**Featured Article**

**Superior Activity of the Combination of Histone Deacetylase Inhibitor LAQ824 and the FLT-3 Kinase Inhibitor PKC412 against Human Acute Myelogenous Leukemia Cells with Mutant FLT-3**

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**ABSTRACT**

**Purpose:** Mutant FLT-3 receptor tyrosine kinase is a client protein of the molecular chaperone heat shock protein 90 and is commonly present and contributes to the leukemia phenotype in acute myelogenous leukemia (AML). LAQ824, a cinnamyl hydroxamate histone deacetylase inhibitor, is known to induce acetylation and inhibition of heat shock protein 90. Here, we determined the effects of LAQ824 and/or PKC412 (a FLT-3 kinase inhibitor) on the levels of mutant FLT-3 and its downstream signaling, as well as growth arrest and cell-death of cultured and primary human AML cells.

**Experimental Design:** The effect of LAQ824 and/or PKC412 treatment was determined on the levels of FLT-3 and phosphorylated (p)-FLT-3, on downstream pro-growth and pro-survival effectors, e.g., p-STAT5, p-AKT, and p-extracellular signal-regulated kinase (ERK) 1/2, and on the cell cycle status and apoptosis in the cultured MV4–11 and primary AML cells with mutant FLT-3.

**Results:** Treatment with LAQ824 promoted proteasomal degradation and attenuation of the levels of FLT-3 and p-FLT-3, associated with cell cycle G0/G1 phase accumulation and apoptosis of MV4–11 cells. This was accompanied by attenuation of p-STAT5, p-AKT, and p-ERK1/2 levels. STAT-5 DNA-binding activity and the levels of c-Myc and oncostatin M were also down-regulated. Cotreatment with LAQ824 and PKC412 synergistically induced apoptosis of MV4–11 cells and induced more apoptosis of the primary AML cells expressing mutant FLT-3. This was also associated with more attenuation of p-FLT-3, p-AKT, p-ERK1/2, and p-STAT5.

**Conclusions:** The combination of LAQ824 and PKC412 is highly active against human AML cells with mutant FLT-3, which merits in vivo studies of the combination against human AML.

**INTRODUCTION**

Mutations in the receptor tyrosine kinase FLT-3 have been reported to occur in approximately one-third of patients with acute myelogenous leukemia (AML; Refs. 1 and 2). These include the activating length mutation, e.g., the internal tandem duplication of the juxtamembrane domain, and the point mutation at the aspartate 835 within the tyrosine kinase domain, occurring in approximately 25 and 7% of AML, respectively. Both mutations lead to the autophosphorylation and activation of FLT-3 tyrosine kinase (1, 2). When transduced into the primary murine bone marrow progenitor 32D and BaF3 cells, FLT-3 mutations induce a myeloproliferative disorder and result in leukemia transformation (3, 4). The presence of FLT-3 length mutation or tyrosine kinase domain mutations also confers poor prognosis in AML (1, 2, 5, 6). Several relatively specific inhibitors of FLT-3 kinase have been developed and are being tested in AML (2, 7–9).

The staurosporine derivative PKC412 (4′-N-benzylostaurosporine) was earlier identified as an inhibitor of protein kinase C, but later, it was also shown to inhibit other kinases including the kinase insert domain receptor (vascular endothelial growth factor receptor 2), the receptor for platelet-derived growth factor, the receptor for the stem cell factor, c-kit, and FLT-3 (7, 10–12). Exposure to PKC412 has also been reported to induce accumulation in the G2-M phase of the cell cycle associated with increased polyploidy and apoptosis of tumor cells (13). PKC412 exerts a potent in vivo antitumor activity as single agent and inhibits vascular endothelial growth factor-dependent angiogenesis due to inhibition of kinase insert domain receptor and protein kinase C (10). PKC412 directly inhibits FLT3 tyrosine kinase, which selectively induced G1 arrest and apoptosis of mouse myeloid Ba/F3 cell lines expressing mutant FLT3 (IC50 < 10 nM; Ref. 7). Also, progressive leukemia was prevented in PKC412-treated BALB/c mice transplanted with marrow transduced with FLT3-internal tandem duplication-expressing retrovirus. Recently, PKC412 has been evaluated in the treatment of...
patients with AML (14). However, unlike imatinib in CML, treatment with PKC412 or the other FLT-3 kinase inhibitors alone has thus far yielded limited clinical benefit (2, 9, 15).

A variety of histone deacetylase inhibitors have been shown to induce p21, promoting growth arrest and apoptosis of AML cells (16, 17). LAQ824 is a potent histone deacetylase inhibitor belonging to the class of hydroxamic acid analogs, which are known to inhibit class I, IIA, and IIB histone deacetylases (18, 19). Treatment with approximately 100 nM LAQ824 for 24–48 h induces histone acetylation, p21 levels, cell cycle G1-phase accumulation, and apoptosis of human acute leukemia cells (20, 21). Importantly, LAQ824 induces more apoptosis of leukemia versus normal bone marrow progenitor cells (20). LAQ824 has also been shown to exert in vivo antitumor effects in xenograft animal models (19). Recently, LAQ824 has been shown to also induce acetylation of heat shock protein 90 (hsp90; Ref. 20). This inhibited the ATP binding and chaperone association of hsp90 with its client proteins (e.g., Bcr-Abl and mutant FLT-3), resulting in polyubiquitylation and proteasomal degradation of the client proteins (20, 22, 23). This novel histone deacetylase inhibitor is currently in clinical trials as an anticancer agent. We have previously reported that treatment with the histone deacetylase inhibitor, LAQ824 or SAHA, attenuates Bcr-Abl levels and significantly enhances imatinib-induced apoptosis of CML cells (20, 24). Would treatment with LAQ824 also attenuate the level of mutant FLT-3 and sensitize AML cells containing the FLT-3 length mutation or tyrosine kinase domain mutation to PKC412? Present studies were carried out to address this issue.

MATERIALS AND METHODS

Reagents. LAQ824 and PKC412 were provided by Novartis Pharmaceuticals, Inc. (East Hanover, NJ). Antibodies for the immunoblot analyses were purchased as follows: FLT-3, STAT5, and c-Myc from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); p-FLT-3 and p-ERK1/2 from Cell Signaling Technology (Beverly, MA); p-STAT5 from Upstate Biotechnology, Inc. (Lake Placid, NY); and Oncostatin M from R&D Systems, Inc. (Minneapolis, MN). The source of the other antibodies used in these studies has been described previously (20, 21, 24–26).

Cells. Acute leukemia MV4–11 (containing a 30-bp-long internal tandem duplication in the exon 14 of FLT-3) and RS4–11 (containing wild-type FLT-3) cells were obtained from American Tissue Culture Collection (Manassas, VA) and maintained in culture as described previously (20–22). Primary leukemia MV4–11 and RS4–11 cells were treated with the indicated concentrations of LAQ824 for 48 h. After this, the percentage of annexin V-stained apoptotic cells was determined by flow cytometry. *, values (represented by the open bars) to be significantly different from those represented by closed bars. B, Western blot analyses of p-FLT-3, FLT-3, p21, p-AKT, AKT, p-ERK1/2, ERK1/2, p-STAT5, STAT5, c-Myc, oncostatin M, and poly(ADP-ribose) polymerase (PARP) were performed in the cell lysates from MV4–11 and RS4–11 cells after treatment with the indicated concentrations of LAQ824 for 24 h. The levels of β-actin served as the loading control. C, LAQ824 attenuates FLT-3 and tyrosine-phosphorylated FLT-3. After exposure of MV4–11 cells to the indicated concentrations of LAQ824 or PKC412 for 24 h, FLT-3 was immunoprecipitated and immunoblotted with the antiphosphotyrosine (anti-p-Tyr) or anti-FLT-3 antibody.
kemia blasts from six patients with AML in relapse were harvested and purified as described previously (21), according to a protocol sanctioned by the local institutional review board.

**Flow Cytometry for Cell Cycle Status and Apoptosis Assessment.** Flowcytometric evaluation of the cell cycle status and sub-G1, apoptotic population of cells was performed as described previously (20, 27).

**Assessment of Percentage of Nonviable and Apoptotic Cells.** Primary AML cells were stained with trypan blue (Sigma, St. Louis, MO). Numbers of nonviable cells were determined by counting the cells that showed trypan blue uptake in a hemocytometer and reported as the percentage of untreated control cells. The percentage of apoptotic cells was determined by flow cytometry as described previously (20, 21). Analysis of synergism between LAQ824 and PKC412 in inducing apoptosis of MV4–11 cells was performed by median dose-effect analysis using commercially available software (Calcusyn; Biosoft, Ferguson, MO).

**Western Blot Analysis.** Western analyses of proteins from untreated and drug-treated cells were performed as described previously (27, 28).

**Autophosphorylation of FLT-3.** The immunoprecipitates of FLT-3 were subjected to SDS-PAGE and immunoblotted with antiphosphotyrosine antibody (PharMingen, San Diego, CA) as described previously (20).

**Reverse Transcription-PCR Assay for FLT-3 mRNA Levels.** Reverse transcription-PCR analysis was performed as described previously (5). To detect FLT-3 internal tandem dupliciation, the primer sequences were as follows: forward primer, 5′-TGT CGA GCA GTA CTC TAA ACA-3′; and reverse primer, 5′-ATC CTA GTA CCT TCC CAA ACT C-3′. For β-actin, the primer sequences were: forward primer, 5′-CTA CAA TGA GCT GCG TGT GG-3′; and reverse primer, 5′-AAG GAA GCC TGG AGT AGT GC-3′. The size of the amplified products was 395 bp for the FLT-3 and 527 bp for the β-actin product, respectively.

**Electrophoretic Mobility Shift Assay for STAT5a.** Untreated or LAQ824- and/or PKC412-treated cells were lysed, nuclear extracts were obtained, and the electrophoretic mobility shift assay for the DNA-binding activity of STAT5a was performed as described previously (25).

### RESULTS AND DISCUSSION

**Effects of LAQ824 on Cell-Cycle Status and Apoptosis in MV4–11 and RS4–11 Cells.** First, we compared the cell cycle and apoptotic effects of LAQ824 on MV4–11 versus RS4–11 cells. Exposure to 10–50 nM LAQ824 for 48 h induced a dose-dependent increase in apoptosis, along with increased poly(ADP-ribose) polymerase cleavage, in MV4–11 and RS4–11 cells (Fig. 1A and B). Exposure to 10–50 nM LAQ824 significantly induced more apoptosis of MV4–11 than RS4–11 cells (P < 0.05). Treatment with LAQ824 (20 nM for 24 h) also significantly increased the percentage of cells in the G1 phase of the cell cycle in MV4–11 (from 55.9 ± 1.3 to 71.3 ± 2.9%) and RS4–11 cells (40.1 ± 2.2 to 51.1 ± 2.9%; P < 0.05). This was also accompanied by a significant increase in the sub-diploid apoptotic population of cells in both cell types (P < 0.01; data not shown). In these studies, the doses of LAQ824 used are clinically achievable, because a preliminary pharmacokinetic evaluation of LAQ824 in a Phase I trial has revealed the peak plasma concentrations of LAQ824 to be in the micromolar range.

**Effect of LAQ824 on FLT-3 Levels in MV4–11 and RS4–11 Cells.** Treatment with LAQ824 induced p21, a likely reason for cell cycle G1-phase accumulation of the cells (Fig. 1A; Ref. 20). In contrast, treatment with LAQ824 markedly attenuated the expressions of FLT-3 and p-FLT-3 in MV4–11 and FLT-3 levels in RS4–11 cells (Fig. 1B). LAQ824-mediated decline in the p-FLT-3 levels may be due to inhibition of the autophosphorylation of FLT-3 (1, 2). Although data in Fig. 1C suggest that LAQ824 may attenuate the autotyrosine phosphorylation of FLT-3, this result can also be due to the induction of a phosphatase activity. Treatment of MV4–11 cells with 50 nM LAQ824 for up to 8 h did not inhibit the mRNA transcript levels of FLT-3 (Fig. 2A). Longer exposure interval, up to 24 h, also did not reduce the transcript level of FLT-3 (data not shown), ruling out the possibility that transcriptional down-regulation is a significant contributory mechanism toward LAQ824-mediated repression of FLT-3 in MV4–11 cells. Recently, FLT-3 was shown to have a chaperone association with hsp90 (22). Moreover, the inhibitors of hsp90 disrupted this association, directing FLT-3 to polyubiquitylation and proteasomal degradation (22, 23). Also, our recent findings have

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Footnote: 3 P. Atadja, personal communication.
demonstrated that treatment with LAQ824 in addition to causing acetylation of histones H3 and H4 also results in the acetylation of hsp90 (20, 29). This inhibits the chaperone function of hsp90 and promotes the proteasomal degradation of its client proteins, e.g., Bcr-Abl, especially if it has a mutant conformation (20, 22, 24, 25, 30). To determine whether LAQ824 would also induce proteasomal degradation of mutant FLT-3, we evaluated the effect of LAQ824 and/or the proteasome inhibitor PS-341 in MV4–11 cells. Treatment with LAQ824 alone attenuated FLT-3 levels, whereas PS-341 treatment alone led to accumulation of the FLT-3 levels in the NP40 (a weak detergent)-soluble fraction of MV4–11 cells (Fig. 2B). Consistent with this, cotreatment with PS-341 restored LAQ824-mediated attenuation of FLT-3 to levels observed in the untreated MV4–11 cells. These findings support the conclusion that LAQ824 treatment promotes proteasomal degradation of mutant FLT-3 in the MV4–11 cells.

Effect of LAQ824 on Signaling Molecules Downstream of FLT-3. Previous studies have shown that the autophosphorylation and binding of the cytosolic domain of FLT-3 to the p85 subunit of phosphoinositidylinositol 3'-kinase, GRB2, SRC, and Src homology and collagen results in the activation of Ras-Raf-ERK1/2, phosphoinositidylinositol 3'-kinase-AKT, p85 subunit of phosphoinositidylinositol 3'-kinase, GRB2, SRC, and Src homology and collagen results in the activation of Ras-Raf-ERK1/2, phosphoinositidylinositol 3'-kinase-AKT, and STAT5a signaling pathways, which promote cell growth and survival (1, 2, 31). The importance of STAT5a in this signaling is highlighted by the observations that FLT-3 ligand is able to induce proliferation of STAT5a++/ or STAT5b++/ but not of STAT5a−/− hematopoietic progenitor cells (1, 2). STAT-5 can up-regulate several genes that confer proliferative and/or survival advantage. These notably include c-Myc, oncostatin M, and Pim-2 (32, 33). This pro-growth and pro-survival function of STAT5a may also be bolstered by the downstream phosphorylation and activity of ERK1/2 and AKT (1, 2), which are also known to promote the survival of leukemia cells. In the present studies, LAQ824 mediated down-regulation of FLT-3 was associated with attenuation of the levels of p-STAT5 and p-AKT in MV4–11 and RS4–11 cells (Fig. 1B). However, p-ERK1/2 levels were inhibited only in MV4–11 cells. Inhibition of p-STAT5 by LAQ824 was accompanied by attenuation of its DNA-binding activity by approximately 45%, as assessed by the densitometric analysis (see below; Fig. 4B). Consistent with this, treatment with LAQ824 down-regulated c-Myc and oncostatin M levels, which are growth promoting and transactivated by STAT5a in MV4–11 and RS4–11 cells (Fig. 1B). However, it is also possible that LAQ824-mediated down-modulation of c-Myc may be due to LAQ824-induced G1 accumulation of MV4–11 cells.

Cotreatment with LAQ824 Enhances Anti-FLT-3 Effects of PKC412 in MV4–11 Cells. We next determined whether cotreatment with LAQ824 would sensitize MV4–11 cells to growth inhibition and apoptosis induced by PKC412. Fig. 3A clearly shows that exposure to PKC412 inhibits growth in cell numbers over 72 h more than the exposure to LAQ824, and combined treatment with LAQ824 and PKC412 completely abrogated the growth of MV4–11 cells. Additionally, cotreatment with 10 or 20 nM LAQ824 enhanced PKC412-induced apoptosis (Fig. 3B). PKC412 is known to be 99% plasma protein-bound, and in a recent report on pharmacokinetics of PKC412 administered orally to patients with AML, peak levels of PKC412 were found to be approximately 25 μM (14). This
means that the free plasma levels of PKC412 (250 nM) were clearly in the range of those used in the present studies, i.e., 100 nM. Importantly, exposure to the combination of LAQ824 and PKC412 exerted synergistic apoptotic effect on MV4–11 cells, as determined by median dose-effect analysis, which revealed combination index values <1.0 (Fig. 3C). Compared with either agent alone, cotreatment with LAQ824 (10 nM) and PKC412 (100 nM) was associated with a greater decline in the expression of p-FLT-3, FLT-3, p-STAT5, p-AKT, p-ERK1/2, and c-Myc as well as increased processing of poly(ADP-ribose) polymerase (Fig. 4A). Compared with the untreated cells, treatment with 10 nM LAQ824 attenuated the DNA binding of STAT5a by approximately by 45% (relative density of 1.0 versus 0.54 by densitometry of the bands; Fig. 4B). Similarly, treatment with 50 nM PKC412 inhibited the DNA-binding activity of STAT5a by 60%. Combined treatment with LAQ824 and PKC412 almost completely inhibited the DNA-binding activity of STAT5a, compared with the treatment with LAQ824 alone (Fig. 4B). These results are representative of two separate experiments and are consistent with the greater decline in the p-FLT-3 and p-STAT5 levels due to the combination (Fig. 4A). Additionally, the correlation between marked decline in the DNA-binding activity of STAT5a and the mutant FLT-3 also suggests the possibility of an autocrine feedback loop in which the inhibition of STAT5a activity further down-regulates FLT-3 levels and activity. Thus the combined treatment with LAQ824 and PKC412 may be superior in interrupting this feedback loop than either agent alone. Availability and use of specific STAT5a inhibitors in combination with PKC412 may help in addressing this issue. A marked decline in the genes downstream of STAT5a that promote cell proliferation and survival coupled with the decline in the levels of p-AKT and p-ERK1/2 may also contribute to the superior outcome after treatment with the combination of LAQ824 and PKC412. Additionally, cotreatment with LAQ824 and PKC412 depleted the levels of the antipoptotic Mcl-1 and XIAP without affecting the levels of Bcl-2 and Bax in MV4–11 cells (Fig. 4C), Bcl-xL levels were undetectable even in untreated MV4–11 cells (data not shown). Collectively, these molecular perturbations may further bolster the antileukemia effect of the treatment with LAQ824 and PKC412. Although, LAQ824 alone induced p21 levels, combined treatment with PKC412 and LAQ824, which induces marked caspase activation and apoptosis, resulted in the concomitant processing and down-regulation of p21 (Fig. 4D; data not shown). Individually, LAQ824 and PKC412 have been shown to exert more cytotoxic effect against leukemia versus normal bone marrow progenitor cells and have not exhibited limiting in vivo bone marrow toxicity (1, 14, 20). However, the in vitro and in vivo cytotoxic effects of the combination of LAQ824 and PKC412 against normal marrow progenitor cells remain to be determined.

**Superior Antileukemia Effect of the Combination of LAQ824 and PKC412 in Primary AML Cells with FLT-3 Mutation.** We next determined whether the combination of LAQ824 and PKC412 would also have superior activity than either agent alone against primary AML cells isolated from the peripheral blood or bone marrow samples from six patients with AML in relapse. Although not shown, sample 1 and 2 cells contained a duplication of 51- and 20-bp sequences in exon 14, respectively, and sample 3 cells contained a point mutation D835Y in FLT-3. Samples 4, 5, and 6 contained the wild-type FLT-3 (data not shown). The table in Fig. 5A indicates that in samples 1, 2, and 3, cotreatment with LAQ824 and PKC412 resulted in a higher percentage of nonviable cells than treatment with either agent alone. In contrast, this was not the case in samples 4, 5, and 6. Although exposure to LAQ824 increased the percentage of nonviable cells in a dose-dependent manner, this was clearly less than in samples 1, 2, and 3. PKC412 treatment also increased the percentage of nonviable cells in samples 1 and 2 in a dose-dependent manner, whereas there was no increase to a minimal increase in the percentage of nonviable cells in samples 4, 5, and 6. Collectively, these data suggest that the combination of LAQ824 and PKC412 exerts more cytotoxicity against mutant versus wild-type FLT-3-containing primary AML cells. We next determined the effect of LAQ824 and/or PKC412 on pFLT-3 and FLT-3. Because inadequate numbers of cells were available from samples 2 and 3, we could perform Western blot analyses only on the total cell lysates of sample 1. Fig. 5B demonstrates that, compared with treatment with either
LAQ824 and PKC412 in AML Cells

A recent report has indicated that in primary AML cells, there is a significant correlation between presence of mutant FLT-3 and the constitutive activation of STAT5 (31). This highlights the FLT3-STAT5 pathway as a potential target for therapy in AML (31). Therefore, combination of agents that attenuate the activity of this pathway needs to be clinically tested against AML with mutant FLT-3. Recently, diverse mutations have been shown to have different sensitivity to FLT-3 kinase inhibitors (34). These findings also generate the rationale to investigate the clinical efficacy of the combination that includes not only a FLT-3 kinase inhibitor but also LAQ824 that lowers the levels of mutant FLT-3.

Although considerable progress has occurred in the treatment of AML with conventional chemotherapy and auto or allogeneic bone marrow transplantation, a majority of patients are either unable to tolerate relatively intense chemotherapy or allogeneic bone marrow transplantation, a majority of patients were treated with the indicated concentrations of LAQ824 and or PKC412 for 48 h and the percentage of nonviable cells was determined (see text). The values represent mean of two experiments performed in duplicate. B. Western blot analyses of p-FLT-3 and FLT-3 were performed on the cell lysates from the cells in sample 1 after treatment with 20 nm LAQ824 and/or 100 nm PKC412 for 24 h.

REFERENCES


Fig. 5  A, Primary AML cells with FLT-3 internal tandem duplication (patient 1) or tyrosine kinase domain mutation (patient 2) or wild type FLT-3 (patients 3 and 4) were treated with the indicated concentrations of LAQ824 and or PKC412 for 48 h and the percentage of nonviable cells was determined (see text). The values represent mean of two experiments performed in duplicate. B, Western blot analyses of p-FLT-3 and FLT-3 were performed on the cell lysates from the cells in sample 1 after treatment with 20 nm LAQ824 and/or 100 nm PKC412 for 24 h.

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agent alone, a greater decline in the p-FLT-3 and FLT-3 levels was seen after treatment with LAQ824 plus PKC412.
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