



## Combination simvastatin and metformin synergistically inhibits endometrial cancer cell growth



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### HIGHLIGHTS

- Combination metformin and simvastatin treatment synergistically inhibited growth in three endometrial cancer cell lines.
- This treatment combination demonstrated apoptosis induction and mTOR pathway inhibition in vitro.
- Drug repurposing using this combination may warrant clinical investigation in endometrial cancer.

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### ABSTRACT

**Objective.** Recent data show that simvastatin (SIM) and metformin (MET) have anti-proliferative effects in endometrial cancer cells. The combination (MET+SIM) inhibits tumor growth and metastasis in prostate cancer cells which possess similar molecular alterations to many early endometrial cancers. We tested the hypothesis that the anti-proliferative effects of MET+SIM in endometrial cancer cells would be greater than the effects of each agent alone.

**Methods.** RL95-2, HEC-1B, and Ishikawa endometrial cancer cell lines were treated with MET and/or SIM. Growth inhibition was measured by MTS cell proliferation assays. Apoptosis was evaluated by caspase-3, Annexin V, and TUNEL assays and by apoptosis markers (BAX, Bcl-2, Bim) using western blot. Bim was silenced using Bim siRNA to confirm this apoptotic pathway. Treatment effects on the mTOR pathway were investigated by western blot using antibodies to phosphorylated (phospho)-AMPK and phospho-S6.

**Results.** MET+SIM synergistically inhibited growth in all three cell lines. The combination induced apoptosis as measured by TUNEL, Annexin V, and caspase-3 assays. Bim siRNA transfection abrogated this effect—silencing Bim in MET+SIM-treated RL95-2 cells rescued cell viability in MTS assays and reduced caspase-3 activity compared with control siRNA-transfected cells. Combination treatment upregulated phosphorylated AMPK and downregulated downstream phosphorylated S6, suggesting mTOR inhibition as a mechanism for these anti-proliferative effects.

**Conclusions.** MET+SIM treatment synergistically inhibits endometrial cancer cell viability. This may be mediated by apoptosis and mTOR pathway inhibition. Our results provide preclinical evidence that the combination of these well-tolerated drugs may warrant further clinical investigation for endometrial cancer treatment.

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### 1. Introduction

Endometrial cancer is the most common gynecologic malignancy in the United States where 63,230 new cases and 11,350 deaths are estimated to have occurred in 2018 [1]. Type I endometrial cancers are of endometrioid histology, are associated with a hyperestrogenic state,

and comprise approximately 80% of all endometrial carcinomas [2]. One important risk factor for the development of type I endometrial cancer is obesity [3,4], and with the growing obesity epidemic over the past few decades, the incidence of endometrial cancer has similarly risen [5]. Epidemiologic data suggest that the anti-diabetic drug metformin (MET) as well as lipid-lowering agents such as simvastatin (SIM) may lower the risk of several cancers [6,7] including endometrial cancer [8,9]. These medications are of particular interest in the endometrial cancer population as many of these patients are obese with obesity-related comorbidities including diabetes, hyperlipidemia, and metabolic syndrome [10].

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Both MET and SIM have demonstrated anti-proliferative effects on endometrial cancer cell lines in vitro and in vivo [11,12], however their combined effects are unknown. In prostate cancer cells, the combination of MET and SIM has shown synergistic inhibition of tumor growth and metastasis in part through effects on the PI3K/Akt/mTOR pathway [13,14]. This pathway is also an important target in endometrial cancer due to the high frequency of PTEN mutations leading to constitutive activation of Akt and overactive mTOR pathway signaling [15]. Importantly, these molecular changes also occur in endometrial hyperplasia and histologically normal proliferative endometrium [16–18] suggesting that PTEN loss may be one of the earliest events in carcinogenesis and thus a compelling target for prevention. Given that both medications are generally well tolerated and relatively inexpensive, if they are proven to have additive or synergistic anti-cancer effects there is great potential for use of these agents in endometrial cancer patients.

In this study, we sought to evaluate the effects of MET and SIM in combination (MET+SIM) on endometrial cancer cell growth in vitro. We hypothesized that the anti-proliferative effects of combination treatment would be greater than the effects of each individual agent alone.

## 2. Materials and methods

### 2.1. Cell culture and reagents

Three human endometrial cancer cell lines were used: RL95-2, HEC-1B (purchased from ATCC, Manassas, VA), and Ishikawa (Sigma-Aldrich, St. Louis, MO). Cell lines were authenticated by DNA short-tandem repeat analysis by the supplier. All cell lines were initially expanded and cryopreserved upon receipt and used within six months of receipt or resuscitation. RL95-2 cells were derived from a moderately-differentiated endometrial adenocarcinoma and were cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 medium supplemented with 10% FBS, insulin, penicillin-streptomycin, and 7.5% sodium bicarbonate. Ishikawa cells were derived from a well-differentiated endometrial adenocarcinoma; cells were cultured in minimum essential medium (MEM) supplemented with 5% FBS, penicillin-streptomycin, L-glutamine, and non-essential amino acids. HEC-1B cells were derived from a moderately-differentiated endometrial adenocarcinoma and were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS and penicillin-streptomycin. All media and supplements were purchased from ThermoFisher Scientific (Waltham, MA) with the exception of insulin (purchased from Sigma-Aldrich) and EMEM (purchased from ATCC). All cells were maintained in a 37 °C, 5% CO<sub>2</sub> humidified incubator and used for experiments at a relatively low passage number (<15 passages).

### 2.2. Cell proliferation assays

The effects of MET, SIM, and combination MET+SIM treatments on proliferation were examined using MTS assays. Ishikawa and HEC-1B cells were plated at 3000 cells/well and RL95-2 cells were plated at 5000 cells/well in 96-well plates in their corresponding media for 24 h then treated with 1–12 μM SIM (Sigma-Aldrich, St. Louis, MO), 2–8 mM MET (Medisca, Plattsburgh, NY), or combination MET+SIM for 72 h. Viability was quantified using the formazan dye-based MTS assay (Promega, Madison, WI) according to the manufacturer's specifications. The plates were incubated at 37 °C until the untreated wells exhibited an A<sub>490</sub> of 0.7–0.9. Wells containing medium alone were used as blanks. Viability was expressed as a percentage of untreated controls on the same 96-well plate. Each experiment was performed in quadruplicate and repeated at least twice to assure consistency.

Data were analyzed isoblographically using CompuSyn software (Biosoft) to characterize the inhibitory effect of treatment agents on

the cell lines. Raw data for each agent or combination was entered singly to generate a median effect plot. From this plot, the combination index (CI) equation was applied to determine whether the drug effects were additive, synergistic or antagonistic. CI values of <1, =1 or >1 indicated synergy, additivity or antagonism, respectively.

### 2.3. TUNEL assay

Apoptosis was assessed by deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) in RL95-2, Ishikawa, and HEC-1B cells seeded into 60 mm dishes and incubated overnight as follows: RL95-2 cells were seeded at  $1.5 \times 10^6$  cells/dish (24 h) and  $1.0 \times 10^6$  cells/dish (48 h). Ishikawa and HEC-1B were seeded at  $4.0 \times 10^5$  cells/dish (24 h) and  $3.5 \times 10^5$  cells/dish (48 h). The culture medium was then replaced with fresh medium containing 4–8 mM MET with and without 1–8 μM SIM, and incubated for 24 and 48 h. All cells, floating and adherent, were collected, washed, fixed, and stained by TUNEL using the APO-Direct kit (BD Pharmingen, San Jose, CA), according to the manufacturers' protocols. Ten thousand cells were measured by FACS Calibur (BD Biosciences) and the data were analyzed using CellQuest Pro 5.2.1 software. Each sample was gated to include only a singlet population using dual parameters of DNA width (x-axis) and DNA area (y-axis). In assessing apoptosis, the resultant gates were used to generate dual parametric graphs of DNA area (x-axis) and FITC-dUTP (y-axis). Gates were based on increased dUTP-FITC labeling compared to the untreated controls. Results are expressed as fold-increase over controls. Experiments were repeated at least three times and/or run in triplicate.

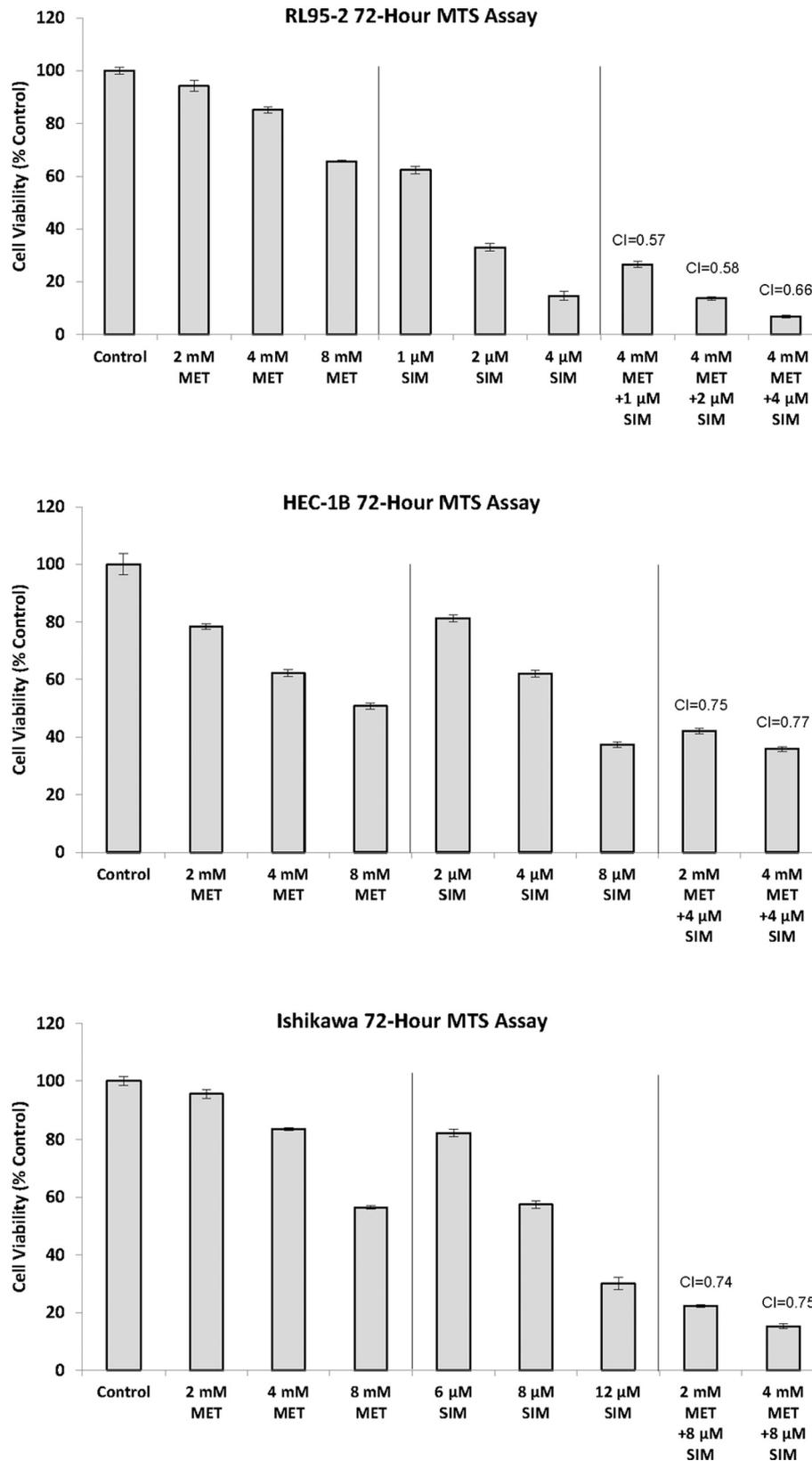
### 2.4. Annexin V apoptosis assay

Apoptosis was independently assessed using Annexin V in RL95-2 cells seeded and treated for 24 h as described above for TUNEL assay. All cells, floating and adherent, were collected, washed and stained by FITC Annexin V Apoptosis Detection Kit 1 (BD BioSciences), according to the manufacturer's protocols. Ten thousand cells were measured by FACS Calibur and the data were analyzed using CellQuest Pro 5.2.1 software. Gates were based on increased Annexin-FITC-only labeling compared to the untreated controls. Results are expressed as fold-increase over controls. Experiments were repeated at least three times and/or run in triplicate.

### 2.5. Caspase-3 apoptosis assay

Apoptosis induction was verified following SIM, MET, and MET+SIM treatments using a fluorometric caspase-3 assay kit (BioVision Technologies, Milpitas, CA) or the Caspase-Glo 3/7 assay (Promega Corporation) as per manufacturer's instructions. These kits detect activity of caspases that recognize the sequence DEVD. For the fluorometric assay, cells were plated in 60 mm dishes at concentrations as described above. Following 24 h of incubation, fresh medium was used to treat cells with SIM (1–8 μM), MET (4 mM), or combination MET+SIM for 24–48 h. Cells were then lysed and protein concentrations measured and loaded equally onto a 96-well plate. Reagents were added as directed and samples were read using a 400 nm excitation filter and 505 nm emission filter. Fold-increase of caspase activity was determined by comparison of treated samples to untreated samples.

For the Caspase-Glo assay, cells were plated in 96-well dishes at 4500 cells/well and allowed to incubate overnight. After transfection (see below) cells were treated with 4 mM MET, 1 mM SIM, and MET+SIM and incubated for 48 h. This caspase assay uses a luminogenic caspase 3/7 substrate which contains DEVD. Changes in caspase activity as indicated by luminescence were determined by comparison of treated samples to untreated samples. Each experiment was run with a minimum of three samples and/or repeated three times.



**Fig. 1.** Metformin (MET) and simvastatin (SIM) individually inhibit growth dose-dependently in all three cell lines, and are synergistic in combination (MET+SIM). MTS assays of cell proliferation inhibition at 72 h post-treatment yielded combination indices (CI) of 0.57–0.77 (CI < 1 indicates synergy) for MET+SIM. Data are presented as mean  $\pm$  SEM. Each experiment was performed in quadruplicate and repeated at least twice.

2.6. Western immunoblotting

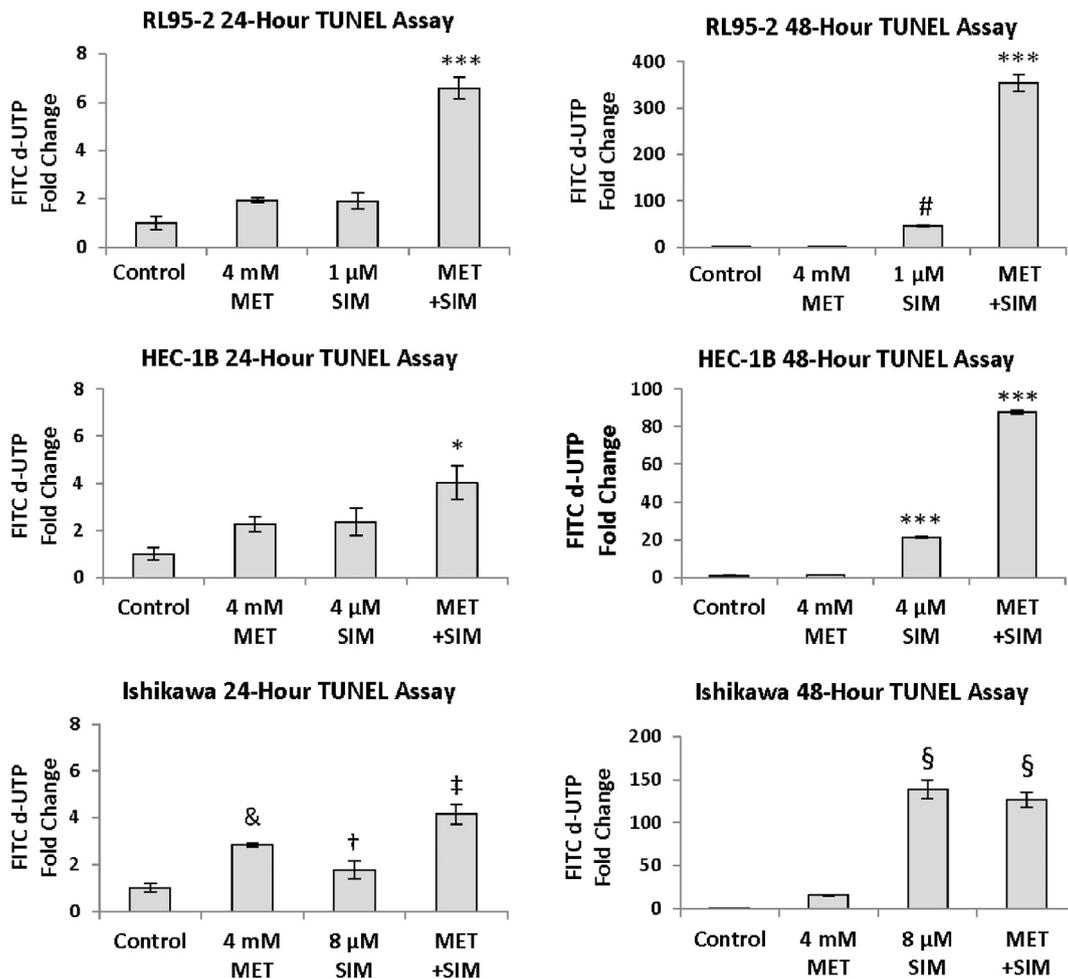
The effects of SIM, MET, and the combination on signaling targets within the PI3K/Akt/mTOR pathway and apoptosis were evaluated with western immunoblotting. Primary antibodies specific for phosphorylated AMPK $\alpha$  (Thr172)(40H9), phosphorylated S6, Bax, Bcl-2, beclin-1 (D40C5), Bim (C34C5) and HRP-conjugated actin (C-11) were used. Cells were plated in 60 mm dishes as described above, incubated for 24 h, and then treated with 1–8  $\mu$ M SIM, 4 mM MET, or MET+SIM for 24 and 48 h. Prior to harvesting the cells for molecular analysis, brightfield images of the cells were captured at 10 $\times$  and 20 $\times$  using a Nikon inverted photo microscope. Cell lysates were prepared using RIPA buffer (Santa Cruz Biotechnology). Protein concentrations were determined by the DC Protein Assay Kit (Bio-Rad Laboratories). Thirty  $\mu$ g of protein per lysate were denatured at 95  $^{\circ}$ C, separated by SDS-PAGE gel electrophoresis, and transferred to a PVDF membrane. Membranes were blocked with 5% nonfat dry milk in TBST for one hour then probed with the primary antibody overnight at 4  $^{\circ}$ C, incubated with the appropriate HRP-conjugated secondary antibody, and proteins detected using either Luminol (Santa Cruz Biotechnology) or SuperSignal West Femto Chemiluminescent Substrate (ThermoFisher). All primary and secondary antibodies were purchased from Cell Signaling with the exception of actin (Santa Cruz). Protein bands were imaged and quantified by densitometry using LabWorks Image Acquisition and Analysis Software (UVP, Upland, CA).

2.7. Bim siRNA

Bim is a Bcl-2 family pro-apoptotic protein that interacts with and activates BAX [19]. To verify apoptosis as a primary molecular pathway underlying reduced cell viability and increased caspase activity following exposure to treatments, Bim siRNA was used. RL95-2 cells were seeded at 4500 cells/well in a clear or white 96-well plate respectively and allowed to adhere for 72 h. Cells were transfected and treated as follows. For each experiment, 40 nM of Bim siRNA (GE Dharmacon, Lafayette, CO) or 40 nM of ON-TARGET control pool siRNA (GE Dharmacon) was incubated in OptiMEM (ThermoFisher) for 5 min in separate tubes. During this incubation, 2 additional tubes of OptiMEM and DharmaFECT I transfection reagent (GE Dharmacon) were incubated at 25  $^{\circ}$ C. Next, the tubes containing the siRNA and its corresponding tubes of DharmaFECT I were combined and incubated for 30 min at 25  $^{\circ}$ C. The reagents were then added to the cells in OptiMEM and transfected for 48 h. Following transfection, the cells were treated for 48 h with 1  $\mu$ M SIM, 4 mM MET, or the combination. Viability was assessed by MTS assay and caspase-3 activity was assessed by Caspase 3/7 Glo Assay, both described above.

2.8. Statistical analysis

Results are presented as mean  $\pm$  SEM and were compared statistically with  $p < 0.05$  considered significant. Multiple group comparisons



**Fig. 2.** Apoptosis is induced in endometrial cancer cells with combination metformin and simvastatin (MET+SIM) treatment. Deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) increased dUTP-FITC label in MET- and SIM-treated cells compared with controls at both 24 and 48 h in all cell lines, consistent with apoptosis. Combination treatment of all three cell lines increased apoptosis dramatically. Ishikawa cells were the only cell line to show equal efficacy of SIM alone and MET+SIM treatment at 48 h. Mean  $\pm$  SEM; \* $p < 0.05$  vs control; \*\*\* $p < 0.001$  vs all other groups; # $p < 0.05$  vs control and MET; & $p < 0.05$  vs control and MET; † $p < 0.05$  vs control and MET+SIM; ‡ $p < 0.01$  vs MET+SIM; § $p < 0.001$  vs control; by ANOVA. Experiments were repeated at least three times and/or run in triplicate.

were made using an ANOVA with post hoc Newman-Keuls, and a Student's *t*-test was used when comparing two groups. All tests were two-tailed. Statistical analysis was performed using GraphPad Prism version 4 (La Jolla, CA).

### 3. Results

#### 3.1. Treatment with MET+SIM synergistically inhibits cell viability

The effects of SIM, MET, and MET+SIM combination treatment on cell viability were examined in the endometrial cancer cell lines RL95-2, HEC—1B, and Ishikawa. Light microscopy evaluation following 24–48 h of each treatment showed evidence of cellular changes consistent with cell death not demonstrated in cells cultured only in control medium. These visible changes included plasma membrane blebbing, cell shrinkage, and cell fragmentation (Supplementary Fig. S1). MTS assays performed at 72 h following treatment demonstrated that SIM and MET individually inhibited growth in all three cell lines dose-dependently (Fig. 1). In addition, the combination of MET+SIM demonstrated synergistic inhibition compared with treatments with the individual agents (combination indices 0.57–0.77; see Fig. 1).

#### 3.2. Combination MET+SIM treatment induces apoptosis in endometrial cancer cells

We sought to investigate the mechanism of growth inhibition by MET+SIM treatment. Apoptosis has been a proposed mechanism in endometrial cancer cells following both SIM [11] and MET [12] treatment individually, and morphologic changes seen with light microscopy appeared most characteristic of apoptotic changes [20]; therefore, we investigated apoptosis induction with combination MET+SIM treatment. TUNEL assay demonstrated a significant increase in dUTP-FITC labeling in MET+SIM-treated cells compared with controls at both 24 and 48 h in all cell lines (Fig. 2 and Supplementary Fig. S2) consistent with apoptosis induction. Combination treatment of RL95-2 cells produced an impressive 6.6-fold and 354-fold increase in apoptosis at 24 and 48 h, respectively (both  $p < 0.001$ ). Combination treatment of HEC-1B cells also increased apoptosis dramatically (4-fold and 88-fold at 24 and 48 h, respectively,  $p < 0.001$ ). Ishikawa cells responded similarly at 24 h but was the only cell line to show equal efficacy of SIM alone and combined treatment at 48 h (139-fold and 127-fold increase, respectively,  $p < 0.001$ ). We further verified apoptosis in RL95-2 cells using an Annexin V assay. Cells treated for 24 h with control medium, 4  $\mu$ M SIM, 8 mM MET, or MET+SIM similarly showed a significant increase in Annexin V labeling (5.8-fold,  $p < 0.001$ ) in combination-treated cells compared with controls (Supplementary Fig. S3). Individual treatment with SIM and MET resulted in much lower Annexin V label (3-fold,  $p < 0.001$  and 1.6-fold,  $p < 0.01$ , respectively).

Caspase-3 assay was then performed as another means of assessing apoptosis induction. Following treatment of RL95-2, HEC—1B, and Ishikawa cells for 24–48 h with control medium, SIM, MET, or MET+SIM, cell lysates were prepared and caspase-3 activity was evaluated. At 24 and 48 h, caspase-3 activity was significantly increased ( $p < 0.001$ ) in combination MET+SIM-treated cells compared with controls in all three cell lines (Fig. 3). In the Ishikawa cell line the caspase activity fold-change observed with combination treatment was less profound than that of the other cell lines and smaller than that of SIM treatment alone.

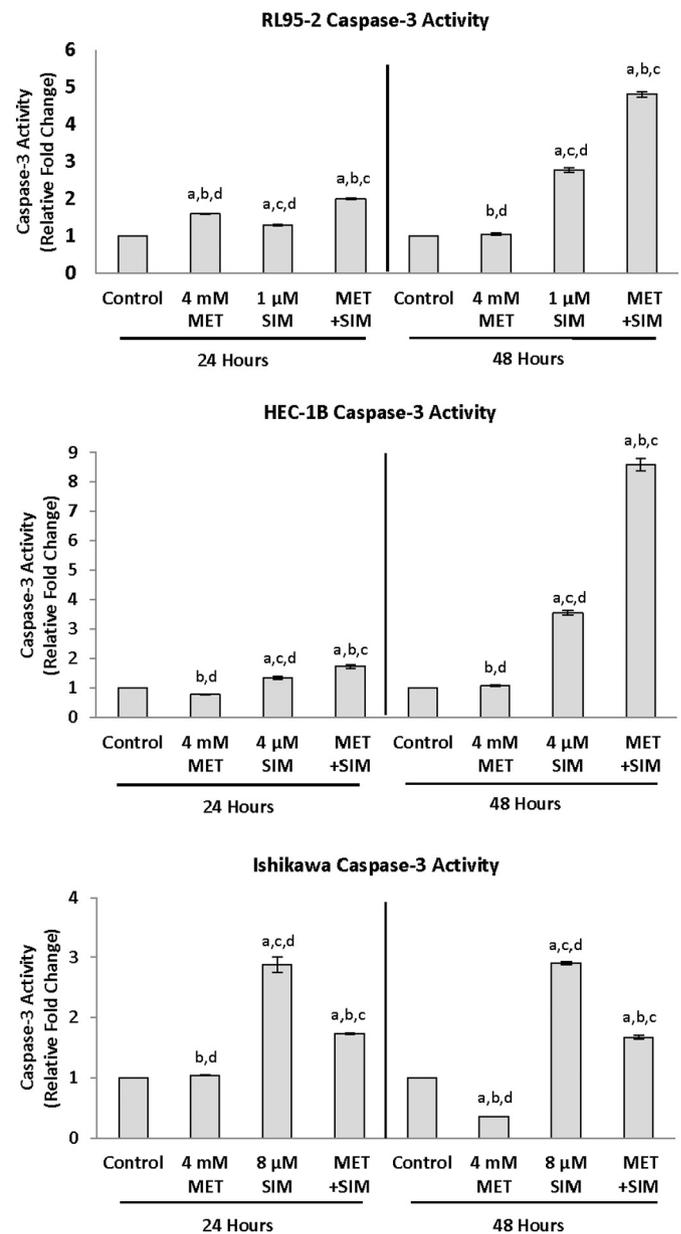
#### 3.3. BAX/Bcl-2 ratio is static in SIM- and MET-treated endometrial cancer cells

To characterize the apoptotic mechanism, RL95-2 cells treated with 1  $\mu$ M SIM, 4 mM MET, or the combination for 48 h were tested by western immunoblotting of BAX and Bcl-2 proteins as this pathway has been implicated in SIM-treated cancer cell lines [21,22]. Both BAX and Bcl-2

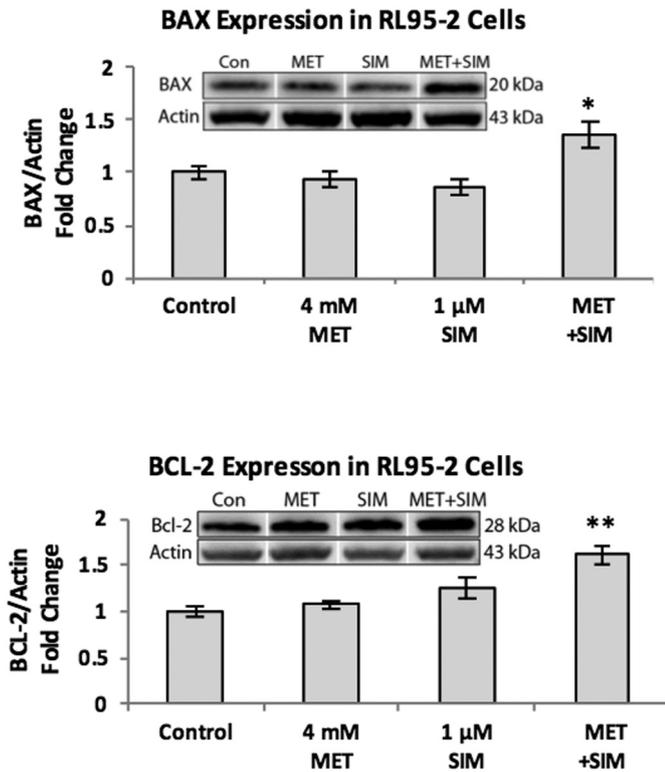
were similarly increased, and the BAX/Bcl-2 ratio was not different between combination-treated ( $0.26 \pm 0.002$ ) and untreated ( $0.27 \pm 0.003$ ) cells (Fig. 4).

#### 3.4. MET and SIM upregulate Bim in RL95-2 cells

SIM has also been reported to induce apoptosis via the Bcl-2 like 11 protein (Bim), which upregulates mitochondrial apoptosis [22]. Because the BAX/Bcl-2 ratio did not change, we therefore performed similar experiments with Bim as with BAX using western immunoblot analysis. The combination treatment showed significant upregulation of all three isoforms of Bim with the greatest significance being due to BimE1 ( $P < 0.001$ ). SIM showed similarly significant effects with BimL and BimS ( $p < 0.001$ ) (Fig. 5A).



**Fig. 3.** Caspase-3 activity increases compared with control following treatment with metformin (MET), (simvastatin) SIM, or the combination (MET+SIM) for 24 and 48 h in all three cell lines. Data are presented as mean  $\pm$  SEM. Letters above the chart bars indicate pair-wise comparisons: a) compared to control, b) compared to SIM, c) compared to MET, and d) compared to MET+SIM; all are significant at  $p < 0.001$ , by ANOVA. Each experiment was run with a minimum of three samples and/or repeated three times.



**Fig. 4.** BAX and Bcl-2 both increase in RL95-2 endometrial cancer cells treated with simvastatin (SIM), metformin (MET), or the combination (MET+SIM) for 48 h. Western immunoblotting of BAX and Bcl-2 showed similar increases in both proteins and a BAX/Bcl-2 ratio that was not different between MET+SIM-treated ( $0.26 \pm 0.002$ ) and control ( $0.27 \pm 0.003$ ) cells. Mean  $\pm$  SEM; \* $p < 0.01$  vs control and MET,  $p < 0.05$  vs SIM; \*\* $p < 0.05$  versus all other treatments; by ANOVA. Ratios of untreated controls are set to 1.0. Each experiment was run with a minimum of three samples and/or repeated three times.

**3.5. Bim silencing reverses MET- and SIM-induced apoptosis and cell growth inhibition**

In order to verify Bim involvement in the apoptotic pathway, RL95-2 cells transfected with Bim siRNA or control siRNA were treated with 1 μM SIM, 4 mM MET, or the combination for 48 h and assessed for caspase-3 activity and viability. MTS assay demonstrated a significant rescue of cell viability in Bim siRNA-transfected combination-treated cells compared with control siRNA-transfected combination-treated cells ( $p = 0.0001$ ) (Fig. 5C). In addition, Bim siRNA transfection resulted in a significant abrogation of caspase-3 activity compared with control siRNA transfection in cells treated with combination MET+SIM ( $p < 0.01$ ) (Fig. 5D). Taken together, these results indicate that apoptosis in MET+SIM-treated endometrial cells is Bim-mediated and further suggest that apoptosis induction is a major mechanism underlying the inhibitory effect of combination treatment on cell growth.

**3.6. Inhibition of endometrial cancer cell growth following MET+SIM treatment is partially mediated by AMPK activation and mTOR pathway effects**

The mTOR pathway has been a proposed key target in MET- and SIM-associated inhibition of endometrial cancer cells as well as in other cell types including breast, prostate, and ovarian cancer. Combination MET+SIM has demonstrated similar anti-proliferative effects in castrate-resistant prostate cancer cells. We therefore evaluated the effects of treatment with SIM, MET, or combination MET+SIM on targets of this pathway including phosphorylated AMPK (P-AMPK) and phosphorylated S6 (P-S6). Phosphorylated AMPK directly phosphorylates the mTOR subunit Raptor which in turn blocks the ability of mTOR

kinase complex from phosphorylating its downstream substrates, including S6 which mediates cell cycle progression [23]. As shown in Fig. 6, western immunoblot analysis demonstrated that effects on phosphorylation of AMPK and S6 following treatment with SIM and MET alone were variable and differed by cell line. Combination MET+SIM treatment, however, increased P-AMPK and decreased P-S6 by 48 h following treatment in all cell lines (Fig. 6).

**3.7. Autophagy is unlikely a significant mechanism of cell death following treatment with MET+SIM in endometrial cancer cells**

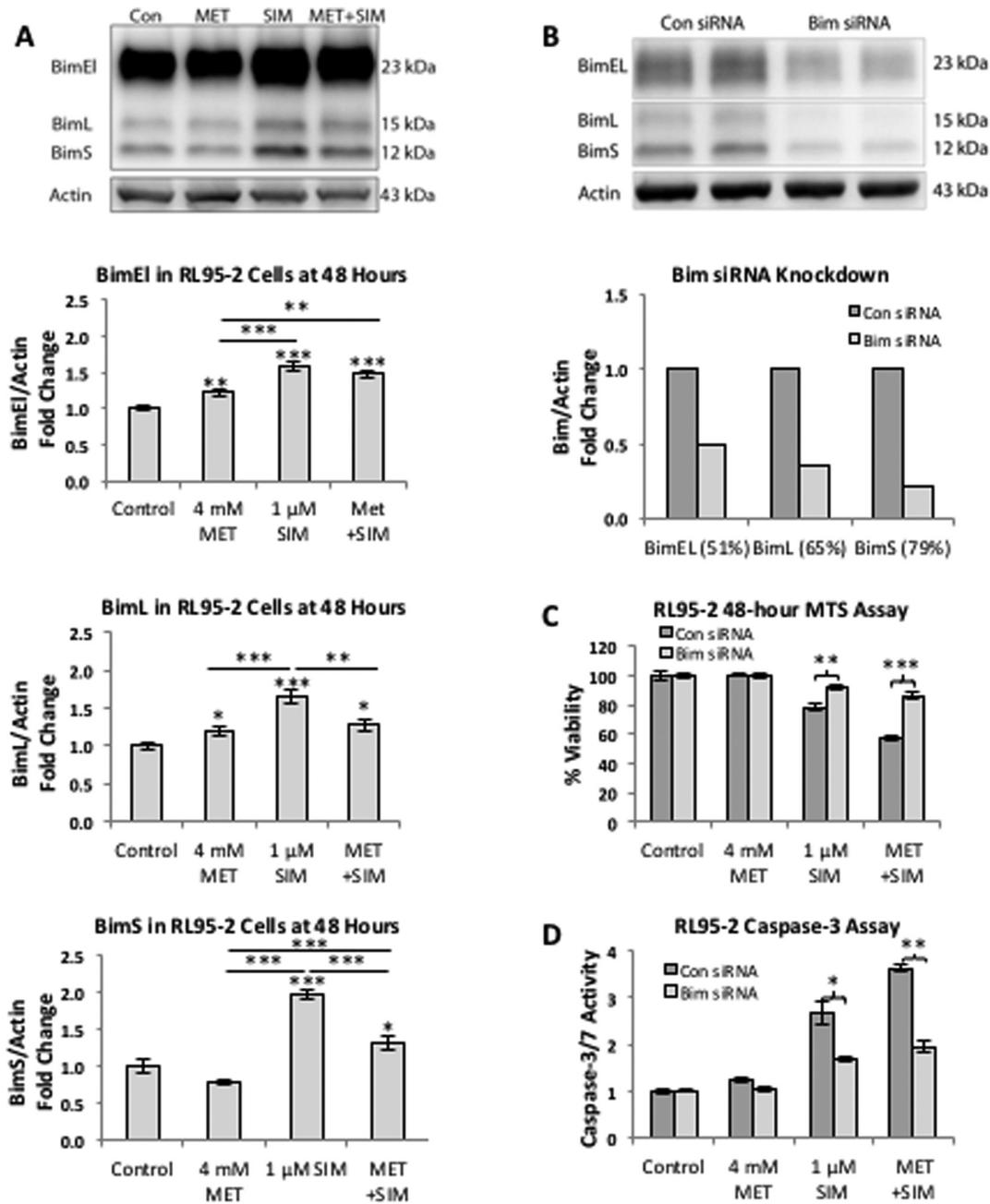
Macroautophagy (hereafter referred to as autophagy) is a lysosomal-dependent process of degradation of cytoplasmic contents, abnormal protein aggregates, and excess or damaged organelles. This process can promote either survival or cell death and is regulated by mTOR in that mTOR inhibition promotes autophagy signaling. Autophagy and apoptosis are interrelated via complex crosstalk between the two mechanisms and this crosstalk is in part caspase-mediated. Given our findings suggestive of apoptosis induction and increased P-AMPK with associated decreased P-S6 along the mTOR pathway, we next sought to determine if autophagy was also being induced. Beclin-1 is a central regulator of autophagy downstream of mTOR and was thus evaluated to determine if significant autophagy induction occurred. Western blot analysis showed that MET+SIM combination treatment resulted in statistically significant but modest increases in beclin-1 levels at 24 h in all cell lines (Supplementary Fig. S4). By 48 h following treatment, these effects were attenuated: combination-treated HEC-1B cells showed a statistically significant but equally modest increase in beclin-1, and there were no longer significant differences in RL95-2 and Ishikawa cells.

**4. Discussion**

Repurposing of inexpensive, commonly used, FDA-approved medications to exploit their anti-cancer effects may yield the development of cost-effective approaches to cancer therapy. Along these lines, MET and SIM have great potential for repurposing in the endometrial cancer population given the high prevalence of comorbid metabolic syndrome, obesity, and hyperlipidemia [10]. We demonstrate here that the combination of MET+SIM treatment resulted in synergistic growth inhibition in three endometrial cancer cell lines. This was associated with profound activation of apoptosis as manifested by markedly increased caspase-3 activity, TUNEL positivity, and Annexin V labeling. Additionally, we showed that the combination activates the AMPK pathway.

Interestingly, apoptosis was not consistently induced in our study by MET alone, yet the effect with combination MET+SIM treatment was robust. One prior study found that MET induces apoptosis at 24 h in Ishikawa cells but only at high concentrations [12]. We replicated this finding in Ishikawa cells though we did not find MET-induced apoptosis in HEC-1B or RL95-2 cell lines. Apoptosis induction has also previously been demonstrated in Ishikawa cells following treatment with SIM [11] alone using similar doses as those used in this study, however the combination of MET+SIM in endometrial cancer has not been previously investigated. To our knowledge, this combination has only been studied in castration-resistant prostate cancer cells in which apoptosis induction with MET+SIM treatment was not observed [14]. This difference may be explained by this prostate cancer cell line's resistance to extrinsic apoptotic cell death due to loss of tumor necrosis factor receptor-associated death domain protein expression.

Silencing Bim in our study reversed both MET+SIM-induced apoptosis and synergistic growth inhibition, suggesting that the dramatic apoptotic effects we observed may explain the synergy seen with this treatment combination. Bim may directly interact with BAX to initiate apoptosis [29]. This mechanism of cell death is consistent with prior investigations with statins in human cancer cells, including ovarian cancer cells [22,30]. Of note, Bim can both activate BAX and bind bcl-2,



**Fig. 5.** Bim is upregulated by combination treatment with metformin and simvastatin and has a primary role in endometrial cancer cell apoptosis. **A**, Western blot analysis shows that the production of all three isoforms of Bim is increased in RL95-2 cells in response to simvastatin (SIM) and combination metformin and simvastatin (MET+SIM). Metformin (MET) alone significantly increased BimEL and BimL. Mean  $\pm$  SEM; symbols above bars indicate comparison to control, symbols above lines designate comparisons between indicated treatment groups; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , by ANOVA. Ratios of untreated controls are set to 1.0. **B**, Western blot images and densitometry chart of RL95-2 cells transfected with Bim siRNA or control siRNA show substantial knockdown of Bim production by Bim siRNA (percent knockdown indicated in parentheses). First two lanes show control siRNA, last two lanes show Bim siRNA (each performed in duplicate with composite data shown here). **C**, RL95-2 cells transfected with Bim siRNA or control siRNA were treated with MET, SIM, or MET+SIM for 48 h. MTS assay demonstrated a significant rescue of cell viability in Bim siRNA-transfected MET+SIM-treated cells compared with control siRNA-transfected MET+SIM-treated cells, as well as in SIM-treated cells. Mean  $\pm$  SEM; \*\* $p < 0.01$ , \*\*\* $p = 0.0001$ , by *t*-test. **D**, MET-, SIM-, and MET+SIM-treated RL95-2 endometrial cancer cells were assessed for caspase-3 activity. Bim siRNA transfection resulted in a significant abrogation of caspase-3 activity compared with control siRNA transfection in cells treated with MET+SIM, as well as in cells treated with SIM alone. Mean  $\pm$  SEM; \* $p < 0.05$ , \*\* $p < 0.01$ , by *t*-test.

effectively changing the ratio of these two molecules without changing their relative expression, thus making BAX the final effector of apoptosis [29]. Bim is also an important molecule in the interaction between apoptosis and autophagy, which we found may play a minor role as demonstrated by initial modest increases in beclin-1 at 24 h with attenuation of this effect by 48 h post-treatment with MET+SIM. Bim has been found to neutralize beclin-1 by sequestering it onto microtubules, thereby inhibiting autophagy [31]. Thus our findings of an only modest

beclin-1 increase may reflect Bim-mediated abrogation of autophagy upregulation. Further elucidation of the complex crosstalk between autophagy and apoptosis pathways in this setting is beyond the scope of this study but would be of interest in future investigations.

Interaction along the AMPK pathway may be another important mechanism accounting for the synergistic effects observed using this drug combination. Synergistic growth inhibition associated with mTOR inhibition with MET+SIM has previously been shown in



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