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TITLE: Targeting TBK1 and CARM1 to combat lung adenocarcinoma

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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

The overall objective of this proposal is to assess if inhibitors of CARM1, TBK1 or Raf-1, alone or in combination, reduces the viability of lung cancer cells through the downregulation of YAP1. A series of biochemical and in vivo experiments will be conducted to assess this. Experiments will also be conducted to elucidate these drugs or their combinations, affects the anti-cancer activity of T-cells in vitro and T-cell infiltration in vivo. The Specific Aims of the study are: Aim 1. To elucidate the molecular mechanisms by which TBK1 and CARM1 modulate the growth and metastasis of lung adenocarcinoma and Aim 2. To test the efficacy of TBK1 and CARM1 inhibitors in targeting K-Ras mutant lung cancer.

**15. SUBJECT TERMS**
None listed.

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1. **INTRODUCTION:**

This proposal attempts to test the efficacy of new drugs and drug combinations in preventing the growth of lung cancer. A large majority of lung cancers are correlated with tobacco smoking, and such cancers tend to have alterations in a cancer promoting gene, K-Ras. We propose to test the theory that using three different inhibitors, or their combination, will prevent the growth of such tumors. We propose to test the efficiency of inhibiting TBK1 kinase, alone or in combination with inhibitors of Raf1 kinase or the arginine methyl transferase, CARM1. We expect that these drug combinations will be effective in combating smoking-related lung cancers.

2. **KEYWORDS:**

Non-Small cell lung cancer, tank binding kinase 1, CARM1, drug efficacy, drug resistance, metabolomics,

3. **ACCOMPLISHMENTS:**

**What were the major goals of the project?**

The major goals of the proposal include the acquisition of cell lines and reagents, obtaining the requisite approvals and conduct of multiple experiments. Specific Aim 1 had four Major Tasks. Major Task 1 included the conduct of metabolomics analysis and RNA seq experiments on lung cancer cell lines, subsequent to the inhibition of TBK1, Raf1 or CARM1. All reagents have been acquired and the IACUC and IRB equivalent approvals have been obtained. The cell treatments for these above experiments are in progress; they are being conducted on A549 cells and will be completed in the coming weeks. About 70% of the listed tasks have been completed.

Major Task 2 inovle the assessment of how combinations of a TBK1 inhibitor with a Raf1 inhibitor or CARM1 inhibitor affect the viability of K-Ras mutant non-small cell lung cancer cells. This Task has been completed; we find that the combinations are highly effective in suppressing the viability of NSCLC cell lines.

Major Task 3 was to assess how TBK1 and CARM1 inhibitors affect the activity of T cells. This Task has been completed 90%. We find that CARM1 inhibitors enhance T cell activation, while TBK1 inhibitors appear to suppress T cell activation, in in vitro experiments. Combination studies remain to be conducted.

Major Task 4 was to examine the potential correlations between the levels of YAP1, TBK1, CARM1 and ERK1 in a lung cancer TMA as well as on sections from primary and metastatic tissues. The stainings have been standardized abd multi-spectral imaging will be conducted soon. The Task has been completed 50%.

Specific Aim 2 had two Major Tasks. Major Task 1 was to assess how CARM1 and TBK1 inhibitors affect the growth of lung cancer organoids. This task has been completed 70% and the studies are in progress. Major Task 2 is to assess the efficacy of TBK1, CARM1 and Raf1 inhibitors alone or in combination, on the growth of lung cancer cells in an immunocompetent model. The studies are in progress and this aim has been completed 30%.

**What was accomplished under these goals?**

A significant amount of the work proposed in Aim 1 has been completed. Experimentally, we have completed most of the in vitro experiments on cell lines, where we have tested the ability of TBK1 inhibitor, alone or in combination, to suppress the growth of NSCLC cells, as measured by MTT assays. As shown in Figure 1, treatment with CARM1 inhibitors reduce YAP1 levels, and how depletion or inhibition suppresses cell growth (Figure 2). Our results show that combination of BX795 with the CARM1 inhibitor, piperidone 1 as well as the general methyltransferase inhibitor, MS023, markedly reduces the viability of A549 and H460 K-Ras mutant cells (Figure 3). In addition, the presence of the CARM1 inhibitor markedly reduces the IC50 of the TBK1 inhibitor, BX795 (Figure 4). We had found that inhibition or depletion of TBK1 induces ERK activity while suppressing AKT (Figure 5). Based on this results, we examined if Sorafenib, the Raf1 inhibitor, or U0126, a MEK inhibitor, reduces cell viability. As showin in Figures 6 and 7, the Raf1 and MEK inhibitors could suppress cell proliferation and significantly enhance the potency of the TBK1 inhibitor, BX795. This confirms that combining Raf1 inhibitors or CARM1 inhibitors with TBK1 inhibitor would be effective in combating NSCLC. The efficacy of CARM1 inhibitors in suppressing tumor growth in vivo was examined. CARM1 inhibitor could reduce the viability of the mouse NSCLC cell line LKR13 (Fig. 8, top panel). Both the CARM1 inhibitor pieridone 1 and EZM2302 could suppress tumor growth in vivo, quite effectively (Figure 8, bottom panel).

A significant amount of in vitro studies have been conducted on how inhibitors of TBK1 and CARM1 affects T cell function. In the first experiment, we tested how TBK1 and CAM1 inhibitors affect T cell proliferation (Figure 9); two TBK1 inhibitors could suppress proliferation, while CARM1 inhibitor did not affect proliferation at low doses (Figure 9). The effect of these inhibitors on T cell activation was examined, by treating human T cells with the appropriate inhibitors and analyzing various surface markers for T cell activation by flow cytometry. Our results clearly show that treatment of T cells with the TBK1 inhibitor, BX795, led to a suppression of T cell activation (Figure 10). Activation of T cells result in induction of granzyme B, perforin, IFNγ etc (granzyme B induction from a publication is shown in Figure 11); treatment with TBK1 inhibitor reduced the expression of granzyme B in both CD4+ and CD8+ T cells (Figure 12). BX795 could also suppress the expression of IFNγ by T cells, after 48 or 72 hr of activation (Figure 13). Suppression of T cell activation by BX795 occurred in a dose-dependent fashion (Figure 14), as seen by the expression of CD25 in CD8+ and CD4+ T cells (top panels), as well as IFNγ expression (lower panel). These results also support the contention that TBK1 inhibitors might be suppressing T cell response, perhaps by enhancing YAP1 levels. Efforts are underway to measure the levels of YAP1 in T cells, following the inhibition of TBK1 or CARM1.

One of our hypothesis was that induction of YAP1 upon TBK1 inhibition will lead to immunosuppression, since high levels of YAP1 are known to enhance the frequency of myeloid-derived suppressor cells as well as Tregs. We next examined if treatment with CARM1 inhibitor can prevent the conversion of CD4+ T cells into Tregs. As shown in Figure 15, T reg conversion was achieved by treatment with αCD3, αCD28, IL2 and TGFβ. It can be seen that CARM1 inhibition suppresses Treg conversion effectively, in a dose-dependent manner. On the contrary, TBk1 inhibition is expected to induce YAP1 and reduce T cell function. We next examined how BX795 affects tumor cell kill mediated by PBMCs. As shown in Figure 16, BX795 could markedly reduce cell kill mediated by PBMCs. Overall, our experiments suggest that suppressing TBK1 alone may not be fully effective in inhibiting tumor growth, due to the concomitant induction of ERK signaling as well as YAP1.

The above results provide ample support to the hypothesis that combining inhibitors of TBK1 with inhibitors of CARM1, or components of the MAPK cascade, have a strong suppressive effect on the growth of non-small cell lung cancer cell lines. Completion of these studies in the coming months will further expand these findings and will also reveal the molecular basis for the observed changes.

**What *training and professional development opportunities* were provided?**

Nothing to report

**How were the results disseminated to communities of interest?**

Nothing to report

**What do you plan to do during the next reporting period to accomplish the goals?**

The next reporting period will involve conducting experiments on the effect of combinations on mouse models, assessing the molecular basis of why TBK1 and CARM1 inhibitors have opposite effects on T cells, examining the correlation between TBK1, YAP1 and components of the MAPK cascade in lung cancer TMAs.

## *4.* IMPACT:

**What was the impact on the development of the principal discipline(s) of the project?**

Nothing to report

**What was the impact on other disciplines?**

Nothing to report?

**What was the impact on technology transfer?**

Nothing to report

**What was the impact on society beyond science and technology?**

Nothing to report

**5. CHANGES/PROBLEMS:**

Nothing to report

**Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to report

**Changes that had a significant impact on expenditures**

Nothing to report

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

**Significant changes in use or care of human subjects**

Nothing to report

**Significant changes in use or care of vertebrate animals**

Nothing to report

**Significant changes in use of biohazards and/or select agents**

Nothing to report

## 6. PRODUCTS:

- **Publications, conference papers, and presentations**
  **Journal publications.**

| Nothing to report |
| --- |

**Books or other non-periodical, one-time publications.**

| Nothing to report |
| --- |

**Other publications, conference papers and presentations**.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**What individuals have worked on the project?**

| | |
|---|---|
| Name: Srikumar Chellappan – PD/PI | -No Change |
| Name: Jaya Padmanabhan -Rsch. Scientist III | -No Change |
| Name: Biswarup Saha – Rsch. Scientist I | -No Change |
| Name: Fatema Khambati – Rsch. Scientist III | -No change |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

None

**What other organizations were involved as partners?**

Nothing to report

## 8. SPECIAL REPORTING REQUIREMENTS

**COLLABORATIVE AWARDS:**

**QUAD CHARTS:**

## 9. APPENDICES:

# APPENDIX

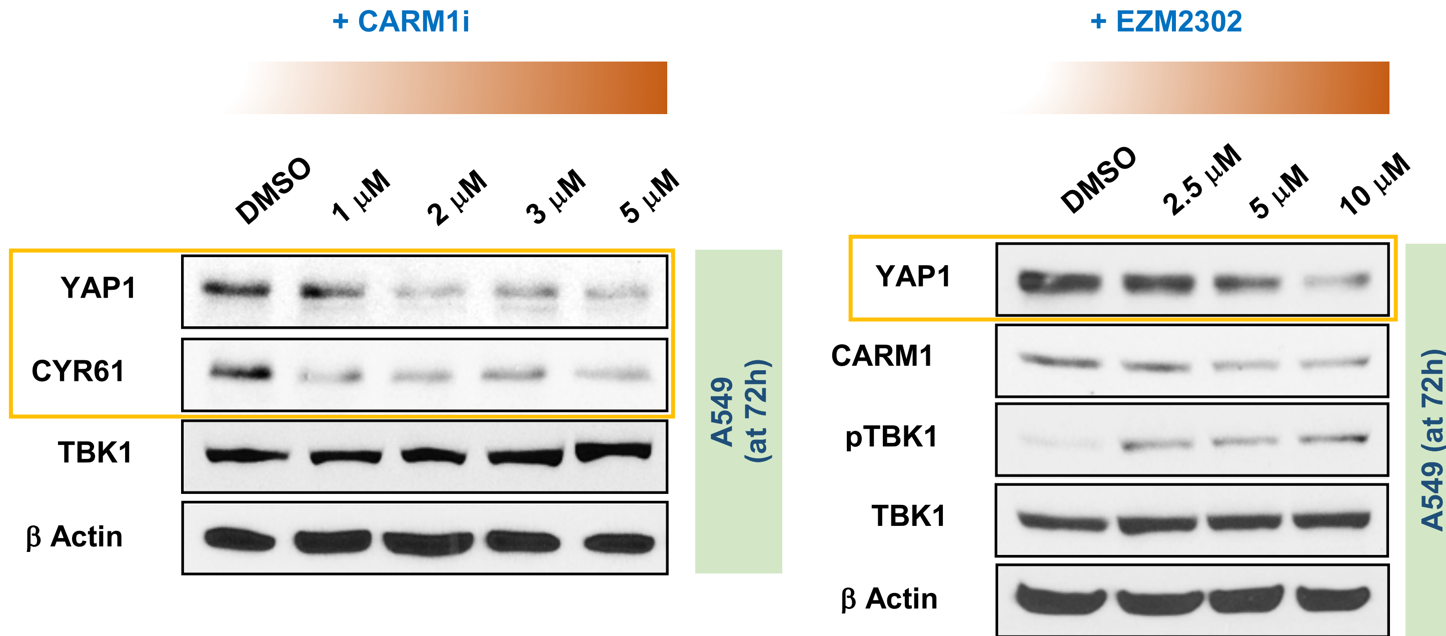
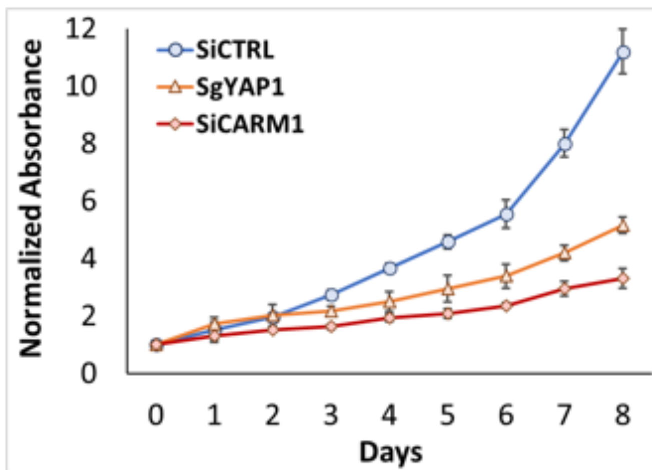



Figure 1. Increasing doses of CARM1 inhibitors, piperidone 1 (left panel), or EZM2302 (right panel) reduce the levels of YAP1 protein in A549 cells. CYR61 is a transcriptional target of YAP1. EZM2032 treatment led to an induction of TBK1 in the cells; the underlying mechanism of this induction or its functional consequences are unknown.



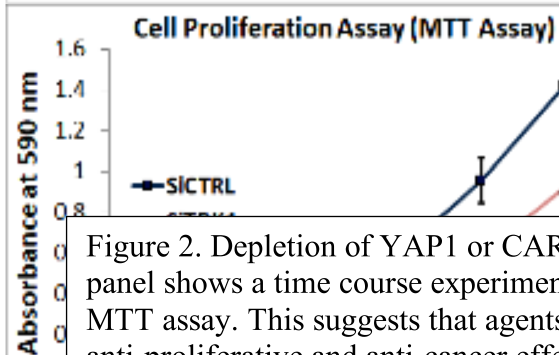




Figure 2. Depletion of YAP1 or CARM1 causes marked suppression of cell proliferation. Left panel shows a time course experiment, where the proliferation of A549 cells was measured by a MTT assay. This suggests that agents that can inhibit CARM1 and thus reduce YAP1 might have anti-proliferative and anti-cancer effects. Right panel shows a growth curve of A549 cells after depletion of TBK1. While proliferation was retarded, the overall reduction in cell growth was not remarkable.

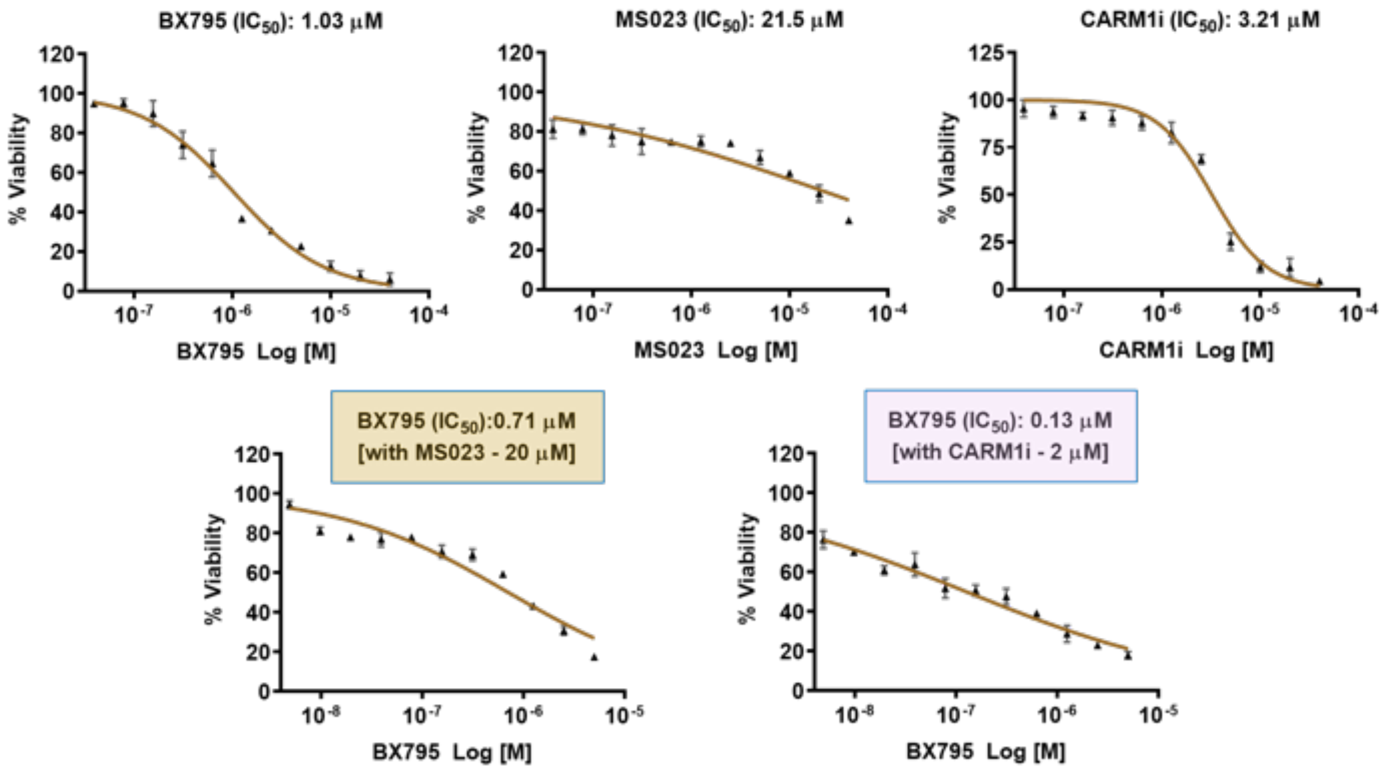



Figure 3. (Top panels) IC50 values were determined to assess the potency of the TBK1 inhibitor, BX795 (left panel, top), a pan-arginine methyl transferase inhibitor MS023 (middle panel, top) and the CARM1 inhibitor piperidone 1 (right most panel, top). BX795 had an IC50 of 1.03 μM, while MS023 and CARM1 inhibitor has IC50 values of 21.5 μM and 3.21μM respectively. (Bottom panels) The effect of combining 20 μM of MS023 with different doses of BX795 was tested . It was found that the presence of 20μM MS023 reduce the IC50 of TBK1 to 0.71μM (bottom left). Remarkably, combining 2 μM CARM1 inhibitor reduced the IC50 of BX795 to 0.13μM (130 nM). This supports our contention that the reduction in YAP1 levels in response to CARM1 inhibitor will enhance the efficacy of TBK1 inhibitors.

| Cell line | A549 | H460 |
|---|---|---|
| BX795 (in μM) | 1.5 ± | 1.03 ± |
| MS023 (in μM) | 30.4 ± | 21.5 ± |
| CARM1i (in μM) | 2.41 ± | 3.21 ± |
| BX795 (in μM) (with MS023 – 20 μM) | 0.18 ± | 0.71 ± |
| BX795 (in μM) (with CARM1i – 2 μM) | 0.15 ± | 0.13 ± |

Figure 4. Summary of combination experiments, showing how arginine methyl transferase inhibitors enhance the potency of the TBK1 inhibitor, BX795. Addition of 20μM MS023 reduced the IC50 of BX795 from 1.5μM and 1.03μM to 0.18μM and 0.71 μM in A549 ad H460 cells respectively. Similarly, inclusion of 2μM CARM1 inhibitor reduced the IC50 of BX795 to 0.15μM and 0.13μM.

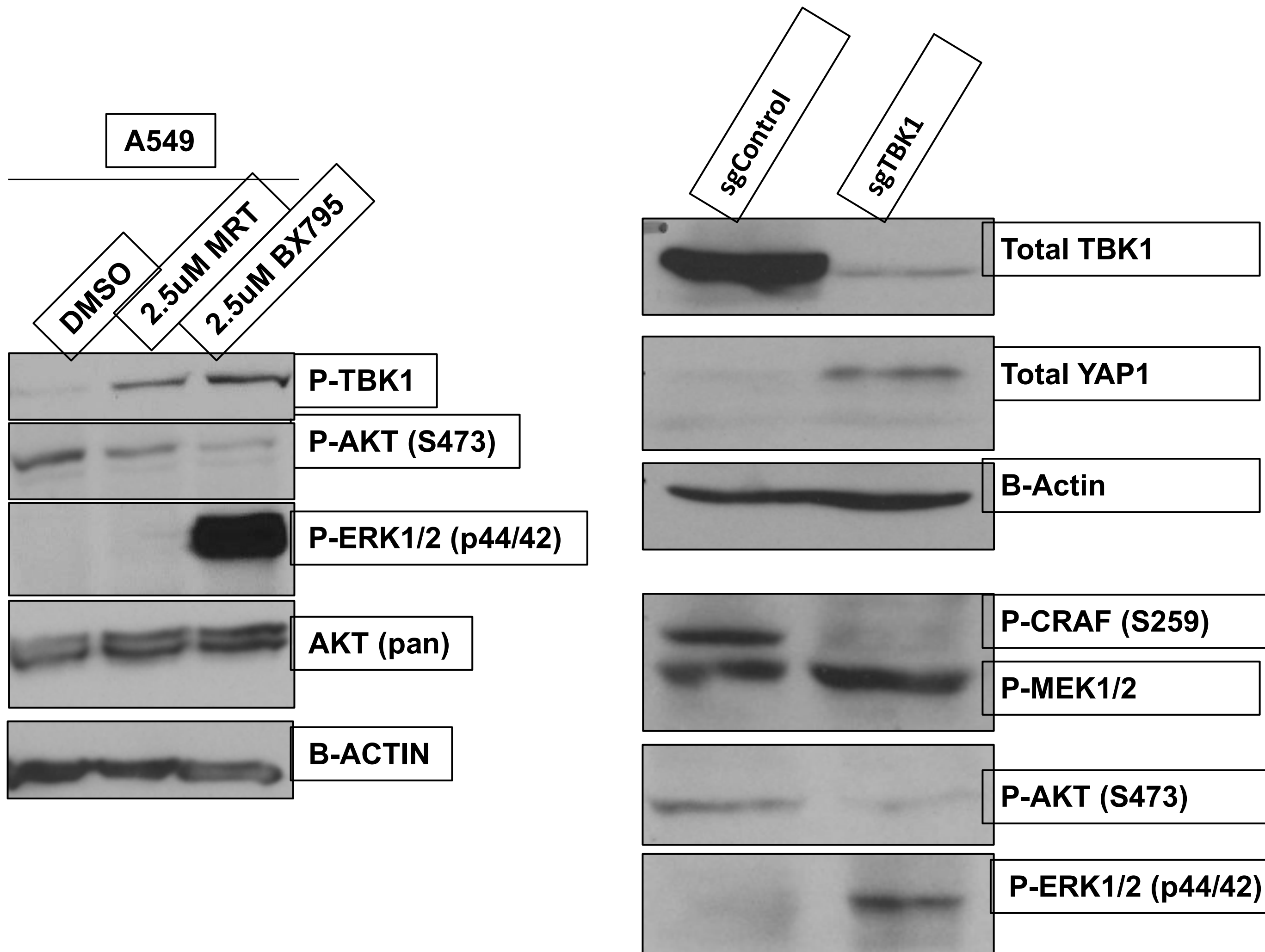
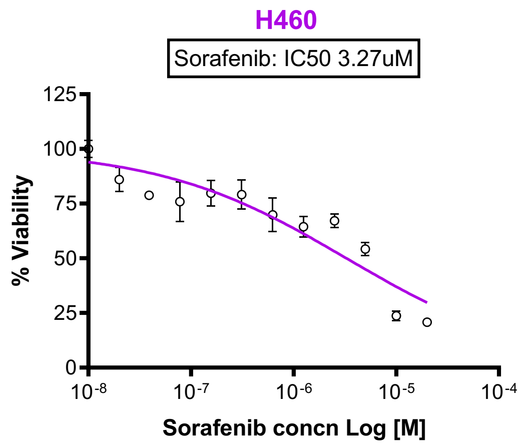
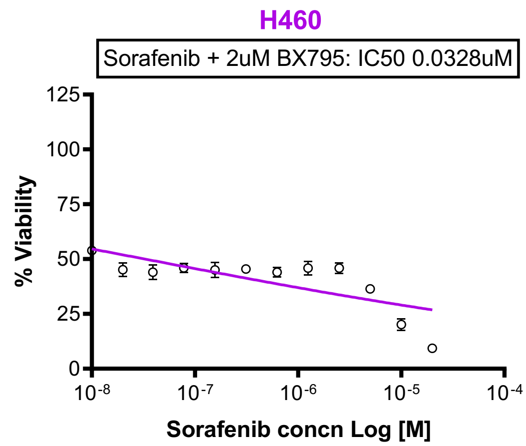
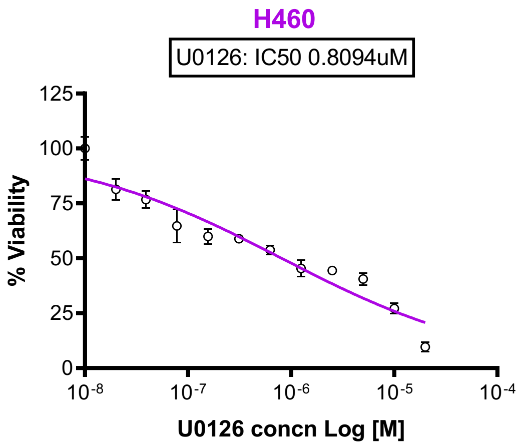



Figure 5. Western blots showing how inhibition or depletion of TBK1 affects downstream signaling cascades. 2.5μM BX795 reduced the levels of pAKT, while significantly inducing pERK activity (left panel). Similar induction of pERK and suppression of Akt activation was observed when TBK1 was depleted in A549 cells (right panel). Induction of YAP1 can also be seen when TBK1 is depleted, as in earlier experiments.

Figure 6. Inclusion of BX795 enhances the efficacy of Raf1 inhibtor, Sorafenib (top panels> Sorafenib had an IC50 of 3.27μM on H460 cells; inclusion of 2μM BX795 reduced the IC50 to 32nM. Similarly, the MEK inhibitor U0126 had an IC50 of 0.8094μM; 2μM of BX795 reduced it to 24nM. These values for the combination treatment are generated by the program and may not be fully accurate, since only about 50% of the cells survived even at the lowest dose of the inhibitors.



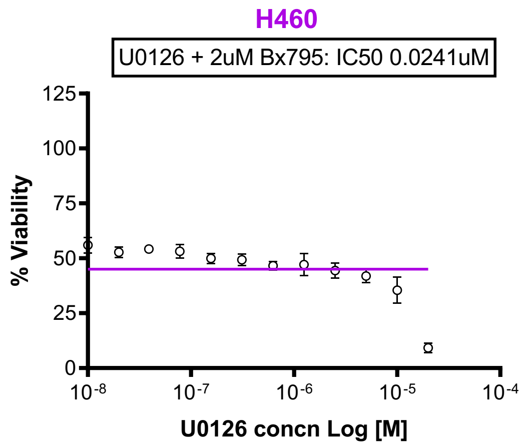
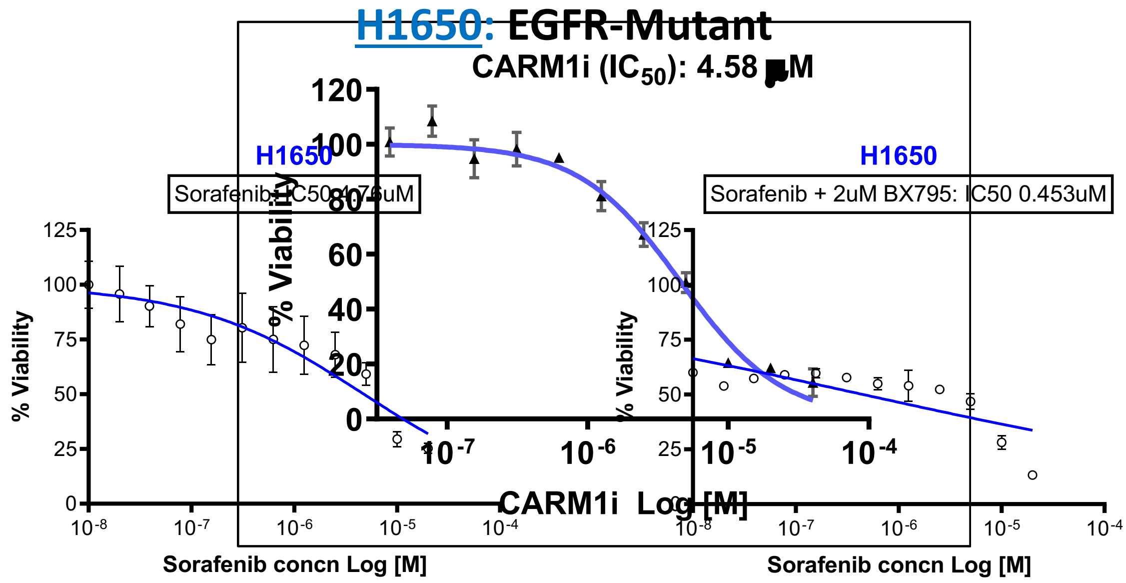




Figure 7. Similar studies as in Figure 6, showing the combination works effectively in EGFR mutant NSCLC cell line, H1650. 2µM BX795 reduced the IC50 of Sorafenib from 4.76µM to 0.453µM, and that ofU0126 from 1.04µM to 0.279µM



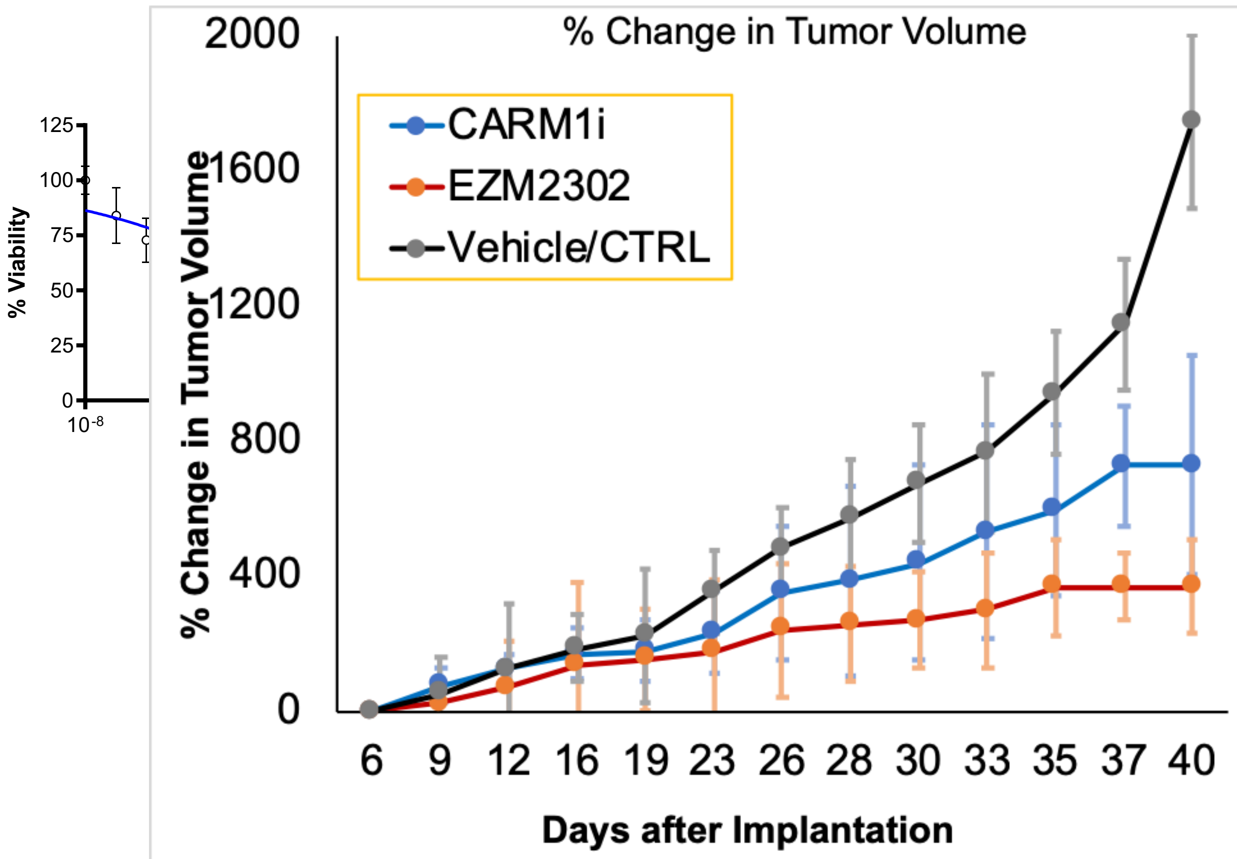




Figure 8. The CARM1 inhibitor, piperidone 1, is effective in reducing the viability of the K-Ras mutant mouse NSCLC cell line, LKR13, with an IC50 of 4.58µM (top panel). Two inhibitors of CARM1, CARM1 inhibitor piperidone 1 and EZM 2032 could suppress the growth of LKR13 cells implanted subcutaneously into 129s mice. The drug treatment was initiated at day 7, at a dose of 50mg/kg; the dose was increased to 75mg/kg on day 28. The mice did not show any significant weight loss (data not shown).

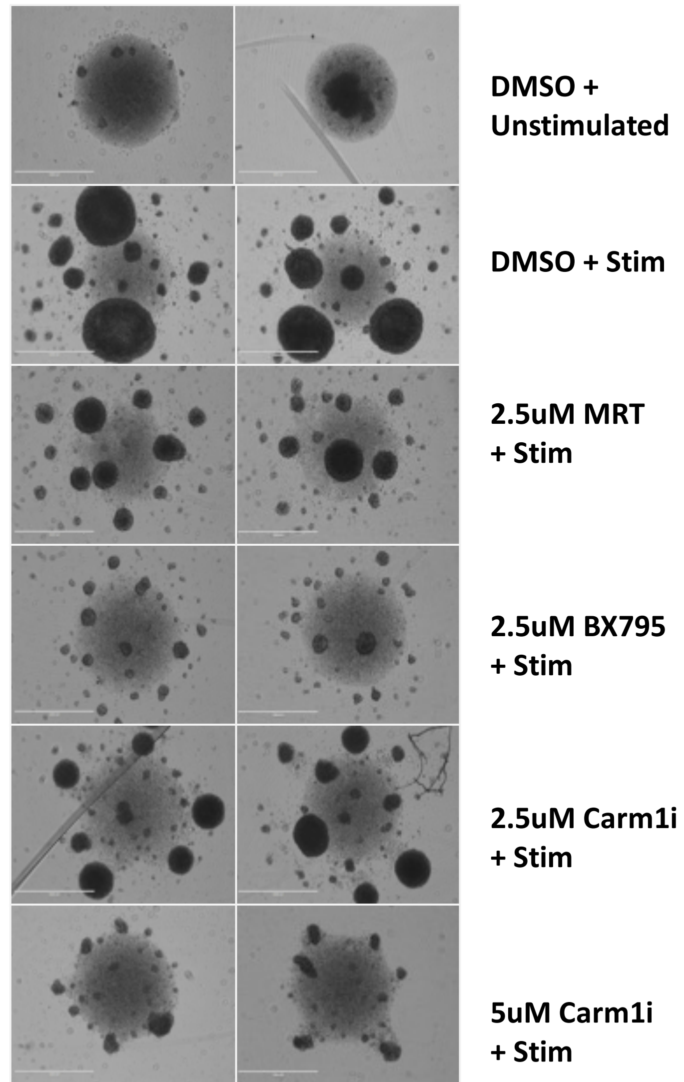



Figure 9. TBK1 inhibitors BX795 and MRT67307 reduce the proliferation of T cells. PBMCs were activated by treatment with CD3 and CD28 antibodies and IL2 for 96 hours, in the presence or absence of BX795 as well as MRT67307; both the inhibitors markedly reduced T cell proliferation. 2.5μM CARM1 inhibitor did not have any notably suppressive effect, but 5μM could suppress proliferation.

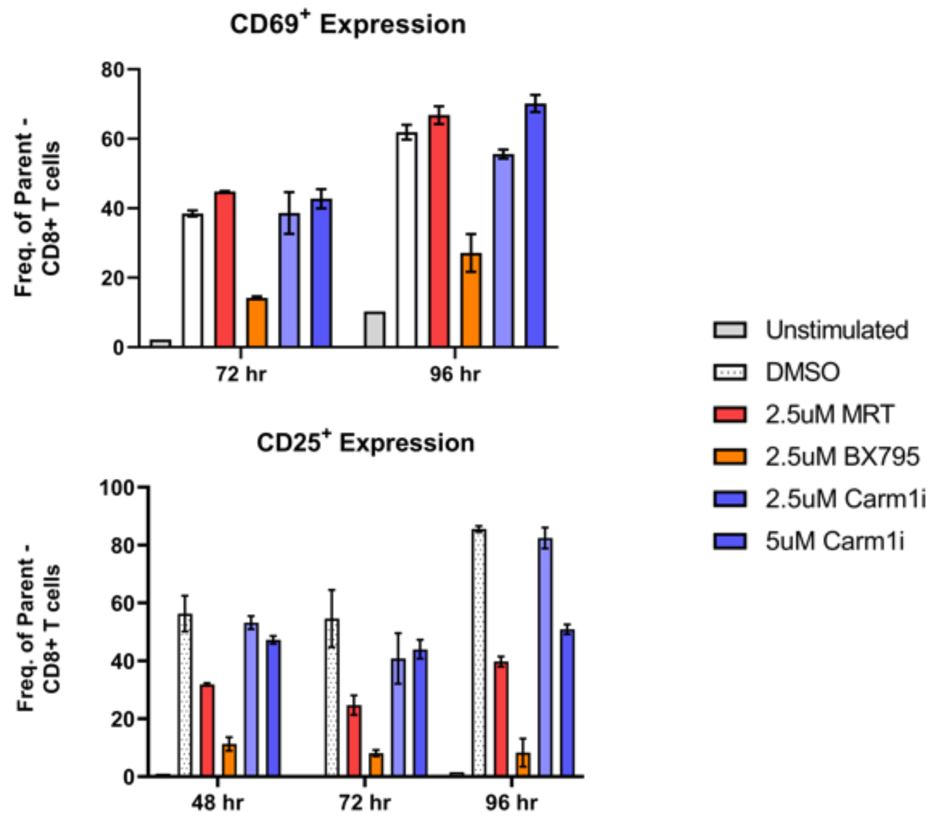
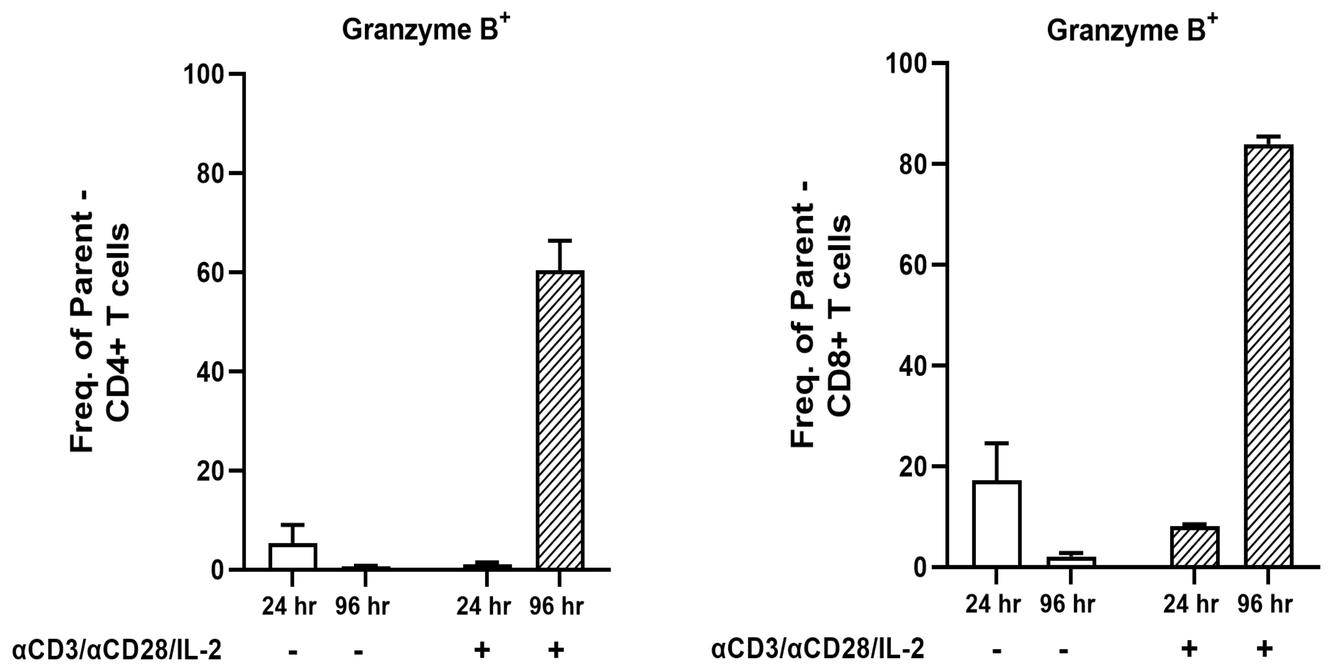



Figure 10. Inhibition of TBK1 can suppress T cell activation by CD3 and CD25 antibodies. FACS analysis showed that 2.5µM BX795 significantly reduced the frequency of CD69+ CD8 T cells, while both BX795 and MRT67307 could reduce the frequency of CD25+ cells.




96 hours of activation results in higher level of Granzyme B Production

Figure 11. Activation of T cells lead to the increase in the frequency of granzyme expressing CD4+ and CD8+ T cells. (FIGURE FOR DEMONSTRATION ONLY: taken from a publication, Smith et al., 2015)

Figure 12. TBK1 inhibitors, but not CARM1 inhibitors, suppress the expression of granzyme B in both CD4+ and CD8+ T cells. T cells were activated in the presence or absence of the inhibitors for 96 hours.

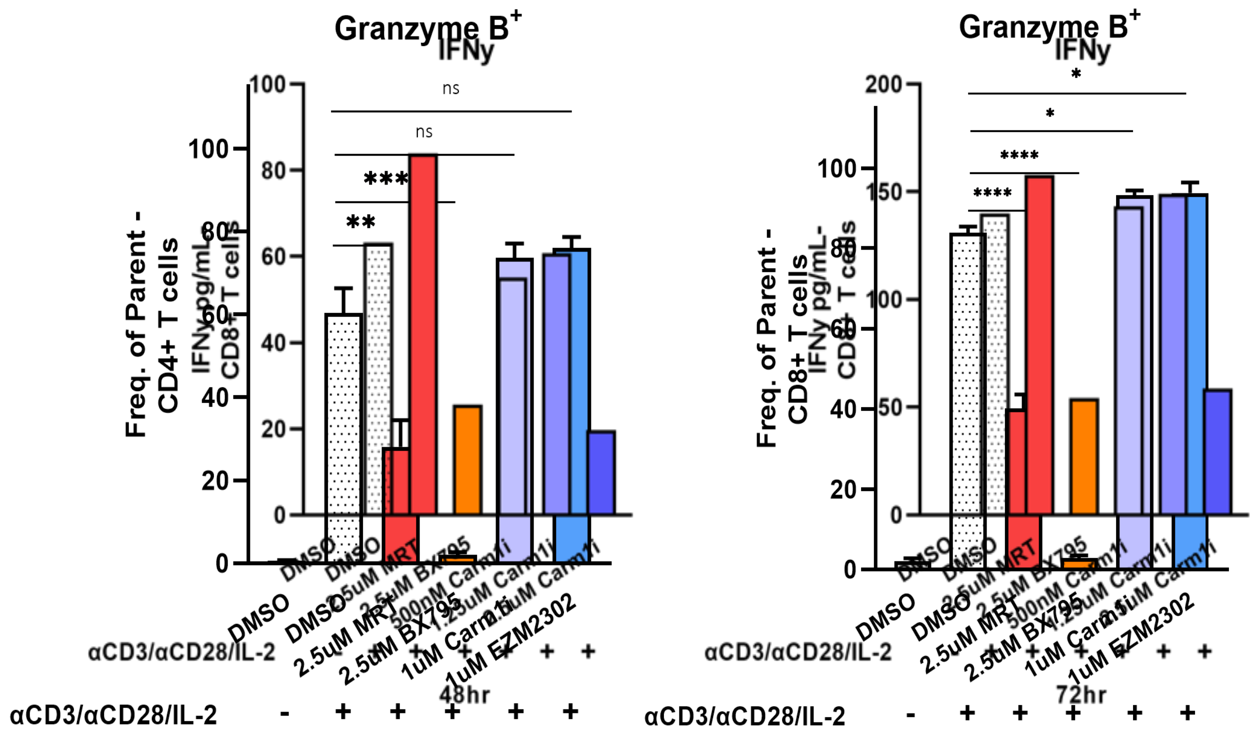



Figure 13. BX795 can suppress the production of IFNγ by CD8+ T cells. T cells were activated as shown, for 48hrs or 72 hrs. 2.5µM of BX795 reduced the frequency of IFNγ expressing T cells. MRT67307 had not observable suppressive effects in this initial experiment; Similarly, high doses of CARM1 inhibitor could suppress IFNγ expression.

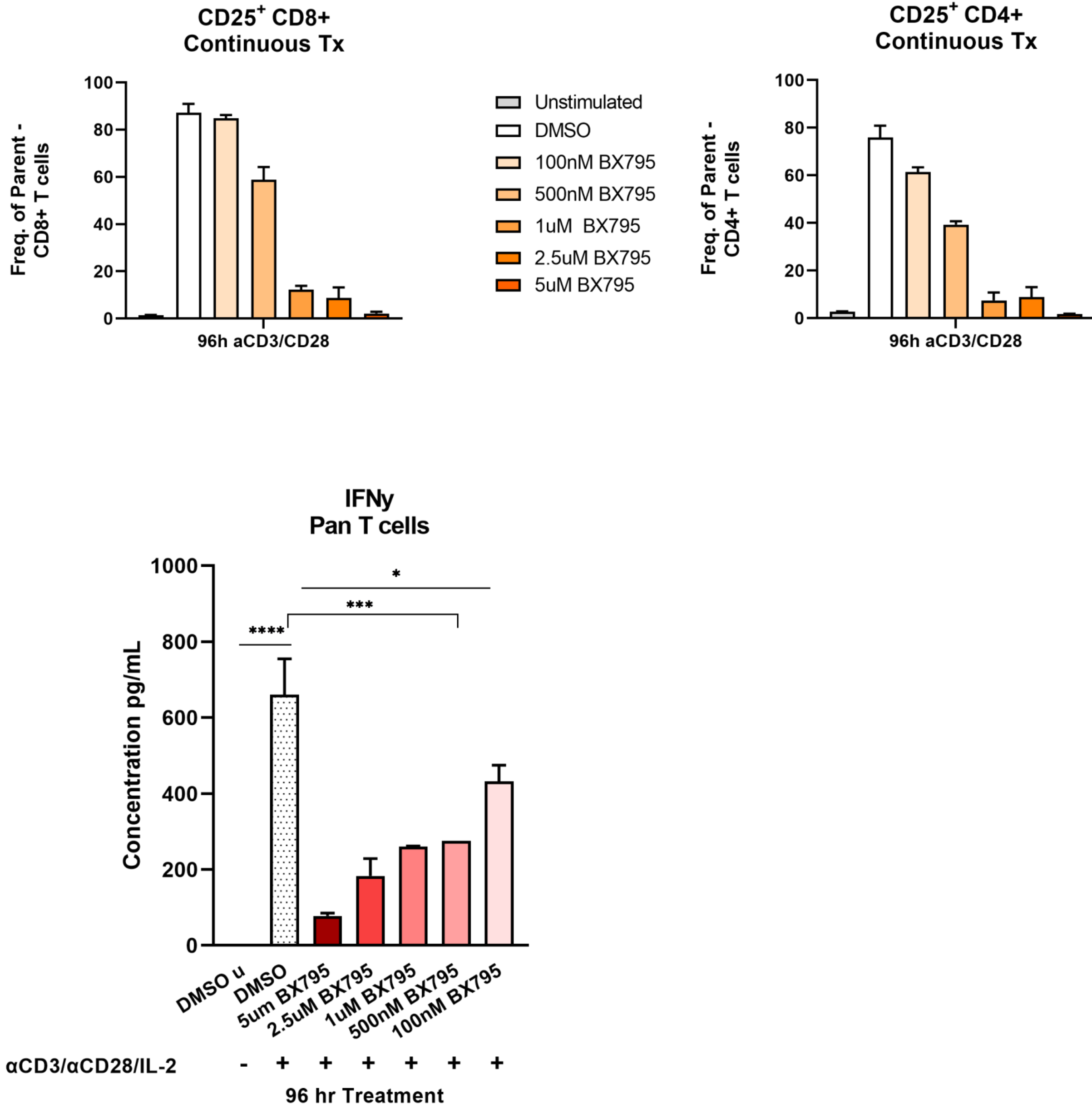
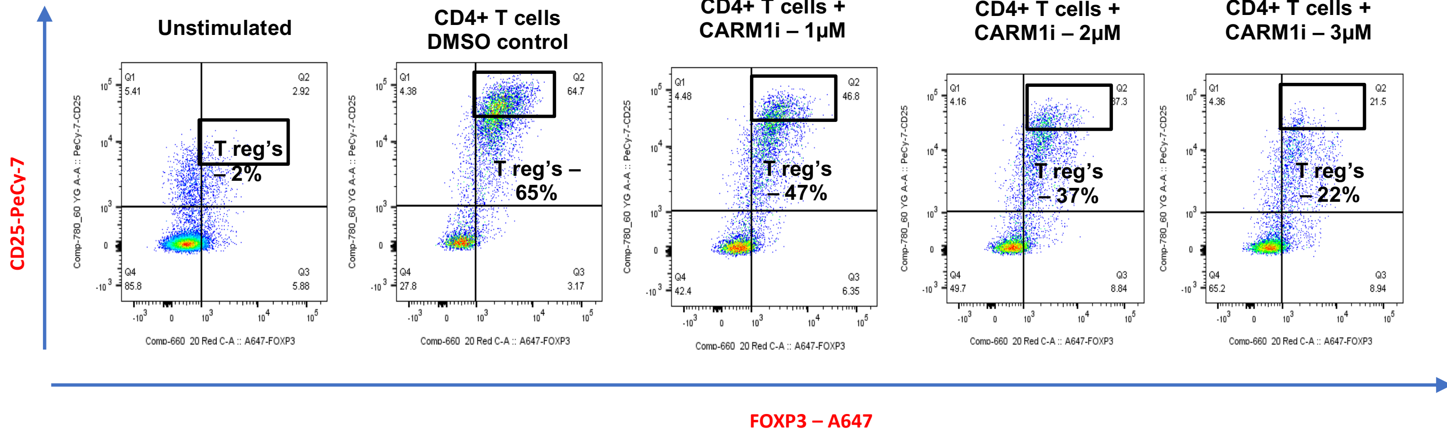
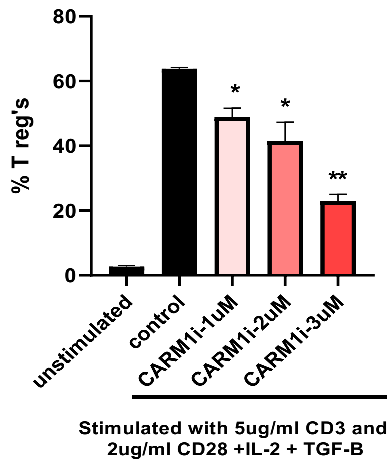



Figure 14. BX795 suppresses T cell activation and IFNγ production in a dose-dependent manner. Frequency of CD4+ and CD8+ T cells expressing the activation marker was reduced in a dose dependent manner. Similarly, the secretion of IFNγ by pan T cells was reduced at doses of BX795 ranging from 500nM to 5μM.



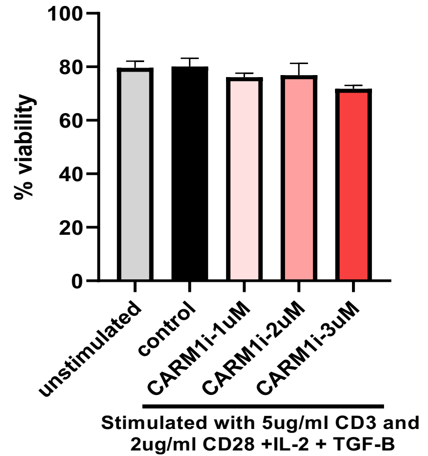




Figure 15. CARM1 inhibitor can prevent the Treg conversion of CD3+ T cells. T reg conversion was induced as shown, and the markers for Tregs are shown on the top of the left panel. CARM1 inhibitor could suppress the Treg conversion, indicating an enhancement of T cell cytotoxic activity. This supports our hypothesis that suppression of YAP1 levels can reduce Treg conversion and inhibiting CARM1 might be a viable strategy to suppress YAP1 and increase T cell function.

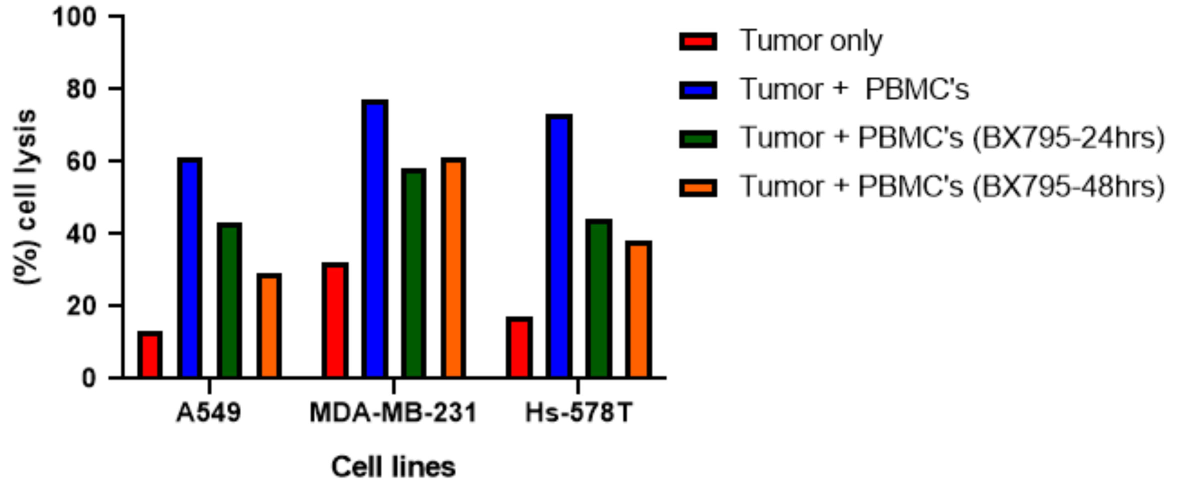



Figure 16. BX795 treatment can reduce the cell kill mediated by PBMCs. We hypothesized that inhibition of TBK1 will elevate YAP1 levels, which is expected to promote the generation of Tregs and MDSCs. Supporting this contention, inhibition of TBK1 reduces the ability of PBMCs to kill tumor cells. This could be one of the mechanisms by which tumors survive the treatment with TBK1 inhibitors.