The Regulation of Apoptosis by Cooperative Src and MAPK Signaling

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Abstract

Introduction: Thyroid cancer is the most common endocrine malignancy with poor survival rates for patients with advanced and anaplastic thyroid cancer due to lack of effective therapies. While genetic alterations in the MAPK pathway account for the majority of driver mutations expressed in thyroid cancer (*BRAF, RAS, RET/PTC),* there has been mixed success in targeting this pathway in the clinic. Our lab has demonstrated that combined Src and MAPK inhibition 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 have further delineated the mechanism(s) of apoptotic regulation by dual Src and MAPK inhibition.

Methods/Case Presentation: Reverse Phase Protein Array (RPPA) was performed on a panel of thyroid cancer cell lines treated with a Src inhibitor and/or a MEK1/2 inhibitor. Western blotting was performed using Odyessy Imaging, growth assays were performed using Sulforhodamine B (SRB) or CellTiter-Glo 2.0 and apoptosis assays were performed using Caspase-Glo 3/7 assay. IC50 values from CellTiter-Glo were calculated in GraphPad Prism 9 using the nonlinear regression analysis with a variable slope.

Results/Discussion: RPPA identified the pro-apoptotic protein BIM as a key regulator of the apoptotic response. Western botting showed a 6-fold induction of BIM in *BRAF*- and *RAS*-mutant cells that are sensitive to combined Src and MEK1/2 inhibition when treated with the combination, and only a 1.5- to 3-fold induction of BIM in cells that are resistant. Ectopic expression of doxycycline inducible BIM in resistance cells promoted sensitivity to growth inhibition driven by combined Src and MEK1/2 inhibition. Sale et al. demonstrated that low Bcl-xL expression in melanoma tumor cells compared to pancreatic tumor cells predicted sensitivity to an MCL1 inhibitor. Here we demonstrate thyroid tumors cells align with pancreatic tumor cells and are thus more sensitive to a Bcl-xL inhibitor.

Conclusion: In summary, BIM is a key pro-apoptotic protein cooperatively regulated by the Src and the MAPK pathways and is sufficient to induce sensitivity to combined Src and MEK1/2 inhibition in a resistant cell. The efficacy of combined Src and MEK1/2 inhibition can be increased through the addition of a BH3 mimetic targeting Bcl-xL.

Introduction

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

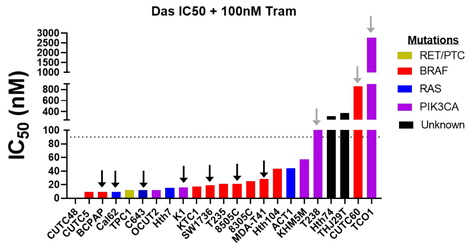

Figure 1: IC50 values of dasatinib in the presence of 100 nanomolar trametinib. 23 thyroid cancer cell lines were treated with increasing doses of dasatinib plus 100 nM of trametinib. Growth curves were measured across the cell lines using either Sulfurhodamine B (SRB) or CellTiter-Glo 2.0 Assay (Promega) 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.

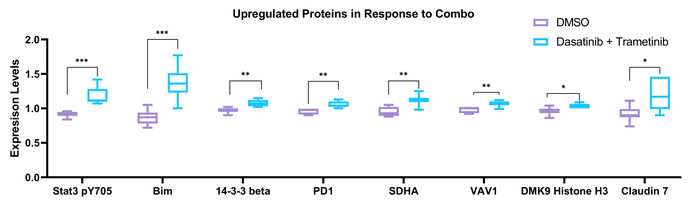

Figure 2: Proteins upregulated in response to combined Src and MEK1/2 inhibition. Protein expression data for the 425 proteins on the RPPA was compared in the sensitive cells treated with either vehicle or combined dasatinib and trametinib for 24 hours. Multiple T tests per row comparing the means between the two treatment groups were performed in GraphPad Prism 9 ****p<0.00005 *** p<0.0005 ** p<0.005 * p <0.05

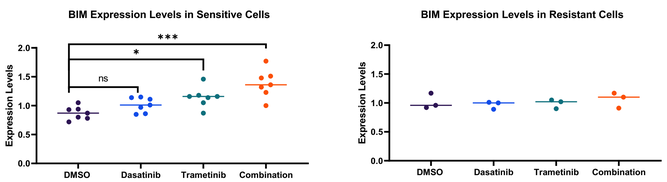

Figure 3: BIM protein expression in response to combined Src and MEK1/2 inhibition. RPPA analysis comparing BIM expression levels in 7 sensitive (*left*) and 3 resistant (*right*) cell lines treated with vehicle, dasatinib 50 nM, trametinib 100 nM, or the combination for 24 hours. One-way ANOVA with multiple comparisons was performed using GraphPad Prism 9 *** p<0.005 * p <0.05

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

Results

Combined Src and MEK1/2 inhibition induces expression of BIM in cells sensitive to Src and MEK1/2 inhibition

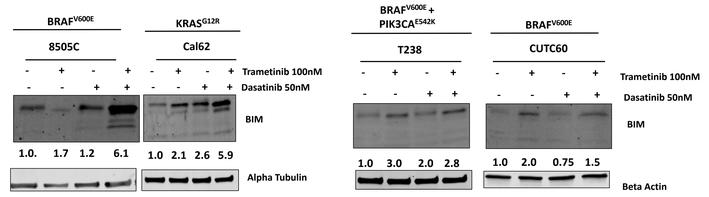

Figure 4: Analysis of BIM expression following treatment with single agents trametinib, dasatinib, or the combination. Cells were treated with indicated doses of either trametinib, dasatinib, or the combination for 24 hours. Lysates were then analyzed by immunoblot analysis and probed for the indicated antibodies. Band intensity was quantified using ImageStudio.

Ectopic Expression of inducible BIM sensitizes a *PIK3CA*-mutant resistant cell to combined Src and MEK1/2 inhibition

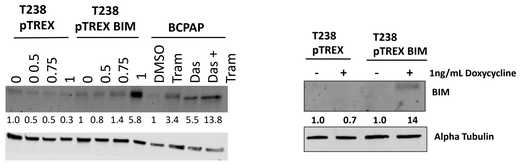

Figure 5: Modulating induction of BIM in low BIM expressing cells. Low BIM expressing *PIK3CA*-mutant T238 cells were transduced with lentivirus expressing Empty Vector (pTREX) or doxycycline-inducible BIM (pTREX BIM). Cells were then treated with indicated doses of doxycycline for 24 hours. Sensitive *BRAF*-mutant BCPAP lysates treated with vehicle, trametinib, dasatinib, or the combination for 24 hours were included as a positive control for high BIM expressing cells. Lysates were then analyzed by immunoblot analysis and probed for the indicated antibodies. Band intensity was quantified using ImageStudio.

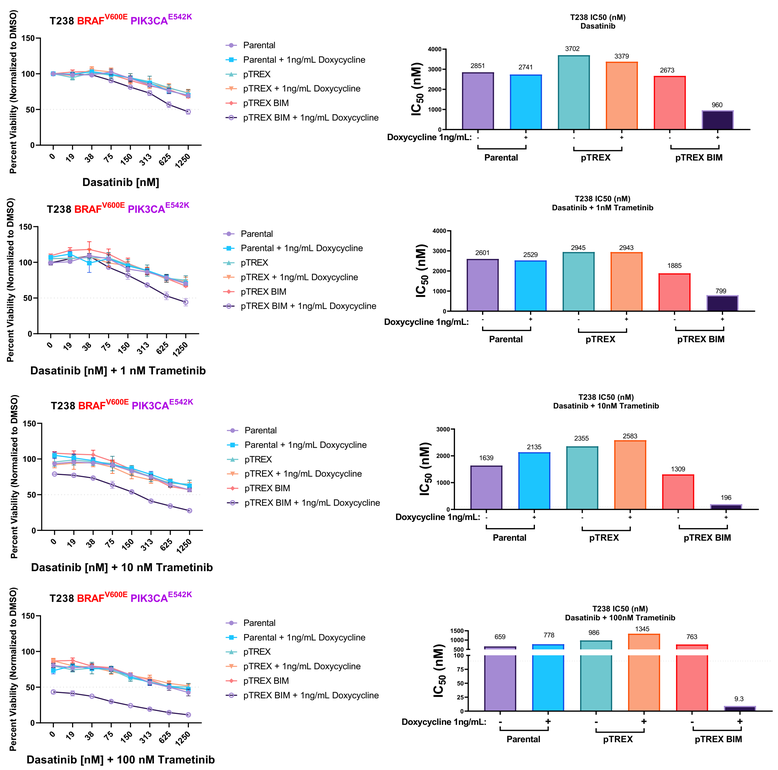

Figure 6: Induction of BIM in low BIM expressing cells sensitizes them to combined Src and MEK1/2 inhibition. *Left: PIK3CA*-mutant T238 parental, empty vector (pTREX), or BIM overexpressing (pTREX BIM) cells were treated with 1ng/mL of doxycycline for 24 hours, then subsequently treated with increasing 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. *Right:* IC50 value of dasatinib in the presence of trametinib was calculated using GraphPad Prism 9. An IC50 cut-off of 90nm was used to mark sensitivity to dasatinib (dashed line).

Results

Combining a Bcl-xL mimetic with Src and MEK1/2 inhibition increases efficacy in a *BRAF*-mutant cell

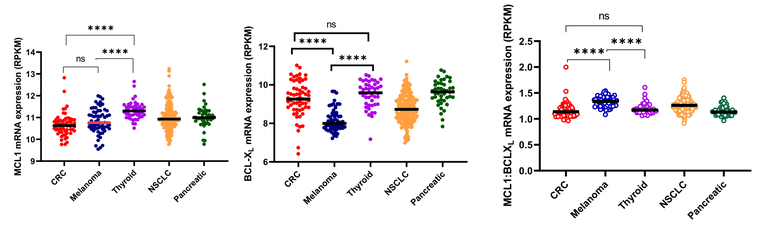

Figure 7: **High Bcl-xL expression in thyroid cancer predicts sensitivity to Bcl-xL inhibition.** RNA-sequencing mRNA expression data from the Cancer Cell Line Encyclopedia for *MCL1 (left) Bcl-xL (middle)* and *MCL1:Bcl-xL (right)* ratio in colorectal cancer (CRC), melanoma, thyroid, non-small cell lung cancer (NSCLC) and pancreatic tumor cells. Values are reads per kilobase million (RPKM) in log$_2$ scale. Median values are indicated by black lines.

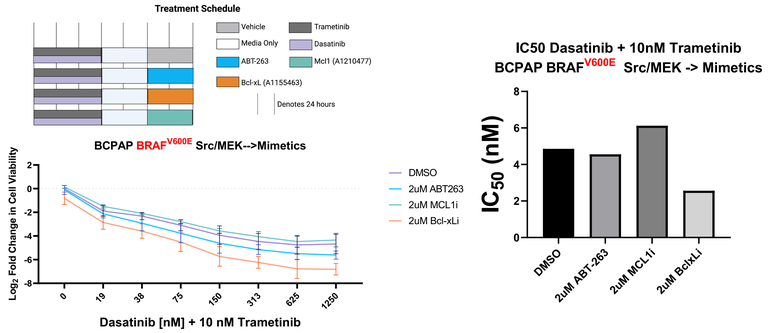

Figure 8: Addition of a Bcl-xL mimetic increases efficacy of combined Src and MEK1/2 inhibition. *Left: BRAF*-mutant BCPAP cells were treated with increasing doses of dasatinib in combination with 10 nM of trametinib. After 72 hours dasatinib and trametinib was removed. 48 hours later dasatinib and trametinib were readded in combination with either vehicle, a pan Bcl-2 (ABT-263), MCL1 (A1210477) or Bcl-xL mimetic (A1155463) for 48 hours (see treatment schedule) and viability was measured using CellTiter-Glo 2.0 (Promega). *Right:* The IC50 value of dasatinib in the presence of 10 nM trametinib was calculated using GraphPad Prism 9.

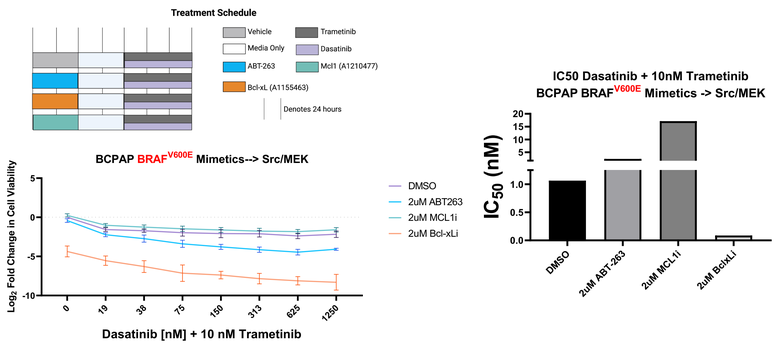

Figure 9: Pre-treatment with a Bcl-xL mimetic increases efficacy of combined Src and MEK1/2 inhibition. *Left: BRAF*-mutant BCPAP cells were treated with either vehicle, a pan Bcl-2 (ABT-263), MCL1 (A1210477) or Bcl-xL mimetic (A1155463) for 48 hours. Drug was removed and after 48 hours mimetics were readded in combination with increasing doses of dasatinib and 10 nM of trametinib for 72 hours (see treatment schedule). Cell viability was measured using CellTiter-Glo 2.0 (Promega). *Right:* IC50 values of dasatinib in the presence 10 nM trametinib were calculated using GraphPad Prism 9

Summary & Conclusions

- RPPA analysis reveals BIM as the only apoptotic protein 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, while this 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 an otherwise resistant cell line
- Thyroid tumor cells align with pancreatic tumor cells and thus are predicted to be sensitive to a Bcl-xL inhibitor
- Addition of a Bcl-xL inhibitor enhances the efficacy of combined Src and MEK1/2 inhibition in a sensitive *BRAF*-mutant cell