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Targeting senescent cholangiocytes and activated fibroblasts with Bcl-xL inhibitors ameliorates fibrosis in Mdr2^{-/-} mice

Anja Moncsek¹, Mohammed S. Al-Suraih², Christy E. Trussoni², Steven P. O'Hara², Patrick L. Splinter², Camille Zuber¹, Eleonora Patsenker¹, Piero V. Valli¹, Christian D. Fingas³, Achim Weber⁴, Yi Zhu⁵, Tamar Tchkonja⁵, James L. Kirkland⁵, Gregory J. Gores², Beat Müllhaupt¹, Nicholas F. LaRusso², and Joachim C. Mertens¹

¹Department of Gastroenterology and Hepatology, University Hospital Zürich, Zürich, Switzerland

²Division of Gastroenterology and Hepatology and Center for Cell Signaling in Gastroenterology, Mayo Clinic, Rochester MN, USA ³Department of General, Visceral, and Transplantation Surgery, University Hospital Essen, Essen, Germany ⁴Department of Pathology and Molecular Pathology, University Hospital Zürich, Zürich, Switzerland ⁵The Robert and Arlene Kogod Center on Aging, Mayo Clinic, Rochester, MN, USA

Abstract

Cholangiocyte senescence has been linked to primary sclerosing cholangitis (PSC). Persistent secretion of growth factors by senescent cholangiocytes leads to the activation of stromal fibroblasts (ASF), which are drivers of fibrosis. The activated phenotype of ASF is characterized by an increased sensitivity to apoptotic stimuli. Here, we examined the mechanisms of apoptotic priming in ASF and explored a combined targeting strategy to deplete senescent cholangiocytes and ASF from fibrotic tissue to ameliorate liver fibrosis. Using a co-culture system, we determined that senescent cholangiocytes promoted quiescent mesenchymal cell activation in a PDGF-dependent manner. We also identified Bcl-xL as a key survival factor in PDGF-activated human and mouse fibroblasts. Bcl-xL was also upregulated in senescent cholangiocytes. *In vitro*, inhibition of Bcl-xL by the small molecule BH3 mimetic A-1331852 or Bcl-xL-specific siRNA induced apoptosis in PDGF-activated fibroblasts but not in quiescent fibroblasts. Likewise, inhibition of Bcl-xL reduced the survival and increased apoptosis of senescent cholangiocytes, compared to non-senescent cells. Treatment of Mdr2^{-/-} mice with A-1331852 resulted in an 80% decrease in senescent cholangiocytes, a reduction of fibrosis-inducing growth factors and cytokines, decrease of α SMA-positive ASF and finally in a significant reduction of liver fibrosis.

Conclusions—Bcl-xL is a key survival factor in ASF as well as in senescent cholangiocytes. Treatment with the Bcl-xL-specific inhibitor A-1331852 reduces liver fibrosis, possibly by a dual

CORRESPONDING AUTHOR: Dr. med. Joachim C. Mertens, M.D., University Hospital Zürich, Department of Gastroenterology and Hepatology, Rämistr. 100, CH-8091 Zürich, Switzerland, Tel.: +41 44 255 9167, Fax: +41 44 255 9933, joachim.mertens@usz.ch.

CONFLICT OF INTEREST

YZ, TT, JLK, and Mayo Clinic have a financial interest related to this research. This research has been reviewed by the Mayo Clinic Conflict of Interest Review Board and is being conducted in compliance with Mayo Clinic conflict of interest policies. The other authors have no conflicts of interest related to this work.

effect on activated fibroblasts and senescent cholangiocytes. This novel mechanism represents an attractive therapeutic strategy in biliary fibrosis.

Keywords

Primary Sclerosing Cholangitis; Apoptotic Priming; Bcl-xL; BH3 Mimetic; Senolytics

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic idiopathic cholangiopathy. While the pathogenesis is still poorly understood, several steps in the disease progression have been identified. One model of pathogenesis suggests that cholangiocyte injury initiates an inflammatory cascade that involves other cell types such as fibroblasts in the periductal stroma. This interplay of injured epithelial cells and subsequently activated stromal fibroblasts leads to the characteristic fibrotic reaction that further promotes the injury by causing biliary strictures and cholestasis. Recent studies have demonstrated that cholangiocyte senescence is a prominent feature in PSC. These senescent cholangiocytes are characterized by growth arrest, but also exhibit features of the senescence-associated secretory phenotype (SASP), including increased secretion of signaling molecules and growth factors such as platelet-derived growth factor (PDGF) (1, 2).

Periductal stromal fibroblasts in the liver are responsible for the synthesis, deposition, and remodeling of the extracellular matrix and play a pivotal role in tissue repair. Upon tissue injury and through their interplay with epithelial cells, fibroblasts become activated by growth factors such as PDGF (3–5). Persistent activation of stromal fibroblasts can lead to fibrosis (6). Activated stromal fibroblasts (ASF) exhibit increased sensitivity to apoptotic stimuli (7). Indeed, states of cell activation or injury can result in increased apoptotic sensitivity, also termed ‘apoptotic priming’ (8). Cellular activation leads to an imbalance of pro- and anti-apoptotic Bcl-2 proteins, often with an overweight of pro-apoptotic family members. In this state, minimal further pro-apoptotic stimuli are sufficient to drive activated cells into cell death. However, the molecular mechanisms of fibroblast activation and apoptotic priming in the liver are not well understood.

Bcl-2 proteins are key regulators of cellular apoptosis. The Bcl-2 protein family comprises pro- and anti-apoptotic members. Among the most prominent anti-apoptotic proteins are Bcl-2, Bcl-xL, and Mcl-1. Pro-apoptotic family members include the effector proteins Bax and Bak, which upon activation form pores in the outer mitochondrial membrane. Additionally, the BH3 only proteins inhibit anti-apoptotic family members and ‘fine tune’ the apoptotic machinery. Anti-apoptotic proteins of the Bcl-2 family keep Bax and Bak in check, while BH3 only proteins in turn regulate the activity of anti-apoptotic Bcl-2 proteins (9). In a healthy cell, these pro- and anti-apoptotic factors are in a finely regulated balance to ensure cell survival.

We have previously shown in a murine model of cholangiocarcinoma (CCA), that ASF in the tumor microenvironment can be selectively targeted with BH3 mimetics (7), small molecules that mimic BH3 protein function and have pro-apoptotic effects. This results in reduced tumor volume and prolonged survival of the animals. The senescent cholangiocyte

phenotype characteristic of PSC (1) may maintain cellular activation in the microenvironment by secretion of activating mediators, thereby fueling the development of biliary fibrosis. Interestingly, several senescent cell types seem to depend on Bcl-xL for survival (10, 11). Very recently small molecules that selectively induce apoptosis in senescent cells have gained interest. The elimination of senescent cells by so called 'senolytics' may reduce detrimental aging processes (12). Thus, ASF as well as senescent human cholangiocytes might be sensitive to pro-apoptotic or senolytic effects of BH3 mimetics, respectively.

In this study, we examined the mechanism of PDGF-mediated apoptotic priming in ASF. Furthermore, we explored whether BH3 mimetics might have a dual therapeutic effect on ASF as well as on senescent cholangiocytes in the *Mdr2*^{-/-} mouse model of biliary liver fibrosis, thereby reducing liver injury and fibrosis.

MATERIALS AND METHODS

In vitro experiments

Fibroblasts were activated with 50 ng/ml recombinant human PDGF-BB (eBioscience, San Diego, CA) for 24 hours. Non-transformed, low passage (less than passage 15) normal human cholangiocytes (NHC) were induced to senesce with ionizing radiation (10Gy) a well-documented approach to induce cellular senescence *in vitro* (13). For apoptosis induction, fibroblasts were treated for 24 hours with following pro-apoptotic BH3 mimetics: 1 μ M navitoclax (ABT-263, specific for Bcl-2, Bcl-xL, and Bcl- ω), 1 μ M A-1195425 (ABT-199, specific for Bcl-2), 1 μ M A-1331852 (specific for Bcl-xL), and 10 μ M A-1210477 (specific for Mcl-1). NHC were treated with 1 μ M A-1331852 for 1h, 48 h post IR-treatment. The set of BH3 mimetics was kindly provided by Abbvie International (Chicago, IL).

Co-culture experiments

NHCs were seeded on the inserts of transwell plates (6 or 24 wells plate), and were induced to senescence by IR. NHCs remained in culture for 4 days to achieve maximum percentage of senescence (60%) as measured by Beta-Gal staining. Human hepatic stellate cells (LX-2) were seeded on the bottom of separate plates (6 or 24 wells plate). LX-2 were incubated with a PDGF neutralizing antibody (R&D Systems, Minneapolis, MN) at the recommended concentration from 1 hour prior to the start of the co-culture. NHCs and senescent cholangiocytes were incubated with LX-2 in the presence or absence of PDGF antibody for 24 hours before harvesting proteins.

For a complete list of employed cell lines see supplemental material.

siRNA

Cells were transfected with 10 nM of Silencer Select Pre-Designed siRNA targeting Bcl-xL or Silencer Select Negative Control No. 2 (both from Ambion) for 24 to 48 hours. Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA) was used according to the manufacturer's protocol.

***In situ* co-staining of mRNA and protein**

Detection of p16^{Ink4a} mRNA and Cytokeratin 19 protein in formalin-fixed paraffin embedded mouse liver tissue was performed as described previously (14). Briefly, tissues were deparaffinized, rehydrated, and subjected to heat-mediated antigen retrieval in 0.01M sodium citrate buffer, pH 6.0. Slides were allowed to cool, washed, and incubated in pre-hybridization solution (3% BSA in 4X SSX) for 20 minutes at 55°C. Tissues were then incubated for 1 h at 55°C with a fluorescein-labeled p16^{Ink4a} LNA probe (Exiqon) diluted to 25 nM in hybridization buffer (10% Dextran Sulfate in 4X SSC). Slides were washed and subjected to a tyramide signal amplification step (Perkin Elmer). Slides were washed again, blocked in 4% BSA, and incubated overnight at 4°C with a primary antibody to Cytokeratin 19 (Abcam). After overnight incubation, slides were washed and incubated with a secondary antibody (Alexa Fluor 568, Thermo Fisher Scientific), washed again, and mounted in Prolong Gold with DAPI (Thermo Fisher Scientific). Slides were analyzed on a Zeiss 710 Confocal Microscope.

Mdr2^{-/-} rodent model of primary sclerosing cholangitis

Mdr2^{-/-} mice were kindly provided by Frank Lammert (Saarland University Medical Center, Department of Medicine II, Homburg, Germany). Balb/cJ wild type mice were purchased from Jackson Laboratory (Bar Harbor, ME). All protocols were approved by the local Animal Care and Use Committees (264/2013). 12 week old female Balb/cJ wild type mice and Mdr2^{-/-} mice were treated during the light cycle with 50 mg/kg/d navitoclax (ABT-263), 25 mg/kg/d A-1331852 (Bcl-xL inhibitor), or vehicle for 14 days by daily oral gavage. Finally, blood was taken for liver enzyme quantification. For fibrosis quantification, animals were sacrificed and livers were excised and snap-frozen in liquid nitrogen. For immunohistochemistry, liver sections were also paraffin-embedded and frozen in Tissue-Tek O.C.T. compound (Sakura, Alphen aan den Rijn, The Netherlands).

Sirius red staining

Paraformaldehyde-fixed, paraffin-embedded liver tissue sections (3 µm) were deparaffinized, hydrated, and incubated in Picro-sirius red solution (0.5% Picro-sirius red in picric acid, both from Sigma-Aldrich, St. Louis, MO). Following a washing step, sections were incubated in Weigert's haematoxylin (Sigma-Aldrich, St. Louis, MO). Sections were rinsed and differentiated with hydrochloric acid. Sections were then dehydrated and mounted. The percentage of Sirius red positive area was quantified using ImageJ and calculated from 10 portal fields (magnification 20×) in each animal.

Hydroxyproline quantification

Hydroxyproline content was quantified colorimetrically using Hydroxyproline Assay Kit (Sigma-Aldrich, St. Louis, MO). Briefly, 100 mg liver tissue from the left liver lobe was homogenized in 1 ml H₂O bidest. using GentleMacs Tissue Homogenizer (Miltenyi Biotec, Bergisch Gladbach, Germany) and subsequently hydrolyzed in 1 ml 12 M hydrochloric acid at 120°C for 3 hours. The hydrolysate was centrifuged at 13.000 × g for 10 min and 20 µl of the supernatant were transferred to a microtiter plate and evaporated to dryness at 60°C. The lyophilisate was rehydrated in 100 µl Chloramine T/Oxidation Buffer and incubated at room

temperature for 5 min. Following addition of 100 μ l of DMAB/perchloric acid/isopropanol solution, samples were incubated at 60°C for 90 min. Absorbance was read at 561 nm (Synergy 2; BioTek, Winooski, VT). Hydroxyproline concentration was calculated from a standard curve prepared with high-purity hydroxyproline.

Statistical analysis

Data represent at least three independent experiments and are expressed as mean $\pm$ SD. Statistical significance was determined using two-tailed Student's *t* tests or ANOVA. Graphs were generated using GraphPad Prism software 6 (La Jolla, CA).

Further materials and methods are described in the supplemental material.

RESULTS

Senescent cholangiocytes exhibit an altered Bcl-2 protein profile and are sensitive to Bcl-xL inhibition

In general, senescent cells upregulate the anti-apoptotic Bcl-2 proteins Bcl- ω and Bcl-xL to maintain their viability (10). In order to study the expression of Bcl-2 family members in senescent cholangiocytes, NHC were experimentally induced to senescence. Western blot analysis confirmed that anti-apoptotic Bcl-xL is upregulated in senescent cholangiocytes as compared to non-senescent cells whereas anti-apoptotic Bcl-2 and pro-apoptotic Bak are unchanged (Fig. 1A).

We then asked if senescent cholangiocytes need Bcl-xL for their survival. Therefore, we exposed IR-treated senescent cholangiocytes to the selective Bcl-xL inhibitor A-1331852, and monitored both apoptosis and cell viability. While non-senescent NHC showed no alterations, senescent cholangiocytes had a reduced level of viable cells (Fig. 1B) and a high level of caspase 3/7 activity (Fig. 1C) after only 1 hour of A-1331852 treatment. Enhanced apoptosis sensitivity in senescent cholangiocytes was confirmed by both cleaved PARP (Fig. 1D) and apoptotic nuclei (suppl. Fig. 1) suggesting that only senescent cholangiocytes are sensitive to A-1331852-induced apoptosis.

In vitro activated fibroblasts are primed for apoptosis and sensitive to BH3 mimetics

In order to investigate apoptotic priming in activated fibroblasts, we stimulated the human HFF-1 fibroblasts with PDGF-BB to achieve an activated phenotype (suppl. Fig. 2A and B) and subsequently exposed them to the pro-apoptotic BH3 mimetic navitoclax *in vitro*. Pretreatment with PDGF-BB significantly increased the sensitivity of fibroblasts to navitoclax-mediated apoptosis as assessed biochemically by caspase-3/7 activity and morphologically using DAPI staining for apoptotic nuclei, whereas non-activated fibroblasts did not response to navitoclax (Fig. 2A, suppl. Fig. 2C). Treatment of PDGF-BB-pretreated human primary fibroblasts (hFB) revealed the same results (Fig. 2A, suppl. Fig. 2C). Furthermore, treatment of 3T3 mouse fibroblasts and human liver fibroblasts obtained from a PSC patient (hFB-PSC) with navitoclax significantly increased caspase-3/7 activity in PDGF-BB-pretreated fibroblasts as compared to quiescent fibroblasts (Fig. 2B). These data

show that quiescent fibroblasts can be activated *in vitro* and that activated fibroblasts can be targeted with the BH3 mimetic navitoclax.

Activation of stromal fibroblasts decreases Bcl-2 protein

We have previously shown that alteration of the Bcl-2 family is responsible for apoptotic priming in cancer-associated fibroblasts (15). Bcl-2 profiling of PDGF-activated fibroblasts revealed a downregulation of Bcl-2 whereas Bcl-xL protein was unchanged (Fig. 3, suppl. Fig. 3). Pro-apoptotic Bax was increased in HFF-1 cells but not changed in activated primary fibroblasts (Fig. 3B).

Survival of activated fibroblasts is Bcl-xL-dependent

To explore the hypothesis that loss of Bcl-2 protein renders activated fibroblasts dependent on other anti-apoptotic Bcl-2 proteins for survival, we exposed PDGF-activated human and mouse fibroblasts to specific Bcl-2 protein inhibitors. The specific inhibition of Bcl-xL solely induced apoptosis in PDGF-activated fibroblasts, whereas specific inhibition of Bcl-2 itself and Mcl-1 did not result in cell death (Fig. 4A). Furthermore, treatment of different human and mouse cell lines and primary human fibroblasts with the Bcl-xL inhibitor A-1331852 did result in significant apoptosis induction in PDGF-activated cells as assessed biochemically by caspase-3/7 activity and morphologically using DAPI staining, whereas non-activated fibroblasts did not respond to Bcl-xL inhibition (Fig. 4B and 4C, suppl. Fig. 4).

To confirm the Bcl-xL dependency of activated stromal fibroblasts, we performed a siRNA knockdown of Bcl-xL in human and mouse fibroblasts. Consistent with our previous observations, treatment of these cells with PDGF induced apoptosis in fibroblasts with Bcl-xL knockdown as shown by increased caspase-3/7 activity and cleaved caspase-3 by Western blot, whereas nonsense siRNA-treated cells did not respond to PDGF treatment (Fig. 4D and 4E). This confirms that the survival of activated fibroblasts is exquisitely dependent on Bcl-xL.

Senescent cholangiocytes exhibit a secretory phenotype and induce fibroblast activation *in vitro*

Senescent cholangiocytes are characteristically found in PSC. Analysis of cell culture media from normal and IR-induced senescent NHCs revealed an increased secretion of CXCL1, IL-6, and IL-8 by senescent NHCs (Fig. 5A). Co-cultivation of the hepatic stellate cell line LX-2 with senescent NHC induced activation of these cells as seen by increased α SMA, compared to co-cultivation with normal NHCs (Fig. 5B). Addition of an anti-PDGF antibody almost completely abrogated this activation. These data confirm that senescent cholangiocytes induce fibroblast activation that is blocked by anti-PDGF antibodies.

Bcl-xL inhibition in the *Mdr2*^{-/-} PSC mouse model depletes senescent cholangiocytes

Based on our *in vitro* data, we treated *Mdr2*^{-/-} mice with the selective Bcl-xL inhibitor A-1331852 to deplete senescent cholangiocytes as well as activated stromal fibroblasts and ideally to reduce biliary liver fibrosis. In the case of cholangiocytes, the number of p16^{Ink4a}-positive senescent cholangiocytes was significantly reduced in animals treated with

A-1331852 (Fig. 6A). In addition, a reduction of SASP proteins in the sera of treated animals was observed (suppl. Fig. 5B).

RNAscope *in situ* hybridization of liver tissue sections confirmed the expression of pro-fibrotic TGF β , PDGF β , and TNF α by cholangiocytes (Fig. 6C). Treatment of Mdr2^{-/-} mice with Bcl-xL inhibitor A-1331852 lead to a significant reduction of expression of pro-fibrotic factors, including TGF β , PDGF, and TNF α , in the liver as compared to vehicle-treated mice (Fig. 6B).

Selective inhibition of Bcl-xL reduces liver fibrosis in Mdr2^{-/-} mice

In line with the reduction of senescent cholangiocytes, we found significantly reduced expression of the fibroblast activation marker α SMA (Fig. 7E), and a decrease in overall fibrotic tissue in A-1331852-treated animals as compared to vehicle-treated controls (Fig. 7A). Morphometric analysis of Sirius red-stained liver sections revealed a significant reduction of periductal fibrosis (Fig. 7B). Quantification of collagen by measuring hydroxyproline confirmed reduction of fibrosis in treated animals (Fig. 7C). Furthermore, Collagen 1A1 and Tenascin C mRNA expression in the liver was reduced in Mdr2^{-/-} mice treated with Bcl-xL inhibitor, as compared to vehicle-treated animals (Fig. 7D).

To better characterize the Mdr2^{-/-} mouse model of PSC, H&E stained tissue sections were examined for inflammation and signs of cholestasis. Mild portal inflammation but no signs of cholestasis or bilirubinostasis were observed in the Mdr2^{-/-} mice.

To assess for cholangiocyte proliferation and ductular reaction, immunohistochemistry for Ki67 and Sox9 (suppl. Fig. 7) was performed. Morphometry of these stainings showed a significantly reduced ductular reaction after treatment with A-1331852.

Complementing TUNEL staining did not show increased cell death of normal bile duct epithelia or hepatocytes following treatment with A-1331852 (suppl. Fig. 6).

DISCUSSION

This study provides new mechanistic insights into the interaction of senescent cholangiocytes and stromal fibroblasts. Our data indicate that: a) senescent cholangiocytes and activated fibroblasts depend on Bcl-xL for survival, b) activation of stromal fibroblasts by PDGF causes loss of pro-survival Bcl-2 with resulting Bcl-xL dependency and apoptotic priming, c) senescent cholangiocytes activate fibroblasts in an PDGF-dependent manner and d) specific inhibition of Bcl-xL by the BH3 mimetic A-1331852 reduces both senescent cholangiocytes and activated stromal fibroblasts, thereby alleviating biliary fibrosis in Mdr2^{-/-} mice.

A large percentage of cholangiocytes in the livers of patients with PSC exhibit a senescent phenotype and SASP (1). Due to secreted signaling molecules, SASP-expressing cholangiocytes alter the periductal microenvironment and induce activation of stromal fibroblasts (1, 2). Recruitment and activation of stromal fibroblasts by PDGF has been shown in both cancer and benign, chronic fibrosing conditions (16–18). Besides other growth factors, PDGF is released into the microenvironment by senescent/SASP signals to

stromal fibroblasts. As previously shown, PDGF activates stromal fibroblasts, induces proliferation and extracellular matrix deposition, but also primes these fibroblasts for apoptosis (15). We demonstrated earlier that ASF become selectively sensitive to the pro-apoptotic BH3 mimetic navitoclax and undergo apoptosis upon treatment (7). As shown here and in previous studies (15), PDGF-mediated fibroblast activation results in downregulation of the anti-apoptotic regulator Bcl-2. This primes ASF for apoptosis and renders their survival dependent on the complementary pro-survival factor Bcl-xL. Consequently, inhibition of Bcl-xL with specific BH3 mimetics effectively induces apoptosis in these cells. Interestingly, after siRNA knockdown of Bcl-xL, PDGF becomes an inducer of apoptosis in stromal fibroblasts and results in increased cell death. To our knowledge, this effect of the growth factor PDGF has not been described before.

Recent data show that like ASF, senescent cells depend on Bcl-xL as a pivotal survival factor (10, 11) and can be efficiently eliminated by the poly-specific BH3 mimetic, navitoclax (ABT-263) (19). This provides the rationale to target both cell types, Bcl-xL-dependent ASF and Bcl-xL-dependent senescent cholangiocytes, with the specific BH3 mimetic, A-1331852, as a dual treatment strategy for biliary fibrosis in PSC. Indeed, in the *Mdr2*^{-/-} mouse model of PSC, inhibition of Bcl-xL by A-1331852 significantly reduced both the number of p16^{Ink4a}-positive senescent cholangiocytes and fibrosis. This reduction in senescent cholangiocytes was reflected by a reduction of SASP-specific signaling molecules in the sera of treated animals (Suppl. Fig. 5B). At the same time, we observed a reduction in pathological ductular reaction following Bcl-xL inhibition, while liver parenchyma and normal bile ducts appeared unaffected (Suppl. Fig. 7).

There was no significant improvement of lab values in the animals (Suppl. Fig. 5A). This was not unexpected, since the approach to inhibit Bcl-xL does not prevent ongoing biliary tract injury, but eliminates cells that have already suffered damage or have already been activated. Moreover, the ultimate goal of PSC therapy is the reduction of hepatobiliary fibrosis. We propose a pathogenetic pathway of epithelial injury, ensuing epithelial senescence, development of a SASP, and subsequent persistent stromal cell activation that result in fibrosis, which further aggravates epithelial injury (Fig. 8). To interrupt this process appears to be a promising therapeutic approach.

Earlier unspecific Bcl-2 inhibitors like Navitoclax showed dose-limiting hematological toxicities. Neutropenia was the primary limiting side effect particularly after combination with standard chemotherapeutics. Pronounced thrombocytopenia was also observed but not dose-limiting. Although still limited, current data indicate that BCL-xL-selective inhibitors such as A-1331852 may actually have an improved safety profile and increased efficacy (20). In addition, the desired therapeutic effects are due to the cytotoxic action of A-1331852 rather than a cytostatic one, therefore intermittent application of the drug for treatment of a human disease seems feasible to reduce potential hematological side effects.

Normal hepatocytes and normal biliary epithelia did not show increased cell death following A-1331852 treatment as assessed by TUNEL staining. This supports the idea that no relevant liver toxicity has to be expected. Moreover, there was a trend to improved liver

values in the animals treated with A-1331852 when compared with animals that received vehicle control (suppl. Fig. 5B).

These findings are novel to the field of PSC. Similar observations have been made previously in cholangiocarcinoma with regard to depletion of activated stromal fibroblasts. From our point of view the current findings underline a link between biliary fibrosis and cancer development. The study employs a new, specific Bcl-xL inhibitor that has not been examined in biliary fibrosis so far. It promotes a possible cancer preventive approach in PSC and as such has clinical relevance.

We therefore suggest combined targeting of both activated stromal fibroblasts and senescent cholangiocytes with BH3 mimetics as a novel therapeutic strategy. Similar mechanisms of interaction between injured, senescent epithelia that activate stromal cells with resulting apoptotic priming may be a pathogenic mechanism in other forms of liver fibrosis and even in other organs. In this regard, Bcl-xL-specific inhibitors may have relevance in anti-fibrotic treatment strategies beyond the liver.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

PSC	Primary sclerosing cholangitis
ASF	activated stromal fibroblast
PDGF	Platelet-derived growth factor
DAPI	4',6-diamidino-2-phenylindole
Mdr2	Multidrug resistance protein 2
BH3	Bcl-2 homology domain 3
Bcl-xL	B-cell lymphoma-extra large
FISH	Fluorescence <i>in situ</i> hybridization
αSMA	α -smooth muscle actin
SASP	senescence-associated secretory phenotype

Bcl-2	B-cell lymphoma 2
CCA	cholangiocarcinoma
HFF-1	human foreskin fibroblasts
Ac-DEVD-AMC	N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-Methylcoumarin
hFB	human fibroblasts

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Author names in bold designate shared co-first authorship.

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Figure 1.

Senescent cholangiocytes upregulate Bcl-xL and exhibit enhanced apoptotic sensitivity to the specific Bcl-xL inhibitor A-1331852.

Normal human cholangiocytes (NHC) were induced to senesce with ionizing radiation (IR; 10Gy). (A) Protein lysates were subjected to Western blot for quantification of selected Bcl-2 family proteins as well as the senescence marker p16^{Ink4a}. (B) Treatment of NHC with A-1331852 resulted in a reduction of cell viability in senescent but not in non-senescent cholangiocytes, as determined by MTT assay. (C) A-1331852 treatment also significantly increased caspase-3/7 activity in senescent cholangiocytes as compared to non-senescent cells. (D) In addition, cleavage of PARP was increased in A-1331852-treated senescent cholangiocytes. Data in B and C are presented as mean $\pm$ S.D. of at least three independent experiments, performed in triplicate.

Figure 2. PDGF-activated fibroblasts are sensitive to navitoclax-induced apoptosis. Human and mouse fibroblasts were activated *in vitro* with 50 ng/ml PDGF-BB for 24 h. Fibroblasts were then treated with the pro-apoptotic BH3 mimetic navitoclax for an additional 24 h to induce apoptosis. (A) Apoptosis induction in human HFF-1 fibroblasts and primary fibroblasts (hFB) was quantified biochemically by measuring caspase-3/7 activity using the fluorogenic Ac-DEVD-AMC substrate and morphologically using DAPI staining. The number of apoptotic nuclei was quantified in at least four high power fields

and is expressed as a percentage of total. (B) Apoptosis induction in murine 3T3 fibroblasts and human primary fibroblasts from PSC (hFB-PSC) was quantified biochemically by measuring caspase-3/7 activity. Data are presented as mean $\pm$ S.D. of at least three independent experiments, performed in triplicate. Abbreviations: ns, not significant; PDGF, platelet-derived growth factor

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Figure 3.

Activation of fibroblasts results in reduction of Bcl-2 protein.

HFF-1 (left panel) and primary human fibroblasts (hFB, right panel) were activated *in vitro* with 50 ng/ml PDGF-BB for 24 h. (A) Gene expression of the Bcl-2 family was assessed by quantitative real time PCR. Changes following PDGF treatment are shown as fold change relative to vehicle. Data are presented as mean $\pm$ S.D. of at least three independent experiments, performed in triplicate. (B) Whole cell lysates were subjected to Western blot for quantification of selected pro- and anti-apoptotic Bcl-2 family proteins. Diagrams

represent data $\pm$ S.E.M. of at least three independent experiments. Abbreviations: ns, not significant; PDGF, platelet-derived growth factor

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Figure 4.

Survival of activated fibroblasts is Bcl-xL-dependent.

(A) Human HFF-1 and mouse 3T3 fibroblasts were activated *in vitro* with 50 ng/ml PDGF-BB for 24 h. Cells were then treated with pro-apoptotic BH3 mimetics specific for different Bcl-2 proteins for additional 24 h. Apoptosis was quantified biochemically by measuring caspase-3/7 activity using the fluorogenic Ac-DEVD-AMC substrate. Treatment of activated fibroblasts with 1 μ M A-1331852, a specific Bcl-xL inhibitor, leads to a significant increase of caspase-3/7 activity, whereas specific inhibition of Bcl-2 using 1 μ M ABT-199 (A-1195425) and Mcl1 using 10 μ M A-1210477 shows no apoptotic effect. (B,C) PDGF-activated fibroblasts were treated with 1 μ M Bcl-xL inhibitor A-1331852 for 24 h. Apoptosis induction was quantified biochemically by measuring caspase-3/7 activity using the fluorogenic Ac-DEVD-AMC substrate and morphologically using DAPI staining. The number of apoptotic nuclei was quantified in at least four high power fields and is expressed as a percentage of total. (D, E) PDGF induces apoptosis in fibroblasts after Bcl-xL knockdown. Fibroblasts were treated with 10 nM Bcl-xL-specific siRNA or nonsense siRNA for 48 h. Cells were then activated with 50 ng/ml PDGF-BB for 24 h. (D) Induction of

apoptosis was quantified biochemically by measuring caspase-3/7 activity using the fluorogenic Ac-DEVD-AMC substrate. (E) Knockdown of Bcl-xL was confirmed by Western blot. Western blot for cleaved caspase-3 shows an increase only in fibroblasts with Bcl-xL-knockdown confirming induction of apoptosis by PDGF in these cells. Caspase activity is presented as mean $\pm$ S.D. of at least three independent experiments, performed in triplicate.

Abbreviations: ns, not significant; PDGF, platelet-derived growth factor

Figure 5.

Senescent cholangiocytes activate stellate cells in a PDGF-mediated manner. Normal human cholangiocytes (NHC) were induced to senescence with ionizing radiation (IR; 10Gy). (A) Cell culture supernatant of normal and senescent NHCs was analyzed for the cytokines and chemokines indicated using a membrane-based antibody array. (B) Normal and senescent NHCs were co-cultured with human hepatic stellate cells (LX-2) for 24 h. Whole cell lysates of LX-2 were analyzed for fibroblast activation marker α SMA. Addition of anti-PDGF antibodies prior co-culture completely blocked LX-2 activation. Diagrams represent mean $\pm$ S.D. of at least three independent experiments. Abbreviations: CXCL1, (C-X-C motif) chemokine; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte/macrophage colony stimulating factor; IL, interleukin; α SMA, alpha smooth muscle actin

Figure 6.

Bcl-xL inhibition eliminates senescent cholangiocytes and decreases pro-fibrotic factors in Mdr2^{-/-} mice.

Female BALB/cJ wild type and Mdr2^{-/-} mice were treated with vehicle or 25 mg/kg/d Bcl-xL inhibitor A-1331852 for 14 days by daily oral gavage starting at week 12 of age. (A) Co-staining of p16^{Ink4a} mRNA and Cytokeratin 19 protein was performed in formalin-fixed paraffin-embedded mouse liver tissue. Representative images (p16^{Ink4A} probe = green; CK19 = red; DAPI = blue) and semi-quantitative analysis of fluorescence intensity demonstrate a significant reduction of senescent cholangiocytes in Bcl-xL inhibitor-treated Mdr2^{-/-} mice compared to vehicle-treated animals. Bar = 50μm (B) Expression of pro-fibrotic cytokines and growth factors was assessed by quantitative real-time PCR. Gene expression is shown as fold change relative to vehicle. Data are presented as mean ± S.D. of at least three independent experiments, performed in triplicate. (C) Expression of pro-fibrotic cytokines and growth factors in cholangiocytes was investigated by chromogenic RNAscope® *in situ* hybridization. Representative images and semi-quantitative assessment of RNAscope® stainings are shown.

Abbreviations: FISH, fluorescence *in situ* hybridization; ns, not significant

Figure 7.

Treatment with Bcl-xL inhibitors reduces liver fibrosis and expression of pro-fibrotic genes in Mdr2^{-/-} mice.

Female BALB/cJ wild type and Mdr2^{-/-} mice were treated with 25 mg/kg/d Bcl-xL inhibitors A-1331852 for 14 days by daily oral gavage starting at week 12 of age. (A) Liver fibrosis was visualized by Sirius red collagen staining. (B) Morphometric analysis revealed a significant reduction of liver fibrosis after A-1331852 treatment. (C) Hydroxyproline quantification of 100 mg liver tissue confirmed the reduction of liver fibrosis. (D) Gene expression analysis of Collagen1A1 (Col1A1) and Tenascin c (TNC) by real-time PCR demonstrated a reduction in A-1331852-treated animals. (E) Quantitative PCR for α SMA, a marker for activated fibroblasts, shows a reduction in mice treated with Bcl-xL inhibitor. Data in D and E are presented as mean $\pm$ S.D. of at least three independent experiments, performed in triplicate.

Figure 8.

Proposed mechanism of liver fibrosis reduction by Bcl-xL inhibition. Persistent injury of cholangiocytes in PSC leads to a pro-inflammatory response, which further results in chronic inflammation, cholestasis, and fibrosis. A large number of these cholangiocytes become senescent and upregulate Bcl-xL for survival. Although senescent cells do not proliferate, they remain metabolically active and produce increased levels of cytokines and growth factors. Pro-fibrotic growth factors such as PDGF are key mediators of fibroblast activation. Persistent fibroblast activation is a hallmark of liver fibrosis. Activated fibroblasts show a reduction of pro-survival Bcl-2 and their survival becomes Bcl-xL-dependent. Since Bcl-xL is pivotal for the survival of both cell types, treatment of liver fibrosis with specific Bcl-xL inhibitors represents a potential dual therapeutic strategy.

Abbreviations: bd, bile duct; cv, central vein; ECM, extracellular matrix ha, hepatic artery; PDGF, platelet-derived growth factor; pv, portal vein; PSC, primary sclerosing cholangitis; ns, not significant