Acute liver failure is a serious clinical problem of which the underlying pathogenesis remains unclear, and for which effective therapies are lacking. The Fas receptor/ligand system, which is negatively regulated by AKT, is known to play a prominent role in hepatocytic cell death. We hypothesized that AKT activation may represent a strategy to alleviate Fas-induced fulminant liver failure. We report here that a novel AKT activator, SC79, protects hepatocytes from apoptosis induced by agonistic anti-Fas antibody CH11 (for humans) or Jo2 (for mice) and significantly prolongs the survival of mice given a lethal dose of Jo2. Under Fas-signaling stimulation, SC79 inhibited Fas aggregation, prevented the recruitment of the adaptor molecule Fas-associated death domain (FADD) and procaspase-8 [or FADD-like IL-1β-converting enzyme (FLICE)] into the death-inducing signaling complex (DISC), but SC79 enhanced the recruitment of the long and short isoforms of cellular FLICE-inhibitory protein at the DISC. All of the SC79-induced hepatoprotective and DISC-interruptive effects were confirmed to have been reversed by the Akt inhibitor LY294002. These results strongly indicate that SC79 protects hepatocytes from Fas-induced fatal hepatic apoptosis. The potent alleviation of Fas-mediated hepatotoxicity by the relatively safe drug SC79 highlights the potential of our findings for immediate hepatoprotective translation. (Am J Pathol 2018, 188: 1171–1182; https://doi.org/10.1016/j.ajpath.2018.01.013)
ligation. The expression of Fas is strongly up-regulated in the livers of patients with fulminant hepatic failure and acute hepatitis. In a mouse model, the administration of a CD95/Fas agonistic monoclonal antibody (Jo2) also causes massive hepatocyte apoptosis and induces severe fulminant hepatitis. Accordingly, pharmacologic inhibition of CD95/Fas-induced hepatic apoptosis and inflammation was developed and has been proven to be clinically relevant in the prevention of fulminant hepatitis, ALF, and endotoxic shock from Fas-associated liver diseases. Mechanistically, the binding of FasL to CD95/Fas transmits cell-death signals via pro-apoptotic adapter protein Fas-associated death domain (FADD), which mediates the activation of procaspase-8, also called FADD-like IL-1β-converting enzyme, to form a death-inducing signaling complex (DISC) and subsequent activation of downstream executioner caspases 3 and 7, leading to hepatocyte apoptosis. FADD-like IL-1β-converting enzyme inhibitory protein (FLIP) is an antiapoptotic cytoplasmic protein with sequence homology to caspase-8. FLIP functions as a dominant-negative inhibitor of caspase-8 to prevent Fas-induced apoptosis because its sequence contains a substitution of a tyrosine for an active-site cysteine, making it unable to undergo cleavage. An antiapoptotic role of FLIP is observed in a variety of cell types, and enhanced FLIP expression could render cells resistant to Fas-induced apoptosis.

AKT is one of the major downstream targets of the phosphoinositide-3 kinase—signaling pathway with anti-apoptotic activity. AKT is a crucial mediator of cell survival, and its deactivation is implicated in various types of stress-induced pathologic cell death, including the pathogenesis of hepatocyte injury. In T lymphocytes, the inhibition of AKT using either phosphoinositide-3 kinase inhibitor or a selective AKT inhibitor up-regulated expression of the FasL gene (FASLG). Moreover, the inhibition of AKT resulted in a corresponding decrease in FLIP expression and an increase in caspase-8 activity, leading to Fas-mediated apoptosis. Recently, a high-throughput chemical genetic screening identified the small molecule SC79 as a unique and specific AKT activator capable of increasing cytosolic AKT activity in various physiologic and pathologic conditions. Therefore, we hypothesized that SC79 might specifically antagonize Fas-mediated liver injury in vitro and in vivo. Pretreatment with SC79 could indeed protect hepatocytes from apoptosis induced by agonistic anti-Fas antibody CH11 (for humans) or Jo2 (for mice) and significantly extend the survival time of the mice administered a lethal dose of Jo2. Mechanistic studies have showed that the hepatoprotective effects of SC79 may act through suppressing Fas aggregation, blocking the recruitment of FADD and procaspase-8 into the DISC, as well as enhancing the recruitment of FLIP_L/S at the DISC. Therefore, SC79 possesses a therapeutic potential for the management of Fas-induced fulminant hepatic injury and may emerge as a lead compound for further development into a clinically applicable drug.

Materials and Methods

Cell Lines and Cell Culture

The human hepatoblastoma cell line HepG2 and primary mouse hepatocytes (PMHs) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and maintained in a humidified atmosphere containing 5% CO2 at 37°C. PMHs were isolated from male C57BL/6 mice as described in Isolation of PMHs.

Treatment of Cells with Chemicals and Antibodies

In HepG2 cells, Fas receptor stimulation was performed with the agonistic monoclonal antibody anti-Fas CH11 (catalog number SY-001; MBL, Nagoya, Japan) at 2 µg/mL plus 0.5 µg/mL actinomycin D (catalog number A1410; Sigma-Aldrich, St. Louis, MO). In PMHs, Fas receptor stimulation was performed with the agonistic monoclonal antibody anti-Fas Jo2 (catalog number 554255; BD Pharmingen, San Diego, CA) alone at 2 µg/mL. To oppose agonistic anti-Fas antibody—induced apoptosis, cells were pretreated with the Fas receptor antagonistic monoclonal antibody anti-Fas ZB4 (catalog number MD-11-3; MBL) at 2 µg/mL or AKT activator SC79 (catalog number 123871; Calbiochem, La Jolla, CA) at 4 µg/mL. To potentiate agonistic Anti-Fas antibody—induced apoptosis, cells were pretreated with the AKT inhibitor LY294002 (catalog number L9908; Sigma-Aldrich) at 50 µmol/L. All of these pretreated cells were then treated with anti-Fas CH11 or Jo2 at 2 µg/mL for an additional 4 hours. IgM (catalog number M079-3; MBL) was used as a control for anti-Fas CH11, and IgG (catalog number M075-3; MBL), for anti-Fas ZB4.

Isolation of PMHs

PMHs were isolated from C57BL/6 male mice by a two-step collagenase-perfusion technique. The animal study protocol was approved by the Fujian Medical University Institutional Animal Care and Use Committee (protocol number M00186). Living hepatic parenchymal cells were separated by 35% Percoll (MilliporeSigma, St. Louis, MO). After seeding for 4 to 6 hours, cells were refreshed with medium and cultured overnight. On the next day, cells were pretreated with dimethyl sulfoxide and SC79 before stimulation with anti-Fas Jo2.

Animal Experiments

Male, age-matched (6- to 8-week—old) C57BL/6 or BALB/c mice (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) weighing 16 to 18 g were used in the animal study. Mice were housed in...
humidity- and temperature-controlled rooms, with free access to food and water. Mice were pretreated with 10 mg/kg SC79 or dimethyl sulfoxide intraperitoneally at 0.5 hour before the i.p. administration of an agonistic anti-Fas Jo2 antibody at a lethal dose of 0.5 and 0.4 mg/kg for C57BL/6 and BALB/c mice, respectively, within 12 hours. Mouse IgG (catalog number 555740; BD Biosciences, San Jose, CA) was used as a control for Jo2. After this lethal challenge, mice were monitored continuously for mortality. For analyses other than mortality, mice were sacrificed at various time points after Jo2 injection. Serum levels of alanine aminotransferase and aspartate aminotransferase were determined using a standard clinical automatic analyzer (model 7020; Hitachi, Kyoto, Japan). Immediately after the blood samples were obtained retro-orbitally, mice were sacrificed by cervical dislocation. The excised liver mass was sectioned, fixed overnight at 4°C in 10% formalin solution, dehydrated, paraffin embedded, cut at 3-mm thickness, and stained with hematoxylin and eosin for histologic examination. Liver tissues were extracted, immediately snap-frozen using liquid nitrogen, and stored at −80°C until analyzed.

Western Blot Analysis

Cell lysates and tissues were prepared using radioimmunoprecipitation assay protein lysis buffer (Pierce Company, Rockford, IL). A total of 40 μg of protein extracts were quantified and then subjected to electrophoresis on a 12% or 15% SDS-PAGE gel. The proteins were transferred to polyvinylidene difluoride membranes and blocked in Tris-buffered saline containing 5% bovine serum albumin. The specific antibodies used in this study included anti-Fas (catalog number 4233;1:1000 dilution; Cell Signaling Technology, Beverly, MA), anti-AKT (catalog number 4691;1:1000 dilution; Cell Signaling Technology), anti-phosphorylated (p)AKT (Thr308, catalog number 4060;1:1000 dilution; Cell Signaling Technology), anti-Fyn (catalog number ab24533, 1:1000 dilution; Abcam, Cambridge, MA), anti-FADD (catalog number 8023; Cell Signaling Technology) and protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA). After immunoprecipitation, the beads were washed four times with immunoprecipitation/lysis buffer, and the immunoprecipitants were eluted from beads with 2 × Laemmli buffer (2.1% SDS, 26.3% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue, 65.8 mmol/L Tris-HCl, pH 6.8) at 95°C for 5 minutes and subjected to SDS-PAGE, followed by immunoblot analysis.

IHC Analysis

Tissues were fixed in 4% neutral buffered formalin, processed, then embedded in paraffin and cut into 5-μm sections. Tissue sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked in 3% H2O2 in phosphate-buffered saline for 10 minutes. After blocking nonspecific sites with 1.5% blocking serum in phosphate-buffered saline for 1 hour at room temperature, tissue sections were incubated for 1 hour at room temperature with the anti-Fas antibody (1:50 dilution; Abcam). After a 30-minute reaction with a biotinylated secondary antibody, slides were washed with phosphate-buffered saline and incubated with streptavidin conjugated with horseradish peroxidase for 10 minutes. The reaction was then revealed with diaminobenzidine. The slides were mounted with Eukitt and observed with an Olympus BX60 microscope (Olympus, Center Valley, PA). Images were captured with IPE software version 6.0 (Aspen Tech, Bedford, MA).

Fas Palmitoylation Detection by Acyl–Biotin Exchange Technique

Protein palmitoylation was detected by a previously described method adapted from the acyl–biotin exchange protocol of Hueber and colleagues.24 Briefly, 1.5 mg of protein lysate (50 mmol/L Tris, pH 7.5, 5.0 mmol/L EDTA, 50 mmol/L NaCl, 2% SDS, and small peptide inhibitors)
Cells were seeded into 96-well plates with 5,000 cells per well and treated with pro-apoptotic chemicals or/and antibodies. Apoptotic cells were labeled using a DeadEnd Fluorometric Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) System kit (Promega, Madison, WI) according to the manufacturer’s instructions. Tissues were dissected and fixed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin, then subjected to TUNEL assay. For quantification of apoptosis, five microscopic fields were randomly selected at high-power magnification (×200), and the mean counts of TUNEL-positive cells were calculated by fluorescence microscopy (Carl Zeiss, Oberkochen, Germany).

Annexin V Binding Assay

Cells were treated with pro-apoptotic chemicals or/and antibodies. Apoptosis was detected using a Fluorescein Isothiocyanate Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer’s instructions. Briefly, cells were harvested and resuspended in 1× binding buffer (1 × 10⁶ cells/mL). Aliquots of 10⁵ cells (100 μL) were mixed with 5 μL of annexin V and 5 μL of propidium iodide. After 15 minutes of incubation at room temperature in the dark, fluorescence was analyzed by flow cytometry (FACSVerse; BD Biosciences) using FACSuite software version 1.0.3 (BD Biosciences).

Real-Time Quantitative PCR Analysis

Total RNA was extracted using the TRIzol reagent (Invitrogen) and reverse-transcribed to cDNA by using the ExScript RT-PCR kit (Takara, Kusatsu, Japan). Real-time quantitative PCR was performed in the Mx3000P Real-Time PCR System (Agilent Technologies, Santa Clara, CA) with the SYBR Premix Ex Taq kit (Takara) following the manufacturer’s instructions. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a reference gene, and relative mRNA levels were calculated using the 2⁻ΔΔCT method. The paired forward and reverse primers were reported previously.

Semiquantitative RT-PCR Analysis of mFas and sFas

Transcribed cDNA was used as a template for PCR amplification. The paired primers P1 and P2 were used for total Fas amplification, and P3 and P5, for membrane-associated/soluble Fas (mFas/sFas) amplification; primer sequences were reported previously. PCR products were analyzed on a 2.5% agarose gel by electrophoresis and ethidium bromide staining. A digital image of the gel was obtained using a SynGene apparatus (SynGene, San Diego, CA) and GeneSnap software version 4.00.00 (SynGene). Individual band intensities were quantitated using Quantity One.
densitometric software (Bio-Rad). sFas or mFas mRNA level was expressed as a ratio of sFas or mFas PCR product signal to that of GAPDH.

Enzyme-Linked Immunosorbent Assay for sFas
sFas in cell culture supernatants was detected using a human sFas enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The absorbance of each well was measured at 450 nm using a microplate reader (Bio-Rad). The concentration of sFas was calibrated from a dose—response curve based on reference standards.

Caspase Enzyme Activity Assay
Activities of caspases 3/7, 8, and 9 were measured by using the Apo-ONE Homogeneous Caspase-Glo 3/7, 8, and 9 assay kits, respectively (catalog numbers G8091, G8201, and G8211; Promega), according to the manufacturer’s instructions. In

Figure 1  SC79 inhibits Fas-mediated apoptosis in HepG2 cells and primary mouse hepatocytes (PMHs). A: SC79 augments AKT phosphorylation (pAKT) at both the Thr308 and Ser473 sites. B: HepG2 cells were pretreated with increasing concentrations of SC79 for 0.5 hour, followed by treatment with 2 μg/mL agonistic Fas antibody CH11 in the presence of 0.5 μg/mL actinomycin D for 12 hours. Cell viability was determined by Cell Counting Kit (CCK)-8 assay (Dojindo Molecular Technologies, Mashiki-machi, Japan). C: Cell viability determined by CCK-8 assay 12 hours after dimethyl sulfoxide (DMSO)- and SC79-pretreated HepG2 cells were treated with increasing concentrations of anti-Fas CH11 in the presence of actinomycin D. D: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining performed on DMSO- or SC79-pretreated HepG2 cells treated with anti-Fas CH11 for 4 hours in the presence of actinomycin D with or without pretreatment with antagonistic anti-Fas ZB4. E: Quantification of apoptotic cells stained with TUNEL and expressed as percentages of the number of TUNEL-positive cells among the total number of DMSO- or SC79-pretreated HepG2 cells counted. F: Representative flow-cytometric analysis of DMSO- or SC79-pretreated HepG2 cells treated with 2 μg/mL agonistic Fas CH11 for 4 hours in the presence of actinomycin D. Cells were doubly stained with propidium iodide (PI) and annexin V. G: Annexin V staining of DMSO- or SC79-pretreated HepG2 cells after CH11 stimulation. Annexin V—positive populations were calculated. H: Measurements of caspase-3/7 enzymatic activity, determined from three independent experiments, expressed as the fold-change relative to that in the DMSO-pretreated HepG2 control cells. I: Cell viability determined by CCK-8 assay 12 hours after DMSO- and SC79-pretreated PMHs were treated with increasing concentrations of anti-Fas Jo2. J: Pretreatment with the phosphoinositide-3 kinase inhibitor LY294002 abolishes SC79-induced Akt activation in PMHs. K: Annexin V staining of DMSO- or SC79-pretreated PMHs after treatment with 2 μg/mL agonistic Fas Jo2 for 4 hours. Annexin V—positive populations were calculated. Data are expressed as means ± SD (B, C, E, G–I, and K). n = 3 (G and K); n = 5 (B, C, E, and I). *P < 0.05.
brief, proluminescence substrate of caspase-3/7, -8, or -9, which consists of aminoluciferin (substrate for luciferase) and the tetrapeptide sequence DEVD, LETD, or LEHD (cleavage site for caspase-3/7, -8, or -9, respectively), were added to cultured cells in each well of a 96-well plate, and the plate was incubated for 60 minutes at room temperature. In the presence of caspase-3/7, -8, or -9, aminoluciferin was liberated from the proluminescence substance and utilized as a substrate for the luciferase reaction. The resultant luminescence in relative light units was measured by using the Orion II Microplate Luminometer (Berthold Detection Systems, Pforzheim, Germany). For in vivo caspase activities, liver lysates were prepared by homogenization in hypotonic buffer (25 mmol/L HEPES, pH 7.5, 5 mmol/L MgCl₂, 1 mmol/L EDTA, and small peptide inhibitors). Homogenates were centrifuged at 12,000 × g for 15 minutes, and the proteins (50 g) were tested in triplicate experiments by caspase activities.

Statistical Analysis

The χ² test was used to analyze the differences between the apoptosis rates. The statistical significance of the differences between mRNA and protein levels was determined by analysis of variance.

Results

SC79 Inhibits Fas-Mediated Apoptosis in HepG2 Cells and Primary Mouse Hepatocytes

AKT is a key cell-survival factor whose deactivation has been implicated in the pathogenesis of hepatocyte injury. Therefore, we hypothesized that AKT activation may protect hepatocytes from Fas-induced apoptosis. SC79, a known Akt-specific activator, was confirmed capable of increasing hyperphosphorylation of Akt at the Thr308 and Ser473 sites (Figure 1A). Next, the effects of SC79 on Fas-induced apoptosis in HepG2 cells were tested by pretreatment of the cells with increasing concentrations of SC79 for 30 minutes before the addition of a fixed cytotoxic concentration of agonistic anti-Fas antibody CH11 and then assessment of cell viability 12 hours later. Indeed, SC79 increased cell survival in a dose-dependent manner up to 4 μg/mL (Figure 1B), which was chosen for subsequent studies. On the other hand, SC79 at the fixed concentration of 4 μg/mL protected HepG2 cells from CH11 cytotoxicity over the various concentrations of CH11 tested (Figure 1C). To determine whether the increased viability of HepG2 cells pretreated with SC79 was due to less induction of apoptosis, we assessed the frequency of apoptotic cells by TUNEL assay. SC79 pretreatment significantly decreased the percentage of apoptotic cells exposed to CH11, whereas treatment with antagonistic anti-Fas ZB4 fully blocked the proapoptotic effects of CH11 irrespective of SC79 (Figure 1D and E). Moreover, a decrease of Fas-induced apoptosis in SC79-pretreated HepG2 cells was also verified by annexin V binding and caspase-3/7 activity assay (Figure 1F–H). The anti-apoptotic effect of SC79 was further extended in PMHs; the results demonstrated that SC79-pretreated PMHs were less sensitive to the cytotoxic effect of the agonistic anti-Fas Jo2 (Figure 1I). To determine whether the protective effect was Akt dependent, the phosphoinositide-3 kinase inhibitor LY294002 was applied to diminish hyperphosphorylation of Akt induced by SC79 (Figure 1J). Expectedly, LY294002 abolished the cytoprotective effect...
SC79 Protects against Fas-Induced ALF

of SC79 (Figure 1K). These results clearly indicate that SC79 is able to protect the HepG2 cells and PMHs from Fas-mediated apoptosis and is likely to act as a suppressor of the Fas/FasL system.

SC79 Suppresses Procaspase-8 Cleavage via Reducing the Formation of SDS-Stable Fas Aggregation and Enhancing the Recruitment of FLIP\textsubscript{L/S} at the DISC

To elucidate the underlying mechanisms by which the AKT activator SC79 executes its Fas-related anti-apoptotic effect, components of the Fas/FasL apoptotic pathway were examined in HepG2 cells treated with CH11 alone or in combination with SC79. FasL binding to the Fas receptor is known to induce the formation of DISC consisting of oligomerized receptors, FADD, procaspase-8, procaspase-10, and FLIP\textsubscript{L/S}.\textsuperscript{15,27} The ratio of procaspase-8 to FLIP\textsubscript{L/S} at the DISC has been reported to have a major impact on the regulation of pro-apoptotic versus anti-apoptotic signaling pathways.\textsuperscript{28,29} Hence, the effect of AKT activation by SC79 on DISC formation was first evaluated in the lysates of CH11-stimulated HepG2 cells pretreated with the vehicle control, SC79, or SC79 plus LY294002 that were resolved using SDS-PAGE and detected by Western blot analysis (Figure 2, A and B). Exposure to CH11 alone resulted in aggregation of SDS-stable high-molecular mass tetrameric Fas. However, SC79 pretreatment completely abolished CH11-induced tetrameric Fas aggregation, whereas co-incubation of SC79 with LY294002 restored the ability of CH11 to promote Fas aggregation. Coincidentally, SC79 treatment also led to the up-regulation of anti-apoptotic FLIP\textsubscript{L/S} expression, which was reversible with LY294002. Furthermore, CH11 treatment alone could efficiently cleave procaspase-8 to generate the active p18 prodomain. In contrast, SC79 treatment before CH11 stimulation processed procaspase-8 only to the p41/43 fragment and failed to produce the active p18 subunit, which can be reversed by cotreatment with the AKT inhibitor LY294002. Next, a Fas antibody was used to immunoprecipitate the DISC from the lysates, which were then separated under nonreducing conditions on a gradient gel and analyzed by Western blot (Figure 2A). Similarly, the decrease in Fas aggregation and the recruitment of FADD and procaspase-8, and the increase in FLIP\textsubscript{L/S} expression, were correlated with the activation of AKT by SC79. In concert with less cleavage or activation of procaspase-8 at the DISC, a significant reduction in the

Figure 3  Stimulation with anti-Fas CH11 in HepG2 cells. Reduced redistribution of Fas aggregation and increased localization of Fas-associated death domain (FADD)–like interleukin-1β–converting enzyme inhibitory protein, long and short isoforms (FLIP\textsubscript{L/S}), into lipid rafts inhibit the recruitment of procaspase-8 by SC79. A–C: Total cell extracts from HepG2 cells pretreated with dimethyl sulfoxide (DMSO) (A), SC79 (B), or SC79 plus the phosphoinositide-3 kinase inhibitor LY294002 (C) were loaded on a discontinuous gradient to isolate detergent-resistant membranes after Fas stimulation. Equal volumes of each fraction were analyzed for lipid raft markers and death-inducing signaling complex proteins on a SDS-PAGE. Lck, proto-oncogene tyrosine-protein kinase LCK.
enzymatic activities of caspase-8 and its downstream effectors caspases 9 and 3/7 was observed (Figure 2C). It may be noteworthy that in addition to its membrane-associated form (mFas), Fas is also present as a soluble molecule (sFas) that can protect cells from Fas-mediated apoptosis. Post-translational modification of Fas by palmitoylation has been reported as necessary for efficient Fas-receptor internalization, DISC assembly, and the subsequent caspase cascade leading to cell death. The effects of SC79 on the expression levels of mFas and sFas, and on the extent of Fas palmitoylation, were examined. SC79 neither influenced the expression of mFas and sFas nor affected Fas palmitoylation (Supplemental Figure S1). Taken together, these data suggest that the activation of AKT by SC79 leads to not only the inhibition of Fas aggregation and FADD recruitment but also augmentation of FLIP<sub>LS</sub> expression and recruitment, resulting in protection of the cells from caspase-8-dependent apoptotic death.

**SC79 Inhibits the Redistribution of DISC into Lipid Rafts**

Fas-mediated signaling largely depends on protein—protein interactions, and the recruitment and concentration of Fas and distinct downstream apoptotic molecules in membrane rafts are essential for the generation and amplification of apoptotic signals. To determine whether SC79 is involved in modulating the redistribution of DISC into lipid rafts, HepG2 cells were pretreated with SC79, and subsequently a redistribution pattern of DISC in the raft and nonraft detergent-resistant membrane was analyzed. As anticipated, in HepG2 cells stimulated with CH11, a significant amount of Fas aggregates were found in the detergent-resistant membrane fraction, likely serving as a hub for the recruitment of FADD, caspase-8, and FLIP<sub>LS</sub> (Figure 3A). When AKT was hyperactivated by SC79, the Fas monomers in detergent-resistant membrane did not change. However, Fas aggregates, FADD, and processed caspase-8 were reduced, accompanied by increased FLIP<sub>LS</sub> in the detergent-resistant membrane fraction (Figure 3B). Notably, SC79-induced inhibition of DISCs into lipid rafts was fully reversible with LY294002 (Figure 3C). These results indicate that SC79 could inhibit the redistribution of DISC into lipid rafts.

**Figure 4**  SC79 protects C57BL/6 mice from Fas-induced fulminant hepatic failure. A: SC79 treatment leads to Akt hyperactivation in the livers of live animals. Protein extracts collected from the livers of untreated and SC79-treated mice were resolved on SDS-PAGE and immunoblotted with indicated antibodies. SC79 was applied via i.p. injection at a concentration of 10 mg/kg of body weight. B: The protective effects of SC79 on an acute and lethal apoptotic hepatic injury induced by an anti-Fas agonist, Jo2 antibody. Of 20 SC79-pretreated mice, 7 survived, whereas all 20 littermates pretreated with dimethyl sulfoxide (DMSO) died within 12 hours after the injection of 0.5 mg/kg Jo2 antibody (P < 0.01). C: Macroscope analysis of representative liver samples, hematoxylin and eosin (H&E) staining, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining 6 hours after Jo2 treatment of the different groups as indicated. In the TUNEL staining, bright yellow indicates apoptotic cells. D–F: Activity assay of caspases 8, 9, and 3/7. G–I: TUNEL assay (G) and analyses of serum alanine aminotransferase (ALT; H) and aspartate aminotransferase (AST; I) show acute and severe cytolyis in the control mice, but delayed and significantly less severe increases in caspase activities, intensity of apoptotic process, and serum aminotransferase levels in SC79-pretreated mice. Return to normal values is observed within 5 days. Data are expressed as means ± SD (D–I). n = 3. *P < 0.05. Original magnification, ×200 (C, middle and bottom rows).

SC79 Protects against Fas-Induced ALF

To examine the therapeutic relevance of SC79, it was investigated whether SC79 could protect hepatocytes in an in vivo ALF mouse model. C57BL/6 mice were injected i.p. with 0.5 mg/kg, a lethal dose, of Jo2 after pretreatment with SC79 at 10 mg/kg. Activation of Akt in the livers of surviving animals from i.p. administration of SC79 was verified (Figure 4A). To ensure that SC79 did not affect endogenous Fas antigen expression, Western blot analysis of membranous fractions from the livers of SC79-pretreated and control mice was performed and demonstrated a similar abundance of mFas expression (Figure 4A). In addition, SC79 pretreatment did not alter the distribution of Fas either in the cytoplasm or in the membrane (Supplemental Figure S2). Treatment of mice with 10 mg/kg of SC79 at 0.5 hour before Jo2 injection increased mouse survival at 12 hours after Jo2 injection from 0% to 35%, and no additional mortality was observed to the end of the 2-month observation period (Figure 4B). Hence, the survival study indicates that SC79 had potent protective effects against lethal ALF in vivo.

The hepatoprotective effects of SC79 in Jo2-injected mice were further characterized by analysis of liver sections for tissue and cellular morphology, presence of apoptotic cells, and in situ caspase activity. The protective effect of SC79 was already evident macroscopically on the liver, with less hemorrhagic response at 6 hours after Jo2 stimulation (Figure 4C). Hematoxylin and eosin staining and histologic analysis revealed that a single dose of SC79 was able to significantly reduce Jo2-mediated liver damage, and normal liver structure was restored on day 14 after Jo2 stimulation (Figure 4C). TUNEL assay confirmed extensive hepatocyte apoptosis in mice treated with Jo2, whereas apoptotic hepatocytes in the livers of SC79-pretreated and surviving mice were substantially reduced (Figure 4C). Consistent with the apoptosis data, cytosolic liver extracts prepared from mice treated with SC79 before Jo2 injection showed a decreased activity of caspases 8, 9, and 3/7 compared with those prepared from mice given Jo2 alone (Figure 4, D–F). Quantification of the apoptotic cells as a function of time revealed that although the number of the apoptotic cells in the SC79-treated mice also peaked at 12 hours, it gradually resolved over the ensuing days and...
returned to nearly normal by 3 days after Jo2 stimulation (Figure 4G). Likewise, changes in the patterns of serum alanine aminotransferase and aspartate aminotransferase levels mirrored the kinetics of apoptotic injury recovery in the SC79-pretreated mice (Figure 4, H and I). Since both C57BL/6 and BALB/c mice are commonly used models of Jo2-induced hepatotoxicity with similar susceptibility, BALB/c mice were used to confirm the equivalent capacity of SC79 in preventing Fas-mediated fulminant hepatic failure from a lethal dose of Jo2 challenge (Supplemental Figure S3).

Discussion

Apoptosis is a cellular self-destruction mechanism that removes senescent and damaged cells for the maintenance of organ homeostasis and protection from external disturbances. Hepatocyte apoptosis plays essential roles not only in the removal of external microorganisms but also in the occurrence and development of various chronic and acute liver diseases, such as viral hepatitis, alcoholic and nonalcoholic liver disease, hepatic ischemia reperfusion, and drug-induced liver injury. Therefore, the elucidation of the molecular mechanisms and regulating factors involved in the initiation and progression of hepatocyte apoptosis is crucial for the treatment of liver diseases.

Hepatocyte apoptosis is mainly induced by the binding of the death ligand to the death receptor on the plasma membrane of the cell. Death ligands belong to the tumor necrosis factor (TNF) superfamily and include FasL, TNF-α, and TNF-related apoptosis—inducing ligand. Upon stimulation by FasL, the death receptor Fas transduces hepatic cell death signaling, and the molecular basis of intracellular death signaling by the FasL/Fas system is well documented. AKT is best known for its function as an indisputable cell-survival factor. Several growth factors and cytokines confer resistance to Fas-induced liver injury through the activation of the AKT pathway. Thus, an elevation of AKT activity is likely to produce a beneficial effect on Fas-related liver diseases. Unfortunately, this endeavor has been impeded due to the absence of specific AKT activators. Recently, from a cell-based high-throughput chemical genetic screening, the small molecule SC79 was identified as a potent and specific AKT activator that enables cytosolic activation of AKT, and such activation recapitulates the primary function of AKT signaling. Research efforts have demonstrated its pro-survival potential as an AKT activator in various experimental settings. For instance, SC79 protects neurons from stroke both in vitro and in vivo. Gong et al demonstrated that SC79-induced activation of AKT signaling protects retinal pigment epithelial cells from UV radiation. Similarly, this novel AKT activator could rescue osteoblasts from dexamethasone cytotoxicity. Here, we demonstrate that SC79 protects against Fas-induced hepatocyte apoptosis both in vitro and in vivo. Pretreatment of hepatoblastoma HepG2 cells and PMHs with SC79 rendered them resistant to Fas-induced apoptosis via interruption of the DISC assembly, enhancing the recruitment of anti-apoptotic FLIP_L/S into DISC and suppressing the redistribution of DISC into lipid rafts. More importantly, the SC79-pretreated mice showed less liver tissue damage with fewer apoptotic hepatocytes and lower caspase activities, lower aminotransferase levels, and lower mortality after a lethal dose of Jo2 challenge. These findings provide novel evidence of an important role of AKT in protection against Fas-induced hepatocyte apoptosis.

It should be recognized that, in addition to FasL, TNF contributes to hepatocyte apoptosis in ALF via the death receptor pathway. During ALF, interactions between TNF and TNF receptor 1 trigger a series of intracellular events, culminating in the activation of caspases 3, 8, and 9. The concurrent activation of the antiapoptotic factor NF-κB is probably the underlying mechanism. However, it appears that, unlike FasL, TNF alone is not powerful enough to initiate the effector caspase cascade and cause liver injury in normal hepatocytes. AKT has been reported to involve in the TNF-mediated activation of NF-κB in HeLa cells and MCF7 breast carcinoma cells, suggesting a possible link between these two pathways. However, Delhase et al failed to detect any involvement of AKT in the signaling pathway through which TNF-α leads to NF-κB activation. Therefore, the role of AKT and the extent to which it affects TNF signaling remain elusive. Our preliminary experiments suggest that the activation of AKT by SC79 also has a protective role in TNF-mediated hepatocyte apoptosis (data not shown). The precise underlying mechanism that connects SC79-induced AKT activation and TNF signaling is currently being investigated.

Our data support a pivotal role of AKT activation in Fas-induced hepatocyte apoptosis. This observation is of important clinical value as Fas-induced apoptosis is implicated in the pathogenesis of hepatitis and hepatic failure. Hepatic infection caused by hepatitis C and hepatitis B viruses is known to influence the Fas/FasL system, whose activities correlate well with disease severity and response to therapy. In patients with chronic hepatitis C, Fas and FasL are increased, and Fas-induced apoptosis is one of the major mechanisms responsible for hepatitis C virus—induced hepatocyte apoptosis. Hepatitis B virus is known to render hepatocytes hypersensitive to Fas signaling, and this alteration may contribute to hepatocarcinogenesis. The Fas-signaling pathway also plays predominant roles in alcoholic liver disease, nonalcoholic steatohepatitis, and cholestatic liver injury. Thus, the modulation of Fas-induced hepatocyte apoptosis by the AKT activator SC79 may have broad clinical implications in various acute and chronic liver diseases.

In summary, we demonstrate for the first time that the novel AKT activator SC79 may be effective in protecting hepatocytes from Fas-induced hepatotoxicity. The results imply that systemic treatment with AKT activators may increase resistance to acute liver injury and thereby prolong the time...
until transplantation is needed, or even allow the endogenous regenerative capacity of the liver to rescue the organ. As SC79 appears to be a relatively safe drug, AKT activators like SC79 and its analogues might represent new pharmacologic interventions for ALF of a variety of etiologies, with the prospect of fast translation into clinical use.

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X.L., X.-J.L., and W.-N.C. conceived and designed the project; W.L., Z.-T.J., S.-X.W., Y.H., and Y.-T.L. performed the experiments; X.L. and W.-N.C. supervised the experiments; W.L., Z.-T.J., S.-X.W., and X.L. analyzed the data; X.-J.L. and X.L. wrote the manuscript; and all authors read and approved the manuscript.

Supplemental Data

Supplemental material for this article can be found at https://doi.org/10.1016/j.ajpath.2018.01.013.

References

49. Pianko S, Patella S, Ostatowicz G, Desmond P, Sievert W: Fas-mediated hepatocyte apoptosis is increased by hepatitis C virus infection and alcohol consumption, and may be associated with hepatic fibrosis: mechanisms of liver cell injury in chronic hepatitis C virus infection. J Viral Hepat 2001, 8:406–413