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REVIEW

Cyclophilin inhibition as a potential treatment for nonalcoholic steatohepatitis (NASH)

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ABSTRACT

Introduction: Cyclophilins are a family of diverse regulatory enzymes that have been studied for over 30 years; they participate in many pathophysiological processes. Genetic deletion or pharmacologic inhibition of cyclophilins has shown therapeutic effects in a wide spectrum of disease models, including liver disorders, and hence may be beneficial in treating nonalcoholic steatohepatitis (NASH).

Areas Covered: This articles briefly describes cyclophilin isomerases and the main classes of cyclophilin antagonists; it then summarizes data showing cyclophilin participation in the major pathophysiological activities that occur in NASH.

Expert Opinion: Optimization of therapeutic outcomes in the treatment of NASH may be best realized by targeting multiple pathologic pathways, especially when treating advanced stages of the disease. A preferred approach for achieving this goal is to use compounds such as cyclophilin inhibitors that simultaneously target multiple disease processes. The pleiotropic benefits of this drug class derive from the extraordinary functionality of prolyl isomerization as a regulatory mechanism and its evolutionary diversification into many biochemical pathways. Nonimmunosuppressive analogs of cyclosporine A are the most thoroughly characterized cyclophilin inhibitors and show significant potential to attenuate several of the major pathophysiological events in NASH – mitochondrial dysfunction, cellular injury and death, inflammation, and in particular, fibrosis.

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1. Introduction

NASH is commonly considered to be the hepatic manifestation of metabolic syndrome, and many candidate therapies are justifiably focused on normalizing the liver's metabolic activities. Reducing steatosis is a common goal and achieved by different strategies such as blocking lipogenesis or stimulating lipolysis. Another therapeutic goal is improvement in glucose and insulin homeostasis since these are closely linked to lipid metabolism. It is important to attenuate these drivers of NASH pathogenesis in order to reverse disease course. However, NASH is a chronic, multifactorial disease and unlikely to be universally responsive to a single type of treatment. Residual pathological features may persist even after addressing the major disease drivers, especially in advanced disease stages where tissue architecture is substantially changed. As precedence, eradication of hepatitis C virus (HCV) from chronically infected individuals significantly reduces the risk of liver-related morbidities such as hepatocellular carcinoma (HCC) but does not eliminate it. Moreover, individuals with cirrhosis or pre-cancerous liver nodules at the time of HCV eradication still retain high risks of HCC following sustained virological response [1–4]. These findings advise us that treatments should also directly target the many pathophysiological features of chronic liver disease such as hepatocyte dysfunction/death, inflammation, and fibrosis if the management of NASH is to be complete [5].

Cyclophilin antagonists are a drug class that offers the opportunity to treat multiple pathological processes. Since the first identification of cyclophilins in 1984 [6,7], there have been over 4000 publications on cyclophilins. Thirty years of investigation has revealed their participation in a wide variety of biochemical and pathophysiological pathways, and genetic or pharmacologic inhibition of cyclophilins has demonstrated therapeutic efficacy in at least 30 animal models of disease. This has propelled the search and development of cyclophilin inhibitors for treating human ailments, but no compound has yet been clinically approved to specifically target cyclophilins. CsA indeed is a relatively potent cyclophilin inhibitor, and some of its clinical actions may occur through this mechanism, but its additional immunosuppressive activity prevents its use for most clinical conditions.

The cyclosporin class of cyclophilin inhibitors are especially good candidates for treating liver diseases due to their first-pass extraction and steady-state accumulation in the liver [8–10], and a previous review by Naoumov (2014) describes their potential in a variety of liver disease [11]. Here we provide a more in-depth and up-to-date focus on NASH-related studies. After an introduction to cyclophilins and their pharmacological antagonists, we summarize the studies that have addressed the role of cyclophilins across a broad spectrum of NASH pathophysiology. Altogether these studies overwhelmingly support the clinical investigation of cyclophilin inhibitors to alleviate disease from the earliest drivers, such as mitochondrial dysfunction, to the latest sequalae such as fibrosis and malignancy.

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Article Highlights

- Cyclophilin isomerases are evolutionarily diverse enzymes that regulate a vast number of biochemical processes by controlling the conformation of proline peptide bonds and in turn protein structure and function.
- Cyclosporine A (CsA) and nonimmunosuppressive CsA analogs are the best characterized cyclophilin antagonists and potently inhibit multiple cyclophilin isoforms due to highly conserved active sites.
- The most studied cyclophilin isoforms, A, B, and D, participate in many if not all of the major pathophysiologic processes that are active in NASH and other chronic liver diseases.
- Genetic knockout or pharmacologic inhibition of cyclophilins preserves mitochondrial function and decreases cell death, inflammation, and fibrosis in well over 30 disease models including NASH-related disease.
- Cyclophilin inhibitors have been pharmaceutically optimized by 35 years of study and hold considerable potential to safely and effectively treat NASH by directly targeting multiple pathologic mechanisms.

This box summarizes key points contained in the article.

2. Cyclophilin isomerases and pharmacological antagonists

Cyclophilins are a family of enzymes that catalyze *cis-trans* isomerization of proline peptide bonds. Two other families of enzymes, FK506 binding proteins (FKBP) and parvulins, also possess this type of enzymatic activity, and collectively the three classes of peptidyl-prolyl enzymes are called immunophilins. Prolyl peptide bonds are distinct from all other peptide bonds in the human repertoire in that they can transition more easily between *cis* and *trans* conformations. Only around 0.3% of non-proline peptide bonds exist in the *cis* conformation, whereas approximately 5.7% of proline bonds exist in this conformation at steady state [12]. Prolines significantly define protein morphology due to the geometric restrictions imposed by proline's rigid pyrrolidine ring. More importantly, protein conformation and function can be regulated by the *cis-trans* flexibility of proline peptide bonds and their catalysis by immunophilins at specific microdomains of proteins. Prolyl isomerization between *cis* and *trans* conformers is a process that is potentially inherent to every protein and modulates not only the individual structure and activity of the protein but also its binding interactions. Uncatalyzed *cis-trans* transitions occur relatively slowly (e.g. minutes time scale), whereas cyclophilin-catalyzed isomerization occur many orders of magnitude faster [13,14]. Thus, cyclophilins substantially improve the temporal orchestration of biochemical processes and have been shown to play roles in folding of nascent peptides into proteins, protection from protein aggregation (chaperone activity), intracellular protein trafficking and secretion, second messenger signaling, and control over a vast array of protein-protein interactions [15–18]. Cyclophilin isoforms are present in all life forms – archaebacteria, bacteria, plants, fungi, protists, animalia. Seventeen cyclophilin isoforms exist in the human proteome and are present in all the major compartments of all cells [19]. Also, many plant and animal viruses including the hepatitis viruses B and C utilize host cyclophilins for replication, immune evasion, and other activities [20]. Considering the broad evolutionary and functional diversities of cyclophilins and similarly, FKBP's, it should come as no surprise that their actions are evident across an equally diverse range of pathophysiological conditions.

The inquiry into cyclophilins and their antagonists is an interesting historical journey. The name, 'cyclophilin', originates from its identification in 1984 as a binding partner of cyclosporine A (CsA) [6], but not until 1989 was it determined that cyclophilins also are prolyl isomerases [21]. Cyclosporine A (CsA) is a fungal cyclic undecapeptide first isolated in 1971. It was discovered to be a potent immunosuppressant, which led to its regulatory approval in 1983 for preventing solid organ graft rejection. CsA is the prototypical inhibitor of cyclophilin isomerase activity, but its immunosuppressive activity is not primarily due to Cyp A binding. Instead, immunosuppression results from the binding of CsA-Cyp A dimers to the phosphatase, calcineurin, which blocks calcineurin-mediated activation of nuclear factor of activated T cells (NFAT) and its downstream signaling. CsA blocks Cyp A isomerase activity with relatively high affinity (Ki ≈ 15 nM). Furthermore, it blocks the activity of most cyclophilin isoforms (e.g. Cyp B, C, D, etc.) due to high evolutionary conservation of the isomerase active sites [19]. Although CsA primarily is utilized for its calcineurin inhibition, it has been tested clinically to inhibit cyclophilins with mixed results in, for example, myocardial infarction and muscular dystrophies [22–27]. However, the use of CsA for conditions other than those that benefit from immunosuppression unnecessarily puts individuals at risk of infections, nephrotoxicity, and other immunosuppression-related toxicities.

Shortly after the discovery of CsA-Cyp A binding, it was found that some synthetic modifications of CsA produced analogs that were largely devoid of immunosuppressive activity but still retained Cyp A binding [28,29]. Several of these nonimmunosuppressive CsA analogs have been made over the past 30 years and have been investigated in many experimental models [30]. The three compounds that have been studied most extensively are NIM811, SCY-635, and alisporivir (Debio-025). All three, and alisporivir most extensively, have been evaluated in in clinical trials for chronic hepatitis C since hepatitis C virus (HCV) replication is dependent on interactions with host cell cyclophilins [31–36]. While largely safe and efficacious, none of the three compounds advanced completely through development for application of regulatory approval. Much has been learned over the years on the structure-activity relationships of CsA analogs, for example on how to minimize drug transporter interactions and how to target the molecules intracellularly and extracellularly [37–40], and compounds continue to be developed and evaluated. CRV431 is one the most recently developed CsA analog and currently in Phase 1 clinical trials. Relevant to liver disease, CRV431 has demonstrated antiviral activity toward hepatitis B and C viruses, anti-fibrotic activity in diet-induced and chemical-induced models of liver fibrosis, and the capacity to decrease NASH-induced liver tumors in mice [41–43]. Other chemical classes also have been explored as cyclophilin inhibitors but generally show disadvantages compared to CsA analogs. For example, the immunosuppressive macrolide, sanglifehrin A, and nonimmunosuppressive sangamide derivatives of it (e.g. NV556), are potent cyclophilin inhibitors but generally have shown poor bioavailability and have not been thoroughly

studied, including their toxicology [44–47]. Many types of small molecules also have been made, and some have shown *in vivo* efficacy, but the cyclophilin inhibition potencies of most of the compounds are lower than those of CsA analogs [48–56]. In similarity to sanglifehrin-derived compounds, no small molecule cyclophilin inhibitors have advanced to clinical trials to our knowledge. Finally, genetic knockdown or knockout technologies have been used extensively to interrogate cyclophilins in experimental models but not yet as clinical therapeutics.

3. Cyclophilins and their antagonists in liver pathophysiology

3.1. Cyclophilin involvement in mitochondrial metabolism

It is generally accepted that NASH liver pathology is driven by maladaptive lipid and carbohydrate metabolism producing imbalances and overproduction of lipids, especially longer chain fatty acids, thus driving cellular toxicities. Obesity, metabolic syndrome, and insulin resistance or diabetes are commonly associated with NASH and act in concert to drive metabolic disturbances. The metabolic changes are accompanied by an array of pathological activities including oxidative/nitrosative stress, endoplasmic reticulum (ER) stress, inflammation, ischemia, apoptotic and necrotic cell death, fibrogenesis, and oncogenesis to varying degrees among individuals.

Mitochondria play a central role in metabolism as the organelle responsible for fatty acid beta-oxidation, pyruvate dehydrogenation, the citric acid cycle, and ATP generation. Considerable evidence has accumulated that cyclophilin D (Cyp D) in the mitochondrial matrix participates directly or indirectly in many of these processes and that pharmacologically targeting Cyp D may improve the metabolic perturbations in NASH. Cyp D levels increase in animal models of obesity and metabolic syndrome and decrease when disease is reversed, for example by exercise [57–61]. The most understood mechanism by which Cyp D regulates mitochondrial function is its role as an inducer of the mitochondrial permeability transition (mPT) which is a common mechanism of cellular injury and death [62] (Figure 1). mPT was observed as early as the 1950's and now is understood to represent the formation of large, high-conductance pores in the inner mitochondrial membrane that results from excessive calcium uptake in combination with elevated oxidative or nitrosative stress and inorganic phosphate (i.e. ATP depletion) [48,63–70]. mPT pores conduct ions and molecules up to 1.5 kDa in size which, in the pathological state, elicits a cascade of mitochondrial depolarization, swelling, reactive oxygen species (ROS) production, and if left unchecked, mitochondrial membrane rupture. Ultimately necrotic cell death ensues and is sometimes accompanied by apoptotic mechanisms due to release of apoptosis-inducing molecules from the mitochondria. Since the triggering events of calcium dysregulation and oxidative stress

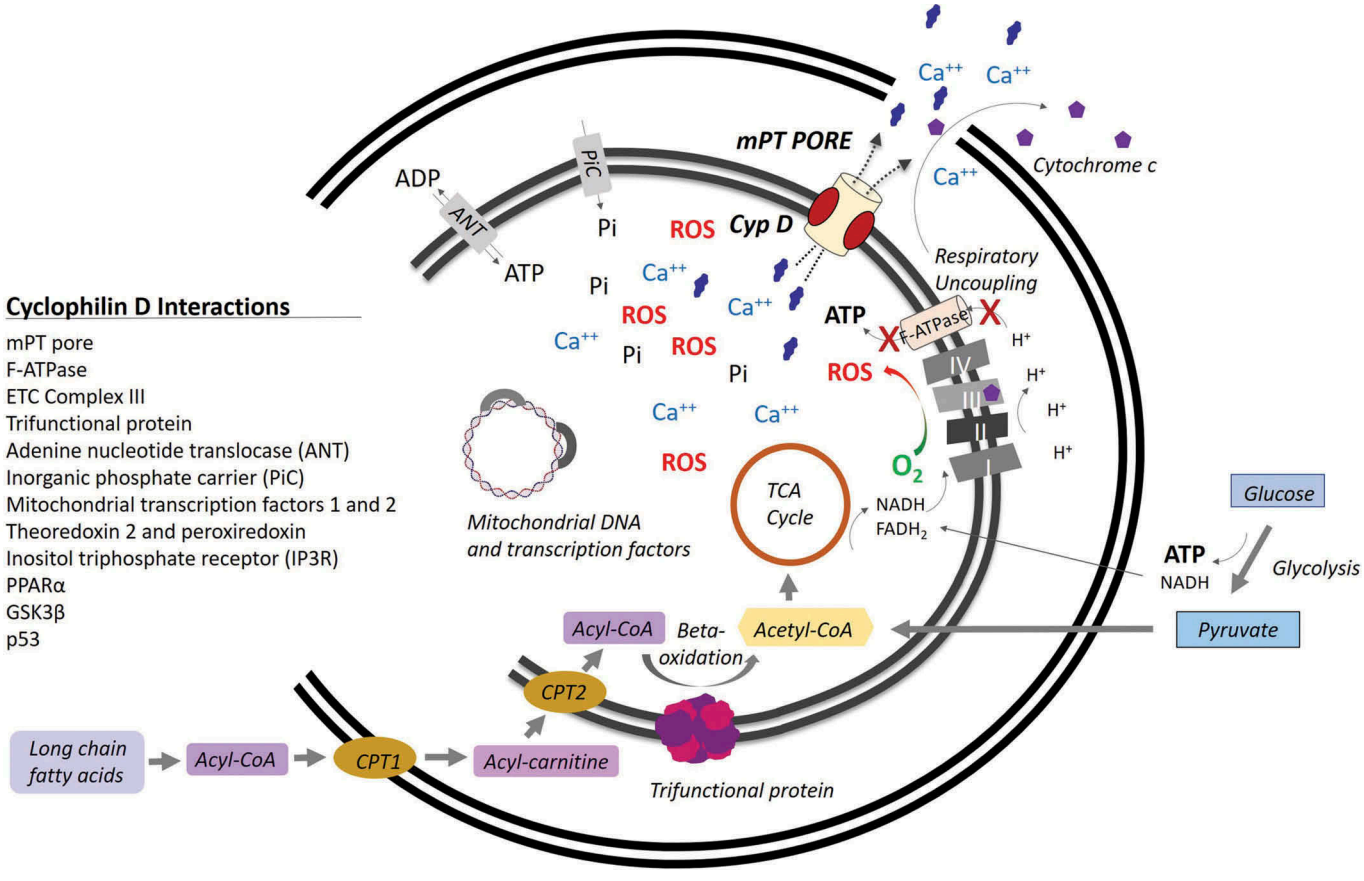

Figure 1. Cyclophilin D regulates mitochondrial metabolism and integrity through binding to multiple mitochondrial proteins, including mitochondrial permeability transition pores (mPT). Cyclophilin D regulates glycolysis, β-oxidation, and ATP production through interactions with several key mitochondrial proteins. In pathological conditions Cyp D is recruited from the matrix to the inner mitochondrial membrane and promotes mPT in response to excessive matris calcium, ROS, and phosphate. Ion and metabolite flux through mPT pores in the inner mitochondrial membrane results in mitochondrial swelling, membrane rupture, and release of pro-apoptotic moleclues. Cell death ensures when mitochondrial injury or loss exceeds a thresold and can be blocked by cyclophilin D inhibition.

arise from many types of tissue insults, mPT has been documented in many disease and injury states, including lipotoxicity and other forms of hepatic injury. Cyp D-regulated pore openings also occur physiologically but at lower amplitudes and appear to act as a homeostatic 'release valves'. However, the consequences of blocking these transient openings appear to be minimal, in contrast to the highly protective effects of blocking pathologic mPT [63,69–74]. Cyp D levels are higher in hepatic and cardiac mitochondria than in other tissues, which translates to higher mitochondrial calcium concentrations and higher sensitivity to mPT induction [75–81]. Cyp D knockout or pharmacological inhibition greatly reduces sensitivity to mPT and thereby prevents cell death, except under the most extreme conditions, and indeed the ability to block cell death with CsA has become the diagnostic hallmark of mPT.

Although Cyp D is best known for its regulation of mPT-mediated cell death, studies also have begun to elucidate a major role in global mitochondrial metabolism. Genetic Cyp D knockout in mice has been found to change metabolic activities in the liver, heart, skeletal muscle, bone, and kidney [80,82–86]. The two major changes are a decrease in fatty acid β-oxidation and an increase in glycolysis. The decline in β-oxidation is characterized by decreases in carnitine palmitoyltransferase I (CPT1) and trifunctional protein activity and increases in acylcarnitine species. Cyp D ablation also changes acetylation levels of many mitochondrial proteins that may favor lower β-oxidation rates [87]. The increase in glycolysis is characterized by elevated levels of lactate, pyruvate, and mRNA of several glycolytic enzymes. Cellular glucose levels were also found to be elevated, suggesting stimulated gluconeogenesis. Concentrations of NADH, NAD+, FAD, and acetyl-CoA decreased slightly, but mRNA levels and activities of Krebs cycle (citric acid cycle) enzymes and respiratory complex enzymes were unchanged in Cyp D -/- mice. The net effects of these metabolic alterations were found to be a slight increase in ATP production due to an increase in glycolysis and/or ATP synthasome activity, yet a reduction in oxygen consumption [82,83,88,89]. These metabolic changes were also evident in an examination of exercise capacity, where Cyp D knockout mice showed higher exercise capacity (longer and faster treadmill activity) but lower oxygen consumption both during exercise and at rest, compared to wildtype mice [90].The mechanisms through which Cyp D deletion results in this shift in metabolism have not been thoroughly studied, but one possibility is by downregulation of mitochondrial gene transcription. Cyp D silencing in cell culture by genetic knockdown or CsA was found to block Cyp D interaction with mitochondrial transcription factors 1 and 2 and in turn decrease mitochondrial RNA synthesis [91]. Other candidate mechanisms require investigation, since Cyp has been shown to bind to many prominent mitochondrial proteins (Table 1) [57]. Included in this list are trifunctional protein which orchestrates β-oxidation, redox-sensitive theoredoxins, and molecules at the core of ATP production such as F-ATPase which also is a lead candidate for the pore-forming molecule of the mPT [92].

The decrease in β-oxidation with Cyp D deletion may seem counter-productive to resolving the lipid overload in NASH but in fact may preserve mitochondria over the long run by preventing the harmful effects of elevated β-oxidation. High rates of β-oxidation necessarily drive high oxygen flux and a proportional spill-over of reactive oxygen species (ROS) production which also is exacerbated by electron transport chain (ETC) uncoupling. Indeed, Koliaki et al. (2015) found that mitochondria from NAFLD livers have high oxygen consumption, a moderate increase in ROS-induced lipid peroxidation, and ETC uncoupling indicative of inefficient ATP production [105]. Upon progression to NASH, mitochondria had even higher levels of ROS, lipid peroxidation, depressed antioxidant defenses, and ETC uncoupling. NASH mitochondrial mass was elevated, presumably as an attempt to compensate for poor ATP production, yet oxygen consumption was reduced compared to NAFLD. Together these observations point to significant mitochondrial dysfunction in NASH and an inability to cope with prolonged steatosis and heightened β-oxidation. Widespread mitochondrial swelling is evident [106], consistent with high ROS- and low ATP-induced mPT. Thus, Cyp D inhibition may be protective in NASH both by preventing mitochondrial exhaustion arising from prolonged β-oxidation and ROS production and later by blocking mPT-mediated cell death.

Higher rates of glycolysis arising from Cyp D silencing also may be advantageous by improving glucose handling systemically. In support of this hypothesis, two studies found better glucose tolerance in high fat diet (HFD)-fed Cyp D -/- mice compared to wildtype mice, whereas two other studies found no improvement [82,107–109]. In the studies where Cyp D ablation improved glucose tolerance, the mice also had slightly higher insulin levels, pancreatic islet β-cell proliferation, and other markers of improved insulin resistance [58,82,107]. Anti-diabetic effects of Cyp D deletion also were observed in murine

Table 1. Cyclophilin D molecular interactions.

Cyp D Interaction	Function of Interacting Protein
F-ATPase	Generates ATP from proton gradient across IMM [89,92–94]
Adenine nucleotide translocase	Exchanges ATP and ADP across the IMM [69,95]
Inorganic phosphate carrier	Transports phosphate ions across IMM [69]
Trifunctional protein	Catalyzes 3 of 4 steps of long-chain and medium-chain fatty acid β-oxidation [82]
Mitochondrial transcription factors 1 and 2	Regulators of mitochondrial gene expression [91]
Electron transport chain complexes III	Electron transport chain [96]
Thioredoxin 2 and peroxiredoxin	Primary molecules in thioredoxin antioxidant system [145,147]
Inositol triphosphate receptor (IP3R)	Calcium channel in IP3R/VDAC/Grp75 complex that transports calcium between mitochondriaand ER at mitochondria-associated ER membranes (MAMs) [97,111]
Tumor suppressor p53	Tumor suppressor whose mutations are common in human cancers [98,99]
Peroxisome proliferator-activated receptor-α	Transcription factor and regulator of lipid metabolism [100]
Glycogen synthase kinase-3β (GSK-3β)	Serine/threonine kinase [101,102]
Apoptosis signal-regulating kinase 1 (ASK-1)	MAPK family kinase activated by cellular stresses [103]
B-cell lymphoma 2 (BCL2)	Regulator of apoptosis [104]

diabetes induced by a deficiency in the islet β-cell transcription factor, pancreatic duodenal homeobox gene-1 [110].

Most studies that have investigated hepatic steatosis have found that Cyp D knockout reduced or completely prevented steatosis or weight gain after HFD feeding [58,82,109,111,112]. In livers of wildtype mice, Cyp D increased with HFD and even preceded triglyceride accumulation [58,59]. Furthermore, adenoviral vector-mediated overexpression of Cyp D induced hepatic steatosis in mice administered a normal diet. Wang et al. (2018) found good evidence in HFD-fed mice and palmitate-treated hepatocytes in culture that Cyp D-mediated steatosis resulted from elevated mPT (mitochondrial swelling and cytosolic calcium), ROS generation, ER stress (IRE1α phosphorylation), p38 MAPK phosphorylation, and upregulation of sterol regulatory element-binding protein-1c (SREBP-1c) [58]. Elevated Cyp D, cytosolic calcium, and ER stress were also found to mediate hepatic steatosis originating from a deficiency of mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH), which is observed in NAFLD patients [60]. mPGDH regulated Cyp D levels by modulating Cyp D ubiquitination and proteasomal degradation. Cyp D knockout *in vivo* and *in vitro* abolished the ER stress and liver steatosis arising from mGPDH deficiency.

Cyp A is another cyclophilin isoform that might contribute to NASH pathophysiology through its participation in the adipocyte-diabetes axis. Cyp A is the most abundant cyclophilin in the body and normally located in the cytosol but also is released from cells by oxidative stress. A proteomics study found that high glucose decreased monocyte cellular levels of Cyp A and increased Cyp A secretion. Similarly, serum Cyp A levels were found to be elevated in diabetic patients, especially in those with cardiovascular disease [113,114]. NAFLD patients also exhibit increased serum concentrations of Cyp A compared to non-NAFLD individuals, and this effect is compounded by diabetes [115]. As described in more detail ahead, elevated extracellular Cyp A has a proinflammatory effect and therefore may serve as one of the mechanistic links between glucose dysregulation and inflammation. In another study, genetic knockout of Cyp A decreased adipocyte size and fat mass in mice fed a normal diet, and reduced the weight of mice administered a high fat diet [116]. Cyclophilin B (Cyp B) also is pro-inflammatory when secreted, and serum levels of Cyp B are elevated in HFD-fed mice and in people with metabolic syndrome compared to healthy controls [117].

In vitro studies with CsA have reinforced findings from genetic knockout/knockdown by showing that cyclophilin inhibition can protect mitochondria from the kinds of metabolic disturbances that occur in NASH. For example, CsA blocked mPT in liver mitochondria following application of short-, medium-, and long-chain fatty acids or lysophosphatidylcholine [118–121]; blocked mPT and significantly reduced mitochondrial ROS, ATP depletion, and death of preadipocytes induced by high fatty acid concentrations [122]; blocked mPT and significantly reduced fructose-induced or high glucose-induced death of INS-1 pancreatic islet cells [123]; blocked mPT and restored the ATP deficit induced by long-chain fatty acids or palmitoyl-L-carnitine in cardiac mitochondria [124–128]; blocked mPT and prevented palmitate-induced insulin resistance in muscle mitochondria [107]; blocked hepatocyte death resulting from high glucose and hydrogen peroxide [129]; and alleviated fatty acid-induced ER stress gene induction, ROS elevation, and death of LO2 hepatocytes [60,130]. Düfer et al. (2001), in contrast, found that CsA diminished glucose-induced insulin secretion of mouse pancreatic islets *in vitro* by inhibiting glucose-stimulated oscillations of the cytoplasmic free calcium concentration [131].

The *in vivo* effects of CsA on lipid and carbohydrate metabolism have been more ambiguous than *in vitro* effects, perhaps due to confounding calcineurin inhibition. In similarity to Cyp D knockout, Wang *et al.* (2018) found that CsA treatment of mice from Weeks 30–36 of a 36-week HFD regimen reduced hepatic steatosis and triglyceride levels by 50% [58]. Another study with mice similarly showed that CsA diminished HFD-induced weight gain, gluconeogenic gene expression, blood glucose concentration, proinflammatory cytokine production, and improved glucose tolerance over a 3 week treatment period [132]. In contrast to these two studies, high doses of CsA administered to mice on a regular diet had opposite effects of Cyp D knockout from the standpoint of mitochondrial metabolism [86]. Also, CsA administration to rats on normal diet resulted in elevated serum triglycerides and characteristics of insulin-resistance [133]. Thus, CsA had beneficial effects in HFD models but not in regular diet models. In clinical practice, new onset diabetes after transplantation (NODAT) is a frequent occurrence in kidney transplant patients administered either CsA or tacrolimus, suggesting that diabetic effects can arise from calcineurin inhibition. Interestingly, less NODAT develops in CsA-treated patients compared to tacrolimus-treated patients [134]. Moreover, in a study where renal transplant patients with diabetes were switched from tacrolimus to CsA immunosuppression, approximately 40% of the patients showed complete diabetes reversal [135]. These findings are consistent with the hypothesis that Cyp D inhibition is not diabetogenic and in fact can improve glucose handling. In support of this hypothesis the non-immunosuppressive CsA analog, alisporivir (Debio-025), was administered to over 2000 individuals during its clinical development for chronic HCV infection and did not induce hyperglycemia or other diabetes characteristics [33–35,136]. Alisporivir-treated patients developed a small degree of hypertension and hypertriglyceridemia but at high doses and always in combination with pegylated interferon. In summary, abundant evidence has accumulated suggesting that inhibition of cyclophilins A, B, and especially D may alleviate metabolic disturbances by shifting the dynamic of glucose and lipid metabolism and by protecting against the mitochondrial exhaustion and demise that occurs in NASH.

3.2. Cyclophilin involvement in cellular injury

The mechanisms by which lipid and metabolic disturbances injure cells in NAFLD and NASH have been detailed in many reviews [137–142]. Mitochondrial dysfunction and destruction play central roles, but other cell-damaging processes also are prominent, such as other types of mitochondrial damage (e.g. outer membrane pores), apoptotic modes of cell death, ROS-mediated damage to other organelles and processes, hypoxia, ER stress, autophagy dysregulation, and inflammation.

As introduced above, Cyp D is the primary regulator of mPT. It's induction of mPT pores in response to elevated

mitochondrial ROS appears to occur through cysteine-203 of the Cyp D molecule. Oxidation of this amino acids activates mPT, and nitrosylation deactivates mPT [143–146]. Cyp D redox function may also be regulated by its phosphorylation, acetylation, and binding to two primary molecules involved in the thioredoxin system – thioredoxin 2 and peroxiredoxin [145,147]. Besides the positive metabolism-related effects of Cyp D inhibition observed in dietary models, many additional studies have documented protection from mPT injury in animal and clinical studies across a spectrum of liver disorders. mPT has been classically described in ischemia-reperfusion injuries, and recently in murine liver ischemia-reperfusion a small molecule cyclophilin inhibitor was shown to reduce hepatocyte ballooning and ALT rise, and nearly completely restore mitochondrial respiratory control [53]. Hepatectomies, such as liver cancer resection or living donor transplantation, sometimes cause liver insufficiency and mortality. In rodent hepatectomy experiments CsA and a first-generation, non-immunosuppressive CsA analog blocked necrosis, apoptosis, ALT rise, and augmented regeneration of the remnant liver which is consistent with a major role of mPT in hepatectomy-induced pathology [148–151]. Hepatitis viral proteins such as HBx and HCV core protein can induce mPT or sensitize mitochondria to mPT induction by ROS, and Cyp D-dependent mechanisms may assist HBV in replication [152–155]. Furthermore, recombinant expression of the complete HCV protein repertoire in cells in the absence of viral replication induces mPT, ROS, and a decline in oxygen consumption, all of which are reversed by alisporivir [156]. Cholestasis-induced mPT was demonstrated in the rat bile duct ligation model, where the cyclophilin inhibitor, NIM811, decreased ALT, necrosis, and apoptosis by 60 to 86% [157]. Acetaminophen overdose toxicity is the most common cause of acute liver failure and occurs because an acetaminophen metabolite (N-Acetyl-p-benzoquinone imine) causes glutathione depletion and mitochondrial protein adducts which in turn lead to elevated oxidant stress and GSK-3β, JNK, and Bax signaling. The ensuing mPT, ROS formation, and cell death can be substantially alleviated by CsA, NIM811, or Cyp D knockout although the degree of protection also depends on acetaminophen dose [158–160]. Chronic alcohol consumption in rodents was found to increase Cyp D, cause mitochondrial swelling, and sensitize mitochondria to mPT in the liver, although mitochondrial depolarization and hepatocyte apoptosis were not sensitive to CsA or Cyp D knockout [161–163]. In a human clinical study on chronic hepatitis C, 14 days of NIM811 administration normalized ALT levels, consistent with hepatic cytoprotection [32]. Thus, mPT-mediated liver pathology and its attenuation by cyclophilin inhibition have been widely documented.

Dyslipidemia in NAFLD and NASH impacts not only mitochondria but also the primary organelle involved in lipid synthesis (triglycerides, lipoproteins, phospholipids, cholesterol), the endoplasmic reticulum. Significant perturbations to lipid synthesis, in similarity to stresses on protein synthesis, can induce the unfolded protein response (UPR). Long chain saturated fatty acids (e.g. palmitate), sphingolipids, and lysophosphatidylcholine can all induce UPR. ROS and calcium perturbations are additional causal factors as well as consequences of ER stress. The UPR attempts to compensate for increased demands on the ER, for example through increases in chaperone proteins, but excessive UPR exacerbates steatosis and can promote inflammation and apoptosis. Intracellular signaling cascades producing these outcomes originate from three ER transmembrane sensors, inositol-requiring enzyme (IRE)-1α, protein kinase RNA-like ER kinase (PERK), and activating transcription factor (ATF)-6α [5,164,165]. ER stress was found to be a prominent mechanism in steatosis exacerbated by mGPDH knockout, and was facilitated by Cyp D, as demonstrated by complete reversal of the UPR response pathways by CsA [60]. In contrast, several studies show that CsA or specific cyclophilin isoform deletions (e.g. Cyp B) induce ER stress and/or UPR. These findings were often observed with cancer cells which may be more susceptible to ER stress due to the need for cyclophilins to support high metabolic demands. Another caveat is that the concentrations of CsA that generated UPR in many of the studies exceeded the CsA half-maximal cytotoxicity concentration ($CC_{50} \approx 12$ μM) [43,166–171]. Therefore, ER stress may indeed be a mechanism of high-dose CsA cytotoxicity, but most studies do not show that cyclophilin inhibitors at therapeutic concentrations induce ER stress.

Autophagy is an important cellular process in which aged or damaged organelles and molecules are degraded and recycled into anabolic processes in order to maintain cellular homeostasis. It can be impacted, however, by mitochondrial dysfunction, ROS, lipid disturbances, and other stresses. Most studies have found impaired autophagy in animal and human NAFLD and have found exacerbated liver disease in animals when autophagy is experimentally lowered in hepatocytes or macrophages. In contrast, lowering autophagy in hepatic stellate cells is beneficial as it decreases their activation and fibrotic activity [172,173]. Autophagic flux is high in many types of cancer, including hepatocellular carcinoma (HCC), in order to sustain their high metabolic activity. Thus, pharmacologically modulating autophagic activity may have positive or negative outcomes, depending on endogenous inhibitors or activators, and the type or functional status of the cells. Blocking Cyp D-mediated mPT can help to maintain or restore normal autophagic activity by preventing excessive ROS and other mitochondria-derived signals from impacting autophagy [174–178]. In contrast, blocking Cyp A or B can decrease autophagy, but this is therapeutic toward several types of cancer that are dependent upon high autophagic activity [166,179,180]. For example, genetic ablation of Cyp A or B or high concentrations of NIM811 were found to increase vacuolation, ER stress and UPR, decrease autophagy, and kill glioblastoma multiforme cells [179]. The heterogeneous effects of autophagy modulation is also evident in the literature about CsA-induced nephrotoxicity, where some investigations found induction of autophagy and some studies found reduction in autophagy [181,182]. Thus, the data suggest that cyclophilin inhibitors may indeed influence autophagic processes in NASH, but the clinical effects will depend on drug dose and the pathophysiological context in which they are acting.

3.3. Cyclophilin involvement in inflammation

Hepatic inflammation distinguishes NASH from NAFLD and occurs through many mechanisms. Cytosolic inflammasomes

are part of the innate immune systems and generate IL-1β and IL-18 when activated by molecular species called damage associated molecular patterns (DAMPs; e.g. mitochondrial DNA or N-formyl peptides) or pathogen-associated molecular patterns (PAMPs; e.g. lipopolysaccharide, LPS). DAMPs derive from damaged organelles or malfunctional molecules and can become more abundant in disease states, for example when autophagy is impeded. PAMPs derive from bacteria, fungi, or other non-human organisms and can become prevalent in metabolic system and fatty liver disease as a result of perturbations of the microbiome. Blocking Cyp D and mPT-mediated cellular damage can suppress oxidized phosphatidylcholine (DAMP)-mediated and LPS (PAMP)-mediated inflammasome activity in macrophages [183–185].

Another, and likely more significant, role for cyclophilin inhibition is in attenuating the infiltration and proinflammatory activation of leukocytes (Figure 2). Cyp A, which is predominantly a cytosolic protein, is released from cells under oxidative stress or other injury conditions concomitant with its acetylation [186]. Cyclophilin B, predominantly an ER protein, is also secreted in certain injury states by unknown mechanisms. Serum levels of Cyp A or B are elevated in many disease states including NAFLD, NASH, metabolic syndrome, and type 2 diabetes [113–115,117,187–192]. In the extracellular milieu cyclophilins act both as leukocyte chemoattractants and as pro-inflammatory cytokines by binding to the transmembrane receptor, CD147 (EMMPRIN), and perhaps other receptors on granulocytes, monocytes, and CD4 T cells [193–197]. The cell surface expression of CD147 itself is dependent upon another intracellular cyclophilin isoform, cyclophilin 60 [198]. Cyclophilin binding to CD147 is partially dependent on a functional isomerase active site and is inhibited by CsA [193,199]. CD147 is also present on hepatic stellate cells and contributes to cell activation and fibrogenesis [200–202]. Signaling downstream of CD147 occurs through ERK1/2, NFκB, and SMAD2 [188,193,203] to generate proinflammatory cytokines (e.g. IL-6, IL-8), matrix metalloproteinases (e.g. MMP2, MMP9), and ROS. Cyclophilin inhibitors block binding to CD147 and these and genetic Cyp A deletion have exerted potent anti-inflammatory activities in animal models of chronic asthma, arthritis, atherosclerosis, acute pulmonary embolism, stroke, thrombosis, aortic aneurysm, myocarditis, coronary artery disease, pulmonary arterial hypertension, amyotrophic lateral sclerosis, and other conditions [192,204–213]. One of the activities of activated leukocytes is the oxidative burst in which NADPH oxidase generates ROS, and Cyp D inhibition has been shown to decrease oxidative burst and its consequences both *in vitro* and *in vivo* [214]. In reference to liver disease, Iordanskaia et al. (2015) used the CsA analog, MM284, to exclusively target extracellular cyclophilins in a mouse model of biliary atresia and found it to robustly attenuate liver inflammation, hyperbilirubinemia, and expression of IL-6, TIMP-4, and MMP-7 [203]. MM284 completely blocked SMAD2 phosphorylation both *in vivo* and in cultured human hepatic stellate cells in which SMAD2 phosphorylation was stimulated by extracellular Cyp A. The latter findings are significant because the SMAD pathway is involved in TGFβ-mediated, profibrotic activities.

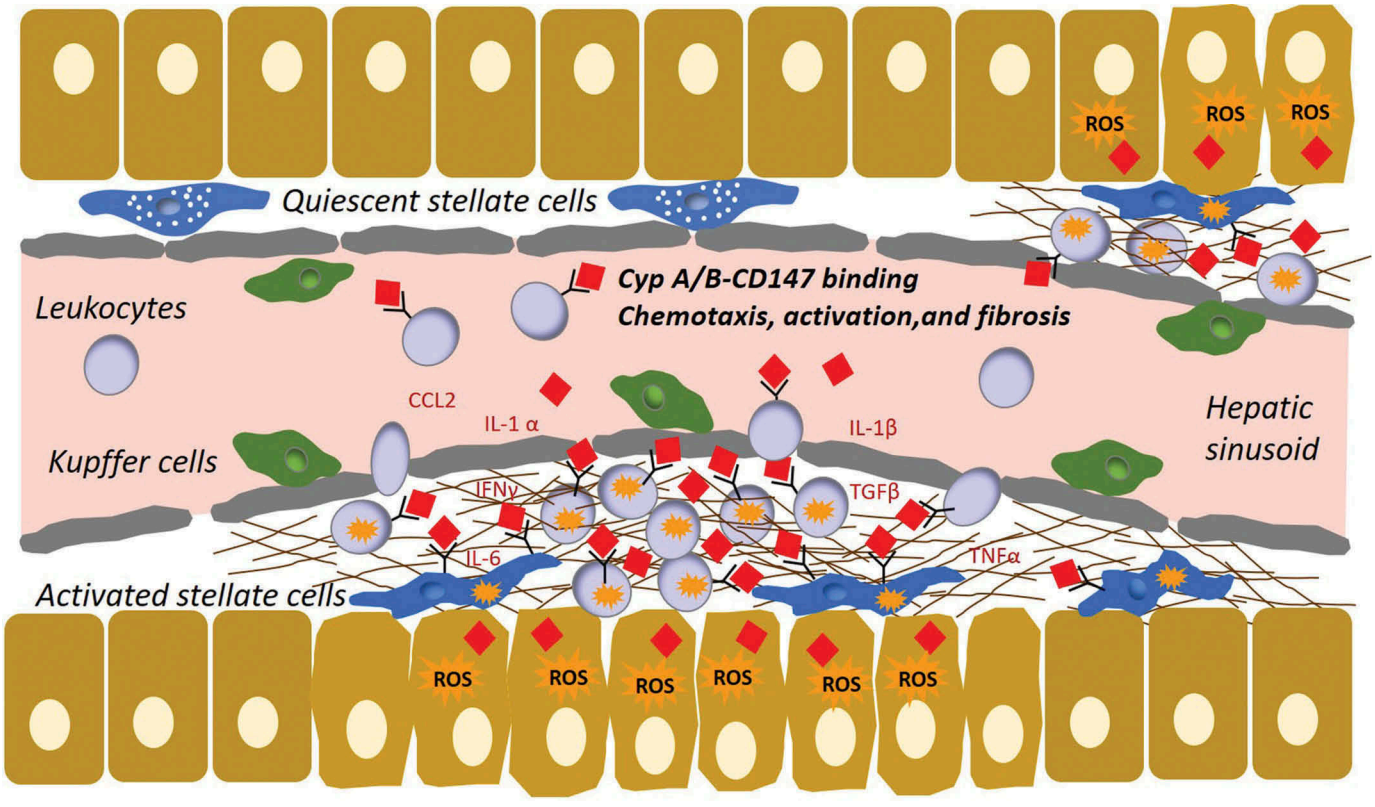

Figure 2. Secreted cyclophilins A and B act as a pro-inflammatory cytokines. Cyco = lophilins A and B are secreted by hepatocytes in response to oxidative and other cellular stresses, Cyp A/B binding to the proinflammatory receptor, CD147, on monocytes, lymphocytes, and granulocytes promotes parenchymal infiltration and activation of inflammatory cells. Stimulation of CD147 on hepatic stellate cells promotes fibrosis.

3.4. Cyclophilin involvement in fibrosis

Liver fibrosis invariably results from persistent hepatocyte injury and inflammation, and regressing fibrosis is one of the highest priorities in treating NASH. Many compounds in development are addressing this goal by targeting upstream (i.e. metabolic) disturbances that eventually lead to fibrotic scarring. Inhibition of Cyp D-mediated mPT and Cyp A/B-mediated inflammatory events fall into this category. Another strategy is to target fibrosis more proximally by modulating the activities of hepatic stellate cells, the primary cell type responsible for extracellular matrix production [215]. Collagens produced by activated stellate cells are the primary components of the fibrotic matrix, and Cyp B is the most catalytically active of several ER prolyl isomerases known to regulate collagen production (Figure 3) [216,217]. The importance of prolyl isomerases derives from the high proline content of collagen. The helical portions of collagen alpha-chains consist of repeating units of glycine-X-Y, where X is usually proline and Y is usually proline that is additionally hydroxylated by prolyl hydroxylases (i.e.hydroxyproline). Hydroxylation of collagen proline and lysine residues is critical to collagen formation because the hydroxylated amino acids provide thermal stability to the collagen helices and serve as the substrates for cross-linking procollagen molecules after they are secreted from cells. Cyp B associates with prolyl-3-hydroxylase isoform 1 (P3H1) and multiple lysyl oxidase isoforms (LH1, LH2, LH3) and thereby assists in many hydroxylation events [218–220]. A second major role for Cyp B is to catalyze *cis*-to-*trans* isomerization of the nascent proline residues (8–16% *cis*) which is required, and indeed is the rate-limiting step for proper folding of collagen alpha-chains into a triple helix [216,218,221]. The consequences of ablating Cyp B have been thoroughly studied in mice and are similar to the effects of rare autosomal recessive mutations in horses and humans which result in hereditary equine regional asthenia (HERDA) and human osteogenesis imperfecta, respectively. In all these conditions there are reductions in the folding rate and total production of collagen, and disruptions in the structural organization of collagen fibrils in skin, tendon, and bone. The skin, for example, shows sparser and disorganized collagen fibrils, hyperextensibility, and decreased stiffness and strength, and bone shows decreased volume, mineral density, and strength. Biochemically, collagen prolyl and lysyl hydroxylations, glycosylations, and crosslinking all are decreased or changed by Cyp B deletion [219,220,222–225]. These observations represent the extremes of Cyp B interference, i.e. complete ablation and interference with collagenous tissue development.

Pharmacological inhibition of cyclophilins has not shown such significant collagenous tissue disturbances as those generated by Cyp B genetic deletion or mutation. For example, osteoporosis is not a major side effect of CsA use in organ transplantation [226–228]. However, cyclophilin inhibitors do reduce collagen production *in vitro* and *in vivo*. CsA, NIM811,

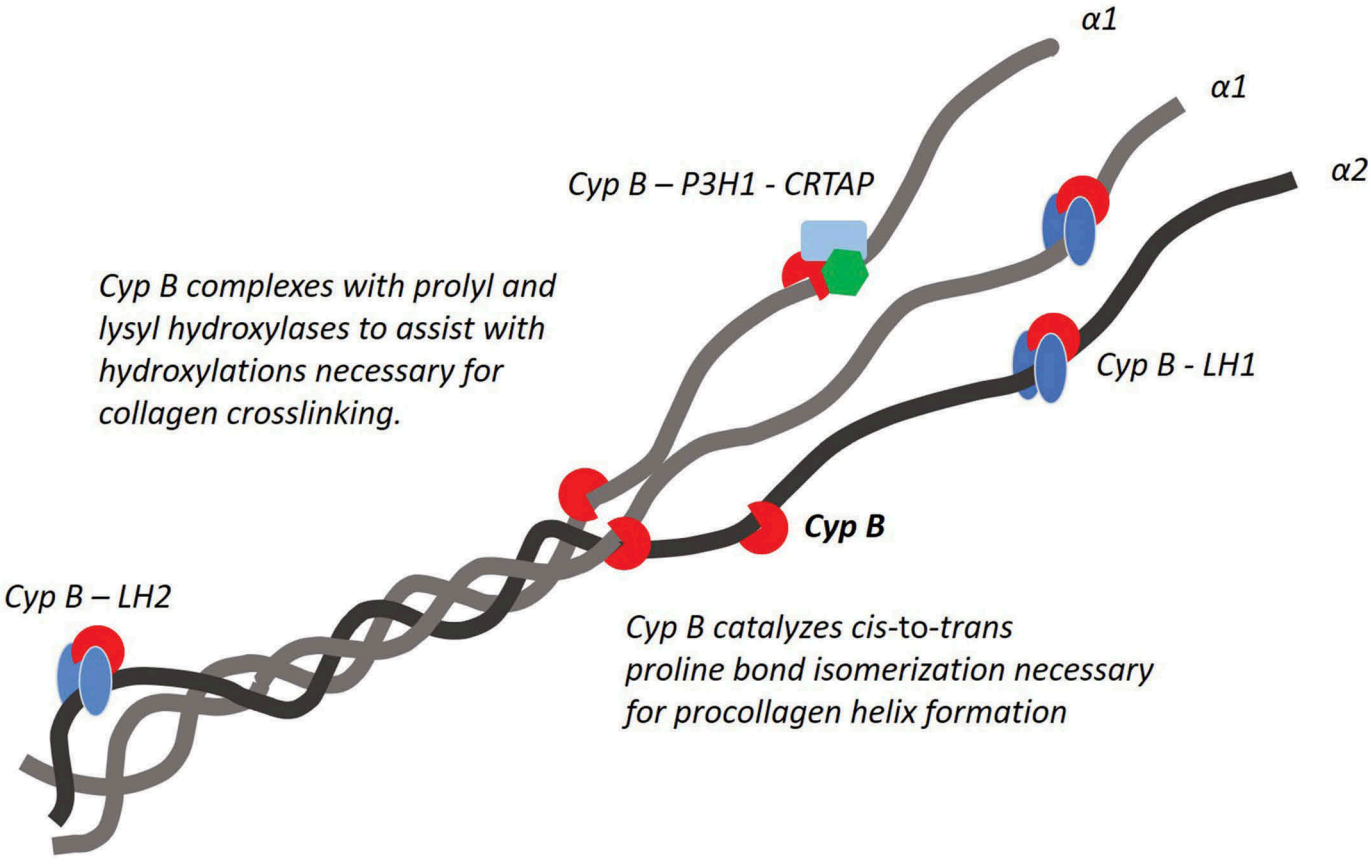

Figure 3. **Cyclophilin B controls collagen synthesis**, Cyclophilin B, alone or in association with prolyl-3-hydroxylase 1 (P3H1) and cartilage associated protein (CRTAP), or with lysyl hydroxylases 1 or 2 (LH1, LH1) binds to proline residues in collagen alpha-chains during procollagen synthesis in the endoplasmic reticulum. Genetic deletion or inhibition of Cyp B results in decreases in prolyl and lysyl hydroxylations, procollagen cynthesis rates, mature collagen fibril density, and tensile strength of collagenous matrices.

and SCY-635 have been shown to decrease collagen secretion from rat hepatic stellate cells, human fibroblasts, and a human stellate cell line by at least 50% and increase MMP1 collagenase production. This effect is accompanied by diminished SMAD2 and 3 phosphorylation (TGFβ signaling pathway) and lower collagen RNA levels [229–232]. In rodent studies CsA and NIM811 decreased carbon tetrachloride (CCl_4)-induced liver fibrosis, and NIM811 additionally decreased serum ALT, AST, liver inflammation, TIMP-1 and TGFβ mRNA [233,234]. CRV431 decreased liver fibrosis both in the murine CCl_4 model and in the streptozotocin-plus-HFD (STAM) mouse model of NASH. CRV431 additionally reduced the development of liver tumors by 50% during a late phase of the STAM model, which might have resulted from its antifibrotic activity or perhaps other anti-cancer mechanisms [43]. In the mouse bile duct ligation model NIM811 did not decrease fibrosis at 2 weeks post-surgery but did reduce serum ALT levels and tissue necrosis and apoptosis markers by 60–86% [157]. NIM811 also decreased cardiac fibrosis by to up 70% in murine coxsackievirus B3-induced myocarditis [205]. Finally, in human studies, CsA was evaluated in the 1990's as a treatment for primary biliary cholangitis. Despite not reducing PBC-related mortality, CsA decreased liver damage biomarkers, slowed cirrhosis progression or regressed cirrhosis in some studies [233,235,236]. In liver transplantation, the REFINE trial compared a 1-year course of CsA to tacrolimus (non-cyclophilin-binding) and found in steroid-free patients no differences between CsA and tacrolimus in immunosuppression-related outcomes but less liver fibrosis in CsA-treated patients. These findings of less collagen production, fibrosis, and cirrhosis following treatment with cyclophilin inhibitors are consistent with the well-established, direct role of Cyp B in collagen production.

4. Conclusion

We have described the primary ways in which the three best-known cyclophilin isoforms, cyclophilins A, B and D, participate in pathophysiological activities prevalent in NASH – dysregulated mitochondrial metabolism, mPT-mediated cell death, inflammatory cell recruitment and activation, and promotion of fibrotic collagen production and maturation. These activities are not unique to NASH or liver diseases; they have been studied in even greater detail in other organ systems and disorders. For example, most of the understanding of mPT has come from studies of ischemia-reperfusion injury in myocardial infarction. Cardiovascular disorders also have been most prominent in deciphering cyclophilin inflammatory activities. Neurological injury is an area of great interest as well. Moreover, fourteen other cyclophilin isoforms exist in the human proteome, suggesting many other regulatory roles. The broad functionality of prolyl isomerization is what links all these disparate activities.

Genetic knockout has been instrumental in defining the roles of individual cyclophilin isoforms, and pharmacological inhibition has confirmed those findings in most cases. CsA and a small number of non-immunosuppressive CsA analogs have been the primary pharmacological antagonists, and their therapeutic actions in many disease models have provided validation that pan-inhibition of cyclophilins, at least in animal studies, is safe and effective. Thirty-five years of clinical use of CsA also can attest to the feasibility of targeting cyclophilins for extended periods of time. The calcineurin-mediated toxicities of CsA have necessitated that dosing levels be kept as low as possible, but in spite of that, therapeutic effects of CsA have been observed that could be attributed to cyclophilin inhibition such as less liver fibrosis in transplant patients and decreases in cirrhosis in primary biliary cholangitis. The biggest question that remains is the extent to which dedicated cyclophilin inhibitors will show therapeutic effects in human disease in comparison to the positive effects of gene deletion in animal models. Dosing and target engagement are major variables, as is the extent to which cyclophilin-mediated pathologies occur in NASH. The large number of these processes present in NASH increases the likelihood of beneficial outcomes.

5. Expert opinion

One of the mysteries surrounding cyclophilins is why genetic knockout of Cyp A, B, or D does not result in lethality or, in the case of Cyp A or D, appreciable phenotype change. On the contrary, Cyp A and D knockouts positively modify disease course in a wide variety of experimental models. Another mystery is why two revolutionizing, immunosuppressive drugs – cyclosporine A derived from a fungus and FK506 (tacrolimus) derived from a bacterium – both inhibit calcineurin but do so by first dimerizing with distinct prolyl isomerases – Cyp A for CsA, and FKBP12 for FK506? These peculiarities seem to inform us that catalysis of prolyl isomerization is not a pivotal 'checkpoint' process whose deletion has catastrophic consequences. A corollary is that their pharmacological inhibition also should carry a lower risk of adverse events in comparison to molecular targets that exhibit knockout lethality. However, these observations also seem to inform us that the multilevel regulation provided by cyclophilins – i.e. the amplitude, timing, and localization of structural and functional events – is an extremely effective means of fine-tuning biochemical processes and adapting them to dynamic environments. This versatility probably explains why cyclophilins and other prolyl isomerases have been evolutionarily recruited into countless biological processes and consequently pathophysiological processes when exposed to supraphysiologic stimuli.

In our opinion the ability to directly target multiple pathophysiological processes in NASH and other complex diseases with a single compound is a major advantage of cyclophilin inhibitors. Dyslipidemia, insulin resistance/diabetes, obesity, hypertension, and dysbiosis all are drivers of NASH, but these phenomena and their pathogenic sequalae – mitochondrial dysfunction and loss, hypoxia, inflammation, fibrosis, malignancy – probably vary in their contributions to NAFLD and NASH among the population. Normalizing as many of the process as possible maximizes the opportunity to ameliorate disease course. Multi-targeting also is advantageous because in theory it reduces the need to administer many narrowly targeted drugs in combination and therefore lowers the cost of treatments and the potential for drug-drug interactions,

which are important considerations in the present development environment.

Multi-targeting carries its own risk of adverse side effects, but at least for CsA analogs this risk has been minimized by applying the lessons learned from 35 years of study and clinical use of this chemical class. The most recently developed CsA analogs possess physicochemical properties that optimize both the safety and efficacy of the compounds and thus provide more flexibility for dosing to clinically appropriate levels [37,43]. CRV431 currently is the only cyclophilin inhibitor in clinical development for NASH, but the many benefits of this drug class could be expected to propel additional compounds into the space. Nonimmunosuppressive CsA analogs present the most favorable risk-benefit ratio from a development standpoint, but patent opportunities in this class are becoming increasingly limited due to decades of research on cyclosporins. Sangamide compounds such as NV556 show some promise as potent inhibitors but they are entirely new entities that have been sparingly researched and not yet thoroughly evaluated in toxicology studies to our knowledge. Small molecule inhibitors offer many potential advantages, such as ease and cost of manufacturing and modification of pharmaceutical properties, but their lower potencies of cyclophilin inhibition may be a detriment to target engagement.

The one pathologic event that we predict will be most impacted by cyclophilin inhibition clinically is liver fibrosis. Collagen production should be universally diminished by cyclophilin inhibitors due to the unequivocal and ubiquitous roles of Cyp B in collagen production, independent of the specific stimuli that activate hepatic stellate cells. The preferred distribution of CsA analogs to the liver makes NASH a good indication for assessment of the antifibrotic potential of these compounds but benefits also are predicted to extend to other fibrotic disorders if sufficient target engagement is achieved in other organs. Fibrosis reduction is a top priority in NASH but few compounds in development directly target the fibrotic process. Therefore, cyclophilin antagonists could be beneficial in many multi-drug combinations. Many investigators have proclaimed a need for cyclophilin inhibitors for a range of clinical disorders, and this need may finally be met by CRV431 or other compounds in the near future.

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Declaration of interest

The authors are employed by Hepion Pharmaceuticals Inc who are working on cyclophin inhibitors for the treatment of liver diseases. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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