Small-molecule HDAC and Akt Inhibitors Suppress Tumor Growth and Enhance Immunotherapy in Multiple Myeloma

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Research

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Abstract

Background: Multiple myeloma (MM) patients may undergo relapse and experience resistance to existing therapies. Cereblon (CRBN) is key mediator of the bioactivities of immunomodulatory drugs (IMiDs), including lenalidomide. Moreover, genetic alteration of CRBN is frequently detected in IMiD-resistant patients and considered to contribute to IMiD resistance. Thus, overcoming resistance to drugs, including IMiDs, is expected to improve clinical outcomes. Here, we examined potential mechanisms of a histone deacetylase (HDAC) inhibitor and Akt inhibitor in treatment of relapsed/refractory MM patients.

Methods: We established lenalidomide-resistant cells by knocking down or knocking out CRBN in MM cells. Additionally, we derived multi-drug (bortezomib, doxorubicin, or dexamethasone)-resistant cell lines and primary cells from relapsed/refractory MM patients. The effects of HDAC and Akt inhibitors on these drug-resistant MM cells were then observed with a particular focus placed on whether HDAC inhibitors enhance immunotherapy efficacy. We also investigated the effect of lenalidomide on CRBN-deficient cells.

Results: HDAC inhibitor suppressed the growth of drug-resistant MM cell lines, and enhanced antibody-dependent cellular cytotoxicity (ADCC) of therapeutic antibodies by upregulating natural killer group 2D (NKG2D) ligands in MM cells. CRBN-deficient cells showed lenalidomide-induced upregulation of glycogen synthase kinase-3 (p-GSK-3) and c-Myc phosphorylation. Meanwhile, HDAC and Akt inhibitors downregulated c-Myc by blocking GSK-3 phosphorylation. HDAC and Akt inhibitors also exhibited synergistic cytotoxic and c-Myc–suppressive effects. Moreover, the dual HDAC and PI3K inhibitor, CUDC-907, exhibited cytotoxic and immunotherapy-enhancing effects for MM cells, including for multi-drug-resistant lines and primary cells including lenalidomide-resistant patients.

Conclusions: Combined HDAC and Akt inhibition represents a promising approach for the treatment of relapsed/refractory MM.

Background

Multiple myeloma (MM) is a plasma cell malignancy that accounts for approximately 10% of all hematological malignancies [1–3]. Currently, there is no curative therapy for this disease, which is accompanied by symptoms including renal failure, anemia, hypercalcemia, and skeletal destruction [2]. However, various novel drugs are available for treating MM, including monoclonal antibodies (mAbs), such as daratumumab [4] and elotuzumab [5]. In addition, proteasome inhibitors (PIs), such as bortezomib [6] and immunomodulatory drugs (IMiDs), including lenalidomide [7] and pomalidomide, have shown efficacy in MM [3, 7, 8]. Notably, the combined use of lenalidomide with PIs [9] and/or mAbs [10] is a key approach in current MM therapy [11].

While most newly diagnosed MM patients respond to lenalidomide, development of resistance is often observed. Cereblon (CRBN) is a component of the CUL4 E3 ligase complex that serves as the primary target of IMiDs [12–14]. Reduced CRBN expression is thought to be associated with IMiD resistance [14–16]. IMiDs selectively bind to CRBN, which mediates the recruitment of Ikaros family zinc finger 1 (IKZF1; Ikaros) or 3 (IKZF3; Aiolos) to E3 ubiquitin ligase for subsequent degradation, resulting in interferon regulatory factor-4 and c-Myc downregulation [17]. DNA samples from the bone marrow of IMiD and PI double-refractory MM patients have been examined using next-generation sequencing, and CRBN pathway-related mutations were identified in 32.5% of patients [18]. Other reports have shown that most patients treated with lenalidomide have downregulated CRBN and upregulated IKZF1 gene expression [19]. CRBN mutations are also associated with pomalidomide resistance in MM [20]. Thus, it is important to develop MM therapies capable of overcoming CRBN-related lenalidomide resistance.

Histone deacetylase (HDAC) inhibitors are known to activate innate immunity [21]. Meanwhile, HDAC inhibition reverses aberrant epigenetic changes contributing to tumor proliferation and enhances tumor immunogenicity [22–24]. HDAC inhibitors also enhance the expression of major histocompatibility complex class I-related chain A and B (MICA and MICB) in leukemic cell lines [25], both of which serve as key natural killer group 2D (NKG2D) ligands [26–28]. NK cells are activated by NKG2D receptor crosslinking on effector cells with NKG2D ligands expressed on tumor cells [26, 29]. In addition to MICA and MICB, NKG2D ligands include UL16-binding proteins (ULBp) [26–28]. Activation of these NKG2D ligands enhances the antibody-dependent cellular cytotoxicity (ADCC) response mounted during mAb therapy for MM [30]. Hence, we proposed a strategy to induce activation of anti-tumor immunity with HDAC inhibitors to overcome resistance in lenalidomide relapse/refractory MM patients.

Moreover, the PI3K/Akt pathway is aberrantly activated in MM [31]. This activation enhances the phosphorylation of glycogen synthase kinase-3 (p-GSK-3) by Akt. Meanwhile, GSK-3 degrades c-Myc, and becomes inactivated by phosphorylation [32, 33]. Hence, increased p-GSK-3 reportedly leads to c-Myc stabilization and MM cell survival [32, 33]. Furthermore, lenalidomide resistance has been attributed to enhanced p-GSK-3 levels [34]. Thus, we also focused on Akt inhibition in lenalidomide-resistant MM patients.

Methods

Cell lines and culture condition

Human MM cell lines (RPMI8226, U266, KMS-11, H929, OPM-2, MM1.S, ADR-R/RPMI8226, KMS-11/BTZ, OPM-2/BTZ, H929 CRBN sh#395, H929 CRBN sh#610, OPM-2 CRBN sh#395, OPM-2 CRBN sh#610, OPM-2 CRBN KO, and Dex-R/MM1.S) were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. KHYG-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin in the presence of 2–20 ng/mL IL-2. Cell lines were acquired as follows: RPMI8226, U266, H929, and OPM-2 cells were obtained from American Type Culture Collection (ATCC Virginia, USA); KMS-11, KMS-11/BTZ, and KHYG-1 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB Osaka, Japan); MM1.S, ADR-R/RPMI8226 and Dex-R/MM1.S cells were kindly provided by Hiroshi Yasui of the University of Tokyo; OPM-2 and OPM-2/BTZ cells were kindly provided by Masaki Ri and Shinsuke Iida of Nagoya City University; OPM-2 CRBN sh#395, OPM-2 CRBN sh#610, and OPM-2 CRBN KO cells were kindly provided by Junichi Yamamoto, Takumi Ito, and Hiroshi Handa of the Tokyo Medical University.

Reagents

Panobinostat (LBH589), romidepsin, ACY-1215 (ricolinostat), ACY-241, and CUDC-907 were obtained from Selleck Chemicals (Houston, Texas, USA). Each chemical was dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium at the indicated concentrations for *in vitro* studies. For *in vivo* experimentation, CUDC-907 was dissolved in DMSO and PEG300, after which Tween80 (polyoxyethylene sorbitan monooleate), and sterile water were added (CUDC-907 5%, PEG300 40%, Tween80 5%, water 50%). Daratumumab for *in vitro* studies was kindly provided by Janssen Pharmaceutical K.K (Beerse, Antwerpen, Belgium). Elotuzumab was obtained from Bristol-Myers Squibb (New York, New York, USA). Daratumumab and elotuzumab were dissolved in sterile water. CHIR-99021 was obtained from Cayman Chemical Company (Ann Arbor, Michigan, USA).

Western blot analysis and immunoprecipitation

For the preparation of cell lysates, collected cells were washed with PBS and lysed in radio-immunoprecipitation assay (RIPA) buffer containing dithiothreitol, rthylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), protease inhibitors, and phenylmethylsulfonyl fluoride (PMSF). Cell lysates were incubated for 5 min at 4 °C, gently shaken for 30 min at 4 °C, and centrifuged at 20 000 × *g* for 10 min. The supernatant was collected and used for SDS-PAGE analysis. Western blotting was performed using anti-caspase-3 (Cell Signaling Technology, Danvers, Massachusetts, USA. #9662), anti-cleaved caspase-3 (Cell Signaling Technology, #9661), anti-caspase-7 (Cell Signaling Technology, #9494), anti-cleaved caspase-7 (Cell Signaling Technology, #9491), anti-Ikaros (Cell Signaling Technology, #5443), and anti-Actin (Sigma-Aldrich, St. Louis, Missouri, USA. A4700-2ML) primary antibodies. Primary antibody labeling was detected using Immobilon Forte Western HRP substrate (Merck Millipore, Burlington, Massachusetts, USA). Images were analyzed with a LAS-4000 mini (FUJIFILM, Tokyo, Japan).

Flow cytometry

MM cells were stained with PE anti-human MICA/MICB (BioLegend, San Diego, California, USA: 320906), anti-hULBP-2/5/6 (R&D systems, Minneapolis, Minnesota, USA: FAB 1298P), and PE anti-human CD38 (BioLegend: 356604) antibodies. The cells were analyzed using a BD FACS Verse flow cytometer (BD Biosciences, East Rutherford, New Jersey, USA).

RNA extraction and cDNA synthesis

Total RNA was extracted from MM cells using a RNeasy Mini Kit (Qiagen, Hilden, Germany. 74104). cDNA was synthesized from total RNA using the Super Script III First-Strand Synthesis System (Thermo Fisher, Waltham, Massachusetts, USA. 18080051).

Quantitative reverse transcription PCR (qRT-PCR)

Real-time PCR was performed in triplicate using mRNA obtained from MM cell lines, treated or untreated with HDAC inhibitor for 0, 1, 2, 4 h. Data are expressed as fold change units normalized to 18S ribosomal RNA expression. qRT-PCR experiments were performed using TaqMan Universal Master Mix II, no UNG (Thermo Fisher) and TaqMan Gene Expression Assays for MICA (Hs.130838), MICB (Hs.731446), IKZF1 (Hs.435949), IKZF3 (Hs.4351372), c-Myc (Hs.4331182), and 18 s (Hs.99999901) on a CFX Connect Real-Time PCR Detection System (Hercules, Bio-Rad, California, USA).

ADCC assay

Luciferase-expressing MM cell lines were exposed to HDAC inhibitor or DMSO for 24 h. MM cells were then treated with 0.001, 0.01, 0.1, 1, or 10 μg/mL daratumumab, elotuzumab, or control (IgG) and co-incubated with NK cells. NK cells were extracted from PBMCs obtained from healthy donors using a human NK Cell Isolation Kit (Miltenyi Biotec, Nordrhein-Westfalen, Germany). Cell counting was performed with a hemocytometer (Erma Inc, Tokyo, Japan). NK cells were collected in RPMI 8226 with 10% FBS and 1% penicillin/streptomycin. Fresh NK cells were added at a ratio of 10:1 to MM cells. Cell death was calculated from the decrease in luciferase activity, which was detected by Steady Glo (Promega, Madison, Wisconsin, USA) or PicaGene (TOYO INK, Tokyo, Japan). Luciferase luminescence in the samples was evaluated using a Nivo spectrophotometer (Perkin Elmer, Massachusetts, USA).

Methyl thiazolyl tetrazolium assay

Each MM cell line was seeded in a 96-well plate and incubated with HDAC inhibitors, Akt inhibitor, GSK-3 inhibitor, and PI for 48–72 h. Cells were exposed to lenalidomide and pomalidomide for 5 days, to doxorubicin for 5 h, or to dexamethasone for one week. Methyl thiazolyl tetrazolium (MTT) assays were performed after staining with a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) for 50 min. Plates were read using a Nivo spectrophotometer. MTT assays were performed in quintuplicates.

In vivo animal experiments

Six-week-old C.B-17/Icr-*scid*/*scid*Jcl mice were purchased from Japan CLEA (Tokyo, Japan). Mice were subcutaneously injected with OPM-2 CRBN-knockout cells to generate SCID mice. After tumor cell injection, SCID mice with tumors over 10 mm in length along the major axis were treated with vehicle or CUDC-907 (50 mg/kg body weight, eight mice per group) three times per week for 2 weeks. The outcome was a change in tumor size compared to the day treatment started. Mice were observed for 14 days after administration. This animal experiment was approved by the Animal Experiment Committee at the Institute of Medical Science of the University of Tokyo (see Study approval). The description was based on The ARRIVE guidelines 2.0 [35].

In vitro experiments using patient samples

Bone marrow samples were collected from MM patients (newly diagnosed or relapse/refractory myeloma). Upon patient consent, an additional 5 mL of bone marrow fluid were collected during a routine bone marrow examination. MM cells were then sorted using FACS Aria (BD Biosciences).

Statistics

For each analysis, P-values < 0.05 were considered to represent statistically significant differences. For qRT-PCR, quantitative data are expressed in box and whisker plots. qRT-PCR was performed in triplicates. Comparisons between two groups were performed by the two-tailed Student's *t*-test. For MTT assays, quantitative data are expressed in box and whisker plots. MTT assays were performed in quintuplicates. Statistical analysis between two groups was performed by the two-tailed Student's *t*-test. Synergistic effects were evaluated by a generalized linear model including the statistical interaction term of two drugs (distribution: normal. link function: log). Effects on ADCC activity and cell viability were evaluated by a general linear regression model with the statistical interaction term of drugs and log-transformed dose or effector/target (E/T) ratio. Repeated measurements in mice were evaluated by a linear mixed regression model with the statistical interaction term of drugs and time (days), considering intra-individual correlation by including random effects. P-values were calculated for the interaction terms in the regression models, which were interpreted as the difference in slope of dose-/time-dependent change between groups.

Results

HDAC inhibitors upregulate NKG2D ligands and enhance mAb effects in MM cell lines

In a previous study, HDAC inhibitors enhanced NKG2D ligand expression in hepatoma cells and sensitized them to NK-mediated cytolysis [36]. Panobinostat reportedly increased CD38 expression in MM cells in a time-dependent manner, enhancing the efficacy of daratumumab [37]. However, it is unknown whether panobinostat alters NKG2D ligand expression on MM cells. We found that panobinostat treatment for 24 h increased CD38 and NKG2D ligand (MICA/MICB and ULBp) expression in RPMI8226 and U266 cells (Fig. 1A). Romidepsin and ACY-1215 also enhanced surface MICA/MICB and ULBp expression (Fig. 1B). Next, we tested whether MICA and MICB expression is altered under HDAC inhibition and observed increased mRNA expression of MICA, but not MICB, for all tested inhibitors (Fig. 1C-D and Supplementary Fig. 1A-D), as summarized in Supplementary Fig. 1E.

IKZF1 and IKZF3 are negative regulators of MICA [38]. We found that HDAC inhibitors downregulated IKZF1 and IKZF3 at the mRNA level (Fig. 1E-F and Supplementary Fig. 1F)

We also found that daratumumab ADCC was significantly upregulated by ACY-1215 treatment (Fig. 1G). Additionally, the ADCC activity of elotuzumab was enhanced by ACY-1215 in the presence of high elotuzumab concentrations (Fig. 1H). Further, ACY-1215 enhanced the direct cytotoxicity of NK cells against MM cells (Fig. 1I).

Analysis of gene expression profiles from 414 newly diagnosed MM patients [39] deposited in an integrated gene expression and disease prognosis database (GenomicScape, http://www.genomicscape.com) revealed that higher MICA expression was significantly associated with better overall survival (OS; p = 0.0049, Fig. 1J). We also analyzed data from a clinical trial that compared the efficacy and safety of bortezomib treatment [40]. The data indicated longer OS of patients with high MICA and ULBp2 expression when compared to patients with low MICA (p = 0.068, Supplementary Fig. 1G) and ULBp2 (p = 3.6e-05, Supplementary Fig. 1H) expression. We hypothesized that upregulation of NKG2D ligands including MICA and ULBp2 is critical for enhanced MM cell recognition by NK cells and contributes to better clinical outcomes.

HDAC inhibitors upregulate NKG2D ligands independently of CRBN expression

We established CRBN-deficient RPMI8226 (Fig. 2A), H929 (Fig. 2B) and OPM-2 cells (Fig. 2C) by RNAi-mediated downregulation of CRBN or CRISPR-Cas9-mediated CRBN knockout to establish lenalidomide-resistant cells. The CRBN-deficient cell lines were experimentally validated using an MTT proliferation assay. CRBN-deficient cells were resistant to lenalidomide cytotoxicity, proliferating under high lenalidomide concentrations (Fig. 2D–F). In contrast, pomalidomide cytotoxicity [41] was observed in both CRBN-knockdown and parent RPMI8226 cells (Fig. 2G).

The TP53RK-mediated Myc inhibitory pathway is activated by pomalidomide, but not by lenalidomide [42]. Pomalidomide was speculated to suppress Myc in CRBN-knockdown cells, not via the CRBN-mediated pathway, but rather via the TP53RK-mediated pathway. However, decreased pomalidomide cytotoxicity was observed in CRBN-deficient H929 CRBN sh#610 cells when compared to their CRBN-intact counterparts (Fig. 2H). For OPM-2 cells, pomalidomide cytotoxicity decreased with lower CRBN expression (Fig. 2I). Therefore, pomalidomide was not effective against all lenalidomide-resistant cells.

Next, we investigated whether HDAC inhibitors are able to overcome IMiD resistance in CRBN-deficient MM cells. ACY-1215 treatment of CRBN-deficient cells resulted in upregulation of MICA mRNA (Fig. 2J-K). Further, ACY-1215 treatment reduced IKZF1 mRNA (Fig. 2L-M). These observations suggest that ACY-1215-dependent IKZF1 downregulation and MICA upregulation occur via a CRBN-independent pathway, as well as that HDAC inhibitors can overcome CRBN-associated IMiD resistance through IKZF1 downregulation.

HDAC and Akt inhibitors are potential treatments for lenalidomide-resistant cells

Enhanced p-GSK-3 α/β expression was previously reported in MM cell lines following extended exposure to lenalidomide, in turn compromising lenalidomide sensitivity [34]. Thus, we examined how treatment with 10 and 30 μM lenalidomide for one month would alter p-GSK-3 α/β levels [34] in H929 and OPM-2 cells when CRBN was downregulated to variable extents. We observed a phosphorylation-dependent mobility shift of GSK-3 α/β and an increase in p-GSK-3 α/β and c-Myc levels in H929 cells after lenalidomide exposure (Fig. 3A). Increased p-GSK-3 α/β and c-Myc levels were also observed in OPM-2 cells (Fig. 3B). These changes were exacerbated when cells were exposed to higher lenalidomide concentrations, implying that CRBN disruption in MM cells could further increase c-Myc levels after extended lenalidomide treatment. Paradoxically, lenalidomide may increase p-GSK-3 and c-Myc expression in MM cells when CRBN expression is reduced.

Since GSK-3 and c-Myc are located downstream of the PI3K/Akt pathway, it was speculated that Akt inhibitors may also be useful for MM therapy. We, therefore, assessed the effects of HDAC inhibitors, Akt inhibitors, and the combination of HDAC inhibitors and Akt inhibitors. Oral Akt inhibitor afuresertib has been clinically tested in patients with advanced MM. Both phase I [43] and II [44] trials of afuresertib were performed in relapsed/refractory malignant

lymphoma patients. However, the efficacy of combined HDAC and Akt inhibitor treatment in MM is currently unknown. We measured GSK-3 α/β, p-GSK-3 α/β, and c-Myc protein levels following HDAC inhibitor and/or Akt inhibitor treatment. ACY-1215 and/or afuresertib decreased p-GSK-3 α/β and c-Myc protein levels in RPMI8226 and H929 cells (Fig. 3C-D), suggesting that HDAC inhibitors and Akt inhibitors may overcome lenalidomide-enhanced c-Myc expression in CRBN-deficient cells. ACY-1215 also downregulated c-Myc mRNA in RPMI8226 cells (Fig. 3E). According to clinical trial data comparing the efficacy and safety of treatment with and without bortezomib [40], patients with high GSK-3 α/β expression had better OS than those with low GSK-3 α ($p = 1.9 \times 10^{-9}$, Supplementary Fig. 2A)/β (p = 0.02, Supplementary Fig. 2B) expression. Further, patients with high Myc expression had poorer OS than those with low expression (p = 0.0043, Supplementary Fig. 2C). These results are suggestive of the negative effect of GSK-3 α/β inactivation-related c-Myc stabilization on MM patient prognosis.

Finally, we investigated the proapoptotic effect of ACY-1215 and afuresertib alone and in combination. Both inhibitors induced apoptosis, and combined treatment had an even stronger effect (Fig. 3F, Supplementary Fig. 2D).

HDAC and Akt inhibitors exhibit cytotoxicity in several MM lines, including drug-resistant MM cell lines

Since HDAC inhibitors and Akt inhibitors were found to induce apoptosis in MM cells, we decided to confirm their cytotoxicity in MM cells by MTT assay. Several MM lines, including drug-resistant cells, received ACY-1215 and/or afuresertib. Combined treatment exerted greater cytotoxic effects on RPMI8226 cells than each inhibitor alone (Fig. 4A). Similar results were observed in doxorubicin-resistant RPMII8226 (Fig. 4B), U266 (Supplementary Fig. 2E), H929 (Fig. 4C), CRBN-deficient H929 (Fig. 4D and Supplementary Fig. 2F), OPM-2 (Fig. 4E), CRBN-deficient OPM-2 (Fig. 4F-H), bortezomib-resistant OPM-2 (Fig. 4I), KMS-11 (Supplementary Fig. 2G), bortezomib-resistant KMS-11 (Supplementary Fig. 2H), MM1.S (Supplementary Fig. 2I), and dexamethasone-resistant MM1.S (Fig. 4J). The synergistic effects of ACY-1215 and afuresertib were observed in RPMI8226, U266, KMS-11, OPM-2, OPM-2 CRBN sh#395, and OPM-2 CRBN sh#610 cells. We then evaluated whether GSK-3 inhibition impairs the anti-MM activity of HDAC and Akt inhibitors by using GSK-3 inhibitor CHIR 99021 [45]. Co-treatment with CHIR 99021 partially counteracted the cytotoxic effect of ACY-1215 and afuresertib in H929 cells (Fig. 4K) suggesting that anti-MM effects of ACY-1215 and afuresertib were partly mediated via GSK-3 activation.

Dual HDAC and PI3K inhibitor CUDC-907 upregulates NKG2D ligands, enhancing daratumumab and elotuzumab activity

CUDC-907 is a dual HDAC and PI3K inhibitor [46, 47] that is currently being evaluated in clinical trials for treatment of MM [48] and lymphoma [49]. CUDC-907 inhibits PI3K, which is an upstream activator of Akt in the PI3K/Akt pathway [33]. We found that CUDC-907 upregulated MICA and ULBp expression as well as CD38 expression in RPMI8226 and U266 cells (Fig. 5A-B). CUDC-907 also upregulated MICA mRNA (Fig. 5C) and downregulated IKZF1 (Fig. 5D), IKZF3 (Fig. 5E), and Myc (Fig. 5F) mRNA levels in RPMI8226 cells. CUDC-907 significantly enhanced the ADCC activity of daratumumab (Fig. 5G) and elotuzumab (Fig. 5H), while also downregulating p-GSK-3 α/β and c-Myc expression (Fig. 5I).

CUDC-907 upregulates NKG2D ligands and exhibits cytotoxicity in CRBN-knockout MM cells

CUDC-907 treatment upregulated MICA mRNA expression (Fig. 6A) and downregulated IKZF1 (Fig. 6B), IKZF3 (Fig. 6C), and Myc (Fig. 6D) mRNA expression in both CRBN-knockout and parent MM cells. Further, CUDC-907 suppressed p-GSK-3 α/β and c-Myc expression in CRBN-knockout cells (Fig. 6E). We performed xenograft mouse model experiments using CRBN-deficient cells resistant to lenalidomide. Mice (n = 8 per group) received vehicle or CUDC-907 (50 mg/kg body weight) orally (Fig. 6F). CUDC-907-treated mice exhibited reduced tumor growth rates compared to vehicle mice (Fig. 6G-H). There was no significant difference in the changes in body weight between vehicle- and CUDC-907-treated mice (p = 0.358, Fig. 6I).

Efficacy of CUDC-907 in primary and drug-resistant MM cells

We examined the efficacy of CUDC-907 on parent MM cell lines, lenalidomide-resistant, bortezomib-resistant, doxorubicin-resistant, and dexamethasone-resistant cells (Supplementary Fig. 3A-O), as well as primary cells from MM patients (Table 1) sensitive to lenalidomide (S1–S6) (Fig. 7A-F) and six patients refractory to lenalidomide (R1–R6) (Fig. 7G-L). Refractory patients were defined as patients with disease that had no response while on lenalidomide-based therapy or experienced progression within 60 days of their last therapy, as per The International Myeloma Working Group criteria [50]. In particular, lenalidomide (5 μM and 10 μM) showed no cytotoxicity in primary cells from R1 (Fig. 7G) and R2 (Fig. 7H) patients. Thus, we confirmed that cells derived from R1 and R2 patients were resistant to lenalidomide even *in vitro*. On the other hand, most cells, including R1 and R2 cells, were sensitive to CUDC-907 after 48 h of treatment (Fig. 7A-K). Although we observed a dose-dependent increase in cytotoxicity, primary cells from Patient R6 were not significantly affected by CUDC-907 (Fig. 7L). One reason for this could be the low absolute number of primary cells.

Table 1
Clinical parameters of MM patients.

Patient	Age	Sex	Type of Ig	Clinical stage			Medical history	Lenalidomide refractory
				D&S	ISS	R-ISS		
S1	68	F	IgA-κ	A			Newly diagnosed	No
S2	78	M	IgG-λ	A			Newly diagnosed	No
S3	78	M	IgG-κ	A		N/A	Newly diagnosed	No
S4	46	M	IgG-λ	A			Newly diagnosed	No
S5	66	M	IgG-κ	A	I		Newly diagnosed	No
S6	51	F	IgG-λ	A			CyBorD,HDCY,BD,MEL,autoPBSCT	No
R1	56	M	IgG-κ/ BJP-κ	IA	I	N/A	VRd	Yes
R2	55	F	IgA-λ	A			VRd,VP16,MEL,autoPBSCT,biweeklyBor,LEN,maintenance,DRd,VTDPACE,DCEP,KRd	Yes
R3	80	M	IgA-κ	A		N/A	CyBorD,Bor biweekly,VTd,VRd, HDCY,MEL,auto PBSCT	Yes
R4	73	F	BJP-λ	B			Ld,Eld,DVd,PCd	Yes
R5	62	M	IgG-κ	A			BD,VRd	Yes
R6	76	M	IgA-λ	A			CyBorD,VRd,LEN maintenance,DVd,PCd,KRd,Kd	Yes

CyBorD; cyclophosphamide, bortezomib, dexamethasone. HDCY; High dose cyclophosphamide. BD; bortezomib, dexamethasone. MEL; melphalan. PBSCT; peripheral blood stem cell transplantation. VRd; bortezomib, lenalidomide, dexamethasone. VP-16; etoposide. Bor; bortezomib. LEN; lenalidomide. DRd; daratumumab, lenalidomide, dexamethasone. VTD-PACE; bortezomib, thalidomide, dexamethasone, cisplatin, doxorubicin, cyclophosphamide, etoposide. DCEP; dexamethasone, cyclophosphamide, etoposide, cisplatin. KRd; carfilzomib, lenalidomide, dexamethasone. VTd; bortezomib, thalidomide, dexamethasone. Ld; lenalidomide, dexamethasone. Eld; elotuzumab, lenalidomide, dexamethasone. DVd; daratumumab, bortezomib, dexamethasone. PCd; pomalidomide, cyclophosphamide, dexamethasone. Kd; carfilzomib, dexamethasone. D&S: Durie-Salmon staging. ISS International Staging System. R-ISS Revised International Staging System.

Discussion

In the current study, we investigated whether small-molecule HDAC and Akt inhibitors could overcome lenalidomide resistance through suppression of tumor growth and enhancement of immunotherapy in multiple MM. HDAC inhibitors reduced tumor growth by downregulating c-Myc in a CRBN-independent manner, while upregulating MICA expression and, thus, enhancing the efficacy of immunotherapy. In particular, HDAC inhibitors enhanced the ADCC activity of daratumumab and elotuzumab.

Lenalidomide is currently used for induction and maintenance therapy in MM treatment. However, development of lenalidomide resistance worsens the prognosis of MM patients. Thus, overcoming this challenge is important for improving clinical outcomes. CRBN is the primary target of IMiDs [12–14], and low CRBN expression is associated with resistance to lenalidomide and pomalidomide monotherapy [15]. Unlike lenalidomide, pomalidomide exhibited cytotoxicity in some CRBN-knockdown cells.

Surprisingly, CRBN-knockdown and knockout cells cultured with lenalidomide showed a concentration-dependent upregulation of p-GSK-3 and c-Myc. These results are consistent with the significant increase in c-Myc following development of lenalidomide resistance, when compared to its levels in bone marrow samples collected from MM patients at diagnosis [16]. Our data indicates that c-Myc is an important therapeutic target in CRBN-deficient MM cells.

As GSK-3 and c-Myc are located downstream of the PI3K/Akt pathway, we explored the effects of Akt inhibition. Akt inhibitor afuresertib downregulated p-GSK-3 α/β and c-Myc at the protein level. Furthermore, combined HDAC and Akt inhibitor treatment induced stronger downregulation of c-Myc than either inhibitor alone. Thus, combined HDAC and Akt inhibition could suppress the proliferation of lenalidomide-resistant cell lines by overcoming c-Myc upregulation.

Based on the synergetic suppression of MM cell proliferation by combined HDAC and Akt inhibition, we evaluated dual HDAC and PI3K inhibitor CUDC-907. CUDC-907 enhanced daratumumab and elotuzumab ADCC, while also upregulating NKG2D ligand expression even in CRBN-knockout cells and suppressing IKZF1, IKZF3, p-GSK-3, and Myc expression. We also confirmed the efficacy of CUDC-907 in SCID mice injected with CRBN-knockout cells. Additionally, CUDC-907 was cytotoxic to primary cells from MM patients, including those resistant to various lenalidomide treatment regimens. According to the data from a

pharmacokinetics study involving CUDC-907 [48], the *in vitro* concentration of CUDC-907 used in the current study was similar to that detected in the plasma of six patients (0–20 ng/mL, 0–39.3 nM). This concentration was observed on day 15 after receiving 60 mg of oral CUDC-907 daily for five days, followed by a 2-day intermittent break [48]. Furthermore, Phase I trial data indicated that the most common adverse effects of CUDC-907 were diarrhea, fatigue, nausea, and thrombocytopenia. However, these effects were relatively rare [thrombocytopenia (18%), neutropenia (7%), hyperglycemia (7%), and diarrhea (5%)] [48].

Conclusions

HDAC inhibitor treatment downregulated IKZF1 and IKZF3, inducing Myc downregulation and MICA upregulation. In turn, MICA upregulation led to enhanced ADCC activity of mAbs and NK cell activity. Moreover, HDAC or Akt inhibitors downregulated p-GSK-3, which functions to stabilize c-Myc. In turn, p-GSK-3 suppression caused c-Myc downregulation. Meanwhile, the dual PI3K and HDAC inhibitor, CUDC-907, enhanced MICA and suppressed Myc (Fig. 8). In summary, HDAC and Akt inhibitors, as well as CUDC-907, are promising drugs for cases of relapse/refractory MM, including lenalidomide resistance.

Abbreviations

MM
Multiple myeloma; CRBN:Cereblon; IMiDs:immunomodulatory drugs; HDAC:histone deacetylase; ADCC:antibody-dependent cellular cytotoxicity; NKG2D:natural killer group 2D; p-GSK-3:phosphorylation of glycogen synthase kinase-3; mAb:monoclonal antibodies; PIs:proteasome inhibitors; IKZF1:Ikaros family zinc finger 1; IKZF3:Ikaros family zinc finger 3; MICA:major histocompatibility complex class I-related chain A; MICB:major histocompatibility complex class I-related chain B; ULBp:UL16-binding proteins; DMSO:Dimethyl sulfoxide; qRT-PCR:Quantitative reverse transcription PCR; MTT:Methyl thiazolyl tetrazolium; E/T:effector/target; OS:overall survival; SD:standard deviation

Declarations

Ethics approval and consent to participate: Animal experimentation protocols were approved by the Animal Experiment Committee at the Institute of Medical Science of the University of Tokyo (H2-04). This project was approved by the Institutional Review Board at the Institute of Medical Science of the University of Tokyo (30-72-A0222). Written informed consent was obtained from participants prior to inclusion in the study.

Consent for publication: Not applicable

Availability of data and materials: The dataset supporting the conclusions of this article are included in the published article and its supplementary information files.

Competing Interests: YI received honoraria from Celgene/Bristol-Myers Squibb and Janssen as well as research funding from Janssen, Celgene/Bristol-Myers Squibb, Okinaka Memorial Institute for Medical Research, and JSPS KAKENHI (Grant Numbers 1 7 K 0 9 9 1 6 and 2 0 K 0 8 7 2 6). TI received honoraria from Ono, Takeda, Celgene/Bristol-Myers Squibb, Janssen. MR received research funding from Celgene/Bristol-Myers Squibb. SI has previously received honoraria from Takeda, Ono, Celgene/Bristol-Myers Squibb, Janssen, Sanofi, Daichi Sankyo, and received research funding from Takeda, Janssen, Abbvie, Celgene/Bristol-Myers Squibb, MSD, as well as endowments from Takeda, Ono, Chugai, Kyowa Kirin, Sanofi. All other authors declare no competing interests.

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Author contributions': MH and YI performed the research, analyzed, and interpreted data, and wrote the manuscript. YI designed the research and edited the manuscript. TM assisted the research. KS and TI provided clinical samples, JY, TI, HH, HY, MR, and SI provided research samples. MH, KS, TI and YI collected the patients' samples, MN performed statistical processing on experimental data. YK, TM, KS, JY, TI, MF, MR, HY, YK, NG, SI and HH checked and revised the manuscript. AT supervised projects and edited the manuscript.

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Figures

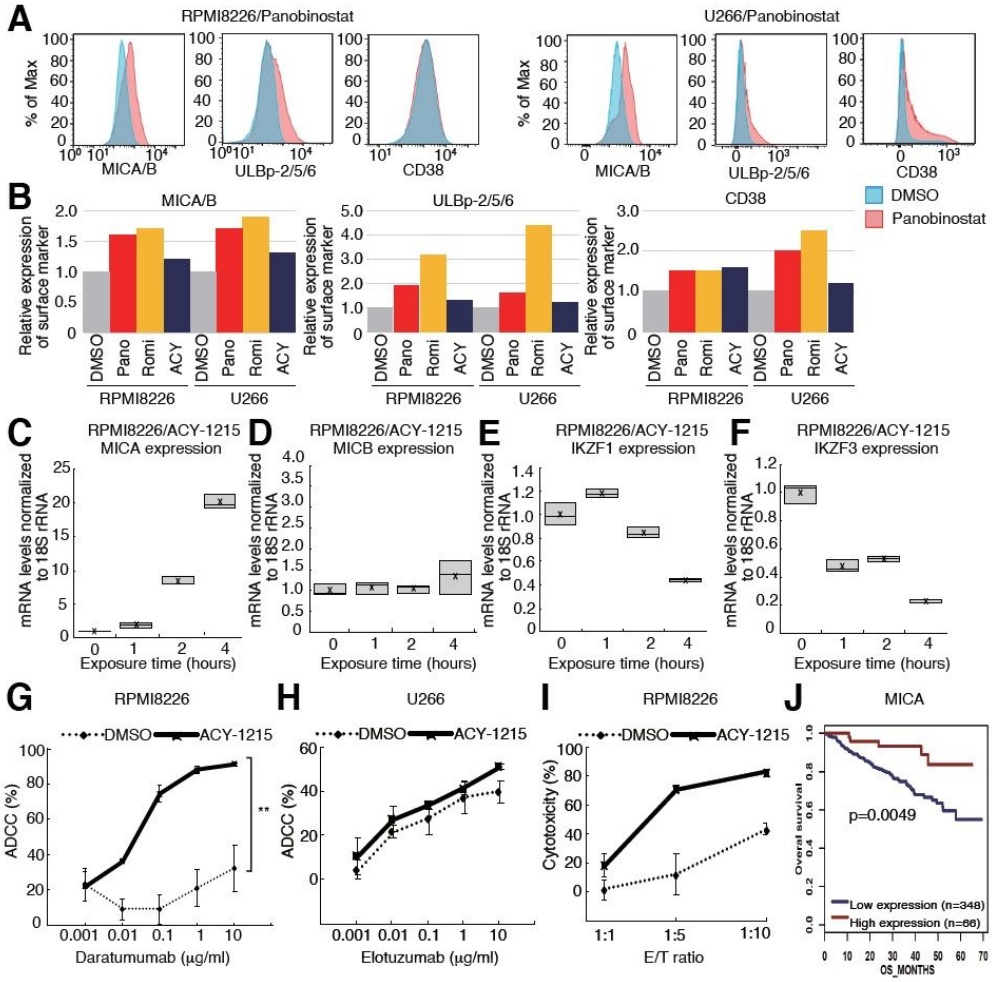
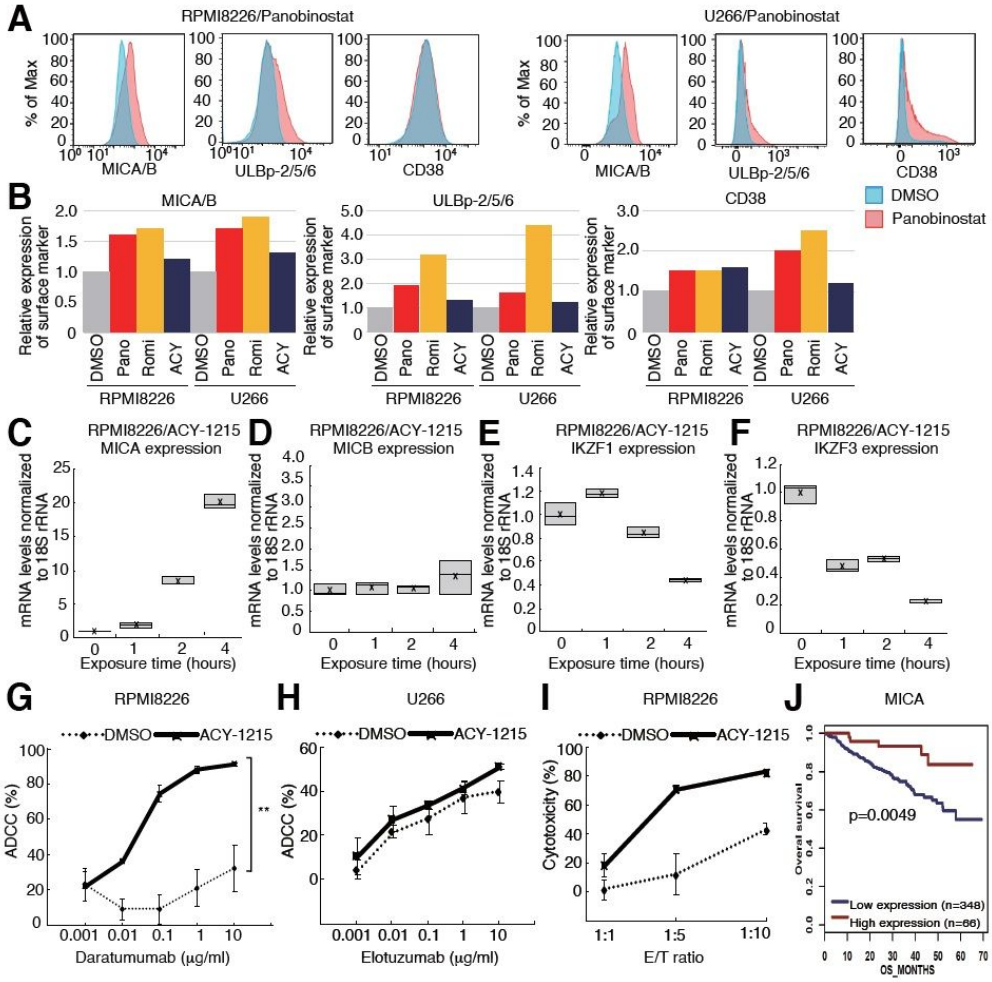


Figure 1

HDAC inhibitors enhance NK cell activity via NKG2D ligand upregulation and IKZF1/3 downregulation. (a) Histograms of CD38, MICA, ULBp expression in RPMI8226 and U266 cells treated with panobinostat for 24 h. (b) Summary of the ratio of mean fluorescence intensity (MFI) for MICA/B, ULBp-2/5/6, and CD38 expression between cells exposed to DMSO and HDAC inhibitors (Panobinostat [Pano], Romidepsin [Romi], ACY-1215 [ACY]). (c–f) MICA, MICB, IKZF1/3 mRNA levels in RPMI8226 cells exposed to ACY-1215. Experiments were performed in triplicate (n = 3). (g–i) ADCC and NK cell assay of daratumumab and elotuzumab in the presence of ACY-1215. Experiments were repeated three times (n = 3). Error bars represent the SD. (**p < 0.01). (j) Prognostic value of MICA expression based on MM patient clinical trial data.



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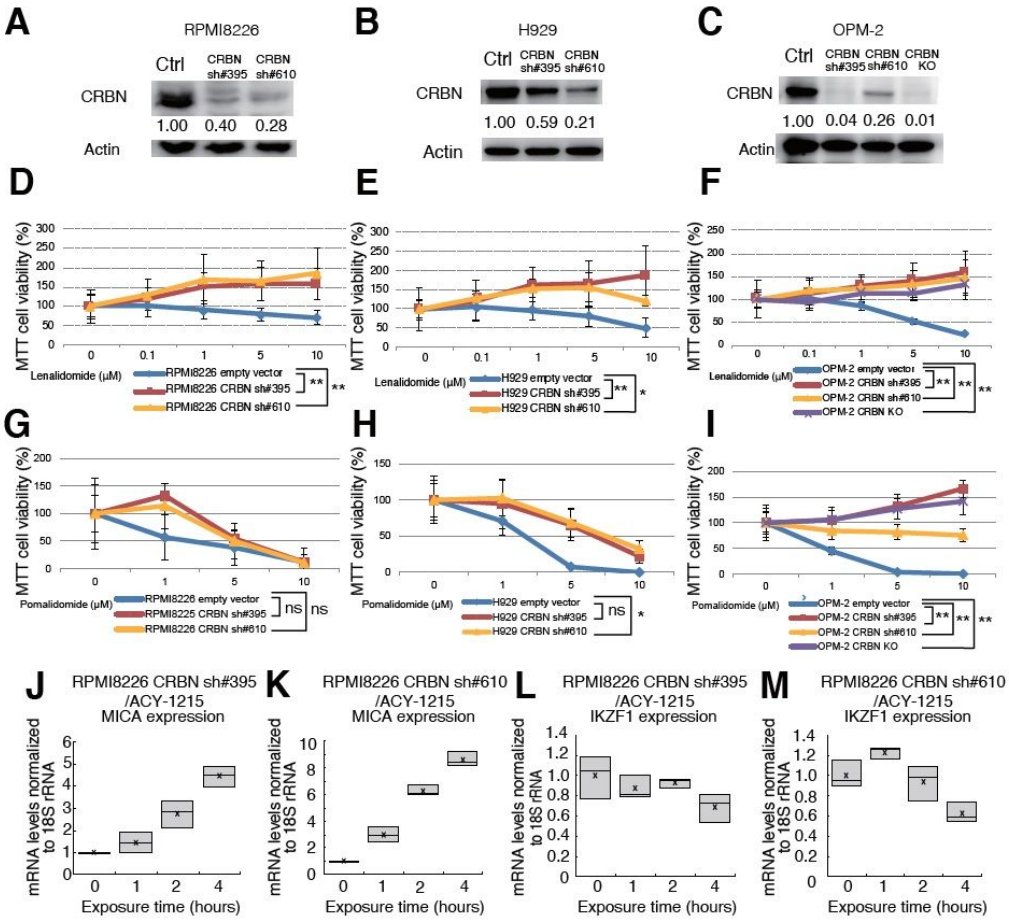
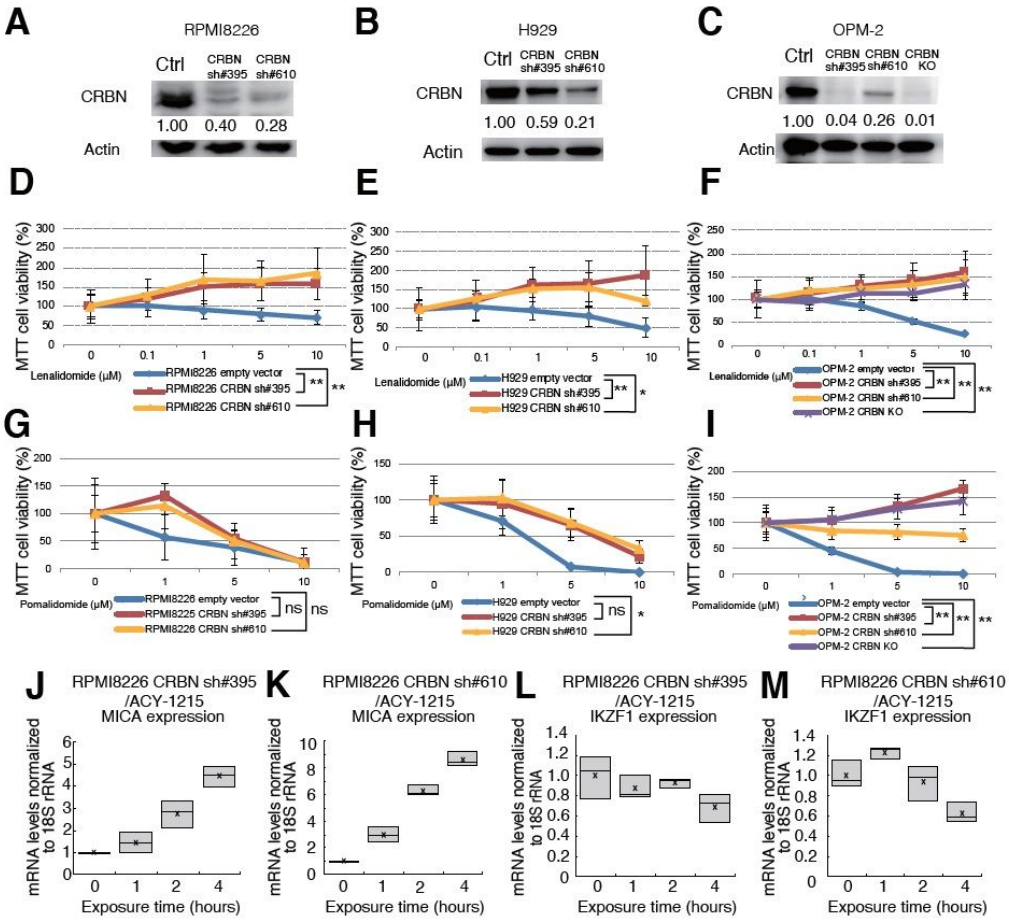


Figure 2

HDAC inhibitors enhance NKG2D ligand expression via a CRBN-independent pathway. (a–c) CRBN protein levels in RPMI8226, H929, and OPM-2 cells. The number below the band shows the density of CRBN normalized to that of actin. (d–i) MTT proliferation assay. The sensitivity of CRBN knockdown and CRBN-knockout MM cell lines to lenalidomide and pomalidomide. Each experiment was performed in quintuplicate (n = 5). Results are shown as the mean ± SD. Data were analyzed using the two-tailed Student's t-test (*p < 0.05, **p < 0.01). "ns" indicates no significant difference. (j-m) MICA and IKZF1 mRNA levels in CRBN-deficient RPMI8226 cells exposed to ACY-1215. Experiments were performed in triplicate (n = 3).



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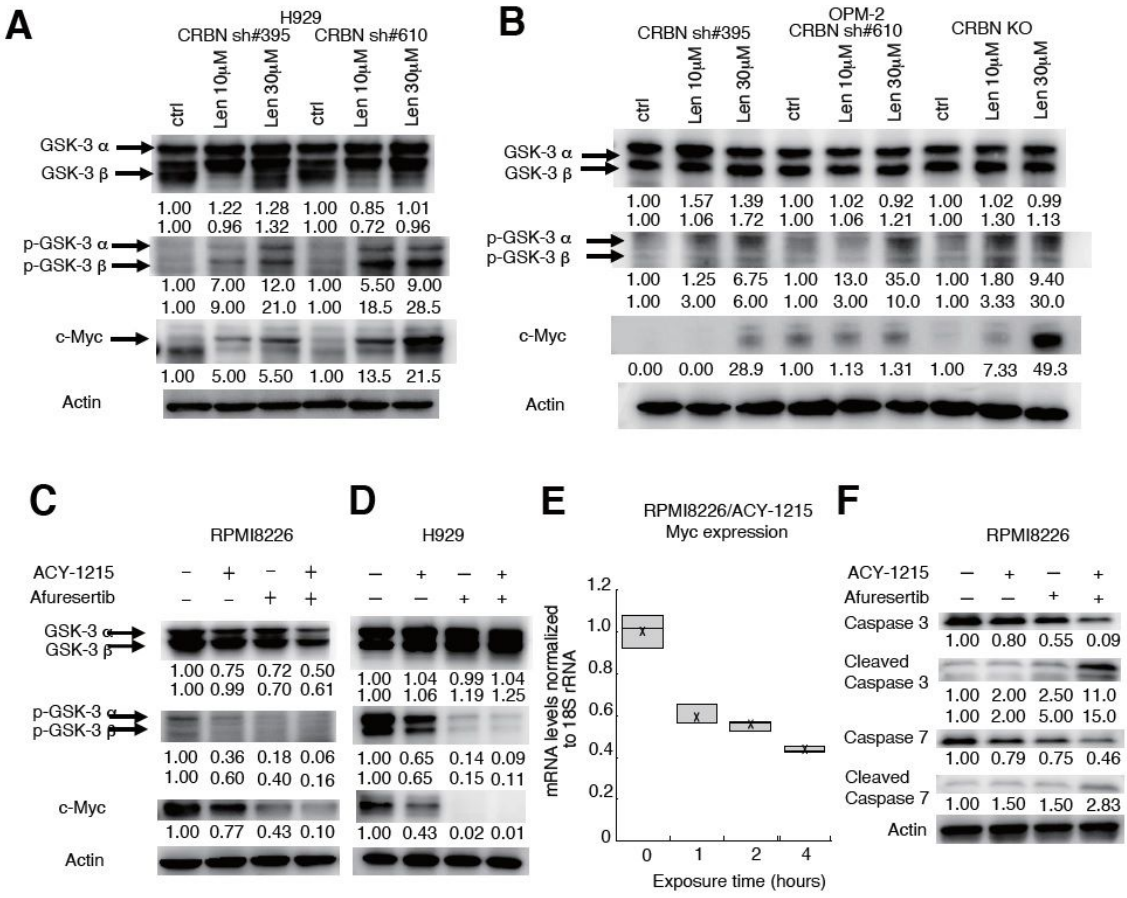
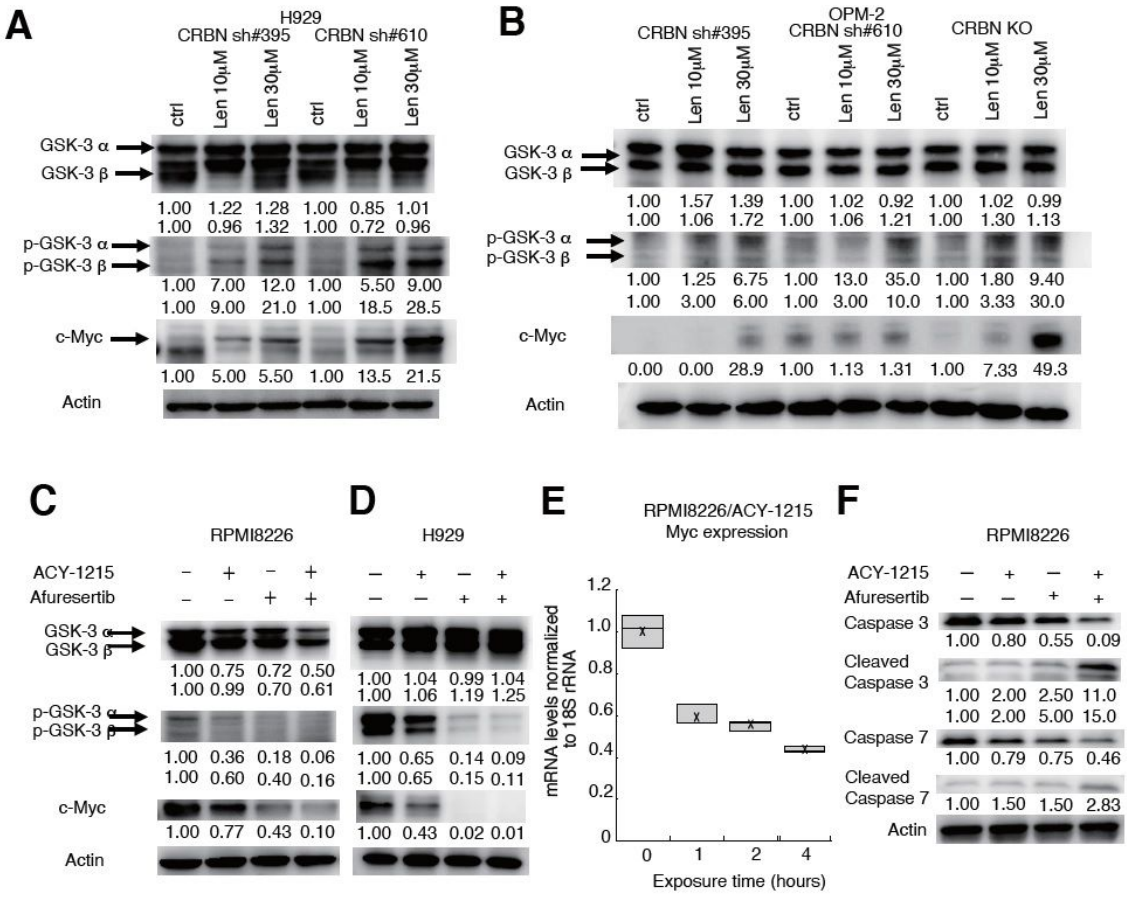


Figure 3

HDAC and Akt inhibitor treatment can overcome c-Myc upregulation by lenalidomide in CRBN-deficient cells. (a-b) Western blot validation of the effects of lenalidomide (Len) on CRBN-deficient cells. c-Myc expression could not be detected in OPM-2 CRBN sh#395 cells exposed to the control drug and Len 10 μM. (c-d) RPMI8226 and H929 cell lysates treated with ACY-1215 and/or afuresertib were immunoblotted with the indicated antibodies (against GSK-3, p-GSK-3, c-Myc, and actin). (e) Myc mRNA levels in RPMI8226 cells treated with ACY-1215. f RPMI8226 cell lysates treated with ACY-1215 and/or afuresertib were immunoblotted with indicated antibodies (against caspase-3, cleaved caspase-3, caspase-7, cleaved caspase-7, actin).



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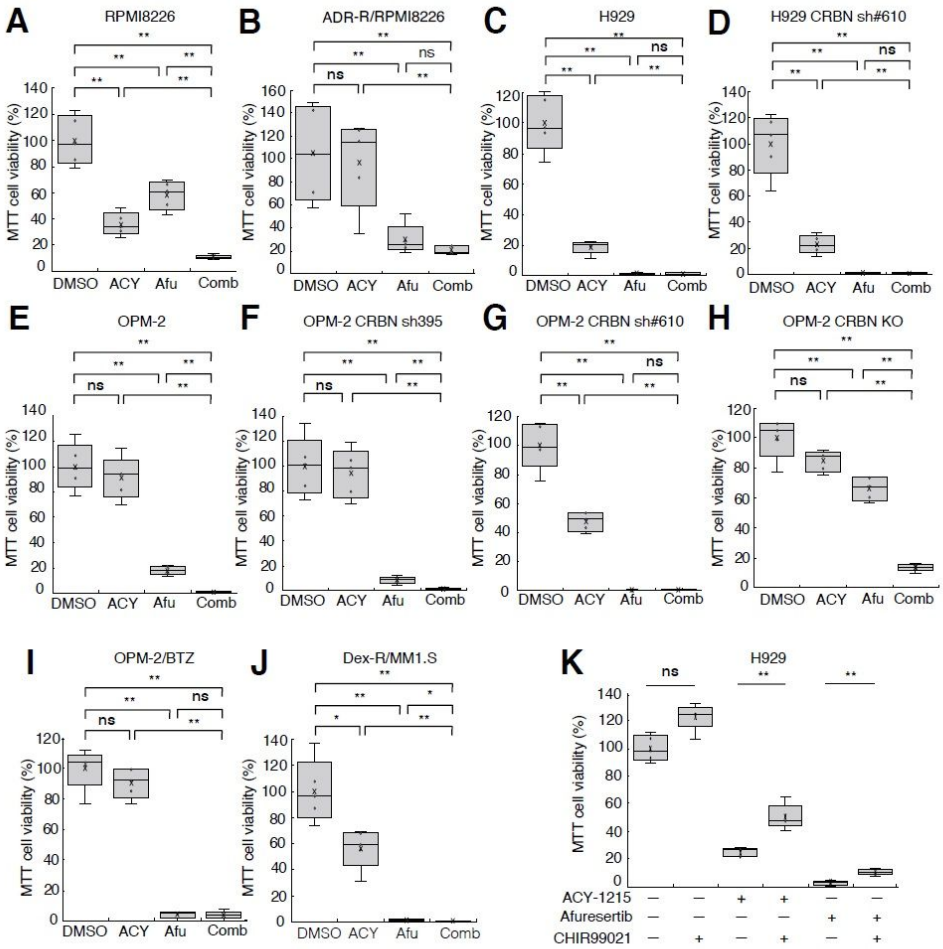
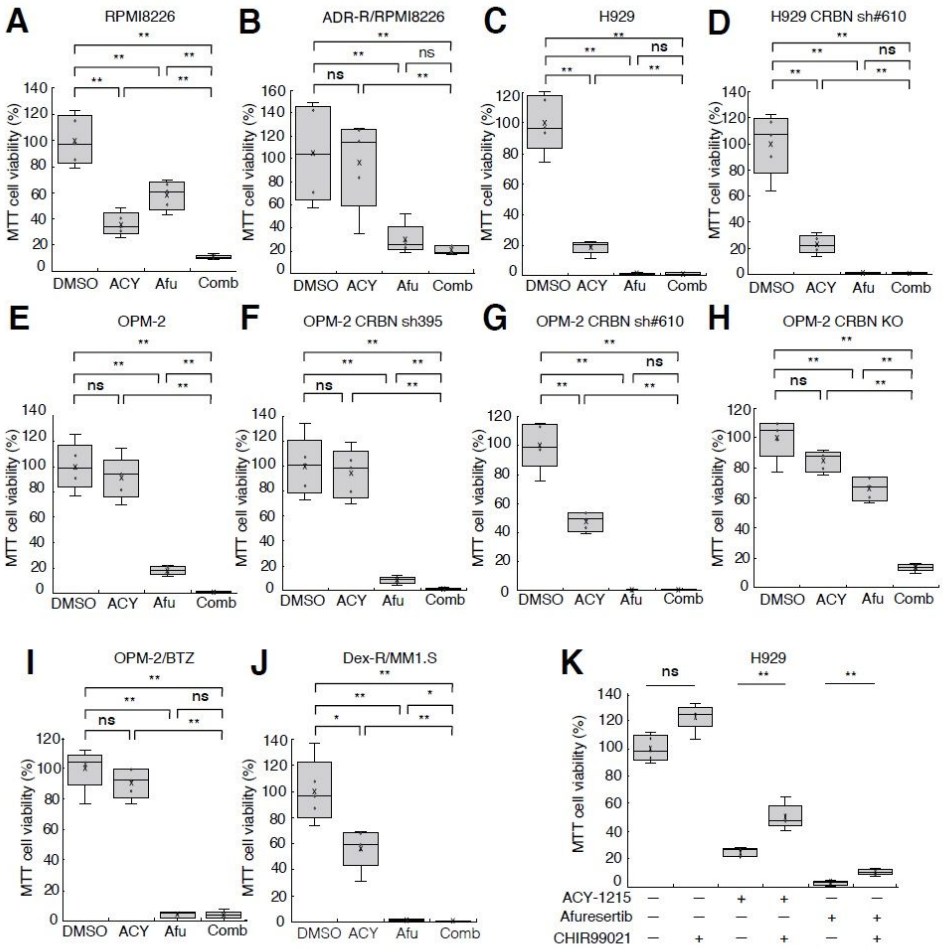


Figure 4

HDAC and Akt inhibitors exhibit cytotoxicity in several MM cell lines. (a-j) MTT proliferation assay. Each cell line was treated with DMSO, 2 μM ACY-1215 (ACY), 4 μM afuresertib (Afu), or a combination of ACY-1215 and afuresertib (Comb) for 72 h. The average was calculated for each experiment performed in quintuplicate (n = 5). Data were analyzed using the paired Student t-test (*p < 0.05, **p < 0.01). “ns” indicates no significant difference. (k) H929 were treated with 2 μM ACY-1215, 4 μM afuresertib with/without 1 μM CHIR 99021 (GSK-3 inhibitor) for 48 h. Experiments were performed in quintuplicate (n = 5). Data were evaluated using the two-paired Student t-test (**p < 0.01). “ns” indicates no significant difference.



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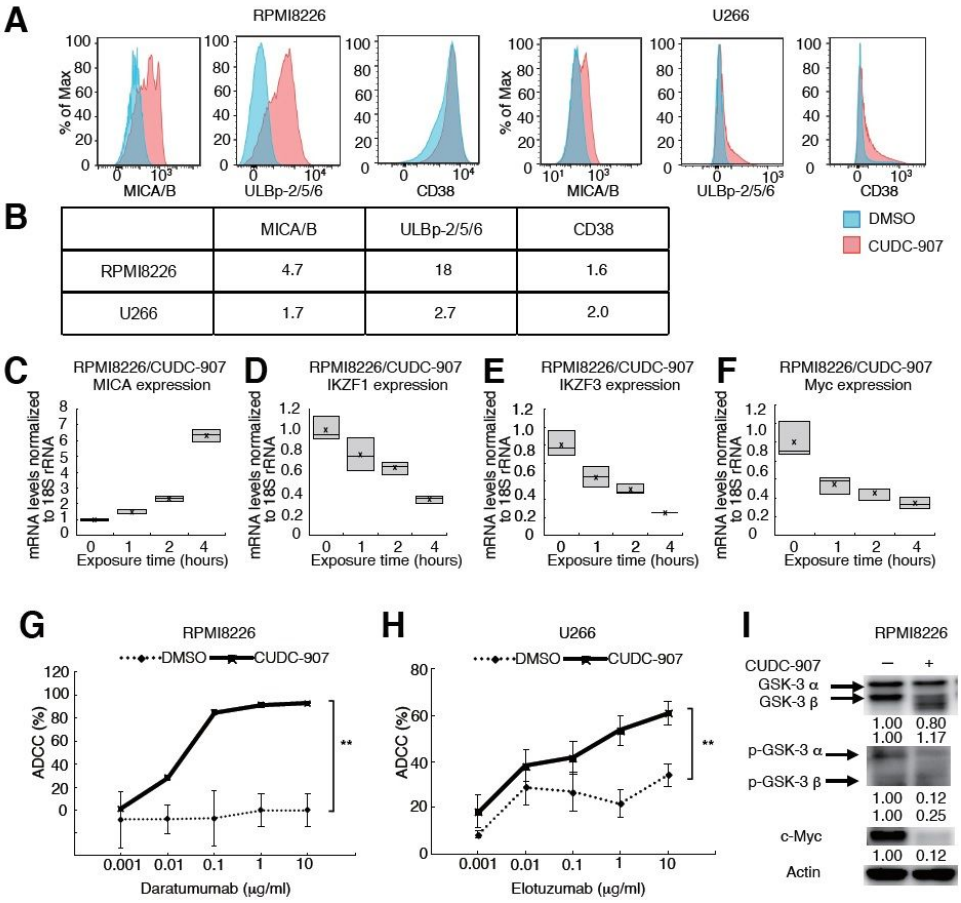
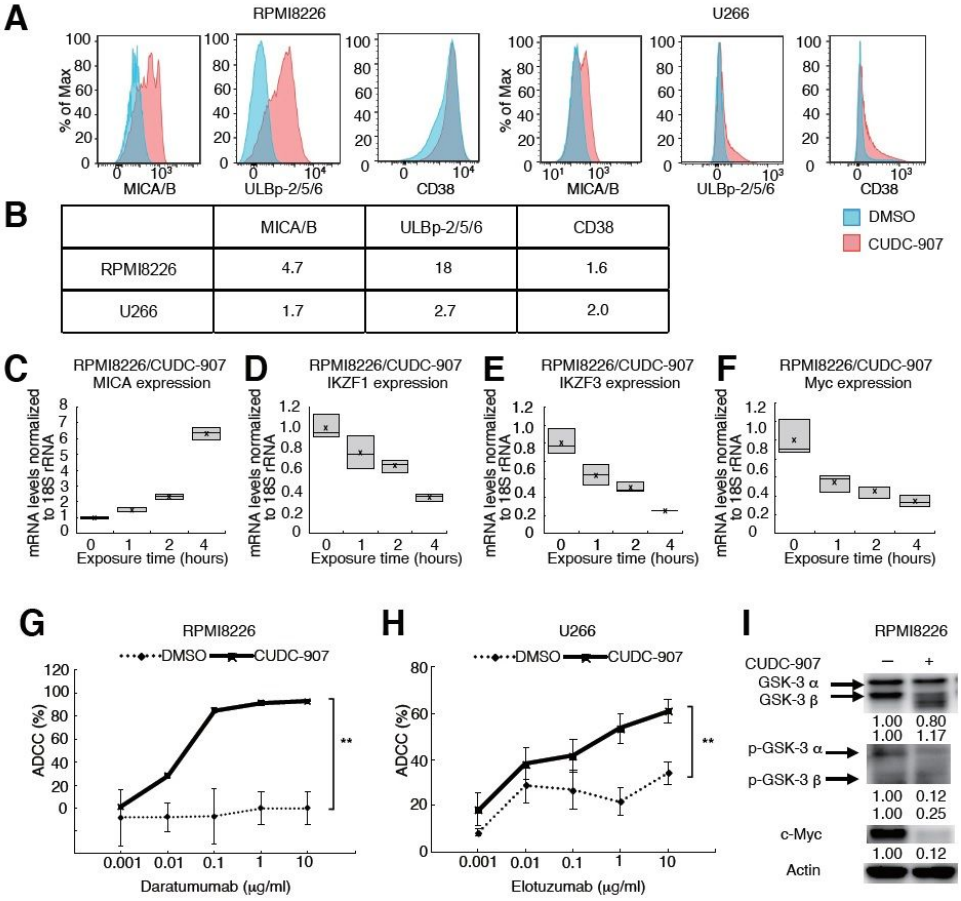


	MICA/B	ULBp-2/5/6	CD38
RPMI8226	4.7	18	1.6
U266	1.7	2.7	2.0

Figure 5

CUDC-907 enhances ADCC of mAbs by upregulating MICA, ULBp-2/5/6, CD38 while downregulating c-Myc. (a) Histograms show MICA/MICB, ULBp, and CD38 expression in RPMI8226 and U266 cells treated with 10 nM CUDC-907. (b) Summary of the ratio of MFI for MICA/B, ULBp-2/5/6, and CD38 expression in cells exposed to CUDC-907. (c-f) MICA, IKZF1/3, Myc mRNA levels in RPMI8226 cells exposed to CUDC-907. Experiments were performed in triplicate (n = 3). (g–h) ADCC assay of daratumumab and elotuzumab. Experiments were performed in triplicate (n = 3), and data are presented as the mean ± SD. P-values were calculated for the interaction terms in the regression models, which are interpreted as the difference in slope of dose-dependent change between the groups (**p < 0.01). (i) Western blot validation of the effects of CUDC-907 on RPMI8226 cells.



	MICA/B	ULBp-2/5/6	CD38
RPMI8226	4.7	18	1.6
U266	1.7	2.7	2.0

Figure 5

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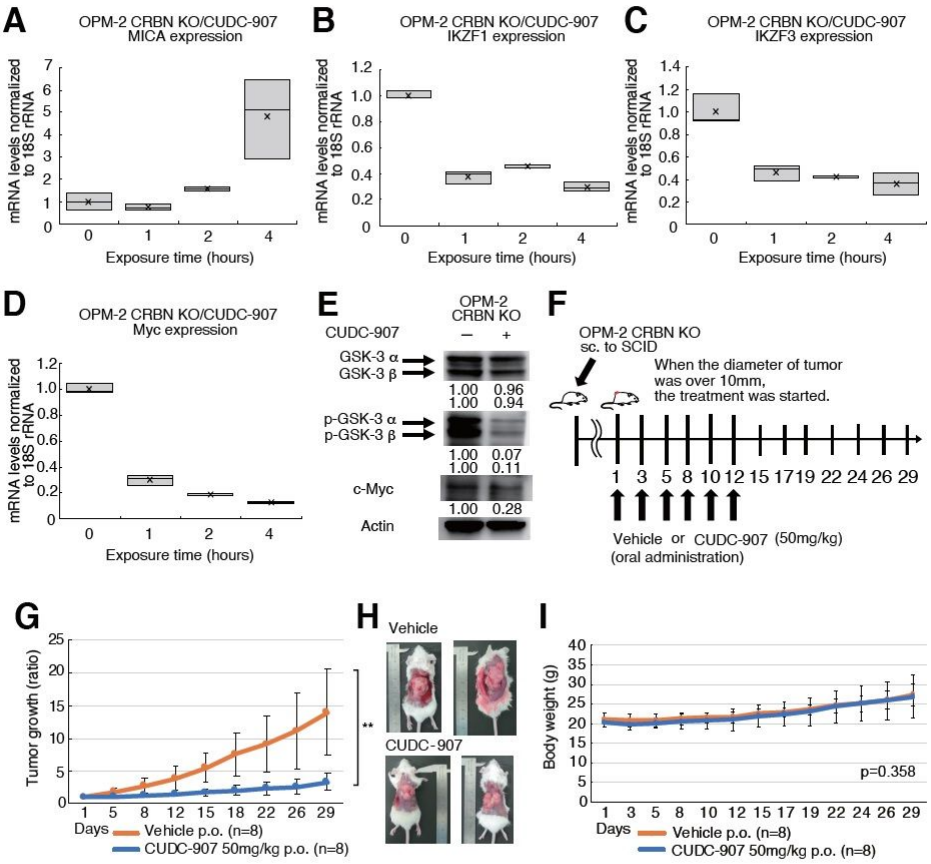
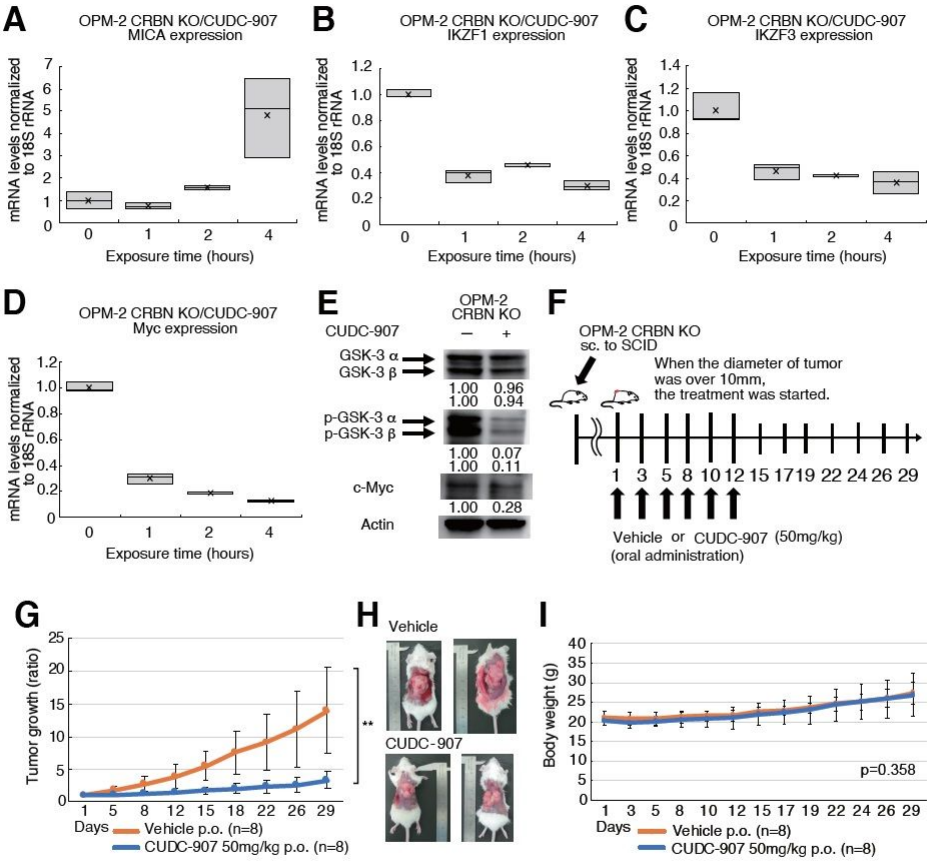


Figure 6

CUDC-907-induced NKG2D ligand upregulation and IKZF1/3 downregulation reduces CRBN-knockout cell growth in vivo. (a–d) mRNA levels of MICA, IKZF1, IKZF3, and Myc in CRBN-knockout OPM-2 cells treated with CUDC-907. Experiments were performed in triplicate (n = 3). (e) OPM-2 CRBN-knockout cell lysate treated with CUDC-907 was immunoblotted with the indicated antibodies. (f) Xenograft mouse model using CRBN-knockout OPM-2 cells. Eight mice were orally administered 50 mg/kg vehicle (n = 8) or CUDC-907 (n = 8) three times a week for 2 weeks. (g) Tumor growth (ratio) of CRBN-knockout OPM-2 cells in SCID mice treated with either vehicle or CUDC-907. P-values were calculated for the interaction terms in the regression models (**p < 0.01). (h) The representative images of tumors of each group at day 29. (i) Body weight was measured on the indicated days. P-values were calculated for the interaction terms in the regression models.



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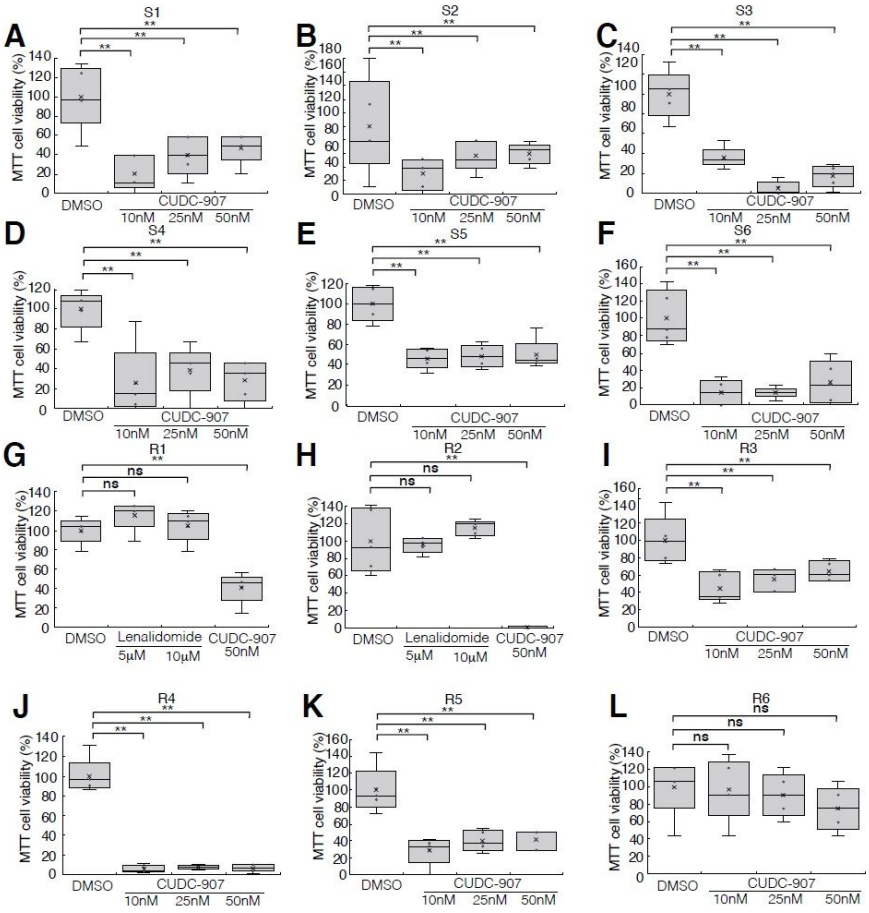
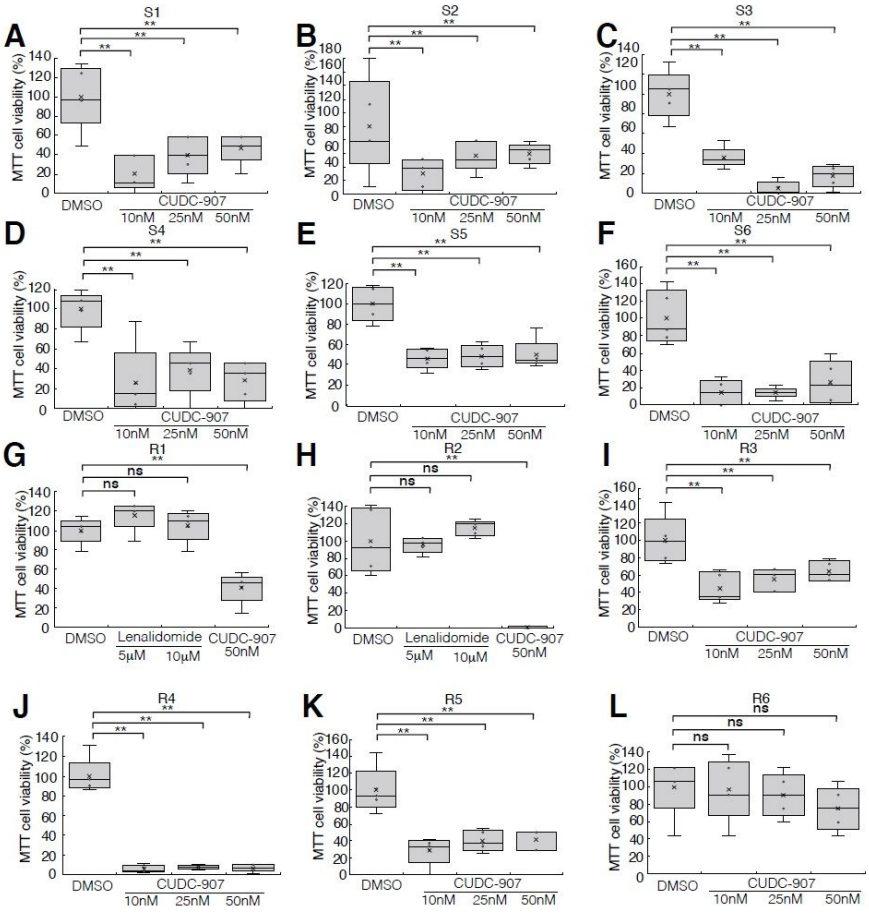


Figure 7

CUDC-907 is effective in primary cells obtained from lenalidomide-resistant patients. (a–g) MTT proliferation assays were performed in primary cells from six lenalidomide-resistant MM patients. Cells were treated with 5 μM lenalidomide, 10 μM lenalidomide, or 10–50 nM CUDC-907 for 48 h.



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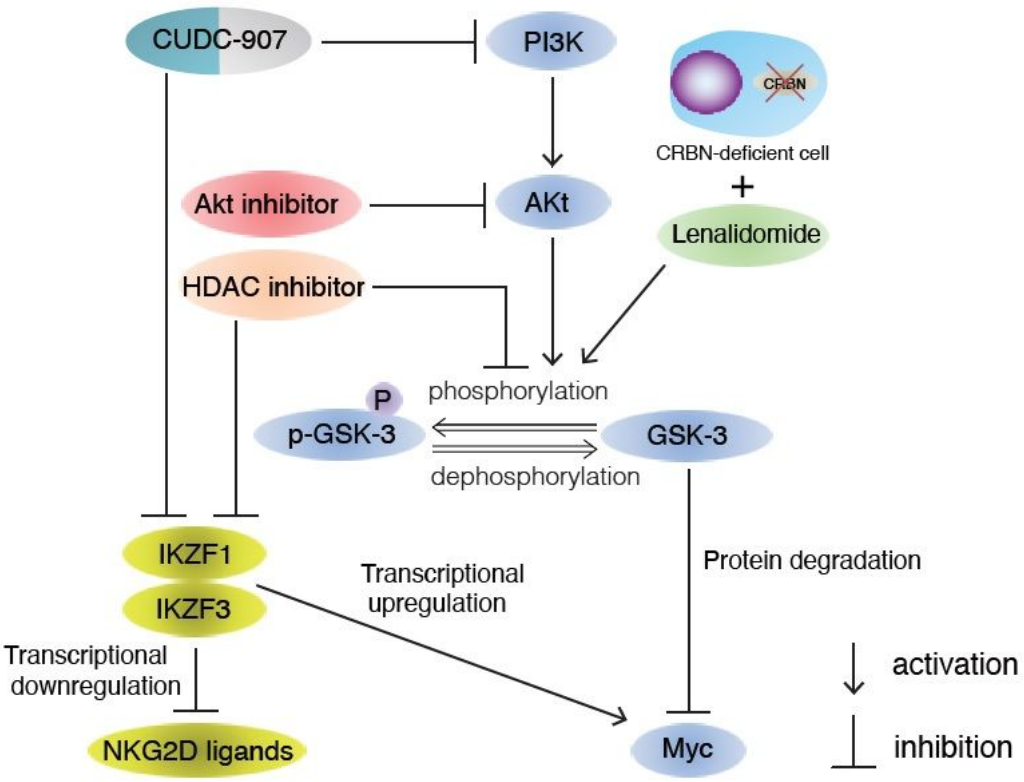
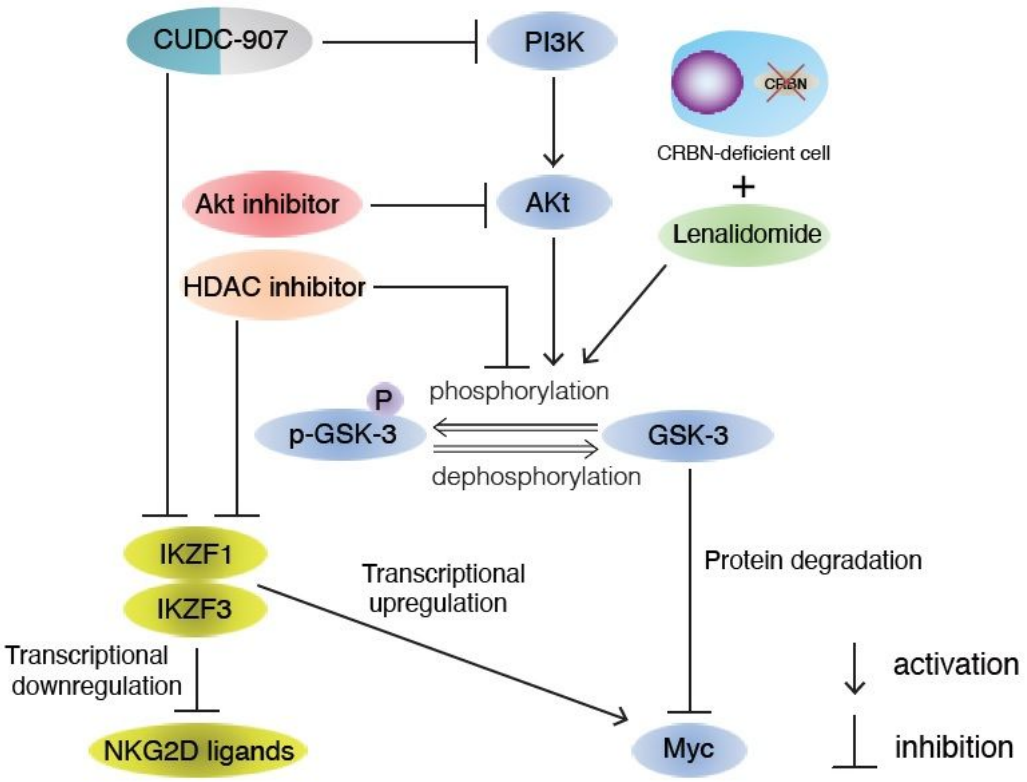


Figure 8

Proposed mechanism of HDAC, Akt, and PI3K inhibition in MM cells. Lenalidomide increases p-GSK-3, which functions to stabilize c-Myc and enhance MM cell survival, when CRBN expression is reduced. HDAC inhibitor treatment downregulates IKZF1/3, inducing c-Myc downregulation and MICA upregulation. PI3K or Akt inhibitors downregulate p-GSK-3 and c-Myc expression in MM cells. HDAC inhibition also downregulates p-GSK-3. CUDC-907 has the ability to enhance MICA and suppress c-Myc. CUDC-907 is a promising drug that has the advantages of dual HDAC and Akt inhibition.



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