Co-targeting BCL-XL and BCL-2 by PROTAC 753B eliminates leukemia cells and enhances efficacy of chemotherapy by targeting senescent cells

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Abstract

BCL-X_L and BCL-2 are key anti-apoptotic proteins and validated cancer targets. 753B is a novel BCL-X_L/BCL-2 proteolysis targeting chimera (PROTAC) that targets both BCL-X_L and BCL-2 to the von Hippel-Lindau (VHL) E3 ligase, leading to BCL-X_L/BCL-2 ubiquitination and degradation selectively in cells expressing VHL. Because platelets lack VHL expression, 753B spares on-target platelet toxicity caused by the first-generation dual BCL-X_L/BCL-2 inhibitor navitoclax (ABT-263). Here, we report pre-clinical single-agent activity of 753B against different leukemia subsets. 753B effectively reduced cell viability and induced dose-dependent degradation of BCL-X_L and BCL-2 in a subset of hematopoietic cell lines, acute myeloid leukemia (AML) primary samples, and *in vivo* patient-derived xenograft AML models. We further demonstrated the senolytic activity of 753B, which enhanced the efficacy of chemotherapy by targeting chemotherapy-induced cellular senescence. These results provide a pre-clinical rationale for the utility of 753B in AML therapy, and suggest that 753B could produce an added therapeutic benefit by overcoming cellular senescence-induced chemoresistance when combined with chemotherapy.

Introduction

Acute myeloid leukemia (AML) is a hematopoietic malignancy involving clonal hematopoiesis and defects in differentiation, proliferation, and cell death, resulting in the accumulation of immature blasts, marrow failure, and rapid death of patients if not treated. Therapeutic progress has been slow despite the recent identification of genomic and epigenetic alterations. Fewer than half of adults with AML (and less than 10-20% of elderly [>60 years old] patients with AML) survive long-term. Overexpression of anti-apoptotic BCL-2 family proteins is a core oncogenic property of leukemia, and is associated with disease progression and resistance to chemotherapy by protecting tumor cells from apoptosis.[1]

Targeting BCL-2 family proteins has been successfully explored as a therapeutic strategy for leukemia, and several small-molecule inhibitors of BCL-2 proteins have been identified.[2,3] Venetoclax (ABT-199), a BCL-2–selective inhibitor, has been approved by the US Food and Drug Administration for treatment of chronic lymphocytic leukemia (CLL) and of AML when combined with low-intensity chemotherapy. However, despite high response rates, the majority of the patients treated with hypomethylating agents and venetoclax eventually relapse.[4] The upregulation of anti-apoptotic proteins other than BCL-2, such as BCL-X_L or MCL-1, have been identified as major determinants of venetoclax resistance in CLL and AML,[2,5-7] consistent with the high efficacy of the dual BCL-2/BCL-X_L inhibitor navitoclax in killing venetoclax-resistant

CLL cells.[7]

However, the clinical utility of navitoclax was hampered by the on-target and dose-limiting thrombocytopenia due to dependence of platelet survival on BCL-X_L.[8] We have previously reported that DT2216, a von Hippel-Landau (VHL)-recruiting proteolysis-targeting chimera (PROTAC) derived from navitoclax, is able to overcome this on-target thrombocytopenia.[9] DT2216 selectively ubiquitinated and degraded BCL-X_L in a VHL E3 ligase- and proteasome-dependent manner in VHL-expressing cells, and was highly effective against tumors that primarily depend on BCL-X_L for survival, such as T-cell acute lymphoblastic leukemia (T-ALL).[9] Since platelets do not express VHL, DT2216 importantly spares platelets. Although DT2216 binds both BCL-2 and BCL-X_L with high affinity, DT2216 degrades only BCL-X_L, not BCL-2. Consequently, DT2216 showed minimal efficacy in cancers that co-depend on both BCL-X_L and BCL-2 for survival, such as certain subsets of leukemia and mantle cell lymphoma (MCL), unless combined with venetoclax or conventional chemotherapy.[10]

We recently reported the first-in-class dual BCL-X_L/BCL-2 PROTAC 753B that induces both BCL-X_L and BCL-2 ubiquitination and degradation selectively in cells expressing VHL, and demonstrated its pre-clinical activity in tumor cells that co-depend on BCL-2 and BCL-X_L.[10] In this study, we characterized its broad activity in AML. In addition, recent findings indicate that chemoresistance in AML is associated with chemotherapy-induced cellular senescence.[11,12] Upregulation of both BCL-X_L and BCL-2 has been reported essential for senescent cell survival.[13] Accordingly, inhibition of BCL-X_L and BCL-2 activity may facilitate clearance of senescent cells.[14] Here, we sought to evaluate the efficacy of 753B as a senolytic agent in leukemia after chemotherapy. Our study showed that 753B effectively eliminates leukemia cells both *in vitro* and *in vivo*, and enhances the efficacy of chemotherapy by targeting senescent cells.

Methods

Drugs and reagents

The drugs 753B, DT2216, and QVD (a pan-caspase inhibitor) were kindly provided by Prof. Zhou at the University of Texas Health Science Center and Prof. Zheng at the University of Florida. Venetoclax (ABT-199), Navitoclax (ABT-263), cytarabine (Ara-C) and S63845 were purchased from Selleckchem (Pittsburgh, PA, USA).

Cell lines and primary acute myeloid leukemia samples

Leukemia cell lines were obtained from ATCC (Manassas, VA, USA) or DSMZ (Braunschweig, Germany) and maintained in RPMI 1640 supplemented with 10-20% heat-inactivated fetal bovine serum (Gibco, Thermo-Scientific). All cell lines were frozen and low passage cells were used for all experiments.

Peripheral blood samples were collected from patients with AML; informed consent was obtained in accordance with the requirements of the Institutional Review Board of the University of Texas MD Anderson Cancer Center. The clinical features of the patients are listed in *Online Supplementary Table S1*.

Western blotting

Cells were lysed and 10-15 mg were prepared,[15] resolved by electrophoresis on NuPAGE 4-12% SDS-PAGE gradient gels (Invitrogen), and transferred to polyvinylidene fluoride membranes (Invitrogen). Immunoblotting was performed with primary antibodies: BCL-2 (DAKO), MCL-1 (Santa Cruz Biotechnology), PARP, Caspase-3, Cleaved caspase-3, BCL-X_L (Cell Signaling Technology), and tubulin (Sigma-Aldrich). Blots were scanned with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

Fluorogenic senescence-associated β-galactosidase activity assay using 5-dodecanoylaminofluorescein di-β-D-galactopyranoside

Senescence-associated β-galactosidase (SA-β-al) activity was assayed as previously described[16] using 20 μM 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}-FDG) (Thermo Fisher Scientific). Briefly, C_{12}-FDG was prepared in pre-warmed media and added to cells with gentle mixing of the cell suspension. Cells were then incubated with C_{12}-FDG and protected from light for 2-4 hours (h) at 37°C and 5% CO_2. SA-β-gal-positive cells were detected and quantified by flow cytometry.

Animal studies

All mouse experiments were approved by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center. Eight-week-old NOD *scid* gamma (NSG) mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were injected with $3x10^6$ AML patient-derived xenograft (PDX) #4138550 cells (which have *FLT3-ITD*, *DNMT3A*, *IDH1*, *KIT*, and *NPM1* mutations) via the tail vein. After leukemia engraftment was confirmed by flow cytometry 33 days after injection, mice were randomized to receive vehicle or 753B (5 mg/kg intraperitoneally, every 4 days) for three weeks. Mouse livers, spleens, and femurs were collected after the treatment. Leukemia burden was quantified by $hCD45^+$ flow cytometry.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8. Results are expressed as mean ± Standard Error of Mean (SEM) of three separate replicate experiments, unless otherwise indicated. Two-sided unpaired Student

t-test was used for comparisons between the means of two groups. Kaplan-Meier test was used to analyze the survival rate in the *in vivo* study and the data were statistically analyzed using log rank (Mantel-Cox) test.

Results

753B is more potent in reducing cell viability in a subset of hematologic cell lines compared with other BCL-X_L and/or BCL-2 targeting agents via degradation of BCL-X_L and BCL-2

We first evaluated the sensitivity of 24 genetically diverse hematologic cell lines to venetoclax, navitoclax, DT2216 (a BCL-X_L selective PROTAC), and 753B (a dual BCL- X_L/BCL-2 PROTAC). Our cohort of cell lines included 17 AML, five T-ALL, and two AML secondary to myeloproliferative neoplasms (MPN-AML) cell lines. Treatment with 753B for 24 h caused a dose-dependent but variable reduction of cell viability in all leukemia cell lines tested, as determined by CellTiter-Glo© (CTG) assay, with half-maximal inhibitory concentration (IC_{50}) values ranging from 0.01 μM to 27.35 μM (*Online Supplementary Table S2*). Both BCL-X_L-dependent cell lines (CCRF-CEM, TF-1) and BCL-X_L/BCL-2-co-dependent cell lines (Kasumi-1, KG-1) were sensitive to 753B (Figure 1A). Median IC_{50} values of 753B (0.35 μM) were lower than those of venetoclax (7.69 μM) and navitoclax (0.61 μM) across all cell lines tested (*Online Supplementary Figure S1A*), indicating that 753B on a molar basis is more potent than other BCL-X_L and/or BCL-2 targeting agents. Notably, 12 AML cell lines (KG-1, Kasumi-1, TF-1, HEL 92.1.7, MV4-11, Kasumi-3, UCSD-AML1, HNT37, MOLM-1, CMK, M-07e, F-36P) and three T-ALL cell lines (Jurkat, PF832, CCRF-CEM) showed greater sensitivity to 753B than to navitoclax (median value: 0.10 μM for 753B *vs.* 0.48 μM for navitoclax; P=0.0005) (Figure 1B). The IC_{50} of 753B in these cell lines was well correlated with that of navitoclax (correlation coefficient: r^2=0.6673) (*Online Supplementary Figure S1B*). Of note, the top seven highly sensitive cell lines to 753B included three AML-EVI-1 (MECOM) rearranged cell lines (Kasumi-3, UCSD-AML1 and HNT37), which represent AML with extremely poor prognosis[17] and are resistant to venetoclax (*Online Supplementary Table S2*, *Online Supplementary Figure S1C*).

We next analyzed the baseline (pre-treatment) expression of the anti-apoptotic proteins BCL-X_L, BCL-2 and MCL-1 in 17 leukemia cell lines by western blotting. BCL-2 was in general highly expressed in AML cell lines with a notable exception for the MPN-AML cell lines SET-2 and HEL 92.1.7 and all T-ALL lines, in which BCL-X_L expression was more prevalent. EVI-1 rearranged lines (e.g., Kasumi-3 and UCSD-AML1) co-expressed BCL-X_L and BCL-2 (*Online Supplementary Figure S1D, E*). To quantify BCL-X_L/BCL-2 protein degradation by 753B, we performed western blotting and densitometry analyses in 22 leukemia cell lines treated with 753B. Notably, 753B effectively and potently induced dose-dependent BCL-X_L degradation in all tested lines within 24 h, with the concentrations at which 50% of the protein was degraded (DC_{50}) ranging from 0.01 μM to 0.54 μM; in 21 of 22 tested cell lines, the DC_{50} of BCL-X_L to 753B was less than 0.15 μM. BCL-2 was also degraded in 21 of the 22 cell lines, but this generally required higher doses of 753B, with DC_{50} ranging from 0.02 μM to more than 1 μM, with the exception of the T-ALL Loucy cell line (no degradation at 24 h) (*Online Supplementary Table S2*, *Online Supplementary Figure S1E*).

To further understand 753B pro-apoptotic effects, we analyzed apoptosis in 753B-treated Kasumi-1 cells harboring AML-ETO rearrangement (BCL-X_L and BCL-2 co-dependent). Consistent with rapid degradation of BCL-X_L that we observed as early as 4 h (Figure 1C), 753B treatment rapidly induced apoptosis in Kasumi-1 cells as determined by Annexin-V staining after 4 h of treatment (Figure 1D). The results were confirmed by analyzing the cleavage of caspase-3 and PARP after 8 h (Figure 1C). The activation of caspase-3 by 753B was also observed in the HL60 cell line (*Online Supplementary Figure S1F*). Moreover, the decrease in cell viability induced by 753B was effectively rescued by the pan-caspase inhibitor Q-VD-OPh (QVD) (Figure 1E, *Online Supplementary Figure S1G*), suggesting that 753B-induced cell death is largely mediated by caspase-dependent apoptosis.

753B is a more potent antitumor agent than DT2216 in both leukemia cell lines and primary patient samples

To determine whether 753B is more potent than the first-generation BCL-X_L-targeted PROTAC DT2216, we exposed 22 cell lines, including AML, T-ALL, and MPN-AML cell lines, to increasing concentrations of DT2216 and 753B for 24 h and then determined their IC_{50} values. We observed that the sensitivity to DT2216 generally tracked with responsiveness to 753B (Figure 2A). We found that the IC_{50} of 753B in these cell lines strongly correlated with but were lower than the IC_{50} of DT2216 (correlation coefficient: r^2=0.8732) (Figure 2B). It is notable that in BCL-X_L-dependent leukemia cell lines, 753B was more potent than DT2216 (median IC_{50} : 0.10 *vs.* 1.16 μM) (*Online Supplementary Figure S2A*), consistent with higher potency of 753B as BCL-X_L degrader due to changing the link-out position on navitoclax and allowing additional lysines on BCL-X_L (K20) and BCL-2 (K17) to interact with the E2 enzyme for ubiquitination.[10] To better evaluate the antitumor efficacy of 753B, we tested 753B efficacy in the BCL-X_L/BCL-2 co-dependent AML cell line KG-1. Compared with DT2216, 753B more potently induced BCL-X_L degradation (DC_{50}, 0.01 *vs.* 0.06 μM) and additionally induced BCL-2 degradation in KG-1 cells (Figure 2C). Moreover, 753B was the most potent tested inhibitor inducing apoptosis (Figure 2D).

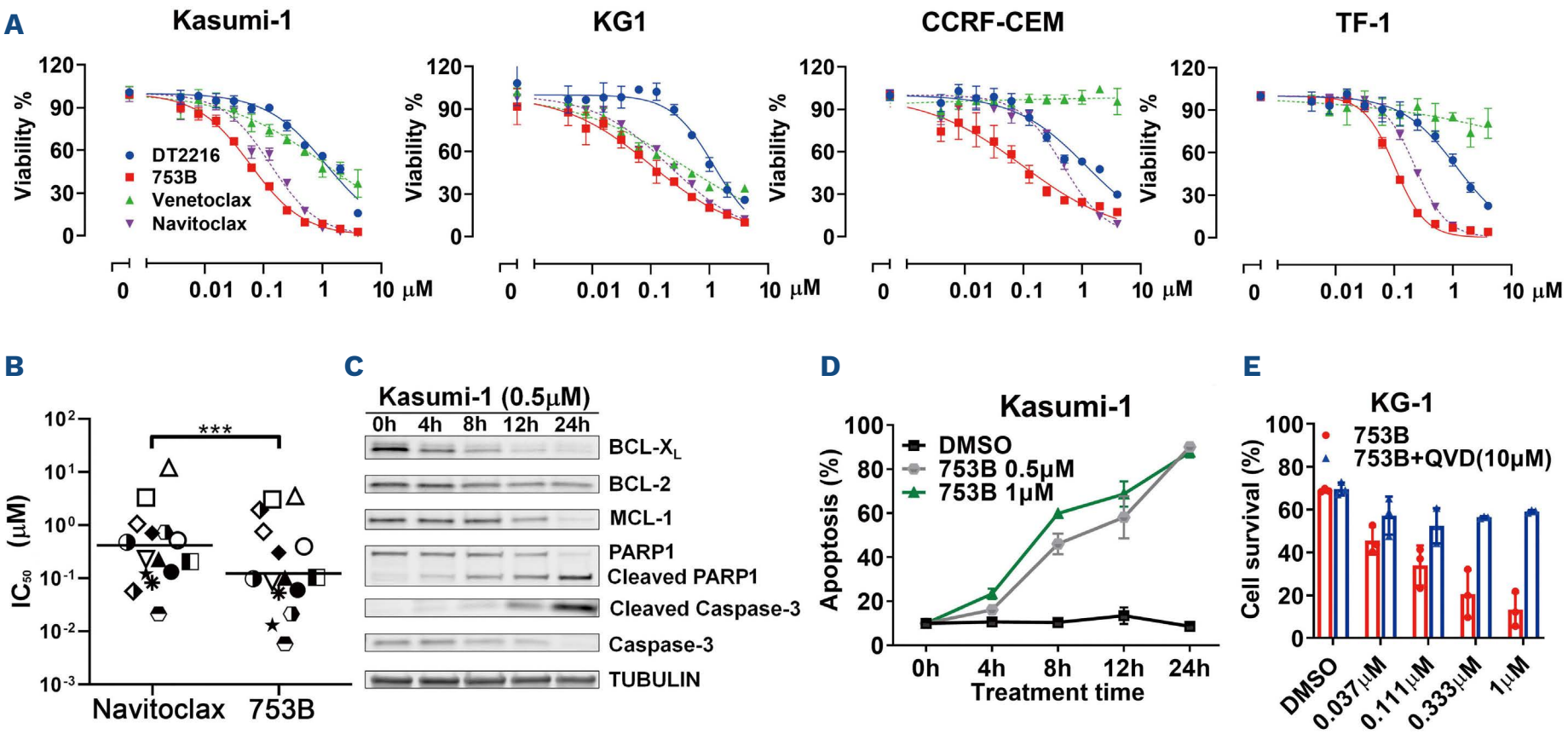

Figure 1. 753B is more potent in reducing cell viability in a subset of hematologic cell lines compared with other BCL-X_L and/or BCL-2 targeting agents via degradation of BCL-X_L and BCL-2. (A) Representative graphs of cell viability of three acute myeloid leukemia (AML) cell lines (Kasumi-1, TF-1 and KG-1) and one T-cell acute lymphoblastic leukemia (T-ALL) cell line (CCRF-CEM). Cell viability was determined by CellTiter-Glo assay following treatment with DT2216, 753B, venetoclax, or navitoclax at the indicated concentrations for 24 hours (h). (B) Comparison of IC_{50} values of 753B and navitoclax in 12 AML cell lines (KG-1, Kasumi-1, TF-1, HEL 92.1.7, MV4-11, Kasumi-3, UCSD-AML1, HNT37, MOLM-1, CMK, M-07e, F-36P) and three T-ALL cell lines (Jurkat, PF832, CCRF-CEM). Symbols represent individual cell lines. (C and D) Apoptosis in Kasumi-1 cells treated with the indicated concentrations of 753B or with DMSO at the indicated serial time points. Expression of apoptosis-related proteins was detected by western blotting (C) and apoptosis induction was determined by Annexin-V/DAPI staining (D). Tubulin served as loading control. (E) Percentage survival of KG-1 cells treated with 753B at the indicated concentrations for 24 h with or without treatment with the pan-caspase inhibitor Q-VD-OPh (QVD). DMSO: dimethyl sulfoxide. Data are presented as mean ± Standard Error of Mean of three repeats, performed in triplicate. ***P<0.001.

We next tested the sensitivity of 16 primary AML samples to 753B, DT2216, venetoclax, and navitoclax. Our cohort included ten AML samples from patients who were relapsed or refractory (R/R) to venetoclax-based therapies. Consistent with the results in leukemia cell lines, 753B more potently reduced cell viability than DT2216. The median IC_{50} value of 753B was 0.23 μM and ranged from 0.02 μM to 2.29 μM with IC_{50} values <0.50 μM in 13 of 16 primary AML patient samples (*Online Supplementary Table S1*). Of note, the IC_{50} of 753B in primary samples was lower than that in AML cell lines. 753B was more potent than DT2216 as evident by the extent of BCL-X_L degradation, higher apoptosis induction, lower IC_{50} with antileukemia potency comparable to that of navitoclax in all tested AML samples, including seven venetoclax-resistant samples (defined as IC_{50} >1 μM) (Figure 2E-G). 753B degraded BCL-X_L in all six tested primary samples, and BCL-2 in 4 out of 6 tested samples, with BCL-X_L DC_{50} of 0.01 to approximately 0.18 μM, and BCL-2 DC_{50} of 0.18 to approximately 3.57 μM (Figure 2E, H, *Online Supplementary Figure S2B-E*). 753B induced apoptosis associated with cleavage of caspase-3 and PARP (Figure 2H).

753B enhances the efficacy of chemotherapy by eliminating senescent leukemia cells

Recent studies suggested that chemotherapy-induced cellular senescence-mediated chemoresistance and disease relapse in AML.[18,19] Tumor cells can undergo senescence in response to stress and therapy, characterized by cell-cycle arrest, increased SA-β-gal activity and acquisition of a Senescence-Associated Secretory Phenotype (SASP) that comprises pro-inflammatory cytokines, chemokines and growth factors. Senescent cells can accumulate, especially under chemotherapy-induced immune suppression, and exhibit a deleterious effect on the tissue microenvironment that drives aging phenotype and potentially promotes tumor growth, relapse, metastasis, and resistance to chemotherapy.[11,19,20] Navitoclax and other BCL-X_L/BCL-2 inhibitors, like ABT-737 and A1331852 are known to function as potent senolytics, small molecules that selectively clear senescent cells in a variety of tissues and solid tumors,[13,14,21] help maximize the efficacy of chemotherapy, and prevent tumor relapse and metastasis.[13] Based on these findings, we hypothesized that 753B might exhibit senolytic activity in leukemia.

To confirm the induction of cellular senescence by Ara-C,

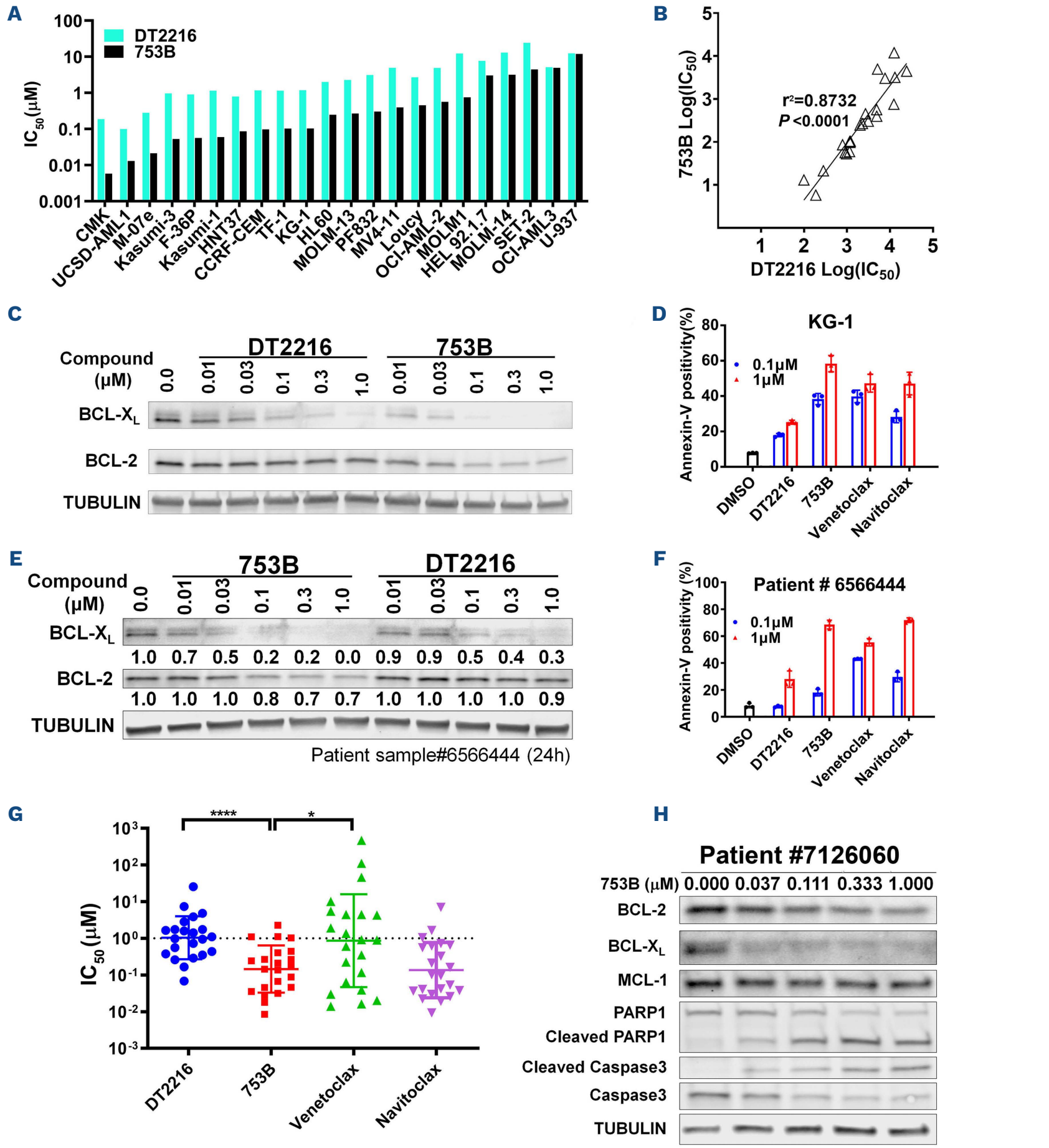

Figure 2. 753B is a more potent antitumor agent than DT2216 in both leukemia cell lines and primary patient samples. (A) IC_{50} values in leukemia cell lines treated with DT2216 or 753B for 24 hours (h). IC_{50} values were based on the percentage of viable cells and normalized to controls. (B) IC_{50} for 753B (y-axis) and DT2216 (x-axis) in hematologic cell lines listed in (A). Linear regression is solid black line. Correlation coefficient is discussed in the text. (C) Representative western blotting showing BCL-2 and BCL-XL protein levels in KG-1 cells treated with the indicated concentrations of DT2216 or 753B for 24 h. (D) Apoptosis in KG-1 cells treated with 0.1 μM or 1 μM DT2216, 753B, venetoclax, or navitoclax for 24 h. Apoptosis was determined using Annexin-V/DAPI staining and flow cytometry. Data are representative of two independent experiments. (E) Western blotting showing protein levels of BCL-2 and BCL-XL in a primary patient sample (#6566444) treated with the indicated concentrations of 753B or DT2216 for 24 h. Tubulin was used as an equal loading control. (F) Apoptosis in a primary patient sample (#6566444) treated with 0.1 μM or 1 μM DT2216, 753B, venetoclax, or navitoclax for 24 h. Apoptosis was determined using Annexin-V/DAPI staining and flow cytometry. Data are presented as mean ± Standard Error of Mean of three repeats, performed in triplicate. (G) IC_{50} values of DT2216, 753B, venetoclax, and navitoclax in primary patient samples (N=16) treated for 24 h. IC_{50} values were calculated based on the percentage of viable cells, normalized to control, as determined by CellTiter-Glo assay. (H) Western blotting showing protein levels of BCL-2 and BCL-XL and apoptosis-related proteins in a primary patient sample (#7126060) treated with the indicated concentrations of 753B. Tubulin was used as an equal loading control. DMSO: dimethyl sulfoxide. *P<0.05; ****P<0.0001.

one of the most used chemotherapy agents in AML, we treated MOLM-14 cells with increasing concentrations of Ara-C for three days. Ara-C-treated cells increased in size and cytoplasmic granularity by flow cytometry (Figure 3A), consistent with previous reports. SA-β-gal is a sensitive marker to identify cells in senescence state,[22] and its fluorogenic substrate C_{12}-FDG is widely used to quantify senescent cells.[16] Consistent with prior reports, the activity of SA-β-gal and C_{12}-FDG median fluorescence intensity (MFI) was drastically increased in viable cells after 0.05 μM Ara-C treatment for 72 h, indicating that Ara-C induced cellular senescence[11] (Figure 3B, C). 753B not only inhibited baseline cell senescence (*Online Supplementary Figure S3A-C*), but also reversed Ara-C-induced cellular senescence, as indicated by reduced SA-β-gal staining and lower MFI of C_{12}-FDG (Figure 3D, E).

To further validate the effect of 753B on cell senescence, we used immunoblotting to analyze the expression of cell cycle regulators such as p16 (CDKN2A), p21 (CDKN1A), and p53 (TP53), which are known to be associated with senescence.[23-25] Expression levels of p16 and p21 were increased after Ara-C treatment but abrogated by 753B (Figure 3B). Consistently, Ara-C treatment also increased the expression levels of pro-inflammatory cytokines and chemokines including interleukin (IL)-8, IL-6, IL-1β, and CCR5, which are SASP markers of senescent cells.[26,27] In contrast, 753B co-treatment prevented the Ara-C-induced expression of these cytokines and chemokines (Figure 3G). The inhibitory effect of 753B on chemotherapy-induced senescence was further confirmed in an additional AML cell line, Kasumi-1 (*Online Supplementary Figure S3D-K*). Taken together, these data indicate that 753B can target chemotherapy-induced senescent AML cells.

Chemotherapy-induced senescent cells express higher levels of BCL-X_L, representing a therapeutic target for 753B

To explore the molecular mechanism behind the chemotherapy-induced senescence phenotype, we FACS-sorted viable C_{12}-FDG-high (senescent) and C_{12}-FDG-low MOLM-14 cells after three days exposure to low doses of Ara-C (0.05 μM or 0.10 μM) (*Online Supplementary Figure S4A*). Compared to that in the C_{12}-FDG-low cells, the expression of BCL-X_L was significantly higher in the C_{12}-FDG-high senescent cells (Figure 4A). Consistently high expression of BCL-X_L was also observed in C_{12}-FDG-high, Ara-C-treated Kasumi-1 cells (*Online Supplementary Figure S4B*). This finding suggested that BCL-X_L is a key anti-apoptotic protein in senescent cells.

To analyze the clearance of senescent cells upon 753B treatment, MOLM-14 cells were incubated with Ara-C (0.05 μM) for three days to induce senescence, followed by 24 h of treatment with 753B. Compared with control cells without Ara-C treatment (non-senescent cells), senescent cells induced by Ara-C were significantly more sensitive to treatment with 753B, with a 75% death rate at the highest concentration tested (Figure 4B). Besides, the combination of Ara-C and 753B induced higher levels of cleaved caspase-3 and cleaved PARP, indicating increased apoptosis in these cells (Figure 4C). Furthermore, the combination of 753B and Ara-C treatment showed a synergistic growth-inhibitory effect (Figure 4D, E), suggesting that targeting BCL-X_L with 753B in combination with chemotherapy may boost chemotherapeutic efficacy in leukemia.

BCL-X_L degradation by 753B is associated with MCL-1 upregulation in selected leukemia cells, and the combination of 753B and an MCL-1 inhibitor induced synergistic cell death

MCL-1 is a member of BCL-2 family and a known resistance factor to venetoclax.[6,28,29] We have previously reported that in subsets of AML cells, short-term BCL-2 inhibition causes MCL-1 upregulation associated with activation of MAPK signaling.[30] The degradation of BCL-X_L and BCL-2 by 753B at the intermediate dose (0.11 μM) was similarly associated with MCL-1 upregulation in 13 of 22 cell lines tested. Out of 13 cell lines, we observed increased expression of MCL-1 at all applied concentrations of 753B in five AML lines (*Online Supplementary Table S3*, *Online Supplementary Figure S1D*). MCL-1 dose-dependent upregulation was observed as early as 4 h after 753B exposure (Figure 5A). Co-immunoprecipitation (Co-IP) and BH3 profiling were performed to explore the functional consequences of an increased level of MCL-1. 753B induced an increase in Bim binding to MCL-1 compared with vehicle treated cells as demonstrated by Co-IP (Figure 5B, C). Dynamic BH3 profiling demonstrated higher release of cytochrome C by the MCL-1-specific peptide MS-1 and the MCL-1 inhibitor S63845, indicating increased MCL-1 dependence following 753B exposure (Figure 5D). To further characterize the relationship between expression of BCL-2 family proteins and sensitivity to 753B, we quantified the expression levels of BCL-X_L, BCL-2 and MCL-1 by western blotting by densitometry of the bands in ten AML cell lines (*Online Supplementary Figure S1D*). Spearman correlation analysis was performed to evaluate the relationship between IC_{50} values and the protein change level of BCL-X_L, BCL-2 and MCL-1 by 753B. We found that the sensitivity to 753B inversely correlated with MCL-1 level when treated with 753B at the concentration of 1 μM at 24 h ($r=-0.8182$, $P=0.0038$) but did not correlate with the degradation level of BCL-X_L or BCL-2 at the same concentrations (Figure 5E). These data further support MCL-1 dependence following 753B treatment. 753B induced cell death in 50% of OCI-AML-2 cells at a concentration of 0.64 μM and in nearly 100% of the cells, when combined with a low dose of S63845 (0.005 μM) at 24 h by CTG assay; induction of

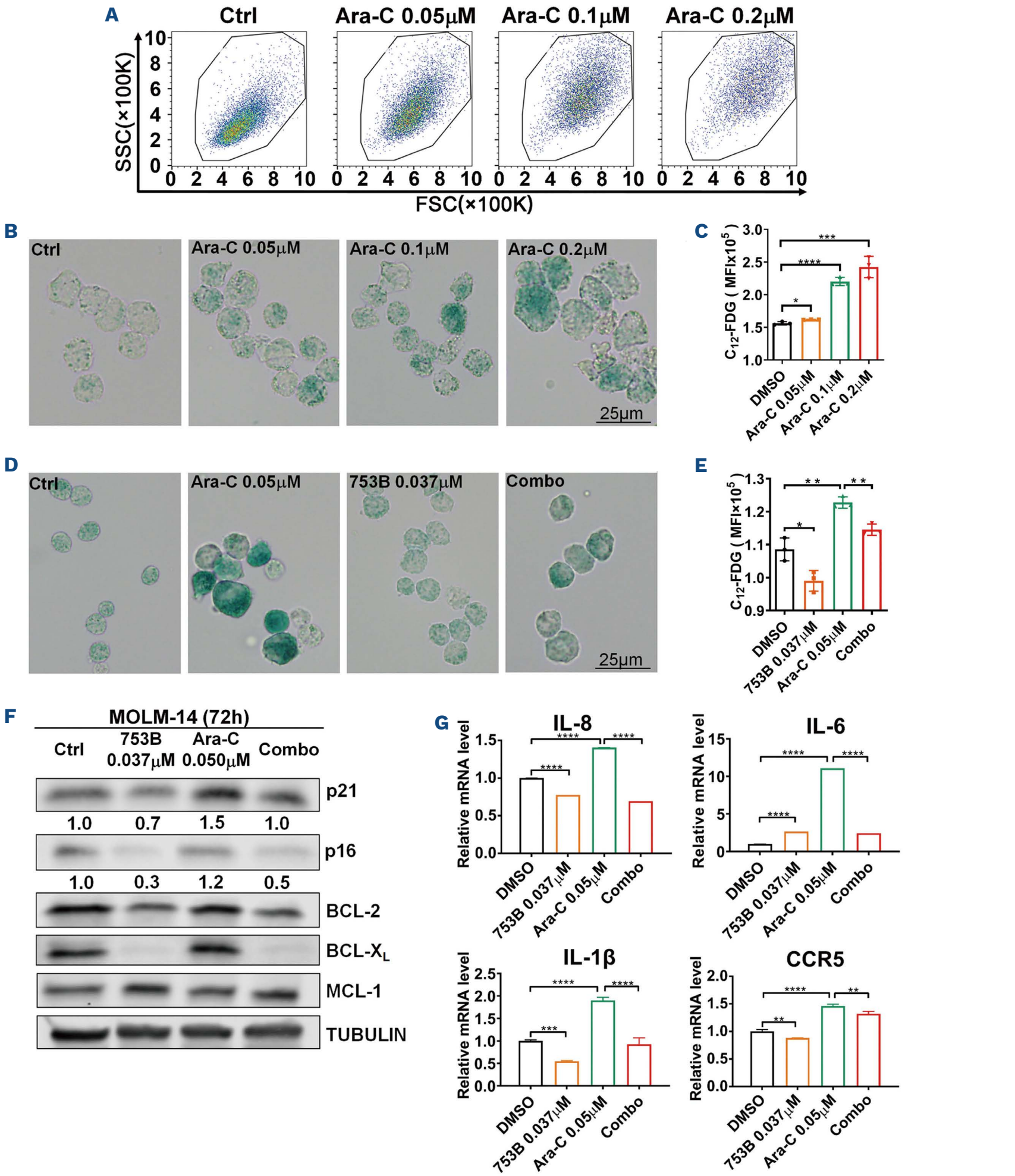

Figure 3. 753B enhances the efficacy of chemotherapy by eliminating senescent leukemia cells. (A) Flow cytometry forward scatter (FSC) *versus* side scatter (SSC) plots showing cell size in MOLM-14 cells treated with the indicated concentrations of cytarabine (Ara-C) for three days. (B) Representative images of SA-β-gal activity in MOLM-14 cells exposed to Ara-C for three days and stained with X-gal. Scale bar = 25 μM. (C) Flow cytometry histogram depicting SA-β-gal activity using the fluorogenic β-galactosidase substrate C_{12}-FDG in MOLM-14 cells after three days of Ara-C exposure (0.05 μM, 0.1 μM or 0.2 μM). (D and E) Images of SA-β-gal staining (D) and flow cytometry histogram of C_{12}-FDG (E) showing induction of senescence in MOLM-14 cells gated on viable cells (by DAPI- exclusion) and treated with Ara-C (0.05 μM), 753B (0.037 μM) or the combination (concomitantly). (F and G) Expression of p21, p16, BCL-2, BCL-XL, and MCL-1 (F) and mRNA expression of SASP markers (*IL-8*, *IL-6*, *IL-1β*, *CCR5*) (G) in MOLM-14 cells exposed to Ara-C, 753B, or the concomitant combination. C_{12}-FDG: 5-dodecanoylaminofluorescein di-β-D-galactopyranoside; SA-β-gal: senescence-associated β-galactosidase; SASP: senescence-associated secretion phenotype. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

cell death was synergistic at multiple concentrations tested (Figure 5F). These data suggest that the dual targeting of MCL-1 and BCL-X_L is highly effective in inducing cell death in AML.

Anti-leukemia efficacy of 753B *in vivo* in acute myeloid leukemia patient-derived xenograft model

To investigate the antileukemia activity of 753B *in vivo*, we developed a PDX model by injecting NSG mice with AML PDX #4138550 (generated from an AML patient harboring *FLT3-ITD*, *DNMT3A*, *IDH1*, *KIT*, and *NPM1* mutations). After confirming engraftment by peripheral blood flow cytometry analysis, mice were randomized into two groups to receive either vehicle or 753B (5 mg/kg intraperitoneally every 4 days) for three weeks. Mice tolerated 753B therapy well with no significant changes in body weight and no significant normal hematopoietic cells, platelet or white blood cell (WBC) toxicity as measured by blood counts (Figure 6A, *Online Supplementary Figure S5A-C*). 753B treatment reduced the circulating leukemia cell burden measured by flow cytometry, reduced liver and spleen weight, and extended overall survival (Figure 6B, C, *Online Supplementary Figure S5D*). The histological evaluation of Hematoxylin & Eosin (HE) stained sections of the bone marrow (BM), liver, and spleen showed reduction in the tumor burden of the BM, spleen and liver after 753B treatment, with no microscopic evidence of liver or spleen damage (Figure 6D). The reduction of leukemia burden induced by 753B was associated with a notable reduction in BCL-X_L expression in leukemia cells harvested after 753B treatment (Figure 6E, F).

Discussion

Both BCL-X_L and BCL-2 are validated therapeutic targets

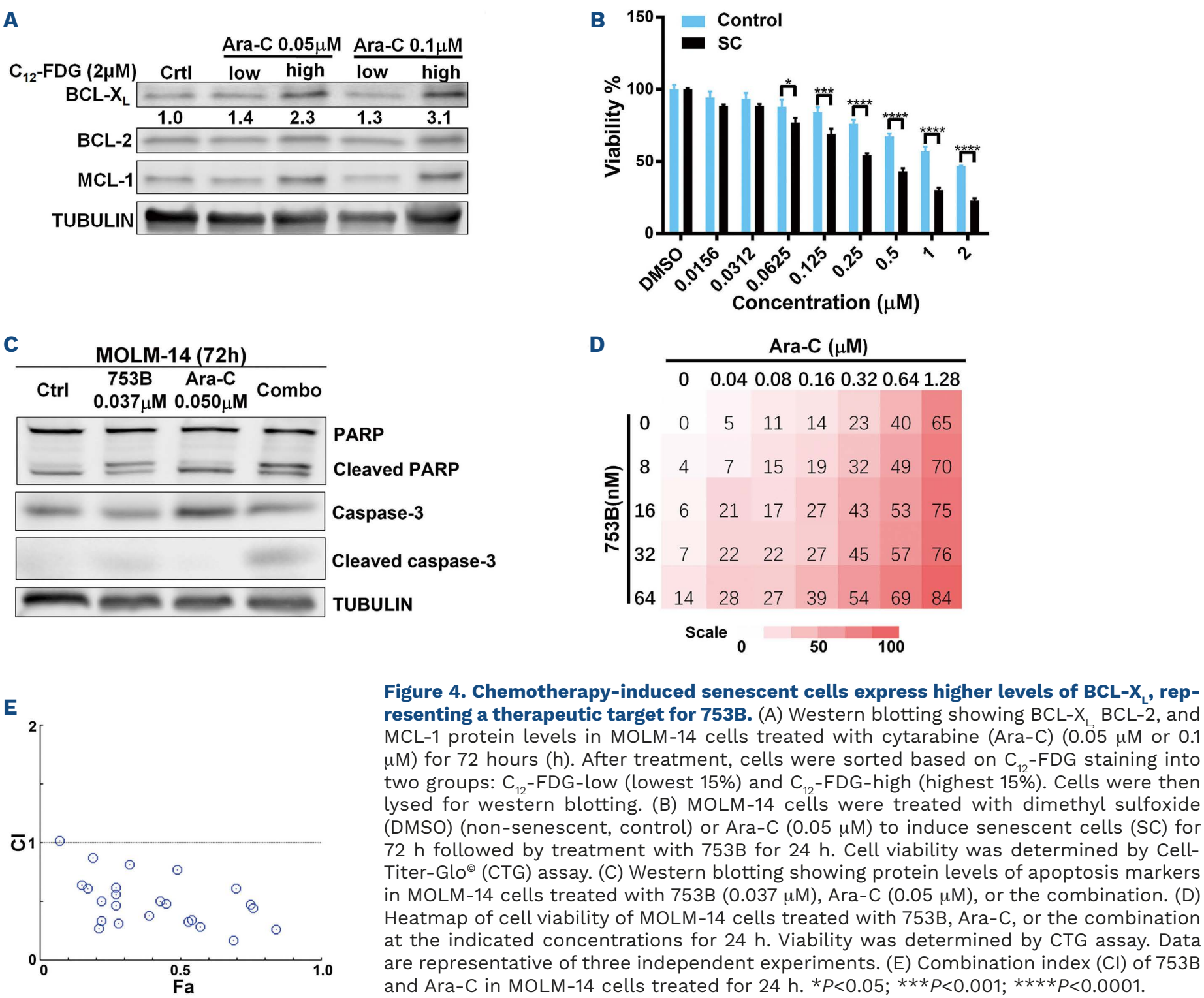

Figure 4. Chemotherapy-induced senescent cells express higher levels of BCL-X_L, representing a therapeutic target for 753B. (A) Western blotting showing BCL-X_L, BCL-2, and MCL-1 protein levels in MOLM-14 cells treated with cytarabine (Ara-C) (0.05 μM or 0.1 μM) for 72 hours (h). After treatment, cells were sorted based on C_{12}-FDG staining into two groups: C_{12}-FDG-low (lowest 15%) and C_{12}-FDG-high (highest 15%). Cells were then lysed for western blotting. (B) MOLM-14 cells were treated with dimethyl sulfoxide (DMSO) (non-senescent, control) or Ara-C (0.05 μM) to induce senescent cells (SC) for 72 h followed by treatment with 753B for 24 h. Cell viability was determined by CellTiter-Glo© (CTG) assay. (C) Western blotting showing protein levels of apoptosis markers in MOLM-14 cells treated with 753B (0.037 μM), Ara-C (0.05 μM), or the combination. (D) Heatmap of cell viability of MOLM-14 cells treated with 753B, Ara-C, or the combination at the indicated concentrations for 24 h. Viability was determined by CTG assay. Data are representative of three independent experiments. (E) Combination index (CI) of 753B and Ara-C in MOLM-14 cells treated for 24 h. *P<0.05; ***P<0.001; ****P<0.0001.

with overexpression noted in a wide range of hematologic malignancies.[31-33] In addition to MCL-1, BCL-X_L is also a key mediator of chemotherapy and venetoclax resistance.[4,5,34] Thus, developing a tolerable and effective BCL-X_L-targeted or BCL-2/BCL-X_L co-targeted therapy is of high priority. Given the caution required in dealing with navitoclax-induced thrombocytopenia, drug discovery efforts have aimed at circumventing this limitation through optimizing navitoclax-based targeting. In this study, we validated the effect of a novel dual BCL-X_L/BCL-2 PROTAC 753B, that was developed using navitoclax as a ligand in genetically diverse leukemia cells. We observed that 753B exhibited a potent anti-leukemia efficacy similar to that of navitoclax, making it a promising therapeutic candidate. We noted that 753B does not degrade BCL-2 as readily as BCL-X_L . We speculate that one of the possible mechanisms for the discrepancy in degradation between BCL-X_L and BCL-2 is at least partially to be attributed to the differential distribution and orientation of lysines on the protein surfaces, as we reported recently.[10] In addition, the

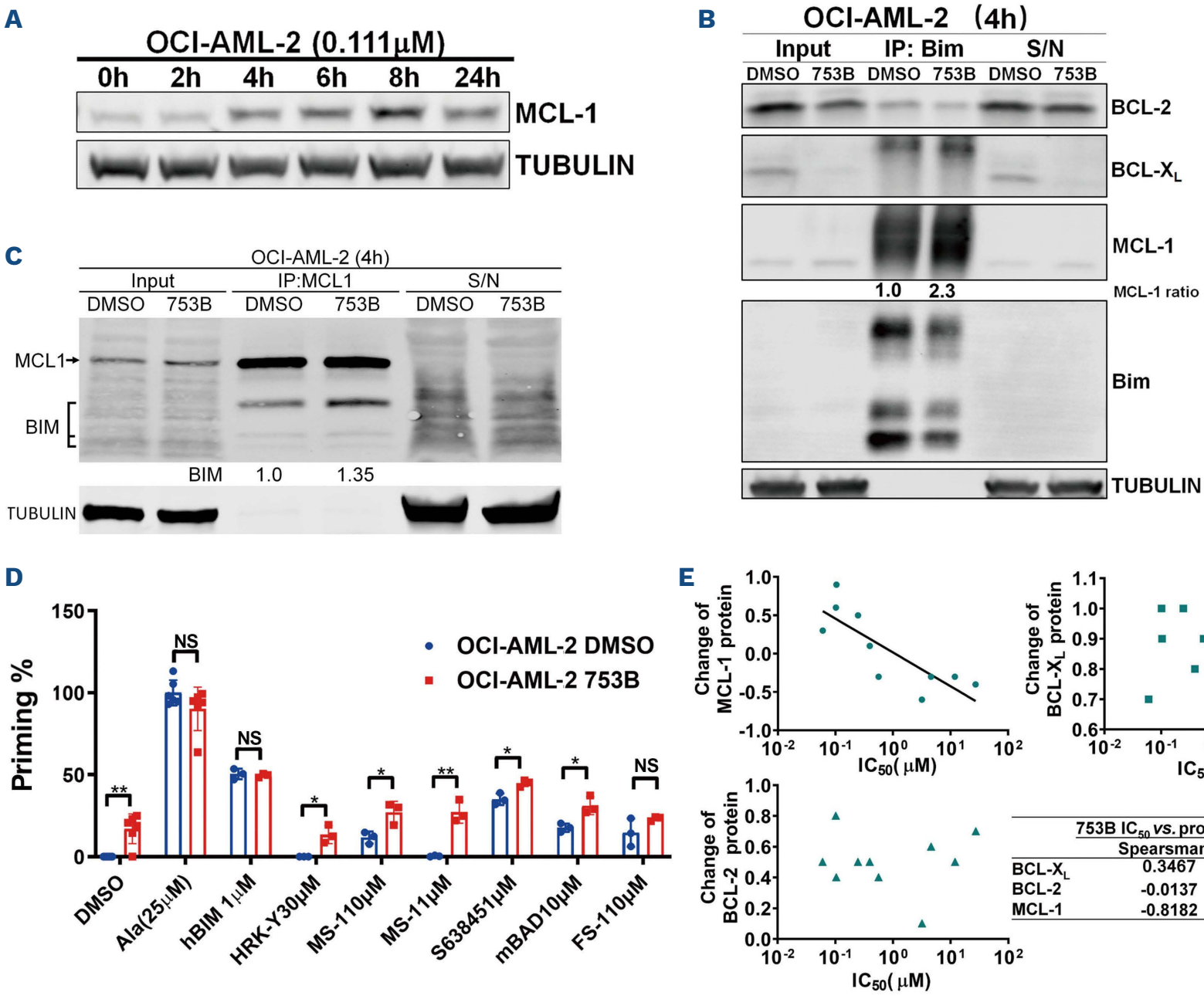

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Figure 5. BCL-X_L degradation by 753B is associated with MCL-1 upregulation in selected leukemia cells, and the combination of 753B and an MCL-1 inhibitor induces synergistic cell death. (A) Western blotting showing protein levels of MCL-1 in OCI-AML-2 cells treated with 753B (0.111 μM) and collected at the indicated time points. (B) OCI-AML-2 cells were treated with dimethyl sulfoxide (DMSO) or 753B (0.111 μM) for 4 hours (h). Immunoprecipitated Bim lysates demonstrate increased Bim and MCL-1 binding after 753B treatment. Immunoprecipitation was carried out with Bim antibody and detected with anti-MCL-1. S/N: supernatant after immunoprecipitation. (C) Co-immunoprecipitation (Co-IP) of OCI-AML-2 cells treated with DMSO or 753B (0.111 μM) for 4 h. Immunoprecipitation was carried out with MCL-1 antibody and detected with anti-BIM. (D) Column graph showing BH3 profiling of OCI-AML-2 cells treated with DMSO or 0.111 μM 753B for 4 h. (E) Correlation analyses of 753B IC_{50} values and the protein change levels of MCL-1, BCL-X_L and BCL-2 in ten AML cell lines. The changed level of certain protein (MCL-1, BCL-X_L or BCL-2) = baseline protein expression level quantified from untreated samples - protein expression level quantified from samples treated with 753B after 24 h at 1 μM. Non-parametric one-tailed Spearman test was used to determine the correlation coefficient r. (F) Heatmap of cell viability of OCI-AML-2 cells treated with 753B, S63845 (MCL-1 inhibitor), or the combination at the indicated concentrations, determined by CTG assay. Data are representative of three independent experiments. **P*<0.05; ***P*<0.01. NS: not significant.

protonated state, flexibility, as well as the other residues around the lysine involved in the E2/POI interface would also be key factors contributing to the difference in lysine ubiquitination effectiveness between BCL-X_L and BCL-2. These differences may render BCL-X_L more susceptible to 753B-induced protein ubiquitination and degradation than BCL-2. The detailed mechanisms and optimization of the dual degradation will require future studies.

753B induced rapid dose-dependent BCL-X_L and BCL-2 degradation in leukemia, which translated into inhibition of cell growth and apoptosis. This effect was observed in 11 BCL-2/BCL-X_L co-dependent cell lines (2/2 MPN-AML and 9/17 AML cell lines including the high-risk EVI-1 rearranged cells) and in the majority of the primary AML samples harboring high-risk mutations (such as *FLT-ITD*, *TP53* and *NRAS*), exhibiting a broader activity than venetoclax. Notably, 10 out of 16 samples were collected from patients, whose disease progressed after venetoclax-containing regimens, and all ten samples responded *in vitro* to navitoclax and 753B. These data indicate a potential utility of BCL-X_L-targeting agents in overcoming resistance to venetoclax. 753B showed preliminary *in vivo* efficacy in AML-derived PDX harboring *FLT3-ITD*, *DNMT3A*, and *IDH1* mutations. We noted the rebound in hCD45 cellularity after the last week of 753B treatment and assume that the rebound of leukemia could result from MCL-1 upregulation or due to a sub-optimal dose of 753B used in the *in vivo* study. Efficacy of 753B or optimized next generation degrader should be tested in additional AML PDX models with diverse genetic backgrounds in future studies. With further optimization of formulation and dosing, 753B has the potential to become the first-in-class platelet-sparing BCL-X_L/BCL-2 targeting antitumor agent.

Chemotherapy works in part by inducing cellular senescence; immune cells then clear senescent cancer cells, leading to tumor stasis or regression. However, these senescent cells can escape from immune surveillance and re-enter the cell cycle after escaping from a resting state with accelerated potential for growth.[18,35,36] These cells can acquire novel stem-cell and self-renewing features, promote disease relapse, and enhance aggressiveness, as evidenced in several mouse tumor models, including AML.[27] Recent studies identified navitoclax and other BCL-X_L/BCL-2 inhibitors (ABT-737 and A1331852) as potent senolytics.[21,37,38] Because BCL-X_L/BCL-2 are key anti-apoptotic proteins in many types of senescent cells, we hypothesized that 753B may eliminate senescent cells before escaping from senescence. In our study, we found that 753B largely reversed Ara-C-induced senescence markers and enhanced the efficacy of Ara-C by targeting senescent AML cells, suggesting an added benefit from targeting BCL-X_L/BCL-2 with 753B in combination with chemotherapy for AML treatment. This finding provides a rationale for sequential treatment of chemotherapy followed by 753B, which could reduce the toxicity of concomitant administration, yet avoid relapse by eliminating chemotherapy-induced senescent tumor cells.

Our mechanistic studies suggest that the sensitivity of AML cell lines to 753B inversely correlates with high MCL-1 expression. MCL-1 expression commonly accounts for resistance to other BH3 mimetics, including venetoclax.[39,40] Our data indicate that 753B has a strong synergistic effect on inducing cell death in MOLM-14 cells when combined with a small molecule inhibitor targeting MCL-1 (Figure 5F). However, the synergy of 753B and MCL-1 inhibitors should be tested in additional cells lines or xenograft models in future studies. Notably, co-administration of MCL-1 inhibitor (VU661013) and BCL-2 inhibitor has been reported to have a reasonable safety profile in AML xenografts models.[41] Besides, co-targeting BCL-X_L and MCL-1 with DT2216 and AZD8055 showed a synergistic effect on inhibiting small-cell lung cancer growth without causing on-target toxicities in mice.[42] On the contrary, the simultaneous use of MCL-1 and BCL-X_L inhibitors caused acute lethality in mice in lung squamous cell carcinomas and pancreatic cancer *in vivo* models.[43,44] Since 753B is a BCL-2/BCL-X_L co-targeted compound, the safety of directly combining an MCL-1 inhibitor with 753B will require careful *in vivo* safety studies in future experiments.

Recognition of the important mechanistic role of BCL-X_L in leukemias sparked renewed interest in therapeutically inhibiting BCL-X_L. In a recently reported phase I study

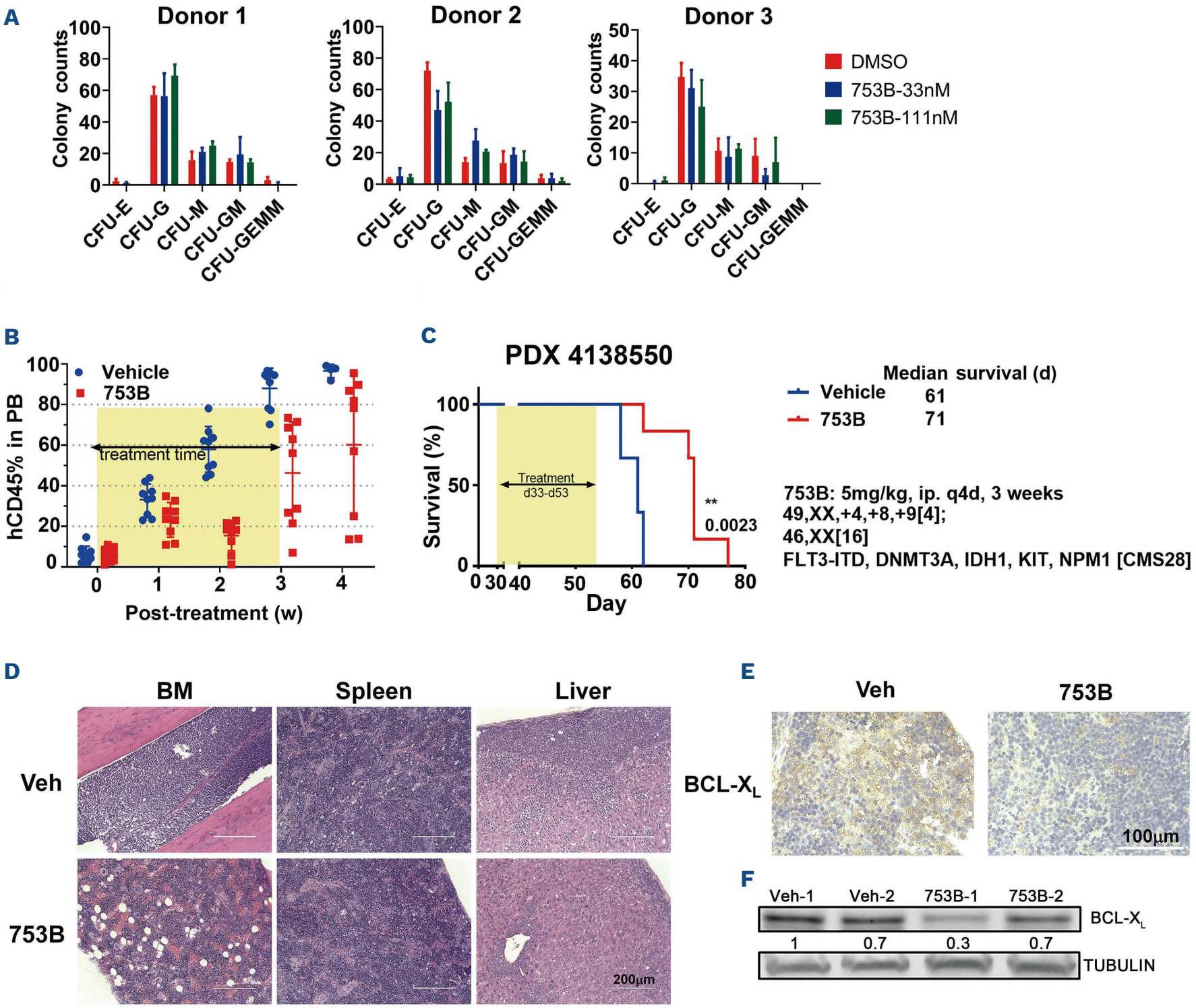

Figure 6. Anti-leukemia efficacy of 753B *in vivo* in acute myeloid leukemia patient-derived xenograft model. (A) CD34⁺ bone marrow (BM) cells were sorted from three individual donors (Donor 1-3) by FACS and colony formation was analyzed for CFU-E, CFU-G, CFU-M, CFU-GM or CFU-GEMM cells treated with indicated concentration of 753B. (B) Circulating tumor burden in an acute myeloid leukemia (AML) patient-derived xenograft (PDX) model (#4138550) treated with vehicle (Veh) or 753B (treatment started on day 33 after AML cell injection and was administered intraperitoneally at a dose of 5 mg/kg, every 4 days for 3 weeks). (C) Kaplan-Meier survival curves for mice treated as described in (A) (N=6/arm). Statistical significance was calculated using the log rank (Mantel-Cox) test (P<0.01). (D) Representative Hematoxylin & Eosin (HE) stained sections of BM, spleen and liver from 753B or vehicle-treated mice. Scale bar on the bottom right indicates 200 μm. (E) Immunohistochemical staining of BCL-X_L in an AML PDX model from 753B or vehicle-treated mice. Scale bar on the bottom right indicates 100 μm. (F) Western blotting showing BCL-X_L protein levels in an AML PDX model (N=2 mice in each group). DMSO: dimethyl sulfoxide; PB: peripheral blood; d: day; w: week.

(clinicaltrials. gov identifier: NCT03181126), co-targeting BCL-X_L and BCL-2 by combination of reduced doses of navitoclax and venetoclax showed promising efficacy in patients with relapsed or refractory ALL who progressed after multiple lines of therapy.[45] The use of navitoclax as an "add-on" approach to the JAK2 inhibitor ruxolitinib in patients with myelofibrosis produced objective responses in a phase II clinical study (clinicaltrials. gov identifier: NCT03222609), although careful dose titration of navitoclax was required due to an overall decrease in platelet counts.[46] AZD0466 is a nanoparticle-formulated novel dual BCL-X_L/BCL-2 inhibitor that is currently in a phase I clinical trial for hematologic malignancies (clinicaltrials. gov identifier: NCT04214093) and it is pharmacologically optimized to minimize thrombocytopenia.[47] In contrast, 753B is a first-in-class dual BCL-X_L/BCL-2 degrader that avoids platelet toxicity due to selective expression of VHL E3 ligase. Because 753B is derived from navitoclax, 753B

is expected to be a safe strategy to target both BCL-2 and BCL-X_L in AML, without resultant thrombocytopenia.
Taken together, our study demonstrates that the single-agent 753B has an anti-tumor activity in a subset of hematologic cell lines, primary patient-derived AML blasts, and a xenograft model via BCL-2 family protein degradation. We demonstrated that the senolytic properties of 753B may enhance the efficacy of chemotherapy by targeting BCL-X_L-expressing senescent cells. Importantly, the first-in-human dose escalation study of BCL-X_L PROTAC DT2216 has opened for patient accrual (clinicaltrials. gov identifier: NCT04886622). We anticipate that the clinical use of PROTAC based on our informative pre-clinical findings will lead to safe and effective co-targeting of BCL-2 and BCL-X_L in AML.

Disclosures

GZ and DZ are co-founders of and have equity in Dialectic Therapeutics, which develops BCL-XL PROTAC to treat cancer. The other authors have no conflict of interests to disclose.

Contributions

YJ conceived, designed and performed most of the biological and biochemical experiments, analyzed and interpreted data, and wrote the manuscript. LH, CR, ZW, CW, LY, SC and HM performed and analyzed some of the biological experiments. WZ, MA, ND, NJ, NP, KB and SM supervised some of the biological studies and revised the manuscript. PZ synthesized 753B, and prepared the formulation of vehicle and 753B for animal study. GZ conceived, designed and supervised the synthesis of BCL-XL/2 PROTAC, and revised the manuscript. DZ conceived, designed and supervised the synthesis of BCL-XL/2 PROTAC, revised the manuscript, and guided senescence studies. QZ and MK conceived, designed, and supervised the study, analyzed and interpreted data, and wrote the manuscript. All authors discussed the results and commented on the manuscript.

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Data-sharing statement

Original data and protocols are available to other investigators upon request by contacting the corresponding author or last author.

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