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# The Novel Phospholipid Mimetic KPC34 Is Highly Active Against Acute Myeloid Leukemia with Activated Protein Kinase C☆

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ABSTRACT

Acute myeloid leukemia (AML) is an aggressive malignancy with poor outcomes. Nucleoside analogs are subject to resistance mechanisms including downregulation of equilibrative nucleoside transporter (ENT1) and deoxycytidine kinase (dCK). KPC34 is a novel phospholipid mimetic that when cleaved by phospholipase C (PLC) liberates gemcitabine monophosphate and a diacylglycerol mimetic that inhibits the classical isoforms of protein kinase C (PKC). KPC34 acts independently of ENT1 and dCK. KPC34 was active against all AML cell lines tested with $IC_{50}$s in the nanomolar range. Enforced expression of PLC increased response to KPC34 *in vivo*. In an orthotopic, xenograft model, KPC34 treatment resulted in a significant increase in survival compared to control animals and those treated with high-dose cytarabine. In a PDX model with activated PKC, there was a significant survival benefit with KPC34, and at progression, there was attenuation of PKC activation in the resistant cells. In contrast, KPC34 was ineffective against a syngeneic, orthotopic AML model without activated PKC. However, when cells from that model were forced to express PKC, there were significantly increased sensitivity *in vitro* and survival benefit *in vivo*. These data suggest that KPC34 is active against AML and that the presence of activated PKC can be a predictive biomarker.
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## Introduction

Acute myeloid leukemia (AML) is characterized by resistance to therapy and poor outcomes. AML is an aggressive malignancy that leads to the accumulation of immature myeloid precursors, resulting in progressive marrow failure and death [1]. It affects approximately 20,000 people per year in the United States, and despite decades of research, the overall 5-year survival remains a disappointing 27% [2–4]. Standard treatment is intensive induction chemotherapy followed by consolidation using chemotherapy or a stem cell transplant [2]. Most patients achieve a complete, but transient, remission. Once AML has relapsed, 1-year survival is estimated at 26% and 5-year survival at 11% [5]. This is secondary to increased resistance with salvage remission rates less than 50% [6]. There have been several new FDA approvals for the treatment of AML in the last several years. These include targeted agents like gilteritinib, enasidinib, and ivosidinib [7–9]. These agents have complete remission rates in the 20% range, have median survivals of less than a year, and are only active in patients that harbor specific mutations [7–9]. There remains a need for additional therapies that circumvent common resistance mechanisms.

Nucleoside analogs are important treatments in AML. Cytarabine, azacytidine, and decitabine are cytidine analogs that are central in the treatment of AML [10–13]. They are hydrophilic molecules that gain entry into cells mainly by facilitated diffusion *via* the human ENT1 protein [14]. As a result, downregulation of ENT1 leads to cytarabine resistance *in vitro* and in patients [15,16]. Once inside the cell, cytidine analogs must be phosphorylated by the rate-limiting enzyme deoxycytidine kinase (dCK), and levels of dCK are prognostic in AML [17] and can alter sensitivity to these agents [18,19].

Protein kinase C (PKC) is a novel target in AML. PKC is a family of at least 12 related proteins with diverse cellular functions whose dysregulation has been implicated in oncogenesis (reviewed by Griner et al. [20]). The classic members (PKCα, β1, β2, and γ) require calcium and diacylglycerol (DAG) for activity. The α and β family members are highly expressed in many AML cell lines and patient samples, and targeting these kinases is cytotoxic [21,22]. PKC isoforms have also been implicated in resistance to DNA damaging agents, including cytarabine in AML cells [23,24]. This is thought to occur *via* phosphorylation and activation of the antiapoptotic protein BCL-2. Active PKCα and BCL-2 phosphorylation is also associated with a poor prognosis in AML [25].

KPC34 is a novel gemcitabine phospholipid conjugate (Supplemental Figure 1) previously shown to have activity in cytarabine-resistant acute lymphoblastic leukemia models [26]. It is orally bioavailable, crosses the blood brain barrier, does not require ENT1 for cellular uptake, and is not

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a substrate for the MDR-1 efflux pump [26,27]. Once acted on by phospholipase C (PLC), it is converted to gemcitabine monophosphate and an amide-containing DAG mimetic that inhibits the classical forms of PKC including the α and β family members. Conversion of the prodrug to the monophosphate form of gemcitabine bypasses the need for dCK. This study reports the preclinical activity of KPC34 against preclinical AML models.

## Materials and Methods

### *Reagents*

KPC34 was synthesized as previously published in [27]. Cytarabine was purchased from NOVAPLUS (New York, NY); gemcitabine was purchased from Millipore-Sigma (St. Louis, MO). For animal studies, all chemotherapeutic agents were dissolved in saline prior to treatment.

### *Cell Culture*

The mouse leukemia cell lines MFL2 and MR2 were maintained in 45% DMEM, 45% IMDM, and 10% FBS supplemented with iL3, iL6, SCF, L-glutamine, penicillin, and streptomycin (stem cell media). The human leukemia cell lines OCI-AML3, HL60, and K562 were maintained in RPMI media (Gibco, Carlsbad, CA) supplemented with 10% FBS, penicillin, and streptomycin. OCI-AML3 cells were luciferase tagged by lentiviral infection with a luciferase-expressing vector by the Cell Virus and Vector Laboratory and subjected to clonal derivation by limiting dilution.

### *PKCα and PLCγ2 Overexpression*

MFL2 cells were grown in stem cell media at 37°C with 5% $CO_2$ as in Pardee et al. [28]. OCI-AML3 and HL60 cells were grown in RPMI media at 37°C with 5% $CO_2$. Cells were infected with the nuclease dead Cas9 VP64 fusion-expressing vector pMSCV-LTR-dCAS9-VP64-BFP (a gift from Stanley Qi & Jonathan Weissman, Addgene plasmid # 46912), and infected cells were selected with puromycin. Resistant cells were then transfected with sgRNA-expressing vector LRG Lenti_sgRNA_EFS_GFP (a gift from Christopher Vakoc, Addgene plasmid # 65656) tagged with GFP targeting either PKCα or PLCγ2. Overexpression was confirmed by Western blot.

### *Patient Samples*

All patient samples were collected during routine clinical care under a protocol approved by the Wake Forest School of Medicine Institutional Review Board. All patients provided written informed consent. Mononuclear cells from bone marrow biopsies or peripheral blood were isolated by Ficoll gradient separation and stored at −80°C until use.

### *Cell Viability*

Viability assays were performed in 96-well plates using the Cell Titer-Glo assay (Promega, Madison, WI) according to the manufacturer's protocol after 72-hour exposure to indicated drug concentration. Murine cell lines were plated at 50,000 cells/ml and human cell lines were plated at 100,000 cells/ml prior to drug exposure. Both types of cells were plated at 100-μl volume in triplicate for each treatment group.

### *Flow Cytometry Assays*

Cells were seeded in 24-well plates at 100,000 cells/ml in 0.5 ml, grown for 2 days, and treated with drug for 48 hours. After centrifugation and washing in cold PBS, cells were stained with propidium iodide (PI) (Sigma Aldrich, St. Louis, MO) and allophycocyanin-conjugated Annexin V in a binding buffer [0.1 M Hepes (pH 7.4), 0.14 M NaCl, and 25 mM $CaCl_2$ solution] (BD Pharmingen, San Jose, CA) according to the manufacturer's protocol. Flow cytometric analysis was conducted on an Accuri C6 cytometer (BD Pharmingen, San Jose, CA) with the FCS Express software (*De Novo* Software, Los Angeles, CA).

### *Western Blotting*

Cells were lysed in Laemmli buffer (1.6 ml 10% SDS, 500 μl 1 M Tris-HCl [pH 6.8], 800 μl glycerol, 400 μl 2-mercaptoethanol, 4.7 ml H2O), and samples separated by SDS-PAGE before transfer to an Immobilon polyvinylidene difluoride membrane (Millipore, Billerica, MA). Primary antibodies against caspase 3 (#9662 Cell Signaling, Beverly, MA), actin (AC-15, 1:10,000; Abcam, Cambridge, MA), and phospho-PKC α/βII (Thr638/641) (#9375, 1:1000; Cell Signaling, Beverly, MA) and a secondary antibody anti-mouse (#7076, 1:5000; Cell Signaling, Beverly, MA) or anti-rabbit (#7074, 1:5000; Cell Signaling, Beverly, MA) were used.

### *Mice*

The Comprehensive Cancer Center of Wake Forest University Institutional Animal Care and Use Committee approved all mouse experiments. Luciferase-tagged OCI-AML3 leukemia cells were transplanted into 6- to 8-week-old recipient NSG or C57Bl/6 mice by tail vein injection of $1 \times 10^6$ viable cells. Mice were monitored by bioluminescent imaging using an IVIS100 imaging system (Caliper LifeSciences, Hopkinton, MA). Mice were injected with 150 mg/kg D-luciferin (Gold Biotechnology, St. Louis, MO), anesthetized with isoflurane, and imaged for 2 minutes. Chemotherapy was initiated upon clear detection of signal. Mice were randomly assigned to treatment groups and treated as described. For the duration of treatment, drinking water was supplemented with 1 mg/ml neomycin. Animals were euthanized upon onset of hind limb paralysis, hunched posture, or difficulty breathing.

### *Statistical Analysis*

Groups of three or more were analyzed using a one-way analysis of variance. All means were compared by a Student's two-tailed *t* test. The *in vivo* survival graphs were generated with the Kaplan-Meier method, with *P* values determined by the log-rank test. Multiple measures were analyzed using a two-way analysis of variance. All analyses were performed using GraphPad Prism Version 6.05 (GraphPad Software). A $P$ value $\leq .05$ was considered to be significant.

## Results

### *KPC34 Is Highly Active Against AML Cell Lines In Vitro*

In order to determine if KPC34 has activity against preclinical models of AML, its activity was tested against several human and murine AML cell lines. HL60, MFL2, KG1a, and OCI-AML 3 cells were incubated with increasing amounts of KPC34 for 72 hours, and then viability was assessed. The $IC_{50}$ values of KPC34 ranged from 22 to 195 nM (Table 1). Previously, KPC34 has been shown to induce apoptosis in acute lymphoblastic leukemia (ALL) cells [26]. In order to determine if apoptosis was being induced, HL60 cells were incubated with KPC34 for 48 hours and stained with PI and Annexin V, and viable cells were assessed by flow cytometry. Consistent with the results in ALL cell lines, KPC34 exposure resulted in a dose-dependent increase of PI and Annexin V–positive cells (Figure 1, *A* and *B*). This was confirmed by treating HL60 cells with KPC34 for 72 hours and Western

**Table 1**
Cell Line $IC_{50}$ Values

| Cell Line | Properties | $IC_{50}$, nM (95% CI) |
|---|---|---|
| HL60 | Human promyelocytic leukemia, p53−, myc+ | 144.9 (85.0-247.0) |
| MFL2 | Murine MLL-ENL AML, FLT3 ITD+ | 54.36 (18.83-157.0) |
| KG1a | Human AML, CD34+, MDR+ | 195.8 (163.5-234.4) |
| OCI-AML3 | Human AML, mutated NPM1 and DNMT3A | 22.44 (17.94-28.07) |

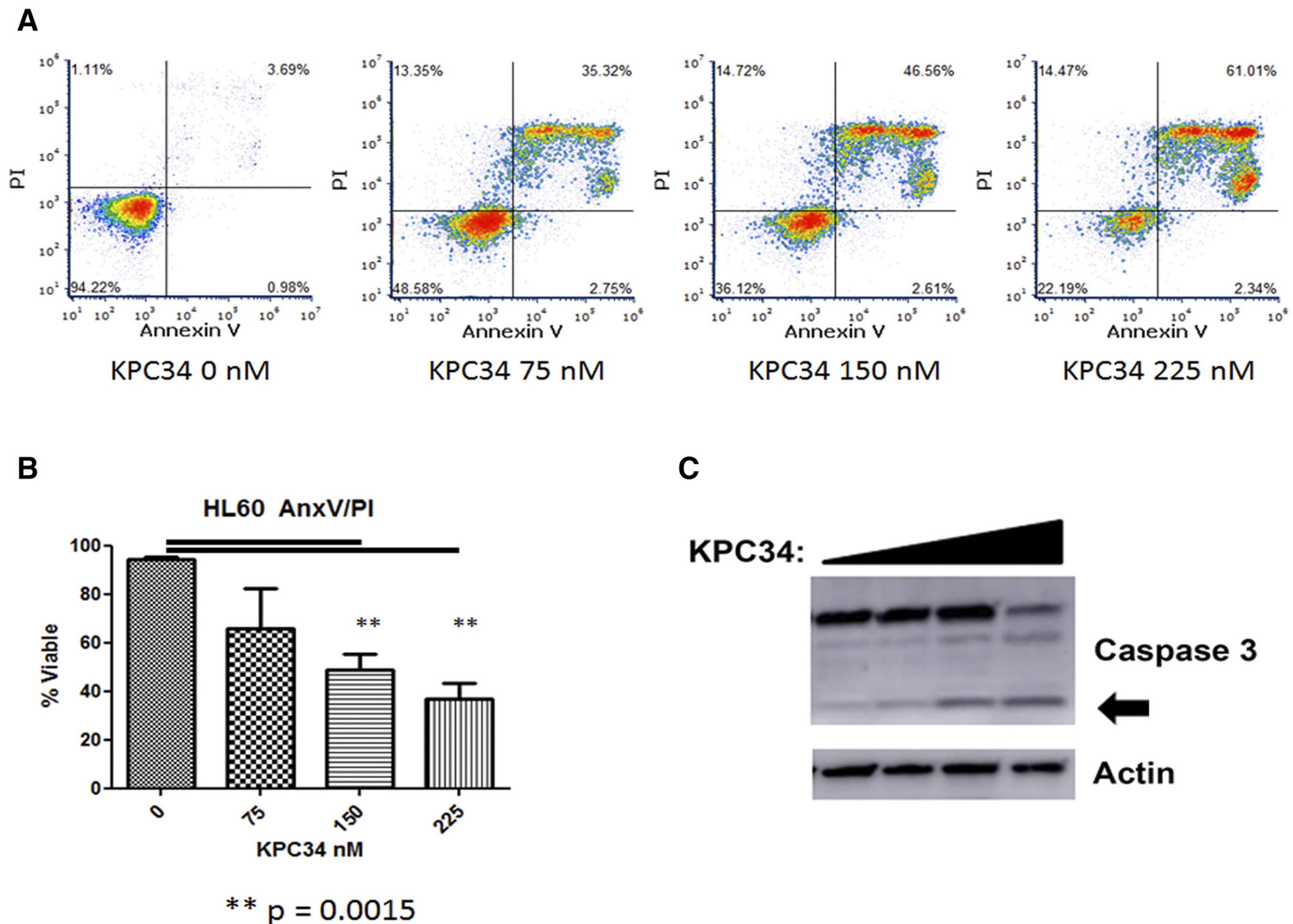


**Figure 1.** KPC34 induces apoptosis in AML cells. (A) Flow cytometry of PI and Annexin V assay. HL60 cells were treated with the indicated amount of KPC34 for 48 hours and then labeled with Annexin V and PI. Representative dot plots are shown. (B) Quantification of apoptosis induction. Mean percentage of dual-negative cells from three independent experiments is shown. ** = *P* value of .0015 (C) Western blot analyses of apoptotic induction. HL60 cells were treated with increasing amounts of KPC34 (0, 75, 150, and 225 nM) for 48 hours and blotted for caspase 3. Arrow denotes cleaved caspase 3. Actin was used as a loading control.

blotting for cleaved caspase 3. There was a dose-dependent increase in cleaved caspase 3, further supporting an apoptotic response to treatment with KPC34 (Figure 1*C*). These data demonstrate that KPC34 efficiently induces apoptosis in AML cell lines with nanomolar concentrations.

*PLC Expression Induces Sensitivity to KPC34*

KPC34 is predicted to be cleaved by PLC into its two active metabolites: gemcitabine monophosphate and a DAG mimetic. If this is the case *in vivo*, then AML cells with higher expression of PLC should be more responsive to KPC34. In order to assess this, MLF2 cells with catalytically dead Cas9 fused to the transcriptional activator VP64 were infected with vectors expressing sgRNAs targeting either the safe harbor locus ROSA26 or PLCγ2. Clones with increased PLC expression were identified (Figure 2*A*). ROSA26- or PLC-expressing cells ($1 \times 10^6$) were then tail vein injected into syngeneic C57Bl/6 mice. Animals were monitored for engraftment and then treated with saline or KPC34 and followed for survival. KPC34 treatment did not result in a significant survival advantage for ROSA26 sgRNA cells. In contrast, there was a significant increase in survival when animals with PLC sgRNA cells were treated with KPC34 (Figure 2*B*). To extend and confirm this result, a human AML model, HL60 cells expressing a Cas9-VP64 fusion, was infected with vectors expressing sgRNAs targeting either PLCγ2 or a control nontargeting sgRNA. Control or PLC-expressing cells ($1 \times 10^6$) were then tail vein injected into NSG mice. KPC34 treatment resulted in a median survival of 31 days for mice injected with control sgRNA cells compared to 34 days for mice with PLC sgRNA cells ($P$ = .0224, Supplemental Figure 2*B*). These data support the role of PLC in activating KPC34 *in vivo*.

*KPC34 Inhibits PKC In Vitro and Is Active Against AML In Vivo*

After being activated by PLC, KPC34 is converted into gemcitabine monophosphate and an amide-containing diacylglycerol mimetic shown to inhibit PKC in ALL [26]. The classical isoforms of PKC are autophosphorylated upon activation. To determine if KPC34 would inhibit the activation of PKCα and β, OCI-AML3 cells were incubated with KPC34 for 4 hours, harvested, and subjected to Western blotting with an antibody specific for the phosphorylated form of PKC. KPC34 incubation resulted in a time-dependent loss of PKC phosphorylation consistent with its inhibition (Figure 3*A*). These data demonstrate that KPC34 can inhibit PKC in AML cells. In order to determine if this would result in antileukemic activity *in vivo*, luciferase-expressing OCI-AML3 cells were tail vein injected into sublethally irradiated NSG recipients. Upon engraftment, mice were treated with saline (control), cytarabine (Ara-C) intraperitoneally (IP) at 50 mg/kg (the maximally tolerated dose), or KPC34 (KPC) orally at 10 mg/kg once daily for 4 days. After completion of treatment, mice were followed for survival. Treatment with KPC34 resulted in a highly significant increase in

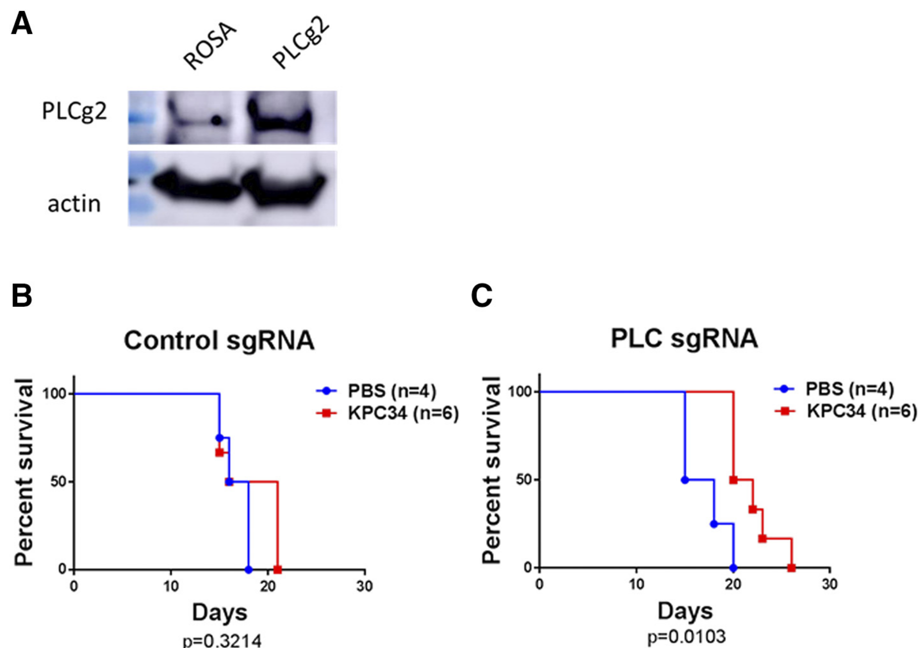


**Figure 2.** PLC-expressing AML is more sensitive to KPC34 *in vivo*. (A) Cas9-VP64 expressing murine AML cell line MFL2 was infected with virus expressing sgRNAs targeting PLCγ2 or the control safe harbor locus ROSA26 were blotted for PLC expression. (B + C) Syngeneic C57Bl/6 mice were injected with $1 \times 10^6$ cells. Upon engraftment, mice were treated with saline (PBS) or 20 mg/kg KPC34 (KPC34) and followed for survival. *P* value from log-rank test.

survival when compared to controls and cytarabine (Figure 3*B*). Treatment with cytarabine at the maximally tolerated dose only provided a 2-day increase in median survival. In contrast, KPC34 treatment resulted in a median survival of 44.5 days ($P = .0007$). Since AML is treated with repeated courses of chemotherapy, NSG animals were injected with OCI-AML3 cells and subjected to the same initial course of four daily treatments of KPC34 followed by two additional treatments administered weekly until criteria for euthanasia were met. Doses were held if animals showed signs of toxicity or weight loss of 10% total body weight or more. Animals treated with the repeat dosing regimen had a highly significant increase in overall survival compared to control-treated and single-course KPC34-treated animals with a median survival of 78.5 days ($P = .0001$, Figure 3*C*). These data demonstrate that KPC34 is highly active against an orthotopic model of AML with activated PKC and that repeat dosing is both well tolerated and provides a significant increase in survival.

### *Activated Protein Kinase Is Detectable in a Subset of AML Patient Samples and Cell Lines*

Previous reports have demonstrated that the classic isoforms of PKC are highly expressed in human AML samples [22,29]; however, what percentage of AML samples and cell lines has activated PKC is less well known. To address this, patient samples and several myeloid leukemia cell lines were subjected to Western blotting for phosphorylated PKC α and $\beta_{II}$ using the commercially available antibody. See Table 2 for patient characteristics. Of the eight patient samples analyzed, five had detectable phosphorylated PKC albeit at highly variable levels (Figure 4*A*). Cell lines likewise displayed variable expression of phosphorylated PKC, with OCI-AML3, K562, and HL60 having detectable pPKC, while the murine lines MFL2 and MR2 did not (Figure 4*B*). Phosphorylated PKC levels for most samples mirrored total PKCα levels, suggesting that this isoform is mainly responsible for the detectable activated PKC. These data demonstrate that activated PKC is detectable in a subset of AML patient samples and cell lines.

### *KPC34 Treatment Selects for Cells with Reduced PKC in a PDX Model*

To further establish the efficacy of KPC34 in AML, a PDX model derived from a 56-year-old female with therapy-related AML in second relapse was tested for response to KPC34. This model was chosen as it displayed readily detectable phosphorylated PKC. NSG mice were sublethally irradiated and injected with $1 \times 10^6$ cells. When human CD33+ cells were detected in peripheral blood, treatment with either saline (control) or KPC34 at 10 mg/kg once daily for 4 days followed by twice weekly was initiated. KPC34 treatment resulted in a significant survival benefit despite the small number of animals treated ($P = .0389$, Figure 5*A*). When moribund animals were euthanized, bone marrow cells were harvested and subjected to Western blotting for phosphorylated PKC. Both control-treated animals displayed similar levels of phosphorylated PKC, while all KPC34-treated animals had lower levels of phosphorylated PKC consistent with selection against high levels of activated PKC in the KPC34-resistant cells (Figure 5*B*).

### *KPC34 Activity Is Affected by Levels of Activated PKC*

To determine if detectable levels of phosphorylated PKC were associated with response to KPC34, C57bl/6 mice were sublethally irradiated and injected with the murine AML cell line MFL2. This line was chosen as it displayed minimal levels of phosphorylated PKC (Figure 4*B*). Upon confirmation of engraftment by luciferase imaging, animals were treated with either saline (control) or KPC34 at 20 mg/kg once daily for 4 days. Treatment with KPC34 was not effective against MFL2-injected animals (Figure 5*C*), consistent with activated PKC playing a role in response to KPC34 *in vivo*. In order to further support this conclusion, MFL2 cells expressing Cas9-VP64 were infected with vectors that expressed an sgRNA targeting PKCα or the safe harbor locus Rosa26. A dramatic increase in phosphorylated PKC was detected in the PKCα-targeted cells compared to controls (Figure 5*D*). When tested against KPC34, there was a significant increase in sensitivity for PKC-expressing cells compared to Rosa26 controls (Figure 5*E*). To further expand this result, the PKC-expressing cells were then tail vein injected into sublethally irradiated C57bl/6 mice. Upon confirmation of engraftment by luciferase imaging, animals were treated with

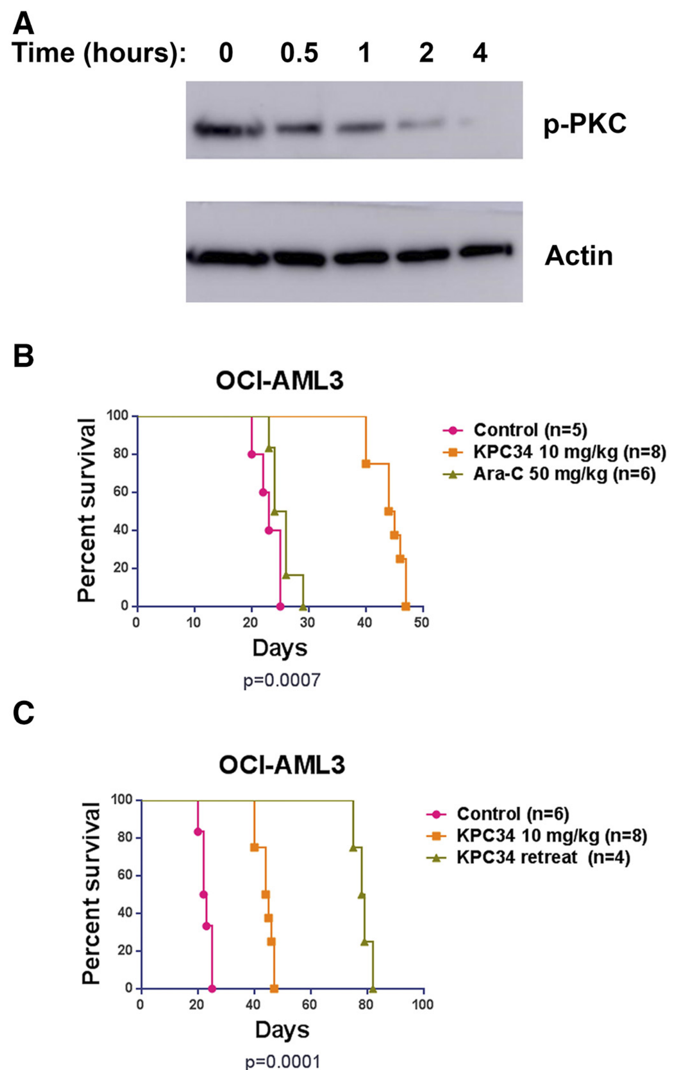


**Figure 3.** KPC34 is active *in vivo* against an orthotopic model of AML expressing phosphorylated PKC. (A) KPC34 inhibits PKC phosphorylation. Western blots of phosphorylated PKCα and $\beta_{II}$. OCI-AML3 cells were treated with 200 nM KPC34 for the indicated time and were blotted with an antibody that recognizes phosphorylated PKCα and PKC$\beta_{II}$ (p-PKC). Actin was used a loading control. (B) Kaplan-Meier curves for transgenic NSG mice tail vein injected with OCI-AML3 cells and treated with saline (control) or KPC34 at 10 mg/kg per os (PO) or cytarabine at 50 mg/kg IP once daily for 4 days. (C) Kaplan-Meier curves for transgenic NSG mice tail vein injected with OCI-AML3 cells and treated with saline (control) or KPC34 at 10 mg/kg PO once daily for 4 days followed by 2 days every week until moribund.

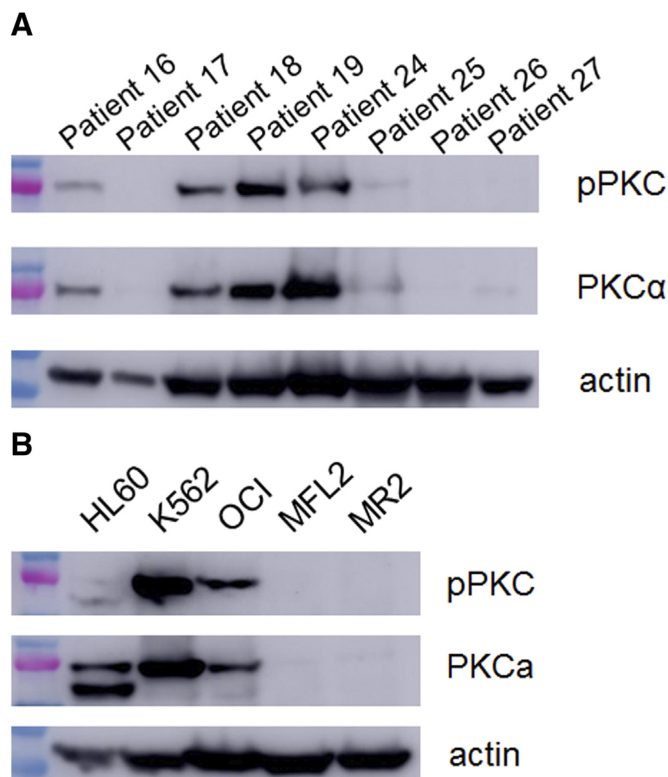


**Figure 4.** A subset of AML patients and cell lines expresses detectable phosphorylated PKC. (A) AML cells from patient samples were subjected to Western blotting with an antibody that recognizes phosphorylated PKCα and PKC$\beta_{II}$ (pPKC). Actin was used a loading control. (B) HL60, K562, MFL2, MR2, and OCI-AML3 cells were harvested and subjected to Western blotting with an antibody that recognizes phosphorylated PKCα/$\beta_{II}$ (pPKC) as well as total PKCα (PKCa). Actin was used a loading control.

either saline (control) or KPC34 at 20 mg/kg once daily for 4 days. In contrast to the parental line, KPC34 treatment resulted in a significant increase in survival (Figure 5*F*). These data suggest that phosphorylated PKC has utility as a predictive marker for AML cells most likely to respond to KPC34.

## Discussion

AML is an aggressive malignancy that is characterized by relapse and resistance to therapy. The outcomes in relapsed AML are extremely poor, and novel therapies are desperately needed.

Recently, the FDA approved the combination of the hypomethylating agent decitabine or azacytidine and the BCL-2 inhibitor venetoclax based on encouraging clinical efficacy in older AML patients [11]. While this therapy provided an impressive response rate and median survival of

**Table 2**
Patient Sample Characteristics

| ID # | Age | Gender | Diagnosis | Source | Karyotype | FLT3 Status |
|---|---|---|---|---|---|---|
| 16 | 65 | F | AML, initial diagnosis | Leukapheresis | 47XX, +8, iso13 | ND |
| 17 | 66 | M | AML, initial diagnosis | Leukapheresis | 46XY, del 6q | FLT3 ITD |
| 18 | 59 | F | AML, initial diagnosis | Leukapheresis | 46XX, inv16 | ND |
| 19 | 33 | F | AML, initial diagnosis | Leukapheresis | 45XX, del 7, inv3 | ND |
| 24 | 53 | M | AML, initial diagnosis | Leukapheresis | 46XY | FLT3 ITD |
| 25 | 89 | F | AML, initial diagnosis | Leukapheresis | 47,XX,der(4) add(4)(p16.1), +8 | ND |
| 26 | 59 | F | AML, initial diagnosis | Leukapheresis | 46XX | ND |
| 27 | 69 | M | AML, initial diagnosis | Leukapheresis | 46XY | FLT3 ITD |
| 210 | 56 | F | AML, second relapse | Bone marrow | 46,XX,t(3;12)(q26;p13) [3] | ND |

*ITD*, internal tandem duplication; *ND*, not determined.

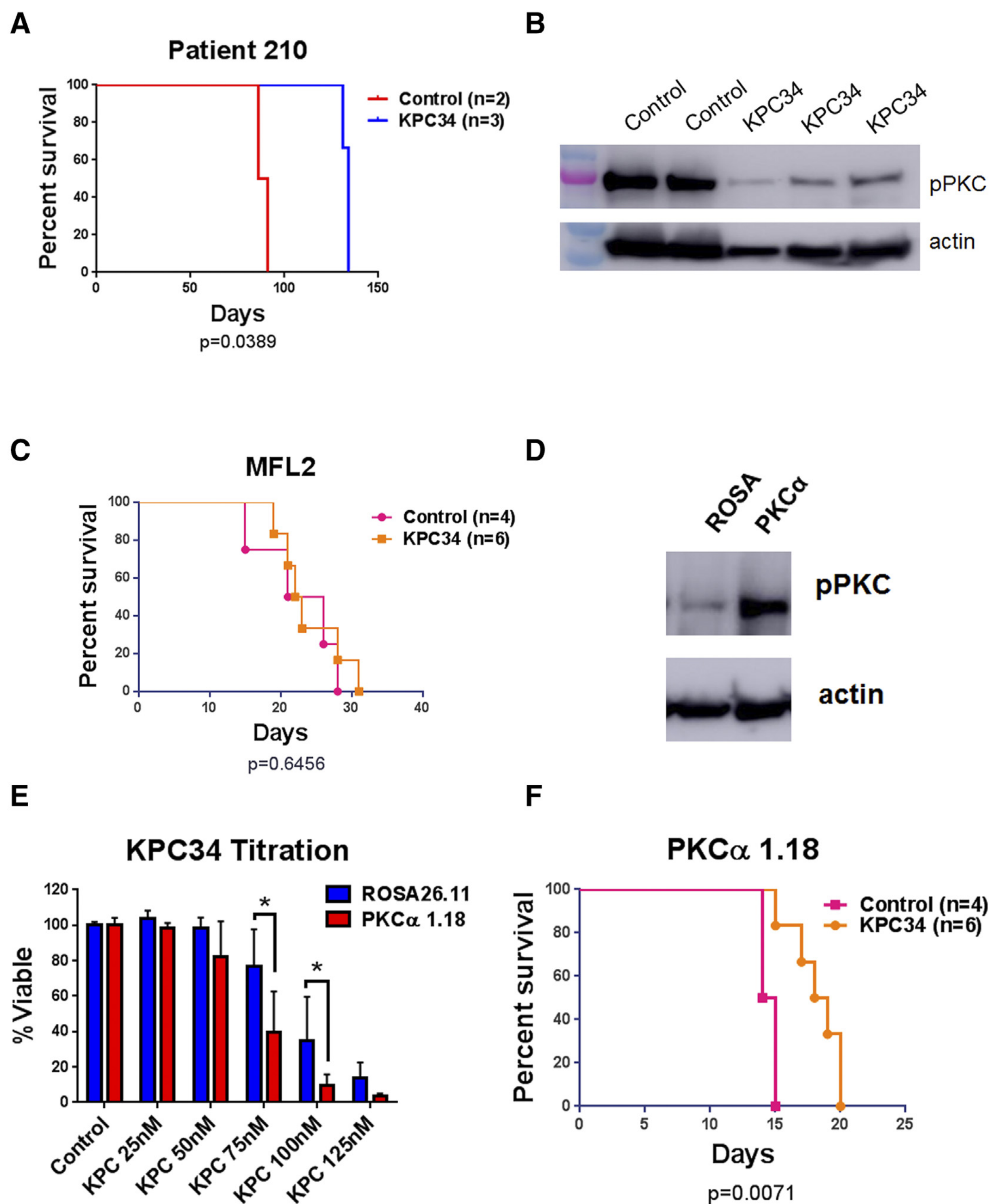


**Figure 5.** KPC34 activity is modulated by PKC status. (A) KPC34 is active *in vivo* against a PDX model of AML expressing phosphorylated PKC. Kaplan-Meier curves for transgenic NSG mice tail vein injected with CD34 selected cells from patient sample #210 and treated with saline (control) or KPC34 at 10 mg/kg PO once daily for 4 days followed by 2 days every week until moribund. (B) KPC34-resistant cells have decreased activated PKC. Cell lysates from harvested bone marrow of moribund mice treated in A were subjected to Western blotting with an antibody that recognizes phosphorylated PKCα and PKC$\beta_{II}$ (pPKC). Actin was used a loading control. PKC phosphorylation status correlates with response to KPC34. (C) Kaplan-Meier curves for C57Bl/6 mice tail vein injected with MFL2 cells and treated with saline (control) or KPC34 at 20 mg/kg PO once daily for 4 days. (D) MFL2 cells expressing Cas9-VP64 and an sgRNA targeting the ROSA or PKCα loci were harvested and subjected to Western blotting with an antibody that recognizes phosphorylated PKCα and PKC$\beta_{II}$ (pPKC). Actin was used a loading control. (E) Viability assays of the cells in D treated with the indicated amount of KPC34 for 72 hours. * = $P$ value $< .05$ by Sidak's multiple-comparisons test. (F) Kaplan-Meier curves for C57Bl/6 mice tail vein injected with PKCα 1.18 cells and treated with saline (control) or KPC34 at 20 mg/kg PO once daily for 4 days.

17.5 months, it is not curative and all patients will progress at some point. Both decitabine and azacytidine are cytidine analogue prodrugs. Additionally, venetoclax in combination with another cytidine analogue prodrug, cytarabine, was also recently approved for older and unfit adults with AML [30]. Therapy for younger patients and fit older patients also includes cytarabine [31]. This means that virtually all AML patients who undergo treatment will be exposed to a cytidine analogue prodrug. Azacytidine, decitabine, and cytarabine all require the activity of hENT1 and dCK for cell entry and activation, and downregulation of these enzymes is a source of resistance [19,32,33]. KPC34 delivers the cytidine analogue gemcitabine monophosphate independent of both ENT1 and dCK, making it an ideal candidate for testing in patients who have failed previous treatment with cytidine analogues.

Additional recent FDA approvals for therapies in AML include gilteritinib for FLT3 mutated patients, ivosidenib for IDH1 mutated patients, and enasidenib for IDH2 mutated patients [7,8,34]. These agents

are important new options for patients and orally administered, adding to patient convenience; however, they are limited to patients with the specific mutations, complete remission rates are in the 20% range, and median survival is less than a year. KPC34 is orally bioavailable and appears to have increased efficacy in AML cells that have activated PKCα or β, making this a possible biomarker that would be independent of the mutational status of the patient.

Expression of the classical isoforms of PKC has been associated with enhanced AML cell survival and worse prognosis [25,35], and its inhibition is cytotoxic [22]. Despite these encouraging preclinical data, only one PKC targeted therapy has been successfully translated into the clinic for the treatment of AML. Ironically, midostaurin (formally PKC412), though initially developed as a PKC inhibitor, was only tried clinically in AML after it was also found to have inhibitory effects against the FLT3 protein kinase [36], and this is currently thought to be the main mechanism of action against AML cells [37]. When used as a single agent, midostaurin produced only transient responses and rapid progression [38]. However, when paired with DNA-damaging agents, significant survival benefits were observed [39], leading to FDA approval. This suggests that kinase inhibition should be paired with DNA-damaging agents, and administration of KPC34 simultaneously delivers both.

Lipid nucleoside conjugates that bypass hENT1 have also been tested in AML. The novel cytarabine lipid conjugate elacytarabine enters the cell independent of hENT1 activity and was tested in patients with advanced AML [40]. In one study, elacytarabine was given by continuous IV infusion on days 1 to 5; there was a response rate of 18%, with a median survival of 5.3 months. However, in a randomized phase 3 study for patients with relapsed AML, elacytarabine was not significantly better than investigators' choice [41]. This study illustrates that while nucleoside lipid conjugates have clinical activity, they are limited by low remission rates and short median survival. Although elacytarabine does not require ENT1-mediated uptake, it still requires the action of dCK. Indeed, one documented resistance mechanism against this compound is downregulation of dCK [42]. This may explain the negative outcome and suggests that agents need to be independent of both ENT1 and dCK, like KPC34, to be maximally effective in the relapse setting.

Gemcitabine causes inhibition of DNA polymerization. However, unlike cytarabine, it has several additional "self-potentiating" activities, including the inhibition of ribonucleotide reductase leading to increased DNA incorporation, chain termination after the addition of another nucleotide leading to "masked" chain termination resistant to proofreading repair enzymes, inhibition of thymidylate synthase, and prolonged cellular retention (reviewed by Mini et al. [43]). KPC34 delivers gemcitabine in a manner that is independent of both ENT1 and dCK. It has the potential to take advantage of the multiple mechanisms of cytotoxicity of gemcitabine while not being subjected to its resistance mechanisms.

In summary, KPC34 represents a novel approach of combining both a targeted agent and a cytotoxic chemotherapy in one molecule. It has activity in multiple preclinical AML models, and activated PKCα or β may serve as a predictive biomarker. This study justifies the further translation of KPC34 in the clinic.

## Declaration of Competing Interest

G. L. K. holds patents on KPC34. G. L. K. and T. S. P. are consultants to Spherix Inc. All other authors have nothing to disclose.

*Availability of Data and Material*

All materials will be made available to requesting scientists.

## Funding

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## Authorship Contributions

Conceived and designed the experiments: P. M. A., T. S. P., G. L. K.. Performed the experiments: P. M. A., K. M. P. Analyzed the data: P. M. A., G. L. K., T. S. P. Wrote/edited the manuscript: P. M. A., T. S. P., G. L. K.

## Appendix A. Supplementary Data

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## References


[1] J.D. Licht, D.W. Sternberg, The molecular pathology of acute myeloid leukemia, Hematology Am. Soc. Hematol. Educ. Program (2005) 137–142.

[2] H. Dohner, E.H. Estey, S. Amadori, F.R. Appelbaum, T. Buchner, A.K. Burnett, H. Dombret, P. Fenaux, D. Grimwade, R.A. Larson, F. Lo-Coco, T. Naoe, D. Niederwieser, G.J. Ossenkoppele, M.A. Sanz, J. Sierra, M.S. Tallman, B. Lowenberg, C.D. Bloomfield, Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet, Blood 115 (2010) 453–474.

[3] R. Siegel, E. Ward, O. Brawley, A. Jemal, Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths, CA Cancer J. Clin. 61 (2011) 212–236.

[4] D.I. Marks, Treating the "older" adult with acute lymphoblastic leukemia, Hematology Am. Soc. Hematol. Educ. Program 2010 (2010) 13–20.

[5] D.A. Breems, W.L. Van Putten, P.C. Huijgens, G.J. Ossenkoppele, G.E. Verhoef, L.F. Verdonck, E. Vellenga, G.E. De Greef, E. Jacky, J. Van der Lelie, M.A. Boogaerts, B. Lowenberg, Prognostic index for adult patients with acute myeloid leukemia in first relapse, J. Clin. Oncol. 23 (2005) 1969–1978.

[6] X. Thomas, E. Raffoux, A. Renneville, C. Pautas, S. de Botton, T. de Revel, O. Reman, C. Terre, C. Gardin, Y. Chelghoum, N. Boissel, B. Quesnel, C. Cordonnier, J.H. Bourhis, M. Elhamri, P. Fenaux, C. Preudhomme, G. Socie, M. Michallet, S. Castaigne, H. Dombret, Outcome of treatment after first relapse in younger adults with acute myeloid leukemia initially treated by the ALFA-9802 trial, Leuk. Res. 36 (2012) 1112–1118.

[7] A.E. Perl, G. Martinelli, J.E. Cortes, A. Neubauer, E. Berman, S. Paolini, P. Montesinos, M.R. Baer, R.A. Larson, C. Ustun, F. Fabbiano, H.P. Erba, A. Di Stasi, R. Stuart, R. Olin, M. Kasner, F. Ciceri, W.C. Chou, N. Podoltsev, C. Recher, H. Yokoyama, N. Hosono, S.S. Yoon, J.H. Lee, T. Pardee, A.T. Fathi, C. Liu, N. Hasabou, X. Liu, E. Bahceci, M.J. Levis, Gilteritinib or chemotherapy for relapsed or refractory FLT3-mutated AML, N. Engl. J. Med. 381 (2019) 1728–1740.

[8] C.D. DiNardo, E.M. Stein, S. de Botton, G.J. Roboz, J.K. Altman, A.S. Mims, R. Swords, R.H. Collins, G.N. Mannis, D.A. Pollyea, W. Donnellan, A.T. Fathi, A. Pigneux, H.P. Erba, G.T. Prince, A.S. Stein, G.L. Uy, J.M. Foran, E. Traer, R.K. Stuart, M.L. Arellano, J.L. Slack, M.A. Sekeres, C. Willekens, S. Choe, H. Wang, V. Zhang, K.E. Yen, S.M. Kapsalis, H. Yang, D. Dai, B. Fan, M. Goldwasser, H. Liu, S. Agresta, B. Wu, E.C. Attar, M.S. Tallman, R.M. Stone, H.M. Kantarjian, Durable remissions with ivosidenib in IDH1-mutated relapsed or refractory AML, N. Engl. J. Med. 378 (2018) 2386–2398.

[9] E.M. Stein, Enasidenib, a targeted inhibitor of mutant IDH2 proteins for treatment of relapsed or refractory acute myeloid leukemia, Future Oncol. 14 (2018) 23–40.

[10] A. Burnett, M. Wetzler, B. Lowenberg, Therapeutic advances in acute myeloid leukemia, J. Clin. Oncol. 29 (2011) 487–494.

[11] C.D. DiNardo, K. Pratz, V. Pullarkat, B.A. Jonas, M. Arellano, P.S. Becker, O. Frankfurt, M. Konopleva, A.H. Wei, H.M. Kantarjian, T. Xu, W.J. Hong, B. Chyla, J. Potluri, D.A. Pollyea, A. Letai, Venetoclax combined with decitabine or azacitidine in treatment-naive, elderly patients with acute myeloid leukemia, Blood 133 (2019) 7–17.

[12] W. Blum, R. Garzon, R.B. Klisovic, S. Schwind, A. Walker, S. Geyer, S. Liu, V. Havelange, H. Becker, L. Schaaf, J. Mickle, H. Devine, C. Kefauver, S.M. Devine, K.K. Chan, N.A. Heerema, C.D. Bloomfield, M.R. Grever, J.C. Byrd, M. Villalona-Calero, C.M. Croce, G. Marcucci, Clinical response and miR-29b predictive significance in older AML patients treated with a 10-day schedule of decitabine, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 7473–7478.

[13] H. Dombret, J.F. Seymour, A. Butrym, A. Wierzbowska, D. Selleslag, J.H. Jang, R. Kumar, J. Cavenagh, A.C. Schuh, A. Candoni, C. Recher, I. Sandhu, T. Bernal del Castillo, H.K. Al-Ali, G. Martinelli, J. Falantes, R. Noppeney, R.M. Stone, M.D. Minden, H. McIntyre, S. Songer, L.M. Lucy, C.L. Beach, H. Dohner, International phase 3 study of azacitidine vs conventional care regimens in older patients with newly diagnosed AML with >30% blasts, Blood 126 (2015) 291–299.

[14] Y. Huang, P. Anderle, K.J. Bussey, C. Barbacioru, U. Shankavaram, Z. Dai, W.C. Reinhold, A. Papp, J.N. Weinstein, W. Sadee, Membrane transporters and channels: role of the transportome in cancer chemosensitivity and chemoresistance, Cancer Res. 64 (2004) 4294–4301.

[15] C.M. Galmarini, X. Thomas, F. Calvo, P. Rousselot, M. Rabilloud, A. El Jaffari, E. Cros, C. Dumontet, In vivo mechanisms of resistance to cytarabine in acute myeloid leukaemia, Brit. J. Haematol. 117 (2002) 860–868.

[16] I. Hubeek, R.W. Stam, G.J. Peters, R. Broekhuizen, J.P.P. Meijerink, E.R. van Wering, B.E.S. Gibson, U. Creutzig, C.M. Zwaan, J. Cloos, D.J. Kuik, R. Pieters, G.J.L. Kaspers, The human equilibrative nucleoside transporter 1 mediates in vitro cytarabine sensitivity in childhood acute myeloid leukaemia, Brit. J. Cancer 93 (2005) 1388–1394.
[17] C.M. Galmarini, X. Thomas, F. Calvo, P. Rousselot, A. El Jafaari, E. Cros, C. Dumontet, Potential mechanisms of resistance to cytarabine in AML patients, Leuk. Res. 26 (2002) 621–629.
[18] N.J. Raynal, L.F. Momparler, G.E. Rivard, R.L. Momparler, 3-Deazauridine enhances the antileukemic action of 5-aza-2′-deoxycytidine and targets drug-resistance due to deficiency in deoxycytidine kinase, Leuk. Res. 35 (2011) 110–118.
[19] A.P. Stegmann, M.W. Honders, M.G. Kester, J.E. Landegent, R. Willemze, Role of deoxycytidine kinase in an in vitro model for AraC- and DAC-resistance: substrate-enzyme interactions with deoxycytidine, 1-beta-D-arabinofuranosylcytosine and 5-aza-2′-deoxycytidine, Leukemia 7 (1993) 1005–1011.
[20] E.M. Griner, M.G. Kazanietz, Protein kinase C and other diacylglycerol effectors in cancer, Nat. Rev. Cancer 7 (2007) 281–294.
[21] M.L. Guzman, X. Li, C.A. Corbett, R.M. Rossi, T. Bushnell, J.L. Liesveld, J. Hebert, F. Young, C.T. Jordan, Rapid and selective death of leukemia stem and progenitor cells induced by the compound 4-benzyl, 2-methyl, 1,2,4-thiadiazolidine, 3,5 dione (TDZD-8), Blood 110 (2007) 4436–4444.
[22] P.P. Ruvolo, L. Zhou, J.C. Watt, V.R. Ruvolo, J.K. Burks, T. Jiffar, S. Kornblau, M. Konopleva, M. Andreeff, Targeting PKC-mediated signal transduction pathways using enzastaurin to promote apoptosis in acute myeloid leukemia-derived cell lines and blast cells, J. Cell. Biochem. 112 (2011) 1696–1707.
[23] T. Jiffar, S. Kurinna, G. Suck, D. Carlson-Bremer, M.R. Ricciardi, M. Konopleva, M. Andreeff, P.P. Ruvolo, PKC alpha mediates chemoresistance in acute lymphoblastic leukemia through effects on Bcl2 phosphorylation, Leukemia 18 (2004) 505–512.
[24] L. Hunakova, J. Duraj, D. Romanova, L. Novotny, J. Sedlak, M.R. Kelley, T. Szekeres, H.N. Jayaram, B. Chorvath, Staurosporine enhanced benzamide riboside-induced apoptosis in human multidrug-resistant promyelocytic leukemia cells (HL-60/VCR) in vitro, Neoplasma 45 (1998) 204–209.
[25] S. Kurinna, M. Konopleva, S.L. Palla, W. Chen, S. Kornblau, R. Contractor, X. Deng, W.S. May, M. Andreeff, P.P. Ruvolo, Bcl2 phosphorylation and active PKC alpha are associated with poor survival in AML, Leukemia 20 (2006) 1316–1319.
[26] P.M. Alexander, D.L. Caudell, G.L. Kucera, K.M. Pladna, T.S. Pardee, The novel phospholipid mimetic KPC34 is highly active against preclinical models of Philadelphia chromosome positive acute lymphoblastic leukemia, PLoS One 12 (2017), e0179798.
[27] K.A. Pickin, R.L. Alexander, C.S. Morrow, S.L. Morris-Natschke, K.S. Ishaq, R.A. Fleming, G.L. Kucera, Phospholipid/deoxycytidine analogue prodrugs for the treatment of cancer, J. Drug Delivery Sci. Technol. 19 (2009) 31–36.
[28] T.S. Pardee, E. Gomes, J. Jennings-Gee, D. Caudell, W.H. Gmeiner, Unique dual targeting of thymidylate synthase and topoisomerase1 by FdUMP[10] results in high efficacy against AML and low toxicity, Blood 119 (2012) 3561–3570.
[29] J. Beck, R. Handgretinger, T. Klingebiel, R. Dopfer, M. Schaich, G. Ehninger, D. Niethammer, V. Gekeler, Expression of PKC isozyme and MDR-associated genes in primary and relapsed state AML, Leukemia 10 (1996) 426–433.
[30] A.H. Wei, S.A. Strickland Jr., J.Z. Hou, W. Fiedler, T.L. Lin, R.B. Walter, A. Enjeti, I.S. Tiong, M. Savona, S. Lee, B. Chyla, R. Popovic, A.H. Salem, S. Agarwal, T. Xu, K.M. Fakouhi, R. Humerickhouse, W.J. Hong, J. Hayslip, G.J. Roboz, Venetoclax combined with low-dose cytarabine for previously untreated patients with acute myeloid leukemia: results from a phase Ib/II study, J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 37 (2019) 1277–1284.
[31] H. Dohner, E. Estey, D. Grimwade, S. Amadori, F.R. Appelbaum, T. Buchner, H. Dombret, B.L. Ebert, P. Fenaux, R.A. Larson, R.L. Levine, F. Lo-Coco, T. Naoe, D. Niederwieser, G.J. Ossenkoppele, M. Sanz, J. Sierra, M.S. Tallman, H.F. Tien, A.H. Wei, B. Lowenberg, C.D. Bloomfield, Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel, Blood 129 (2017) 424–447.
[32] P. Macanas-Pirard, A. Leisewitz, R. Broekhuizen, K. Cautivo, F.M. Barriga, F. Leisewitz, V. Gidi, E. Riquelme, V.P. Montecinos, P. Swett, P. Besa, P. Ramirez, M. Ocqueteau, A.M. Kalergis, M. Holt, M. Rettig, J.F. DiPersio, B. Nervi, Bone marrow stromal cells modulate mouse ENT1 activity and protect leukemia cells from cytarabine induced apoptosis, PLoS One 7 (2012), e37203.
[33] C.M. Galmarini, X. Thomas, K. Graham, A. El Jafaari, E. Cros, L. Jordheim, J.R. Mackey, C. Dumontet, Deoxycytidine kinase and cN-II nucleotidase expression in blast cells predict survival in acute myeloid leukaemia patients treated with cytarabine, Br. J. Haematol. 122 (2003) 53–60.
[34] E.M. Stein, C.D. DiNardo, D.A. Pollyea, A.T. Fathi, G.J. Roboz, J.K. Altman, R.M. Stone, D.J. DeAngelo, R.L. Levine, I.W. Flinn, H.M. Kantarjian, R. Collins, M.R. Patel, A.E. Frankel, A. Stein, M.A. Sekeres, R.T. Swords, B.C. Medeiros, C. Willekens, P. Vyas, A. Tosolini, Q. Xu, R.D. Knight, K.E. Yen, S. Agresta, S. de Botton, M.S. Tallman, Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia, Blood 130 (2017) 722–731.
[35] J. Zabkiewicz, L. Pearn, R.K. Hills, R.G. Morgan, A. Tonks, A.K. Burnett, R.L. Darley, The PDK1 master kinase is over-expressed in acute myeloid leukemia and promotes PKC-mediated survival of leukemic blasts, Haematologica 99 (2014) 858–864.
[36] E. Weisberg, C. Boulton, L.M. Kelly, P. Manley, D. Fabbro, T. Meyer, D.G. Gilliland, J.D. Griffin, Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412, Cancer Cell 1 (2002) 433–443.
[37] R.M. Stone, P.W. Manley, R.A. Larson, R. Capdeville, Midostaurin: its odyssey from discovery to approval for treating acute myeloid leukemia and advanced systemic mastocytosis, Blood Adv. 2 (2018) 444–453.
[38] R.M. Stone, D.J. DeAngelo, V. Klimek, I. Galinsky, E. Estey, S.D. Nimer, W. Grandin, D. Lebwohl, Y. Wang, P. Cohen, E.A. Fox, D. Neuberg, J. Clark, D.G. Gilliland, J.D. Griffin, Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412, Blood 105 (2005) 54–60.
[39] R.M. Stone, S.J. Mandrekar, B.L. Sanford, K. Laumann, S. Geyer, C.D. Bloomfield, C. Thiede, T.W. Prior, K. Dohner, G. Marcucci, F. Lo-Coco, R.B. Klisovic, A. Wei, J. Sierra, M.A. Sanz, J.M. Brandwein, T. de Witte, D. Niederwieser, F.R. Appelbaum, B.C. Medeiros, M.S. Tallman, J. Krauter, R.F. Schlenk, A. Ganser, H. Serve, G. Ehninger, S. Amadori, R.A. Larson, H. Dohner, Midostaurin plus chemotherapy for acute myeloid leukemia with a FLT3 mutation, N. Engl. J. Med. 377 (2017) 454–464.
[40] S. O'Brien, D.A. Rizzieri, N. Vey, F. Ravandi, U.O. Krug, M.A. Sekeres, M. Dennis, A. Venditti, D.A. Berry, T.F. Jacobsen, K. Staudacher, T. Bergeland, F.J. Giles, Elacytarabine has single-agent activity in patients with advanced acute myeloid leukaemia, Brit. J. Haematol. 158 (2012) 581–588.
[41] G.J. Roboz, T. Rosenblat, M. Arellano, M. Gobbi, J.K. Altman, P. Montesinos, C. O'Connell, S.R. Solomon, A. Pigneux, N. Vey, R. Hills, T.F. Jacobsen, A. Gianella-Borradori, O. Foss, S. Vetrhusand, F.J. Giles, International randomized phase III study of elacytarabine versus investigator choice in patients with relapsed/refractory acute myeloid leukemia, J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 32 (2014) 1919–1926.
[42] A.D. Adema, N. Losekoot, K. Smid, I. Kathmann, F. Myhren, M.L. Sandvold, G.J. Peters, Induction of resistance to the lipophilic cytarabine prodrug elacytarabine (CP-4055) in CEM leukemic cells, Nucleos Nucleot. Nucl. 29 (2010) 394–399.
[43] E. Mini, S. Nobili, B. Caciagli, I. Landini, T. Mazzei, Cellular pharmacology of gemcitabine, Ann. Oncol. 17 (Suppl 5) (2006) v7–12.