mTORC1 inhibition activates PI3K/Akt by up-regulating IGF-1R signaling in acute myeloid leukemia: rational for therapeutic inhibition of both pathways

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Running title: Akt upregulation following mTORC1 inhibition in AML
Abstract

The PI3K/Akt and mTORC1 pathways are frequently activated, representing potential therapeutic targets in acute myeloid leukaemia (AML). In 19 AML samples with constitutive PI3K/Akt activation, the rapamycin derivative inhibitor everolimus (RAD001) increased Akt phosphorylation. This mTORC1-mediated Akt up-regulation was explained by an IGF-1/IGF-1 receptor autocrine loop: a/ blast cells expressed functional IGF-1 receptors, and IGF-1-induced Akt activation was increased by RAD001, b/ a neutralizing anti-IGF-1R alpha-IR3 monoclonal antibody reversed the RAD001-induced Akt phosphorylation, c/ autocrine production of IGF-1 was detected in purified blast cells by quantitative RT-PCR and immunofluorescence. This RAD001-induced PI3K/Akt upregulation was due to an up-regulated expression of the IRS2 adaptor. Finally, we observed that concomitant inhibition of mTORC1 and PI3K/Akt by RAD001 and IC87114 induced additive anti-proliferative effects. Our results suggest that dual inhibition of the mTORC1 complex and the IGF-1/IGF-1R/PI3K/Akt pathway in AML may enhance the efficacy of mTOR inhibitors in treatment of this disease.
Introduction

Acute myeloid leukemia (AML) is associated with a low survival rate. Therefore, new therapeutic strategies may prove effective in addition to chemotherapy. The deregulation of several signal transduction pathways is a common feature in AML. The PI3K/Akt pathway is activated in AML blast cells. We previously showed that the p110delta isoform of PI3K is a potential therapeutic target, and that the p110 delta-selective inhibitor IC87114 blocks AML cell proliferation. The mammalian target of rapamycin (mTOR) is activated in response to stimuli activating the PI3K pathway, and mTORC1 inhibitors may have therapeutic value in the treatment of AML patients; however, rapamycin alone led to a modest antileukemic activity. We investigated the effect of the mTORC1 inhibitor RAD001 (Everolimus, Novartis Pharmaceuticals) in 19 bone marrow samples from newly diagnosed patients with AML. We show that mTORC1 inhibition with RAD001 increased Akt activating phosphorylation, as a result of up-regulated expression of the IRS-2 protein adaptor that promoted IGF-1/IGF-1R signaling. Moreover, we show that the enhanced activation of Akt was dependent on the IGF-1 autocrine production by leukemic cells. Our results provide a rationale for combined inhibition of both mTORC1 and PI3K/Akt pathways in AML, and we observed an additive effect of both RAD001 and IC87114 on blast cell proliferation.

Materials and Methods

Bone marrow (BM) samples were obtained from 19 newly diagnosed AML patients, all treated in the AML2001 chemotherapy trial, initiated by the French Multicenter Group, Groupe Ouest Est des Leucémies et Autres Maladies du Sang (GOELAMS). All biological studies were approved by the GOELAMS Institutional Review Board and signed informed consent was provided according to the Declaration of Helsinki. The clinical characteristics of patients are summarized in Table S1 (see the Supplemental Materials). All patients presented a constitutive activation of Akt at diagnosis, a previously reported. Cells were incubated with the following inhibitors: RAD001 (Everolimus) kindly provided by Novartis (Basel, Switzerland), IC87114 purchased from ICOS Corporation (Bothell, WA), LY294002 from Sigma (St Louis, MO), alphaIR3 from Calbiochem (La Jolla, CA). IGF-1 was from Sigma. Expression of total and phosphorylated proteins was detected by Western Blot (WB) analysis.
as previously reported 7. The references for the antibodies are summarized in Table S2 (see Supplementary Data). Immunofluorescence staining for IGF-1 expression and quantitative RT-PCR were performed on blast cells, sorted according to their CD45low expression and side scatter (see Supplemental Materials for details). Blast cell proliferation was assessed by [3H]-thymidine incorporation as reported previously 7.

**Results and discussion**

This study was conducted in an attempt to inhibit Akt and mTORC1 phosphorylation in AML samples. We compared the effect of three kinase inhibitors: 1) IC87114 7, 2) LY294002, and 3) the rapamycin derivative RAD001, in fresh BM blast cells from patients with AML presenting constitutive PI3K activation 4,7. We observed that IC87114 and LY294002 totally suppressed Akt phosphorylation whereas, in contrast, RAD001 substantially increased Akt activation. The enhancement of Akt phosphorylation in the presence of RAD001 (mean 86%) was detected in all 19 AML samples (Figure 1A). Furthermore, increased Akt activation was maintained after a 24 hr incubation with 10 nM RAD001 (Figure 1B, sample G192), and also with RAD001 at higher concentrations (Figure 1B, sample G194). Thus, RAD001 treatment led to an increased Akt activation in all AML samples presenting constitutive PI3K activation. Since mTORC1 has been reported to inhibit Insulin/IGF-1 signaling 11, we hypothesized that the IGF-1/IGF-1R pathway could play a role in the RAD001-induced Akt activation in AML cells. We found that exogenous IGF-1 stimulation increased Akt phosphorylation and that RAD001 increased IGF-1-stimulated Akt phosphorylation in AML samples (Figure 1C). Because BM cells were cultured in serum-free medium and harbored a functional IGF-1R, we analyzed the production of IGF-1 inside the leukemic cells. To that end, we purified the blast cell population by flow cytometry cell sorting as described previously 4. IGF-1 expression was detected at the mRNA level and the IGF-1 protein was detected by immunofluorescence analysis in all samples tested (Figure 1D). From these data, we concluded that an autocrine production of IGF-1 was constantly observed in primary AML blast cells. We inhibited the interaction between IGF-1 and its receptor with a neutralizing monoclonal antibody alpha-IR3, directed against the alpha-subunit of IGF-1 receptor 12. As shown in Figure 1E, blocking the IGF-1/IGF-1R interaction reversed the RAD001-induced increase of Akt phosphorylation. These data strongly suggest that the enhancement of Akt phosphorylation in response to RAD001 treatment involved the IGF-1R in leukemic cells.
mTORC1 activity down-regulates insulin/IGF-1 signaling through proteasome-mediated decrease of IRS adaptor proteins. To confirm the involvement of the IGF-1R in the mechanism of Akt activation, a variation of IRS-2 expression following treatment with RAD001 was evaluated in AML samples. RAD001 treatment led to a significant increase of IRS2 protein expression, which in each case paralleled the level of RAD001-induced Akt phosphorylation (Figure 1F).

Previously, we showed that IC87114, a PI3K p110δ-selective inhibitor, suppressed AML cell proliferation. On the other hand, we observed that the blockage of PI3K by IC87114 did not inhibit mTORC1, as assessed by the persistence of P70S6K phosphorylation (see Figures 1A and 2A). Thus, these pathways seem to be independent in AML, thereby allowing us to assess the rationale of a treatment combining two inhibitors. We tested the effect of the association of RAD001 and IC87114 on blast cell proliferation. In our series of 19 patients, RAD001 and IC87114, when used alone, inhibited blast cell proliferation to the same level. However, the concomitant inhibition of both pathways with RAD001 and IC87114 resulted in a significant additive anti-proliferative effect, when compared to the effect of each compound alone (p<0.001) (Figure 2A). The additive effect of PI3K and mTOR inhibitors was confirmed in dose response curves for the two compounds, in 4 AML samples (Figure 2B).

In summary, our study shows that mTORC1 inhibition enhanced Akt activation in AML cells, and may contribute to limit the efficacy of Rapamycins when used in monotherapy, as described in other systems. Recently, it was reported that a 24 hr exposure to rapamycin may impair mTORC2 assembly and, subsequently downregulate Akt phosphorylation. Similar observations were made by this group in primary AML samples treated with CCI-779 (tensirolimus, Wyeth Pharmaceuticals). In our hands, Akt activation was unchanged after 24h hr incubation with RAD001, even at higher RAD001 concentrations (see Figure 1B). The reasons for this discrepancy are unknown. It is possible that the culture conditions for primary blast cells were different; alternatively, CCI-779 could be more active on a prolonged incubation period than RAD001 or could have additional side inhibitory effects.

In conclusion, our results show that autocrine IGF-1 signaling is present in primary AML blast cells and is, at least, partly responsible for the effect of RAD001 on mTORC1-mediated PI3K/Akt up-regulation. Our results provide a molecular basis for understanding the increased Akt activation induced by mTORC1 inhibitors, and suggest that a combined therapeutic strategy targeting both PI3K and mTORC1 pathways might be useful in AML patients.
Acknowledgments

We acknowledge the Novartis Institutes for Medical Research Basel, Oncology for the supply of RAD001. We thank all participating investigators from the GOELAMS. We thank Dr David Fruman (University of California-Irvine) for helpful criticism of the manuscript. This work was supported by grants from the Ligue Nationale contre le Cancer (LNCC, laboratoire associé), the Association pour le Recherche contre le Cancer (ARC), the Institut National du Cancer (INCA), and the Association Laurette Fugain.

Author Contribution Statement: JT performed research, analyzed data and wrote the paper, NC, PS, VB, SP and LW performed research and analyzed data; NI and FD contributed AML patient samples and analyzed clinical data; CL and PM analyzed data and wrote the paper; DB designed research, analyzed data and wrote the paper.

Conflict of Interest Disclosure: The authors declare no other competing financial interests.
Figure Legends

Figure 1. mTORC1 inhibition with RAD001 induces Akt activation in primary AML samples by activation of the IGF-1/IGF1-R signaling pathway, dependent on an IGF-1 autocrine loop.

1A- Bone marrow (BM) blast cells from 19 AML patients were starved 4 hr in cytokine and serum-free MEM, with or without the following kinase inhibitors: 25µM LY294002, 10µM IC87114 or 10nM RAD001, added during the last hour of starvation. Western Blot (WB) analysis were performed with anti-phospho-Akt (ser 473), anti-phospho-p70S6K (thr 389) and anti-Akt antibodies. Quantification of phospho-Akt signal intensity was normalized to Akt signal intensity. Each histogram of the graph represents the phospho-Akt signal intensity in RAD001-treated blast cells, expressed as percentage of signal intensity in control cells (M, Medium without inhibitors).

1B- BM blast cells from patients G192 and G194 were collected after ficoll-hypaque density gradient separation, then washed once in PBS buffer. 5.10⁵/mL blast cells from patient G192 were starved 4 hr in cytokine and serum-free MEM, then incubated without or with 10nM RAD001 for 1 hr. In independents experiments, 5.10⁵/mL blast cells from patients G192 and G194 were incubated without or with RAD001 during 24 hr, in αMEM with 10% FCS. RAD was used at 10nM for sample G192, and at 10, 50 or 100nM for sample G194. WB analysis were performed with anti-phospho-Akt (ser 473), anti-phospho-p70S6 kinase (thr 389), anti-phospho-S6R (Ser 235/236) and anti-Akt antibodies.

1C- RAD001 increases IGF-1-stimulated Akt phosphorylation in AML blast cells. BM blast cells from patients G179, G194 and G149 were starved 4 hr in serum-free MEM, without or with 10nM RAD001, then stimulated or not with 50 ng/mL IGF-1 for 10 minutes. WB analyses were performed with anti-phospho-Akt (Ser 473) and anti-Akt antibodies.

1D- AML blast cells express IGF-1 at the RNA and protein level. BM blast cells from 8 patients were highly purified by flow cytometry cell sorting according to CD45low expression and side scatter. The MV4-11 AML cell line was used as negative control and the OPM2
myeloma cell line was used as a positive control for IGF-1 expression. IGF-1 mRNA expression was quantified in the purified blast cells by quantitative RT-PCR and their levels were expressed relative to HPRT (hypoxanthine phosphoribosyl transferase) mRNA levels. Similar results were obtained using another housekeeping gene, UBCV (C ubiquitin, data not shown). Immunofluorescence staining was performed on purified blasts for the same eight patients mentioned above, and on MV4-11 and OPM2 cell lines, using a mouse monoclonal anti-IGF-1 antibody and FITC-conjugated goat anti-mouse antibody. Nuclei were stained with DAPI. Images obtained from the representative patient G102 and from MV4-11 and OPM2 cell lines are presented.

1E- The mTORC1-mediated positive feedback on PI3K/Akt activity involves the IGF-1 receptor. BM blast cells from patients G179, G194, G199 and G205 were starved for 4 hr in serum-free MEM. Cells were incubated in the following conditions: medium alone, 10 nM RAD001, 10 nM RAD001 for 1 hr and 5µg/mL αIR3 (added 30 minutes before RAD001). WB were performed with anti-phospho-Akt (Ser 473) and anti-Akt antibodies. αIR3 is a blocking mouse monoclonal antibody directed against the alpha subunit of the IGF-1 receptor and was obtained from Calbiochem (La Jolla, CA).

1F- mTORC1 inhibition by RAD001 increases the expression of the IRS2 adaptor. BM blast cells from patients G72, G99, G189 and G191 were starved 4 hr in serum-free MEM, then incubated with or without RAD001 (10nM) for 1 hr. WB were performed with anti-IRS2 and anti-actin antibodies.

Figure 2. Concomitant inhibition of mTORC1 and p110δ PI3K activity with RAD001 and IC87114, respectively, induces additive inhibition of blast cells proliferation.

2A- 10⁵/mL Blast cells from the 19 patients were incubated 48h hr in duplicate in 5% FCS MEM under the following conditions: control (C), 10nM RAD001 (R), 10μM IC87114 (IC), 10nM RAD001 and 10μM IC87114 (R+IC), and pulsed for 6 hr with [3H] thymidine (1µCi, [37 kBq]). A Student’s t test was performed to compare proliferation rates between the different conditions. Significance was: C/R P<0.001, C/IC P<0.001, R/R+IC P<0.001, IC/R+IC P<0.001. The WB analysis shows the effect of inhibitors on Akt (Ser 473) and P70S6K (Thr 389) phosphorylation.
2B- $10^5$/mL BM blast cells from 4 AML samples were incubated 48 hr in triplicate in 5% FCS MEM, with or without inhibitors as described below. They were then pulsed 6 hr with $[^3]H$-thymidine 1µCi, [37 kBq]. The amount of radioactivity incorporated was determined by trichloracetic acid precipitation. In the upper panel, increasing amounts (from 1.25nM to 20nM) of RAD001 were used, without (upper line) or with a constant concentration of IC87114 (10µM). In the lower panel, increasing (from 1.25µM to 20µM) amounts of IC87114 were used, without (upper line) or with a constant concentration of RAD001 (10nM). Error bars indicate standard deviations.
References

Figure 1.
Figure 1.
Figure 2.

A.

![Graph showing Thymidine incorporation (% of Control) for different treatments: C, RAD001, IC87114, and RAD + IC.]

B.

![Graph showing % of Control for different concentrations of RAD001 and IC87114.]

Figure 2.
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