Research Square

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Bone morphogenetic protein (BMP) receptor inhibitor JL5 synergizes with Ym155 to induce Apoptosis-Inducing Factor (AIF) caspase independent cell death

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Abstract

Background: Bone morphogenetic protein (BMP) is an evolutionarily conserved morphogen that is reactivated in lung carcinomas. BMP receptor inhibitors promote cell death of lung carcinomas by mechanisms not fully elucidated. The studies here reveal novel mechanisms by which the “survivin” inhibitor Ym155 in combination with the BMP receptor inhibitor JL5 synergistically induces death of lung cancer cells.

Methods: This study examines the mechanism by which Ym155 in combination with JL5 downregulates BMP signaling and induces cell death of non-small cell lung carcinoma (NSCLC) cell lines. Validation experiments were performed on five passage 0 primary NSCLC cell lines.

Results: We found that Ym155, which is reported to be a survivin inhibitor, potently inhibits BMP signaling by causing BMPR2 mislocalization into the cytoplasm and its decreased expression. The combination of Ym155 and the BMP receptor inhibitor JL5 synergistically causes the downregulation of BMP Smad-1/5 dependent and independent signaling and the induction of cell death of lung cancer cell lines and primary lung tumors. Cell death involves the nuclear translocation of apoptosis inducing factor (AIF) from the mitochondria to the nucleus. This causes DNA double stranded breaks independent of caspase activation, which occur only when JL5 and Ym155 are used in combination. Knockdown of BMPR2 together with Ym155 also induced AIF localization to the nucleus.

Conclusions: These studies suggest that inhibition of BMPR2 together with Ym155 can induce AIF caspase-independent cell death. AIF caspase-independent cell is an evolutionarily conserved cell death pathway that has never been targeted to induce cell death in cancer cells. These studies provide mechanistic insight of how to target AIF caspase-independent cell death using BMP inhibitors.

Background

The bone morphogenetic proteins (BMP) are evolutionarily conserved cytokines that regulate a plethora of signaling events throughout development. Following the development of the fetal lung there is a decrease in BMP signaling, with little BMP activity seen in the mature lung [1, 2]. BMP signaling is reactivated in lung and many other carcinomas [3]. In non-small cell lung carcinomas (NSCLC), the BMP-2 ligand is over-expressed 17-fold higher in comparison to normal lung tissue and benign lung tumors [4]. A number of studies have shown that BMP signaling has significant tumorigenic effects that involve the regulation of cell survival, migration, proliferation, stemness, and angiogenesis, and that high ligand expression correlates with a worse prognosis [3, 5-10].

The BMP ligands signal through receptor serine/threonine kinases [11]. BMP ligands bind to the BMP type 1 receptors (BMPR1) (alk2, alk3, or alk6) promoting phosphorylation by the constitutively active BMP type 2 receptors (BMPR2) (BMPR2, ActR-IIA, ActRIB) [11]. The BMP complex phosphorylates Smad-1/5, which translocates to the nucleus and regulates transcriptional events including the inhibitor of differentiation proteins (Id1, Id2, and Id3) [12-14]. Id1 has tumorigenic effects regulating self-renewal and

migration of cancer cells [15-19]. Recent studies suggest that the BMP signaling cascade mediates cell survival independent of its regulation of Smad-1/5 transcription. Inhibition of BMPR2 decreases the expression of the anti-apoptotic factor X chromosome-linked inhibitor of apoptosis protein (XIAP) and transforming growth factor beta (TGFb) activated kinase 1 (TAK1) [7, 20, 21]. The inhibition of XIAP is a mechanism by which the BMP inhibitor JL5 creates synergistic cell death in lung cancer cells in combination with TRAIL [22]. Inhibition of BMPR2 and not the type I BMP receptors increases the release of the pro-apoptotic mitochondrial proteins Smac/DIABLO and cytochrome c into the cytosol[22].

Here we report that the BMP receptor inhibitor JL5 and the “survivin” inhibitor Ym155 synergistically promote cell death of lung cancer cell lines and primary tumors. We identified novel mechanisms by which Ym155 promotes cell death in cancer cells, which involves the inhibition of BMPR2 Smad-1/5 independent signaling. The combination of Ym155 and JL5 synergistically decreases BMP independent signaling by inhibiting BMPR2. Cell death induced by the combination of Ym155 and BMPR2 inhibition occurs independent of caspase activation and involves the translocation of AIF to the nucleus. These studies provide novel insights into how to target BMPR2 Smad-independent signaling to induce cell death of lung cancer cells.

Methods

Cell culture and reagents

The A549, H1299, and U1752 lung cancer cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma Aldrich, Saint Louis, MO, USA) with 5% fetal bovine serum (R&D Systems). JL5 and DMH2 were synthesized by David Augeri, John Gilleran, and Jacques Roberge, Rutgers School of Pharmacy. Ym155 was purchased from Selleckchem. Z-VAD-FMK was purchased from R&D Systems. SB-505124 and necrostatin-1 were purchased from Sigma Aldrich (Saint Louis, MO, USA).

Western blot analysis

Western blot analysis was performed as previously reported [3]. In brief, total cellular protein concentration was determined using the BCA method then separated by SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). The primary antibodies used were rabbit monoclonal anti-XIAP, rabbit monoclonal anti-Smac/DIABLO, rabbit monoclonal anti-cytochrome c, rabbit monoclonal anti-c-IAP1, rabbit monoclonal anti-activated caspase-3, rabbit monoclonal anti-PARP, rabbit monoclonal anti-AIF, rabbit monoclonal anti-pSmad1/5, (Cell signaling Technology, MA, USA), rabbit monoclonal anti-Id1 (Calbioreagents, San Mateo, CA), rabbit polyclonal anti-Smad 1/5 (Upstate Biotechnology, NY, USA), rabbit polyclonal survivin (Novus Biologicals, CO, USA), rabbit anti-actin, an affinity isolated antigen specific antibody (Sigma, Saint Louis, MO), rabbit polyclonal anti-GAPDH (Sigma) and mouse monoclonal anti-Spectrin (EMD Millipore, CA, USA).

Blots suggesting regulation of loading controls actin or GAPDH by Ym155 and/or JL5 were then probed with the spectrin, which is also a cytoskeletal protein.

Cell viability

Cells were plated in duplicate into 6-well plates and treated the next day for the designated period of time. Cell counts were determined using the automated cell counter Vi-CELL cell analyzer (Beckman Coulter). Approximately 500 cells per sample were analyzed and trypan blue dye exclusion determined number of dead cells. The experiment was replicated three times in our laboratory.

Combined drug effects

The median-effect principle of Chou and Talalay was used to evaluate synergy between JL5 and Ym155 [23]. The combination index (CI) values were calculated using Compusyn software to determine mode of interaction with CI < 1.0 indicating synergism, 1.0 additive and > 1.0 antagonistic response.

Transient knockdown

Validated select siRNA was used to knockdown BMPR2 (Life Technologies). The ID numbers for the siRNA are: BMPR2 (s2044 and s2045). Silencer Select negative control siRNA (4390843) was used to evaluate selectivity. Transfections of the siRNA were performed in duplicate using Lipofectamine® RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Cells were transfected with 6 nM BMPR2 or 6 nM of siRNA control. The experiment was replicated three times in our laboratory.

Cytosol extraction

Cytosolic protein extraction was performed using Mitochondria/Cytosol fractionation kit as per manufacturer's instructions (Enzo Life Sciences, NY, USA). Cell pellets were resuspended in 100µl of ice-cold Cytosol Extraction Buffer Mix containing dithiothreitol (DTT) and Protease Inhibitors. After a 10-minute (min) incubation on ice, cells were homogenized. The homogenates were collected to a fresh 1.5ml tube and centrifuged at 700 x g for 10 min at 4°C. The supernatant was collected as the cytosolic fraction and used for further experiments.

TUNEL assay

DNA double strand breaks (DSB) after treatment were analyzed by using FlowTACS In Situ TUNEL-based apoptosis detection kit (Trevigen) according to the manufacturer's protocol. Cells were treated in duplicate. After treatment, cells were trypsinized and the cell pellet was fixed with 4% formaldehyde and permeabilized with cytonin for 30 min. Cells were washed with labeling buffer and resuspended in reaction mix for 1 hour (h), then stained with strep-fluorescein solution and analyzed using flow cytometry (LSRII, BD Biosciences). The experiment was replicated three times in our laboratory.

Immunofluorescence staining

Cells were seeded for 24h onto microscope cover glasses in a 6-well plate then treated. Cells were fixed with 4% formaldehyde and permeabilized with 0.5% triton-X. Cells were blocked with CAS-block for 1h;

cells were stained with anti-BMPR2 antibody that recognizes an extracellular epitope (Sigma-Aldrich) or AIF for 1h at room temperature. To assess for BMPR2 on the plasma membrane the cells were fixed but not permeabilized prior to staining. Cells were then washed with phosphate buffered saline (PBS) and stained with Alexa Flour 488 conjugated secondary antibody for 1h at room temperature. After washing with PBS, the nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 10 min. Fluorescent images were captured using a Nikon eclipse TE300 inverted epifluorescent microscope and a Cool Snap black and white digital camera. IP Lab imaging software was used to assign pseudo-color to each channel. All of the immunofluorescence experiments except the primary tumors cells were replicated three times in our laboratory. The AIF immunofluorescence study using primary tumor cell lines was performed once for each of the 5 tumors.

Primary lung carcinomas

Primary tumor tissues were obtained within 30 min of surgery from The Cancer Institute of New Jersey (CINJ) as approved by the Research Ethics and Institutional Review Board (Protocol Number: 001608). Consecutive non-small cell lung carcinomas were used. The tissues were washed with PBS and then cut into small pieces. Human tissue dissociation kit (Miltenyl Biotec) was used to dissociate the tumor tissues according to the manufacturer's protocol. Dissociated cells were grown in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 20% Fetal Bovine Serum (FBS) and 1% antibiotic-antimycotic for approximately 10 days without splitting. At 70% confluence, cells were trypsinized and plated for all the experiments at the same time.

Tumor xenograft studies

Tumor xenografts from H1299 cells were established by injecting 2 million cells into the intradermal space of the flanks of 6 week old female NOD-*scid* IL2Rgamma^{null} (NSG) mice (bred in-house and randomly assigned to groups without blinding). When tumors reached approximately 4mm x 4mm, mice were treated with 3 or 10 mg/kg of JL5 twice daily (or DMSO control) for four days. Eight mice were used in these studies, 4 DMSO control and 4 JL5 treated. Treated mice were not blinded. At the end of the experiment mice were euthanized using CO2. Experiments were conducted in compliance with ethical regulations and approved by Rutgers IACUC.

Statistical Analysis

The mean of the control group was compared to the mean of each treated group using a paired student t-test assuming unequal variances. Differences with p values <0.05 were considered statistically significant.

Results

Ym155 decreases BMP signaling

H1299 cells were injected into the intradermal space of the flanks of NSG mice and were treated with DMSO or JL5 for 4 days. We previously reported that JL5 decreases the expression of Id1 but not XIAP in tumor xenografts [8]. Survivin belongs to the family of anti-apoptotic proteins that have been reported to be regulated by BMP signaling and are known to stabilize the expression of XIAP [24]. We found for the first time that inhibition of BMP signaling with JL5 causes a significant increase in the expression of survivin in tumor xenografts (Fig. 1A).

We explored whether Ym155, a reported survivin inhibitor [25], would have synergy when used in combination with a BMP inhibitor. First, we looked at the effects of Ym155 on the expression of survivin and XIAP. Ym155 induced a significant decrease in the expression of XIAP in H1299 cells (Fig. 1B) and A549 cells (Fig. 1C) with little change in the expression of survivin. This is consistent with prior studies suggesting that Ym155 mediates its effects independent of survivin [26].

Since BMPR2 regulates XIAP through Smad-1/5 independent mechanisms, we examined whether Ym155 regulated BMP signaling. The effects of Ym155 on BMP Smad-1/5 dependent signaling was assessed by examining changes in the phosphorylation of the BMP transcription factor Smad-1/5 and its downstream transcriptional target Id1. Ym155 caused a significant decrease in the activity of Smad-1/5 and Id1 in both the H1299 (Fig. 1D) and A549 (Fig. 1E) cells at nanomolar concentrations. H1299 cells were stably transfected with the Id1 promoter driving the expression of the luciferase reporter. Ym155 caused a dose responsive decrease in the Id1 reporter (Fig. 1F). Transfection of constitutively active BMPR1A (caBMPR1A) activates Smad-1/5-Id1 signaling. Ym155 inhibited caBMPR1A activation of Smad-1/5 (Fig. 1G). These studies show that Ym155 regulates both Smad-1/5 dependent and independent signaling, raising the possibility it regulates BMPR2.

We examined the effects of Ym155 on cell survival and the induction of cell death. After 48 h the majority of A549 cells treated with 100 nM were dead (Fig. 1H). Examining the IC50 at 24 h with concentrations below 100 nM, we found the IC50 to be 89 nM and 19 nM for A549 and H1299, respectively (Fig. 1I). An increase in the percent dead cells was not seen in the A549 cells at these concentrations after 24 h however; a small increase in dead cells was seen in H1299 cells at 40 nM and above (Figs. S1A-B). In comparison, the BMP inhibitor JL5's IC50 was higher with an IC50 of 12 mM and 7 mM for A549 and H1299 cells, respectively (Fig. 1J) with few dead cells seen except at high concentrations (10 mM) (Figs. S1C-D).

Ym155 synergizes with JL5 to decrease BMP signaling and induce cell death

Since Ym155 does not target the BMP receptors, we examined whether it would induce synergy with JL5. To test for potential synergy, we used concentrations of Ym155 and JL5 that were below their IC50 and had no increase in cell death. JL5 caused significantly more cell death when used in combination with Ym155 compared to either compound alone in both H1299 and A549 cells (Figs. 2A-B). The combination index (CI) [23] was calculated to be <1.0 (0.94 H1299 cells and 0.16 A549 cells) indicating the combined effect was synergistic. Morphologically, the nuclei were significantly smaller and demonstrated chromatin condensation when treated with both JL5 and Ym155 in comparison to either compound

alone (Figs. S1E-F). By Western blot analysis, there was little change in BMP signaling with JL5 and Ym155 after 24 h. The combination of Ym155 and JL5 caused a greater decrease in the expression of Id1 and XIAP in comparison to each compound alone in both the A549 and H1299 cells (Figs. 2C-F). Ym155 alone induced apoptosis as demonstrated by the expression of activated caspase-3, with 17 kd and 19 kd fragments and cleavage of its downstream target poly ADP ribose polymerase (PARP) (Fig. 2G). We found no activation of caspase-3 or caspase-9 when examined at 1, 3, and 24 h (Fig. 2G and data not shown). The pan-caspase inhibitor Z-VAD-FMK did not affect cell death induced by JL5 and Ym155 in combination (Fig. 2H). Necrostatin, which inhibits necrosis induced by receptor-associated adaptor kinase 1 (RIP1) [27], also had no effect on cell death induced by JL5 and Ym155 when used in combination (Fig. 2H). These studies suggest that Ym155 together with a BMP receptor inhibitor synergistically mediates cell death by mechanisms independent of the caspases or RIP1 induced necrosis.

Ym155 together with JL5 increases cytosolic AIF and Smac/DIABLO

Since apoptosis is not induced with JL5 and Ym155, we explored whether cell death involved the mitochondrial release of AIF [28]. In cells undergoing AIF induced cell death, AIF is cleaved producing 67 kd and 57 kd fragments, which are then rapidly transported to the nucleus. Nuclear AIF induces DNA fragmentation and chromatin condensation leading to cell death [28]. The combination of Ym155 and JL5 synergistically enhanced the release of Smac/DIABLO into the cytosol as early as 3h and persisted for at least 24h in A549 cells (Figs. 3A-B). In the H1299 cells, we did not identify cytosolic AIF after 3h but identified cytosolic AIF after 24h in cells treated with JL5 and Ym155 in combination (Figs. 3C-D). Cytosolic AIF was not observed when JL5 and Ym155 were used alone. Smac/DIABLO causes the inhibition and degradation of anti-apoptotic proteins cellular inhibitor of apoptosis 1 (c-IAP-1). Consistent with an increase in cytosolic Smac/DIABLO, JL5 together with Ym155 caused a significant decrease in the expression of c-IAP-1 (Figs. 3E-F).

BMP inhibitors with YM155 cause DNA double stranded breaks and nuclear localization of AIF

A hallmark of AIF induced cell death is the induction of DNA double stranded breaks (DSB) and its localization to the nucleus. The TUNEL assay was used to determine DNA-DSB. Three hours following treatment with JL5 or Ym155 alone or in combination no DNA-DSB were found (Figure 4A). After 24h, very few cells treated with JL5 or Ym155 alone demonstrated DNA-DSB. When JL5 was used in combination with Ym155, approximately 40% and 65% of cells demonstrated DNA-DSB in A549 and H1299 cells, respectively (Fig. 4B). DMH2 is similar to JL5, having potent inhibition of BMP type 1 receptors with some inhibition of BMPR2 [8]. DMH2 treated cells also demonstrated an increase in DNA-DSB when used in combination with Ym155 (Fig. 4C). To assess whether synergy also occurred by inhibiting TGFb signaling, we used the selective TGFb receptor inhibitor SB-505124 [29]. SB-505124 had no effect on DNA-DSB when used alone or in combination with Ym155 (Figure 4C). Using immunofluorescent imagining, we examined whether AIF localized to the nucleus following treatment with JL5 and Ym155. JL5 and Ym155 alone did not cause the localization of AIF to the nucleus in either

the H1299 or A549 cells but the combination of JL5 and Ym155 showed an increase in AIF localization to the nucleus (Figs. 4D-G).

Ym155 decreases BMPR2 expression and promotes BMPR2 mislocalization to the cytoplasm

Since Ym155 decreases the expression of XIAP and Id1, which are both regulated by BMPR2, we examined if Ym155 regulated the expression of BMPR2. Ym155 caused a dose-related decrease in the expression of BMPR2 that was associated with a corresponding decrease in the expression of XIAP in both the A549 and H1299 cells (Figs. 5A-B). At lower concentrations of Ym155 (20 nM), which has no effect on the level of BMPR2 expression, an enhanced decrease in expression of BMPR2 and XIAP was observed when Ym155 was used in combination with JL5 (Figs. 5C-D). As previously reported, JL5 caused the mislocalization of BMPR2 to the cytoplasm (Fig. 5E) [22]. We show for the first time that Ym155 alone also caused an increase in the localization of BMPR2 to the cytosol (Fig. 5E), which was greatly increased when JL5 and Ym155 were used in combination (Fig. 5E).

To assess whether the increased nuclear localization of AIF in cells treated with JL5 in combination with Ym155 was mediated by the inhibition of BMPR2, we knocked down the expression of BMPR2 with siRNA in H1299 cells then treated the cells with a low concentration of Ym155 (20 nM). The knockdown of BMPR2 together with Ym155 (20 nM) significantly enhanced AIF localization to the nucleus in comparison to siRNA control cells treated with Ym155 (20 nM) (Figs. 5F-G). These studies show that Ym155 decreases BMP signaling, which involves the downregulation of the expression of BMPR2 expression and its mislocalization to the cytoplasm. The data also suggests that the inhibition of BMPR2 is required for AIF localizing to the nucleus.

Synergy of Ym155 and JL5 in primary NSCLC

Primary NSCLC cells were obtained directly from 5 surgically resected lung tumors. Tumors 1, 2, 3 and 5 were adenocarcinomas and tumor 4 was a squamous carcinoma. Tumors were immediately gently digested and plated for cell culture. After approximately 10 days the cells were treated with JL5 and Ym155 alone and in combination for 48h. Primary cancer cells were examined for changes in cell survival, regulation of BMP signaling, and nuclear localization of AIF.

The combination of JL5 and Ym155 caused an increase in cell death compared to DMSO control in 5 of 5 tumors (Fig. S5A). In 3 of 5 tumors, the combination of JL5 and Ym155 induced cell death that was greater than either compound alone (Fig. 6A). XIAP was utilized as a marker of the downregulation of BMPR2 Smad-1/5 independent signaling. The combination of JL5 and Ym155 synergistically decreased the expression of XIAP in 3 of 5 tumors (tumors 1-3), which corresponded to the tumors that JL5 in combination with Ym155 resulted in enhanced cell death (Figs. 6A-C). In tumors 3 and 4, Ym155 alone caused a decrease in the expression of XIAP that was not enhanced further with JL5 (Figs. 6 A-C).

AIF nuclear localization was examined in primary lung tumors 2-5. AIF nuclear staining was not seen in primary tumors treated with DMSO or JL5 (Figs. 6 D-F). Only a small percentage of cells from the primary

lung tumors treated with Ym155 demonstrated nuclear staining with AIF (0-10%). AIF nuclear staining occurred predominantly in primary tumors that demonstrated increased cell death with the combination of JL5 and Ym155 (tumors 2 and 3, 67% and 25% of cells, respectively) (Figs. 6D-E).

Discussion

Our prior studies suggested that targeted inhibition of BMPR2 induces cell death mechanisms not seen with the inhibition of BMP type 1 receptors [22]. Knockdown of BMPR2 decreases the expression of XIAP in lung cancer cell lines and increases cytosolic cytochrome c and Smac/DIABLO [22]. The BMP inhibitors JL5 and DMH2 decrease the expression of XIAP and cause more cell death than the BMP inhibitors DMH1 and LDN [7, 8]. JL5 and DMH2 both demonstrate some inhibition of BMPR2 with in vitro phosphorylation kinase assays with an IC50 of approximately 8 mM [8]. Despite JL5 having a relatively low IC50 for BMPR2 it causes its mislocalization to the cytoplasm at 2.5 mM. This mislocalization does not occur with BMP inhibitors DMH1 or LDN, which have no activity for BMPR2 [22]. The over-expression of XIAP attenuates cell death induced by JL5 [22]. These studies suggest that cell death induced by JL5 and/or DMH2 involves the inhibition of BMPR2 and the downregulation of XIAP [22].

In tumor xenograft studies, JL5 caused some tumor regression, however when tumors were examined after 21 days there was little cell death [8]. Tumors examined after 4 days of treatment showed that Id1 expression was decreased but there was no decrease in the expression of XIAP [8]. This suggested that signaling pathways were induced that stabilized the expression of XIAP and/or BMPR2 was not sufficiently inhibited. We show in the present study that the expression of survivin is significantly increased in tumor xenografts treated with JL5. Survivin expression is increased by BMP signaling [30] and is known to increase XIAP stability against ubiquitin-dependent degradation [31]. Surprisingly, Ym155, which is reported to be a survivin inhibitor [25], caused a significant decrease in Smad-dependent and independent signaling at nanomolar concentrations. We show that Ym155 causes a significant decrease in the expression of BMPR2 and its mislocalization to the cytoplasm, which are likely mechanisms leading to the decrease in BMP signaling. In lung cancer cells, the BMP type 1 receptors only regulate Smad-dependent signaling while BMPR2 regulates both Smad-dependent and independent BMP signaling. The mechanism by which Ym155 causes a decrease in BMPR2 expression is not known but may involve the trafficking of BMPR2 to the lysosome and subsequent degradation.

Our studies suggest that inhibition of BMPR2 together with Ym155 promotes AIF caspase-independent cell death. AIF is an evolutionary conserved protein that has two independent functions; biogenesis of the electron transport chain and cell death [32-34]. An increase in permeability of the outer mitochondrial membrane (OMM) is required for its release into the cytosol [35]. AIF is transported to the nucleus where it induces large-scale DNA fragmentation and cell death [28, 34, 35]. AIF caspase-independent cell death occurs in response to ischemic-reperfusion injury in neurons and myocardium and has only infrequently been reported in cancer cells.

Ym155 was originally reported to be a survivin inhibitor [25]. A subsequent study showed that Ym155 resulted in dose-dependent induction of yH2AX and pKAP1, which are markers of DNA damage, at a concentration lower than required to decrease the expression of survivin [26]. This report concluded that Ym155 is a DNA damaging agent and the suppression of survivin is a secondary event. The mechanism by which Ym155 initiates the downregulation of BMP signaling and promotes cell death needs further investigation. Although Ym155 has not shown much efficacy in human trials it causes significant cell death of cancer cells in vitro. Ym155 has a short half-life and its pharmacokinetic properties could hinder its activity in human tumors. The identification of the mechanism by which Ym155 regulates cell death may lead to the identification of other drugs that could be used in combination with BMPR2 inhibitors.

Conclusions

We demonstrate that Ym155 decreases BMP signaling that is associated with a downregulation of BMPR2 signaling in lung cancer cells. We show that a BMPR2 inhibitor and Ym155 can synergistically target BMPR2 signaling to induce cell death. Cell death induced by Ym155 and BMPR2 inhibition involves AIF caspase-independent cell death mechanisms in lung cancer cells. These studies provide further insight into how to target BMP signaling as a therapeutic in cancer and supports further drug development of more specific and potent BMPR2 inhibitors.

Declarations

Ethics of approval and consent to participate

Institutional review board of Rutgers University of New Jersey approved tissue to be obtained from patients' resected tumors. Consent was obtained from an honest broker who kept patient personal information anonymous and secure. Mice experiments were conducted in compliance with ethical regulations and approved by Rutgers IACUC.

Consent for publication

Our manuscript does not contain any individual person's data in any form.

Availability of data and materials

The datasets obtained and analyzed for this study will be made available from the corresponding author in a reasonable request.

Competing interests

A provisionary patent application was sent for BMP inhibitor JL5. The full patent application for JL5 has been withdrawn and is no longer being pursued. There are no active or pending financial agreements regarding JL5 nor has any money been received or is pending.

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Authors' contributions

Conception and design: JL

Design and synthesis of JL5 and DMH2: DA, JG, JR

Development of methodology: AM, EL

Acquisition of data: AM, EL, RN, DG, MS, LL, JG, DA, AZ

Analysis of data: JL, AM, RN, YP

Writing, review, and/or revision of the manuscript: JL, RN, AM.

All authors have read and approved the manuscript

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Disclosure and Potential Conflicts of Interest

The authors have no active conflicts of interests with the material in this manuscript. A patent has been issued for DMH2 but this compound is no longer being pursued for further development by any of the authors.

Abbreviations

Bone morphogenetic protein (BMP); Bone morphogenetic protein receptor 2 (BMPR2); Apoptosis inducing factor (AIF); X-linked inhibitor of apoptosis protein (XIAP);

Transforming growth factor beta (TGFb) activated kinase 1 (TAK1); Reactive oxygen species (ROS); Tumor-necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL);

Non-small cell lung carcinomas (NSCLC); Z-VAD-FMK (VAD); LDN-193189 (LDN);

DNA double stranded breaks (DNA-DSB); Inhibitor of apoptosis proteins (c-IAP); mitochondrial DNA (mDNA); NOD SCID IL2Rgamma^{null} (NSG); Poly ADP ribose polymerase (PARP); Receptor-associated

adaptor kinase 1 (RIP1);

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Figures

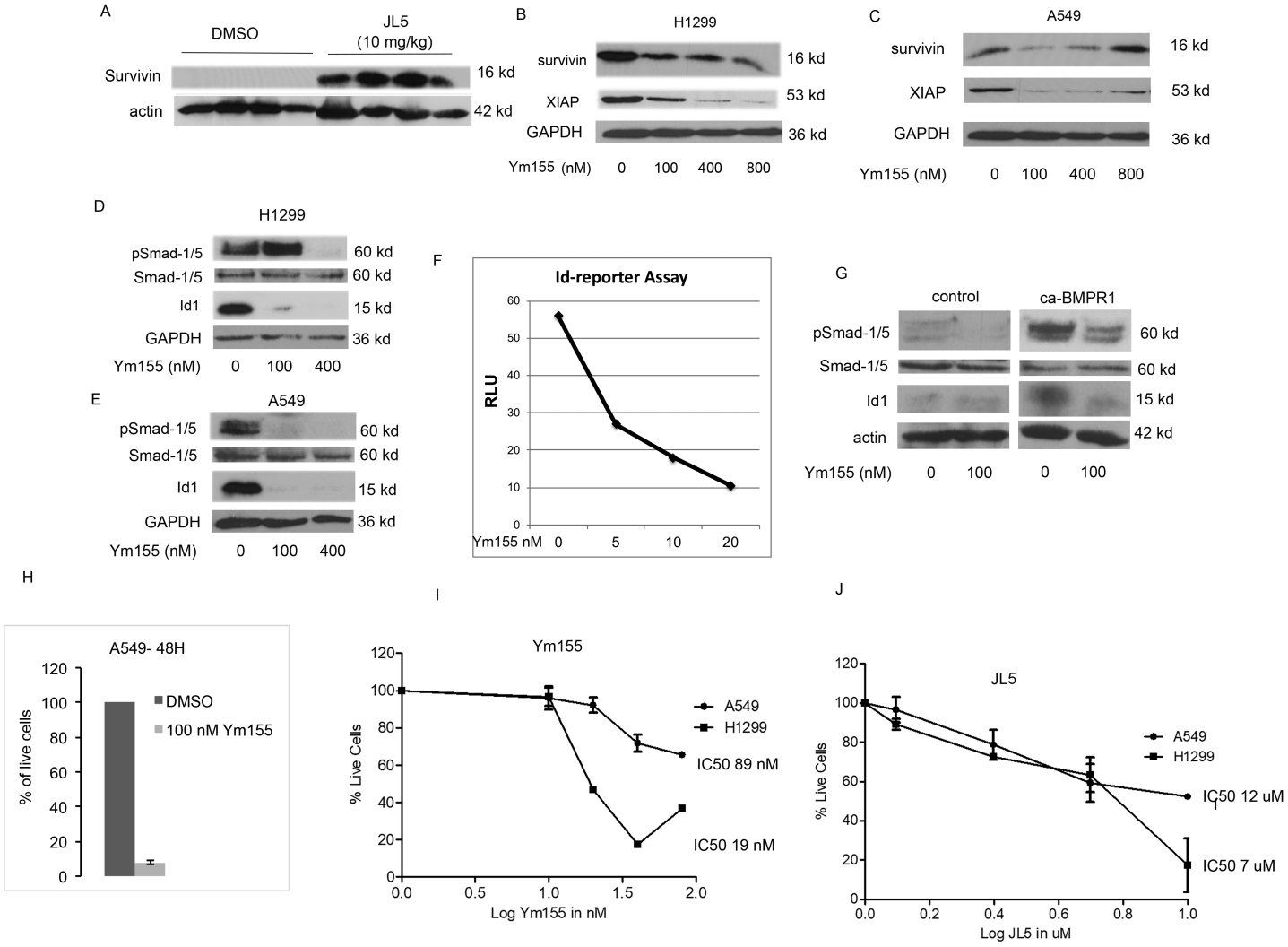

Figure 1

Ym155 regulates BMP signaling. H1299 cells were injected intradermally into NSG mice. When tumors reached approximately 0.5 mm2, mice were treated with DMSO or JL5 for 4 days. (A) Western blot analysis shows JL5 treated tumors had a significant increase in the expression of survivin. (B-C) H1299 and A549 cells were treated with Ym155 for 24 hours. Western blot shows Ym155 causes a decrease in Smad 1/5 independent signaling as demonstrated by a decrease in expression of XIAP. (D-E) Western blot showing Ym155 causes a decrease in Smad 1/5 dependent signaling in both the (D) H1299 and (E) A549 cells. (F) H1299 cells stably transfected with Id1-luciferase reporter were treated with Ym155 for 24 hours and relative luminescence units (RVU) measured. Data shown is the mean of 2 independent experiments. (G) H1299 cells were transiently transfected with control vector or caBMPR1 then treated with Ym155 for 24 hours. Western blot analysis showing Ym155 inhibits caBMPR1 activation of Smad-1/5 signaling. (H-1) Data shows the mean of percent live cells of 3 independent experiments after being treated with Ym155 for 48 hrs. (J) Dose response curves of the number of live cells after being treated with Ym155 (I) or JL5 for 24 hrs. Full-length Western blots are shown in Supplementary Figure 2. Lanes were removed

from original Western blots in Figure 1A and Figure 1G using Adobe Photo shop as shown in Supplementary Figure 2.

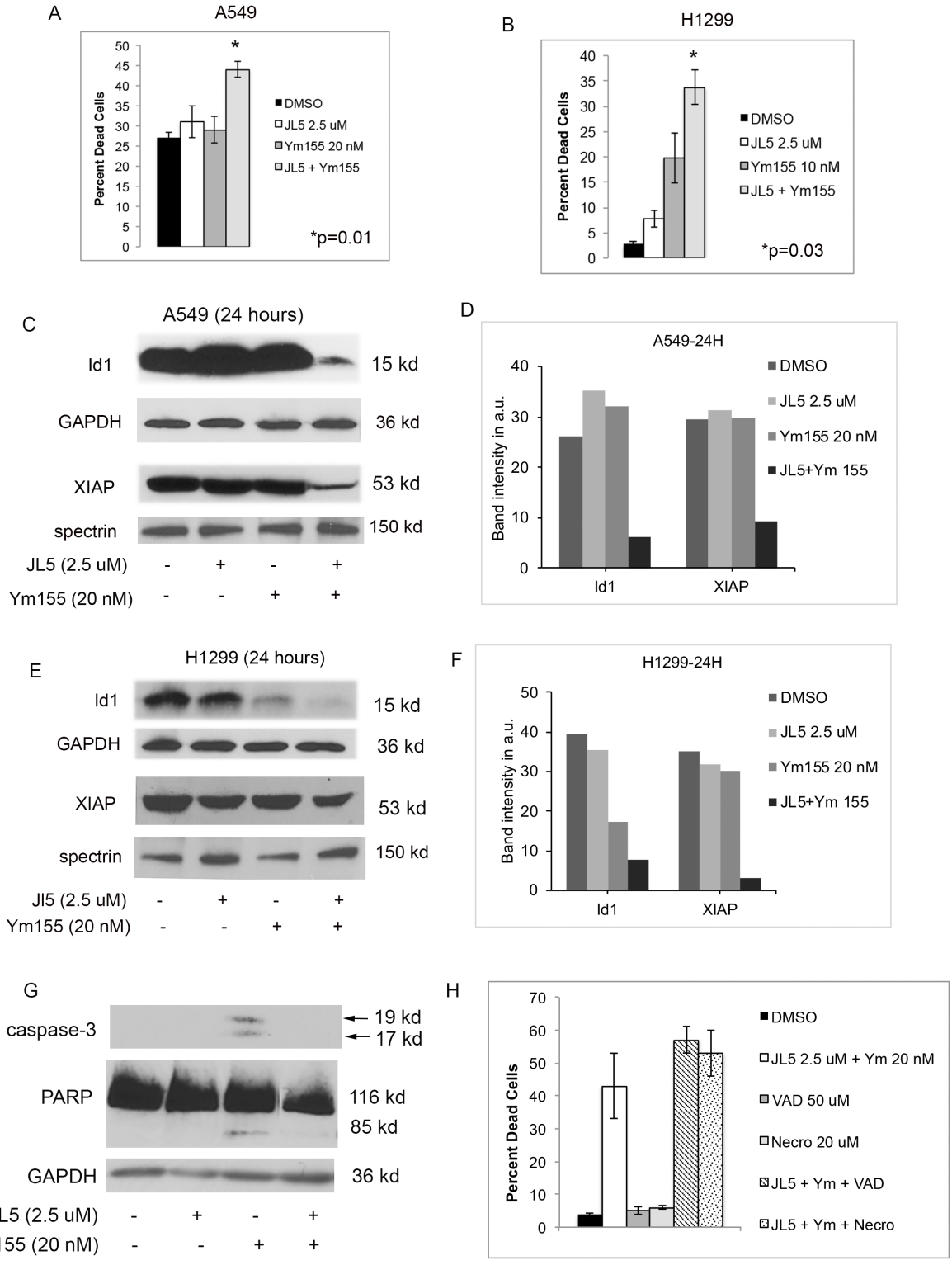

Figure 2

Ym155 synergizes with JL5. (A-B) Lung cancer cells were treated with Ym155 and JL5 alone and in combination for 24 hours and cell counts performed. (A-B) Represent the mean of four independent experiments. (C-F) Western blot analysis assessing the BMP signaling cascade in response to cells

treated with Ym155 and JL5 alone and in combination for 3 or 24 hours. (D, F) Band intensity for Id1 and XIAP was quantified using ImageJ and normalized to spectrin. (G) Western blot analysis of H1299 cells treated for 24 hours. Activated caspase-3 and PARP cleavage only occurred in cells treated with Ym155. (H) The mean percentage of dead H1299 cells after treatment with JL5 and Ym155 in combination for 24 hours with and without Z-VAD-FMK or necrostatin. Data represents the mean of 4 independent experiments. Full-length Western blots are shown in Supplementary Figure 3. Lanes were removed from original Western blots in Figure 2G using Adobe Photo shop as shown in Supplementary Figure 2.

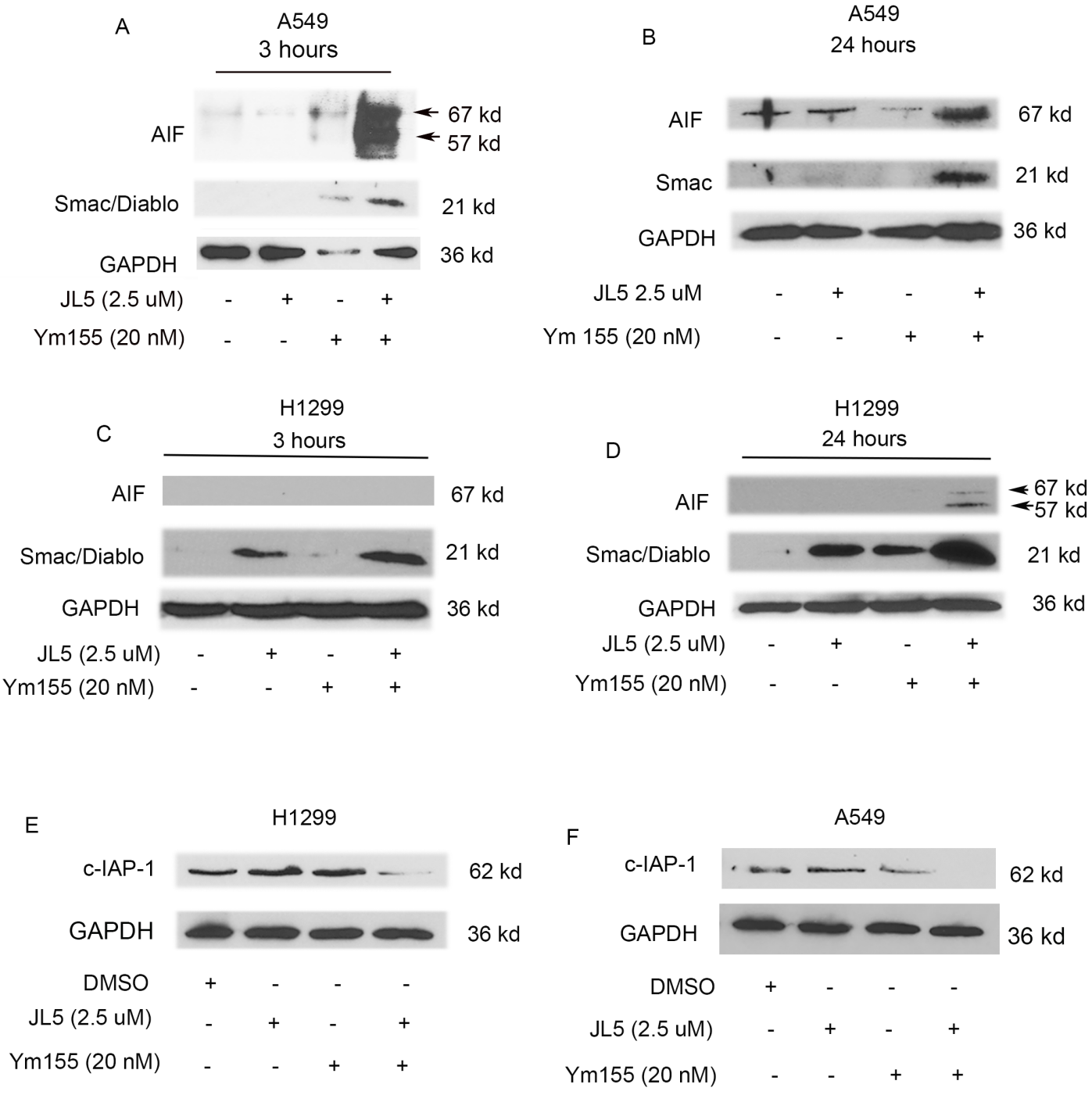

Figure 3

Ym155 in combination with JL5 increases cytosolic AIF. (A-D) Western blot of cytosol of A549 and H1299 cells treated with JL5 and Ym155 alone and in combination for 3 or 24 hours. Arrowheads demonstrate the 67 kd and 57 kd fragments of AIF, which only occur in cells treated with JL5 and Ym155 in combination. (E-F) Western blot analysis of cells treated for 24 hours demonstrating a decrease expression of anti-apoptotic proteins when cells are treated with both JL5 and Ym155 in combination. Full-length Western blots are shown in Supplementary Figure 2.

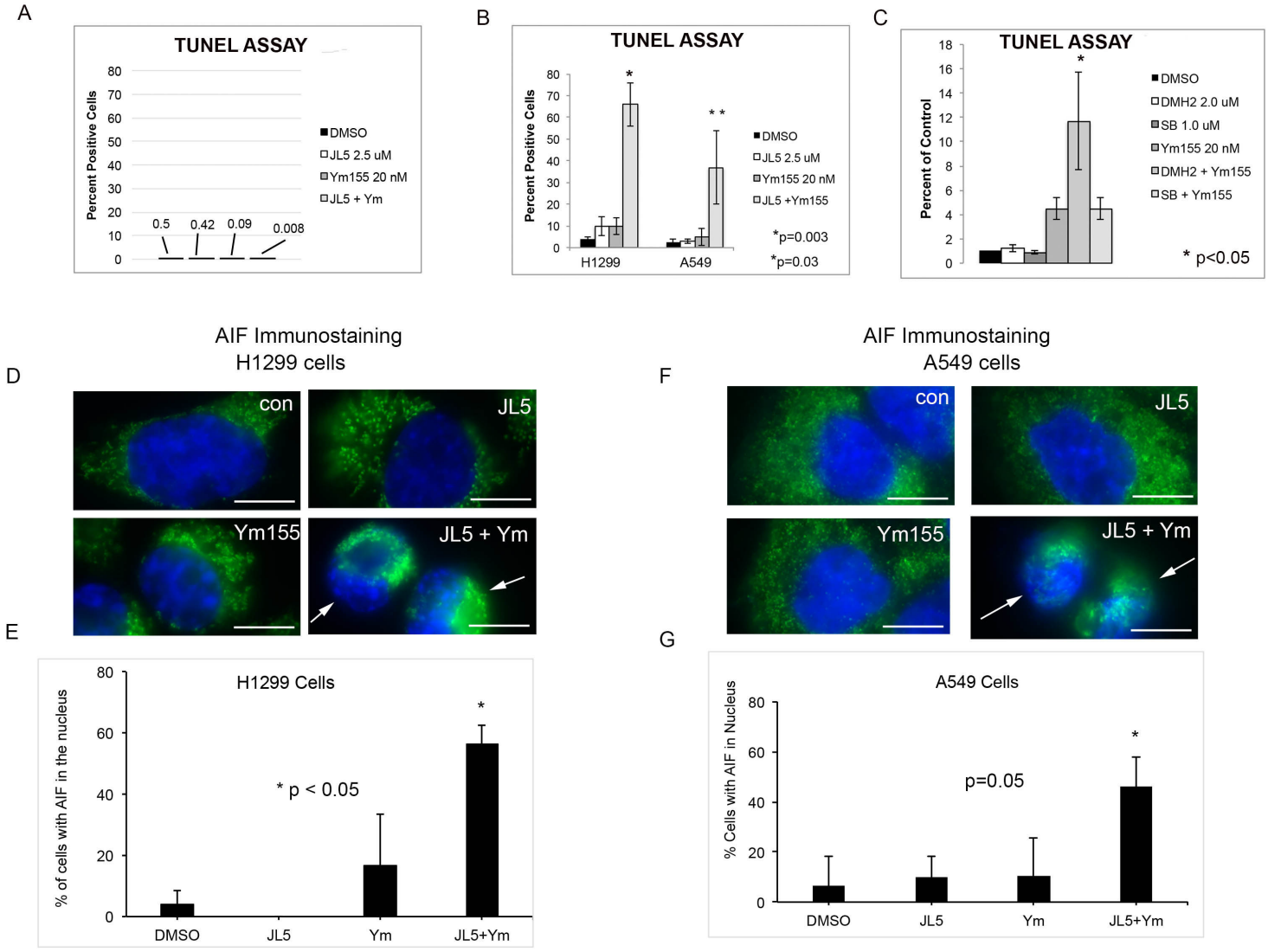

Figure 4

BMP inhibitors together with Ym155 increase DNA-DSB and AIF localization to the nucleus. (A) TUNEL assay of H1299 cells were treated with JL5 and Ym155 alone and in combination for 3 hours. (B) TUNEL assay of H1299 and A549 cells treated with JL5 and Ym155 alone and in combination for 24 hours. (C) TUNEL assay of H1299 cells treated with DMH2, SB-505124 (SB), alone or in combination with Ym155 for 24 hours. (A-C) Data represents the mean of 4 independent experiments. (D-G) AIF Immunostaining after cells being treated with JL5 and Ym155 alone and in combination for 24 hrs. (D, F) Representative immunofluorescent images of AIF (green) with nucleus stained with DAPI (blue). Arrows show cells with

AIF in the nucleus and condensation of the nuclei. (E, G) Graphs depicting the percentage of cells with AIF localized to the nucleus for each cell line. Scale bar is equal to 10 μm.

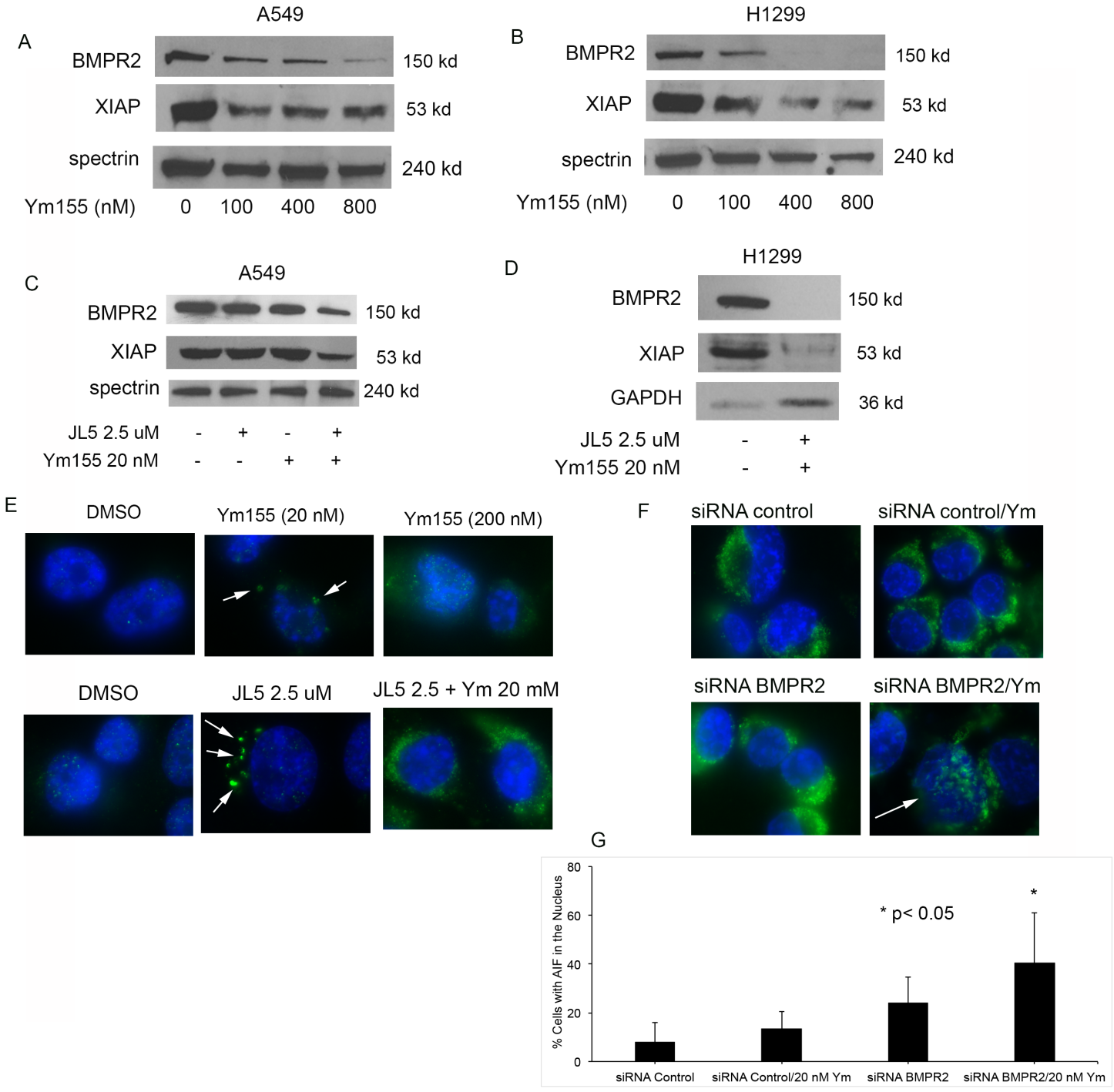

Figure 5

Ym155 regulates BMPR2 expression and causes it to localize to the cytoplasm. (A-B) Western blot of cells treated with Ym155 for 24 hours demonstrating a decrease in the expression of BMPR2 and XIAP. (C-D) Western blot of cells treated with JL5 and Ym155 alone and in combination for 24 hours. (E) Immunofluorescent imaging for cytoplasmic BMPR2 (green) with nucleus stained with DAPI (blue) of H1299 cells treated for 24 hours. BMPR2 localized to the cytoplasm when treated with Ym155 or JL5 2.5

M alone, which increased significantly when compounds were used in combination. (F-G) H1299 cells were transfected with siRNA control or siRNA BMPR2 then treated with DMSO or Ym155 20 nM for 24 hours. (F) AIF localization to the nucleus increased in siRNA BMPR2 cells treated with Ym155 compared to siRNA control cells treated with Ym155. (G) Over 100 cells were quantified for nuclear localization of AIF and presented as percent positive cells. Scale bar is equal to 10 m. Original Western blots are shown in Supplementary Figure 2.

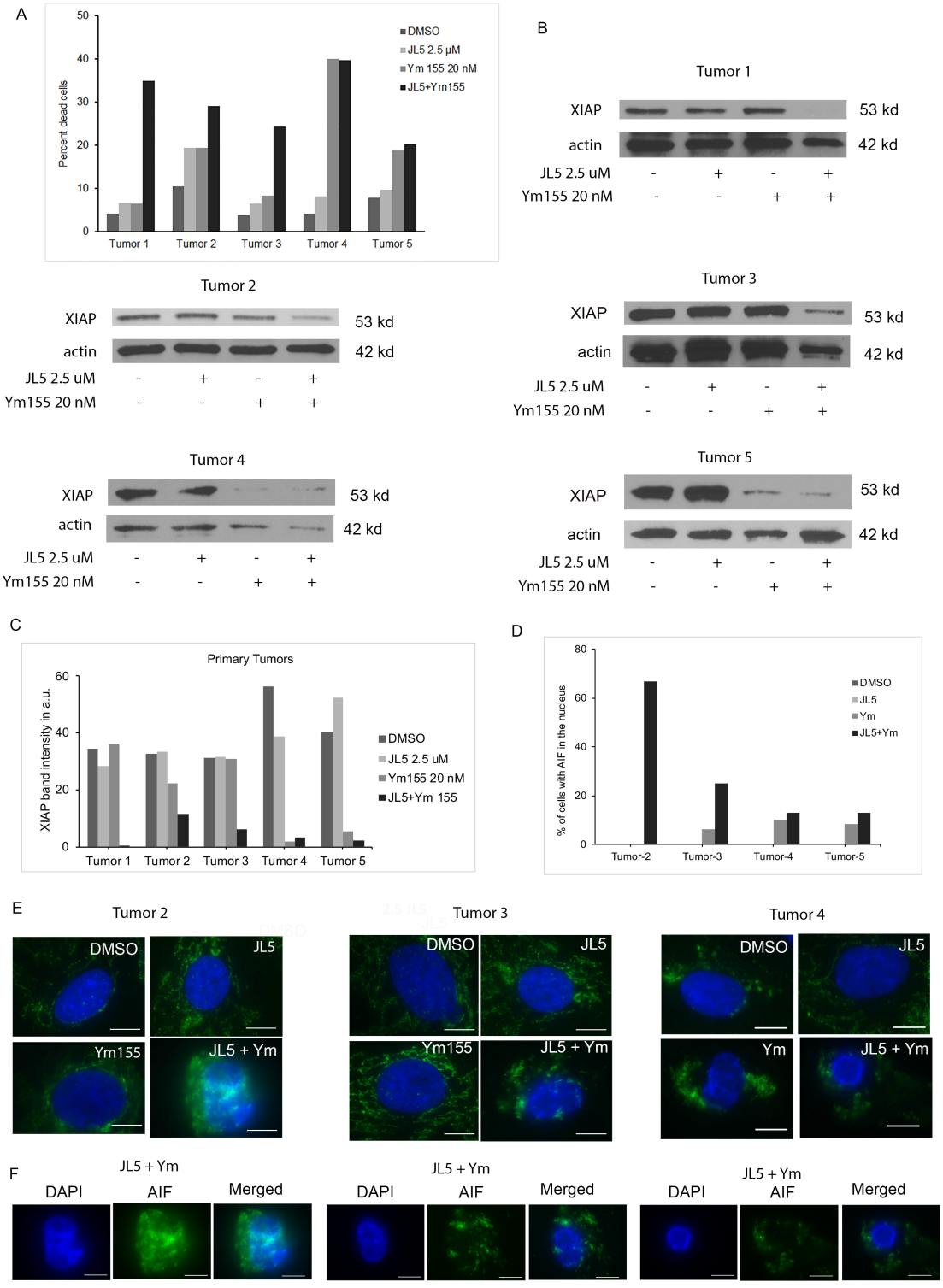

Figure 6

JL5 and Ym155 enhance cell death of primary lung tumors. Five NSCLC samples resected from patients were gently digested and placed into cell culture. When the cells reached confluence (approximately 10 days) they were split and the next day treated with JL5 2.5 M and Ym155 20 nM alone and in combination for 48 hours. (A) The percent dead cells after treatment. Data depicts the average of two experiments. (B) Western blot analysis of tumors 1-5. (C) Immunoblots were scanned and band intensity for XIAP was quantified by ImageJ and normalized to actin (D) The percentage of cells with nuclear staining with AIF after treatment for 48 hours. (E) Immunofluorescent imaging for AIF (green) and counterstained with DAPI (blue) for tumors 2-4. Approximately 50 cancer cells were counted per tumor for each condition. Scale bar is equal to 10 m. (F) DAPI, AIF, and merged images from tumors 2-4 treated with JL5 and Ym155. Full-length Western blots are shown in Supplementary Figure 2.

Supplementary Files

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