86% even at lower doses of CBD (6.25 mg) and myo-inositol (125 mg) as shown in Figure 7c. CBD even at the lowest dose of 3.125 mg in combination with caffeine showed a considerable combination effect of 68% (Figure 7e).

DISCUSSION

The pain induced in phase 2 with formalin treatment is caused by inflammation. Therefore, we proposed that drugs that can inhibit pain in phase 2 may be used to treat inflammatory pain. Our results suggest that both omega-3 (as krill oil) and CBD can be used to treat inflammatory pain. Endocannabinoids (eCBS) are a large group of structurally related fatty acids that play an important role in cellular processes, particularly in resolving pain and inflammation. Many omega-3 derivatives of DHA and EPA such as docosahexaenoylethanolamide (DHA-EA or synaptamide), 2-docosahexaenoyl-glycerol (2-DHG) eicosapentanoyl-glycerol (EPG), and eicosapentaenoylethano-

https://doi.org/10.1021/acsomega.2c06949
ACS Omega 2023, 8, 9445−9453

lamide (EPA-EA) from eicosatetraenoic ethanolamide (ETA-EA) are chemically and functionally similar to CBS (anandamide and 2-AG). These eCBS affect many cellular functions including the attenuation of inflammation and pain. In the LPS-treated adipocytes, the addition of DHA-EA reduced the production of proinflammatory cytokines, IL-6 and monocyte chemotactic protein-1 (MCP-1). Receptor inhibition studies in adipocytes have shown that DHA-EA exhibits an anti-inflammatory activity by non-CB receptor-mediated PPARγ pathways.[16]

EPA and DHA in krill oil are reported to reduce pain through induction of autophagy by multiple mechanisms. Ethanolamide derivatives of DHA and EPA, namely, DHA-EA and EPA-EA phosphorylate Bcl-2, trigger disassociation of Beclin-1 from Bcl-2, resulting in the induction of autophagy since binding of Bcl-2 to Beclin-1prevents Beclin-1 from forming phagosomes.[17] The second mechanism to induce autophagy by krill oil (omega-3) occurs through upregulation of SQSTM1/p62, which is needed to recruit ubiquitinated proteins and organelles.[18] The third mechanism is via PTEN−AKT−mTOR energy metabolism pathway. DHA-EA and EPA-EA enhance PPARγ expression, which in turn activates the PPARγ response element-dependent genes, particularly PTEN (phosphatase and tensin homolog on chromosome ten). PTEN inhibits AKT−mTOR (mammalian target of rapamycin) pathways, resulting in the upregulation of autophagy.[19] Our EI analysis identified the network of this interlink of signaling events. The fourth mechanism by which EPA and DHA suppress inflammation is through production of distinct anti-inflammatory molecules such as resolvins, protectins, and maresins. The proresolving lipid mediators, such as 15-epi-LXA4 and resolvin D1 (RvD1), are shown to promote autophagy in murine and human macrophages. These resolvins induce degradation of SQSTM1 and formation of MAP1LC3, a central protein in autophagosome formation, which also promotes the fusion of autophagosomes with lysosomes.[20] These reports strongly suggest that autophagy plays an essential role in inhibiting inflammation by omega-3 via resolvins. Many dietary clinical trials, mostly open-labeled trials, showed that a high intake of omega-3 is effective in alleviating various chronic inflammatory diseases such as rheumatoid arthritis, psoriasis, Crohn's disease, lupus erythematosus, multiple sclerosis, ulcerative colitis, and migraine headaches.

Our results on CBD in inflammatory response were consistent with the previously reported studies. Many recent studies focusing on the clinical uses of phytocannabinoids,[21−25] particularly CBD, shed light on the anti-inflammatory mechanism of actions. CBD treatment is reported to be beneficial in alleviating chronic pain associated with various conditions such as schizophrenia, bipolar mania, social anxiety disorder, cancer, cancer anorexia, Huntington's disease, insomnia, epilepsy, migraine, fibromyalgia, peripheral neuropathy, kidney transplantation, multiple sclerosis, spinal cord injury, brachial plexus injury, and limb amputation. The dosage for treating those diseases ranged from 20 to 1500 mg per day.[26−29] It has been reported that CBD's anti-inflammatory activity is due to binding to CBD receptors (CB1 and CB2), which activate autophagy in multiple ways. CBD induces autophagy by inhibiting the AKT/mTOR pathway and by inducing autophagy-related proteins needed to complete the process. *In vitro* studies with glioblastoma cell lines showed that CBD induces autophagy by simultaneous upregulation of the autophagy gene, Beclin-1, and increasing the level of phosphorylation of the Bcl2 protein, which helps release Beclin-1 from Bcl2 to form autophagosomes by Beclin-1. Furthermore, CBD induces LC3-II, a component of autophagosomes, which are needed for the downstream autophagosome maturation process.[30] The anti-inflammatory activities of CBD are due to induction of autophagy, which results in reduction in the activity of many pro-inflammatory cytokines, inhibition of T cell proliferation, increased T cell apoptosis, and reduction of migration and adhesion of immune cells.[31] In rodent models, CBD attenuates pain induced by diabetic neuropathy and chemotherapeutic agents such as paclitaxel.[32−35] A study on the high-glucose (HG)-induced effect on the human coronary artery endothelial cells showed that CBD significantly attenuated HG-induced inflammatory mediators such as mitochondrial superoxide, NF-κB, iNOS, and adhesion molecules, 3-NT formation, and monocyte-endothelial adhesion.[36−38] In macrophages, CB2 activation reduces TNF-α levels and inflammation.[39] All these results support the use of CBD for treating inflammatory pain.

Among the natural substances identified by EI analysis to promote autophagy and treat chronic pain, myo-inositol and caffeine are found to be promising. In our pain-induced animal model, myo-inositol alleviated pain both as a single agent and in combination with endocannabinoid ligands (Figure 7b,c). Consistent with this finding, MI supplementations were reported to reduce inflammation significantly in bronchial lesions of heavy smokers, and autophagy activation was suggested to be a mechanism of action.[39,40] This was later confirmed by *in vitro* studies with immortalized human bronchial cells, which showed that MI decreases endogenous and tobacco carcinogen-induced activation of Akt and Erk, the autophagy inhibitors. The same study showed that treatment of MI resulted in decreased cell proliferation and induced G (1)-S phase cell cycle arrest.[39] In a study, mice fed with a diet supplemented with MI showed a significant decrease in tumor development and concomitant reduction in cytokines, IL-6 and LIF. IL-6 is downregulated indirectly by myo-inositol through PI3K75, a key factor in the transduction of the IL-6 signals.[41−43] Myo-inositol can mitigate pain in patients during their postoperative days after being treated with 1,2,6-IP3. The requirements of opioid analgesics were significantly reduced during the first three postoperative days.[44,45] Our EI analysis and the results obtained in the formalin-induced pain models also suggest that myo-inositol can be safely used to treat inflammatory pain.

We tested caffeine to understand whether it can be added as an adjuvant to enhance the pain reduction property of omega-3 and CBD endocannabinoid products. As higher doses of caffeine create unwanted side effects such as jittering and insomnia, we used only 65 mg (HED) in our experiments, as this dose is being safely used in other pain products such as Anacin. Our results showed that caffeine by itself can reduce pain by 50% in phase 2 (inflammatory pain), suggesting that it can be used to treat pain in conjunction with other natural anti-inflammatory products. There is a correlation between coffee consumption and the reduction of several metabolic diseases. This is reflected in a decrease in overall mortality, which prompted an exploration into understanding the effect of caffeine in the biological system. Many *in vitro* and *in vivo* studies revealed that caffeine induces autophagy.[46,47] In mice, autophagy is induced in 1−4 h after coffee consumption. In those animals, analysis of liver, muscle, and heart tissues

https://doi.org/10.1021/acsomega.2c06949
ACS Omega 2023, 8, 9445−9453

showed that autophagic flux was observed due to an increase in LC3B's lipidation and the autophagic substrate sequestosome 1 p62/SQSTM1's reduction. As these two proteins are consumed in autophagy, they are reduced when autophagy is activated. In the same experiment, the autophagic inhibitor mTORC1's enzymatic activity was reduced, which resulted in reduced phosphorylation of p70S6K and global deacetylation of cellular proteins detectable by the immunoblot.[48] Moreover, treatment with caffeine inhibits the Prion protein (PrP), which mediates neuronal apoptosis by activating Akt.[49] Caffeine inhibits kinase activities including PI3K and mTOR. However, Erk 1/2 phosphorylation was increased by caffeine, indicating dual mechanisms: inhibition of the Akt/mTOR/p70S6K pathway and activation of the Erk1/2 autophagy pathway.[50,51] One study showed that autophagy was suppressed by insulin treatment due to phosphorylation of Akt, an inhibitor of autophagy. However, this was completely abolished by a caffeine supplement.[49] Further, it is shown that the expression of LC3II, an essential protein and indicator of autophagy, was increased after caffeine treatment.[52] The caffeine-induced increase in LC3II levels was inhibited by the autophagy inhibitors, 3-MA and wortmannin, as measured by Western blot and densitometric analyses.[53] In addition, caffeine influences the biological system by regulating Ca^{2+}. At high concentrations, caffeine is shown to increase Ca^{2+} transiently through the release of Ca^{2+} ions from the sarcoplasmic reticulum, which is independent of external Ca^{2+}. However, at low concentrations of caffeine, Ca^{2+} release is dependent on extracellular calcium.[54,55] The release of the internal storage of calcium into the cytoplasm activates the CaMKK kinase activity, leading to activation of AMPK. This, in turn, inhibits mTOR, leading to the activation of autophagy.[49,56] The four natural products studied in our experiments showed pain reduction in phase 2 of the formalin-induced pain model. This reveals that they can be used to treat inflammation and pain in many acute and chronic conditions. As autophagy is the main regulator of inflammation and these four natural products are known to induce autophagy, it can be assumed that inhibition of inflammation in our animal pain model might be due to activation of autophagy.

Our results established that inflammatory pain can be modulated by autophagy, induced by natural products. The introduction of these natural products with standard pain drugs can be more effective in controlling pain than standalone treatments with widely used pain medications on the market. The optimal doses of the natural products used in the combinations were less than or equivalent to the doses normally used as a supplement without any adverse events. The krill oil dose (1 to 2 g) used in supplements is shown to have clinical benefits in various diseases ranging from inflammatory diseases such as CVD and CNS. The CBD doses (50 to 1600 mg) used in supplements and at doses up to 2000−3000 mg did not show adverse effects. In all the combinations, the maximum doses of krill oil that we used were 800 and 12.5 mg for CBD.

Further experiments on myo-inositol with varied concentrations show that a dose of 500 mg elicited the highest pain inhibitory effect and in combination with 250 mg of myo-inositol was adequate to inhibit the pain. The maximum dose of myo-inositol that can be used without any adverse effects was shown to be 10 g/day. The caffeine dose of 65 mg (HED) used in our combination studies was much lower than the quantity of caffeine (100 mg) in a cup of coffee. These results show that the combinations that we examined can be safely used as a supplement to alleviate pain, especially in inflammatory pain. Moreover, combination therapy has more advantages than single drug usage because more than one pathway can be targeted simultaneously. This also helps reduce the dose of the standalone drug while increasing the efficacy of the drug. However, the study explained above discussed combined treatment only in inflammatory pain. In addition, the compounds that we used may also have a pleiotropic effect in modulating a wide range of pain, an added advantage to treat chronic pain effectively. Furthermore, the natural ingredients used in our study are commonly used as food supplements with many health benefits.

CONCLUSIONS

Overall, our findings imply positive implications for using these natural products in treating chronic pain without much adverse effects. These products have the potential to replace existing opioids and NSAIDs used for pain. Though NSAIDs are effective for both acute and chronic pain, they are responsible for 30% of hospital admissions due to their adverse events including GI ulcers, bleeding, heart attack, stroke, and renal damage. It is reported that 16,500 persons are dying annually from these complications.[57] Thus, the natural products that we chose are nonaddictive and can be a safe replacement for existing toxicity-inducing NSAIDs and addictive narcotic pain medications. In the future, this study can be continued to optimize doses in human clinical trials, which may help replace many over-the-counter (OTC) drugs and prescribed pain drugs. Most importantly, it is a natural approach to treat pain, and these combinations have the potential to be used as "food as medicine".

ASSOCIATED CONTENT

Data Availability Statement

We author declare that the data supporting the findings of this study are available within the paper. The data are also available from the corresponding author upon request.

AUTHOR INFORMATION

Corresponding Authors

Bhuvarahamurthy Venugopal − *Department of Medical Biochemistry, Dr. A.L.M. Postgraduate Institute of Basic Medical Sciences, University of Madras, Chennai 600113 Tamil Nadu, India*; orcid.org/0000-0001-8684-9133; Email: b.murthymedbio@gmail.com, murthyboston@gmail.com

Anton Franz Fliri − *SystaMedic, Inc., Groton, Connecticut 06355, United States*; Email: afliri01@snet.net

Authors

Palaniyandi Manivasakam − *SystaMedic, Inc., Groton, Connecticut 06355, United States*

Atchayaa Ravi − *Department of Medical Biochemistry, Dr. A.L.M. Postgraduate Institute of Basic Medical Sciences, University of Madras, Chennai 600113 Tamil Nadu, India*

Janani Ramesh − *Department of Medical Biochemistry, Dr. A.L.M. Postgraduate Institute of Basic Medical Sciences, University of Madras, Chennai 600113 Tamil Nadu, India; Renal Division, Brigham and Women's Hospital, BWH, Boston, Massachusetts 02115-6195, United States;* orcid.org/0000-0002-2137-500X

https://doi.org/10.1021/acsomega.2c06949
ACS Omega 2023, 8, 9445−9453

Divyarathna Bhuvarahamurthy − *Boston University, Boston, Massachusetts 02215, United States*
Kalaiyarasi Kasirajan − *Department of Medical Biochemistry, Dr. A.L.M. Postgraduate Institute of Basic Medical Sciences, University of Madras, Chennai 600113 Tamil Nadu, India*
Singaravel Vijayapoopathi − *Department of Medical Biochemistry, Dr. A.L.M. Postgraduate Institute of Basic Medical Sciences, University of Madras, Chennai 600113 Tamil Nadu, India*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c06949

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors gratefully acknowledge the partial financial assistance from RUSA 2.0 Research, Innovation and Quality Improvement, University of Madras, and also, we would like to acknowledge research fellows (Dr. Gothandaraman, Ms. Pooja Asani, and Mr. Vinothkumar) of Dr. V. Bhuvarahamurthy's Research Laboratories, Department of Medical Biochemistry, University of Madras, Taramani, Chennai 600113, for their assistance in this study.

■ REFERENCES

(1) Turk, D.-C.; Okifuji, A. Psychological factors in chronic pain: evolution and revolution. *J. Consult. Clin. Psychol.* **2002**, *70*, 678−690.
(2) Dydyk, A.-M.; Ngnitewe Massa, R.; Mesfin, F.-B. Disc Herniation. in *StatPearls* (Treasure Island (FL), Last update 18 January 2021).https://www.ncbi.nlm.nih.gov/books/ NBK441822/#:~:text=A%20herniated%20disc%20is%20a,pain%20and%20spinal%20cord%20dysfunction. (Accessed 21 December 2022)
(3) Tsang, A.; Von Korff, M.; Lee, S.; Alonso, J.; Karam, E.; Angermeyer, M.-C.; Borges, G.-L.-G.; Bromet, E.-J.; De Girolamo, G.; De Graaf, R.; et al. Common chronic pain conditions in developed and developing countries: gender and age differences and comorbidity with depression-anxiety disorders. *J. Pain.* **2008**, *9*, 883−891.
(4) Bair, M.-J.; Sanderson, T.-R. Coanalgesics for chronic pain therapy: a narrative review. *Postgrad. Med.* **2011**, *123*, 140−150.
(5) Yaksh, T. L.; Woller, S. A.; Ramachandran, R.; Sorkin, L. S. The search for novel analgesics: targets and mechanisms. *F1000Prime Rep.* **2015**, *7*, 1−27.
(6) Szklarczyk, D.; Gable, A.-L.; Lyon, D.; Junge, A.; Wyder, S.; Huerta-Cepas, J.; Simonovic, M.; Doncheva, N.-T.; Morris, J.-H.; Bork, P.; et al. STRING v11: protein−protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* **2019**, *47*, D607−D613.
(7) Fliri, A.-F.; Kajiji, S. Functional characterization of nutraceuticals using spectral clustering: Centrality of caveolae-mediated endocytosis for management of nitric oxide and vitamin D deficiencies and atherosclerosis. *Front. Nutr.* **2022**, *9*, No. 885364.
(8) Hunskaar, S. Similar effects of acetylsalicylic acid and morphine on immediate responses to acute noxious stimulation. *Pharmacol. Toxicol.* **1987**, *60*, 167−170.
(9) Wheeler-Aceto, H.; Cowan, A. Standardization of the rat paw formalin test for the evaluation of analgesics. *Psychopharmacology* **1991**, *104*, 35−44.
(10) Weng, W.; Yao, C.; Poonit, K.; Zhou, X.; Sun, C.; Zhang, F.; Yan, H. Metformin relieves neuropathic pain after spinal nerve ligation via autophagy flux stimulation. *J. Cell. Mol. Med.* **2019**, *23*, 1313−1324.
(11) Steinfeld, N.; Lahiri, V.; Morrison, A.; Metur, S.-P.; Klionsky, D.-J.; Weisman, L.-S. Elevating PI3P drives select downstream membrane trafficking pathways. *Mol. Biol. Cell* **2021**, *32*, 143−156.
(12) Shi, D.; et al. The precursor of PI(3,4,5)P3 alleviates aging by activating daf-18(Pten) and independent of daf-16. *Nat. Commun.* **2020**, *11*, 4496.
(13) Hughes, M.-S.-A.; Dosa, D.-M.; Caffrey, A.-R.; Appaneal, H.-J.; Jump, R.-L.; Lopes, V.; LaPlante, K. L. Antibiograms Cannot Be Used Interchangeably Between Acute Care Medical Centers and Affiliated Nursing Homes. *J. Am. Med. Dir. Assoc.* **2020**, *21*, 72−77.
(14) Graham, G.-G.; Davies, M.-J.; Day, R.-O.; Mohamudally, A.; Scott, K.-F. The modern pharmacology of paracetamol: therapeutic actions, mechanism of action, metabolism, toxicity and recent pharmacological findings. *Inflammopharmacology* **2013**, *21*, 201−232.
(15) Bonnefont, J.; Courade, J. P.; Alloui, A.; Eschalier, A. Mécanisme de l'action antinociceptive du paracétamol [Antinociceptive mechanism of action of paracetamol]. *Drugs* **2003**, *63*, 1−4.
(16) Balvers, M.-G.; Verhoeckx, K.-C.; Plastina, P.; Wortelboer, H.-M.; Meijerink, J.; Witkamp, R.-F. Docosahexaenoic acid and eicosapentaenoic acid are converted by 3T3-L1 adipocytes to N-acyl ethanolamines with anti-inflammatory properties. *Biochim. Biophys. Acta* **2010**, *1801*, 1107−1114.
(17) Ramesh, J.; Ronsard, L.; Gao, A.; Venugopal, B. Autophagy Intertwines with Different Diseases-Recent Strategies for Therapeutic Approaches. *Diseases* **2019**, *7*, 15.
(18) Muilenburg, D.; Parsons, C.; Coates, J.; Virudachalam, S.; Bold, R.-J. Role of autophagy in apoptotic regulation by Akt in pancreatic cancer. *Anticancer Res.* **2014**, *34*, 631−637.
(19) Bonofiglio, D.; Giordano, C.; De Amicis, F.; Lanzino, M.; Ando, S. Natural Products as Promising Antitumoral Agents in Breast Cancer: Mechanisms of Action and Molecular Targets. *Mini. Rev. Med. Chem.* **2016**, *16*, 596−604.
(20) Prieto, P.; Rosales-Mendoza, C.-E.; Terrón, V.; Toledano, V.; Cuadrado, A.; López-Collazo, E.; Bannenberg, G.; Martín-Sanz, P.; Fernández-Velasco, M.; Boscá, L. Activation of autophagy in macrophages by pro-resolving lipid mediators. *Autophagy* **2015**, *11*, 1729−1744.
(21) Nakamoto, K.; Nishinaka, T.; Mankura, M.; Fujita-Hamabe, W.; Tokuyama, S. Antinociceptive effects of docosahexaenoic acid against various pain stimuli in mice. *Biol. Pharm. Bull.* **2010**, *33*, 1070−1072.
(22) Norling, L.-V.; Perretti, M. The role of omega-3 derived resolvins in arthritis. *Curr. Opin. Pharmacol.* **2013**, *13*, 476−481.
(23) Simopoulos, A. P. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp. Biol. Med.* **2008**, *233*, 674−688.
(24) Simonetto, M.; Infante, M.; Sacco, R.-L.; Rundek, T.; Della-Morte, D. A Novel Anti-Inflammatory Role of Omega-3 PUFAs in Prevention and Treatment of Atherosclerosis and Vascular Cognitive Impairment and Dementia. *Nutrients* **2019**, *11*, 2279.
(25) Maroon, J.-C.; Bost, J. W. Omega-3 fatty acids (fish oil) as an anti-inflammatory: an alternative to nonsteroidal anti-inflammatory drugs for discogenic pain. *Surg. Neurol.* **2006**, *65*, 326−331.
(26) Cuñetti, L.; Manzo, L.; Peyraube, R.; Arnaiz, J.; Curi, L.; Orihuela, S. Chronic Pain Treatment With Cannabidiol in Kidney Transplant Patients in Uruguay. *Transplant. Proc.* **2018**, *50*, 461−464.
(27) Xu, D.-H.; Cullen, B.-D.; Tang, M.; Fang, Y. The Effectiveness of Topical Cannabidiol Oil in Symptomatic Relief of Peripheral Neuropathy of the Lower Extremities. *Curr. Pharm. Biotechnol.* **2020**, *21*, 390−402.
(28) van de Donk, T.; Niesters, M.; Kowal, M.-A.; Olofsen, E.; Dahan, A.; van Velzen, M. An experimental randomized study on the analgesic effects of pharmaceutical-grade cannabis in chronic pain patients with fibromyalgia. *Pain* **2019**, *160*, 860−869.
(29) Zhornitsky, S.; Potvin, S. Cannabidiol in humans-the quest for therapeutic targets. *Pharmaceuticals* **2012**, *5*, 529−552.
(30) Ivanov, V.-N.; Grabham, P.-W.; Wu, C.-C.; Hei, T.-K. Inhibition of autophagic flux differently modulates cannabidiol-induced death in 2D and 3D glioblastoma cell cultures. *Sci. Rep.* **2020**, *10*, 2687.
(31) Jean-Gilles, L.; Braitch, M.; Latif, M.-L.; Aram, J.; Fahey, A.-J.; Edwards, L.-J.; Robins, R.-A.; Tanasescu, R.; Tighe, P.-J.; Gran, B

Effects of pro-inflammatory cytokines on cannabinoid CB1 and CB2 receptors in immune cells. *Acta Physiol.* **2015**, *214*, 63−74.
(32) Toth, C. C.; Jedrzejewski, N.-M.; Ellis, C.-L.; Frey, W. H. Cannabinoid-mediated modulation of neuropathic pain and microglial accumulation in a model of murine type I diabetic peripheral neuropathic pain. *Mol. Pain* **2010**, *6*, 16.
(33) Ward, S.-J.; McAllister, S.-D.; Kawamura, R.; Murase, R.; Neelakantan, H.; Walker, E.-A. Cannabidiol inhibits paclitaxel-induced neuropathic pain through 5-HT(1A) receptors without diminishing nervous system function or chemotherapy efficacy. *Br. J. Pharmacol.* **2014**, *171*, 636−645.
(34) King, K.-M.; Myers, A.-M.; Soroka-Monzo, A.-J.; Tuma, R.-F.; Tallarida, R.-J.; Walker, E.-A.; Ward, S. J. Single and combined effects of Delta(9) -tetrahydrocannabinol and cannabidiol in a mouse model of chemotherapy-induced neuropathic pain. *Br. J. Pharmacol.* **2017**, *174*, 2832−2841.
(35) Jesus, C.-H.-A.; Redivo, D.-D.-B.; Gasparin, A.-T.; Sotomaior, B.-B.; de Carvalho, M.-C.; Genaro, K.; Zuardi, A.-W.; Hallak, J.-E.-C.; Crippa, J.-A.; Zanoveli, J.-M.; et al. Cannabidiol attenuates mechanical allodynia in streptozotocin-induced diabetic rats via serotonergic system activation through 5-HT1A receptors. *Brain Res.* **2019**, *1715*, 156−164.
(36) −9 (2007).doi: Rajesh, M.; Mukhopadhyay, P.; Bátkai, S.; Haskó, G.; Liaudet, L.; Drel, V.-R.; Obrosova, I.-G.; Pacher, P. Cannabidiol attenuates high glucose-induced endothelial cell inflammatory response and barrier disruption. *Am. J. Physiol. Heart. Circ. Physiol.* **2007**, *293*, H610.
(37) Burstein, S. Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorg. Med. Chem.* **2015**, *23*, 1377−1385.
(38) Pellati, F.; Borgonetti, V.; Brighenti, V.; Biagi, M.; Benvenuti, S.; Corsi, L. Cannabis sativa L. and Nonpsychoactive Cannabinoids: Their Chemistry and Role against Oxidative Stress, Inflammation, and Cancer. *Biomed. Res. Int.* **2018**, 1691428.
(39) Han, K.-H.; Lim, S.; Ryu, J.; Lee, C.-W.; Kim, Y.; Kang, J.-H.; Kang, S.-S.; Ahn, Y.-K.; Park, C.-S.; Kim, J.-J. CB1 and CB2 cannabinoid receptors differentially regulate the production of reactive oxygen species by macrophages. *Cardiovasc. Res.* **2009**, *84*, 378−386.
(40) Lam, S.; McWilliams, A.; LeRiche, J.; MacAulay, C.; Wattenberg, L.; Szabo, E. A phase I study of myo-inositol for lung cancer chemoprevention. *Cancer Epidemiol. Biomarkers Prev.* **2006**, *15*, 1526−1531.
(41) Unver, N.; Delgado, O.; Zeleke, K.; Cumpian, A.; Tang, X.; Caetano, M.-S.; Wang, H.; Katayama, H.; Yu, H.; Szabo, E.; Wistuba, I. I.; Moghaddam, S. J.; Hanash, S. M.; Ostrin, E. J. Reduced IL-6 levels and tumor-associated phospho-STAT3 are associated with reduced tumor development in a mouse model of lung cancer chemoprevention with myo-inositol. *Int. J. Cancer* **2018**, *142*, 1405−1417.
(42) Zhang, Y.; Li, C.; Zhang, W.; Zheng, X.; Chen, X. Decreased Insulin Resistance by Myo-Inositol Is Associated with Suppressed Interleukin 6/Phospho-STAT3 Signaling in a Rat Polycystic Ovary Syndrome Model. *J. Med. Food* **2020**, *23*, 375−387.
(43) Piranlioglu, R.; Lee, E.; Ouzounova, M.; Bollag, R.-J.; Vinyard, A.-H.; Arbab, A.-S.; Marasco, D.; Guzel, M.; Cowell, J.-K.; Thangaraju, M.; et al. Primary tumor-induced immunity eradicates disseminated tumor cells in syngeneic mouse model. *Nat. Commun.* **2019**, *10*, 1430.
(44) Tarnow, P.; Cassuto, J.; Jonsson, A.; Rimback, G.; Hedman, C. Postoperative analgesia by D-myo-5inositol-1,2,6-trisphosphate in patients undergoing cholecystectomy. *Anesth. Analg.* **1998**, *86*, 107−110.
(45) Shi, Y.; Azab, A.-N.; Thompson, M.-N.; Greenberg, M.-L. Inositol phosphates and phosphoinositides in health and disease. *Subcell. Biochem.* **2006**, *39*, 265−292.
(46) Sinha, R.-A.; Farah, B.-L.; Singh, B.-K.; Siddique, M.-M.; Li, Y.; Wu, Y.; Ilkayeva, O.-R.; Gooding, J.; Ching, J.; Zhou, J.; et al. Caffeine stimulates hepatic lipid metabolism by the autophagy-lysosomal pathway in mice. *Hepatology* **2014**, *59*, 1366−1380.
(47) Liu, Y.-W.; Yang, T.; Zhao, L.; Ni, Z.; Yang, N.; He, F.; Dai, S.-S. Activation of Adenosine 2A receptor inhibits neutrophil apoptosis in an autophagy-dependent manner in mice with systemic inflammatory response syndrome. *Sci. Rep.* **2016**, *6*, 33614.
(48) Pietrocola, F.; Malik, S.-A.; Mariño, G.; Vacchelli, E.; Senovilla, L.; Chaba, K.; Niso-Santano, M.; Maiuri, M.-C.; Madeo, F.; Kroemer, G. Coffee induces autophagy in vivo. *Cell Cycle* **2014**, *13*, 1987−1994.
(49) (2011).doi: Saiki, S.; Sasazawa, Y.; Imamichi, Y.; Kawajiri, S.; Fujimaki, T.; Tanida, I.; Kobayashi, H.; Sato, F.; Sato, S.; Ishikawa, K.; Imoto, M.; Hattori, N. Caffeine induces apoptosis by enhancement of autophagy via PI3K/Akt/mTOR/p70S6K inhibition. *Autophagy* **2011**, *7*, 176−187.
(50) Foukas, L.-C.; Daniele, N.; Ktori, C.; Anderson, K.-E.; Jensen, J.; Shepherd, P.-R. Direct effects of caffeine and theophylline on p110 delta and other phosphoinositide 3-kinases. Differential effects on lipid kinase and protein kinase activities. *J. Biol. Chem.* **2002**, *277*, 37124−37130.
(51) Kudchodkar, S.-B.; Yu, Y.; Maguire, T.-G.; Alwine, J.-C. Human cytomegalovirus infection alters the substrate specificities and rapamycin sensitivities of raptor- and rictor-containing complexes. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 14182−14187.
(52) Li, Y.; Chen, Y.; Huang, H.; Shi, M.; Yang, W.; Kuang, J.; Yan, J. Autophagy mediated by endoplasmic reticulum stress enhances the caffeine-induced apoptosis of hepatic stellate cells. *Int. J. Mol. Med.* **2017**, *40*, 1405−1414.
(53) Moon, J.-H.; Lee, J.-H.; Park, J.-Y.; Kim, S.-W.; Lee, Y.-J.; Kang, S.-J.; Seol, J.-W.; Ahn, D.-C.; Park, S.-Y. Caffeine prevents human prion protein-mediated neurotoxicity through the induction of autophagy. *Int. J. Mol. Med.* **2014**, *34*, 553−558.
(54) O'Neill, S.-C.; Eisner, D.-A. A mechanism for the effects of caffeine on Ca2+ release during diastole and systole in isolated rat ventricular myocytes. *J. Physiol.* **1990**, *430*, 519−536.
(55) Ahmed, I.-A.; Hopkins, P.-M.; Winlow, W. Low concentrations of caffeine raise intracellular calcium concentration only in the presence of extracellular calcium in cultured molluscan neurons. *Gen. Pharmacol.* **1997**, *28*, 245−250.
(56) Dunn, D.-M.; Munger, J. Interplay Between Calcium and AMPK Signaling in Human Cytomegalovirus Infection. *Front. Cell Infect. Microbiol.* **2020**, *10*, 384.
(57) Fine, M. Quantifying the impact of NSAID-associated adverse events. *Am J Manag Care* **2013**, *19*, s267−s272.

https://doi.org/10.1021/acsomega.2c06949
ACS Omega 2023, 8, 9445−9453