

Bone Morphogenetic Protein Receptor 2 (BMPR2)/TAK1-d signaling regulates the phosphorylation of Ser76 in MCUb to control mitochondrial Ca^{2+} levels and survival of cancer cells

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Abstract

Background

Inhibiting bone morphogenetic protein receptor 2 (BMPR2) signaling downregulates TAK1-d that increases mitochondrial Ca^{2+} (mtCa²⁺) levels. BMPR2 inhibition in non-small cell lung carcinoma (NSCLC) cells and mouse hippocampal cells increases mitochondrial bioenergetics. In leukemia cells, BMPR2 inhibition causes more cell death, higher mtCa²⁺ levels and less ATP production compared to NSCLC. In this study, we examined whether BMPR2/TAK1-d signaling phosphorylates Ser92 in the mitochondrial uniporter (MCU) and/or Ser76 in MCUB, a dominant-negative MCU paralog, to control mtCa²⁺ levels. In addition, we explored whether combining a BMPR2 inhibitor with a BCL-2 inhibitor induces mtCa²⁺ overload and cell death. Furthermore, the effects of phosphorylating Ser76 MCUB on ATP levels was studied.

Methods

mtCa²⁺ and mitochondrial superoxide anion (mtROS) levels were assessed with Rhod-2AM and MitoSox Red staining respectively and quantified by flow cytometry. BMPR2 inhibitor JL189, TAK1-d inhibitor (5Z-7-Oxozeaenol), siRNA targeting BMPR2, TAK1, MCU, and MCUB, cDNA overexpression, and genetic deletions were used to determine whether BMPR2/TAK1-d signaling phosphorylated Ser92 in MCU and/or Ser76 in MCUB. MCUB knockout cells reconstituted with wild-type MCUB, kinase inactive Ser76/A MCUB, or constitutively active Ser76/E MCUB were used to assess the effects of the phosphorylation of Ser76 MCUB on mtCa²⁺ levels. CellTiter Glo was used to determine ATP levels.

Results

JL189 combined with BCL-2 inhibitors synergistically induces cell death of NSCLC and leukemia cells associated with a synergistic increase in mtROS and mtCa²⁺ levels. Inhibiting BMPR2/TAK1-d signaling enhances mtCa²⁺ influx induced by ROS. BMPR2/TAK1-d signaling phosphorylates both Ser76 in MCUB and Ser92 in MCU. Phosphorylation of Ser76 MCUB decreases mtCa²⁺ levels and ATP levels, while decreasing the phosphorylation of Ser76 MCUB increases mtCa²⁺ levels. Importantly, chemotherapy resistant p53 mutated acute myeloid cells leukemia are highly sensitive to the combination of JL189 and venetoclax.

Conclusion

Our studies suggest that inhibiting BMPR2/TAK1-d signaling promotes synergistic cell death when combined with BCL-2 inhibitors and other therapeutics by inducing mtCa²⁺ overload which is mediated in

part, by decreasing the phosphorylation of Ser76 MCUB. Furthermore, our studies suggest that BMPR2/TAK1-d regulation of Ser76 in MCUB are pleiotropic, regulating both cell survival and mitochondrial bioenergetics.

Introduction

Bone morphogenetic proteins (BMP) are highly conserved multifunctional signaling proteins that are required for cell fate decision of stem cells during embryonic development. Post development BMP signaling becomes silent in most tissues [1]. BMP ligand expression increases with inflammation and is overexpressed in several age-related diseases including Alzheimer's disease (AD), obesity [2, 3], cancer [4–7], metabolic syndrome [8], cardiovascular disease [9], and anemia of chronic disease [10]. There are more than 20 bone morphogenetic protein (BMP) ligands that signal through type 1 (Alk2, Alk3, Alk6) receptors (BMPR1) and type 2 (BMPR2, ACVR2A, and ACVR2B) serine-threonine kinase receptors. BMPR2 signals through binding of the type 1 receptors in the canonical pathway or independent of the type 1 receptors in the noncanonical pathway [11–14]. Tumorigenic pathways upregulated by BMPR2 noncanonical signaling include X-linked inhibitor of apoptosis (XIAP), TGF β activated kinase (TAK-1), and PI3K/mTOR signaling [12–15].

We recently reported that BMPR2 inhibition (BMPR2i) by genetic deletion and with the BMPR2 inhibitor JL189 induces Ca²⁺ influx into the mitochondria (mt Ca²⁺), which is dependent on the mitochondrial uniporter (MCU) [16]. BMPR2 signaling activates TAK1-d splice variant, which inhibits mtCa²⁺ influx [16]. BMPR2i increased mtCa²⁺ levels in non-small cell lung cancer cells (NSCLC), leukemia cells, triple-negative breast cancer cells, mouse hippocampal neuronal cells, and in *C. elegans*, suggesting that BMPR2 regulation of mtCa²⁺ influx is conserved [16]. The response to mtCa²⁺ influx induced by BMPR2i varies between cell types. In NSCLC and mouse hippocampal cells, BMPR2i increases mitochondrial bioenergetics [16]. In leukemia cells, BMPR2i induces higher mtCa²⁺ levels, less ATP production, and promotes more cell death compared to NSCLC cells [16]. The TAK-d activator TAB1, co-immunoprecipitated with TAK1-d and a 30 kd protein recognized by an anti-phospho Ser92 MCU antibody [16]. Ser92 is in the N-terminal domain redox sensing region of the MCU. MCUB is a dominant-negative paralog of MCU [17]. The N-terminal redox region is conserved in MCU and MCUB. Ser92 of MCU is conserved as Ser76 in MCUB [17]. This suggested that BMPR2/TAK1-d signaling may regulate Ser92 in MCU and/or Ser76 in MCUB to regulate mtCa²⁺ levels.

The uptake of Ca²⁺ into the mitochondria is a conserved mechanism that is essential for increasing mitochondrial bioenergetics. However, when mtCa²⁺ levels become too high it induces programmed cell death pathways [18]. Entry of Ca²⁺ into the mitochondrial intermembrane space is controlled by the voltage dependent anion channel (VDAC1)[19]. The anti-apoptotic regulators BLC-2 and BLC-xL attenuate mtCa²⁺ overload by inhibiting VDAC1 transport of Ca²⁺ into the mitochondria intermembrane space [20, 21]. The MCU complex is composed of the MCU, MCUB, mitochondrial calcium uptake 1–3 (MICU1-3), and EMRE [22]. The MCU regulates the rapid entry of Ca²⁺ from the intermembrane space into the

mitochondrial matrix, which is inhibited by MCUB [22]. The mechanisms regulating the transport of Ca^{2+} by the MCU complex is poorly understood. It is also unknown whether the MCU complex can be pharmaceutically targeted to induce mtCa^{2+} overload and cell death of cancer cells.

Studies here reveal that the combination of the BMPR2 inhibitor JL189 [23] and BCL-2 inhibitors venetoclax and navitoclax synergistically induce cell death and mtCa^{2+} overload, which is more prominent in leukemia cells compared to NSCLC. Importantly, chemotherapy resistant p53 mutated AML cell lines are highly sensitive to the combination of JL189 and venetoclax. Inhibiting BMPR2/TAK1-d signaling enhances mtCa^{2+} uptake induced by reactive oxygen species (ROS). Our studies show that BMPR2/TAK1-d signaling phosphorylates both Ser76 in MCUB and Ser92 in MCU. Phosphorylation of Ser 76 in MCUB decreases mtCa^{2+} levels and ATP levels, while decreasing the phosphorylation of Ser76 in MCUB increases mtCa^{2+} levels. Our studies suggest that inhibition of BMPR2/TAK1-d signaling enhances the uptake mtCa^{2+} by decreasing the phosphorylation of Ser76 in MCUB, which sensitizes cancer cells to mtCa^{2+} overload and cell death when combined with a BCL-2 inhibitor or chemotherapeutics that generate ROS. Furthermore, our studies suggest that the effects of phosphorylating Ser76 in MCUB are pleiotropic, promoting cell survival in leukemia cells and decreasing mitochondrial bioenergetics in neuronal cells.

Material and Methods

Chemicals and reagents

The compound JL189 was designed and synthesized at the Rutgers Molecular Design and Synthesis facility by John Gilleran and Jacques Roberg. The reagents are purchased from the commercial source as follows: Rhod-2 AM (R1245MP), Fluo-4 AM (F14201), Pluronic F-127 (P3000MP), MitoTracker Green (M7514), Cell ROX (C10444), MitoSOX Red (M36008), RNAiMAX (13778), and Lipofectamine 3000 reagent (L3000) from Invitrogen (Waltham, MA, USA). 5Z-7-Oxozeaenol (HY-12686), Vitamin E (HY-N0683), cisplatin (HY-17394), ABT-263 (HY-10087), ABT-199 (HY-15531), vincristine (HY-N0488), vinblastine (HY-13780), paclitaxel/taxol (HY-B0015) from MedChemExpress (Monmouth Junction, NJ, USA). DDKTrap Anti-DDK Affinity Resin (AR100028S) from OriGene (Rockville, MD, USA). Nondenaturing lysis buffer (9803) and protease/phosphatase inhibitor cocktail (5872) from Cell Signaling Technology (Danvers, MA, USA) and RIPA lysis buffer (R0278) from Sigma-Aldrich (St. Louis, MO, USA). BMP2 ligand (355-BEC) from R&D systems.

Cell lines and cell culture

The cancer cell lines used in this study were established from human tumor tissues. The commercial source of the cell lines is as follows: NSCLC cell lines Calu-1 (KRAS G12C mutation, homozygous p53 deletion), NSCLC cell lines H1299 (p53 homozygous deletion, NRAS Q61K heterozygous mutation), and A549 (KRAS G12C homozygous mutation, LKB1 deletion), H2009, and H2030 are from American Type Culture Collection (ATCC) (Manassas, VA, USA). A549 BMPR2 KO cells with a 104 bp deletion of exon 11

in the kinase domain are from FenicsBio (Helethorpe, MD, USA). Chronic myelogenous leukemia cell lines K562 are from ATCC and HAP-1 WT and HAP-1 VDAC1 KO cells are from Horizon Discovery (Cambridge, UK). The HT-22 mouse hippocampal neuronal cell line is from Millipore (Burlington, MA, USA). Acute myeloid leukemia (AML) cell lines, THP-1 cells (p53 heterozygous mutation, MLL-AF9 fusion), and Kasumi-1 cells (p53 homozygous mutation, c-Kit activating mutation), MV4-11, and HEK293 cells are from ATCC and HL-60 (p53 homozygous deletion, NRAS mutation) from DSMZ (Brunswick, Germany). Acute lymphoblastic leukemia (ALL), Jurkat cells (Activating Notch1 A1627H heterozygous mutation, p53 heterozygous mutation), and Jurkat MCU KO cells were kindly provided by Mohamed Trebak (University of Pittsburgh, USA), and DND-41 from DSMZ. The HT-22 mouse hippocampal neuronal cell line is from Millipore (Burlington, MA, USA). Cell lines were authenticated by the suppliers.

All cell lines were cultured at 37 °C in a humidified incubator with 5% CO₂ in media supplemented with 10% FBS, 1% antibiotic, and 1 x L-glutamine, unless otherwise specified. THP-1, K562, Kasumi-1, Jurkat, H2009, and H2030 cells were cultured in RPMI-1640. Additionally, 0.05 mM 2-Mercaptoethanol was added to THP1 cells, and 20% FBS was added to Kasumi-1 cells. A549 WT, A549 BMPR2 KO, and H1299 cells were cultured in DMEM low glucose and 5% FBS, and HT-22 cells were grown in DMEM high glucose. Calu-1 cells were cultured in McCoy's medium, and HAP-1 WT and VDAC1 KO cells were cultured in IMDM medium.

Cell viability analysis

Cell death was analyzed by flow cytometry with FITC Annexin V staining using the Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's protocol. Briefly, cells were treated for the desired time points and then washed with PBS. Cells were stained with 100 µL staining solution containing Annexin V-FITC and Propidium iodide (PI) in 1X binding buffer for 30 minutes in the dark at room temperature. After incubation, 400 µL buffer was added to each sample, and the samples were analyzed by flow cytometry at the CINJ Rutgers Central Facility (Cytex Aurora).

Cell viability was performed by Vi-CELL BLU cell viability analyzer (Beckman Coulter Brea, CA, USA), using the trypan blue dye exclusion principle. Briefly, cells were seeded in a 6-well plate, treated at the desired time point, and live and dead cells were counted. GraphPad Prism was used to calculate IC₅₀ values.

Antibodies

Rabbit monoclonal antibodies phospho-Smad1/5 (Cat. No. 9516), MCU (Cat. No. 14997), and phospho-TAK1 (Thr184/187) (Cat. No. 4508) were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibodies phospho-TAK1 (Ser412) (Cat. No. 9339) and cleaved caspase 3 (Cat. No. 9661) were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibody anti-actin (Cat. No. A2066) were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal antibody MCUb (Cat. No. AP50412) was purchased from OriGene (Rockville, MD, USA), and MCUb (Cat No. 20387-1-AP) was purchased from Proteintech (Rosemont, IL, USA). Rabbit polyclonal antibody phospho-Ser92

MCU (Cat. No. 14297) was purchased from Signalway Antibody (Greenbelt, MD, USA). Goat polyclonal anti-rabbit IgG (H+L) HRP secondary antibody (Cat. No. 31460) is from Invitrogen (Waltham, MA, USA). Goat anti-mouse IgG (H+L) HRP secondary antibody (Cat. No. 1706516) is from BioRad (Hercules, CA, USA).

Western blot

The Western blot was performed as previously described protocol [6]. Protein was extracted using a radioimmunoprecipitation assay (RIPA) lysis buffer. Briefly, cells were washed with ice-cold PBS and lysed in RIPA buffer supplemented with phosphatase and protease inhibitor cocktails. The cell lysate was then centrifuged at $14,000 \times g$ for 10 minutes at 4°C, and the supernatant obtained. Protein concentration was determined by the BCA assay. Samples were subjected to SDS-PAGE and then transferred onto a 0.2 µm nitrocellulose membrane overnight at 30V at 4 °C. Membranes were blocked with 5% non-fat dry milk or BSA in TBST and incubated with primary antibody overnight at 4 °C. After primary antibody incubation membrane was washed with TBST and incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) and detected using an X-ray film developer.

siRNA and cDNA

The siRNAs and cDNA are purchased from a commercial source as follows: MCUb siRNA (SR324347), TAB1 siRNA (SR307092), and BMPR2 siRNA (SR319490) from OriGene (Rockville, MD, USA). MCU siRNA (4392420) from Ambion Thermo Fisher's (Waltham, MA, USA) and TAB1-Flag cDNA expression vector (HG17460) is from Sinobiological (Beijing, China).

MCUb and MCU KO

MCUb and MCU knockouts were generated using the non-homology-mediated CRISPR-Cas9 technique according to the manufacturer's protocol (Origene). Briefly, cells were seeded in a 6-well plate, and 1 µg of the donor DNA vector and 1 µg of MCUb gRNA (KN401057G1-TGGCAAGGTAAGTGTTACCA) or MCU gRNA (KN406952G1-TATCTGTGAGGCTACCATCC) were transfected using Lipofectamine 3000 Reagent. After 48 hours, the cells were split 1:10 for 5 passages, then antibiotic selection was performed. After antibiotic selection, individual cell colonies were isolated by limiting dilution in a 96-well plate. Genetic knockdown was confirmed by Sanger sequencing at GENEWIZ Azenta Life Sciences (South Plainfield, NJ).

Mitochondrial respiration assay

Mitochondrial function was analyzed by the Seahorse XF Cell Mito Stress assay following the manufacturer's instructions. In brief, cells were plated onto assay plates, and prior to the assay, the culture medium was exchanged for Seahorse assay medium. Oligomycin (1 µM), FCCP (1 µM), rotenone, and antimycin A (1 µM) were then added in sequence, and corresponding changes in oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were recorded. The resulting OCR and

ECAR measurements were normalized against cell numbers. Four independent biological replicates were included for each experimental condition.

Mitochondrial membrane potential analysis by flow cytometry

Mitochondrial membrane potential was analyzed by TMRM staining using flow cytometry. Briefly, cells were treated at the desired time points, then stained with 20 nM TMRM for 30 minutes at 37°C, 5% CO₂. For positive control, the cells were treated with 1 µL of 50 mM CCCP for 5 minutes. After staining, cells were washed with PBS, resuspended in 500 µL PBS, and analyzed by flow cytometry.

Mitochondrial Calcium analysis by flow cytometry

Mitochondrial calcium was analyzed by Rhod-2 AM staining using flow cytometry as described previously [16]. Briefly, cells were treated at the desired time points, then stained with Rhod-2AM (2 µM) for 45 minutes at 37°C, 5% CO₂. 0.02% w/v nonionic detergent Pluronic F-127 was added in Rhod-2 AM solution. DAPI was used to gate for [16] dead cells. After staining, cells were washed with PBS, resuspended in 500 µL PBS, and analyzed using a Cytex Aurora and an Attune I flow cytometer.

Mitochondrial superoxide analysis by flow cytometry

Mitochondrial superoxide levels were assessed by MitoSOX Red staining using flow cytometry, as described previously. Briefly, cells were treated at the desired time points and subsequently stained with 5 µM MitoSOX Red for 30 minutes at 37°C in a 5% CO₂ incubator. After staining, cells were washed with PBS, resuspended in 500 µL PBS, and analyzed using a Cytex Aurora and an Attune I flow cytometer.

Oxidative stress detection by CellROX

Reactive oxygen species (ROS) were analyzed by CellROX green staining by flow cytometry, according to the manufacturer's protocol. Cells were stained with 5 µM Cell ROX green for 30 minutes at 37°C in a 5% CO₂ incubator. After staining, the cells were washed with PBS, resuspended in 500 µL PBS, and analyzed using a Cytex Aurora flow cytometer.

Immunoprecipitation (IP)

The cells were transfected with the Flag-tagged TAB1 expression vector for 24 hours, and then total protein was extracted under non-denaturing conditions. 40 µL of DDKTrap™ resin slurry (20 µL bed volume) was used for each sample. Resin was washed in a non-denaturing lysis buffer, and the clarified protein lysate was incubated with the resin overnight at 4 °C with gentle rotation. The resin was then washed 4 times with a non-denaturing buffer to remove the unbound fraction. The bound protein was eluted in 2× SDS sample buffer at 95 °C for 5 min. Samples were centrifuged at 14,000×g for 1 min, and the supernatant was collected for western blot analysis. The whole cell lysate was used as input.

Cellular ATP measurement

Cellular ATP levels were measured using the CellTiter-Glo assay (Promega). Cells were seeded in a 96-well clear-bottom white plate and treated for the desired time points. CellTiter-Glo reagent and plate were equilibrated at room temperature for 30 minutes. Then, CellTiter-Glo reagent was added directly into the culture medium and mixed for 2 minutes to induce cell lysis. To stabilize the luminescent signal, the plate was incubated for 10 minutes at room temperature in the dark. Luminescence was recorded on a luminometer with an integration time of 1 second.

Lung cancer xenograft

Mice study was approved by Rutgers Rutger's Institutional Animal Care and Use Committee (IACUC), ID number TR202400021. Mice were euthanized for tumors exceeding 15 mm × 15 mm, pain, or loss of 20% body weight, as approved by Rutgers IACUC regulations, which were not met during the study. H1299 cells were mixed with 50% Matrigel in PBS, and 2×10^6 cells were injected into the flanks of NCr nude mice (Taconic Biosciences). Tumors were isolated, and a single-cell suspension was prepared. A total of 1×10^6 cells were injected subcutaneously into the mice with 50% Matrigel. After visible tumor development, the mice were randomized into vehicle, JL189, ABT-263, and JL189 + ABT263 groups. JL189 was dissolved in 5% NMP, 5% Solutol HS-15 and 90% citric acid, and ABT263 was dissolved in 30% PPG, 5% T80, 3.30% D5W, and 1% DMSO. JL189 (30 mg/kg twice a day) and ABT263 (12 mg/kg once a day) were injected by intraperitoneal (IP) injection 5 days a week for 3 weeks. Tumor size and volume were measured.

Pharmacokinetics

The pharmacokinetic properties of JL189 were examined in BALB/c mice following intraperitoneal injection as previously described [15](Sai Life Science, Pune India).

hERG and CYP inhibition

hERG testing was done using a manual patch clamp performed with JL189 at 10 mM in duplicate. Inhibition of CYP isoforms was done using liver microsome with JL189 at decreasing doses to determine the IC_{50} . Samples were analyzed by LC-MS/MS. Studies were done at BioDura, Irvine CA.

Statistical analysis

Statistical analysis was performed in Excel (Microsoft) and GraphPad Prism software (GraphPad Software, San Diego, CA). Data are presented as the mean $\pm$ the standard error. When comparing two groups a paired Student's t test, assuming unequal variances, and one-way analysis of variance (ANOVA) was used to determine statistical significance between two groups and multiple groups respectively. Differences with p values <0.05 were considered to indicate statistical significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

BMPR2i synergistically enhances cell death when combined with BCL-2 inhibitors.

We examined whether the BMPR2 small molecule inhibitor JL189 [23] together with BCL-2/BCL-xL inhibitors ABT-263 or ABT-199 enhanced cell death. ABT-263 (Navitoclax) inhibits both BCL-2 and BCL-xL and ABT-199 (Venetoclax) is selective for BCL-2 inhibition [24]. Synergy was determined by calculating the combination index (CI) [25]. A CI of < 1.0 indicates synergy [25]. JL189 combined with ABT-263 synergistically induced cell death in all five RAS mutated NSCLC cell lines tested (Fig. 1A and **Table S1**). The combination of JL189 and ABT-263 synergistically induced cell death in different subtypes of leukemia cell lines including T-acute lymphoblastic leukemia (T-ALL) (Jurkat, DND-41), acute myeloid leukemia (AML) (Kasumi-1, THP-1) and chronic myeloid leukemia (CML) (HAP-1) cell lines (Fig. 1B and **Table S1**). The percentage of live cells remaining after treatment with JL189 and ABT-263 for 48 hr was lower in the leukemia cell lines as compared to the NSCLC cell lines (**Table S1**). Cells treated with JL189 and ABT-199 induced synergistic cell death in the leukemia cells but not the NSCLC cell lines (**Fig S1**). CML cell lines, HAP-1 and K562, were less cells to cell death induced by JL189 and ABT-199 compared to AML cell lines (**Fig S1, Table S1**). Importantly, all 3 AML cell lines harboring p53 mutations (Kasumi-1, THP-1, HL-60) were highly sensitive to the combination of JL189 and ABT-199 (**Fig S1**). The immortalized human embryonic kidney cells (HEK293) were not responsive to the combination of JL189 and ABT-263 (Fig. 1C), suggesting that cancer cells are more susceptible to cell death.

BMPR2i combined with ABT-263 induces mitochondrial cell death.

Activated caspase-3 and cleaved PARP-1 expression was higher in Jurkat and NSCLC cells (H1299, A549) after being treated with the combination of JL189 and ABT-263 compared to either compound alone (Fig. 1D). JL189 by itself increased mitochondrial membrane potential (MMP) in Jurkat, mouse hippocampal (HT-22) and H1299 cells (Fig. 1E-G), which is likely from the increase in mtCa^{2+} levels. Jurkat cells demonstrated a significant decrease in mitochondrial membrane potential (MMP) when JL189 was combined with ABT-263 and not with either compound alone (Fig. 1E). HT-22 cells and H1299 cells had no decrease in MMP after treatment with JL189 combined with ABT-263 (Fig. 1F-G). Mitochondrial respiration was significantly suppressed in NSCLC cell lines treated with the combination of JL189 with ABT-263 compared to each treatment alone. (Fig. 1H-I). These data suggest that the combination of JL189 and a BCL-2 inhibitor induces synergistic cell death, which is mitochondrial mediated and is more pronounced in leukemia cells compared to NSCLC cells.

BMPR2i synergistically enhances cell death when combined with microtubule targeting drugs.

We examined whether BMPR2i promotes synergy with commonly used chemotherapeutics that target the microtubules (MT). Vincristine and vinblastine are MT destabilizing agents used to treat leukemias. Vincristine combined with JL189 synergistically induced cell death in leukemia cell lines (**Table S1**). Taxol, a MT stabilizing agent used to treat NSCLC, when combined with JL189 synergistically induced cell death in RAS mutated NSCLC cell lines (**Table S1**). To confirm that BMPR2i is required for the observed synergistic cell death, A549 WT and A549 BMPR2 KO cells were examined. Both Taxol and

Vinblastine induced significantly more cell death in the A549 BMPR2 KO cells compared to A549 WT cells (Fig. 1J).

BMPR2 inhibition combined with BCL-2 inhibitors increases mtCa²⁺ levels.

We examined whether JL189 combined with BCL-2 inhibitors synergistically increase mtCa²⁺ levels. After treatment, cells were loaded with mitochondrial Ca²⁺ sensor Rhod2AM and DAPI [16]. DAPI negative cells were analyzed to exclude dead cells. Significantly higher mtCa²⁺ levels were demonstrated in H1299 and A549 cells treated with the combination of JL189 and ABT-263 compared to each compound alone (Fig. 2A-B). In Calu-1 cells, both JL189 and ABT-263 alone significantly increased mtCa²⁺ levels without a significant increase when the compounds were used together (Fig. 2C). Significantly higher mtCa²⁺ levels were also demonstrated in the Jurkat and THP-1 leukemia cells lines treated with the combination of JL189 and ABT-263 compared to each compound alone (Fig. 2D-E). The combination of JL189 and ABT-199 (Venetoclax) also enhanced mtCa²⁺ levels in THP-1 cells but not H1299 cells (Fig. 2F-G).

BMPR2i combined with Taxol synergistically increases mtCa²⁺ levels.

To examine if the synergistic rise in mtCa²⁺ levels is specific for BCL-2 inhibitors, we examined synergy with MT targeting agents. H1299 and Calu-1 cells treated with JL189 combined with Taxol had higher mtCa²⁺ levels than either compound alone (Fig. 2H-I). MT targeting agents are not known to increase mtCa²⁺ levels. However, MT targeting agents do increase ROS levels, which can increase mtCa²⁺ uptake through the MCU [26]. This raised the question whether BMPR2i enhanced mtCa²⁺ uptake induced by ROS.

BMPR2i combined with ABT-263, or MT targeting agents synergistically increases ROS levels, which regulates mtCa²⁺ levels and cell death.

Increasing mtCa²⁺ levels promote oxidative phosphorylation, causing a rise in superoxide anion (mtO₂^{•-}) levels [27]. Free radicals increase the transport of Ca²⁺ across the MCU [27], which can amplify mitochondria Ca²⁺ transport leading to high mtCa²⁺ and ROS levels [27]. If our drug combinations were causing an amplifying effect on mtCa²⁺ transport and ROS production, we would also expect a synergistic increase in mtO₂^{•-} levels. MitoSox Red, a fluorescent mitochondrial superoxide indicator, was used to measure mtO₂^{•-} levels. JL189 increased mtO₂^{•-} levels in Calu1, H1299, and Jurkat cells (Fig. 3A-C). The combination of JL189 and ABT-263 induced significantly higher mtO₂^{•-} levels in Calu-1 (Fig. 3A), H1299 (Fig. 3B), and Jurkat (Fig. 3C) cells compared to either compound alone. CellRox Green, which measures both mitochondrial and cytosolic O₂^{•-} levels, also resulted in significantly greater O₂^{•-} levels in Calu-1 cells treated with the combination of JL189 and ABT-263 (Fig. 3D).

The free radical scavenger vitamin E was used to determine the role of ROS in regulating mtCa²⁺ levels and cell death. Vitamin E effectively attenuates the increase in total O₂^{•-} (Fig. 3D) and mtO₂^{•-} levels

induced by the combination of JL189 and ABT-263 (Fig. 3D-E). Vitamin E also attenuates the increase in mtCa^{2+} levels in Calu-1 cells induced by the combination of JL189 and ABT-263 (Fig. 3F). Vitamin E significantly decreased cell death induced by JL189 combined with ABT-263 in Calu-1, A549, H1299, and Jurkat cells (Fig. 3G-J). Taxol increased $\text{mtO}_2^{\bullet}$ levels in Calu-1 cells (Fig. 3K). The combination of JL189 and Taxol increases $\text{mtO}_2^{\bullet}$ levels significantly higher than either compound alone (Fig. 3K). Vitamin E effectively attenuates the increase in $\text{mtO}_2^{\bullet}$ levels induced by JL189 combined with Taxol (Fig. 3L). Taxol also significantly increased mtCa^{2+} levels, which is attenuated with vitamin E (Fig. 3M). Compared with A549 WT cells, A549 BMPR2-KO cells have higher total $\text{O}_2^{\bullet}$ levels (Fig. 3N). Cisplatin is frequently used to treat advanced NSCLC. A549 BMPR2 KO cells exhibited a greater increase in $\text{O}_2^{\bullet}$ levels when treated with cisplatin compared to A549 WT cells (Fig. 3O). Vitamin E significantly decreases cell death of A549 BMPR2 KO cells treated with cisplatin (Fig. 3P). These data suggest that BMPR2i enhances ROS levels when combined with cancer therapeutics, which contributes to elevated mtCa^{2+} levels and cell death.

BMPR2 signaling regulates mitochondria Ca^{2+} transport induced by ROS.

In Calu-1 cells, JL189 induces a rapid increase in mtCa^{2+} levels with peak levels after 90 seconds and returning to baseline within 5 minutes [16]. However, after 2 hr of treatment with JL189, the baseline mtCa^{2+} levels remain elevated (Fig. 4A). Pretreatment with vitamin E attenuates JL189 induced increase in the basal mtCa^{2+} levels (Fig. 4A). These data suggest that the sustained increase in mtCa^{2+} levels after treatment with JL189 involves an increase in ROS levels. JL189 does not increase $\text{mtO}_2^{\bullet}$ levels during the first 3 minutes after treatment in Calu-1 cells, suggesting that mtCa^{2+} influx proceeds the rise of $\text{mtO}_2^{\bullet}$ levels following treatment with JL189 (Fig. 4B).

Next, we examined whether BMPR2 signaling regulated mtCa^{2+} transport induced by ROS. Calu-1 cells were treated with 500 μM of hydrogen peroxide (H_2O_2) with and without JL189. Extracellular levels of H_2O_2 at 500 μM is considered a high physiologic dose [28]. mtCa^{2+} levels were higher in cells treated with JL189 and H_2O_2 together compared to either compound alone (Fig. 4C). Conversely, stimulating BMPR2 signaling with BMP2 ligand decreases mtCa^{2+} influx induced by H_2O_2 (Fig. 4D). Knockdown (KD) of BMPR2 in Calu-1 cells with siRNA significantly increased mtCa^{2+} influx induced by H_2O_2 (Fig. 4E). These studies demonstrate that BMPR2 signaling regulates ROS-induced mtCa^{2+} influx.

BMPR2 downstream target TAK1-d regulates mtCa^{2+} influx induced by ROS.

BMPR2i induces the influx of mtCa^{2+} by downregulating the activity of TAK-d splice variant [16]. Utilizing the TAK-1 inhibitor 5Z-7-Oxozeaenol (5Z-7-Oxo), we examined if TAK-1 regulates mitochondria Ca^{2+} influx is induced by ROS. Calu-1, THP-1, and HT-22 cells treated with 5Z-7-Oxo demonstrated higher baseline mtCa^{2+} levels by 2 hr (Fig. 4F-H). Pretreatment with 5Z-7-Oxo enhanced mtCa^{2+} influx induced by H_2O_2 in Calu-1, THP-1, and HT-22 cells (Fig. 4F-H). TAB1 is required for TAK1 activation and its transfection in THP-1 cells activates TAK1-d splice variant [16]. THP-1 cells transfected with TAB1 significantly

suppressed H₂O₂ induced mtCa²⁺ influx (Fig. 4I). These data suggest that BMPR2/TAK1-d signaling suppresses the influx of mtCa²⁺ induced by ROS and when BMPR2/TAK1-d signaling is inhibited the influx of mtCa²⁺ induced by ROS is enhanced.

BMPR2/TAK1-d signaling regulates the phosphorylation of a 30 kd MW protein detected by an anti-phospho Ser92 MCU antibody.

We recently reported that TAB1-Flag co-immunoprecipitated with TAK1-d and a 30 kd protein that is detected by an anti-phospho Ser92 MCU antibody [16]. First, we examined whether BMPR2 and TAK1-d signaling regulates the phosphorylation of the proteins detected by the anti-phospho-Ser92 MCU antibody. For clarity, we refer to proteins detected by the anti-phospho-Ser92 MCU antibody as pSer92 MCU even though it may also represent pSer76 in MCUB. JL189 significantly decreased the expression of pSer92 MCU at 30 kd in Calu-1, THP-1, and K562 cells (Fig. 5A-C). Activation of TAK1-d by TAB1 transfection increased the expression of 30 kd pSer92 MCU in THP-1 cells (Fig. 5D). To inhibit TAK1-d signaling, TAB1 expression was KD with siRNA [16], which caused a significant decrease in the expression of 30 kd pSer92 MCU (Fig. 5E). These studies establish that BMPR2/TAK1-d signaling regulates the phosphorylation of the 30 kd MW protein detected by the anti-phospho-Ser92 MCU antibody.

BMPR2/TAK1-d signaling regulates higher MW proteins detected by phospho-Ser92 MCU antibody in NSCLC but not leukemia cell lines.

On further analysis, TAB1-Flag also co-immunoprecipitated 52 kd and 77 kd MW proteins recognized by the anti-phospho-Ser92 MCU antibody (Fig. 5F). Immunoblots show that the anti-phospho-Ser92 MCU antibody detects phosphorylated 77 kd, 52 kd, 39 kd, and 30 kd MW proteins in both the NSCLC and leukemia cell lines. On light exposure, the immunoblot highlights that the 77 kd, 52 kd, and 30 kd MW proteins are expressed higher in NSCLC cell lines compared to leukemia cell lines, while the 39 kd MW protein is expressed higher in leukemia cell lines (Fig. 5G).

To test whether the different MW pSer92 MCU proteins are regulated by BMPR2/TAK1-d signaling, cells were treated with 5Z-7-Oxo. In Calu-1 cells, 5Z-7-Oxo decreased the expression of 52 kd and 30 kd pSer92 MCU (Fig. 5H). KD of BMPR2 with [16] siRNA also significantly decreased the expression of 52 kd and 30 kd pSer92 MCU in Calu-1 cells (Fig. 5I). In H1299 cells, 5Z-7-Oxo decreased the expression of 39 kd and 30 kd pSer92 MCU (Fig. 5J). In THP1 and K562 cells, 5Z-7-Oxo significantly decreased the expression of only 30 kd pSer92 MCU (Fig. 5K-L). The estimated MW of MCU and MCUB are approximately 30–39 kd, therefore, the different MW proteins are most likely post-transcriptional modifications such as glycosylation [29]. These studies suggest that in NSCLC, BMPR2/TAK1-d signaling regulates 52 kd, 39 kd, and 30 kd pSer92 proteins. While in leukemia cells, BMPR2/TAK1-d signaling only regulates the 30 kd protein detected by the anti-phospho-Ser92 MCU antibody.

MCUB KO in THP-1 and Calu-1 cells.

To assess whether BMPR2/TAK1-d signaling phosphorylates Ser76 in MCUB and/or Ser92 in MCU, we utilized CRISPR-Cas9 gRNA targeting MCUB exon 3 in THP-1 and Calu-1 cells. THP-1 MCUB KO clones have multiple missense mutations in exon 3 (**Fig S2A**). Early passage of THP-1 MCUB KO cells showed decreased expression of pSer92 MCU at 52 kd and 30 kd in 2 of 3 clones (Fig. 6A-B). Sequencing of MCUB KO clones in Calu-1 cells revealed a single missense mutation at in exon 3 (**Fig S2B**). There were doublets in the sequencing suggesting not all the alleles of MCUB were KO. MCUB in Calu-1 clones showed a decreased expression of 52 kd pSer92 MCU and an increased expression of 30 kd pSer92 MCU (Fig. 6C). KD of MCUB with siRNA in Calu-1 cells decreased the expression of 52 kd pSer92 MCU with a variable response in the expression of 30 kd pSer92 MCU (Fig. 6D). In some studies, 30 kd pSer92 MCU expression was increased and others it was decreased. These studies suggest that 52 kd pSer92 MCU is MCUB. However, the origin of the 30 kd pSer92 MCU band needed further investigation. Although the 30 kd pMCU band was not consistently downregulated or even upregulated in MCUB KO clones this could be due to a compensatory increase in the phosphorylation of Ser92 in the MCU.

Phosphorylated 30 kd Ser92 MCU regulated by BMPR2/TAK1-d is MCUB

siRNA targeting MCUB or MCU was used to further identify the origin of 30 kd protein detected by the anti-phospho-Ser92 MCU antibody. In Calu-1 WT cells, knockdown (KD) of MCU did not change the expression of 30 kd pSer92 MCU (Fig. 6E). However, in Calu-1 MCUB KO cells, KD of MCU significantly decreased the expression of 30 kd pSer92 MCU (Fig. 6F). In THP-1 MCU KO3 cells but not THP-1 WT cells, KD of MCU also decreased the expression of 30 kd pSer92 MCU (Fig. 6G). We utilized K562 cells since they have a high transfection efficiency without cell death. In K562 cells, KD of MCUB significantly decreased the expression of the 30 kd protein detected by the anti-phospho-Ser92 MCU antibody (Fig. 6H), while KD of MCU significantly increased the expression of the 30 kd pSer92 MCU band (Fig. 6I). The siRNA KD studies suggest that the 30 kd phosphorylated protein recognized by our anti-phospho-Ser92 MCU antibody and regulated by BMPR2/TAK1-d signaling is Ser76 in MCUB. The studies also suggest that decreasing the expression of MCUB causes a compensatory increase in the phosphorylation of Ser92 in MCU.

Decreased expression of MCU causes a compensatory increase in phosphorylation of Ser76 in MCUB

We utilized the haploid chronic myelogenous leukemia (CML) cell line HAP-1 to KO MCU. HAP-1 MCU KO cells demonstrated an increase in the expression of 30 kd pSer92 MCU (Fig. 6J). In HAP-1 MCU KO cells, the KD of MCUB decreased the expression of the phosphorylated 30 kd protein detected by the anti-phospho-Ser96 MCU antibody (Fig. 6K). These data suggest that decreasing the expression of MCU causes a compensatory increase in the phosphorylation of Ser76 MCUB. Next, we examined whether the compensatory increase in the phosphorylation of Ser76 in MCUB in HAP-1 MCU KO cells is mediated by

TAK1. In HAP-1 MCU KO cells, 5Z-7-Oxo caused a significant decrease in the expression of the 30 kd protein detected by the anti-phospho-Ser92 MCU antibody (Fig. 6L).

BMPR2/TAK1-d signaling phosphorylates Ser76 in MCUB.

To confirm that BMPR2/TAK1-d signaling specifically phosphorylates Ser76 in MCUB, Ser76 in MCUB was mutated to alanine (Ser76/A) to inactivate the phosphorylation site. Interestingly, we discovered that MCUB cDNA is reported in GenBank as having two different open reading frames (ORF). In ORF-1 (NCBI GenBank Sequence ID, NM_017918), the ATG start codon is in exon 1 of chromosome 4. ORF 1 is full length and transcribes the conserved N-terminal redox region containing Ser76, which is in exon 3. ORF 2 (NCBI GenBank Sequence ID, KJ902740.1), does not contain the N-terminal redox region with transcription starting 3' to Ser76 in exon 3 (**Fig S2B**). Transfection of MCUB ORF-1 increased the expression pSer92 MCU at 30 kd in THP-1 (Fig. 6M) and K562 cells (Fig. 6N). Transfection of MCU-Flag and Ser76/A MCUB of ORF-1 did not increase expression of the 30 kd protein detected by the anti-phospho-Ser92 MCU antibody (Fig. 6N). MCUB ORF 2 containing a c-terminal HA tag transfected into K562 cells migrated at approximately 27 kd and was not detected by the anti-phospho-Ser92 MCU antibody (Fig. 6O). These studies strongly support that BMPR2/TAK1-d signaling phosphorylates Ser76 in MCUB.

It is reported that there is no reliable commercially available anti-MCUB antibody [29]. We tested two commercially available anti-MCUB antibodies and neither detected a 30 kd protein and did not detect a change in expression of any band following transfection with MCUB cDNA, despite a clear increase in expression of the 30 kd protein detected by the anti-phospho-Ser92 MCU antibody (**Fig S3B-C**). Furthermore, although there is a clear decrease in expression of the 30 kd protein detected by the anti-phospho-Ser92 MCU antibody following transfection with siRNA targeting MCUB, there was no decrease in MCUB expression using these antibodies (**Fig S3D-E**). Therefore, we also conclude that in our cell lines, the commercially available anti-MCUB antibodies tested are not reliable in monitoring changes in expression of MCUB.

The phosphorylation of Ser76 MCUB regulates mtCa²⁺ levels.

Calu-1 MCUB and THP-1 KO clones have higher basal mtCa²⁺ levels compared to WT cells (Fig. 7A-B). Calu-1 MCUB and THP-1 knockout cells also demonstrated significantly higher mtO2• levels compared to WT cells (Fig. 7C-D). In Calu-1 MCUB KO cells, ROS (H₂O₂) induced greater influx of mtCa²⁺ compared to WT cells (Fig. 7E). Thapsigergin, a sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA) inhibitor increases cytosol Ca²⁺ levels that causes mtCa²⁺ levels to rise [30]. Thapsigergin increased mtCa²⁺ influx more in THP-1 MCUB KO compared to WT cells (Fig. 7F). Transfection of THP-1 MCUB KO cells with MCUB WT decreased mtO2• levels (Fig. 7G). These studies are consistent with other reports that MCUB inhibits mtCa²⁺ uptake and the production of mtO2• [17, 31], and supports that the mutations generated by CRISPR-Cas9 induced functional KOs of MCUB. To determine the effects of the phosphorylation of Ser76 MCUB on the regulation of mtCa²⁺ levels, THP-1 MCUB KO cells were

transiently transfected with insertless, MCub WT, and Ser76/A MCub expression vectors. Transfection with MCub WT but not control or Ser76/A MCub expression vectors into THP-1 MCub KO cells decreased mtCa^{2+} levels (Fig. 7H). Ser76 MCU was mutated to glutamate (Ser76/E) to make it constitutively active. Transfection of K562 cells with MCub WT decreased mtCa^{2+} levels, which trended lower with Ser76/E MCub compared to control vector (Fig. 7I). These studies suggest that decreased phosphorylation of Ser76 in MCub increases mtCa^{2+} levels.

KO of MCub enhances cell death induced by BCL-2 inhibitors.

THP-1 and Calu-1 MCub KO cells treated with BCL-2 inhibitors ABT-199 and ABT-263 respectively, demonstrated more cell death compared to WT cells (Fig. 7J-K). We examined whether KO of MCub enhanced the influx of mtCa^{2+} induced by ABT-199. The percent increase in mtCa^{2+} levels from control was not different between cell lines treated with ABT-199. However, since basal mtCa^{2+} levels are higher in MCub KO cells compared to WT cells, the mtCa^{2+} levels after treatment with ABT-199 were higher in MCub KO cells (Fig. 7L). These data suggests that the higher mtCa^{2+} levels in the MCub KO cells may contribute to the increased cell death following treatment with ABT-199.

BMPR2/TAK1-d signaling regulates the phosphorylation of both Ser76 MCub and Ser92MCU.

5Z-7-Oxo induced significantly higher mtCa^{2+} levels in THP-1 WT cells compared to THP-1 MCub KO cells (Fig. 7M). This result is consistent with TAK1-d phosphorylating Ser76 in MCub to increase mtCa^{2+} levels. Although the increase in mtCa^{2+} levels was less in the MCub KO cells, there was still significant increase in mtCa^{2+} levels compared to vehicle control (Fig. 7M). This suggested that in MCub KO cells, TAK1-d may also be regulating the phosphorylation of Ser92 in MCU. 5Z-7-Oxo significantly decreased the phosphorylation of the 30 kd protein detected by the anti-Ser92 MCU antibody in both THP-1 WT and THP-1 MCub KO cells (Fig. 7N).

In K562 cells transfected with MCub Ser76/E expression vector, 5Z-7-Oxo induced a significantly lower increase in mtCa^{2+} levels compared to control vector but increased compared to vehicle (Fig. 7O). Together our data suggested that BMPR2/TAK1-d phosphorylated both Ser92 in MCU and Ser76 in MCub and there is a compensatory increase in the phosphorylation of Ser 92 in MCU following the downregulation of MCub (Fig. 6F). We next examined whether the compensatory phosphorylation of Ser92 in MCU regulated cell survival and mtCa^{2+} levels. THP-1 WT and MCub KO cells were treated with 5Z-7-Oxo and ABT-199 alone and in combination. The combination of 5Z-7-Oxo and ABT-199 induced more cell death (Fig. 7P) and induced higher mtCa^{2+} levels (Fig. 7Q) in MCub KO cells compared to WT. These data suggest that BMPR2/TAK1-d may phosphorylate both Ser92 MCU and Ser76 MCub to regulate mtCa^{2+} levels and cell survival.

Phosphorylation of Ser76 MCub decreases ATP levels.

Out recent studies suggest that inhibiting BMPR2/TAK-1 signaling in NSCLC cell lines and HT-22 mouse hippocampal neuronal cells increases mtCa²⁺ levels leading to an increase in mitochondrial bioenergetics [16]. HT-22 cells treated with 5Z-7-Oxo demonstrated an increase in ATP levels (Fig. 7R). Transfection of HT-22 cells with Ser76/E MCub suppressed ATP levels greater than MCub WT expression vector (Fig. 7S). Interestingly, THP-1 MCub KO cells have higher ATP levels compared to WT cells (Fig. 7T). Transfection of Ser76/E MCub in THP-1 MCub KO caused a greater decrease in ATP levels compared to control and MCub WT expression vectors (Fig. 7U). These studies show that phosphorylation of Ser76 in MCub decreases ATP levels, suggesting that BMPR2/TAK1-d signaling phosphorylation of Ser76 in MCub decreases mitochondrial bioenergetics.

Treatment with JL189 combined with ABT-263 synergistically decreased the growth of lung tumor xenografts in mice.

We examined synergy in tumor xenografts in mice using H1299 cells. There was an approximately a 50% greater reduction in the tumor weight in mice treated with the combination of JL189 and ABT-263 compared to mice treated with vehicle or each compound alone (**Fig S4**). This tumor xenograft study supports synergy between JL189 and ABT-263. However, JL189 as a single agent also did not have significant anti-tumor properties, which is likely from its poor drug properties. JL189 *in vitro* IC₅₀ for BMPR2 is only 130 nM and its serum half-life is only 60 minutes in mice (**Table S2**).

JL189 and ABT-263 were also studied in a T-ALL mouse model. However, the combination JL189 and ABT-263 was not tolerated in this mouse model and the study was stopped. We identified that JL189 has other liabilities, including prohibitively high inhibition of the cardiac potassium channel hERG as well as all 6 isotypes of liver cytochrome 450 enzymes (CYP), which is very likely to have contributed significantly to the observed toxicity (**Table S3**). Although JL189 is a good tool compound for *in vitro* studies, it does not have the drug properties necessary to evaluate BMPR2 inhibition *in vivo*.

Discussion

We recently reported that BMPR2i increases the uptake Ca²⁺ into the mitochondria that is mediated by TAK1-d splice variant [16]. The effects of BMPR2i induced increase in mtCa²⁺ levels were different between NSCLC and leukemia cell lines. In NSCLC cell lines, BMPR2i increased mtATP levels, TCA cycle intermediates, and oxidative phosphorylation. In leukemia cells, BMPR2i caused more cell death and had higher Ca²⁺ levels with less ATP production. The KO of the MCU attenuated cell death in Jurkat cells treated with JL189. These studies suggested that the effects of BMPR2/TAK1-d induced increase in mtCa²⁺ levels were dependent on the cell type. Although these studies suggested that BMPR2/TAK1-d signaling regulates the MCU complex, the mechanism of regulation was not elucidated.

The present study suggests that BMPR2/TAK1-d signaling phosphorylates Ser76 in MCub and Ser92 in MCU to regulate mtCa²⁺ levels. siRNA KD of MCub but not the KD of MCU in WT cells decreased the phosphorylation of the 30 kd protein recognized by our anti-phospho-Ser92 antibody. In addition,

transfection of MCUB but not MCU cDNA increased the phosphorylation of the 30 kd protein recognized by our anti-phospho-Ser92 antibody. These studies suggest that BMPR2/TAK1-d signaling predominantly phosphorylates WT Ser76 MCUB compared to Ser92 MCU in leukemia cells. In MCUB KO cells, there was a compensatory increase in the phosphorylation of Ser92 in MCU, which is regulated by BMPR2/TAK1 signaling. Likewise, KO of MCU resulted in an increase phosphorylation of Ser76 in MCUB, which is also regulated by BMPR2/TAK1-d signaling. These studies suggest that BMPR2/TAK1-d signaling regulates both Ser76 in MCUB and Ser92 in MCU. Future studies are needed to determine whether BMPR2/TAK-d signaling preferentially phosphorylation Ser76 in MCUB or Ser92 in MCU in NSCLC and other cell types.

We demonstrate a novel mechanism by which BMPR2/TAK1-d signaling when combined with other cancer therapeutics synergistically mediates mtCa^{2+} overload and cell death. Our studies suggest that synergistic cell death is mediated, at least in part, by decreasing the phosphorylation of Ser76 in MCUB, which increases the transfer of Ca^{2+} into the mitochondria. Our data suggest that downregulating the phosphorylation of Ser76 in MCUB decreases its inhibitory effect on the MCU, leading to an increase in mtCa^{2+} levels. This was demonstrated by the high mtCa^{2+} levels in THP-1 MCUB KO cells being rescued by MCUB WT but not the kinase dead Ser76/A MCUB. Transfection of constitutively active Ser76/E MCUB decreased mtCa^{2+} levels, which is also consistent with the phosphorylation of Ser76 MCUB decreasing mtCa^{2+} uptake. MCUB is known to inhibit ROS induced transfer of Ca^{2+} by the MCU [31]. Our studies show that inhibition of BMPR2/TAK1-d signaling enhances mtCa^{2+} uptake induced by ROS. Most chemotherapeutics increase ROS levels that can increase mtCa^{2+} levels [32]. Additional studies are needed to determine whether the downregulation of the phosphorylation of Ser76 in MCUB and/or Ser92 in MCU are required to promote ROS induced mtCa^{2+} overload.

MCUB KO cells demonstrated increased sensitivity to BCL-2 inhibitors associated with high mtCa^{2+} levels. Leukemia cells are less sensitive to ROS (H_2O_2) induced increase in mtCa^{2+} levels compared to NSCLC cells. In leukemia cells treated with BMPR2 and BCL-2 inhibitors, cell death and mtCa^{2+} overload may not be dependent on elevated ROS levels. BCL-2 and BCL-xL inhibit VDAC and ER Ca^{2+} channels, which transfer Ca^{2+} into the mitochondrial intermembrane space [20, 21]. Therefore, inhibiting BCL-2 proteins will increase Ca^{2+} levels in the mitochondrial intermembrane space and when combined with a BMPR2 inhibitor increase the flow of Ca^{2+} into the mitochondrial matrix. The role of the phosphorylation of Ser92 in MCU is not completely elucidated. A prior study suggested the phosphorylation of Ser92 in MCU decreases uptake of Ca^{2+} [33]. Our studies suggest that inhibiting BMPR2/TAK1-d signaling also decreases the phosphorylation of Ser92 in MCU to regulate mtCa^{2+} levels and cell survival. MCUB KO cells treated with TAK1 inhibitor decreased phospho-Ser92 MCU levels and induced more cell death and higher mtCa^{2+} levels compared to WT cells. These studies suggest that both phospho-Ser92 in MCU and phospho-Ser76 in MCUB could regulate mtCa^{2+} levels and cell survival. However, the effects of only decreasing the phosphorylation of Ser92 in MCU was not studied.

Leukemia cells are more sensitive to the combination of BCL-2 inhibitor venetoclax (ABT-199) and the BMPR2 inhibitor JL189 compared to NSCLC cell lines. Importantly, JL189 combined with the FDA approved venetoclax (ABT-199) synergistically induces cell death in p53 mutated AML cell lines. p53 mutated AML has a dismal prognosis with a median survival of only 8 months, demonstrating an urgent need for better treatment options [34]. These data support the continued development of BMPR2 inhibitors and evaluation in patients with p53 mutated AML. Although leukemia cells are more sensitive to BMPR2 inhibition compared to NSCLC cell lines, a BMPR2 inhibitor may be effective in enhancing the effects of cancer therapeutics in lung and other types of cancers. Despite JL189 having limited pharmacokinetic properties and hERG and CYP liabilities, synergy was seen in H1299 NSCLC xenografts in mice treated with taxol and JL189. Future studies should examine whether a more potent BMPR2 inhibitor enhances the effectiveness of current standard of care. For NSCLC, it would be adding a BMPR2 inhibitor to the standard of care therapy such as carboplatin combined with taxol, which may provide better results. Furthermore, future studies should examine whether a BMPR2 inhibitor in combination with therapeutics which specifically increase ROS levels, such as metformin could potentially substitute for chemotherapy agents or allow for a reduction of current dose thereby decreasing toxicity.

Our prior studies demonstrated that BMPR2/TAK1-d signaling negatively effects mitochondrial bioenergetics in NSCLC and mouse HT-22 hippocampal neurons. BMP ligand expression is significantly increased in other age-related diseases including Alzheimer's disease [35]. Mitochondrial bioenergetics is suppressed in Alzheimer's disease (AD) and other neurodegenerative diseases. The transfer of Ca^{2+} into the mitochondria is essential for increasing and maintaining mitochondrial bioenergetics. In the present study, we show that transfection of constitutively active Ser76/E MCub suppresses ATP in HT-22 and THP-1 cells. This study suggests that BMPR2/TAK1-d signaling suppresses mitochondrial bioenergetics by phosphorylating Ser76 in MCub. Future studies are needed to prove this hypothesis. These data suggest that BMPR2/TAK1-d signaling may have pleotropic effects on different cell types through differential regulation of mitochondrial Ca^{2+} transport.

This manuscript also provides insight into the complexities of the regulation of mtCa^{2+} transport. In addition to the phosphorylation of MCU and MCub to regulate Ca^{2+} homeostasis, we highlight that there are two ORF of MCub. How they interact with each other and the MCU are not known. Furthermore, our studies show higher MW proteins detected by the anti-phospho-Ser92 antibody, suggesting MCub is post-transcriptionally modified. Glycosylation of MCub may affect mtCa^{2+} transport and may be a mechanism by which different cell types uniquely regulate mtCa^{2+} transport.

Conclusion

Our data suggests that BMPR2 signaling mediated through TAK1-d promotes the phosphorylation of Ser76 in MCub, which inhibits Ca^{2+} influx through the MCU complex. Inhibiting BMPR2/TAK1-d signaling decreases the phosphorylation of Ser76 in MCub leading to an increase in mtCa^{2+} levels. When a BMPR2 inhibitor is combined with BCL-2 inhibitor it induces mtCa^{2+} overload and cell death, in which p53

mutated AML cell lines are highly sensitive. Our studies suggest a novel mechanism(s) by which MCU complex can be pharmaceutically regulated using a small molecule targeting BMPR2 to regulate cell survival and mitochondrial bioenergetics. These studies support further drug development of BMPR2 inhibitors and their evaluation as a chemotherapy sensitizer for the treatment of cancer.

Abbreviations

BMP
Bone morphogenetic protein
BMPR2
Bone morphogenetic protein type 2 receptor
BMPR2i
Bone morphogenetic protein type 2 receptor inhibition
ID1
Inhibitor of differentiation
XIAP
X-linked inhibitor of apoptosis
TAK
TGFB β activated kinases
MTs
microtubule
AMPK
AMP-activated kinase
mtCa⁺⁺
mitochondrial calcium
TCA
tricarboxylic acid cycle
MCU
mitochondrial uniporter
ROS
reactive oxygen species
KO
Knockout
WT
Wild type
ER
endoplasmic reticulum
VDAC
Voltage-dependent anion channel
OXY

oxidative phosphorylation
AIF
apoptosis-inducing factor
NSCLC
Non-small cell lung carcinoma
T-ALL
T-cell acute lymphoblastic leukemia
AML
Acute myelogenous leukemia
NSLC
Non-small cell lung carcinoma
CML
Chronic myeloid leukemia
FOP
Fibrodysplasia ossificans progressive
AD
Alzheimer's disease
siRNA
small interfering ribonucleic acid
DNA
deoxyribonucleic acid

Declarations

Ethics approval

Studies did not include human participants, human data or tissue. Mice studies were approved by Rutgers IACU.

Consent for publication

Not applicable

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 provisional patent has been filed for developing BMP small molecule inhibitors. There are no active or pending financial agreements regarding these compounds.

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Authors' contributions

JL contributed to the study conception and design, analysis and interpretation of the data and drafted the manuscript. MS, HC, AT and DJ performed the experiments, analyzed the data, and edited the manuscript. JR and JG synthesized JL189, analyzed PK and hERG studies. DH performed leukemia mouse model study.

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