

Ceritinib overcomes proteasome inhibitor resistance in multiple myeloma by suppressing the protein folding response

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SUPPLEMENTARY DATA

SUPPLEMENTARY METHODS

Cell culture

The myeloma cell lines (AMO-1, L363, RPMI-8226 and ARH-77) and their derivatives were maintained in 10% FCS-supplemented RPMI-1640 with 1% penicillin/streptomycin (all Merck/Sigma–Aldrich, Buchs, Switzerland). U2-OS cell line was maintained in 10% FCS-supplemented Dulbecco's Modified Eagle's Medium (DMEM) with 1% penicillin/streptomycin (all Merck/Sigma–Aldrich). Cell lines were routinely tested for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland) and verified by STR-typing (DSMZ, Braunschweig, Germany).

Chemicals

Proteasome inhibitors: bortezomib (PS-341; HY-10227) and carfilzomib (PR-171; HY-10455), ALK-inhibitors: crizotinib (PF-02341066; HY-50878), ceritinib (LDK378; HY-15656), brigatinib (AP-26113; HY-12857), entrectinib (NMS-E628; HY-12678), alectinib (CH5424802; HY-13011), lorlatinib (PF-06463922; HY-12215) and ensartinib (X-396; HY-103714) ; FOXO-1 inhibitor (AS-1842856; HY-100596), IGF-1R/INSR inhibitor (NVP-AEW541; HY-50866), IGF-1R/INSR inhibitor (BMS-536924; HY-10262), were purchased from MedChem Express (Monmouth Junction, NJ, USA).

Viability assays

Cytotoxicity of the treatments in cell lines was assessed using the Cell Counting Kit-8 (CCK-8) colorimetric assay (GLPBio, Montclair, CA, USA). Cells were seeded into clear, flat-bottom 96-well plates and treated with the indicated doses and drug combinations for 48 hours. Following treatment, CCK-8 reagent was added, and absorbance was measured at 492 nm using a Tecan microplate reader (Tecan, Männedorf, Switzerland).

For primary cells, cytotoxicity was evaluated using the CellTiter-Glo luminescence assay (Promega, Madison, WI, USA). Cells were seeded in white, flat-bottom 96-well plates and treated under the same conditions. After 48 hours, luminescence was measured using a Tecan reader.

Dose-response curves and IC₅₀ values were generated using non-linear regression analysis. The coefficient of drug interaction (CDI) in the combination treatment was calculated as

previously described.¹ To identify the doses generating the strongest interaction, multiple drug combinations were tested in the indicated cell lines, and only the data showing the lowest CDI for each cell line are presented. CDI values between 0.0 and 0.8 indicate synergistic effects, values between 0.8 and 1.0 suggest additive effects, and values greater than 1.0 indicate antagonism.

Determination of proteasome activity by active site labeling with activity-based probes

Proteasome-specific affinity-based probes, each targeting a specific proteasome subunit, were used to evaluate the activity of all constitutive and immunoproteasome subunits as described.² In brief, equal amounts of total protein were resolved by SDS-PAGE using bis-tris 12% gels (Thermo Fisher Scientific, MA, USA), and labeled active β -subunits were directly visualized in the gel using Fusion Solo S (Vilber Lourmat, France).

Western blots

Briefly, samples were lysed with lysis buffer, and 20 μ g of protein per lane was separated by SDS-PAGE on Bis-Tris 10% gels (Thermo Fisher Scientific, MA, USA). After SDS-PAGE, proteins were transferred onto PVDF membranes. The membranes were then blocked with RotiBlock (Roth, Germany) and incubated with primary antibodies. The following day, the membranes were washed, incubated with secondary antibodies, and the chemiluminescence signal was acquired after treatment with the HCL substrate using the Fusion S Solo camera (Vilber Lourmat, France).

Antibody	Source	ID
HSP70 (rabbit)	Invitrogen	#PA1-014A; RRID:AB_559362
HSPA5 (GRP78, BiP) (mouse)	Invitrogen	#MA3-006; RRID:AB_325454
phospho-p70 S6 Kinase (rabbit)	Cell Signaling Technology	#9204; RRID:AB_2265913
p70 S6 Kinase (rabbit)	Cell Signaling Technology	#9202; RRID:AB_331676
phospho-S6 Ribosomal protein (rabbit)	Cell Signaling Technology	#4858; RRID:AB_916156
S6 Ribosomal protein (rabbit)	Cell Signaling Technology	#2217; RRID:AB_331355
Ub-K48 Recombinant Rabbit Monoclonal Antibody (ARC0811)	Invitrogen	#MA5-35382; RRID:AB_2849283
HRP-conjugated β -actin (mouse)	Proteintech	#60008; RRID:AB_2819183
HRP-conjugated α -tubulin (mouse)	Proteintech	#66031; RRID:AB_2687491
Peroxidase AffiniPure Goat Anti-Rabbit IgG	JacksonImmunoResearch	#111-035-003; RRID:AB_2313567
Peroxidase AffiniPure™ Sheep Anti-Mouse IgG	JacksonImmunoResearch	#515-035-003; RRID:AB_2340295

RNA seq data analysis of MM cell lines

RNA sequencing data from multiple MM cell lines were obtained from publicly available sources under the accession number GSE160572, as previously described.³

RNA sequencing after drug treatment and data analysis

AMO-1 cells were treated for 8 h with ceritinib (4 μ M), carfilzomib (5 nM) or drug combinations. Subsequently, the cells were washed with PBS and snap-frozen in liquid nitrogen. RNA sequencing analysis was performed by the BGI Tech Solutions (Hong Kong) using NovaSeq (Illumina). Samples were sequenced using paired-end 75 bp reads; NEBNext Ultra II Directional RNA Library Prep Kit for Illumina + polyA selection (New England Biolabs, Ipswich, MA, USA). The obtained data passed quality control. Differential gene expression was calculated by DESeq2.⁴ Differently expressed genes with adjusted p value < 0.05 were taken into further analyses. All data related to differentially expressed genes between treatment conditions are presented in the **Supplementary Table S6**.

Kinase inhibitor selectivity data

Kinase inhibitor selectivity data was retrieved from ChEMBL version 30. All activity data available for the clinical inhibitors were downloaded through the web interface and aggregated on a protein level using KNIME Analytics Platform (version 4.5). Proteins were then sorted based on the count of assigned 'ACTIVE' in ChEMBL, corresponding to any determined inhibition constant for the inhibitor protein pair. This resulted in 33 proteins for which at least 3 of 6 inhibitors were known to be active, with ALK as expected targeted by all 6.

Multiple Myeloma Research Foundation CoMMpass dataset analysis

Multiple Myeloma Research Foundation (MMRF) provided an access to the CoMMpass study. Data were accessed via the MMRF Researcher Gateway (IA18 release) on 20th of March, 2020. The salmon aligned untraded transcripts, survival data and patients' information was used to analyze the expression of INSR, IGF-1R, ALK, FLT3, BLK, LCK, ZAP70, FES, BTK, SRC, NRPL2, NRPL3, TSC1, TSC2, DDIT4, FOXO1 and DDIT4 in plasma cells from 544 MM patients at the time of diagnosis. The data was filtered to exclude patients that died from unrelated causes, had illnesses that were reported to interfere with the study assessment, were not compliant with the study procedure, were removed from the study due to untreated health problems or had a stem-cell transplant done after the first line of therapy while including only patients with ages between 45 and 85 years. The majority of patients were treated with

proteasome inhibitors. For INSR, IGF1R, NPRL2, NPRL3, DDIT4, FOXO1 and PDCD4, the association with overall survival and progression-free survival was estimated using Kaplan-Meier survival analysis,⁵ the statistical significance being tested through the Tarone-Ware test.⁶ Gene expression levels were stratified into four groups based on quartile splits, allowing for a comparison of survival outcomes across different expression levels. For extracting the expression level information, raw gene counts were normalized using the DESeq2 pipeline. For these analyses, the following R packages were used: readr,⁷ stringr,⁸ DESeq2,⁴ ggplot2,⁹ survival,¹⁰ tidyverse,¹¹ xlsx,¹² clusterProfiler,¹³ survminer,¹⁴ coin¹⁵ and edgeR.¹⁶

Relapsed/refractory dataset analysis

A bulk RNA-seq data from RRMM patients from University Hospital Würzburg was used to analyze the expression changes in plasma cells obtained from 75 MM patients at relapse, as previously described.¹⁷ The study was approved by the Internal Review Board at the University hospital of Würzburg (Wü8/21) and adhered to the tenets of the Declaration of Helsinki. RRMM was defined as per current IMWG criteria.¹⁸ To compare expression of INSR and IGF1R between newly diagnosed patients from CoMMpass and dataset of RRMM from Würzburg, first a Spearman correlation was computed between the 2 studies datasets and the top 75 samples with the highest median correlation from the MMRF datasets with respect to the Würzburg dataset were selected. MMRF and Würzburg datasets were aggregated together and filtered to exclude entries with a total sum in all experiments below 400 and that have more than 3 missing values. Moreover, entries with TPM values above 10000 in at least one experiment were removed to help normalize the distributions (these were considerable outliers). An initial list of stable genes was extracted by performing a Kolmogorov-Smirnov test on the log-transformed data, corrected using the BH method for multiple testing, and selecting the genes with an FDR higher than 0.25 (around 400 genes out of 9500). These genes were used as the baseline information for RUVg batch correction algorithm on the normal scale using only one factor of unwanted variation (k). Afterward, the data was normalized using the median of ratios normalization on the log2 scale. Lastly, a limma trend analysis pipeline was applied to identify differentially expressed elements. This consists of estimating the weights for each sample before performing the linear modeling. An intensity-dependent prior trend was used as well as a robust identification of the prior variance and degrees of freedom against outliers. A gene was deemed as differentially expressed if it had an FDR value below 10^{-5} and an absolute log2FC value above 0.75.

Cancer Dependency Map data analysis

The expression and dependency scores of BTK (Bruton Tyrosine Kinase), IGF-1R (Insulin-Like Growth Factor 1 Receptor), INSR (Insulin Receptor), and SRC (SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase) genes were analyzed using data from the DepMap Public 24Q4 dataset. Gene expression levels were assessed using RNA-seq-derived transcripts per million (TPM), normalized across cell lines. Gene dependency was evaluated using CRISPR-Cas9 loss-of-function screening data, specifically the Chronos dependency scores, where lower scores indicate stronger dependency. Analysis was conducted across a panel of 24 MM cell lines.

Untargeted metabolomics analysis

AMO-1 cells were treated for 8 h with ceritinib (4 μ M), carfilzomib (5 nM) or drug combinations. Subsequently, the cells were washed with PBS and snap-frozen in liquid nitrogen. Untargeted metabolomics analysis was performed by the BGI Tech Solutions (Hong Kong). A Waters UPLC I-Class Plus (Waters, USA) tandem Q Exactive high-resolution mass spectrometer (UPLS-MS) (Thermo Fisher Scientific, USA) was used for separation and detection of metabolites. Data quality control was ensured and is described in detail in supplementary methods. Data pre-processing was performed using metaX,¹⁹ to obtain all forms of compounds and their intensities for the next step of data analysis. Classification and functional annotation of the identified metabolites were performed according to their KEGG ID and HMDB ID, and search in respective databases. For the comparison of two groups (treatment vs. control), univariate analysis was used: fold change (ratio between two groups in analysis) was calculated, and the p value was obtained by the t-test. Only metabolites with a p-value < 0.05 and FC ≥ 1.2 or ≤ 0.83 were considered statistically different. For better visualization, the data are presented as log₂ (FC) values. All metabolites identified in the treatment conditions and compared between the conditions are presented in the **Supplementary Table S10**. Enrichment analysis of statistically different metabolites was performed using the MetaboAnalyst analysis toolkit.²⁰

PamGene Kinase screening

The PamGene assays measure kinase activity in cell and tissue lysates by measuring the phosphorylation of peptide representations of kinase targets/ substrates (referred hereafter as phosphosites) that are immobilized on the PamChip® microarrays (PamGene International, s-Hertogenbosch, The Netherlands). Two types of PamChip® microarrays have been used to evaluate the activity of ceritinib in AMO-1 cells following 30 min incubation with 4 μ M ceritinib;

one, which evaluates phosphosites of 196 protein tyrosine kinase (PTK) and the other one, which evaluates phosphosites of 144 serine/threonine kinase (STK). In brief, whole-cell lysates were prepared using lysis buffer that contained inhibitors of proteases and phosphatases and were stored immediately in a -80°C freezer. Protein quantification was performed with the bicinchoninic acid assay according to the manufacturer's instructions (BCA; Merck KGaA, Darmstadt, Germany). Per array 5 μg of protein and 400 μM ATP were applied. Sequence-specific peptide tyrosine and serin–threonin phosphorylation were detected by the fluorescein-labeled antibody PY20 (Exalpha, Maynard, MA, USA) and a CCD camera using the Evolve software (1200PTKlysv04.PS12Protocol; PamGene International). Data were analyzed using the Bio Navigator software (PamGene International). To identify significant differences in PTK phosphosites between control and ceritinib-treated cells, an ANOVA with Dunnett post-test was used, for analysis of differences in STK phosphosites, LIMMA was applied.

Apoptosis determination

The AMO-1 cells were exposed to the indicated drugs for 24 h. The cells were washed and stained using an Annexin V/FITC Detection Kit (Vazyme Biotech, Nanjing, China), according to the manufacturer's instructions. The fluorescence intensity of the cells was analyzed using a BD LSR Fortessa cell analyzer (BD Biosciences, New Jersey, USA). The data were analyzed using the BD FACS DIVA Software (BD Biosciences).

Evaluation of autophagic flux

AMO-1 cells were transduced with a lentiviral vector expressing the pHluorin-mKate2-hLC3 construct (FUGW-PK-hLC3, RRID:Addgene_61460, kindly provided by Isei Tanida²¹), enabling simultaneous quantitative assessment of autophagosome and lysosome formation. This system leverages the pH-sensitive quenching of GFP within lysosomes, combined with the pH-stable fluorescence of mKate2. As a result, autophagosomes appear yellow (due to combined GFP and RFP signals), while lysosomes appear red (due to loss of GFP fluorescence and persistence of RFP), allowing differentiation based on the RFP/GFP fluorescence ratio. Lentivirus production followed the protocol described above. Transduced cells were sorted based on stable RFP expression and subcloned. Single-cell-derived clones with the highest RFP signal were selected for subsequent experiments. To measure autophagic flux, cells were treated with the indicated drugs for 8 hours, washed with PBS, and analyzed for RFP and GFP fluorescence using a BD LSR Fortessa cell analyzer (BD Biosciences). Data were processed and analyzed using BD FACSDiva Software (BD Biosciences).

Analysis of UPR induction

AMO-1 cells were equipped with ER-stress sensor, an XBP1 splicing fluorescent reporter with mNeonGreen²² (RRID:Addgene_115968; a gift from David Andrews). Lentivirus was produced as described above. Transduced cells were sorted for fluorescence intensity after ER stress and subcloned. The single-cell-derived clones with the highest positivity were used in the experiments.

To measure UPR induction, the cells were treated with the drugs for 8 h, washed with PBS and the fluorescence intensity of the reporters was determined using a BD LSRFortessa cell analyzer (BD). The data were analyzed using the FACSDiva Software (BD Biosciences).

Analysis of mTORC1 activity

AMO-1 cells were equipped with mVenus-TOSI, a fluorescent reporter for mTORC1 signaling (monitoring PDCD4 degradation, RRID:Addgene_172491, a gift from David Scadden) using retroviral transduction. Retrovirus was produced using pUMVC and pCMV-VSV-G plasmids (RRID:Addgene_8448 and RRID:Addgene_8454, both gifts from Bob Weinberg) in HEK-293-LentiX cell line (Clontech/Takara Bio; Saint-Germain-en-Laye, France). Transduced cells were sorted for fluorescence intensity and subcloned. The single-cell-derived clones with the highest positivity were used in the experiments.

To measure mTORC1 inhibition, the cells were treated with the drugs for 12 h, washed with PBS and the fluorescence intensity of the PDCD4-reporter was determined using a BD LSRFortessa cell analyzer (BD Biosciences). The data were analyzed using the FACSDiva Software (BD Biosciences).

Aggresome formation

Formation of aggresomes, inclusion bodies of aggregated, misfolded proteins that form when the ubiquitin-proteasome protein-degradation machinery is overwhelmed, was evaluated 20 h after treatment using the PROTEOSTAT® Aggresome Detection Kit (Enzo Life Sciences, Farmingdale, NY, USA), according to the manufacturer's recommendations. The intensity of fluorescence was determined using a BD LSRFortessa cell analyzer (BD Biosciences). The data were analyzed using the FACSDiva Software (BD Biosciences).

Analysis of proteasome function by Ub-G76V-GFP

AMO-1 cells were stably expressing Ub-G76V-GFP were generated previously.²³ Accumulation of GFP fluorescence in cells 12 h after respective treatment was acquired using a BD LSRFortessa cell analyzer (BD Biosciences). The data were analyzed using the FACSDiva Software (BD Biosciences).

Generation of U-2 OS cells with full FOXO1-Clover

U-2 OS cells were equipped with fluorescently-tagged FOXO-1 construct using lentiviral transduction, as described above. pLenti-FoxO1-Clover (RRID:Addgene_67759, a gift from Peter Rotwein), allows assessment of Akt activity in single cells²⁴ using FoxO-1, a well-characterized Akt kinase substrate.²⁵ The transduced cells were enriched for GFP positivity and subcloned. The single-cell-derived clones with stable and uniform GFP fluorescence were further used. To identify nuclear translocation of FOXO-Clover, cells were equipped with nuc-mCherry-pTRIP (RRID:Addgene_163520, a gift from Thomas Weber).

Incucyte measurement

Real-time monitoring of FOXO-1 localization was performed using the Incucyte® S3 Live-Cell Analysis System (Sartorius, Göttingen, Germany), following the Real-Time Kinetic Analysis of Akt Activity protocol provided by Sartorius. Cells were imaged and analyzed over a 12-hour period every hour. In this setup, nuclear regions were identified via the mCherry signal, while FOXO-1 localization was tracked through the Clover (GFP) signal. Quantification of nuclear FOXO-1 was performed by measuring the GFP fluorescence intensity within the mCherry-defined nuclear area. Translocation was expressed as 1 - normalized nuclear GFP intensity relative to time 0, indicating FOXO-1 ratio in nucleus to the cytoplasm over time.

Quantification and statistical analysis

Statistical analysis was performed as indicated in specific method description or in figure legends. A p value < 0.05 was considered to indicate statistical significance.

Unless otherwise specified, all data were evaluated in GraphPad Prism v10 (GraphPad Software, La Jolla, CA). Graphical abstract was generated using CorelDRAW Graphics Suite (Alludo, 2025), v 2018.

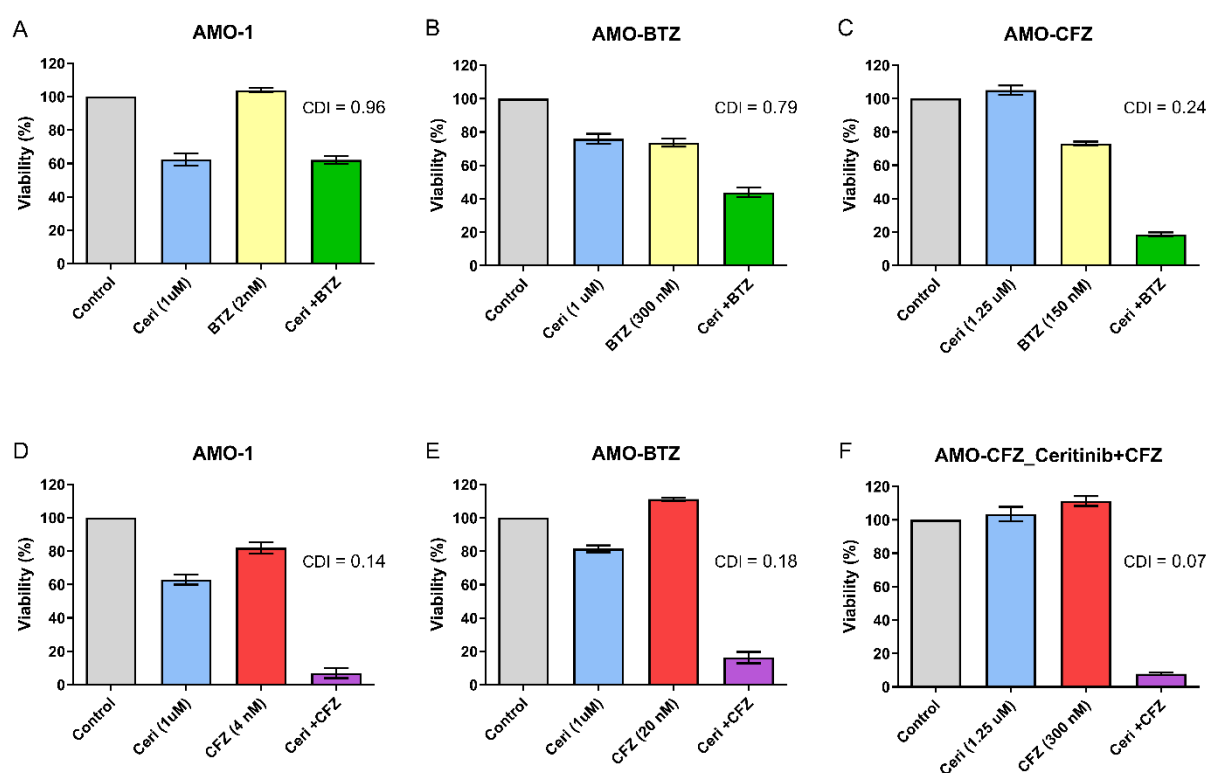
SUPPLEMENTARY FIGURES

Figure S1: Cytotoxicity of ceritinib in combination with proteasome inhibitors in the AMO-1 model, represented as a decrease in viability relative to control cells.

(A, B, C) Cytotoxicity of ceritinib in combination with bortezomib in AMO-1, AMO-BTZ and AMO-CFZ cells.

(D, E, F) Cytotoxicity of ceritinib in combination with carfilzomib in AMO-1, AMO-BTZ and AMO-CFZ cells.

Cell viability was assessed 48 h after continuous treatment. Data represent the mean $\pm$ SD from at least 3 independent experiments. The coefficient of drug interaction (CDI) for the combination treatments was calculated and is presented in Table 1.



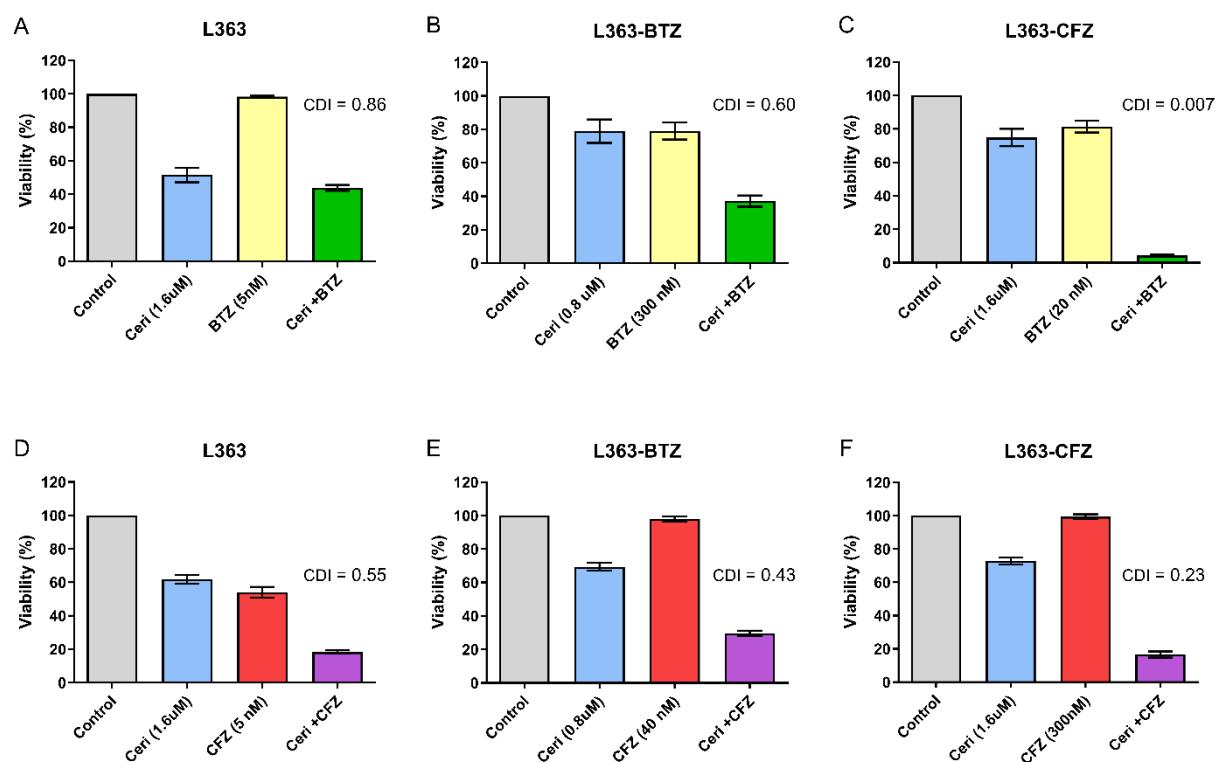
BTZ: bortezomib; Ceri: ceritinib; CFZ: carfilzomib.

Figure S2: Cytotoxicity of ceritinib in combination with proteasome inhibitors in the L363 model, represented as a decrease in viability relative to control cells.

(A, B, C) Cytotoxicity of ceritinib in combination with bortezomib in L363, L363-BTZ and L363-CFZ cells.

(D, E, F) Cytotoxicity of ceritinib in combination with carfilzomib in L363, L363-BTZ and L363-CFZ cells.

Cell viability was assessed 48 h after continuous treatment. Data represent the mean $\pm$ SD from at least 3 independent experiments. The coefficient of drug interaction (CDI) for the combination treatments was calculated and is presented in Table 1.



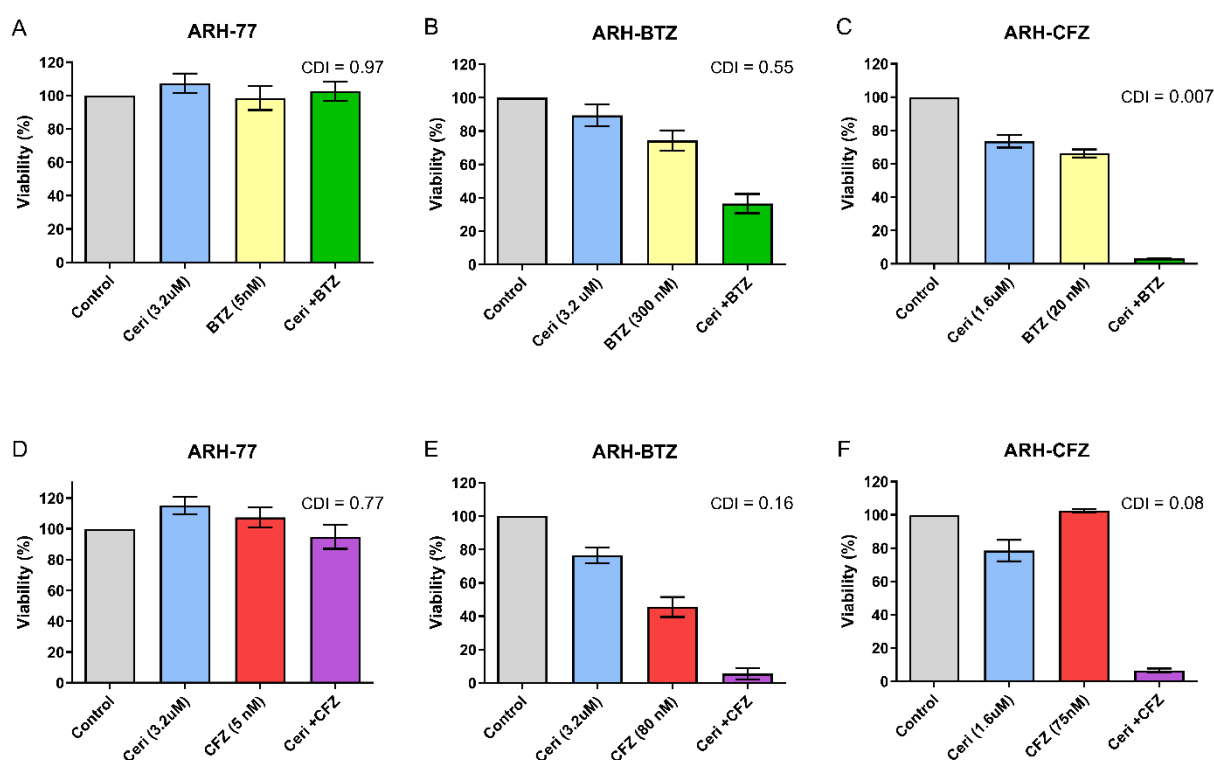
BTZ: bortezomib; Ceri: ceritinib; CFZ: carfilzomib.

Figure S3: Cytotoxicity of ceritinib in combination with proteasome inhibitors in the ARH-77 model, represented as a decrease in viability relative to control cells.

(A, B, C) Cytotoxicity of ceritinib in combination with bortezomib in ARH-77, ARH-BTZ and ARH-CFZ cells.

(D, E, F) Cytotoxicity of ceritinib in combination with carfilzomib in ARH-77, ARH-BTZ and ARH-CFZ cells.

Cell viability was assessed 48 h after continuous treatment. Data represent the mean $\pm$ SD from at least 3 independent experiments. The coefficient of drug interaction (CDI) for the combination treatments was calculated and is presented in Table 1.



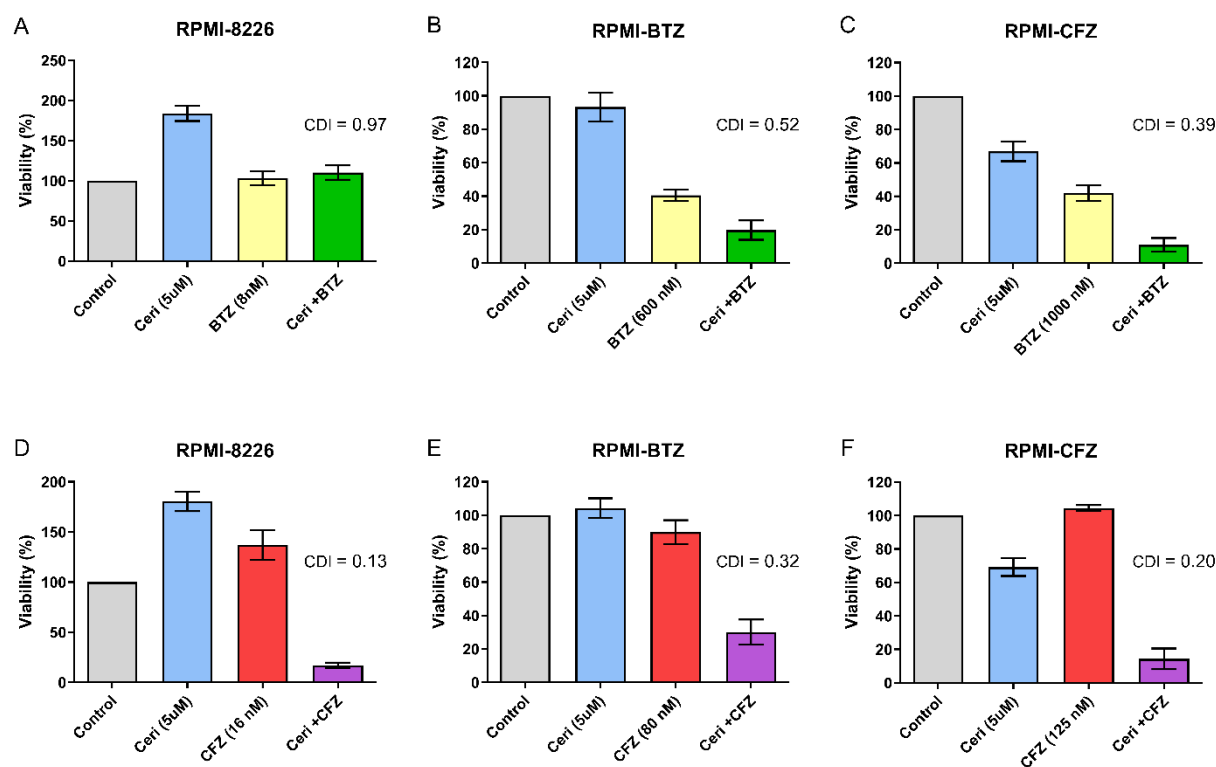
BTZ: bortezomib; Ceri: ceritinib; CFZ: carfilzomib.

Figure S4: Cytotoxicity of ceritinib in combination with proteasome inhibitors in the RPMI-8226 model, represented as a decrease in viability relative to control cells.

(A, B, C) Cytotoxicity of ceritinib in combination with bortezomib in RPMI-8226, RPMI-BTZ and RPMI-CFZ cells.

(D, E, F) Cytotoxicity of ceritinib in combination with carfilzomib in RPMI-8226, RPMI-BTZ and RPMI-CFZ cells.

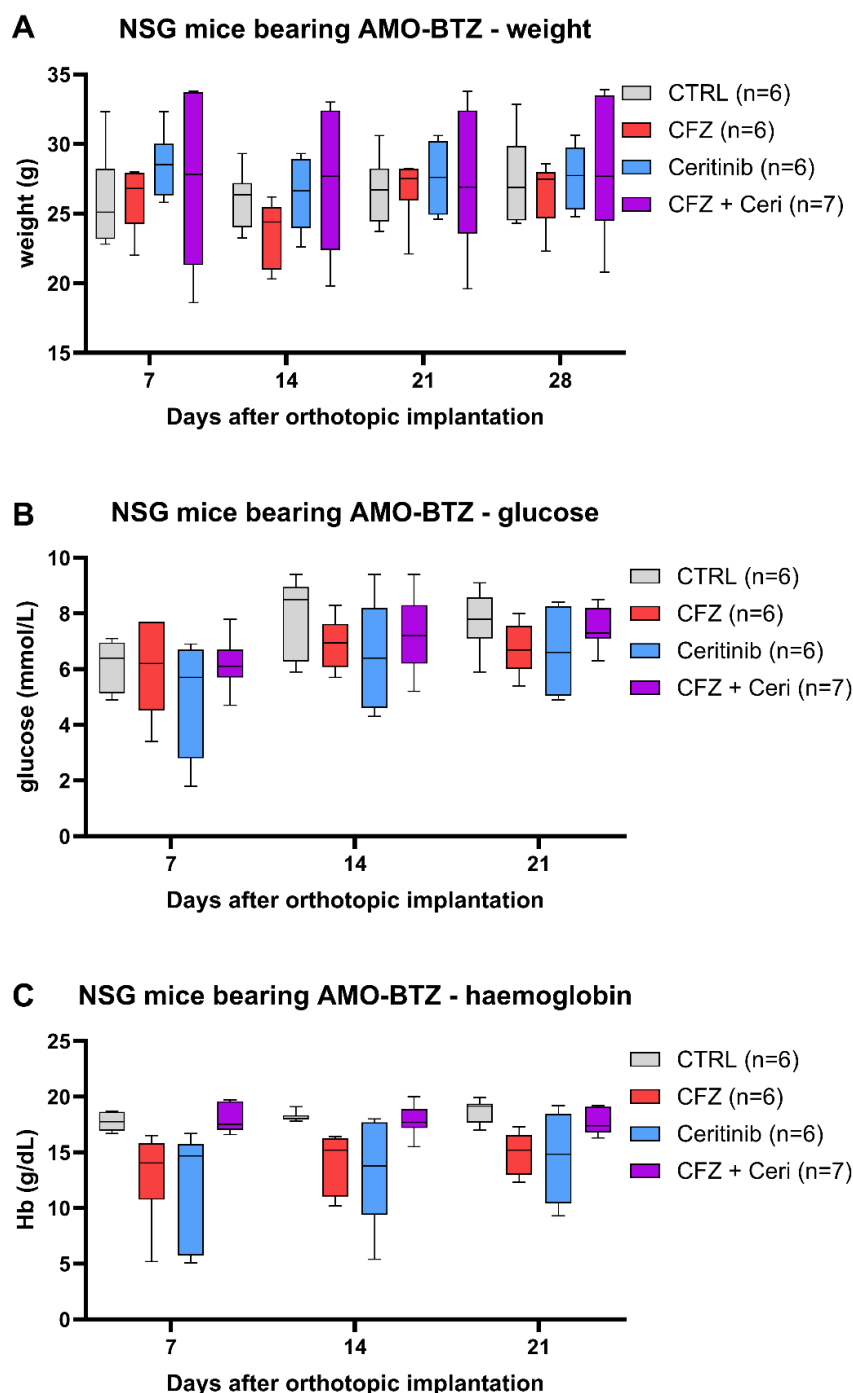
Cell viability was assessed 48 h after continuous treatment. Data represent the mean $\pm$ SD from at least 3 independent experiments. The coefficient of drug interaction (CDI) for the combination treatments is presented in Table 1.



BTZ: bortezomib; Ceri: ceritinib; CFZ: carfilzomib.

Figure S5: Effect of ceritinib, carfilzomib and their combination on mice well-being.

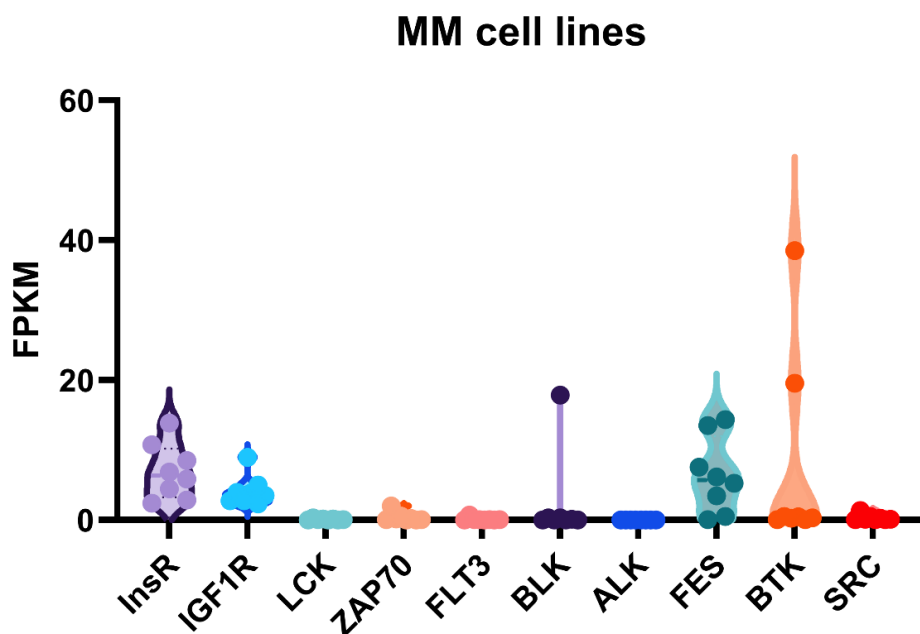
NSG mice were injected intrafemorally with AMO-BTZ cells. Seven days after orthotopic implantation, treatment was initiated with ceritinib (25 mg/kg, orally, 5 days/week), carfilzomib (4 mg/kg, intravenously, 2 days/week), or their combination. Captisol was used as the vehicle control. Mice were treated for 2 weeks and monitored for changes in **(A)** body weight, **(B)** blood glucose levels, and **(C)** hemoglobin levels. Statistical significance was tested with two-way ANOVA and Tukey's posttest, no significant differences were obtained.



Ceri: ceritinib; CFZ: carfilzomib; CTRL: vehicle control (Captisol); Hb: haemoglobin; NSG: NOD/Scid gamma

Figure S6: Expression of kinases in MM cell lines.

Violin plot representing expression of kinases identified to be candidates for ceritinib inhibition in a set of MM cell lines (AMO-1, AMO-BTZ, AMO-CFZ, L363, L363-BTZ, L363-CFZ, RPMI-8226 and RPMI-BTZ). Data represent FPKM (Fragments Per Kilobase Million) values.



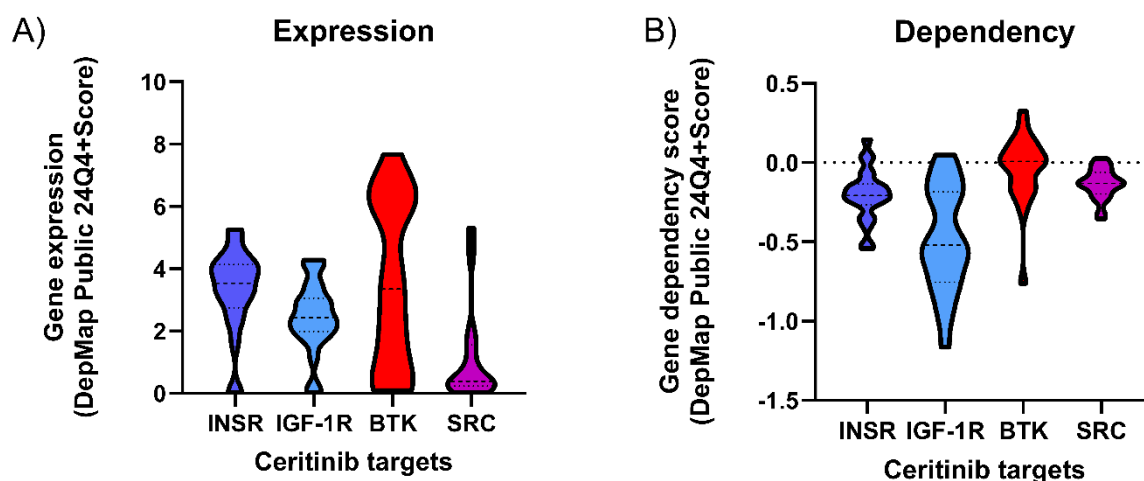
ALK: Anaplastic Lymphoma Receptor Tyrosine Kinase; BLK: B Lymphoid Tyrosine Kinase; BTK: Bruton Tyrosine Kinase; FES: FES Proto-Oncogene, Tyrosine Kinase; FLT3: Fms Related Receptor Tyrosine Kinase 3; IGF-1R: Insulin Like Growth Factor-1 Receptor; INSR: Insulin Receptor; LCK: LCK Proto-Oncogene, Src Family Tyrosine Kinase; SRC: SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase; ZAP70: Zeta Chain Of T Cell Receptor Associated Protein Kinase 70.

FPKM: Fragments Per Kilobase Million

Figure S7: Expression and dependency on putative targets of ceritinib, obtained from Cancer Dependency Map.

(A) Expression of INSR, IGF-1R, BTK and SRC in 24 MM cell lines.

(B) Dependency on INSR, IGF-1R, BTK and SRC in 24 MM cell lines. For dependency, the lower is Gene dependency score, the more essential is the gene for viability. List of cell lines and expression of genes is available in Supplementary Table S4.



BTK: Bruton Tyrosine Kinase; IGF-1R: Insulin Like Growth Factor-1 Receptor; INSR: Insulin Receptor; SRC: SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase

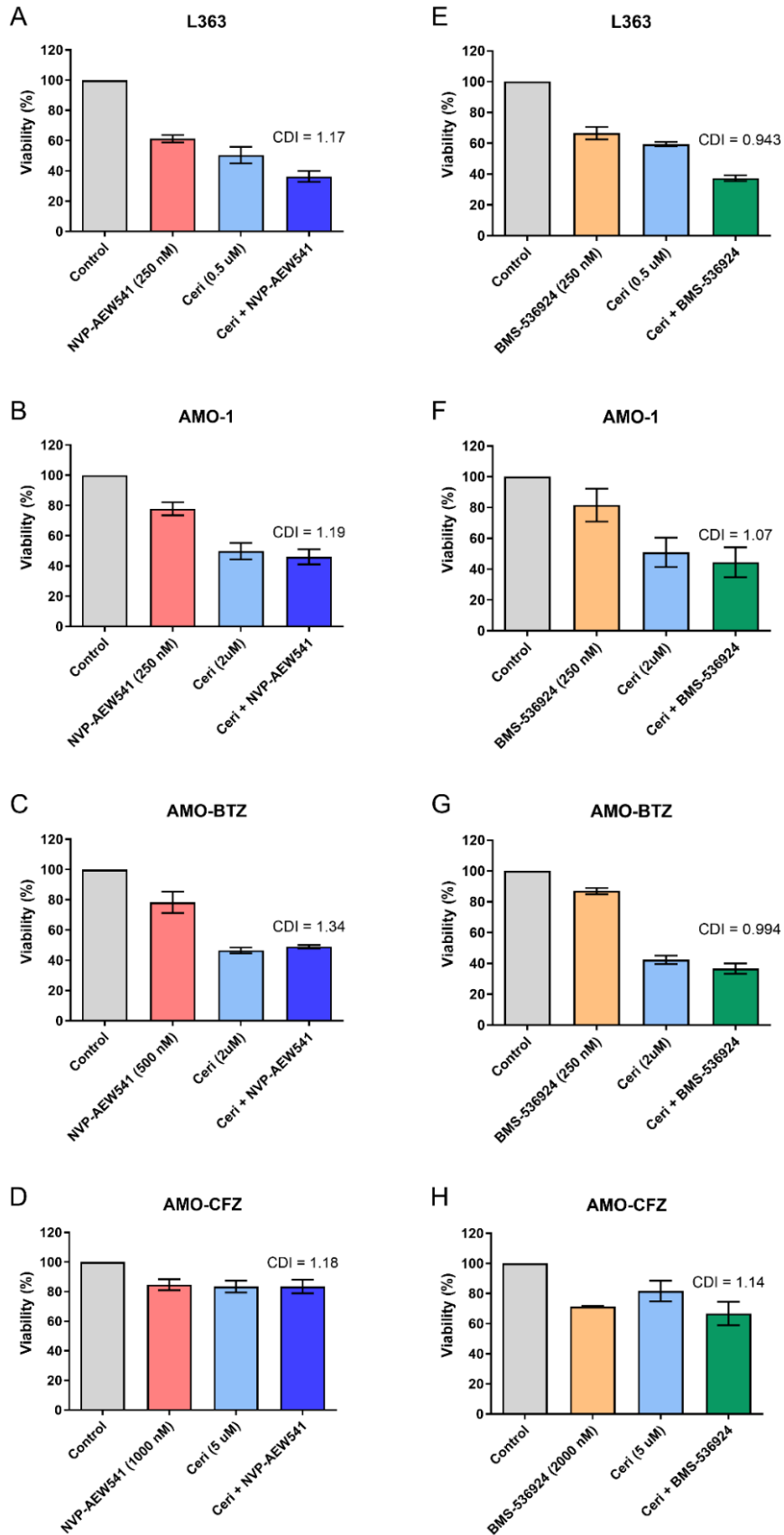
Figure S8: Cytotoxicity of ceritinib in combination with IGF-1R/INSR inhibitors in MM cells, represented as a decrease of viability relative to control cells.

(A-D) Cytotoxicity of ceritinib in combination with IGF-1R/INSR inhibitor NVP-AEW541 in AMO-1 (A), AMO-BTZ (B), AMO-CFZ (C) and L363 (D) cells.

(E-H) Cytotoxicity of ceritinib in combination with IGF-1R/INSR inhibitor BMS-236924 in AMO-1 (E), AMO-BTZ (F), AMO-CFZ (G) and L363 (H) cells.

In all experiments, viability was assessed 48 hours post continuous treatment. Data represent the mean $\pm$ SD of at least 3 independent experiments.

The coefficient of drug interaction (CDI) for the combination treatments was calculated.



BTZ: bortezomib; CFZ: carfilzomib; IGF-1R: Insulin Like Growth Factor-1 Receptor; INSR: Insulin Receptor

Figure S9: Phosphorylation of p70 S6 kinase and ribosomal protein S6.

Quantification of changes in phosphorylation of p70 S6 kinase (p70S6K) and ribosomal S6 protein in AMO-1 cells following western blot analysis, complementing Figure 3G. Data represent mean $\pm$ SD of three independent experiments. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

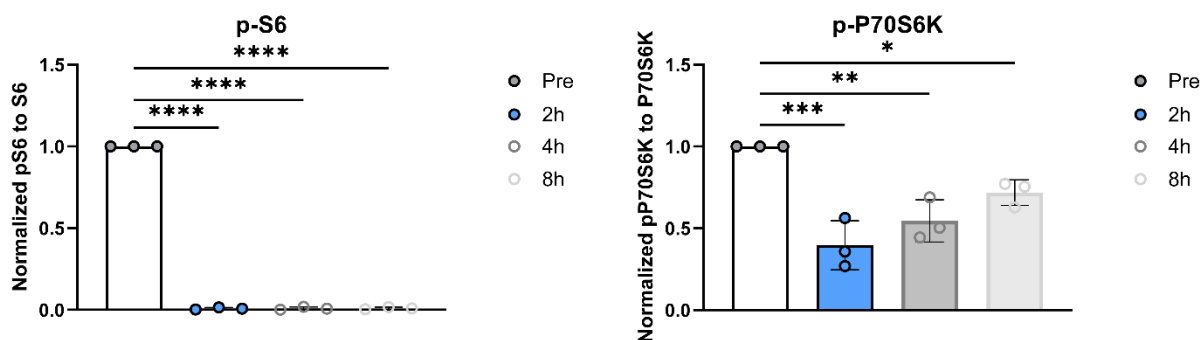


Figure S10: GSEA of ceritinib, carfilzomib, and combination treatments.

Enrichment plot showing significantly deregulated biological processes, based on GSEA followed by GO BP classification, in AMO-1 cells 8 hours after treatment with 4 μ M ceritinib.

(A) GSEA enrichment plot showing negative enrichment of the E2F target gene set following ceritinib treatment.

(B) Heatmap illustrating the leading-edge deregulated genes ($\log_2$ FC $\geq$ -3 for ceritinib treatment) under the control of the E2F transcription factor.

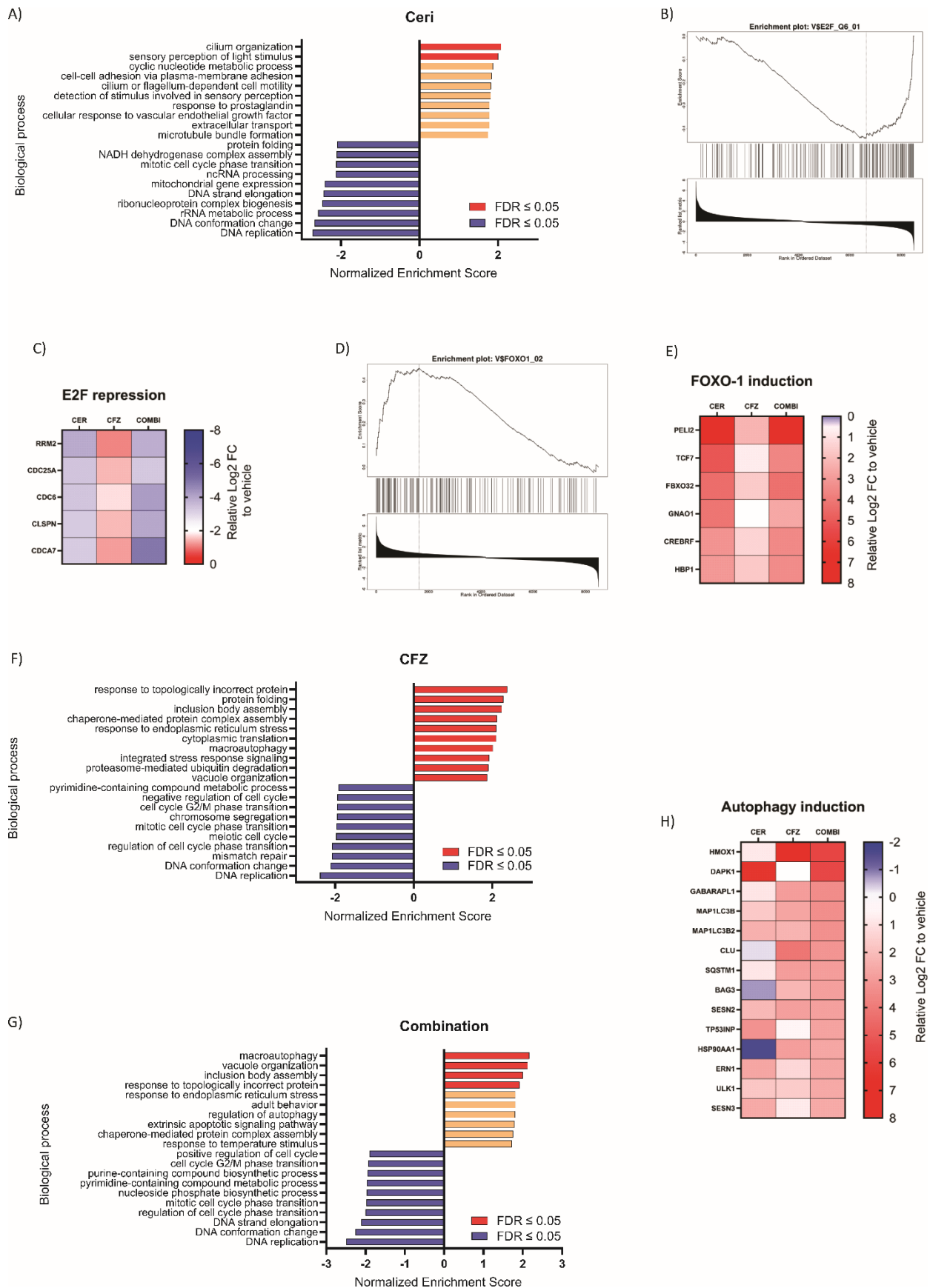
(C) Enrichment plot depicting significantly deregulated biological processes in AMO-1 cells 8 hours after treatment with 5 nM carfilzomib, based on GSEA and GO BP classification.

(D) GSEA enrichment plot showing positive enrichment of the FOXO1 target gene set following ceritinib treatment.

(E) Enrichment plot showing significantly deregulated biological processes in AMO-1 cells 8 hours after treatment with the combination of 4 μ M ceritinib and 5 nM carfilzomib, based on GSEA and GO BP classification.

(F) Heatmap illustrating the leading-edge deregulated genes ($\log_2$ FC $\geq$ 3 for ceritinib treatment) under the regulation of the FOXO1 transcription factor.

(G) Heatmap of leading-edge genes associated with the autophagy biological process identified through GSEA.



CER: ceritinib; CFZ: carfilzomib; COMBI: combination of ceritinib and carfilzomib; E2F: E2F transcription factor; FOXO1: Forkhead Box O1; GSEA: Gene Set Enrichment Analysis; Log2 FC: Log2 fold change

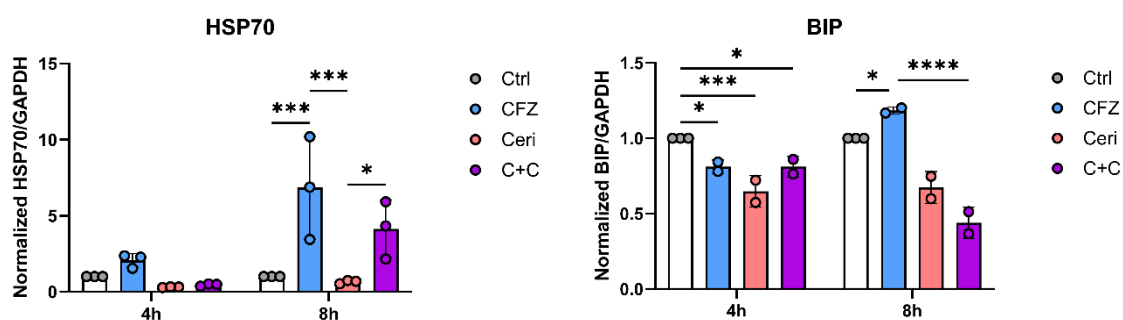
Figure S11: Levels of HSPA5 (BiP) and HSP70 in cell lines. Quantification of HSPA5 (GRP78/BiP) and HSP70 following western blot analysis, complementing Figures 4H–I.

(A) Changes in HSP70 and HSPA5 levels in AMO-1 cells at 4 and 8 hours after treatment with 4 μ M ceritinib, 5 nM carfilzomib, or their combination (C+C).

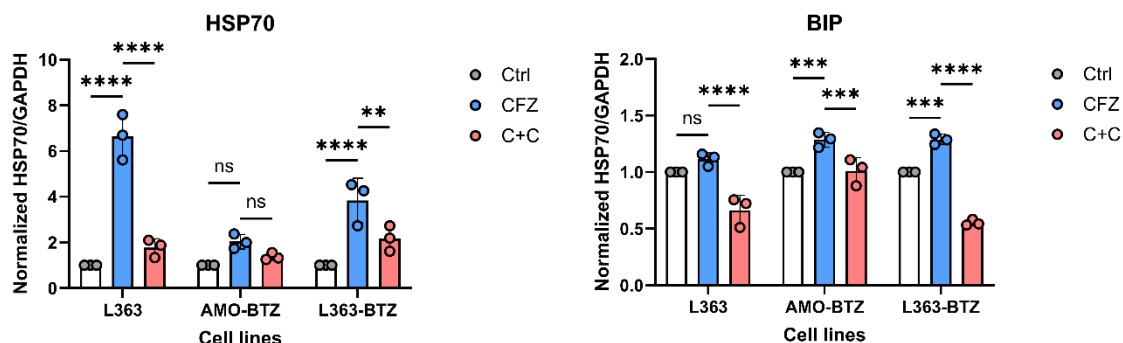
(B) Changes in HSP70 and HSPA5 levels in L363, AMO-BTZ, and L363-BTZ cells 8 hours after treatment with 5 nM carfilzomib or the combination with 4 μ M ceritinib (C+C).

Data represent mean $\pm$ SD of three independent experiments. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

A)



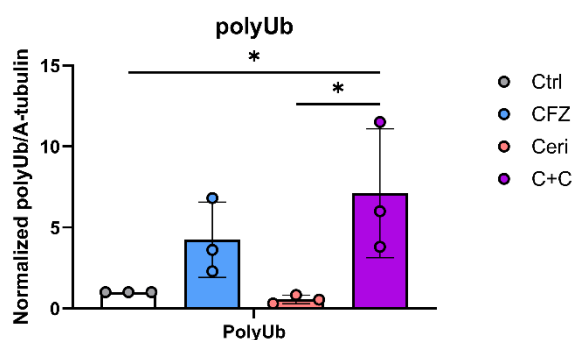
B)



Ceri: ceritinib; CFZ: carfilzomib; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; HSPA5/BiP: Heat Shock 70kDa Protein 5 (Glucose-Regulated Protein, 78kDa)/ Binding-Immunoglobulin Protein; HSP70: Heat Shock 70 KDa Protein.

Figure S12: Levels of PolyUb protein.

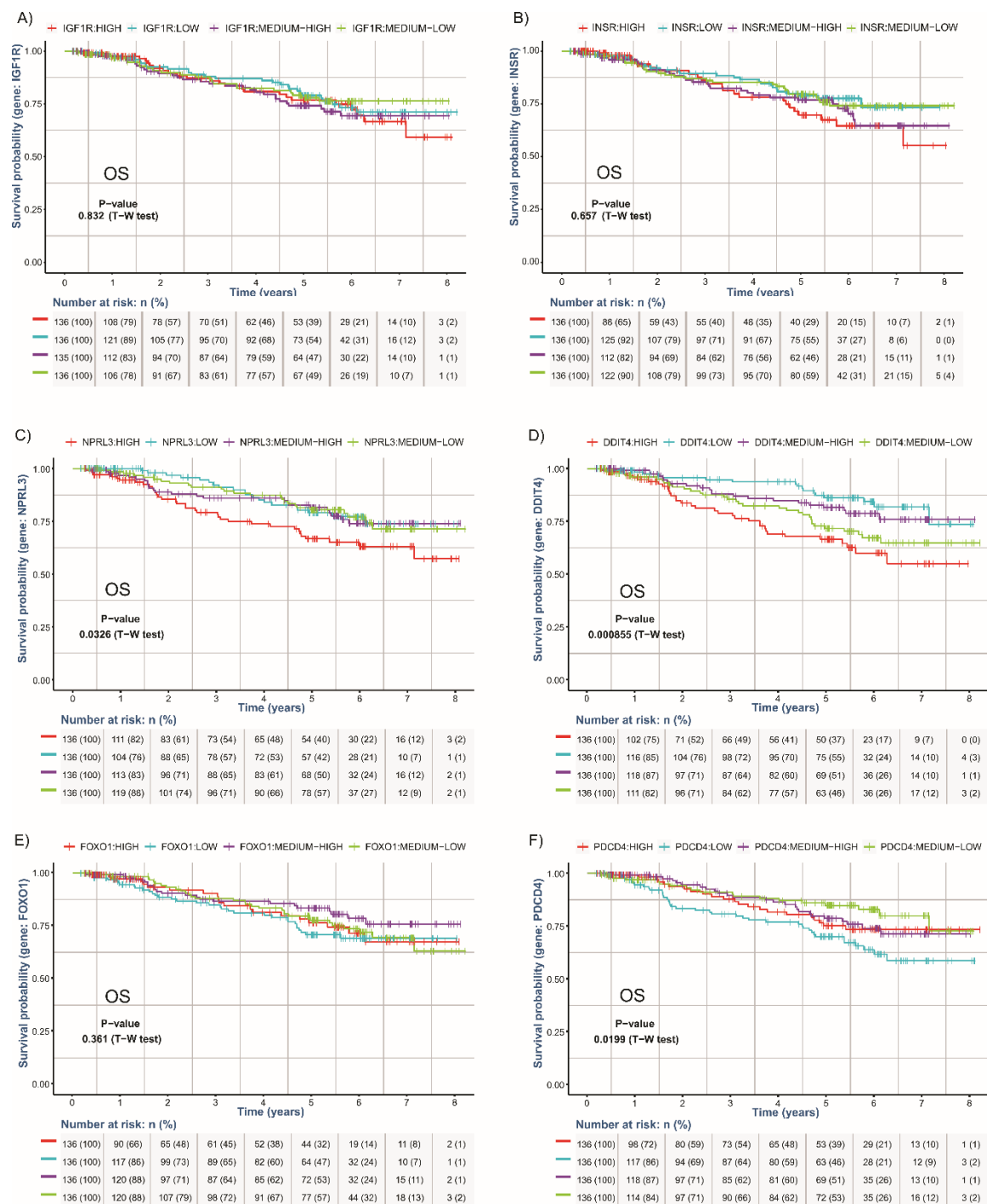
Quantification of polyUb following western blot analysis, complementing Figure 5A. Presented are changes in polyUb level in AMO-1 cells at 8 hours after treatment with 4 μ M ceritinib, 5 nM carfilzomib, or their combination (C+C). Data represent mean $\pm$ SD of three independent experiments. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test: * $p < 0.05$.



Ceri: ceritinib; CFZ: carfilzomib; polyUb: Ub-K48-linked polyubiquitination

Figure S13: Survival outcomes in newly diagnosed MM patients from the CoMMpass dataset.

Kaplan–Meier survival curves illustrating overall survival of 544 newly diagnosed MM patients stratified by expression levels (high, medium-high, medium-low or low) of **(A)** IGF1R, **(B)** INSR, **(C)** NPRL3, **(D)** DDIT4, **(E)** FOXO1, **(F)** PDCD4. P value was considered statistically significant at the level $p < 0.05$, statistical significance was assessed by Tarone-Ware test.



IGF-1R: Insulin Like Growth Factor-1 Receptor; INSR: Insulin Receptor, NPRL3: NPR3 Like, GATOR1 Complex Subunit, DDIT4: DNA Damage Inducible Transcript 4, FOXO1: Forkhead Box O1, PDCD4: Programmed Cell Death 4.

SUPPLEMENTARY TABLES

Supplementary Table S1: Characteristics of MM and PCL samples

MM PATIENTS			
	MM 35	MM 42	MM 68
AGE AT SAMPLE COLLECTION	84	74	81
SEX	Male	Female	Female
RELAPSED AFTER	Len, BTZ, CFZ	Len, BTZ, CFZ	Len, BTZ
PCL PATIENTS			
	PCL 12	PCL 13	PCL 14
AGE AT SAMPLE COLLECTION	69	51	67
SEX	Female	Female	Female
RELAPSED AFTER	BTZ	Len, Pom, BTZ, CFZ	BTZ, Len

BTZ: Bortezomib; CFZ: Carfilzomib; Len: Lenalidomide; MM: Multiple Myeloma; PCL: Plasma Cell Leukemia; Pom: Pomalidomide

Supplementary Table S2: IC50 values of ALK inhibitors in MM cell lines

Proteasome inhibitor-naïve cell lines (AMO-1 and L363) and bortezomib resistant cell lines (AMO-BTZ and L363-BTZ) were treated with ALK inhibitors for 48h. The IC50 values were determined from the dose-response curves shown in Figure 1A.

Cell line	ALK inhibitors IC50 (μM)						
	Ceritinib	Brigatinib	Entrectinib	Crizotinib	Ensartinib	Alectinib	Lorlatinib
L363	1.031	2.472	3.474	3.324	3.7	6.119	21.85
L363-BTZ	0.7891	2.308	4.395	4.151	5.402	6.221	18.53
AMO-1	2.089	1.463	3.511	3.917	4.501	14.13	25.88
AMO-BTZ	2.77	2.667	4.383	6.474	6.252	26.73	37.77

BTZ: bortezomib

Supplementary Table S3: Data from the ChEMBL database on the known on-target and off-target activities of various ALK inhibitors.

The drugs are categorized based on their activity in multiple myeloma: the upper section lists ALK inhibitors that are active in MM (Table S1), while the lower section includes those that are less active or inactive. For each drug, the table also specifies the dose required to inhibit the activity of the identified target.

Target (ChEMBL ID)	Actives	Target Name	Brigatinib (nM)		Ceritinib (nM)		Crizotinib (nM)		Entrectinib (nM)	
CHEMBL4247	6	ALK	Active	1	Active	17	Active	103	Active	12
CHEMBL3982	5	FER	Unknown	x	Active	214	Active	269	Active	5
CHEMBL5568	5	ROS	Active	2	Active	9	Active	18	Active	6
CHEMBL1957	4	IGF-1R	Active	25	Active	395	Active	335	Active	152
CHEMBL1974	4	FLT3	Active	2	Active	516	Active	761	Active	226
CHEMBL1981	4	INSR	Active	195	Active	135	Active	527	Active	209
CHEMBL2041	4	RET	Unknown	x	Active	955	Active	1641	Active	124

Target (ChEMBL ID)	Actives	Target Name	Alectinib (nM)		Ensartinib (nM)		Lorlatinib (nM)	
CHEMBL4247	6	ALK	Active	3	Unknown		Active	3
CHEMBL3982	5	FER	Active	3802	Unknown		Active	3
CHEMBL5568	5	ROS	Unknown	x	Unknown		Active	1
CHEMBL1957	4	IGF-1R	Inactive	30000	Unknown		Unknown	x
CHEMBL1974	4	FLT3	Inactive	30000	Unknown		Unknown	x
CHEMBL1981	4	INSR	Inactive	30000	Unknown		Unknown	x
CHEMBL2041	4	RET	Active	41	Unknown		Unknown	x

ALK: Anaplastic Lymphoma Kinase; FER: Feline Encephalitis Virus-Related Kinase; ROS: ROS Proto-Oncogene 1, Receptor Tyrosine Kinase; IGF-1R: Insulin Like Growth Factor 1 Receptor, FLT3: Fms Related Receptor Tyrosine Kinase 3; INSR: Insulin receptor; RET: Ret Proto-Oncogene, Receptor Tyrosine Kinase

Supplementary Table S4: List of ceritinib sensitivity and resistance candidate genes

This table lists the ceritinib sensitivity and resistance candidate genes identified from the genome-wide CRISPR-Cas9 knockout library screen in AMO-1 cells. The genes are ranked based on their false discovery rate (FDR < 0.01) and their overall ranking determined by MAGeCK-VISPR analysis.

Candidate sensitivity genes				
Gene ID	number of gRNA	FDR (negative)	Rank (negative)	Log2 FC
PELO	4	0.002475	1	-1.1182
NDUFA6	4	0.002475	2	-1.2656
Candidate resistance genes				
Gene ID	number of gRNA	FDR (positive)	Rank (positive)	Log2 FC
FOXO1	4	0.000495	1	2.6339
ZFP36L2	4	0.000495	2	1.6974
FOXO3	4	0.000495	3	1.6506
DDIT4	4	0.000495	4	1.1467
ID3	4	0.000495	5	1.2037
KICS2	4	0.000495	6	2.0332
NPRL3	4	0.000495	7	2.645
NPRL2	4	0.000495	8	1.7576
FAM122A	4	0.000495	9	1.2447
XPNPEP1	4	0.000495	10	1.4268
EIF2AK3	4	0.001142	12	1.3858
RUNX2	4	0.001142	13	1.7436
FIBP	4	0.00165	14	1.4161
SQLE	4	0.00165	15	1.5256
PTEN	4	0.004368	17	1.266
TSC1	4	0.005226	18	1.7607
TSC2	4	0.006514	19	1.0931

PELO, Pelota MRNA Surveillance And Ribosome Rescue Factor; NDUFA6, NADH:Ubiquinone Oxidoreductase Subunit A6; FOXO1, Forkhead Box O1; ZFP36L2, ZFP36 Ring Finger Protein Like 2; FOXO3, Forkhead Box O3; DDIT4, DNA Damage Inducible Transcript 4; ID3, Inhibitor Of DNA Binding 3; KICS2, KICSTOR Subunit 2; NPRL3, NPR3 Like, GATOR1 Complex Subunit; NPRL2, NPR2 Like, GATOR1 Complex Subunit; FAM122A = PABIR1, PP2A Aalpha (PPP2R1A) And B55A (PPP2R2A) Interacting Phosphatase; XPNPEP1, X-Prolyl Aminopeptidase 1; EIF2AK3 = PERK, Eukaryotic Translation Initiation Factor 2 Alpha Kinase 3; RUNX2, RUNX Family Transcription Factor 2; FIBP, FGF1 Intracellular Binding Protein; SQLE, Squalene Epoxidase; PTEN, Phosphatase And Tensin Homolog; TSC1, TSC Complex Subunit 1; TSC2, TSC Complex Subunit 2.

FDR, false discovery rate

Supplementary Table S5: Gene expression and CRISPR-Cas9 dependency scores for BTK, SRC, IGF-1R and INSR

Gene expression and CRISPR-Cas9 dependency scores from the DepMap (DepMap Public 24Q2) for selected cell lines. Each pair of columns represents the CRISPR dependency score and expression level (RNA-seq TPM) for an individual gene across the listed cell lines. Lower CRISPR scores indicate higher gene dependency (essentiality). Expression values are $\log_2(\text{TPM} + 1)$ normalized. DepMap IDs and corresponding cell line names are provided for reference.

DepMap ID	Cell Line	BTK		SRC		IGF-1R		INSR	
		CRISPR	Expression	CRISPR	Expression	CRISPR	Expression	CRISPR	Expression
ACH-001539	KML1	-0.121	6.921	-0.129	1.556	-0.184	0.189	0.144	0.029
ACH-000889	KMM1	0.139	0.669	-0.138	0.299	-0.754	4.218	-0.227	2.748
ACH-002214	BC1	-0.016	0.098	-0.061	0.189	-0.176	2.436	-0.080	1.623
ACH-002478	BC2	-0.160	0.138	-0.149	0.176	-0.042	1.736	-0.172	0.029
ACH-000024	OPM2	0.095	0.098	-0.130	0.506	-0.484	2.705	-0.134	5.153
ACH-001677	U2904	-0.301	6.360	-0.111	0.333	0.047	2.390	-0.212	2.245
ACH-000363	SKMM2	-0.004	2.087	-0.075	0.151	-0.771	2.094	-0.400	5.250
ACH-000124	OCILY19	-0.108	6.137	-0.164	1.757	-0.687	3.536	-0.243	3.900
ACH-002678	WILL1	-0.233	7.660	-0.354	0.070	-0.522	2.014	-0.155	4.164
ACH-002677	U2946	-0.763	6.245	-0.319	0.029	-0.203	1.157	-0.246	3.058
ACH-000183	L363	0.324	2.325	-0.201	0.390	-0.799	3.445	-0.210	4.129
ACH-000167	KE97	0.016	6.121	-0.242	4.852	-0.007	1.722	-0.055	3.274
ACH-000763	MM1S	0.150	3.014	-0.198	2.087	-0.913	2.870	-0.543	3.285
ACH-000714	KMS11	0.021	0.163	-0.140	0.239	-0.980	4.275	-0.356	3.698
ACH-000838	AMO1	-0.173	4.216	-0.052	0.799	-1.164	2.325	-0.516	3.992
ACH-000829	HUNS1	-0.026	6.292	-0.100	4.320	-0.114	0.029	0.021	3.540
ACH-000821	EJM	0.025	6.017	-0.220	0.275	-0.213	2.138	-0.153	4.139
ACH-000817	RPMI8226	0.104	3.909	-0.016	0.575	-0.543	2.850	-0.152	2.425
ACH-000512	INA6	-0.183	6.624	0.005	5.304	-0.612	2.444	-0.207	4.496
ACH-000541	KMS34	0.234	0.444	-0.118	0.322	-0.365	3.051	0.046	4.548
ACH-000653	JJN3	0.015	1.124	-0.134	1.202	-0.588	3.053	-0.266	3.457
ACH-000576	KMS27	0.088	3.352	-0.021	0.895	-0.596	1.986	-0.193	3.048
ACH-000588	KMS26	0.007	1.718	0.026	0.275	-0.511	4.055	-0.344	3.904

Supplementary Table S6: Differentially expressed genes identified by NGS

A list of the differentially expressed genes between control cells and cells treated with ceritinib, carfilzomib, or the drug combination. Data is provided in a separate Excel sheet.

Supplementary Table S7: Top 10 positive and top 10 negative enriched biological process categories deregulated by ceritinib treatment

A list of the top 10 positively and top 10 negatively enriched biological process categories, as determined by Gene Ontology (GO) analysis. The categories were derived from Gene Set Enrichment Analysis (GSEA) of differentially expressed genes between ceritinib-treated and control cells. Significantly deregulated processes are highlighted in bold (positive in light red, negative in light blue).

Gene Set	Description	Size	Leading Edge Number	NES	FDR
GO:0044782	cilium organization	232	110	2.0769	0.022359
GO:0050953	sensory perception of light stimulus	54	28	1.9992	0.031542
GO:0009187	cyclic nucleotide metabolic process	15	7	1.8727	0.11312
GO:0098742	cell-cell adhesion via plasma-membrane adhesion molecules	60	19	1.8372	0.13056
GO:0001539	cilium or flagellum-dependent cell motility	48	29	1.8203	0.12824
GO:0050906	detection of stimulus involved in sensory perception	22	12	1.8152	0.11286
GO:0034694	response to prostaglandin	10	4	1.7802	0.14818
GO:0035924	cellular response to vascular endothelial growth factor stimulus	31	10	1.7786	0.13156
GO:0006858	extracellular transport	19	15	1.7777	0.11809
GO:0001578	microtubule bundle formation	45	24	1.748	0.14789
GO:0006457	protein folding	143	67	-2.0946	0.0057124
GO:0010257	NADH dehydrogenase complex assembly	45	37	-2.1062	0.0056961
GO:0044772	mitotic cell cycle phase transition	288	77	-2.1241	0.0051265
GO:0034470	ncRNA processing	311	156	-2.1325	0.0052311
GO:0140053	mitochondrial gene expression	137	100	-2.4146	2.20E-16
GO:0022616	DNA strand elongation	32	20	-2.4453	2.20E-16
GO:0022613	ribonucleoprotein complex biogenesis	350	241	-2.4779	2.20E-16
GO:0016072	rRNA metabolic process	196	117	-2.5847	2.20E-16
GO:0071103	DNA conformation change	72	36	-2.6748	2.20E-16
GO:0006260	DNA replication	208	98	-2.7215	2.20E-16

GeneSet: ID of the gene set; **Description:** Name/Description of the gene set; **Size:** Number of genes in the set after filtering; **Leading Edge Number:** Number of genes in the leading edge; **NES:** Normalized Enrichment Score, enrichment score normalized to average enrichment score of all permutations; **FDR:** p value corrected for multiple testing

Supplementary Table S8: Top 10 positive and top 10 negative enriched biological process categories deregulated by carfilzomib treatment

A list of the top 10 positively and top 10 negatively enriched biological process categories, as determined by Gene Ontology (GO) analysis. The categories were derived from Gene Set Enrichment Analysis (GSEA) of differentially expressed genes between carfilzomib-treated and control cells. Significantly deregulated processes are highlighted in bold (positive in light red, negative in light blue).

Gene Set	Description	Size	Leading Edge Number	NES	FDR
GO:0035966	response to topologically incorrect protein	112	31	2.3745	2.20E-16
GO:0006457	protein folding	143	45	2.2825	4.90E-04
GO:0070841	inclusion body assembly	17	8	2.2393	6.54E-04
GO:0051131	chaperone-mediated protein complex assembly	18	7	2.1186	1.72E-03
GO:0034976	response to endoplasmic reticulum stress	179	44	2.0997	1.77E-03
GO:0002181	cytoplasmic translation	106	64	2.094	1.47E-03
GO:0016236	macroautophagy	227	62	2.0113	0.0046237
GO:0140467	integrated stress response signaling	34	8	1.92	0.020106
GO:0043161	proteasome-mediated ubiquitin-dependent protein catabolic process	306	105	1.901	0.023103
GO:0007033	vacuole organization	154	42	1.8661	0.029334
GO:0072527	pyrimidine-containing compound metabolic process	48	21	-1.9225	0.0068098
GO:0045786	negative regulation of cell cycle	239	82	-1.9448	0.0056466
GO:0044839	cell cycle G2/M phase transition	104	34	-1.9475	0.0059713
GO:0007059	chromosome segregation	287	108	-1.9583	0.0047915
GO:0044772	mitotic cell cycle phase transition	288	93	-1.9629	0.0052513
GO:0051321	meiotic cell cycle	132	51	-1.9774	0.0056918
GO:1901987	regulation of cell cycle phase transition	283	97	-2.0795	0.00076229
GO:0006298	mismatch repair	22	12	-2.0809	0.0010164
GO:0071103	DNA conformation change	72	32	-2.1136	0.00050819
GO:0006260	DNA replication	208	89	-2.3881	2.20E-16

GeneSet: ID of the gene set; **Description:** Name/Description of the gene set; **Size:** Number of genes in the set after filtering; **Leading Edge Number:** Number of genes in the leading edge; **NES:** Normalized Enrichment Score, enrichment score normalized to average enrichment score of all permutations; **FDR:** p value corrected for multiple testing.

Supplementary Table S9: Top 10 positive and top 10 negative enriched biological process categories deregulated by combination treatment

A list of the top 10 positively and top 10 negatively enriched biological process categories, as determined by Gene Ontology (GO) analysis. The categories were derived from Gene Set Enrichment Analysis (GSEA) of differentially expressed genes between combination-treated and control cells. Significantly deregulated processes are highlighted in bold (negative in light blue, positive in light red).

Gene Set	Description	Size	Leading Edge Number	NES	FDR
GO:0016236	macroautophagy	227	94	2.1637	2.83E-03
GO:0007033	vacuole organization	154	65	2.1237	0.0047086
GO:0070841	inclusion body assembly	17	7	2	0.015695
GO:0035966	response to topologically incorrect protein	112	32	1.915	0.038846
GO:0034976	response to endoplasmic reticulum stress	179	45	1.815	0.10284
GO:0030534	adult behavior	49	21	1.8136	0.087266
GO:0010506	regulation of autophagy	234	87	1.8016	0.083679
GO:0097191	extrinsic apoptotic signaling pathway	125	33	1.7927	0.082518
GO:0051131	chaperone-mediated protein complex assembly	18	6	1.7563	0.10704
GO:0009266	response to temperature stimulus	92	24	1.7186	0.13796
GO:0045787	positive regulation of cell cycle	180	58	-1.8999	0.011061
GO:0044839	cell cycle G2/M phase transition	104	42	-1.9322	0.010624
GO:0072522	purine-containing compound biosynthetic process	159	100	-1.9539	0.0081846
GO:0072527	pyrimidine-containing compound metabolic process	48	22	-1.9629	0.0075721
GO:1901293	nucleoside phosphate biosynthetic process	181	110	-1.9724	7.62E-03
GO:0044772	mitotic cell cycle phase transition	288	106	-1.9793	7.90E-03
GO:1901987	regulation of cell cycle phase transition	283	105	-2.0056	6.24E-03
GO:0022616	DNA strand elongation	32	19	-2.1177	2.20E-16
GO:0071103	DNA conformation change	72	29	-2.2566	2.20E-16
GO:0006260	DNA replication	208	98	-2.4918	2.20E-16

GeneSet: ID of the gene set; **Description:** Name/Description of the gene set; **Size:** Number of genes in the set after filtering; **Leading Edge Number:** Number of genes in the leading edge; **NES:** Normalized Enrichment Score, enrichment score normalized to average enrichment score of all permutations; **FDR:** p value corrected for multiple testing.

Supplementary Table S10: List of identified metabolites and their differential expression. (Separate excel file).

Column Headings:

Metabolite ID: Ion ID, consisting of RT and m/z, ie "RT_m/z" (retention time_mass-to-charge ratio).

Fold change: Fold change (variation multiple), according to the group ratio in the file name.

Log2 FC: Log2 of fold change.

Change: change in the treatment condition vs the control.

P value: The test and statistical significance used to screen for differential ions.

Q value: The test and statistical significance used to screen for differential ions corrected for multiple testing.

VIP: Variable important for the projection, indicating the contribution of m/z to the PLSDA model.

Sample: Comparison group.

Ion Mode:

RT: retention time, (in min), indicates the time in the liquid chromatograph when the maximum concentration of the component appears after sample entering LC for separation.

Molecular Weight: Metabolite molecular weight

Formula: Metabolite chemical formula

Name: Substance Name

Database: Classification according to KEGG or HMDB

KEGG ID: Kyoto Encyclopedia of Genes and Genomes (KEGG) database number.

HMDB ID: Human Metabolome Database (HMDB) database number.

Super class: Substance classification information.

Sub Class: Second-level classification information.

Class: Third-level classification information.

Super Pathway: Substance classification information based on the metabolic pathways

Sub Pathway: Second-level classification information based on the metabolic pathways

Pathway: Third-level classification information based on the metabolic pathways

State: Significant or non-significant classification, based on the p value

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