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Caspase Inhibitors for the Treatment of Hepatitis C

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Apoptosis and Liver Injury

Apoptosis, a highly regulated form of cell death, represents the physiologic counterpart to mitosis, and together they provide the necessary regulation of cell number to maintain homeostatic cell turnover in adult organisms. Virtually any alteration in this balance, either toward excessive proliferation or excessive cell death, leads to the development of a pathologic condition. Apoptosis is also the most effective process employed by the immune system to eliminate mutated or infected cells. The apoptotic machinery can be activated externally by engagement of a subset of cell surface receptors belonging to the tumor necrosis factor (TNF) receptor super-family, called death receptors, or internally by activation and release of mitochondrial proteins (Fig. 1). The common end point of both these pathways is the activation of a cascade of intracellular proteolytic enzymes called caspases (cysteiny aspartate-specific proteases).

Caspases are cysteine proteases, which play a critical role in initiating and executing the apoptotic program by degrading several cellular components, resulting in the apoptotic phenotype. Apoptotic cells are then fragmented into small apoptotic bodies and engulfed by phagocytes. Engulfment of apoptotic bodies by stellate cells results in their activation, transformation to myofibroblasts, and increases their expression of collagen I.¹ In an analogous manner, phagocytosis of apoptotic bodies by Kupffer cells, the resident macrophages in the liver, increases their expression of death ligands for the death receptors.² Kupffer cell expression of death ligands may in turn promote further apoptosis of death receptor-expressing hepatocytes resulting in a feedforward mechanism whereby apoptosis begets more apoptosis. Thus, apoptosis promotes liver damage and fibrogenesis.³ Inhibition of caspases often abrogates apoptosis. Caspases are synthesized as proenzymes and subsequently activated either by autocatalytic processing or by proteolytic cleavage by other caspases at an aspartate in the p1 position of the substrate. Several caspases have been identified, and, among them, caspases 2, 3, 6, 7, 8, 9, 10 have been demonstrated to play a role in apoptosis.⁴

Caspase Inhibition will not Promote Hepatocarcinogenesis

Clinicians may be concerned that inhibition of apoptosis promotes cancer. Therefore, this issue needs to be discussed. As mentioned above, there are 2 canonical pathways of apoptosis, death receptor (the so-called extrinsic pathway) versus non-death receptor

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(termed intrinsic pathway). The death receptor pathway is dependent on caspase 8 (see Fig. 1).⁵ In the liver, the genetic absence of caspase 8 attenuates hepatitis in experimental models mediated by Fas or TNF- α .⁶ The intrinsic or non-death receptor-mediated apoptosis is regulated by the B cell lymphoma 2 (Bcl-2) family of proteins and induces mitochondrial dysfunction resulting in activation of caspase 9, which in turn activates the effector caspase 3, 6, and 7. However, inhibition of caspase activation down stream of mitochondria only delays and does not prevent cell death.^{7,8} Thus, pan-caspase inhibitors will likely only prevent cell death by death receptors. Animals with genetic deletions of death receptors do not develop spontaneous cancers.^{9–11} Moreover, by blocking apoptosis, caspase inhibitors may actually reduce carcinogenesis in the liver, which requires cell turnover. Inhibition of apoptosis reduces cell turnover and in fact may be an antiapoptotic strategy. For example, apoptosis inhibition actually reduces hepatocarcinogenesis in a transgenic murine model of hepatitis B virus (HBV).¹² Therefore, caspase inhibitors in humans should only block death receptor-mediated apoptosis and should not promote cancer development.

Hepatitis C Virus and Apoptosis

Increased apoptosis is a hallmark of active hepatitis C infection, and the level of hepatocyte apoptosis correlates with the histologic activity grade.¹³ It has been suggested that hepatocytes apoptosis may be a mechanism for viral shedding and thus inhibition of apoptosis could ameliorate hepatitis C.¹⁴ Hepatitis C virus (HCV) infection has been shown to influence the death receptor-mediated pathway and the mitochondrial apoptotic pathway.¹⁵ Consistently, increased caspase-cleaved products of cytokeratin-18 (CK-18), the major intermediate filament in hepatocytes, are present in serum and liver biopsies of HCV-infected patients compared with healthy controls, demonstrating that caspases are activated during HCV infection.¹⁶ Moreover, caspase activation correlates with the degree of inflammatory liver injury, that is, necroinflammatory activity, in chronic hepatitis C infection.^{16,17} The mechanism through which this increase in apoptosis is mediated is still being defined, but several lines of evidence suggest that it is primarily immune-mediated. Although HCV itself has mild cytopathic effects on the infected host cells, the extensive tissue damage associated with viral hepatitis is generally the result of host immune response to the viral antigen. During viral hepatitis, specific classes of cytotoxic T lymphocytes (CTL) recognize and kill viral antigen-presenting, HCV-infected hepatocytes to clear the virus from the liver. This process causes the initial liver damage, which is exacerbated by the influx of antigen-nonspecific inflammatory cells. The antigen-primed CTL and CD8⁺ T lymphocytes directly kill HCV-infected hepatocytes by cell-cell contact, and release of cytotoxic or antiviral cytokines such as interferon- γ (IFN- γ) and TNF- α . The killing occurs by apoptosis, as demonstrated by the presence of apoptotic bodies, once referred to as Councilman bodies, in close proximity to infiltrating CD8⁺ T lymphocytes in the liver of patients with viral hepatitis.¹³ Death receptor-mediated apoptosis, in particular, is critical in HCV associated liver injury. Immune effector cells express the death ligands, Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL), and secrete TNF- α . Consistently, upregulation of the main death receptors and their ligands has been described in HCV infection.^{13,18}

If a death receptor is engaged by its cognate ligand (Fas ligand, TRAIL, or TNF- α), a series of conformational changes occurs that leads to the recruitment of intracellular cytoplasmic adaptor molecules, which provides a platform for recruitment and activation of the initiator caspases 8 or 10. The activated initiator caspases, in turn, start a proteolytic cascade resulting in activation of other caspases referred to as effector caspases (caspase 3, 6 and 7), either by direct cleavage of the effector caspase or indirectly by cleavage and activation of other substrates (ie, the Bcl-2 protein Bid), which promotes mitochondrial permeabilization and release of caspase-activating factors (ie, cytochrome *c*) (see Fig. 1). Among the death

receptors, the Fas/FasL (CD95/CD95L) system plays the most important pathogenic role in this process.¹⁹ Upregulation of Fas in hepatocytes and FasL induction in T lymphocytes have been observed in the liver of patients with chronic hepatitis C, and directly correlate with disease activity such as periportal and intralobular inflammation.^{20–24} In addition to Fas, other death receptors have been implicated in the pathogenesis of HCV-mediated liver injury. TNF- α expression is increased in the liver of HCV-infected patients, and HCV-specific CTLs have been shown to secrete TNF- α in vitro.^{25,26} Moreover, TNF- α and the soluble form of its receptor, TNF-receptor 1 (TNF-R1), are often elevated in the serum of patients with HCV infection, especially during fulminant hepatitis, suggesting this system may play a more important role in acute inflammation than in chronic hepatitis.^{27,28} TRAIL is also increased in hepatocytes from patients with chronic hepatitis C,²⁹ and expression of TRAIL in CD8+ T cells and CD68+ macrophages has been reported to be increased in the immediate vicinity of apoptotic hepatocytes in HCV-infected liver.³⁰ TRAIL expression is increased in chronic hepatitis C independent of the extent of lymphocyte infiltration.^{31,32} Unlike FasL, TRAIL induces apoptosis only in infected cells, demonstrating that other cytokines or perhaps the virus itself sensitizes hepatocytes to TRAIL-mediated apoptosis.³³ It is not clear whether Fas or TRAIL expression is mainly regulated by virus-specific proteins or by inflammatory cytokines, such as interleukin-1 (IL-1), generated after the first immune response. In general, the role of the individual HCV proteins in apoptosis remains controversial, as multiple proteins encoded by the HCV genome have been demonstrated to have pro- or antiapoptotic effects.³⁴ Because of the long-time unavailability of tissue culture systems or animal models suitable for HCV replication, most of these data have been generated in studies employing either in vitro overexpression systems or transgenic animal models, which do not adequately reproduce the clinical situation in HCV-infected patients; the viral proteins are usually present at low levels, and therefore the results must be interpreted cautiously. However, the recent establishment of an efficient cell culture system that hosts the complete viral life cycle in human hepatocyte-derived target cells has allowed the study of HCV-host interaction and apoptosis in a more appropriate model.^{35,36} Using this model, recent studies have demonstrated that HCV infection renders hepatocytes sensitive to TRAIL-induced apoptosis, suggesting an essential contribution of the mitochondrial pathway of apoptosis in HCV-mediated sensitization to TRAIL.^{18,30,37} Caspase-dependent apoptosis of HCV-specific CD8+ T cells has also been demonstrated in acute and chronic HCV infection and may play a role in preventing an effective T cell response and viral persistence.^{38–40}

Chronic HCV infection is characterized by variable degrees of hepatic inflammation, damage and fibrosis with progressive risk of developing liver cirrhosis and hepatocellular carcinoma. Hepatocytes apoptosis during HCV infection might directly promote liver fibrosis by activation of stellate cells and Kupffer cells.³ Engulfment of apoptotic bodies by stellate cells in vitro increases expression of collagen and transforming growth factor β (TGF- β) in a cell culture model.¹ Similarly, Kupffer cell engulfment of apoptotic bodies promotes inflammation and fibrogenesis.² Therefore, inhibition of caspase activation and apoptosis during HCV may be beneficial not only to reduce liver damage but also to prevent the onset of liver fibrosis.

Caspase Inhibitors in Preclinical Studies of Liver Disease

Treatment with the pan-caspase inhibitor ZVAD-fmk resulted in decreased mortality in the rat massive hepatectomy model of acute liver failure.⁴¹ Infusion of the pan-caspase inhibitor IDN-6556 ((3-{2-[(2-tert-butyl-phenylaminoxalyl)-amino]-propionylamino}-4-oxo-5-(2,3,5,6-tetrafluoro-phenoxy)-pentanoic acid)) has also been shown to reduce ischemia-reperfusion injury in rodent models.^{42–44} IDN-6556 has been shown to be effective in reducing transaminase elevation and apoptosis in rodent models of acute liver injury.⁴⁵

More importantly, a caspase inhibitor also reduced hepatic fibrosis in the bile duct ligated mouse.⁴⁶ This latter study provided solid preclinical data indicating that caspase inhibitors can mitigate hepatic fibrogenesis in addition to simply reducing the magnitude of apoptosis. Deletion of the death receptor Fas or the death ligand TRAIL also attenuate hepatic fibrogenesis in the bile duct ligated animal. Thus, the data to date indicate that inhibition of death receptor-mediated apoptosis attenuates liver injury in preclinical models.

Caspase Inhibitors in Human Liver Injury

Data employing caspase inhibitors in humans are scant. Caspase inhibitors for human use were developed by IDUN Pharmaceuticals, Gilead Sciences, Inc, and Vertex Pharmaceuticals. However, only 1 proof of concept trial has been performed in patients with HCV.⁴⁷ This study employed IDN-6556, which was developed by IDUN Pharmaceuticals, later purchased by Pfizer. IDN-6556 is an irreversible pan-caspase inhibitor. IDN-6556 also inhibits caspase 1, which has been implicated in inflammation; thus, the drug may also inhibit inflammation in addition to blocking apoptosis. The study design was a multicenter, double-blind, placebo-controlled, dose-ranging study with only a 14-day dosing period. At the time of the study design and implementation, long-term animal toxicity data were not available, so the FDA appropriately limited the duration of the study. A total of 105 patients were enrolled in the study; 79 received active drug, 80 patients had chronic hepatitis C, and 25 had other liver diseases including nonalcoholic steatohepatitis (NASH), hepatitis B, primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC). The doses of IDN-6556 in the study ranged from 5 to 400 mg orally on a daily basis. In the HCV patient population, all doses of IDN-6556 significantly lowered alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Fig. 2). Declines in aminotransferase activity were also observed in 4 patients with NASH (Fig. 3). No adverse reactions to the drug were noted in this 2-week study. Mean HCV RNA levels were not altered by drug administration compared with baseline values. Thus, oral IDN-6556 does lower serum aminotransferase activity in HCV patients. Longer-term studies will be required to assess the potential effects of IDN-6556 on liver inflammation and fibrosis.

A human trial with IDN-6556 was also conducted in organ preservation injury.⁴⁸ What was reported from the study was a post hoc analysis of a Phase II, multicenter, randomized, placebo-controlled, double-blinded, parallel group study. Subjects were assigned to 4 treatment groups: Group 1 (organ storage/flush, placebo-recipient, placebo); Group 2 (organ storage/flush, 15 µg/mL-recipient, placebo); Group 3 (organ storage/flush, 5 µg/mL-recipient, 0.5 mg/kg); and Group 4 (organ storage/flush, 15 µg/mL-recipient, 0.5 mg/kg). Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay and caspase 3/7 immunohistochemistry were performed to measure liver apoptosis. Liver injury was assessed by serum AST/ALT determinations. Serum markers of the CK18Asp396 (M30) neo-epitope (a cleavage product of caspase 3 and therefore a biomarker for hepatocyte apoptosis) were reduced in all groups receiving drug compared with placebo. However, serum AST/ALT levels were only consistently reduced in Group 2 (drug exposed to organ only). As to why the caspase inhibitor was not additive when also given to the recipient, is likely to be explained by the increase in neutrophils accumulating in the liver of those recipients receiving active drug. Perhaps inhibition of neutrophil apoptosis permitted their accumulation in the liver following ischemia/reperfusion injury. The neutrophils would then further injure the liver. These observations illustrate the complexity of inhibiting apoptosis in multiple cell types in this form of liver damage. Nonetheless, IDN-6556, when administered in cold storage and flush solutions during liver transplantation, seems to offer local therapeutic protection against cold ischemia/warm reperfusion liver injury.

Summary

Several preclinical and early clinical studies suggest a potential benefit for caspase inhibitors in liver injury. However, significant methodologic issues including small sample size, short follow-up, and use of surrogate markers, is present in all the current studies. In particular, none of these agents has been shown to consistently decrease the critical outcomes of mortality or time to transplant. Although the results are intriguing, additional carefully designed studies with adequate methodology, patient sample size, and follow-up need to be performed before any of these medications may be recommended for the treatment of hepatitis C. Nonetheless, caspase inhibitors are extremely promising hepatoprotective agents and their further study is strongly encouraged.

Acknowledgments

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Fig. 1.

The extrinsic pathway is initiated by the engagement of a death receptor by its cognate ligand, which results in initiator caspases 8 and 10. Activated initiator caspases cleave the BH3-only member of the Bcl-2 family Bid, which, in turn, causes mitochondrial dysfunction. The intrinsic pathway can be triggered by different intracellular stresses converging on the mitochondria. Both pathways lead to mitochondrial permeabilization with release of pro-apoptotic mitochondrial proteins, resulting in caspase 9 activation, and, subsequently, through a proteolytic cascade, activation of the effector caspases 3, 6 and 7, responsible for the degradation of numerous cellular components. Inhibition of caspases is an effective tool to block the extrinsic or death receptor pathway of cell death. Blocking caspase 9/3/6/7 will not prevent cell death as mitochondrial dysfunction, a lethal event, has already occurred.

Fig. 2. Patients received IDN-6556 orally once a day at the dose indicated. Changes in serum ALT values are expressed as percent change from baseline. QD dosing indicates once a day drug administration. Data are expressed as the means $\pm$ standard error of the mean (SEM).
(Reprinted from Pockros PJ, Schiff ER, Shiffman ML, et al. Oral IDN-6556, an antiapoptotic caspase inhibitor, may lower aminotransferase activity in patients with chronic hepatitis C. *Hepatology* 2007;46(2):326; with permission.)

Fig. 3.

Four patients with NASH were treated with IDN-6556 at a dose of 100 mg orally twice a day. Changes in the serum ALT values are expressed as a percent change from baseline. Results are the mean $\pm$ SEM in 4 patients. (*Reprinted from Pockros PJ, Schiff ER, Shiffman ML, et al. Oral IDN-6556, an antiapoptotic caspase inhibitor, may lower aminotransferase activity in patients with chronic hepatitis C. Hepatology 2007;46(2):327; with permission.*)