A Temporal PROTAC Cocktail-Mediated Sequential Degradation of AURKA Abrogates Acute Myeloid Leukemia Stem Cells

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AURKA is a potential kinase target in various malignancies. The kinase-independent oncogenic functions partially disclose the inadequate efficacy of the kinase inhibitor in a Phase III clinical trial. Simultaneously targeting the catalytic and noncatalytic functions of AURKA may be a feasible approach. Here, a set of AURKA proteolysis targeting chimeras ( PROTACs) are developed. The CRBN-based dAurA383 preferentially degrades the highly abundant mitotic AURKA, while cIAP-based dAurA450 degrades the lowly abundant interphase AURKA in acute myeloid leukemia (AML) cells. The proteomic and transcriptomic analyses indicate that dAurA383 triggers the “mitotic cell cycle” and “stem cell” processes, while dAurA450 inhibits the “MYC/E2F targets” and “stem cell” processes. dAurA383 and dAurA450 are combined as a PROTAC cocktail. The cocktail effectively degrades AURKA, relieves the hook effect, and synergistically inhibits AML stem cells. Furthermore, the PROTAC cocktail induces AML regression in a xenograft mouse model and primary patient blasts. These findings establish the PROTAC cocktail as a promising spatial-temporal drug administration strategy to sequentially eliminate the multifaceted functions of oncoproteins, relieve the hook effect, and prevent cancer stem cell-mediated drug resistance.

1. Introduction

Aurora kinase A (AURKA, Aurora-A) is mainly localized in the spindle poles and plays a crucial role in proper bipolar spindle formation in the mitotic process. AURKA is frequently overexpressed in various malignancies, including acute myeloid leukemia (AML). A high level of AURKA is associated with a higher tumor grade and poorer prognosis. Recently, we and others have demonstrated that AURKA is highly expressed in CD34+/CD38- AML stem cells and augments stemness. The genetic depletion of AURKA inhibits proliferation, impairs self-renewal and induces apoptosis of AML stem cells. Therefore, AURKA is considered a potential therapeutic cancer target in AML. We previously developed a set of AURKA kinase inhibitors with anticancer effects in various cancers, including AML. A series of AURKA kinase inhibitors, including...
results indicate that AURKA kinase inhibitors maybe unable to abrogate kinase-independent oncogenic functions. We found that the nuclear translocation of AURKA in the interphase facilitates stem-cell-like phenotypes via directly activating c-Myc \cite{22} and FOXM1 transcription, \cite{23} or by enhancing N6-methyladenosine modification of DROSHA mRNA to activate STC1 transcription \cite{24} in a kinase-independent manner. Other groups have found that AURKA directly binds to c-Myc \cite{25} and N-myc \cite{26,27} proto-oncoproteins thereby noncatalytically protecting them from proteasomal degradation. Thus, a strategy of simultaneously targeting the kinase-dependent and kinase-independent functions of AURKA to induce mitotic catastrophe and impair cancer stemness is proposed to be a more effective therapeutic approach.

The emerging proteolysis targeting chimeras (PROTACs) provide an opportunity to target both the catalytic and noncatalytic functions of kinases via artificial ubiquitylation and subsequent degradation by proteasomes. The bifunctional small molecule PROTACs are comprised of two chemical moieties. One binds to a target of interest and the other to a cellular E3-ubiquitin ligase. Mitotic AURKA naturally undergoes ubiquitylation by anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase with its coactivator CDH1, \cite{29} or Skp1-Cul1-F-box proteinubiquitin ligase with its coactivator FBXL7/FBXW7 \cite{26,30,31} and is subsequently degraded upon mitotic exit. The von Hippel-Lindau (VHL) is effectively degrade AURKA, cause an S-phase defect \cite{33} and prevent fragmentation of the mitochondrial network. \cite{34} Yet, these AURKA PROTACs confront the problem of the hook effect, and no evidence indicates that AURKA PROTACs could eliminate the stemness promoting function of interphase AURKA.

Here, we have developed a set of AURKA PROTACs based on the E3 ligases CRBN, cIAP, and VHL. The CRBN-based PROTAC dAurA383 preferentially degrades the highly abundant mitotic AURKA, while cIAP-based PROTAC dAurA450 degrades the lowly abundant interphase AURKA. The combination of dAurA383 and dAurA450 sequentially degrades the mitotic and interphase AURKA, impairs AML proliferation, relieves the hook effect, and abrogates acute myeloid leukemia stem cells. This study extends the spatial-temporal drug administration strategy of PROTACs.

2. Results

2.1. Design and Characterization of Different E3 Ubiquitin Ligase-Based AURKA PROTACs

We checked the crystal structures of AURKA in the Protein Data Bank \cite{35} to evaluate its degradation potential and to find its binding scaffold (Figure 1A, PDB code 2x81). Considering that lysine residues on protein surface are accessible for modification, \cite{36,37} the abundant surface lysine residues suggest AURKA is a potential candidate for PROTAC mediated ubiquitination degradation. MLN8054 has been reported to selectively bind to the pocket of AURKA \cite{18,39}. MLN8237 is a close analogue of MLN8054 with greater tolerable safety profile in multiple clinical studies \cite{40} (Figure S1A, Supporting Information). Considering the greater binding affinity of MLN8237 \cite{41} we chose MLN8237 as the AURKA binding scaffold. The commonly used CRBN ligand, VHL ligand, and cIAP ligand were attached to MLN8237 through different linkers to synthesize a series of AURKA PROTACs (Figure 1B; Figure S1B, Supporting Information). The structure and purity of the compounds are shown in the Experimental Section. We set out to investigate the degradation activity of these PROTACs against AURKA by Western blot. As shown in Figure S1C,D of the Supporting Information, CRBN-based dAurA383 and dAurA1071 exhibit the best degradation activity in all of the PROTACs. Besides, the VHL-based dAurA425 and the cIAP-based dAurA450 exhibit moderate degradation activity. We chose dAurA383, dAurA425, and dAurA450 with distinct E3 ligase as the potential candidates for further evaluation.
Figure 1. Design and characterization of different E3 ubiquitin ligase-based AURKA PROTACs. A) Crystal structure of AURKA in complex with MLN8054 (PDB code 2кс1). The lysine residues on the surface of AURKA are highlighted. B) Chemical structures of AURKA PROTAC based on MLN8237 and the ligands of CRBN, VHL, and cIAP. MW, molecular weight (Daltons). C) SPR sensorgrams and KD values of MLN8237 and AURKA PROTACs binding to the AURKA protein. D) Dual-colored fluorescence SPPIER (DF-SPPIER) imaging of ternary complex formation in HEK293T cells with PROTACs and E3 ligase induction (500 \times 10^{-9} m) for 3 h. Fluorescence histogram of the line across the cells (normalized fluo., normalized fluorescence intensity) and the log10 normalized sum of pixel fluorescence intensity (normalized SPPIER, log_{10} (intensity + 1)) of yellow droplets in each cell are shown on the right. Scale bar: 5 \mu m. E) Degradation of endogenous AURKA in KG1A cells following 6 h treatment with the indicated concentration of PROTACs. F) Degradation of endogenous AURKA in KG1A cells following 6 h treatment with the indicated PROTACs (500 \times 10^{-9} m) and E3 ligase ligands. G) Degradation of endogenous AURKA in KG1A cells with or without doxycycline (DOX, 0.5 \mu g mL^{-1}) treatment for 72 h following 6 h treatment with the
enzyme inhibitor), Bortezomib, and MG132 (proteasome inhibitors) (Figure S1J, Supporting Information).

To further examine the selective degradation effect of the three PROTACs, we performed tandem mass tags (TMTs)-based quantitative proteomic analysis after treatment with dAurA383, dAurA425, dAurA450 or DMSO vehicle in KG1A cells. dAurA383 displays a highly specific degradation activity on AURKA, while dAurA425 and dAurA450 are less specific (Figure 1H; Figure S1K and Table S1, Supporting Information).

Although the results of the SPR and SPPIER assays demonstrate that all of dAurA383, dAurA425, and dAurA450 bind AURKA and form ternary complexes with E3 ligases, they have significantly different degradation efficiencies. We speculated that the cell cycle-dependent expression of AURKA and E3 ligases might contribute to this discrepancy and examined the cell cycle phase distribution of AURKA, CRBN, VHL, and cIAP1 in KG1A and Kasumi-1 cells (Figure 1I, J; Figure S2A, B, Supporting Information). The protein expression patterns of AURKA, CRBN, cIAP1, and VHL are summarized in Figure 1K and Figure S2C (Supporting Information). The expression of CRBN increases when cells enter the S phase and reaches a maximum level at the S/G2 boundary (KG1A cells) or G2/M boundary (Kasumi-1 cells), then reduces along with mitosis progression and increases again when cells enter the next cell cycle. This observation suggests CRBN-based dAurA383 could timely and effectively degrade the newly synthesized AURKA proteins from the S phase to mitosis. The expression of cIAP1 and VHL increases when cells exit mitosis and enter the G1 phase. The expression patterns of the CRBN, cIAP1, and VHL in the cell cycle support the CRBN-based PROTAC dAurA383 effectively degrades the highly abundant mitotic AURKA, while the cIAP-based PROTAC dAurA450 degrades the lowly abundant interphase AURKA.

2.2. AURKA PROTACs Inhibit Cell Growth and Induce Apoptosis of AML Cells In Vitro

To study the anticancer activity of these PROTACs in vitro, KG1A, Kasumi-1, ML160, NB4, U937, and THP1 cells were treated with dAurA383, dAurA425, and dAurA450. The inhibition of cell proliferation was evaluated by CCK8 assays. In KG1A cells, the IC_{50} doses are 3.04 ± 0.28 × 10^{-9} m (dAurA383), 9.23 ± 2.00 × 10^{-9} m (dAurA425) and 3.41 ± 0.28 × 10^{-9} m (dAurA450). In Kasumi-1 cells, the IC_{50} doses are 1.17 ± 0.08 × 10^{-9} m (dAurA383), 3.75 ± 0.32 × 10^{-9} m (dAurA425), and 3.19 ± 0.35 × 10^{-9} m (dAurA450) (Figure 2A, B). In other AML cells, the IC_{50} dose-levels are between 0.42 × 10^{-9} and 1.70 × 10^{-9} m for dAurA383, between 7.28 × 10^{-9} and 11.99 × 10^{-9} m for dAurA425, and between 1.95 × 10^{-9} and 2.77 × 10^{-9} m for dAurA450 (Figure S3, Supporting Information). The apoptotic induction function of the three PROTACs was further assessed by Annexin V assays. dAurA383 and dAurA450 induce apoptosis of both KG1A and Kasumi-1 cells, while dAurA425 weakly induces apoptosis of Kasumi-1 cells (Figure 2C, D). In addition, methylcellulose-based colony-forming cell (CFC) assays demonstrate that the PROTACs significantly suppress the colony formation of KG1A cells (Figure 2E, F).

2.3. Characterization of Cellular Responses to PROTACs in AML Cells

To explore the anticancer mechanism of the three PROTACs, we examined the cell cycle changes upon the PROTAC treatment. The carboxyfluorescein succinimidyl amino ester (CFSE) staining assays demonstrate that dAurA383 and dAurA450 obviously delay the division of KG1A and Kasumi-1 cells (Figure 3A). The propidium iodide (PI) staining assays show that dAurA383 arrests the cell cycle in mitosis in KG1A and Kasumi-1 cells, while dAurA450 does not (Figure 3B; Figure S4A, Supporting Information). Additionally, neither knockdown of E3 ligases nor treatment with E3 ligands changes the cell cycle phase distribution (Figure S4B, C, Supporting Information), suggesting the bifunctional PROTACs change the cell cycle through AURKA.

We profiled the transitory response of proteomics underlying dAurA383, dAurA425, and dAurA450 treatment (Table S1, Supporting Information). The differentially expressed proteins in each group (p < 0.05, abundance ratio < 0.5) are presented in a heatmap (Figure 3C). The top 100 decreased proteins in each group were subject to the g:Profiler[44] for gene ontology (GO) analysis. The processes of “heterocyclic compound,” “cellular responses to stress,” and “cellular component biogenesis” are enriched in all the groups. Consistent with the cell cycle analysis, the processes of “cell cycle phase transition” and “mitotic cell cycle” are enriched in dAurA383 treated cells. The process of “oxidative phosphorylation” is enriched in dAurA450 treated cells (Figure 3D), supporting the emerging functions of interphase AURKA in mitochondrial dynamics and energy production.[45, 46]

We next evaluated the transcriptional responses of PROTAC treatment. The transcripts per million for each gene are listed in Table S2 of the Supporting Information. Principal component analysis of the top 1000 differentially expressed genes indicates a reasonable distribution and correlation for each sample (Figure S4D, Supporting Information). Gene set enrichment analysis (GSEA) shows dAurA383 treatment triggers “mitotic spindle” and “stem cell” processes, while dAurA450 treatment inhibits “MYC/E2F targets,” “stem cell,” and “oxidative phosphorylation” processes (Figure 3E). The transcriptomic profiling supports the finding that dAurA383 degrades mitotic AURKA, while dAurA450 degrades interphase AURKA. Consistent with changes of stemness in proteomics and transcriptomics, a population of KG1A cells increase cell surface CD34 (hematopoietic stem/progenitor cell marker[47]) in response to dAurA383
Figure 2. AURKA PROTACs inhibit cell growth and induce apoptosis of AML cells in vitro. A) Dose–response curves of KG1A or Kasumi-1 treated with AURKA PROTACs. Cells were treated with various concentrations of AURKA PROTACs for 72 h and were stained with CCK8. For IC50 in KG1A cells, dAurA383 = 3.04 ± 0.28 × 10⁻⁶ m, dAurA425 = 9.23 ± 2.00 × 10⁻⁶ m, dAurA450 = 3.41 ± 0.28 × 10⁻⁶ m. For IC50 in Kasumi-1 cells, dAurA383 = 1.17 ± 0.08 × 10⁻⁶ m, dAurA425 = 3.75 ± 0.32 × 10⁻⁶ m, dAurA450 = 3.19 ± 0.35 × 10⁻⁶ m. B) Cell proliferation of KG1A or Kasumi-1 treated with AURKA PROTACs. Cells were treated with AURKA PROTACs (1 × 10⁻⁶ m) and were stained with CCK8. C,D) Cell apoptosis of KG1A cells treated with AURKA PROTACs (1 × 10⁻⁶ m) for 48 h. E,F) Methylcellulose-based colony forming cell (CFC) assays show the effects of AURKA PROTACs (1 × 10⁻⁶ m) on KG1A cells for 14 days. Sphere number (diameter > 50 μm, left panel) and size (diameter, right panel) were calculated. Scale bar: 50 μm. Statistics, significance: one-way ANOVA with Bonferroni correction (B,D,F); ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.

treatment, while most of the cells decrease cell surface CD34 in response to the dAurA450 treatment (Figure 3F).

We further investigated the effect of PROTACs on AURKA-related signaling pathways. Consistent with the previous study of CRBN-based PROTAC-D, dAurA383 induces a marked degradation of the spindle associated AURKA, while preserves the centrosome associated AURKA (Figure 3G). Meanwhile, MLN8237 disrupts the localization of AURKA at the centrosome and spindle (Figure 3G). Furthermore, AURKB and cyclin B1 slightly decrease in mitotic arrest cells after dAurA383 treatment, suggesting a mitotic slippage in dAurA383 treated cells (Figure 3H). Although the expression of AURKA binding partners TPX2 and TACC3 are not affected, phosphorylated TACC3 (pTACC3 on S558) is significantly decreased in dAurA383 and MLN8237 treated cells (Figure 3H), implying that AURKA inhibition destroys the stabilization of parallel spindle microtubules.[48]

2.4. A dAurA383 and dAurA450 Cocktail Synergistically Inhibit the Growth and Stemness of AML Cells

The results from the CD34 flow cytometry assays and the global changes in proteomics and transcriptomics indicate that dAurA383 degrades the mitotic AURKA but triggers the stemness program activation in some cells, and that dAurA450 degrades interphase AURKA and confronts the stemness program.
Figure 3. Characterization of cellular response to PROTACs in AML cells. A) KG1A and Kasumi-1 cells were stained with a carboxyfluorescein succinimidyl amino ester (CFSE) probe and cultured with AURKA PROTACs ($1 \times 10^{-6}$ m) for 72 h. CFSE fluorescence was analyzed by flow cytometry. MFI, mean fluorescence intensity. B) KG1A cells were treated with AURKA PROTACs ($1 \times 10^{-6}$ m) for 48 h. The cell cycle profile was assayed by FACS with propidium iodide (PI) staining. The cell cycle phase distribution was analyzed by FlowJo software. C) A heatmap of the relative normalized abundance of proteins in TMT-based quantitative proteomic assays. D) TMT-based quantitative proteomics after treatment with PROTACs ($500 \times 10^{-9}$ m) or the DMSO Vehicle for 6 h in KG1A cells. The top 100 decreased proteins were subjected to g:Profiler to perform gene ontology (GO) analysis. E) Gene set enrichment analysis (GSEA) of RNA-seq data from KG1A cells treated with AURKA PROTACs ($1 \times 10^{-6}$ m) for 48 h. F) KG1A cells were treated with AURKA PROTACs ($1 \times 10^{-6}$ m) or ATRA ($0.6 \times 10^{-6}$ m) for 48 h. Cell surface CD34 expression was analyzed by FACS. MFI, mean fluorescence intensity. G) KG1A cells were arrested with nocodazole ($0.1 \mu$g mL$^{-1}$) for 16 h followed by PROTACs or MLN8237 treatment with indicated concentration for 6 h. AURKA localization were detected by immunofluorescence. Scale bar: 5 µm. H) KG1A cells were arrested with nocodazole ($0.1 \mu$g mL$^{-1}$) for 16 h followed by DMSO, PROTACs ($1 \times 10^{-6}$ m) or MLN8237 ($50 \times 10^{-9}$ m) treatment for indicated times. The expression of AURKA and related proteins were detected. Statistics, significance: one-way ANOVA with Bonferroni correction (A,F); ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.
Therefore, we propose a PROTAC cocktail of dAurA383 and dAurA450 to synergistically induce AML regression by eliminating the proliferative cells and abrogating leukemia stem cells. The ratios of the PROTACs in cocktail were assessed. As shown in Figure S5A of the Supporting Information, the cocktail of dAurA383 and dAurA450 exerts the best cytotoxic effect at the ratio of either 1:1 or 0.75:1.25 in KG1A cells, while exhibits the best cytotoxic effect at the ratio of either 1.25:0.75, 1:1 or 0.75:1.25 in Kasumi-1 cells. As these optimal ratios are close to 1:1, we used 1:1 to conduct all the PROTAC cocktail studies.

We investigated the cytotoxic effect of single and cocktail of PROTACs by CCK8 assays. PROTAC cocktail displays better antiproliferative action than that of the single drugs (Figure 4A). These data were subjected to CompuSyn software to calculate the combination index (CI). An index less than 0.8, between 0.8 and 1.2, and more than 1.2 represents synergy, additivity, and antagonism, respectively. As shown in Figure 4B, dAurA383 and dAurA450 act synergistically to inhibit KG1A and Kasumi-1 cells. The cocktail of dAurA383 and dAurA450 severely inhibits cell division (Figure 4C), arrests AML cells in mitosis and generates aneuploid cancer cells (Figure 4D). Annexin V assays further demonstrate that the PROTAC cocktail significantly triggers the apoptosis of KG1A and Kasumi-1 cells compared with the single agent administration (Figure 4E).

The PROTAC cocktail effectively decreases cell surface CD34 (Figure 4F), suppresses both primary and secondary colony formation (Figure 4G) and reduces the ratio of aldehyde dehydrogenase (ALDH) positive AML stem cells (Figure 4H,I). The stemness markers c-Myc, NANOG, STAT5A, and FOXM1 are decreased in the PROTAC cocktail treated AML cells (Figure 4I). Consistent with our finding that AURKA transcriptionally activated c-Myc, the cocktail decreases c-Myc at transcriptional level (Figure 4J).

We further examined the specificity of the PROTAC cocktail. The treatment of PROTAC cocktail does not reduce the expression of AURKB, TPX2, and TACC3 in both proliferating and mitotic arrest cells (Figure 5D,E). The TMT-based quantitative proteomic analysis confirms the specific degradation of AURKA by PROTAC cocktail (Figure 5F). The degradation activity of PROTAC cocktail is blocked by MLN4924 and Bortezomib (Figure 5G).

2.6. The AURKA PROTAC Cocktail Induces AML Regression in a Xenograft Mouse Model and Primary AML Blasts

We evaluated the in vivo anticancer effects of AURKA PROTAC cocktail in a xenograft model using MLN8237 as a positive control. Nude mice harboring KG1A xenografts were subjected to dAurA383, dAurA450, or the PROTAC cocktail by daily intraperitoneal injection. As shown in Figure 6A,B and Figure S7A,B (Supporting Information), at the dose level of 30 μmol kg⁻¹ day⁻¹, AURKA PROTAC cocktail significantly suppresses KG1A cell growth in vivo. We further isolated the KG1A cells from the dissected tumor tissues. AURKA PROTAC cocktail decreases the expression of hematopoietic stem/progenitor cell marker CD34 and increases the expression of myeloid-monocytic lineage differentiation marker CD11b (Figure 6C). Meanwhile, AURKA PROTAC cocktail more effectively degrades AURKA and induces cancer cell apoptosis (Figure 6D). The body weights show no significant differences between the PROTAC treated and the vehicle treated mice (Figure 6E), while show a slight decrease in MLN8237 treated mice (Figure 5C, Supporting Information). Additionally, the plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), and blood urea nitrogen (BUN) in the PROTAC cocktail treatment group are not statistically different from that in vehicle control group (Figure 6F).

Finally, we assessed the inhibition effect of the AURKA PROTAC cocktail in malignant bone marrow mononuclear cells (BMMCs) obtained by gradient centrifuged from three pathologically confirmed de novo AML patients. Western blot analysis shows AURKA PROTAC cocktail effectively degrades AURKA

Figure 4. dAurA383 and dAurA450 synergistically inhibit the growth and stemness of AML cells. A,B) KG1A and Kasumi-1 cells were treated with various concentrations of dAurA383, dAurA450 alone or in combination for 72 h. The growth inhibitory effects were determined by CCK8 staining. The CI value was calculated with CompuSyn software. C) KG1A and Kasumi-1 cells were stained with carboxyfluorescein succinimidyl amino ester (CFSE) probe and cultured with PROTACs (500 × 10⁻⁹ m) for 72 h. CFSE fluorescence was analyzed by flow cytometry. MFI, mean fluorescence intensity. D) KG1A and Kasumi-1 cells were treated with AURKA PROTACs (500 × 10⁻⁹ m) for 48 h. The cell cycle profile was assayed by FACS with propidium iodide (PI) staining. The cell cycle phase distribution was analyzed by Flowjo software. E) The apoptosis of KG1A and Kasumi-1 cells treated with the indicated PROTACs (500 × 10⁻⁹ m) for 48 h. The rate of Annexin V positive cells was calculated. F) KG1A cells were treated with AURKA PROTACs (500 × 10⁻⁹ m) for 48 h. Cell surface CD34 expression was analyzed by FACS. MFI, mean fluorescence intensity. G) Methylcellulose-based colony forming cell (CFC) assays to examine the stemness of KG1A cells treated with PROTACs (500 × 10⁻⁹ m) for 14 days. Sphere number (diameter > 50 μm, left panel) and size (diameter, right panel) were calculated. Scale bar: 50 μm. H,I) KG1A cells were treated with AURKA PROTACs (500 × 10⁻⁹ m) for 48 h. ALDH positive cells were analyzed by flow cytometry assays. J&K. KG1A and Kasumi-1 cells were treated with AURKA PROTACs (1 × 10⁻⁹ m) and MLN8237 (50 × 10⁻⁹ m) for 48 h. Western blot analysis of AURKA as well as stemness markers c-Myc, NANOG, STAT5A, and FOXM1. Statistics, significance: one-way ANOVA with Bonferroni correction (A,C,E,F,G,I); ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 5. An AURKA PROTAC cocktail relieves the hook effect. A) Scheme depicting the strategy to combine dAurA383 and dAurA450 to relieve the hook effect. B) Degradation of endogenous AURKA in KG1A and Kasumi-1 cells following 12 h treatment with the indicated concentration of PROTACs. C) Relative AURKA protein levels in panel B were quantified using the ImageJ software. D) KG1A and Kasumi-1 cells were treated with PROTAC cocktail (4 × 10⁻⁶ M) for 12 h. The expression of AURKA and related proteins was detected. E) KG1A cells were arrested with nocodazole (0.1 μg mL⁻¹) for 16 h followed by PROTAC cocktail (4 × 10⁻⁶ M) treatment for indicated times. The expression of AURKA and related proteins were detected. F) TMT-based quantitative proteomics after treatment with dAurA383 and dAurA450 cocktail (1 × 10⁻⁶ M) or the DMSO Vehicle for 6 h in KG1A cells. The differentially expressed proteins are presented in the volcano plot. G) Degradation of endogenous AURKA in KG1A and Kasumi-1 cells following 12 h treatment with PROTAC cocktail (4 × 10⁻⁶ M), NEDD8-activating enzyme inhibitor MLN4924 (1 × 10⁻⁶ M) or proteasomal inhibitor Bortezomib (25 × 10⁻⁹ M).
Figure 6. AURKA PROTAC cocktail induces AML regression in a xenograft mouse model and primary blasts. A) Nude mice bearing KG1A xenografts were intraperitoneally injected with dAurA383, dAurA450, and cocktail (30 μmol kg⁻¹ day⁻¹). The tumor volume from 1 to 14 days is plotted versus time. B) Left panel, tumors resected from the mice in each group are shown. Right panel: statistical analysis of the tumor weight. C) The surface CD34 and CD11b of dissected tumors were analyzed by FACS assays. D) The indicated proteins of dissected tumors were detected by Western blot. E) The body weight of the mice were measured and plotted against time. F) Plasma ALT, AST, and BUN of the mice were measured. The dotted lines display the normal reference values of BALB/c nude mice in Charles River Laboratories. G) AURKA expression was detected by Western blot in AML primary blasts treated with AURKA PROTACs (1 × 10⁻⁶ M) for 6 h. H) Cell apoptosis was analyzed in AML primary blasts treated with AURKA PROTACs (1 × 10⁻⁶ M) for 48 h. Statistics, significance: one-way ANOVA with Bonferroni correction (A,B,C,F); ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.
protein in AML patient derived BMMCs (Figure 6G). The AURKA PROTAC cocktail significantly induces apoptosis of patient derived AML blasts (Figure 6H).

3. Discussion

PROTAC is an emerging drug discovery paradigm.[53] In recent years, researchers have focused on developing new compounds to degrade various target proteins such as kinases,[54] transcription factors,[55–57] and "undruggable" proteins.[58] However, the spatial-temporal distribution and multifaceted functions of target proteins have hindered the effectiveness of the first generation PROTACs. In this study, we have developed a set of AURKA PROTACs based on different E3 ligases. The CRBN-based dAurA383 preferentially targets canonical functions of mitotic AURKA, while cIAP-based dAurA450 preferentially targets the emerging noncanonical functions of interphase AURKA. We conclude that the dAurA383 and dAurA450 cocktail would both eliminate the proliferative AML cells and abrogate AML stem cells (Figure 7). The spatial-temporal drug administration strategy was validated as a feasible approach in animal xenografts and the patient derived primary blasts. Based on our findings, we propose a PROTAC cocktail strategy as a second generation of PROTACs to overcome the multifaceted oncogenic functions of targets.

The proteomic and transcriptomic analyses support the cell cycle phase specific functions of dAurA383 and dAurA450. For example, dAurA383 treatment triggers the mitotic related processes such as "mitotic cell cycle," echoing that dAurA383 arrests AML cells at mitosis by removing the spindle pool of AURKA, decreasing the phosphorylation of TACC3 and disrupting spindle microtubules.[59] However, dAurA383 treatment induces stemness related processes such as "CONRAD stem cell," which is consistent with our finding that dAurA383 induces a population of CD34 high stem cells. dAurA450 treatment suppresses the stemness related processes such as "hallmark MYC/E2F targets," “EPPERT Progenitor,” “WONG Embryonic stem cell core,” and “Oxidative phosphorylation,” supporting previous studies that interphase AURKA facilitates stem-cell-like phenotypes[22–24] and increases energy production.[45,46] and in line with that dAurA450 induces CD34 low cells and suppresses the expression of stem cell markers including c-Myc, NANOG, STAT5A, and FOXM1. The stemness inhibition may also explain the result that dAurA450 delays the cell division but not obviously changes the cell cycle phase distribution.

Besides targeting the multifunctions of AURKA, another advantage of PROTAC cocktail is reliving the hook effect, a phenomenon manifesting the loss of degradation activity of PROTACs at higher concentrations.[51] PROTAC overdose generates saturated individual [E3 ligase-PROTAC] and [PROTAC-target protein] binary complexes which compete for the formation of the [E3 ligase-PROTAC-target protein] ternary complex.[60] Employing more available E3 ligase partially explains how the cocktail alleviates the hook effect. Other mechanisms by which the different E3 ligase-based PROTACs degrade AURKA levels in different degrees and how PROTAC cocktail synergistically degrades AURKA through the ubiquitin-proteasome system warrant further investigations. Theoretically, all PROTAC molecules exhibit the "hook effect" at high concentrations. Considering the differences of drug bioavailability including absorption, metabolism, and excretion, the effective dose range of single PROTACs and cocktail to degrade AURKA varies in different cells. Nevertheless, the cocktail strategy might expand the therapeutic window of PROTACs.

The E3 ligase ligands binding to CRBN, cIAP, VHL, and MDM2 are frequently used to design PROTAC degraders. However, how to choose the E3 ligand rationally is an unsettled issue in PROTAC design. We finally obtained 5 CRBN-based PROTACs, 3 VHL-based PROTACs, and 4 cIAP-based PROTACs with the commonly used E3 ligands[61] and different linkers. Generally, the CRBN-based PROTACs have better degradation activity on AURKA. With similar linker length, CRBN-based dAurA383,
VHL-based dAurA1067, and cIAP-based dAurA1072 exhibit disparate degradation activity, suggesting the predominant role of E3 ligase in PROTAC activity. Besides, the degradation activity of PROTACs increases with the extension of the linker length (from 6 atoms to 15 atoms) in CRBN-based group, which is in line with the published finding that linker length is a critical parameter in PROTAC activity.[34] Meanwhile, all of the reported CRBN-based AURKA PROTACs (dAurA383 with a 12-atom linker, dAurA1071 with a 15-atom linker, PROTAC-D with a 21-atom linker,[34] and JB170 with a 12-atom linker[33]) with more than about 11 atoms can effectively degrade AURKA, which implies when the linker reaches a certain length, the benefit from further linker extension is limited. By summary, the correlation between the expression of E3 ligases and the distribution of target protein in cell cycle deserves the preferential consideration in choosing E3 ligand to design PROTACs. Besides, the physical properties of linkers such as linker length are also crucial for PROTAC activity.

Although all of dAurA383, dAurA425, and dAurA450 effectively bind to AURKA as indicated by the SPR and SPR-IER assays, only the CRBN-based dAurA383 degrades the vast majority of AURKA. By blocking the ubiquitin-proteasome pathway including suppression of NEDD8-activating enzyme E1, knockdown of E3 ligases, and inhibition of proteasome, we proved that dAurA425 and dAurA450 also induce AURKA degradation through the ubiquitin-proteasome system. The distribution of E3 ligases in cell cycle contributes to the inadequate degradation efficiency of dAurA425 and dAurA450. Other reasons for the low efficiency of dAurA425 and dAurA450 such as the “degron” motifs[62] recognized by VHL and cIAP within AURKA need to be further investigated.

Taken together, this study is the first to describe a PROTAC combination strategy to overcome the multifaceted oncogenic functions of AURKA and relieve the hook effect of PROTACs. The concept of the secondary generation PROTAC cocktail is worthy to be tested on more targets in future.

4. Experimental Section

Synthesis of PROTAC dAurA383, dAurA425, and dAurA450. AURKA PROTACs were designed by the laboratory and synthesized from MLN8237 and the derived ligands bound to one of E3 ubiquitin ligases CRBN, VHL, and cIAP. AURKA PROTACs were purified by silica gel column chromatography and resolved in DMSO as a stock solution (10 × 10⁻³ m) at −20 °C. They are abbreviated as dAurA379, dAurA380, dAurA383, dAurA393, dAurA408, dAurA425, dAurA448, dAurA449, dAurA450, dAurA1067, dAurA1071, and dAurA1072 (Figure 1B; Figure S1B, Supporting Information). Their synthesis is exemplified by compounds dAurA383, dAurA425, and dAurA450. The rest PROTACs are synthesized using similar schemes as given in the Supporting Information.

MLN8237 (GLPBI0, Cat# GC12690) and compounds 1-7 were purchased from commercial vendors or prepared following the reported works.[63,64] In this study, all the biologically tested compounds were >95% pure by HPLC analysis. ¹H NMR (400 or 500 MHz) and ¹³C NMR (101 or 125 MHz) spectra were obtained by Bruker Avance spectrometer 400/500. Chemical shifts are given in ppm (δ) that internally referenced to CDCl₃ with 7.26 for ¹H and 77.16 for ¹³C, d₆-DMSO with 2.50 for ¹H and 39.5 for ¹³C, and D₄-MeOD with 3.31 for ¹H and 49.0 for ¹³C. Multiplicity of ¹H NMR signals was reported as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. HPLC analysis was performed on an Agilent 1260 with a C18 column (4.6 mm × 150 mm, 1.8 μm) with the flow rate 1 mL min⁻¹. High-resolution mass spectra were conducted by Thermo Fisher QExactive. The progress of the reactions was monitored by thin-layer chromatography on a glass plate coated with silica gel with fluorescent indicator (GF254). Column chromatography was performed on silica gel (200–300 mesh).

Synthesis of dAurA383: Compound 2 was prepared following the previous report.[63] After cleavage of Boc in compound 2 under 10% TFA solution in dichloromethane, to a solution of the freshly prepared free amine (35.0 mg) in DMF (1.0 mL) was added DIPEA (63 μL), MLN8237 (40.0 mg), and HATU (29.3 mg). The reaction mixture was stirred for 2 h at room temperature. EtOAc was added, and the mixture washed with water three times. The organic layer was dried over MgSO₄, filtered, and...
evaporated in vacuo. The resulting residue was purified by silica column chromatography (MeOH/CH$_2$Cl$_2$ = 1/20) to provide dAurA383 as a yellow solid (39 mg, 52%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 10.59 (s, 1H), 8.62 (brs, 1H), 8.53 (s, 1H), 8.25-8.22 (m, 2H), 8.14 (d, $J = 8.7$ Hz, 1H), 7.69-7.54 (m, 3H), 7.64 (dd, $J = 7.3$ Hz, 1H), 7.34-7.27 (m, 3H), 6.76 (m, 2H), 5.00 (dd, $J = 12.5$, 5.4 Hz, 1H), 4.39-4.31 (m, 2H), 3.77 (s, 3H), 3.69-3.65 (m, 10H), 3.50-3.20 (m, 2H), 2.90 (m, 2H), 2.76 (m, 1H), 2.20-2.09 (m, 1H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 171.7, 170.2, 167.2, 165.9, 159.2, 158.4, 156.5, 156.4, 154.4, 138.1, 136.6, 136.0, 135.3, 133.8, 133.1, 131.1, 130.9, 130.5, 130.3, 123.6, 119.6, 117.2, 116.2, 114.8, 110.7, 108.5, 108.3, 107.1, 101.4, 77.1, 120.8, 70.6, 70.2, 69.7, 69.4, 56.2, 50.4, 49.4, 45.9, 39.5, 31.6, 22.7. HRMS (m/z, ESI) for C$_{48}$H$_{46}$O$_{11}$N$_7$ClF (M$^+$+H$^+$): calcd 950.29224; found 950.29195. HPLC purity: 95.99%.

**Synthesis of dAurA425:** Compounds 4 and 5 were prepared following the previous report.$^{[63]}$ To a solution of 5 (40.0 mg) in DMF (1.0 mL) was added DIPEA (77.0 $\mu$L), 4 (29.4 mg), and HATU (35.3 mg). The reaction solution was stirred for 2 h at room temperature. EtOAc was added, and the mixture washed with water three times. The organic layer was dried over MgSO$_4$, filtered, and evaporated in vacuo. The resulting residue was purified by column chromatography (MeOH/CH$_2$Cl$_2$ = 1/20) to provide WZM421 as a yellow solid (20.0 mg, 34%).

TFA (100 $\mu$L) was added to a solution of WZM421 (20 mg) in dichloromethane (1 mL). The reaction mixture was stirred for 3 h at room temperature, and then it was evaporated in vacuo to provide Boc-cleaved free amine that was used directly for the next step without purification. To a solution of the freshly prepared free amine in DMF (1.0 mL) was added DIPEA (17.2 $\mu$L), MLN8237 (18.0 mg), and HATU (13.2 mg). The reaction solution was stirred for 2 h at room temperature. EtOAc was added, and the mixture washed with water three times. The organic layer was dried over MgSO$_4$, filtered, and evaporated in vacuo. The resulting residue was purified by column chromatography (MeOH/CH$_2$Cl$_2$ = 1/20) to provide dAurA425 as a yellow solid (5.0 mg, 13%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.66 (s, 1H), 8.51 (s, 1H), 8.20 (m, 2H), 8.09 (d, $J = 8.2$ Hz, 1H), 7.88 (s, 1H), 7.57 (d, $J = 8.4$ Hz, 1H), 7.53 (s, 1H), 7.41 (d, $J = 8.4$ Hz, 1H), 7.37-7.27 (m, 6H), 7.05 (d, $J = 7.3$ Hz, 1H), 6.79 (m, 2H), 4.87 (brs, 1H), 4.60-4.45 (m, 4H), 4.07-4.00 (m, 4H), 3.93 (s, 3H), 3.72-3.65 (m, 7H), 3.57 (d, $J = 10.2$ Hz, 1H), 3.23-3.19 (m, 3H), 2.47 (m, 3H), 2.04 (t, $J = 10.6$ Hz, 1H), 1.39 (t, $J = 7.2$ Hz, 3H), 0.92 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 171.2, 171.0, 167.2, 165.9, 159.4, 158.7, 156.8, 150.4, 148.5, 148.0, 144.0, 138.3, 136.2, 135.2, 132.9, 131.8, 131.1, 131.0, 130.1, 130.0, 130.4, 130.30, 129.6, 128.7, 128.3, 124.1, 115.4, 111.0, 107.4, 107.2, 107.2, 101.6, 100.2, 81.6, 71.6, 70.5, 70.5, 70.2, 58.6, 57.2, 56.9, 56.0, 50.5, 43.3, 39.6, 36.2, 35.6, 26.5, 16.2. HRMS (m/z, ESI) for C$_{55}$H$_{60}$O$_{9}$N$_9$ClFS (M$^+$+H$^+$): calcd 1076.39018, found 1076.39003. HPLC purity: 99.73%.

**Synthesis of dAurA450:** DIPEA (34.1 $\mu$L), MLN8237 (40.0 mg), and HATU (29.3 mg) were added to a solution of compound 6 (17.8 mg) in DMF (0.5 mL). The reaction solution was stirred for 2 h at room temperature. EtOAc was added, and the mixture washed with water three times. The organic layer was dried over MgSO$_4$, filtered, and evaporated in vacuo. The resulting residue was purified by column chromatography (MeOH/CH$_2$Cl$_2$ = 1/20) to provide WZM444 as a yellow solid (55.0 mg, 96%).

TFA (100 $\mu$L) was added to a solution of WZM444 (55.0 mg) in dichloromethane (1 mL). The reaction solution was stirred for 3 h at room temperature before it was concentrated in vacuo to provide free amine. To
A solution of the freshly prepared free amine (47.0 mg) in DMF (0.5 mL) was added DIPA (33.0 μL) compound 7 (36.5 mg), and HATU (34.0 mg). The reaction solution was stirred for 2 h at room temperature. EtOAc was added, and the mixture was washed with water three times. The organic layer was dried over MgSO4, filtered, and evaporated in vacuo. The resulting residue was purified by column chromatography (MeOH/CH2Cl2 = 4:3:1). 1H NMR (500 MHz, CDCl3) δ 4.88 (s, 1H), 8.22 (d, J = 8.5 Hz, 1H), 8.14 (d, J = 8.5 Hz, 1H), 7.96 (s, 1H), 7.85 (m, 1H), 7.71 (s, 1H), 7.56 (dd, J = 8.5, 2.0 Hz, 1H), 7.32 (m, 1H), 7.29 (d, J = 6.7 Hz, 1H), 7.24-7.21 (m, 4H), 7.18 (d, J = 6.8 Hz, 1H), 7.14 (d, J = 8.4 Hz, 1H), 6.74 (brs, 2H), 6.52 (s, 1H), 5.88 (d, J = 6.2 Hz, 1H), 5.16 (d, J = 8.4 Hz, 1H), 4.49-4.36 (m, 1H), 4.15-4.00 (m, 2H), 3.98 (s, 3H), 3.42 (m, 3H), 3.24 (m, 2H), 3.11-2.99 (m, 3H), 1.81 (m, 3H), 1.69 (m, 1H), 1.60-1.55 (m, 4H), 1.45-1.42 (m, 3H), 1.36 (s, 9H), 1.30-1.25 (m, 4H), 0.91 (d, J = 6.0 Hz, 3H), 0.88 (s, J = 6.0 Hz, 3H). LCMS (m/z, ESI): 1035.3 (M+H+).

WZM446 (6.20 mg) was added to HCl solution in MeOH (1 mL, 1M), and the reaction solution was stirred for 5 h at room temperature. The solution was evaporated in vacuo to provide dAurA445 as a yellow solid (4.70 mg, 100%). 1H NMR (400 MHz, MeOD) δ 6.84 (s, 1H), 8.42 (d, J = 8.5 Hz, 1H), 8.00 (d, J = 1.7 Hz, 1H), 7.92 (d, J = 8.7 Hz, 1H), 7.83 (d, J = 8.5, 2.1 Hz, 1H), 7.53 (dd, J = 15.2, 8.5 Hz, 1H), 7.40-7.27 (m, 7H), 6.90 (brs, 2H), 4.35 (dd, J = 8.3, 6.4 Hz, 1H), 4.12 (d, J = 3.1 Hz, 1H), 4.01 (s, 3H), 3.78 (m, 1H), 3.40 (t, J = 7.1 Hz, 2H), 3.19-3.08 (m, 3H), 2.92 (m, 1H), 1.65-1.58 (m, 1H), 1.48 (m, 2H), 1.40-1.30 (m, 10H), 0.97 (d, J = 10.8 Hz, 3H), 0.95 (d, J = 10.8 Hz, 3H). HRMS (m/z, ESI) for C51H61O6N8ClF (M+H+): calcd 794.4381, found 793.4382. HPLC purity: 96.88%.

SPPR Assay: The binding of MLN8237, AURKA PROTACs or E3 binding ligands with AURKA was detected by the intermolecular interaction and analysis system Biacore T200. In brief, chemicals and AURKA proteins were prepared. Homemade AURKA protein was previously purified and embedded on a CMS microarray. Chemicals MLN8237, dAurA433, dAurA445, dAurA450, and E3 binding ligands were serial diluted to 50, 25, 12.5, 6.25, 3.12, 1.56 × 10−6 M. After Equipment debugging, the microarray was successively flowed over different concentrations of small molecules MLN8237, AURKA PROTACs or E3 binding ligands to determine the binding kinetics and affinity with AURKA proteins.

Cell Lines and Culture Conditions: Human leukemia cell lines HL60, U937, and NB4 were obtained from the laboratory repository and cultured in Roswell Park Memorial Institute (RPMI) 1640 (C11875500BT, Gibco) containing 10% fetal bovine serum (FBS, 10270106, Gibco). Human leukemia cell lines KG1 and Kasumi-1 were donated by Prof. Zhijie Long (The Third Affiliated Hospital of Sun Yat-sen University) and cultured in RPMI 1640 containing 20% FBS. Human leukemia cell lines THP1 were donated by Prof. Guangming HU (Zhongshan University Cancer Center) and cultured in RPMI 1640 containing 10% heat-inactivated FBS (30 min at 56 °C) and 55 × 10−6 M 2-mercaptoethanol (21985-023, Gibco). Human embryonic kidney cell line HEK293T was from the laboratory repository and cultured in Dulbecco’s modified Eagle medium (C11995500BT, Gibco) containing 10% FBS. Cell lines were cultured in a constant humidity incubator containing 5% CO2 at 37 °C and were mycoplasma-free.

Antibodies: Antibodies against GAPDH (ET1601-4, Huabio), AURKA (144755, Cell Signaling Technology), PARP (9532S, Cell Signaling Technology), Myc (22906, Beyotime), pH3510 (97015, Cell Signaling Technology), H3 (4499, Cell Signaling Technology), Bcl-2 (15071, Cell Signaling Technology), CRBN (AF6564, Beyotime), cIAP1 (GT1X110087, GeneTex), VHL (68547, CST), cyclin E1 (AF2491, Beyotime), cyclin B1 (AF1606, Beyotime), STAT5A (AF2038, Beyotime), NANOG (ab203919, Abcam), FOXM1 (AF6924, Beyotime), AURKB (GTX132702, GeneTex), TACC3 (AF1345, Beyotime), TPX2 (GTX115654, GeneTex), Phospho-TACC3 (Ser558) (AF4506, Affinity Biosciences), and Phospho-Aurora A (Thr288)/Aurora B (Thr232)/Aurora C (Thr198) (29145, CST) were used for the Western blot analysis. PE antihuman CD34 (143506, Biolegend) and Alexa Fluor 700 antihuman/humauc (101222, Biolegend) were used for the flow cytometry analysis. AURKA (144755, Cell Signaling Technology), α-Tubulin (AT819, Beyotime), Alexa Fluor 488 goat antirabbit IgG (H+L) (A10344, Invitrogen), Alexa Fluor 546 goat antimouse IgG (H+L) (A1003, Invitrogen) were used for immunofluorescence assay.
Roche), and incubated at 37 °C for 30 min in the dark. The cells were then washed, resuspended with 200 μL PBS and detected with flow cytometry under the PI/PE channel. The cell cycle phase distribution was analyzed using FlowJo software.

**CFSE Staining Assay**: Briefly, cells were prewashed with CFSE probe (58269, Selleck, 0.5 × 10^{-6} M) in a serum free medium for 15 min, washed with PBS and then suspended and cultured in fresh medium with indicated concentrations of AURKA PROTACs for 72 h. Then, cells were collected, washed, and resuspended in 250 μL PBS. CFSE fluorescence was detected by flow cytometry under the FITC channel. The relative mean fluorescence intensity (MFI) of CFSE were analyzed by CyExpert and FlowJo software.

**Apoptosis Analysis**: The effect of AURKA PROTACs on apoptosis of leukemia cell lines and primary blasts were detected by flow cytometry according to the manufacturer’s protocol with some modifications. Primary blasts were enriched with Ficoll-Hypaque solution (LTS1077, TBD) by density gradient centrifugation and then washed and counted for further treatment. AURKA PROTACs treated leukemia cells or primary blasts were collected, centrifuged at 800 RPM for 5 min, and washed with PBS. For Annexin V assays, Annexin V-PE/PI apoptosis kit (AP006, Yi Shan Biotech) was used. The cells were resuspended in a 100 μL 1X Annexin V binding buffer with 5 μL Annexin V-PE and 10 μL PI and incubated at room temperature for 15 min in the dark. Then, 400 μL 1X Annexin V binding buffer was added into each sample and Annexin V positive cells were detected by flow cytometry. The results were analyzed by CyExpert software.

**Methycellulose-Based Colony Forming Cell Assay**: The clonogenic activity of leukemia stem cells was determined by methycellulose-based CFC assay. For the primary colony formation, leukemia cells were pretreated with indicated concentration of AURKA PROTACs or DMSO for 12 h and then counted and uniformly resuspended in a 250 μL cell culture medium containing twice the concentration of indicated drugs at a density of 500 cells per well. The mixture was then mixed with an equal volume of methyl cellulose (M0512, Sigma) and inoculated into 24-well cell culture plates to culture. After 14 days, images were acquired with microscope, and the number and size of colonies formed in each treatment were counted. The secondary colony formation was conducted with the same procedure using cells collected from primary colonies.

**Western Blot Analysis**: PROTACs, E3 ligands, MLN8237 (S1133, Selleck), MLN4924 (B1036-5, APEXBio), and Bortezomib (T2399, Target-Mol) treated samples as well as released synchronized cells were subject for immunoblot analysis. Whole cell lysates were extracted using Ficoll-Hypaque solution (LTS1077, TBD) by density gradient centrifugation and then washed and counted for further treatment. AURKA PROTACs treated leukemia cells or primary blasts were collected, centrifuged at 800 RPM for 5 min, and washed with PBS. For Annexin V assays, Annexin V-PE/PI apoptosis kit (AP006, Yi Shan Biotech) was used. The cells were resuspended in a 100 μL 1X Annexin V binding buffer with 5 μL Annexin V-PE and 10 μL PI and incubated at room temperature for 15 min in the dark. Then, 400 μL 1X Annexin V binding buffer was added into each sample and Annexin V positive cells were detected by flow cytometry. The results were analyzed by CyExpert software.

**Real-Time PCR**: Total RNA was extracted from leukemia cells using the HiPure Total RNA Plus Mini Kit (R4121-02, Magen). Using total RNA as a template, reverse transcription was conducted and cDNA was synthesized with TransScript All-in-one first-strand cDNA Synthesis SuperMix for qPCR (AT341-01, TransGen). According to the instructions of ChamQ Universal SYBR qPCR Master Mix (Q311-03, Vazyme), qPCR was performed using a Bio-RAD CFX96 fluorescence quantitative PCR instrument and the relative expression of the interested genes was calculated by the 2^-ΔΔCt method. Primers were designed according to the PrimerBank website (https://pga.mgh.harvard.edu/primerbank/). qPCR primers are shown in Table S4 of the Supporting Information.

**Evaluation of Drug Interactions–Combination Index (CI) Calculation**: The CompuSyn software (CompuSyn, Paramus, NJ, USA) was used to evaluate the CI of cells that responded to the drugs in combination. In brief, the percentage of restrained cells upon a series concentration of single PROTACs or cocktail was acquired from the CCK-8 assay and subjected to CompuSyn software. CI values below 0.8, between 0.8 and 1.2, and over 1.2 were defined as synergistic, additive, and antagonistic, respectively. The relative cell survival and CI values of each treatment were plotted.

**Xenograft Mouse Model**: BALB/c nude mice (female, 6 weeks old) were used to evaluate the effects of AURKA PROTACs in vivo. Wild-type KG1A cells were resuspended in PBS with a density of 5 × 10^4/100 μL and subcutaneously inoculated into the right flank of the mice. After 6 days, dAurA383, dAurA450, PROTAC cocktail, and MLN8237 were intraperitoneally injected with an equal-molar dosage of 30 μmol kg^{-1} day^{-1} (28.51 mg kg^{-1} day^{-1} for dAurA383, 29.16 mg kg^{-1} day^{-1} for dAurA450, and 15.57 mg kg^{-1} day^{-1} for MLN8237) in a 100 μL vehicle (DMSO 10%, PEG300 40%, Tween-80 5%, saline 45%). The long axis (A) and short axis (B) of the tumor were measured every 2 days, the tumor volume V = A × B × 1/2 was calculated, and the time–volume curve of tumor growth was plotted. After 2 weeks, blood was sampled from the mice’s eyes for ALT, AST, and BUN testing. Then the mice were sacrificed and the tumors were dissected, weighted, and digested into single cells for flow cytometry and Western blot analysis.

**Cell Surface CD34 and CD11b Analysis**: An equal number of cells were used for cell surface CD34 and/or CD11b staining together with the dead cell marker PI. For immunofluorescence, AURKA PROTACs, and ATRA (A9120, Solarbio) treated leukemia cells from the in vitro and in vivo assays were collected, counted, and washed with PBS. Subsequently, cells were resuspended and stained in a 100 μL staining buffer containing 1:100 diluted PE anti-human CD34 (343056, Biolegend) and/or 1:100 diluted Alexa Fluor 700 anti-human CD11b (101222, Biolegend) together with 1:500 diluted Zolimbi in the dark for 30 min at room temperature. After being washed with PBS, the cells were subjected to flow cytometry, while the relative cell surface CD34 and CD11b expression (MFI) were analyzed by CyExpert and FlowJo software.

**RNA Sequencing and Analysis**: The AURKA PROTACs and DMSO treated cells were used for total RNA extraction with the HiPure Total RNA Plus Mini Kit and sent to the Novogene company. All samples prepared for the RNA-SEQ library had RIN values greater than 9.5. An Illumina HiSeq2000 (150 bp, paired-end) was used for sequencing. RNA-seq data were analyzed using the RINACocktail toolkit. GSEA was executed with the GSEA algorithm.

**Mass Spectrum and Analysis**: The AURKA PROTACs and DMSO treated cells were collected, lyzed, and quantified. An equal amount of peptide from each treatment was used for TMT labeling as DMSO-TMT-126, dAurA383-TMT-127, dAurA425-TMT-128, dAurA450-TMT-129, and cocktail-TMT-131. Labeled samples were mixed together and subjected to mass spectrum identification. Data were analyzed with the MaxQuant software and protein abundance was compared in the AURKA PROTACs.
treated versus DMSO treated cells. The top 100 decreased proteins were subjected to gProfiler to execute GO analysis.

**ALDEFLUOR Assays:** ALDH positive leukemia cells were analyzed with the ALDEFLUOR Stem Cell Identification Kit (01700, Stemcell) according to the manufacturer’s protocol. In brief, leukemia cells were pretreated with the indicated concentration of AURKA PROTACs or DMSO for 2 days. Subsequently, cells were collected, washed, supplied with a 5 μL activated ALDEFLUOR substrate reagent and divided into two tubes as either the experimental group or the inactivated control group. The inactivated control group was further supplied with 5 μL ALDEFLUOR DEAB. All samples were incubated at 37 °C for 40 min in dark, then centrifuged and resuspended in 500 μL ALDEFLUOR buffer and subjected to flow cytometry. The proportion of ALDH positive cells was analyzed with DEAB treated group as control.

**Data Analysis:** The top 100 decreased proteins were analyzed with CyExperts software.

**Study Approval:** All animal studies and human subject research were approved by the ethical committee of Sun Yat-sen University Cancer Center (IRB Approval Nos. L02021017222R and GZ2020-041).

**Statistical Analysis and Data Availability:** Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc., LaJolla, CA, USA). Unpaired Student’s t-test was used to perform statistical analysis between the two groups. One-Way ANOVA was used for comparisons between the two groups in multiple groups. The level of significance was set at a $p < 0.05$, $p < 0.01$, and $p < 0.001$. The data generated during and/or analyzed in this current study are available from the corresponding author upon reasonable request. The raw data for RNA-Seq are available in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics) database with the accession number: HRA001384. For manuscript reviewing, the shared URL is https://ngdc.cnbc.ac.cn/gsa-human/s/7F76qzm9 (temporary).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Author Contributions**

F.L., X.W., J.L.D., Z.J.H., Z.M.W., and L.L.L. contributed equally to this work. Conceptualization: Z.F.W., S.J.W., Q.L., and F.L.; Methodology, F.L., Z.F.W., X.W., J.L.D., Z.J.H., Z.M.W., L.L.L., H.Q.L., D.H., Y.F.R., Y.W., X.Y.L., J.X.Z., Z.J.Z., B.H., M.Y., H.M.Y. and L.H.Z.; Investigation, Q.L., Z.F.W., S.J.W. and J.S.Y.; Software, Z.F.W. and F.L.; Data Analysis, Z.F.W. and Q.L.; Writing-Review & Editing, Z.F.W., F.L., Q.L., and S.J.W.; Supervision, Q.L. and Z.F.W.; Funding Acquisition, L.Q., S.J.W., Z.F.W. and M.Y.

**Data Availability Statement**

The data that support the findings of this study are openly available in Genome Sequence Archive at https://ngdc.cnbc.ac.cn/gsa-human/s/7F76qmzr, reference number 1384.

**Keywords**

acute myeloid leukemia stem cells, Aurora kinase A (AURKA), PROTAC cocktail, E3 ubiquitin ligase

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