The Regulation of Apoptosis by Cooperative Src and MAPK Signaling

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Abstract

Introduction: Advanced thyroid cancer (TC) patients have poor survival rates due to lack of effective therapies. We've shown that combined Src (Srci) and MAPK inhibition (MEKi) results in synergistic inhibition of growth *in vitro* and *in vivo*, and increased apoptosis in *BRAF*- and *RAS*-mutant cells, while *PIK3CA*-mutants are resistant. Here we further delineated the mechanism(s) of apoptotic regulation by dual Srci and MEKi.

Methods/Case Presentation: Reverse Phase Protein Array (RPPA) was performed on a panel of TC cell lines treated with Srci and/or MEKi. Western blotting was performed using Odyssey Imaging, growth and apoptosis assays were performed using Sulforhodamine B or CellTiter-Glo, and flow cytometry assays. Knockdown experiments were performed using siRNA. All stats were calculated in GraphPad Prism 9.

Results/Discussion: Western blots showed a 6-fold induction of BIM in cells that are sensitive to combined Srci and MEKi, and a ≤3-fold induction of BIM in resistant cells. Knockdown of BIM in sensitive cells promoted resistance to combined Srci and MEKi. Overexpression of BIM in resistance cells promoted sensitivity to combined Srci and MEKi.

Conclusion: In summary, BIM is a key pro-apoptotic protein necessary for cellular response to combined Src and MEK1/2 inhibition as knockdown of BIM promoted resistance. BIM is also sufficient to induce a response to combined Src and MEK1/2 inhibition as overexpression of BIM in resistant cell lines promoted sensitivity.

Introduction

RPPA analysis reveals BIM as a potential mediator in response to Src and MEK1/2 inhibition

Figure 1: IC50 values of dasatinib in the presence of 100 nanomolar trametinib. 23 thyroid cancer cell lines were treated with increasing doses of dasatinib plus 100 nM of trametinib. Growth curves were measured across the cell lines using either Sulfurhodamine B (SRB) or CellTiter-Glo 2.0 Assay (Promega) and the IC50 values were calculated. An IC50 cut-off of 90 nM was used to determine cell lines sensitive and resistant to dasatinib (dashed line). Arrows denote cell lines used in RPPA Analysis, black arrows denote sensitive cell lines and grey arrows denote resistant cell lines.

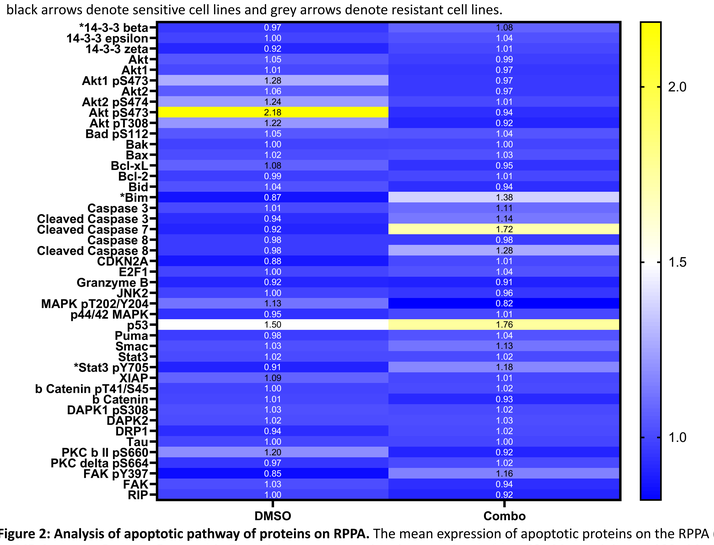

Figure 2: Analysis of apoptotic pathway of proteins on RPPA. The mean expression of apoptotic proteins on the RPPA (as defined by MD Anderson Pathway Browser) in 7 cell lines treatment with either vehicle (left) or 50 nM dasatinib and 100 nM trametinib (right) for 24 hours. T-tests were performed to compare the means between vehicle treated and combination treated, adjusting for multiple comparisons. Asterisk indicates p-values <0.05

Hypothesis: Pro-apoptotic protein BIM is necessary for mediating sensitivity to combined Src and MEK1/2 inhibition.

Results

BIM expression is increased in cells that are sensitive to combined Src and MEK1/2 inhibition and necessary for promoting sensitivity

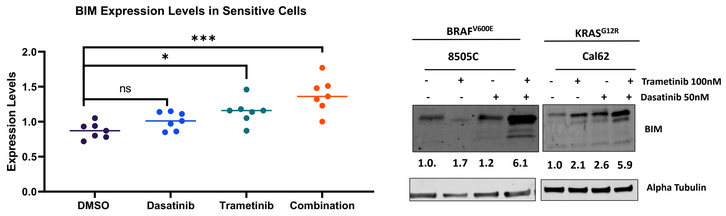

Figure 3: Analysis of BIM expression following treatment with single agents trametinib, dasatinib, or the combination in sensitive cells. *Left:* RPPA analysis comparing BIM expression levels in 7 sensitive cell lines treated with vehicle, dasatinib 50 nM, trametinib 100 nM, or the combination for 24 hours. One-way ANOVA with multiple comparisons was performed using GraphPad Prism 9 *** p<0.005 * p <0.05 *Right:* Cells were treated with indicated doses of either trametinib, dasatinib, or the combination for 24 hours. Lysates were then analyzed by immunoblot analysis and probed for the indicated antibodies. Band intensity was quantified using ImageStudio.

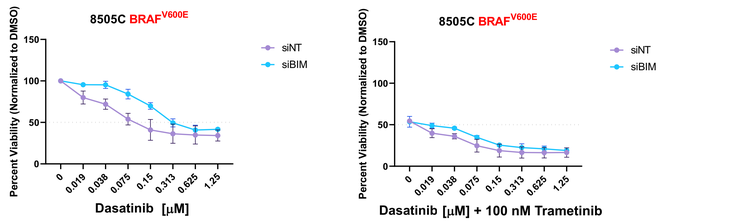

Figure 4: Knockdown of BIM promotes resistance to combined Src and MEK1/2 inhibition. *BRAF*-mutant 8505C cells were plated in 96-well plate for reverse transfection of either SMARTpool non-targeting siRNA (siNT) or BIM (siBIM) for a final concentration of 50 nM. 24 hours later cells were treated with increasing doses of dasatinib with or without 100 nM trametinib for 72 hours. Viability was measured using CellTiter-Glo 2.0 (Promega). The viability of cells treated with DMSO alone was set to 100% and inhibition of growth by 50% is represented by the dashed line. Results shown are the means +/- SEM from two independent experiments.

Ectopic Expression of inducible BIM sensitizes resistant cells to combined Src and MEK1/2 inhibition

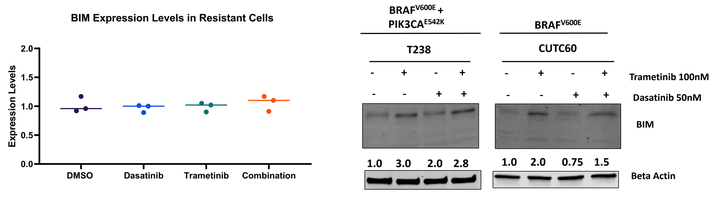

Figure 5: Analysis of BIM expression following treatment with single agents trametinib, dasatinib, or the combination in resistant cells. *Left:* RPPA analysis comparing BIM expression levels in 3 resistant cell lines treated with vehicle, dasatinib 50 nM, trametinib 100 nM, or the combination for 24 hours. One-way ANOVA with multiple comparisons was performed using GraphPad Prism 9. *Right:* Resistant cell lines *PIK3CA*-mutant T238 and *BRAF*-mutant CUTC60 were treated with indicated doses of trametinib, dasatinib, or the combination for 24 hours. Lysates were then analyzed by immunoblot analysis and probed for the indicated antibodies. Band intensity was quantified using ImageStudio.

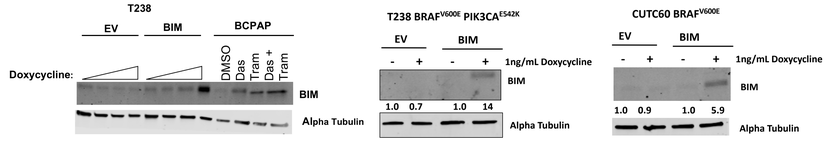

Figure 6: Ectopic expression of doxycycline inducible BIM in low BIM expressing cells. *Left:* T238 cells were transfected with a doxycycline inducible BIM plasmid. Cells were then treated for 24 hours with increasing concentrations of doxycycline (0, 0.5, 0.75, or 1 ng/mL) to induce BIM expression to levels that are similar to those found in sensitive cell line BCPAP. Lysates were then analyzed by immunoblot analysis and probed for the indicated antibodies. *Center and right:* T238 and CUTC60 empty vector (EV) and BIM expressing cells were treated with 1 ng/mL of doxycycline for 24 hours. Lysates were then analyzed by immunoblot analysis and probed for the indicated antibodies. Band intensity was quantified using ImageStudio

Results

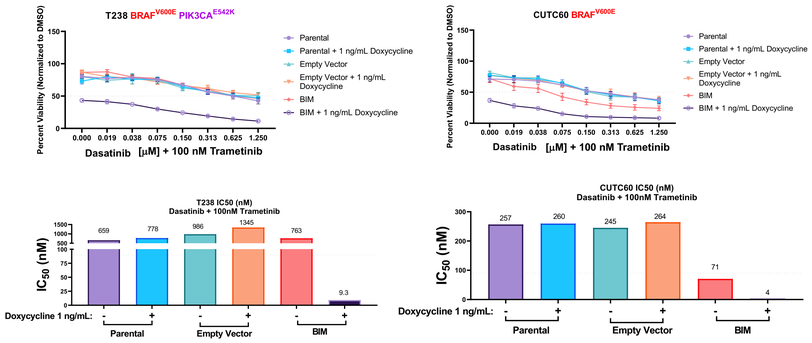

Figure 7: Induction of BIM in low BIM expressing cells sensitizes them to growth inhibition induced by Src and MEK1/2 inhibition. *Top:* T238 and CUTC60 parental, empty vector, or BIM overexpressing cells were treated with 1ng/mL of doxycycline for 24 hours, then subsequently treated with increasing doses of dasatinib and the indicated dose of trametinib for 72 hours. Viability was measured using CellTiter-Glo 2.0 (Promega). The viability of cells treated with DMSO alone was set to 100% and inhibition of growth by 50% is represented by the dashed line. Results shown are the means +/- SEM from three independent experiments. *Bottom:* IC50 values of dasatinib in the presence of 100 nM trametinib were calculated using GraphPad Prism 9. An IC50 cut-off of 90 nm was used to mark sensitivity to dasatinib (dashed line).

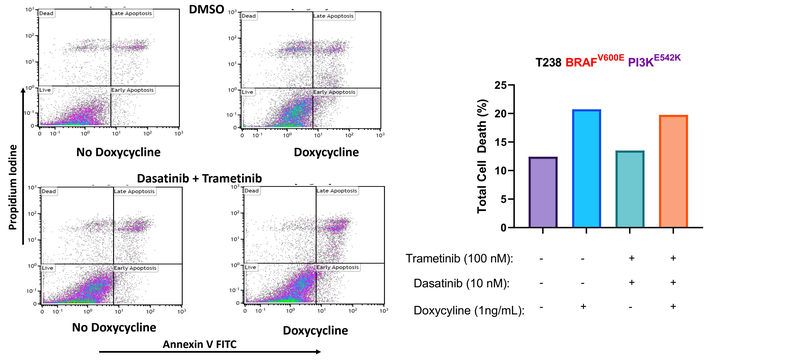

Figure 8: Induction of BIM in a low BIM expressing cell sensitizes it to apoptosis induced by Src and MEK1/2 inhibition. BIM overexpressing T238 cells were treated with or without 1 ng/mL of doxycycline. The following day cells were treated with either vehicle or 10 nM dasatinib and 100 nm trametinib in low serum conditions and harvested for flow cytometry 48 hours later. Quantification was performed of total cell death by adding the percent population of cells with high annexin V/ low propidium iodine and high annexin v/ high propidium iodine. Results are from one independent experiment.

Summary & Conclusions

- RPPA analysis reveals BIM as an apoptotic protein significantly changed in response to Src and MEK1/2 inhibition in cells that are sensitive to Src and MEK1/2 inhibition
- BIM is induced 6-fold in response to Src and MEK1/2 inhibition in cells that are sensitive to Src and MEK1/2 inhibition
- Knockdown of BIM increased resistance to combined Src and MEK1/2 inhibition in an otherwise sensitive cell line
- BIM induction is blunted to 1.5-3-fold in cells that are resistant to Src and MEK1/2 inhibition
- Overexpression of BIM promotes sensitivity to growth inhibition induced by Src and MEK1/2 inhibition in otherwise resistant cell lines
- Overexpression of BIM promotes induction of apoptosis induced by Src and MEK1/2 inhibition in a resistant cell line