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Original Article

mTOR inhibition by AZD2014 alleviates BCR::ABL1 independent imatinib resistance through enhancing autophagy in CML resistant cells

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Abstract: Chronic myeloid leukemia (CML) is a common hematopoietic malignancy in adults. Great progress has been made in CML therapy with imatinib. However, resistance to imatinib may occur during treatment. BCR::ABL1 dependent imatinib resistance has been well resolved with more potent tyrosine kinase inhibitors, but BCR::ABL1 independent resistance still remains to be resolved. This study is devoted to find novel targets for BCR::ABL1 independent imatinib-resistant patients. It is reported BCR::ABL1 independent resistance is mainly related to the activation of alternative survival pathway, and mTOR is an important regulator for cell growth especially in tumor cells. Hence, we explored the role of mTOR in BCR::ABL1 independent resistance, the possibility of mTOR to be a therapeutic target for imatinib resistant patients and the related mechanism. We found mTOR was upregulated in imatinib-resistant cells. mTOR inhibition by AZD2014 led to growth inhibition and synergized with imatinib in apoptosis induction in K562/G01. AZD2014 exerted its anti-leukemia effect through enhancing autophagy. mTOR signal pathway is poorly inhibited by imatinib and AZD2014 shows little effect on BCR::ABL1 signal pathway, which indicates that mTOR is involved in imatinib resistance via a BCR::ABL1 independent manner. Taken together, mTOR represents a potential target to overcome BCR::ABL1 independent imatinib resistance.

Keywords: mTOR, AZD2014, autophagy, chronic myeloid leukemia, imatinib resistance

Introduction

Chronic myeloid leukemia (CML) is a common hematopoietic malignancy in adults. This disease is manifested as an increased and uncontrolled growth of myeloid cells in the bone marrow, which then accumulate in the peripheral blood [1, 2]. The pathogenesis is attributed to BCR::ABL1 fusion gene, which is originated from the translocation of t(9;22)(q34;q11) [3] and encodes BCR::ABL1 oncoprotein with constitutive activated tyrosine kinase activity, which can activate diverse signal pathway of pro-proliferative and anti-apoptosis, such as RAS/MAPK, PI3K/AKT/mTOR, and JAK/STAT [4]. Imatinib, a tyrosine kinase inhibitor (TKI) targeted BCR::ABL1, is recommended as the first-line drug for CML therapy [5]. About 70% CML patients gets benefit from imatinib, but the other 30% responds not so well due to resistance or intolerance to imatinib [6]. The mechanisms of imatinib resistance have been identified and can be sorted into BCR::ABL1 dependence and independence. Mutation or amplification of BCR::ABL1 leads to BCR::ABL1-dependent resistance, which has been well researched by previous studies and resolved by the second- or third-generation of TKIs [7-10]. However, imatinib resistance independent of BCR::ABL1 remains to be resolved. It is reported that BCR::ABL1 independent imatinib resistance may be mainly related to the activation of alternative survival pathway [11]. Therefore, it is promising to find new survival targets and corresponding chemicals to overcome BCR::ABL1 independent resistance in CML.

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Mammalian target of rapamycin (mTOR) is an important regulatory molecule for cell growth especially in tumor cells [12]. It has been confirmed that down regulation of mTOR is highly correlated to cancer remission [13, 14]. mTOR is the core component of two multi-subunit protein complexes with different functions named as mTORC1 (mTOR complex 1) and mTORC2 (mTOR complex 2) [15]. mTORC1 is an important switch for most energetically processes in the cell, such as driving cell growth and building cellular biomass under nutrient sufficiency. It also regulates the autophagic recycling of cellular components under nutrient limitation. The autophagy process is under dynamic balance in organisms, and conversely, it could cause apoptosis when autophagy is overactivated. The mechanism of mTOR blocking autophagy is to directly suppress the early steps of the process, as well as to control the lysosomal degradative capacity by inhibiting the transactivation of genes encoding structural, regulatory, and catalytic factors [16]. The autophagy is suppressed as a result of the transmission of the PI3K/AKT signal to mTOR [17]. Rapamycin is a classical mTORC1 inhibitor, which shows antitumor effect on many malignancies originated in liver, lung, ovary and breast [18, 19]. Meanwhile, the mTOR pathway was also found to be activated in BCR::ABL1 positive leukemic cells, such as K562, SUP-B15 and LAMA cell lines [20, 21]. However, whether mTOR participates in imatinib resistance and the related mechanism have not yet been well investigated [22]. By analyzing the data from SRA database, we were surprised to find that mTOR signaling was significantly up-regulated in imatinib-resistant K562/G01 cells compared to imatinib-sensitive K562 cells. Hence whether the overexpression of mTOR is associated with imatinib resistance would be explored in this study.

Rapamycin shows significant antitumor effect by only inhibiting mTORC1. AZD2014 is a novel mTOR inhibitor with strong lysosome activation and favorable pharmacokinetic properties by inhibiting both mTORC1 and mTORC2 [23]. Therefore, it is expected AZD2014 exerts more effective antitumor effect than rapamycin theoretically. It has been reported that AZD2014 shows potential anti-neoplastic activity in ovarian cancer and prostate cancer [24, 25]. But the antitumor effect of AZD2014 on CML cells, especially on BCR::ABL1 independent resistance cells still needs to be explored.

In this study, we intend to explore whether mTOR participates in BCR::ABL1 independent resistance, if mTOR would be a potential target for TKI resistant CML therapy, and the antitumor mechanism of AZD2014 on imatinib resistant CML cells.

Materials and methods

Reagents and antibodies

AZD2014 (purity >98%) or imatinib was purchased from TargetMol (Massachusetts, USA) and dissolved in DMSO. Anti-BCR::ABL1, anti-PARP, anti-STAT5, and anti-Cyclin D1 were purchased from Cell Signaling Technology (Massachusetts, USA). Anti-c-Myc, anti-p27, anti-Bad, and anti-Bcl-2 were purchased from Abmart (Shanghai, China). Anti-mTOR, anti-p-mTOR, anti-4EBP1, anti-p-4EBP1, anti-p62, anti-Beclin-1, anti-ATG12, anti-ULK1, anti-TFEB, anti-Histone-H3, and anti-GAPDH were purchased from Zengbio (Chengdu, China). Anti-β-actin was purchased from Zsbio (Beijing, China).

Cell lines and culture conditions

HS-5 is a cell with fibroblast morphology that was isolated from the bone marrow stroma of a White male patient and cultured in DMEM medium with 10% fetal bovine serum (FBS) (Gibco, Massachusetts, USA). K562 derived from a 53-year-old female CML patient in blast crisis [26] and was purchased from National Collection of Authenticated Cell Cultures of China. K562/G01 is a cell resistant to imatinib and was established by culturing K562 with low concentration of imatinib for months. Imatinib is not necessary to maintain the resistant phenotype for K562/G01. The molecular basis underlying the K562/G01 resistant to imatinib is defined as overexpression of BCR::ABL1 without BCR::ABL1 mutation [27-29]. K562 and K562/G01 were cultured in RPMI 1640 medium with 10% FBS.

Clinical samples

All peripheral blood samples were collected from the Department of Laboratory Medicine, the First Affiliated Hospital of Chongqing Medical University, China. Peripheral blood

Table 1. Patient information

Samples	Diagnosis	Gender	Age	Sensitive/Resistant to imatinib	Additional information
N1	Healthy	Male	24	-	-
CML1	CML	Female	49	Sensitive	-
CML2	CML	Male	52	Resistant	Acquired resistance

Table 2. Primer sequences for detecting target gene by Q-PCR

Gene	Sequence (5'-3')
mTOR	F: ACAGTGAAAGTGAAGCCGAGAG
	R: CAAGGAGATAGAACGGAAGAAGC
ULK1	F: CAGCAAAGGCATCATCCAC
	R: GGTTTGCGTTGCACTAGGG
Beclin-1	F: GAGATTGGACCAGGAGGAAGCT
	R: GTGCCAAACTGTCCGCTGTG
ATG12	F: TAGAGCGAACACGAACCATCC
	R: CACTGCCAAAACACTCATAGAGA
β-actin	F: ACTTAGTTGCGTTACACCCTTT
	R: TGTCACCTTCACCGTTCC

F stands for forward primer; R stands for reverse primer.

mononuclear cells were separated by density gradient centrifugation with mononuclear cell separation reagent (Solarbio, Beijing, China). Informed consent was conducted for human samples. The information of patients was exhibited in **Table 1**.

CCK-8 assays

Cell viability was evaluated by CCK-8 assay (Top science, China). HS-5, K562 or K562/G01 cells (5000 cells/well) were plated in 96-well plates, and treated with AZD2014 in combination with or without imatinib for indicated time. Then, 10 μl CCK-8 reagent was added to each well and cultivated for hours. Absorbance was measured at 460 nm with microplate reader (Eon, BioTek, USA). IC50 was calculated with GraphPad Prism 8.0 software.

Immunofluorescence assay

Cells were smeared evenly on the slides, followed by fixation with 4% paraformaldehyde. Afterward, cells were permeabilized with 1% Triton X-100 and blocked with goat serum. Then, cells were incubated with primary antibody at 4°C overnight, subsequently Cy3-labeled secondary antibody (Invitrogen, California, USA) at 37°C for 1 h. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI). Eventually, the smears were sealed with glycerol and observed with confocal laser scanning microscope (Leica, Wetzlar, Germany).

Quantitative real time polymerase chain reaction (Q-PCR)

Total RNA was extracted with TRIZOL (Takara, Kyoto, Japan). The reverse transcription was executed with the prime Script RT reagent Kits (Takara, Kyoto, Japan). Q-PCR was performed with the TB Green PCR Kit (Takara, Kyoto, Japan). Relative expression of target mRNA to β-actin was calculated with the $2^{-\Delta\Delta Cq}$ method. The primer sequences for target gene are listed in **Table 2**.

Western blot (WB)

Cells were collected and lysed with RIPA (CST, Massachusetts, USA) with 1% PMSF, 1% NaF and 1% Na_3VO_4 to prevent protein from dephosphorylation. 80 μg total protein was electrophoresed through sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Then target protein was transferred onto PVDF membrane (Millipore, Massachusetts, USA). The membrane was blocked with 5% skim milk, then incubated with corresponding primary antibody overnight and then secondary antibody for 1 hour. Finally, the target protein was developed with ChemistarTM high-sig ECL Western Blot Substrate (Tanon, Shanghai, China).

Colony formation assay

Cell self-renewal ability was assessed by the colony formation assay. K562 or K562/G01 cells were plated into the semisolid medium with AZD2014 in 96-well plates (100 cells/well). The colonies were photographed and counted with an inverted microscope 7 days later. Three replicates were set for each group.

Cell apoptosis and cell cycle analysis

Cells (8×10^5) were planted in 6-well plates, treated with AZD2014 for 48 h and then collected. For cell apoptosis analysis, cells were

double-labeled with annexin V and PI (Sungene Biotech, Tianjin, China), then measured by flow cytometry (FCM) according to the manufacturer's instructions. For cell cycle analysis, cells were fixed in 70% ethanol at 4°C overnight, then re-suspended with 500 μl PI/RNase solution for detection by FCM (Beckman Coulter, Californian, USA). The data were analyzed by CytExpert.

Murine leukemia model

All the female mice used in this study was purchased from Huachuang Cigna Pharmaceutical Technology Co., Ltd. (Jiangsu, China) and fed in individual ventilating cage (IVC) in Chongqing Medical University Animal Center (SPF grade). The temperature of the Animal Center was maintained at 20°C-26°C, with a humidity of 40%-70% and well-ventilated conditions. After adapting to the new environment for one week, six-week-old NOD-SCID mice were received 250 cGy X-ray radiation and then injected with K562/G01 cells (5×10^6 cells per mouse) through the tail vein. After seven days, the mice were divided into four groups randomly (n=6 for each group). Each group was received administration of imatinib (50 mg/kg), AZD2014 (50 mg/kg), or imatinib + AZD2014 (25 mg/kg) through intraperitoneal injection twice a week for consecutive three weeks respectively. The mouse health condition, body weight, and white blood cell counts were examined weekly. Diseased mice were executed by cervical dislocation in time. The observation period lasted for 90 days. The mice that still survived at 90-day were sacrificed and dissected. The livers and spleens were removed and weighed. Leukemic infiltration in the liver, spleen and bone marrow was analyzed by HE staining and immunofluorescence assay.

Database analysis

SRA files (SRR11452004-11452009, SSR12-755782-SRR12755787) of K562 and K562/G01 cells were downloaded from NCBI, and converted to fastq files using the fastq-dump in sratoo lkit. The acquired sequencing data of transcriptome were performed quality control by using the FastQC package and were further filtered by using Trimmomatic to obtain high-quality data. Then the high-quality sequencing data were analyzed via using HISAT2+ Stringtie+Ballgown package for gene annotation and differential gene. The obtained differential genes were analyzed through using R language packages, such as ggplot2 and pheatmap, for visualization. The GSEA function in the clusterProfiler package was then used to explore the biological processes affected by the differential genes of the two cell lines. Lastly, the GO function annotation and KEGG signaling pathway analysis were performed through the https://metascape.org/website.

Statistical analysis

All experiments conducted in this study were replicated for three times. The statistical differences among each group were analyzed by t test (two groups) or one-way ANOVA (more than two groups) with GraphPad Prism 8.0. $P<0.05$(*) is defined as statistically significant.

Results

mTOR signaling pathway is upregulated in imatinib-resistant CML cells

By analyzing the data from SRA database (SRR11452004-11452009 and SSR127557-82-SRR12755787, https://www.ncbi.nlm.nih.gov/sra?linkname=bioproject_sra_all&from_uid=616389), we found the expression of mTOR signal pathway was higher in imatinib-resistant K562/G01 cells compared to imatinib-sensitive K562 cells (**Figure 1A**). In consideration of the vital role of mTOR as a survival factor for various tumor cells and its higher expression in K562/G01 cells [13, 14], we wondered if mTOR mediated imatinib resistance in CML cells. To verify this hypothesis, we detected the mRNA level of mTOR in a normal donor, an imatinib-sensitive CML patient (CML1) and an imatinib-resistant CML patient (CML2). The results showed that the mRNA of mTOR was higher in CML patients especially in the imatinib-resistant patient (**Figure 1B**). Similarly, we observed a higher expression of mTOR mRNA in K562/G01 than that of K562 (**Figure 1C**). We found that there was no statistical difference between K562 and K562/G01 in the expression of AKT although it was slightly up-regulated in K562/G01. Furthermore, the activation of mTOR (p-mTOR) was higher in K562/G01, which in turn stimulated AKT activation (p-AKT) (**Figure 1D**). The higher expression of p-mTOR in K562/G01 was con-

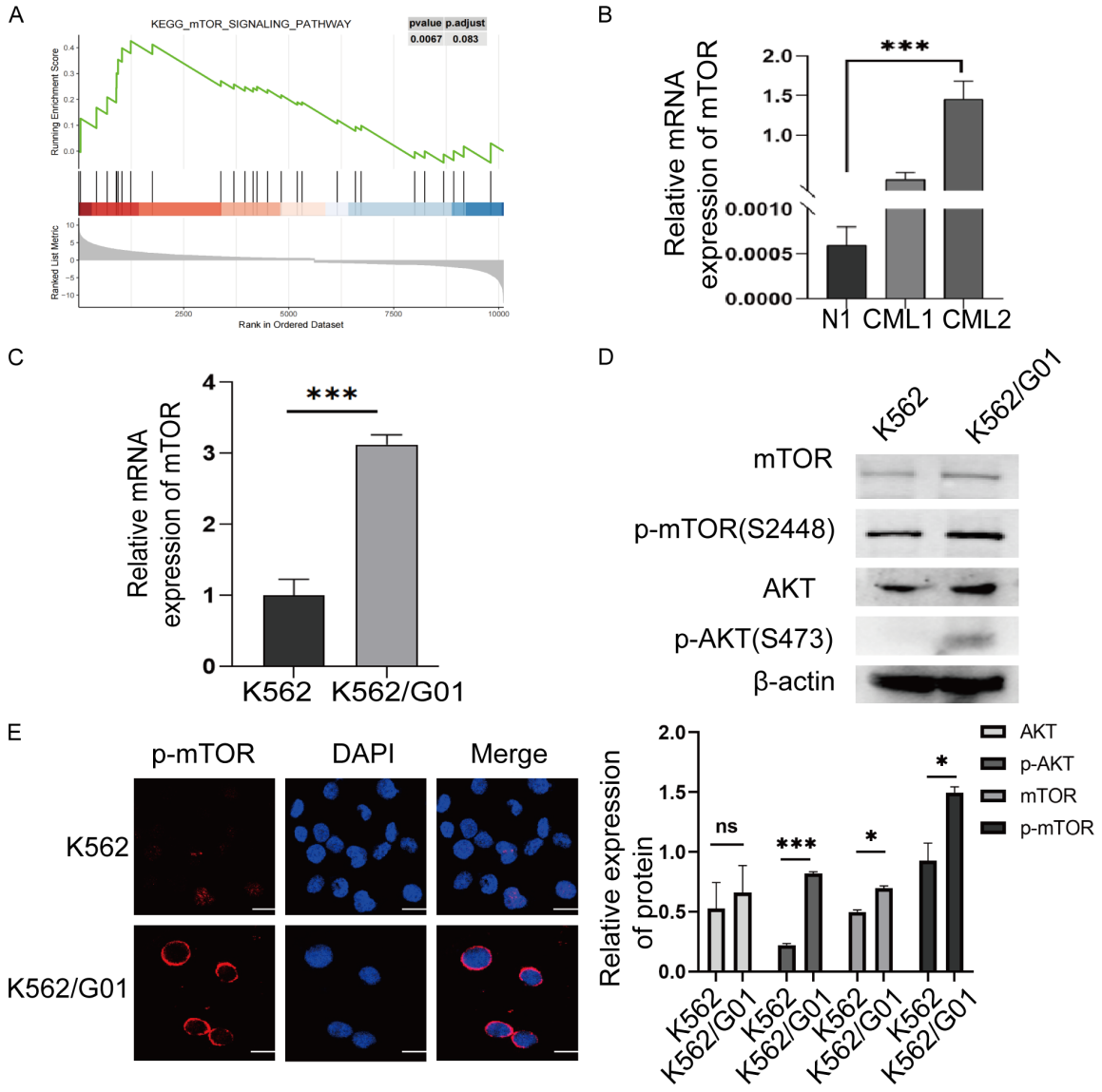

Figure 1. mTOR signal pathway is upregulated in imatinib-resistant chronic myeloid leukemia (CML) cells. (A) The expression of mTOR signaling pathway in K562 and K562/G01 cells was analyzed with SRA database (SRR11452004-11452009 and SSR12755782-SRR12755787, https://www.ncbi.nlm.nih.gov/sra?linkname=bioproject_sra_all&from_uid=616389). (B) The mRNA level of mTOR in the peripheral blood mononuclear cells (PBMCs) from normal donor (N1), imatinib-sensitive patient (CML1), or imatinib-resistant patient (CML2) was detected by Q-PCR. The mRNA expression of mTOR was normalized to β-actin. Results represent the mean ± standard deviation (SD) of three independent experiments. ***P<0.001 compared to control. (C) The mRNA level of mTOR in K562 or K562/G01 cells was also detected according to the method in (B). (D) The expression of p-mTOR or p-AKT was examined in K562 and K562/G01 cells by western blot. Then the relative expression level was analyzed by Image J. (E) The expression of p-mTOR in K562 or K562/G01 cells was visualized by immunofluorescence assay.

firmed by immunofluorescence assay (**Figure 1E**). Taken together, mTOR signal pathway is upregulated in imatinib-resistant cells compared to imatinib-sensitive cells, which suggests that mTOR may be involved in imatinib resistance.

Inhibition of mTOR by AZD2014 inhibits the proliferation of CML cells especially imatinib-resistant cells

To test the role of mTOR in CML cells, AZD2014 which is a specific mTORC1/C2 dual inhibitor

was applied to inhibit mTOR, then the effects on CML cells were analyzed. K562 and K562/G01 cells were treated with series concentration of AZD2014 for 24 h or 48 h, and then cell proliferation was detected by CCK-8 assay. The results showed that the inhibitory effect of AZD2014 on CML cell proliferation was in a dose and time dependent manner. The IC50 value was much lower in K562/G01 than in K562 (1.7 μM vs 6.5 μM for 24 h, and 0.6 μM vs 2.1 μM for 48 h) (**Figure 2A**). The inhibitory effect of mTOR inhibition on CML cell proliferation was also confirmed by colony formation assay. As shown in **Figure 2B**, the number and size of colonies in both K562 and K562/G01 were significantly decreased by mTOR inhibition with AZD2014. The more important discovery was that the inhibitory effect was much stronger in K562/G01 than K562 under the same concentration of AZD2014. The effect of mTOR inhibition by AZD2014 on CML cell cycle was detected by flow cytometry. As shown in **Figure 2C**, G1-phase population increased and S-phase population decreased in a dose-dependent manner both in K562 and K562/G01 under the AZD2014 treatment. Meanwhile, the G1-phase arrest was more effective in K562/G01 than K562. The G1-phase percentage in K562 was 31.07%, 42.21%, and 48.73% at the concentration of 4 μM, 8 μM and 16 μM AZD2014 respectively (vs 29.39% in control group). While the G1-phase percentage in K562/G01 cells reached to 49.44% only with 1 μM AZD2014 (vs 28% in control group). To clarify the mechanism of G1-phase arrest induced by AZD2014, we examined the expression of downstream molecules of mTOR (p-S6K and p-GSK3β) and G1-phase regulator (Cyclin D1 and p27). It has been proven that mTOR signaling pathway plays a pivotal role in cell cycle regulation [30]. Moreover, Cyclin D1 is a key regulator that drives cells from G1 phase to S phase [31, 32]. As shown in **Figure 2D**, p-S6K, p-GSK3β and Cyclin D1 reduced with the AZD2014 treatment both in K562 and K562/G01, and p27 increased. These results suggested that mTOR inhibition by AZD2014 induced cell cycle arrest in G1 phase through mTOR-S6K-GSK3β axis, which downregulated Cyclin D1 and upregulated p27 to inhibit the binding of Cyclin D1 with CDK2. Taken together, these results above illustrate that inhibition of mTOR by AZD2014 inhibits the proliferation of CML cells especially imatinib-resistant K562/G01 cells.

mTOR inhibition by AZD2014 synergizes with imatinib to overcome imatinib-resistance

Inhibiting mTOR by AZD2014 showed vigorous growth inhibition in CML cells. However, AZD2014 could not significantly increase apoptosis either in K562 or K562/G01 cells (Figure S1A). Nonetheless, AZD2014 significantly increased the apoptotic cell percentage in K562 (**Figure 3A**) and K562/G01 cells (**Figure 3B**) in combination with imatinib. The apoptosis induction effect was a little stronger in K562/G01 than K562 (34.42% *vs* 29.04% in the AZD2014 + imatinib treatment group). Correspondingly, the treatment of AZD2014 in combination with imatinib made PARP cleave, c-Myc and Bcl-XL down-regulate, Bad up-regulate in K562 (**Figure 3C**) and K562/G01 cells (**Figure 3D**). Meanwhile, obvious nuclear fragmentation was observed in K562/G01 through DAPI staining under the treatment of AZD2014 plus imatinib (Figure S1B). We further tested whether combination of AZD2014 with imatinib had a synergistic effect on the proliferation of CML cells. After treatment with AZD2014 and imatinib, the cell viability of K562 or K562/G01 was tested, and then the combination index (CI) was calculated. All CI values in K562/G01 were less than 1 (**Figure 3F**), and partial CI values in K562 cells were little higher than 1 (**Figure 3E**) (CI<1, =1, and >1 represent synergistic, additive, and antagonistic effect, respectively). These results suggested AZD2014 had a more significant synergistic effect with imatinib on K562/G01 than K562. Taken together, these data suggest that AZD2014 synergizes with imatinib to induce apoptosis and inhibit proliferation in CML cells, and the synergistic effect is more remarkable in K562/G01 cells.

AZD2014 synergizes with imatinib to show anti-leukemia activity in imatinib-resistant CML-like mouse

NOD-SCID mice were injected with K562/G01 cells to develop imatinib-resistant CML-like disease, then AZD2014 or imatinib was injected intraperitoneally. The observation lasted for 90 days. The initial and final body weight of each mouse were recorded. As shown in **Figure 4A**, the mouse body weight in AZD2014 plus

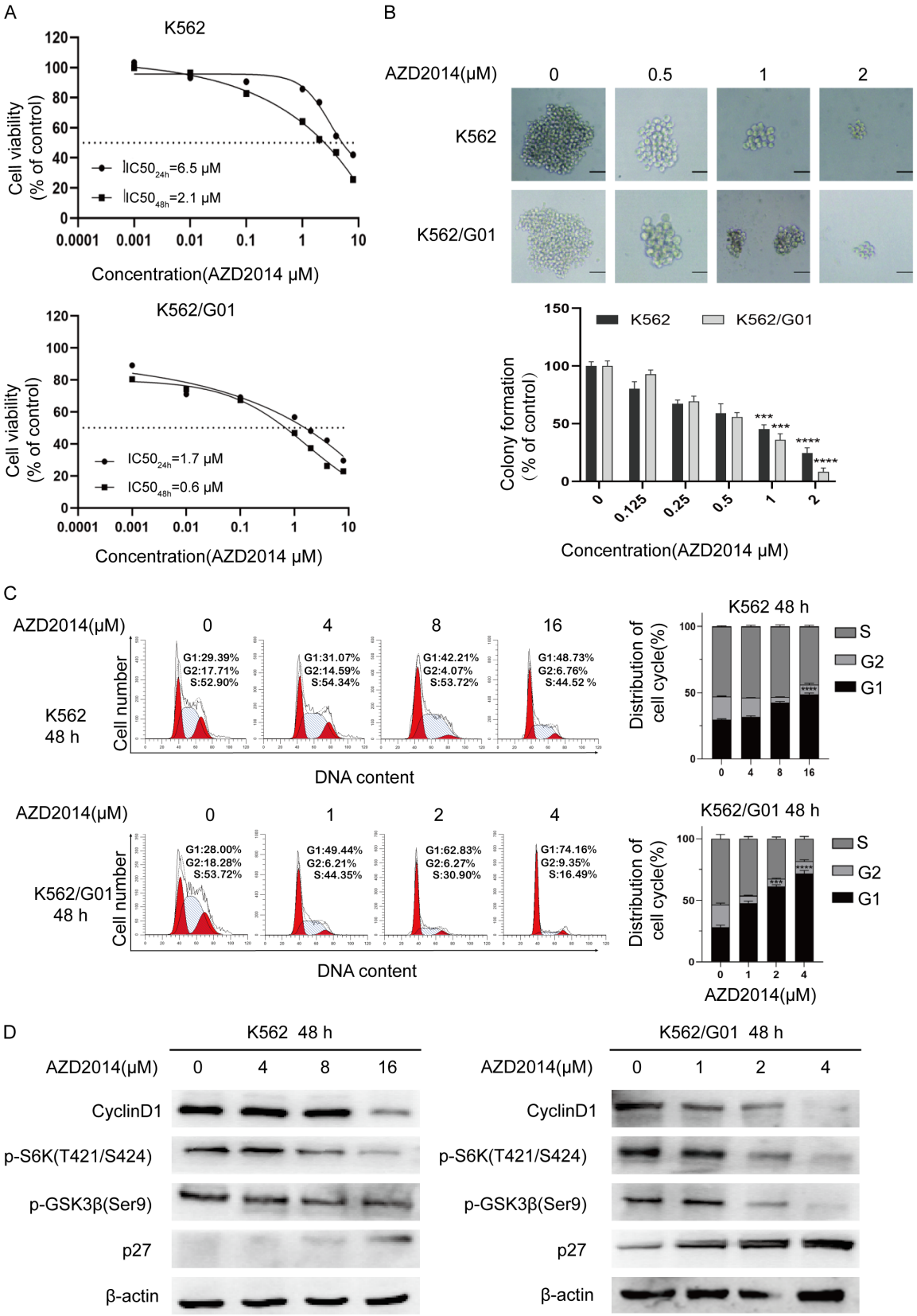

Figure 2. Inhibition of mTOR by AZD2014 inhibits the proliferation of CML cells especially imatinib-resistant cells. A. K562 and K562/G01 cells were treated with indicated dose of AZD2014 for 24 or 48 h, then cell viability was determined by CCK-8 assay. The IC50 value of AZD2014 for each cell line was calculated, and cell viability was shown as

percentage to control group. B. K562 and K562/G01 cells were planted in the semi-medium with indicated concentration of AZD2014 for 7 days, then the colonies were photographed and calculated. The pictures in the left panel display the representative colonies. The histogram in the right panel exhibits the mean ± SD of three independent experiments. ***P<0.001, ****P<0.0001 compared to control group. C. K562 and K562/G01 cells were treated with indicated dose of AZD2014 for 48 h, then cell cycle distribution was analyzed by flow cytometry (FCM). The pictures in left panel show representative result of each group. The histogram in the right panel exhibits the mean ± SD of three independent experiments. ***P<0.001, ****P<0.0001 compared to control group. D. K562 and K562/G01 cells were treated with indicated concentration of AZD2014 for 48 h, then the level of p-S6K, p-GSK3β, CyclinD1 or p27 was examined by western blot.

imatinib group increased slightly, while mouse body weight in other groups showed different degrees of weight loss, especially in the control group. The white blood cells (WBC) were the highest in the control group, AZD2014 or imatinib could decrease the WBC in mice, and AZD2014 plus imatinib significantly decreased WBC in imatinib-resistant CML-like mice (**Figure 4B**). The diseased mice were executed in time and the living mice in each group were executed at the end of 90 days. Bone marrow cells were separated, liver and spleen were dissected for leukemic infiltration analysis. CD45 is a hallmark of human white blood cells which does not express in murine white blood cells. Hence the proportion of CD45+ cell reflects the number of K562/G01 cells infiltrated and proliferated in murine bone marrow. As shown in **Figure 4C**, the proportion of CD45+ cell reached almost 80% in the control group. AZD2014, imatinib and AZD2014 plus imatinib reduced the proportion of CD45+ cell to 40%, 55% and 16%, respectively. These results suggested AZD2014 plus imatinib effectively inhibited the proliferation and expansion of K562/G01 cells in murine bone marrow. Similarly, the change of liver weight (**Figure 4D**) or spleen weight (**Figure 4E**) in each group was consistent with the change of WBC. As shown in **Figure 4F**, mice in control group, AZD2014 group and imatinib group developed obvious hepatosplenomegaly, while hepatosplenomegaly was not obvious in AZD2014 plus imatinib group. The expression of BCR::ABL1 in bone marrow, liver and spleen cells were detected by immunofluorescence assay to reflect K562/G01 infiltration. The results showed that fewer leukemic cells infiltrated into bone marrow, liver and spleen in the AZD2014 plus imatinib group compared to the other groups (**Figure 4G**). Similar results were observed by Wright's staining of these cells (**Figure 4H**) and HE staining of liver and spleen tissues (**Figure 4I**). Accordingly, AZD2014 plus imatinib significantly prolonged the survival of imatinib-resistant CML-like mice (**Figure 4J**). In summary, these results demonstrate that AZD2014 synergizes with imatinib to alleviate disease symptoms, reduce leukemic infiltration into organs and prolong lifespan in mice with imatinib-resistant CML-like disease.

AZD2014 is safety in vivo and vitro

As shown in **Figure 5A**, it was hardly to detect the expression of mTOR in HS-5 cells on contrast to the overexpression of mTOR in K562 and K562/G01 cells. We tested the effect of mTOR inhibition by AZD2014 on HS-5 cell growth. It was demonstrated that AZD2014 showed little growth inhibition on HS-5 cells as detected by CCK-8 assay (**Figure 5B**). Moreover, we tested the influence of AZD-2014 on kidney and liver function in healthy Balb/c mice to reflect the bio-safety of AZD2014. Consistent with the limited effect on HS-5 cell growth in vitro, AZD2014 just slightly increased the levels of BUN, ALT and AST in mice serum, but the value still remained in the normal reference range (**Figure 5C**). In summary, these results proved that AZD2014 possesses bio-safety in vitro and in vivo.

mTOR inhibition by AZD2014 works in a BCR::ABL1 independent manner in CML cells

To clarify the potential mechanism by which mTOR mediates imatinib resistance, the state of molecules associated with mTOR or BCR::ABL1 signal pathway was examined in CML cells after treatment with imatinib or AZD2014. As shown in **Figure 6A**, p-mTOR, p-S6K and p-4EBP1 had no obvious change when K562/G01 was treated with imatinib. This result suggests that mTOR signal pathway is not regulated by BCR::ABL1 in imatinib resistant CML cells. Meanwhile, p-BCR::ABL1, p-STAT5 and p-CRKL also had no obvious change when K562/G01 was treated with

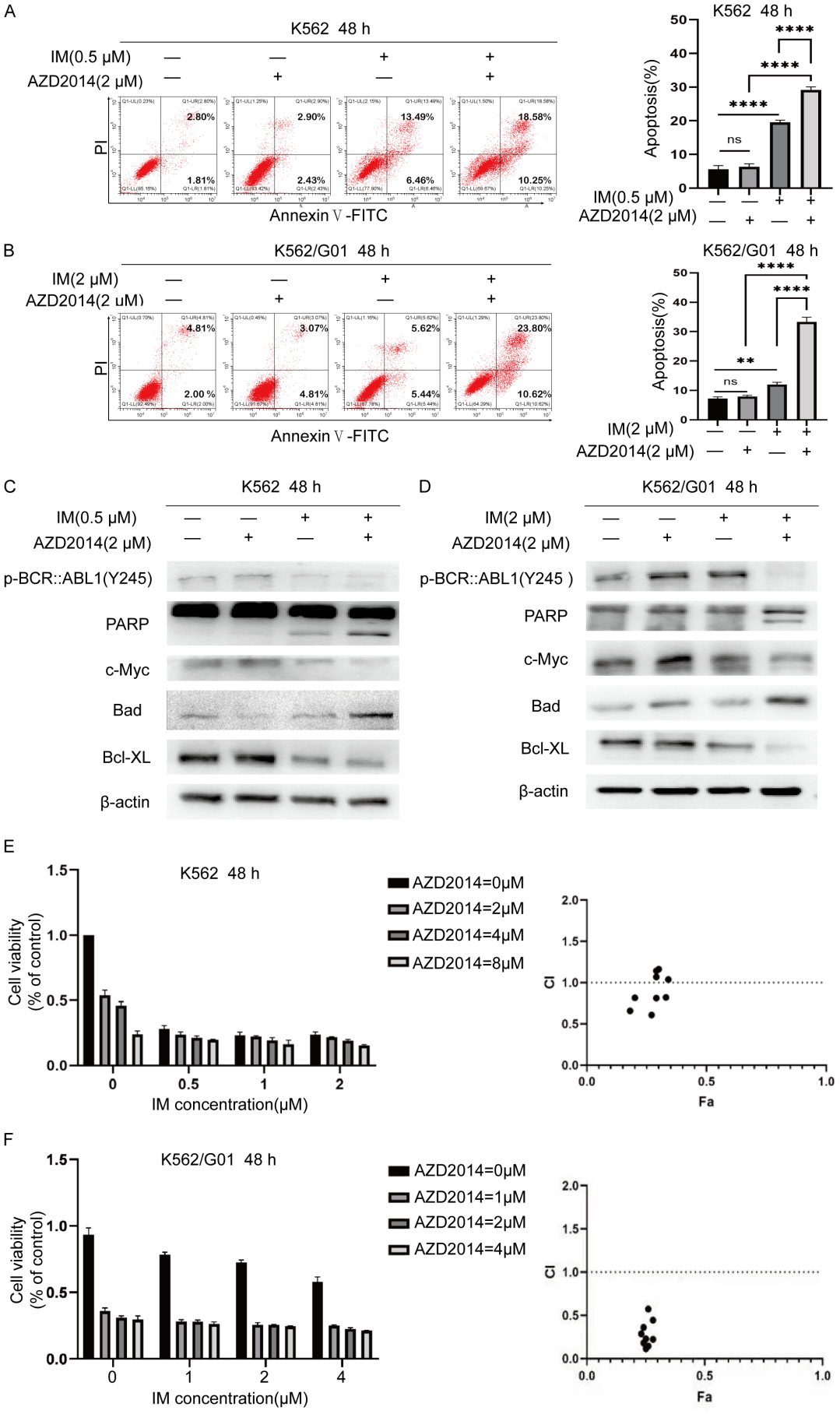
A
K562 48 h
IM(0.5 μM)
AZD2014(2 μM)
PI
Annexin Ⅴ-FITC
2.80%
1.81%
2.90%
2.43%
13.49%
6.46%
18.58%
10.25%
Apoptosis(%)
ns
****
B
K562/G01 48 h
IM(2 μM)
AZD2014(2 μM)
4.81%
2.00 %
3.07%
4.81%
5.62%
5.44%
23.80%
10.62%
**
C
K562 48 h
p-BCR::ABL1(Y245)
PARP
c-Myc
Bad
Bcl-XL
β-actin
D
K562/G01 48 h
p-BCR::ABL1(Y245)
E
K562 48 h
Cell viability (% of control)
IM concentration(μM)
AZD2014=0μM
AZD2014=2μM
AZD2014=4μM
AZD2014=8μM
CI
Fa
F
K562/G01 48 h
AZD2014=0μM
AZD2014=1μM
AZD2014=2μM
AZD2014=4μM

Figure 3. mTOR inhibition by AZD2014 synergizes with imatinib to overcome imatinib-resistance. K562 (A) and K562/G01 cells (B) were treated with imatinib, AZD2014 or imatinib plus AZD2014 respectively for 48 h, then cell apoptosis was analyzed by FCM. The pictures in left panel show representative result of each group. The histogram in the right panel exhibits the mean ± SD of three independent experiments. **P<0.01 compared to control. The expressions of PARP, c-Myc, Bad, and Bcl-XL were detected in K562 (C) and K562/G01 (D) by western blot. The combination effect of AZD2014 and imatinib on the proliferation of K562 (E) and K562/G01 (F) was determined by CCK-8 assay. Combination index (CI) value was calculated with CompuSyn software.

AZD2014 (**Figure 6B**). This result suggests that BCR::ABL1 signal pathway is not influenced by mTOR in imatinib resistant CML cells and AZD2014 executes the anti-cancer effect through a BCR::ABL1 independent manner. As expected, AZD2014 could inhibit the activation of mTOR signal pathway including mTOR, S6K, 4EBP-1 and AKT both in K562 and K562/G01 (**Figure 6C**). Interestingly, we found that the expression of AKT was significantly down-regulated in K562 and K562/G01 treated with AZD2014. It might be related to that AKT was the intercross of mTOR and BCR::ABL1 signal pathways. Taken together, these data demonstrate that mTOR activation in imatinib resistant CML cells is independent on BCR::ABL1, and AZD2014 executes its anti-leukemia effect by inhibition of mTOR but not BCR::ABL1.

mTOR inhibition by AZD2014 reinforces autophagy in imatinib-resistant cells and mouse model

It is reported that mTOR is strongly associated with autophagy [33-35]. Therefore, we evaluated the effect of AZD2014 on autophagy in K562/G01 cells. Firstly, the expression of p62 and LC3 which are two key markers of autophagy was assayed. We found that p62 as the autophagy substrate reduced, and the ratio of LC3 II/I increased (**Figure 7A**, **7B**). LC3 expression was also confirmed by immunofluorescence assay and it was proven to elevate (**Figure 7C**). These data confirm that AZD2014 boosts autophagy in K562/G01 cells. Furthermore, decreased p-mTOR and p62, and increased LC3 II/I were observed in the bone marrow cells of AZD2014, or AZD2014 plus imatinib treated mice (**Figure 7D**), which indicated the anti-cancer effect of AZD2014 in vivo was through up-regulating autophagy by mTOR inhibition. To clarify the specific molecular mechanism, the interaction molecules with mTOR was analyzed in STRING network (http://string-db.org). We found that ULK1 was closely correlated with autophagy, followed by ATG12 and Beclin-1 deeply relevant to ULK1 (**Figure 7E**). We performed experiments to verify the information predicted by the STRING network. It was shown that the transcription (**Figure 7F**) and translation (**Figure 7G**) of ULK1, ATG12 and Beclin-1 were all increased in K562/G01 with AZD2014 treatment. Meanwhile, AZD-2014 could induce the translocation of TFEB from cytoplasm to nuclei in which TFEB acts as the main transcription factor for autophagy (**Figure 7H**). Nuclear translocation of TFEB was also observed with immunofluorescence assay (**Figure 7I**). Generally speaking, these results indicated that mTOR inhibition by AZD2014 could induce TFEB into nuclei and then transcriptionally activate ATG12, Beclin-1 and ULK1, which reinforce the autophagy in K562/G01 cells.

The anticancer effect of mTOR inhibition by AZD2014 on imatinib resistant K562/G01 cells is reversed by autophagy inhibitor

To verify whether autophagy is responsible for the anticancer effect of mTOR inhibition by AZD2014 in K562/G01 cells, 3-MA was applied to inhibit autophagy, and then autophagy markers and anticancer effect of AZD2014 were detected. As shown in **Figure 8A**, the enhancement of LC3 II/I was reversed by 3-MA in AZD2014 treated K562/G01 cells. It was proved that 3-MA almost reversed the apoptosis induced by AZD2014 plus imatinib in K562/G01. On the contrary, the apoptotic percentage reduced by 3-MA in AZD2014 plus imatinib treated K562 cells was not so significant (**Figure 8B**, **8C**). Meanwhile, the decrease of p-BCR::ABL1 and c-Myc was recovered, and PARP cleavage disappeared by application of 3-MA in K562/G01 (**Figure 8E**). However, 3-MA could not absolutely reverse the decrease of p-BCR::ABL1 and c-Myc, and PARP cleavage induced by AZD2014 plus imatinib in K562 cells (**Figure 8D**). Besides, 3-MA partially prevented CML cells from cell cycle arrest induced by AZD2014 (**Figure 8F**). Taken together, these data indicate that the elevated autophagy induced by AZD2014 is responsible for its

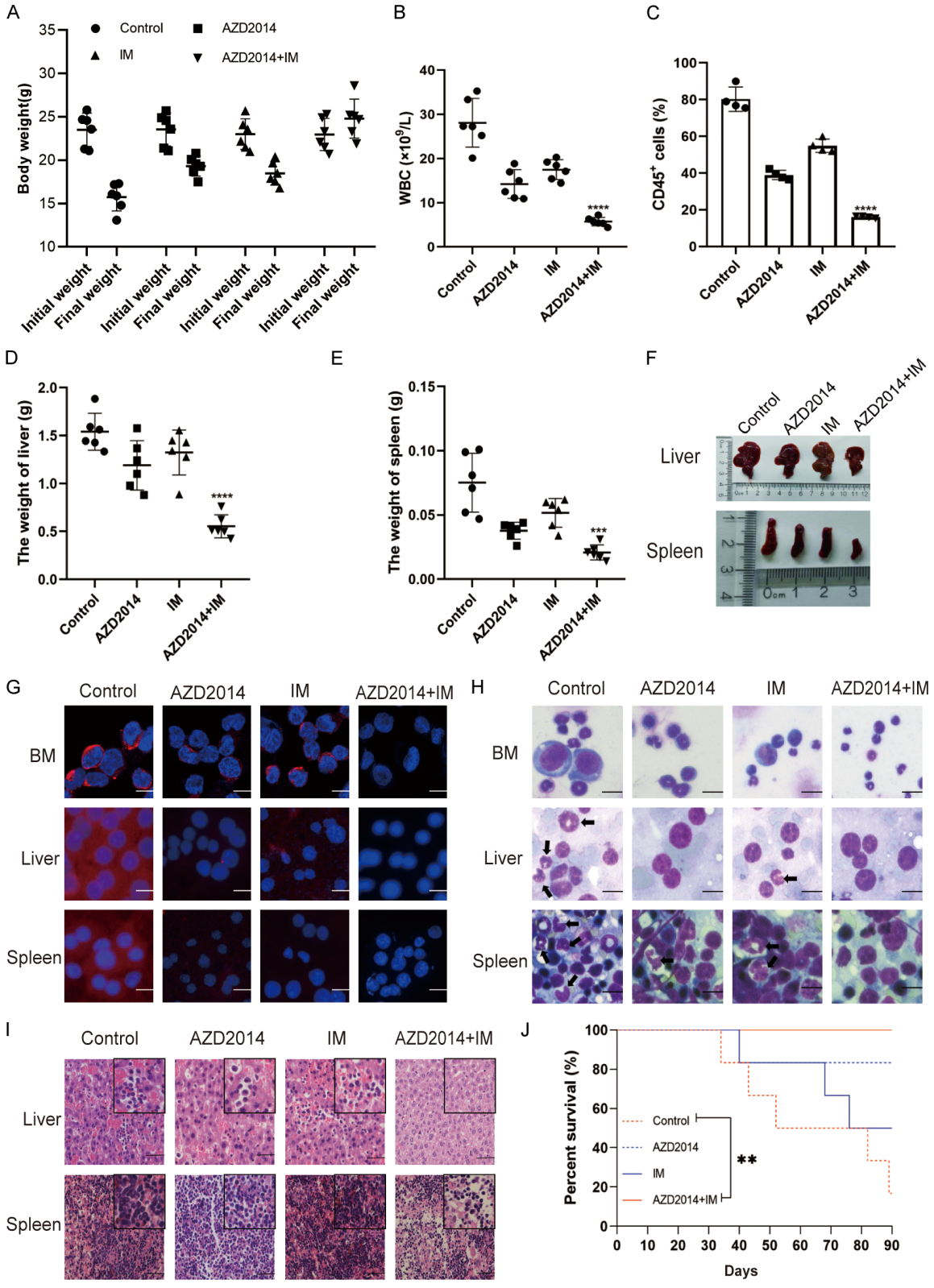

Figure 4. AZD2014 synergizes with imatinib to show anti-leukemia activity in imatinib-resistant CML-like mouse. A. The initial and final body weight of each mouse during the observation period were recorded. B. The maximum white blood cell (WBC) of each mouse was recorded. ****P<0.0001 compared to control. C. The percentage of CD45+ cells in murine bone marrow was detected by FCM. ****P<0.0001 compared to control. D, E. The liver or spleen

weight of each mouse was measured after execution. ***P<0.001, ****P<0.0001 compared to control. F. The representative liver and spleen from each group were displayed. G. The expression of BCR::ABL1 in bone marrow, liver or spleen was detected by immunofluorescence assay with imprint smear. The scale bar represents 10 μm. H. Tissue infiltration of K562/G01 cells into murine bone marrow, liver, or spleen was analyzed by Wright's staining. The arrows indicate representative leukemic cells. The scale bar represents 20 μm. I. Tissue infiltration of K562/G01 cells into liver or spleen was analyzed by hematoxylin and eosin (HE) staining. The scale bar represents 50 μm. J. The mice survival curves were analyzed by the Kaplan-Meier curve.

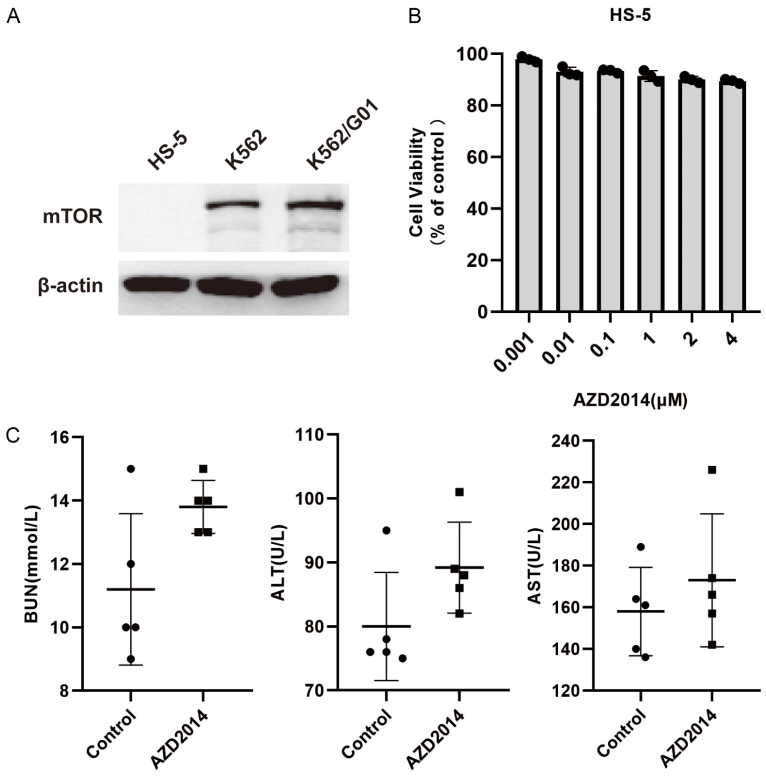

Figure 5. AZD2014 is safety in vitro and in vivo. A. The expression of mTOR in HS-5, K562 and K562/G01 cells were detected by western blot. B. The cell viability of HS-5 treated by different concentrations of AZD2014 for 48 h was detected by CCK8 assay. This experiment was performed triplicate. C. AZD2014 was administered by intraperitoneal injection into Balb/C mice twice a week for consecutive three weeks at dose of 50 mg/kg, then blood urea nitrogen (BUN), alanine transaminase (ALT) and aspartate transaminase (AST) in the peripheral blood serum were detected.

anticancer effect on imatinib resistant K562/G01 cells.

Discussion

Imatinib makes CML to be a curable disease. However, imatinib resistance hampers the favorable prognosis for a part of CML patients. The main mechanisms for imatinib resistance are classified as BCR::ABL1 dependent and independent [36]. BCR::ABL1 dependent resistance which is caused by BCR::ABL1 mutation or amplification has been well resolved by the second or third generation of TKIs [9, 10]. However, BCR::ABL1 independent resistance which mainly caused by activation of alternative survival pathways has not yet been resolved [37]. Hence, we focused on alternative survival pathway which may endow CML cells to develop imatinib resistance. mTOR is an important regulatory molecule for cell growth especially in tumor cells. What's more, mTOR is found to be activated in CML cell lines [20, 21]. Therefore, we researched the role of mTOR in imatinib resistance, and the possibility of mTOR inhibitor as a therapeutic candidate for imatinib-resistant CML patients.

We found that the expression of mTOR was higher in imatinib-resistant CML patient and cell line than imatinib-sensitive CML patient and cell line, which is consistent with the findings of Mitchell R [22]. We demonstrated that inhibiting mTOR by AZD2014 impeded the growth both of K562 and K562/G01, and the inhibitory effect was stronger in K562/G01 than K562. Meanwhile, AZD2014 induced G1-phase arrest, and the cell cycle retardation effect was stronger in K562/G01 than K562. However, the exact mechanism by which AZD2014 functions more effectively in K562/G01 cells remains to be further illustrated. We speculate that mTOR functions as an alternative survival factor for K562/G01 to sustain imatinib resistance. Therefore, K562/G01 cells were more sensitive to mTOR inhibition by AZD2014. These data suggest

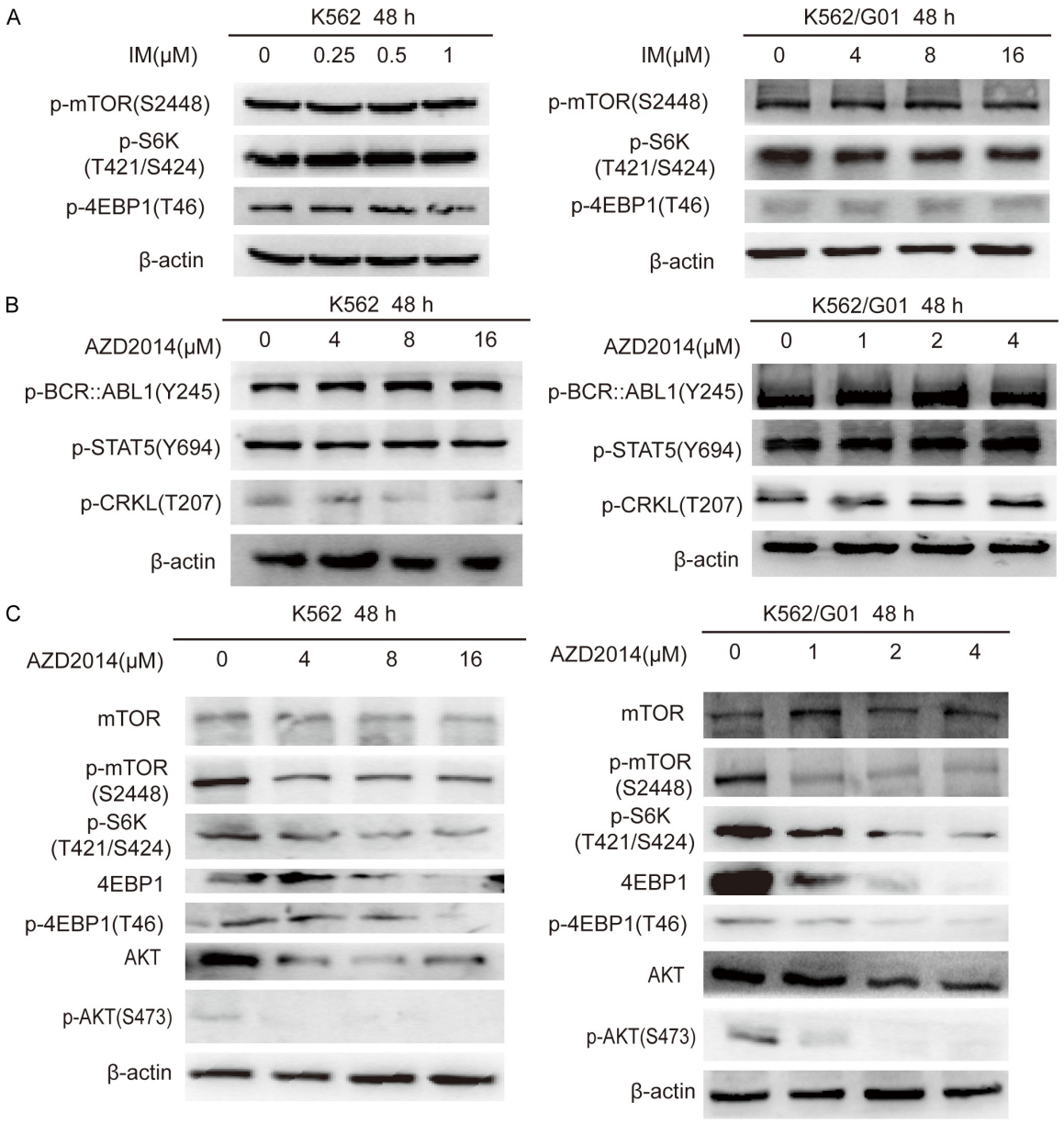

Figure 6. TOR inhibition by AZD2014 works in a BCR::ABL1 independent manner in CML cells. A. K562/G01 and K562 cells were treated with indicated dose of imatinib for 48 h, and then the activation state of mTOR signal pathway including p-mTOR, p-S6K and p-4EBP1 was examined by western blot. B. K562/G01 and K562 cells were treated with indicated dose of AZD2014 for 48 h, and then the activation state of BCR::ABL1 signal pathway including p-BCR::ABL1, p-STAT5 and p-CRKL was examined by western blot. C. K562 and K562/G01 cells were treated with indicated dose of AZD2014 for 48 h, and then the activation state of mTOR signal pathway including p-mTOR, p-S6K, p-AKT and p-4EBP1 was examined by western blot.

that mTOR could be a potential target to overcome imatinib resistance.

It has been reported that mTOR is activated in CML cells. Nevertheless, little is known about the association of mTOR activation with imatinib resistance [38, 39]. In this study, we verified imatinib inhibited BCR::ABL1 and its downstream molecules but did not inhibit mTOR and its downstream molecules in K562/G01 cells. On the other hand, AZD2014 inhibited mTOR signaling pathway (including mTOR, S6K, 4EBP-1 and AKT), but had no effect on BCR::ABL1 signaling pathway. These data suggest that mTOR pathway participates in imatinib resistance in BCR::ABL1 independent manner.

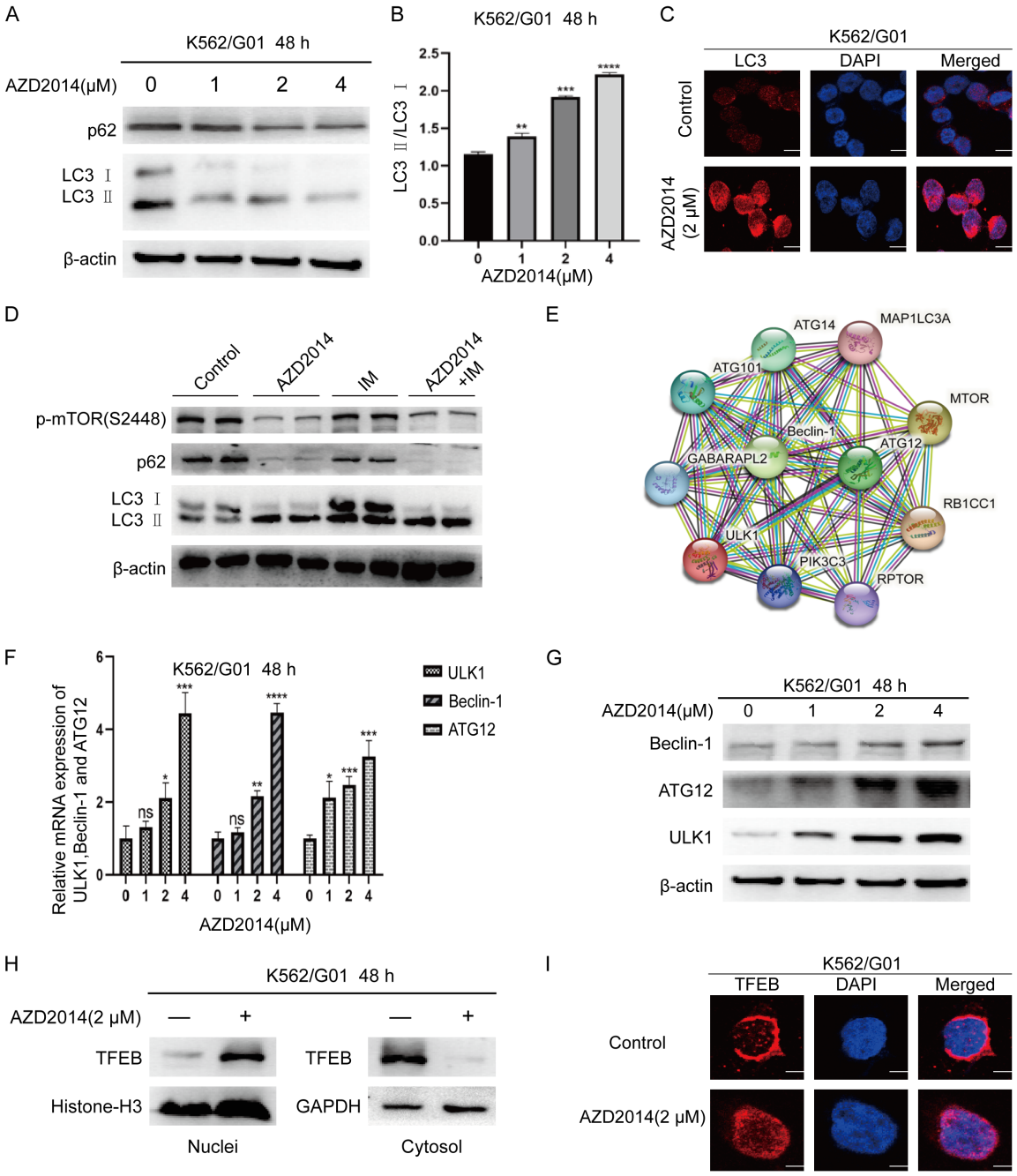

Figure 7. mTOR inhibition by AZD2014 reinforces autophagy in imatinib-resistant cells and mouse model. A. K562/G01 cells were treated with indicated dose of AZD2014 for 48 h, then the expression of p62 and LC3 were detected by western blot. B. The ratio of LC3 II/I was measured with Image J software. Results represent the mean ± SD of three independent experiments. **P<0.01, ***P<0.001, ****P<0.0001 compared to control. C. The expression of LC3 was also examined by immunofluorescence assay. D. The expression of p-mTOR, p62 or LC3 in murine bone marrow was detected by western blot. E. Molecules interacting with mTOR were analyzed with STRING network, and the interaction signal network predicted by STRING was presented. F, G. K562/G01 cells were treated with indicated dose of AZD2014 for 48 h, and then the mRNA and protein level of ULK1, ATG12, and Beclin-1 were detected by Q-PCR or western blot respectively. β-actin was used as an internal control. Results represent the mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared to control. H. K562/G01 cells were treated with 2 μM AZD2014 for 48 h, and then the portion of TFEB in nuclei or cytoplasm was detected by western blot. I. The cellular location of TFEB was detected by immunofluorescence assay.

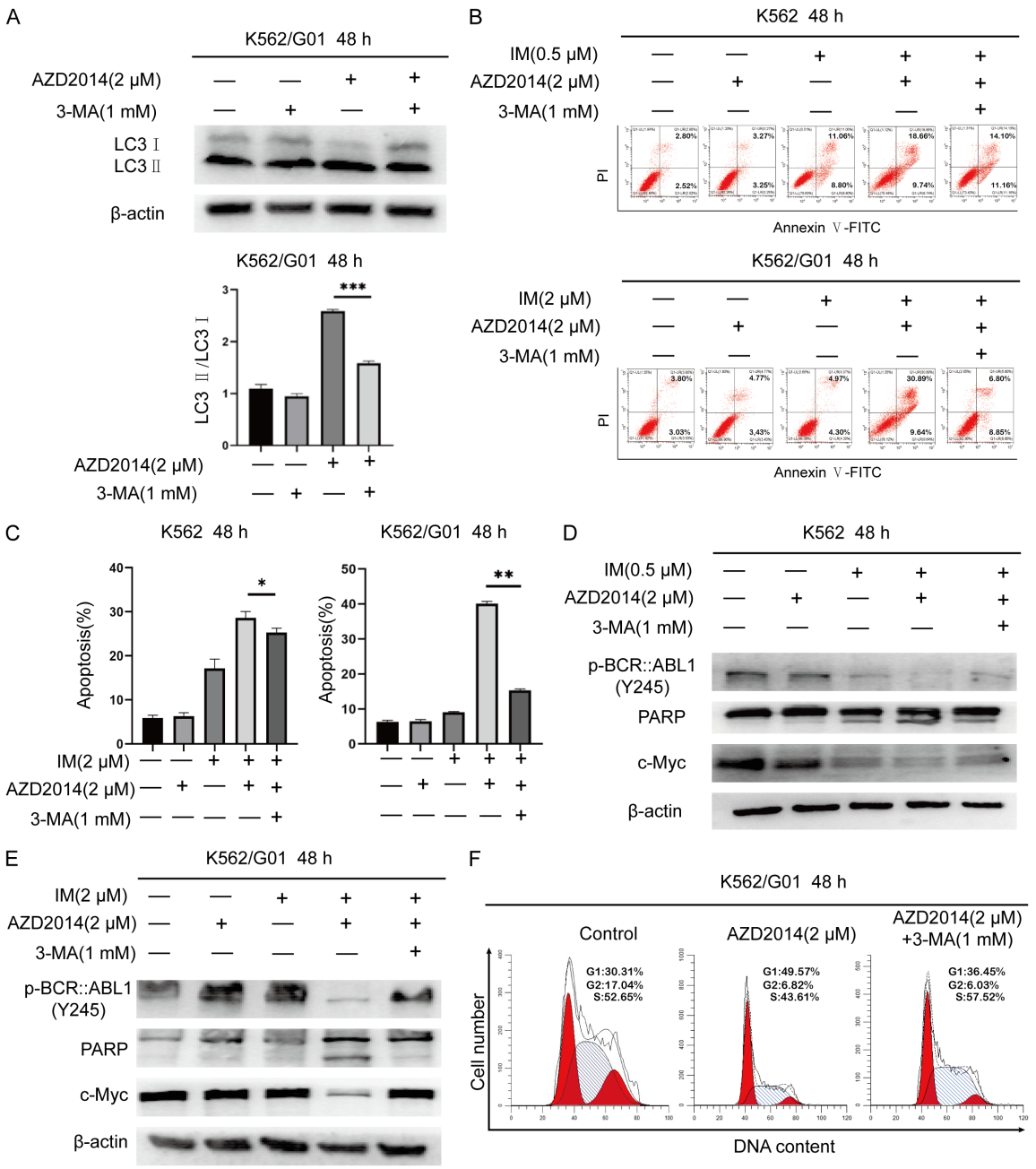

Figure 8. The anticancer effect of mTOR inhibition by AZD2014 on imatinib resistant K562/G01 cells is reversed by autophagy inhibitor. A. K562/G01 cells were treated with AZD2014, 3-MA, or AZD2014 plus 3-MA for 48 h, and then the expression of LC3 was detected by western blot. The ratio of LC3 II/I was analyzed with Image J software. B, C. K562 and K562/G01 were treated with imatinib, AZD2014, or imatinib plus AZD2014 with or without 3-MA for 48 h respectively, and then cell apoptosis was examined by FCM. The histogram represents the mean ± SD of three independent experiments. *P<0.05, **P<0.01 compared to control. D, E. K562 and K562/G01 were treated with imatinib, AZD2014, or imatinib plus AZD2014 with or without 3-MA for 48 h respectively, and then the level of p-BCR::ABL1, PARP, and c-Myc was detected by western blot. F. K562/G01 cells were treated by AZD2014 with or without 3-MA for 48 h, and then cell cycle was analyzed by FCM. The histogram represents the mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared to control.

It has been reported that mTOR is closely associated with autophagy [40, 41]. mTOR takes part in autophagy through regulating autophagy regulators, such as ATG12, ULK1 and Beclin-1. Two ubiquitin-like conjugations are required for autophagosome formation, the

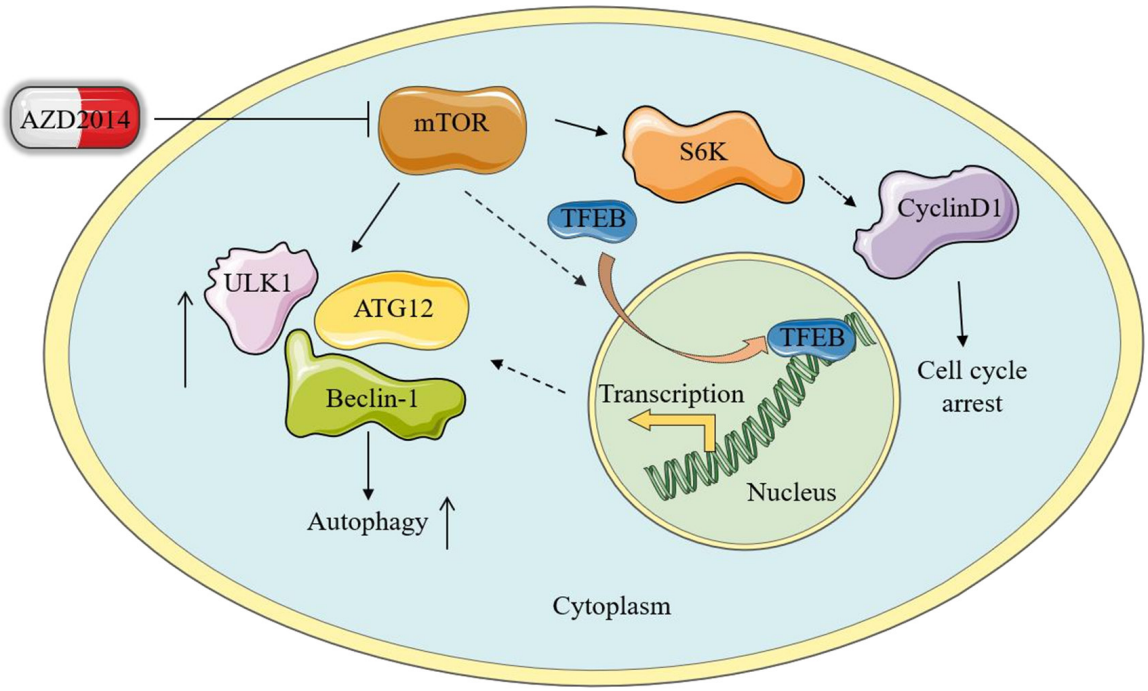

Figure 9. Schematic illustration of anti-cancer function of AZD2014 in CML.

first of which is to attach ATG12 to ATG5. This conjugate interacts with the Bro1 and V domains and blocks the inhibitory effect of the C-terminal proline rich domain of PDCD6IP, and then promotes membrane abscission event and controls the spatial distribution of late endosomes [42]. However, the expression of ATG12-ATG5 complex is inhibited by mTOR [43]. What's more, the autophagy-initiating ULK1 complex is under the repressive control of mTORC1, which binds ULK1 via its RAPTOR (regulatory-associated protein of mTOR) subunit and catalyzes the phosphorylation of ULK1 at Serine637 and Serine757 [44]. Due to the excessive activation of ATG12 and ULK1 via mTOR inhibition, the balance of intracellular autophagy is broken. Beclin-1 is a master regulator of autophagy. It executes its autophagy regulating function by facilitating PI3K to recruit other factors to the autophagy membrane then promoting autophagosomes production. However, the function of Beclin-1 can be inhibited by mTOR [45, 46].

There has recently reported that autophagy is a potential target in Ph+ leukemias, including CML [47-52]. In this study, we confirmed that mTOR inhibition by AZD2014 could push TFEB, a key regulator of autophagy and lysosome biogenesis [53, 54], into nuclei and raise the expression of ATG12, Beclin-1 and ULK1, which reinforce autophagy in CML cells. Notably, 3-MA could confront the effect of apoptosis induction and proliferation inhibition by AZD2014 in K562/G01. Meanwhile, AZD2014 showed antileukemia effect in imatinib-resistant CML-like disease mice as demonstrated by less severe symptom, less leukemic infiltration, less weight loss and longer lifespan, which was proved to be related to the elevated autophagy induced by AZD2014.

In general, great clinical progress has been made in CML patients with the application of more potent TKIs [55]. The main challenge is to resolve BCR::ABL1 independent imatinib resistance [11]. The data presented in this study have proven that targeting mTOR by AZD2014 could alleviate imatinib resistance and enhance the sensitivity of CML cells to imatinib. Our study presents evidence that targeting mTOR by AZD2014 is a potent complementary therapy for CML patients with BCR::ABL1 independent imatinib resistance.

Conclusions

The data presented in this study have proven that targeting mTOR by AZD2014 could allevi-

ate imatinib resistance and enhance the sensitivity of CML cells to imatinib. Our study presents evidence that targeting mTOR by AZD2014 is a potent complementary therapy for CML patients with BCR::ABL1 independent imatinib resistance (**Figure 9**).

mTOR is upregulated in imatinib-resistant CML cells. Inhibition of mTOR by AZD2014 leads to growth inhibition of K562/G01. AZD2014 synergizes with imatinib in apoptosis induction. Moreover, AZD2014 induces autophagy in K562/G01 cells, which is responsible for its anti-leukemia effect. Interestingly, mTOR signal pathway is poorly inhibited by imatinib and AZD2014 shows little effect on BCR::ABL1 signal pathway, which indicates that mTOR is involved in imatinib resistance via a BCR::ABL1 independent manner. Taken together, mTOR may represent a potential target to overcome BCR::ABL1 independent imatinib resistance.

Our study proved that weakening mTOR signaling pathways by AZD2014 alleviates BCR::ABL1 independent resistance through enhancing autophagy in imatinib-resistant CML cells. We also demonstrated that AZD2014 possessed the potential to improve CML cell susceptibility to imatinib. Our study provides a research basis for AZD2014 as a solution for BCR::ABL1 independent imatinib-resistant patients.

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Informed consent was obtained from all subjects involved in the study.

Disclosure of conflict of interest

None.

Abbreviations

CML, Chronic myeloid leukemia; mTOR, mammalian target of rapamycin; Q-PCR, Quantitative real-time PCR; FBS, Fetal bovine serum; FCM, Flow cytometry; BM, Bone marrow; HE, Hematoxylin and eosin; SD, Standard deviation; IM, Imatinib; PBMCs, Peripheral blood mononuclear cells; TKIs, Tyrosine kinase inhibitors; CI, Combination index; WBC, White blood cell; BUN, blood urea nitrogen; ALT, alanine transaminase; AST, aspartate transaminase.

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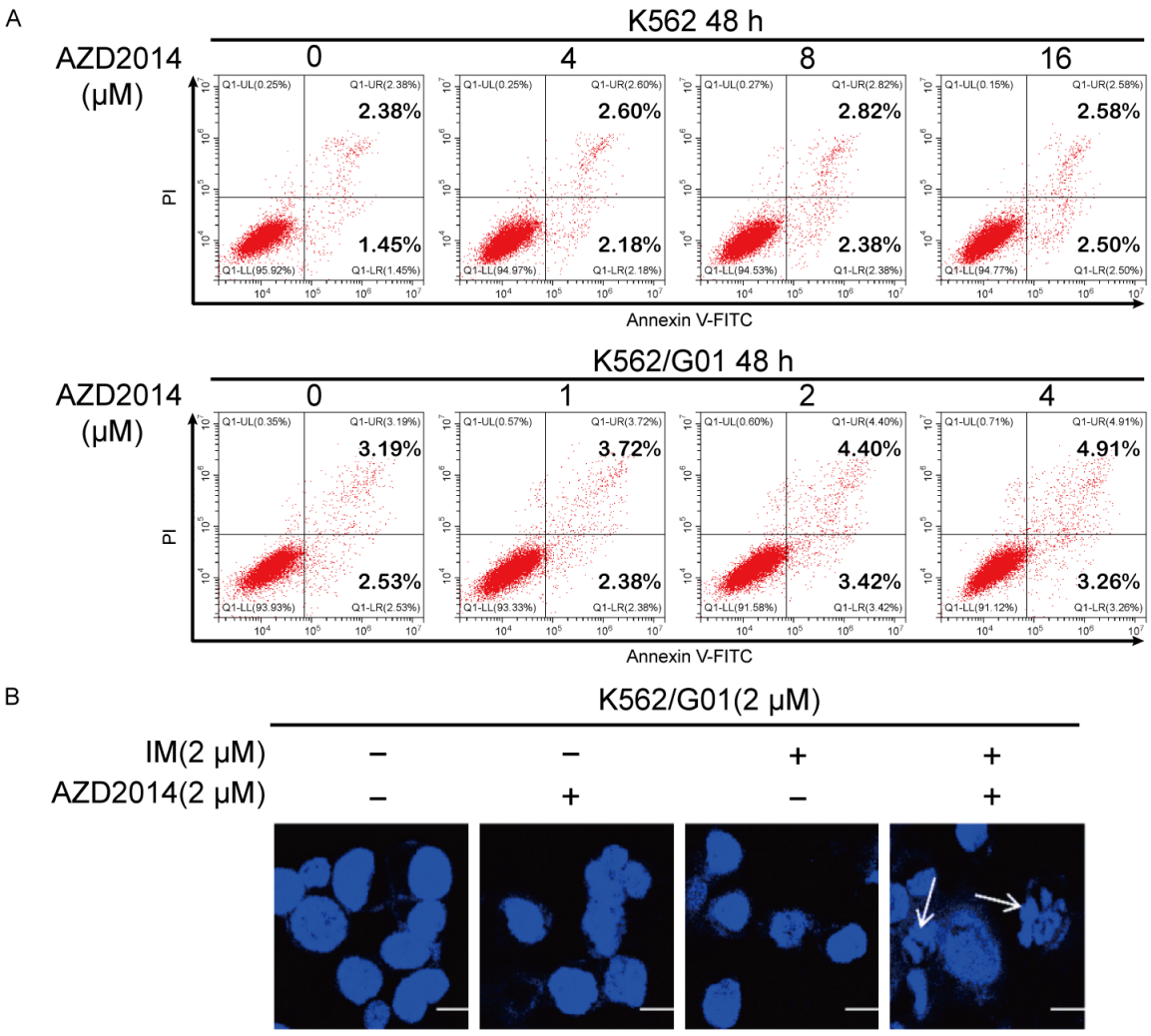

Figure S1. AZD2014 could not induce obvious apoptosis both in imatinib-sensitive and -resistant CML cells but caused obvious nuclear fragmentation in imatinib-resistant CML cells in combination with imatinib. A. K562 and K562/G01 cells were treated with indicated concentration of AZD2014 for 48 h, and then cell apoptosis was analyzed by FCM. B. K562/G01 cells were treated with AZD2014 and imatinib for 48 h, and then cell nuclei were stained with DAPI. The typical apoptotic cells were indicated with white arrows. The scale bar represents 10 μm.