Small Molecule Inhibitor YM155-Mediated Activation of Death Receptor 5 Is Crucial for Chemotherapy-Induced Apoptosis in Pancreatic Carcinoma

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Abstract

Despite much effort, pancreatic cancer survival rates are still dismally low. Novel therapeutics may hold the key to improving survival. YM155 is a small molecule inhibitor that has shown antitumor activity in a number of cancers by reducing the expression of survivin. The aim of our study is to understand the mechanisms by which YM155 functions in pancreatic cancer cells. We established the antitumor effect of YM155 with in vitro studies in cultured cells, and in vivo studies using a mouse xenograft model. Our data demonstrated that YM155 reduced the expression of survivin; however, downregulation of survivin itself is insufficient to induce apoptosis in pancreatic cancer cells. We showed for the first time that treatment with YM155 increased death receptor 5 (DR5) expression in pancreatic cancer cells. We found that YM155 induced apoptosis by broad-spectrum inhibition of IAP family member proteins (e.g., CIAP1/2 and FLIP) and induced proapoptotic Bak protein upregulation and activation; the antitumor effect of YM155 treatment with either the DR5 agonist lexatumumab or gemicitabine on pancreatic cancer cells was synergistic. Our data also revealed that YM155 inhibits tumor growth in vivo, without apparent toxicity to the noncancerous human pancreatic ductal epithelial cell line. Together, these findings suggest that YM155 could be a novel therapeutic agent for pancreatic cancer.

Introduction

Pancreatic cancer has a 5-year survival rate of less than 5% and is the fourth leading cause of cancer-related death in the United States (1, 2). Recent increases in patient survival are due to improvements in surgical procedures and the development of adjuvant chemotherapy (3). Only 20% of patients with pancreatic cancer have operable tumors and only 15% to 20% of those patients survive (2). New and effective systemic chemotherapeutic strategies are urgently needed for the treatment of advanced stage pancreatic cancer.

Apoptosis plays an important role in the maintenance of homeostasis, and elimination of damaged cells in multicellular organisms (4). Evasion of apoptosis is a characteristic feature of cancer (5). Currently, induction of apoptosis is one of the most attractive strategies for cancer therapy (6). Of note, TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) is a member of the TNF superfamily able to specifically induce programmed death in tumor cells without causing toxicity to normal healthy tissue is a very promising concept for cancer therapy including pancreatic cancer, where there is a clear unmet need (9, 10).

YM155 (sepantronium bromide) is a novel small molecule (chemical structure, see Fig. 1A) that has been reported to specifically suppress survivin expression at the transcriptional level (11–13). YM155 shows potent antitumor activity in non–small cell lung carcinoma (NSCLC) xenograft models, and induces in vivo antitumor activity without systemic toxicity in mice. Patient clinical trials also suggest beneficial applications of YM155 (14, 15). YM155 sensitizes tumors to radiation and other chemotherapeutics such as platinum compounds or taxanes to induce apoptosis in human NSCLC (16, 17). YM155 is also a broad-spectrum antitumor agent among a wide variety

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of human cancer cell lines (11). It has been previously reported that YM155 induces apoptosis in pancreatic cancer cells, but the molecular mechanisms have yet to be fully elucidated (18, 19). Recognizing that YM155 may be acting as a broad-spectrum antitumor agent, the current study sought to characterize the effects of YM155 on pancreatic cancer cells, and to identify the molecular pathways involved, by the use of cell culture models of pancreatic cancer and a murine xenograft model. The results of our study reveal that YM155-induced apoptosis is associated with DR5 upregulation and Bak activation; YM155 enhances the therapeutic effect of either lexatumumab or gemcitabine in a synergistic manner; YM155 exhibits tumor growth inhibition in vivo, but has no significant toxicity to normal pancreatic cells.

Materials and Methods
Cell culture and reagents
Human pancreatic cancer cells Panc-1, PC-3, and immortalized human pancreatic ductal epithelial (HPDE) cells were provided by Dr. Steve Hochwald (Roswell Park Cancer Center, Buffalo, New York, NY) in April 2010. Pancreatic cancer cells and HPDE cells were authenticated by using short tandem repeat analysis and amelogenin analysis. Panc-1 and PC-3 cells were grown in DMEM with 10% FBS (Sigma) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37°C in 5% CO2. HPDE cells were grown in keratinocyte serum-free media and supplemented with bovine pituitary extract and EGF as per the manufacturer’s instructions (Life Technologies). Anti–caspase-9, anti–caspase-8, anti–caspase-3, anti-Bid, anti-PARP, anti-FLIP, anti-CIAP1/2, anti-survivin, anti–Bcl-2, anti–Mcl-1, anti-Bak, anti-Bim, anti-DR5, and anti-DR4 primary antibodies were obtained from Cell Signaling Technology; anti–β-actin monoclonal antibody, Hoechst 33258 staining (cells exemplifying apoptotic nuclei are demarcated by white arrows). Panc-1 cells were treated with YM155 (10 nmol/L) for an additional 24 hours or not, control cells had neither YM155 treatment nor transfection with siRNA. Apoptosis was assessed by Hoechst 33258 staining (cells exemplifying apoptotic nuclei are demarcated by white arrows), E, Panc-1 cells were treated as in C, and the ratio of apoptotic cells was assessed by counting the number of cells with apoptotic nuclei. Each experiment was conducted in triplicate and repeated twice independently (*, P < 0.05). F, Panc-1 cells were treated as in C. Apoptosis was assessed by a DNA ladder assay. G, Panc-1 cells were treated as in C and cell lysates were prepared for Western blotting to detect survivin and cleaved caspase-3. β-Actin was assessed as the control for equal loading of protein.

Figure 1. Survivin downregulation is not sufficient to trigger apoptosis. A, chemical structure of YM155. B, Panc-1 cells were treated with YM155 and cell lysates were prepared for Western blotting to detect survivin. β-Actin was assessed as the control for equal loading of protein. C, Panc-1 cells were transfected with either survivin-specific siRNA or scramble-siRNA as negative control. Forty-eight hours after transfection, cell lysates were prepared for Western blotting to examine survivin. β-Actin was assessed as the control for equal loading of protein. D, Panc-1 cells were initially transfected with survivin-specific siRNA. Forty-eight hours after transfection, cells were either treated with YM155 (10 nmol/L) for an additional 24 hours or not, control cells had neither YM155 treatment nor transfection with siRNA. Apoptosis was assessed by Hoechst 33258 staining (cells exemplifying apoptotic nuclei are demarcated by white arrows). E, Panc-1 cells were treated as in C, and the ratio of apoptotic cells was assessed by counting the number of cells with apoptotic nuclei. Each experiment was conducted in triplicate and repeated twice independently (*, P < 0.05). F, Panc-1 cells were treated as in C. Apoptosis was assessed by a DNA ladder assay. G, Panc-1 cells were treated as in C and cell lysates were prepared for Western blotting to detect survivin and cleaved caspase-3. β-Actin was assessed as the control for equal loading of protein.

YM155 Induces Apoptosis via DR5 in Pancreatic Cancer
Lexatumumab was kindly provided by Human Genome Science Inc. YM155 and gemcitabine were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. Lexatumumab was dissolved in water and stored at −80°C.

siRNA-mediated knockdown

siRNA knockdown was performed as previously described (20). Panc-1 cells were transfected with survivin siRNA duplex mixtures in the presence of Lipofectamine RNAiMax (Invitrogen). A nonspecific scramble siRNA (Cell Signaling Technology) was also transfected at the same concentration as a control.

Hoechst staining

For Hoechst 33258 staining, cells were plated at a density of 5 × 10⁵ cells per milliliter in a 6-well plate and treated with YM155. After treatment, cells were stained with Hoechst 33258 for 15 minutes at room temperature in the dark. Cells were then analyzed using a fluorescence microscope. For quantitative analysis, the cell death ratio was assessed by counting the number of apoptotic cells with condensed nuclei in 6 to 8 randomly selected areas.

DNA ladder assays

A DNA ladder assay was performed as previously described with modification (21). Briefly, cells were treated under different experimental conditions and then washed twice in PBS and harvested. Genomic DNA was then separated by electrophoresis.

Western blotting analysis

Western blotting was performed as previously described (22). Cells were washed twice with sterile PBS and harvested. Cell lysates were prepared and samples were subjected to SDS-PAGE on 12% gels. Bands were detected using enhanced chemiluminescence. Protein molecular weight was determined by comparison with prestained protein markers.

Cross-linking of Bak protein

The Bak cross-linking assay was performed as described previously (23, 24). After treatment with YM155, cells were washed with conjugating buffer and DSS was added to a final concentration of 2 mmol/L. After incubating at room temperature for 30 minutes, the cross-linker was quenched by Tris-HCl. Bak was detected by Western blotting with anti-Bak polyclonal antibody.

MTS assays

Cell viability assays were performed as previously described (25). Briefly, cells were treated with gemcitabine with or without YM155. Cell viability was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit according to the manufacturer’s protocol.

Colony formation assays

Cells were seeded in 6-well plates at a density of 1 × 10³ cells. Cells were tested under various experimental conditions for 48 hours. After being rinsed with fresh medium, cells were allowed to grow to form colonies, which were then stained with 0.4 g/L crystal violet (Sigma). Clonogenic assay was used to elucidate the possible differences in long-term effects the combination on human pancreatic cancer cells.

Tumor growth inhibition

Mice were managed humanely in line with criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH publication 85-23, revised 1985). Eight-week-old male SCID mice were divided into two groups. Each mouse (5/group) was inoculated subcutaneously with Panc-1 cells in PBS in the right flank. Treatment with YM155 was initiated by daily tail vein injection of YM155 or vehicle only when tumors were palpable, each for a total of 7 days. The effect of drug treatment on tumor growth was analyzed statistically with individual group comparison and evaluated by student paired t test by SPSS 15.0.

Results

Downregulation of survivin in pancreatic cancer cells does not induce apoptosis

It has been reported that YM155 induces apoptosis through the inhibition of survivin expression in a number of cancer cells (26, 27). We tested the effects of YM155 on pancreatic cancer cells. Cells were treated with YM155 for 48 hours and apoptosis was measured by Hoechst 33258 staining, DNA ladder assay, and Western blot analysis. Our data showed that YM155 induced apoptosis in Panc-1 cells (Supplementary Fig. S1A and S1B). The treatment induced apoptosis in Panc-1 cells in both time- and dose-dependent manners as determined by treatment with different concentrations of YM155 or by treatment over a time course (Supplementary Fig. S1C and S1D). To assess intracellular apoptotic events, Panc-1 cells were treated and Western blotting was performed. The results showed that YM155 induced activation of caspase-8, Bid, caspase-3, and caspase-9 (Supplementary Fig. S1E and S1F). Cleaved PARP was also detected in YM155-treated Panc-1 cells (Supplementary Fig. S1E and S1F). We also treated PC-3 pancreatic cancer cells with YM155, to identify whether YM155 had the same effect in other pancreatic cancer cell lines. Treatment of PC-3 cells with YM155 induced apoptosis as observed by nuclear condensation detected by Hoechst staining (Supplementary Fig. S2A). Treatment of PC-3 cells over 24 hours with different doses of YM155 downregulated expression of survivin and increased expression of DR5 (Supplementary Fig. S2B). These data suggest that YM155 induces apoptosis via activation of components of the extrinsic and intrinsic apoptotic signaling pathways in pancreatic cancer cells.

To determine whether YM155-induced apoptosis is indeed mediated by survivin downregulation, Panc-1 or PC-3 cells were treated with YM155 for up to 48 hours, followed by Western blot analysis of survivin expression. Survivin expression was almost completely ablated after treatment for 48 hours (Fig. 1B and Supplementary Fig. S2B). To establish the causal relationship between survivin suppression and apoptosis, a survivin-specific siRNA was used to knockdown survivin in Panc-1 cells. As shown in Fig. 1C, survivin expression was effectively depleted by the siRNA knockdown. Induction of apoptosis was then assessed by siRNA knockdown. Cells were either treated by survivin–siRNA transfection or with YM155. Untreated Panc-1 cells were used as the control. Hoechst staining demonstrated that there was no overt apoptosis in Panc-1 cells transfected with survivin siRNA (Fig. 1D and E). We also compared the effect of survivin knockdown and YM155-mediated survivin downregulation on apoptosis using the DNA ladder assay. Consistent with the Hoechst staining test, genomic DNA extracted from control cells or cells

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YM155 transfects with survivin–siRNA had no DNA fragmentation, while cells treated with YM155 did (Fig. 1F). Similarly, caspase-3 cleavage was only detected in cells treated with YM155, but neither control cells nor cells transfected with survivin–siRNA showed cleavage of caspase-3 (Fig. 1G).

YM155 induces DR5 upregulation

We first examined functional expression of the death receptors DR4 and DR5 at the protein level. We found that YM155 treatment significantly increased the expression of DR5 in Panc-1 cells (Fig. 2A and B). We next tested whether YM155 is able to regulate DR5 and DR4 expression at the mRNA level in Panc-1 cells. As shown in Fig. 2C and D, YM155 treatment increased DR5 mRNA and decreased DR4 mRNA, which is consistent with the protein expression pattern. To determine whether the effect of YM155 on DR5 and DR4 is related to survivin expression, we knocked down survivin expression using a siRNA approach. Western blotting results showed that siRNA-mediated survivin gene silencing had no effect on DR4 or DR5 expression (Fig. 2E). To evaluate the role of DR5 overexpression in YM155-induced apoptotic signaling in pancreatic cancer cells, a known DR5 monoclonal antibody, lexatumumab, that

Figure 2.
YM155 induces death receptor 5 upregulation. A, Panc-1 cells were treated with YM155 for 48 hours and cell lysates were prepared for Western blotting to detect DR4 and DR5. β-Actin was assessed as the control for equal loading of protein. Band density was measured by ImageJ software. B, Panc-1 cells treated with YM155 and cell lysates were prepared for Western blotting to detect DR4 and DR5 expression. β-Actin was used for equal total protein amounts loading control. Band density was measured by ImageJ software. C, Panc-1 cells treated with YM155 for 48 hours were harvested and total mRNA was extracted. Reverse transcription PCR and quantitative real-time PCR were performed to examine DR5 mRNA levels with specific primers. Statistical analysis was performed to show the effects of YM155 on DR5 mRNA levels. Each experiment was conducted in triplicate and repeated twice independently (#, P < 0.05). D, Panc-1 cells were treated with YM155 and cell lysates were prepared for Western blotting to detect caspase-8, Bid, and caspase-3 activation. I, cells were treated as in A and apoptosis was measured. Statistical analysis was performed to determine the ratio of apoptotic cells by counting the number of apoptotic cells with condensed nuclei. Each experiment was conducted in triplicate and repeated twice independently (*, P < 0.05).
can activate DR5 to induce apoptosis was used to cotreat cells with YM155. Pretreatment of cells with YM155 for 1 hour followed by lexatumumab for another 12 hours induced significant apoptosis, whereas treatment with either YM155 for 1 hour or lexatumumab for 12 hours did not cause apoptosis as assessed by Hoechst 33258 staining and DNA ladder assay (Fig. 2F and G). The synergistic effect of lexatumumab and YM155 is further confirmed by Western blot analysis of caspase-3, caspase-8, and Bid (Fig. 2H). Combined treatment with lexatumumab induced the most potent apoptosis in 30% of cells as observed by Hoechst staining (Fig. 2I). These data suggest that DR5 upregulation plays a role in YM155-induced apoptosis.

YM155 exerts broad-spectrum IAP inhibition and induces oligomerization of Bak

We examined the effect of YM155 on IAP family protein expression. Western blotting analysis showed that YM155 inhibits survivin expression in Panc-1 cells (Fig. 3A) and PC-3 cells (Supplementary Fig. S2B). Treatment with YM155 also reduced CIAP1/2 and FLIP expression. Bcl-2 family protein expression analysis indicated that Bcl-2 and Bim were not affected by YM155, whereas Mcl-1 was decreased and Bak was upregulated (Fig. 3A). The oligomerization of Bak has previously been reported to occur only in apoptotic cells. Oligomerization of Bak is required for the formation of pores which release cytochrome c from the mitochondria (28). We next investigated whether YM155 could trigger Bak oligomerization. After treatment with YM155, Panc-1 cells were exposed to the membrane-permeable cross-linking agent DSS and subjected to Western blotting for the analysis of Bak oligomerization. The detection of a Bak band at 52 to 56 kDa indicates the presence of Bak homodimer. The elevation of Bak homodimer formation correlated with YM155 concentration increase (Fig. 3B). Moreover, we performed nonreducing SDS-PAGE to confirm Bak dimerization. Bak homodimers were detected 48 hours after treatment with YM155 (Fig. 3C). Homodimerization of Bak correlated with the detection of tBid, the increase in Bak homodimerization indicates that YM155 induces apoptosis via the intrinsic pathway.

YM155 enhances gemcitabine-induced apoptosis in Panc-1 cells

Gemcitabine is a common agent used in the clinical treatment of pancreatic cancer (29). We studied mono and combination treatment using YM155 and gemcitabine. Quantitative MTS assays showed that the presence of sublethal quantity of YM155 increased gemcitabine cytotoxicity (Fig. 4A); chromatin condensation assays indicated that gemcitabine and YM155 worked...
synergistically to induce apoptosis (Fig. 4B); colony formation assays revealed that gemcitabine and YM155 combination treatment effectively inhibits cell proliferation (Fig. 4C). These data suggest that YM155 enhanced the antitumor effects of gemcitabine.

YM155 has no apoptotic effects on noncancerous pancreatic cells

HPDE cells are a model for noncancerous pancreatic cells (30). HPDE cells were treated with YM155 and Hoechst 33258 staining was performed. YM155 treatment induced apoptosis in the Panc-1 cells (positive control), but not in HPDE cells (Fig. 5A). To further assess YM155-induced apoptosis in HPDE and Panc-1 cells Western blotting was performed to measure caspase-3 activation. Our results indicated that YM155 indeed induced no apoptotic toxicity in HPDE cells, whereas Panc-1 cells, as the positive control underwent apoptosis (Fig. 5B). These data suggest that YM155 appears to be relatively safe to normal healthy pancreatic cells in vitro.

YM155 inhibits tumor growth in vivo

Finally, we investigated the antitumor effects of YM155 in vivo. Pancreatic cancer xenografts were established in SCID mice. Mice were injected with Panc-1 cells to form xenograft tumors. One group of mice was treated with PBS buffer (control group) and other group was treated with YM155. The result showed that the control mice had developed much larger and more highly vascularized tumors than those mice treated with YM155 (Fig. 6A). There was a significant difference in tumor mass with at least a 10-fold decrease in YM155-treated mice when compared with untreated control mice (Fig. 6B). To identify whether or not the DR5 pathway was upregulated by YM155 in tumor tissue, tumor tissues from untreated or treated groups were analyzed to examine DR5 protein levels. Our results indicated that, in comparison with control samples (N), tumor tissue from YM155-treated mice (T), had elevated expression of DR5 (Fig. 6C). These data suggest that YM155 effectively inhibits malignant pancreatic tumor growth in vivo and the mode of action is similar to that which we have observed in the cell culture experiments.

Discussion

Pancreatic cancer is a complex disease. Effective therapeutic strategies to treat this cancer require a more in-depth understanding of the molecular mechanisms underlying survival and
apoptotic pathways in pancreatic cancer cells. In this study, we have investigated the mechanism of YM155-induced apoptosis in pancreatic cancer cells and tumors. We found that survivin downregulation is insufficient to initiate apoptosis in pancreatic cancer cells. We showed that expression of a number of IAPs as well as survivin is suppressed by YM155. Surprisingly, YM155 treatment induces DR5 upregulation. Our results suggest that YM155 has a much broader function on regulating pro- and antiapoptotic proteins. Consistent with this notion, YM155 indeed enhances the therapeutic effect of gemcitabine and death receptor agonist lexatumumab.

YM155 was initially identified as a survivin inhibitor through high-throughput screening. In vitro studies consistently demonstrated its suppression on survivin expression. Previous reports showed that YM155 can induce apoptosis in prostate cancer cells and non-Hodgkin lymphoma cells (27–31). YM155 has entered a few early-stage clinical trials for the treatment of advanced cancers. The preliminary results have shown a potent antitumor growth activity (11, 12, 32, 33). However, YM155 has yet to be fully tested in human pancreatic cancer. In the current study, we demonstrate YM155 can induce apoptosis in pancreatic cancer cells at clinically relevant doses. The reported plasma concentration is approximately 15 nmol/L (12, 13, 34).

Our study suggests that YM155 may have potential use as a systemic therapy for pancreatic cancer.

Consistent with previous reports that YM155 is an effective survivin suppressor (13, 14), YM155 indeed induced a dramatic survivin downregulation in Panc-1 and PC-3 cells. However, our siRNA-mediated knockdown experiments provided evidence to support the notion that downregulation of survivin protein expression alone is insufficient to trigger apoptosis in pancreatic cancer cells, which raises interesting questions regarding the mechanisms by which YM155 induces robust apoptosis. In searching for answers, we analyzed the molecular events related to YM155-induced apoptosis. Our experiments demonstrated that caspase-8, Bid, and caspase-9 were significantly activated in YM155-treated pancreatic cancer cells. This is similar to death receptor-mediated intrinsic or extrinsic apoptosis signal pathway activation (35–37). We then examined the death receptor DR4 and DR5 expression upon YM155 treatment. We found that YM155 induces expression of DR5 at both mRNA and protein levels and activates the DR5-mediated intrinsic apoptotic pathway in Panc-1 cells, whereas the DR4 expression is suppressed. This observation is confirmed in a xenograft pancreatic cancer mouse model. Further experiments confirmed that the effect on DR5 and DR4 is not caused directly by survivin, as knockdown of survivin did not affect DR5 or DR4 expression. There are previous reports showing that chemotherapeutic agents could result in DR5 upregulation to induce apoptosis (38–40); ectopic overexpression of DR5 in cells has been shown to trigger apoptosis without additional stimuli (41–43). The role of DR4 suppression in YM155-treated cells is further demonstrated by tests with the monoclonal antibody specifically against DR5 (lexatumumab). Neither YM155 nor lexatumumab single treatment induced apoptosis as evidenced by the lack of caspase-8, caspase-3, and Bid activation, which are hallmarks of YM155-induced apoptosis. However, combination treatment was able to induce apoptotic events in a similar pattern to YM155-induced apoptosis. These findings support the notion that DR5 activation may be a mechanism for YM155-induced apoptosis. Nevertheless, key questions remain: How does YM155 induce DR5 transcription and suppress DR4 transcription? Since the discovery of YM155, the detailed mechanisms by which YM155 downregulates survivin RNA have not been defined. Our study indicates that the impact of YM155 on gene transcription machinery may be broader than survivin itself. The underlying mechanism of YM155 is likely to be complex and may involve the regulation of transcription at more genes than originally thought. This presents an opportunity for further investigation. We are currently working on the structural basis of YM155 interaction with DNA regulatory elements. We hope this effort will provide further insights into the specific mechanism by which YM155 regulates transcription of survivin and DR5 mRNA.

Our study also demonstrates the impact of YM155 on other genes expressed in pancreatic cancer cells. In addition to the inhibition of survivin expression in pancreatic and other cancers (11, 26, 44), YM155 downregulates two additional IAP family members CIAP1/2 and FLIP, suggesting that YM155 may be an IAP family inhibitor. This is not in agreement with a previous study that reported selective inhibition of survivin by YM155 without affecting CIAP level. However, that study only analyzed survivin expression inhibition and did not analyze the expression of other IAP family members (11). We also found

Figure 5.
YM155 has no apparent apoptotic cytotoxicity to normal pancreatic cells. A, HPDE cells grown in 48-well plates were treated with YM155 (0 nmol/L) for 48 hours. Panc-1 cells treated under the same conditions were used as a positive control. Apoptosis was measured by Hoechst 33258 staining. B, HPDE cells and Panc-1 cells (a positive control) grown in 6-well plates were treated with YM155 for 48 hours. Cell lysates were prepared for Western blotting to show caspase-3 activation. β-Actin was assessed as a control for equal protein loading.
that YM155 decreased the expression of Mcl-1. Moreover, YM155 treatment increased Bak expression but not Bim expression, suggesting some degree of Bak-specific activation by YM155. Both Bak and Bim are BH3 domain containing pro-apoptotic proteins. Bak activation in apoptosis has been reported to occur via the oligomerization of Bak dimers that plays a role in cytochrome c release (28, 45). Our experiments demonstrated dose-dependent Bak dimerization, which occurred in cells treated with YM155. Similar findings were reported in studies using small molecule compounds such as doxorubicin and gossypol that induce apoptosis by activating Bak/Bax (22, 46). We observed a decrease in Bak expression at 20 nmol/L after 48 hours, which may be caused by substantial cell death and subsequent protein degradation. All the data suggest that YM155 is a broad-spectrum IAP family inhibitor and is not survivin specific.

Because of the broader impact of YM155 on expression of genes related to apoptosis, it is conceivable that YM155 may have synergistic treatment effect on cancer cells when combined with other chemotherapeutic agents. Gemcitabine remains one of the most widely used agents in the treatment for various solid tumors, especially pancreatic cancer (47). We tested this idea using a cell culture model of pancreatic cancer in combination with gemcitabine. Our data demonstrate that YM155 exerts cell growth inhibition effects synergistically with gemcitabine. This is consistent with reports that YM155 enhances the antitumor effects of other chemotherapeutic agents including cisplatin, rituximab, and paclitaxel (14, 19, 34, 48, 49). Our study reveals several novel findings: (i) YM155 effectively induces apoptosis in pancreatic cancer cells both in vitro and in vivo; (ii) YM155 has broad-spectrum impact on the expression of genes that are related to pro- or anti-apoptotic pathways including upregulation of DR5 and Bak; (iii) YM155 exhibits synergistic therapeutic effects when combined with other chemotherapeutic agents including gemcitabine and leatumumab; (iv) nonspecific cytotoxicity is not observed in noncancerous pancreatic epithelial cells and treated animals. On the basis of our investigation, we believe YM155 has a potential for pancreatic cancer therapy either as monotherapy agent or in combination with other agents. Moreover, through better understanding of the molecular mechanisms underpinning YM155 function, we may be able to design better small molecules for cancer therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: X. Zhao, C. Liu
Development of methodology: D.A. Ostrov, C. Liu
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Liu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Zhao, Z. Lu, D.A. Ostrov, K.D. Robertson, C. Liu
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W.M. Puszyk, C. Liu
Study supervision: C. Liu

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Figure 6. YM155 induces tumor growth inhibition in vivo. A, BALB/c SCID mice were inoculated subcutaneously with 5 × 10⁶ Panc-1 cells. The mice were treated with 10 mg/kg body weight of YM155 via tail vein injection until day 14. At the end point of the experiment, mice were euthanized and tumors were excised. Pictures of excised tumors from xenograft mice show larger tumors from untreated (control) mice and smaller tumors from YM155-treated mice. B, the mass of tumors from the control group and the YM155-treated group were compared. Statistical analysis was performed to show inhibitory effects of YM155 on the mass of implanted tumors (*, P < 0.05). C, xenograft pancreatic tumors from untreated groups and YM155-treated groups were collected and lysates were prepared. Western blotting was performed to detect DR5 expression. β-Actin was assessed as an equal protein loading control. The five pairs of tissues tested were randomly selected sample pairs, from untreated or YM155-treated tumor mice.
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References

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38. Baritaki S, Huerta-Yepez S, Sakai T, Spanidios DA, Bonavida B. Chemo-
therapeutic drugs sensitize cancer cells to TRAIL-mediated apoptosis: up-
sensitizes tumor necrosis factor-related apoptosis-inducing ligand
(TRAIL)-resistant hepatoma cells to TRAIL-induced apoptosis through
40. Thomas SA, Vasudevan S, Thamkachy R, Lekohmi SI, Samthoshkumar TR,
Rajaekhanan KN, et al. Upregulation of DR5 receptor by the diaminothia-
zole DAT1 [4-amino-5-benzoyl-2-(4-methoxy phenyl amino) thiazole] 
triggers an independent extrinsic pathway of apoptosis in colon cancer cells 
with compromised pro and antiapoptotic proteins. Apoptosis 2013;18: 
apoptosis and limits adenovirus gene therapy product expression in the 
TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis 
43. Wu GS, Burns TF, Zhan Y, Alinemsi ES, El-Deiry WS. Molecular cloning 
and functional analysis of the mouse homologue of the KILLER/DR5 tumor 
necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor. 
YM155, a selective survivin suppressant, inhibits tumor spread and pro-
longs survival in a spontaneous metastatic model of human triple negative 
45. Dewson G, Kratina T, Czabotar P, Day CL, Adams JM, Kluck RM. Bak 
activation for apoptosis involves oligomerization of dimers via their 
46. Panaretakis T, Piskorskaika K, Shoshan MC, Grander D. Activation of Bak, 
Bax, and BH3-only proteins in the apoptotic response to doxorubicin. J Biol 
47. Liao ZI, Guo YH, Zhao Z, Yao JT, Xu R, Nan KI. Gemcitabine inhibits the 
micrometastasis of non-small cell lung cancer by targeting the EpCAM-
positive circulating tumor cells via the HGF/cMET pathway. Int J Oncol 
2014;45:651–8.
Combination of YM155, a survivin suppressant, with bendamustine and 
rutinimab: a new combination therapy to treat relapsed/refractory diffuse 
YM155 reverses cisplatin resistance in head and neck cancer by decreasing 

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