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

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

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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 G₁-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.
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-nm stock solutions in dimethylsulfoxide for in vitro studies or normal saline in vivo studies. Lyso-phosphatidic acid (LPA), C5a, anti-IgM, LY294002 and wortmannin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The appropriate primary antibodies to p110δ, p110β, p110γ and p110δ, AKT, p-5250, S6, p-P70S6K, P70S6K, p-AKT, p-aktT308 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).

In vivo study. Female nude mice (Balb/c-A 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 × 10⁶ cells. When tumors reached a volume of 100–300 mm³, 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 cytarbaine was administered intravenously (i.v.), and co-treatment groups also received oral puquitinib daily. Tumor volume was calculated as (length × width²)/2.

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 electrophobted onto polyvinylidene 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).

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 IC₅₀ values of 992.8, 959.2, 89.8 and 3.3 nM for p110α, p110β, p110γ and p110δ, 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 IC₅₀ values of 992.8, 959.2, 89.8 and 3.3 nM for p110α, p110β, p110γ and p110δ, 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
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. Despite the decrease in LPA- or C5a ligand-stimulated phosphorylations of AKT in PC-3 or RAW 264.7 cells by puitinib was observed, the decrease was much weaker than that of Raji cells. No obvious inhibitory activity of p110α/AKT phosphorylation by puitinib was observed in SK-BR-3 cells (Fig. 1b). This pharmacological profile of puitinib is similar to that of CAL-101 in these cell-based assays. Thus, these data indicate that puitinib is a potent, selective inhibitor of PI3Kδ.

To understand the mechanisms underlying the isoform selectivity of this inhibitor, we further investigated the site of puitinib binding to PI3Kδ using structural modeling. The crystal structure of a PI3Kδ–PIK-39 complex generated a PI3K template structure for docking analysis. 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 and 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

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**Table 1. Kinase inhibition profile of puquitinib**

| Drugs  | Biochemical IC₅₀ (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 IC₅₀.

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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. Notably, puquitinib inhibited the proliferation of these p110δ-positive AML cell lines with the mean IC₅₀ 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 G1-phase cell-cycle arrest in p110δ-positive MV4;11 cells. The proportion of G1-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).

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
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...
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 \textit{in vivo} anti-tumor 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 iludarabine, 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 \textit{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).
Puquitinib establishes 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. 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-isom 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. 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.

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

| Drugs           | MV4;11 CI (mean ± SEM) | RS4;11 CI (mean ± SEM) |
|-----------------|------------------------|------------------------|
| 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. Recently, several new PI3Kδ isoform-selective inhibitors showing improved selectivity and potency have been reported. 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. 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 provided two hydrogen bonds in the ATP-binding pocket 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. 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-isom selectivity of puquitinib translated well to cell-based assays, in which puquitinib inhibited p110δ-dependent responses at low nanomolar concentrations.

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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.
with B cell malignancies or AML. (32) AKT, are frequently activated in leukemic blasts from patients with newly diagnosed AML are offered the combination of puquitinib 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 PI3K and its downstream target, AKT, are frequently activated in leukemic blasts from patients with B cell malignancies or AML. (33–34) 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 ERK 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, (23,35) 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.
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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.