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BH3 mimetics as antifibrotic therapy: unleashing the mitochondrial pathway of apoptosis in myofibroblasts

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Highlights

- Persistent activation of scar-forming myofibroblasts drives non-resolving pathological fibrosis.
- Myofibroblasts are “primed for death” and “addicted” to antiapoptotic proteins to ensure survival.
- BH3 mimetic drugs unleash the mitochondrial pathway of apoptosis in primed for death myofibroblasts, reversing fibrosis in experimental models.
- BH3 profiling is a functional tool to predict sensitivity of myofibroblasts to BH3 mimetic drugs in patients with fibrotic disease.

Abstract

Organs and tissues in mammals can undergo self-repair following injury. However, chronic or severe tissue injury leads to the development of dense scar tissue or fibrosis at the expense of regeneration. The identification of novel therapeutic strategies aiming at reversing fibrosis is therefore a major clinical unmet need in regenerative medicine. Persistent activation of scar-forming myofibroblasts distinguishes non-resolving pathological fibrosis from self-limited physiological wound healing. Thus, therapeutic strategies selectively inducing myofibroblast apoptosis could prevent progression and potentially reverse established fibrosis in fibrotic diseases. In this Review, we discuss recent findings that have demonstrated that activated myofibroblasts, traditionally viewed as apoptosis-resistant cells, are actually “primed for death”. In this state, mitochondria of activated myofibroblasts are loaded with proapoptotic BH3 proteins, which creates a cellular “addiction” to individual antiapoptotic proteins to block prodeath signaling and ensure survival. This creates a novel therapeutic opportunity to treat organ fibrosis by inducing myofibroblast apoptosis with the so-called BH3 mimetic drugs, which have recently showed potent antifibrotic activities in experimental models. Finally, we
discuss the potential use of BH3 profiling as a functional tool to diagnose myofibroblast addiction to individual antiapoptotic proteins, which may serve to guide and assign the most effective BH3 mimetic drug for patients with fibrotic disease.

Introduction
Adult mammalian tissues can undergo self-repair following injury [1]. However, severe or chronic tissue injury results in the development of extensive scar tissue or fibrosis at the expense of organ regeneration [2]. Tissue fibrosis, characterized by excessive deposition and crosslinking of extracellular matrix (ECM) components, leads to loss of organ architecture, end-stage organ failure and ultimately death [3-7]. It is estimated that fibrotic disorders such as idiopathic pulmonary fibrosis (IPF), chronic kidney disease (CKD), liver cirrhosis, cardiac fibrosis and systemic sclerosis (SSc, scleroderma) account for 45 percent of deaths in the developing world [8]. Therefore, there is a high unmet clinical need for the development of safe and effective antifibrotic therapies capable of preventing and/or reversing the progression of organ fibrosis [9]. The initiation of organ fibrosis is thought to result from aberrant wound-healing responses to chronic or severe tissue injury, leading to activation of the effector cells in fibrosis, the myofibroblasts. Persistent activation of myofibroblasts distinguishes non-resolving pathological fibrosis from self-limited physiological wound healing [10]. The cellular and molecular mechanisms shifting the tissue repair program towards organ fibrosis are unclear, but certainly involve the activation of molecular pathways perturbing the biology of fibroblasts in the context of chronic or severe tissue injury [11]. Two fundamental mechanisms related to abnormal fibroblast biology have been linked to the development of tissue fibrosis: (a) sustained activation of myofibroblasts, and (b) failure of myofibroblasts to undergo apoptosis or revert to a quiescent phenotype [12-17].

Over the last two decades, tremendous efforts have been made to understand the molecular mechanisms controlling myofibroblast activation. These important studies have revealed several genes and pathways driving the activation of myofibroblasts, and have unveiled numerous therapeutic targets that are being currently tested in clinical trials for various
fibrotic diseases [17]. However, less is known about the pathways promoting myofibroblast persistence in fibrotic diseases, which likely include those blocking myofibroblast apoptosis or impeding their reversion to a quiescent phenotype, as these are two biological processes required for the resolution of the normal tissue repair program. In this Review, we discuss recent findings of the molecular mechanisms leading to myofibroblast persistence and discuss novel therapeutic strategies aimed at inducing myofibroblast apoptosis, which could not only prevent the progression of organ fibrosis but potentially reverse established fibrosis.

Myofibroblast persistence in tissue repair and fibrosis

Over 40 years ago, Giulio Gabbiani first identified myofibroblasts in the granulation tissue of healing wounds [18]. During the normal tissue repair program these cells orchestrate many aspects of the healing process, including the synthesis and remodeling of ECM components and contraction of the granulation tissue, leading to wound closure [19]. Once the wound is closed, myofibroblasts are no longer needed, and are predominantly eliminated by apoptosis, although other mechanisms such as reversion to a quiescent phenotype or senescence have also been described [12, 14, 16]. Therefore, the activation and elimination of myofibroblasts during tissue repair is a temporally and spatially regulated process [20]. Both biochemical and biophysical cues have been implicated in the activation of myofibroblasts during the normal tissue repair program. Perturbations in the duration and intensity of these signals have been shown to promote sustained activation and survival of myofibroblasts in the wound, leading to over-exuberant production of highly cross-linked ECM and development of fibrosis [17].

A major insight into the mechanisms by which myofibroblasts acquire a persistently profibrotic phenotype has been the discovery that matrix stiffness and mechanical stress in severely damaged tissues initiate a positive feedback loop between myofibroblasts and their surrounding microenvironment that perpetuates fibrosis [21, 22]. In this loop, increased matrix stiffness promotes mechanoactivation of myofibroblasts via mechanotransduction, a process in which cells transform mechanical cues into biochemical signals [23]. Stiffness-
activated myofibroblasts express alpha-smooth muscle actin (α-SMA), which further increases the contractile activities of these cells [24, 25]. In this hypercontractile state, mechanoactivated myofibroblasts contract and remodel the ECM, leading to further increases in matrix stiffness, completing this amplification loop. The precise pathways allowing this amplification loop to be terminated during physiological wound healing but to persist in progressive fibrotic disorders are still poorly understood. Previous studies have shown that reducing matrix stiffness, releasing mechanical stress, or inhibiting mechanotransduction pathways induce myofibroblast apoptosis or their reversion to quiescence [12, 26-32]. These important studies have provided new therapeutic avenues to promote myofibroblast apoptosis by forcing these cells out of the mechanical positive feedback loop maintaining their activated phenotype. We and others have recently demonstrated that interrupting mechanical signaling pathways induces apoptosis of myofibroblasts and reduces organ fibrosis in experimental models, thus restoring the normal tissue repair program [33-37]. In contrast, in nonactivated fibroblasts inhibition of mechanotransduction pathways does not induce apoptosis. These findings suggest that mechanoactivated myofibroblasts, traditionally viewed as apoptosis-resistant cells, depend on continuous mechanical signals for survival and persistence. The mechanistic basis for this phenomenon have however remained largely unknown. Here, we discuss recent findings that have described the importance of mitochondrial apoptotic priming as a critical determinant of myofibroblast fate.

**Primed for Death: a cellular state of activated myofibroblasts**

Matrix stiffening, commonly viewed as an end point of organ fibrosis, is now appreciated as a critical regulator of fibrosis progression by promoting myofibroblast activation and persistence [38]. As mentioned above, we and others have shown that inhibiting the ability of activated myofibroblasts to sense or respond to biophysical signals such as increased matrix stiffness induces their apoptosis. Thus, in fibrotic diseases increased matrix stiffness paradoxically also gives drugs that target mechanotransduction pathways such as focal
adhesion kinase (FAK) or Rho-associated protein kinase (ROCK) the ability to induce myofibroblast apoptosis, potentially widening the therapeutic index of these agents in fibrotic diseases. The cellular and molecular mechanism(s) responsible for this effect of increased tissue stiffness in myofibroblasts, however, had not been fully elucidated. Recent work from our laboratory indicates that this effect can be explained by relative differences in the “mitochondrial priming” between quiescent fibroblasts and activated myofibroblasts [39]. First defined by Anthony Letai and colleagues, mitochondrial priming measures the proximity of a cell to the apoptotic threshold [40-43]. Mitochondrial priming is determined by the relative balance between proapoptotic and antiapoptotic members of the BCL-2 family of proteins. We have shown that the phenotypic transformation of quiescent fibroblasts into activated myofibroblasts driven by matrix stiffness increases the mitochondrial priming of these cells, effectively pushing them closer to the apoptotic threshold. How myofibroblast mitochondrial priming is dynamically regulated by soluble pro-fibrotic factors derived from fibrogenic cell types such as macrophages or injured epithelial cells is an important open question that should be further investigated. In our recent studies, we have shown that mitochondria in stiffness-activated myofibroblasts, but not quiescent fibroblasts, are loaded with proapoptotic proteins, thus increasing their mitochondrial priming. In this “primed for death” state, stiffness-activated myofibroblasts are more prone to undergo apoptosis and become addicted to antiapoptotic proteins for their survival. As we will discuss in more detail below, pharmacological blockade of these antiapoptotic proteins rapidly unleashes the mitochondrial apoptotic pathway in myofibroblasts but not quiescent fibroblasts, providing a therapeutic window to selectively trigger myofibroblast death and reversion of tissue fibrosis without affecting normal healthy tissues.

The molecular basis of a cell’s mitochondrial priming rests on relative levels of proapoptotic and antiapoptotic proteins of the BCL-2 family, which is composed of at least 24 members [44]. Based on their function, the BCL-2 family of proteins is divided by specific function into four groups: (I) effectors (BAX and BAK), (II) activators (BIM and BID), (III) antiapoptotic proteins (BCL-2, BCL-XL, BCL-W, MCL-1 and BFL-1) and (IV) sensitizers (PUMA, NOXA,
BAD, HRK, BMF and BIK) (Figure 1A). A common feature of the structure of the BCL-2 family of proteins is the expression of one or more conserved BCL-2 homology (BH) domains, named BH1, BH2, BH3 and BH4 [45] (Figure 1B). Activator and sensitizer proteins solely express the homology domain BH3 and are therefore called BH3-only proteins; while effector and antiapoptotic proteins are multi BH-domain relatives. The relative abundance of sensitizer, antiapoptotic, activator and effector proteins, as well as their net interactions, direct cell fate. These interactions are governed by specific affinities between members of the BCL-2 family of proteins, which are differentially modulated by prosurvival and proapoptotic signaling pathways [46]. For example, exposure of cells to proapoptotic stimuli forces BH3-only activators bound to antiapoptotic proteins to leave those complexes and instead bind proapoptotic effectors, triggering apoptosis. This process of changing metaphorical dancing partners thus determines cell survival or death, and has been described as the “Dance of Death” [47]. The structural and molecular basis for the BCL-2 family “interactome” are still under investigation, but both functional and structural studies have shed light on the role of this complex interplay between proapoptotic and antiapoptotic proteins in both setting a cell’s apoptotic threshold and activating the mitochondrial pathway of apoptosis [48]. Briefly, mitochondrial apoptosis is initiated by proapoptotic effectors BAX and BAK, which triggers apoptosis by the formation of pores upon their homo-oligomerization, an irreversible step called mitochondrial outer membrane permeabilization (MOMP) [49]. Mitochondrial cytochrome c is released through these pores into the cytosol, promoting the activation of caspases and ultimately cell apoptosis [50] (Figure 1A). A major advance in the apoptosis field was the discovery of a step-wise mechanism controlling MOMP. In this model, BH3-only activator proteins BIM and BID bind (via their BH3 domains) and activate both BAK and BAX, triggering their oligomerization and subsequent release of cytochrome c [51-53]. Therefore, cell death via the mitochondrial apoptotic pathway requires expression of both activator and effector BCL-2 proteins. However, a cell can still prevent apoptosis and assure their survival even in the presence of these proteins. Biochemical and genetic studies have demonstrated that both activator and effector proteins can bind to and
be sequestered by multidomain antiapoptotic proteins, thus preventing their critical interaction and the initiation of MOMP [54-56]. Further, a group of proteins known as BH3-only sensitizers promote apoptosis by binding and blocking antiapoptotic proteins, thus releasing formerly bound proapoptotic activator and effector proteins [57]. The concept of BH3-only sensitizers functioning exclusively as indirect promoters of apoptosis is currently under debate, since some BH3-only sensitizers have recently been shown to directly activate BAX and BAK [58]. Moreover, recent in vitro studies have even questioned the requirement of BH3-only proteins to induce MOMP, as the authors were able to induce apoptosis in HCT-116 human cancer colon cells lacking all BH3-only sensitizer and activator proteins [59].

Taken together, these complex interactions demonstrate that the activation of the mitochondrial pathway of apoptosis is more than a simple binary yes/no decision. Instead, activation functions as a molecular rheostat in which the relative balance between pro- and antiapoptotic proteins reflects an integrated summary of pro- and antiapoptotic signals, ultimately instructing a cell to survive or undergo apoptosis. Thus, relative mitochondrial priming can be used to group cells by different cellular states (Figure 1C). Lack of expression of proapoptotic BH3-only activator and/or effector proteins leads to an apoptotic resistant phenotype, in which cells can not be killed via the mitochondrial apoptotic pathway. Low mitochondrial priming due to low expression of proapoptotic proteins likewise sets mitochondria far away from the apoptotic threshold, making cells highly resistant to apoptosis as well. Conversely, high expression of proapoptotic proteins results in high mitochondrial priming, a state called being “primed for death”. In this state, cells become dependent to one or more antiapoptotic proteins to sequester proapoptotic members and ensure survival. Genetic or pharmacological inhibition of antiapoptotic proteins results in the release of proapoptotic proteins, leading to cell death. Thus, pharmacological blockade of antiapoptotic factors can be exploited to kill primed for death cells such as cancer cells or activated myofibroblasts via the mitochondrial pathway of apoptosis.

Previous studies have shown upregulation of antiapoptotic proteins in cultured myofibroblasts isolated from patients with fibrotic disease, leading to a common
misconception that these cells are resistant to apoptosis [33, 60]. In contrast, stiffness-activated myofibroblasts undergo apoptosis when mechanosensitive FAK or ROCK signaling pathways are inhibited [33, 36]. Contrary, quiescent fibroblasts plated on soft substrates are resistant to apoptosis induced by inhibition of mechanical signaling pathways. The observation that stiffness-activated myofibroblasts depends on continual mechanotransduction suggests that mitochondrial priming is regulated during fibroblast-to-myofibroblast differentiation induced by matrix stiffness. Using BH3 profiling, a technique to measure mitochondrial priming (discussed in detailed below), we have recently demonstrated that matrix stiffness increases the mitochondrial priming of these activated cells [39] (Figure 2A). Expression of the proapoptotic BH3-only activator protein BIM is highly upregulated in stiffness-activated myofibroblasts, which become addicted to the antiapoptotic protein BCL-XL to sequester BIM and ensure survival. Genetic knockdown of BCL-XL induced BIM-dependent apoptosis of stiffness-primed myofibroblasts, while fibroblasts cultured on soft matrices showed low mitochondrial priming and remained resistant to apoptosis induced by BCL-XL knockdown. Together, these data indicate that expression of proapoptotic proteins such as BIM is increased during fibroblast-to-myofibroblast differentiation driven by matrix stiffness, a mechanism that would predispose myofibroblasts to be rapidly eliminated by apoptosis upon completion of the normal tissue repair program. Since stiffness-activated myofibroblasts are exquisitely sensitive to death upon removal of stiffness-induced prosurvival signaling [28], we hypothesize that in normal healing increased mitochondrial priming in activated myofibroblasts may provide an effective mechanism for their rapid elimination during the resolution of tissue repair, in which matrix stiffness decreases. Furthermore, dysregulation of this system may lead to persistence of activated myofibroblasts in injured tissues, leading to progressive tissue fibrosis. Therefore, understanding the molecular basis behind increased mitochondrial priming in activated myofibroblasts may ultimately prove quite rewarding in allowing development of novel anti-fibrotic therapies to selectively unleash the mitochondrial apoptotic pathway in myofibroblasts.
BH3 mimetics as antifibrotic therapy: unleashing the mitochondrial pathway of apoptosis in myofibroblasts

The realization that activated myofibroblasts become addicted to expression of antiapoptotic proteins to block mitochondrial prodeath signaling and ensure survival has paved the way to directly target these cells for apoptosis in cancer and fibrotic disease. We and others have recently demonstrated that targeted apoptosis of myofibroblasts not only prevents progression of organ fibrosis but also reverse established fibrosis in experimental models [39, 61, 62]. Here, we briefly review recent efforts aimed at triggering mitochondrial apoptosis in myofibroblasts by targeting antiapoptotic BCL-2 proteins with the so-called BH3 mimetic drugs (BH3 mimetics). BH3 mimetics are small molecules designed to disrupt the interaction between antiapoptotic and proapoptotic proteins. The structure of BH3 mimetics allows them to occupy a shallow-hydrophobic groove in antiapoptotic proteins, thus preventing these proteins from binding and blocking their proapoptotic relatives. Importantly, structural differences in this shallow-hydrophobic groove of different antiapoptotic proteins have led to the discovery and development of effective and specific inhibitors of antiapoptotic BCL-2 proteins [63-66]. The BH3 mimetics ABT-737 and its orally available analogue, ABT-263, also called navitoclax, display subnanomolar binding affinity for BCL-2, BCL-X₅ and BCL-W [64]. ABT-737 and ABT-263 have shown proapoptotic activities in primed cancer cells as a single agent in xenografts models [64]. Of note, we have recently shown that daily administration of navitoclax in a therapeutic treatment regimen reversed established dermal fibrosis through induction of myofibroblast apoptosis [39]. Mechanistically, ABT-263 displaces BCL-X₅ that is bound to BIM, allowing BIM to activate apoptosis, as observed in both stiffness-primed myofibroblasts and dermal fibrotic fibroblasts isolated from patients with scleroderma, an autoimmune fibrotic disease characterized by multiorgan fibrosis (Figure 2B). In Mdr2/- mice, a model of biliary liver fibrosis characterized by the presence in the liver of BCL-X₅-dependent myofibroblasts, treatment with the BCL-X₅-specific BH3 mimetic A-1331852 treated liver fibrosis by inducing myofibroblast apoptosis [61]. Similarly, ABT-263 showed proapoptotic effects on senescent lung myofibroblasts, major effector cells in idiopathic pulmonary fibrosis (IPF), a fatal aging-associated lung disease [67]. Persistent
fibrosis in IPF is characterized by the accumulation of senescent type II alveolar epithelial cells and apoptosis-resistant myofibroblasts. Intriguingly, ABT-263 has also been shown to reverse established lung fibrosis in mice via induction of apoptosis in senescent type II alveolar epithelial cells, indicating that BH3 mimetic drugs also have potent senolytic activities [62]. In this regard, ABT-263 has recently shown to rejuvenate mice in a mouse model of premature aging by clearing out senescent cells [68]. Together, BH3 mimetic drugs seem to have both fibrolytic and senolytic activities, which support the potential use of these drugs for the treatment of age-related fibrotic diseases. Clinically, the BH3 mimetic ABT-263 has already demonstrated therapeutic efficacy in hematological malignancies as both monotherapy and in combination with other cytotoxic agents [69-72]. However, in chronic lymphocytic leukemia dose-limiting thrombocytopenia and the demonstration that BCL-2 rather than BCL-XL is the ABT-263 target has led to the development of novel BCL-2-specific BH3 mimetics [73]. Treatment with the BCL-2-specific BH3 mimetic ABT-199 caused significantly less thrombocytopenia than ABT-263 treatment and has been recently approved for chronic lymphocytic leukemia in patients with a specific chromosomal abnormality [74].

The frequent observations that solid tumors and cancer-associated fibroblasts more often up-regulate BCL-XL than BCL-2, provides ongoing interest in using ABT-263 to target BCL-XL. The risk of thrombocytopenia is seen as manageable if the clinical efficacy of ABT-263 in solid tumors or fibrotic diseases, is high enough to leave a wide therapeutic window [73]. Furthermore, the oral bioavailability of ABT-263 should provide dosing flexibility to maximize clinical utility in the context of fibrotic disorders. However, as of yet the therapeutic efficacy of ABT-263 as a single agent or in combination regimens has not yet been clinically evaluated in patients with fibrotic diseases. Collectively, this compelling recent data demonstrates the potential efficacy of targeting activated myofibroblasts for apoptosis with ABT-263 in experimental models of fibrosis, suggesting that this drug could be a selective, safe and potent anti-fibrotic tool to reverse organ fibrosis.
BH3 profiling: a functional tool to predict responses to BH3 mimetics

The design of selective and effective antifibrotic therapies aiming at targeting only myofibroblast apoptosis requires a detailed understanding of the molecular basis behind a potential drug’s therapeutic index. Previous studies have shown that the ability of BH3 mimetics to induce cell apoptosis depends on that cell’s mitochondrial apoptotic priming [40-42]. Thus, the development of biomarkers able to rapidly assess mitochondrial priming would help predict cell sensitivity to apoptosis induced by BH3 mimetics. While genetic and mathematical models have been used to predict cell sensitivity to BH3 mimetics, BH3 profiling has emerged as a robust, functional, predictive assay that enables rapid assessment of mitochondrial priming in human cells and tissues [75, 76] (Figure 3A). In the BH3 profiling assay, single cell suspensions are treated with low concentrations of digitonin, which relies on a membrane’s cholesterol content to induce pore formation [77]. Since cell membranes have a higher content compared to mitochondria membranes, digitonin treatment induces a fast permeabilization of the cell membrane while leaving the mitochondrial membrane intact. After digitonin exposure, cells are exposed to a panel of synthetic BH3 peptides derived from the α-helical BH3 domains of BH3-only activators and sensitizers. These peptides then can diffuse into cell’s mitochondria and bind the various members of the BCL-2 family of proteins. These BH3 peptides mimic the proapoptotic activities of BH3-only activators and sensitizers including the induction of MOMP, which is determined in the BH3 profiling assay by measuring mitochondrial depolarization using the fluorescent dye JC-1 [78]. Thus, mitochondrial depolarization induced by BH3 peptides provides information above diverse aspects of the mitochondrial apoptotic pathway. For example, BH3 peptides derived from BH3-only activators BIM and BID directly bind and activate the proapoptotic effectors BAX and BAK, so the amount of these peptides needed to induce MOMP reflects both the expression levels of BAX and BAK and their functionality. The BH3 peptide derived from the BH3-only sensitizer PUMA, which binds and blocks all antiapoptotic proteins but cannot activate BAX and BAK, is used to directly measure overall mitochondrial priming (Figure 3B). Since all antiapoptotic proteins are blocked by the PUMA
BH3 peptide, the degree of MOMP induced by this peptide reflects the amount of freed proapoptotic activators and effectors in the studied mitochondria. Highly primed cells in which mitochondria are loaded with BIM and/or BID require low concentrations of PUMA BH3 peptide to induce MOMP, while a high concentration of the PUMA BH3 peptide is needed to induce MOMP in unprimed cells. Thus, by titrating PUMA BH3 peptide one can rapidly assess differences in mitochondrial priming between two cell populations. Further, by using BH3 peptides with specific affinities for antiapoptotic proteins, BH3 profiling can also identify which antiapoptotic protein(s) control cell survival. BAD BH3 peptide binds and blocks BCL-2, BCL-X<sub>L</sub>, or BCL-W, whereas HRK peptide binds only BCL-X<sub>L</sub>. The MS1 peptide specifically blocks MCL-1. Consequently, MOMP induced by these peptides can reveal the cellular reliance on specific antiapoptotic proteins.

Using BH3 profiling, we have recently shown that activated myofibroblasts from patients with the autoimmune fibrotic disease scleroderma are primed for death [39]. Of note, BH3 profiling identified different patient subgroups based on their BH3 profile, suggesting that BH3 mimetics targeting different antiapoptotic proteins may be required by different individual scleroderma patients (Figure 3C). In addition BH3 profiling of dermal fibroblasts in skin biopsies may help stratify scleroderma patients and facilitate individualized treatment with BH3 mimetics. BH3 profiling can not only assess mitochondrial priming and cell survival requirements using BH3 derived peptides but also predict apoptotic responses by direct measurement of MOMP induced by BH3 mimetic drugs. Previous studies have already shown that the extent of mitochondrial priming assessed by BH3 profiling predicts clinical responses to certain drug treatments, for example in anti-cancer therapies [75, 76, 79]. More recently, we have demonstrated that BH3 profiling similarly predicts the specific BH3 mimic that is most effective in fibroblasts from individual scleroderma patients, and consequently can be used to tailor the most effective antifibrotic therapy for each individual patient (Figure 3C). For example, we found that myofibroblasts from a subset of patients rely on BCL-X<sub>L</sub> for survival and are therefore highly sensitivity to apoptosis induced by ABT-263. In this subset of patients, the extent of cell apoptosis induced by ABT-263 treatment correlated dramatically
with the BH3 profiling measurements of MOMP induced by the BCL-X\textsubscript{L}-specific HRK peptide. This indicates that mitochondrial priming measured by BH3 profiling can be used as a tool to predict cell apoptotic response to BH3 mimetics in scleroderma patients. Interestingly, BH3 profiling also identified another subset of patients in which their primed myofibroblasts relied on both BCL-X\textsubscript{L} and MCL-1 expression for survival. ABT-263 was not able to induce apoptosis in the cells, indicating MCL-1 overexpression contributes to resistance to ABT-263-induced apoptosis in these cells. Importantly, pretreatment of these cells with a BH3 mimetic drug targeting MCL-1 makes these cells sensitive to apoptosis induced by ABT-263, indicating that therapeutic targeting of MCL-1 is a promising strategy to overcome resistance of myofibroblasts to apoptosis induced by ABT-263. Based on these findings, we envision a personalized medicine approach to fibrotic diseases such as scleroderma, in which myofibroblast BH3 profiling not only is used to stratify scleroderma patients into more homogeneous cohorts but also to predict and prescribe the most effective BH3 mimetic drug for patients with fibrotic disease.

**Conclusions and Perspectives**

Targeting myofibroblasts for apoptosis is emerging as a novel therapeutic strategy to reverse established fibrosis. We and others have recently demonstrated that inhibition of the antiapoptotic protein BCL-X\textsubscript{L} with BH3 mimetic drugs including ABT-263 or A-1331852 induces myofibroblast apoptosis and reverses fibrosis in mouse models, which could potentially translate into novel anti-fibrotic therapies for the treatment of human diseases such as scleroderma, idiopathic pulmonary fibrosis or liver cirrhosis. The pleiotropic role of BCL-X\textsubscript{L} in cell survival, however, has raised concerns about on-target adverse effects of its inhibition including dose-limiting toxicity of thrombocytopenia. The development of “smart” delivery vehicles such as targeted nanoparticles that can deliver drugs to specific tissues or cells will be able to maximize the therapeutic effects of BH3 mimetic drugs for the treatment of organ fibrosis. The use of smart nanomedicines could be a “game changer” and likely has multiple advantages: limited on-target adverse effects, reduced toxicity, controlled release.
and increased efficacy and selectivity over free drug form. Together, we envision a personalized medicine approach for the treatment of fibrotic diseases with BH3 mimetic drugs, in which BH3 profiling is used to identify and predict the most effective BH3 mimetic drug and smart delivery systems are used to specifically shuttle these drugs to myofibroblasts. Overall, targeted apoptosis of myofibroblasts with BH3 mimetic drugs is a promising therapeutic strategy for the treatment of fibrotic diseases; however, further research is needed to translate these preclinical studies into effective clinical therapies.

**Figure Legends**

**Figure 1. The BCL-2 family of proteins controls mitochondrial priming.** (A) Overview of BCL-2 family member interactions and control of the mitochondrial pathway of apoptosis. Mitochondrial apoptosis is induced through release of mitochondria-stored cytochrome c into the cytosol, which promotes the activation of effector caspases and ultimately cell death. Mitochondrial outer membrane permeabilization (MOMP) precedes cytochrome c release and is controlled by homo-oligomerization of the proapoptotic “effector” proteins BAX and BAK, which are activated by the proapoptotic “activator” proteins BIM and BID. “Antiapoptotic” proteins (BCL-2, BCL-XL, BCL-W, MCL-1 and BFL-1) can bind and sequester both activators and effectors, thereby preventing their interaction and the induction of MOMP. “Sensitizer” proteins are a distinct group of proteins that promote apoptosis by binding and blocking antiapoptotic proteins, thus releasing formerly bound proapoptotic activator and effector proteins. (B) Members of the BCL-2 family of proteins are classified based on their structural homology. The multi-domain group includes effectors and antiapoptotic family members, which share four homology regions (BH1-4). BH3-only proteins exclusively express the BH3 homology domain. Localization to mitochondria can be achieved via transmembrane (TM) domains. (C) Mitochondrial priming is determined by the relative balance between proapoptotic and antiapoptotic members of the BCL-2 family of proteins. Lack of proapoptotic activators and/or effectors leads to an apoptosis “resistant” phenotype.
“Unprimed” cells express low levels of proapoptotic proteins. Increased expression of proapoptotic proteins results in high mitochondrial priming and shifts a cell towards a “primed for death” state. In this state, cells become addicted to one or more antiapoptotic proteins to sequester proapoptotic members and ensure survival. Cell death occurs if the sequestration capacity of antiapoptotic proteins is exceeded by levels of proapoptotic proteins.

**Figure 2. BH3 mimetic drugs induce apoptosis of activated myofibroblasts. (A)** Fibroblast-to-myofibroblast differentiation induced by matrix stiffness increases the mitochondrial priming of these activated cells. (B) Myofibroblast activation associates with increased levels of proapoptotic activator BIM, thus priming these cells for death. In this state, myofibroblasts become “addicted” to the expression of antiapoptotic proteins such as BCL-X<sub>L</sub> to sequester BIM and ensure survival. The BH3 mimetic drug ABT-263 binds to BCL-X<sub>L</sub> and displaces BIM. Freed BIM then interacts with effector proteins BAX and BAK, activating the mitochondrial pathway of apoptosis in primed for death myofibroblasts. Nonactivated fibroblasts are not sensitive to ABT-263 due to their low expression levels of proapoptotic activators.

**Figure 3. BH3 profiling as functional tool to predict responses to BH3 mimetics in fibrotic diseases. (A)** Data acquisition. Patient-derived tissue biopsy is digested to obtain single cell suspensions. After cell membrane permeabilization and JC-1 staining, cells are exposed to different BH3 peptides derived from BCL-2 proteins. Peptide exposure and sample measurements via fluorescence is accomplished within 3 hours. (B) Schematic illustrating how BH3 peptides in BH3 profiling determine mitochondrial priming and cellular addiction to antiapoptotic proteins. BIM BH3 peptide directly binds to effector proteins, thus MOMP induction by this peptide provides information about expression and function of BAX and BAK. PUMA BH3 peptide inhibits all antiapoptotic proteins, thus MOMP induction by this peptide directly reflects the amount of freed proapoptotic activators and effectors. Specific
inhibition of individual proapoptotic proteins provides insight on cell's addiction to specific antiapoptotic proteins. (C) Schematic diagram of BH3 profiling in clinical practice. BH3 profiling can help to predict responses to BH3 mimetics and assign the most effective BH3 mimetic drug for patients with fibrotic disease.

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Keywords: Fibrosis, Myofibroblast, Apoptosis, BH3 mimetic, BH3 profiling

Abbreviations

ECM, extracellular matrix; SSc, systemic sclerosis or scleroderma; IPF, idiopathic pulmonary fibrosis; CKD, chronic kidney disease; α-SMA, alpha-smooth muscle actin; FAK, focal adhesion kinase; ROCK, rho associated protein kinase; BH, BCL-2 homology; MOMP, mitochondrial outer membrane permeabilization.

References


Figure 1

A

**ACTIVATORS**
- BIM
- BID

**EFFECCTORS**
- BAX
- BAK

**SENSITIZERS**
- BAD
- NOXA
- PUMA
- HRK
- MCL-1
- BCL-W
- BFL-1

- MOMP
- Cyt c
- Caspase 3/7
- Apoptosis

C

- “Resistant”
- “Unprimed”
- “Primed for Death”
- “Death”

- “Mitochondrial Priming”
  (Proximity to the apoptotic threshold)

B

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Figure 2

A Fibroblast

- Soft matrix

Mechanoactivation of fibroblasts by matrix stiffness

Mitochondrial Priming

Myofibroblast

- Stiff matrix

B Fibroblast Mitochondria

- + ABT-263

- Efector (BAX/BAK)

- Anti-Apoptotic (BCL-\(X_L\))

- Activator (BIM)

- ABT-263

Resistance

Myofibroblast Mitochondria

- + ABT-263

- ABT-263

Apoptosis

Figure 2
### Figure 3

**A. Data Acquisition**

- **Skin biopsy**
  - Digestion (45 min)
  - Permeabilization and JC-1 staining
  - Peptide Exposure
  - Readout and Analysis

**B. BH3 profiling analysis**

- HRK Peptide
- MS1 Peptide
- BAD Peptide
- BCL-2
- BCL-X_L
- BCL-W
- MCL-1
- BFL-1

- PUMA BH3 Peptide
- Anti-apoptotic
  - Activators
  - Effectors
  - MOMP
  - JC-1

**C. BH3 mimetic assignment**

- Clinical Evaluation
  - Patient A
  - Patient B
- Personalized Medicine