Canurta

mPGES-1/5-LO Whitepaper

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Date: Sept 14, 2022

Issue: The Problem We Solve

When injured or infected with disease-causing agents, the body mounts the essential physiological response known as acute inflammation. When the inflammatory process persists, it can contribute to a variety of diseases and turn into chronic inflammation. At the time of writing, more than 50% of all deaths are attributable to inflammation-related diseases, establishing chronic inflammatory disease as the most significant cause of death globally [1].

A critical network involved in the initiation, progression, and resolution of inflammation is the eicosanoid network which produces locally acting signalling lipids including arachidonic acid (AA) [2]. Eicosanoids can be further subdivided into the cyclooxygenase (COX)-derived prostanoids, such as prostaglandin E_2 (PGE_2) as well as the lipoxygenase (LO)-derived (poly)hydroxylated and/or epoxydated PUFAs, including leukotrienes [2]. Early research established PGE_2 and leukotrienes as inflammation-promoting eicosanoids.

Traditionally, the most widely used drugs to treat inflammation, fever, and pain include non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit eicosanoid biosynthesis [2]. Although commercially successful, long-term usage of NSAIDs has been proven to elicit adverse events (AE). There is increasing demand for innovative, effective, and safe anti-inflammatory drug candidates that can combat chronic disease.

This document details the mechanism of action of cannflavins, novel anti-inflammatory agents, across the pharmacologically relevant target enzymes microsomal prostaglandin E2 synthase-1 (mPGES-1) and 5-Lipoxygenase (5-LO), which are critical for the biosynthesis of pro-inflammatory and non-resolving PGE_2 and leukotrienes, respectively. This document also describes recommendations for future clinical exploration of specific disease states of interest.

Based on the available evidence: what impact on health and disease does dual inhibition of mPGES-1 and 5-LO have via cannflavin action, and what is their commercial potential for various inflammatory diseases?

Analysis

Summary of biological function

mPGES-1 and 5-LO are critical enzymes in the biosynthesis of pro-inflammatory eicosanoids such as PGE_2 and leukotrienes, establishing them as the main target molecules for pharmacological intervention of inflammatory disease.

To produce PGE_2, cyclooxygenases (COX)-1 and 2 first convert AA to PGH_2, a substrate for various prostanoid synthases that produce bioactive prostaglandins and thromboxane A2 [(Tx)A2], as well as three terminal PGES enzymes that convert PGH_2 into PGE_2 [3]. mPGES-1 is the terminal enzyme involved in the biosynthesis of prostanoids from AA into PGE_2. It is an inducible, membrane-bound enzyme often co-expressed with the inducible COX-2 enzyme, and appears to be responsible for elevated PGE_2 formation at inflammatory sites [4]. PGE_2 has vital homeostatic roles in maintaining blood pressure, renal function, and mucosal integrity of the GI tract, but it also plays a critical role in diseases with inflammatory components [5]. PGE_2 drives

inflammation via inducing local vasodilation and vascular permeability, promoting leukocyte infiltration to inflammatory sites upon early inflammatory challenge [6]. Inflammation typically resolves when the inflammatory stimuli are removed, however, PGE_2 also induces cytokines such as IL-10 to limit non-specific inflammation, resulting in an immunosuppressive state that when persistent can yield chronic inflammation [7].

NSAIDs inhibit the COX enzymes, which has problematic long-term effects since COX-derived prostanoids possess crucial homeostatic functions. Inhibiting the constitutively expressed homeostatic enzyme COX-1 subsequently decreases the formation of cytoprotective PGE_2 and prostacyclin (PGI_2) by the epithelium [4]. The adverse events caused by long-term treatment of chronic diseases with NSAIDs are thus associated with severe cardiovascular, renal, and gastrointestinal (GI) side effects due to the suppression of beneficial homeostatic prostanoids like GI protective PGE_2 and antithrombotic and vasodilatory PGI_2 [3]. COX-2-selective inhibitors called coxibs were designed in an attempt to circumvent GI complications but faced a similar limitation by shifting the ratio of PGI_2 to pro-thrombotic (Tx)A2 [8]. Thus, coxibs increase the risk for adverse cardiovascular events including myocardial infarction, stroke, systemic and pulmonary hypertension, congestive heart failure, and sudden cardiac death [8]. This has caused several coxibs, such as rofecoxib (VIOXX1) and valdecoxib (BEXTRA1), to be withdrawn from the market [4].

Leukotrienes have also been implicated in a wide spectrum of inflammatory diseases. 5-LO is the initial catalytic enzyme involved in the biosynthesis of leukotrienes such as leukotriene A4 (LTA4) from AA, after which distinct enzymatic reactions convert LTA4 to LTB4 or the cysteinyl-LTs (CysLTs) C4, D4 and E4 which

have been established in the literature to play critical roles in inflammatory reactions [9]. In fact, NSAIDs appear to induce GI toxicity and evoke asthma not only by decreasing the production of gastroprotective prostanoids but by redirecting the COX substrate AA into leukotriene biosynthesis, ultimately causing vasoconstriction of the airways and gastric mucosa due to leukotriene recruitment and activation of immune cells [2]. Although dual COX/5-LO inhibitors have been explored, they appear to suppress beneficial prostanoids as previously mentioned, rendering their long-term use problematic.

Consequently, there is an urgent demand for safe and efficacious anti-inflammatory drugs due to the clinical complications associated with NSAIDs and coxibs. Interestingly, many studies have suggested that by selectively targeting both mPGES-1 and 5-LO, effectively avoiding COX-1/2, this approach could inhibit pro-inflammatory PGE_2 and leukotriene synthesis while sparing homeostatic PGI_2. A dual mPGES-1/5-LO inhibitor has the potential to be a therapeutic agent that mostly, if not only, suppresses the formation of PGE_2, LTB4, and CysLTs, bypassing interference with the biosynthesis of physiologically relevant prostanoids. Several mPGES-1 inhibitors have been previously investigated, and they seem to preferentially inhibit excessive pro-inflammatory PGE_2 production while maintaining basal levels required for homeostatic processes [10]. The implications of such a dual target inhibitor are vast, as there is suggested synergy in anti-inflammatory efficacy [4]. An mPGES-1/5-LO dual inhibitor also has the potential to have a more favourable drug safety profile as it acts on related targets within pro-inflammatory lipid mediator biosynthesis [4]. Overall, the capacity to suppress pro-inflammatory prostanoids instead of beneficial ones makes this an attractive therapeutic target. Several natural products and

synthetic derivatives have been identified as dual mPGES-1/5-LO inhibitors, including cannflavins A and B.

In 2014, Werz et al. demonstrated that cannflavins A and B exerted inhibitory effects on both mPGES-1 and 5-LO in a substrate concentration-independent and reversible manner [11]. They did not substantially inhibit COX-1/2, ultimately confirming that cannflavins inhibit PGE_2 synthesis by directly interfering with mPGES-1 and that they inhibit leukotriene synthesis via 5-LO interference [11]. Cannflavins mechanism of inhibiting pro-inflammatory prostanoid and leukotriene synthesis via mPGES-1/5-LO dual inhibition therefore provides a scientific basis for commercializing hemp-derived health food or novel anti-inflammatory therapeutics.

Affected disease states and rationale for targeting

PGE_2 and leukotrienes are key lipid mediators involved in the pathogenesis of several inflammatory diseases such as rheumatoid arthritis, dermatitis, asthma, and cancer [2]. There are also other diseases for which there are certain unmet medical needs alongside associations with mPGES-1 and 5-LO, a few of which will be explored further.

<u>CHAGAS DISEASE</u>

Chagas disease is a neglected tropical disease in northern industrialized nations caused by infection with the protozoan parasite *Trypanosoma cruzi* and is endemic in Latin American countries. 6-7 million individuals worldwide are estimated to be infected with *T. cruzi*, with 75 million people at risk of infection according to the World Health Organization (WHO) [12]. Chagas disease involves a

continuous inflammatory process resulting in the replacement of functional health tissues by connective tissue, and subsequent loss of function in tissues and organs, which can be fatal [13]. The acute phase of the disease usually lasts 2-3 months, is often asymptomatic, with associated high parasitemia and is typically undiagnosed, in contrast to the chronic phase which can manifest symptomatically [14]. In the chronic phase, 30-40% of infected individuals develop heart or gastroenteric manifestations that can result in death [14].

The most severe manifestation and most frequently found form of those symptomatic with Chagas disease is known as chronic Chagas cardiomyopathy (CCC) [15]. CCC is developed from a parasite-driven systemic inflammatory profile that leads to cardiac tissue damage [15]. Increased frequencies of circulating T lymphocytes are found in the chronic phase, which secrete pro-inflammatory mediators involved in local and systemic responses against the parasite, thus the use of immunomodulators has been proposed as a rational alternative in treating CCC [15]. In particular, the AA pathway has emerged as a critical player in the pathogenesis of CCC, so there is emerging interest in the pharmacological intervention of eicosanoid production as a novel treatment opportunity [16].

Chagas disease presents a dual treatment opportunity for mPGES-1 inhibition as PGE_2 production is known to correlate with pathogenic lipid droplet formation which modulates parasite growth, and myocarditis [13]. Previous works have also documented lipid bodies as sites for *in situ* production of leukotrienes and prostaglandins within activated cells in an inflammatory environment [13]. 5-LO has been shown to potentiate cardiac parasitism and be a key determinant of acute myocardial inflammation and mortality in those with Chagas disease [19]. For 5-LO

deficient mice, *T. cruzi* infection resulted in improved survival rate, and overall reduced inflammation compared to WT mice [19]. These results suggest that the reduced myocarditis in the 5-LO deficient mice appears to efficiently control *T. cruzi* infection [19].

Furthermore, it has been demonstrated that *T. cruzi* infection promotes the selective upregulation of the mPGES-1 and COX-2 enzymes, which produce prostanoids in heart tissue [17]. One study previously investigated COX-2 inhibition in Chagas disease to test its ability to decrease PGE_2 synthesis and found that it was successful [18]. The reduction in PGE_2 ultimately reduced the cardiac damage observed during the acute phase of Chagas disease via a reduction of inflammatory filtrate, parasite nets, cardiac fibrosis, and fewer COX-2 positive cells in obtained cardiac tissue from mice [18]. Taken together, the severity of inflammatory injury in response to tissue parasitism for those with Chagas disease appears to be influenced by the production of PGE_2 and leukotrienes, indicating that dual inhibition is a reasonable approach.

Carrying out this effort with natural products has also been attempted previously. For example, one study investigated if curcumin could interfere with the pathogenesis of Chagas myocarditis in an *in vivo* study using a murine model of acute *T. cruzi* infection and *in vitro* experiments [20]. The curcumin treatment hindered leukocyte recruitment and activation of the eicosanoid pathway without modifying parasite burden in the heart, demonstrating a clear anti-inflammatory effect [20]. Curcumin inhibited COX-2 and mPGES-1 induction, and subsequent PGE_2 production in parasite-infected cardiomyocytes, effectively hindering BNP expression, a strong indicator of cardiac pathogenesis in Chagas disease [20].

Curcumin conferred 100% survival after infection and proved effective in attenuating relevant inflammatory processes in the heart of acutely infected mice [20]. Of particular interest is the fact that curcumin substantially decreased inflammatory infiltrate production, which is traditionally accepted as a potent source of inflammatory agents mediating organ dysfunction in Chagas disease [20]. Curcumin demonstrated higher efficacy in preventing the overproduction of inflammatory mediators than a standard trypanocidal drug for Chagas disease. Therefore, it appears a decrease in inflammatory damage can be achieved upon inhibition of key myocardial enzymes such as mPGES-1 and 5-LO in Chagas disease, however further experimentation is required in models of its chronic phase.

ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammatory disease that causes lesions in medium-sized arteries which may be clinically silent for decades before resulting in pathological conditions like acute myocardial infarction, unstable angina or sudden cardiac death [21]. It is known as one of the most prevalent cardiovascular disorders globally and is considered one of the top reasons for premature adult death [22]. It is well-established in the literature that polyphenols exhibit a cardioprotective effect on atherosclerosis. Low-density lipoprotein (LDL) oxidation is considered a key mechanism in the pathogenesis of atherosclerosis, and polyphenols are potent inhibitors of it [21]. In addition, polyphenols are cardioprotective via antioxidant, anti-platelet, anti-inflammatory effects as well as increasing HDL, improving endothelial function, and contributing to stabilizing atheroma plaque [21]. An excellent review evaluated the effectiveness of polyphenolic compounds in the form of dietary or

pharmaceutical supplements from various plant sources in atherosclerosis and related complications from clinical trial data [22]. It found overall beneficial effects on multiple cardiovascular parameters of atherosclerosis via different mechanisms [22]. In addition, focusing on addressing inflammation as a driver of atherosclerosis has prompted the exploration of novel anti-inflammatory therapeutics, given recent findings that such interventions can delay atherosclerotic complications [23].

There is an abundance of research demonstrating the involvement of mPGES-1-derived PGE_2 in atherosclerosis. Significantly elevated levels of mPGES-1 and COX-2 have been observed in atherosclerotic plaque of normal mice suggesting mPGES-1 has a role in its pathogenesis and may modulate inflammatory processes related to plaque stability [24]. Experimental studies using PTGES knockout animals have demonstrated significantly reduced atherosclerotic lesions, where mPGES-1 disruption delayed the development of atherogenesis, and a significant improvement concerning the atherosclerotic condition of mice kept on a high-fat diet over 3-6 months [25, 26]. The authors of this study suggested this result reflected the rediversion of accumulated PGH_2 (a COX product) to increase vascular PGI_2, which plays a key role in protecting cardiac and vascular function [25]. mPGES-1 inhibition may also stabilize plaques and prevent acute ischemic syndromes in patients with atherosclerotic disease, as PGE_2 is known to be a predominant eicosanoid that induces the expression of crucial enzymes (MMP-2 and MMP-9) in the degradation of plaque stability [27]. Essentially, mPGES-1 deletion appears to decrease systemic biosynthesis of PGE_2 and augment that of PGI_2, overall implicating it as a promising cardiovascular drug target.

5-LO-derived leukotrienes, notably LTB4 as it has strong chemoattractant activity for neutrophils in atherosclerotic lesions, are similarly important in the pathogenesis of atherosclerosis. [28] is an excellent review that details leukotriene involvement in atherosclerosis and can be viewed for further reading. Of interest, 5-LO has been significantly expressed in human atherosclerotic plaque, 5-LO deficiency was observed to have a significant protective effect against atherosclerosis in ApoE- and/or LDLR-deficient mice, 5-LO mRNA levels have been found to correlate with the clinical stage of atherosclerotic disease and symptoms of plaque instability, and morphological and genetic evidence further supports their role [29-31]. Ultimately, targeting 5-LO and mPGES-1 and its metabolites appears to be a scientifically sound mechanism for decreasing vascular inflammation in atherosclerosis.

CELIAC DISEASE:

Celiac disease is a chronic intestinal autoimmune disorder where the ingestion of dietary gluten products causes chronic inflammation of the small intestine, exhibiting a global prevalence of 1.4% [32]. A disease prevalence of 1%-2% has been estimated for Europe, the United States, and Canada [32]. Although the only practical option for celiac patients is to adhere to a gluten-free diet (GFD) as there are no commercial treatments, it has been suggested that plant flavonoids could preserve intestinal barrier integrity and play a protective role against toxic gliadin peptides, the main component of gluten, via inhibition of eicosanoid generating enzymes [33]. Gliadins in particular appear to be a notable protein target for polyphenol interactions as they are rich in proline residues and have protein

motifs that preferentially interact with polyphenols [34]. One seminal study provided evidence of biological efficacy for three polyphenols [gallic acid (GA), epigallocatechin gallate (EGCG) and theaflavin (TF)] in the treatment of celiac disease, demonstrating stable gliadin-polyphenol complexes throughout digestion and prevention of inflammation *in vitro* [35]. The findings suggested that the structural conformation of phenolic ligands influenced their capacity to bind to the uniquely immunostimulatory peptide α2-gliadin, exerted protective effects on the gut barrier, and suppressed the secretion of pro-inflammatory cytokines IL-6 and IL-8 [35].

In addition to observed anti-inflammatory activity in the context of celiac disease making polyphenols an appealing option for therapeutic development, prostanoids and leukotrienes have also been scientifically associated with its pathogenesis. One study examined jejunal PGE_2 secretion in adult patients with histologically active celiac disease under basal conditions and after local gliadin challenge [36]. It found a five-fold higher basal jejunal secretion of PGE_2 compared to healthy controls and that after gliadin challenge of the jejunal segment, a significant increase in PGE_2 secretion was noted in celiac disease patients [36]. Elevated PGE_2 synthesis therefore appears to be involved in the pathophysiological processes initiated by gliadin in celiac disease patients.

Another study corroborated these findings, in addition to finding significantly elevated LTB4, LTC4, LTD4, and LTE4 in the small bowel mucosa of children with celiac disease on a gluten-containing diet in comparison to controls [37]. The CystLT pathway has previously been linked to various allergic disorders and was recently found to be involved in celiac disease, suggesting that drugs targeting the CystLT

pathway have the potential to disrupt the end effector response responsible for tissue damage [38]. The findings also suggested that COX inhibitors may exacerbate celiac disease via elevating leukotriene production [38]. The CystLT upregulation was demonstrated only in patients with active celiac disease and not in healthy controls or patients on a GFD [38]. Therefore, the elevated production of AA metabolic products appears to be involved in celiac disease pathogenesis, suggesting that targeting mPGES-1 and 5-LO may be intriguing targets.

Potency

The following table summarizes synthetic derivatives and natural phenolic compounds that have been identified as dual mPGES-1/5-LO inhibitors. Potencies, as determined via cell-free assays and relevant *in vivo* models that demonstrate anti-inflammatory efficacy if applicable (ex. carrageenan-induce pleurisy in rats, zymosan-induced peritonitis in mice), are summarized. The inhibitors highlighted in yellow are synthetic, and the ones in green are of natural origin. There are also a set of acylphloroglucinols and non-phenolic acidic structures that are natural dual mPGES-1/5-LO inhibitors which can be found in [2], but for the purposes of this document, only the phenolic compounds have been included.

Table 1. Dual mPGES-1/5-LO inhibitors.

Substance	**IC_{50} (mPGES-1) cell-free**	**IC_{50} (5-LO) cell-free**	**PGE_2 and LTB_4 reduction *in vivo***	**Reference**
Licofelone	6 μM	>30 1.7 μM (cell-based)	Yes (dogs)	[40], [41], [42]

Licofelone analogue-9 (LFA-9)	0.87 μM (human); 0.52 μM (mouse); 1.40 μM (rat)	2.75 μM (human);2.64 μM (mouse); 0.89 μM (rat)	Yes (rat paw edema)	[57]
Pirinixic acid, cmpd **7b**	1.3 μM	1 μM		[43]
Pirinixic acid, YS121	3.4 μM	6.5 μM	Yes (rat pleurisy)	[44]
Pirinixic acid, cmpd **16**	0.4 μM	0.3 μM	Yes (mouse peritonitis)	[45]
Cinnamic acid, cmpd **29**	1.1 μM	0.8 μM		[46]
Benzo[g]-indole-3-carboxylate, cmpd **11a**	0.6 μM	0.09 μM	Yes (rat pleurisy)	[47], [48]
N-phenyl-benzenesulfonamide derivative, cmpd **47**	0.7 μM	2.3 μM	Yes (mouse peritonitis and air pouch sterile inflammation)	[58]
4,5-diarylisoxazol-3-carboxylic acid scaffold, cmpd **18**	0.16 μM	0.39 μM	Yes (mice paw edema)	[59]
EGCG (from *Camellia sinensis*)	1.8 μM	50-65 μM		[49]
Curcumin (from *Curcuma longa*)	0.3 μM	0.7 μM		[50], [51]
Carnosol, carnosic acid (from *Salvia officinalis; Rosmarinus officinalis)*	5 μM, 5 μM	0.1 μM, 1.0 μM		[52], [53]
Perlatolic acid (from *Cetrelia monachorum*)	0.4 μM	0.4 μM		[54], [55]
(+)-conocarpan (from *Krameria lappacea*)	19.5 μM	18.4 μM		[56]
Cannflavin A, Cannflavin B (from *Cannabis sativa*)	1.8 μM, 3.7 μM	0.9 μM, 0.8 μM		[11]

Other drugs on the market

There are currently no dual mPGES-1/5-LO inhibitors commercially available, however, several respective leads are available for clinical trials [4]. Certain natural compounds and synthetic derivatives have been identified as potent dual inhibitors

in literature as shown in Table 1, but none are available for clinical use [2]. There are also no single target mPGES-1 inhibitors commercially available, however, zileuton is the only 5-LO inhibitor on the market for the treatment of chronic asthma [39].

CHAGAS DISEASE

For Chagas disease, only two drugs are available for its treatment, both have been used since the 1970s: nifurtimox (Lampit) and benznidazole (Rochagan) which act as pro-drugs that are activated within the parasite and ultimately result in nitrile production that reacts with cellular components to yield parasite toxicity [60]. It should be noted that these drugs are only effective in the acute/early infection phases. In addition, therapy discontinuation has occurred in 10-30% of treated patients due to adverse events such as anorexia, nausea, vomiting, headache, central nervous system depression or maniacal symptoms, seizures, vertigo, paresthesia, peripheral polyneuropathies, and dermatitis [61]. Benznidazole seems to have no benefit for arresting disease progression in patients with chronic Chagas cardiomyopathy according to the results from the BENEFIT trial, a prospective, multicenter, randomized study involving 2854 patients with Chagas' cardiomyopathy [62].

Another factor that has complicated the pharmacological management of this disease and is thus a cause of treatment failure is the resistance of different parasite strains to the currently available drugs. For example, one study analyzed the sensitivity of benznidazole on Colombian *T. cruzi* strains and revealed that 36% were sensitive, 48% moderately sensitive, and 16% resistant to the drug [63]. Another recent study has shown that a dormant, non-reproducing form of the parasite allows the infection to persist even after treatment with benznidazole for up to a month,

which demonstrates that current treatments often fail to cure Chagas despite extended drug exposure [64]. Thus, there is an urgent need for new therapeutic options that are greater in efficacy and have a better safety profile to treat Chagas disease, especially for those suffering in the chronic phase.

ATHEROSCLEROSIS

Currently available therapeutics for atherosclerosis (such as statins, niacin, aspirin, clopidogrel, prasugrel, ticagrelor, β-blockers, renin-angiotensin system inhibitors etc.) mainly alleviate hypertension and hyperlipidemia or control hemostasis to prevent thrombotic complications, but these strategies do not directly address the inflammatory mechanisms driving atheroprogression [65]. Anti-inflammatory statins like atorvastatin and rosuvastatin are marketed with the aim to inhibit cholesterol synthesis, but despite their widespread use, there is a large residual risk for atherothrombosis [66]. In addition, patients with multiple medical co-morbidities have demonstrated an increased risk of adverse events from long-term statin use [67]. Furthermore, a recently published meta-analysis asserted that long-term statin therapy is associated with the risk of new-onset diabetes [68]. Ultimately, despite current treatments for atherosclerosis mostly being antihyperlipidemic to manage dyslipidemia as the key player in its pathogenesis, only 30–40% of cardiovascular events are preventable with this therapeutic regimen [69]. As few FDA-approved drugs act on the inflammatory mechanism of atherosclerosis, treatments targeting this aspect warrant further attention to fight this disease successfully.

CELIAC DISEASE

As mentioned earlier, there are no commercial therapeutics that treat celiac disease, with the only option being adopting a GFD which requires considerable patient education, motivation, and follow-up. In addition to GFD limitations such as increased food costs, there appear to be adverse nutritional implications. One study tracking adolescents on the GFD showed macronutrient imbalance by excessive protein and fat consumption, and low amounts of carbohydrates, fiber, calcium, and iron [70]. Thus, adoption of the GFD potentially does not help resolve absorption-related nutritional deficiencies that celiac disease patients manifest at diagnosis [70]. There are a few treatments in current and ongoing clinical trials such as probiotics, antibiotics, anti-inflammatory therapeutics, immunomodulators, zonulin antagonists, TG2 inhibitors, dietary replacements, enzyme supplements, and anti-gliadin antibody supplements, though most appear to be ineffective, yield mixed data, or result in adverse effects [34]. At present, polyphenols appear to be the only common dietary component being explored as a potential treatment for celiac disease compared to an abundance of synthetic pharmaceuticals which involve extensive safety and tolerance testing before determining efficacy [34]. Since there are no celiac disease treatments in development that have attempted to inhibit eicosanoid synthesis and a lack of effective interventions in development, there is a unique opportunity to develop a novel treatment option for this disease.

Reasons for drug failure

As discussed prior, there are no dual mPGES-1/5-LO inhibitors on the market, though a few have been in development that are worth discussing. Licofelone is an anti-inflammatory drug that was initially reported to dually inhibit the prostaglandin

and leukotriene biosynthesis pathway via COX/5-LO inhibition but never reached the market despite promising results from Phase III clinical trial data for osteoarthritis. It is unclear why it was not submitted for regulatory approval, but some potential reasons include the inadequacy of published data on the elderly [71]. Although licofelone was proposed initially as a dual COX/5-LO inhibitor, it was later shown to directly inhibit mPGES-1 without affecting COX-2, moderately interfere with COX-1, and instead suppress cellular leukotriene formation by interference with 5-lipoxygenase activating protein (FLAP) instead of directly inhibiting 5-LO [72]. The moderate interference with COX-1 is another potential reason why licofelone failed to reach the market, as there were COX-associated side effects, safety concerns, and renal function disruption noted [73]. It is therefore crucial that novel therapeutics exhibit strong anti-inflammatory efficacy as well as high selectivity against mPGES-1 and 5-LO.

Only a few mPGES-1 inhibitors have been tested in humans, the most notable being the Eli Lilly compound LY3023703 which dose-dependently inhibited PGE_2 production in human whole blood *ex vivo* as demonstrated from a Phase I clinical trial [74]. Despite promising results, one subject developed drug-induced liver injury upon taking 30 mg/day of LY3023703 for 28 days [74]. Thus, a follow-up compound called LY3031207, was tested in a Phase I trial but the study was terminated when several subjects developed drug-induced liver injury due to a reactive metabolite common to both compounds [75]. Interestingly, investigators concluded in a follow-up study that the toxicity was likely caused by the conversion of the inhibitors into toxic metabolites, and not driven by an mPGES-1 or PGE_2 adverse effect [76]. The compound showed potent PGE_2 inhibition and elevated cardioprotective

prostacyclin formation during mPGES-1 inhibition, suggesting that this approach could have promising implications if non-toxic molecules were to be used.

As summarized in Table 1, several dual inhibitors have been identified from synthetic and natural sources but their pharmacological assessment, and efficacy *in vivo* and within clinical trials have been incompletely investigated. Bioavailability and pharmacokinetic properties such as water solubility, absorption, metabolic stability, and plasma protein binding after oral application have not been adequately studied in most of the natural products presented [2]. Some natural compounds such as curcumin and EGCG which have demonstrated dual mPGES-1/5-LO inhibition also indicate poor physicochemical and pharmacokinetic features, which has perhaps hampered their therapeutic development [2]. Clinical trial data with natural products such as extracts from willow bark, nettle, devil's claw etc. are also known to be insufficient in validating efficacy in inflammatory or painful disorders like osteoarthritis, rheumatoid arthritis, or back pain, which poses another hurdle for phytomedicine-derived therapeutic development [2].

Risks/Adverse effects

The main risk associated with mPGES-1/5-LO dual inhibition is that PGE_2 also has homeostatic functions, so there may be potential for mPGES-1 intervention to alter certain physiological processes. For example, PGE_2 is a natriuretic which regulates renal sodium and water excretion, so it is possible that its decline would result in hypertension, a fact which has been observed in PTGES knockout animals that demonstrated elevated blood pressure and hypertension [77-79]. PGE_2 also has a homeostatic role in maintaining synaptic transmission and long-term plasticity

(LTP), so it has been suggested that mPGES-1 inhibition may reduce LTP and impair memory. COX-2-derived PGE_2's contribution to memory and cognition has also been well evidenced by *in vivo* experiments, and COX-2 inhibitors have been shown to impair memory acquisition, consolidation, passive avoidance memory, and spatial memory retention, so mPGES-1 inhibitors may have similar effects [80-83]. mPGES-1 also promotes angiogenesis which is important in ulcer healing, so the use of mPGES-1 inhibitors in those with gastric ulcers needs to be observed [84].

Thus, the most substantial risk associated with mPGES-1/5-LO inhibition is that several downstream products of the PGE_2 pathway are vital for normal physiological functioning [85]. For autoimmune conditions like celiac disease, the effects of lowering PGE_2 levels should be cautiously considered. Constitutive expression of mPGES-1 has been evidenced in distinct organs in the gastric mucosa such as the stomach, and one experiment noted it seemed to contribute 80% of basal PGE_2 production [86]. There was a hypothesized prediction that mPGES-1 null mice in the study would develop spontaneous gastric or intestinal lesions based on this finding, but gross macroscopic examinations of stomachs from the null mice did not reveal any abnormality [86]. These results are promising as it reinforces that mPGES-1 inhibition could be safe for use in the gastric mucosa, though further studies will need to be done, especially under pathological or stress conditions like celiac disease.

Regardless, targeting mPGES-1/5-LO offers better safety profiling compared to existing inflammatory molecular targets like COX-1/2, as their deletion in animal and cell models have resulted in no severe adverse effects [85].

Assay recommendations

Several assays and techniques can be employed to determine mPGES-1/5-LO dual inhibitory efficacy and selectivity:

- ***In vitro* cell-free enzymatic assays using fluorometric/colorimetric methods.** Most of these assays are based on microsomes derived from mPGES-1-(over)expressing cell lines such as human A549, transfected HEK293, and HeLa cells or murine RAW264.7 macrophages, or recombinant human mPGES-1 is used as enzyme source [3]. The aim is to directly inhibit mPGES-1 in such microsomes by assessing the generation of PGE_2 from a substrate such as PGH_2 [3]. Cell-free assays allow analysis of direct interference of enzyme activity with a test compound and are suitable for inhibitor screening approaches [3]. It should be noted that including the detergent triton-X100 (0.1%) in the assays helps to exclude nuisance inhibition, which is relevant for highly lipophilic compounds such as cannflavins which may form colloid-like aggregates and inhibit mPGES-1 without specific interaction [3]. There are also fluorometric-based 5-LO inhibitor screening kits that can be used to conduct cell-free 5-LO assays, for specific methods refer to [57]. Cell-free assays are also compatible with HTS systems.
- ***In vitro* cell-based assay.** Cell-based assays principally consist of either primary cells (e.g., peripheral blood mononuclear cells and monocytes), cell lines (e.g., A549, RAW264.7; HEK293), or human whole blood [4]. To induce mPGES-1 expression and PGE_2 formation, cells/blood are treated with pro-inflammatory agents such as LPS, TNFa or IL- 1b (ex, for 12–24 h), followed in some cases by short-term challenge with a second stimulus (e.g., Ca2+-

ionophore), in the presence or absence of exogenous AA as a substrate for enzymes. Cell-based assays can be used to evaluate the effect of inhibitor compounds on PGE_2, LTB_4, and PGI_2 production.

- **Human whole blood assay and prostanoid profiling using LC–MS/MS.** This assay can be used to assess a compound's capacity to inhibit LPS-induced PGE_2 production in a complex biological matrix [87].
- **MTT assay.** MTT assays are used to assess the cytotoxic effects of inhibitor compounds on normal cells such as colonocytes of rats [57]. Results can be expressed as % viability at various doses of each compound.
- **Binding assay via isothermal titration calorimetry (ITC).** ITC is a well-established technique that can be used to determine thermodynamic parameters such as binding interaction affinity between an enzyme and small molecule and the binding affinity dissociation constant (KD) can be calculated accordingly [57].

Application to Canurta

Canurta's molecules

Canurta's IP defends technology that allows for the extraction of polyphenolic compounds in various ingredients such as powdered hemp-polyphenols, highly concentrated extracts, and pure, single polyphenols. Of particular interest in Canurta's ingredient portfolio are cannflavins A and B as they dually inhibit mPGES-1 and 5-LO, but there are other polyphenols in hemp and other plants which can be explored in the future. Many natural compounds have demonstrated anti-inflammatory activity in preclinical and clinical studies as mPGES-1/5-LO inhibitors

which encourages further investigation into cannflavins and other phytochemicals [2].

Canurta's molecules pose a competitive advantage in multiple ways. Prenylated flavonoids like cannflavins A and B demonstrate a long elimination half-life, so it can be rationally assumed that by regularly consuming hemp products containing them, bioactive concentrations can be achieved in plasma and tissues [88]. The dual inhibition mechanism can also be reasonably assumed to result in improved selectivity for treating diseases characterized by elevated PGE_2 and leukotriene levels.

The literature suggests that mPGES-1/5-LO dual inhibitors have a better renal and cardiovascular safety profile than NSAIDs and coxibs [26]. The nonselective inhibition of other prostaglandins by NSAIDs leads to several adverse effects such as GI complications (upper GI bleeding, gastric ulceration, life-threatening perforations of stomach and duodenum), renal complications (acute renal failure, hypertension, and electrolyte imbalance), cardiovascular complications (coronary heart disease), and reduced platelet aggregation [26]. Thus, the main advantage of applying cannflavins to disease states like Chagas disease, atherosclerosis, and celiac disease, is that Canurta will be capitalizing on the notion that these molecules will not yield adverse side effects via dual inhibition of mPGES-1 and 5-LO. Such side effects would be extremely detrimental for these disease states, offering a valuable opportunity to shape Canurta's future therapeutic development programs accordingly.

Successful/unsuccessful drugs

It is reasonable for Canurta to believe novel therapeutics can be developed for the disease states discussed in this document via dual mPGES-1/5-LO inhibition as there are a few drugs in development following a similar approach.

Of note, VIA-2291 (Atreleuton), a compound derived from the structural optimization of zileuton, has completed Phase II clinical trials for atherosclerosis and cardiovascular disease establishing it as one of the leading 5-LO inhibitors in clinical development [89]. The Phase II trial conducted by Tallikut Pharmaceuticals Inc. compared the effect of VIA-2291 vs. placebo on atherosclerotic vascular inflammation, involving 191 participants and took two years to complete [90]. Its primary outcome measure was the *ex vivo* LTB4 synthesis change from baseline in whole blood [90].

In addition, BHB-TZD, a dual COX/5-LO inhibitor, effectively attenuated atherosclerosis in a mouse model [91]. Although the study evaluated COX's role in plaque instability, it demonstrates that a dual inhibitor for mPGES-1/5-LO could have the potential to reduce the size of atherosclerotic lesions by reducing the production of inflammatory prostanoids such as PGE_2 or leukotrienes like LTB4 [91]. Licofelone, the dual COX/5-LO inhibitor mentioned earlier, has also been demonstrated to attenuate atherosclerosis in a rabbit model [92]. These drugs substantiate the argument for targeting the PGE_2 and leukotriene biosynthesis pathway and targeting mPGES-1 instead of COX would also yield fewer adverse effects, especially in the cardiovascular context as alluded to earlier. Though Canurta should be aware that these drugs may be commercialized in the future and may pose competition, Canurta still defends the advantage of a better safety profile via mPGES-1/5-LO inhibition as opposed to COX inhibition.

Though 5-LO inhibitors such as zileuton exist on the market, there are several reasons why mPGES-1 inhibitors have been difficult to develop. High lipophilicity impedes efficiency in biologically relevant test systems like serum or whole blood due to strong protein binding and aggregation [4]. In addition, most compounds have poor bioavailability and pharmacokinetics, and there are interspecies differences for some promising leads, resulting in a lack of suitable animal models [4]. One way to overcome this hurdle however is to use guinea pigs or knock-in human mPGES-1 mice models instead of rats or wild-type mice [4]. Another complication with designing dual inhibitors is the sensitive balance of the lipid mediator profile which depends upon species, gender, diet, and individuals, thus there is a strong need for relevant models [4].

Ideas for future clinical exploration

The following section will discuss preliminary ideas for formulations, regulatory incentives, and market analysis in the context of the aforementioned disease states.

<u>CHAGAS DISEASE</u>

Since Chagas disease is caused by the parasite *T. cruzi*, Canurta must develop it as an adjunct therapy that can be combined with a drug that has established anti-parasitic effects. Most investigators currently believe that a critical factor in causing inflammation and developing chronic myocarditis in Chagas disease is parasite persistence [94]. It has been previously established in one study involving animal models of *T. cruzi* infection that tissue parasite load correlates with the intensity of

inflammation [95]. Although two commercial anti-parasitic drugs are available, numerous phytochemicals have established anti-parasitic effects for Chagas disease which may be worth exploring in the future, as summarized in a recent review [14]. Of note, eupatorin and 5-desmethylsinensetin, two isolated flavonoids, have shown potent activity against *T. cruzi* in various life cycle stages in an *in vitro* setting [96]. Neither flavonoid showed toxicity in Vero cells, a tissue culture cell line derived from monkey kidney epithelial cells, indicating that these compounds can inhibit parasite growth without exhibiting significant toxicity on the host's cell [96]. There are also other cannabis components which have prominent anti-parasitic activity including THCA, CBDA, CBGA, alpha-pinene, and nerolidol which could be further explored by Canurta to treat Chagas disease [117]. Thus, these promising candidates could also be explored by Canurta and potentially be scaled up for commercialization. Developing a multi-target drug for Chagas disease has been suggested by experts to greatly increase treatment success, reduce adverse effects and toxicity, and avoid problems of resistance [14]. Taken together, an mPGES-1/5-LO dual inhibitor could potentially address the inflammation and pain associated with CCC, and it could be co-administered orally with an antichagasic drug to eliminate *T. cruzi*.

Exploring Chagas disease would be an interesting route for Canurta to pursue. It is established that targeting PGE_2 production is a reasonable approach, and Canurta would have the advantage of inhibiting mPGES-1 instead of COX enzymes which typically yield adverse cardiovascular events. Though most of the aforementioned studies looked at mice infected with *T. cruzi* in the acute phase of the disease, there is potential for treatment with an mPGES-1/5-LO dual inhibitor to

favour a reduction in parasitemia and inflammation. As Chagas progresses into cardiomyopathy in its chronic form, this advantage should be considered.

Chagas disease in particular has a regulatory advantage as it may yield an orphan drug designation, thus allowing for accelerated drug approval. Though it is endemic to 21 Latin American countries, it has been found in regions such as the European Union, United States, Canada, Japan, and Australia due to globalization and migration, making it a growing public health concern [99]. The highest rates of infection are seen in rural economically disadvantaged and marginalized populations in endemic countries, so economic hardship and political instability have driven prevalence in non-endemic regions of the world [99]. For Health Canada to authorize orphan drugs for sale for rare diseases, submission needs to be filed for review indicating the details of the drug's safety, efficacy, and quality, and various regulatory resources can be used during this process. The U.S FDA has accelerated approval regulations for drugs for serious conditions that fill an unmet medical need that can be approved based on a surrogate endpoint, which is a measure thought to predict clinical benefit.

In 2017, the FDA granted accelerated approval to benznidazole for use in children, making it the first treatment approved in the U.S for Chagas disease [100]. Its manufacturer, Chemo Research, S.L., was awarded a Tropical Disease Priority Review Voucher per a provision included in the Food and Drug Administration Amendments Act of 2007 [100]. The clinical trials involved treating 235 children with chronic Chagas disease with either benznidazole or placebo tablets twice daily for 60 days, with a follow-up period of 3-4 years [100]. The endpoint measurement was the number of patients who lost antibodies to the *T. cruzi* parasite, which ranged from

55-60% seroconversion compared to 5-14% in the placebo group [100]. In 2020, nifurtimox was approved under the same accelerated approval program by the FDA based on evidence from one clinical trial involving 219 children with Chagas, where they were randomly treated with tablets three times a day for 60 days and followed for one year [101]. 32% of patients in the trial conducted by Bayer HealthCare Pharmaceuticals Inc. exhibited seroconversion [101]. As these two drug approvals were relatively recent, it is clear that Canurta could justifiably explore developing a novel therapeutic for Chagas disease.

Several experts in the Drugs for Neglected Diseases initiative (DNDi) have published a target product profile (TPP) for Chagas disease, indicating that new treatments should involve simple treatment regimens, be safe and well-tolerated, and have a low probability of resistance and interaction with other drugs [14]. Interestingly, it has been very recently determined that Chagas disease has been aggravated by climatic hazards such as fires, ocean climate change, precipitation, storms, and warming, so with the ongoing emission of greenhouse gases (GHGs), its impact on human health must be mitigated [102].

Concerning market analysis, in 2016 the global Chagas treatment disease market was valued at $5.67 million USD, and it is expected to witness a CAGR (compound annual growth rate) of 7.3% over a forecast period of 2017-2025, surpassing $10.29 million USD by 2025 [103]. Benznidazole currently holds the largest market share at 88.63%, and some of the key players operating in the Chagas disease treatment market include Nortec Quimica SA, Bayer AG, Laboratorio Elea Phoenix SA, Maprimed S.A., and Laboratório Farmacêutico de Pernambuco S/A [103]. One of the major factors that is expected to generate market growth soon are increasing

approvals for Chagas disease treatment drugs, with North America expected to hold the dominant position in the global market [103]. According to a recent report on clinical developmental success rates by BIO, Informa Pharma Intelligence, and QLS Advisors, rare disease therapies demonstrated particular success with an overall likelihood of approval (LOA) of 17%, which is nearly three times higher than the 5.9% LOA for chronic, high prevalence diseases [116]. Taken together, there is a strong case for the development of a novel therapeutic to treat Chagas disease and benefit its affected populations through Canurta's molecules.

ATHEROSCLEROSIS

Since atherosclerosis is a multifactorial disease, developing an adjunct therapy would be ideal. Since there is a close association between inflammation and atherothrombosis, developing an adjunct therapy that targets inflammation could potentially reduce clinical events in atherosclerosis. Oral supplementation could be explored to use Canurta's molecules to mute inflammation and complement traditional atherosclerotic drugs that target lipids and hypertension.

Pursuing atherosclerosis as a potential disease state is also reasonable due to a wealth of scientific evidence validating dual inhibition of both the prostaglandin and leukotriene biosynthesis pathways. For decades, atherosclerosis was traditionally considered to be a lipid-driven disease characterized by lipid deposition in the arterial wall, so prior therapies focused on the prevention of relevant risk factors like hyperlipidemia and hypertension [104]. Millions of deaths occur around the world despite the commercial availability of such therapies, so the understanding of atherosclerosis has transitioned to be a chronic, lipid-driven, low-

grade inflammatory disease of the arterial wall [104]. Although statins are to this day considered the most effective treatment for atherosclerosis by exhibiting anti-inflammatory effects and reducing atherogenic lipoprotein levels, treatments targeting the inflammatory nature of atherosclerosis are still lacking and deserve to be pursued to fight this multifactorial disease successfully [104].

Canurta has a strong scientific basis for pursuing a dual mPGES-1/5-LO inhibitor for atherosclerosis as it is well evidenced that inhibition of the COX enzymes suppresses antithrombotic and vasodilatory PGI_2 and leads to adverse cardiovascular effects. COX-2 inhibitors or enzyme deletion have been shown in various mouse models to retard, accelerate, or leave the development of atherosclerosis unaltered [105]. Prominent authors in the field have suggested that selective inhibitors of mPGES-1 may reduce the likelihood of hypertension and predisposition to thrombosis associated with COX-2 inhibitors, retain clinical efficacy, and potentially confer cardiovascular benefits during sustained dosing [25]. Because mPGES-1 inhibition is not associated with accelerated thrombogenesis like COX-2 is, and has been shown in multiple knockout disease models to diminish vascular inflammation, there is a compelling case for Canurta to pursue atherosclerosis. The scientific basis is encouraging but further experiments would need to be conducted by Canurta to determine if a dual inhibitor can retain the advantageous properties conferred by mPGES-1 deletion.

Since there are various treatments available on the market for atherosclerosis, Canurta could enter the market through a nutraceutical or dietary supplement approach to provide a safe alternative to mute the inflammatory aspects of this disease and complement traditional drugs. As suggested by the Norn group's age-

related disease overview, clinical trials lasting 64 months with 7106 people in total are the recommendations for trial design for atherosclerosis, though their guidance is not definitive or error-free, so these values should only be taken as suggestions [115]. It should be noted that cardiovascular drug programs typically have the longest Phase III durations due to large patient population sizes and long-term evaluation of cardiovascular outcomes [116]. Cardiovascular disease areas have demonstrated a large number of failed Phase II transitions for novel drug candidates, exhibiting an overall LOA of just 21%, thus Canurta must focus on conducting strong proof-of-concept studies that detail a compelling relationship between atherosclerosis and the dual inhibitors mechanism and perhaps focus on a natural health product pathway instead [116].

Key drivers of the atherosclerosis market include increasing risk factors for disease pathogenesis, such as cigarette smoking, unhealthy eating habits, sleep deprivation, and sedentary lifestyles. According to Technavio, a market research firm, the atherosclerosis therapeutics market share is expected to grow by $3.44 billion USD from 2021 to 2026, progressing at a CAGR of 5.66% [106]. Key players include Amgen Inc, AstraZeneca, Eli Lilly and Co, Roche, and Dr. Reddys Laboratories Ltd [106]. Of interest, the report indicates that the small molecules segment will yield significant market share growth during the forecast period, as the manufacturing and regulatory approval process of small molecules are relatively easy and their use can significantly reduce the risk of atherosclerotic complications [106]. North America is also expected to have substantial market share growth during this period [106]. Taken together, Canurta could enter as a new player in the atherosclerosis

market and cater to this increasing demand for small molecule drugs as well, making future therapeutic possibilities broad.

CELIAC DISEASE

Celiac disease is also a multifactorial disease in nature, so developing an adjunct to the GFD may be the best route to take in terms of therapeutic development. An adjunct has the potential to address accidental gluten exposure (ex. via cross-contamination) by decreasing intestinal inflammation and symptoms. Dual mPGES-1/5-LO inhibition could address intestinal inflammation, but it does not target the digestion of gluten peptides or genetic factors associated with celiac disease pathogenesis, making a nutraceutical dietary supplement an ideal formulation. Gluten is in more than 80% of food and interestingly, many patients adhering to the GFD remain symptomatic for celiac disease and experience small intestinal inflammation, indicating that inadvertent gluten consumption regularly occurs despite best efforts to follow the GFD, thus there is a huge demand for adjunct treatments [97]. Since individuals with celiac disease experience intestinal inflammation after ingestion of gluten products, perhaps making a superfood marketed as something that can be ingested consistently would be worth exploring. Hemp is a naturally gluten-free plant, so there is great potential for it to be marketed as a safe superfood for those with celiac disease. It would be important however for Canurta to ensure the hemp does not come into contact with gluten at any point in the process of growing to harvesting, shipping, storing, or processing it.

However, if Canurta were interested in taking the pharmaceutical route to develop a monotherapy, one idea would be to create a capsule supplement that is

either orally or sublingually administered. Though the sublingual route would avoid destruction by gastric juices or metabolism by the liver, applying an enteric coating to the surface of the capsule could be explored for an orally administered drug. For example, the synthetic polymer cellulose acetate phthalate (CAP) is insoluble at low pH (<5) but soluble at intestinal pH (>6) and is commonly used for commercialized drugs [98]. As cannflavins have poor bioavailability, using an enteric coating may address this limitation as well.

The main advantage of Canurta pursuing celiac disease in the future is the fact that there is a huge market need for novel therapeutics. The GI tract is where the concentration of polyphenols might achieve their highest values, and given that celiac disease predominantly affects the small intestinal mucosa, it is a rational notion that using cannflavins may hold great promise as a safe, effective, and natural strategy to modulate critical regulatory pathways associated with celiac disease [107]. Furthermore, it is known that therapeutics involving COX inhibition suppresses homeostatic GI-protective PGE_2, indicating that Canurta's cannflavin molecules have the potential to suppress only the excessive PGE_2 formation related to inflammation, but maintain basal PGE_2 levels that are required for GI homeostasis [3]. Long-term treatment with COX-2 inhibitors is also problematic for those with impaired GI healing, and NSAIDs are known to induce GI toxicity and vasoconstriction leading to injury, which further elevates the case for developing cannflavins as a safer therapeutic alternative.

There are also various regulatory advantages associated with pursuing celiac disease as a disease state. Firstly, there is an opportunity to apply for federal funding through the National Institutes of Health (NIH). This would be an appropriate choice

to fund the therapeutic as the NIH recently released a Notice of Special Interest (NOSI) for potential applicants to inform particular interest in developing disease ameliorating therapies for celiac disease [108]. There are also various research partnership opportunities, such as the Celiac Disease Foundation, which offers the largest U.S database of celiac disease patients and a focused patient registry called iCureCeliac, making them uniquely equipped to help recruit qualified candidates for clinical trials [109]. Of great interest is the fact that the FDA very recently issued draft guidance for industry on developing adjunct therapeutics to treat celiac disease with the GFD [110]. The guidance details eligibility criteria, trial design, considerations for efficacy and safety, and clinical outcome assessments. It notably suggests clinical trials should be randomized, double-blind, placebo-controlled, and that treatment duration should ideally be at least 52 weeks to characterize the drug's safety profile and durability of response [110]. According to DelveInsight Business Research, the celiac disease market size is set to prosper at a CAGR of 9.18% during the study period 2018-2030, which is very promising [111]. 9 Meters Biopharma, Immuno-genX, Provention Bio, Takeda, and Cour Pharma are the key companies working in the celiac disease market and the launch of pipeline therapies, so Canurta should be aware of these competitors [111]. Taken together, as there are many regulatory resources available that can help navigate the clinical development of novel celiac disease drugs as an adjunct to the GFD and a prosperous market outlook, Canurta could reasonably pursue this disease state.

Risks

Bioavailability and pharmacokinetics

One of the main limitations of flavonoids like cannflavins is their poor bioavailability. The majority of *in vitro* and *in vivo* studies suggest that proteins, dietary fibre, and minerals may hamper flavonoid bioavailability, whereas lipids, digestible carbohydrates, vitamins, alkaloids, carotenoids and other flavonoids are likely to improve flavonoid bioavailability [112]. Co-administration of flavonoids with food does appear to produce better absorption from the gut, so using approaches like a cyclodextrin complex or lipid/carbohydrate-flavonoid conjugate may be investigated in the future [113]. If Canurta were to develop therapies in a food matrix, these aspects should be considered.

Flavonoid delivery is also challenging due to poor solubility, run-down permeability, low bioavailability, instability in the biological environment, and extensive first-pass metabolism [113]. Several absorption-enhancing techniques have been recently developed to improve oral bioavailability and efficacy of poorly absorbable flavonoids via increasing solubility or GI permeability and preventing metabolic degradation [113]. Some of these strategies include structurally modifying the parent compound, nano-formulation, matrix complex formation, co-crystal techniques, dispersion techniques, and using colloidal drug delivery systems (CDDS), which may be explored to enhance the bioavailability and pharmacokinetics of cannflavins in the future [113].

Flavonoid interactions with clinically used drugs

Some dietary flavonoids may have the potential to interact adversely with clinically used drugs as a few have been found to alter their pharmacokinetic profiles [113]. Though this likely will not be a major concern with the consumption of hemp

extracts or cannflavin supplements as they typically produce low concentrations of flavonoids during daily dietary intake, such interactions and pharmacokinetic alterations are unpredictable. Canurta must therefore prioritize establishing a solid understanding of the timing and dosing of cannflavins to maximize its benefits and minimize adverse effects, especially if developing adjunct therapies as suggested prior.

Conclusion

Canurta as a startup biotech company can handle the drug discovery phase and complete *in vitro* preclinical studies and small-scale proof of concept studies in association with university researchers soon. In the long term, collaborating with larger pharmaceutical companies may be necessary to successfully complete clinical trials, have regulatory expertise and filing fees, conducting health technology assessments, have market access expertise, and manufacturing guidance. In terms of markets to pursue, it is clear that there is a significant market need for novel therapies for celiac disease, chronic Chagas disease, and atherosclerosis. 40% of celiac disease patients are interested in exploring novel therapeutics as they are unsatisfied with maintaining their alimentary regimen, and similar patient trends may exist in other disease states [114]. Rare disease R&D as would be the case for Chagas also appears to be an appealing venture as it combines a large unmet medical need with commercial potential, regulatory incentives, and above-average clinical success rates [116].

Canurta should therefore focus on entering the market with adjunct therapies for these disease states either through developing pharmaceutical drugs or

pursuing a natural health product route. Being able to incorporate a natural health product, dietary supplement, or super-food that can be taken consistently will be able to modulate systemic inflammation in individuals suffering from these disease states and complement traditional treatments or diets. Multifactorial diseases need therapies to perform multiple points of attack to be successfully managed or treated and Canurta can enter the market with this notion. There are also valuable opportunities that may streamline the preclinical development process, such as using *in vitro* organoid models that more accurately reflect disease pathology. HUB Organoids for example offers the only technology capable of generating *in vitro* models and specific assays that can study inflammatory conditions such as inflammatory bowel diseases (IBD) (Crohn's disease and ulcerative colitis). These models and assays would be invaluable in studying intestinal barrier integrity and immunomodulatory function, particularly for celiac disease. As the main issue with mPGES-1/5-LO dual inhibitor design, and with developing therapeutics for inflammatory diseases at large, is a lack of suitable preclinical models, using organoids to provide greater predictive validity for human efficacy would be in Canurta's best interests during the R&D process. Since there are no previously approved drugs that achieve mPGES-1/5-LO dual inhibition, clinical validation of the targets will be an important factor for Canurta to consider.

Therefore, it is sound to conclude that the potent anti-inflammatory effects, affordability, and outstanding safety profile evidenced by Canurta's cannflavin molecules deserve further consideration as complementary therapies to the limited array of treatments available for these disease states. This is an exciting time to use the scientific basis behind dual mPGES-1/5-LO inhibition and commercialize natural

compounds like cannflavins to revolutionize the next generation of anti-inflammatory drugs.

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This whitepaper was based on a search strategy examining peer-reviewed literature on mPGES-1, 5-LO, PGE_2, leukotrienes, cannflavins, polyphenols, and flavonoids in general in relation to chronic inflammation and specific disease states such as Chagas disease, atherosclerosis, and celiac disease. Clinical trial data was obtained from FDA Drug Trial Snapshots and market analysis was obtained from independent market research firm reports.

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