The Regulation of Apoptosis by Cooperative Src and MAPK Signaling

Madison M. Rose, N Pozdeyev, MC Hofmann, LA Pike, VL Espinoza, and RE Schweppe
Department of Endocrinology, University of Colorado Anschutz Medical Campus

University of Colorado Anschutz Medical Campus

University of Colorado Cancer Center
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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 Cell Titer Glo and apoptosis assays were performed using Caspase-Glo 3/7 assay.

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 constitutively active AKT in sensitive cells promoted resistance to growth inhibition, apoptosis, and blunted BIM induction. While ectopic expression of doxycycline inducible BIM in resistance cells promoted sensitivity to growth inhibition driven by combined Src and MEK1/2 inhibition

Conclusion: In summary, dual inhibition of Src and MEK1/2 synergistically inhibits growth and induces apoptosis through key signaling nodes: Src/FAK, MEK/ERK, and AKT; and BIM is a key pro-apoptotic protein cooperatively regulated by the Src and the MAPK pathways.

Introduction

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

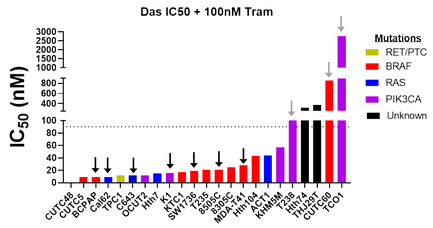


Figure 1: IC50 values of Dasatinib plus 100 nanomolar Trametinib. 23 thyroid cancer cell lines were treated with increasing doses of dasatinib plus 100 nm of trametinib. Growth curves were measured across the cell lines using CellTiter-Glo Assay (Promega) and the IC50 values were calculated using GraphPad Prism8. An IC50 cut-off of 90nm was used to determine cell lines sensitive and resistant to dasatinib. Arrows denote cell lines used in RPPA Analysis where black are sensitive cell lines and grey are resistant cell lines

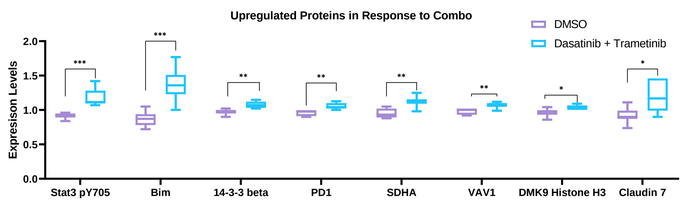


Figure 2: Proteins Upregulated in Response to Combined Src and MEK1/2 Inhibition. Protein expression data for the 425 proteins on the RPPA was compared in the sensitive cells treated with either vehicle or combined dasatinib and trametinib for 24 hours. Multiple T tests per row comparing the means between the two treatment groups were performed in GraphPad Prism 8 ****p<0.00005 *** p<0.0005 ** p<0.005 * p <0.05

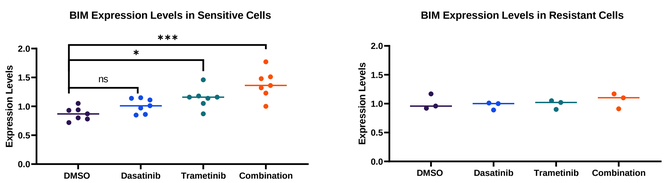


Figure 3: BIM Protein Expression in Response to Combined Src and MEK1/2 Inhibition. RPPA analysis comparing BIM expression levels in either 7 sensitive (left) or 3 resistant (right) cell lines treated with vehicle, dasatinib 50 nM, trametinib 100 nM, or the combination for 24 hours. One-way ANOVA with multiple comparisons was performed using GraphPad Prism8 *** p<0.005 * p <0.05

Hypothesis: Inhibition of growth by combined Src and MEK1/2 inhibition is mediated by the upregulation of BH3 proteins to induce apoptosis and through the inhibition of key signaling nodes FAK/Src, MEK/ERK, and AKT

Results

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

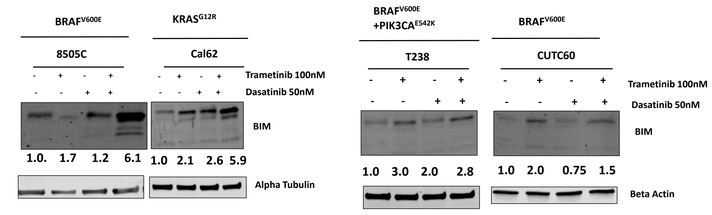
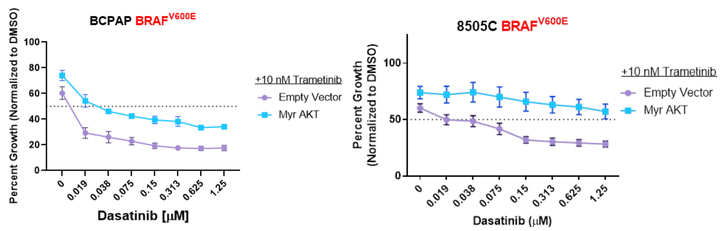


Figure 4: Analysis of BIM Expression Following Treatment with Single Agents Trametinib and Dasatinib or the Combination. Cells were treated with indicated doses of either trametinib, dasatinib, or the combination for 24 hours. Lysates were then analyzed by immunoblot analysis and probed for the indicated antibodies. Band intensity was quantified using ImageStudio

Ectopic expression of a constitutively active AKT induces resistance to combined Src and MEK1/2 inhibition and blunts apoptotic response in *BRAF*-mutant cells



Trametinib uM	Empty Vector IC50 uM	Myr AKT IC50 uM	Fold Increase in IC50
0.01	0.00012	0.02	167

Trametinib uM	Empty Vector IC50 uM	Myr AKT IC50 uM	Fold Increase in IC50
0.01	0.02	6.8	357

Figure 5: Constitutively Active AKT increases resistance to Combined Src and MEK1/2 inhibition in *BRAF*-mutant cells. *BRAF*-mutants BCPAP and 8505C cells ectopically expressing Empty Vector or Myristoylated-AKT (Myr-AKT) were treated with increasing doses of dasatinib in combination with 10 nM of trametinib for 72 hours and viability was measured using Cell Titer Glo (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.

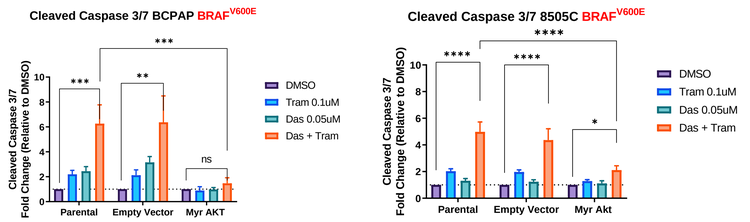


Figure 6: Constitutively Active AKT blunts apoptotic response to combined Src and MEK1/2 inhibition in *BRAF*-mutant cells. Cells were treated with indicated concentrations of either trametinib, dasatinib, or the combination for 24 hours, and caspase activity was measured using the Caspase-Glo 3/7 Assay (Promega). All measurements are relative to their respective DMSO control. Results shown are the mean +/- SEM of 3 biologic replicates. ***p<0.001 **p<0.01

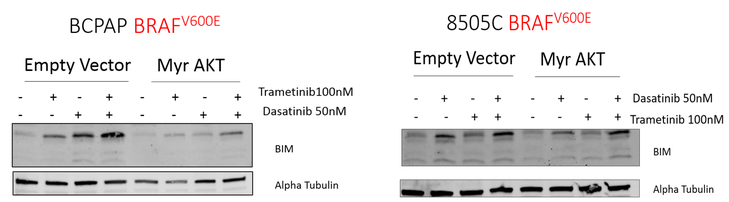


Figure 7: Constitutively Active AKT blunts increase of BIM in response to combined Src and MEK1/2 inhibition in *BRAF*-mutant cells. Cells were treated with indicated doses of either trametinib or dasatinib for 24 hours. Lysates were then analyzed by immunoblot analysis and probed for the indicated antibodies.

Results

Ectopic expression of a constitutively active AKT has mixed response to Src and MEK1/2 inhibition in *RAS*-mutant cells

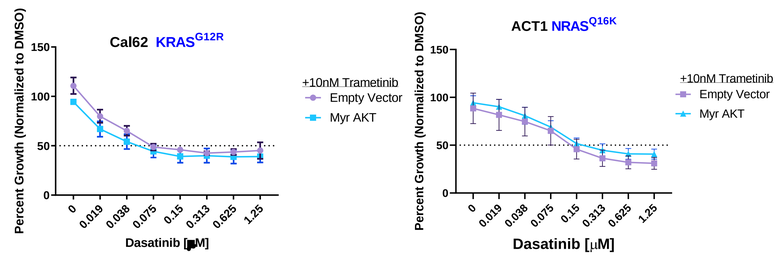


Figure 8: Constitutively Active AKT fails to increase resistance in *RAS*-Mutant Cell Lines Treated with Combined Dasatinib and Trametinib. *RAS*-mutant Cal62 and ACT1 ectopically expressing Empty Vector or Myristoylated AKT (Myr AKT) cells were treated with increasing doses of dasatinib in combination with 10 nM of trametinib for 72 hours and viability was measured using Cell Titer Glo (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.

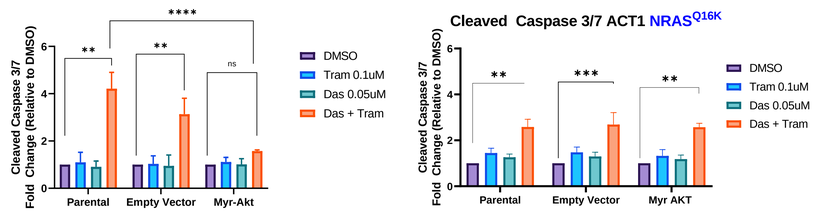


Figure 9: Constitutively Active AKT has mixed apoptotic response in *RAS*-Mutant Cell Lines Treated with Combined Dasatinib and Trametinib. *RAS*-mutant Cal62 and ACT1 ectopically expressing Empty Vector or Myristoylated AKT (Myr AKT) cells were treated with indicated concentrations of either trametinib, dasatinib, or the combination for 24 hours, and caspase activity was measured using Caspase-Glo 3/7 Assay (Promega). All measurements are relative to their respective DMSO control. Results shown are the mean +/- SEM of 3 biologic replicates. ****p<0.0001 ***p<0.001

Ectopic Expression of inducible BIM increases sensitivity to to combined Src and MEK1/2 inhibition in a *BRAF*-mutant cell

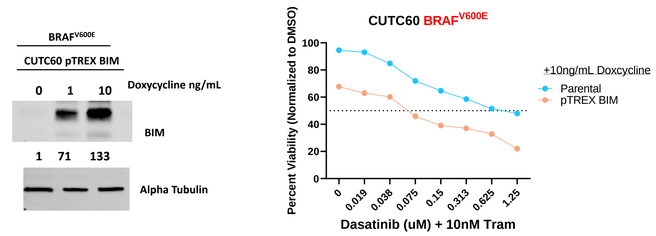


Figure 10: Overexpression of BIM. *Left: BRAF*-mutant CUTC60 cells ectopically expressing a doxycycline inducible BIM plasmid (pTREX BIM) were treated for 24 hours with indicated concentration of doxycycline to induce BIM expression. Lysates were then analyzed by immunoblot analysis and probed for the indicated antibodies. Band intensity was quantified using ImageStudio. *Right: BRAF*-mutant CUTC60 cells expressing pTREX BIM were plated in the presence of 10ng/mL of doxycycline. 24 hours later cells were treated with increasing doses of dasatinib in combination with 10 nM of trametinib for 72 hours and viability was measured using Cell Titer Glo (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.

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
- Ectopic expression of myristoylated AKT promotes resistance to growth inhibition and apoptosis, and blunts BIM induction in response to combined Src and MEK1/2 inhibition in *BRAF*-mutant cells
- Ectopic expression of myristoylated AKT fails to promote resistance to growth inhibition in response to combined Src and MEK1/2 inhibition in *RAS*-mutant cells
- Ectopic expression of myristoylated AKT has mixed apoptotic response in response to combined Src and MEK1/2 inhibition in *RAS*-mutant cells
- Overexpression of BIM promotes sensitivity to growth inhibition induced by Src and MEK1/2 inhibition