Therapeutic Effects of Deleting Cancer-Associated Fibroblasts in Cholangiocarcinoma

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Abstract

Cancer-associated fibroblasts (CAF) are abundant in the stroma of desmoplastic cancers where they promote tumor progression. CAFs are "activated" and as such may be uniquely susceptible to apoptosis. Using cholangiocarcinoma as a desmoplastic tumor model, we investigated the sensitivity of liver CAFs to the cytotoxic drug navitoclax, a BH3 mimetic. Navitoclax induced apoptosis in CAF and in myofibroblastic human hepatic stellate cells but lacked similar effects in quiescent fibroblasts or cholangiocarcinoma cells. Unlike cholangiocarcinoma cells, neither CAF nor quiescent fibroblasts expressed McI-1, a known resistance factor for navitoclax cytotoxicity. Explaining this paradox, we found that mitochondria isolated from CAFs or cells treated with navitoclax both released the apoptogenic factors Smac and cytochrome c, suggesting that they are primed for cell death. Such death priming in CAFs appeared to be due, in part, to upregulation of the proapoptotic protein Bax. Short hairpin RNA-mediated attenuation of Bax repressed navitoclax-mediated mitochondrial dysfunction, release of apoptogenic factors, and apoptotic cell death. In a syngeneic rat model of cholangiocarcinoma, navitoclax treatment triggered CAF apoptosis, diminishing expression of the desmoplastic extracellular matrix protein tenasin C, suppressing tumor outgrowth, and improving host survival. Together, our findings argue that navitoclax may be useful for destroying CAFs in the tumor microenvironment as a general strategy to attack solid tumors. Cancer Res; 73(2); 897–907. © 2012 AACR.

Introduction

Cancer progression is a complex, multifaceted process that involves not only intrinsic genetic changes unique to the malignant cell but also dynamic reciprocal communication between the cancer cell and stromal cells within the tumor microenvironment (1). As cancers progress, the stromal cells become reactive, phenotypically mimicking tissues undergoing wound healing or chronic inflammation. Indeed, many solid human cancers are characterized by desmoplasia, with high numbers of myofibroblasts (2). These myofibroblasts often termed cancer-associated fibroblasts (CAF), express tenasin C (Ten C), periostin, seprase, matrix metalloproteinases (MMP), and other proteins promoting tumor progression, invasion, and metastases (3–5). Notably, Ten C has been implicated in tumor progression by facilitating metastasis (6).

In liver, CAFs likely originate from hepatic stellate cells, although CAFs may also be derived from portal fibroblasts or potentially even from bone marrow–derived precursor cells (3). Activation of hepatic stellate cells not only converts these cells into matrix-producing myofibroblasts but also enhances their susceptibility to apoptosis analogous to the activation-induced cell death observed in T lymphocytes (7). For example, resolution of the wound-healing response in acute liver injury is associated with enhanced myofibroblast apoptosis, thereby reducing their absolute numbers in the liver (8). By analogy, one may predict that CAFs may also be uniquely sensitive to proapoptotic stimuli, suggesting that selective deletion of CAFs with proapoptotic therapies could potentially abrogate their support of cancer cells. This in turn would be expected to negatively affect tumor survival, growth, and progression. However, selectively targeting CAF as anticancer strategy remains largely unexplored.

Apoptosis is regulated by members of the Bcl-2 family of proteins, which control mitochondrial outer membrane permeabilization (MOMP; ref. 9). A proapoptotic subset of these proteins displays only a single Bcl-2 homology domain termed the BH3 domain. BH3-only proteins act as initiators of cell death by either promoting activation of the multidomain proapoptotic Bcl-2 proteins Bax and Bak (10) or by neutralizing the antiapoptotic Bcl-2 proteins (9). Given the ability of

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BH3-only proteins to initiate cell death pathways, the pharmaceutical industry has developed BH3-only protein mimetics such as navitoclax (ABT-263; ref. 11). This small organic molecule may exert single-agent activity in selected cells despite its inability to directly activate Bax or Bak. The direct cytotoxicity by a sensitizing BH3 mimic can be explained if the mitochondria of these cells are "primed" for cell death (12). In one model of priming, the mitochondrial antiapoptotic proteins are nearly saturated with activator BH3-only proteins. In this model, the BH3 mimic may displace and liberate preexisting bound activators, thus allowing them to trigger Bax or Bak oligomerization and MOMP. In an alternative model termed the "embedded together" model, antiapoptotic proteins inhibit not only BH3-only proteins but also Bax or Bak within the outer mitochondrial membrane (13, 14). In this latter model, BH3 mimetics may alter the competing equilibrium between the various Bcl-2-binding partners permitting Bax or Bak activation and MOMP.

Herein, we used cholangiocarcinoma as a model of a highly desmoplastic cancer to examine the role of proapoptotic signaling in targeting CAFs. Our results suggest that compared with quiescent fibroblasts and cholangiocarcinoma cells, CAFs are significantly more susceptible to cell death by navitoclax in vitro and in vivo. Moreover, our data indicate that mitochondria from CAFs appear to be primed for cell death. These mechanistic insights expand the role of proapoptotic therapies in cancer by suggesting that they may target the tumor microenvironment as well as the cancer cells.

Materials and Methods

Cell lines and culture

The human cholangiocarcinoma cell lines HuCCT-1 (15), Mz-Cha-1 (16), KMCH (17), and KMBC (18); the human hepatic myofibroblast LX-2 (19), quiescent human fibroblasts (hFB) kindly provided by V.H. Shah (Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN) and R.S. Bahn (Division of Endocrinology); the rat cholangiocarcinoma cell line BDEneu (20), BDEneu tumor-associated myofibroblasts (rCAF; ref. 21), and quiescent rat fibroblasts (rFB) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) under standard conditions. Cell lines were authenticated in June (HuCCT-1, MzCha-A, KMCH, KMBC) and July 2011 (LX-2) by Genetica DNA Laboratories using an AmpF/STR Identifier kit and GeneMapper v3.2 software. Three primary human cholangiocarcinoma-associated myofibroblasts cell lines (hCAF) were isolated and cultured. These cell lines were obtained in full compliance and approval of the Mayo Institutional Review Board. Briefly, liver cancer tissue was processed with the BD MediMachine (BD Biosciences). Disaggregated cells were cultured in RPMI medium supplemented with 5% PLTMax (Mill Creek Life Sciences; ref. 22). The hCAF cell lines were karyotyped to confirm a normal karyotype. Expansion media consisted of DMEM with 10% FBS, 100 U/mL penicillin, and 100 g/mL streptomycin.

Immunoprecipitation of Bcl-X<sub>L</sub> and associated BH3-only proteins

LX-2 cells were grown on 20-cm tissue culture dishes to subconfluency and treated with navitoclax (1 μmol/L) for 24 hours. Cells were subsequently lysed in cold CHAPS lysis buffer [1% CHAPS, 150 mmol/L NaCl, 20 mmol/L HEPES, 1% glycerol, 3% thioglycolic acid, 1 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 1 × protease inhibitor mix, 100 mmol/L sodium fluoride, 25 mmol/L microcystin]. Lysates were centrifuged for 15 minutes at 15,000 × g to pellet cellular debris. Protein concentration was determined via Bradford assay. About 400 μg of total protein was precleared by incubation with 40 μL agarose A/G beads (Invitrogen) for 1 hour at 4°C. Bcl-X<sub>L</sub> (clone N-20, Santa Cruz Biotechnology and control rabbit IgG (BD Pharmingen) crosslinked beads were prepared as previously described (23). About 40 μL of control IgG or Bcl-X<sub>L</sub> crosslinked beads were added to the precleared 400 μg of total lysate protein and incubated rotating overnight at 4°C. The beads were then pelleted by 2 minutes of centrifugation at 8,000 × g, washed 4 times in CHAPS lysis buffer, resuspended in sample buffer [4 mmol/L urea, 2% SDS, 62.5 mmol/L Tris-HCl (pH 6.8), 1 mmol/L EDTA, and 5% 2-mercaptoethanol], and boiled for 20 minutes to release immunoprecipitated proteins. The samples were centrifuged for 2 minutes at 8000 × g, and the supernatant was subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane as previously described.

Bax activation by navitoclax

Cells were grown on glass slides and treated with navitoclax or vehicle. Slides were then washed with PBS fixed for 30 minutes with 4% paraformaldehyde and permeabilized with 0.01% CHAPS in PBS. After a further washing, slides were incubated with a conformation-specific antibody against activated Bax 6A7 (1:100) overnight at 4°C, washed, and incubated with secondary antibody (goat-anti mouse Alexa 488 1:1,000; ref. 24).

Generation of stable transfectants

HEK 293T cells were transfected with pCMV-VSV-G (Addgene), pCMV-dR8.2 dvpr (Addgene), and the lentiviral shBax or shBak constructs (Open Biosystems, V2LHS_240441 and V2LHS_94682), respectively, using Lipofectamine LTX reagent (Invitrogen) to amplify the shBax or shBak containing lentivirus. Target LX-2 cells were grown to 50% confluence and infected with lentivirus-containing medium from the HEK 293T cells. Medium was previously passed through a 0.45-μm pore filter, and polybrene (Sigma Aldrich) was then added to a final concentration of 8 μg/mL. LX-2 cells were incubated with infectious medium for 3 hours before medium was replaced with fresh noninfected medium. Infection was again repeated 24 hours after the initial exposure. Infected LX-2 cells were split into selection medium containing 10 μg/mL puromycin. Cell lysates were prepared from shBax and shBak cells to confirm knockdown of Bax or Bak protein by Western blotting. Stably transfected shMel-1 KMCH clones were generated as previously described in detail (25). LX-2 cells were stably transfected...
with an S-peptide tagged Mcl-1 construct as previously described for HuH-7 cells (26). The S-tag results in a slight increase in molecular weight for Mcl-1, which can readily be identified by immunoblot analysis to verify stable expression of the protein.

**Syngeneic rodent model of intrahepatic cholangiocarcinoma**

All animal experimentation described in this study was carried out in accordance with and approved by the Institutional Animal Care and Use Committee. Syngeneic, in vivo cell transplantation was conducted in adult Fischer 344 male rats (Harlan) with initial mean body weights ranging between 180 and 230 g, as we have previously described in detail (27). Buprenorphine (0.05 mg/kg SubQ) was used for postoperative analgesia. For apoptosis studies, navitoclax (5 mg/kg) or vehicle was given intraperitoneally (i.p.) once daily for 2 consecutive days starting 7 days after tumor implantation. Twenty-four hours after receiving the second treatment, the rats were euthanized, and the livers were removed for analysis. For tumor size and metastasis analysis, navitoclax (5 mg/kg) or vehicle was given i.p. once daily for 10 consecutive days starting 7 days after tumor implantation. Animals were sacrificed at day 18, and the livers were removed for analysis. Survival studies were conducted as regulated by the Institutional Animal Care and Use Committee, and animals were euthanized according to defined endpoints (weight loss >25% of original body weight, disabilitating tumor mass, inability to reach food or water, moribund appearance).

**Statistical analysis**

Data represent at least 3 independent experiments using cells from a minimum of 3 separate isolations and are expressed as means ± SEM. Differences between groups were compared using 2-tailed Student t tests or χ² tests. Survival data were analyzed, and Kaplan–Meier graphs were generated using GraphPad Prism software 6 (GraphPad Software).

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**Figure 1.** Navitoclax induces apoptosis in human CAFs but not cholangiocarcinoma cells. Quiescent hFBs, human primary CAFs from 3 different patients with cholangiocarcinoma (hCAF 1, 2, and 3), and activated hepatic myofibroblasts (LX-2) were plated onto multiwell plates and grown to approximately 70% confluency. Cells were treated as indicated with increasing doses of navitoclax for 48 hours. Cells were analyzed for apoptotic nuclear morphology by 4′,6-diamidino-2-phenylindole (DAPI) staining and quantitation of apoptotic nuclei by fluorescence microscopy (A; mean ± SEM; n = 3; †, P < 0.01). hFB, hCAF, LX2, as well as the human cholangiocarcinoma cell lines HuCCT-1, Mz-ChA-1, KMCH, and KMBC were treated with navitoclax (1 µmol/L) or vehicle for 48 hours. Apoptosis was measured by DAPI staining with quantitation of apoptotic nuclei by fluorescence microscopy (B, top graph; mean ± SEM; n = 3; ‡, P ≤ 0.01) or fluorometric analysis of caspase-3/7 activity displayed as fold change compared with vehicle control (B, bottom graph; mean ± SEM; n ≥ 5; ‡, P ≤ 0.05).
Other materials and methods
Other materials and methods for PCR, cell death assays, immunoblot analysis, mitochondrial membrane depolarization, Smac release, and immunofluorescence are described in the Supplementary Materials and Methods.

Results
Navitoclax selectively induces myofibroblast apoptosis
CAFs are characterized by the expression of α-smooth muscle actin (α-SMA) as a hallmark of the myofibroblast phenotype and expression plus secretion of Ten C protein (4, 28). We confirmed α-SMA and Ten C and mRNA expression in hCAFs, human liver–derived myofibroblastic LX-2 cell line, and their shBAX- and shBAK-modified clones, as well as the absence of these markers in quiescent hFBs and human cholangiocarcinoma cell lines (Supplementary Fig. S1). Next, we examined the potential, single-agent, proapoptotic effects of navitoclax on hCAFs, LX-2, and hFBs (Fig. 1A). As assessed by both morphologic and biochemical criteria, navitoclax markedly induced apoptotic cell death in CAF and LX-2 cells, whereas quiescent fibroblasts were resistant to navitoclax cytotoxicity. To confirm that CAFs were in fact undergoing caspase-dependent apoptosis, the pan-caspase inhibitor QVD was used. QVD (5 mmol/L) effectively reduced navitoclax induced apoptosis in CAF cells (<5% apoptotic cells after 48 hours of 1 μmol/L navitoclax treatment; data not shown).

Figure 2. Activated hepatic myofibroblasts exhibit alterations in their Bcl-2 protein profile and are sensitized to navitoclax by Bax. Whole-cell lysates were prepared from quiescent hFBs, hepatic myofibroblasts LX-2, and hCAFs, as well as quiescent rat fibroblasts (rFB), rCAFs, and the malignant erbB-2/neu transformed rat cholangiocyte cell line (BDEneu) that is used in the described in vivo model of cholangiocarcinoma. Cell lysates were subject to immunoblot analysis of Bcl-2 proteins. Except where indicated by white lines, all lanes were adjacent on the membranes; in some cases, additional lanes originally run between those shown are omitted for clarity (A and B). All full-length blots/gels are presented in Supplementary Fig. S6. hFB, LX-2, and hCAF cells were grown to approximately 50% confluence on glass chamber slides and treated with navitoclax (1 μmol/L) for the indicated time. Cells were then analyzed by fluorescence microscopy using a conformation-specific antibody (6A7) against activated Bax. Bax-positive cells are plotted as percentage of all cells (C; mean ± SEM; n = 4; **, P ≤ 0.01). Wild-type LX-2 cells as well as stably transfected shBax and shBak LX-2 cells were treated with navitoclax (1 μmol/L, 24 hours), and apoptosis was assessed by DAPI staining and fluorescence microscopy (D, top graph; mean ± SEM; n = 4; **, P ≤ 0.01) as well as fluorometric measurement of caspase-3/7 activity (D, bottom graph; mean ± SEM; n = 6; **, P ≤ 0.01).
contrast to the myofibroblasts, human cholangiocarcinoma cells were relatively resistant to navitoclax-mediated cell death (Fig. 1B, top and bottom), although the HuCCT-1 cell line displayed a moderate increase in caspase-3/7 activity following exposure to navitoclax. The partial sensitivity of HuCCT-1 cells to navitoclax-induced apoptosis may be due to their enhanced Bax plus reduced Bcl-2 and Bcl-XL expression altering the complex balance of anti- and proapoptotic regulators (Supplementary Fig. S2). Finally, treatment of quiescent fibroblasts with TGF-β not only resulted in their activation, as observed by induction of α-SMA expression, but also sensitized these cells to navitoclax-induced apoptosis (Supplementary Fig. S3). Taken together, these data suggest navitoclax selectively induces apoptosis in CAFs as compared with cholangiocarcinoma cells.

**CAFs do not express Mcl-1 and upregulate Bax**

We next explored the potential mechanisms resulting in selective CAF sensitivity to navitoclax-mediated apoptosis. We first profiled CAFs for antiapoptotic multidomain Bcl-2 proteins (Bcl-2, Bcl-XL, and Mcl-1), proapoptotic Bcl-2 proteins (Bax and Bak), and BH3-only proteins (Bad, Bim, Bid, Noxa, and PUMA; Fig. 2A). Expression of these proteins was quite variable between the different cell lines. Interestingly, Mcl-1, a known resistance factor for navitoclax cytotoxicity (29), was minimally overexpression in LX-2 cells confers resistance to navitoclax-induced apoptosis (Supplementary Fig. S2). Finally, treatment of quiescent hepatic myofibroblast LX-2 cells (Western blotting for Mcl-1; A, inset) and cells were treated with navitoclax (1 µmol/L) or vehicle for 48 hours. Apoptosis was measured by 4′,6-diamidino-2-phenylindole (DAPI) staining and fluorescence microscopy (A, top graph; mean ± SEM; n = 3; **, P ≤ 0.01) or fluorometric analysis of caspase-3/7 activity displayed as fold change compared to vehicle control (A, bottom graph; mean ± SEM; n ≥ 5; ***, P ≤ 0.001). Mcl-1 was overexpressed in the activated hepatic myofibroblast LX-2 (Western blotting for Mcl-1; B, inset) and cells were treated with navitoclax (1 or 5 µmol/L) or vehicle for 48 hours. Apoptosis was assessed by DAPI staining and measurement of caspase-3/7 activity as described above (mean ± SEM; n = 3; **, P ≤ 0.01).

Navitoclax is incapable of directly inducing Bax activation (11, 31). Moreover, quiescent fibroblasts were also resistant to navitoclax cell killing despite failure to express Mcl-1. To
reconcile these observations, we postulated that CAF mitochondria were primed for cell death (32). Mitochondrial priming refers to the empiric observation that activated or transformed cells have a reduced threshold for BH3 peptide-or BH3 mimic-induced mitochondrial release of apoptogenic factors such as Smac and cytochrome c (33). For these studies, we used the LX-2 cells, given their ability to be easily trans-

![Image](image-url)

Figure 4. Navitoclax selectively induces release of mitochondrial proapoptotic factors in activated myofibroblasts. Heavy membranes enriched in mitochondria were isolated from LX-2 cells and incubated with navitoclax (10 μmol/L) or vehicle. Mitochondria were separated from supernatant by centrifugation and fractions were subject to immunoblot analysis to assay for Smac release from the mitochondria into the supernatant (A). LX-2 cells were treated with navitoclax (1 μmol/L) and separated into cytoplasmic and mitochondria-containing fractions (pellet—loading control) by differential centrifugation after selective digitonin permeabilization. Fractions were analyzed for Smac and cytochrome c by immunoblotting (B). Cytoplasmic- and mitochondria-containing fractions (pellet) of shBax and shBak LX-2 cells and human cholangiocarcinoma cell lines MzChA-1 and HuCCT-1 treated with navitoclax (1 μmol/L) are shown in C and D, respectively. Note in these short-term incubation studies, the pool of cytochrome c and/or Smac released was limited and did not significantly influence the amount of these proteins in the pellet. hFBs, LX-2, shBax LX-2, and Mz-CHA-1 cholangiocarcinoma cells were treated with navitoclax or vehicle for 24 hours. After tetramethylrhodamine methyl ester (TMRM, 100 nmol/L) loading, cells were analyzed for mitochondrial depolarization and subsequent loss of fluorescence by flow cytometry. TMRM fluorescence (excitation wavelength, 544 nm) intensity was measured in 50,000 cells (E).
Primed for cell death, the LX-2 cells, but not quiescent fibroblasts, nor cholangiocarcinoma cells underwent mitochondrial membrane depolarization following navitoclax treatment and this effect was abolished in shBax LX-2 cells (Fig. 4E). To further examine mechanisms responsible for this observed priming, mitochondrial-enriched heavy membrane fractions obtained from cholangiocarcinoma, LX-2, and hCAF cells were profiled for activator BH3-only proteins (Bid, Bim, and Puma), and Bax and Bak by immunoblot analysis (Fig. 5A). Increased Bax, Bid, and Bim were identified in the mitochondrial fraction of myofibroblasts but not cholangiocarcinoma cells. In accordance with other studies of priming (30), navitoclax treatment of LX-2 cells decreased Bim binding to Bcl-XL as examined by immunoprecipitation studies (Fig. 5B). These observations support the concept that the activated state of CAFs is associated with translocation of Bax and BH3-only proteins to mitochondria. The presence of Bim association with Bcl-XL in the presence of Bax on mitochondria supports an embedded together model that primes the cells for cell death by navitoclax (13).

**Navitoclax reduces tumor burden and metastasis in vivo**

To ascertain whether the effects of navitoclax on CAFs from cholangiocarcinoma observed in vitro may be translated to an in vivo model, we used a syngeneic, orthotopic rodent model of cholangiocarcinoma (34). First, cytotoxicity assays for the used BDeneu tumor cells and rCAFs previously isolated from orthotopic rat tumors were conducted analogous to the previous assays with human cells (Supplementary Fig. S4A and S4B). These studies showed that rat CAFs, similar to human CAFs, are more sensitive to navitoclax than the corresponding cholangiocarcinoma cells. The number of α-SMA–positive cells which were TUNEL-positive was increased compared with tumors from vehicle-treated animals (Fig. 6B) and was greater than the TUNEL-positive/CK7 cells. Consistent with this observation, there was also a net reduction in quantitative immunofluorescence staining for the tumor stroma markers α-SMA and Ten C (Fig. 6C). This observation was also confirmed by quantitative analysis of α-SMA and Ten C mRNA expression in the tumor samples (Fig. 6D). Finally, we examined the potential tumor-suppressing effect of navitoclax treatment in this model. In vehicle- and navitoclax-treated rats, tumor weight and metastases were examined after 10 days of treatment, initiated 7 days after tumor implantation. Mean gross tumor wet weight (Fig. 7A) and mean tumor: liver weight ratio (Fig. 7B) were reduced in the navitoclax-treated animals. Peritoneal metastases were also reduced in this group (Fig. 7C). The navitoclax treatment–associated tumor suppressive effects also translated into an improved animal survival (Fig. 7D). The morphometric analysis revealed an altered tumor composition after 10 days of treatment, namely, a reduction in the ratio of α-SMA- and Ten C–positive stroma to tumor area (Fig. 7E). Histologically, the tumors appeared less desmoplastic with larger contiguous areas of tumor cells rather than isolated tumor glands typical for cholangiocarcinoma (Fig. 7F). Finally, we were able to show Bax activation and loss of mitochondrial Smac in CAFs following navitoclax treatment for 48 hours, thereby suggesting the mechanisms of navitoclax-mediated CAF cytotoxicity in vivo parallels that observed in vitro (Supplementary Fig. S5). Taken together, these observations indicate that navitoclax reduces tumor burden and metastasis in vivo.
suggest navitoclax impairs tumor growth and metastasis by depleting the tumor stroma of CAFs, thereby reducing the extent of growth-supporting cholangiocarcinoma tumor stroma.

Discussion

The results of this study provide new mechanistic insights about therapeutic targeting of CAFs within the tumor microenvironment as an anticancer strategy. These data indicate that (i) liver CAFs display sensitivity to single-agent cell killing by the BH3 mimetic navitoclax; (ii) the sensitivity of CAFs to navitoclax is associated with enhanced Bax expression and mitochondrial priming for cell death; and (iii) targeting CAFs for cell death in the tumor microenvironment is therapeutic in a syngeneic rodent in vivo model of cholangiocarcinoma. These findings are discussed in greater detail below.
Navitoclax mimics the binding characteristics of the BH3-only protein Bad and like Bad does not bind to the anti-apoptotic protein Mcl-1 (14); indeed, Mcl-1 expression is a well-documented resistance factor for navitoclax (29). Given that cholangiocarcinoma expresses Mcl-1 (25), their resistance to navitoclax is not surprising. However, resistance to navitoclax is unlikely to be mediated solely by a single Bcl-2 protein given the complexity of the interactions between pro- and antiapoptotic members of this family. For example, the HuCCT cells displayed partial sensitivity to navitoclax despite Mcl-1 expression, likely due to their increased Bax plus reduced Bcl-2 and Bcl-XL expression. Alternatively, lack of Mcl-1 phosphorylation at Ser-64 in these cells could potentially explain the insufficiency of Mcl-1 in preventing navitoclax cytotoxicity (35).

The absence of Mcl-1 expression by CAFs was unexpected but partially explains their sensitivity to navitoclax-mediated apoptosis. The mechanism for silencing Mcl-1 expression by CAFs is beyond the scope of this study, given the complexity of Mcl-1 regulation (36). Because navitoclax cannot directly activate Bax or Bak, the absence of Mcl-1 expression is not sufficient to explain the sensitivity of CAFs to navitoclax killing. Indeed, quiescent fibroblasts were also resistant to navitoclax cytotoxicity despite their lacking of Mcl-1 expression. These observations suggest that CAFs may also be primed for cell death.

In one model, mitochondrial priming is considered to be a state in which the capacity of antiapoptotic Bcl-2 proteins to sequester proapoptotic BH3-only proteins or bind Bax/Bak is almost completely exhausted (32). In this situation, minimal
shifts of the Bcl-2 equilibrium can cause cells to rapidly enter apoptosis (37). By binding to the antiapoptotic Bcl-2 proteins Bcl-2 and Bcl-XL, navitoclax displaces sequestered BH3 proteins such as Bim from these antiapoptotic proteins and enables them to activate Bax and Bak, inducing apoptosis (14). Indeed, we were able to show Bim displacement from Bcl-XL following navitoclax treatment in our current studies. In an alternative model, coined the “embedded together” model, antiapoptotic proteins inhibit not only BH3-only proteins but also Bax or Bak all embedded within the outer mitochondrial membrane (13, 14). In this model, BH3 mimetics may alter binding between the various Bcl-2–binding polypeptides allowing Bax or Bak activation and MOMP. Our data are perhaps most consistent with an embedded together model as we observed that Bim and Bax were already associated with mitochondria under basal conditions in myofibroblasts. Following navitoclax treatment, Bim was released from Bcl-XL, suggesting that BH3 mimic altered the complex binding dynamics in the membrane in such a manner as to favor Bax activation and MOMP.

In our in vivo studies, despite the more prominent cytotoxic effects on CAFs than cholangiocarcinoma cells, navitoclax treatment was sufficient to markedly reduce tumor size. These observations support the concept that tumor stroma is crucial for tumor development and even metastasis (38). Quantitative depletion of CAFs from the stroma by navitoclax also reduced the expression of typical components of the tumor extracellular matrix, such as Ten C, a protein recently found to support cancer growth (6, 39, 40). Thus, therapeutic induction of CAF apoptosis can be coupled to a decrease in tumor-promoting components of the extracellular matrix. The noticeable reduction in metastasis might be a result of this depletion particularly of Ten C from the microenvironment, which has just recently been described as important matrix component of the metastatic niche (6, 41).

In this study, we used cholangiocarcinoma as a model of a highly desmoplastic cancer containing abundant CAFs and tumor-specific extracellular matrix (4). This cancer is similar to other desmoplastic cancers, such as prostate, breast, and pancreatic cancer, where tumor stroma contributes to tumor development and progression (42, 43). Thus, the results of this study not only are germane to cholangiocarcinoma but also may provide information relevant to desmoplastic cancers in general (2, 44). While cancer therapy targeting the cancer cells becomes increasingly more individualized, reflecting the multitude of genetic and cell biologic alterations an individual tumor may present, the stromal reaction of the organism appears more uniform across many different types of malignancies (45). This suggests the tumor stroma to be a viable and attractive target for combination anti-cancer therapy. Our results further suggest that CAF priming to select proapoptotic therapies targeting this cell population represents a novel anticancer strategy. In this regard, navitoclax may be a clinically relevant therapeutic agent.

Disclosure of Potential Conflicts of Interest
M.P. Gustafson and A.B. Dietz have patent applications regarding culture of primary human cells and A.B. Dietz holds shares in Millcreek Life Sciences. No potential conflicts of interest were disclosed by the other authors.

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