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LOR-253 Overcomes Resistance to ABT-199 by Targeting MTF1 in AML

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Abstract **Introduction:** Chemoresistance is one of the major challenges for the acute myeloid leukemia (AML) treatment. Venetoclax (ABT-199), a selective small molecule BCL-2 inhibitor, is being clinically vetted and is an effective therapy for some B-cell lymphomas, yet many patients who initially respond to ABT-199 develop resistance. Thus, enhancing the sensitivity of resistant cells to chemotherapy is a great interest to clinical trial. **Method:** The resistant cell lines were generated by culturing in the medium containing ABT-199. CCK8 analysis was used to detect the cell viability. Flowcytometric analysis with Annexin-V/PI was used to test the apoptosis. CRISPR/Cas9 by lentivirus delivering well-validated shRNAs in pLKO.1 vector was used to knockout the expression of MTF1. Western blot with the antibodies was used to determine the expression of the molecules. Clonogenic growth assay was used to determine the growth of parental and DTEP cells. **Results:** Here we report that resistance to the BCL-2 targeting drug ABT-199 in AML cell lines evolves from outgrowth of persister clones harbor BCL2. Furthermore, persister status is generated via adaptive super-enhancer remodeling that reprograms transcription and offers opportunities for overcoming ABT-199 resistance. Notably, pharmacogenomic screens revealed that persisters are vulnerable to inhibition of metal regulatory transcription factor 1 (MTF1), which is essential for the transcriptional reprogramming that drives and sustains ABT-199 resistance. **Conclusion:** LOR-253, which is a MTF1-targeting agent, add novel insights to overcome ABT-199-resistance in B-cell lymphomas.

Keywords: *acute myeloid leukemia, ABT-199, resistance, MTF1, transcriptional reprogramming*

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1. Introduction

Acute myeloid leukemia (AML) is an aggressive haematopoietic malignancy, characterized by the expansion of progenitor myeloid cells within the bone marrow and peripheral blood [1,2,3]. It is the most common form of leukaemia among adults with 4.3 per 100000 every year of incidence after adjusting age in United States [4]. Chemotherapy is the main treatment for AML [5], but chemoresistance has promoted to explore new therapies to enhance chemosensitivity or restrain chemoresistance [6].

BCL-2 has major roles as an anti-apoptotic protein in hematological malignancies. In particular, B cell lymphomas, such as mantle cell lymphoma (MCL) and double-hit lymphoma (DHL) often have dysregulated BCL-2 and are addicted to this oncoprotein to variable degrees [7]. Venetoclax (ABT-199), a novel, potent, and selective small molecule BCL-2 inhibitor, is being clinically vetted and is an effective therapy for some B-cell lymphomas [8,9]. Indeed, ABT-199 has the potential to be the standard of care for B-cell lymphomas, including MCL, yet many patients who initially respond to ABT-199 develop resistance [10,11]. Thus, there is an urgent need to define the mechanisms of ABT-199 resistance.

Emerging evidence suggests that, to evade drug pressure, small subpopulations of cancer cells entered a largely quiescent drug-tolerant "persister" (DTP) state after drug treatment. Furthermore, some DTP cells can then expand in the presence of drug to become drug-tolerant expanded persisters (DTEP) [12]. Importantly, DTP cells survive and serve as the reservoir for the expansion of subpopulations of cells, maintain resistance after therapy and then expand and lead to relapse. Moreover, several studies have demonstrated that identified extremely large enhancer domain termed super-enhancer (SEs), which were identified based on epigenetic modification such as histone H3 lysine 27 acetylation (H3K27ac). SEs also specifically regulate genes associated with cell identity and disease, including oncogenes. Accordingly, finding new strategies that disable DTP and the emergence of DTEP would have a major impact in the clinic.

Amounting researches have indicated MTF1 is overexpressed in lung, colorecta, breast and cervical cancers, implying a vital effect on malignancy [13]. A study of advanced stage head and neck carcinoma have

shown that MTF1 can be acted a biomarker to predict disease recurrence. MTF1 is a highly conserved zinc (Zn)-dependent transcription factor containing six zinc finger domains, which initially expressed in the cytoplasm in normal conditions while it translocates into the nucleus when activated by redox stress and cytokines. It plays an important role in maintaining metal homeostasis by binding to the metal-responsive elements (MREs) to regulate gene transcription, including zinc efflux protein ZnT-1, zinc influx regulator ZIP-1, matrix metalloproteinases (MMPs), and metal binding protein metallothionein (MT1) [14]. A study of advanced stage head and neck carcinoma have shown that MTF1 can be acted a biomarker to predict disease recurrence. However, the role of MTF1 in AML is still unknown.

In this study, we constructed an anti-tumor metabolic compound library which customized 50 compounds approved by Food and Drug Administration (FDA), and performed the CCK8 to screen out compounds that can affect the survival rate of ABT-199 resistant cell lines on a large scale. Here, we screened that LOR-253, a small molecule inhibitor of MTF1, could inhibits the viability of ABT-199-resistant cell lines via targeting MTF1 in AML models.

2. Materials and Methods

2.1. Animals

Six- to eight-week-old male NOD SCID gamma (NSG) mice were purchased from Jackson Laboratory and were subcutaneously injected with 0.7 million ABT-199-resistant MOLM-13 cells. After confirmation of engraftment experiments, mice were randomized to four groups (8 mice per group) and then treated with vehicle, ABT-199(100 mg/kg), LOR-253 (50 mg/kg) or the combination daily by oral gavage. ABT-199 and LOR-253 was dissolved in DMSO and further diluted in 5% DMSO + 5% Tween 80+50% 0.9% NaCl + 40% PEG 300. All animal studies were performed according to protocols approved by Fudan University.

2.2. Generation of ABT-199 Drug-Resistant Cell Lines

MOLM-13 and MV4-11 parental cells were cultured in the RPMI-1640 medium supplemented with 10% fetal bovine serum and were seeded into 96-well collagen-coated plates (4,000 cells per well) for 24 h. After culturing in the medium containing ABT-199 with initiation at IC50 dose, only a few isolated cells were on survival. Every 2 days, the cells were resuspended in fresh medium containing ABT-199. Then cell viability was detected by CCK8. When cell viability was ≥90%, the ABT-199 dose doubled to obtain more than 30-fold drug-resistant cells compared with their parent cells.

2.3. Western Blot

Cells were harvested and subjected to lysis in RIPA lysis (Beyotime, Shanghai, China) supplemented with protease and phosphatase inhibitor cocktail. The protein was separated by SDS-PAGE on a 10% or 12% polyacrylamide gel. Then the protein was transferred to a PVDF membrane at 250 mA. Next, the membrane was blocked at room temperature in 5% fat-free milk and incubated at 4 ℃ overnight with the following primary antibodies: Bcl-2 (sc-7382), Mcl-1 (sc-819) from Santa Cruz Biotechnology; Bax (50599-2-Ig), β-Actin (66009-1-Ig) and MTF1 (25383-1-AP) from Proteintech. MYC (ab32072) from Abcam, CKD7 (2916) from Cell Signaling Technology. Then, the membrane was washed in the TBST and probed with secondary antibodies (Proteintech, China). Finally, the membrane was imaged by imaging system (Tanon, Shanghai, China).

2.4. CRISPR/Cas9 Editing of MTF1

MTF1 were knocked down by lentivirus delivering well-validated shRNAs in pLKO.1 vector. gRNAs targeting MTF1 or GFP were cloned into a vector encoding espCas9. gRNA sequences targeting MTF1 are 5'-GCCATTTGAGTGTGACGTGC-3' and 5'-CCTTCGTGTGCACTCGCACG-3'. Briefly, lentivirus was generated by co-transfection of HEK293T cells with packaging vectors pMD2.G (Addgene) and transfer vector psPAX2 using Lipofectamine 3000 transfection reagent according to the manufacturer's instructions (ThermoFisher, USA). Forty eight hours after transfection, lentiviral supernatants were harvested and centrifugated at a speed of 800 g for 10 min, room temperature. Then the supernatant was filtered to remove cells debris. 8 μg/mL Polybrene (Chemicon, Temecula, CA, USA) and the virus stock was added to infect cells. After 3 days, infected cells were selected with 0.5 μg/mL puromycin (Invitrogen, USA). Cell clones were obtained and verified the knockdown efficiency by Western Blot using MTF1 antibodies.

2.5. CCK8 Assay

The cytotoxicity effect of LOR-253 were assessed using the CCK8 (Sigma, USA) as instructed by the manufacturer. Briefly, MOLM-13 and MV4-11 cells were placed into 96-well plates at a density of 2.5×10^4 cells/well. Then cells were incubated with indicated concentration of LOR-253 for designed time, while control cells were treated with 0.1% dimethyl sulfoxide. Subsequently, cells were added with 10 μl of cell counting kit-8 stock solution and incubated for 4 h at 37°C. A microplate reader was used to measure absorbance at 450 nm. All experiments were performed three times and each experiment contained three replicates.

2.6. Flow Cytometric Analysis of Apoptotic Cells

Flow cytometry analysis for cell phenotype and apoptosis. Cells were incubated for 25 min at 4°C with fluorescein-conjugated monoclonal Abs in labeling solution. Fluorescein-conjugated isotype controls were used to establish the background. Flow cytometry analyses were conducted on Fortessa X20 (BD Biosciences). Data was analyzed with FlowJo 7.6 software.

2.7. Statistical Analysis

Data are shown as mean ± SD and are representative of three independent experiments.

3. Results

3.1. Evolution of ABT-199-resistant Cells in AML Models

To define the mechanisms of drug resistance in AML, we used MV4-11 and MOLM-13, two AML cell lines sensitive to the ABT-199 to model the response to ABT-199. The vast majority of parental MV4-11 and MOLM-13 cells ((MOLM-13-P and MV4-11-P) were killed with exposure to ABT-199 in a dose and time dependent manner. However, very marginal apoptosis happened in the surviving resistant MOLM-13 and MV4-11 cells (MOLM-13-R and MV4-11-R, Figure 1A and Figure 1B). The IC50 values from Figure A were calculated based on live cell numbers (Table 1). The ABT-199 IC50 increased by more than 14- and 30-fold in MOLM-13-R and MV4-11-R cells, respectively. Compared with parental cells, the resistant cells expressed increased levels of MCL-1 and unchanged levels of Bcl-2 (Figure 1C), indicating that the elevated expression of MCL-1 at least partly contributed to the resistance to ABT-199 in the surviving cells. Consistently with the results, flowcytometric analysis was performed to confirm the apoptosis of the parental and resistant MOLM-13 and MV4-11 cells, the data showed that the percentage of early apoptotic cells (Annexin-V-positive) was comparable in parental and resistant cells (Figure 1D). To test if resistance was a stable phenotype, DTEP cells were cultured without ABT-199 for 6-8 weeks and then retested for their sensitivity to the drug. The slope of the drug-sensitivity curves revealed that subpopulations of these "drug holiday" (dh) DTEP cells partially re-acquired sensitivity to ABT-199 (Figure 1E). Therefore, rewired signaling programs that alter the expression of apoptotic regulators are associated with ABT-199 resistance in MCL models.

Table 1. The IC50 values were calculated with Calcusyn software based on live cell numbers

Cell	IC50
MOLM-13-P	56
MV4-11-P	49
MOLM-13-R	>800
MV4-11-R	>1500

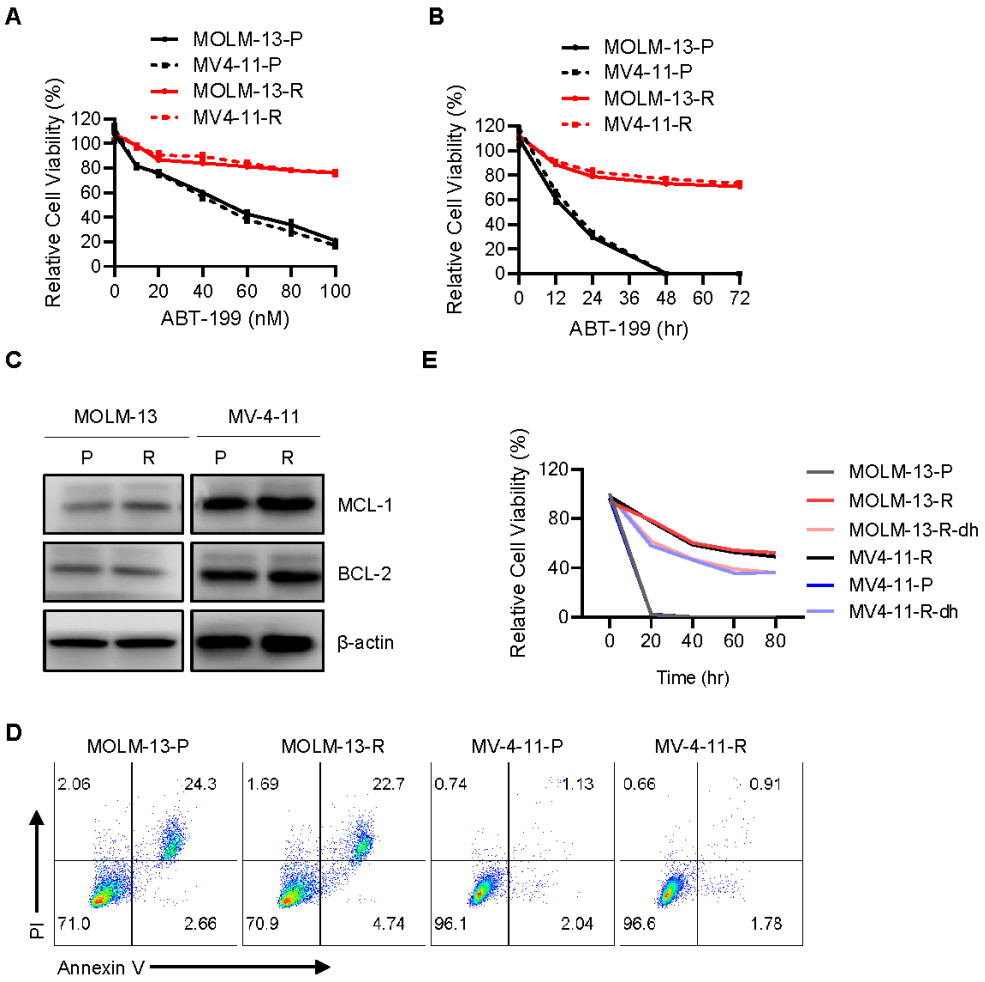

Figure 1. Phenotypes of ABT-199-resistant AML cell lines. (A and B) CCK8 analysis of relative cell viability in parental MOLM-13 and MV4-11 cells and DTEP cells treated with ABT-199 in dose- (72 hr) (A) or time-(100 nM) (B) response manner with technical replicates at each indicated dose. (C) Western blot analysis of the protein level of MCL-1 and BCL-2 in parental and resistant MOLM-13 and MV4-11 cells. (D) Flowcytometric analysis (FACS) of apoptosis in parental and resistant MOLM-13 and MV4-11 cells. (E) CCK8 analysis of relative cell viability in parental cells, DTEP cells, and (''drug holiday'' [dh]) DTEP cells without ABT-199 treatment for 6-8 weeks to ABT-199 (100 nM)

3.2. MTF1 is a Vulnerability for ABT-199-Resistant AML Models

To identify effectors that drive and sustain DTEP, a drug sensitivity screen was performed on MOLM-13-R and MV4-11-R cells and their parental cells using a panel of 50 small molecule inhibitors and epigenetic drugs. We found DTEP cells were most vulnerable to LOR-253, a metal regulatory transcription factor 1 (MTF1) inhibitor that controlling the expression of a raft of metallothioneins. Low doses of LOR-253 rapidly compromised the survival of all DTEP cells, yet had only modest activity against MOLM-13-R and MV4-11-R cells (Figure 2A-2D). Similarly, low doses of LOR-253 abolished the colony-forming capacity of DTEP cells in methylcellulose, but had no effect on MOLM-13-P and MV4-11-P cells (Figure 2E).

MTF1 heads a hierarchy of zinc sensors, and through controlling the expression of a raft of metallothioneins and other key proteins involved in controlling intracellular zinc levels (e.g. ZnT1) alters zinc buffering capacity and total cellular zinc content. Furthermore, LOR-253 treatment downregulated the expression of MCL-1, cell proliferation associated-genes, such as CKD7 and Myc, increased in resistant cells, but LOR-253 treatment upregulated the expression of cell apoptosis associated-genes, such as BAX and BAK decreased in resistant cells (Figure 2F).

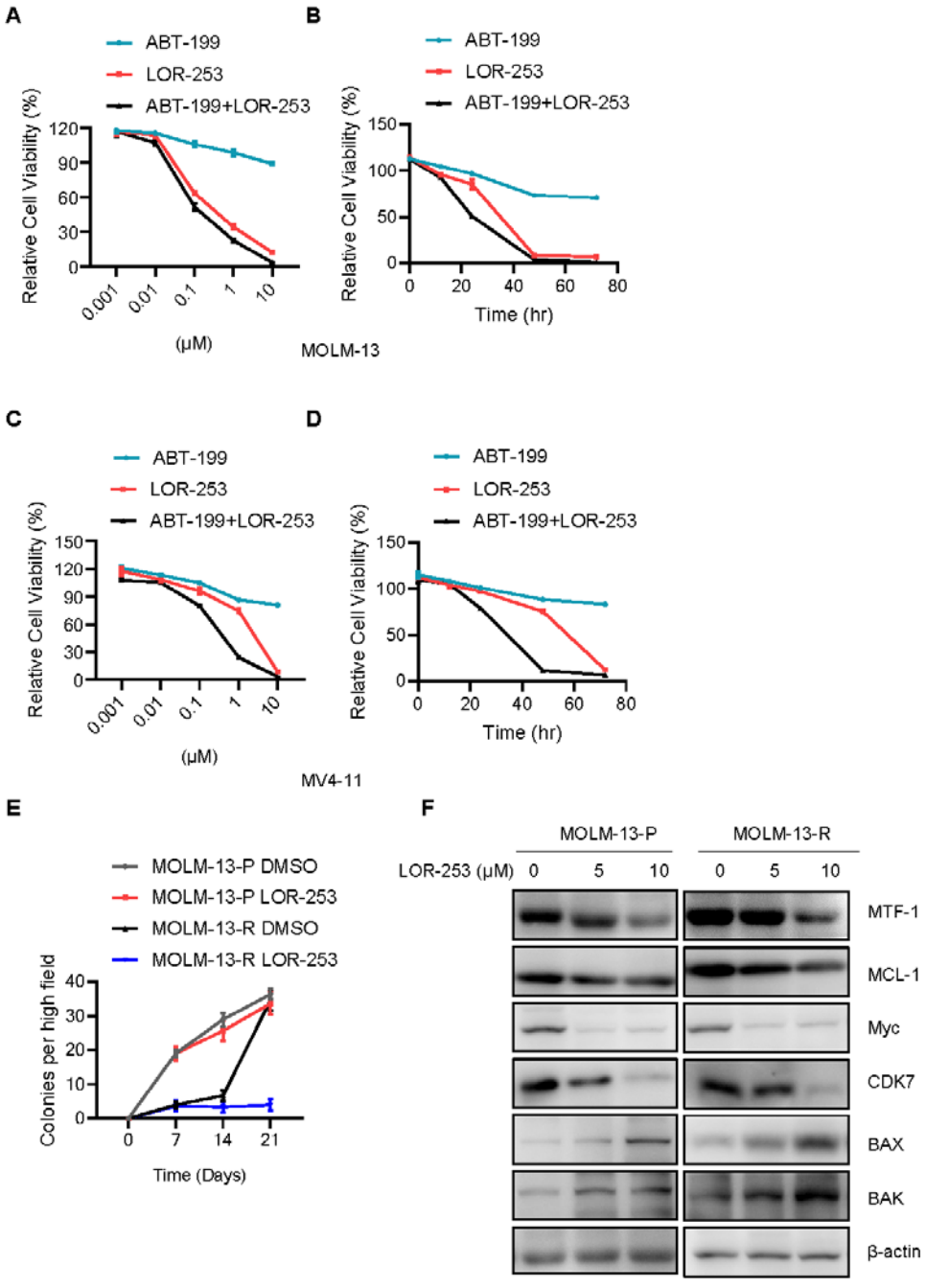

Figure 2. MTF1 is a vulnerability for ABT-199-resistant AML models. (A to D) CCK8 analysis of relative cell viability in MOLM-13-R (A and B) and MV4-11-R (C and D) cells and DTEP cells treated with ABT-199 and LOR-253 (5 μM) in dose- (72 hr) (A and C) or time-(100 nM) (B and D) response manner with technical replicates at each indicated dose. (E) Clonogenic growth assay of parental and DTEP cells treated with DMSO or LOR-253 (5 μM) for the indicated time points. (F) Western blot analysis of the protein level of indicated proteins in parental and resistant MOLM-13 cells treated with LOR-253 (5 μM and 10 μM) for 2 h.

To test this in vivo, we injected the MOLM-13-R cells into NOD SCID gamma (NSG) mice. The mice were randomly divided to four groups (n = 8/group) and treated with vehicle, ABT-199, LOR-253, or both ABT-199 and LOR-253. The mice bearing resistant MOLM-13 cells showed sensibility to LOR-253 treatment compared with resistance to ABT-199 treatment. Furthermore, the ABT-199 + LOR-253 combination showed superior tumor regression in MOLM-13-R-derived xenografts (Figure 3A). After injection, MOLM-13-R cells quickly killed vehicle and ABT-199-treated mice approximately at the same time However, LOR-253 increased survival by 6 days, while the combination apparently prolonged survival by 14 days (Figure 3B). These results indicated that LOR-253 overcome the ABT-199 resistance of MOLM-13-R cells in vitro and in vivo. Notably, the ABT-199+LOR-253 combination had superior efficacy in tumor regression.

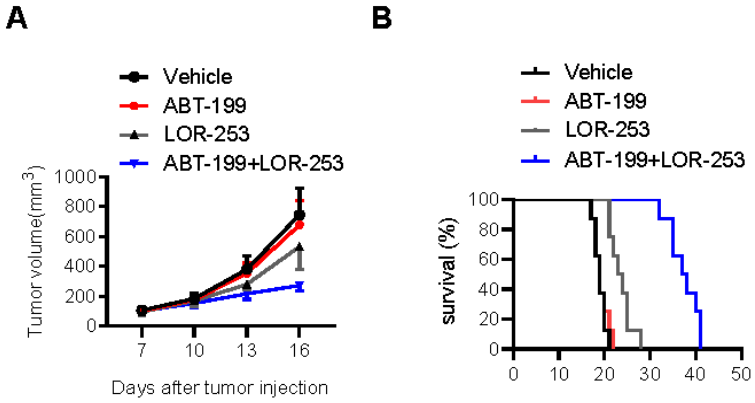

Figure 3. ABT-199 combination with LOR-253 suppresses the tumor growth. (A and B) Tumor growth (A) and survival curves (B) in Vehicle-, ABT-199-, LOR-253- or ABT-199 + LOR-253-treated mice after MOLM-13-R inoculation (n = 8 mice per group)

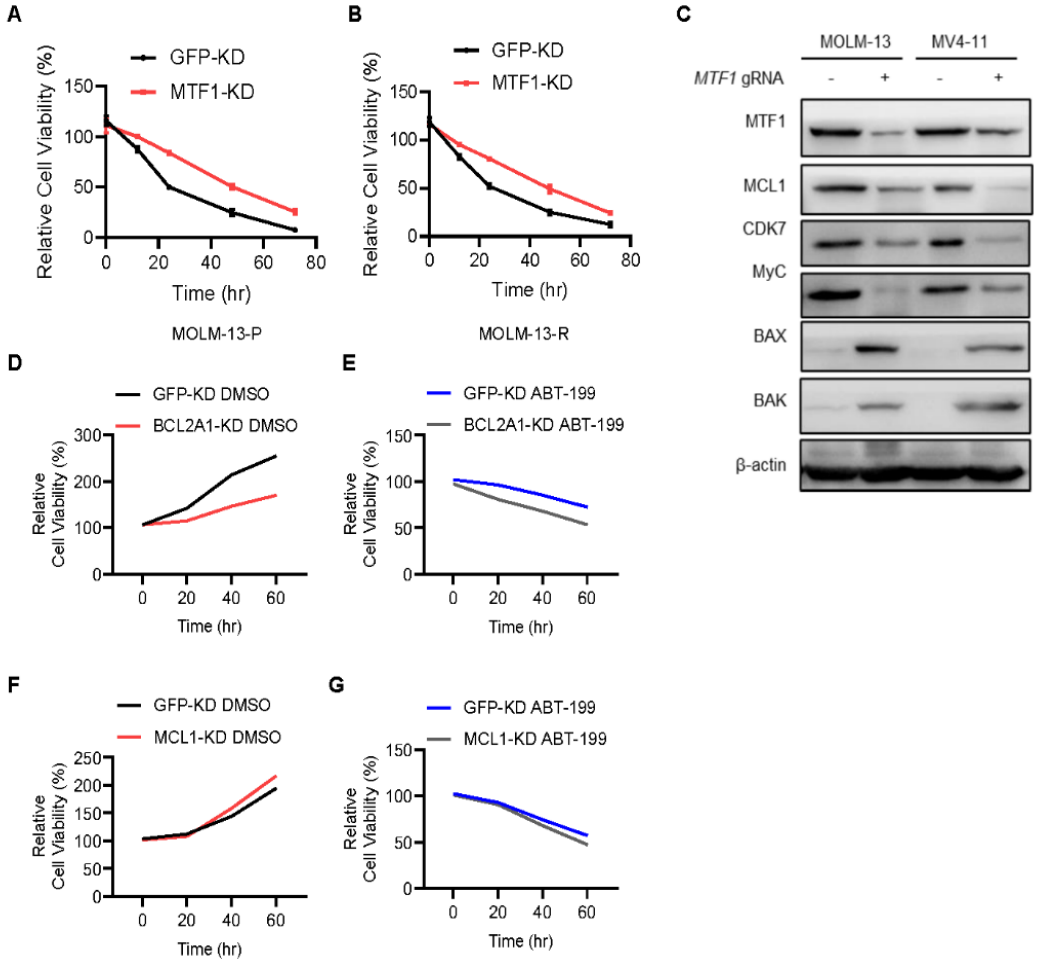

Figure 4. MTF1 is necessary to sustain SE-Driven transcriptional reprogramming in ABT-199-resistant AML cells. (A and B) CCK8 analysis of relative cell viability of control (GFP) or MTF1 knockdown derivatives of MOLM-13-P (A) and MOLM-13-R (B) cells treated with LOR-253 (5 μM) with technical replicates at each indicated dose. (C) Western blot analysis of the protein level of indicated proteins in resistant MOLM-13 and MV4-11 cells treated with MTF1 gRNA knockdown. (D-G) CCK8 analysis of relative cell viability in MOLM-13-R cells of control (GFP) or BCL2 (D and E), MCL1 (F and G) knockdown treated with DMSO (D and F) or ABT-199 (10 μM) (E), (3.3 μM) (G) with technical replicates at each indicated dose

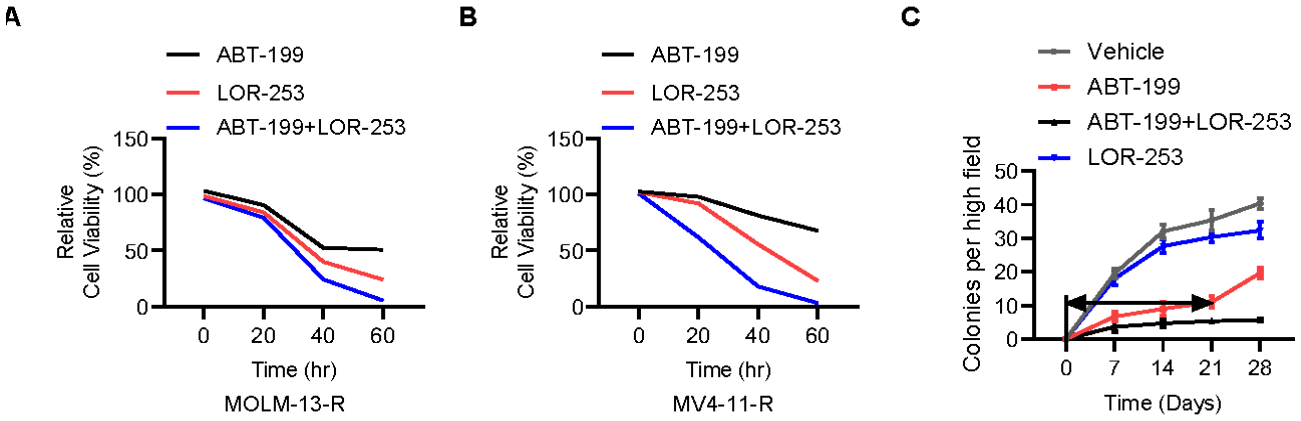

Figure 5. Dual MTF1/BCL-2 inhibition disables maintenance of ABT-199 resistance. (A and B) CCK8 analysis of relative cell viability in MOLM-13-R (A) or MV4-11-R (B) cells treated with ABT-199, LOR-253 or ABT-199 + LOR-253 with technical replicates at each indicated dose. (C) Clonogenic growth assay of parental MOLM-13-R cells was assessed at the indicated intervals following treatment with vehicle, ABT-199 (20 nM), LOR-253 (5 μM), or ABT-199 + LOR-253 (20 nM and 5 μM, respectively) for the indicated time points.

3.3. MTF1 is Necessary to Sustain SE-Driven Transcriptional Reprogramming in ABT-199-resistant AML Cells

To confirm the effects of MTF1 were on target, CRISPR editing was used to knock down MTF1 in DTEP and parental AML cells. As predicted, transient MTF1 knockdown attenuated the cytotoxic effects of MTF1 treatment on these cells (Figure 4A and Figure 4B). Furthermore, MTF1 knockdown led to reductions in expression of the SE-associated regulators MCL1, CDK7 and Myc upregulated in DTEP cells and rise in expression of the apoptosis associated-genes BAX and BAK downregulated in DTEP cells (Figure 4C). Individual knockdown of IRF5, FOXC1, and MCL-1 had little-to-modest effects on DTEP growth or survival, whereas BCL2A1 knockdown effectively blocked DTEP cell proliferation (Figure 4D-4G). Loss of the DTEP-associated gene was sufficient to restore the sensitivity of at DTEP cells to ABT-199 (Figure 4D–4G). Thus, these SE-driven transcriptional targets converge and cooperatively contribute to ABT-199 resistance in this AML model.

3.4. Dual MTF1/BCL-2 Inhibition Disables Maintenance of ABT-199 Resistance

Given the essential role of BCL-2 in AML, MTF1-dependent transcriptome reprogramming, and the low basal levels of BCL-2 still manifest in DTEP, we evaluated the effects of combined ABT-199 + LOR-253 treatment in DTEP lines. There were clear synergistic effects of combined ABT-199 + LOR-253 in two resistant cells (Figure 5A and Figure 5B). To assess if MTF1 inhibition would block the emergence of ABT-199 resistance, parental MOLM-13-R cells were cultured in the presence or absence of high doses of ABT-199 and assessed for anchorage-independent colony formation. As expected, ABT-199 treatment triggered initial growth suppression, and this was then followed by the growth of drug-resistant DTEP colonies (Figure 5C). In contrast, LOR-253 co-treatment abolished the emergence of ABT-199-resistant DTEP colonies (Figure 5C). Thus, LOR-253 activity is also necessary for the emergence of ABT-199 resistance in this AML model.

4. Discussion

The results of this study demonstrated that inhibition of MTF1 expression in ABT-199-resistant AML cell lines reduces the proliferation of ABT-199-resistant cell in vitro and in vivo. Accumulating studies have shown that adaptive SE-associated transcriptional programs promote resistance of solid tumors to targeted therapies, and that MTF1 inhibition disrupts these programs to provoke a therapeutic response [15,16]. While our present study revealed a similar adaptive MTF1-dependent, SE-associated transcriptional reprogramming as a novel mechanism of drug resistance to ABT-199 in AML cell lines. Moreover, combined treatment with an MTF1 inhibitor and ABT-199-resistant cell lines significantly increased ABT-199-induced AML cell death. Recent studies showed that resistance to ATB-199 is associated with upregulation of MCL-1 [17], consistent with this study, we identified and established the roles of reprogrammed SE-associated genes needed to sustain ABT-199 resistance. Excitingly, ABT-199 + LOR-253 combination therapy is now shown to prevent the ABT-199 resistance in aggressive lymohoma models and to terminate drug-resistant cells.

Because of the high levels of BCL-2 protein, the majority of MCL cells may induced apoptosis after ABT-199 treatment [18,19]. However, small subsets of these DTP cells will survive, and some of which then survive and expand as ABT-199 DTEP cells. Previous studies have showed that loss of the 19q21 amplicon is not a requisite for ABT-199 resistance by analyzing the ABT-199-resistant cells from single cells, and DTEP clones can be derived from cells that retain the amplicon [17]. However, due to ABT-199-dependent selection for loss of apoptotic regulators, the independent DTEP derivates having distinct deletions or reductions indicates that these rare clones could be the most fit in the population, and that such clones would likely contribute to ABT-199 resistance in relapsed patients. Together, these results demonstrate that once ABT-199 achieves clinical approval for treating B-cell lymphomas, it will be critical to assess if the mechanism is manifest in resistant B-cell lymphomas.

Amounting evidence have showed that LOR-253 induced KLF4 expression and enhances apoptosis induced by cisplatin in both SKOV3 and OVCAR3 cells [20]. LOR-253 also causes DNA damage in Raji cells or in MV4-11 cells. Recent studies have demonstrated that LOR-253 suppresses the proliferation of AML cell lines and various forms of lymphoma cell lines, causes G0/G1 cell cycle arrest, induces apoptosis, and down regulates MYC RNA and protein expression in AML lines [21]. However, there remains unknown in ABT-199 resistant lymphoma cells. Our study firstly revealed that LOR-253 overcomes the sensitivity of lymphoma cells to ABT-199 treatment, supporting that LOR-253 is a hopeful clinical combination agent in ABT-199-resistant AML patients.

Abbreviations

AML: acute myeloid leukemia
MTF1: metal regulatory transcription factor 1
MCL: mantle cell lymphoma
DHL: double-hit lymphoma
DTP: drug-tolerant persister
DTEP: drug-tolerant expanded persisters
H3K27ac: histone H3 lysine 27 acetylation
MRE: metal-responsive elements
MMP: matrix metalloproteinases
MT1: metal binding protein metallothionein
FDA: Food and Drug Administration

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Statement of Competing Interests

The authors have no competing interests.

Availability of Data

All data obtained are transcribed in this manuscript. For ethical and legal reasons individual data collected could not be made publicly available.

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