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Viral/Nonviral Chimeric Nanoparticles To Synergistically Suppress Leukemia Proliferation *via* Simultaneous Gene Transduction and Silencing

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S Supporting Information

ABSTRACT: Single modal cancer therapy that targets one pathological pathway often turns out to be inefficient. For example, relapse of chronic myelogenous leukemia (CML) after inhibiting BCR-ABL fusion protein using tyrosine kinase inhibitors (TKI) (e.g., Imatinib) is of significant clinical concern. This study developed a dual modal gene therapy that simultaneously tackles two key BCR-ABL-linked pathways using viral/nonviral chimeric nanoparticles (ChNPs). Consisting of an adeno-associated virus (AAV) core and an acid-degradable polymeric shell, the ChNPs were designed to simultaneously induce pro-apoptotic BIM expression by the AAV core and silence pro-survival MCL-1 by the small interfering RNA (siRNA) encapsulated in the shell. The resulting BIM/MCL-1 ChNPs were able to efficiently suppress the proliferation of BCR-ABL+ K562 and FL5.12/p190 cells *in vitro* and *in vivo* via simultaneously expressing BIM and silencing MCL-1. Interestingly, the synergistic antileukemic effects generated by BIM/MCL-1 ChNPs were specific to BCR-ABL+ cells and independent of a proliferative cytokine, IL-3. The AAV core of ChNPs was efficiently shielded from inactivation by anti-AAV serum and avoided the generation of anti-AAV serum, without acute toxicity. This study demonstrates the development of a synergistically efficient, specific, and safe therapy for leukemia using gene carriers that simultaneously manipulate multiple and interlinked pathological pathways.

KEYWORDS: hybrid vector, core-shell nanoparticles, AAV transduction, RNA interference, BIM expression, MCL-1 silencing, synergistic leukemia gene therapy

The development and progression of cancer are driven by an unbalanced homeostasis that is often collaboratively attributed to the interference of apoptosis while at the same time elevation of survival pathways.^{1–3} Cancer therapy in general aims to correct those pathways using anticancer agents and methods (*i.e.*, chemotherapeutics and radiation). For example, tyrosine kinases (TKs) are promising targets in cancer therapy due to their crucial roles in driving cellular proliferation, apoptosis, and angiogenesis.^{4,5} A well-documented, TK-mediated pathogenic example is Philadelphia chromosome positive (Ph+) chronic myelogenous leukemia (CML).^{6–8} The Ph+ genetic lesion is a reciprocal translocation [t(9;22)(q34;q11)] between the genes encoding the break-point cluster region (BCR) and Abelson murine leukemia viral

oncogene homologue 1 (ABL),^{9–11} resulting in a fusion protein BCR-ABL endowed with constitutive kinase activity allowing for increased cell proliferation while inhibiting apoptosis.^{12,13} This particular chimeric kinase, though highly linked with CML, can also be found in other forms of leukemia including acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML).^{14,15} Characterization of TK molecular structure and understanding their roles in tumorigenesis led to the first TK-specific cancer therapeutic drug, Imatinib, which was FDA-approved for first-line treatment of CML in 2002.¹⁶

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Imatinib binds close to the ATP binding site of constitutively activated BCR-ABL and inhibits the proliferation of Ph+ cells as well as inducing apoptosis.^{17–19} Despite the impressive clinical results of this targeted drug, CML patients still face the possibility of relapse either from the development of mutations in the kinase domain of BCR-ABL or the activation of compensatory survival pathways.^{20–23} This suggests that the current tyrosine kinase inhibitor (TKI)-mediated chemotherapy does not eradicate all CML cells and over time selects for mutated clones resistant to TKIs. In this study, we developed a dual-modal gene therapy approach to that dilemma by simultaneously manipulating two components of the cell survival machinery in order to achieve synergistically enhanced and safe therapy for Ph+ leukemia. This approach could be broadened to other cancers including Ph-like ALL, FLT3/ITD AML.^{24,25} Ph+ leukemia was used as the model disease for this proof-of-concept study because its survival mechanisms are well characterized.

Recent progress in deciphering the BCR-ABL pathways has led to a new therapeutic approach to apoptosis-mediated gene therapy.^{26–28} It is well documented that BCR-ABL-mediated leukemic potential is directly regulated by B-cell lymphoma-2 (Bcl-2) family proteins such as Bcl-2-interacting mediator of cell death (BIM)^{29,30} and myeloid cell leukemia-1 (MCL-1);^{31,32} specifically, BIM provokes the apoptosis of leukemia cells but is readily inactivated by MCL-1.³³ Pro-survival MCL-1 and pro-apoptotic BIM counteract each other in the apoptosis pathway, and therefore, the antileukemic effect caused by simultaneous BIM expression and MCL-1 silencing is greater than the effects caused by BIM restoration or MCL-1 silencing alone. Therefore, simultaneously restoring BIM and silencing MCL-1 (dual modal gene manipulation) could synergistically facilitate the apoptosis of BCR-ABL+ cells. Inspired by this rationale, herein we developed a gene therapy that effectively suppresses the proliferation of BCR-ABL+ cells in a synergistic and targeted manner.

RESULTS AND DISCUSSION

Design, Synthesis, and Characterization of Viral/Nonviral Chimeric Nanoparticles That Simultaneously Express BIM and Silence MCL-1 (BIM/MCL-1 ChNPs). Gene delivery carriers that allow simultaneous BIM expression and MCL-1 silencing were synthesized by incorporating the advantages of viral and nonviral vectors. In this study we used adeno-associated virus (AAV), which is known to be efficient in expressing a transgene with the benefit of having no known diseases reportedly linked with an AAV infection.^{34–36} The AAV encoding BIM (isoform L) was then shelled in the nonviral vector component which consisted of acid-degradable polyketal polymers that were designed to (1) shield the AAV core from the immune system, (2) encapsulate small interfering RNA (siRNA) against MCL-1, and (3) rapidly release siRNA and the AAV core into the cytoplasm from the endosome (Figure 1A). The resulting BIM-expressing, MCL-1 silencing (BIM/MCL-1) chimeric nanoparticles (ChNPs) are thus shielded from inactivation by the immune system, protected against degradation of siRNA by nucleases, and incapable of provoking immunity against the AAV core. Upon endocytosis, MCL-1 siRNA is released into the cytoplasm from the endosome/lysosome, making the cells susceptible to apoptosis. Concurrently, the AAV core is trafficked to the nucleus for BIM expression, triggering cellular apoptosis. Therefore, BCR-ABL+ cells are synergistically forced to undergo apoptosis *via*

Figure 1. Synthesis of viral/nonviral chimeric nanoparticles that simultaneously express BIM and silence MCL-1 (BIM/MCL-1 ChNPs) and hypothetical process of BIM/MCL-1 ChNPs in BCR-ABL+ cell for synergistically induced apoptosis. (A) Synthesis of BIM/MCL-1 ChNPs and acid degradation in a cell. BIM-encoding AAV (serotype 2) conjugated with eosin (photoinitiator) was surrounded by an acid-degradable polyketal (PK) shell *via* photopolymerization of acid-cleavable amino ketal monomers and cross-linkers. During the polymerization, MCL-1 siRNA premixed with the amino ketal monomers was added for concurrent encapsulation in the PK shell, resulting in viral/nonviral nanoparticles with a BIM AAV core and acid-degradable, MCL-1 siRNA-encapsulating PK shell (BIM/MCL-1 ChNPs). The PK shell is synthetically programmed to degrade in the mildly acidic endosome/lysosome, releasing MCL-1 siRNA and BIM AAV for facilitated intracellular processes. (B) Extra- and intracellular processes of BIM/MCL-1 ChNPs for simultaneous BIM expression and MCL-1 silencing in BCR-ABL+ leukemia cell. The AAV core and siRNA in BIM/MCL-1 ChNPs are shielded from the immune system and degradation by nucleases, respectively, during circulation. Upon endocytosis, the PK shell rapidly degrades in the mildly acidic endosome/lysosome and releases BIM AAV core and MCL-1 siRNA into the cytoplasm for facilitated intracellular processes. The siRNA released into the cytoplasm silences the

Figure 1. continued

expression of pro-survival MCL-1. Meanwhile, the AAV is transported into the nucleus for the expression of BIM, whose transcription and lifespan are interfered by BCR-ABL-initiated pathways. Resensitization to BIM-mediated apoptosis by MCL-1 silencing in simultaneous combination with restored apoptosis by BIM expression synergistically suppressed the proliferation of BCR-ABL⁺ leukemia cells.

simultaneous BIM expression and MCL-1 silencing (Figure 1B).

The hydrodynamic size and the zeta potential of BIM/MCL-1 ChNPs were measured to be 187.3 ± 10.7 nm in diameter and $+24.8 \pm 3.8$ mV, while those of free BIM AAVs were 31.2 ± 8.5 nm and -12.4 ± 6.2 mV, respectively (Figure S1A), indicating encapsulated BIM AAV particles in an approximately 80 nm-thick polyketal shell. In addition, TEM images confirmed spherical BIM/MCL-1 ChNPs before acid-hydro-

lysis and free BIM AAVs released from a polyketal shell upon incubation at an acidic pH of 5.0 (Figure S1B). The amounts of BIM AAV and MCL-1 siRNA released from BIM/MCL-1 ChNPs, which were synthesized using 6.0×10^{10} GC AAV and $3.0 \mu\text{g}$ siRNA, were quantified to be $6.08 \pm 0.21 \times 10^{10}$ GC and $2.8 \pm 0.4 \mu\text{g}$ (Figure S2), respectively. This result indicates highly efficient encapsulation of both BIM AAV and MCL-1 siRNA in ChNPs via surface-initiated photopolymerization. Since the polymerization was initiated by eosin conjugated only on the BIM AAV surface (*i.e.*, no polymerization in bulk) and the resulting BIM/MCL-1 ChNPs were relatively homogeneous in size (PDI = 0.212 with no peak representing the size of BIM AAV) (Figure S1), it was speculated that one BIM AAV particle was encapsulated in the core of BIM/MCL-1 ChNPs and approximately 2,000 siRNA molecules were loaded in the polyketal shell.

BIM/MCL-1 ChNP-Mediated, Simultaneous BIM Expression, and MCL-1 Silencing in BCR-ABL⁺ Cells. To confirm the synergistic antileukemic effect on BCR-ABL⁺ cells

Figure 2. Synergistically induced apoptosis of BCR-ABL⁺ human chronic myelogenous leukemia (CML) K562 cells *in vitro* and *in vivo* via simultaneous BIM expression and MCL-1 silencing by ChNPs. (A, B) Restored BIM expression and simultaneously silenced MCL-1 expression in K562 cells at mRNA and protein levels confirmed by (A) RT-PCR and (B) Western blot. Cell lysates collected 3 days after K562 cells with PBS, BIM AAV alone, MCL-1 siRNA alone, BIM/MCL-1 ChNPs, BIM/Scr ChNPs, Null/MCL-1 ChNPs, and Null/Scr ChNPs, where Scr and Null represent siRNA with a scrambled sequence and AAV encoding no transgene, respectively. Consistent doses of 1.0×10^{10} genome copies (GC) BIM AAV/mL and 125 nM MCL-1 siRNA were used, and 10% serum was used for the entire period of the experiment. The relative levels of BIM and MCL-1 mRNA and proteins were quantified by densitometry analysis and further normalized by β -tubulin mRNA and protein levels, respectively. The dotted line indicates the averaged background mRNA and protein levels (*e.g.*, BIM mRNA and protein levels in the cells incubated with PBS, MCL-1 siRNA alone, Null/MCL-1 ChNPs, and Null/Scr ChNPs; MCL-1 mRNA and protein levels in the cells incubated with PBS, BIM AAV alone, BIM/Scr ChNPs, and Null/Scr ChNPs). (C) Growth of K562 cells incubated with PBS, BIM AAV alone, MCL-1 siRNA alone, Null/Scr ChNPs, BIM/Scr ChNPs, Null/MCL-1 ChNPs, and BIM/MCL-1 ChNPs. The cells were stained with Annexin V Alexa Fluor 488 and propidium iodide (PI), and only viable cells were counted by flow cytometry at different incubation time points. (D) Evaluation of antileukemic effects of BIM/MCL-1 ChNPs in a K562 xenograft animal model. Luciferase-expressing K562 cells (K562/Luc; 2.5×10^6 cells per mouse) were transplanted in 16–20 week-old immunodeficient NOD SCID gamma (NSG) mice via intravenous injection in the tail. After 1 week, PBS, BIM AAV alone, MCL-1 siRNA alone, and BIM/MCL-1 ChNP were injected intravenously (5.0×10^{11} GC BIM AAV and $25 \mu\text{g}$ MCL-1 siRNA per mouse; 3 mice per group). The proliferation of K562/Luc cells was evaluated by bioluminescence imaging exposed for 5 min after the animals were intraperitoneally injected with 2 mg of synthetic luciferin. The overlapped bioluminescence and bright-field images of the most representative mouse from each group are shown along with averaged total photon flux of each treatment group at 0, 14, and 25 days after treatment.

via simultaneous BIM expression and MCL-1 silencing, we prepared various ChNPs, including those composed of (1) BIM AAV and MCL-1 siRNA (BIM/MCL-1 ChNPs), (2) BIM AAV and siRNA with scrambled sequence (BIM/Scr ChNPs), (3) Null AAV (no insert gene in promoters) and MCL-1 siRNA (Null/MCL-1 ChNPs), and (4) Null AAV and Scr siRNA (Null/Scr ChNPs), and used them to transduce/transfect (by AAV/siRNA; transfect herein) K562 human CML cells. Additional controls for the study were cells treated with no ChNPs but instead with free BIM AAV alone and free MCL-1 siRNA alone. The lysates of all the K562 cells were then analyzed for BIM and MCL-1 expression at the mRNA and the protein levels by reverse-transcription polymerase chain reaction (RT-PCR) and Western blot. The expression of BIM was significantly increased at both mRNA ($p < 0.01$) and protein levels ($p < 0.01$) in cells transfected with BIM AAV or BIM AAV-containing ChNPs (e.g., BIM/MCL-1 and BIM/Scr ChNPs), as compared to that of BIM AAV-free groups (e.g., phosphate buffered solution [PBS], MCL-1 siRNA, Null/MCL-1 ChNPs, and Null/Scr ChNPs) (Figure 2A,B). MCL-1 siRNA-containing ChNPs (e.g., BIM/MCL-1 and Null/MCL-1 ChNPs) also showed benefit by efficiently reducing the expression of the antiapoptotic MCL-1 at both mRNA and protein levels, while no or insignificant changes in MCL-1 expression were observed when the cells were treated with free MCL-1 siRNA alone (i.e., without a transfection agent), or Scr siRNA alone (Figure S3).^{37,38}

Synergistically Induced Apoptosis and Suppressed Proliferation of BCR-ABL+ Leukemia *in Vitro* and *in Vivo* by BIM/MCL-1 ChNPs. Evaluation of whether simultaneous BIM expression and MCL-1 silencing induced the apoptosis of K562 cells was also investigated. The cells incubated with BIM/MCL-1 ChNPs showed a significant decrease ($p < 0.01$) in proliferation throughout the entire incubation period, compared to other treatment groups (Figure 2C). Notably, free BIM AAV alone did not affect the proliferation of K562 cells, while BIM/Scr ChNPs moderately reduced the number of viable cells ($p < 0.01$), although as mentioned previously, both BIM AAV and BIM/Scr ChNPs increased BIM expression at mRNA and protein levels (Figure 2A,B). This implies that ChNP formulation improved intracellular processes and kinetics/duration of BIM expression as partially hinted in a previous report.³⁹ In addition, increased BIM expression induced by BIM/Scr ChNPs slightly inhibited the proliferation of the cells, but MCL-1 silencing alone by Null/MCL-1 ChNPs did not affect the cells. This suggests that BIM expression plays a slightly more crucial role than MCL-1 silencing in inducing apoptosis of K562 at the tested dose. However, codelivery of BIM gene and MCL-1 siRNA by ChNPs at the corresponding doses used in single gene regulations did synergistically reduce the number of BCR-ABL+ K562 cells. The proliferation of the cells incubated with Null/Scr ChNPs was similar to that of PBS-incubated cells, indicating lack of nonspecific cytotoxicity by the ChNPs themselves. As shown in Figures 2C and 3B, the antileukemic effect of BIM/MCL-1 ChNPs is greater than the simply summed antileukemic effects of BIM/Scr or Null/MCL-1 ChNPs alone. The viability of K562 cells incubated with BIM/MCL-1 ChNPs was significantly lower than that of the cells incubated with a mixture of BIM/Scr-1 and Null/MCL-1 ChNPs at the same concentrations ($p < 0.01$) (Figure S4). This result suggests that codelivery of BIM AAV and MCL-1 siRNA on the same platform is crucial for achieving synergistically

Figure 3. BCR-ABL-targeted, IL-3-independent therapy for leukemia by BIM/MCL-1 ChNPs. (A, B) Relative viability of BCR-ABL- FL5.12 and BCR-ABL+ FL5.12/p190 cells incubated with PBS and various ChNPs (Null/Scr, Null/MCL-1, BIM/Scr, and BIM/MCL-1 ChNPs) for 3 days at 1.0×10^{10} GC BIM AAV/mL and 125 nM MCL-1 siRNA. Since FL5.12 cells proliferate only in the presence of IL-3, both cells were incubated with 5 ng/mL IL-3, and viable cells were quantified by MTT assay. (C–F) Growth of BCR-ABL- FL5.12 and BCR-ABL+ FL5.12/p190 cells in the presence of or absence from IL-3. Viable cells treated with PBS or various ChNPs (Null/Scr, Null/MCL-1, BIM/Scr, and BIM/MCL-1 ChNPs) were counted by flow cytometry after double stained with Annexin V Alexa Fluor 488 and PI.

enhanced antileukemic effect *via* simultaneous BIM expression and MCL-1 silencing.

Effective antileukemic activity of BIM/MCL-1 ChNPs in an animal model was also investigated. K562 cells that were transduced with lentivirus to express firefly luciferase (K562/Luc cells) were intravenously injected into immunodeficient NOD-SCID-gamma common chain knockout (NSG) mice. After 1 week, PBS, BIM AAV alone, MCL-1 siRNA alone, and BIM/MCL-1 ChNPs were intravenously injected into the mice ($n = 3$), and the expansion of K562/Luc cells in the animals was monitored by *in vivo* luminescence imaging. Compared to the groups of animals treated with PBS, BIM AAV alone, and MCL-1 siRNA alone, a noticeably lower bioluminescence (i.e., inhibited leukemia expansion) was observed in the animals injected with BIM/MCL-1 ChNPs than the other groups (Figure 2D). In addition, the bioluminescence of the mice treated with PBS (~370-fold), BIM AAV (~200-fold), and MCL-1 siRNA (~6-fold) increased more rapidly than that of

Figure 4. Synergistically enhanced therapy for BCR-ABL+ FL5.12/p190 leukemia by BIM/MCL-1 ChNPs in fully immune-competent animals and avoided immune recognition and activation against the BIM AAV core. (A) Effectively inhibited proliferation of BCR-ABL+ cells loaded in Balb/c mice after intravenously injected with BIM/MCL-1 ChNPs. Balb/c mice ($n = 5$; 6–7 week-old female) were intravenously injected in the tail vein with firefly luciferase-expressing FL5.12/p190 (FL5.12/p190/Luc) cells (1.0×10^5 cells per mouse). After 1 week, PBS, BIM AAV alone, MCL-1 siRNA alone, mixture of BIM AAV and MCL-1 siRNA, Null/Scr ChNPs, BIM/Scr ChNPs, and BIM/MCL-1 ChNPs were intravenously injected in the tail vein at the dose of 5.0×10^{11} GC BIM AAV and $25 \mu\text{g}$ MCL-1 siRNA per mouse. The proliferation of FL5.12/p190/Luc cells in the animals was observed by bioluminescence imaging every 2 days, as described in Figure 2. Total photon fluxes of each mouse at 3, 5, and 7 days after treatment are shown along with averaged total photon flux of each treatment group (indicated by short horizontal solid bars). Animals started dying at day 9 when comparing the bioluminescence of the same number of animals from each treatment group was no longer attainable. (B) The most representative bioluminescence images of Balb/c mice described in (A). Full bioluminescence images of all animals are shown in Figure S9. (C) Kaplan–Meier survival chart of FL5.12/p190/Luc cell-carrying Balb/c mice treated with PBS, BIM AAV alone, MCL-1 siRNA alone, mixture of BIM AAV and MCL-1 siRNA, Null/Scr ChNPs, BIM/Scr ChNPs, and BIM/MCL-1 ChNPs. (D) Relative transduction/transfection of HeLa cells by GFP-expressing AAV and GFP/Scr ChNPs in the presence of serum collected from Balb/c mice injected with PBS, GFP AAV, Scr siRNA, and GFP/Scr ChNPs. Balb/c mice ($n = 3$) were intravenously injected in the tail vein with PBS (no treatment), GFP AAV, Scr siRNA, and GFP/Scr ChNPs at the doses of 5.0×10^{11} GC AAV and $25 \mu\text{g}$ siRNA per mouse. After 2 weeks, blood was collected from the lateral saphenous vein of the animals and processed to generate serum. Then HeLa cells were incubated with either GFP AAV or GFP/Scr ChNPs in the media containing 10% serum collected from the animals or in

Figure 4. continued

serum-free media for 1 day. Transduction by GFP AAV or GFP/Scr ChNPs, which was quantitated by flow cytometry, was normalized by those obtained without using the serum collected from the animals. In addition, the cells were also transduced/transfected by GFP AAV or ChNPs in the media that contained serum collected from the animals without being injected with any reagents.

BIM/MCL-1 ChNP treated ones (~ 1.5 -fold) from days 14–25, as quantified by a total photon flux (photons/second) in individual mice. It was confirmed that BIM/Scr ChNPs showing the greatest antileukemic effect *in vitro* among control groups (Figure 2C) were significantly less effective in suppressing the proliferation of leukemia in animals than BIM/MCL-1 ChNPs ($p < 0.05$) (Figure S5). The results of this study indicated that BIM/MCL-1 ChNPs were able to effectively codeliver the BIM gene and MCL-1 siRNA to K562 cells and inhibit the expansion of the cells after overcoming multiple delivery barriers. This demonstrates the potential of BIM/MCL-1 ChNPs to be an effective carrier for Ph⁺ leukemia gene therapy.

BCR-ABL-Specific Antileukemic Effects of BIM/MCL-1 ChNPs. Xenograft models using human cells established in immunodeficient animals, also employed in this study (Figure 2D), have been extensively used as a central tool in evaluating the response to drugs as well as associated parameters (e.g., stability, distribution, and toxicity). However, immunodeficient animals (e.g., NSG mice) have defects in T, B, and natural killer cells as well as other immune cells, thereby lacking pivotal factors in cancer biology. This could be of significant concern pertinent to immune response to cancer cells and particularly when potentially immunogenic materials (e.g., proteins and viruses) are administered. To address this important limitation of using K562 xenograft in NSG mice, we investigated the antileukemic activity of BIM/MCL-1 ChNPs on murine leukemia, interleukin-3 (IL-3)-dependent FL5.12 cells and BCR-ABL⁺ FL5.12/p190 cells not only *in vitro* but also in a syngeneic animal model. First, we tested whether synergistically suppressed proliferation of leukemia cells *via* simultaneous BIM expression and MCL-1 silencing was specific to BCR-ABL⁺ cells. The viability of BCR-ABL[−] FL5.12 cells was not affected no matter what ChNPs were incubated with them (Figure 3A). In contrast, a significant number of BCR-ABL⁺ FL5.12/p190 cells were killed after incubated with BIM/Scr or BIM/MCL-1 ChNPs, while no changes were observed in the cells incubated with Null/Scr or Null/MCL-1 ChNPs (Figure 3B). Notably, BIM/MCL-1 ChNPs were significantly more potent in suppressing the proliferation of FL5.12/p190 than BIM/Scr ChNPs ($p < 0.05$). These results indicate that the synergistically suppressed proliferation by BIM/MCL-1 ChNPs was specific to BCR-ABL⁺ cells, as an ABL-targeted TKI (e.g., Dasatinib). BIM/Scr ChNPs resulted in a detectable death of FL5.12/p190 cells only at a high dose (1.0×10^{10} GC/mL; 125 nM siRNA) (Figure S6), whereas Null/MCL-1 ChNPs did not affect the cells. Along with the result shown in Figure 3B, this suggests that BIM expression plays a more significant role than MCL-1 silencing in triggering the death of FL5.12/p190 cells, similar to K562 cells *in vitro* (Figure 2C). We further assessed the targeted, efficient antileukemic effects of BIM/MCL-1 ChNPs against BCR-ABL⁺ cells by testing them with FL5.12 and FL5.12/p190 cells in the presence or absence of a proliferative cytokine, IL-3. Human serum contains approximately 2 pg/mL of IL-3⁴⁰ and could rescue BCR-ABL⁺ cells from BIM/MCL-1 ChNP-mediated apoptosis *in vivo*. The result shown in Figure 3 demonstrates that the antileukemic

effect of BIM/MCL-1 ChNPs on BCR-ABL⁺ FL5.12/p190 was only slightly ($\sim 10\%$) lowered by the presence of IL-3 at a concentration of 5 ng/mL, while a significant decrease ($\sim 30\%$) in antileukemic effect was observed with the cells incubated with BIM/Scr ChNPs (Figure 3C,F) in the presence of IL-3. In contrast, the proliferation of BCR-ABL[−] FL5.12 cells was entirely determined by the presence of IL-3, independent of incubation with BIM/MCL-1 and other control ChNPs (Figure 3C,E). This result implies that simultaneous BIM expression and MCL-1 silencing is a promising strategy to effective and targeted therapy for BCR-ABL⁺ leukemia not only *in vitro* but also *in vivo*.

Suppressed Proliferation of BCR-ABL⁺ Leukemia in Fully Immune-Active Animals by BIM/MCL-1 ChNPs. In order to confirm the antileukemic effect of BIM/MCL-1 ChNPs under fully active immune conditions, luciferase-expressing FL5.12/p190/Luc cells were intravenously injected into Balb/c mice, followed by injections of various ChNPs, free AAVs, siRNA, and their mixture in 7 days. The circulation time of BIM/MCL-1 ChNPs in blood ($t_{1/2} = \sim 2.2$ h) was significantly longer than that of free BIM AAV ($t_{1/2} = \sim 0.4$ h), possibility attributed to the polyketal shell that increased the ChNP size and prevented the protein absorption on the surface (Figure S7). Luciferase signals (expansion of FL5.12/p190/Luc cells) sharply increased from 3 to 7 days in the mice treated with PBS, BIM AAV alone, MCL-1 alone, mixture of BIM AAV and MCL-1 siRNA, and Null/MCL-1 ChNPs (Figure 4A,B). However, average luminescence signals in mice treated with BIM/MCL-1 ChNPs barely increased for 7 days. The proliferation of FL5.12/p190 cells in Balb/c mice (represented by luminescence) treated with BIM/MCL-1 ChNPs was synergistically suppressed when quantitatively compared with those injected with BIM/Scr or Null/MCL-1 ChNPs (Figure 4A). A dose-dependent inhibition of leukemia expansion in the animals injected with BIM/MCL-1 ChNPs was shown by bioluminescence imaging (Figures S8 and S9). Kaplan–Meier analysis showed a significantly extended survival of the mice treated with BIM/MCL-1 ChNPs (37 days) in comparison to those treated with PBS (19 days), BIM AAV alone (18 days), MCL-1 siRNA alone (20 days), BIM+MCL-1 mixtures (21 days), Null/MCL-1 ChNPs (19 days), and BIM/Scr ChNPs (29 days) (Figure 4C). These results demonstrate that simultaneous BIM expression and MCL-1 silencing by ChNPs can efficiently suppress the proliferation of BCR-ABL⁺ cells in fully immune-active animals.

Avoided Immune Provocation Against the AAV Core in BIM/MCL-1 ChNPs. A key obstacle that impedes the use of gene therapy through viral vectors in clinical trials is triggering an immune response to the viruses.^{41,42} Past strategies to overcome this complication, particularly on repeated treatments, often involved the administration of immunosuppressants, which resulted in additional significant clinical risks (e.g., opportunistic infections). Many studies have attempted to resolve this challenge by engineering viral surface molecules and grafting synthetic polymers;^{43,44} however, these solutions compromise the activity of viral vectors. The BIM AAV core in ChNPs is surrounded by a polyketal shell that was designed to

shield the AAV from immune inhibition (e.g., inactivation by AAV-neutralizing antibodies),^{45,46} while enhancing intracellular trafficking.³⁹ Incubation of GFP-encoding AAV with HeLa cells in the presence of the serum collected from mice injected with free AAV almost entirely inhibited AAV transduction (<8% GFP expression by AAV compared to the serum-free transduction) (Figure 4D). However, the same serum did not affect the transfection by ChNPs, indicating that the polyketal shell of ChNPs efficiently protected the AAV core from the inactivation factors in the serum (e.g., AAV-neutralizing antibodies). Furthermore, the serum collected from mice injected with ChNPs did not affect the transduction by free AAV and ChNPs. This implies that the AAV core of ChNPs was not efficiently processed to prime the immune system, which likely was contributed to the inability to recognize AAV proteins buried in ChNPs. The fact that ChNPs did not generate immunity against AAV (no immunogenicity by ChNPs) suggests the high feasibility for multiple ChNP administrations for repeated treatments. BIM/MCL-1 ChNPs were able to repeatedly suppressed the proliferation of BCR-ABL+ cells without a change in efficiency (Figure S10). In addition, no acute severe cytopenic toxicity was correlated with use of BIM/MCL-1 ChNPs based on corresponding complete blood count (CBC) tests (Figure S11). No obvious toxicity in the spleen and the liver was observed in the animals injected with BIM/MCL-1 ChNPs, in comparison with those injected with PBS (Figure S12).

CONCLUSION

In conclusion, we developed a strategy to suppress the proliferation of leukemia in an efficient and targeted manner using gene carriers that simultaneously expressed and silenced two linked molecular pathways for the facilitated death of target cells. This multimodal gene therapy approach was found to be collaboratively efficient, specific to cancer cells dependent on a model oncogene (i.e., BCR-ABL), immunologically compatible, and safe. Similar strategies could be applied to a broad range of diseases including many forms of malignant, neurodegenerative, and infectious diseases where a molecular pathway propels the progression of the disease along with a silenced antagonistic pathway. The gene carriers developed in this study can be further evolved to codeliver not only genetic materials but also chemotherapeutic agents for combined gene and chemotherapy.

MATERIALS AND METHODS

Materials. AAV vectors (GFP and Null; serotype 2) were purchased from Vector Biolabs (Philadelphia, PA, U.S.A.). BIM-encoding AAV vectors were prepared by Vector Biolabs using BIM (isoform L) plasmid construct gifted by Dr. Jeff Rathmell (Duke University). MCL-1 siRNA, siRNA with scrambled sequences, and primers for MCL-1 mRNA were obtained from Qiagen (Venlo, Netherlands). Eosin-5-isothiocyanate and N-hydroxysuccinimide (NHS)-functionalized polyethylene glycol (NHS-PEG, 5 kDa) were purchased from Invitrogen (Carlsbad, CA, U.S.A.) and Creative PEG Works Inc. (Winston Salem, NC, U.S.A.), respectively. PD mini size-exclusion columns (5 kDa MWCO) were purchased from GE Healthcare (Pittsburgh, PA, U.S.A.), and Amicon Ultra Centrifugal filters (100 kDa MWCO) were supplied from EMD Millipore (Darmstadt, Germany). Recombinant mouse interleukin 3 (IL-3) was purchased from BioLegend, Inc. (San Diego, CA, U.S.A.). Acid-degradable amino ketal methacrylamide monomers and acid-degradable ketal bismethacrylamide cross-linkers were synthesized as previously reported.^{34,39} Human chronic myelogenous leukemia K562

cells were purchased from ATCC (Manassas, VA, U.S.A.). Murine pro-B-cell lymphoid FL5.12 cells and FL5.12/p190 cells were obtained from Dr. Aimee Edinger (UC Irvine). Both K562 and FL5.12/p190 cells were transduced to express firefly luciferase by lentiviral vectors purchased from Capital Bioscience (Gaithersburg, MD, U.S.A.).

Cell Culture. K562 cells and luciferase-expressing K562 cells (K562/Luc cells) were cultured in Iscove's modified Dulbecco's medium (MediaTech, Herndon, VA, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, U.S.A.) and 1% antibiotics (MediaTech, Herndon, VA, U.S.A.). FL5.12/p190 and luciferase-expressing FL5.12/p190 cells (FL5.12/p190/Luc cells) were grown in RPMI 1640 containing 10% FBS and 1% antibiotics. FL5.12 cells were cultured in the same media supplemented with 5 ng/mL IL-3. All cells were cultured at 37 °C with 5% CO₂ and 100% humidity.

Preparation of BIM-Expressing, MCL-1 Silencing Chimeric Nanoparticles (BIM/MCL-1 ChNPs). BIM/MCL-1 ChNPs were synthesized by a previously reported method,³⁹ with slight modifications. Briefly, BIM-encoding AAV vectors (1.0×10^{11} genome copy [GC]) in 5 mL of 10 mM sodium bicarbonate buffer (pH 8.0) were reacted with 2 mg of eosin-5-isothiocyanates in 10 μ L of dimethyl sulfoxide (DMSO) with mild agitation. After 3 h incubation at RT, the residual eosin-5-isothiocyanates were removed using a PD mini size-exclusion column. The eosin-conjugated AAV vectors (6.0×10^{10} GC) were suspended in 1 mL of 10 mM HEPES buffer (pH 7.4) containing 10 mg of ascorbic acids. Ten mg of amino ketal methacrylamide monomers and 3.0 μ g of siRNA were premixed in 50 μ L of 10 mM HEPES buffer for 30 min at RT. The resulting monomers/siRNA solution was then added to eosin-conjugated AAV solution, followed by photopolymerization with mild stirring under a halogen lamp at 700 klux. After 10 min, 10 mg of amino ketal methacrylamide monomers and 4 mg of ketal bismethacrylamide cross-linkers were added and further polymerized for another 5 min. Ascorbic acids, unreacted monomers, and cross-linkers were removed by centrifugal filtration (100 kDa MWCO) of the resulting solution at 3000 rpm for 30 min at 4 °C. For *in vivo* study, ChNPs were in 1 mL of 10 mM HEPES buffer were incubated with 0.5 mg of NHS-PEG (5 kDa) in 20 μ L of DMSO for 4 h at 4 °C. Unreacted NHS-PEG was removed using centrifugal filtrations (100 kDa MWCO) at 3000 rpm for 30 min at 4 °C.

Characterization of BIM/MCL-1 ChNPs. The hydrodynamic size and the zeta potential of BIM/MCL-1 ChNPs (0.8×10^{10} GC AAV/mL in deionized water) were measured using a Malvern Zetasizer Nano ZS (Malvern Instruments, Westborough, MA). BIM/MCL-1 ChNPs (0.3×10^{10} GC AAV in 5 μ L of in deionized water) were spotted on a carbon-coated grid (Ted Pella, Redding, CA) and dried in a vacuum chamber for 2 h at room temperature. The grid was further stained with 1% uranyl acetate, dried in a vacuum chamber for 4 h, and observed under a Philips CM20 TEM (Philips Electronic Instruments, Mahwah, NJ) at 80 kV. The concentrations of siRNA and AAV encapsulated in BIM/MCL-1 ChNPs were determined as previously reported.³⁹ Briefly, BIM/MCL-1 ChNPs in 100 mM acetate buffer (pH 5.0) were incubated for 4 h at 37 °C with mild stirring, followed by centrifugation at 3000 rpm for 10 min using an Amicon Ultra centrifugal filter (MWCO 100 kDa; EMD Millipore, Darmstadt, Germany). The eluted free siRNA was quantified using a Quant-iT RiboGreen RNA Assay Kit (Invitrogen, Carlsbad, CA). The amount of BIM AAV released from acid-hydrolyzed BIM/MCL-1 ChNPs was quantified using a QuickTiter AAV Quantitation Kit (Cell Biolabs, San Diego, CA).

Cell Proliferation/Apoptosis and Viability Assays. K562 cells (5.0×10^4 cells/well) were seeded in a 24-well plate. After 24 h, the cells were incubated with PBS, BIM AAV alone, MCL-1 siRNA alone, mixture of BIM AAV and MCL-1 siRNA, and various ChNPs (Null/Scr, Null/MCL-1, BIM/Scr, BIM/MCL-1 ChNPs) at 1.0×10^{10} GC AAV/mL and 125 nM siRNA. After 24 h, the medium was replaced with AAV- and siRNA-free media, and the cells were further maintained by replacing the old media with fresh media on a daily basis. The cells were counted by flow cytometry (Guava Technologies, Hayward, CA, U.S.A.) after stained by Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen, Carlsbad, CA, U.S.A.) according

to manufacturer's recommendation. The proliferation/apoptosis of BCR-ABL-negative FL5.12 cells and BCR-ABL-positive FL5.12/p190 cells was also carried out as described above.

FL5.12 and FL5.12/p190 cells were seeded in a 48-well plate at a density of 2.0×10^4 cells/well with/without IL-3 (5 ng/mL). After 24 h, PBS, BIM AAV alone, MCL-1 siRNA alone, mixture of BIM AAV and siRNA, and various ChNPs (Null/Scr, Null/MCL-1, BIM/Scr, BIM/MCL-1 ChNPs) were added to the cells at different BIM AAV and MCL-1 siRNA concentrations. To measure cell viability, the cells maintained with fresh media everyday were pelleted by centrifugation (2000 rpm, 5 min, 4 °C) and resuspended in fresh media. The cells in 90 μ L medium then were transferred into a 96-well plate where MTT (5 mg/mL in 10 μ L PBS) was subsequently added. After 4 h of incubation at 37 °C, the well plate was centrifuged at 2000 rpm and 4 °C for 5 min, and the media was replaced by 100 μ L DMSO per well. The relative cell viability was quantified by comparing absorptions at 570 nm using a microplate reader (Synergy H1, Biotek), based on the absorption in the wells containing nontreated cells.

Western Blot. K562 cells were seeded on a 6-well plate (3.0×10^{10} cells per a well) for 24 h before being incubated with PBS, BIM AAV alone, MCL-1 siRNA alone, and various ChNPs (Null/Scr, Null/MCL-1, BIM/Scr, and BIM/MCL-1 ChNPs) at concentrations of 1.0×10^{10} GC BIM AAV and 125 nM MCL-1 siRNA. After 3 days, the cells were collected by centrifugation (2000 rpm, 5 min, 4 °C) and lysed in IP buffer (Pierce, Rockford, IL, U.S.A.). The protein concentration in cell lysates were measured and normalized by β -tubulin ELISA (MyBioSource, San Diego, CA, U.S.A.), following the manufacturer's protocol. Equal amounts of protein were loaded and separated in 12% Mini-PROTEAN TGX Precast gel (Bio-Rad, Hercules, CA, U.S.A.) for 1 h at 200 V, followed by being transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, U.S.A.). The membrane was then blocked with 5% milk in TBST buffer (10 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.05% Tween 20) for 1 h at RT with gentle shaking and washed three times with TBST buffer. Rabbit IgG against BIM, MCL-1, and β -tubulin primary antibodies (Cell Signaling Technology, Beverly, MA, U.S.A.) were added to the membrane in TBST buffer and incubated overnight at 4 °C, followed by rinsing three times with TBST. Then, horseradish peroxidase conjugated antirabbit IgG secondary antibodies (Promega, Madison, WI, U.S.A.) were added and incubated overnight at 4 °C. After the membrane was rinsed three times with TBST, the protein bands were visualized using enhanced chemiluminescence detection Kit (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturers' instructions. The chemiluminescence signals were visualized and analyzed using a UV transilluminator and its software (Alpha Innotech, Santa Clara, CA, U.S.A.).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Entire RNA in K562 cells, which were incubated with AAV, siRNA, and ChNPs as in Western blot, was isolated using NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) and was reversely transcribed to cDNA using the reverse transcription system kit (Promega, Madison, WI, U.S.A.) according to the manufacturers' protocol. The RT-PCR was performed using an ABI 7900HT fast real-time PCR system (Applied Biosystems, Carlsbad, CA, U.S.A.) in a 384-well plate. The RT-PCR was carried out at 50 °C for 30 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The resulting samples were electrophoresed in a 1% agarose gel containing 1 μ g/mL ethidium bromide (EtBr) in TBE buffer at 110 V for 30 min. The bands were visualized and quantitatively compared using a UV transilluminator and its software (Alpha Innotech, Santa Clara, CA, U.S.A.).

In Vivo Bioluminescence Imaging. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at University of California, Irvine (UCI). In xenograft model, K562/Luc cells (2.5×10^6 cells in 100 μ L PBS per mouse) were intravenously injected in the tail vein of 16–20 1 week-old female. After 1 week, PBS, BIM AAV alone, MCL-1 siRNA alone, and BIM/MCL-1 ChNPs were intravenously injected into mice at doses of 5.0×10^{11} GC AAV and 25 μ g siRNA per mouse. In immune-competent syngeneic model, 6–7 weeks-old Balb/c female mice were

intravenously injected with BCR-ABL-positive FL5.12/p190/Luc cells (1.0×10^5 cells per mouse) in the tail vein. After 1 week, the mice were intravenously injected with PBS, BIM AAV alone, MCL-1 siRNA alone, mixture of BIM AAV and MCL-1 siRNA, and various ChNPs (Null/MCL-1, BIM/Scr, and BIM/MCL-1 ChNPs) at doses of 5.0×10^{11} GC AAV and 25 μ g siRNA per mouse. For *in vivo* studies, ChNPs tethered with PEG (5 kDa) as described earlier were injected. The expansions of K562/Luc and FL5.12/p190/Luc cells were visualized by imaging mice intraperitoneally (IP) injected with 2 mg of synthetic firefly luciferin in 100 μ L PBS (Promega, Madison, WI, U.S.A.) using an IVIS Lumina system (Caliper Life Sciences, Hopkinton, MA, U.S.A.) for 5 min. Bioluminescence signal intensities in mice were quantified using Living Image 3.2 software associated with the imaging system.

Anti-AAV Sera Assay. The 4–6 week-old Balb/c mice were intravenously injected with PBS, GFP AAV alone, Scr siRNA alone, and GFP/Scr ChNPs at doses of 5.0×10^{11} GC AAV and 25 μ g siRNA per mouse. After 2 weeks, blood was harvested in a serum separation tube (BD Biosciences, Franklin Lakes, NJ, U.S.A.) from the lateral saphenous vein. The isolated mice serum was then incubated in K562 cells for 1 h before GFP AAV or GFP/Scr ChNPs were added for transduction and transfection. After 48 h, the cells were washed by cold PBS twice and analyzed for GFP expression by flow cytometry (Guava Technologies, Hayward, CA, U.S.A.).

Statistical Analysis. All triplicate experimental data collected from independently repeated measurements were represented as mean $\pm$ standard deviation. Statistical analysis was performed with Student's *t* Test and statistical significance was at *p*-values lower than 0.05. Kaplan–Meier survival curve was analyzed using MedCalc statistical software.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.6b04155.

Characterization of BIM/MCL-1 ChNPs for size, morphology, and siRNA encapsulation; BIM and MCL-1 expression in K562 cells incubated with additional control group materials, cytotoxicity *in vitro* including augmented eradication by repeated treatments, and proliferation *in vivo*; dose-dependent antileukemic effects of BIM/MCL-1 ChNPs on FL5.12/p190 cells *in vitro* and *in vivo*; clearance and toxicity of BIM/MCL-1 ChNPs *in vivo* (PDF)

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Author Contributions

^vThese authors contributed equally. C.A.H. and S.K.C. synthesized ChNPs, conducted *in vitro* and *in vivo* evaluations, and wrote the manuscript. J.K., B.P., and D.I., and J.E. carried out Western blot, synthesized acid-cleavable monomers and cross-linkers used for ChNP synthesis, performed TEM analysis of ChNPs and developed animal model, and performed *in vivo* studies. A.C. wrote the manuscript, and D.F. participated in the design of the study. Y.J.K. directed the study, designed experiments, and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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