Supplementary Material for

mTOR Inhibition Specifically Sensitizes Colorectal Cancers with KRAS or BRAF Mutations to BCL-2/BCL-XL Inhibition by Suppressing MCL-1


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Supplementary Figures

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Supplementary Figure Legends

Supplementary Table 1. Human colorectal cancer cell lines and drug sensitivity. 27 human CRCs were genotyped for KRAS, BRAF, PIK3CA, PTEN and p53 status and assayed for drug sensitivity as previously described (1). IC50s were determined as previously described (1) and the cataloguing of this and other data has been recently described (2). All data were acquired as of early 2013.

Supplementary Figure 1. Similar drug profiles between KRAS or BRAF mutant and KRAS/BRAF wild-type colorectal cancers. IC50s for 27 human colorectal cancer cell lines mutant for KRAS or BRAF or wild-type were treated with 5-Fluorouracil, cis-Diammineplatinum(II) dichloride, Etoposide, Vinorelbine, Vinblastine Sulphate, BMS-754807 and 17-AAG were analyzed by a Student’s-t test. All the data were obtained from www.cancerRxgene.org as of early 2013, with the exception of the 5-Fluorouracil data that are only available in Supp. Table 1, as are brief descriptions of each drug’s primary target(s). Please note: P=NS, no significant difference between the two groups for all drugs.

Supplementary Figure 2. MCL-1 is expressed at similar levels in KRAS MT and KRAS WT colorectal cancers, and BRAF MT colorectal cancers downregulate MCL-1 following TORC1/2 inhibition. (A) Boxplot showing MCL-1 RNA expression differences between KRAS MT (n=28) and KRAS/BRAF WT CRC (n=7) Expression data from the Broad-Novartis Cancer Cell Line Encyclopedia (3). A Wilcoxon rank sum test was used to assign a P value for differential expression between the MT and WT cell lines (NS= no significant difference between the two groups). (B) Lysates from the indicated KRAS MT colorectal cell lines and KRAS/BRAF WT cells were subjected to Western blot analyses with the indicated antibodies. (C) KRAS/BRAF WT colorectal CW-2 cells were transfected with 50 nM scrambled (sc) or 50 nM MCL-1 siRNA for 24 hours. An aliquot of transfected cells was used to prepare lysates for Western blot analyses with the indicated antibodies (upper panels) or re-seeded and the next day treated for 48 hours with vehicle (no Rx) or 1 μM ABT-263; percent of apoptotic cells was quantified by FACS (n=3). The amount of apoptosis induced is shown
relative to the no-drug treatment in sc-transfected controls. (D) *BRAF* MT COLO-205 and LS-411N CRC cell lines were treated for 16 hours with no drug (No Rx), 500 nM AZD8055, 1 μM ABT-263, or combination ABT-263/AZD8055, and lysates were subjected to Western blot analyses with the indicated antibodies. The numbers represent the ratio of MCL-1 to GAPDH normalized to no drug (No Rx) control. MCL-1 and GAPDH expression were probed for from the same membrane.

**Supplementary Figure 3.** TORC1/2 inhibition suppresses MCL-1 levels in *KRAS* or *BRAF* MT CRCs.

*KRAS* MT SW620, SW837 and LS-1034 CRC cell lines, and *BRAF* MT COLO-205 and LS-411N CRC cell lines were treated for 16 hours with no drug (No Rx), 500 nM AZD8055, 1 μM ABT-263, or combination ABT-263/AZD8055, and lysates were subjected to Western blot analyses with the indicated antibodies. The lysates were independent of those probed for in Figures 1D and Supplemental Figure 2D. MCL-1 and GAPDH expression were probed for from the same membrane.

**Supplementary Figure 4.** TORC 1/2 inhibition leads to growth arrest in both *KRAS* mutant and wild-type colorectal cancer cells. (A) *KRAS* MT CRC SW620 and SW480 cells and *KRAS/BRAF* WT CRC HT-55 and CW-2 cells were treated for 16 hours with no drug (No Rx), 500 nM AZD8055, 1 μM ABT-263, or combination ABT-263/AZD8055, gently lysed, RNAse treated, and stained with propidium iodide. Cell cycle profiles were obtained by FACS. Error bars are the S.D. * = P <0.05, No Rx versus combination. (B) CRC cell lines (n=27) were treated with increasing concentrations of AZD8055 for 72 hours, and cell viability and IC50s were determined as described(1) (and converted to natural log, x axis). IC50s of *KRAS/BRAF* MT CRCs (n=21) were compared to *KRAS/BRAF* WT CRCs (n=6). Student’s t test was performed to assign a P value between the two groups (NS = not significant).

**Supplementary Figure 5.** MCL-1 RNA is not decreased by TORC1/2 inhibition in *KRAS* mutant or *KRAS* WT colorectal cancer cells. *KRAS* MT CRC lines SW620 and H747 and *KRAS* WT CRC lines CW-2 and HT-55 cells were treated for 16 hours with no drug (No Rx) or 500 nM AZD8055, and the abundance of *MCL-1*
RNA in relation to $\beta$-ACTIN RNA was determined by quantitative PCR. Error bars are the S.D. As a control, abundance of MCL-1 RNA was also determined in MCL-1 siRNA-transfected SW620 cells and sc siRNA-transfected cells (50 nM each) and normalized to $\beta$-ACTIN RNA. (B) KRAS MT SW620 cells and KRAS/BRAF WT CW-2 cells were treated with 500 nM AZD8055 for the indicated times, and lysates were subjected to Western blot analyses with the indicated antibodies. (C) KRAS/BRAF WT CW-2 CRC cells expressing doxycycline-inducible KRAS shRNA vectors were treated with or without 50 ng/mL doxycycline (DOX) for 72 hours, and lysates were subjected to Western blot analyses with the indicated antibodies.

Supplementary Figure 6. Exogenous MCL-1 expression in SW620 cells and analysis of BCL-2 protein complexes prior to and following drug treatments. SW620 cells engineered to overexpress MCL-1 (“High MCL-1” cells from Fig. 2D) were treated for 16 hours with either no drug (No Rx), 500 nM AZD8055, 1 $\mu$M ABT-263, or combination, and lysates were subjected to Western blot analyses. MCL-1 levels were compared to that of GFP control cells treated with the combination (that did not express exogenous MCL-1). (B) KRAS/BRAF WT CW-2 cells underwent immunoprecipitation (IP) with antibodies targeting BIM or IgG (left panel), following six hours of no drug (no Rx), 500 nM AZD8055, 1 $\mu$M ABT-263 or combination therapy (263/8055). Precipitates were analyzed by Western blot analyses with the indicated antibodies. Arrow indicates BCL-XL that migrates slower than the light chain. Whole cell extracts were set aside prior to IP and were subjected to Western blot analyses with the indicated antibodies (right panel).

Supplementary Figure 7. In KRAS mutant non-small cell lung cancers, AZD8055 does not downregulate MCL-1 protein or RNA nor combine with ABT-263 to induce marked apoptosis. (A) The indicated KRAS MT NSCLCs were treated with (+) or without (-) 500 nM AZD8055 for 16 hours and lysates were subjected to Western blot analyses with the indicated antibodies (all from the same membrane). (B) The KRAS MT lung cancer cell lines were treated with no drug, 500 nM AZD8055, 1 $\mu$M ABT-263, or the combination of AZD-8055/ABT-263 for 72 hours, and apoptosis was quantified by FACS. Each bar graph represents the amount of apoptosis treatment induced over no drug. Error bars are the S.D. (n=3). The percent of apoptosis of KRAS MT
SW620 CRC cells undergoing identical treatments and FACS analysis (shown originally in Fig. 2A) is included for comparison. (C) KRAS MT NSCLC H2030 cells were transfected with scrambled (sc) or MCL-1 siRNA and after 24 hours aliquots of cells were lysed and subjected to Western blot analyses with MCL-1 antibody to ensure knockdown (right panel), or cells were re-seeded and the next day treated with the indicated drugs for 48 hours [same doses as (B)], and apoptosis was determined by FACS as in (B). The amount of apoptosis induced is shown relative to the no-treatment sc-transfected controls. (D) KRAS MT NSCLC lines H2030 and H2009 were treated for 16 hours with no drug (No Rx) or 500 nM AZD8055, and the abundance of MCL-1 RNA in relation to β-ACTIN RNA was determined by quantitative PCR. The comparative data from SW620 cells originally presented in Supplemental Figure 5A are included for comparison. Error bars are the S.D. (E) KRAS MT H358 and H2009 NSCLC cell lines expressing doxycycline-inducible KRAS shRNA vectors were treated with (+) or without (-) 50 ng/mL doxycycline (DOX) for 72 hours, and lysates were subjected to Western blot analyses with the indicated antibodies.

Supplementary Figure 8. The stability of MCL-1 protein is similar in KRAS MT CRCs as KRAS MT non-small cell lung cancers. KRAS MT CRC lines (SW620 and SW837) or KRAS MT NSCLC lines (H2009 and H2030) were treated with No Rx (-) or 50ug/ml cycloheximide (CHX) for 1, 2 or 4 h and cells were lysed and subjected to Western blot analyses with the indicated antibodies. The numbers represent the ratio of MCL-1 to GAPDH normalized to no drug (No Rx) control. MCL-1 and GAPDH expression were probed for from the same membrane.

Supplementary Figure 9. KRAS MT SW620 colorectal cancer cells are resistant to other combination approaches, but are sensitive to combined ABT-263/AZD8055 and undergo robust apoptosis following this combination. (A) KRAS MT SW620 colorectal tumors (Fig. 3A) were harvested approximately three hours after final drug treatment, fixed, and sectioned. Sections underwent immunohistochemical (IHC) analysis for cleaved caspase 3 (CC3) staining. Images were taken at 40X magnification and representative images are
shown. (B) KRAS MT SW620 CRC xenografts were treated with either a combination of an IGFR inhibitor, R1507, (12 mg/kg twice per week via i.p. injection) and a MEK inhibitor, AZD6244, (25 mg/kg/qd), an EGFR inhibitor (cetuximab, 40 mg/kg twice per week via i.p. injection) and AZD6244 (25 mg/kg/qd), Cetuximab (40 mg/kg twice per week via i.p. injection) and R1507 (12 mg/kg twice per week via i.p. injection), or a combination of a dual PI3K/mTORC inhibitor (NVP-BEZ235, 40 mg/kg/qd) and AZD6244 (25 mg/kg/qd). The results presented in Fig. 3A from combination AZD8055 (16 mg/kg/qd) and ABT-263 (80 mg/kg/qd) for the first 14 days of treatment are included for comparison.

Supplementary Figure 10. KRAS MT colorectal cancer cells remain sensitive to AZD8055/ABT-263 combination therapy when doses of AZD8055 are decreased. (A) KRAS MT SW620 and SW837 CRC cells were subjected to increasing doses of AZD8055 in the presence of 100 nM ABT-263 and cell viability was assayed 72 h later by Cell-Titer Glo. Viable cell numbers under the red-dotted lines represent a net decrease in cell number. (B) KRAS MT SW620 and SW837 CRC cells were subjected to increasing doses of AZD8055 for 16 h and cells were lysed and subjected to Western blot analyses with the indicated antibodies. (C) KRAS MT SW620 CRC xenografts were treated with either no drug (control), AZD8055 (2mg/kg/qd) or the combination of AZD8055 (2 mg/kg/qd) and ABT-263 (80 mg/kg/qd) and the average tumor measurements of each cohort was plotted. Error bars are S.E.M.

Supplementary Figure Legend

References

