A small molecule compound IX inhibits telomere and attenuates oncogenesis of drug-resistant leukemia cells

Yinghui Li, Jiali Gu, Yahui Ding, Huier Gao, Yafang Li, Yue Sun, Mei He, Wenshan Zhang, Jingjing Yin, Cuigai Bai, Yingdai Gao

1State Key Laboratory of Experimental Hematology, PUMC Department of Stem Cell and Regenerative Medicine, CAMS Key Laboratory of Gene Therapy for Blood Diseases, National Clinical Research Center for Blood Diseases, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin, China
2State Key Laboratory of Medicinal Chemical Biology, College of Pharmacy and Tianjin Key Laboratory of Molecular Drug Research, Nankai University, Tianjin, China
3High-throughput Molecular Drug Discovery Center, Tianjin International Joint Academy of BioMedicine, Tianjin, P. R. China

Correspondence
Cuigai Bai, High-throughput Molecular Drug Discovery Center, Tianjin International Joint Academy of BioMedicine, Tianjin 300457, P. R. China. Email: baicuigai@tjab.org

Yingdai Gao, State Key Laboratory of Experimental Hematology, PUMC Department of Stem Cell and Regenerative Medicine, CAMS Key Laboratory of Gene Therapy for Blood Diseases, National Clinical Research Center for Blood Diseases, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin 300020, China. Email: ydgao@ihcams.ac.cn

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INTRODUCTION

The development of targeted treatment of leukemia has progressed markedly to improve overall survival in leukemia patients, but drug resistance remains as one of the major challenges in leukemia therapy. The advent of imatinib mesylate (IM), the first-generation selective tyrosine kinase inhibitor (TKI), effectively induced remissions, and improved prognosis in patients. Unfortunately, not all patients with Bcr-Abl+ leukemia cells benefit from imatinib treatment. Relapse usually occurs after TKIs therapy, even with the more potent TKIs such as dasatinib, nilotinib, and bosutinib. The conventional chemotherapeutic drugs, including targeted drug TKIs, fail to eradicate quiescent LSCs, which is concerned as the root of drug resistance and disease relapse. Hence, targeting the eradication of LSCs should be one of the strategies for the treatment of refractory leukemia.

The previous study has indicated that abnormal telomere is related to the sensitivity of cancer cells to therapy. About 80%-90% of tumor cells including acute and chronic leukemias cells depend on telomerase activity (TA) to stabilize telomeres and immortalization. The activity of telomerase is associated with the uncontrolled self-renewal of LSCs. The cancer cells become more sensitive to drugs when telomerase is inhibited. Those observations implicate that telomerase plays an important role in the chemosensitivity of leukemia cells. In addition, 15% of cancers are telomerase-negative and achieve immortality via alternative lengthening of telomere (ALT), which depends on homologous recombination. ALT mechanism was detected in cancer stem cells and suggested playing an important role in tumorigenesis in vivo. It was concerned that the occurrence of the ALT mechanism in drug-resistant cells may disturb the effect of telomerase inhibitors in human cancer treatment. Therefore, targeting aberrant telomere elongation is a promising strategy for leukemia therapy.

Wnt pathway controlled various biologic processes, including the balance of cell stemness and cell differentiation. Canonical and non-canonical Wnt signaling plays complex roles in hematopoietic homeostasis. Consequently, the Wnt signaling was recognized to be crucial for leukemogenesis. The interaction between telomerase and Wnt pathway in cancer is well-known and the cooperation between Wnt/β-catenin signaling and telomerase is concerned as an important factor in cancer stem cell renewal regulating. Moreover, mutation in β-catenin can lead to the stabilization of telomeres, which is one of the hallmarks of tumorigenesis. Targeting this cancer-relevant interaction may be a promising drug target for leukemia therapy.

In the present study, we identified a reported small molecule compound IX, an imatinib derivative with a replacement fragment of a telomerase inhibitor, could selectively eradicate LSCs while sparing normal HSCs. Furthermore, compound IX could not only affect telomere elongation mechanism, but also inhibit the canonical and non-canonical Wnt pathways. These characteristics suggested that compound IX may serve as a potential therapeutic candidate for drug-resistant leukemia.

MATERIALS AND METHODS

2.1 | Cell culture and compound treatment

The CML cell lines K562 and K562/G were grown in suspension in RPMI 1640 medium (Gibico) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (Gibico), and 1% penicillin/streptomycin (Thermo Fisher Scientific) in a humidified 5% CO2 at 37°C. A stock solution of imatinib (Meilunbio) and compound IX at a concentration of 20 mM was prepared by dissolving the compound in sterile DMSO and stored at −20°C until use.

2.2 | Primary samples

Primary samples of blast crisis phase CML patients (n = 52) and umbilical cord blood of healthy donors (n = 3) are provided by the Institute of Hematology and Blood Diseases Hospital (China Tianjin). This study is carried out in accordance with the declaration of Helsinki and approved by the Ethics Review Board of the Institute of Hematology and Blood Diseases Hospital and Chinese Academy of Medical Sciences (China Tianjin). Patient mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation and shortly cultured in Iscove's Modified Dulbecco's medium (HyClone, Thermo Scientific, USA) with 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (Gibico).
2.3 | Transplant of mouse AML cells

For in vivo experiments of IX on mouse AML cells, GFP positive cells were harvested from bone marrow of AML mice model and cultured with compounds or vehicle control (DMSO) at their IC50 concentration (2.5 μM IX, 5 μM IM, 5 μM DMSO) for 18 hours before injection. After 18 hours of treatment, 2 x 10^5 live cells per mice were counted with trypan blue and transplanted into recipient mice. Peripheral blood was taken from the tail vein on Day0, Day10, and Day20. Mice were sacrificed 3 weeks later. Bone marrow was analyzed for the percentage of GFP positive cells by flow cytometry.

For Limiting Dilution Assay, GFP positive cells of different concentration gradients were injected into recipient mice, the survival times of each group were recorded to calculate the LSC frequencies by L-Calc™ Limiting Dilution Software (Stem Cell Technologies).

2.4 | Mice and leukemia mouse model

C57BL/6j mice were purchased from The Jackson Laboratory. The plasmid MSCV-MLL-AF9-IRES-GFP was co-transfected into the 293T packaging cell line using Lipofectamine 2000 (Invitrogen, Grand Island, NY) to produce virus. The virus supernatants were harvested 72 hours after transfection. Lineage-negative (Lin−) cells from the BM of 8-week-old C57BL/6j mice (CD45.2+) were enriched and transduced with virus supernatants. Bone marrow mononucleated cells (BMMNCs, 10^6/host) from 8-week-old B6.SJL mice (CD45.1+) with 1 x 10^6 MLL-AF9 virus transduced CD45.2+Lin− cells were transplanted into lethally irradiated (9.5 Gy) B6.SJL mice. The primary MLL-AF9 AML cells were isolated from the BM and spleen of end-stage recipient mice for further experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Hematology, Chinese Academy of Medical Science and all mice were hosted in the SPF facilities in the same institute.

2.5 | Colony-forming assay

For K562/G cells, the cells were co-cultured with target compounds at different concentrations for 18 hours, then the cells were seeded in triplicate in methylcellulose (H4434, Stem Cell Technologies) according to the manufacturer’s protocols and the colonies were counted 10 days after culture. Similarly, cells from one typical drug-resistant CML patient volunteer were selected to conduct the colony-forming units (CFU) assay.

For mice AML cells CFU Assay, murine leukemia GFP positive cells, after cultured with target compounds and vehicle control, were seeded in triplicate in methylcellulose (M3434, Stem Cell Technologies) according to the manufacturer's protocols. The concentrations of both compounds were 2.5 μM. The numbers of colonies were counted 5-7 days after culture.

2.6 | Xenograft transplantation experiments

Primary cells, isolated from patients with refractory or blast crisis phase CML, were injected via the lateral tail vein into sublethally irradiated (280 cGay) NOD/SCID mice 1 week before drug treatment. IX, IM, and vehicle control were administered orally every other day, 75 mg/kg each time for a total of six times. About 12 weeks after transplantation, mice were sacrificed to analyze the bone marrow.

2.7 | Telomerase activity measurement

Telomerase activity of compound-treated cells was determined by Telo TAGGG telomerase PCR ELISAplus kit (Roche, Manheim, Germany) according to the manufacturer’s protocol.

2.8 | Measurement of cell death

Cell viability was determined 18 hours after cultured with compounds by apoptosis assays. Cells were washed with cold phosphate-buffered saline (PBS) twice and then using the Annexin V-APC Apoptosis Analysis Kit (Tianjin Sungene Biotech) according to the manufacturer's protocol. Then analyzed the cell samples by flow cytometry within 1 hour. The samples were analyzed on a BD LSRII flow cytometer (BD Biosciences).

2.9 | RNA extraction and preparation of cDNA

Total RNA from K562/G cells was isolated 18 hours after treated with compounds by TRIzol. The quantity of RNA samples was determined by Nanodrop 2000 (Nanodrop Technologies). The following RT-reaction was conducted as described by the same reagents.25

2.10 | Quantitative real-time PCR

Real-time PCR was performed by 5 μL SYBR Premix Ex Taq technology (Takara BIO), 1 μL of cDNA product, 0.25 μL of each forward and reverse primers (20 μM), and 3.5 μL of
were downloaded from the genome website directly. Index of quality. Reference genome and gene model annotation files downstream analyses were based on the clean data with high Q30, and GC content the clean data were calculated. All the and low quality reads from raw data. At the same time, Q20, scripts. In this step, clean data (clean reads) were obtained by FASTQ format were first processed through in-house perl paired-end reads were generated. Raw data (raw reads) of sequenced on an Illumina Hiseq platform and 125 bp/150 bp tions. After cluster generation, the library preparations were performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer’s instruc-
tions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and 125 bp/150 bp paired-end reads were generated. Raw data (raw reads) of FASTQ format were first processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, Q20, Q30, and GC content the clean data were calculated. All the downstream analyses were based on the clean data with high quality. Reference genome and gene model annotation files were downloaded from the genome website directly. Index of the reference genome was built using Hisat2 v2.0.5 and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5. We selected Hisat2 as the mapping tool for that Hisat2 can generate a database of splice junctions based on the gene model annotation file and thus a better mapping result than other non-splice mapping tools. FeatureCounts v1.5.0-p3 was used to count the reads numbers mapped to each gene. And then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM, expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced, considers the effect of sequenc-
ing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels. Differential expression analysis of two conditions/groups (two biological replicates per condition) was performed using the DESeq2 R package (1.16.1). DESeq2 provides statistical routines for determining differential ex-
pression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg’s approach for controlling the false discovery rate. Genes with an adjusted P-value <.05 found by DESeq2 were assigned as differentially expressed. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-
level information, especially large-scale molecular datasets generated by genome sequencing and other high-through put experimental technologies (http://www.genome.jp/kegg/). We used clusterProfiler R package to test the statistical enrichment of differential expression genes in KEGG pathways.

2.12 Western blotting

Cells were harvested and lysed in RIPA buffer (Thermo Scientific, USA) after cultured with compounds. The protein level of each sample was quantified and normalized by a BCA protein Assay (Pierce, Thermo Scientific, USA). An equal amount of protein (20 µg/lane) was separated by SDS-PAGE, transferred to a PVDF membrane (Millipore, UK), blocked by 5% non-fat milk, and incubated with primary antibodies (all from Cell Signaling Technology, Danvers, MA, USA). After incubation with HRP-conjugated secondary antibodies, the signals were detected using an enhanced ECL substrate (GE Healthcare, Chicago, USA) and visualized using the ChemiDoc Touch Western Imager (Bio-Rad, USA).

2.13 Enzyme and uptake assays

Kinase activities were measured by a non-radioisotopic method. The IC50 values (concentration causing a
half-maximal inhibition of control specific activity), EC50 values (concentration producing a half-maximal increase in control basal activity), and Hill coefficients (nH) were determined by non-linear regression analysis of the inhibition/concentration-response curves generated with mean replicate values using Hill equation curve fitting: \( Y = D + ((A - D)/((1 + (C/C50)^{nH}))) \). Y = specific activity, A = left asymptote of the curve, D = right asymptote of the curve, C = compound concentration, C50 = IC50 or EC50, and nH = slope factor.

### 2.14 C-circle assay

CC Assay was conducted as Henson JD\(^{26}\) described. Genomic DNA was digested with 4 U/µg HinFl and Rsal restriction enzymes and 25 ng/µg RNase (Dnase-free; Roche). Sample, 10 µL, was combined with 10 µL of 0.2 mg/mL BSA, 0.1% Tween, 1 mM each dATP, dGTP and dTTP, 1 × Φ29 Buffer and 7.5 U Φ29 DNA polymerase (BioLabs) and incubated at 30°C for 8 hours then 65°C for 20 minutes. For quantification, the reaction products were diluted to 60 µL with 2 × SSC and dot-blotted onto a 2 × SSC-soaked positively charged nylon membrane (Roche). DNA was UV-cross-linked onto the membrane, which was then hybridized at 37°C with DIG-labeled telomere specific hybridization probe (Roche) and DIG Easy Hyb Granules (Roche). Incubation with Anti-DIG-Alkaline Phosphatase and then perform chemiluminescence detection using the ChemiDoc Touch Western Imager (Bio-Rad, USA).

### 2.15 Cell cycle analysis

K562, K562/G, and mice primary GFP\(^{+}\) cells (1 × 10\(^6\) cells/10 mL) were incubated with compound IX or IM for 24 hours. Then cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), and fixed with 75% ethanol. After centrifugation, the fixed cells were resuspended in propidium iodide (PI) solution (25 µg/mL) (BD Biosciences), and incubated in the dark for 30 minutes at 4°C to be available for analysis using BD LSRII flow cytometer. Data were analyzed using the ModFit software.

### 2.16 Statistical analysis

Statistical analysis was performed using Prism software (GraphPad) and Student's t tests were used to analyze the significance between two experimental groups. Kaplan-Meier method and Log-rank test were used for survival analysis. A \( P \)-value <0.05 was considered statistically significant in all cases.

## 3 RESULTS

### 3.1 Compound IX induced apoptosis of drug-resistant leukemia cells

Compound IX was reported as an analog of IM and was able to inhibit Bcr-abl kinase in the K562 cell line\(^{24}\) (Supporting Figure S1A). To evaluate the cytotoxic effect of compound IX, we first adopted K562 cells and representative imatinib-resistant K562/G cells.\(^{27}\) We treated cells with IX and IM separately at various concentrations and then measured cell viability by Annexin V/PI dual staining. The result on K562 showed that compound IX demonstrated only slightly cytotoxicity at the lower concentration, but when the concentration reached 10 µM, it showed the ability to rapidly eliminate viable cells. IM induced cell apoptosis in a dose-dependent manner (Figure 1A,B). Compared with IM, compound IX induced a remarkable reduction of K562/G cells when it reached the concentration of 10 µM (Figure 1C,D). Furthermore, compound IX did not affect the cell cycle phases of either K562 cells or K562/G cells (Figure 1E,F). In contrast, IM induced an obvious cell cycle arrest in the G0/G1 phase in K562 cells but not in K562/G cells (Figure 1E,F), suggesting that apoptosis induction by IM in K562 cells may be related to the cell cycle arrest. These results indicated that the mechanism of compound IX may be different from IM. In order to evaluate the activity of compound IX on leukemia initiation cells, primary cells were isolated from refractory and blast crisis phase CML patients and cultured with compounds separately. Compound IX induced a significant decrease in viable primary leukemia cells at both concentrations. In contrast, IM barely induced any reduction in primary leukemia cell viability (Figure 1H). The representative flow cytometric data of blast crisis phase CML patients after compound treatment are shown in Figure 1G. These results indicated that compound IX exhibited a stronger inhibitory effect than IM on both drug-resistant cells and primary initiation leukemia cells.

### 3.2 Compound IX could inhibit the self-renewal and survival of leukemic stem cells in AML mouse model

Since drug resistance is believed to be caused by cancer stem cells, we suspected that compound IX could affect the survival of LSCs. We then adopted the MLL-AF9 mouse leukemia model which is believed to be caused by LSCs\(^{28}\) to further explore the stem cells eradicating ability of compound IX. We first tested the killing capacity of compound IX on primary AML cells isolated from the bone marrow of the AML mouse model in vitro. Compared with IM, compound IX significantly reduced the survival of primary AML cells (GFP\(^{+}\) cells) in vitro (Figure 2A,B). In addition, compound IX did not affect the cell
Compound IX induces apoptosis in drug-resistant cells and primary LSC-like cells in vitro. A-D, Viabilities of K562 (A and B) after pretreatment with IM at 0.25, 0.5, 1 μM and compound IX at 2.5, 5 and 10 μM for 18 h and K562/G (C and D) after pretreatment with IM and compound IX at 2.5, 5 and 10 μM for 18 h were measured by Annexin V/PI staining. E and F, Cell cycle distribution of K562 (E) and K562/G cells(F) treated with compound IX (5 μM for K562 cells, 5 μM for K562/G cells) or IM (0.5 μM for K562 cells, 5 μM for K562/G cells) for 24 h was analyzed by propidium iodide staining and flow cytometry. G, Representative flow cytometric data for Annexin V/PI staining in primary cells isolated from blast crisis phase CML patient after IM and compound IX treatment. H, Viabilities of monocular cells of patients (n = 52) with refractory or blast crisis phase CML after treatment with IM or compound IX at 5 and 10 μM for 18 h. *P < .05, **P < .01, ***P < .001, ****P < .0001 by two-sided Student’s t test. n.s., not significant (P > .05)

3.3 | impaired human LSCs in PDX model

Since the compound IX exhibited great potential in eradicating LSCs in AML mouse model, we then examined whether compound IX could affect the function of K562/G cells and human primary leukemia cells. We first analyzed the clonogenic potential of K562/G cells. The results showed that compound IX at 2.5 μM could induce a significant reduction of the K/562G cell colony formation. The clonogenic potential of K562/G cells was almost completely abolished when compound IX reached the concentration of 10 μM, while IM showed only slight inhibition of K562/G cells CFA (Figure 3A). As for primary leukemia cells derived from patients with blast crisis phase CML, the majority of them were inhibited by both compounds. However, compound IX still had a better outcome than IM (Figure 3B). This result is consistent with the previous study that leukemia stem cells are less sensitive to kinase inhibitors.30 These results suggested that compound IX can effectively eradicate human leukemia stem or progenitor cells.

To further investigate whether compound IX treatment could be effective in vivo, we adopted a PDX model (Figure 3C). The results showed that both compound IX and IM could relieve the tumor burden of the mice and prolong the life span of mice compared with vehicle treatment. However, mice in the compound IX group survived longer than those in the IM group (Figure 3D). Besides, mice treated with compound IX showed a more significant reduction in the percentage of leukemic cells in the bone marrow of mice was determined using FACS analysis and the representative flow cytometric data were shown in Figure 3F. These results indicated that compound IX could effectively inhibit leukemia and at least partially reduce the survival of LSC in vivo. More importantly, IX had little effect on normal monocular cells and CD45+ CD34+ CD38− cells from healthy umbilical cord blood within its effective concentration 5 μM (Figure 3G). The apoptosis of one representative normal blood sample was shown in Supporting Figure S1E. As HSCs and LSCs have a lot of features in common, it is important that the candidate compounds selectively acting on LSCs and sparing normal HSCs. Therefore, these characteristics of IX make it a promising candidate for drug-resistant leukemia therapy.

3.4 | Compound IX inhibited telomerase activity and Wnt pathway

IX is an analog of IM that has a replacement fragment of a telomerase inhibitor.24 As is known to all, IM is a tyrosine
Compound IX decreases leukemia cells in the AML mice model and affects LSCs function. A and B. Viabilities of GFP+ cells after pretreatment with IM and compound IX at 2.5, 5 μM for 18 h were measured by Annexin V/PI staining. C. Cell cycle distribution of GFP+ cells treated with compound IM (5 μM) or IX (1.25 μM) for 24 h was analyzed by propidium iodide staining and flow cytometry. D. Colony numbers of different colony types of IM (5 μM) and compound IX (1.25 μM) for 18 h pretreatment mouse AML cells were counted 1 week later. E. Representative colony pictures of different colony types. F-H. The percentage of GFP+ cells (F and G) and C-kit/Gr-1 staining LSCs (H) in the bone marrow of AML mouse models (n = 5 per group) was determined 3 weeks after the injection of DMSO, IM (5 μM) and IX (2.5 μM) pretreated AML cells. I. Limiting dilution assays comparing the frequencies of LSCs in vehicle-treated and IX-treated BM cells. (Experiments were conducted two additional times for validation.) *P < .05, **P < .01, ***P < .001, ****P < .0001 by two-sided Student’s t test. n.s., not significant (P > .05).

kinase inhibitor which represents a milestone in CML treatment, we first investigated the kinase activity inhibition ability difference between IX and IM. The result of enzyme and uptake assay showed no difference compared with IM (Figure 4A). The influence of compounds on the drug efflux pump has no obvious difference either (data not shown). We then thought the difference in response of drug-resistant cells and LSCs to the compounds may be caused by the altered structure between IX and IM. Preview studies have shown that the acquisition of unlimited cellular proliferation of cancer cells is closely related to the maintenance of telomere. Besides, telomerase activity was conceded relating to the drug resistance of cancer cells. Our result also showed that the IM-resistant K562/G cells had a higher telomerase activity (Figure 4B). To determine the effect of IX on telomerase, both the transcriptional activity of hTERT and the enzymatic activity of telomerase were determined. We first measured the hTERT expression after IX treatment by RT-PCR. The relative mRNA level of hTERT was dramatically decreased (Figure 4C). The TA result also showed a great decline in K562/G cells treated with compound IX (Figure 4D). The IM-treated cells only showed moderate inhibition. The results indicated that IX-treated cells exhibited a remarkable reduction in both hTERT expression and TA. Additionally, there are 15% telomerase-negative cancers depend on the alternative telomere-lengthening mechanism (ALT) to achieve immortality. 11 We then conducted the C-circle assay on the reported ALT positive cell line U2-OS, the first ALT assay that recognized to have a quantitative relationship with ALT activity levels, to determine the effect of compound IX on ALT. 26 The reduced C-circle level of compound IX co-cultured cells suggested this new compound could affect the ALT activity (Figure 4E), but the underlying mechanism still needs further investigation. These results together identified the aberrant elongation of telomere mechanism may be the target of IX. Though telomerase is thought to be active in normal stem cells, our previous data showed that compound IX did not have detrimental effects on the normal stem and progenitor cells which may due to the effects on the ALT mechanism.

The gene expression changed in the K562/G cell line was assessed by RNA sequencing after treated with compound IX, revealing 5910 differentially expressed genes (Supporting Figure S1F). The KEGG analysis did not show any stem cell-related signaling pathway (Supporting Figure S1G). However, the Wnt pathway has been widely reported to be critical in cancer development by affecting CSCs and essential for LSCs in AML. 21,29,34 Wnt signaling in drug resistance cancer is important as well. 35,36 Besides, the regulation between telomerase and Wnt/β-catenin pathway in stem cells and cancer cells is a general biological feature. 23 We then performed gene set enrichment analysis (GSEA) to determine whether the expression of Wnt related genes was changed. By the RNA-Seq result of K562/G cells treated with compound IX and the Reactome Wnt gene set (https://reactome.org), we found that the Wnt signaling pathway was down-regulated in compound IX-treated cells (Figure 4F). We then investigated the mRNA levels of the Wnt signaling pathway-related factors by quantitative RT-PCR. Both canonical and noncanonical Wnt pathways were affected by compound IX. Canonical Wnt pathway relies on the activity of beta-catenin in nucleus, the ligands binding to Wnt receptors results in the activation of DVL protein, which promotes beta-catenin accumulating and entering the nucleus. After co-culture with compound IX, the mRNA level of β-Catenin and DVL2 was markedly decreased (Figure 4G). Compared to the other two groups, the receptors of Wnt ligands in IX-treated cells including LRP5, LRP6, FZD5, and FZD7 were noticeably decreased on the transcriptional level, the protein level of LRP5 was markedly downregulated as well (Figure 4G, H). Meanwhile, the expression of NFKD which inhibits the activation of DVL was increased (Figure 4H). Moreover, one of the non-canonical Wnt pathway, Wnt5/Ca2+ pathway which remains unclear in mammalian cells especially in human cancers was affected as well. 37 Some studies support that WNT5 related non-canonical Wnt pathway suppresses tumor progression, 38 while others insist it promotes oncogenesis. 37 It has been reported that the Wnt5/Ca2+/NFAT pathway plays a role in the maintenance of Bcr-Abl+ leukemia cells. When ligands bind to FZD and co-receptor Ryk, PLCB will be activated and finally result in the activation of NFAT, which promotes the survival of leukemia cells. 37 Our results showed that the transcription level of WNT5B, one of the agonists of the Wnt5/Ca2+ pathway, and PLCB1 was markedly downregulated by compound IX (Figure 4G). It is worth noting that compound IX significantly decreased the expression of WNT5 and PLCB1, while IM barely affects either of them (Figure 4H). Meanwhile, in compound IX-treated K562/G
cells, the expression of WNT3 that initiates the canonical Wnt signaling pathway was inhibited by both compound IX and IM, but the expression in IX-treated group is lower than IM-treated groups (Figure 4H). The expression of the Wnt pathway in mouse MLL-AF9 cells was measured as well. The variation of the Wnt pathway caused by compound IX was
Compound IX inhibits CFA of primary cells, relieves tumor burden, and prolongs the survival of PDX model mice. A and B. Colony Numbers of IM and compound IX pretreatment K562/G cells (A) and primary cells isolated from refractory or blast crisis phase CML patients (n = 22) (B). C. Schematic outline of the in vivo experiment of PDX models. D. Kaplan-Meier survival curves of xenograft mice (n = 5 in control group, n = 6 in IX and IM group). Mice received 5 × 10⁶ primary CML cells isolated from primary refractory CML patients. *P < .05, **P < .01, ***P < .001, ****P < .0001, two-sided log-rank test. (Experiments were conducted two additional times for validation.) E and F, leukemia engraftment in BM 12 weeks after injection (E). Representative data for flow cytometric analysis of hCD45 staining in the BM of PDX model (F). G, Viability of normal cells (CD34⁺CD38⁻ normal cells (up panel) and normal mononuclear cells (down panel) from three umbilical cord blood samples) in response to IM and compound IX at 5 and 10 μM. *P < .05, **P < .01, ***P < .001, ****P < .0001 by two-sided Student’s t test. n.s., not significant (P > .05)

4 | DISCUSSION

Although the emergence of TKIs making great progress in CML treatment, the following evolved drug resistance still makes CML an intractable disease all over the world. The new generations of TKIs, such as dasatinib and nilotinib, ease the symptom to some extent at the beginning, but fail to truly overcome this issue for the mutation of Bcr-Abl. As we all know, the reactivation of Bcr-Abl kinase is the major reason for TKIs resistance in CML and some Bcr-Abl negative CML patients have a poor response to IM or other TKIs as well. Another non-negligible reason is that CML stem cells are insensitive to kinase inhibitors. Therefore, it is urgent to explore new agents that can overcome drug resistance and targeting LSCs. In this study, we adopted different stem cell function assays to evaluate the effect of compound IX on LSC cells. Both CFU assay and LDA assay results indicated that compound IX could result in LSC functional defects. The transplantation experiment showed that compound IX could delay the development of leukemia cells and decrease the disease engraftment in recipient mice. Besides, the LSK population and C-kit⁺Gr-1⁻ cell population in mice BM, which reported to enrich with stem cells in AML, all exhibited a significant decline by IX treatment. These evidences demonstrated that compound IX could decrease LSCs in vitro and in vivo. Moreover, it could effectively prolong the life of the PDX model mice. As HSCs and LSCs have a lot of features in common, it is important that candidate compounds selectively act on LSCs. According to the apoptosis examination on three different normal umbilical cord blood samples, toxicity on normal CD34⁺CD38⁻ cells or normal healthy mononuclear cells were not observed within the effective concentration of compound IX (5 μM). The reasons that IX exerts this specific targeting character may be related to the different telomerase activity between LSCs and HSCs.

Numerous studies have indicated that telomerase is related to the sensitivity of cancer cells to therapy. In some drug-resistant cancer cells, the expression of telomerase was increased. When the telomerase was inhibited, the cancer cells would be more sensitive to drugs. Those observations implicate that telomerase may play an important role in downregulation of chemosensitivity in cancer cells. Thus, telomerase is concerned as a promising target for cancer therapy. It has been reported that telomerase reduction could interfere with the development of CML induced by Bcr-Abl. Consistent with the report, our results showed that imatinib-resistant K562/G cells had a higher telomerase activity than K562 cells, and IX could significantly decrease the transcriptional level of hTERT and enzyme activity of telomerase in K562/G cell line. In this study, we demonstrated a novel small molecule compound IX with a fragment of telomerase inhibitor as a potential refractory CML drug candidate.

It has been demonstrated that oncogenic growth in leukemias of both myeloid and lymphoid lineage is dependent on Wnt signaling. Both chronic and acute subtypes of myeloid leukemias were reported to be regulated by the Wnt signaling pathway. The blast crisis cells isolated from therapy-resistant CML patients display activated Wnt signaling. Moreover, the Wnt pathway participates in the regulation of progenitors and stem cells self-renewal. Overactivation of Wnt signaling would enable granulocyte-monocyte precursors (GMPs) to develop from short-term renewal capacity to long-term renewal. Canonical Wnt pathway, as well as non-canonical Wnt pathway, participates in the stem cell...
function, but they perform differently in some contexts. Therefore, the antagonism between them may be a novel target for cancer therapy. β-catenin is the central protein in the Wnt/β-catenin pathway. Wnt ligand, like Wnt3, binding to the Fzd receptor as well as LRP5/6 receptors initiates the transcription of β-catenin. The RT-PCR and Western blot
FIGURE 4  Compound IX inhibits abnormal telomere elongation mechanism and Wnt signaling pathway in drug-resistant cells. A, The Enzyme and Uptake Assays show the IC_{50} values of compound IX against each kinase. B, The telomerase activity of K562 and K562/G cells measured by PCR-based TRAP (Telomerase repeat amplification protocol) assay. *P-value < .05; C, The relative mRNA level of hTERT was measured using RT-PCR in K562/G cells after treated with 10 μM compound IX for 24 h. D, The inhibition of telomerase activity by IM and compound IX in K562/G cells measured after treatment with compounds at 10 μM for 24 h. E, Dot blot of the C-circle assay on 20ng genomic DNA from vehicle (DMSO), IM (10 μM), and IX (10 μM) treated-K562/G cells. Compound IX-treated cells had lower C-circle levels compared to vehicle and IM-treated cells. F, The gene expression profiles of K562/G cells incubated with compound IX were compared to WNT related genes derived from Reactome gene set. Enrichment score (ES), normalized enrichment score (NES), false discovery rate (FDR), and P-values were calculated using gene set enrichment analysis (GSEA) software. G, Cells incubated for 18 h in the presence of 10 μM IM, 10 μM IX or DMSO. β-catenin, DVL2, NKD2, LRP5, FZD5, FZD7, WNT5B, and PLC-beta1 relative mRNA level measured by RT-PCR. Data are mean ± SD of three independent experiments. H and I, Western Blot on the protein levels of WNT related molecules in K562/G cells (H) treated with IX (10 μM) and IM (10 μM), and in mouse MA9 cells (I) treated with IX (2.5 μM) and IM (5 μM). B-actin was used as the reference proteins. *P < .05, **P < .01, ***P < .001. ****P < .0001 by two-sided Student's t test. n.s., not significant (P > .05)

results demonstrated that compound IX could decrease the expression level of ligands and receptors in Wnt/β-catenin signaling in the K562/G cell line. Particularly, the inhibition of β-catenin worthy noting. The interaction between β-catenin and telomerase is complicated. It had been reported that β-catenin could directly regulate hTERT expression in cancer cells. Others claimed β-catenin and telomerase could reinforce each other in the stem cell niche or cancer cells. Our results showed that compound IX could inhibit both telomerase and β-catenin. Wnt/Ca^{2+} pathway is one of the non-canonical Wnt pathway, which remains secretory in mammalian cells, especially its role in human cancers. Some studies support that the non-canonical Wnt pathway suppresses tumor progression, while others insist it promotes oncogenesis. Gregory had proved that the inhibition of this pathway could sensitize blast crisis CML cells to imatinib. In present works, the results suggested that the Wnt/Ca^{2+} pathway was related to the drug-resistance issue of CML. The application of IX on K562/G cells could induce significantly mRNA and protein expression reduction of WNT5a, which is the activator of the Wnt/Ca^{2+} signaling pathway. The Wnt receptor FZD was inhibited as well. These data suggested that IX effectively acting on drug-resistant cells not only relays on telomerase inhibition, but also depends on the inhibition of the Wnt/Ca^{2+} signaling pathway. However, the mechanism of the Wnt/Ca^{2+} signaling pathway contributing to resistance to imatinib therapy remains unknown. The phenomenon could be observed in mouse MA9 cells too. The protein level of both Wnt3 and Wnt5 was decreased and their receptors were inhibited as well. Noticeably, the downregulation of Wnt/β-catenin signaling proteins caused by compound IX in MA9 cells was not as significant as in K562/G cells. Meanwhile, IM hardly showed the inhibition of Wnt/β-catenin signaling in MA9 cells, which totally inconsistent with the results in K562/G cells. This phenomenon perhaps could explain the eradication ability difference against MA9 cells between compound IX and IM. After compound IX treatment, we could see an obvious decline in Wnt5 and PLCB1 in the MA9 cell just like in the K562/G cell line. Accordingly, we speculated that the inhibition of the Wnt5-related non-canonical Wnt pathway plays an important role in the procedure of compound IX inhibiting leukemia.

In conclusion, this work suggests a novel small molecule compound IX can eradicate drug-resistant leukemia cells or LSCs, while has little effect on the normal stem cells and progenitor cells in vitro. Unlike traditional TKIs inhibitors that target Bcr-Abl kinase, IX can inhibit the abnormal elongation of the telomere. It not only effectively decreases the telomerase activity of drug-resistant leukemia cells, but also exerts inhibition activity on the ALT mechanism. Moreover, IX can significantly suppress the Wnt signaling pathway. These evidence suggesting that IX may serve as a safe and promising candidate for refractory leukemia therapy.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Y. Li, J. Gu, C. Bai, Y. Gao designed the research; C. Bai provided the compounds; Y. Li, J. Gu analyzed the data; J. Gu, Y. Ding, Y. Li, M. He, W. Zhang, J. Yin collected primary samples; Y. Li, J. Gu performed animal experiments and collected data; Y. Gao, Y. Li, J. Gu wrote and revised the manuscript; and all authors read and approved the final manuscript.


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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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