Cancer Science
Japanese Cancer Association JCA
Open Access

Puquitinib, a novel orally available PI3Kδ inhibitor, exhibits potent antitumor efficacy against acute myeloid leukemia

Chengying Xie, Ye He, Mingyue Zhen, Yulan Wang, Yongping Xu and Liguang Lou

Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China

Key words

Acute myeloid leukemia, CAL-101, PI3K signaling, PI3Kδ, puquitinib

Correspondence

Liguang Lou, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Rd, Shanghai 201203, China.
Tel: +86-21-50806056; Fax: +86-21-50806056;
E-mail: lglou@mail.shcnc.ac.cn

Funding Information

The National Natural Science Foundation of China (Grant/Award Number: 30801404, 81273546) and the National Key Research & Development Plan (Grant/Award Number: 2016YFC1201003).

Received February 15, 2017; Revised March 22, 2017; Accepted April 10, 2017

Cancer Sci **108** (2017) 1476–1484

doi: 10.1111/cas.13263

The PI3Kδ isoform (PIK3CD), also known as P110δ, is predominately expressed in leukocytes and has been implicated as a potential target in the treatment of hematological malignancies. In this report, we detailed the pharmacologic properties of puquitinib, a novel, orally available PI3Kδ inhibitor. Puquitinib, which binds to the ATP-binding pocket of PI3Kδ, was highly selective and potent for PI3Kδ relative to other PI3K isoforms and a panel of protein kinases, exhibiting low-nanomolar biochemical and cellular inhibitory potencies. Additional cellular profiling demonstrated that puquitinib inhibited proliferation, induced G_1-phase cell-cycle arrest and apoptosis in acute myeloid leukemia (AML) cell lines, through downregulation of PI3K signaling. In *in vivo* AML xenografts, puquitinib alone showed stronger efficacy than the well-known p110δ inhibitor, CAL-101, in association with a reduction in AKT and ERK phosphorylation in tumor tissues, without causing noticeable toxicity. Furthermore, the combination of puquitinib with cytotoxic drugs, especially daunorubicin, yielded significantly stronger antitumor efficacy compared with each agent alone. Thus, puquitinib is a promising agent with pharmacologic properties that are favorable for the treatment of AML.

The phosphatidylinositol 3-kinase (PI3K) subfamily is divided into three classes (I, II and III) based on their structural characteristics and substrate specificity.[1,2] Of these, the most commonly studied are the class I enzymes, which are activated directly by cell surface receptors and are associated with tumorigenesis. There are four members of class I, designed p110α, p110β, p110γ and p110δ.[3] The different class I isoforms have nonredundant roles and different expression profiles in different cell types.[4–6] The p110α and p110β isoforms are ubiquitously expressed, whereas the expression of p110γ, which has a particular role in T-cell activation, is largely restricted to hematopoietic cells, where it is critically involved in B-cell homeostasis and function.[7] PI3Kδ and its downstream target, AKT, are reported to be frequently activated in leukemic blasts from patients with B-cell malignancies or acute myeloid leukemia (AML).[8,9] Constitutive activation of the PI3K/AKT pathway in malignant leukocytes results in sustained proliferation and survival of tumor cells.[10] Therefore, the development of selective inhibitors of PI3Kδ with a high therapeutic index offers a new approach for treating hematological malignancies. Over the past 10 years, the development of PI3Kδ inhibitors has made great leaps forward.[11] Currently, there are several PI3Kδ inhibitors at different stages of clinical development, including AMG319, GSK226955 and INCB040093.[12,13] Idelalisib (GS-1101, CAL-101), a more potent derivative of the highly p110δ-selective inhibitor, IC87114, was approved recently to treat patients with relapsed follicular B-cell non-Hodgkin lymphoma and relapsed small lymphocytic lymphoma, another type of non-Hodgkin lymphoma.[14]

Acute myeloid leukemia is the most common acute leukemia in adults and is among the most lethal. There have been significant research efforts aimed at improving outcomes in AML, but the standard therapy for most subtypes of newly diagnosed AML has remained unchanged for the past four decades, and developing new therapies for AML has been challenging.[15] Thus, there remains a clear need for newer therapies and a more individualized approach for the treatment of AML. P110δ, the only class I PI3K isoform consistently expressed in AML blast cells, is the main contributor to PI3K activity in AML blasts.[16] Although IC87114 inhibited AML cell proliferation, it showed weaker inhibitory activity towards p110δ in AML cells compared with B-cell acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia cells.[14]

Puquitinib, a novel selective PI3Kδ inhibitor, exhibited notable safety, tolerability and efficacy against hematological malignancies in phase I clinical trials, and is currently undergoing phase II clinical trials in China. In the present study, we characterized the biochemical and cellular properties of puquitinib, and investigated its potency and selectivity for inhibition of the PI3Kδ pathway. Puquitinib alone or in combination with cytotoxic agents showed superior antitumor efficacy against

AML compared with CAL-101, both *in vitro* and *in vivo*. Thus, these studies provide a rationale for the clinical development of puquitinib as a PI3Kδ inhibitor for treating AML.

Materials and Methods

Materials. Puquitinib and CAL-101, provided by Zhejiang Medicine (Zhejiang, China), were prepared as 50-mM stock solutions in dimethylsulfoxide for *in vitro* studies or normal saline *in vivo* studies. Lysophosphatidic acid (LPA), C5a, anti-IgM, LY294002 and wortmannin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The appropriate primary antibodies to p110α, p110β, p110γ, AKT, p-$S6^{235/6}$, S6, p-$P70S6K^{T389}$, P70S6K, p-AKT^{S473}, p-AKT^{T308} and p-ERK1/2 were purchased from Cell Signaling (Beverly, MA, USA). Antibodies specific for p110δ and ERK1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. RAW264.7, Raji, SK-BR-3, PC-3, MM.1R (multiple myeloma), human AML MV4;11, RS4;11, TF-1, Kasumi-1, U-937, THP-1 and KG-1a cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Set-2, EOL-1 and Molm-16 human AML cell lines were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (GmbH, Braunschweig, Germany). Mo7e AML cell line was provided by the Genetics Institute (Boston, MA, USA). All cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS at 37°C in a humidified 5% CO_2 atmosphere.

Cell proliferation assay. Cell proliferation was evaluated by MTT assay.[17] Briefly, cells in 96-well plates were treated in triplicate with different concentrations of drugs and incubated at 37°C for 72 h. Cytotoxicity was assessed by measuring the conversion of MTT (Sigma-Aldrich) to a colored product. GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA) curve-fitting software was used to calculate half-maximal inhibitory concentration (IC_{50}) values.

Cell cycle analysis. After drug treatment, cells were fixed in ice-cold 70% ethanol overnight at −20°C. Fixed cells were stained with 50 μg/mL of propidium iodide containing 50 μg/mL of DNase-free RNase A at 37°C for 30 min. The DNA content of cells (10 000 cells/experimental group) was analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) using ModFit LT Mac V3.0 software.

***In vitro* kinase profiling.** *In vitro* kinase profile assays were analyzed using Life Technologies' SelectScreen Profiling Service (Thermo Fisher, Waltham, MA, USA). PI3K ELISA were performed using a PI3Kinase Activity/Inhibitor Assay Kit (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. Briefly, recombinant class I PI3K isoforms and drugs were prepared in kinase reaction assay buffer containing phosphatidylinositol 4,5-bisphosphate and incubated in a glutathione-coated 96-well plate. The plate was then treated with biotin-phosphatidylinositol 3,4,5-trisphosphate and glycine-rich protein 1 working solution. After washing, a streptavidin-HRP (SA-HRP) working solution was added, followed by addition of the HRP substrate, tetramethylbenzidine. The absorbance of each well was then measured at 450 nm using a microplate reader.

Molecular docking. Molecular modeling calculations were based on crystallographic data for the PI3K inhibitor PIK-039 in complex with PI3Kδ (PDB code 2WXF).[18] Docking studies were performed using Glide software (version 5.5).[19] The obtained docked poses were analyzed with Maestro, PyMOL and LigPlot.[20]

Western blotting. Cells were collected at the end of treatment and lysed in SDS sample buffer (100 mM Tris–HCl pH 6.8, 2% SDS, 20% glycerol, 1 mM dithiothreitol). Equal amounts of whole-cell lysates were separated by SDS-PAGE and electroblotted onto polyvinylidine difluoride membranes (Millipore). Blots were probed with primary antibodies, and then incubated with the appropriate secondary antibodies (Millipore). Immunoreactive proteins were visualized using enhanced chemiluminescence reagents (Millipore).

***In vivo* study.** Female nude mice (Balb/cA-nude, 5–6 week old) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). Human carcinoma xenografts were established by inoculating nude mice subcutaneously with 5–9 × 10^6 cells. When tumors reached a volume of 100–300 mm^3, mice were randomly assigned to control and treatment groups. Control groups were given vehicle alone, and treatment groups received oral puquitinib or CAL-101 daily. Daunorubicin or cytarabine was administered intravenously (i.v.), and co-treatment groups also received oral puquitinib daily. Tumor volume was calculated as (length × $width^2$)/2.

Pharmacokinetic/pharmacodynamic studies were carried out as described previously.[17] Mice bearing MV4;11 tumors received a single dose of 60 mg/kg puquitinib or vehicle, and tumor tissues and blood were collected at different times post-dosing. Concentrations of puquitinib in plasma and tissue were determined by HPLC/tandem mass spectrometry. Tumor samples were analyzed by western blotting. Animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee guidelines of the Shanghai Institute of *Materia Medica*, Chinese Academy of Sciences.

Data analysis. Data are presented as means ± SEM and were plotted using GraphPad Prism Version 5. An unpaired two-tailed Student's *t*-test was used to test for significance, where indicated. Differences were considered significant at $P < 0.05$.

Results

Puquitinib is a potent, selective inhibitor of PI3Kδ. Puquitinib (Fig. 1a) was synthesized following a high-throughput screen of a chemical library comprising compounds likely to target class I PI3K. There are four subtypes of p110, designated p110α, p110β, p110γ and p110δ, corresponding to the catalytic subunit of PI3Kα, -β, -γ and -δ, respectively.[3] Thus, we first examined the subtype specificity of puquitinib in a biochemical kinase assay. As shown in Table 1, puquitinib inhibited the activities of all four purified enzymes, but with substantially different potencies, exhibiting IC_{50} values of 992.8, 959.2, 89.8 and 3.3 nM for p110α, -β, -γ and -δ enzymes, respectively. These results indicate that puquitinib is selective for p110δ relative to other PI3K class I enzymes. Furthermore, puquitinib had little or no inhibitory activity against a panel of other protein kinases at a concentration of 1000 nM (Table S1).

We next investigated the potency of puquitinib against individual PI3K class I isoforms in cell-based assays by monitoring the phosphorylation of AKT.[21,22] Genetic and pharmacologic approaches that specifically inactivate the p110δ isoform have demonstrated its important role in B-cell signaling.[23,24] Inhibition of p110δ by puquitinib was then investigated in B-lymphocyte Raji cells. Consistent with biochemical results, puquitinib, similar to CAL-101, specifically inhibited PI3Kδ-dependent signaling in anti-IgM–stimulated Raji cells, as reflected in a concentration-dependent decrease

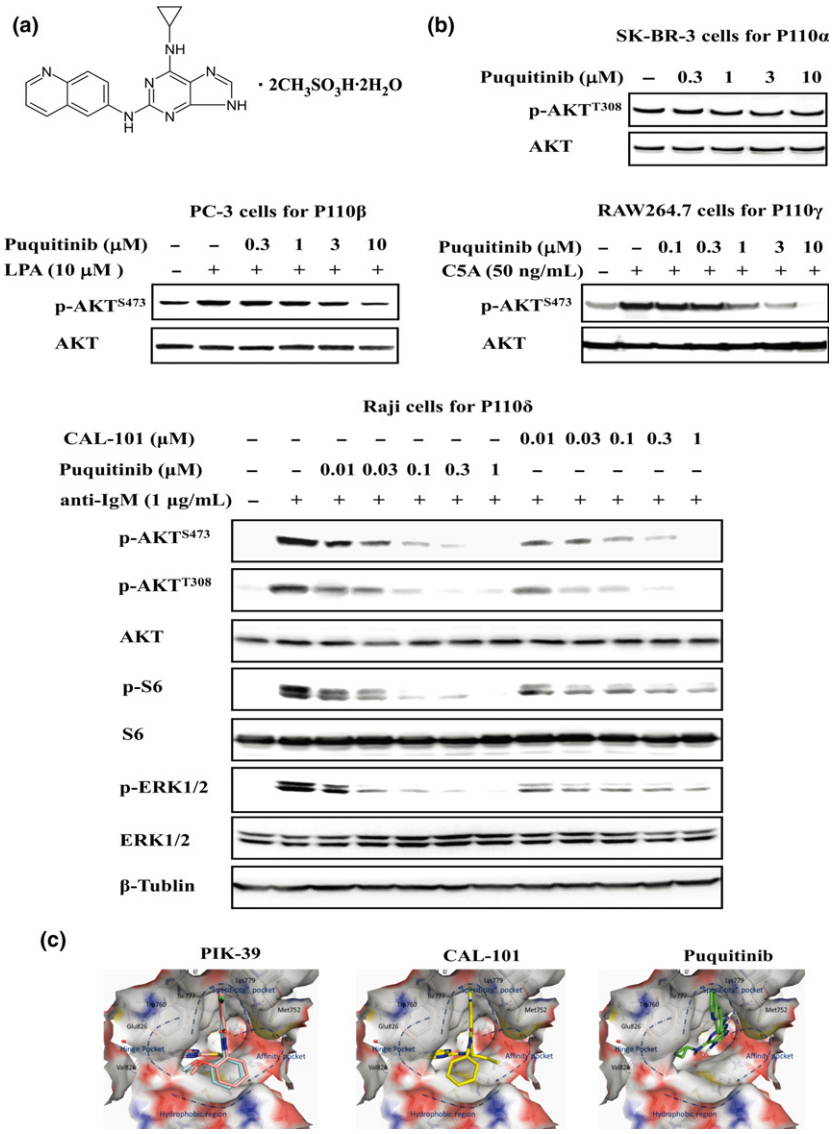

Fig. 1. Puquitinib inhibits PI3Kδ with high selectivity. (a) Chemical structure of puquitinib. (b) Potency of puquitinib in PI3K class I isoform-specific cell-based assays. p110α: SK-BR-3 cells were treated with puquitinib for 3 h. p110β/γ/δ: Following stimulation of PC-3 cells, RAW264.7 cells or Raji cells with LPA (10 μM), C5a (50 ng/mL) or anti-IgM (1 μg/mL), respectively, for 10 min, cells were treated for an additional 3 h with puquitinib or CAL-101. Cell lysates were probed with the indicated antibodies. (c) Molecular modeling of the PI3Kδ-puquitinib complex.

Table 1. Kinase inhibition profile of puquitinib

Drugs	Biochemical IC_{50} (nM, ± SEM)			
	p110α	p110β	p110γ	p110δ
Puquitinib	992.8 ± 319.9	959.2 ± 367.4	89.8 ± 14.0	3.3 ± 0.6
CAL-101	976.3 ± 78.8	711.5 ± 122.3	86.6 ± 6.3	2.8 ± 0.7

The potency of puquitinib against recombinant enzymes of class I PI3Ks *in vitro*, expressed as biochemical IC_{50}.

in the phosphorylation of the PI3Kδ targets, AKT, ribosomal protein S6 (S6) and extracellular signal-regulated kinase (ERK) (Fig. 1b). LPA and C5a stimulate G-protein coupled receptor signals via PI3Kβ and PI3Kγ, respectively.[25,26] Despite the decrease in LPA- or C5a ligand-stimulated phosphorylations of AKT in PC-3 or RAW 264.7 cells by puquitinib was observed, the decrease was much weaker than that of Raji cells. No obvious inhibitory activity of p110α/AKT phosphorylation by puquitinib was observed in SK-BR-3 cells (Fig. 1b). This pharmacological profile of puquitinib is similar to that of CAL-101 in these cell-based assays.[21,22] Thus, these data indicate that puquitinib is a potent, selective inhibitor of PI3Kδ.

To understand the mechanisms underlying the isoform selectivity of this inhibitor, we further investigated the site of puquitinib binding to PI3Kδ using structural modeling. The crystal structure of a PI3Kδ–PIK-39 complex generated a PI3K template structure for docking analysis.[18] The docking complex revealed that the purine group of PIK-39 forms hydrogen bonds with the hinge residues, Glu826 and Val828. In this structure, the quinazolinone moiety is sandwiched into a hydrophobic pocket composed of two parts, with Trp760 and Ile777 on one side, and Met752 and Pro758 on the other side. Puquitinib yielded an interaction pattern with the ATP-binding pocket of PI3Kδ that was slightly different from that of PIK-39 or CAL-101 (Fig. 1c). Although puquitinib was found to occupy the same specificity pocket of PI3Kδ, it did not form hydrogen bonds with the hinge residues Glu826 or Val828. In contrast, the purine group of puquitinib established hydrogen bonds in the ATP-binding pocket of PI3Kδ with two residues, Tyr813 and Lys779.

Puquitinib exerts cytotoxicity against acute myeloid leukemia cell lines. Given the broad importance of the p110δ isoform in

homeostasis, we next sought to determine its expression profile and the effect of puquitinib on proliferation in a panel of AML cell lines. As shown in Figure 2a, p110δ was observed in all these tested AML cell lines and there was no correlation between the expression of p110δ and the other isoforms (p110α, -β and -γ). MM.1R multiple myeloma cell line was used as a negative control in this experiment which lacked p110δ expression.[16] Notably, puquitinib inhibited the proliferation of these p110δ-positive AML cell lines with the mean IC_{50} value of 0.3 μM; in contrast, minimal cytotoxicity was noted in p110δ-negative MM.1R cell line (Fig. 2a). These results suggest that sensitivity to puquitinib is associated with constitutive p110δ expression in AML.

To further analyze the mechanism of puquitinib-induced cytotoxicity, we next examined the effect of puquitinib on the cell cycle profile. Treatment with puquitinib for 24 h induced a concentration-dependent G_1-phase cell-cycle arrest in p110δ-positive MV4;11 cells. The proportion of G_1-phase cells was increased from the control level of 63.3–83.0%, and 70.7% by 1 μM puquitinib and 1 μM CAL-101, respectively (Fig. 2b).

Next, we measured apoptosis induced by puquitinib in MV4;11 cells. With prolonged treatment with puquitinib for 48 h, Annexin V-positive cells increased in a dose-dependent manner (Fig. S1). The apoptosis induction by puquitinib was further evidenced by increased levels of cleaved caspases and poly-(ADP-ribose) polymerase (PARP) both in MV4;11 and RS4;11 cells (Fig. 2c). This apoptosis-inducing effect was significant following treatment with 1 μM puquitinib and became more pronounced at a concentration of 10 μM, while CAL-101 treatment caused weaker induction of apoptosis (Fig. 2c).

Puquitinib inhibits constitutive PI3K signaling in acute myeloid leukemia cells. A previous study showed that inhibition of p110δ by CAL-101 triggers cytotoxicity in association with inhibition of AKT and ERK phosphorylation.[27] Thus, we next examined the effect of puquitinib on these pathways in AML cell lines. As expected, puquitinib significantly inhibited phosphorylation of AKT and ERK in p110δ-positive Kasumi-1 and EOL-1 AML cells, but did not affect phosphorylation of AKT or ERK in MM.1R cells with low expression of p110δ (Fig. 3a). Puquitinib also inhibited constitutive PI3K signaling in MV4;11 cells in a concentration-dependent and time-dependent fashion (Fig. 3b). However, in this study, CAL-101 preferentially inhibited phosphorylation of AKT relative to ERK in these AML cells.

Consistent with a previous report, PI3K activity following inhibition by LY294002 rapidly recovered after removal of the drug, but did not recover following treatment with the irreversible inhibitor wortmannin (Fig. 3c). The effects of puquitinib, similar to those of CAL-101, readily reversed following compound removal, with the levels of phosphorylated AKT (pAKT) and those of downstream factors returning to control values (Fig. 3c). Thus, puquitinib appears to act through both PI3K/AKT and MEK/ERK pathways in these AML cells.

***In vivo* antitumor activity of puquitinib alone in acute leukemia xenografts.** Given its encouraging activity *in vitro*, we next investigated the antitumor efficacy of puquitinib *in vivo*, initially conducting a pharmacokinetic/pharmacodynamic study in the MV4;11 tumor xenograft model. After a single oral dose of 60 mg/kg puquitinib, plasma and tumors were collected at various time points over a 24-h period. As shown in Figure 4a, puquitinib appeared rapidly in plasma and tumor tissue. The phosphorylation of AKT and ERK in tumor tissue was inhibited in a time-dependent manner from 0.5 to 24 h after compound administration (Fig. 4b), a finding that was concordant with the changes in plasma and intratumoral concentrations of puquitinib (Fig. 4a).

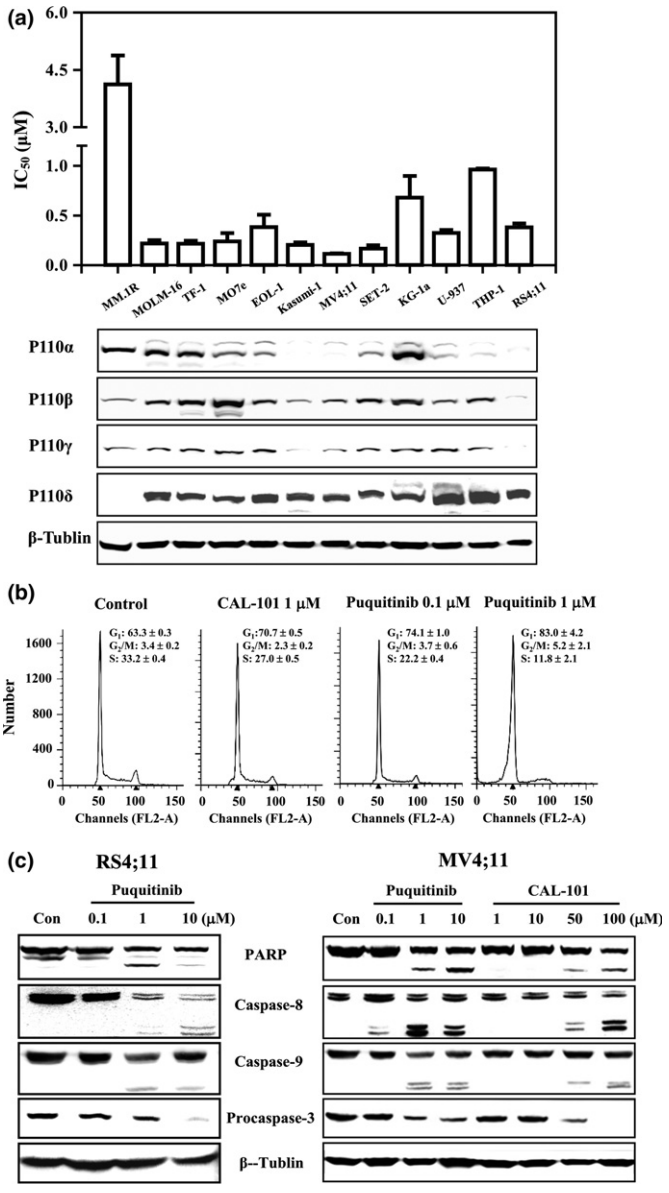

Fig. 2. Cytotoxicity of puquitinib against acute myeloid leukemia (AML) cells. (a) A panel of hematologic tumor cell lines were treated with different concentrations of puquitinib for 72 h, and cell viability was determined by MTT assay. IC_{50} values are presented as means ± SEM of three independent experiments. Expression of p110α, β, γ and δ in tumor cell lines was detected by immunoblotting using specific antibodies. An anti-β-Tubulin monoclonal antibody served as a loading control. (b) Representative cell-cycle phase histograms of MV4;11 cells following treatment with puquitinib or CAL-101 at the indicated concentration for 24 h (n = 3; error bars denote SEM). (c) MV4;11 and RS4;11 cells were treated with puquitinib or CAL-101 at the indicated concentrations for 48 h. Total cell lysates were subjected to immunoblotting with anti-PARP and anti-caspase-3, anti-caspase-8 and anti-caspase-9 antibodies.

We next examined the antitumor activity of puquitinib against MV4;11 xenografts. Oral administration of puquitinib significantly inhibited the growth of MV4;11 xenografts in a dose-dependent manner (Fig. 4c). Administration of puquitinib at a dose of 30 or 60 mg/kg inhibited tumor growth by 72 and 103%, respectively, on the final treatment day. Complete

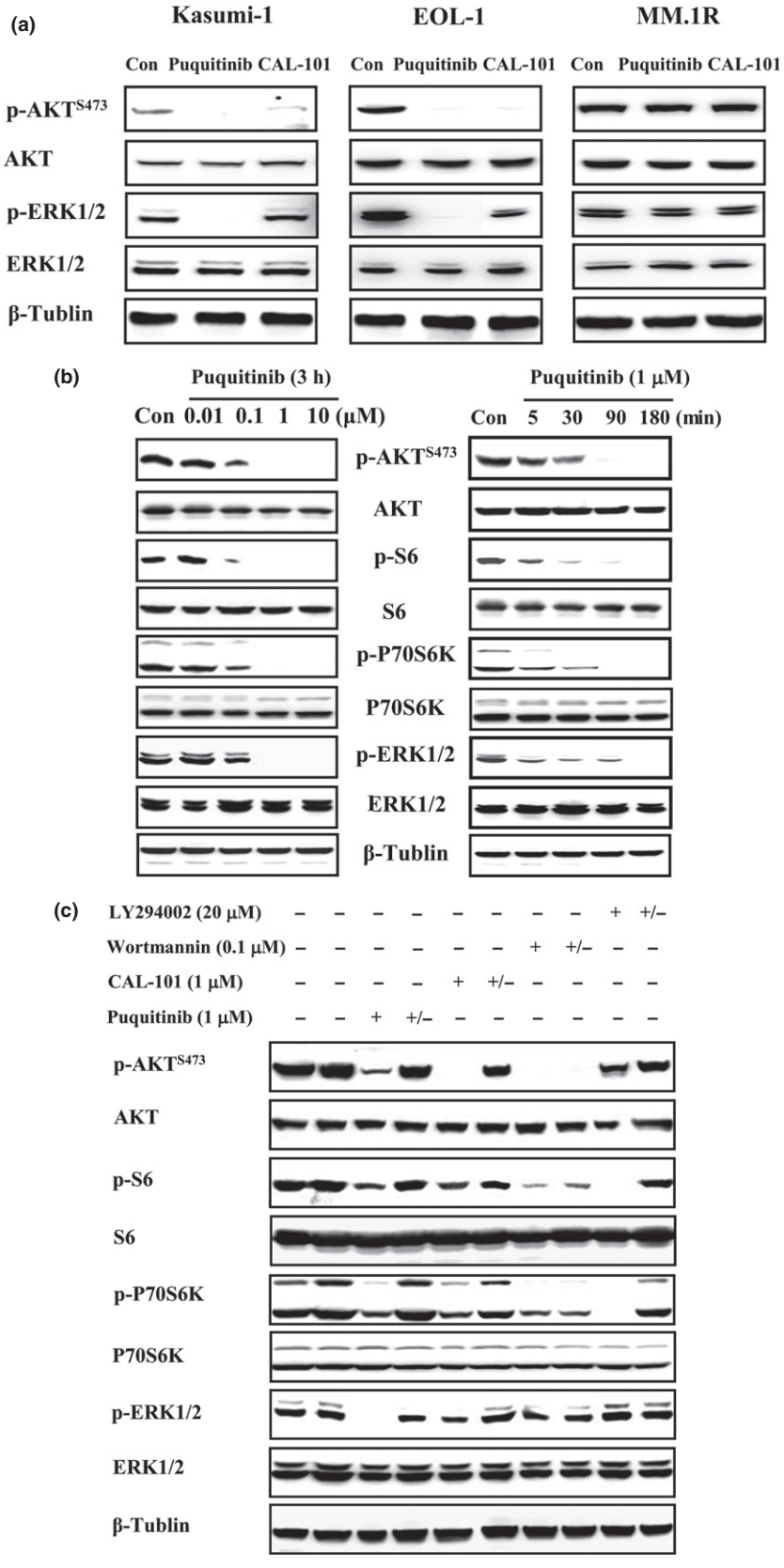

Fig. 3. Inhibition of constitutive PI3K/AKT and ERK activity by puquitinib. (a) MV4;11, EOL-1 and MM.1R cells were treated with 1 μM puquitinib or CAL-101 for 3 h. (b) MV4;11 cells were treated with increasing concentrations of puquitinib (left) or 1 μM puquitinib for the indicated time (right). (c) Reversibility of PI3K signaling. MV4;11 cells were treated with 1 μM puquitinib, 1 μM CAL-101, 20 μM LY294002 or 0.1 μM wortmannin for 3 h (+), or further cultured in drug-free medium for another 30 min after washing with PBS (+/–). Total cell lysates were analyzed by western blotting using the indicated antibodies.

tumor regression was observed in three of six tumors at the higher dosage. This growth inhibition was superior to that produced by CAL-101, which inhibited tumor growth by 50 and 61% following oral administration of a dose of 90 or 180 mg/kg, respectively. The *in vivo* antitumor activity of puquitinib was further examined in RS4;11 xenografts. Administration of 30 and 60 mg/kg puquitinib inhibited tumor growth by 50 and 69%, respectively (Fig. 4c). These treatments were well

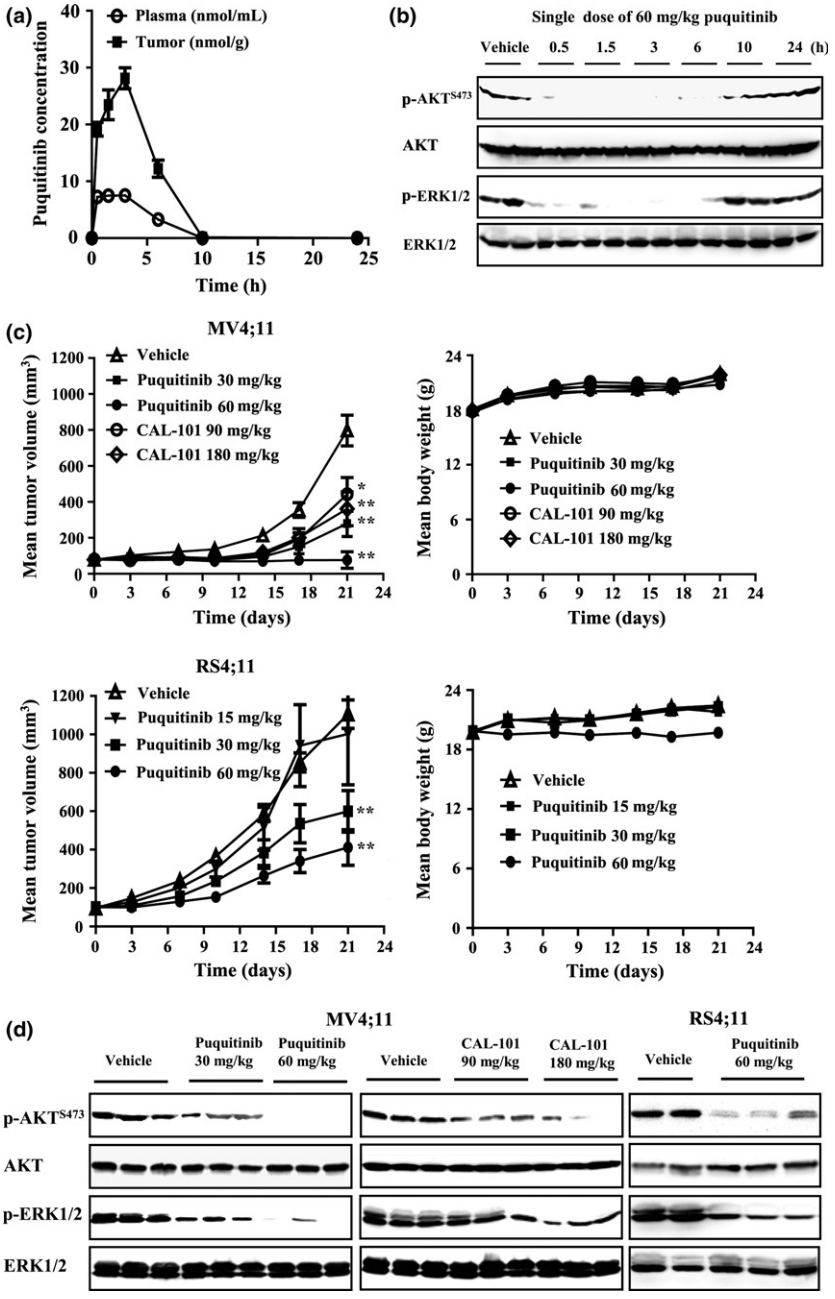

Fig. 4. *In vivo* antitumor activity of puquitinib alone against acute leukemia xenografts. (a,b) MV4;11 tumor-bearing mice were orally administered a single 60-mg/kg dose of puquitinib and killed at the indicated times. The concentration of puquitinib in blood plasma and tumor tissues was determined (a). In parallel, tumor extracts were analyzed by western blotting (b). (c) MV4;11 and RS4;11 tumor-bearing mice were orally administered vehicle ($n = 12$) or puquitinib ($n = 6$) daily for 21 days at the indicated doses. Tumor volumes were measured. $*P < 0.05$, $**P < 0.01$ versus vehicle. (d) Mice were killed 3 h after the last dose, then tumors were removed and analyzed by western blotting.

tolerated, as evidenced by the absence of significant body weight loss or other obvious signs of toxicity during the course of the experiment in all groups. Furthermore, the *in vivo* antitumor activity was also well correlated with the inhibition of PI3K signaling. In both tumor models, puquitinib treatment significantly decreased the levels of pAKT and pERK in tumor tissues (Fig. 4d).

Combined puquitinib with cytotoxic drugs mediates enhanced effects in acute myeloid leukemia. Cytotoxic drugs are widely used alone or in combination to treat leukemia. Thus, we investigated whether inhibition of p110δ by puquitinib combined with the cytotoxic drugs, daunorubicin, aclarubicin or fludarabine, exerted a potentiating effect on antitumor activity. The contribution of combination treatment to cellular activity compared with the effect of the two agents alone was evaluated by calculating the combination index (CI) using Calcusyn software, where CI < 1 denotes synergy, CI = 1 indicates an additive effect and CI > 1 reflects antagonism. As shown in Table 2, puquitinib showed synergistic effects in inhibiting the growth of MV4;11 cells when combined with each of the tested cytotoxic drugs, effects that were also confirmed in RS4;11 cells (Table 2).

Finally, we investigated the efficacy of combined treatment with puquitinib and daunorubicin or cytarabine in MV4;11 xenografts. Consistent with the *in vitro* results, puquitinib, combined with either of these two drugs, produced enhanced antitumor efficacy compared with each single agent (Fig. 5a, b). This was especially notable for puquitinib combined with daunorubicin, which produced an antitumor efficacy that was significantly superior to that of the corresponding single agents ($P < 0.01$). The combination of puquitinib with daunorubicin or cytarabine was generally well tolerated without additional observed toxicity (Fig. 5a,b).

Table 2. Combination of puquitinib with cytotoxic drugs in acute leukemia cells

Drugs	CI (mean ± SEM)	
	MV4;11	RS4;11
Fludarabine	0.58 ± 0.06	0.85 ± 0.11
Daunorubicin	0.87 ± 0.06	0.89 ± 0.03
Cytarabine	0.81 ± 0.04	ND
Mitoxantrone	0.82 ± 0.05	0.89 ± 0.10
Homoharringtonine	0.67 ± 0.06	0.89 ± 0.12
Aclarubicin	0.91 ± 0.06	0.91 ± 0.03

The combination of puquitinib with cytotoxic drugs in acute leukemia cell lines. MV4;11 and RS4;11 cells were plated in 96-well plates and incubated with different concentrations of each compound or their combinations for 72 h in triplicate. Values represent means ± SEM (n = 3). CI, combination index; ND, not determined.

Discussion

The PI3Kδ isoform is the most important isoform in hematologic cells and has been implicated as a potential target for the treatment of hematological malignancies.[28] Recently, several new PI3Kδ isoform-selective inhibitors showing improved selectivity and potency have been reported.[12,13] In the current study, puquitinib was characterized as a novel PI3Kδ inhibitor, and was shown to significantly and selectively inhibit PI3Kδ activity, notably outperforming CAL-101 both *in vitro* and *in vivo* against AML. This promising pharmaceutical activity may support the potential clinical use of puquitinib for the treatment of AML.

A previous study suggested that almost all selective inhibitors create a new specificity pocket in the enzyme that can be exploited to augment their potency towards PI3Kδ.[18,29] Our docking result revealed that puquitinib bound persistently to PI3Kδ, and interacted with the specificity pocket and affinity pocket in the active site of PI3Kδ. The purine group of puquitinib established two hydrogen bonds in the ATP-binding pocket of PI3Kδ (one with Tyr813 and one with Lys779) instead of forming hydrogen bonds with Glu826 and Val828, as is the case for CAL-101.[29] This suggests that the specific pattern of hydrogen bonding accounts for the potency and selectivity of puquitinib for PI3Kδ kinase compared with other protein kinases. Indeed, the PI3Kδ inhibitory activity of puquitinib, with an IC_{50} value of 3.3 nM, was comparable to that of CAL-101. Puquitinib was also 27–300-fold more potent against PI3Kδ than other class I PI3K isoforms, and was highly selective when profiled against other protein kinases. Furthermore, the PI3Kδ-isoform selectivity of puquitinib translated well to cell-based assays, in which puquitinib inhibited p110δ-dependent responses at low nanomolar concentrations.

The p110δ isoform is highly expressed in cells of hematopoietic origin, and has important roles in promoting proliferation and survival.[8] A total of 11 AML cell lines were randomly chosen for direct evaluation of the cytotoxicity of puquitinib *in vitro*. Puquitinib showed cytotoxicity against all tested p110δ-positive AML cell lines with the mean IC_{50} value of 0.3 μM, indicating that puquitinib is more potent than CAL-101. In contrast, puquitinib was minimally cytotoxic towards p110δ-negative cancer cell lines, and showed no cytotoxicity against peripheral blood mononuclear cells even at a concentration of 10 μM (data not shown). Of these cell lines, Flt3-ITD MV4;11 was the most sensitive to puquitinib, with an IC_{50} value of 0.1 μM; by comparison, the IC_{50} value for CAL-101 in this cell line was 2.4 μM (data not shown). Additional studies showed that puquitinib exerted greater cell-cycle arrest and apoptosis effects than CAL-101 in MV4;11 cells. These results suggest that sensitivity to puquitinib is associated with constitutive p110δ expression and imply a favorable therapeutic window.

Acute myeloid leukemia is associated with poor long-term survival. The development of new therapeutic strategies against specific targets is an area of intense interest, and such approaches may prove effective as adjunct treatments in combination with traditional chemotherapy.[15] P110δ is

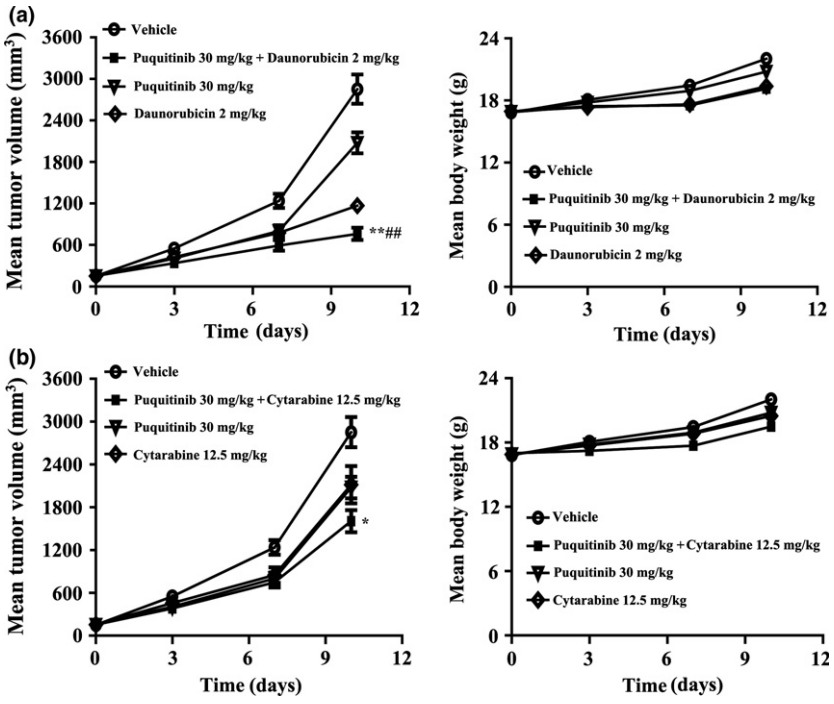

Fig. 5. Antitumor activity of puquitinib combined with cytotoxic drugs in acute myeloid leukemia (AML) MV4;11 xenografts. (a) MV4;11 tumor-bearing mice administered vehicle (n = 12), 30 mg/kg puquitinib, 2 mg/kg daunorubicin or their combination (n = 8 mice/group). (b) MV4;11 tumor-bearing mice administered vehicle, 30 mg/kg puquitinib, 12.5 mg/kg cytarabine or their combination. Tumor volumes were measured and mice body weights were determined. Data are presented as means ± SEM. *P < 0.05, **P < 0.01 versus puquitinib; #P < 0.05, ##P < 0.01 versus either single agent alone (daunorubicin or cytarabine).

consistently expressed at high levels in AML blast cells, and is the main contributor to PI3K activity in AML blasts.[8] Oral administration of puquitinib alone inhibited the growth of MV4;11 xenografts in a dose-dependent manner and caused complete regression of three of six tumors at a dose of 60 mg/kg. Cytotoxic drugs, such as VP16, daunorubicin and aclarubicin, are widely used, alone or in combination, for the treatment of AML.[30] Outside a clinical trial setting, most patients with newly diagnosed AML are offered the combination of standard-dose cytarabine together with an anthracycline (daunorubicin or idarubicin).[31] In the current study, puquitinib combined with these cytotoxic drugs, especially daunorubicin, produced a greater effect than treatment with each agent alone, without producing added toxicity. Thus, puquitinib was well tolerated, caused disease stasis when administered orally, and enhanced efficacy when used in combination with conventional chemotherapeutic agents, suggesting that puquitinib is a good alternative option for the treatment of AML in the clinic.

It has been reported that PI3Kδ and its downstream target, AKT, are frequently activated in leukemic blasts from patients with B cell malignancies or AML.[32–34] In the present study, PI3K/AKT and ERK signaling were selectively inhibited by puquitinib in p110δ-positive AML cells, but not in p110δ-negative MM.1R cells, which was demonstrated in anti-IgM-stimulated Raji cells, suggesting that puquitinib inhibited EKR activation in a p110δ-dependent manner. Although ERK is not a classical downstream target of PI3K signaling, sporadic studies have reported that the inhibition PI3Kδ inhibition might lead to inactivation of ERK.[21,22] It has been reported that CAL-101 blocks ERK phosphorylation in myeloma and chronic lymphocytic leukemia cells,[27,35] which is consistent with our results in anti-IgM-stimulated Raji cells. Interestingly, despite the overexpression of PI3Kδ in AML, CAL-101 does not significantly inhibit ERK signaling. In contrast, puquitinib significantly inhibits ERK activation in AML, suggesting that these two drugs may bear different pharmacological profiles besides the PI3Kδ in AML, which may result in puquitinib's superior antitumor activity. PI3K signaling disrupts insulin signaling, and hyperglycemia has been considered a toxic side effect of PI3K inhibition;[36,37] however, a previous study revealed no puquitinib-related hyperglycemia.[38] Thus, the specific targeted inhibition of PI3Kδ by puquitinib preserved PI3K signaling in normal and non-neoplastic cells, which may result in minimal toxicity.

Taken together, the results of our study demonstrate that puquitinib, a novel orally available PI3Kδ inhibitor, has significant antitumor activity against AML both *in vitro* and *in vivo*, with evidence of target modulation, and this activity is enhanced when combined with cytotoxic agents. Given that puquitinib exhibits reduced nephrotoxicity and superior antitumor activity in AML, puquitinib, as well as puquitinib-based regimens, likely warrant further clinical investigation for the treatment of AML.

Acknowledgments

This research was supported by grants from the National Natural Science Foundation of China (No. 30801404 and No. 81273546) and the National Key Research & Development Plan (2016YF1201003 to M.Z.).

Disclosure Statement

The authors have no conflict of interest to declare.

References

1 Fruman DA, Meyers RE, Cantley LC. Phosphoinositide kinases. *Ann Rev Biochem* 1998; **67**: 481–507.
2 Katso R, Okkenhaug K, Ahmadi K, White S, Timms J, Waterfield MD. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Ann Rev Cell Dev Biol* 2001; **17**: 615–75.
3 Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B. The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol* 2010; **11**: 329–41.
4 Anzai K, Sekine-Suzuki E, Ueno M *et al.* Effectiveness of combined treatment using X-rays and a phosphoinositide 3-kinase inhibitor, ZSTK474, on proliferation of HeLa cells *in vitro* and *in vivo*. *Cancer Sci* 2011; **102**: 1176–80.
5 Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov* 2009; **8**: 627–44.
6 Tamura N, Hazeki K, Okazaki N *et al.* Specific role of phosphoinositide 3-kinase p110alpha in the regulation of phagocytosis and pinocytosis in macrophages. *Biochem J* 2009; **423**: 99–108.
7 Burger JA, Quiroga MP, Hartmann E *et al.* High-level expression of the T-cell chemokines CCL3 and CCL4 by chronic lymphocytic leukemia B cells in nurselike cell cocultures and after BCR stimulation. *Blood* 2009; **113**: 3050–8.
8 Herman SE, Gordon AL, Wagner AJ *et al.* Phosphatidylinositol 3-kinase-delta inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. *Blood* 2010; **116**: 2078–88.
9 So L, Fruman DA. PI3K signalling in B- and T-lymphocytes: new developments and therapeutic advances. *Biochem J* 2012; **442**: 465–81.
10 Garcia-Martinez JM, Wullschleger S, Preston G *et al.* Effect of PI3K- and mTOR-specific inhibitors on spontaneous B-cell follicular lymphomas in PTEN/LKB1-deficient mice. *Br J Cancer* 2011; **104**: 1116–25.
11 Fruman DA, Rommel C. PI3Kdelta inhibitors in cancer: rationale and serendipity merge in the clinic. *Cancer Discov* 2011; **1**: 562–72.
12 Cushing TD, Hao X, Shin Y *et al.* Discovery and *in vivo* evaluation of (S)-N-(1-(7-fluoro-2-(pyridin-2-yl)quinolin-3-yl) ethyl)-9H-purin-6-amine (AMG319) and related PI3Kdelta inhibitors for inflammation and autoimmune disease. *J Med Chem* 2015; **58**: 480–511.
13 Down K, Amour A, Baldwin IR *et al.* Optimization of novel indazoles as highly potent and selective inhibitors of phosphoinositide 3-kinase delta for the treatment of respiratory disease. *J Med Chem* 2015; **58**: 7381–99.
14 Yang Q, Modi P, Newcomb T, Queva C, Gandhi V. Idelalisib: first-in-class PI3K delta inhibitor for the treatment of chronic lymphocytic leukemia, small lymphocytic leukemia, and follicular lymphoma. *Clinical Cancer Res* 2015; **21**: 1537–42.
15 Smith M, Barnett M, Bassan R, Gatta G, Tondini C, Kern W. Adult acute myeloid leukaemia. *Critical Rev Oncol Hematol* 2004; **50**: 197–222.
16 Sujobert P, Bardet V, Cornillet-Lefebvre P *et al.* Essential role for the p110delta isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia. *Blood* 2005; **106**: 1063–6.
17 Zhao Z, Zhu J, Quan H *et al.* X66, a novel N-terminal heat shock protein 90 inhibitor, exerts antitumor effects without induction of heat shock response. *Oncotarget* 2016; **7**: 29648–63.
18 Berndt A, Miller S, Williams O *et al.* The p110 delta structure: mechanisms for selectivity and potency of new PI(3)K inhibitors. *Nat Chem Biol* 2010; **6**: 117–24.
19 Friesner RA, Banks JL, Murphy RB *et al.* Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J Med Chem* 2004; **47**: 1739–49.
20 Laskowski RA, Swindells MB. LigPlot+: multiple ligand–protein interaction diagrams for drug discovery. *J Chem Inf Model* 2011; **51**: 2778–86.
21 Wang X, Zhang X, Li BS *et al.* Simultaneous targeting of PI3Kdelta and a PI3Kdelta-dependent MEK1/2-Erk1/2 pathway for therapy in pediatric B-cell acute lymphoblastic leukemia. *Oncotarget* 2014; **5**: 10732–44.
22 Liu X, Wang A, Liang X *et al.* Characterization of selective and potent PI3Kdelta inhibitor (PI3KDIN- 015) for B-Cell malignances. *Oncotarget* 2016; **7**: 32641–51.
23 Puri KD, Gold MR. Selective inhibitors of phosphoinositide 3-kinase delta: modulators of B-cell function with potential for treating autoimmune inflammatory diseases and B-cell malignancies. *Front Immunol* 2012; **3**: 256.
24 Bernal A, Pastore RD, Asgary Z *et al.* Survival of leukemic B cells promoted by engagement of the antigen receptor. *Blood* 2001; **98**: 3050–7.

25 Jia S, Liu Z, Zhang S *et al.* Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis. *Nature* 2008; **454**: 776–9.
26 Guillermet-Guibert J, Bjorklof K, Salpekar A *et al.* The p110beta isoform of phosphoinositide 3-kinase signals downstream of G protein-coupled receptors and is functionally redundant with p110gamma. *Proc Nat Acad Sci USA* 2008; **105**: 8292–7.
27 Ikeda H, Hideshima T, Fulciniti M *et al.* PI3K/p110{delta} is a novel therapeutic target in multiple myeloma. *Blood* 2010; **116**: 1460–8.
28 Wang X, Ding J, Meng LH. PI3K isoform-selective inhibitors: next-generation targeted cancer therapies. *Acta Pharmacol Sin* 2015; **36**: 1170–6.
29 Somoza JR, Koditek D, Villasenor AG *et al.* Structural, biochemical, and biophysical characterization of idelalisib binding to phosphoinositide 3-kinase delta. *J Biol Chem* 2015; **290**: 8439–46.
30 Dobashi N, Asai O, Yano S *et al.* Aclarubicin plus behenoyl cytarabine and prednisolone for previously treated acute myeloid leukemia patients. *Leuk Lymphoma* 2006; **47**: 2203–7.
31 Kadia TM, Ravandi F, Cortes J, Kantarjian H. New drugs in acute myeloid leukemia. *Ann Oncol* 2016; **27**: 770–8.
32 Min YH, Eom JI, Cheong JW *et al.* Constitutive phosphorylation of Akt/PKB protein in acute myeloid leukemia: its significance as a prognostic variable. *Leukemia* 2003; **17**: 995–7.
33 Tamburini J, Elie C, Bardet V *et al.* Constitutive phosphoinositide 3-kinase/Akt activation represents a favorable prognostic factor in *de novo* acute myelogenous leukemia patients. *Blood* 2007; **110**: 1025–8.
34 Park S, Chapuis N, Tamburini J *et al.* Role of the PI3K/AKT and mTOR signaling pathways in acute myeloid leukemia. *Haematologica* 2010; **95**: 819–28.
35 Hoellenriegel J, Meadows SA, Sivina M *et al.* The phosphoinositide 3′-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. *Blood* 2011; **118**: 3603–12.
36 Courtney KD, Corcoran RB, Engelman JA. The PI3K pathway as drug target in human cancer. *J Clin Oncol* 2010; **28**: 1075–83.
37 Foukas LC, Claret M, Pearce W *et al.* Critical role for the p110alpha phosphoinositide-3-OH kinase in growth and metabolic regulation. *Nature* 2006; **441**: 366–70.
38 Yang H, Wang Y, Zhan J *et al.* Puquitinib mesylate, an inhibitor of phosphatidylinositol 3-kinase p110delta, for treating relapsed or refractory non-Hodgkin's lymphoma. *Oncotarget* 2015; **6**: 44049–56.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Inhibitory activity of puquitinib against protein kinases.

Fig. S1. Puquitinib induced cell apoptosis in MV4;11 cells.