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Ciclo XXVI

Targeted disruption of Aurora and IKK kinases interaction as therapeutic strategy to sensitize Multiple myeloma cells to Apo2L/TRAIL

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2. ABSTRACT

Recombinant human (TNF)-related apoptosis-inducing ligand (Apo2L/TRAIL) ligand and agonistic mAbs have shown remarkable promise as an anticancer agent. However, TRAIL signaling also activates survival signaling that can compromise its efficacy, and TRAIL resistance is now recognized as a common and unfortunate out come. Constitutive activation of the canonical and non-canonical NF-κB pathways is frequent in Multiple Myeloma (MM) and can compromise sensitivity to TRAIL. Aurora A and B are frequently overexpressed in human cancers including multiple myeloma and have been implicated in oncogenic transformation, including development of chromosomal instability and derangement of multiple tumor suppressor and oncoprotein regulated pathways. Because Aurora kinases may play an important function in regulating the activation of NF-κB-directed gene expression and NF-kB plays an important role in MM cell survival, tumorigenesis and drug resistance, the aim of this study was to investigated whether the pharmacological blockade of Aurora kinases sensitizes MM cells to TRAIL.

We demonstrate that targeting Aurora A and B kinases with selective pan-Aurora kinase inhibitors (pan-AKIs) strikingly enhances TRAIL-induced cytotoxicity in TRAIL-sensitive and -resistant MM cell lines, as well as patient-derived MM cells through a caspase-dependent mechanism. Furthermore we demonstrate that Aurora kinases physically and functionally interact with the key regulators of canonical and non-canonical NF-κB pathways IκB Kinase β (IKKβ) and IKKα to activate NF-κB in MM, and the pharmacological blockade of Aurora kinase activity induces TRAIL sensitization because it abrogates

TRAIL-induced activation of NF-κB. We specifically found that TRAIL induces prosurvival signaling by increasing the phosphorylation state of both Aurora and IKK kinases and their physical interactions, and the blockade of Aurora kinase activity by pan-AKIs disrupts TRAIL-induced survival signaling by effectively reducing Aurora-IKK kinase interactions and NF-kB activation. Pan-AKIs consistently blocked TRAIL induction of the antiapoptotic NF-kB target genes A1/Bfl-1, Mcl-1, cellular inhibitor-of apoptosis protein 1 (cIAP1), cIAP2, and/or X-chromosome-linked inhibitor-of-apoptosis protein (XIAP), all important targets for TRAIL sensitization in MM cells. In summary, these results identify a novel interaction between Aurora and IKK kinases and show that these pathways can cooperate to promote TRAIL resistance in MM through NF-κB activation. Finally, combining pan-AKIs with TRAIL in vivo showed dramatic efficacy in a multidrug-resistant human myeloma xenograft model. These findings suggest that combining Aurora kinase inhibitors with TRAIL may have therapeutic benefit in MM.

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4. INTRODUCTION

Multiple myeloma (MM) is a malignancy of plasma cells that accumulate in the bone marrow (BM). Although novel drugs such as bortezomib and thalidomide have extended the overall survival of MM patients they often do not achieve lasting cures, providing an impetus to search for novel therapeutic modalities[1]. TRAIL (also known as Apo2L) is a member of the TNF family of death receptor ligands and has significant potential for use in cancer therapy because of its potent ability to selectively kill cancer cells while sparing normal cells[2]. A variety of preclinical data demonstrated that TRAIL exerts a remarkable antitumor activity both in vitro and in vivo[2,3].

Despite these promising data, TRAIL resistance, both inherent and acquired, is now recognized as a common and unfortunate outcome, and Phase 1/2 clinical trials have demonstrated a good toxicity profile for TRAIL but limited evidence of antitumor activity[4].

Resistance to TRAIL-mediated apoptosis can occur through multiple mechanisms including increased expression of TRAIL decoy receptors, overexpression of intracellular inhibitory proteins, and activation of mitogenic and prosurvial signals[5,6]. Strategies have been developed to bypass TRAIL resistance in diverse preclinical models and are based on the combination of TRAIL and numerous conventional and investigational anticancer agents[2,3,6-11].

The Aurora kinases A, B, and C are highly conserved serine/threonine kinases that play essential and distinct roles in mitosis[12]. Both Aurora A and B are frequently overexpressed in human cancers and have been implicated in oncogenic transformation including development of chromosomal instability

and derangement of multiple tumor suppressor and oncoprotein regulated pathways[12,13]. In this preclinical study we demonstrate, both in vitro and in vivo, a potent and specific antimyeloma activity of the combination of pan-Aurora Kinase inhibitors (pan-AKIs) with TRAIL and elucidate the multiple molecular mechanisms, most of them converging on NF-κB pathway, by which Aurora Kinase inhibition increases the therapeutic effect of TRAIL in MM cells. We propose a novel mechanism linking Aurora and IKK signaling in MM and suggest that the targeted disruption of this interaction can be an effective approach to increase TRAIL sensitivity in MM.

5. MATERIALS AND METHODS

Study approval

Approval for the study was obtained from the Institutional Review Board of the Department of Clinical Sciences, University of Parma. Approval for studies on human tissue samples was obtained from informed consent of all patients and healthy donors in accordance with the Declaration of Helsinki protocol. All procedures involving animal models were performed in accordance with national and international laws and policies. The experimental protocol was approved by the University of Parma Ethics Committee and by the Italian Ministry of Health.

Reagents

Human recombinant KillerTRAIL™ was purchased from Alexis, San Diego, CA. Pan-Aurora kinases inhibitor MK-0457 was provided by Merck & Co. (Rahway, NJ). Pan-Aurora kinases inhibitor PHA-680632 was kindly provided by dr. Jürgen Moll from Nerviano Medical Sciences, Nerviano, Italy. For in vitro experiments pan-AKIs were dissolved in dimethyl sulfoxide (DMSO) (100 mM stock solution) and stored at -20°C until use. Recombinant human interleukin 6 (rhIL-6) and Insulin-like growth factor 1 (IGF-1) were obtained from Endogen (Woburn, MA). Dexamethasone (DEX) from Sigma-Aldrich (St Louis, MO) was dissolved in absolute ethanol and used at the final concentration of 2μM. The peptide control Cbz-Phe-Ala-fluoromethyl ketone (Z-FA-FMK), the selective caspase-9 inhibitor Cbz-Leu-Glu(Ome)-His-Asp(Ome)-fluoromethyl ketone (Z-LEHD-FMK), the selective caspase-8 inhibitor Cbz-Ile-Glu(Ome)-Thr-Asp(Ome)-fluoromethyl ketone (Z-IETD-FMK) and the pancaspase inhibitor Cbz-Val-Ala-Asp(Ome)-fluoromethyl ketone (Z-

VAD-FMK) were from Alexis (San Diego, CA, USA). All caspase inhibitors were dissolved in dimethyl sulfoxide (DMSO), stocked in aliquots at -20°C, and used at the final concentration of 30μM. The IκB kinase (IKKβ) inhibitor PS-1145 was purchased from Sigma-Aldrich (St. Louis, MO) and used at the final concentration of 5μM. The NF-κB inhibitory peptide SN50 and its mutant control peptide SN50M was obtained from Enzo Life Sciences (Plymouth Meeting, PA) and were used at the final concentration of 10μM.

Cell cultures

Human Myeloma cell lines (HMCLs) RPMI 8226, OPM-2, U266 and JJN3 were purchased from DSM (Braunschweig, Germany). The multi drug resistant RPMI 8226/R5 cell line was established by continuous exposure of 8226 parental cells to increasing concentrations of R115777 for over 6 months[14]. The purification of myeloma cells was performed using a MACS separator (Miltenyi Biotec, Germany) with magnetic microbeads coupled to CD138 monoclonal antibody according to the manufacturer's protocol. Only cell populations with purity greater than 95% monitored by flow cytometry analysis with anti-CD138-PC5 antibodies were used for experiments. Peripheral blood mononuclear cells (PBMCs) from healthy subjects and primary bone marrow stromal cells (BMSCs) from MM patients were obtained by Ficoll-Hypaque density sedimentation. BMSCs were obtained after 2-4 weeks of culture of adherent cells in α-MEM medium supplemented with 15% FCS and 2mM glutamine. Both HMCLs ($2.0x10^5$ cells/mL) and fresh purified MM ($2.5x10^5$ cells/mL) cells were seeded in fresh RPMI 1640 medium (GIBCO-BRL, Grand Island, NY), supplemented with 10% fetal calf serum,

2mM L-glutamine, penicillin G (100 U/mL), streptomycin (100mg/mL) (GIBCO-BRL) and pretreated for 3 hours with Pan-AKIs or vehicle and then incubated with TRAIL at the range of concentration described in the text for further 24-48 hours. Similarly HMCLs were incubated in presence of IL-6 (20ng/mL) or IGF-1 (50ng/mL) and treated with MK-0457 or PHA-680632 and/or TRAIL with the same experimental design.

To determine Pan-AKIs/TRAIL Combination index (CI), HMCLs were treated sequentially with escalating doses of MK-0457 (15-2000 ng/mL; 0.032-4.3 μM) or PHA-0680632 (15-2000 ng/mL; 0.03-4 μM) for 3 hours, and subsequently with TRAIL (0.5-50 ng/mL) alone or in combination with pan-AKIs at a fixed ratio of 25:1 for MK-0457:TRAIL and 50:1 for PHA-680632:TRAIL (in combination with pan-AKIs TRAIL was used at 0.6, 1.2, 2.4, 4.8, 9.6, 19.2 ng/mL). After 24 hours, apoptosis was measured by annexin-V binding. CI plots were then generated using Chou-Talalay method. CI values lesser than 1.0 indicates synergism; CI value equal 1.0 indicates additive effect; CI more than 1.0 indicates antagonistic effect.

Co-culture system between MM cells and adherent BMSCs (ratio 5:1) was performed in RPMI-1640 medium at 10% FCS in the presence or absence of Pan-AKIs and/or TRAIL for 24-48 hours. After the treatments the cells were detached with EDTA 0.05% (Sigma-Aldrich), stained with anti-CD38/CD138-PC5 (Beckman Coulter, Miami, F, USA) and the apoptotic MM cells were determined by flow cytometry as CD38/CD138$^+$ annexin-V$^+$ cells.

Apoptosis assays, siRNA transfections, molecular and statistical analysis

Cytofluorimetric analysis was performed to evaluate the percentage of apoptotic cells by Sub-G1 DNA content, Mitochondrial Transmembrane Potential ($\Delta\Psi m$) and Annexin V-FITC/PI or Annexin V-PE/7-AAD staining was performed by Flow cytometry assay to evaluate cell cycle[15].

ELISA

The DNA-binding activity of NF-κB subunits was quantified by the TransAm NFκB family ELISA kit according to the manufacturer's protocols (Active Motif, Rixensart, Belgium).

siRNA transfections

Prior to electroporation, cells were washed twice with serum-free Opti-MEM (Gibco BRL Paisley, United Kingdom) and resuspended to a final concentration of $24x10^6$ cells/mL in Opti-MEM (Gibco BRL). Subsequently, 0.4 mL of cells suspension was mixed either with 1 nmol of a smart pool small interfering double-stranded RNAs (siRNA) against non-specific control (001206), or Aurora A (003545), or Aurora B (003326), or Mcl-1 (004501), or IκB-α (4765) (all obtained from Dharmacon Tech, Lafayette, Co), or 10 μg of siRNA against IKKα or IKKβ or A1/Bfl-1 or cIAP1 or cIAP2 or XIAP or Bcl-xL or Bcl-2 (from Sigma-Aldrich, St. Louis, MO) and electroporated in a 0.4-cm cuvette using the Gene Pulser electroporation apparatus (Bio-Rad Laboratories Inc., Herculex, California, USA) using a single-pulse protocol (voltage 250 V and Capacitance 1050 μF). HMCLs were harvested for

Immunoblotting to monitor knockdown protein levels by siRNA forty-eight hours after siRNA transfection.

Plasmids and transfections

Lentivirus infection of MM cells: pLenti-CMV-RFP-2A-Puro-Blank (control), pLenti-GIII-CMV-hMCL1-RFP-2A-Puro, pLenti-GIII-CMV-hBCL2A1-RFP-2A-Puro Lentiviral Vectors and Lentiviral Packaging Mix were from Applied Biological Materials Inc. (Vancouver, BC, Canada). Lentiviral particles were produced by cotransfection of each lentiviral vector with the Lentiviral Packaging Mix into 293T cell line according to the manufacturer's protocols. RPMI 8226/R5 cells were infected by the lentiviral particles obtained and the transfection efficiency was assessed by flow cytometry. Briefly, RPMI 8226/R5 cells were infected for 18 hours at 37°C. After infection, the medium containing lentivirtal particles was replaced with fresh medium; infected cells were expanded in complete medium without drug selection for 3 days after which the medium was replaced with selection medium containing 0.6 μg/mL puromycin. After cell growth was stable, the cells were used in the experiments described.

Electroporation of plasmids: RPMI 8226/R5 and JJN3 cells were electroporated with 10 μg of pLenti-CMV-GFP-2A-Puro-Blank Lentiviral Control Vector or with pLenti-GIII-CMV-hBCL2-GFP-2A-Puro Lentiviral Vector or pLenti-III-CMV-hBCL2L1-GFP-2A-Puro Lentiviral Vector or pLenti-GIII-CMV-hMCL1-GFP-2A-Puro Lentiviral Vector (Applied Biological Materials Inc.). All lentviral expression vectors co-expressed GFP to monitor the

transfection by flow cytometry. After electroporation cells were expanded in complete medium for 3 days after which the medium was replaced with selection medium containing 0.6 µg/mL puromycin for selection of stable transfected pools. Up to 95% of GFP-positive cells were obtained. HMCLs stably transfected were continuously cultured under puromycin selection. After cell growth was stable, the cells were used in the experiments described.

Western blot and Antibodies

Proteins from whole cell lysates and nuclear fractions were quantified by Bradford assay and separated by SDS-PAGE and analyzed by Western blotting with the indicated antibodies below.The interaction between Aurora A or Aurora B and IKKα or IKKβ was evaluated by coimmunoprecipitation analysis by using ExactaCruz kits (Santa Cruz Biotechnology, Santa Cruz, CA) as per manufacturer's instructions. For immunoprecipitation, the following antibodies were used: mouse monoclonal IKKα (B-8), mouse monoclonal IKKβ (H-4), mouse monoclonal ARK-1 (35C1) and mouse monoclonal ARK-2 (13E8A7) all provided by Santa Cruz Biotechnology. For immunoblotting, the following antibodies were used: rabbit monoclonal anti-Aurora A/AIK, rabbit polyclonal anti-Aurora B/AIM1, rabbit monoclonal anti-phospho Aurora A (Thr288)/ Aurora B (Thr232)/ Aurora C (Thr198), rabbit polyclonal anti-PARP, rabbit polyclonal anti-Cleaved PARP, rabbit polyclonal anti-Bid, rabbit polyclonal anti-Cleaved Caspase-8, rabbit polyclonal anti-Cleaved Caspase-9, rabbit polyclonal anti-Cleaved Caspase-3, rabbit monoclonal anti-NF-κB p65, rabbit polyclonal anti-NF-κB1 p105/p50, rabbit polyclonal anti-NF-κB2 p100/p52, rabbit polyclonal anti-c-Rel, rabbit polyclonal anti-RelB (C1E4),

mouse monoclonal anti-Histone H2B (53H3), rabbit polyclonal anti-IKKα, rabbit polyclonal anti-IKKβ (L570), rabbit monoclonal anti-pospho-IKKα/β (ser176/180) (16A6), mouse monoclonal anti-IκB-α (L35A5), mouse monoclonal anti-IκB-α (112B2), rabbit polyclonal anti-p-IκB-α (ser32), rabbit polyclonal phospho-NF-κB2 p100 (Ser866/870), rabbit polyclonal anti-Mcl-1, rabbit polyclonal anti-A1/Bfl-1, rabbit polyclonal anti-Bcl-xL, rabbit polyclonal anti-cIAP1, rabbit monoclonal anti-cIAP2 (58C7), rabbit polyclonal anti-XIAP all provided by Cell Signaling Technology; mouse monoclonal anti-Lamin A/C (JoL3), mouse monoclonal anti-β Tubulin (D-10) and goat polyclonal anti-human actin all provided by Santa Cruz Biotechnology; mouse monoclonal anti-Bcl-2 clone 124 (Upstate Biotechnology, Lake Placid, NY); goat anti-mouse IgG (H+L)-HRP conjugated (Bio-Rad); goat anti-rabbit IgG (H+L)-HRP conjugated (Bio-Rad); donkey anti-goat IgG (H+L)-HRP conjugated (Santa Cruz Biotechnology).

Animal studies, Histology and Immunohistochemistry

Five-week-old nonobese diabetic (NOD) severe combined immunodeficiency (SCID) NOD.CB17-Prkdcscid/J (NOD-SCID) mice were obtained from the JACKSON LABORATORY (Bar Harbour, ME) and maintained under the same specific pathogen-free conditions. NOD-SCID mice were subcutaneously inoculated in the left flank with $1.0x10^7$ RPMI 8226/R5 cells in 200μL of PBS. NOD-SCID mice (n=5) injected with PBS alone were used as negative controls. When tumors reached volumes of 250 mm^3, approximately 5-6 weeks after MM cell injection, mice were randomly assigned (10/group) to receive vehicle or drugs at various doses. Mice were treated with daily doses

of pan-AKIs for 11 days and two doses of TRAIL on the third and sixth day.

Mice were evaluated for weight loss, tumor mass and overall appearance every 2 days. Tumor size was in 2 dimensions using calipers, and tumor volume was calculated using the following formula: Volume = 0.5 x a x b^2, where “a” and “b” are the long and short diameter of the tumor, respectively.

At different time point of drug treatment or when tumors reached 2 cm^3, animals were killed by CO_2 euthanasia. Tumors were explanted 6-8 hours after the last drug administration. Immediately after euthanasia necropsies were performed and subcutaneous neoplasia (xenotransplantation) were collected, photographed and measured. Splanchnic organs from thoracic, peritoneal and cranial cavities as well as the right femur were also collected.

The tumour masses were submitted in part to Western blotting analysis, in part to morphological and immunohistochemical studies. Histomorphological investigations were performed on organs of splanchnic cavities as well as bone marrow collected from femur. To prepare lysates, tumor tissue was homogenized in lysis buffer and then processed as described above. For immunohistochemical studies, xenograft tumors were fixed overnight in paraformaldahyde followed by dehydration in graded ethanols.

Both Pan-AKIs were formulated in 30% PEG300, 70% 50 mM sodium phosphate buffer pH3.3 (Sigma, St Louis, MO) and 50 mg/Kg of the two inhibitors were administered by intraperitoneal injection. TRAIL was prepared in PBS and administered at 300 μg/mouse by intraperitoneal injection. In Pan-AKIs plus TRAIL-treated mice, MK-0457 or PHA-680632 were administered 6 hours before TRAIL. Controls received a daily intraperitoneal injection of 30% PEG300, 70% 50 mM sodium phosphate buffer vehicle and/or PBS.

Necropsies were performed on all subjetcs and specimens of neoplasia, splanchnic organs and femur bone marrow were collected and formalin-fixed. 5 µm thick sections were obtained from samples and hematoxylin and eosin (H&E) stained to evaluate the morphology of neoplastic cells. The expression of Caspase-3 was determined with immunohistochemical staining using the Kit of Cell Signaling Technology (Signal Stain Cleaved Caspase-3 IHC # 8120) according to manufacturer's protocols. Immunohistochemical staining of phospho-Histone H3 was performed using rabbit polyclonal Phospho-Histone H3 (ser10) #9701 from Cell Signaling Technology.

Statistical analysis

The Chou-Talalay method and Calcusyn software were used to assess synergistic or additive or antagonist effects of combined therapies. The median effective dose (ED_{50}, dose producing 50% of cytotoxicity) was estimated using Median Effect Equation of Chou. For multiple comparisons a statistical analysis was performed using analysis of variance for repeated measurements followed by a Tukey-Kramer or Dunnet post-tests using JMP version 7.0 statistical software (SAS Institute, Cary, NC). Survival curves were derived by the Kaplan-Meier method and compared using the log-rank test, and *P* values for the log rank statistic were adjusted for multiple testing by the Bonferroni method (6 groups, 15 pairwise comparisons) using JMP software.

6. RESULTS

6.1 Blockade of Aurora kinase activity enhances the cytotoxic effect of TRAIL on MM cells

We first analyzed the pharmacologic interactions between pan-AKIs (MK-0457 or PHA-680632) and TRAIL using a fixed-ratio experimental design on HMCLs displaying different degrees of sensitivity to TRAIL (Table 1). We found that the combined treatment of pan-AKIs plus TRAIL resulted in the synergistic induction of apoptosis in TRAIL-sensitive MM cells (Figure 1Ai); moreover, pretreatment with pan-AKIs significantly enhanced TRAIL-induced cell death in highly TRAIL-resistant JJN3 cells (Figure 1Aii); apoptotic cell death of the drug-treated HMCLs was evaluated by either sub-G1 or Annexin V/PI analysis (Figure 1B representative data).

Neither IL-6 (20 ng/mL) nor IGF-1 (50 ng/mL) blocked pan-AKIs/TRAIL-triggered apoptosis in HMCLs (Figure 1C). Furthermore, exogenous IL-6 conferred resistance to TRAIL-mediated apoptosis in RPMI 8226 and OPM-2, but pretreatment with pan-AKIs was able to restore TRAIL sensitivity in both HMCLs (Figure 1C).

Furthermore, consistent with previous studies we found that adherence of MM cells to bone marrow stromal cells (BMSCs) significantly increased their resistance to TRAIL-induced cell death as compared to exogenous IL-6 [16,17](Figure 1C); however, pan-AKIs were able to completely or almost completely reverse the BMSCs-mediated TRAIL resistance in RPMI 8226/R5 and OPM-2 (Figure 1C); whereas in RPMI 8226 and U266 HMCLs that previously has been reported to strongly increase the caspase-8 inhibitor FLIP when cocultured with BMSCs[16], pan-AKIs partially (but significantly) overcame

stroma-mediated TRAIL resistance when TRAIL was utilized at 4-fold higher concentrations (9.6 or 19.2 ng/mL, respectively) than those utilized in absence of stroma (Figure 1C). Similarly to HMCLs, we found that the treatment with pan-AKIs significantly enhanced apoptosis of fresh purified MM cells induced by TRAIL ($P < .05$ $n = 5$) (Figure 1Di and 1Dii). The characteristics of these patients are summarized in Table 2.

In contrast, no significant cytotoxicity in PBMCs from 3 healthy volunteers was observed after pan-AKIs/TRAIL treatment (Figure 1Diii).

Finally, the functional knock-out of Aurora-A and -B gene expression by small interfering (si)RNAs recapitulated the ability of pan-AKIs to sensitize HMCLs to TRAIL (Figure 1E and representative data Figure 1F), thereby confirming the significant role of these kinases in mediating TRAIL sensitization.

Taken together these data indicate that the combination of pan-AKIs/TRAIL triggers significant antitumor activity even against MM cells in the BM milieu.

6.2 Combined exposure to pan-AKIs and TRAIL induces caspase dependent MM cell apoptosis

To determine whether pan-AKIs/TRAIL-induced cytotoxicity is mediated via activation of caspase and PARP degradation, HMCLs were treated with pan-AKIs and/or TRAIL for 24 h and caspase activation and PARP fragmentation were analyzed by Western blotting. Treatment of pan-AKIs strongly potentiated the TRAIL-induced cleavage/activation of caspase-8, -9, and -3 and PARP fragmentation in all HMCLs (Figure 2A). Consistent with these finding we found that the combined treatment induced a robust cleavage/activation of Bid, a substrate of active caspase-8, which is a key

protein involved in the cross-talk between the intrinsic and extrinsic apoptotic pathways in all tested HMCLs (Figure 2A).

To further investigate which caspases were responsible for pan-AKIs/TRAIL-induced apoptosis we used a peptide inhibitor approach: HMCLs were cultured with pan-AKIs and/or TRAIL in the presence of caspase-8 (Z-IETD-FMK), caspase-9 (Z-LEHD-FMK), or pancaspase (Z-VAD-FMK) inhibitors. Either Z-IETD-FMK or Z-LEHD-FMK inhibitors significantly reduced pan-AKIs/TRAIL-induced cytotoxicity in all tested HMCLs except for OPM-2 HMCLs in which only Z-IETD-FMK but not Z-LEHD-FMK significantly affected pan-AKIs/TRAIL-induced cell death. Moreover, the pancaspase inhibitor Z-VAD-FMK protected MM cells from pan-AKIs and/or TRAIL-induced apoptosis, confirming that caspase activity was indispensable for the two drugs-induced cell death (Figure 2B).

6.3 Pan-AKIs disable TRAIL-directed survival pathways by targeting NF-κB

RPMI 8226/R5 cells are insensitive to a diverse group of clinically relevant antitumor agents, including Bortezomib, Melphalan, Doxorubicin, Etoposide, and their multidrug-resistant phenotype, and resistance does not correlate with K-Ras prenylation, farnesyl transferase activity, Ras mutation status, increased expression of P-glycoprotein, or elevated expression of heat shock proteins[14]. Therefore, we focused our molecular analyses on RPMI 8226/R5 and its parental RPMI 8226 with the aim to identify resistance mechanisms that are clinically relevant and the potential targets of the combination pan-AKIs/TRAIL.

Because nuclear factor-κB (NF-κB) plays an important role in MM cell survival, tumorigenesis, and drug resistance[17], and Aurora-A may play an important function in regulating the activation of NF-κB-directed gene expression[18], we investigated whether the combination of pan-Aurora kinase inhibitors/TRAIL could induce apoptosis by interfering with NF-κB pathway in HMCLs with different NF-κB profile[19,20] (Table 1).

In agreement with previous studies demonstrating that TRAIL induces prosurvival signal by activating NF-κB[5,6], we found that TRAIL increased nuclear localization and DNA binding activities of p65, p50, p52, and RelB (Figure 3Ai and ii), suggesting that both canonical and non-canonical NF-κB pathways were activated by TRAIL in MM cells. Furthermore, the blockade of Aurora kinase activity reduced the basal and/or TRAIL-mediated activation of both canonical and non-canonical NF-κB in all tested HMCLs (Figure 3Ai and Aii).

Because in NF-κB signaling cascades, IκB Kinase α (IKKα) and IKKβ are key molecules that predominantly mediate non-canonical and canonical pathways, respectively, we examined whether pan-AKIs and/or TRAIL-treatment could affect their expression levels and/or phosphorylation/activation status.

We found that both the pan-AKIs partially, but significantly ($P < .05$), reduced the basal amounts of active/phosphorylated IKKα/β in all HMCLs except for OPM2, which showed low basal IKKα/β activity (Figure 3B and C) and low NF-κB index[19,20] (Table1); TRAIL strongly increased the phosphorylation/activation of IKKα/β in RPMI 8226 and OPM-2, whereas in RPMI 8226/R5, U266 and JJN3 HMCLs, which have elevated constitutive IKKs activity, this increment was less evident (Figure 3B and C). Moreover,

pretreatment with pan-AKIs significantly reduced the amounts of active/phosphorylated IKKα/β of TRAIL-treated HMCLs (Figure 3B and C); notably, the reduced phosphorylation of IKKα/β in pan-AKIs/TRAIL-treated MM cells correlated with their decreased protein expression in all HMCLs except for OPM-2 (Figure 3B); Consistent with these data we found that basal and/or TRAIL-induced phosphorylation of NF-κB2/p100 and IκB-α, the two downstream direct targets of IKKα and IKKβ that are involved in the activation of non-canonical and canonical pathways respectively, were diminished in HMCLs treated with pan-AKIs (Figure 3B), indicating that pan-AKIs act through the inhibition of TRAIL-induced NF-κB activation in MM cells; accordingly, chemical and/or genetic disruption of IKKα/β functions, or pretreatment with NF-κB inhibitor SN50, significantly enhanced TRAIL sensitivity of MM cells (Figure 3D and 3E).

Because pan-AKIs promote IκB-α accumulation by reducing its phosphorylation and its subsequent degradation (Figure 3Fi and 3Fii)[18], we examined whether its silencing affects the MM cells' responses to pan-AKIs and/or TRAIL. Consistently, siRNA knockdown of endogenous NF-κB inhibitor IκB-α disabled pan-AKIs-triggered IκB-α accumulation/ p65/NF-κB inhibition (Figure 3FII and 3Fiii), and protected MM cells from pan-AKIs/TRAIL-induced apoptosis (Figure 3G), therefore arguing that inhibition of NF-κB activity by pan-AKIs contributes functionally to TRAIL sensitization of MM cells.

These data suggest that NF-κB is an important constituent of cell survival pathways for MM and confers protection against TRAIL-induced apoptosis, and agents inhibiting NF-κB are potent sensitizers of TRAIL in MM[21].

To determine which Aurora was required for IKKα and IKKβ phosphorylation/activation, and to rule out the possibility that the pan-AKIs effects was because of the inhibition of other kinases, we repressed Aurora A or B by siRNA.

Notably, the RPMI 8226 knockdown of Aurora A increased the protein level of Aurora B and the amounts of phosphorylated IKKα/β, IκB-α, and NF-κB2, whereas knockdown of Aurora B decreased the phosphorylation of IKKα/β, IκB-α, and NF-κB2 (Figure 3H); in RPMI 8226/R5 we found that the repression of either Aurora A or Aurora B resulted in decreased phosphorylation of IKKα/β, IκB-α, and NF-κB2 (Figure 3H).

Taken together these knockdown experiments indicate that both Aurora A and Aurora B may be involved in the phosphorylation of IKKα and IKKβ in MM cells.

6.4 Aurora kinases physically and functionally interact with IKK kinases to activate NF-κB in MM

A previous study has demonstrated that Aurora A activates NF-κB via IκB-α phosphorylation[18], a downstream target of IKKβ, and we found in our experiments that the blockade of Aurora kinase activity, either with pan-AKIs or siRNA suppression, affects IKKα/β phosphorylation. Therefore, we next investigated, by co-immunoprecipitation/Western blot analysis, whether there were physical interactions between Aurora and IKK kinases.

As depicted in Figure 4A reciprocal immunoprecipitations (IP) using antibodies directed against IKKα, IKKβ, Aurora A, or Aurora B from lysates of untreated RPMI 8226/R5 showed association of Aurora A with IKKα and β, and Aurora

B primarily with IKKα; TRAIL significantly enhanced the association of both Aurora A and B with IKKβ, and Aurora B with IKKα, but pretreatment with pan-AKIs completely abolished these TRAIL-induced increments (Figure 4A).

To determine whether the TRAIL-induced activating interactions between Aurora and IKK kinases correlated with Aurora kinases activation, we analyzed Aurora A (Thr288) and Aurora B (Thr232) phosphorylation[22] by Western blot and densitometric analysis; we found that TRAIL also increased the amounts of phosphorylated Aurora A and B in MM cells, and these phosphorylations were abolished in the presence of pan-AKIs (Figure 4B).

6.5 Aurora kinases activity is important for TRAIL-induced Aurora-IKK kinases interactions

Finally, to determine whether TRAIL-induced Aurora-IKK kinases interactions also depends on IKK activity, we selectively inhibited IKK activity in MM cells by using PS-1145, a specific small molecule inhibitor of IKK; because PS-1145 is highly selective for IKKβ, with more than 1000-fold greater inhibitory activity for IKKβ than for 30 other cellular kinases, including IKKα[23], we evaluated its effects on TRAIL-induced IKKβ phosphorylation/activation and IKKβ-Aurora kinases interactions. PS-1145 markedly diminished the basal and TRAIL-mediated phosphorylation of IKKβ in MM cells (Figure 5A) but failed in reducing TRAIL-induced IKKβ-Aurora kinases interactions (Figure 5B); conversely pan-AKIs, that prevented TRAIL-induced phosphorylation/activation of Aurora kinases, completely abrogated TRAIL-induced Aurora-IKK kinases interactions (Figure 5B). Because we found that PS-1145 treatment alone also increased IKKβ-Aurora kinases interactions

(Figure 5B), we asked whether this IKKβ inhibitor was able to modulate Aurora kinases activity; as expected we found that PS-1145 significantly increased the activity of both Aurora A and B in MM cells (Figure 5C). Moreover, consistent with the biological significance of targeting Aurora-IKK interactions for enhancing TRAIL sensitivity in MM cells, PS-1145, that was unable to negatively affect TRAIL-induced Aurora-IKK interactions, resulted ineffective in sensitizing MM cells to TRAIL (Figure 5D).

In summary, these data strongly suggest that Aurora kinases activity is relevant for TRAIL-induced Aurora-IKK kinases interactions and support a model in which both Aurora A and B, activated by TRAIL, can physically and functionally interact with IKKs to activate the canonical and non-canonical NF-κB pathways. The blockade of Aurora kinase activity by pan-AKIs can effectively reduce the TRAIL-induced NF-κB transcriptional activation because of disrupted AURORA-IKK kinases interactions (Figure 5E).

6.6 Combined exposure to Pan-AKIs and TRAIL negatively modulates the expression of NF-κB anti-apoptotic target genes

We next analyzed the effects of treatment with pan-aurora inhibitors and/or TRAIL on the levels of A1/Bfl-1, Mcl-1, cIAP1, cIAP2, and XIAP that are well-characterized transcriptional targets of NF-κB.

Treatment for 24 h with TRAIL increased the protein levels of A1/Bfl-1 in all tested HMCLs, and Mcl-1 in RPMI 8226/R5 and, to a lesser extent, in U266 and JJN3 HMCLs (Figure 6A); pretreatment with pan-AKIs prevented TRAIL induction of A1/Bfl-1 and Mcl-1 (Figure 6A) and significantly enhanced

mitochondrial depolarization induced by TRAIL alone in all tested HMCLs Figure 6B).

Using RNAi strategies we found that the knockdown of either A1/Bfl-1 or Mcl-1 significantly increased TRAIL-induced apoptosis in the majority of HMCLs analyzed (Figure 6C and representative data in Figure 6D), whereas retroviral overexpression of either A1/Bfl-1 or Mcl-1 protected MM (RPMI 8226/R5) cells from TRAIL and pan-AKIs/TRAIL-induced apoptosis (Figure 6E) arguing that both A1/Bfl-1 and Mcl-1 are important targets for TRAIL sensitization in MM cells; in RPMI 8226, OPM-2 and JJN3 HMCLs Mcl-1 knockdown produced a large amount of background apoptosis (over 65%) already evident 6 h after transfection that masked the citotoxic effect of TRAIL (data not shown), therefore confirming its pivotal role in controlling MM cell survival and apoptosis[24].

Furthermore, pan-AKIs either as single agents or in combination with TRAIL did not significantly affect Bcl-xL and Bcl-2 protein expression in MM cells (Figure 7A), suggesting that these inhibitors do not act through the modulation of these antiapoptotic Bcl-2 proteins; accordingly, NF-κB inhibitor SN50 did not reduce Bcl-xL and Bcl-2 levels in MM cells[25], and the siRNA knockdown of endogenous Bcl-xL or Bcl-2 did not significantly increase TRAIL-induced apoptosis in HMCLs (Figure 7B and 7C); moreover, overexpression of either Bcl-2 or Bcl-xL in MM cells failed to protect them from TRAIL[26] and pan-AKIs/TRAIL-induced apoptosis (Figure 7D).

Consistent with our findings that pan-AKIs potentiate TRAIL-induced apoptosis through NF-κB inhibition we found that the treatment with pan-AKIs significantly reduced the basal and/or TRAIL-induced protein levels of X-

chromosome-linked inhibitor-of-apoptosis protein (XIAP), cellular inhibitor-of-apoptosis protein 1 (cIAP1) and/or cIAP2, a well known NF-κB target genes that have been demonstrated to play a pivotal role in inhibiting death receptor and mitochondrial signaling[27-29] (Figure 8A). Moreover, the siRNA knockdown of XIAP (in all the tested HMCLs) and cIAP1 or cIAP2 (in RPMI 8226 and JJN3 respectively) significantly increased TRAIL-induced apoptosis, thereby confirming that IAPs can be important targets for TRAIL sensitization in MM cells (Figure 8B).

6.7 Pan-AKIs combined with TRAIL cause regression of multidrug-resistant tumors in vivo

To assess the in vivo efficacy of combining TRAIL and pan-AKIs, we tested these compounds using mouse tumor human plasmacytoma xenograft model in which the multidrug-resistant RPMI 8226/R5 cells (1.0×10^7 cells per mouse) were injected subcutaneously into NOD-SCID mice. When the tumors reached approximately 250 mm^3, mice bearing RPMI 8226/R5 tumors were randomized (n = 10/group) to receive vehicle or MK-0457 or PHA-680632 at 50 mg/kg or TRAIL (300 μg/per mouse) or MK-0457/TRAIL or PHA-680632/TRAIL. Both pan-AKIs and TRAIL were administered by intraperitoneal injection. Mice were treated with daily doses of pan-AKIs for 11 days, and 2 doses of TRAIL. Treatment of RPMI 8226/R5 MM-tumor-bearing mice with pan-AKIs significantly ($P < .01$) reduced MM-tumor growth as compared to control, while TRAIL had minimal effect on the growth of tumors, which increased as in control mice (Figure 7Ai). When pan-AKIs were

combined with TRAIL, there was a significant ($P < .001$) reduction in tumor growth relative to either treatment alone (Figure 9Ai).

Furthermore, as depicted in Figure 9Aii, the combination of pan-AKIs/TRAIL significantly ($P = .0015$, after Bonferroni correction) prolonged survival compared with treatment with either drug alone (mean survival time of MK-0457/TRAIL: 90 days, 95% confidence interval (CI) = 29–102 days; PHA-680632/TRAIL: 94.5 days, 95% CI = 71–103 days; MK-0457: 33.5 days, 95% CI = 14–42 days; PHA-680632: 29.5 days, 95% CI = 15–40 days; TRAIL: 17.5 days, 95% CI = 13–19; untreated: 14 days, 95% CI = 12–16 days) and was well tolerated without significant weight loss (data not shown). We next investigated the in vivo effects of the drug combination on proliferation and apoptosis; whole tumor-cell tissues from mice treated for 6 days (n = 3/group) were subjected to histopathologic examination and immunohistochemical staining to assess in vivo phosphorylation of histone H3 on Ser10 (phospho-H3), a proliferation marker whose phosphorylation during mitosis has been previously used to demonstrate inhibition of Aurora kinase activity[30], and cleaved caspase-3. Tumor tissues from pan-AKIs treatments resulted in profound phospho-histone H3 inhibition compared with tumor tissues from vehicle control or TRAIL-treated animals (Figure 9B), thereby confirming that either MK-0457 or PHA-680632 were acting through Aurora inhibition in tumor cells, and the growth retardation observed in pan-AKIs-treated mice. Either pan-AKIs or TRAIL alone slightly increase caspase-3 cleavage/activation compared with tumors from control cohorts. However, the combination pan-AKIs/TRAIL dramatically increased caspase-3 cleavage/activation in tumors (Figure 9C).

Evidence for tumor reduction was demonstrated by pan-AKIs/TRAIL-induced nearly complete disappearance of tumor cell mass accompanied by substantial fibrosis (Figure 9C).

Finally, histopathological and immunohistochemical findings were additionally confirmed by parallel Western blot analysis of tumor lysates that revealed a stronger activation of caspase-3 and PARP fragmentation in tumors from pan-AKIs/TRAIL-treated mice as compared with either treatment alone (Figure 9D).

Taken together these findings suggest that combining pan-AKIs with TRAIL induces both cytostatic and cytotoxic responses in vivo resulting in regression of tumors, prolongs survival in vivo and is well tolerated in vivo.

7. DISCUSSION

Recombinant human TRAIL ligand and agonistic mAbs have shown remarkable promise as an anticancer agent[2,21]. However, TRAIL signaling also activates survival signaling that can compromise its efficacy[5,6]. In the present study we demonstrate that pan-AKIs disrupt TRAIL-induced survival signaling by effectively reducing TRAIL-mediated activation of both canonical and non-canonical NF-κB pathways and by blocking TRAIL-induction of the anti-apoptotic NF-κB target genes A1/Bfl-1 and/or Mcl-1 in MM cells.

NF-κB plays a crucial role in the pathogenesis of various cancers[31-33], and it has been recently shown that the relationship between canonical and non-canonical NF-κB activity and genetic abnormalities in MM[20,34] suggesting the biologic significance of both NF-κB pathways in MM pathogenesis.

Furthermore, canonical and non-canonical pathways show much interplay, overlap and cross-talk[35]; previous reports have shown that specific IKKβ inhibitors have modest anti-MM activity in vitro and in vivo because they specifically inhibit canonical NF-κB signaling but do not block total NF-κB activity[36]; yet, IKKβ inhibitors may activate non-canonical pathway in MM cells, and vice versa, the down-regulation of IKKα may enhance phosphorylation of IKKβ, suggesting the existence of compensatory cross-talk between canonical and non-canonical pathways[36-38].

Consistent with previous findings reporting that IKKβ can antagonize Aurora-A signaling by promoting its degradation in the ubiquitin-proteasome pathway[39], we demonstrated that IKKβ inhibitors increased the activation of both Aurora A and B and their physical interactions with IKKβ, and were ineffective in sensitizing MM cells to TRAIL. Moreover, we found that simultaneous

inhibition of both IKKβ and IKKα by specific inhibitors and/or siRNA approaches or by pan-AKIs that disrupt NF-κB signaling by abrogating TRAIL-induced Aurora-IKK kinases interactions and activation, was more effective in sensitizing MM cells to TRAIL than inhibition of either kinase alone, thus suggesting that strategies that target both canonical and non-canonical NF-κB pathways may enhance the anti-myeloma effects of TRAIL[36].

Furthermore, in our experiments we demonstrated that the siRNA-mediated depletion of either Aurora A or Aurora B affected the phosphorylation/activation of both IKKα and IKKβ in MM, thus suggesting the need of targeting both Aurora A and B because the inhibition of either kinase alone may be insufficient to block NF-κB activity in MM cells.

All together these findings reveal additional complexity in the signaling network regulating NF-κB pathway and suggest the existence of possible reciprocal regulatory roles between IKK and Aurora kinases.

Given that mutations affecting NF-κB inducing kinase (NIK) levels (NIK, TRAF2, TRAF3, cIAP1, and cIAP2) frequently occur in HMCLs and primary MM patient samples[19,20] (Table 1), and NIK accumulation activates NF-κB signaling through a NIK-mediated activation/phosphorylation of IKK[19,20], we speculate that the NIK-triggered activation of IKK may interfere with and reduce the inhibitory effects exerted by pan-AKIs on the basal IKK activity of MM cells. This could explain the partial IKK inhibition observed in the majority of pan-AKIs-treated HMCLs; however, in all tested HMCLs, irrespective of their NF-κB profile and NIK levels[19,20], TRAIL enhanced the basal phosphorylation/activation state of Aurora and IKK kinases, their physical

interactions and NF-κB activation, and the pharmacological blockade of aurora kinases abrogated TRAIL-induced NF-κB survival signaling by effectively reducing Aurora-IKK kinases phosphorylation/activation and interactions, suggesting that these kinases can cooperate to promote TRAIL resistance in MM mainly through NF-κB activation; consistently, Aurora B has been previously reported to be involved in cancer cells' resistance to TRAIL[40,41].

Previous studies have shown that TRAIL can activate both NF-κB and the Ras/Raf/MEK/ERK mitogen-activated protein kinase (MAPK) cascade signaling pathways[5,6,42,43] and that Aurora A can be a downstream target of ERK[44]. The results of these studies, together with our findings of TRAIL-induced activation of both Aurora and IKK kinases, define an important role for Aurora kinases in connecting MAPK signaling to NF-κB activity.

We also found that pan-AKIs blocked TRAIL-induction of the anti-apoptotic NF-κB target genes A1/Bfl-1 and/or Mcl-1 and demonstrated that either A1/Bfl-1 or Mcl-1 can exert significant protection against TRAIL, thereby indicating that both these antiapoptotic molecules can be critical mediators of TRAIL resistance in MM cells. A1/Bfl-1 and Mcl-1 have been reported to interact with and antagonize the activity of pro-apoptotic Bcl-2 family members apoptotic proteins including Bim and Bid[45]. Bim can physically interact with both DR4 and DR5 TRAIL receptors and contribute to the activation of both extrinsic and intrinsic apoptotic pathways[15]. In addition, as compared with TRAIL treatment alone, co-treatment with pan-AKIs and TRAIL significantly decreased the protein levels of caspase-8 inhibitors cIAP1/2[28] and caspases-3, -7, and -9 inhibitor XIAP[29].

For our in vitro and in vivo experiments, we used two potent and selective small-molecule inhibitors of Aurora A and B kinases, VX-680/MK0457[12,46] and the preclinical candidate PHA-680632[12,47], which has been reported to have both in vitro and in vivo similar activity against the Aurora kinases of its close analog PHA-739358, currently in Phase II clinical trials[12]. In this study, both drugs exhibited similar antitumor activities as single agents or in combination with TRAIL. Furthermore, Aurora kinase inhibitors have recently been studied as potential novel therapeutic targets in solid and hematological malignancies[12,13,48-50] and few of them are currently undergoing Phase I and II clinical trials as both monotherapy and combination therapy in relapsed or refractory MM[51,52].

Finally, the clinical observation that TRAIL monotherapy can be associated with limited efficacy and resistance, coupled with our present preclinical in vitro and in vivo findings demonstrated that TRAIL together with pan-AKIs trigger a more potent anti-MM effect in vitro and in vivo without increased toxicity, provide the framework for testing pan-AKIs and TRAIL combination therapy in clinical trials aimed to improve patients outcome in MM.

Our data provide evidence for a novel functional link between Aurora and IKK kinases, underscoring a critical role of these pathways in MM drug resistance, and the potential therapeutic benefit of targeting Aurora kinases as a strategy to overcome Aurora-IKK crosstalk-related resistance to TRAIL.

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9. REFERENCES

1. Bensinger W. Stem-cell transplantation for multiple myeloma in the era of novel drugs. *J Clin Oncol* 2008; 26(3): 480-492.

2. Ashkenazi A, Herbst RS. To kill a tumor cell: the potential of proapoptotic receptor agonists. *J Clin Invest* 2008; 118(6): 1979-1990.

3. Wiezorek J, Holland P, Graves J. Death receptor agonists as a targeted therapy for cancer. *Clin Cancer Res* 2010; 16(6): 1701-1708.

4. Herbst RS, Eckhardt SG, Kurzrock R, et al. Phase I dose-escalation study of recombinant human Apo2L/TRAIL, a dual proapoptotic receptor agonist, in patients with advanced cancer. *J Clin Oncol* 2010; 28(17): 2839-2846.

5. Thorburn A, Behbakht K, Ford H. TRAIL receptor-targeted therapeutics: resistance mechanisms and strategies to avoid them. *Drug Resist Updat* 2008; 11(1-2): 17-24.

6. Ricci MS, Kim SH, Ogi K et al. Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer Cell* 2007; 12(1): 66-80.

7. Jin H, Yang R, Fong S, et al. Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand cooperates with chemotherapy to inhibit orthotopic lung tumor growth and improve survival. *Cancer Res* 2004;64(14): 4900-4905.

8. Romagnoli M, Desplanques G, Maïga S, et al. Canonical nuclear factor kappaB pathway inhibition blocks myeloma cell growth and induces apoptosis in strong synergy with TRAIL. *Clin Cancer Res* 2007;13(20): 6010-6018.

9. Perez LE, Parquet N, Meads M, Anasetti C, Dalton W. Bortezomib restores stroma-mediated APO2L/TRAIL apoptosis resistance in multiple myeloma. *Eur J Haematol* 2010;84(3): 212-222.

10. Liu Q, Hilsenbeck S, Gazitt Y. Arsenic trioxide-induced apoptosis in myeloma cells: p53-dependent G1 or G2/M cell cycle arrest, activation of caspase-8 or caspase-9, and synergy with APO2/TRAIL. *Blood* 2003; 101(10): 4078-4087.

11. Gobbi G, Mirandola P, Carubbi C, et al. Phorbol ester-induced PKCepsilon down-modulation sensitizes AML cells to TRAIL-induced apoptosis and cell differentiation. *Blood* 2009; 113(13): 3080-3087.

12. Lens SM, Voest EE, Medema RH. Shared and separate functions of polo-like kinases and aurora kinases in cancer. *Nat Rev Cancer* 2010; 10(12): 825-841.

13. Katayama H, Sen S. Aurora kinase inhibitors as anticancer molecules. *Biochim Biophys Acta* 2010; 1799(10-12): 829-839.

14. Buzzeo R, Enkemann S, Nimmanapalli R, et al. Characterization of a R115777-resistant human multiple myeloma cell line with cross-resistance to PS-341. *Clin Cancer Res* 2005; 11(16): 6057-6064.

15. Lunghi P, Giuliani N, Mazzera L, et al. Targeting MEK/MAPK signal transduction module potentiates ATO-induced apoptosis in multiple myeloma cells through multiple signaling pathways. *Blood* 2008; 112(6): 2450-2462.

16. Perez LE, Parquet N, Shain K, et al. Bone marrow stroma confers resistance to Apo2 ligand/TRAIL in multiple myeloma in part by regulating c-FLIP. *J Immunol* 2008; 180(3):1545-1555.

17. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. *Nat Rev Cancer* 2007; 7(8): 585-598.

18. Briassouli P, Chan F, Savage K, Reis-Filho JS, Linardopoulos S. Aurora-A regulation of nuclear factor-kappaB signaling by phosphorylation of IkappaBalpha. *Cancer Res* 2007; 67(4): 1689-1695.

19. Demchenko YN, Glebov OK, Zingone A, Keats JJ, Bergsagel PL, Kuehl WM. Classical and/or alternative NF-kappaB pathway activation in multiple myeloma. *Blood* 2010; 115(17): 3541-3552.

20. Annunziata CM, Davis RE, Demchenko Y, et al. Frequent engagement of the classical and alternative NF-kappaB pathways by diverse genetic abnormalities in multiple myeloma. *Cancer Cell* 2007; 12(2): 115-130.

21. Mitsiades CS, Treon SP, Mitsiades N, et al. TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. *Blood* 2001; 98(3): 795-804.

22. Bayliss R, Sardon T, Vernos I, Conti E. Structural basis of Aurora-A activation by TPX2 at the mitotic spindle. *Mol Cell* 2003; 12(4): 851-862.

23. Bain J, Plater L, Elliott M, et al. The selectivity of protein kinase inhibitors: a further update. *Biochem J* 2007; 408(3): 297-315.

24. Zhang B, Gojo I, Fenton RG. Myeloid cell factor-1 is a critical survival factor for multiple myeloma. *Blood* 2002; 99(6): 1885-1893.

25. Mitsiades N, Mitsiades CS, Poulaki V, Anderson KC, Treon SP. Intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human multiple myeloma cells. *Blood* 2002; 99(6): 2162-2171.

26. Gazitt Y, Shaughnessy P, Montgomery W. Apoptosis-induced by TRAIL and TNF-alpha in human multiple myeloma cells is not blocked by BCL-2. *Cytokine* 1999; 11(12): 1010-1019.

27. Gyrd-Hansen M, Meier P. IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. *Nat Rev Cancer* 2010; 10(8): 561-574.

28. Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* 1998; 281(5383): 1680-1683.

29. Shiozaki EN, Chai J, Rigotti DJ, et al. Mechanism of XIAP-mediated inhibition of caspase-9. *Mol Cell* 2003; 11(2): 519-27.

30. Crosio C, Fimia GM, Loury R, et al. Mitotic phosphorylation of histone H3: spatio-temporal regulation by mammalian aurora kinases. *Mol Cell Biol* 2002; 22(3): 874-885.

31. Perkins ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* 2007; 8(1): 49-62.

32. Van Waes C. Nuclear factor-kappaB in development, prevention, and therapy of cancer. *Clin Cancer R*es 2007; 13(4): 1076-1082.

33. Li ZW, Chen H, Campbell RA, Bonavida B, Berenson JR. NF-kappaB in the pathogenesis and treatment of multiple myeloma. *Curr Opin Hematol* 2008; 15(4): 391-399.

34. Keats JJ, Fonseca R, Chesi M, et al. Promiscuous mutations activate the noncanonical NF-kappaB pathway in multiple myeloma. *Cancer Cell* 2007; 12(2): 131-144.

35. Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF-kappaB signaling pathways. *Nat Immunol* 2011; 12(8): 695-708.

36. Hideshima T, Chauhan D, Kiziltepe T, et al. Biologic sequelae of IkappaB kinase (IKK) inhibition in multiple myeloma: therapeutic implications. *Blood* 2009; 113(21): 5228-5236.

37. Lam LT, Davis RE, Ngo VN, et al. Compensatory IKKalpha activation of classical NF-kappaB signaling during IKKbeta inhibition identified by an RNA interference sensitization screen. *Proc Natl Acad Sci U S A* 2008; 105(52): 20798-20803.

38. Yamamoto Y, Yin MJ, Gaynor RB. IkappaB kinase alpha (IKKalpha) regulation of IKKbeta kinase activity. *Mol Cell Biol* 2000; 20(10): 3655-3666.

39. Irelan JT, Murphy TJ, DeJesus PD, et al. A role for IkappaB kinase 2 in bipolar spindle assembly. *Proc Natl Acad Sci U S A* 2007; 104(43): 16940-16945.

40. Yoon MJ, Park SS, Kang YJ, et al. Aurora B confers cancer cell resistance to TRAIL-induced apoptosis via phosphorylation of survivin. *Carcinogenesis* 2012; 33(3): 492-500.

41. Li J, Anderson MG, Tucker LA, Shen Y, Glaser KB, Shah OJ. Inhibition of Aurora B kinase sensitizes a subset of human glioma cells to TRAIL concomitant with induction of TRAIL-R2. *Cell Death Differ* 2009; 16(3): 498-511.

42. Morel J, Audo R, Hahne M, Combe B. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces rheumatoid arthritis synovial fibroblast proliferation through mitogen-activated protein kinases and phosphatidylinositol 3-kinase/Akt. *J Biol Chem* 2005; 280(16): 15709-15718.

43. Tran SE, Holmstrom TH, Ahonen M, Kahari VM, Eriksson JE. MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors. *J Biol Chem* 2001; 276(19): 16484-16490.

44. Furukawa T, Kanai N, Shiwaku HO, Soga N, Uehara A, Horii A. AURKA is one of the downstream targets of MAPK1/ERK2 in pancreatic cancer. *Oncogene* 2006; 25(35): 4831-4839.

45. Chen L, Willis SN, Wei A, et al. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 2005; 17(3): 393-403.

46. Harrington EA, Bebbington D, Moore J, et al. VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo. *Nat Med* 2004; 10(3): 262-267.

47. Soncini C, Carpinelli P, Gianellini L, et al. PHA-680632, a novel Aurora kinase inhibitor with potent antitumoral activity. *Clin Cancer Res* 2006; 12(13): 4080-4089.

48. Hose D, Reme T, Meissner T, et al. Inhibition of aurora kinases for tailored risk-adapted treatment of multiple myeloma. *Blood* 2009; 113(18): 4331-4340.

49. Chng WJ, Braggio E, Mulligan G, et al. The centrosome index is a powerful prognostic marker in myeloma and identifies a cohort of patients that might benefit from aurora kinase inhibition. *Blood* 2008; 111(3): 1603-1609.

50. Görgün G, Calabrese E, Hideshima T, et al. A novel Aurora-A kinase inhibitor MLN8237 induces cytotoxicity and cell-cycle arrest in multiple myeloma. *Blood* 2010; 115(25): 5202-5213.

51. http://clinicaltrials.gov/ct2/show/NCT00697346 accessed data February 2013.

52. http://clinicaltrials.gov/ct2/show/NCT01034553 accessed data February 2013.

10. TABLES

Table 1. TRAIL Sensitivity and NF-κB profile of HMCLs used in this study.

HMCLs	ED_{50} of TRAIL	NF-κB pathway	NF-κB index	NF-κB mutation
OPM-2	4.7 ± 0.9 ng/mL	Weak canonical	9.03	—
RPMI 8226	4.8 ± 0.4 ng/mL	Both canonical and non-canonical	10.44	TRAF3
U266	55.2 ± 6.8 ng/mL	Both canonical and non-canonical	10.41	TRAF3
RPMI 8226/R5	50.2 ± 7.1 ng/mL	Both canonical and non-canonical	ND	ND
JJN3	>1000 ng/mL	Both canonical and non-canonical	10.80	NIK

HMCLs were cultured in presence of escalating doses of TRAIL (0.6 ng/mL-2000 ng/mL). After 24 hours cell death was measured by annexin V staining and the median effective dose (ED_{50}, dose producing 50% of cytotoxicity) was estimated using Median Effect Equation of Chou. Values represent mean ± SD of four independent experiments.

Contribution of the canonical and non-canonical NF-κB pathways in HMCLs was estimated from steady-state levels of NF-κB subunits and/or effect of IKK β inhibiton on nuclear localization and DNA binding activity of the NF-kB p65 and p52 subunits [19,20]. The NF-κB index was calculated using the average expression of 10 NF-κB target genes [19] ("Genes comprising the NF-κB index in myeloma"). NF-κB mutations were reported previously[19,20]: — indicates no mutation identified, ND indicates non-detected.

Table 2. Clinical characteristics of the MM patients

MM PATIENT no.	Age	PATIENT STAGE	BM PLASMA CELLS (%)	STATE OF DESEASE
1	80	II	85%	DIAGNOSIS
2	59	III	65%	DIAGNOSIS
3	67	III	60%	DIAGNOSIS
4	73	III	98%	RELAPSE
5	73	III	60%	RELAPSE

Primary $CD138^+$ MM cells were isolated from BM aspiration of MM patiens at the diagnosis or relapse and were treated *in vitro* with MK-0457 (0.4 μM) or PHA-680632 (0.8 μM) and TRAIL (9.6 ng/mL) for 24 hours.

11. FIGURE LEGENDS

Figure 1. Pan-Aurora Kinase inhibitors potentiate the TRAIL-induced cell death in MM cells.

(A) (i) HMCLs seeded at $2.0x10^5$ cells/mL were treated sequentially with escalating doses of MK-0457 (15-2000 ng/mL; 0.032-4.3 μM) or PHA-680632 (15-2000 ng/mL; 0.03-4 μM) for 3 hours, and subsequently with TRAIL (0.5-50 ng/mL) alone or in combination with pan-AKIs at a fixed ratio of 25:1 for MK-0457:TRAIL and 50:1 for PHA-680632:TRAIL (in combination with pan-AKIs TRAIL was used at 0.6, 1.2, 2.4, 4.8, 9.6, 19.2 ng/mL). After 24 hours, cell death was measured by annexin-V binding. Combination index (CI) plots was then generated using the Calcusyn software. CI values lesser than 1.0 indicates synergism; CI value equal 1.0 indicates additive effect; CI more than 1.0 indicates antagonistic effect. **(ii)** JJN3 were seeded at $2.0x10^5$ cells/mL in the presence of DMSO (vehicle) or MK-0457 (0.4 μM) or PHA-680632 (0.8 μM) for 3 hours and then incubated with TRAIL at the concentration of 300 ng/mL. After 24 hours, apoptosis was measured by annexin-V labeling. Values represent means ± SD of 4 independent experiments. (*P< .01 versus either treatment alone; Dunnet test. (**B**) R5 were seeded in the presence of DMSO (vehicle) or PHA-680632 (0.8 μM) for 3 hours and then incubated with TRAIL at the concentration of 9.6 and 19.2 ng/mL. After 24 hours, apoptosis was detected by measurement of sub-G1 DNA content and annexin V binding. **(C)** HMCLs wew treated with Pan-AKIs for 3 hours and then were incubated with TRAIL at the indicated doses (ng/mL) in the presence or absence of IGF-1 (50 ng/mL), IL-6 (20 ng/mL) or BMSCs (HMCLs in direct contact to BMSCs monolayer in a 5:1 ratio). After 24 hours HMCLs were

harvested and apoptotic HMCLs cells were determined by flow cytometry as CD38/CD138+ annexin-V+ cells; data represent means ± SD of quadruplicate experiments. Dexamethasone (DEX) (2μM) was used as control to monitor the protective effects of growth factors and stromal cells. (*P< .05, **P< .005; Tukey-Kramer HSD test). **(D) (i-ii)** CD138-purified plasma cells from five patients with MM were isolated from BM and then seeded at $2.5x10^5$ cells/mL. Samples from #1 to #3 were pretreated with MK-0457 (0.4 μM), samples #4 and 5# with PHA-680632 (0.8 μM) and then incubated with TRAIL (9.6 ng/mL) for 24 hours. Sample #4 was cultured in presence or absence of Bone Marrow Stromal Cells (BMSCs). The cell death was measured by annexin-V staining. **(i)** Results are expressed as the net apoptosis induction [percentage of apoptosis in treated cells minus percentage of apoptosis in DMSO (vehicle-treated cells)] of all 5 primary samples tested. **(ii)** Histogram represents the mean percentage of apoptosis ± SD (expressed as net difference between the percentages of apoptosis in treated cells minus percentage of apoptosis in vehicle-treated cells) of the results obtained in the 5 different patient samples. (*P< .05 versus either treatment alone, Dunnet test). **(iii)** Peripheral blood mononuclear cells (PBMCs) from 3 healthy volunteers were treated with DMSO (vehicle) or MK-0457 (0.4 μM) or PHA-680632 (0.8 μM) for 3 hours and then incubated with TRAIL (9.6 ng/mL). After 24 hours of treatment cell death was then measured by annexin-V staining. **(E)** Transfection of Aurora A and/or Aurora B, but not the unrelated non-specific control siRNA, led to a decrease in Aurora kinases protein expression in HMCLs without affecting the levels of the unrelated protein actin. 48 hours after siRNA transfection HMCLs were treated with TRAIL at the indicated doses (ng/mL) for 48 hours and the

percentages of annexin-V+ apoptotic cells was then measured. Values are mean ± SD of 3 independent experiments (*P< .001 versus TRAIL Non-specific si-RNA [CONT]; Dunnet test). (**F**) HMCLs were transfected with non-specific control siRNA (CONT) or with Aurora A and Aurora B siRNA. 48 hours after siRNA transfection HMCLs were treated with TRAIL at the indicated doses (ng/mL) for 48 hours and the percentages of annexin-V+ apoptotic cells was then measured.

Figure 2. Coadministration of Pan-AKIs and TRAIL activates the caspase cascade in MM.

(A) HMCLs were treated with Pan-AKIs for 3 hours and then were incubated with TRAIL (2.4 ng/mL in RPMI 8226 and OPM-2, 9.6 ng/mL in RPMI 8226/R5 and U266, 300 ng/mL in JJN3) for 24 hours, after which cells were lysed and subjected to Western blot analysis to monitor the expression of PARP, cleaved-PARP, cleaved caspase-8, Bid, truncated Bid (tBid), cleaved caspase-9 and cleaved caspase-3; blots were subsequently reprobed for actin expression to ensure equivalent loading and transfer of proteins. **(B)** HMCLs were cultured with Pan-AKIs and TRAIL at the doses indicated in panel A in the presence or absence of 30μM caspase inhibitors for 24 hours, after which the percentage of apoptotic cells was determined by the annexin-V method; data represent means ± SD of 3 independent experiments. (*P< .05, **P< .005 versus Pan-AKIs/TRAIL Z-FA-FMK; Dunnet test). Z-FA-FMK indicates peptide control; Z-IETD-FMK, selective caspase-8 inhibitor; Z-LEHD-FMK, selective caspase-9 inhibitor; Z-VAD-FMK, pancaspase inhibitor.

Figure 3. Pan-AKIs block TRAIL-induced canonical and non-canonical NF-κB activation in MM cells.

(A) HMCLs were treated with Pan-AKIs for 3 hours and then were incubated with TRAIL (2.4 ng/mL in RPMI 8226 and OPM-2, 9.6 ng/mL in RPMI 8226/R5 and U266, 300 ng/mL in JJN3) and after 24 hours nuclear extracts were prepared. **(i)** Nuclear extracts from RPMI 8226 and RPMI 8226/R5 were immunoblotted against NF-κB p65, NF-κB1 p50, NF-κB2 p52, c-Rel, RelB and laminin or histone H2B as nuclear loading control. Bands were subjected to densitometric scanning using the TINA 2 software and the number below each lane represents the relative amount of the indicated proteins normalized to laminin or histone H2B expression. **(ii)** HMCLs nuclear extracts were tested for DNA binding activity of the NF-κB p65, p50, p52, c-Rel and RelB subunits using the TransAM NF-κB ELISA kit. Results were normalized to the untreated control. Values represent mean ± SD of three separate experiments performed in triplicate. **(B)** HMCLs were cultured with Pan-AKIs and/or TRAIL as previously described. Endogenous p-IKKα/β, p-NF-κB2 p100, p-IκB-α, IKKβ and IKKα from whole cell lysates were revealed by Western Blot analysis. Anti-actin immunoblotting was performed as loading control. Bands were subjected to densitometric scanning and p-IKKα/β, p-NF-κB2 p100 and p-IκB-α protein expression levels were normalized to actin; the number below each lane represent relative amount of the indicated proteins.

(**D**) (i) siRNA silencing of IKKα and/or IKKβ, but not the non-specific control siRNA, led to a decrease in IKKα and/or IKKβ protein expression without affecting the levels of the unrelated protein actin. 48 hours after siRNA transfection HMCLs were lysed and the silencing effect of IKKα and/or IKKβ

siRNA at the protein level was monitored by western blot. (ii) HMCLs electroporated with IKKα or non-targeting siRNA (CONT) were treated with IKKβ inhibitors (PS-1145 at 5μM or IKKβ siRNA) and then incubated with TRAIL at the indicated doses. The cytotoxic effects of IKK inhibition were compared to those of MK-0457. After 24 hours the percentages of annexin-V+ apoptotic cells was measured. Values are mean ± SD of three independent experiments (*P< .05, **P< .01; Tukey-Kramer test) (**E**) HMCLs were pretreated with 10 μg/mL of NF-κB inhibitory peptide SN50 or control peptide SN50M for 3 hours followed by treatment with TRAIL (2.4 ng/mL in RPMI 8226 and OPM-2, 9.6 ng/mL in RPMI 8226/R5 and U266, 300 ng/mL in JJN3). After 24 hours the percentages of annexin-V+ apoptotic cells was measured. Values are mean ± SD of three independent experiments (*P< .01, versus either treatment alone; Dunnet test). **(F) (i)** Lysates from vehicle- and MK-treated RPMI 8226/R5 cells were subjected to western blot analysis to monitor the expression and phosphorylation of IκB-α; anti-actin immunoblotting was performed as loading control. **(Ii-iii)** HMCLs were electroporated with IκB-α or non-targeting siRNA (CONT) and 24 hours after siRNA transfection the cells were treated with MK-0457. After 24 hours of treatment cytoplasmic and nuclear extracts were prepared. (**ii**) Cytoplasmic cell lysates were immunoblotted against IκB-α and tubulin as marker of cytoplasmic separation as well as loading control; **(iii)** Nuclear extracts were tested for DNA binding activity of the NF-κB p65 subunit using the TransAM NF-κB ELISA kit. Results were normalized to the untreated control. Values represent mean ± SD of three separate experiments performed in triplicate. **(iii)** Transfection of IκB-α, but not the unrelated non-specific control siRNA, led to a decrease in IκB-α

protein expression in HMCLs without affecting the levels of the unrelated protein actin. 24 hours after siRNA transfection HMCLs were treated with Pan-AKIs for 3 hours and then were incubated TRAIL (2.4 ng/mL in RPMI 8226 and OPM-2, 9.6 ng/mL in RPMI 8226/R5 and U266, 300 ng/mL in JJN3) for 24 hours and the percentages of annexin-V+ apoptotic cells was measured. Values are mean ± SD of three independent experiments (*P< .01 versus pan-AKIs/TRAIL Non-specific siRNA [CONT]; Dunnet test). **(H)** Transfection of Aurora A or B, but not the unrelated non-specific control siRNA, led to a decrease in Aurora kinases protein expression in RPMI 8226 and RPMI 8226/R5 without affecting the levels of the unrelated protein actin. 48 hours after siRNA transfection cells were lysed for immunoblot analysis to monitor the expression of Aurora A and B, p-IKKβ, p-IKKα, p-IκB-α, p-NF-κB2 p100, IKKβ, IKKα and actin as loading control. Bands were subjected to densitometric scanning and p-IKKα/β, p-NF-κB2 p100 and p-IκB-α protein expression levels were normalized to actin; the number below each lane represent relative amount of the indicated proteins.

Figure 4. Aurora and IKK kinases interact in MM cells.

(A) RPMI 8226/R5 were treated with PHA-680632 for 3 hours and then incubated with TRAIL (9.6 ng/mL). After 24 hours of treatment cells were lysed in CHAPS lysis buffer and subjected to immunoprecipitation (IP) using mouse monoclonal IKKα (B-8) or mouse monoclonal IKKβ (H-4) or mouse monoclonal ARK-1 (35C1) (Aurora A) or mouse monoclonal ARK-2 (13E8A7) (Aurora B) or control antibody (IgG) and immunoblotted (IB) with either Aurora A or Aurora B or IKKα and IKKβ antibodies. Bands were subjected to

densitometric scanning and the histogram shows average quantification results ± SD of the association Aurora A/IKKα or Aurora A/IKKβ or Aurora B/IKKα or Aurora B/IKKβ from three blots (*P< .01, versus untreated control cells, Tukey-Kramer test). (B) HMCLs were cultured with Pan-AKIs and/or TRAIL as previously reported for 24 hours. Endogenous phospho-Aurora A (Thr288) and phospho-Aurora B (Thr232) from whole cell lysates were revealed by Western Blot analysis. Anti-actin immunoblotting was performed as loading control. The relative amount of Phospho-Aurora A (Thr288) and phospho-Aurora B (Thr232) was determined by densitometry and normalized to that of actin. Histograms represent the mean ± SD of the ratio Phospho-Aurora A (Thr288)/actin or phospho-Aurora B (Thr232)/actin normalized to the untreated control from blots of three separate experiments.

Figure 5. Aurora Kinases activity is important for Aurora-IKK interactions in Myeloma cells.

(A) HMCLs were cultured with DMSO (vehicle) or with IκB kinase (IKKβ) inhibitor PS-1145 (5μM) or with MK-0457 (0.4 μM) for three hours and then incubated with TRAIL. After 24 hours cells were lysed and subjected to western blot analysis to monitor the expression and phosphorylation of IKKβ; anti-actin immunoblot was performed as loading control. **(B)** The same cell lysates were subjected to immunoprecipitation (IP) using mouse monoclonal IKKβ (H-4) or control antibody (IgG) and immunoblotted (IB) with either IKKβ or Aurora A or Aurora B antibodies. Bands were subjected to densitometric scanning and the histogram shows average quantification results ± SD of the association Aurora A/IKKβ or Aurora B/IKKβ from three blots (*P< .05, **P<

.01, versus untreated control cells, Tukey-Kramer test). **(C)** RPMI 8226 and 8226/R5 cells were cultured with DMSO (vehicle) or with PS-1145 at 5μM for 24 hours. Endogenous pospho-IKKβ, phospho-Aurora A (Thr288) and phospho-Aurora B (Thr232) from whole cell lysates were revealed by Western Blot analysis. Anti-actin immunoblotting was performed as loading control. The relative amount of Phospho-Aurora A (Thr288) and phospho-Aurora B (Thr232) was determined by densitometry and normalized to that of actin. Histograms represent the mean ± SD of the ratio Phospho-Aurora A (Thr288)/actin or phospho-Aurora B (Thr232)/actin normalized to the untreated control from blots of two blots (*P< .01, Tukey-Kramer test). **(D)** HMCLs cells were cultured with DMSO (vehicle) or with PS-1145 at 5μM for 3 hours and then were incubated with TRAIL (2.4 ng/mL in RPMI 8226, 19.2 ng/mL in RPMI 8226/R5 and 300 ng/mL in JJN3) and after 24 the percentage of cell death was determined by the annexin-V method. Values are mean ± SD of three independent experiments. **(E)** Model of TRAIL action; TRAIL induces prosurvival signal in MM cells by activating both Aurora A and B kinases leading to increased Aurora/IKK binding, IKK phosphorylation/activation, NF-κB activation and induction of the anti-apoptotic NF-κB target genes A1/Bfl-1, Mcl-1 and IAPs; Aurora kinases inhibitors disable TRAIL-directed survival pathways because abrogate TRAIL-induced Aurora-IKK kinases interactions and NF-κB activation.

Figure 6. Pan-AKIs sensitize MM cells to TRAIL by inhibiting TRAIL-induced Mcl-1 and A1/Bfl-1 experssion. (A) HMCLs were treated with MK-0457 for 3 hours and then were incubated with TRAIL (2.4 ng/mL in RPMI

8226 and OPM-2, 9.6 ng/mL in RPMI 8226/R5 and U266, 300 ng/mL in JJN3) for 24 hours after which endogenous A1/Bfl-1 and Mcl-1 were revealed by immunoblotting analysis; blots were subsequently reprobed for actin expression to ensure equivalent loading and transfer of protein. Histograms represent the mean value ± SD of the ratio of A1/Bfl-1 or Mcl-1 to actin normalized to the untreated control from blots of three independent experiments (*P< .001; Tukey-Kramer test). **(B)** Transfection of A1/Bfl-1 or Mcl-1, but not the unrelated non-specific control siRNA (CONT), led to a decrease in A1/Bfl-1 or Mcl-1 protein expression in HMCLs without affecting the levels of the unrelated protein actin (insets): 30 minutes after siRNA transfection the cells were treated with TRAIL at the indicated doses (ng/mL) for the indicated time. Apoptosis was measured by annexin-V staining. Values are mean ± SD of 3 independent experiments [*P< .005, **P< .001 versus non-specific control siRNA (CONT); Dunnet test]. **(C)** RPMI 8226/R5 cells were infected with an empty lentiviral vector or with lentivirus expressing A1/Bfl-1 or Mcl-1. All lentviral expression vectors co-expressed RFP to monitor the infection by flow cytometry. Plot represents comparable RFP expression of RPMI 8226/R5s infected with the parental retroviral vector or those encoding for Mcl-1 or A1/Bfl1. Whole-cell lysates of uninfected or virus-infected RPMI 8226/R5 cells were prepared and analyzed by western blot to confirm the overexpression of A1/Bfl-1 or Mcl-1 (inset). Pools of RPMI 8226/R5 were expanded after infection and treated with PHA-680632 (0.8 μM) and then incubated with TRAIL at the indicated doses for 24 hours. Cell death was measured by annexin-V staining. Values are mean ± SD of 3

independent experiments (*P< .005, versus PHA/TRAIL empty vector; Dunnet test).

Figure 7. Pan-AKIs and TRAIL combination does not act through Bcl-xL and Bcl-2 anti-apoptotic proteins. (A) HMCLs were treated with pan-AKIs for 3 hours and then were incubated with TRAIL (2.4 ng/mL in RPMI 8226 and OPM-2, 9.6 ng/mL in RPMI 8226/R5 and U266, 300 ng/mL in JJN3) for 24 hours after which endogenous Bcl-xL and Bcl-2 were revealed by immunoblotting analysis; blots were subsequently reprobed for actin expression to ensure equivalent loading and transfer of protein. **(B)** Transfection of Bcl-xL, but not the unrelated non-specific control siRNA (CONT), led to a decrease in Bcl-xL protein expression in HMCLs without affecting the levels of the unrelated protein actin: 30 minutes after siRNA transfection the cells were treated with TRAIL at the indicated doses (ng/mL). After 24 hours apoptosis was measured by annexin-V staining. Values are mean ± SD of 3 independent experiments. **(C)** Transfection of Bcl-2, but not the non-specific control siRNA (CONT), led to a decrease in Bcl-2 protein expression without affecting the levels of the unrelated protein actin: 24 hours after siRNA transfection the cells were treated with TRAIL at the indicated doses (ng/mL). After 24 hours apoptosis was measured by annexin-V staining. Values are mean ± SD of 3 independent experiments. **(D)** RPMI 8226/R5 and JJN3 were stably transfected with an empty vector or with plasmids expressing Bcl-2 or Bcl-xL or Mcl-1. Pools of stable clones were obtained by selection with puromycin. All expression vectors co-expressed GFP to monitor the transfection by flow cytometry. Plot represents

comparable GFP expression of HMCLs transfected with empty vector or those encoding for Bcl-2 or Bcl-xL or Mcl-1. Whole-cell lysates were prepared and analyzed by western blot to confirm the overexpression of Bcl-2 or Bcl-xL or Mcl-1 (inset). Stable pools of HMCLs expanded after electroporation and selection were treated with MK-0457 (0.4 μM) and then incubated with TRAIL at the indicated doses. Pool#1 and pool#2 of Bcl-2-expressing RPMI 8226/R5 cells were derived from two different transfection experiments and expressed different amount of Bcl-2 protein. After 24 hours cell death was measured by Annexin V-PE/7-AAD staining. Values are mean ± SD of 3 independent experiments (*P< .05, **P< .001; Tukey-Kramer test).

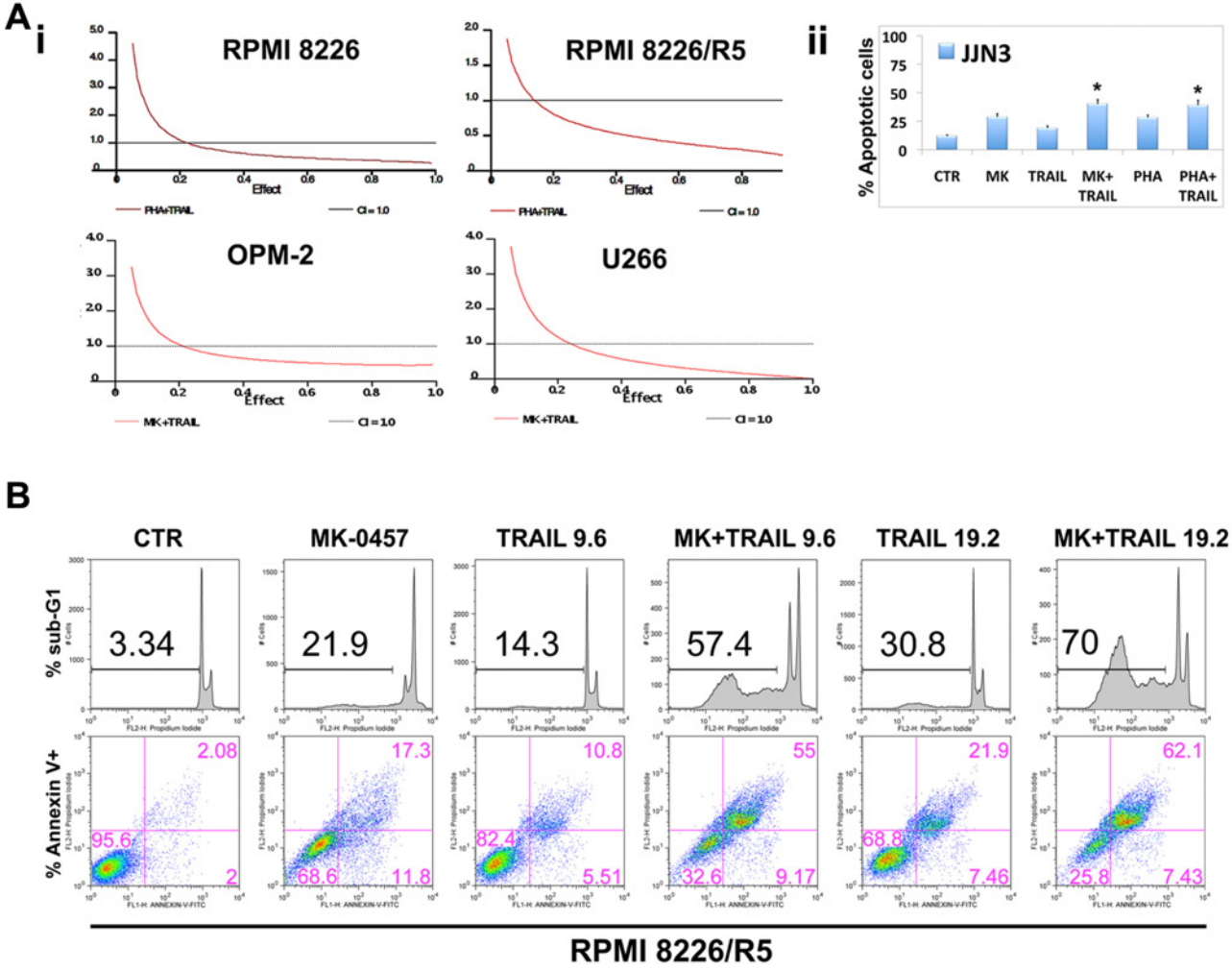
Figure 8. Pan-AKIs sensitize MM cells to TRAIL by inhibiting TRAIL-induced IAP proteins. (A) HMCLs were treated as described in panel A and endogenous cIAP1, cIAP2, XIAP and actin proteins were revealed by immunoblotting analysis. Bands were subjected to densitometric scanning and histograms represent the mean value ± SD of the ratio of cIPA1, cIAP2 or XIAP to actin normalized to the untreated control from blots of three independent experiments (*P< .01; **P< .001; Tukey-Kramer test). (**B**) Transfection of cIAP1 or cIAP2 or XIAP but not the non-specific control siRNA (CONT), led to a decrease in cIAP1 or cIAP2 or XIAP protein expression without affecting the levels of the unrelated protein actin. HMCLs were electroporated with cIAP1 or cIAP2 or XIAP or non-targeting siRNA (CONT). After 24 hours HMCLs were treated with TRAIL at the indicated doses for 24 hours. Values are mean ± SD of 3 independent experiments (*P< .05, **P<.01, Tukey-Kramer test).

Figure 9. Pan-AKIs and TRAIL combination therapy has potent antitumor activity in vivo against drug-resistant human multiple myeloma xenograft model.

(A) (i) When tumor size reached 250 mm^3, mice were randomly assigned (n=10/group) to receive vehicle alone, MK-0457 (50mg/kg), PHA-680632 (50mg/kg), TRAIL (300μg per mouse) or the combination MK-0457/TRAIL or PHA-680632/TRAIL. TRAIL was administered once on days 3 and 6. Pan-AKIs were administered once on each day for 11 days. Results are tumor volume, mean ± SD mm^3, plotted against time. (P< .001 MK-0457/TRAIL or PHA-680632/TRAIL versus either treatment alone; Dunnet test). Inset shows tumors resected from control (vehicle) and Pan-AKIs/TRAIL-treated mice after 11 days of treatment (endpoint). **(ii)** Kaplan-Meier survival curve was evaluated from the first day of treatment until death or sacrifice using JMP version 7.0 statistical software (SAS Institute, Cary, NC). Survival was significantly prolonged in MK-0457/TRAIL-treated animals versus control (*P*= .0015 after Bonferroni correction). The black bar on the abscissa represents the 11-day period of treatment. After six days of treatment, mice from each treatment group (n=3/group) were humanely killed, and the tumors were removed for assay. RPMI 8226/R5-derived tumors were analyzed by immunohistochemical staining for **(B)** phospho-Histone H3 (10x, 20x, and 40x magnification), **(C)** hematoxylin and eosin (H & E) and cleaved caspase-3 (4x, 10x and 20x magnification). The microphotographs shown are representative of similar observations in 3 different mice receiving the same treatment. **(D)** Tumor tissues from mice treated for six days were harvested and processed, and lysates were analyzed by immunoblotting analysis for PARP, cleaved-

PARP and cleaved caspase-3. Anti-actin immunoblotting was performed as loading control.

Figure 1


A
i
RPMI 8226
RPMI 8226/R5
OPM-2
U266
Effect
PHA+TRAIL
MK+TRAIL
CI = 1.0
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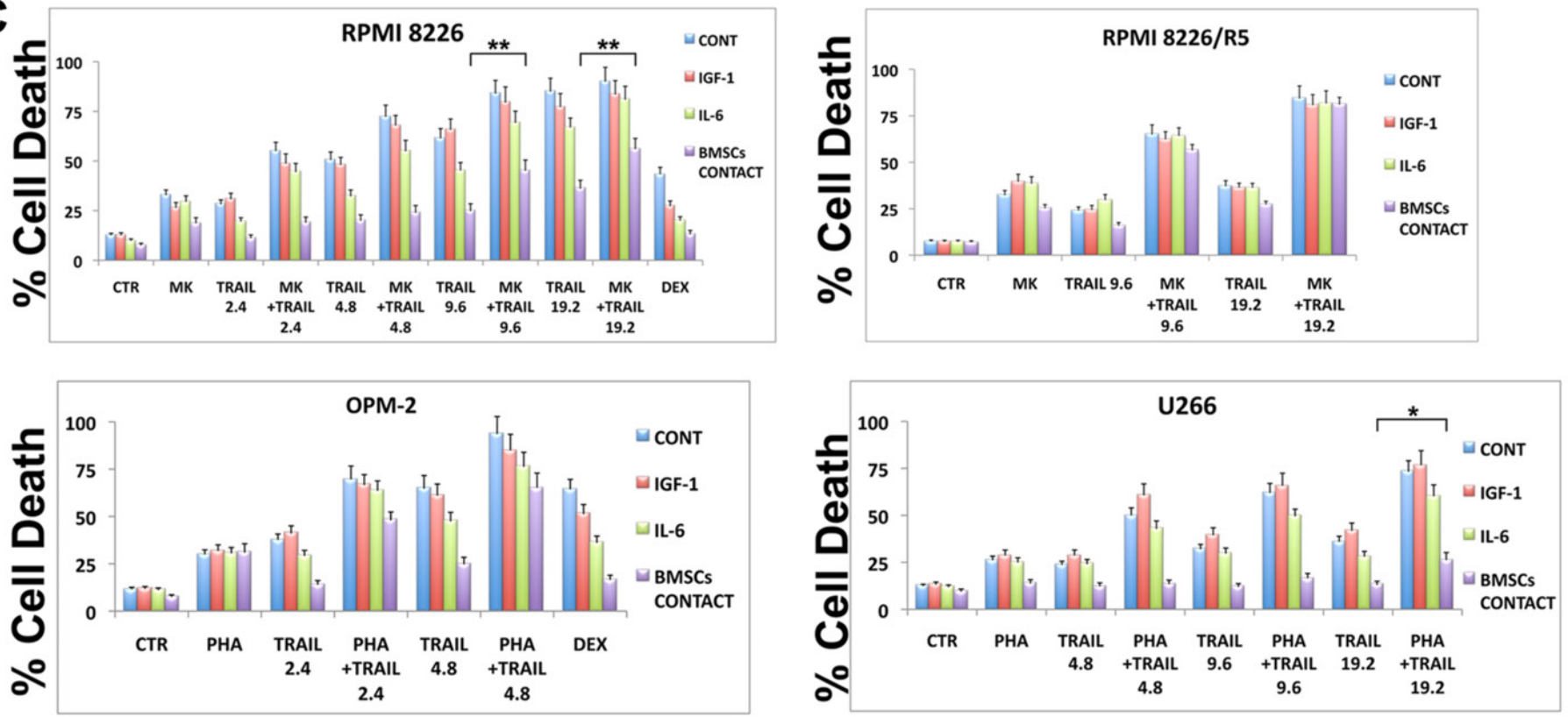
JJN3
% Apoptotic cells
CTR
MK
TRAIL
MK+ TRAIL
PHA
PHA+ TRAIL
B
CTR
MK-0457
TRAIL 9.6
MK+TRAIL 9.6
TRAIL 19.2
MK+TRAIL 19.2
% sub-G1
3.34
21.9
14.3
57.4
30.8
70
% Annexin V+
2.08
95.6
2
17.3
68.6
11.8
10.8
82.4
5.51
55
32.6
9.17
21.9
68.8
7.46
62.1
25.8
7.43
RPMI 8226/R5

Figure 1

C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% Cell Death
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75
50
25
0
**
**
CONT
IGF-1
IL-6
BMSCs CONTACT
CTR
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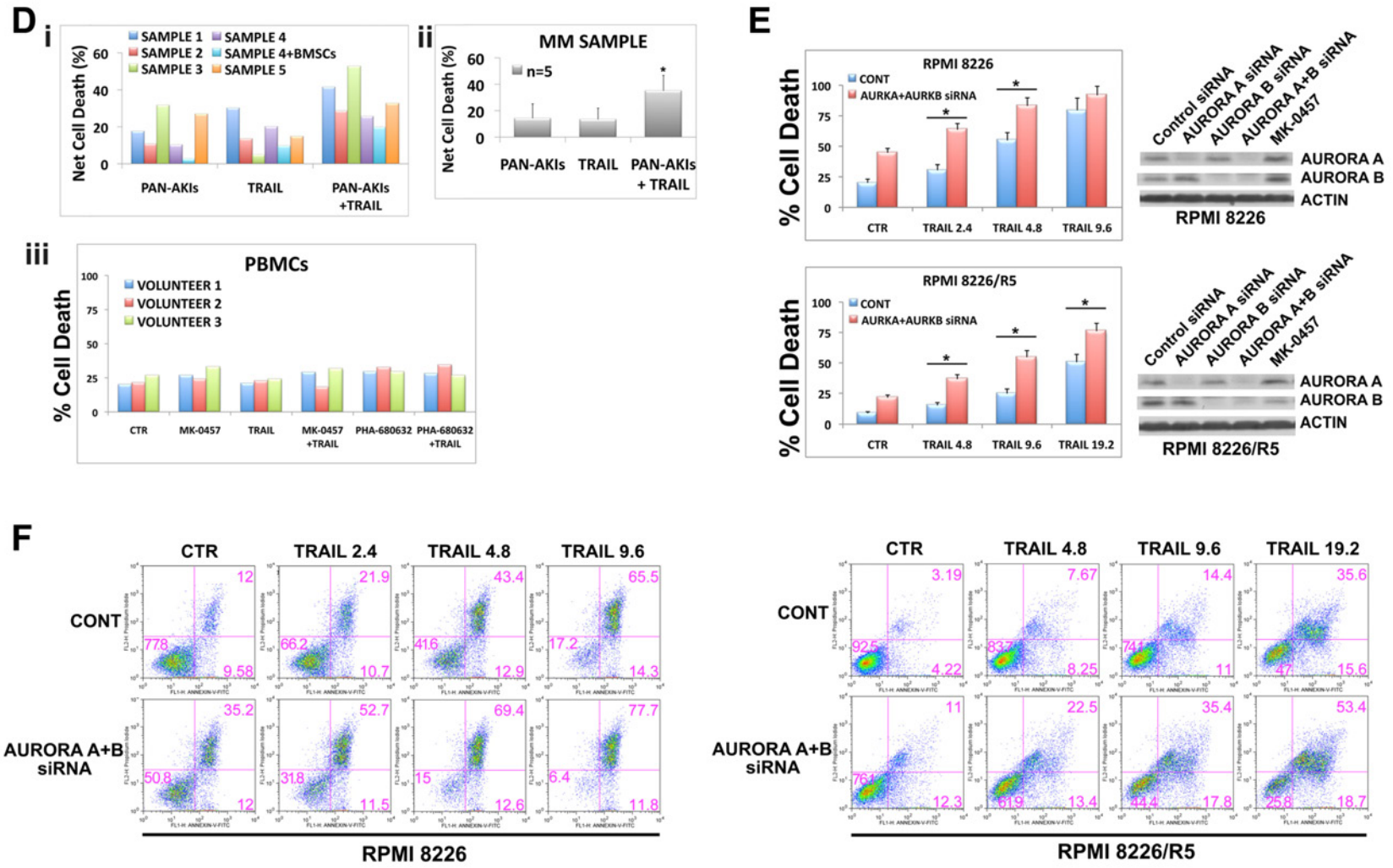
TRAIL 2.4
MK +TRAIL 2.4
TRAIL 4.8
MK +TRAIL 4.8
TRAIL 9.6
MK +TRAIL 9.6
TRAIL 19.2
MK +TRAIL 19.2
DEX
RPMI 8226/R5
% Cell Death
CONT
IGF-1
IL-6
BMSCs CONTACT
CTR
MK
TRAIL 9.6
MK +TRAIL 9.6
TRAIL 19.2
MK +TRAIL 19.2
OPM-2
% Cell Death
CONT
IGF-1
IL-6
BMSCs CONTACT
CTR
PHA
TRAIL 2.4
PHA +TRAIL 2.4
TRAIL 4.8
PHA +TRAIL 4.8
DEX
U266
% Cell Death
*
CONT
IGF-1
IL-6
BMSCs CONTACT
CTR
PHA
TRAIL 4.8
PHA +TRAIL 4.8
TRAIL 9.6
PHA +TRAIL 9.6
TRAIL 19.2
PHA +TRAIL 19.2

Figure 1


D
i
SAMPLE 1
SAMPLE 2
SAMPLE 3
SAMPLE 4
SAMPLE 4+BMSCs
SAMPLE 5
Net Cell Death (%)
PAN-AKIs
TRAIL
PAN-AKIs +TRAIL
ii
MM SAMPLE
n=5
Net Cell Death (%)
PAN-AKIs
TRAIL
PAN-AKIs + TRAIL
iii
PBMCs
VOLUNTEER 1
VOLUNTEER 2
VOLUNTEER 3
% Cell Death
CTR
MK-0457
TRAIL
MK-0457 +TRAIL
PHA-680632
PHA-680632 +TRAIL
E
RPMI 8226
CONT
AURKA+AURKB siRNA
% Cell Death
CTR
TRAIL 2.4
TRAIL 4.8
TRAIL 9.6
Control siRNA
AURORA A siRNA
AURORA B siRNA
AURORA A+B siRNA
MK-0457
AURORA A
AURORA B
ACTIN
RPMI 8226
RPMI 8226/R5
CONT
AURKA+AURKB siRNA
% Cell Death
CTR
TRAIL 4.8
TRAIL 9.6
TRAIL 19.2
Control siRNA
AURORA A siRNA
AURORA B siRNA
AURORA A+B siRNA
MK-0457
AURORA A
AURORA B
ACTIN
RPMI 8226/R5
F
CTR
TRAIL 2.4
TRAIL 4.8
TRAIL 9.6
CONT
AURORA A+B siRNA
12
778
9.58
35.2
50.8
12
21.9
66.2
10.7
52.7
31.8
11.5
43.4
41.6
12.9
69.4
15
12.6
65.5
17.2
14.3
77.7
6.4
11.8
RPMI 8226
CTR
TRAIL 4.8
TRAIL 9.6
TRAIL 19.2
CONT
AURORA A+B siRNA
3.19
92.5
4.22
11
76.1
12.3
7.67
83.7
8.25
22.5
61.9
13.4
14.4
74.1
11
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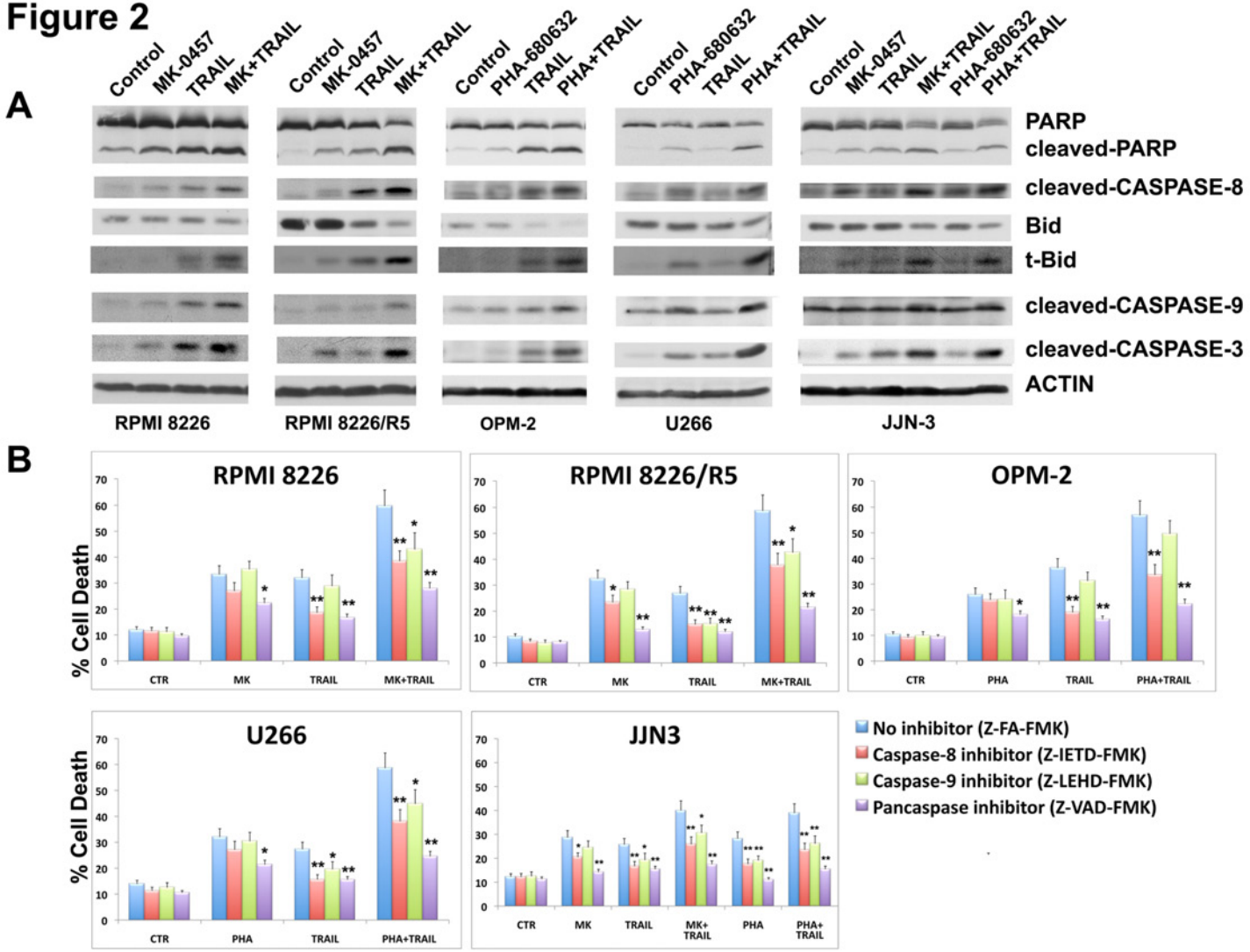
35.6
47
15.6
53.4
25.8
18.7
RPMI 8226/R5

Figure 2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Control
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MK+TRAIL
Control
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TRAIL
PHA+TRAIL
Control
MK-0457
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MK+TRAIL
PHA-680632
PHA+TRAIL
PARP
cleaved-PARP
cleaved-CASPASE-8
Bid
t-Bid
cleaved-CASPASE-9
cleaved-CASPASE-3
ACTIN
RPMI 8226
RPMI 8226/R5
OPM-2
U266
JJN-3
B
RPMI 8226
RPMI 8226/R5
OPM-2
U266
JJN3
% Cell Death
CTR
MK
TRAIL
MK+TRAIL
PHA
PHA+TRAIL
MK+ TRAIL
PHA+ TRAIL
No inhibitor (Z-FA-FMK)
Caspase-8 inhibitor (Z-IETD-FMK)
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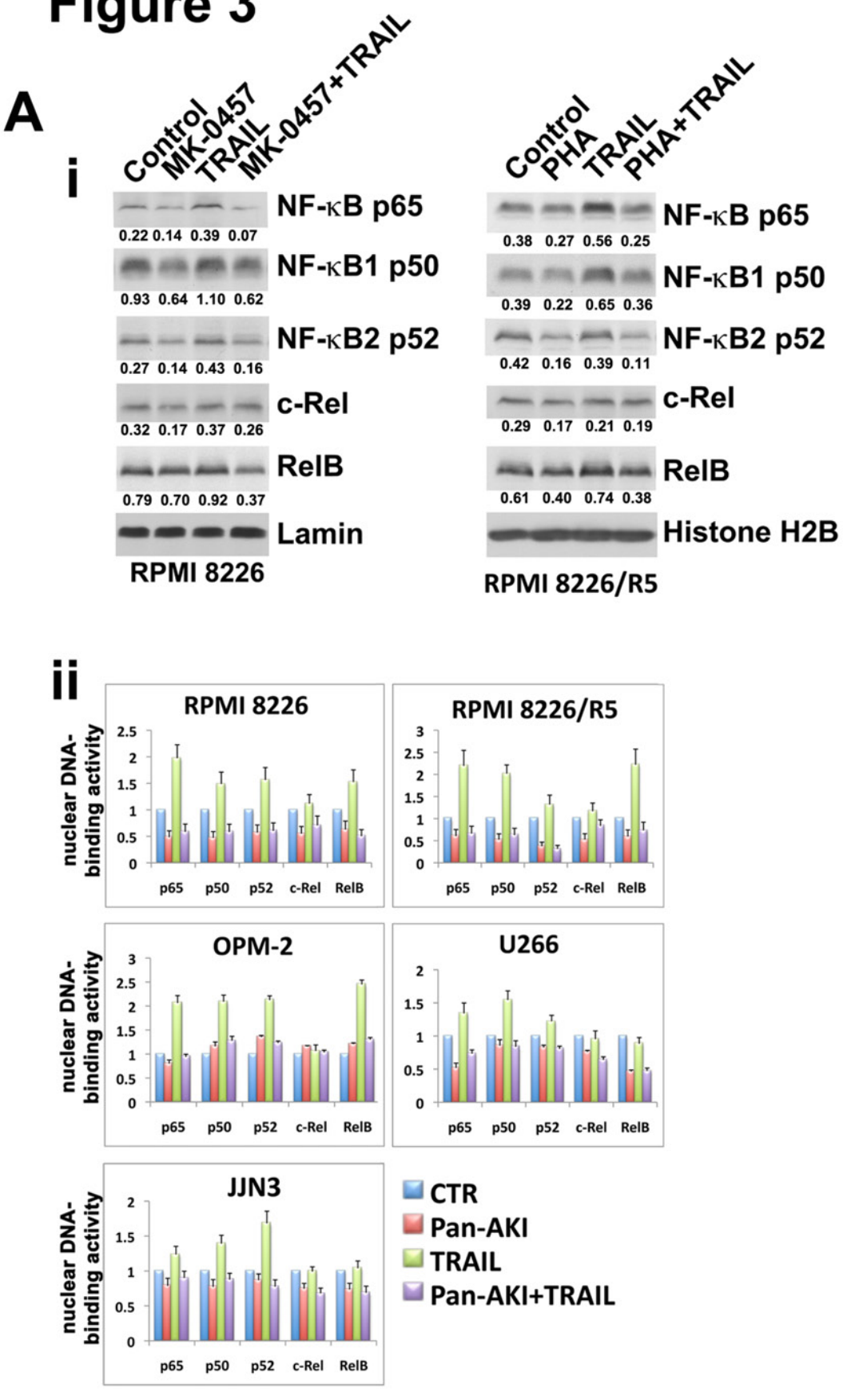
Pancaspase inhibitor (Z-VAD-FMK)

Figure 3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TRAIL
MK-0457+TRAIL
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NF-κB2 p52
0.27 0.14 0.43 0.16
c-Rel
0.32 0.17 0.37 0.26
RelB
0.79 0.70 0.92 0.37
Lamin
RPMI 8226
Control
PHA
TRAIL
PHA+TRAIL
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NF-κB1 p50
0.39 0.22 0.65 0.36
NF-κB2 p52
0.42 0.16 0.39 0.11
c-Rel
0.29 0.17 0.21 0.19
RelB
0.61 0.40 0.74 0.38
Histone H2B
RPMI 8226/R5
ii
RPMI 8226
RPMI 8226/R5
OPM-2
U266
JJN3
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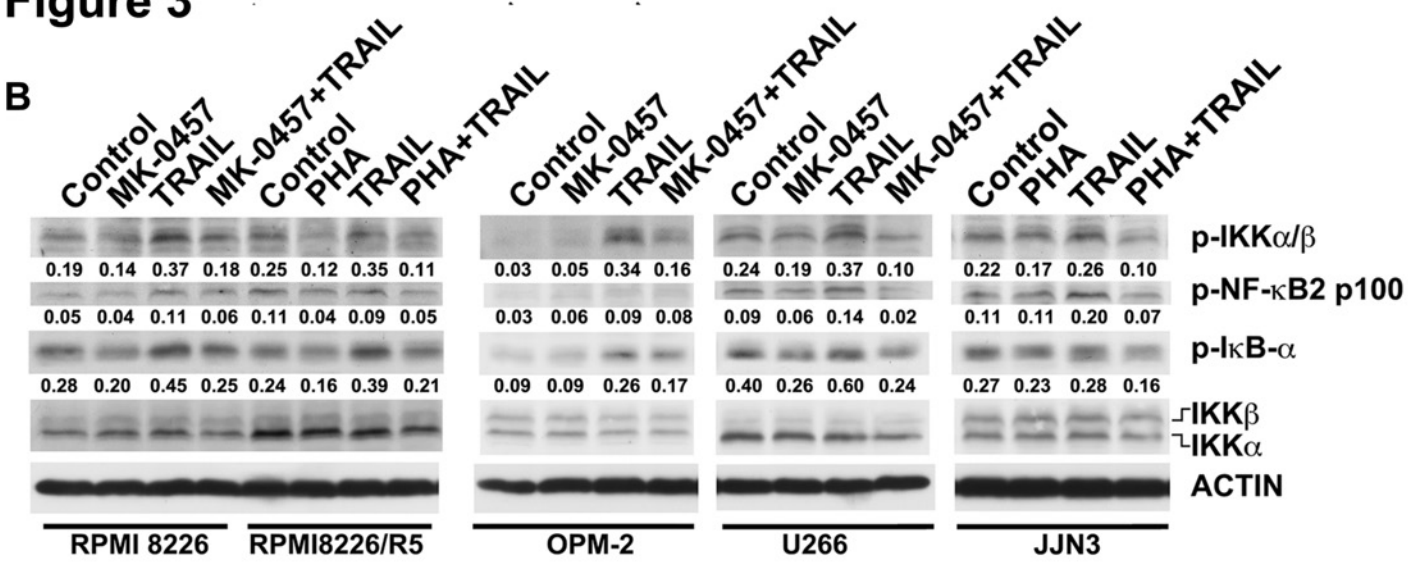
p50
p52
c-Rel
RelB
CTR
Pan-AKI
TRAIL
Pan-AKI+TRAIL

Figure 3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TRAIL
MK-0457+TRAIL
Control
PHA
TRAIL
PHA+TRAIL
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0.03 0.05 0.34 0.16
0.24 0.19 0.37 0.10
0.22 0.17 0.26 0.10
p-IKKα/β
0.05 0.04 0.11 0.06 0.11 0.04 0.09 0.05
0.03 0.06 0.09 0.08
0.09 0.06 0.14 0.02
0.11 0.11 0.20 0.07
p-NF-κB2 p100
0.28 0.20 0.45 0.25 0.24 0.16 0.39 0.21
0.09 0.09 0.26 0.17
0.40 0.26 0.60 0.24
0.27 0.23 0.28 0.16
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OPM-2
U266
JJN3

C

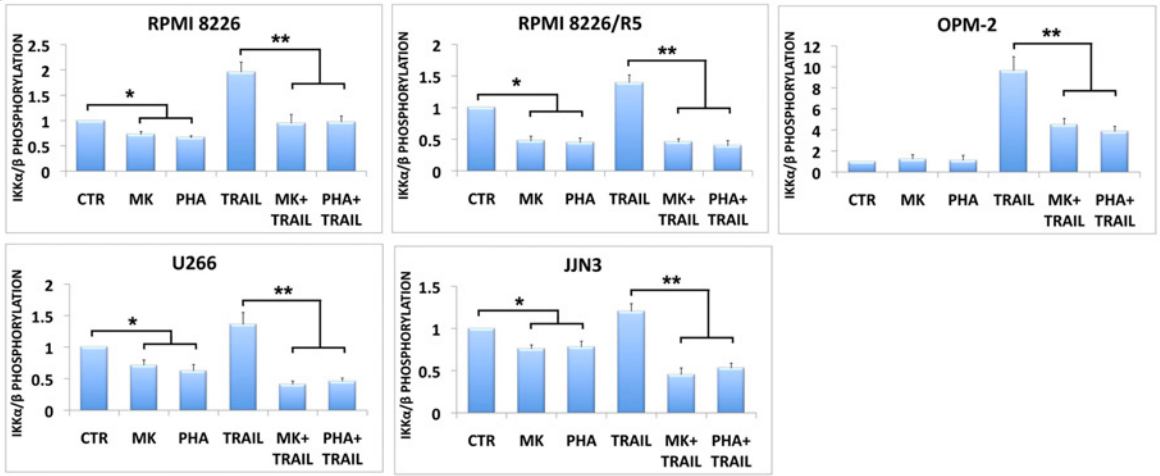
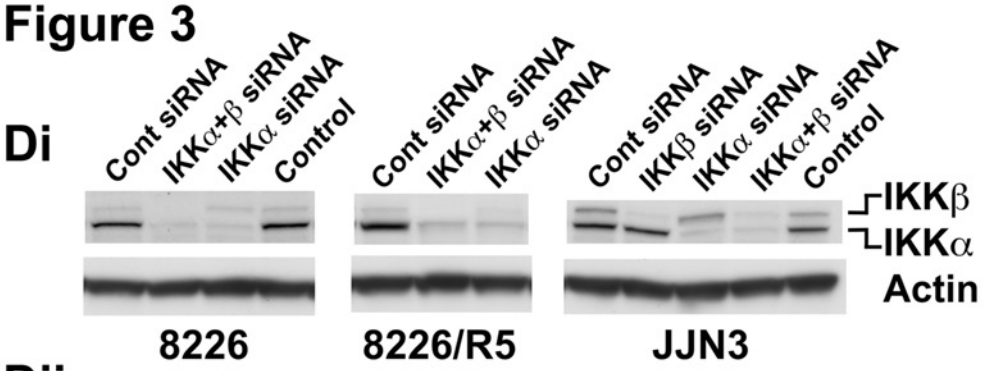

RPMI 8226
RPMI 8226/R5
OPM-2
U266
JJN3
IKKα/β PHOSPHORYLATION
CTR
MK
PHA
TRAIL
MK+ TRAIL
PHA+ TRAIL
*
**

Figure 3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Cont siRNA
IKKα+β siRNA
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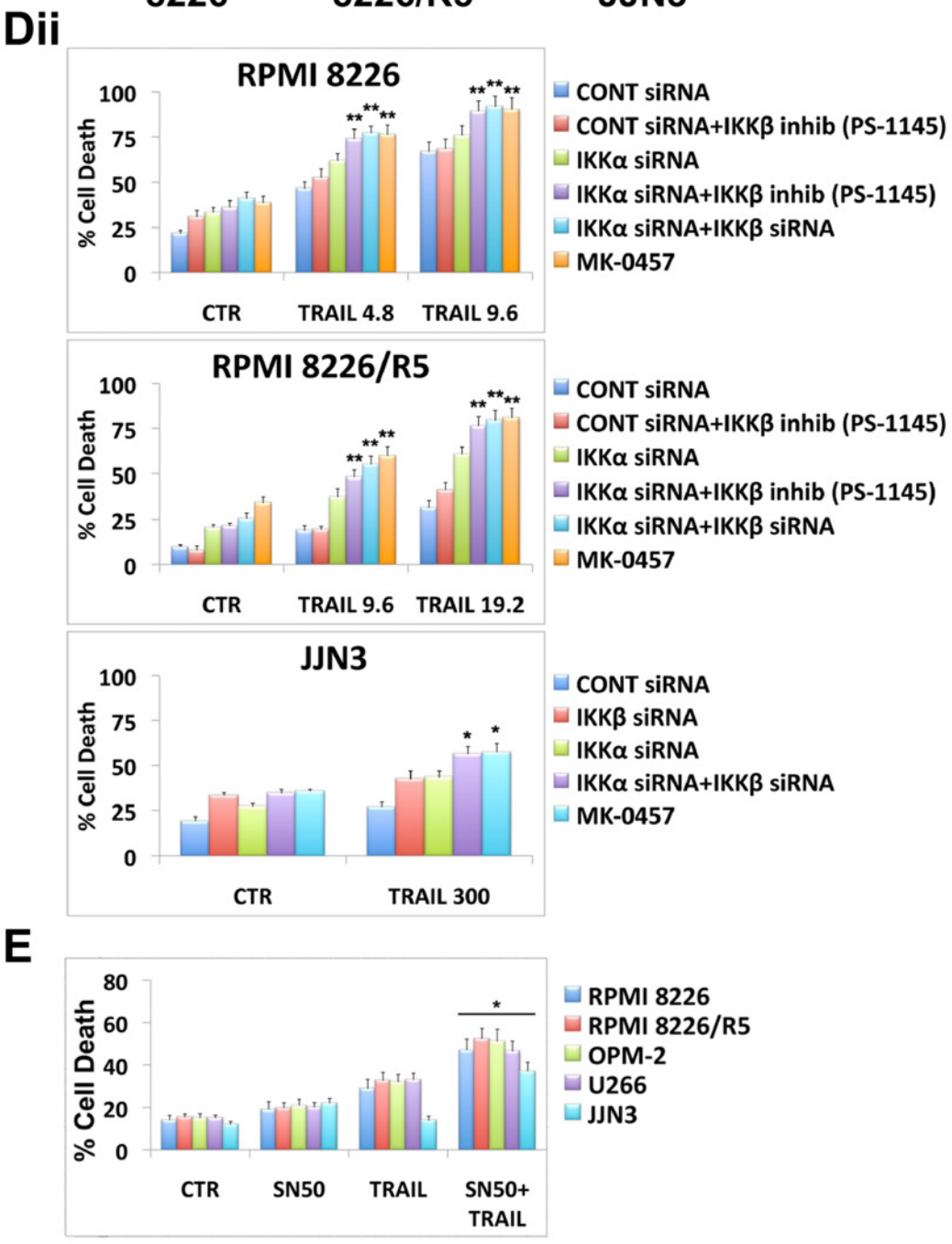
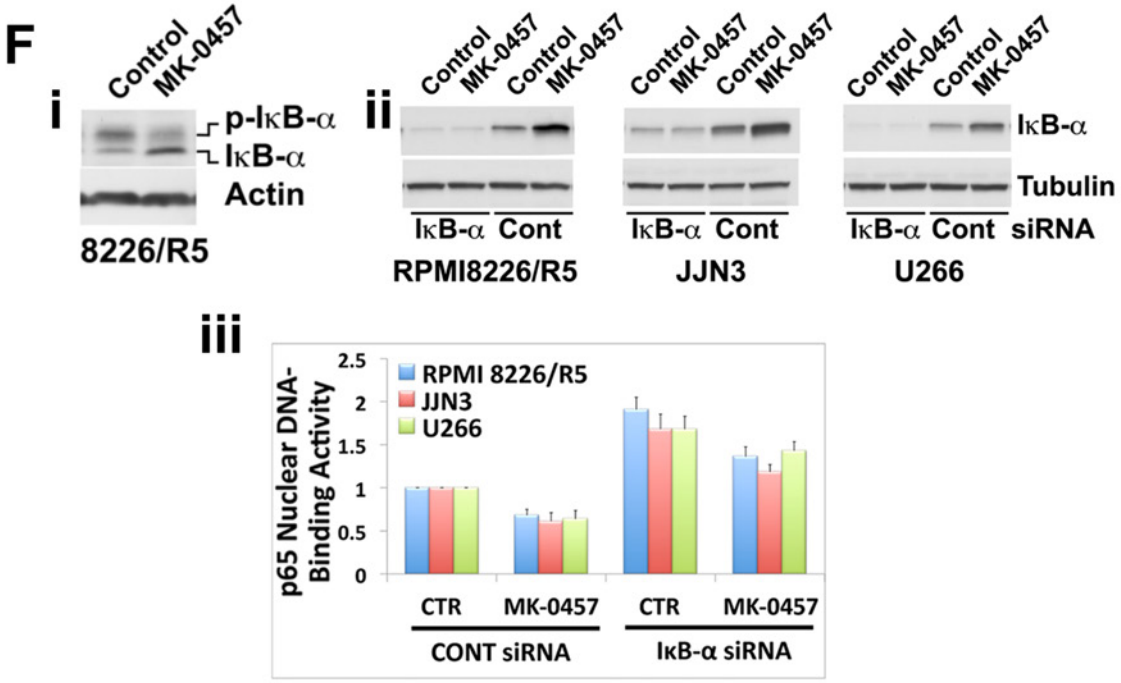
Control
IKKβ
IKKα
Actin
8226
8226/R5
JJN3


Dii
RPMI 8226
% Cell Death
100
75
50
25
0
CTR
TRAIL 4.8
TRAIL 9.6
CONT siRNA
CONT siRNA+IKKβ inhib (PS-1145)
IKKα siRNA
IKKα siRNA+IKKβ inhib (PS-1145)
IKKα siRNA+IKKβ siRNA
MK-0457
RPMI 8226/R5
% Cell Death
CTR
TRAIL 9.6
TRAIL 19.2
CONT siRNA
CONT siRNA+IKKβ inhib (PS-1145)
IKKα siRNA
IKKα siRNA+IKKβ inhib (PS-1145)
IKKα siRNA+IKKβ siRNA
MK-0457
JJN3
% Cell Death
CTR
TRAIL 300
CONT siRNA
IKKβ siRNA
IKKα siRNA
IKKα siRNA+IKKβ siRNA
MK-0457

E
% Cell Death
80
60
40
20
0
CTR
SN50
TRAIL
SN50+ TRAIL
RPMI 8226
RPMI 8226/R5
OPM-2
U266
JJN3

Figure 3


F
i
Control
MK-0457
p-IκB-α
IκB-α
Actin
8226/R5
ii
Control
MK-0457
Control
MK-0457
IκB-α
Cont
RPMI8226/R5
Control
MK-0457
Control
MK-0457
IκB-α
Cont
JJN3
Control
MK-0457
Control
MK-0457
IκB-α
Cont
siRNA
U266
iii
p65 Nuclear DNA-Binding Activity
2.5
2
1.5
1
0.5
0
RPMI 8226/R5
JJN3
U266
CTR
MK-0457
CTR
MK-0457
CONT siRNA
IκB-α siRNA

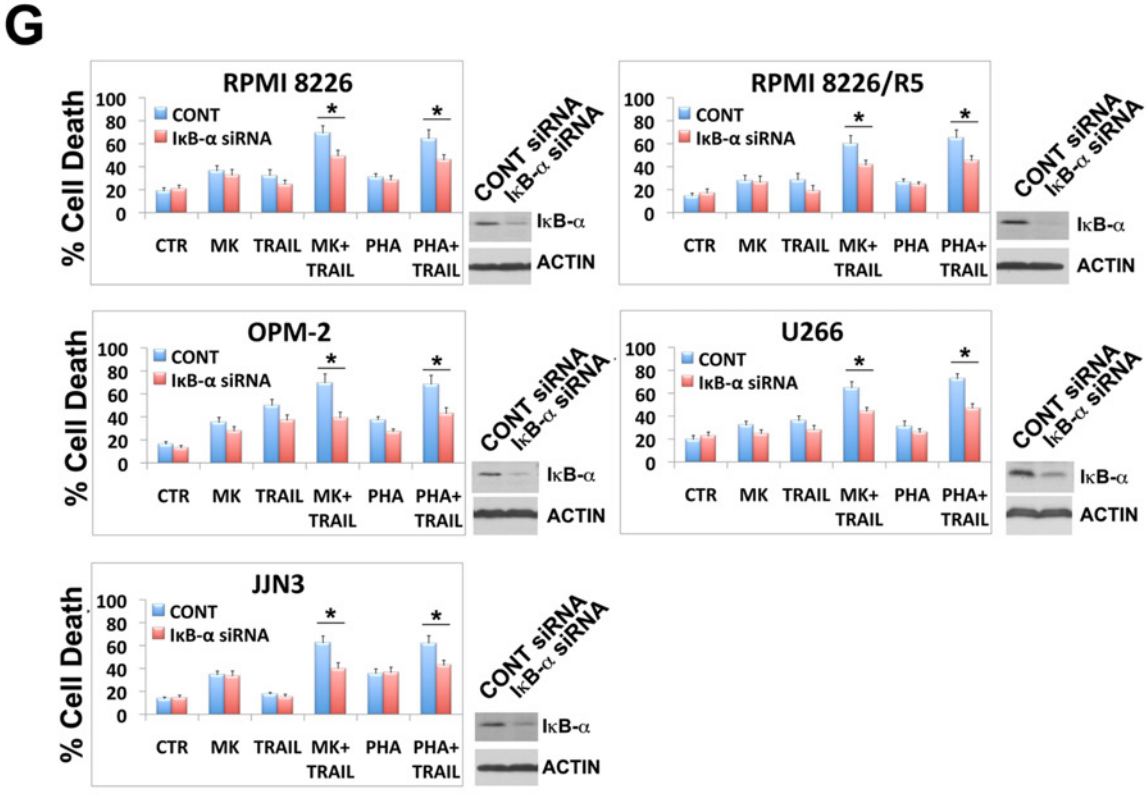

G
RPMI 8226
% Cell Death
CONT
IκB-α siRNA
CTR
MK
TRAIL
MK+ TRAIL
PHA
PHA+ TRAIL
CONT siRNA
IκB-α siRNA
IκB-α
ACTIN
RPMI 8226/R5
OPM-2
U266
JJN3

Figure 3

H

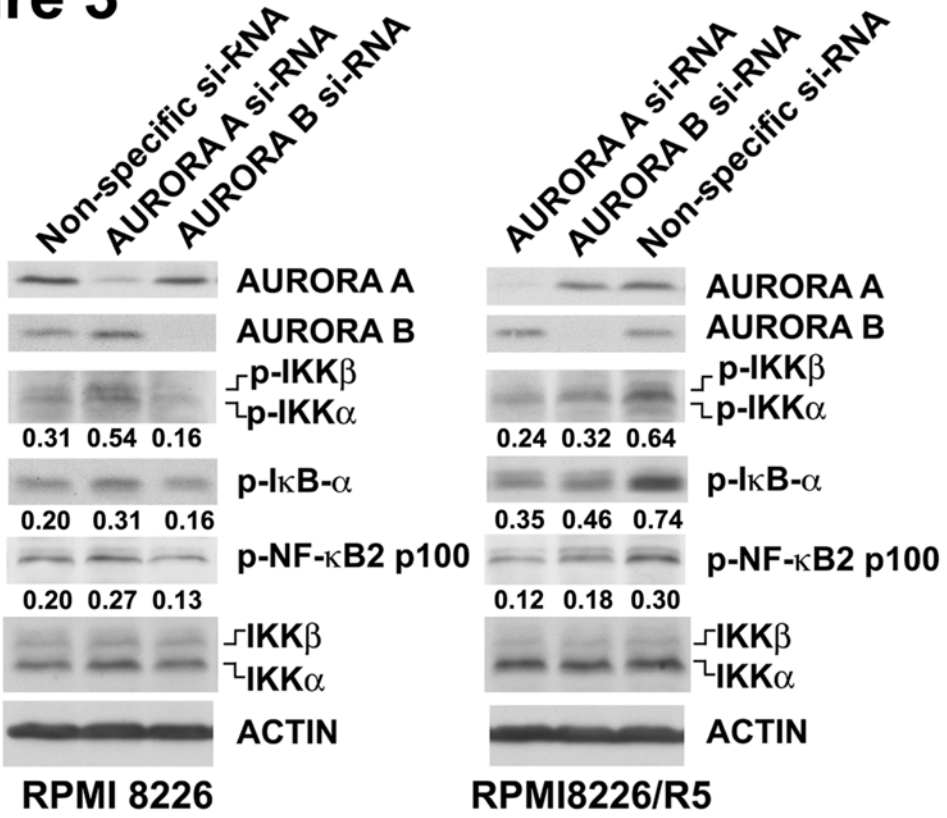

Non-specific si-RNA
AURORA A si-RNA
AURORA B si-RNA
AURORA A
AURORA B
p-IKKβ
p-IKKα
0.31 0.54 0.16
p-IκB-α
0.20 0.31 0.16
p-NF-κB2 p100
0.20 0.27 0.13
IKKβ
IKKα
ACTIN
RPMI 8226
AURORA A si-RNA
AURORA B si-RNA
Non-specific si-RNA
AURORA A
AURORA B
p-IKKβ
p-IKKα
0.24 0.32 0.64
p-IκB-α
0.35 0.46 0.74
p-NF-κB2 p100
0.12 0.18 0.30
IKKβ
IKKα
ACTIN
RPMI8226/R5

Figure 4

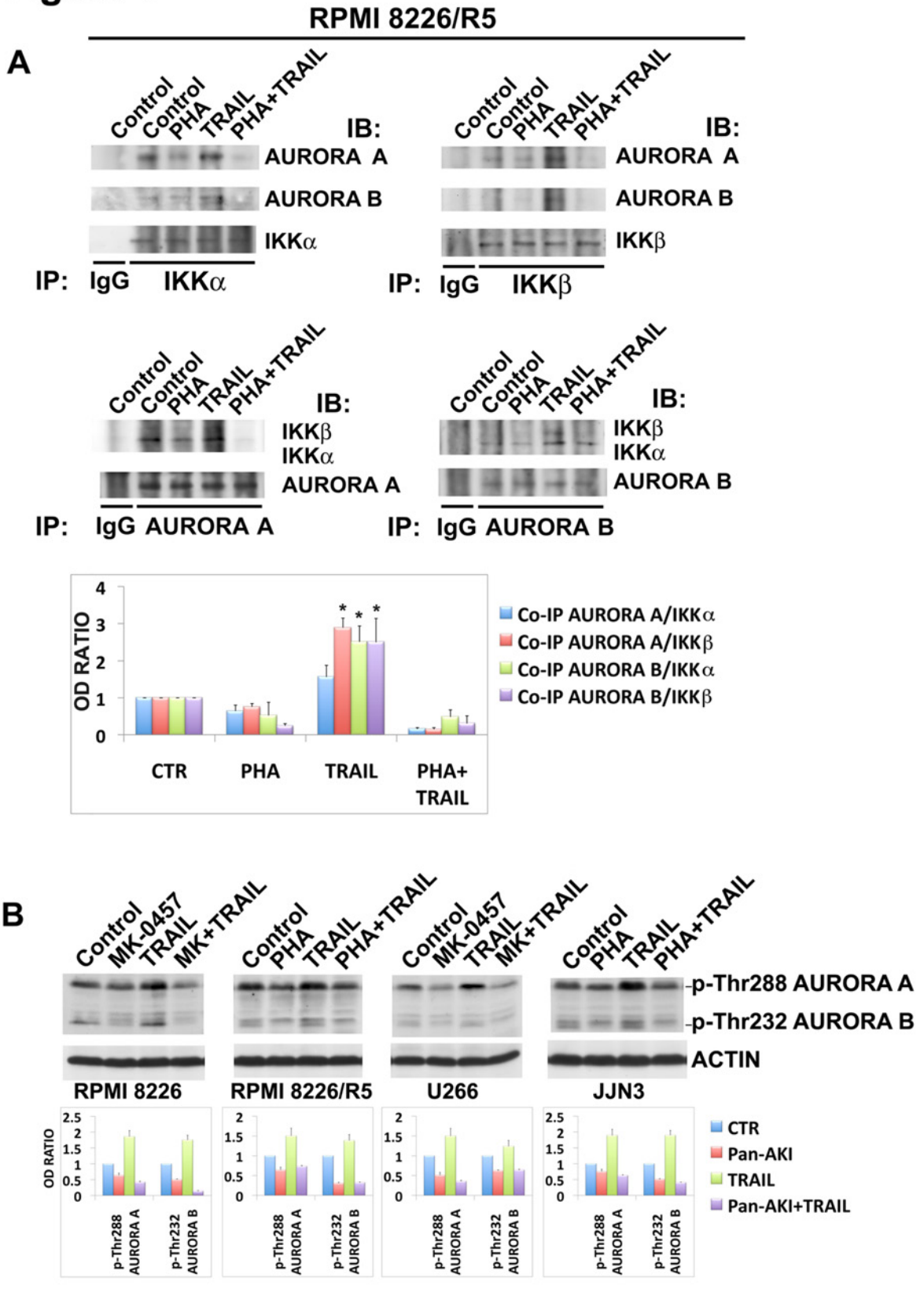

RPMI 8226/R5
A
Control
Control
PHA
TRAIL
PHA+TRAIL
IB:
AURORA A
AURORA B
IKKα
IKKβ
IP: IgG IKKα
IP: IgG IKKβ
IKKβ
IKKα
IP: IgG AURORA A
IP: IgG AURORA B
OD RATIO
CTR
PHA
TRAIL
PHA+ TRAIL
Co-IP AURORA A/IKKα
Co-IP AURORA A/IKKβ
Co-IP AURORA B/IKKα
Co-IP AURORA B/IKKβ
B
MK-0457
MK+TRAIL
p-Thr288 AURORA A
p-Thr232 AURORA B
ACTIN
RPMI 8226
RPMI 8226/R5
U266
JJN3
p-Thr288 AURORA A
p-Thr232 AURORA B
CTR
Pan-AKI
TRAIL
Pan-AKI+TRAIL

Figure 5

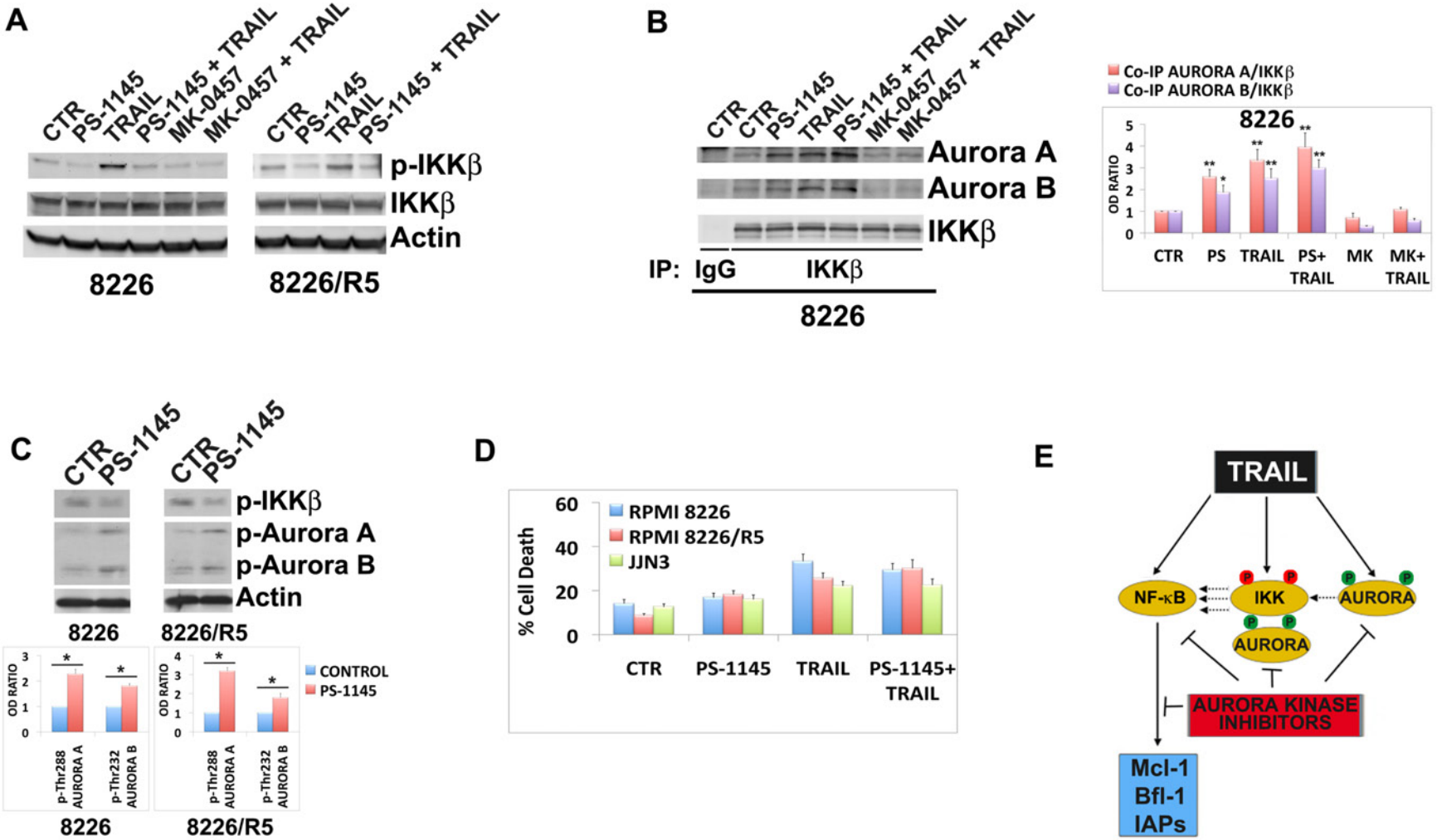

A
CTR
PS-1145
TRAIL
PS-1145 + TRAIL
MK-0457
MK-0457 + TRAIL
p-IKKβ
IKKβ
Actin
8226
8226/R5
B
Aurora A
Aurora B
IKKβ
IP: IgG IKKβ
8226
Co-IP AURORA A/IKKβ
Co-IP AURORA B/IKKβ
OD RATIO
CTR
PS
TRAIL
PS+ TRAIL
MK
MK+ TRAIL
C
p-IKKβ
p-Aurora A
p-Aurora B
Actin
CONTROL
PS-1145
p-Thr288 AURORA A
p-Thr232 AURORA B
D
% Cell Death
RPMI 8226
RPMI 8226/R5
JJN3
E
TRAIL
NF-κB
IKK
AURORA
AURORA KINASE INHIBITORS
Mcl-1
Bfl-1
IAPs

Figure 6

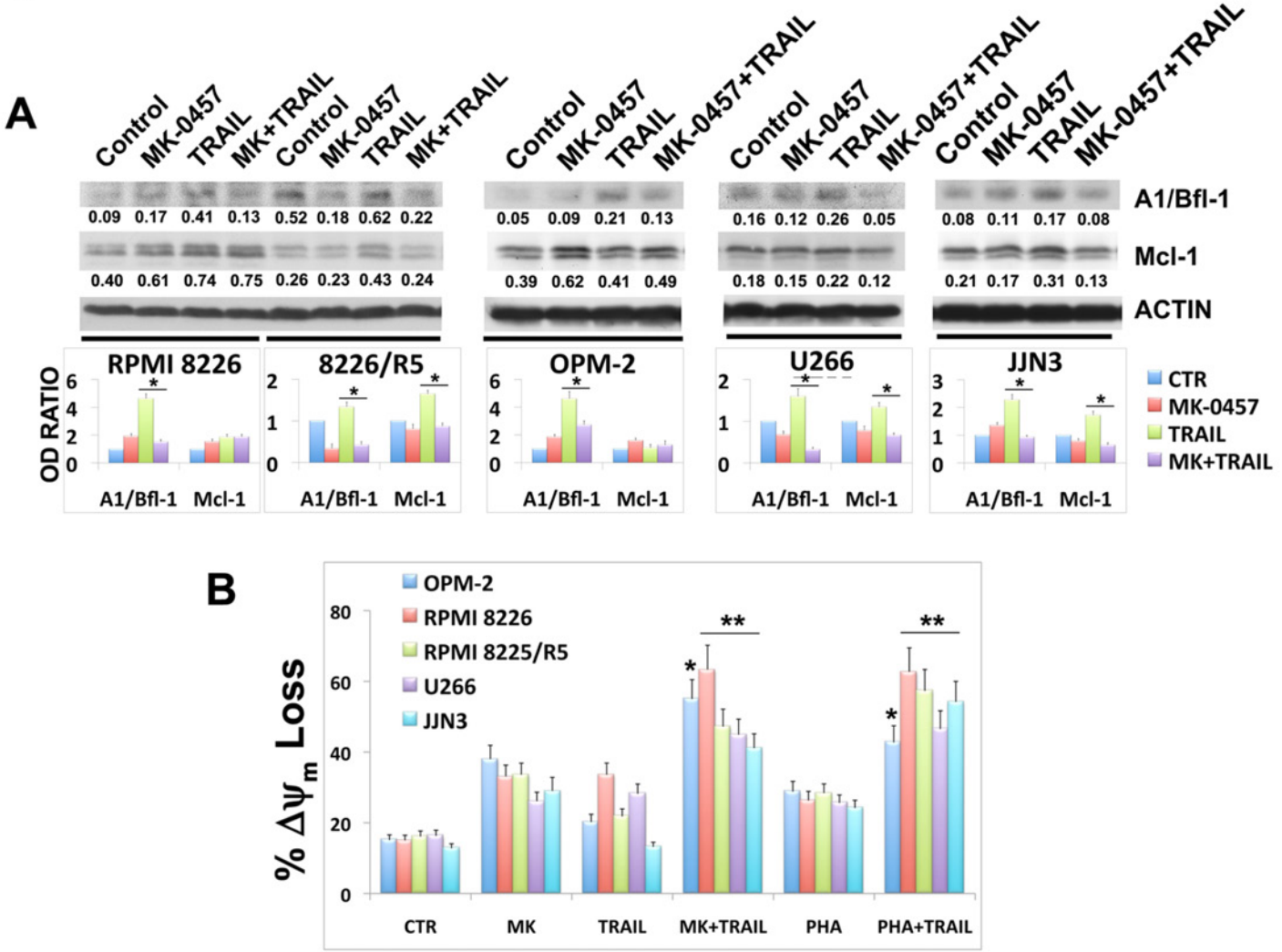

A
Control
MK-0457
TRAIL
MK+TRAIL
MK-0457+TRAIL
0.09 0.17 0.41 0.13 0.52 0.18 0.62 0.22
0.05 0.09 0.21 0.13
0.16 0.12 0.26 0.05
0.08 0.11 0.17 0.08
A1/Bfl-1
0.40 0.61 0.74 0.75 0.26 0.23 0.43 0.24
0.39 0.62 0.41 0.49
0.18 0.15 0.22 0.12
0.21 0.17 0.31 0.13
Mcl-1
ACTIN
RPMI 8226
8226/R5
OPM-2
U266
JJN3
OD RATIO
A1/Bfl-1
Mcl-1
CTR
MK-0457
TRAIL
MK+TRAIL
B
OPM-2
RPMI 8226
RPMI 8225/R5
U266
JJN3
% Δψm Loss
CTR
MK
TRAIL
MK+TRAIL
PHA
PHA+TRAIL

Figure 6

C

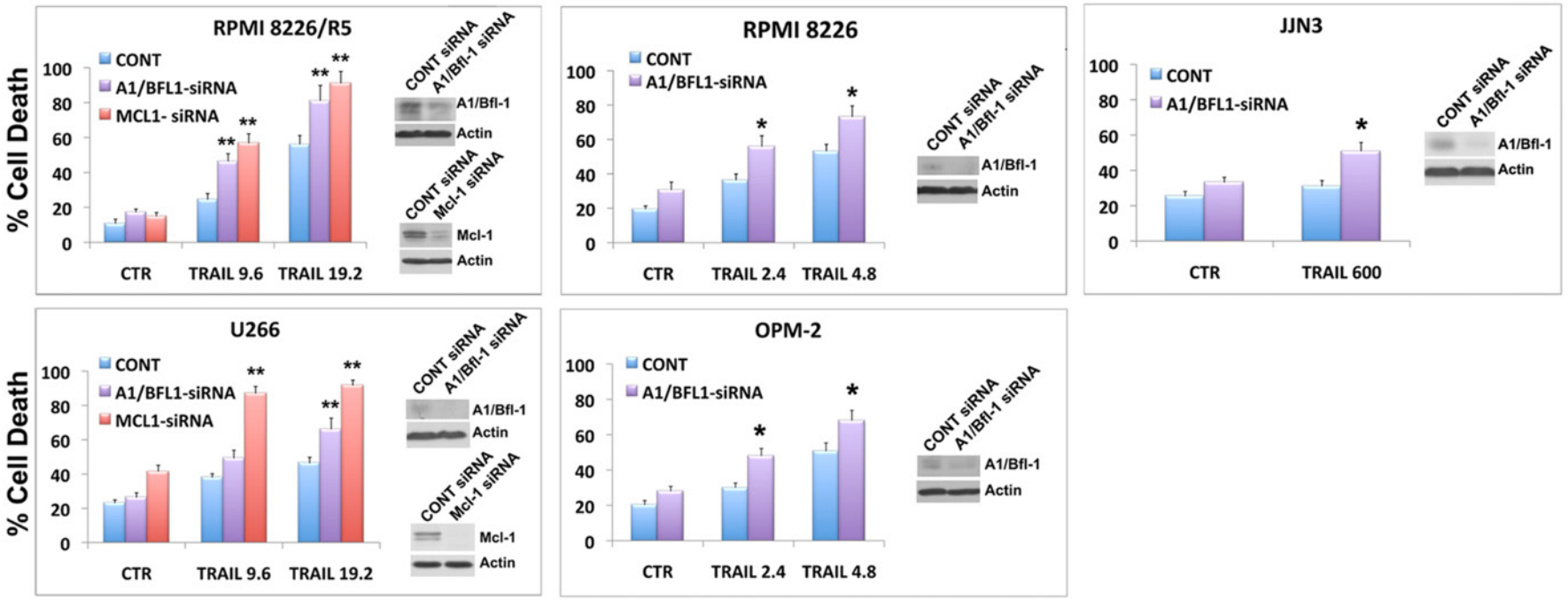

RPMI 8226/R5
% Cell Death
CONT
A1/BFL1-siRNA
MCL1- siRNA
CTR
TRAIL 9.6
TRAIL 19.2
CONT siRNA
A1/Bfl-1 siRNA
A1/Bfl-1
Actin
Mcl-1 siRNA
Mcl-1
RPMI 8226
MCL1-siRNA
TRAIL 2.4
TRAIL 4.8
JJN3
TRAIL 600
U266
OPM-2

Figure 6

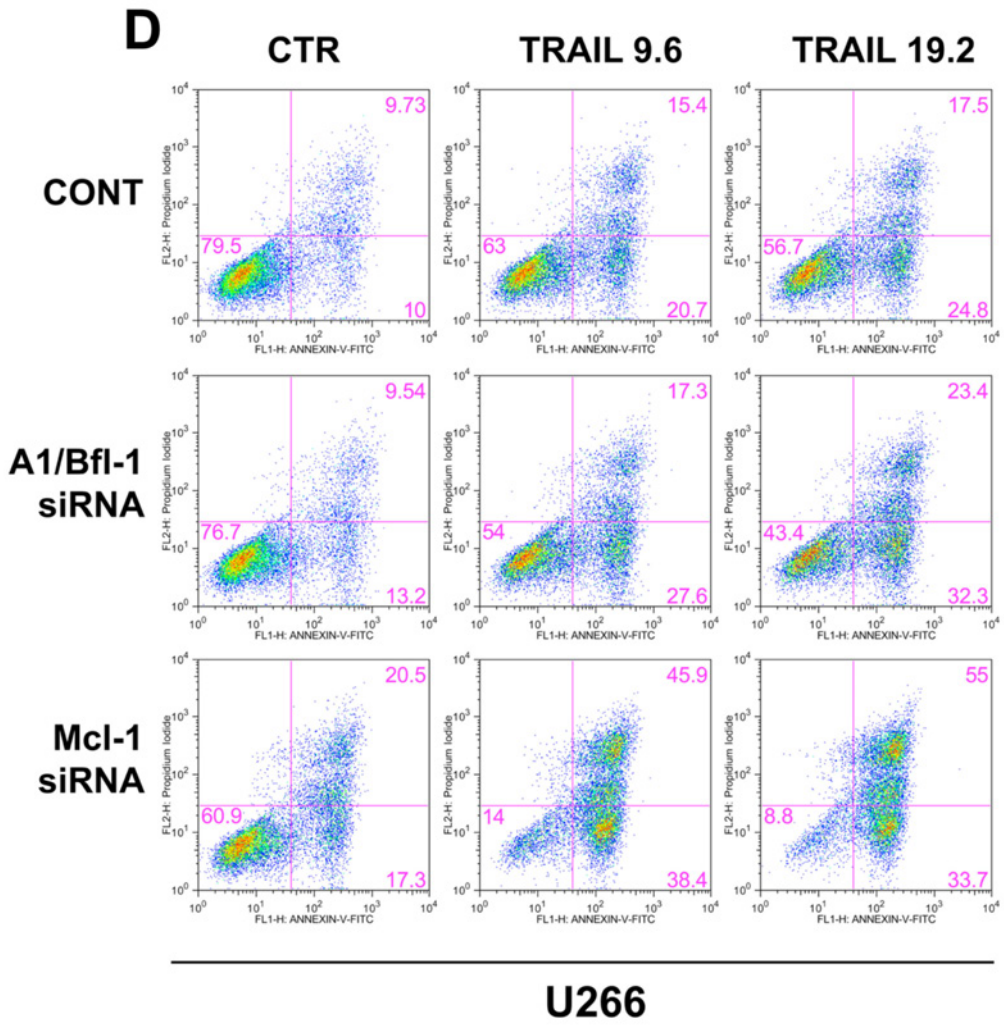
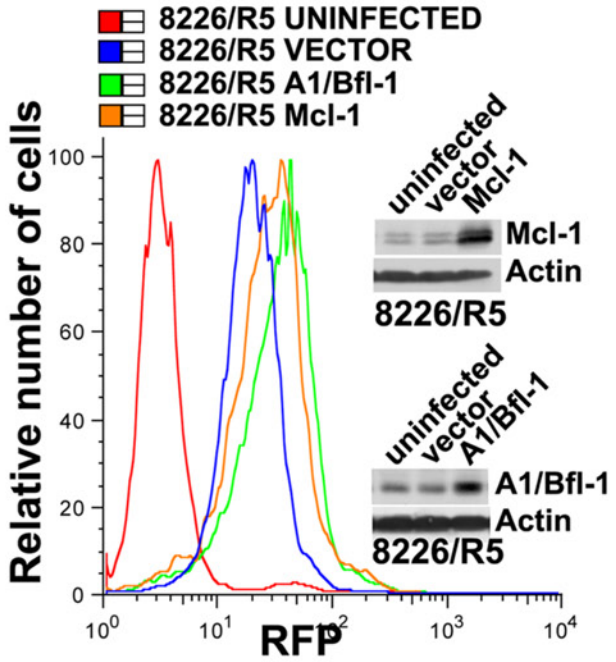

D
CTR
TRAIL 9.6
TRAIL 19.2
CONT
A1/Bfl-1 siRNA
Mcl-1 siRNA
9.73
79.5
10
15.4
63
20.7
17.5
56.7
24.8
9.54
76.7
13.2
17.3
54
27.6
23.4
43.4
32.3
20.5
60.9
17.3
45.9
14
38.4
55
8.8
33.7
FL2-H: Propidium Iodide
FL1-H: ANNEXIN-V-FITC
U266

Figure 6

E


8226/R5 UNINFECTED
8226/R5 VECTOR
8226/R5 A1/Bfl-1
8226/R5 Mcl-1
Relative number of cells
RFP
uninfected
vector
Mcl-1
Mcl-1
Actin
8226/R5
uninfected
vector
A1/Bfl-1
A1/Bfl-1
Actin
8226/R5

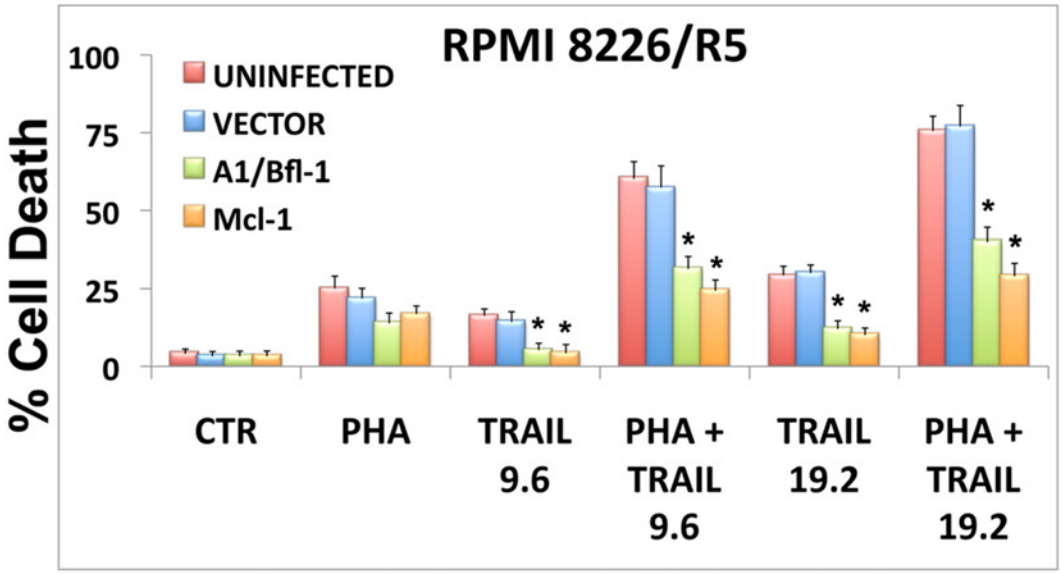

RPMI 8226/R5
% Cell Death
UNINFECTED
VECTOR
A1/Bfl-1
Mcl-1
CTR
PHA
TRAIL 9.6
PHA + TRAIL 9.6
TRAIL 19.2
PHA + TRAIL 19.2

Figure 7

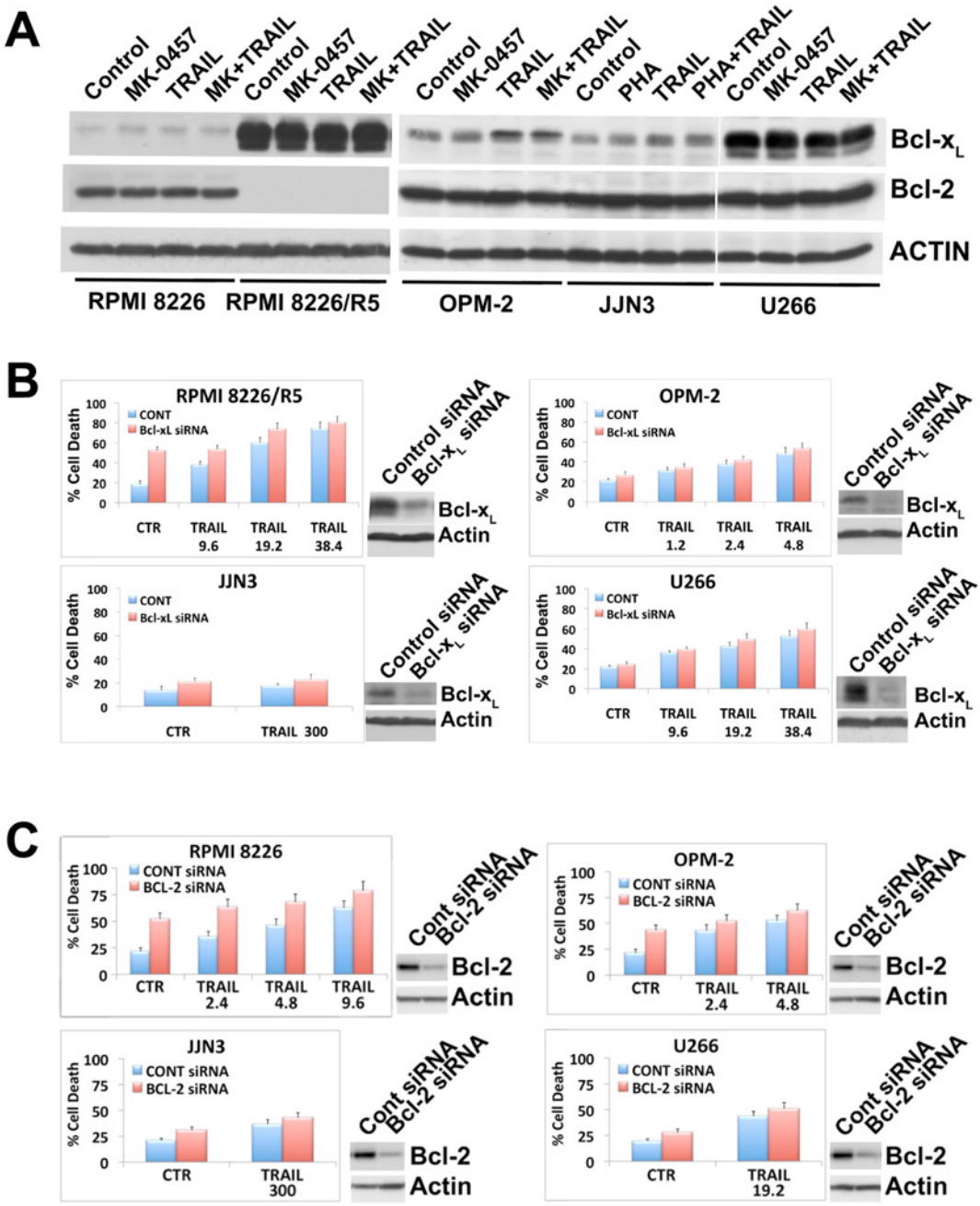

A
Control
MK-0457
TRAIL
MK+TRAIL
Control
MK-0457
TRAIL
MK+TRAIL
Control
MK-0457
TRAIL
MK+TRAIL
Control
PHA
TRAIL
PHA+TRAIL
Control
MK-0457
TRAIL
MK+TRAIL
Bcl-x$_L$
Bcl-2
ACTIN
RPMI 8226
RPMI 8226/R5
OPM-2
JJN3
U266
B
RPMI 8226/R5
% Cell Death
CONT
Bcl-xL siRNA
CTR
TRAIL 9.6
TRAIL 19.2
TRAIL 38.4
Control siRNA
Bcl-x$_L$ siRNA
Bcl-x$_L$
Actin
OPM-2
CTR
TRAIL 1.2
TRAIL 2.4
TRAIL 4.8
JJN3
CTR
TRAIL 300
U266
CTR
TRAIL 9.6
TRAIL 19.2
TRAIL 38.4
C
RPMI 8226
% Cell Death
CONT siRNA
BCL-2 siRNA
CTR
TRAIL 2.4
TRAIL 4.8
TRAIL 9.6
Cont siRNA
Bcl-2 siRNA
Bcl-2
Actin
OPM-2
CTR
TRAIL 2.4
TRAIL 4.8
JJN3
CTR
TRAIL 300
U266
CTR
TRAIL 19.2

Figure 7

D

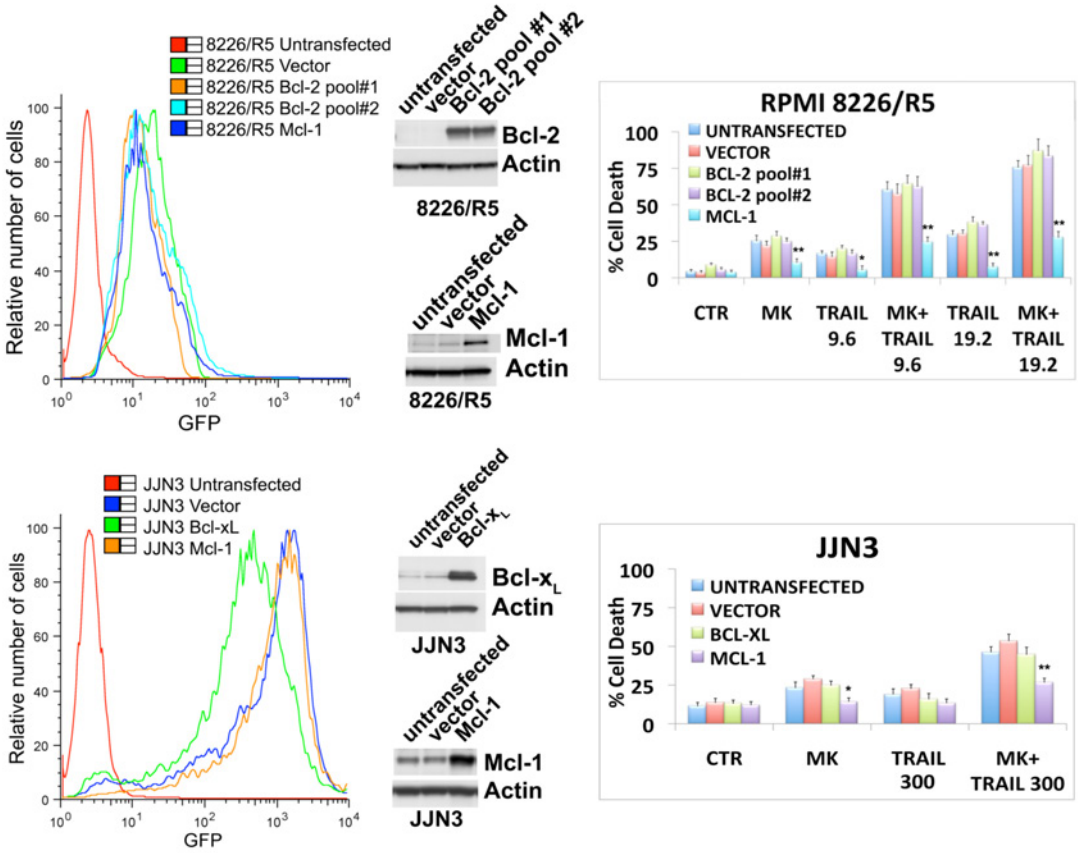

8226/R5 Untransfected
8226/R5 Vector
8226/R5 Bcl-2 pool#1
8226/R5 Bcl-2 pool#2
8226/R5 Mcl-1
Relative number of cells
GFP
untransfected
vector
Bcl-2 pool #1
Bcl-2 pool #2
Bcl-2
Actin
8226/R5
untransfected
vector
Mcl-1
Mcl-1
Actin
8226/R5
RPMI 8226/R5
UNTRANSFECTED
VECTOR
BCL-2 pool#1
BCL-2 pool#2
MCL-1
% Cell Death
CTR
MK
TRAIL 9.6
MK+ TRAIL 9.6
TRAIL 19.2
MK+ TRAIL 19.2
JJN3 Untransfected
JJN3 Vector
JJN3 Bcl-xL
JJN3 Mcl-1
Relative number of cells
GFP
untransfected
vector
Bcl-xL
Bcl-xL
Actin
JJN3
untransfected
vector
Mcl-1
Mcl-1
Actin
JJN3
JJN3
UNTRANSFECTED
VECTOR
BCL-XL
MCL-1
% Cell Death
CTR
MK
TRAIL 300
MK+ TRAIL 300

Figure 8

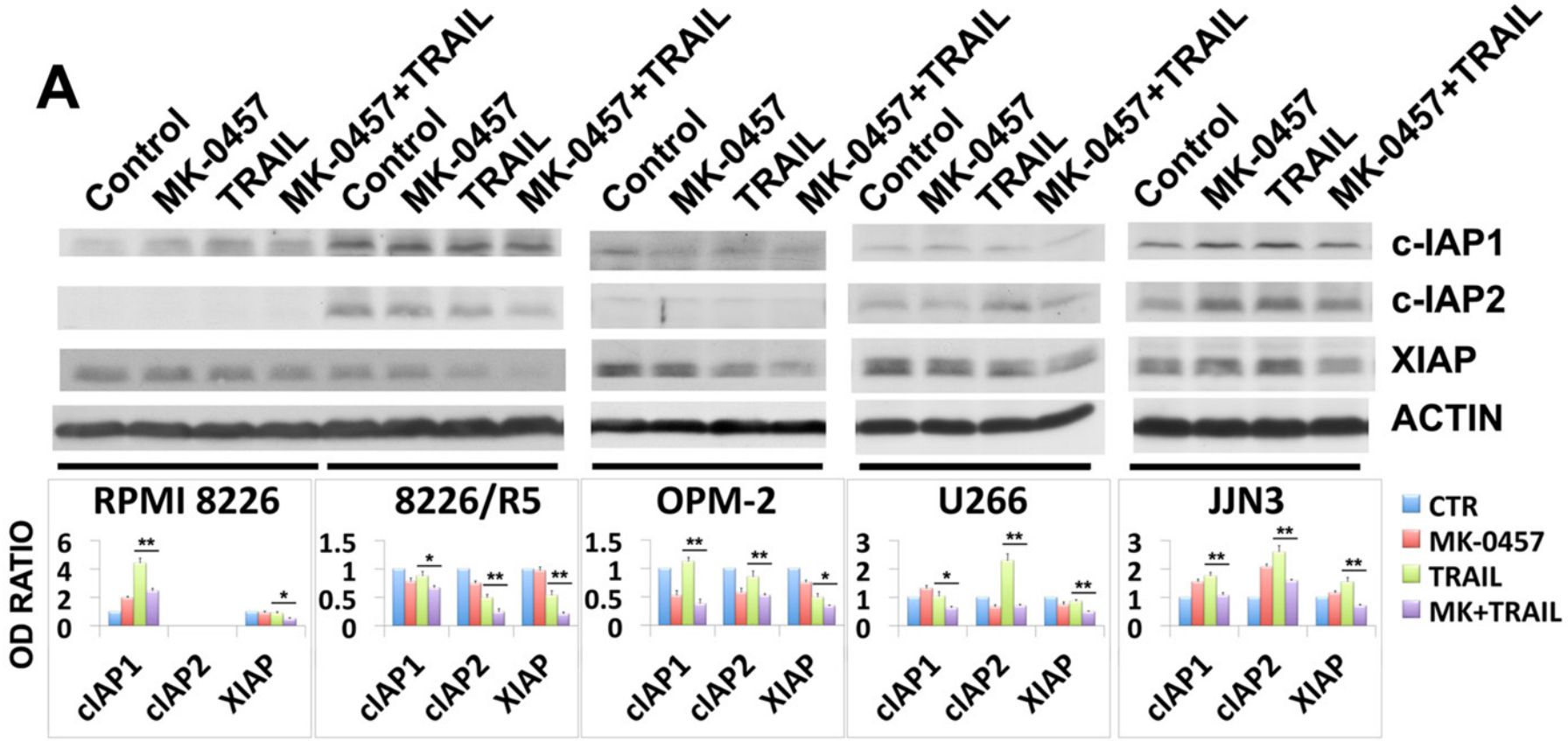

A
Control
MK-0457
TRAIL
MK-0457+TRAIL
c-IAP1
c-IAP2
XIAP
ACTIN
RPMI 8226
8226/R5
OPM-2
U266
JJN3
OD RATIO
cIAP1
cIAP2
XIAP
CTR
MK-0457
TRAIL
MK+TRAIL

Figure 8

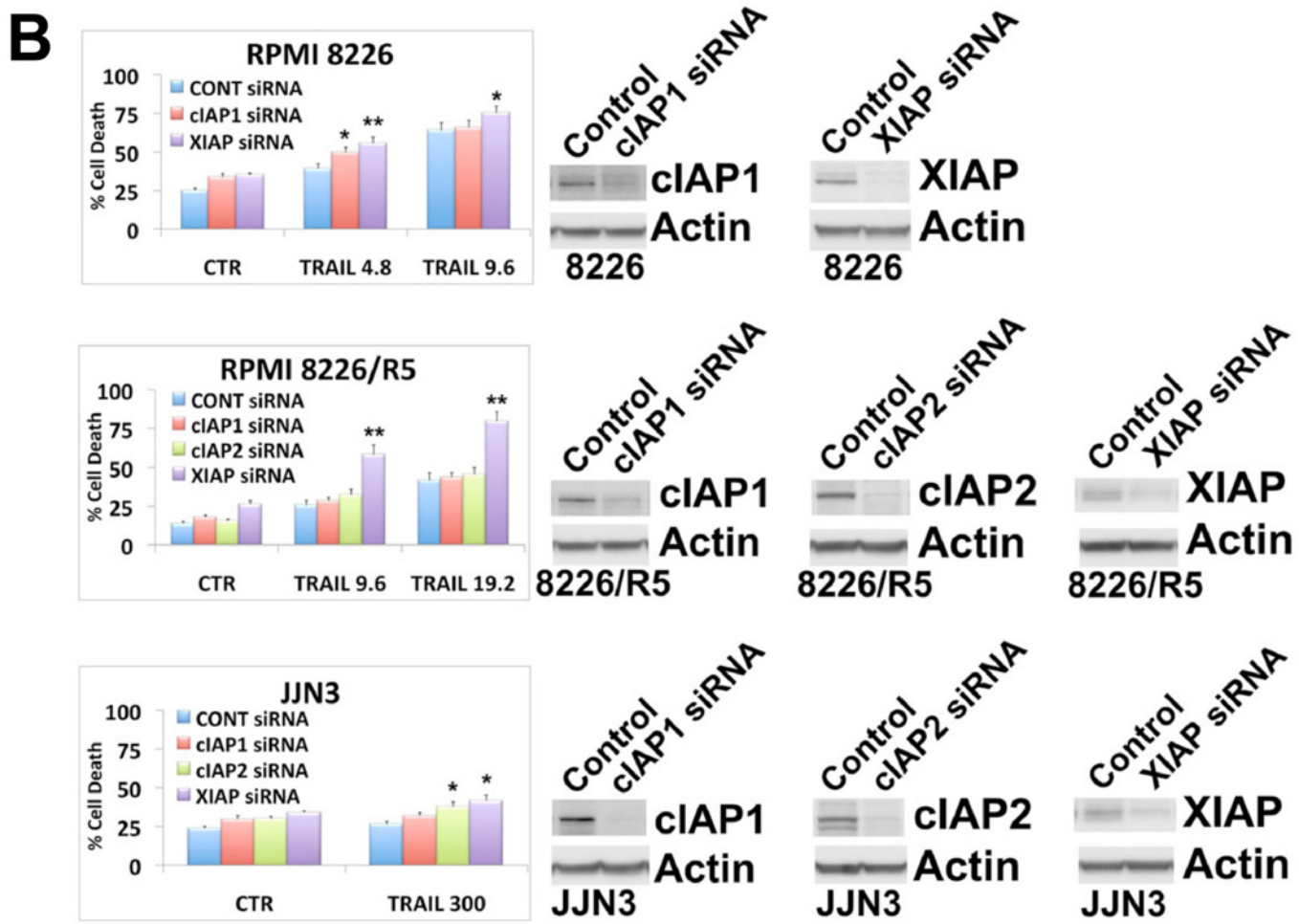

B
RPMI 8226
% Cell Death
100
75
50
25
0
CONT siRNA
cIAP1 siRNA
XIAP siRNA
*
**
*
CTR
TRAIL 4.8
TRAIL 9.6
Control
cIAP1 siRNA
cIAP1
Actin
8226
Control
XIAP siRNA
XIAP
Actin
8226
RPMI 8226/R5
% Cell Death
100
75
50
25
0
CONT siRNA
cIAP1 siRNA
cIAP2 siRNA
XIAP siRNA
**
**
CTR
TRAIL 9.6
TRAIL 19.2
Control
cIAP1 siRNA
cIAP1
Actin
8226/R5
Control
cIAP2 siRNA
cIAP2
Actin
8226/R5
Control
XIAP siRNA
XIAP
Actin
8226/R5
JJN3
% Cell Death
100
75
50
25
0
CONT siRNA
cIAP1 siRNA
cIAP2 siRNA
XIAP siRNA
*
*
CTR
TRAIL 300
Control
cIAP1 siRNA
cIAP1
Actin
JJN3
Control
cIAP2 siRNA
cIAP2
Actin
JJN3
Control
XIAP siRNA
XIAP
Actin
JJN3

Figure 9

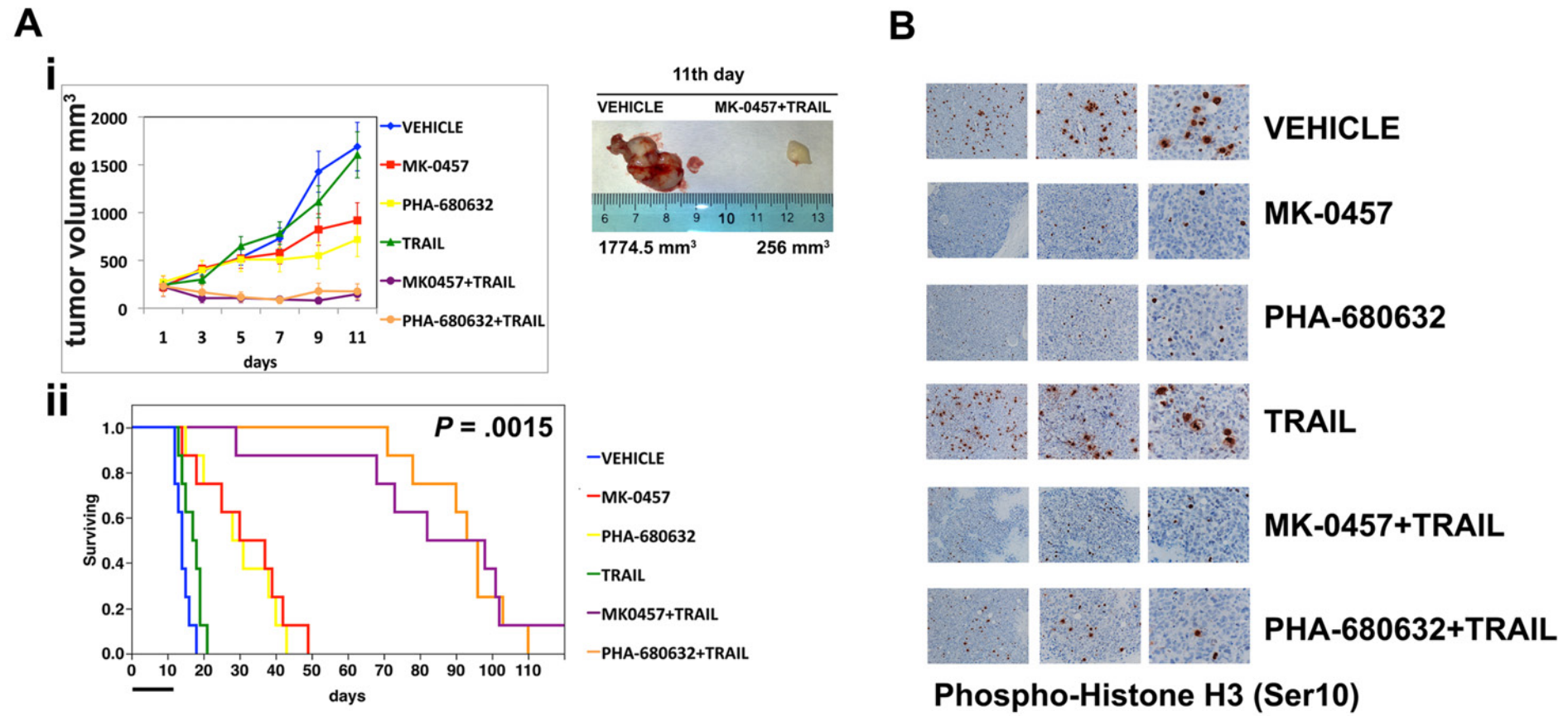
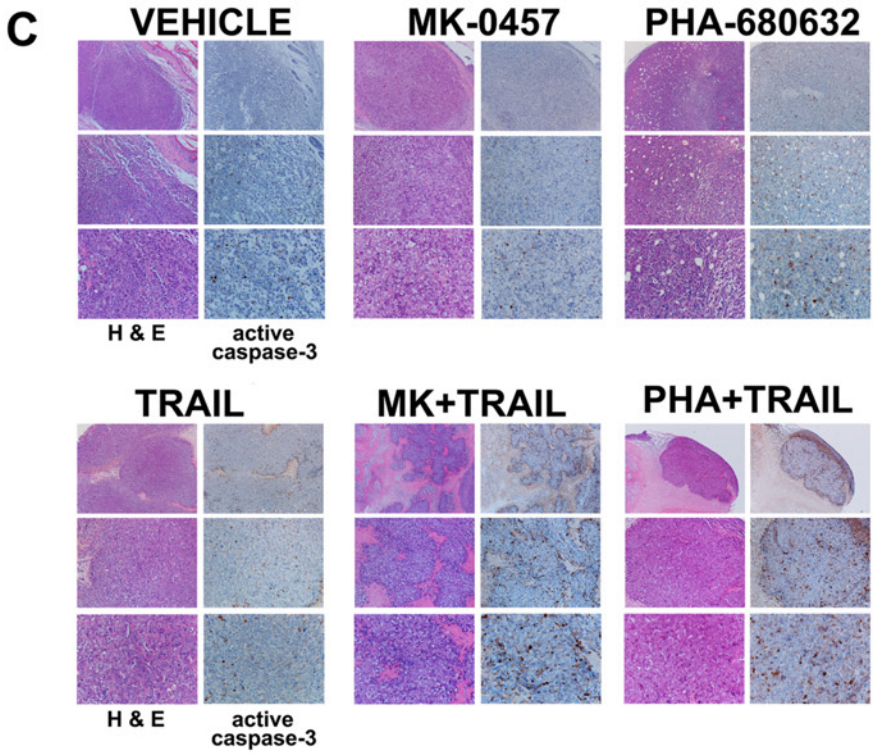
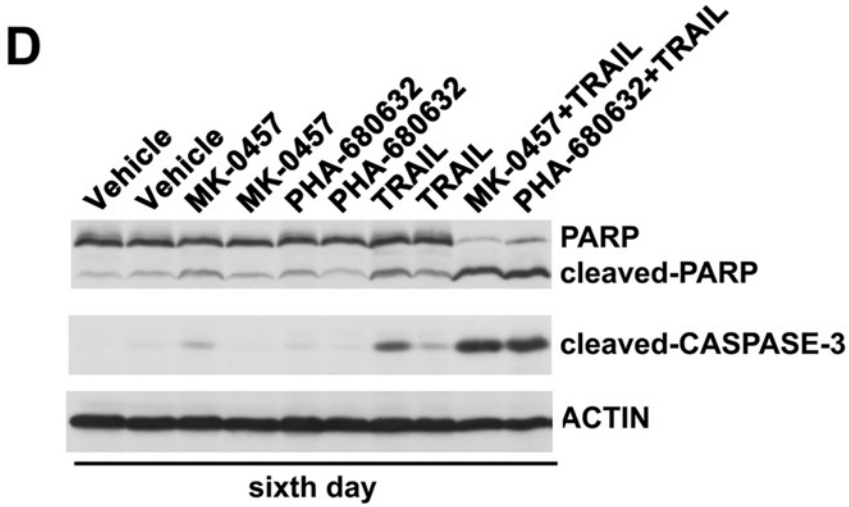

A
i
tumor volume mm3
2000
1500
1000
500
0
1
3
5
7
9
11
days
VEHICLE
MK-0457
PHA-680632
TRAIL
MK0457+TRAIL
PHA-680632+TRAIL
11th day
VEHICLE
MK-0457+TRAIL
6
7
8
9
10
11
12
13
1774.5 mm3
256 mm3
ii
P = .0015
Surviving
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
90
100
110
days
VEHICLE
MK-0457
PHA-680632
TRAIL
MK0457+TRAIL
PHA-680632+TRAIL
B
VEHICLE
MK-0457
PHA-680632
TRAIL
MK-0457+TRAIL
PHA-680632+TRAIL
Phospho-Histone H3 (Ser10)

Figure 9


C
VEHICLE
MK-0457
PHA-680632
H & E
active caspase-3
TRAIL
MK+TRAIL
PHA+TRAIL
H & E
active caspase-3


D
Vehicle
Vehicle
MK-0457
MK-0457
PHA-680632
PHA-680632
TRAIL
TRAIL
MK-0457+TRAIL
PHA-680632+TRAIL
PARP
cleaved-PARP
cleaved-CASPASE-3
ACTIN
sixth day