Multifunctional nanoparticle-mediated SHARP1 knockdown in MLL-AF6 acute myeloid leukemia

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**Abstract**

Acute myeloid leukemia (AML) has an extremely poor prognosis and high relapse and fatality rates. We targeted *SHARP1* using multifunctional small interfering RNA (siRNA) and bortezomib (BTZ)-loaded cRGD-guided PEGylated cationic liposomal nanoparticles to monitor their antileukemic activity in MLL-AF6 AML cells. Efficient siRNA/BTZ co-delivery by the nanoparticles significantly inhibited cell viability, decreasing clonogenic growth of AML cells and stimulating robust apoptosis. We hypothesized that *SHARP1* downregulation induced nonfunctional MLL-AF6, DOT1L, MEN1, and LEDGF fusion protein accumulation, preventing MLL-AF complex formation and downregulating RAS-GTP and Bcl-2, consequently triggering autophagy and apoptosis. The BTZ combination substantially augmented therapeutic synergy leading to enhanced autophagic and apoptotic events. Our findings demonstrate a state-of-the-art biodegradable nanoplatform for siRNA/BTZ co-delivery with targeted *SHARP1* knockdown, demonstrating a potential therapeutic option for MLL-AF6 AML.

**Main Text**

Acute myeloid leukemia (AML) is a heterogeneous, aggressive blood cancer caused by unusual differentiation and proliferation of myeloid blasts beginning at the spleen, peripheral blood, and bone marrow. AML survival rates are poor in elderly and pediatric patients with multiple mutations, and patients generally experience high relapse rates. *MLL* (mixed lineage leukemia; chromosome 11q23) rearrangements are common chromosomal aberrations correlated with AML. *MLL* translocation results in *MLL*-FP complex production, wherein the genomic portion encoding the amino-terminus of *MLL* fuses to the carboxyl-terminal of a group of fusion partner proteins, leading to distinctive leukemogenic transcriptional machinery. *MEN1, LEDGF, AF4*, and over 70 partner genes of *MLL* have been characterized in MLL-rearranged (MLLr) AML. *MLL-AF6* is the most common leukemogenic MLL fusion protein, recruiting both elongation-assisting protein (EAP) and distributor of telomeric silencing 1-like (DOT-1L) complexes.

Identifying therapeutic mechanisms for molecular targeting and effective targeted delivery are crucial for developing a much-needed AML cure. It is known that *SHARP1* is a circadian clock transcription factor that is implicated in the regulation of several cancer types such as breast, colon, and thyroid carcinomas. However, so far, it has not been adequately studied regarding AML regulation. Recently, a study has demonstrated the crucial function of *SHARP1* in MLL-AF6 AML survival, which is greatly regulated via MLL-AF6/DOT1L activity. However, the oncogenic role of *SHARP1* in MLL-AF6 AML growth and maintenance is still unclear. In this study, *SHARP1* knockout in ML-2 cells (specifically MLL-AF6 AML cells, which are more sensitive to *SHARP1*) was investigated to determine *SHARP1* role in stimulating autophagy and apoptosis and to define *SHARP1*-knockdown therapeutics for MLL-AF6 AML. Multifunctional biodegradable lipid nanoparticles engineered to efficiently co-deliver siRNA and bortezomib (BTZ) have ameliorated most challenges of conventional therapeutics. Furthermore, BTZ
combined with proteasome inhibition disrupted key regulatory signaling pathways through several mechanisms, including suppression of nuclear factor-κB (NF-κB) and stimulation of apoptosis in multiple myeloma\textsuperscript{11}, chronic lymphocytic leukemia\textsuperscript{12}, and non-small cell lung cancer\textsuperscript{13}. BTZ induced cleavage of myeloid cell leukemia 1 protein (Mcl-1) and stabilization of Bax, p53, and c-Jun N-terminal kinase (JNK) overexpression, leading to ER stress-dependent cell death\textsuperscript{14,15}.

Incorporating an inert polymer, polyethylene glycol (PEG), reinforces surface functionalization of cationic nanoliposome-siRNA complexes (lipoplexes), forming sterically stabilized nanoparticles\textsuperscript{16,17}. For selective targeting, surface ligands such as cyclic RGD (arginine-glycine-aspartate), which specifically binds with high affinity to αvβ3 integrin, promotes cell–nanomaterial interactions, allowing targeted cargo penetration\textsuperscript{18}. An RGD(d-Phe)(Lys(PEG-Mal)) sequence was synthesized by linking c(RGDfK) to thiolated PEG (NHS-PEG6-maleimide) with head-to-tail cyclic modification, conferring a bioengineered surface that allows optimal nanostructure functionalization.

Here, we exhibit the first preclinical experimental research for \textit{SHARP1}-based AML therapy using therapeutic siRNA nanodelivery (Fig. 1a,b). We show that \textit{SHARP1} is an MLL-AF6-dependent leukemogenic driver, consistent with multifunctional bioengineered nanoparticle activity in \textit{SHARP1} downregulation, revealing a potential approach for human MLL-AF6 AML treatment.

Transmission electron microscopy (TEM) images (Fig. 1c) revealed that the prepared nanoparticles were spherical with regular structure and configuration. Scanning electron microscopy (SEM) also revealed Lipo-siRNA-BTZ-PEG-cRGD uniform spherical shape and narrow size distribution (Fig. 1d). The average hydrodynamic diameters of Lipo-siRNA-BTZ, Lipo-BTZ-PEG-cRGD, and Lipo-siRNA-BTZ-PEG-cRGD were 78.9 ± 13.8 nm, 101.1 ± 15.2 nm, and 138.6 ± 18.5 nm (Fig. 1e), respectively, as estimated by dynamic light scattering. The zeta potentials of the nanoparticles were 26.6 ± 2.1 mV, 13.1 ± 1.1 mV, and 24 ± 1.9 mV, respectively, indicating that the negative charges of siRNA in the nanoformulation are masked (Supplementary Fig. 1 and Supplementary Table 1). These results showed that therapeutic siRNA was encapsulated in the nanoparticles, without bulky aggregation or degradation.

We used confocal microscopy to evaluate Lipo-siRNA-BTZ-PEG-cRGD uptake by ML-2 cells (see Supplementary Fig. 2a). ML-2 cells were transfected with ATTO 550 red-labeled Lipo-siRNA-BTZ-PEG-cRGD, which was observed in the cytoplasm after 4 h of incubation. The comprehensive cellular uptake study showed aggregated and dense ATTO 550 red-labeled Lipo-siRNA-BTZ-PEG-cRGD surrounding the nucleus. Moreover, 3D images (Supplementary Fig. 2b) revealed a noteworthy boost of ATTO 550 red-labeled Lipo-siRNA-BTZ-PEG-cRGD. Flow cytometry with fluorescence intensity quantification further confirmed the intracellular uptake efficiency of Lipo-siRNA-BTZ-PEG-cRGD (Supplementary Fig. 3 and 4). Furthermore, the visible colocalization between cell nuclei (blue) and ATTO 550-labeled Lipo-siRNA-BTZ-PEG-cRGD (red) showed effective nanoparticle transfection in ML-2 cells (Supplementary Fig. 2c and Supplementary Fig. 5). Notably, lipid bilayer fusion, endocytosis, drug conjugation, and facilitated diffusion of a lipofectamine-based formulation\textsuperscript{19} with further surface modification by PEGylation allowed targeting of the αvβ3 integrin ligand\textsuperscript{20}. These mechanisms were used to allow Lipo-siRNA-BTZ-PEG-cRGD
to deliver cargo to ML-2 cells, elucidating how multifunctional bioengineered nanoparticles improve therapeutic efficiency and safety by optimizing delivery in MLL-AF6 AML. Naked siRNA cannot passively diffuse across cell membranes owing to charge instability, high molecular weight, water solubility, and intracellular enzyme degradation\textsuperscript{21}. Peptide-guided PEGylated cationic nanoliposomes are effective for siRNA delivery\textsuperscript{22}; we showed this strategy is effective for \textit{SHARP1} targeting. Consistently, our cellular uptake results demonstrated that Lipo-siRNA-BTZ-PEG-cRGD undergoes αvβ3 receptor-mediated endocytosis.

To elucidate \textit{SHARP1} inhibition in MLL-AF6 AML cells, naked siRNA and the nanoparticles were compared with a control during knockdown experiments. Western blotting, qPCR, and immunofluorescence confirmed knockdown efficiency, displaying that nanoparticle-mediated siRNA delivery significantly reduced \textit{SHARP1} expression. \textit{SHARP1} protein expression was significantly downregulated for cells treated with Lipo-siRNA-BTZ and Lipo-siRNA-BTZ-PEG-cRGD (Fig. 2a,b). We observed 60% and 95% of \textit{SHARP1} suppression by Lipo-siRNA-BTZ and Lipo-siRNA-BTZ-PEG-cRGD nanoparticles ($p < 0.05$), respectively. This suppression was further evidenced by our results on mRNA-level \textit{SHARP1} expression in ML-2 cells (Fig. 2c). Lipo-siRNA-BTZ-PEG-cRGD caused the highest \textit{SHARP1} downregulation: approximately 80% lower expression than that in other treatments. However, we observed no significant change ($p < 0.05$) in \textit{SHARP1} expression level in naked siRNA- or Lipo-BTZ-PEG-cRGD-treated cells. We further assessed the antileukemic activity of naked siRNA and nanoparticles with viability and colony formation assays. Immunofluorescence results (Supplementary Fig. 6 and 7) confirmed successful \textit{SHARP1} silencing in Lipo-siRNA-BTZ-PEG-cRGD-treated cells relative to the control; we observed remarkable reductions in the number of anti-\textit{SHARP1}-positive cells in each group upon Lipo-siRNA-BTZ-PEG-cRGD nanoparticle treatment. Immunofluorescence analysis of anti-\textit{SHARP1}-positive cells showed that Lipo-siRNA-BTZ-PEG-cRGD nanoparticles remarkably suppressed \textit{SHARP1} in ML-2 cells, curbing MLL-AF6 leukemogenicity. Cytotoxicity assays revealed that Lipo-siRNA-BTZ, Lipo-BTZ-PEG-cRGD, and Lipo-siRNA-BTZ-PEG-cRGD nanoparticle treatment considerably reduced survival rates of ML-2 cells by 68%, 75%, and 50%, respectively, compared to those of untreated cells ($p < 0.001$) (Fig. 2d). For colony formation assays (Fig. 2e), Lipo-siRNA-BTZ-PEG-cRGD treatment correlated with the lowest average colony numbers: ~70% reduction than that found in the control ($p < 0.001$). The relative number of colonies formed under Lipo-siRNA-BTZ, Lipo-BTZ-PEG-cRGD, and Lipo-siRNA-BTZ-PEG-cRGD were 310 ± 15, 346 ± 13, and 171 ± 15, respectively. Lipo-siRNA-BTZ-PEG-cRGD nanoparticles showed the highest reduction of viable cells and the lowest relative clonogenic growth, promoting the antileukemic effect of \textit{SHARP1} silencing with proteasome inhibition by targeted siRNA/BTZ co-delivery. Numata \textit{et al.} also reported the oncogenic role of \textit{SHARP1} in MLL-AF6 leukemogenesis\textsuperscript{8}. After demonstrating the cytotoxicity of nanoparticles in MLL-AF6 AML cells, we assessed their therapeutic activity on cell apoptosis. Fluorescence microscopic images and flow cytometry plots revealed robust ML-2 cell apoptosis in Lipo-siRNA-BTZ- and Lipo-siRNA-BTZ-PEG-cRGD-treated cells relative to control, naked siRNA-, and Lipo-BTZ-PEG-cRGD-treated cells (Fig. 2f–h). Collectively, the cell apoptosis study illustrated that Lipo-siRNA-BTZ-PEG-cRGD induced the earliest and latest apoptosis. Live imaging showed cell number and growth reductions in Lipo-siRNA-BTZ-PEG-cRGD-treated cells relative to untreated cells.
(Supplementary Fig. 8, 9 and Supplementary Movie 1, 2). These findings corroborate reports of the protective role of \textit{SHARP1} against p53 pathway-mediated DNA-targeting chemotherapeutic cytotoxicity\textsuperscript{23}. \textit{SHARP1} knockdown led to p53 pathway-related gene overexpression and ML-2 cell apoptosis. Combination with BTZ inhibits proteasomes, selectively blocking NF-κB target gene expression and activating and stabilizing p21, p27, and p53 signaling\textsuperscript{24,25}. These effects suppress cell proliferation and migration and augment synergistic nanoparticle-induced apoptosis.

To investigate the roles of \textit{SHARP1} downregulation-induced autophagy in MLL-AF6 AML, we studied the mRNA expression of MLL-AF6 regulation and DOT1L oncogenic genes affected by \textit{SHARP1} knockdown in ML-2 cells. Mechanistically, DOT1L and MLL-AF6 are oncogenic proteins that directly regulate \textit{SHARP1} expression. It has been reported that downregulation of \textit{SHARP1} does not affect the expression of DOT1L gene\textsuperscript{8}. Therefore, it was suggested that \textit{SHARP1} together with the inhibition of DOT1L may be a favorable treatment modality for MLL-AF6 AML\textsuperscript{9}, since it is known that downregulation of DOT1L can inhibit the regulation of MLL-AF6 AML\textsuperscript{26,27}. However, so far, there are no reports regarding the downregulation of \textit{SHARP1} affecting DOT1L and MLL-AF6 regulation. Here, we hypothesized that DOT1L/MLL-AF6 expression would be suppressed by multifunctional \textit{SHARP1}-targeted Lipo-siRNA-BTZ-PEG-cRGD in MLL-AF6 AML cells. The mRNA expression of DOT1L and MLL-AF6 were profoundly downregulated in Lipo-siRNA-BTZ- and Lipo-siRNA-BTZ-PEG-cRGD-treated cells, while those treated with naked siRNA and Lipo-BTZ-PEG-cRGD were not affected at \( p < 0.05 \) (Fig. 3a,b). Lipo-siRNA-BTZ-PEG-cRGD inhibited DOT1L and MLL-AF6 the most (approximately 80% and 50%, respectively), emphasizing induction of autophagy and indicating a new oncogenic role of \textit{SHARP1} in MLL-AF6 and DOT1L functions in MLL-AF6 AML cells. The MLL-AF6-driven transcriptional machinery correlating with \textit{SHARP1}-related genes for MLL-AF6 AML growth and maintenance are shown in Fig. 3c. Therefore, three fundamental regulatory mechanisms initiated the autophagic signals that eradicated MLL-AF6 AML cells. First, BTZ combination disrupted the ubiquitin-proteasome signaling pathway, causing accumulation of unfolded ubiquitin, triggering autophagy\textsuperscript{28}. Second, selective \textit{SHARP1} inhibition, which results in the breakdown of oncogenic fusion proteins responsible for DOT1L-dependent MLL-AF6-MEN1-LEDGF complex synthesis crucial for leukemogenesis, was significantly abrogated, strengthening autophagy\textsuperscript{29}. Eventually, MLL-AF6 downregulation resulted in considerable RAS-GTP pathway interference. RAS-GTP pathway is necessary for promoting cancer cell survival through binding RAS-GTP selectively to RALB-GTP for overexpressing Bcl-2\textsuperscript{30}. Our nanoparticles primarily interfered with this important oncogenic pathway, leading to autophagic signals. Thus, Lipo-siRNA-BTZ-PEG-cRGD nanoparticles may be RAS inhibitors, potentiating MLL-AF6 AML therapy. Based on these findings, we plotted an ML-2 cell growth curve (Supplementary Fig. 10) to measure cell proliferation, which was strikingly attenuated upon Lipo-siRNA-BTZ-PEG-cRGD treatment. Manipulating autophagy may provide powerful evidence for the effect of Lipo-siRNA-BTZ-PEG-cRGD as a multifunctional targeted therapy in MLL-AF6 AML cells. We provided a comprehensive \textit{in vitro} study on new multifunctional bioengineered smart nanoparticles using well-characterized cRGD-conjugated thiolated PEG (NHS-PEG6-maleimide) to effectively co-deliver siRNA/BTZ for targeted \textit{SHARP1} silencing in MLL-AF6 AML cells. These findings indicate that animal experiments may provide useful data for the development of nanomedicines for AML treatment. We demonstrated the
effects of SHARP1 downregulation on DOT1L and MLL-AF6 expression levels and highlighted a new vital oncogenic role of SHARP1 in MLL-AF6 AML growth and maintenance. Lipo-siRNA-BTZ-PEG-cRGD are multifunctional particles that reveal versatile regulatory mechanisms, including SHARP1 silencing, MLL-AF6/DOT1L inhibition, p53 activation, RAS suppression, proteasome inhibition, and autophagy/apoptosis induction. This approach should open new avenues for applying smart biocompatible re-engineered nanostructures in in vivo studies and further clinical translation to produce advanced MLL-AF6 AML-targeting therapeutics.

Methods

Preparation of nanoparticles

The siRNA duplex solution was prepared at 200 µM (3 mg/mL) and gently mixed with complexation buffer at 1:1 mol ratio for use as diluted siRNA (naked siRNA) with invivofectamine 3.0/lipofectamine RNAiMAX reagents (Invitrogen, Carlsbad, CA, USA), which were vortex dispersed immediately with 3 mM BTZ (EMD Millipore, St. Louis, MO, USA) diluted in Opti-MEM medium (Gibco, Waltham, MA, USA) just before use. The complex was incubated for 30 min at 50 °C, sonicated for 1 h, centrifuged at 500 × g and 25 °C for 5 min, and washed three times with deionized water to collect siRNA/BTZ-loaded cationic liposomal nanoparticles (Lipo-BTZ-siRNA). To fabricate siRNA/BTZ-loaded cRGD-guided PEGylated cationic liposomal nanoparticles (Lipo-siRNA-BTZ-PEG-cRGD), c(RGDFK) linked with thiolated PEG (NHS-PEG6-maleimide) associated with head-to-tail cyclic modification for cell surface αvβ3 receptor targeting to form the RGD{d-Phe}{Lys(PEG-Mal)} sequence construct, which was purchased from GenScript (peptide-081102 ID: J7777DK130; Piscataway, NJ, USA). This was dissolved in PBS solution (pH 7.4) and stored as a stock. Subsequently, 100 µL of this stock solution was added to previously prepared lipid nanoparticles containing siRNA and BTZ. The resultant mixture was continuously stirred for 24 h at 25 °C in the dark, and the nanoparticles were finally recovered by centrifugation at 100 × g and 25 °C for 10 min and washed three times with deionized water. The siRNA/BTZ-loaded cRGD-mediated PEGylated cationic nanoliposomes (Lipo-BTZ-PEG-cRGD) were formulated using the same protocol without the addition of diluted siRNA solution. Fluorescently labeled Lipo-siRNA-BTZ-PEG-cRGD nanoparticles were also prepared using the same protocol with siRNA conjugated to red fluorescent dye ATTO 550 (Sigma-Aldrich, St. Louis, MO, USA) solution. Briefly, 20 µL of siRNA duplex solution was mixed with 60 µL of DNase/RNase-free water (Invitrogen), 10 µL of binding solution (Thermo Fisher Scientific, Waltham, MA, USA), and 10 µL of ATTO 550 solution to obtain 100 µL of the final complex.

Characterization

The sizes and zeta potentials of different treatment nanostructures were measured by dynamic light scattering using a Malvern Nano ZS90 Zetasizer (Malvern Instruments, Malvern, UK). Transmission electron microscopy (TEM) was performed using a 1200 EX transmission electron microscope (JEOL Ltd., Akishima, Japan). The morphologies of siRNA/BTZ-loaded cRGD-tagged PEGylated cationic nanoliposomes (Lipo-siRNA-BTZ-PEG-cRGD) were studied by scanning electron microscopy (SEM) using
a JSM-7200F scanning electron microscope (JEOL Ltd.). Nanoparticle characterization by DLS measurements showed optimal physicochemical properties evidenced by hydrodynamic diameter and zeta potential values. TEM findings indicated efficient siRNA encapsulation by the PEGylated modified surface conjugated with the targeted cRGD ligand. Furthermore, the narrow size distribution and regular spherical structure of Lipo-siRNA-BTZ-PEG-cRGD demonstrated by SEM indicated assembly and morphology.

**Cell culture**

The current study was performed on a human ML-2 cell line (ACC 15) obtained from DSMZ (Braunschweig, Germany). The cells were cultured in RPMI-1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and maintained at 37 °C in a 5% CO₂ atmosphere.

**Delivery study**

Silencer Select pre-designed siRNA targeting SHARP1 was obtained from Invitrogen (antisense sequence, 5′-UAUACAAAGAGGAAUAGUCCA-3′; sense sequence, 5′-GACUAUUCCUUUUGUAUATT-3′). ML-2 cells were transfected with naked siRNA and the indicated structured nanoparticles and investigated for SHARP1 knockdown efficiency by western blotting and qPCR at 2 d or 3 d post transfection.

**Intracellular uptake**

ML-2 cells were seeded in 35-mm glass-bottom dishes (Corning) (4 × 10⁴ cells per well) 1 d prior to transfection. Lipo-siRNA-BTZ-PEG-cRGD nanoparticles were prepared with ATTO-550 (Sigma-Aldrich) fluorophore (red) was used to track intracellular location of nanoparticles. After a 4-h incubation at 37 °C, cells were fixed with 4% paraformaldehyde in PBS (Sigma-Aldrich) for 15 min and directly stained with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) and Concanavalin A-FITC (Sigma-Aldrich) for nuclei (blue) and cell membrane (green) labeling, respectively. Cells were imaged using a TiE-A1R confocal laser scanning microscope (Nikon, Tokyo, Japan). The Z series images and 3D snapshots of cells were taken using Ni-E Z Drive for Z-stack mode measurements. Image data were analyzed using Nikon imaging software (NIS-Elements Viewer 4.50). For flow cytometric analysis, ML-2 cells were seeded in 96-well plates and harvested after 4 h using ATTO-550 (Sigma-Aldrich) fluorophore (red), followed by flow cytometry on FACS Canto II (BD Biosciences, Franklin, NJ, USA).

**Western blot analysis**

Cells were treated with naked siRNA or the indicated treatments for 3 d, and further cultured for 2 d before lysis. The cells were suspended in a radioimmunoprecipitation (RIPA) lysis buffer (Santa Cruz Biotechnology, Dallas, TX, USA) for whole cell lysis. Proteins were separated by SDS-PAGE and blotted onto PVD membrane (Millipore). Images were captured and chemiluminescent signals were analyzed using ImageQuant LAS 4010 (GE Healthcare, Chicago, IL, USA). Western blot experiments were performed using the following antibodies: anti-SHARP1 (sc-373763, 1:1000 working dilution, overnight shaking.
incubation at 4 °C), Cruz Marker molecular weight standards (sc-2035), and β-actin (sc-47778, 1:5000 working dilution, overnight shaking incubation at 4 °C) from Santa Cruz Biotechnology and a secondary horseradish peroxidase (HRP)-conjugated antibody from Abcam (ab205718, 1:2000 working dilution, incubation at 25 °C for 1 h).

Quantitative PCR

RNA was extracted using an RNeasy kit (QIAGEN, Hilden, Germany) and reverse-transcribed using the QuantiTech Reverse Transcription kit (QIAGEN). PCR was performed using SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich) and quantitatively assessed on a Mx3000P (Agilent Technologies). For each sample, transcript levels of tested genes were normalized to GAPDH using the $2^{-\Delta\Delta CT}$ method. The highest expression was arbitrarily set to 1 and expressions in the other samples were normalized to this value. All experiments were performed in triplicate. PCR was performed using cDNA and primer sequences listed in Supplementary Table 2.

Cytotoxicity assay and colony forming assay

For cytotoxicity assay, 1 × 10^4 cells were seeded in 96-well plates one day prior to transfection, incubated at 37 °C for 2 d, and, on the third day, subjected to CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Colony formation assay was performed by plating 1,000 cells per well on a 6-well plate, transfected, and incubated for 7 d. The colonies were fixed with methanol:acetic acid 3:1 (v/v) and stained with 0.5% crystal violet in 20% methanol for 15 min.

Cell apoptosis study

Cell apoptosis was evaluated using an Annexin V-FITC apoptosis detection kit following the manufacturer’s protocol (Thermo Fisher Scientific) and analyzed with a FACS Cantoll flow cytometer (BD Biosciences). For fluorescence imaging, ML-2 cells were seeded into 35-mm glass-bottom dishes (Iwaki AGC Techno Glass, Japan) and treated with naked siRNA and the indicated nanoparticles for 24 h. After two washes with PBS, the cells were stained with 5 µL of Annexin V-FITC and PI and incubated at 25 °C in the dark for 15 min. For nuclei staining, stained cells were counterstained with 4,6-Diamidino-2-phenylindole (DAPI; D1306, Invitrogen) and visualized using an inverted fluorescence microscope (Keyence BZ-9000, Osaka, Japan).

Immunofluorescence and image analysis

For immunofluorescence (IF) staining, ML-2 cells were treated with Lipo-siRNA-BTZ-PEG-cRGD nanoparticles, fixed with 4% paraformaldehyde for 20 min, and permeabilized in PBS containing 0.1% Tween 20 (9809S, Cell Signaling Technology, Danvers, MA, USA) for 10 min. For the immune reaction, cancer cells were incubated in a solution containing the primary antibody, anti-SHARP1 (sc-373763, 1:200, Santa Cruz Biotechnology), in staining buffer overnight at 4 °C. Cells were washed in PBS and then
incubated with a goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor 568 (Thermo Fisher Scientific; A-11036, 4 µg/mL) in staining buffer for 1 h. For nuclei staining, stained cells were counterstained with DAPI (D1306, Invitrogen) for 1 h before visualization by TiE-A1R confocal laser scanning microscope (Nikon). Image data were analyzed by Nikon imaging software (NIS-Elements Viewer 4.50), and the number of anti-SHARP1-positive cells was counted using MATLAB R2020b software (MathWorks, Natick, MA, USA).

**Live cell imaging and cell growth assay**

For phase contrast, ML-2 cells were seeded into 96-well plates (~40,000 cells/well), incubated for 4 h, and then were treated with Lipo-siRNA-BTZ-PEG-cRGD. After washing twice with PBS, the cells were suspended in fresh media and monitored using the IncuCyte ZOOM (Essen BioScience, Ann Arbor, MI, USA) acquiring images at 30 min intervals for 48 h. For the fluorescence-based technique, cells were seeded into 96-well plates (~40,000 cells/well), incubated for 4 h, and then treated with Lipo-siRNA-BTZ-PEG-cRGD. After two PBS washes, the cells were stained with IncuCyte NucLight Rapid Red Reagent for nuclear labeling (Essen Bioscience) and monitored using the IncuCyte ZOOM (Essen BioScience) acquiring images at 1 h intervals for 48 h. To assess cell growth, transfected cells were seeded in 96-well plate (5,000 cells/well) and imaged every 1 h using IncuCyte ZOOM (Essen Bioscience). The confluence was analyzed by the IncuCyte ZOOM 2016A software (Essen Bioscience).

**Statistical analysis**

The statistical significances were analyzed by Student's t-test and ANOVA using JMP Pro 15 software (SAS Institute Inc., Cary, NY, USA), and the data are presented as the mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001.

**Declarations**

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**Author Contributions:**

Conceptualization: SAM, YJ.

Methodology: SAM.

Investigation: YJ.

Writing – original draft: SAM.
Writing – review & editing: YJ.

Visualization: SAM.

Funding acquisition: SAM, YJ.

Supervision: YJ.

**Competing Interests:** The Authors Declare That They Have No Competing Interests.

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Supplementary Materials

Supplementary materials were not provided with this version of the manuscript.

Figures

Figure 1
Synthesis and characterization of therapeutic nanoparticles for efficient delivery.  
a, A schematic illustration of naked siRNA, Lipo-siRNA-BTZ, Lipo-BTZ-PEG-cRGD and Lipo-siRNA-BTZ-PEG-cRGD for codelivery of therapeutic siRNA and bortezomib in MLL-AF6 AML cells.  
b, Proposed cellular uptake mechanisms of Lipo-siRNA-BTZ-PEG-cRGD and subcellular siRNA and BTZ release to induce cancer cell apoptosis.  
c, Transmission electron microscope (EM) images of naked siRNA, Lipo-siRNA-BTZ, Lipo-BTZ-PEG-cRGD and Lipo-siRNA-BTZ-PEG-cRGD. Scale bar: 100 nm, n = 3.  
d, Scanning EM image of Lipo-siRNA-BTZ-PEG-cRGD. Scale bar: 100 nm, n = 3.  
e, Dynamic light scattering plot of Lipo-siRNA-BTZ-PEG-cRGD (n = 3).
Figure 2

Therapeutic activities of synthesized nanoparticles in ML-2 cells. a, Western blots showing SHARP1 protein expression in ML-2 cells transfected with naked siRNA, Lipo-siRNA-BTZ, Lipo-BTZ-PEG-cRG, and Lipo-siRNA-BTZ-PEG-cRGD compared to untreated cells (n = 3). b, Quantification of SHARP1 protein expression in ML-2 cells. Data are presented as mean ± S.E.M. (n = 3 biological replicates); *p < 0.05. c, qPCR for SHARP1 mRNA expression in ML-2 cells (mean ± S.E.M., n = 3); *p < 0.05. d, Cytotoxicity (via
viability assay) and e, clonogenic growth were assayed in ML-2 cells. Data are presented as mean ± S.E.M. (n = 5); *p < 0.05, **p < 0.01, ***p < 0.001. f, Fluorescence imaging of ML-2 cells Annexin V, PI and DAPI staining. Scale bar: 20 µm. g, Representative flow cytometry plots of treated cells relative to untreated for Annexin V and PI. h, Percentage of Annexin V+ PI- and Annexin V+ PI+ apoptotic cells. The graphs show mean ± S.E.M of three independent experiments; *p < 0.05, **p < 0.01, ***p <0.001.

Figure 3

SHARP1 knockdown and proteasome inhibition induce robust autophagy and apoptosis in MLL-AF6 AML cells. a, qPCR for DOT1L mRNA expression in ML-2 cells using all treatments relative to untreated cells (mean ± S.E.M., n = 3); *p < 0.05. b, qPCR for MLL-AF6 mRNA expression in ML-2 cells (mean ± S.E.M., n = 3); *p < 0.05. c Schematic of SHARP1 knockdown and proteasome inhibition-oncogenic functions in MLL-AF6 AML cells. SHARP1 inhibition hindered MLL-FP complex construction following MLL-AF6, LEDGE, and MEN1 accumulation, leading to DOT1L-mediated cell death. BTZ inhibited proteasomes, preventing ubiquitin-proteasome complex formation and causing cell death by accumulation of misfolded ubiquitin proteins.