Pancreatic Cancer Combination Therapy Using a BH3 Mimetic and a Synthetic Tetracycline

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Abstract

Improved treatments for pancreatic cancer remain a clinical imperative. Sabutoclax, a small-molecule BH3 mimetic, inhibits the function of antiapoptotic Bcl-2 proteins. Minocycline, a synthetic tetracycline, displays antitumor activity. Here, we offer evidence of the combinatorial antitumor potency of these agents in several preclinical models of pancreatic cancer. Sabutoclax induced growth arrest and apoptosis in pancreatic cancer cells and synergized with minocycline to yield a robust mitochondria-mediated caspase-dependent cytotoxicity. This combinatorial property relied upon loss of phosphorylated Stat3 insofar as reinduction of activated Stat3-rescued cells from toxicity. Tumor growth was inhibited potently in both immune-deficient and immune-competent models with evidence of extended survival. Overall, our results showed that the combination of sabutoclax and minocycline was highly cytotoxic to pancreatic cancer cells and safely efficacious in vivo. Cancer Res; 75(11); 2305-15. ©2015 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an extremely aggressive cancer that is predicted to cause almost 40,000 deaths in the United States this year (NCI 2014). PDAC is fairly resistant to most standard therapies and results in a 5-year survival rate of about 4% (1). These dire statistics, combined with the fact that there have been minimal new therapies developed for PDAC over the last decade, highlight the need for new approaches to effectively treat this invariably fatal disease.

The aggressive nature and dismal prognosis of patients with pancreatic cancer results partly from the plethora of molecular changes that occur during PDAC development, one of which is overexpression of the antiapoptotic proteins of the Bcl-2 family (2–5). Cancer cells exploit this overexpression to evade cell death and as a mechanism promoting resistance to diverse chemotherapeutic agents. Apoptosis reflects a balance between pro- and antiapoptotic proteins within cells. The ability of a cancer cell to shift the balance toward survival promotes resistance to toxic factors (6).

Consequently, the antiapoptotic Bcl-2 proteins have emerged as a novel therapeutic target. Although multiple strategies have attempted to target these molecules, BH3 mimetics have shown significant promise. Proapoptotic or antiapoptotic effects in cells arise, ultimately, as a consequence of physical interactions between anti- and proapoptotic Bcl-2 proteins (7, 8). On the basis of modeling predictions of these interactions, small molecules have been developed that mimic the BH3 domain of proapoptotic proteins and bind to the antiapoptotic proteins, thereby impeding their ability to inhibit apoptosis. These drugs represent a novel and exciting new strategy in cancer therapeutic development (6).

Sabutoclax (BI-97C1) is a novel Apogossypol derivative BH3 mimetic developed by Wei and colleagues (9–12). This compound binds to the Bcl-2 antiapoptotic proteins Bcl-2, Mcl-1, Bcl-XL, and Bfl-1. It was originally identified on the basis of its ability to bind Bcl-XL with low to submicromolar binding affinity (11). We have previously shown that sabutoclax shows efficacy against prostate and colorectal cancers, two cancers that also overexpress antiapoptotic Bcl-2 proteins (13, 14).

Minocycline is a synthetic tetracycline antibiotic that displays marginal activity against multiple cancers (15–19). However, less than stellar outcomes have diminished enthusiasm for using these drugs in cancer research. The marginal single-agent effects of minocycline against cancer may be due to the fact that it also impedes cell death in the face of toxicity or injury by inhibiting mitochondrial apoptosis and upregulation of Bcl-2 (20–22). In an attempt to develop a unique therapeutic strategy for PDAC, we hypothesized that sabutoclax and minocycline might show therapeutic efficacy against this disease when used in combination because of both the reliance of PDAC on the Bcl-2 proteins for survival as well as the theoretical ability of sabutoclax to counteract the antiapoptotic effects of minocycline, thereby
uncovering the true therapeutic potential of this previously overlooked drug.

Materials and Methods

Human cell lines

MIA PaCa-2, Panc-1, BxPC-3, AsPC-1, and HPNE cells were all obtained from the ATCC. LT2 cells were purchased from Millipore. MIA PaCa-2 and Panc-1 were maintained in DMEM plus 10% FBS. BxPC-3 and AsPC-1 cells were maintained in RPMI plus 10% FBS. HPNE and LT2 cells were maintained with media according to the distributor’s instructions. Cell lines were expanded and cryopreserved at early passages and new vials were thawed out and used for experiments approximately every 3 months.

Creation of KPC mouse cell lines

Cell lines were derived from the ascites of tumor-bearing KPC mice. At the time of necropsy, ascitic fluid was collected from the mice and centrifuged to pellet tumor cells. The pellet was repeatedly washed in PBS and centrifuged before being resuspended in RPMI supplemented with 4% FBS and placed in culture. These media were used to maintain these cell lines.

Drugs and drug administration

For all in vitro studies, sabutoclax (produced by Dr. Maurizio Pellecchia, Sanford-Burnham Medical Research Institute, La Jolla, CA) was diluted in DMSO and minocycline (Sigma) in PBS. For combination treatments, sabutoclax and minocycline were administered to cells simultaneously. zVAD-FMK (20 μmol/L; Promega) was incubated with cells for 1 hour before treatment with sabutoclax and minocycline. Caspase-8–specific inhibitor, z-IETD–FMK (20 μmol/L; BD Pharmingen) was also incubated with cells for 1 hour before treatment with sabutoclax and minocycline.

Proliferation studies

A total of $5 \times 10^5$ cells were plated in 96-well plates and treated with sabutoclax and/or minocycline for 72 hours. Proliferation was assessed by MTT assay as previously described (23). All data were normalized to the control.

Cell death assays

For Trypan blue, $5 \times 10^5$ cells were plated in 6-cm dishes, treated as indicated for 48 hours, and then assayed as previously described (24).

LDH cytotoxicity assays

A total of $5 \times 10^5$ cells were plated in 6-cm dishes and treated as described. After 48 hours, media was collected from each dish and assayed according to the manufacturer’s instructions [Cytotoxicity Detection Kit (LDH); Roche].

Cell-cycle studies

A total of $1 \times 10^5$ cells were plated in 10-cm dishes and cultured in normal media with 0.5% serum for 48 hours. Cells for the zero hour time point were collected and fixed at this point. Remaining plates were kept in either normal media or 750 mmol/L sabutoclax for indicated time points. Once all cells were collected and fixed, they were incubated with propidium iodide and FACS was used for cell-cycle analysis. Cell-cycle studies were done as previously described (25).

Colonies-forming assay

MIA PaCa-2 cells were treated with sabutoclax (500 nmol/L), minocycline (50 μmol/L), or a combination of both for 24 hours. Cells were then trypsinized and 1,000 cells were plated into 6-cm plates in triplicate. Cells were allowed to grow and form colonies in normal media for approximately 14 days. Plates were then fixed and stained with Giemsa.

Western blotting

A total of $5 \times 10^5$ cells were plated in 6-cm dishes and treated as described previously. After 48 hours, whole-cell lysates were prepared and Western blotting analysis was carried out as previously described (24). Primary antibodies used for these studies are PARP (1:1,000), Stat3 (1:1,000), pStat3 (1:750), Mcl-1 (1:1,000), survivin (1:750), p21 (1:750), p27 (1:1,000), cyclin D1 (1:500), caspase-2 (1:1,000), caspase-3 (1:1,000), caspase-6 (1:1,000), caspase-7 (1:1,000), caspase-8 (1:1,000), caspase-10 (1:1,000), caspase-12 (1:1,000), AIF (1:1,000), pRB (1:750; Cell Signaling Technology), EF1-α (1:1,000; Millipore), and actin (1:5,000; Sigma). Densitometric analysis was done using ImageJ software (NIH).

Constructs and transfection

Stat3Y705F clones. MIA PaCa-2 or Panc-1 cells were transfected with a plasmid expressing a mutated form of Stat3 (pRc.CMV.Stat3Y705F; Addgene). Clones were selected with Neomycin for approximately 2 weeks and then picked and grown up individually. Whole-cell lysates were made and samples were used for Western blotting to characterize clones.

Luciferase clones. MIA PaCa-2 or Panc-1 cells were transfected with pGL3.CMV.luc (Promega). Transfections used Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

Immunohistochemistry

Tumors were fixed in formalin, embedded in paraffin, and sectioned for staining. Staining was done as previously described (26) with anti–p-Stat3 (1:100; Abcam), anti–PCNA (1:100; Abcam), and anti–Mcl-1 (1:100; Abcam) per the manufacturer’s instructions.

Combination index calculation

Combination index (CI) values were determined for the combination of sabutoclax and minocycline in MIA PaCa-2 cells. Values were calculated using CompuSyn software (ComboSyn, Inc.) according to the Chou–Talalay method.

In vivo studies

Subcutaneous xenograft studies A total of $5 \times 10^6$ MIA PaCa-2 and $3.5 \times 10^6$ Panc-1 cells were used to establish bilateral subcutaneous tumors on the flanks of 8- to 10-week-old male athymic nude mice. Studies were done as previously described (13). Treatment began when tumors reached approximately 100 mm$^3$. Sabutoclax was given at a dose of 1 mg/kg for both studies and was dissolved in a 10:10:80 solution of 100% ethanol:Cremophor:PBS. Minocycline was given at a dose of 20 mg/kg and dissolved in PBS. Both drugs were given via i.p. injection three times per week; n = 5 mice per group.
Quasi-orthotopic xenograft studies

Low tumor burden. In this model, we injected $1 \times 10^6$ MIA PaCa-2-luc cells i.p. into 8- to 10-week-old male athymic nude mice. We allowed 1 week for tumor cells to attach and grow and then began treatment with PBS, 1 mg/kg sabutoclax, 20 mg/kg minocycline, or both sabutoclax and minocycline. Sabutoclax and minocycline were given via i.p. injection 3× per week. Mice were imaged by bioluminescence imaging (BLI) at 3 weeks after treatment was initiated and then sacrificed at 4 weeks. At time of sacrifice, mice were imaged prenecropsy. After necropsy, organs from a few mice per group were imaged to determine tumor specificity; $n = 7$ mice per group.

High tumor burden. A total of $5 \times 10^6$ MIA PaCa-2-luc cells were injected i.p. into 8- to 10-week-old male athymic nude mice. We allowed 1 week for tumor cells to attach and grow and then began treatment with PBS, 1 mg/kg sabutoclax, 20 mg/kg minocycline, or both sabutoclax and minocycline. Sabutoclax and minocycline were given via i.p. injection 3× per week. Mice were sacrificed at 4 weeks. The pancreas from each mouse was removed and weighed during necropsy; $n = 10$ mice per group.

Syngeneic mouse study

KPC (Pdx-1-Cre/K-rasLSL-G12D/p53fl/fl) mouse cell line 48 was injected s.c. into both flanks of nontumorigenic KPC mice (Pdx-1-Cre negative/K-rasLSL-G12D/p53fl/fl). Tumors were given 1 week to establish before treatment was initiated. Mice were treated with PBS, 1.5 mg/kg sabutoclax, 10 mg/kg minocycline, or both sabutoclax and minocycline. Sabutoclax was dissolved in a 10:10:80 solution of 100% ethanol:Cre-morphor:PBS. Minocycline was dissolved in PBS. Mice were treated with sabutoclax, minocycline, or both drugs every 2 to 3 days via i.p. injection for a total of 6 injections. Tumors were measured with calipers to obtain tumor volumes; $n = 5$ mice per group.

Survival study

KPC mice (Pdx-1-Cre/K-rasLSL-G12D/p53fl/fl) were started on a sabutoclax and minocycline treatment regimen at 1 month of age. Mice were treated with a combination of sabutoclax (1.5 mg/kg) and minocycline (10 mg/kg) via i.p. injection three times per week. Mice were kept on this treatment until reaching a moribund status. At this point, mice were sacrificed and necropsied. Tumor sections were obtained from these mice and subjected to H&E. Mice were treated with either PBS, 1.5 mg/kg sabutoclax, 10 mg/kg minocycline, or both sabutoclax and minocycline. Sabutoclax was showed profound growth inhibition following sabutoclax treatment. Furthermore, sabutoclax displayed greater growth inhibitory effects than the BH3 mimetic ABT-737 (Supplementary Fig. S1).

Sabutoclax induces G1–S phase arrest

Sabutoclax induces cell death in MIA PaCa-2 cells, without promoting potent cell death in either AsPC-1 or PANC-1. Despite this, there is still a dramatic reduction in cell growth after drug treatment. To interrogate the mechanism of growth suppression in these cell lines, we performed cell-cycle analysis (Fig. 2A; Supplementary Fig. S2A–S2C). Sabutoclax increased MIA PaCa-2 sub-G1 phase after 24 and 48 hours (Fig. 2B), without affecting cell cycle, which is consistent with our previous data. In PANC-1 and AsPC-1, however, potent G1–S phase cell-cycle arrest was evident. These experiments only evaluated cells up to 48 hours posttreatment. A second set of experiments also included 72 and 96 hours time points (Fig. 2C, Supplementary Fig. S2D and S2E) to determine whether longer drug incubations promoted cell-cycle arrest or resulted in a switch to apoptosis. In AsPC-1, the G1–S phase arrest was sustained throughout all time points evaluated. However, at 72 and 96 hours, PANC-1 showed an increase in the sub-G1 population of cells, indicating that there is an initial cell-cycle arrest in these cells that later switches to apoptosis (Fig. 2D).

These results emphasize the complexity of responses observed in PDAC to a single agent, such as sabutoclax.

Evaluation of cell-cycle protein markers (Fig. 2E) confirmed decreased cyclin D1 and increased p27 expression in AsPC-1 and PANC-1 cells. In addition, there was a dramatic decrease in levels of phosphorylated Rb (Ser780). This corresponded with the observed G1–S phase arrest. Interestingly, although levels of p21 in AsPC-1 cells increased, p21 levels decreased in PANC-1 cells. This difference may account for the switch from growth arrest to apoptosis in these cells (27,28).

Sabutoclax synergizes with synthetic tetracycline, minocycline, in PDAC

It is now almost axiomatic that to successfully combat cancer multiple targeting strategies may be necessary. Cancer cells develop resistance to initially effective treatments and acquire avoidance mechanisms preventing toxicity. Accordingly, combinatorial approaches attacking multiple pathways in a cancer cell can increase drug efficacy, reduce toxicity, and increase the time to resistance. On the basis of this concept, we sought to identify an agent that would promote synergy when combined with sabutoclax. Minocycline, a commonly used antibiotic, can negatively affect cancer growth and survival (15–19). Despite this, it paradoxically also protects cells in the face of an insult through inhibition of mitochondrial apoptosis and upregulation of Bcl-2 (20–22). For these reasons, we hypothesized that these drugs might work well in combination, given their cancer-selective toxicities as single agents and the potential of sabutoclax to counteract the prosurvival effects of minocycline.
Minocycline is fairly nontoxic to several PDAC cells (MIA PaCa-2 and PANC-1), but is inhibitory in others (AsPC-1 and BxPC-3; Supplementary Fig. S3). In resistant cells, the combination of sabutoclax and minocycline is very toxic. It significantly reduces cell proliferation and overall cell number, and induces cell death to a much greater extent than either agent alone in MIA PaCa-2 (Fig. 3A and Supplementary Fig. S4). CI values also demonstrate synergy between these two compounds (Supplementary Fig. S5). It also promotes increased apoptosis and reduced colony formation in PDAC cells. Importantly, this synergistic effect is not evident in the normal cell line, HPNE (Fig. 3E-G).

We next sought to define the mechanism underlying this synergy. The combination of subtoxic doses of sabutoclax and minocycline induced potent cell death that was partially abrogated by pretreatment with zVAD, indicating that this toxic effect is, in part, caspase mediated. This rescue phenotype is evident in the morphology of treated cells (Fig. 4A; Supplementary Fig. S4C) and through reductions in LDH activity, indicating lower levels of cell death (Fig. 4B; Supplementary Fig. S4B). Furthermore, PARP cleavage was reduced in cells pretreated with zVAD (Fig. 4C; Supplementary Fig. S4A). When specific caspases were evaluated, the combination treatment showed caspase-3 cleavage and loss of full-length caspase-8, which was not found in zVAD-treated samples.

The cytotoxic effect of sabutoclax and minocycline is Stat3 dependent

Stat3 activation is clinically relevant for PDAC as constitutive activation of Stat3 has been reported in 30% to 100% of human tumor specimens, and is crucial for PDAC initiation, progression, and maintenance (29). Sabutoclax treatment resulted in a potent loss of Stat3 phosphorylation (Tyr 705), as well as a loss of downstream Stat3 targets, such as survivin (Fig. 4D). Minocycline alone induced similar changes (Fig. 4D). Subtoxic doses of each drug lowered Stat3 activation, but the combination of both drugs almost completely eliminated pStat3 expression (Fig. 4E). To determine whether this would affect the cytotoxicity of the combination, we created MIA PaCa-2 clones stably expressing an activated Stat3 mutant, Stat3 Y705F, for example, C 13 (Fig. 4F). As compared with the parental cell line, C 13 showed enhanced resistance to combination treatment (Fig. 4G). A similar elevated resistance was observed in other Stat3 Y705F-overexpressing MIA PaCa-2 clones (data not shown). Because pretreatment with zVAD

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**Figure 1.**
Sabutoclax inhibits cell growth and induces apoptosis in pancreatic cancer cells. A, sabutoclax dose–response curves via MTT proliferation assays in pancreatic fibroblast cell line, LT2, and pancreatic cancer cell lines PANC-1, AsPC-1, MIA PaCa-2, and BxPC-3. Experiments were done in triplicate and were independently repeated three times. B, sabutoclax dose–response curve via MTT proliferation assay in pancreatic epithelial-derived cell line, HPNE. Experiments were done in triplicate and were independently repeated three times. C, Western blotting for PARP and EF1-a (loading control). Data representative of three independent experiments. D, Trypan blue assays in AsPC-1, PANC-1, and MIA PaCa-2; **P < 0.001 as compared with untreated (UT) sample. Experiments done in triplicate and independently repeated three times.
Sabutoclax causes a G1–S phase cell-cycle arrest. A and C, cell-cycle analysis. B and D, the percentage of cells analyzed in the sub-G1 phase, indicating cell death. E, Western blotting of whole-cell lysates for cyclin D1, cyclin E, p27, p21, and phospho-Rb. EF1-α was used as a loading control. Data representative of three independent experiments.

BH3 Mimetic and Tetracycline Therapy for Pancreatic Cancer

Sabutoclax alone reduces tumor growth in a xenograft model of pancreatic cancer and this effect is enhanced with the addition of minocycline

Luciferase-expressing MIA PaCa-2 cells, displaying a similar phenotype as parental cells (Fig. 5A), were established as subcutaneous xenografts in athymic nude mice and were treated with sabutoclax (1 mg/kg), minocycline (20 mg/kg), or both agents. Minocycline treatment alone showed minimal effects, whereas sabutoclax alone showed greater reductions in tumor weight and growth (Fig. 5B and C). Despite this, animals treated with a combination of sabutoclax and minocycline showed a synergistic reduction in tumor growth, with a significantly smaller tumor size (Fig. 5D).

Sabutoclax and minocycline induce a cytotoxic phenotype through activation of the intrinsic pathway of apoptosis.

Caspase-8 activation is an integral part of the extrinsic pathway of apoptosis. However, it can also be activated independent of this pathway by other caspases (30). To determine whether caspase-8 involvement was due to extrinsic pathway activation or downstream intrinsic pathway activation, we used Z-IETD–FMK to specifically inhibit caspase-8 before drug treatment. Unlike the pan-caspase inhibitor, zVAD, pretreatment with Z-IETD–FMK did not protect cells from sabutoclax- and minocycline-induced cell death (Fig. 4I; Supplementary Fig. S6A). This indicates that caspase-8 activation is dispensable for sabutoclax- and minocycline-induced cell death and is most likely a secondary effect of other caspases. Therefore, our data support the hypothesis that sabutoclax and minocycline induce a cytotoxic phenotype through activation of the intrinsic pathway of apoptosis.

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The combination of sabotoclax and minocycline produces synergistic cytotoxic effects. A, MTT proliferation assay in MIA PaCa-2 cells 72 hours posttreatment. Experiments were done in triplicate and were independently repeated three times. B, Trypan blue assays evaluating cell death and cell numbers in MIA PaCa-2 cells 48 hours posttreatment; **, P < 0.0001. Experiments were done in triplicate and were independently repeated three times. C, Western blotting of whole cell lysates for PARP expression in MIA PaCa-2 cells 48 hours posttreatment. Actin was used as a loading control. Data representative of three independent experiments. D, colony-forming assay in MIA PaCa-2 cells. Experiments were done in triplicate and were independently repeated three times. E, Western blotting for PARP using whole-cell lysates from HPNE cells 48 hours posttreatment. Data representative of three independent experiments. F, MTT proliferation assays in HPNE cells 72 hours posttreatment; **, P = 0.0001. Experiments were done in triplicate and were independently repeated three times. G, LDH cytotoxicity assay in pancreatic epithelial cell line, HPNE. Experiments were done in triplicate and were independently repeated three times.

-growth rate, even as compared with sabotoclax alone (Fig. 5C). This was confirmed with BLI (Fig. 5D). A similar experiment was conducted using PANC-1-luc cells, resulting in the same trend, with the combination group showing significant inhibition of tumor growth (Supplementary Fig. S7).

IHC of tumor sections (Fig. 5E) demonstrated higher intensity PCNA staining in control-, sabotoclax-, and minocycline-treated groups as compared with the combination-treated group. Furthermore, combination-treated tumors showed significantly less phosphorylated Stat3 expression, consistent with in vitro observations.

Quasi-orthotopic xenograft mouse model

Although subcutaneous xenograft studies are useful in evaluating in vitro observations in an in vivo setting, flank tumors do not accurately mimic natural disease states. To study the effect of our drugs in a more natural setting, we used a quasi-orthotopic model of PDAC (31). We found that MIA PaCa-2-luc cells, when injected i.p., specifically homed to the pancreas, with the majority of tumors or tumor nodules found in this organ. Other locations in which we found tumors (liver, peritoneal lining) are common places for this cancer to metastasize and more closely mimic the clinical picture of this disease.

In an initial set of experiments, we injected $1 \times 10^6$ cells i.p. and allowed 1 week for tumor establishment before treating mice with sabotoclax and minocycline, alone or in combination. Consistent with our previous data, minocycline as a single agent did not have any effect and mice presented similarly to control animals. Sabutoclax showed a potent single-agent effect, with fewer animals showing evidence of disease. The combination group showed even more promise (Fig. 6C), with only 1 of 7 mice showing evidence of disease. The combination group showed evidence of disease.

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significant differences, though the trend supported our other observations (data not shown).

A second quasi-orthotopic study was conducted using similar drug-dosing parameters. Tumors, though, were initiated with the injection of $5 \times 10^6$ cells as opposed to $1 \times 10^5$. In our first study, the use of fewer cells allowed testing whether sabutoclax and minocycline could prevent tumor formation. However, the use of fewer cells also means that the overall tumor burden was less when therapy was initiated, which prevented determining the true magnitude of the effect sabutoclax and minocycline would have in the context of a greater tumor burden, which might more closely imitate the clinical setting.

As anticipated, injecting a greater number of cells resulted in a larger overall tumor burden in the animals. Mice from all groups...
developed tumors or tumor nodules in the pancreas. However, the weight of the pancreas from mice treated with both sabutoclax and minocycline was significantly less than those from animals in all three other groups (Fig. 6D).

**Sabutoclax and minocycline in a transgenic mouse model of PDAC (KPC)**

The KPC transgenic PDAC mouse model is used frequently in PDAC research (32–36). This animal spontaneously develops precursor pancreatic lesions, PanINs, which progress and eventually develop into invasive disease. Tumors form as a result of activated K-ras and functional loss of p53 in the pancreas, genetic changes also seen in a high percentage of human tumors. Histologic analysis shows extensive local invasion as well as metastasis in a subset of animals (37).

Pancreatic tumors from these mice overexpress Mcl-1 as shown by IHC (Supplementary Fig. S8A). This is consistent with the disease observed in humans (2–5) and provides a rationale for using sabutoclax in these animals. In addition, cell lines derived from these animals show combination effects with sabutoclax and minocycline (Supplementary Fig. S8B). These cells were injected s.c. into the flanks of control KPC mice (Pdx-1-Cre-negative/K-rasLSL-G12D/p53lox/wt) and allowed to grow for approximately 1 week. Mice were treated with sabutoclax, minocycline, or both drugs every 2 to 3 days via i.p. injection for a total of 6 injections. Sabutoclax or minocycline alone did not significantly affect tumor growth. The combination of drugs, however, significantly inhibited tumor growth as compared with controls (Fig. 6E). IHC of tumor sections demonstrated decreased staining for PCNA and pStat3 in combination-treated tumors (Fig. 6F and G).

In addition, a survival study was done using KPC mice (Pdx-1-Cre-negative/K-rasLSL-G12D/p53lox/flox) to determine the effects of sabutoclax and minocycline. Mice were treated with sabutoclax (1 mg/kg), minocycline (10 mg/kg), or a combination of both three times a week via i.p. injection starting at around 1 month of age and continuing until animals reached a moribund state. Mice receiving combination treatment showed a significant survival

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**Figure 5.** Sabutoclax reduces tumor growth in a subcutaneous xenograft model and is enhanced by the addition of minocycline; n = 5 mice/group. A, MTT proliferation assay of MIA PaCa-2 cells stably expressing luciferase after treatment with sabutoclax and/or minocycline; ***: P < 0.0001. Experiments were done in triplicate and were independently repeated three times. B, tumor weight as normalized to the control animals at the end of the experience. C, tumor growth kinetics of MIA PaCa-2-luc cell subcutaneous tumors on the flanks of athymic mice; **: P < 0.04 as compared with all other groups. D, BLI of tumors and image quantification; exposure time, 5 seconds. E, tumors were fixed in formalin, embedded in paraffin, and sectioned for staining. Representative images of IHC stained with p-Stat3 Y705 and PCNA. Arrows in PCNA images show margin of negatively stained tumor area at the periphery of each tumor. Data representative of three independent experiments.
advantage as compared with control mice (Fig. 6H). Tumors from these animals were isolated, sectioned, and stained for pStat3Y705. In correlation with our other studies, tumors from animals treated with sabutoclax and minocycline showed significantly less staining as compared to control tumors (Supplementary Fig. S9). Importantly, no toxicity was seen from either drug in any of the animals studies conducted.

Discussion

Pancreatic cancer is one of the most lethal cancers remaining largely untreatable. Moreover, advances in therapy have been minimal over the last 15 years, due in part to the aggressive nature of PDAC and the difficulty in developing selective and effective therapeutics. We presently describe an efficacious novel drug combination for PDAC that uses a new BH3 mimetic and uncovers the hidden therapeutic potential of minocycline as an anticancer agent.

Considering overexpression of the antiapoptotic Bcl-2 proteins in PDAC, we initially evaluated the efficacy of sabutoclax, a BH3 mimetic that targets these antiapoptotic proteins inhibiting their function. Sabutoclax was effective as a single agent. It induced cancer-inhibitory effects in multiple genetically diverse PDAC cell lines. Potent apoptosis-inducing activity was evident in some PDAC cell lines and in those that only showed minor increases in death, we found instead a potent cell-cycle arrest. Tumor heterogeneity is a problem manifested to some degree in all cancers, but is especially relevant in PDAC, contributing to the global resistance seen in this cancer to conventional chemotherapeutics. A beneficial aspect of sabutoclax is its anticancer activity in multiple PDAC cell lines, irrespective of genetic background.
Despite the potential of sabutoclax as a single-agent, there is a pressing need for combination therapy in the clinical setting. Cancer is an adaptive disease and effectively combating it requires a multifaceted approach. Because of this, we sought to find a second drug that would potentially synergize with sabutoclax to further promote its clinical applicability. We focused on the antibiotic, minocycline, which has a small literature base supporting a novel role for this drug in the treatment of cancer 

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In summary, we describe an innovative combinatorial therapeutic approach with remarkable activity against pancreatic cancer cells in vitro and in vivo in three animal PDAC models. Considering the paucity of effective therapies for PDAC, it is clear that new approaches are mandatory to impact clinically on this invariably fatal cancer. The ability to target the Bcl-2 family for inactivation, using sabutoclax, and combining this with a simple synthetic tetracycline antibiotic, such as minocycline, opens up new areas of research with the potential to lead to an effective therapy for pancreatic cancer.

Disclosure of Potential Conflicts of Interest
Maurizio Pellechia has ownership interest (including patents) in AncoreX Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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