Combinatorial strategies targeting NEAT1 and AURKA as new potential therapeutic options for multiple myeloma

Noemi Puccio,[1,2] Gloria Manzotti,[1] Elisabetta Mereu,[3] Federica Torricelli,[1] Domenica Ronchetti,[4] Michela Cumerlato,[3] Ilaria Craparotta,[5] Laura Di Rito,[5] Marco Bolis,[5,6] Valentina Traini,[4] Veronica Manicardi,[1] Valentina Fragliasso,[1] Yvan Torrente,[7,8] Nicola Amodio,[9] Niccolò Bolli,[4,10] Elisa Taiana,[10] Alessia Ciarrocchi,[1] Roberto Piva[3] and Antonino Neri[11]

[1]Laboratory of Translational Research, Azienda USL-IRCCS di Reggio Emilia, Reggio Emilia, Italy; [2]Clinical and Experimental Medicine PhD Program, University of Modena and Reggio Emilia, Modena, Italy; [3]Department of Molecular Biotechnology and Health Sciences, University of Turin, Turin, Italy; [4]Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy; [5]Computational Oncology Unit, Oncology Department, Mario Negri IRCCS, Milan, Italy; [6]Bioinformatics Core Unit, Institute of Oncology Research (IOR), Bellinzona, Switzerland; [7]Stem Cell Laboratory, Dino Ferrari Center, Department of Pathophysiology and Transplantation, University of Milan, Milan, Italy; [8]Neurology Unit, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Milan, Italy; [9]Department of Experimental and Clinical Medicine, Magna Graecia University of Catanzaro, Catanzaro, Italy; [10]Hematology Unit, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Milan, Italy and [11]Scientific Directorate, Azienda USL-IRCCS di Reggio Emilia, Reggio Emilia, Italy

Correspondence: A. Neri
antonino.neri@ausl.re.it

Received: March 22, 2024.
Accepted: July 2, 2024.
Early view: July 11, 2024.

https://doi.org/10.3324/haematol.2024.285470

Abstract

Multiple myeloma (MM) is a dreadful disease, marked by the uncontrolled proliferation of clonal plasma cells within the bone marrow. It is characterized by a highly heterogeneous clinical and molecular background, supported by severe genomic alterations. Important de-regulation of long non-coding RNA (lncRNA) expression, which can influence progression and therapy resistance, has been reported in MM patients. NEAT1 is a lncRNA essential for nuclear paraspeckles and is involved in the regulation of gene expression. We showed that NEAT1 supports MM proliferation, making this lncRNA an attractive therapeutic candidate. Here, we used a combinatorial strategy integrating transcriptomic and computational approaches with functional high-throughput drug screening to identify compounds that synergize with NEAT1 inhibition in restraining MM cell growth. AURKA inhibitors were identified as top-scoring drugs in these analyses. We showed that the combination of NEAT1 silencing and AURKA inhibitors in MM profoundly impairs microtubule organization and mitotic spindle assembly, finally leading to cell death. Analysis of the large publicly available CoMMpass dataset showed that, in MM patients, AURKA expression is strongly associated with reduced progression-free survival (P<0.0001) and overall survival (P<0.0001) probabilities and patients with high levels of expression of both NEAT1 and AURKA have a worse clinical outcome. Finally, using RNA-sequencing data from NEAT1 knockdown MM cells, we identified the AURKA allosteric regulator TPX2 as a new NEAT1 target in MM and as a mediator of the interplay between AURKA and NEAT1, therefore providing a possible explanation for the synergistic activity observed upon their combinatorial inhibition.

Introduction

Multiple myeloma (MM) is an incurable bone marrow-resident hematologic malignancy, characterized by uncontrolled proliferation of clonal plasma cells.[1] It is the second most common type of blood cancer, after non-Hodgkin lymphoma, accounting for 10% of all hematologic tumors.[2] MM has a highly heterogeneous clinical and genetic background, characterized by both numerical and structural chromosomal abnormalities and gene mutations.[3,4]

Long non-coding RNA (lncRNA) are a heterogeneous class of transcripts that partake in all levels of genome organization.[5] These molecules are involved in the regulation of cell differentiation, development, response to DNA damage and regulation of metabolic processes. In cancer, lncRNA contribute to altering cell growth potential, invasion and metastatic ability, to impairing cell death mechanisms, and to increasing antitumor drug resistance.[6,7] Due to their biological relevance, it is not surprising that these molecules are regarded as new potential targets for innovative

cancer treatments.

In MM, the lncRNA expression profile was described to be significantly different between malignant and normal plasma cells, suggesting their pro-oncogenic function in this scenario.[8] Aberrant lncRNA expression in myeloma cells can further contribute to the acquisition of genomic instability, inducing cell transcriptome modification and interfering with chromatin structure.[9,10]

NEAT1 (nuclear paraspeckle assembly transcript 1) is a mono-exonic lncRNA transcribed from the MEN type I locus, localized on human chromosome 11q13. The *NEAT1* gene produces two different isoforms that share the same 5' terminus: a short and polyadenylated isoform (NEAT1_1) and a longer one (NEAT1_2) lacking a poly-A tail but endowed with a triple-helix structure that protects the transcript from degradation.[11,12] As in other types of cancer, increased NEAT1 expression has been shown to be a hallmark of MM and plasma cell leukemia.[13]

NEAT1 mainly localizes in cell nuclei. The long NEAT1 isoform (NEAT1_2) acts as an essential architectural scaffold for stress-induced paraspeckles.[14] Paraspeckles are sub-nuclear membrane-less organelles that regulate gene expression through three main mechanisms: by acting as RNA binding protein hubs, as microRNA sponges, and by promoting mRNA retention.[15-17] By contrast, the short NEAT1 isoform (NEAT1_1) is the more abundant isoform but it is dispensable for paraspeckle assembly and functioning, suggesting a putative independent role.[18] It has already been proven that NEAT1 is required to support MM cell proliferation and viability, both *in vitro* and *in vivo*.[19,20] In line with this observation, NEAT1 silencing causes decreased resistance to standard myeloma treatments such as bortezomib, carfilzomib and melphalan and improves sensitivity to olaparib, making this lncRNA an attractive candidate for the development of novel anti-myeloma strategies.[19]

In this work, we took advantage of an integrated approach combining computational predictive tools with a high throughput functional screening to identify small compounds that cooperate with NEAT1 inhibition in boosting its lethal effect on MM cell viability and growth. We identified Aurora kinase A (AURKA) inhibitors as top-scoring candidates in both analyses and validated this synergy using functional assays. Finally, we derived a potential model to explain the cooperation between NEAT1 and AURKA in controlling MM biology, providing novel insights into the pathophysiology of this disease.

Methods

Full details of gymnotic delivery of gapmeRs, cell viability assessment, live cell imaging, cell cycle and drug synergy analyses, reverse transcription and quantitative polymerase chain reaction, western blotting, immunofluorescence, differentially expressed genes, CoMMpass and survival analyses are provided in the *Online Supplementary Methods*.

Multiple myeloma cell lines and drugs

AMO-1, NCI-H929, and MM1.S were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). Using a CRISPR/Cas9 synergistic activation mediator (SAM) system and guide RNA (gRNA) we obtained AMO-1 SAM gSCR and AMO-1 SAM gN#8 cell lines as previously described.[20] Human MM cell lines were immediately expanded and frozen upon arrival and used from the original stock within 6 months. These cell lines were cultured in RPMI-1640 medium (Gibco®, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Euroclone, Milan, Italy) at 37°C in a 5% CO_2 atmosphere.

All cell lines were routinely tested for *Mycoplasma* contamination using the Lonza Mycoalert Mycoplasma Detection Kit (Euroclone, Milan, Italy). Re-authentication by single nucleotide polymorphism profiling at Multiplexion GmbH (Heidelberg, Germany) was performed for AMO-1, NCI-H929, and MM1.S cell lines in 2023. Alisertib and AURKA inhibitor I (AURKAi-I) were purchased from Selleckem and resuspended in dimethylsulfoxide at a stock concentration of 10 mM.

High throughput screening

A primary screening using a library of 320 small-molecule inhibitors targeting 123 key proteins was conducted on AMO-1 cells, as previously described.[21,22] The cells were treated with gapmeR NEAT1 (gNEAT1 5 μM) or a control at day 0 and after 24 hours were exposed to the inhibitor library at three different concentrations or to dimethylsulfoxide (vehicle). Cell viability was measured by CellTiter-Glo (Promega) luminescence assay at day 0 (d=0) and 3 days after treatment (d=3), in duplicate. Full details are reported in the *Online Supplementary Methods*.

RNA sequencing

RNA-sequencing libraries were obtained starting from 500 ng of total RNA following the TruSeq Stranded Total RNA (San Diego, CA, USA) protocol. Sequencing was performed with a NextSeq 500 sequencer (Illumina) using a 2 × 150 high-output flow cell with 8 samples/run.). Details are described in the *Online Supplementary Methods*.

Ethical approval

All patients' data are derived from the publicly available CoMMpass dataset.

Results

AURKA inhibition mimics the NEAT1 knockdown transcriptomic signature

To identify small compounds that could potentiate the ef-

fect of NEAT1 inhibition in MM, we developed an integrated combinatorial strategy (Figure 1A).

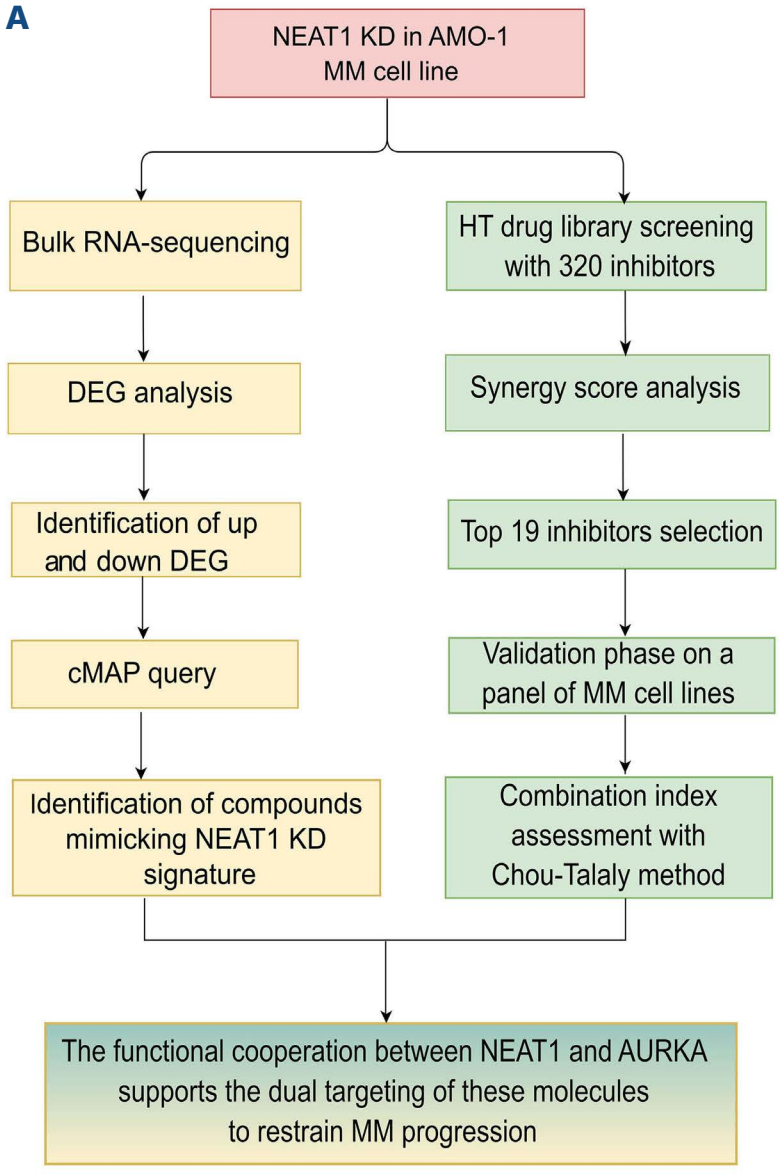
First, we used a computational approach to select drugs whose activity could recapitulate the transcriptional perturbations induced by NEAT1 inhibition in MM cells. To this end, we performed bulk RNA sequencing in the NEAT1 knockdown (KD) AMO-1 cell line and the relative control. NEAT1 KD was obtained using LNA-gapmeR (gNEAT1), as previously described[19] (*Online Supplementary Figure S1*). Differential analysis showed that NEAT1 KD strongly affected the gene expression program in MM cells. A total of 1,710 genes resulted significantly deregulated (false discovery rate ≤0.05) in NEAT1-silenced AMO-1 cells as compared with control cells (Figure 1B). Of these, 753 genes (44%) were downregulated, and 957 genes (56%) were upregulated upon NEAT1 KD (Figure 1C).

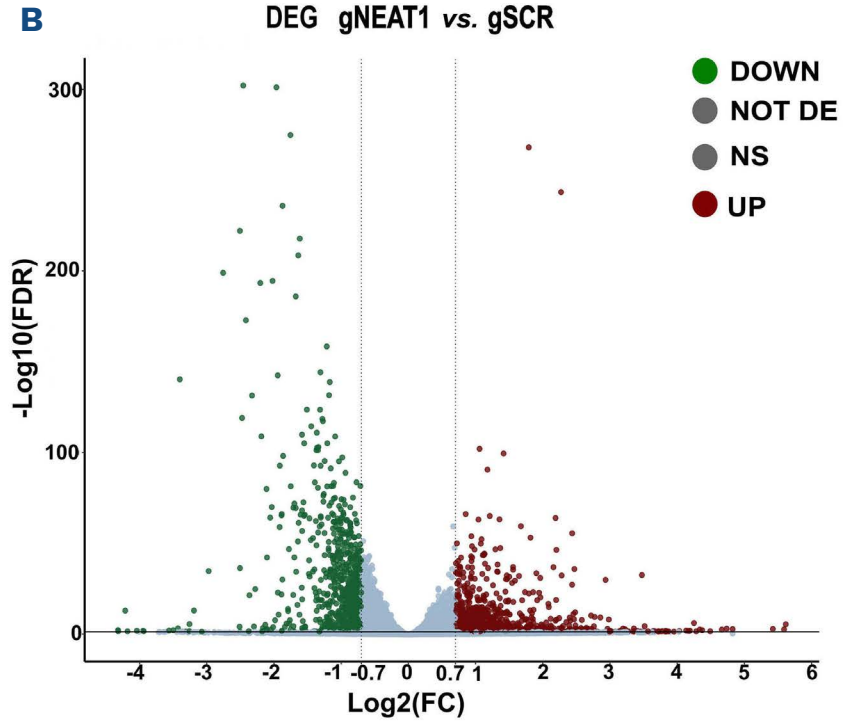
We used this list to query the Connectivity Map database, searching for compounds that could mimic the transcriptional perturbation caused by NEAT1 silencing. We identified 66 small molecules as significantly associated with NEAT1 transcriptomic profiles, including CDK inhibitors, MTOR inhibitors, and AURK inhibitors.

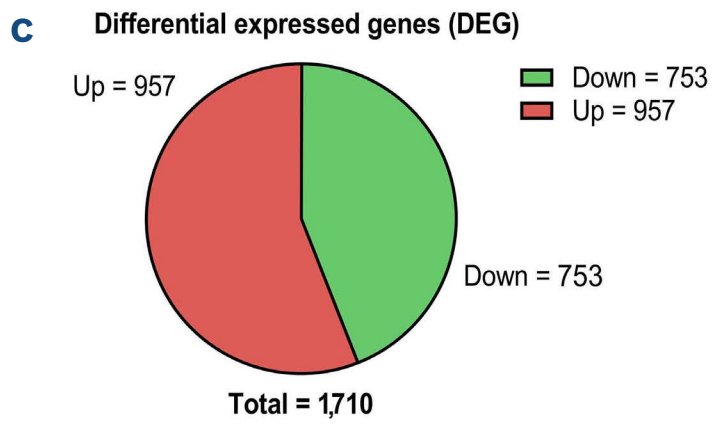
Of note, the AURKA inhibitor alisertib, identified among the top-scoring drugs, has been used clinically in combination with proteasome inhibitors in the treatment of MM patients[23] (Figure 1D, E).

High-throughput drug screening identified that AURKA inhibitors synergize with NEAT1 inhibition in the AMO-1 cell line

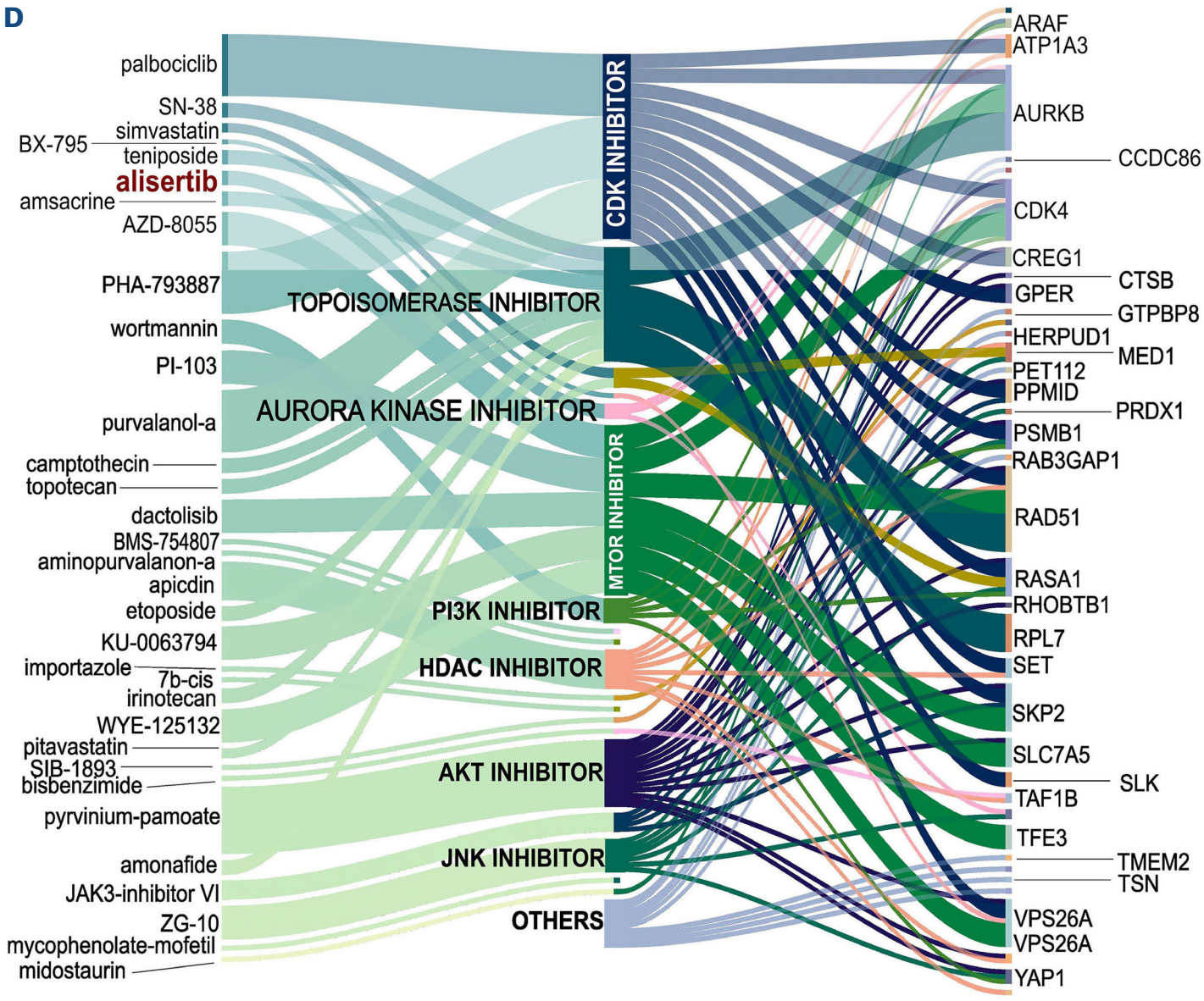
To identify drugs that could potentiate the anti-tumor effects of NEAT1 inhibition in MM cells and to support our computational analyses, we conducted a functional high-throughput screening using a library of 320 small-molecule inhibitors covering 123 pivotal signaling targets. *Online Supplementary Table S1* lists the compounds included in the screening. Figure 2A illustrates the experimental timeline. Briefly, AMO-1 cells were exposed to the drugs at three different concentrations (10 μM, 1 μM, or 100 nM) in the presence or absence of previous NEAT1 silencing and were evaluated at day 0 and 72 h after treatment, using an ATP-based luminescent metabolic assay (Cell TiterGlo). The combination effect assessed by the Excess over Bliss (EOB) score with an arbitrary cut-off of EOB >0.2 defined 35 synergistic candidates, including four Aurora kinase inhibitors (Figure 2B, C). A subsequent independent validation in AMO-1 cells measuring both metabolic activity and apoptosis by tetramethylrhodamine methyl ester staining







Continued on following page.



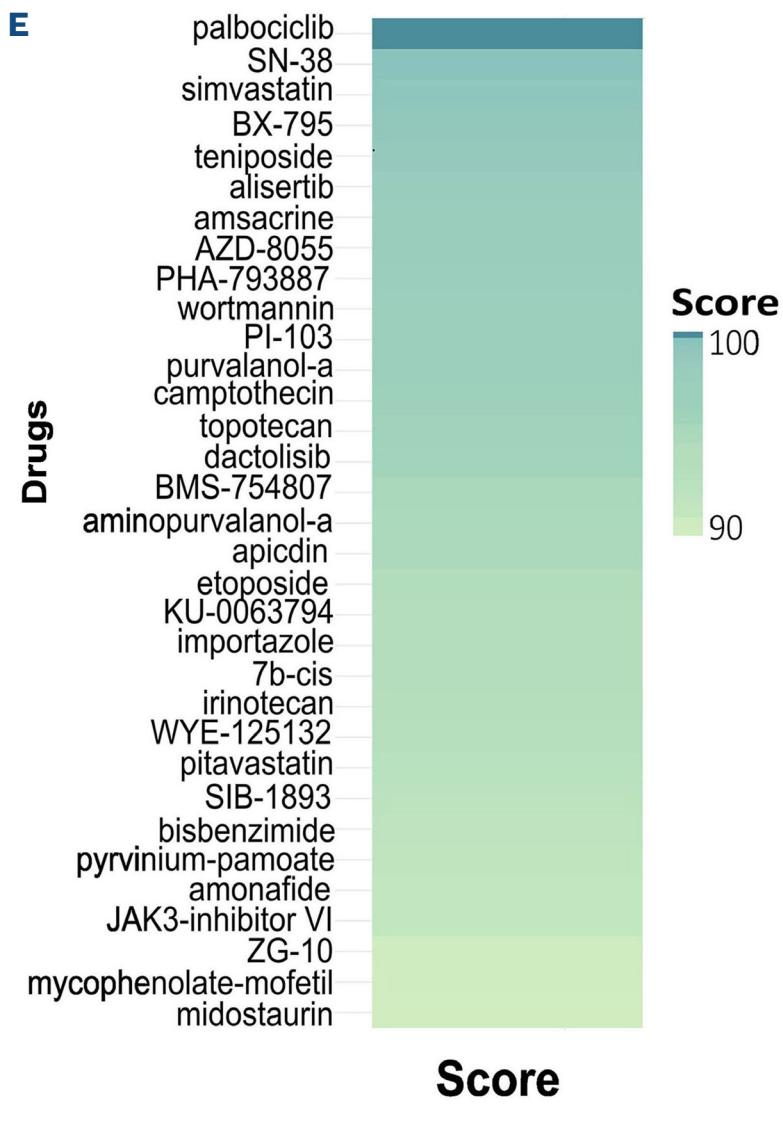
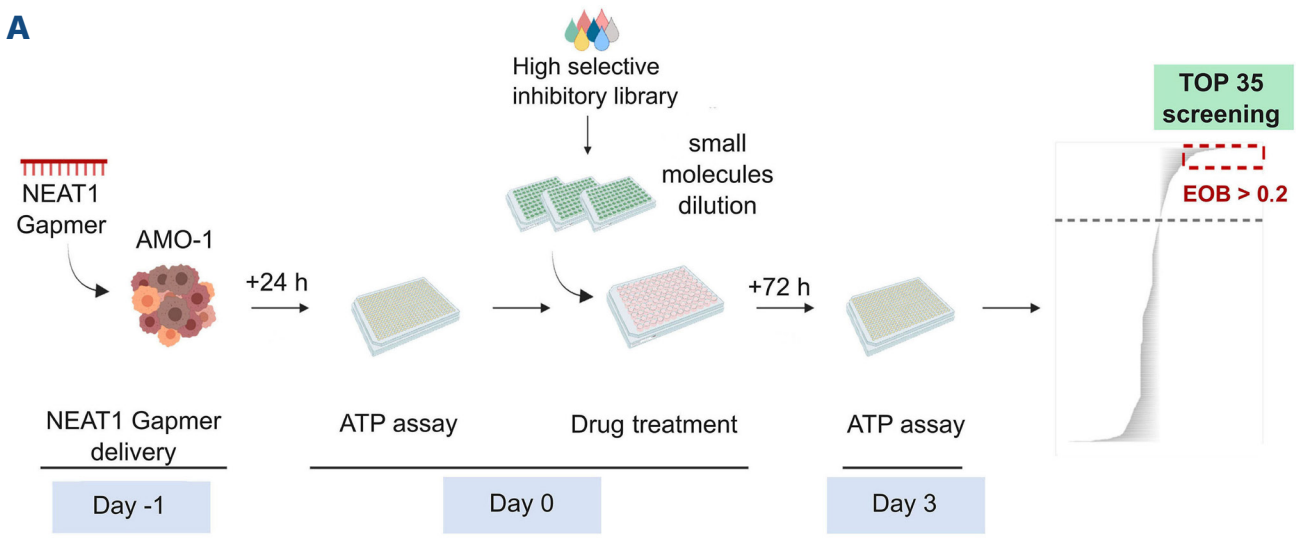
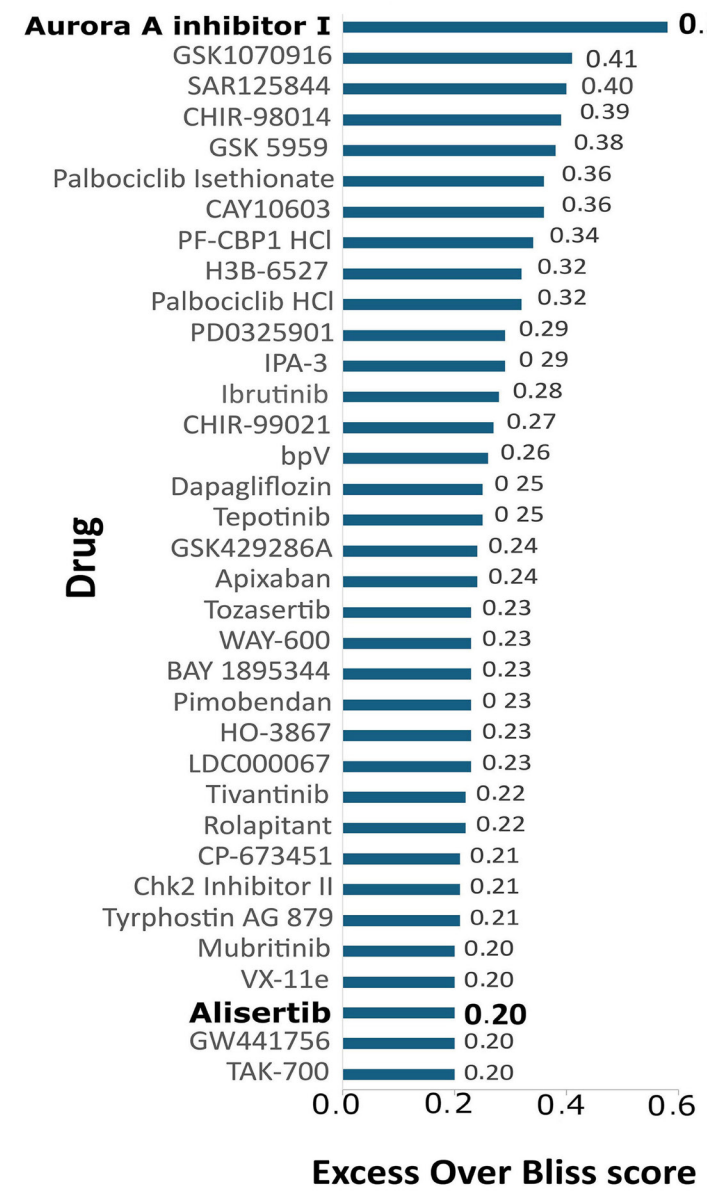
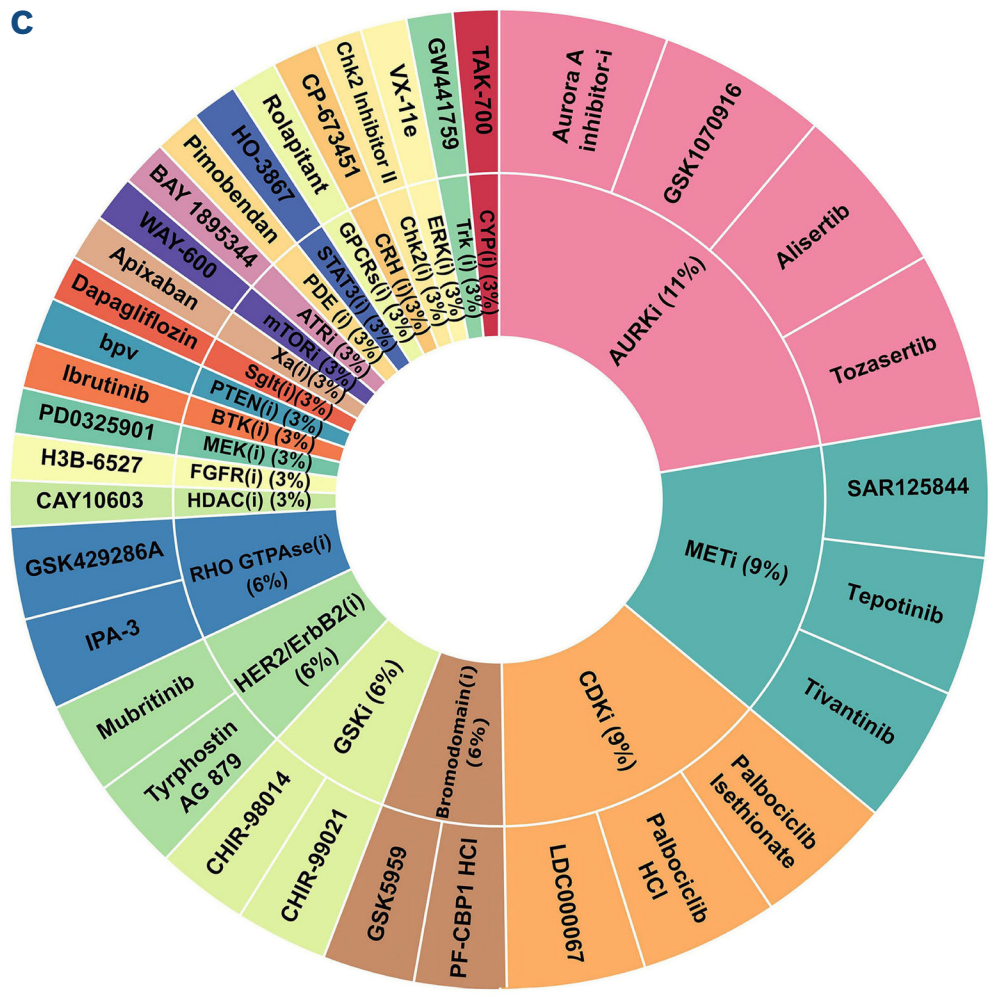
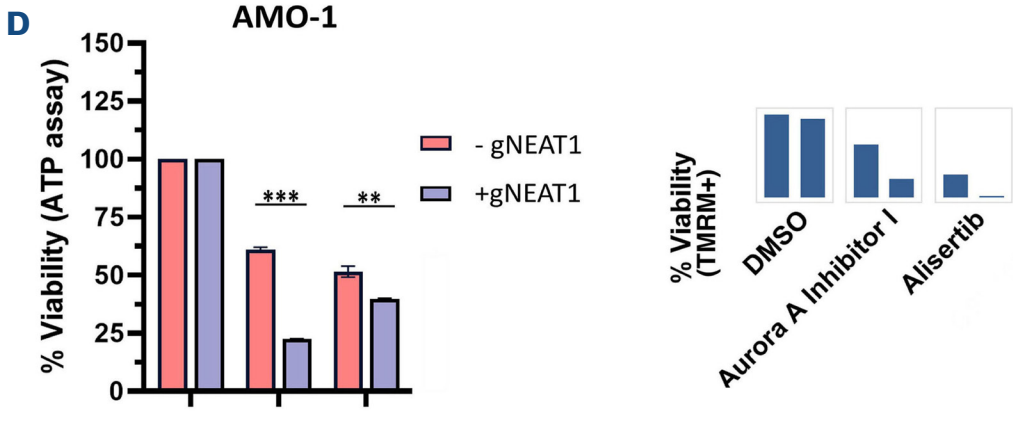


Figure 1. AURKA inhibition mimics the NEAT1 knockdown transcriptomic signature. (A) Framework overview: computational and functional approaches adopted to identify compounds exerting a synergistic activity with total NEAT1 silencing in multiple myeloma cells. (B) Volcano plot showing significantly down- (green) and up- (red) regulated genes in AMO-1 NEAT1 knockdown cells compared to the scramble condition (lnFC ≤ |0.7| *P*adj ≤0.05). (C) Pie chart showing the number of significantly down- and up-regulated genes in AMO-1 NEAT1 knockdown cells compared to the scramble condition lnFC ≤ |0.7| *P*adj ≤0.05 (D) Alluvial plot depicting top scoring molecules derived from the connectivity map query, in particular: inhibitors (left), the class of perturbation to which they belong (middle), and their target genes (right). (E) Heatmap showing the relative cMap score of the 33 top-scoring candidate compounds with a score >90. KD: knockdown; MM: multiple myeloma; HT: high throughput; cMAP: connectivity map; DEG: differentially expressed genes; FDR: false discovery rate; FC: fold change; gSCR: scramble guide RNA; NOT DE: not differentially expressed; NS: not significantly different.









Continued on following page.

Figure 2. High throughput drug screening identifies AURKA inhibitors as promising synergistic agents when combined with NEAT1 inhibition. (A) Experimental overview of the drug screening. AMO-1 cells were seeded and silenced for NEAT1 expression (day -1) through gymnotic delivery of LNA-GapmeR (gNEAT1). Cell viability was assessed using an ATP assay after 24 hours (day 0) followed by treatment with three different concentrations of compounds. At 96 hours of NEAT1 silencing and 72 hours of treatment with the compounds (day 3), cell viability was assessed by ATP assay and NEAT1 expression was quantified through quantitative real-time polymerase chain reaction. The effect of the combined drugs was determined by Excess over Bliss (EOB) analysis. (B) Diagram illustrating the top 35 candidates (EOB >0.2) exhibiting a synergistic effect when combined with NEAT1 silencing. (C) Sunburst diagram depicting the categories of the compounds and the names of the drugs that exert a synergistic activity with NEAT1 knockdown. (D) The viability of AMO-1 cells was evaluated by ATP assay (upper diagram) in duplicate and flow-cytometry (lower diagram) in the presence or absence of gNEAT1. Statistical significance was measured with the Student *t* test. ***P*<0.01, ****P*<0.001. TMRM: tetramethylrhodamine methyl ester; DMSO: dimethylsulfoxide.

confirmed the AURKA inhibitors alisertib and AURKAi-I among the most effective drugs (Figure 2D), consistent with the results of the *in-silico* predictions.

Aurora kinase inhibitors increase the cytostatic effect of NEAT1 inhibition in multiple myeloma cells

To explore the potential synergy between NEAT1 and AURKA inhibition, we performed an in-depth validation. The half maximal inhibitory concentrations (IC_{50}) for alisertib and AURKAi-I were assessed in three different MM cell lines (AMO-1, NCI-H929, and MM1.S). All three cellular models showed robust and consistent sensitivity to both drugs with IC_{50} values in the range of nanomoles for alisertib and micromoles for AURKAi-I (*Online Supplementary Table S2*). NEAT1 silencing was effective in all three cell models tested (*Online Supplementary Figure S1*)

Due to the role of AURKA in promoting mitosis, we evaluated the effect of the drugs on the cell cycle profile in the three MM cell lines, through flow cytometry (Figure 3A, B and *Online Supplementary Figure S2A, B*). As expected, the drugs induced a significant increase in the percentage of cells in G2/M, supported by a reduction of the pAURKA/AURKA ratio (Figure 3C) and, as already described, an increase of PLK1 and CyB1,[24] (Figure 3D) confirming the proper on-target effects in our system.

To assess the level of synergism between NEAT1 and AURKA inhibition, we calculated the synergy score based on cell proliferation. Two different sublethal concentrations of anti-NEAT1 oligos and three different drug concentrations (corresponding to IC_{20}, IC_{50} and IC_{70}) were used (Figure 4A, B). The combination matrix showed a global moderate synergism between NEAT1 inhibition and both AURKA inhibitors in all three cell models tested (*Online Supplementary Figure S3A, B*).

To corroborate these data, we monitored the effect of the combination treatment on cell proliferation over time, using a sublethal concentration of both targeting agents. Figure 5A-C shows the proliferation curves obtained in these experiments. In all three MM cell models, the combination of NEAT1 KD and alisertib produced a major effect compared to the effect of the single agents alone. Similarly, AURKAi-I used in combination with NEAT1 KD improved the proliferation inhibition in AMO-1 and NCI-H929 cells, but not in MM1.S cells.

NEAT1 transactivation reduces the effect of AURKA inhibition on multiple myeloma cell proliferation

Given the synergy observed between NEAT1 and AURKA inhibition in MM cells, we aimed to assess whether NEAT1 overexpression could rescue the cell growth inhibition caused by AURKA inhibitors. Of note, we recently showed that a high level of NEAT1 expression in MM cells provides a pro-survival advantage upon cellular stressor stimuli.[20] To this end, we used an AMO-1 cell line engineered with CRISPRa exploiting the SAM system to constitutively transactivate NEAT1.[20]

As already reported by us, a 2-fold increase of NEAT1 expression in transactivated cells, namely AMO-1 SAM gN#8 cells, as compared to the scramble condition, namely AMO-1 SAM gSCR, was observed (*Online Supplementary Figure S4*). Of note, transactivation of NEAT1 determined an increased resistance to AURKA inhibitor as shown by the higher value of IC_{50} observed in NEAT1 overexpressing cells as compared to the scramble condition (Figure 6A, B) Evaluation of the number of cells 72 hours after treatment showed that NEAT1 transactivation exerted a significant protective effect on MM cell viability at all doses of drugs tested (Figure 6C).

This evidence is in line with the reported role of NEAT1 in promoting drug resistance[19] and supports the hypothesis of a potential interplay between AURKA and NEAT1 in sustaining MM cell growth.

NEAT1 controls AURKA activity through modulation of TPX2 transcription

We performed immunofluorescence staining using α-tubulin antibodies to monitor the biological effects of NEAT1 KD on mitosis in NCI-H929 and AMO-1 cells treated with alisertib. We confirmed, as already reported,[25] that alisertib administration impaired proper bipolar mitotic spindle formation by causing abnormal and unfunctional structures (Figure 7Aii). Interestingly, NEAT1 inhibition induces prominent reorganization of microtubules within the cells with evident local alterations in mitotic spindle orientation (Figure 7Ai). Notably, NEAT1 KD in alisertib-treated cells resulted in a dramatic increase of spindle abnormalities, which worsened the effect of the drug alone. In particular, we observed a marked increase in the number of monopolar and/or multipolar spindles, as well as the presence of

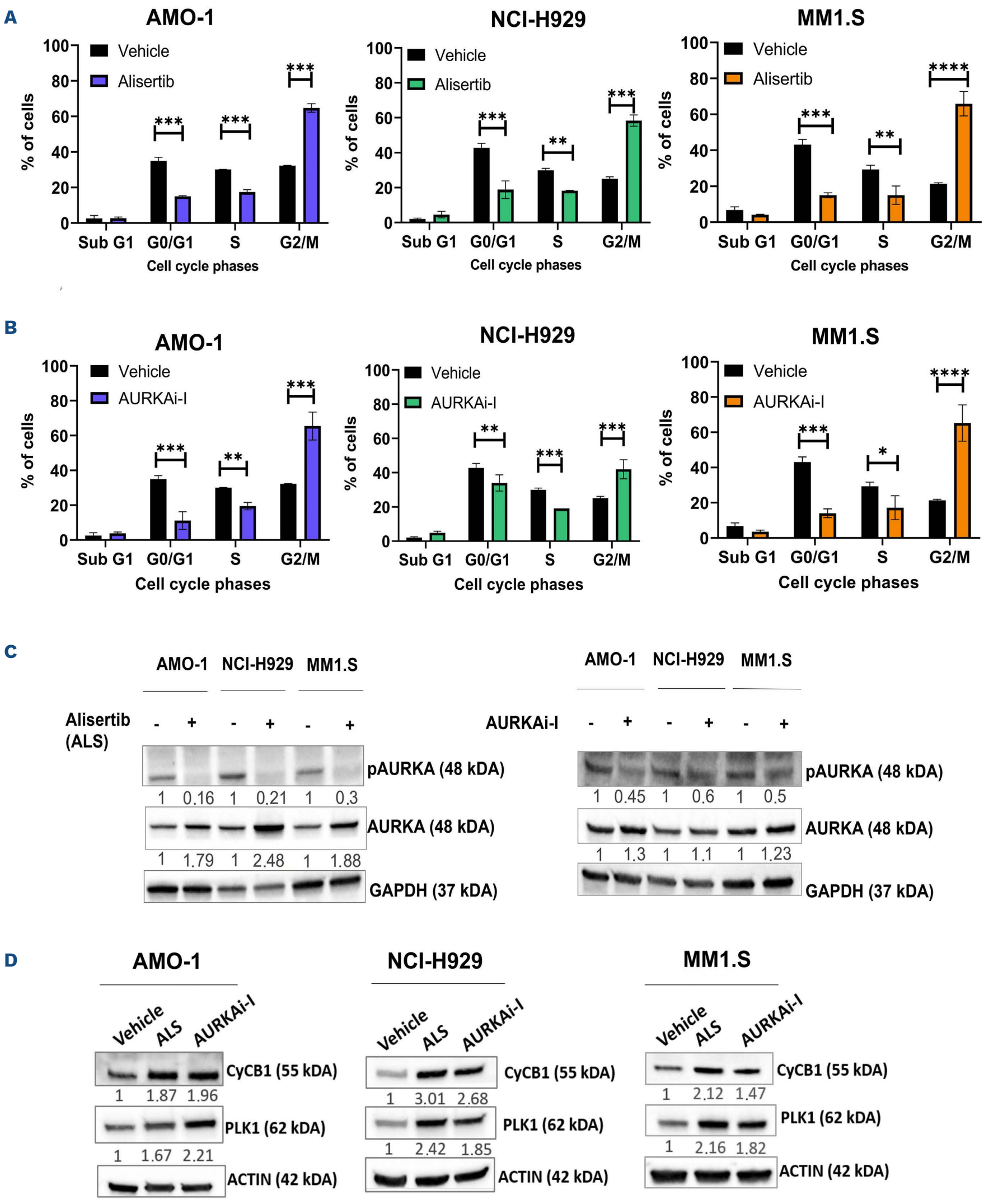


Figure 3. Human myeloma cell lines showed robust sensitivity to AURKA inhibitors. (A, B) Fluorescence activated cell sorting analysis of the distribution of cell cycle phases after treatment with alisertib (A) and an Aurora kinase A inhibitor I (AURKAi-I) (B) for 24 hours in AMO-1, NCI-H929 and MM1.S cells. The histograms show the percentages of cell cycle distribution; the standard deviation of three replicates is reported. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, Student *t* test. (C) Western blot analyses showing pAURKA and AURKA protein expression after treatment with alisertib (24 hours) and AURKAi-I (6 hours) in AMO-1, NCI-H929 and MM1.S cells. (D) Western blot analyses showing CyCB1 and PLK1 cell cycle checkpoint proteins after treatment with alisertib and AURKAi-I (24 hours).

spindles with disorganized microtubules (Figure 7Aiii, B). These morphological alterations are indicative of a defective mechanism of division, in line with the observed impairment of proliferation. Besides, these data indicate a previously underscored interplay between NEAT1 and AURKA in MM cells.

Our hypothesis was further confirmed by the analysis of the RNA-sequencing data obtained in AMO-1 upon NEAT1 silencing (Figure 1B). Gene ontology analysis of the genes downregulated upon NEAT1 KD highlighted several relevant biological processes affected by this lncRNA. In particular, microtubular cytoskeleton and mitotic spindle organization upon cell division scored among the top ten enriched pathways in this analysis (Figure 7C). Several genes involved in microtubular organization during mitosis were observed to be significantly downregulated upon NEAT1 KD in AMO-1 cells (*Online Supplementary Table S3*). Most of these genes were also validated through quantitative real-time polymerase chain reaction analysis in both AMO-1 and NCI-H929 cell lines (*Online Supplementary Figure S5A, B*). Furthermore, we took advantage of our gene expression profiling data previously obtained in NEAT1 KD NCI-H929 cells[19] to perform gene ontology analysis. The results of this analysis revealed the same enriched biological processes also in NCI-H929 cells upon NEAT1 silencing (*Online Supplementary Figure S6*).

Among these genes, we identified *TPX2*, which is an allosteric regulator of AURKA and serves to position AURKA at the level of the mitotic spindle during cell division[26,27] (Figure 7D). We confirmed that upon NEAT1 KD, TPX2 is downregulated at both the transcript and protein levels in AMO-1 and NCI-H929 cells (Figure 7E). Besides, NEAT1 transactivation in AMO-1 cells resulted in a significant trend toward upregulation of TPX2 transcript and protein confirming a positive regulation of NEAT1 on this gene (Figure 7F). NEAT1-mediated perturbation of genes as *TPX2*, involved in the control of AURKA activity could further destabilize cell division upon AURKA inhibitors administration, thus explaining the combinatorial effect observed in drug screening.

AURKA and NEAT1 expression stratifies survival in patients with multiple myeloma

We took advantage of the publicly available CoMMpass dataset to explore the association of AURKA expression with genetic and clinical features of MM patients. To assess AURKA expression profiles in relation to major molecular aberrations in MM, we investigated 660 MM patients from the CoMMpass cohort for whom data on expression, non-synonymous somatic mutations, and copy number alterations were available (*Online Supplementary Methods* and *Online Supplementary Table S4*). Significantly higher AURKA expression levels were observed in MM patients carrying markers of highly aggressive disease, such as 1q gain/amp, 1p loss, 17p deletions, *TP53* alterations, *MAF* and *MYC* translocations, and 13q deletion, whereas lower expression levels were evidenced in hyperdiploid cases (*Online Supplementary Figure S7*). No significant differences in AURKA expression levels were observed in relation to t(11;14) and t(4;14) translocations, or the occurrence of non-synonymous somatic mutations in *RAS/BRAF*, *TRAF3*, *DIS3*, or *FAM46C* genes (*data not shown*). We investigated the clinical impact of AURKA starting from a dataset of 761 patients for whom survival data were available, focusing on those with low (first quartile) and high (fourth quartile) expression of AURKA. Survival curve analysis showed that AURKA expression was associated with both reduced progression-free survival and overall survival probabilities (*Online Supplementary Figure S8A, B*).

To verify whether high AURKA expression levels may be an independent variable for predicting overall and progression-free survival, we tested high AURKA expression and other main molecular or clinical features by Cox regression univariate analysis in 489 MM patients for whom all information was available.

Concerning overall survival, a significantly higher risk of death was observed for cases with higher AURKA expression level (hazard ratio=1.54, 95% confidence interval: 1.11-2.13, Benjamini-Hochberg adjusted *P* value=0.030). With regards to progression-free survival, higher AURKA expression level was associated with a significantly higher risk of disease progression (hazard ratio=1.57, 95% confidence interval: 1.23-2.01, Benjamini-Hochberg adjusted *P* value=0.0016) (*Online Supplementary Table S5A, B*). Interestingly, when all significant variables were tested in multivariate analysis, we observed that AURKA expression retained its independent prognostic impact on progression-free survival, but not on overall survival (*Online Supplementary Figure S9A, B*).

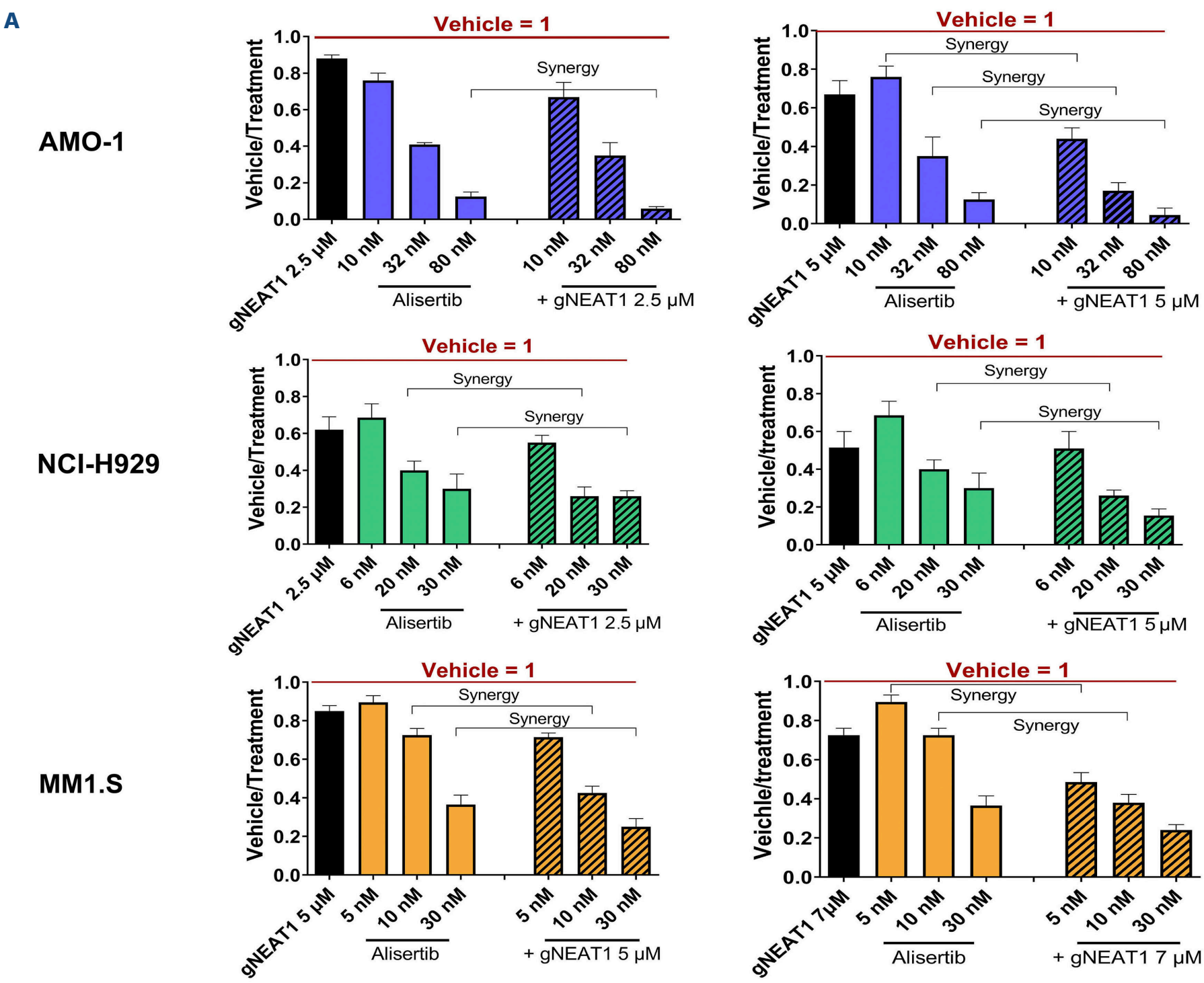
Finally, we evaluated whether AURKA and NEAT1 expression levels, considered together, could affect patients' clinical outcomes. Despite the finding that NEAT1 expression levels alone do not have a significant impact on patients' survival,[13] patients with both high AURKA and high NEAT1 expression levels displayed the worst survival curve (*Online Supplementary Figure S10*).

Discussion

Despite the significant improvement in treatment opportunities for MM observed over the past years, most patients with this malignancy suffer from relapse and frequently develop highly aggressive disease, experiencing drug resistance to almost all currently available therapeutic options.[28,29] De-regulation of non-coding transcripts contributes to the progression of this disease by affecting essential plasma cell biological processes.[8] We previously described that MM patients frequently show altered expression of lncRNA, which contribute to tumor progression.[30] Among them, we showed that the NEAT1 lncRNA is crucial

in promoting the survival of MM cells and enhancing their resistance to stress stimuli.[13] NEAT1 represents the essential architectural structure of nuclear paraspeckles and is involved in several types of cancer. Besides having a role in transcription regulation, this lnc-RNA has been described to modulate the expression of genes that are fundamental for the subsistence of cancer cells, increasing their ability to withstand treatments.[31] We previously demonstrated that NEAT1 silencing reduces MM cell viability by modulating several genome-associated processes. In particular, we observed that NEAT1 is essential for a proficient activity of the homologous recombination DNA repair process and that its downregulation caused increased genomic damage.[19] Furthermore, we showed that NEAT1 is essential for the maintenance of the genome integrity, which it controls through at least two separate mechanisms. On the one hand, NEAT1 promotes paraspeckle assembly by sustaining the stabilization of essential paraspeckle proteins such as NONO, SFPQ and FUS. On the other hand, NEAT1 exercises a tight regulation of the DNA damage response by modulating the activation of the molecular axis involving ATM, DNA-PK kinases and their direct targets pRPA32 and pCHK2.[20] Taken together, this evidence supports the rationale that MM patients with high genomic instability and with higher NEAT1 expression levels could benefit from NEAT1 inhibition. For this reason, NEAT1 is currently regarded as an attractive candidate for therapeutic intervention in MM, prompting the need to develop strategic approaches to counteract its pro-tumoral function. Therapeutic modalities targeting lncRNA in cancer are currently under investigation, mostly in *in vivo* models.[32] Encouraging results have been obtained after the implementation of delivery systems for antisense oligonucleotides and antagomirs which can be conjugated with cholesterol or delivered with lipid nanoparticles and liposomes to improve intracellular affinity for target-specific lncRNA.[33]

Despite the advances in targeting lncRNA in human diseases, it is currently challenging to find a robust strategy



Continued on following page.

B

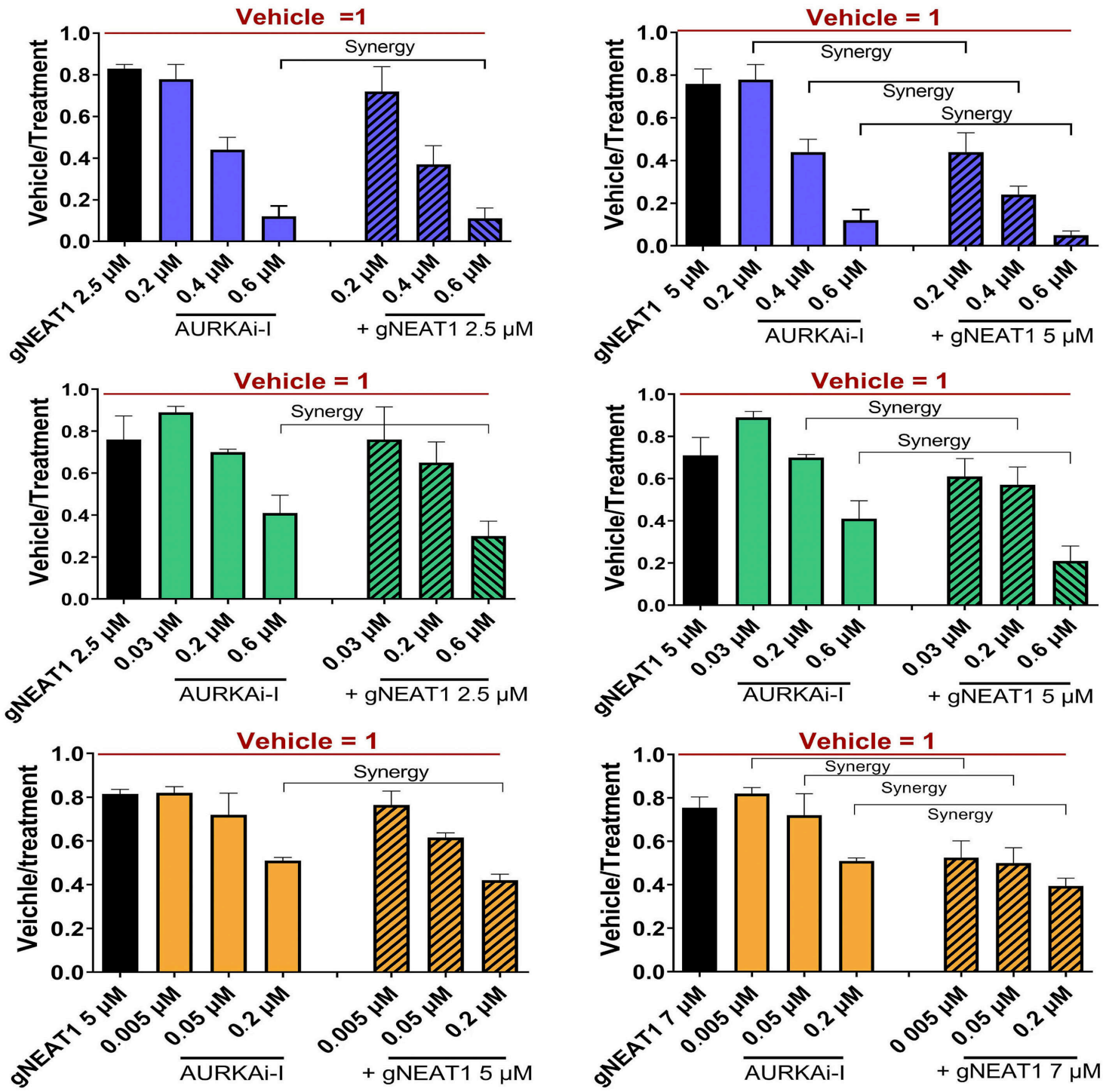


Figure 4. Synergy assessment through the calculation of combination indexes. (A, B) Histograms depicting raw data of combination indexes, i.e., the fraction of viable AMO-1, NCI-H929, and MM1.S cells after 4 days of NEAT1 silencing and 3 days of treatment with the indicated concentrations of alisertib (A) or Aurora kinase A inhibitor I (AURKAi-I) (B).

that counteracts their action in RNA-based therapies in clinical practice. Given the complexity of targeting RNA molecules, approaches such as the one developed in this work may be of relevance to identify combinations able to boost the effect of specific lncRNA antisense oligonucleotides, as well as alternative strategies that mimic the transcript inhibition. Within this framework, we developed an integrated computational and functional approach aimed at identifying drug-based strategies that could potentiate NEAT1 inhibition in impairing MM growth and survival. Two separate strategies were employed. The first was based on the transcriptional alterations induced in MM cells by NEAT1 silencing. The NEAT1 KD-associated gene expression profile was used to search for compounds that could recapitulate the NEAT1 KD transcriptional phenotype, by employing a computational strategy. The second was based on functional high-throughput screening of over 300 small compounds, searching for molecules that could amplify the cytostatic effect of NEAT1 KD on MM cell growth. Both these very different approaches converged on identifying, for the first time, AURKA inhibitors as a promising cooperating agent for NEAT1 inhibition. AURKA has already been implicated in the progression of MM by regulating the activation of autophagy, which represents one of the main causes of drug resistance in MM.[34-37] Indeed, AURKA inhibitors were tested in clinical trials in combination with bortezomib in relapsed MM, confirming that targeting AURKA can potentially overcome the issue of therapy resistance, likely by restraining the activation of autophagy when the proteasome is impaired.[23]

AURKA is a central serine/threonine kinase for regulating the cell cycle and promoting mitosis, participating in the correct

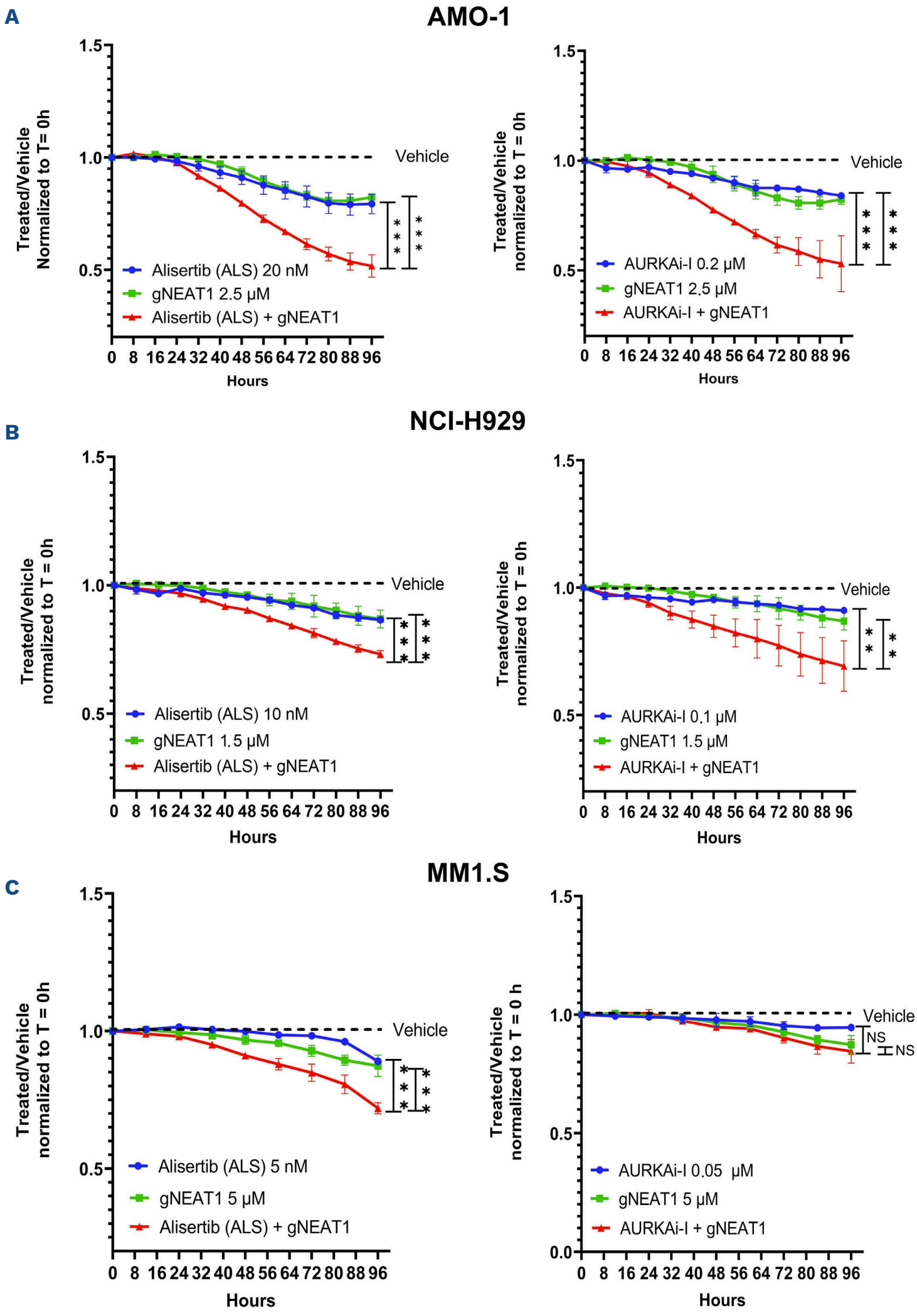


Figure 5. AURKA inhibition increases the cytostatic effect of NEAT1 inhibition in multiple myeloma over time. (A-C) By live cell imaging analysis, the proliferation rate was measured relative to T=0 h, in AMO-1 (A), NCI-H929 (B), and MM1.S (C) multiple myeloma cell lines with Incucyte S3 Live Cell Analysis (Sartorius). NEAT1 expression in the AMO-1, NCI-H929 and MM1.S cells was silenced with different concentrations of GapmeR and then the cells were treated with an inhibitory concentration (IC_{20}) of alisertib or Aurora kinase A inhibitor I (AURKAi-I). Values are represented as the ratio between the treated sample over the vehicle. The graph shows the mean ± standard error of the mean of two independent experiments. Statistical significance was determined with the Student *t* test, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

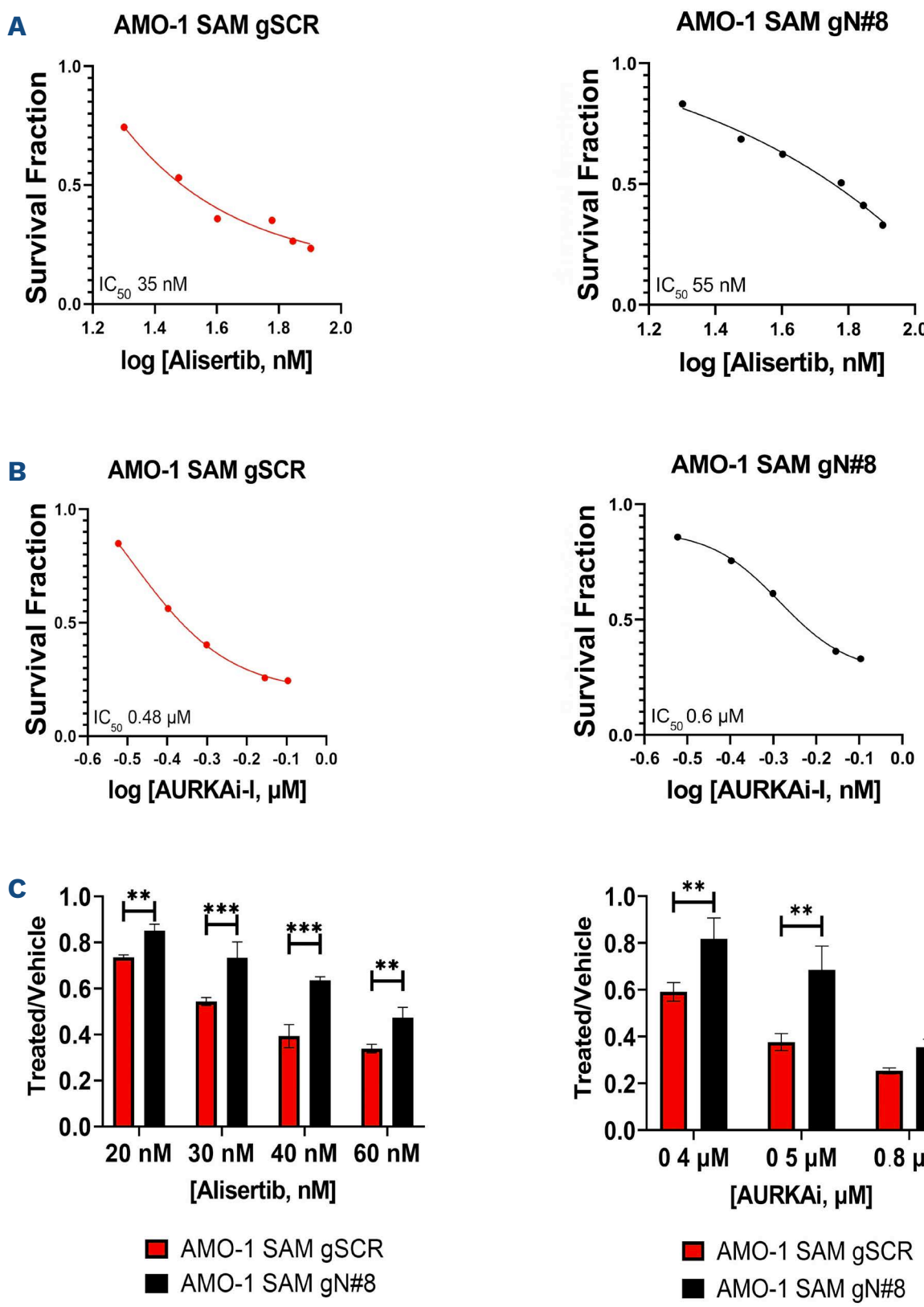
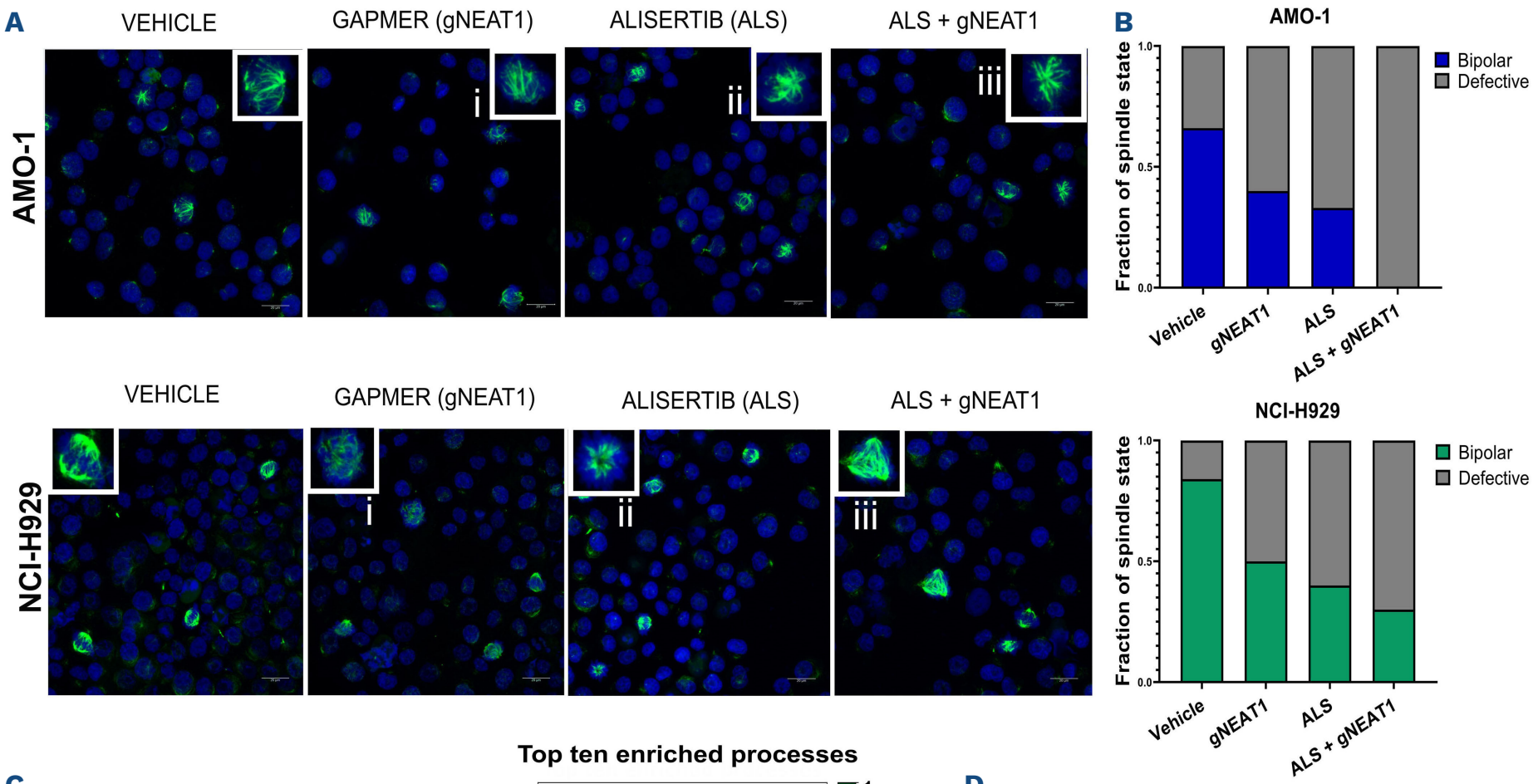


Figure 6. NEAT1 transactivation determines increased resistance to AURKA inhibitors. (A, B) Half maximal inhibitory concentration (IC_{50}) curves of aisertib and Aurora kinase A inhibitor I (AURKAi-I) in AMO-1 SAM cell lines. The IC_{50} value was calculated at 72 hours of treatment using Compusyn software. The fraction of alive cells (%) is provided on the vertical axis and the log (concentration) [µM] of alisertib (A) and AURKAi-I (B) on the horizontal axis. (C) Histograms showing the biological effects obtained in AMO-1 SCR and AMO-1 N#8 SAM cells treated with alisertib and AURKAi-I. Values are represented as the ratio between the treated samples over the vehicle. The graph shows the mean ± standard error of the mean of two independent biological replicates. Statistical significance was determined with the Student *t* test, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

maintenance of genome information. In mitotic cells, phosphorylation at Tyr288 promotes the activity of AURKA, which localizes at centrosomes to control their maturation and at the mitotic spindle to modulate microtubule dynamics and chromosome segregation.[38] The full activation of AURKA requires interaction with allosteric regulators, which favors its proper activity during mitosis.[39,40] Due to its central role in cell cycle regulation, AURKA is considered as a pan-essential gene for cancer cells that need to maintain high-speed cell proliferation.[41] For this reason, several AURKA inhibitors have been developed and used in different clinical settings. In our drug screening, four different compounds (over 20% of all drugs identified) targeting this protein were identified. Of these, we fully validated two distinct compounds, alisertib and AURKAi-I, using three different cell lines. Both drugs target the active loop of AURKA, in proximity of Tyr288, blocking its catalytic activity. When administered to MM cells, both drugs resulted in a relevant inhibition of cell growth,



Continued on following page.

Figure 7. NEAT1 is involved in mitotic spindle dynamics by controlling AURKA activity through TPX2 transcriptional modulation. (A) Representative images of metaphase spindles. AMO-1 and NCI-H929 cells were treated with vehicle, gNEAT1, alisertib, or alisertib + gNEAT1. α-Tubulin was stained in green and DAPI was used to stain cell nuclei. Scale bar 20 µm. (B) Bar plots representing the percentages of mitotic cells with defective spindles in AMO-1 and NCI-H929 cells treated with vehicle, gNEAT1, alisertib, or alisertib + gNEAT1. (C) Dot plot of the top ten significantly down-regulated biological processes (false discovery rate <0.05) obtained in AMO-1 NEAT1 knockdown cells. (D) STRING node depicting functional and physical protein-protein interactions among the down-regulated genes related to mitotic spindle and microtubule organization in AMO-1 NEAT1 knockdown cells. (E) Quantitative real-time polymerase chain reaction of *TPX2* in AMO-1 and NCI-H929 cells silenced for NEAT1 expression (gNEAT1) and in the relative control condition (gSCR), after GapmeR delivery and western blot analysis of TPX2 protein in AMO-1 and NCI-H929 cells silenced for NEAT1 expression (gNEAT1) and in the relative control condition (gSCR), after GapmeR delivery (N=3). (F) Quantitative real-time polymerase chain reaction of *TPX2* in AMO-1 SAM gSCR and AMO-1 SAM gN#8 cells and western blot analysis of TPX2 protein in AMO-1 SAM gSCR and AMO-1 SAM gN#8 cells (N=2).

independently of the MM cellular model used. These effects were maximized upon NEAT1 silencing. Indeed, combination of NEAT1 KD and AURKA inhibition in all three models showed the strongest impairment of cell proliferation, supporting and further validating the synergistic action of these agents. Conversely, we also demonstrated that overexpression of NEAT1 reduces the inhibitory effect of AURKA inhibitors on cell proliferation, confirming the potential interplay between the mitotic kinase and this lncRNA.

Searching for the molecular basis of this cooperation, we observed that NEAT1 KD deregulated a large set of genes involved in cytoskeleton organization and microtubular assembly during mitosis. Notably, when we looked at the morphology of MM cells under treatment, we observed that the combination of AURKA inhibitors and NEAT1 KD led to severe cytoskeleton abnormalities with the appearance of monopolar and multipolar spindles, abnormal mitotic structure, and incomplete cytokinesis. Consistent with this, among the genes that were significantly altered upon NEAT1 silencing in MM cells, we observed several genes that partake in these functions and that affect AURKA function directly or indirectly. Of particular interest, we identified *TPX2* as a NEAT1 target in MM. This gene encodes a microtubule-associated protein that co-localizes at the spindle level during the M-phase. TPX2 acts as an allosteric regulator of AURKA helping its correct positioning at the mitotic spindle and promoting the active conformational state of the protein.[25,42,43] Downregulation of this mediator, as the consequence of NEAT1 silencing, could further compromise AURKA activity supporting the effect of the drugs. Indeed, it has already been shown that alisertib also destabilizes the binding selectivity of TPX2 for AURKA.[44] Even if preliminary and requiring further investigation, this evidence points to a new potential nuclear function of NEAT1 in controlling the cytoskeleton dynamics associated with cell division. A potential involvement of NEAT1 in cytoskeleton dynamics was previously suggested in the context of Alzheimer disease, in which this lncRNA was shown to modulate microtubule polymerization *in vitro* and *in vivo*. Specifically, NEAT1 KD mediates the depolymerization of microtubules by regulating the FZD3/GSK3β/p-tau pathway, thus exerting a relevant role in the etiology of the disease.[45] Furthermore, in hepatocellular carcinoma reactive oxygen species-stress induction promotes nuclear paraspeckle disassembly and NEAT1 translocation to the cytosol, where it interacts with the kinesin KIF11 enhancing protein degradation and thus contributing to defective spindle formation.[46] Since AURKA is the master regulator of the structural apparatus of mitosis, the observation that NEAT1 controls the cytoskeleton during cell division offers a potential readout of the synergistic effects that we observed by inhibiting both and highlights the existence of a functional interplay between them. However, we cannot exclude that the interaction between these two molecules can occur also at different levels. In particular, the functions of NEAT1 of maintaining genomic stability and orchestrating the DNA damage response could be relevant. Indeed, it has been reported that AURKA inhibition, in ovarian cancer models, unbalances the DNA damage repair system towards the non-homologous end-joining error-prone pathway by DNA-PKC activity. In the meantime, AURKA inhibition impairs the mechanism of homologous recombination, through the downregulation of PARP, mimicking a BRCA-ness condition.[47]

In line with a previous report, we confirmed that AURKA is an unfavorable prognostic factor for MM patients, being negatively associated with overall survival and progression-free survival.[48] Conversely, NEAT1 alone, as previously described, has limited impact on disease progression and patients' prognosis.[13] However, based on the CoMMpass dataset, MM patients who have high levels of both AURKA and NEAT1 have the worst outcome, compared with other categories, suggesting that high expression of NEAT1 worsens the prognostic effect of AURKA expression on MM patients. Although this is not direct proof, this observation strongly supports the hypothesis that NEAT1 and AURKA functionally interact in supporting MM growth and progression, and that combinatorial approaches to target both may represent an effective strategy and a new opportunity in the treatment of MM patients.

Disclosures

No conflicts of interest to disclose.

Contributions

NP, GM, IC, LR, MB, VM and VF performed experiments and analyzed the data. FT and DR analyzed the CoMMpass data. EM and MC performed high throughput experiments. NP per-

formed live cell-imaging experiments. NP, ET and VT performed confocal experiments. ET, YT, NA, NB and RP provided critical evaluation of experimental data and of the manuscript. NP, AC and AN conceived the study and wrote the manuscript.

Acknowledgments

YT acknowledges support from the "Piattaforme cellulari per la Ricerca e lo Sviluppo di Terapie Avanzate in Life Science" - Fondo Europeo di Sviluppo Regionale 2014-2020. POR FESR 2014-2020. IC and LR acknowledge the CINECA award under the ISCRA and ELIXIR-IV initiative, for the availability of high-performance computing resources and support.

Funding

The research leading to these results has received funding from AIRC under IG 2020 – ID. 24365 – P.I. Neri Antonino. Also, ET is supported by AIRC-MFAG 2022-ID.27606, VF is supported by AIRC-MFAG 2023-ID. 28974 , NA is supported by AIRC-IG-24449), RP is suppoterted by AIRC- IG21585, NB is funded by the European Research Council under the European Union's Horizon 2020 research and innovation program (grant agreement no. 817997). NP received a fellowship by the PhD program in Clinical and Experimental Medicine of University of Modena and Reggio Emilia. VT received a fellowship by the PhD program in Experimental Medicine of the University of Milan.

Data-sharing statement

Data are available at ArrayExpress; access code: E-MTAB-13925.

References

1. Palumbo A, Anderson K. Multiple myeloma. N Engl J Med. 2011;364(11):1046-1060.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016;66(1):7-30.
3. Rajkumar SV. Multiple myeloma: 2020 update on diagnosis, risk-stratification and management. Am J Hematol. 2020;95(5):548-567.
4. Egan JB, Shi CX, Tembe W, et al. Whole-genome sequencing of multiple myeloma from diagnosis to plasma cell leukemia reveals genomic initiating events, evolution, and clonal tides. Blood. 2012;120(5):1060-1066.
5. Quinn JJ, Chang H. Unique features of long non-coding RNA biogenesis and function. Nat Rev Genet. 2016;17(1):47-62.
6. Wang KC, Chang HY. Molecular mechanisms of long noncoding RNAs. Mol Cell. 2011;43(6):904-914.
7. Chi Y, Wang D, Wang J, Yu W, Yang J. Long non-coding RNA in the pathogenesis of cancers. Cells. 2019;8(9):1015.
8. Ronchetti D, Agnelli L, Taiana E, et al. Distinct lncRNA transcriptional fingerprints characterize progressive stages of multiple myeloma. Oncotarget. 2016;7(12):14814-14830.
9. Taiana E, Gallo Cantafio ME, Favasuli VK, et al. Genomic instability in multiple myeloma: a "non-coding RNA" perspective. Cancers (Basel). 2021;28;13(9):2127.
10. Statello L, Guo CJ, Chen LL, Huarte M. Gene regulation by long non-coding RNAs and its biological functions. Nat Rev Mol Cell Biol. 2021;22(2):96-118.
11. Guru SC, Agarwal SK, Manickam P, et al. A transcript map for the 2.8-Mb region containing the multiple endocrine neoplasia type 1 locus. Genome Res. 1997;7(7):725-735.
12. Naganuma T, Nakagawa S, Tanigawa A, Sasaki YF, Goshima N, Hirose T. Alternative 3'-end processing of long noncoding RNA initiates construction of nuclear paraspeckles. EMBO J. 2012;31(20):4020-4034.
13. Taiana E, Ronchetti D, Favasuli V, et al. Long non-coding RNA NEAT1 shows high expression unrelated to molecular features and clinical outcome in multiple myeloma. Haematologica. 2019;104(2):e72-e76.
14. Clemson CM, Hutchinson JN, Sara SA, et al. An architectural role for a nuclear noncoding RNA: NEAT1 RNA is essential for the structure of paraspeckles. Mol Cell. 2009;33(6):717-726.
15. Chen LL, Carmichael GG. Altered nuclear retention of mRNAs containing inverted repeats in human embryonic stem cells: functional role of a nuclear noncoding RNA. Mol Cell. 2009;35(4):467-478.
16. Hirose T, Virnicchi G, Tanigawa A, et al. NEAT1 long noncoding RNA regulates transcription via protein sequestration within subnuclear bodies. Mol Biol Cell. 2014;25(1):169-183.
17. Jiang L, Shao C, Wu QJ, et al. NEAT1 scaffolds RNA-binding proteins and the microprocessor to globally enhance pri-miRNA processing. Nat Struct Mol Biol. 2017;24(10):816-824.
18. Li R, Harvey AR, Hodgetts SI, Fox AH. Functional dissection of NEAT1 using genome editing reveals substantial localization of the NEAT1_1 isoform outside paraspeckles. RNA. 2017;23(6):872-881.
19. Taiana E, Favasuli V, Ronchetti D, et al. Long non-coding RNA NEAT1 targeting impairs the DNA repair machinery and triggers anti-tumor activity in multiple myeloma. Leukemia. 2020;34(1):234-244.
20. Taiana E, Bandini C, Favasuli VK, et al. Activation of lncRNA NEAT1 leads to survival advantage of multiple myeloma cells by supporting a positive regulatory loop with DNA repair proteins. Haematologica. 2020;108(1):219-233.
21. Bandini C, Mereu E, Paradzik T, et al. Lysin (K)-specific demethylase 1 inhibition enhances proteasome inhibitor response and overcomes drug resistance in multiple myeloma. Exp Hematol Oncol. 2023;12(1):71.
22. Mereu E, Abbo D, Paradzik T, et al. Euchromatic histone lysine methyltransferase 2 inhibition enhances carfilzomib sensitivity and overcomes drug resistance in multiple myeloma cell lines. Cancers (Basel). 2023;15(8):2199.
23. Rosenthal A, Kumar S, Hofmeister C, et al. A phase Ib study of the combination of the Aurora kinase inhibitor alisertib (MLN8237) and bortezomib in relapsed multiple myeloma. Br J Haematol. 2016;174(2):323-325.
24. Metselaar DS, du Chatinier A, Meel MH, et al. AURKA and PLK1 inhibition selectively and synergistically block cell cycle progression in diffuse midline glioma. iScience. 2022;25(6):104398.
25. Fathi AT, Wander SA, Blonquist TM, et al. Phase I study of the aurora A kinase inhibitor alisertib with induction chemotherapy in patients with acute myeloid leukemia. Haematologica. 2017;102(4):719-727.

26. Kufer TA, Silljé HH, Körner R, Gruss OJ, Meraldi P, Nigg EA. TPX2 is required for targeting Aurora-A kinase to the spindle. J Cell Bio. 2022;158(4):617-623.
27. Eyers PA, Erikson E, Chen LG, Maller JL. A novel mechanism for activation of the protein kinase Aurora A. Curr Biol. 2023;13(8):691-697.
28. Yang WC, Lin SF. Mechanisms of drug resistance in relapse and refractory multiple myeloma. Biomed Res Int. 2015;2015:341430.
29. Vo JN, Wu YM, Mishler J, et al. The genetic heterogeneity and drug resistance mechanisms of relapsed refractory multiple myeloma. Nat Commun. 2022;13(1):3750.
30. Ronchetti D, Agnelli L, Pietrelli A, et al. A compendium of long non-coding RNAs transcriptional fingerprint in multiple myeloma. Sci Rep. 2018;8(1):6557.
31. Pistoni M, Rossi T, Donati B, Torricelli F, Polano M, Ciarrocchi A. Long noncoding RNA NEAT1 acts as a molecular switch for BRD4 transcriptional activity and mediates repression of BRD4/WDR5 target genestranscriptional repression via lncRNA NEAT1/BRD4 interaction. Mol Cancer Res. 2021;19(5):799-811.
32. Ling H, Fabbri M, Calin GA. MicroRNAs and other non-coding RNAs as targets for anticancer drug development. Nat Rev Drug Discov. 2013;12(11):847-865.
33. Roberts TC, Langer R, Wood MJA. Advances in oligonucleotide drug delivery. Nat Rev Drug Discov. 2020;19(10):673-694.
34. Xie Y, Zhu S, Zhong M, et al. Inhibition of Aurora kinase A induces necroptosis in pancreatic carcinoma. Gastroenterology. 2017;153(5):1429-1443.
35. Wang P, Gong Y, Guo T, et al. Activation of Aurora A kinase increases YAP stability via blockage of autophagy. Cell Death Dis. 2019;10(6):432.
36. Tang A, Gao K, Chu L, Zhang R, Yang J, Zheng J. Aurora kinases: novel therapy targets in cancers. Oncotarget. 2017;8(14):23937-23954.
37. Murga-Zamalloa C, Inamdar KV, Wilcox RA. The role of aurora A and polo-like kinases in high-risk lymphomas. Blood Adv. 2019;3(11):1778-1787.
38. Naso FD, Boi D, Ascanelli C, et al. Nuclear localisation of Aurora-A: its regulation and significance for Aurora-A functions in cancer. Oncogene. 2021;40(23):3917-3928.
39. Garrido G, Vernos I. Non-centrosomal TPX2-dependent regulation of the Aurora A kinase: functional implications for healthy and pathological cell division. Front Oncol. 2016;6:88.
40. Mou PK, Yang EJ, Shi C, Ren G, Tao S, Shim JS. Aurora kinase A, a synthetic lethal target for precision cancer medicine. Exp Mol Med. 2021;53(5):835-847.
41. Hose D, Rème T, Hielscher T, et al. Proliferation is a central independent prognostic factor and target for personalized and risk-adapted treatment in multiple myeloma. Haematologica. 2011;96(1):87-95.
42. 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.
43. Levinson NM. The multifaceted allosteric regulation of Aurora kinase A. Biochem J. 2018;475(12):2025-2042.
44. Lake EW, Muretta JM, Thompson AR, et al. Quantitative conformational profiling of kinase inhibitors reveals origins of selectivity for Aurora kinase activation states. Proc Natl Acad Sci U S A. 2018;18;115(51):E11894-E11903.
45. Zhao Y, Wang Z, Mao Y, et al. NEAT1 regulates microtubule stabilization via FZD3/GSK3β/P-tau pathway in SH-SY5Y cells and APP/PS1 mice. Aging (Albany NY). 2020;12(22):23233-23250.
46. Chen D, Wang J, Li Y, et al. LncRNA NEAT1 suppresses cellular senescence in hepatocellular carcinoma via KIF11-dependent repression of CDKN2A. Clin Transl Med. 2023;(9):e1418.
47. Do TV, Hirst J, Hyter S, Roby KF, Godwin AK. Aurora A kinase regulates non-homologous end-joining and poly(ADP-ribose) polymerase function in ovarian carcinoma cells. Oncotarget. 2019;8(31):50376-50392.
48. Hose D, Rème T, Meissner T, et al. Inhibition of aurora kinases for tailored risk-adapted treatment of multiple myeloma. Blood. 2009;113(18)4331-4340.