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PRECLINICAL STUDIES

CDKI-73: an orally bioavailable and highly efficacious CDK9 inhibitor against acute myeloid leukemia

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Summary

Acute myeloid leukemia (AML) is the most common form of acute leukemia with dismal long-term prognosis with age. The most aggressive subtype of AML is MLL-AML that is characterized by translocations of the mixed-lineage leukemia gene (*MLL*) and resistance to conventional chemotherapy. Cyclin dependent kinase 9 (CDK9) plays a crucial role in the *MLL*-driven oncogenic transcription, and hence, inhibiting activity of CDK9 has been proposed as a promising strategy for treatment of AML. We investigated the therapeutic potential of CDKI-73, one of the most potent CDK9 inhibitors, against a panel of AML cell lines and samples derived from 97 patients. CDKI-73 induced cancer cells undergoing apoptosis through transcriptional downregulation of anti-apoptotic proteins Bcl-2, Mcl-1 and XIAP by majorly targeting CDK9. Contrastively, it was relatively low toxic to the bone marrow cells of healthy donors. In MV4–11 xenograft mouse models, oral administration of CDKI-73 resulted in a marked inhibition of tumor growth ($p < 0.0001$) and prolongation of animal life span ($P < 0.001$) without causing body weight loss and other overt toxicities. The study suggests that CDKI-73 can be developed as a highly efficacious and orally deliverable therapeutic agent for treatment of AML.

Keywords CDKI-73 · CDK9 · AML · MLL-AML · MV4–11 xenograft · Apoptosis

Introduction

Acute myeloid leukemia (AML) is a clinically heterogeneous blood neoplasm characterized by clonal expansion of immature, non-functional blast cells in the myeloid lineage. It is the most common acute leukemia in adults, with an increasing incidence and long-term dismal prognosis with age [1, 2]. The most devastating subtypes namely MLL-AML are characterized by translocations of the mixed lineage leukemia gene (*MLL*) in chromosome band 11q23, and the MLL-rearrangement in AML leads to chemoresistance [3, 4], resulting in a short overall median survival of only 9 months in patients treated with currently available therapy. Several studies have confirmed that MLL fusion proteins (MLL-AF4, MLL-AF9 and MLL-ENL) directly control the transcription and expression of multiple leukemic oncogenes including *Mcl-1*, *Hoxa9* and *Myc* [5–9]. Cyclin dependent kinase 9 (CDK9) in complex with cyclin T forms the essential regulator of transcriptional elongation which is also required for oncogenic MLL-driven transcription [10]. CDK9 inhibitor directly targets the transcriptional machinery required for MLL-fusion proteins and reduces the transcription of multiple MLL-driven leukemic oncogenes, leading to apoptosis [11, 12].

Several CDK9 inhibitors have been advanced into clinical trials for treating leukemia. However, either their toxicity or unsatisfactory pharmacokinetic properties have hampered their therapeutical applications [13–15]. CDKI-73 is one of

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the most potent CDK9 inhibitors (K_i ~ 3 nM) identified. It induced cell death in chronic lymphocytic leukemia B-cells and ovarian cancer cells potently with low toxicity to noncancerous cells [16–18]. These features along with its favourable pharmaceutical properties and oral bioavailability (F = 54–85%), facilitating drug delivery in vivo [17, 19], make CDKI-73 an excellent drug candidate for development towards the clinic.

This study explores the potential of CDKI-73 as a new therapeutic option for treating AML. We investigated the effectiveness of CDKI-73 on the viability and proliferation of AML cell lines and patient samples, as well as its cellular mechanism of action. Preclinical pharmacology and in vivo anti-tumor efficacy of CDKI-73 were also assessed.

Materials and methods

Chemical compounds

CDKI-73 tartrate was kindly provided by Changzhou Le Sun Pharmaceuticals, Jiangsu, China. Its synthesis has been described previously [16]. All other reagents were purchased from Sigma-Aldrich Pty. Ltd., NSW, Australia.

Cell culture

The leukemia cell lines were kindly provided by Prof. R. D'Andrea (Acute Leukemia Laboratory, University of South Australia, Australia). Bone marrow samples were obtained from newly diagnosed or relapsed AML patients ($n = 97$) admitted to Qilu Hospital, Shandong University, China, from March 2015 to December 2016. Patient characteristics are summarized in Table 2. Normal bone marrow samples were obtained from seven healthy individuals (Supplementary Table 1). Samples from patients and healthy donors were obtained with proper written consent following protocols approved by the institution. Mononuclear cells were separated by Ficoll-Hypaque density-gradient centrifugation and CD34+ cells further positively selected using a magnetic activated sorting system (Miltenyi, Bergish Gladbach, Germany) according to the manufacturer's instructions. Purity of the CD34+ cells was determined as 92–97% with flow cytometry. All of the leukemia cell lines and CD34+ cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco, Grand Island, NY, USA) at 37 °C in a humidified atmosphere containing 5% CO_2 [18].

In vitro assays

The viability of leukemia cell lines and primary cells was determined by resazurin (Sigma-Aldrich, Australia) assay [20] and cell counting Kit-8 (CCK8) (Bestbio, Shanghai, China) assay [21], respectively. Flow cytometry (e.g. cell cycle, apoptosis assays), RT-qPCR and western blot were performed following standard protocols. Detailed materials and methods are provided in the Supplementary methods.

In vivo studies

All the animal experiments were conducted in accordance with the guidelines and approval (i.e. AEC91/12 and AECU03–14 for pharmacokinetics and in vivo efficacy, respectively) from the ethics committee of SA Pathology, Adelaide, Australia. Pharmacokinetic (PK) parameters of CDKI-73 in Balb/C mice were determined by a method reported previously [22]. To establish the xenografts, 5×10^6 MV4–11 human leukemic cells suspended in 1:1 RPMI-1640 medium and Matrigel (BD Biosciences, NSW, Australia) were injected subcutaneously into the hind flanks of female nude (nu/nu) Balb/C mice aged 6–8 weeks [23]. Detailed methods and equations to calculate different parameters are provided in the Supplementary methods. The procedures of all animal work complied with the relevant Commonwealth and State or Territory legislation and the requirements of the Australian Code of Practice for the care and use of animals for scientific purposes, 8th Edition 2013.

Statistical analysis

All the in vitro experiments were performed at least three times, unless mentioned separately and representative figures are shown. Data are presented as mean ± standard deviation (SD) with the exception of data from the in vivo xenograft study which is presented as mean ± standard error of the mean (SEM). Analysis of significant differences in tumor volume and body weight loss was performed by 2-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The significance level was set as $p \leq 0.05$. Student's t test was used to determine the significant differences between two groups, while one-way ANOVA was used for determining differences between more than two groups. Any correlations were examined by the Spearman test. All statistical analyses were performed using GraphPad Prism 6.03 (La Jolla, California, USA).

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

CDKI-73 inhibits the growth of AML cell lines and cells derived from patients with AML

We used a panel of twelve leukemia cell lines, including ten AML cell lines, to examine the effect of CDKI-73 on cell viability. CDKI-73 was highly effective against all cell lines tested with an IC_{50} in the range of 0.012–0.517 μM; in particular three MLL-AML cell lines, namely MOLM13, MV4–11 and THP-1, were highly sensitive to CDKI-73 with IC_{50} values <0.062 μM (Table 1).

The activity of CDKI-73 was further demonstrated in patient-derived samples ($n = 97$), including the cells derived from 74 newly diagnosed and 23 relapsed AML patients (Table 2). In all instances, CDKI-73 elicited potent growth inhibition with an overall mean IC_{50} value of 0.058 μM after 48 h exposure. In comparison, bone marrow cells from healthy donors ($n = 7$) were 158-fold less sensitive to CDKI-73 with a mean $IC_{50} = 9.16$ μM (Supplementary Table 1). No statistically significant differences in the responses to CDKI-73 were found between cells from patients under and over 50 years of age ($p = 0.600$), nor male and female patients ($p = 0.129$). Remarkably, CDKI-73 was highly potent against the cells irrespective of whether they were wild type or mutant with FLT3/ITD ($P = 0.548$) or NPM1 ($P = 0.602$) mutations (Table 2). Additionally, blasts derived from MLL positive vs. negative patients did not show statistically significant difference in sensitivity to CDKI-73 (Table 2).

Moreover, the subtypes of disease did not significantly affect the sensitivity to CDKI-73 when comparing primary CD34+ cells derived from patients diagnosed at stages M1, M2, M4, M5 and M6 (Fig. 1a, $P = 0.688$). No correlation was observed between the response to CDKI-73 and white blood cell counts (Fig. 1b, $r^2 = 0.0058$, $P = 0.457$) or percentage of blasts in bone marrow at diagnosis (Fig. 1c, $r^2 = 0.014$, $P = 0.240$). CD34+ cells of relapsed patients were as susceptible to CDKI-73 as those of newly diagnosed patients (Table 2, $P = 0.152$).

Table 1 Anti-proliferative activity of CDKI-73 against a panel of leukemia cell lines

Leukemia cell line	IC_{50} (μM) ± SD *
HL-60	0.033 ± 0.013
KG-1	0.045 ± 0.005
KYO-1	0.467 ± 0.011
Kasumi-1	0.038 ± 0.001
ME-1	0.363 ± 0.026
MOLM-13	0.033 ± 0.010
MV4–11	0.034 ± 0.005
NB4	0.054 ± 0.004
PL-21	0.037 ± 0.003
SET-2	0.477 ± 0.068
THP-1	0.062 ± 0.002
U937	0.012 ± 0.007

*Anti-proliferative activity was determined by 72 h resazurin assays. The data given are the mean ± SD derived from at least two replicates

Table 2 Characteristics of AML patients and the anti-proliferative activity of CDKI-73 against $CD34^+$ cells purified from samples of their bone marrow

Characteristic	Number (%)	IC_{50} (nM) ± SD*	*p* value**
Age (years old)			0.600
<50	35 (36.1)	0.056 ± 0.030	
>50	62 (63.9)	0.060 ± 0.023	
Sex			0.129
Female	49 (50.5)	0.062 ± 0.026	
Male	48 (49.5)	0.054 ± 0.025	
FAB classification			
M0	0 (0)	–	
M1	15 (15.5)	0.054 ± 0.030	
M2	22 (22.7)	0.056 ± 0.018	
M3	0 (0)	–	
M4	26 (26.8)	0.063 ± 0.028	
M5	31 (32.0)	0.058 ± 0.026	
M6	3 (3.1)	0.062 ± 0.027	
M7	0 (0)	–	
Types of patients			0.152
Newly diagnosed	74 (76.3)	0.056 ± 0.023	
Relapsed	23 (23.7)	0.067 ± 0.033	
FLT3/ITD status			0.548
Wild type	79 (81.4)	0.057 ± 0.026	
FLT3/ITD positive	14 (14.4)	0.062 ± 0.032	
Unknown***	4 (4.1)	–	
NPM1 status			0.602
Wild type	71 (72.4)	0.059 ± 0.027	
Mutant	18 (18.6)	0.063 ± 0.027	
Unknown***	8 (8.2)	–	
MLL			
Positive	7 (7.2)	0.045 ± 0.015	0.110
Negative	79 (81.4)	0.060 ± 0.024	
Unknown	11 (11.3)	–	

* Anti-proliferative activity was determined by 48 h cell counting Kit-8 assays. The data represent the mean ± SD derived from at least two replicates. ** *p* values were determined by Student's *t*-tests. ***Unknown means that these patients were not tested

CDKI-73 induces apoptosis effectively in primary and cultured AML cells

To investigate the apoptotic effect of CDKI-73, we used annexin V/propidiumiodide (PI) double staining. We chose MV4–11 cell line, one of the most commonly used MLL-AML cell lines, and incubated with various concentrations

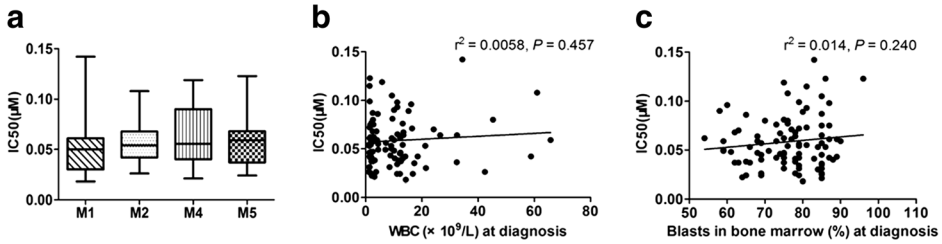


Fig. 1 The anti-proliferative role of CDKI-73 in AML patient blasts. **a** Comparison of IC_{50s} among the four common non-M3 sub-types of AML patient samples (M1, M2, M4, M5; $P = 0.688$). The bar represents the maximum and minimum of each group. **b** Correlation of IC_{50s} with white blood cells (WBC) at diagnosis. **c** Correlation of IC_{50s} with the percentage of blasts in bone marrow at diagnosis

of CDKI-73 for 24 h. Induction of apoptosis was observed as 38% annexin V+/PI+ cells detected compared to untreated cells (9% annexin V+/PI+ cells) at the concentration of 0.1 μM CDKI-73. The apoptotic effects were enhanced with 0.25 and 0.5 μM of CDKI-73 with 88 and 92% apoptotic cell death, respectively (Fig. 2a). When exposed to the same concentrations of CDKI-73 for 48 h, the levels of apoptosis were increased to 30%, 90 and 93%, respectively (Fig. 2b). In vitro kinase assays showed that CDKI-73 also inhibits CDK1 and CDK2 [17], hence cell cycle analysis was performed in MV4–11 cells after exposure for 24 h. There was minimal effect on the distributions of cells in the G1, S and G2/M phases of the cycle, but there was a shift in percentages of cells from G1 to the sub-G1 phase, indicating apoptosis (Fig. 2c).

The induction of apoptotic cell death by CDKI-73 was further confirmed in CD34+ blast cells derived from twenty-five randomly selected non-M3 AML patients. Characteristics of these patients are summarized in the

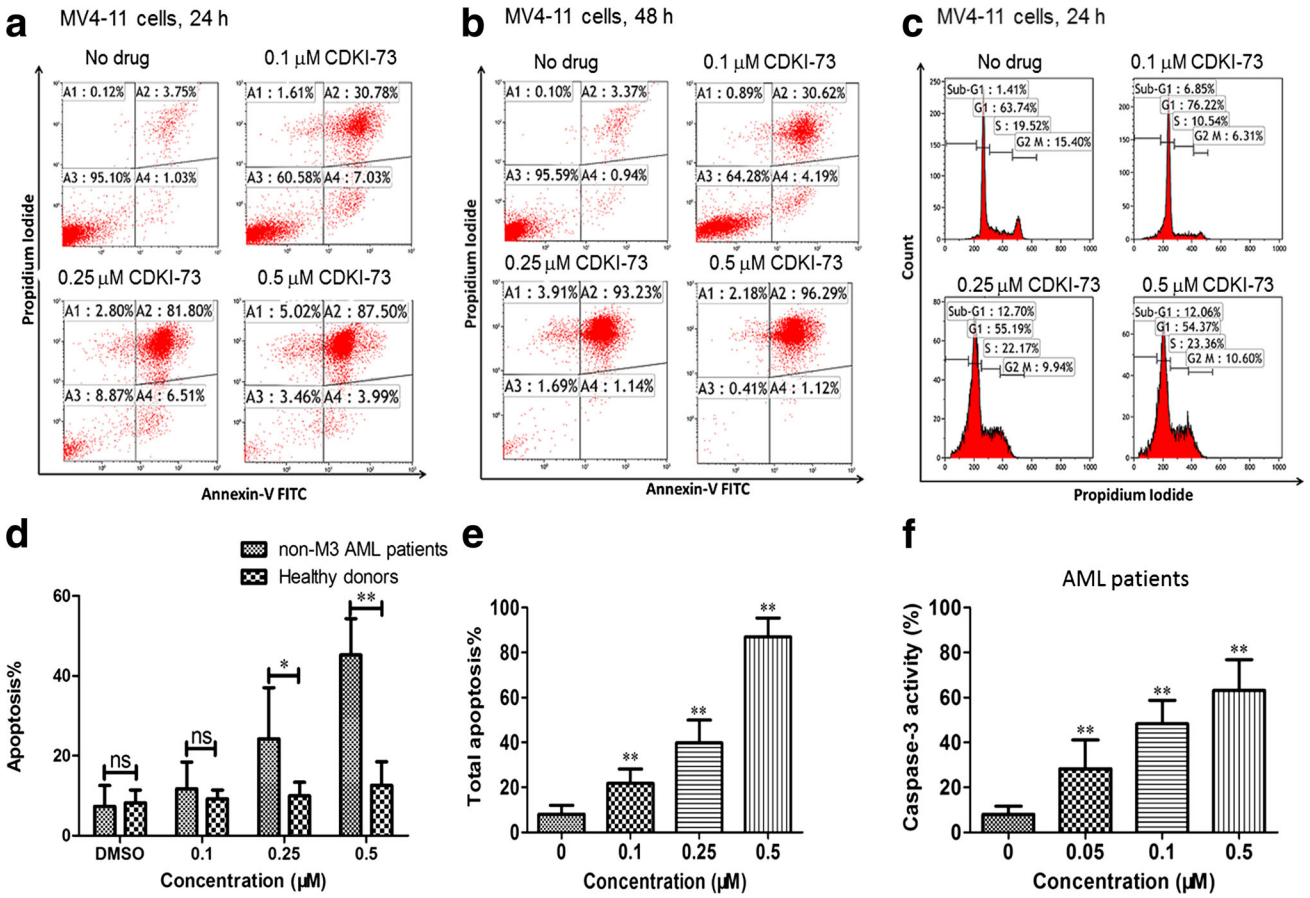


Fig. 2 Effects of CDKI-73 on apoptosis and cell cycle. **a** MV4–11 cells were exposed to CDKI-73 for 24 h at the indicated concentrations and apoptosis was detected using annexin V/PI double staining. **b** Apoptosis analysis after 48 h treatment. **c** Cell cycle analysis following exposure to CDKI-73 for 24 h at the indicated concentrations. **d** Apoptosis analysis in CD34+ cells purified from 25 non-M3 AML patients and 7 healthy donors after 24 h exposure to CDKI-73 (*$P < 0.05$, **$P < 0.01$). **e** Apoptosis analysis in non-M3 AML patient samples ($n = 15$) after 48 h exposure to CDKI-73 (**$P < 0.01$). **f** The activity of caspase-3 in CD34+ cells from five non-M3 AML patients was measured after expoure to the indicated concentrations of CDKI-73 for 24 h (**$P < 0.01$)

Supplementary Table 2. After 24 h exposure at concentrations of 0.25 or 0.5 μM, the percentage of cells undergoing apoptosis was much higher (up to 40% compared to untreated) than that of the cells obtained from bone marrow of seven healthy donors (only up to 7% compared to untreated) (Fig. 2d). These data suggested that CDKI-73 preferably targeted the primary AML cells. When exposure was extended to 48 h, apoptosis of AML blasts increased in a dose-dependent manner up to 80% (Fig. 2e). Moreover, there was a dose-dependent increase in the activity of caspase-3/7 in patient primary blast samples exposed to CDKI-73 for 24 h (Fig. 2f).

CDKI-73 inhibits kinase activity of CDK9 and downregulates anti-apoptotic proteins

Western blotting was used to examine the biological response arising from inhibition of kinase activity by CDKI-73. Phosphorylation of the C-terminal domain of RNA polymerase II (RNAPII CTD) at Ser2 and Ser5 residues (p-RNAPII^{S2} and p-RNAPII^{S5}), the biomarker of CDK9 kinase activity [24–26], was potently inhibited by CDKI-73. As shown in Fig. 3a, exposure of MV4–11 cells to CDKI-73 for 4 h reduced the p-RNAPII^{S2} level in a dose-dependent manner. This was followed by down-regulation of Bcl-2, Mcl-1 and XIAP anti-apoptotic proteins and increased cleavage of PARP protein, confirming induction of apoptosis. RT-qPCR analysis confirmed the anticipated transcriptional downregulation of the anti-apoptotic proteins Bcl-2, Mcl-1 and XIAP after 4 h of exposure to CDKI-73 (Fig. 3b). MV4–11 cells were further exposed to CDKI-73 (0.25 μM) over different periods of time (Fig. 3c, top), and reduction in the level of p-RNAPII^{S2} and p-RNAPII^{S5} was observed within 1 h of the treatment, confirming targeting of CDK9 as the major mechanism of CDKI-73 action. Concurrently, the level of Mcl-1 protein was reduced by 1 h ($P < 0.001$, after 1 h onwards) and undetectable by 12 h of the treatment. There was also significant fold changes in the expression of Bcl-2 and XIAP anti-apoptotic proteins (Fig. 3c, bottom). The expression levels of CDK9, CDK1 (as well its substrates PP1α and p-PP1α) and CDK2 were reduced by 12 h post exposure to CDKI-73 (Fig. 3c, top), likely due to the CDK9-targeted transcriptional inhibition of CDKI-73. Cleavage of PARP protein was observed from 12 h onwards ($P < 0.001$; Fig. 3c, bottom), accompanied by a decrease in caspase-3.

The levels of Mcl-1 mRNA were significantly higher in AML patient samples ($n = 16$) compared to those from healthy volunteers ($n = 16$) (Fig. 4a). To confirm whether CDKI-73 down regulated the expression of Mcl-1 in primary AML blasts in accordance with observation from the MV4–11 cell line, cells selected randomly from nine non-M3 AML patients of >50 years old were exposed to CDKI-73 for 8 h. Not only the mRNA levels of Mcl-1 but also Bcl-2 and XIAP were reduced in a dose-dependent manner (Fig. 4b). Exposure to CDKI-73 for 4 h dose-dependently inhibited phosphorylation of serine-2, and serine-5 to some extent, of RNAP II CTD, whereas expression of CDK1 as well as CDK2 was not affected by CDKI-73 (Fig. 4c). By extending the treatment time to 24 h, a dose-dependent reduction in the levels of Mcl-1 and increased cleavage of PARP was observed (Fig. 4d).

CDKI-73 is orally bioavailable, highly efficacious against MLL-AML MV4–11 xenografts and downregulates anti-apoptotic proteins by targeting CDK9 in vivo

Pharmacokinetic profiles of CDKI-73 in mice after intravenous (IV) at 2 mg/kg and oral (PO) doses at the doses of 10, 20 and 40 mg/kg are shown in Fig. 5a. The maximum concentration (C_{max}) increased from 1.29 to 3.66 μM at a mean time of 1 h and the area under the curve (AUC) of CDKI-73 increased from 3.51 to 12.8 μM.h when the oral dose was escalated from 10 to 40 mg/kg. CDKI-73 was eliminated from plasma with a mean terminal half-life ($T_{1/2}$) of 2 h. Its oral bioavailability (F) ranged from 54 to 85% across the three doses.

To determine the in vivo anti-leukemic efficacy of CDKI-73, two xenograft studies were performed. In the first study, two groups of MV4–11 tumor bearing mice ($n = 8$ per group) were administered vehicle i.e. 1% sodium carboxymethylcellulose (CMC) in water or 25 mg/kg CDKI-73 orally once every day for 33 days. Mice were observed daily during dosing and for a further 50 days afterwards. CDKI-73 caused a remarkable delay in tumor growth (Fig. 5b and c) compared to vehicle-treated mice, as reflected in a percentage for the mean tumor volume in treated to control mice (T/C, Eq. 1,Supplementary methods) of 43% at day 31 ($P < 0.00001$). There was a statistically significant reduction in tumor growth ($P < 0.05$) from day 11 onwards compared to the vehicle treated group, and tumors regressed completely in two out of eight mice. These delays in growth and regression of tumor translated to an increase in life-span (ILS) of 54.5% ($P < 0.001$) for the CDKI-73 treated animals compared to the vehicle treated group (Fig. 5d). CDKI-73 was well tolerated as evident from the absence of loss in body weight and other overt toxicities.

We further examined the efficacy of CDKI-73 at two different dosage regimens. Each group of MV4–11 tumor bearing mice ($n = 9$ per group) was administered with 50 mg/kg or 100 mg/kg of CDKI-73, or vehicle, orally once every 3 days for 21 days. As a positive control, cytarabine (Ara-C) was administered at 20 mg/kg intraperitoneally once every day. CDKI-73 markedly decreased tumor growth in a dose-dependent manner (Fig. 5e and f), resulting in T/C = 31 and 8% ($P < 0.0001$ compared to vehicle treated group) for the groups treated with 50 mg/kg and 100 mg/kg of CDKI-73, respectively, on day 21 (Table 3). Five out of nine animals in

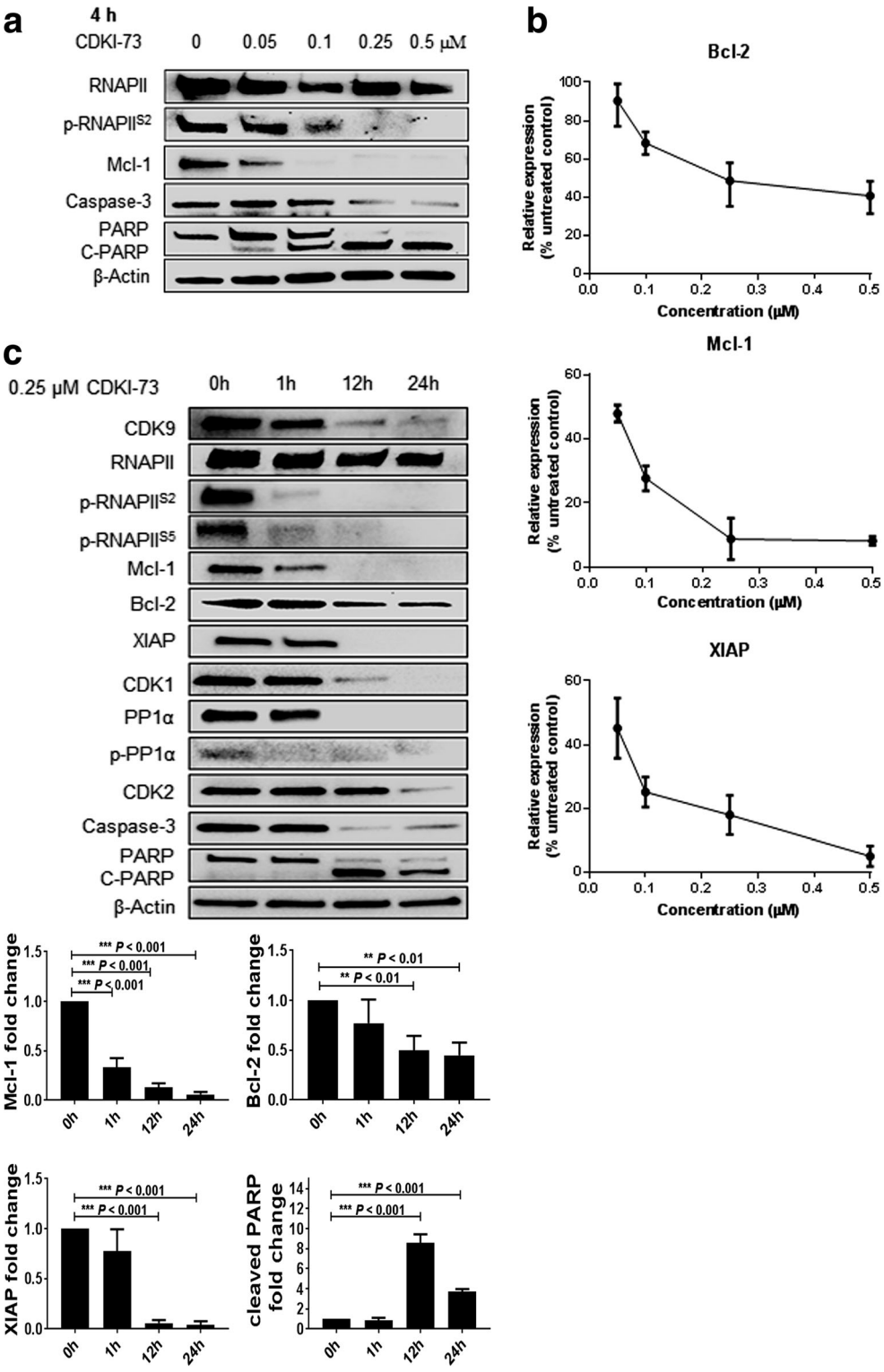


Fig. 3 Inhibition of CDK9 by CDKI-73 leads to the apoptosis of AML cells. a Western blot of MV4–11 cells after exposure to different concentrations of CDKI-73 for 4 h. **b** Quantitative PCR analysis of the mRNA expression of *Bcl-2, Mcl-1* and *XIAP* after exposure to the indicated concentrations of CDKI-73 for 4 h. **c** Time course experiment of exposure to 0.25 μM CDKI-73 for 1, 12 and 24 h. A representative blot was selected from at least two independent experiments. β-Actin was used as internal control (top). Bar graphs showing relative fold change of Mcl-1, Bcl-2, XIAP and cleaved PARP proteins (bottom; normalized to β-Actin; $n = 3$; ** denotes $P < 0.01$ and *** denotes $P < 0.001$)

the 100 mg/kg dosage group were tumor-free, translating to more than 55% tumor-free survival on day 21. In contrast, there was no significant tumor inhibition by Ara-C (Table 3). No significant adverse effect of CDKI-73 on body weight nor any signs of overt toxicity at both dosage regimens were observed (Fig. 5e).

To confirm the in vivo targeting effects of CDKI-73, western blot analysis was performed with the tumors collected from the mice ($n = 3$ per group) treated with vehicle or CDKI-73 from the first xenograft study (Fig. 5b). There was marked reduction of p-RNAPII^{S2} in 25 mg/kg CDKI-73 treatment group compared to the vehicle treated group (Fig. 5g).

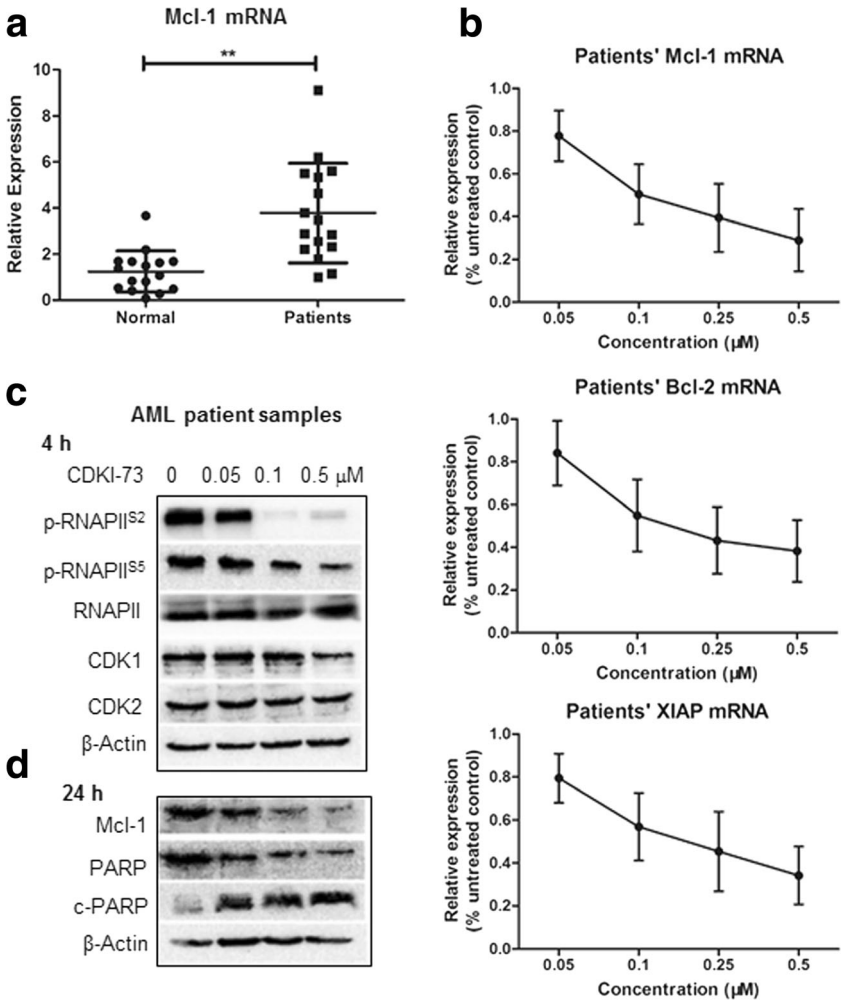


Fig. 4 CDKI-73 reduces anti-apoptotic proteins in a dose dependent manner in AML patient samples. a RT-qPCR analysis of the mRNA levels of *Mcl-1* in mononuclear cells freshly isolated from peripheral blood of AML patients ($n = 16$) and healthy volunteers (n = 16) (**$P < 0.01$). **b** AML primary cells ($n = 9$) were treated with CDKI-73 for 8 h at indicated concentrations and RT-qPCR analysis was done to determine the level of *Mcl-1*, *Bcl-2* and *XIAP*. **c** Western blot analysis of the AML patient samples after exposure to CDKI-73 at the mentioned concentration for 4 h. **d** Western blot analysis of the AML patient samples treated with indicated concentrations of CDKI-73 for 24 h. A representative blot was selected from at least two independent experiments. Dimethyl sulfoxide was used as a vehicle control and β-Actin was used as internal control

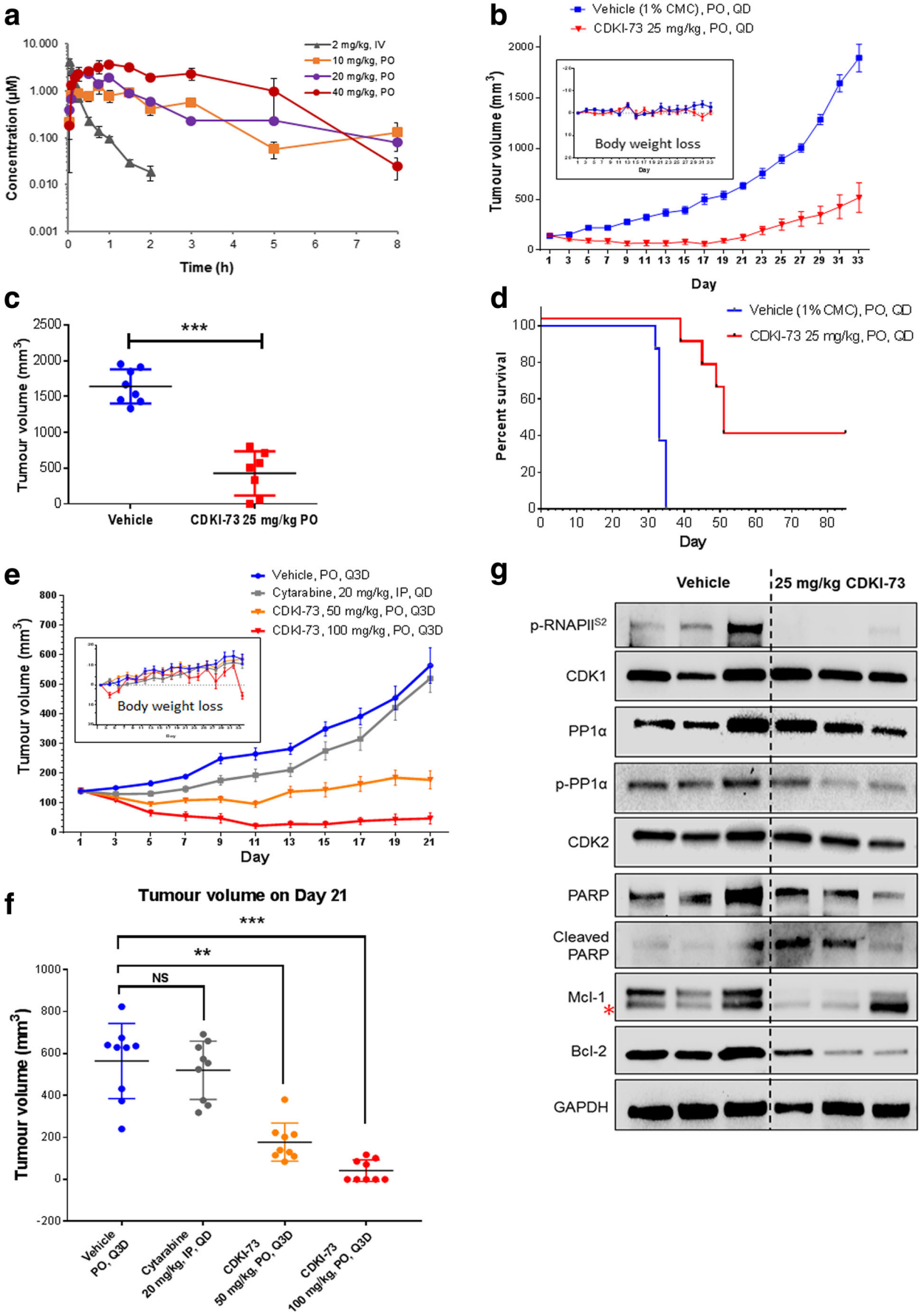
Both Mcl-1 and Bcl-2 were reduced in CDKI-73 treated mice. Interestingly, the truncated Mcl-1 was also reduced in them. The levels of CDK1 and CDK2 were also affected but to a lesser extent, likely due to CDK9-mediated downregulation of these proteins. The increased cleavage of PARP was found in CDKI-73 treated tumors. These suggest that inhibition of CDK9 is the major mechanistic target of antitumor activity of CDKI-73.

Discussion

Targeting transcription factors that co-opt the general transcriptional machinery to sustain the oncogenic state of cancers through pharmacological inhibition of CDK9 has gained support as a promising anti-cancer strategy [27–29]. CDKI-73, as one of the most potent CDK9 inhibitors identified to date, was highly cytotoxic against a broad range of cancer cell lines but showed minimal toxicity towards non-transformed cells [17]. Herein, we assessed the therapeutic potential of CDKI-73 against a panel of AML cell lines and blasts derived from 97 patients with AML that represented different subtypes and stages of the disease.

All the AML cell lines and patient-derived samples were highly sensitive to CDKI-73 (Tables 1 and 2). In agreement with the previous finding of its selectivity in targeting cancer cells [17], CDKI-73 was found to be of low toxic towards the healthy bone marrow cells (mean 158-fold selectivity); on the contrary, it was effective in inducing apoptosis of the AML cell lines and the primary AML blasts (Fig. 2d). Noticeably, the efficacy of CDKI-73 was not affected by patient characteristics and showed potency against samples including those from patients with relapsed AML.

Inhibition of CDK9 confines transcriptional elongation by inhibiting the expression level of p-RNAPII^{S2} and p-RNAPII^{S5} [17, 18, 25]. Western blot data revealed the reduction of both p-RNAPII^{S2} and p-RNAPII^{S5} in the MV4–11 cell line and AML patient samples after exposure to as low concentration as 0.1 μM of CDKI-73; this confirms CDK9 inhibition as the main mechanism of action (Fig. 3a and c). Cancer cells depend on the short half-lived anti-apoptotic proteins for survival which require


a
Concentration (μM)
Time (h)
2 mg/kg, IV
10 mg/kg, PO
20 mg/kg, PO
40 mg/kg, PO
b
Vehicle (1% CMC), PO, QD
CDKI-73 25 mg/kg, PO, QD
Tumour volume (mm3)
Body weight loss
Day
c
Tumour volume (mm3)
***
Vehicle
CDKI-73 25 mg/kg PO
d
Vehicle (1% CMC), PO, QD
CDKI-73 25 mg/kg, PO, QD
Percent survival
Day
e
Vehicle, PO, Q3D
Cytarabine, 20 mg/kg, IP, QD
CDKI-73, 50 mg/kg, PO, Q3D
CDKI-73, 100 mg/kg, PO, Q3D
Tumour volume (mm3)
Body weight loss
Day
f
Tumour volume on Day 21
***
**
NS
Tumour volume (mm3)
Vehicle PO, Q3D
Cytarabine 20 mg/kg, IP, QD
CDKI-73 50 mg/kg, PO, Q3D
CDKI-73 100 mg/kg, PO, Q3D
g
Vehicle
25 mg/kg CDKI-73
p-RNAPII S2
CDK1
PP1α
p-PP1α
CDK2
PARP
Cleaved PARP
Mcl-1
Bcl-2
GAPDH

◀ **Fig. 5 Pharmacokinetic profiles and in vivo anti-leukemic efficacy of CDKI-73. a** Concentrations of CDKI-73 in plasma after single intravenous (IV) (2 mg/kg) and oral (PO) (10, 20 and 40 mg/kg) doses to mice. First xenograft study – **b** Comparison of tumor volume each day after receiving either vehicle or 25 mg/kg CDKI-73; percentage loss of body weight is shown in the inset ($n = 8$). **c** Scatter diagram showing tumor volume for individual mice on day 31. **d** Kaplan–Meier survival plot of mice treated with vehicle and CDKI-73; Mantle–Cox test was performed for statistical analysis ($P = 0.001$). Second xenograft study – **e** Comparison of tumor volume each day after receiving indicated treatments; percentage of body weight lost by different treatment groups is shown in the inset ($n = 9$). **f** Scatter diagram showing tumor volume of individual mice of indicated treatment groups on day 21. Longer lines in scatter diagram indicate the means while smaller lines indicate standard error. Asterisks denote significant differences (**$P < 0.01$, ***$P < 0.001$) and NS denotes non-significant. **g** Western blot analysis of the tumor tissues collected from the first xenograft study. Three individual mice were selected from each treatment group (vehicle and 25 mg/kg CDKI-73) and presented in the figure. Red asterisk sign (*) denotes truncated Mcl-1

continuous expression [27]. The anti-apoptotic protein Mcl-1 has an estimated half-life of less than 1 h and its maintenance depends largely on active transcription [27, 30]. Moreover, overexpression of Mcl-1 is associated with progression and chemoresistance in AML, and Mcl-1 has been considered as a high-priority therapeutic target [31, 32]. Inhibition of Mcl-1 by CDKI-73 (Fig. 3c and 4d) triggered the apoptosis of primary AML blasts and AML cell lines [33, 34], while normal bone marrow cells were much less susceptible [35]. We reason that the higher expression level of Mcl-1 in primary AML cells compared to healthy bone marrow cells (Fig. 4a) makes the AML cells more sensitive to CDKI-73. The role of Bcl-2 and XIAP anti-apoptotic proteins has also been proven to be important in the pathogenesis of AML [31, 36]. In agreement with the previous findings that inhibiting CDK9 downregulated the transcription of multiple anti-apoptotic regulators [27, 37], CDKI-73 was capable of reducing the mRNA level of not only Mcl-1 but also Bcl-2 and XIAP (Fig. 4b). This simultaneous targeting of multiple cancer survivor genes by CDKI-73 provides an excellent opportunity to overcome chemoresistance in AML.

CDKI-73 was well absorbed after oral administration to mice. A high potency towards MV4–11 cells (IC_{50} = 0.034 μM) and induction of apoptosis after only 4 h of exposure in vitro (Fig. 3a), combined with appreciable systemic exposure (as evident from the overall exposure in plasma – AUC), ensured efficacy in the xenograft model of MV4–11 leukemia in mice.

Inhibition of tumor growth ($p < 0.00001$) and prolongation of survival were achieved by 25 mg/kg daily dosing of CDKI-73. The less frequent but higher doses (50 and 100 mg/kg) of CDKI-73 improved the therapeutic potencies without exhibiting any overt signs and symptoms of

Table 3 Summary of tumor growth inhibition (T/C %) in different treatment groups and their corresponding p values compared to vehicle control in the two in vivo MLL-AML xenograft studies

First in vivo MLL-AML xenograft study	Day	CDKI-73 25 mg/kg PO QD (T/C %)	p value	Second in vivo MLL-AML xenograft study	Day	Ara-C 20 mg/kg IP QD (T/C %)	p value	CDKI-73 50 mg/kg Q3D (T/C %)	p value	CDKI-73100 mg/kg PO Q3D (T/C %)	p value
	1	101	0.9977		1	100	>0.9999	101	>0.9999	102	>0.9999
	3	74	0.8542		3	86	0.9939	79	0.9797	72	0.9565
	5	49	0.3495		5	79	0.9744	58	0.8279	40	0.6169
	7	45	0.3391		7	78	0.9579	58	0.7643	29	0.3607
	9	29	0.0568		9	71	0.8091	45	0.3389	19	0.067
	11	27	0.0156		11	73	0.8154	36	0.1666	8	0.0164
	13	23	0.0022		13	75	0.8168	49	0.2884	10	0.0105
	15	26	0.0015		15	79	0.7987	41	0.0591	8	0.0005
	17	20	< 0.0001		17	81	0.7869	42	0.0274	10	0.0001
	19	26	< 0.0001		19	93	0.9786	41	0.0056	10	< 0.0001
	21	30	< 0.0001		21	92	0.9494	31	< 0.0001	8	< 0.0001
	23	37	< 0.0001								
	25	45	< 0.0001								
	27	48	< 0.0001								
	29	46	< 0.0001								
	31	43	< 0.0001								
	33	51	< 0.0001								

PO: oral administration; QD: once every day; IP: intraperitoneal injection; Q3D: once every three days

toxicity. Collectively, our studies demonstrated that CDKI-73 was able to attain efficacious levels of drug exposure with a good window of tolerance and substantiated the potential for CDKI-73 to be developed as a therapeutic agent. Interestingly, despite the relative short half-life (~2 h) in mouse plasma, CDKI-73 was highly efficacious when it was administered once every 3 days, likely due to its accumulation in the tumor tissue. Moreover, the in vivo anti-tumor mechanism of CDKI-73 was similar to that of our in vitro cell based findings of downregulation of short-lived anti-apoptotic proteins (Fig. 3c and 5g). Our data were further supported by previous findings of Bcl-2 family member Mcl-1 as a predictive biomarker associated with anti-tumor response of a CDK9 inhibitor—Dinaciclib [38]. CDKI-73 was able to reduce the both full length and truncated Mcl-1 in vivo. The truncated Mcl-1 retains prolonged anti-apoptotic activity and is associated with extended survival in cancer cells [39]. Thus, reduction of this truncated Mcl-1 by CDKI-73 may enhance further tumor cell death.

In summary, we have investigated the therapeutic potential of CDKI-73 against AML and provided a rationale for developing it as an effective agent for treating AML in the clinic. CDKI-73 exhibited in vitro and in vivo CDK9-targeted mechanism of action, favorable pharmacokinetics and marked anti-cancer efficacy in animal models. With its high oral bioactivity along with appreciable safety profile, CDKI-73 offers a very exciting prospect as a clinical development candidate which has very recently received the approval for clinical trials in AML patients.

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Compliance with ethical standards

Conflicts of interest The authors have no conflicts of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent "Informed consent was obtained from all individual participants included in the study."

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