Lapatinib induces autophagic cell death and differentiation in acute myeloblastic leukemia

Abstract: Lapatinib is an oral, irreversible dual tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR/ErbB1/Her1), ErbB2/Her2/Neu, ErbB3/Her3, and ErbB4/Her4, are common targets for anticancer drugs because their functions are commonly dysregulated in many cancers. Lapatinib, which is taken orally, was originally approved by the US Food and Drug Administration (FDA) in 2007 for the treatment of patients with advanced or metastatic breast cancer. It has also been tested in preclinical studies as targeted therapies for AML. Thus, in recent years, some small-molecule inhibitors, for example, sorafenib, gefitinib, erlotinib, and quizartinib (AC220) have been tested in preclinical studies as targeted therapies for AML. In this study, we examined the effects and mechanism of action of lapatinib on several human leukemia cells, including acute myeloid leukemia (AML) cells. We found that lapatinib inhibited the growth of human leukemia cell lines, including acute myeloid leukemia (AML) cells. We also noted the synergistic effects of the use of lapatinib and cytotoxic drugs in U937 leukemia cells. These results indicate that lapatinib may have potential for development as a novel antileukemia agent.

Keywords: lapatinib, autophagic cell death, leukemia, differentiation, AML

Introduction

Acute myeloid leukemia (AML) is a group of heterogeneous diseases with abnormal active proliferation of hematopoietic precursors, which block patients' normal hematopoiesis, causing neutropenia and anemia. Although more than 50% of acute myeloblastic leukemia cell lines, including acute myeloid leukemia (AML) cells, have been tested in preclinical studies as targeted therapies for AML, only 20%–30% of patients show long-term disease-free survival. Despite being associated with complicated genetic mutations, AML cases correlate with mutations caused by the aberrant activation of receptor tyrosine kinases (eg, FLT3 and c-KIT) or downstream effectors (eg, Ras). Thus, in recent years, some small-molecule inhibitors, for example, sorafenib, gefitinib, erlotinib, and quizartinib (AC220) have been tested in preclinical studies as targeted therapies for AML. In this study, we examined the effects and mechanism of action of lapatinib on several human leukemia cells, including acute myeloid leukemia (AML) cells. We found that lapatinib inhibited the growth of human leukemia cell lines, including acute myeloid leukemia (AML) cells. We also noted the synergistic effects of the use of lapatinib and cytotoxic drugs in U937 leukemia cells. These results indicate that lapatinib may have potential for development as a novel antileukemia agent.
US Food and Drug Administration in combination with capecitabine for advanced breast cancers that have developed resistance to the anti-ErbB2 monoclonal antibody trastuzumab (Herceptin; Genentech, Inc., South San Francisco, CA, USA) or other drugs.6,10,12,13

Differentiation therapies play important roles in leukemia treatment. Differentiation therapies in oncology include changing malignant tumors to curable tumors or terminally differentiated cells that undergo no further proliferation.14 This includes the induction of cancer cell differentiation and tumor eradication by either stopping tumor proliferation or increasing drug sensitivity after differentiation. One successful method of differentiation therapy is treating acute promyelocytic leukemia (APL) with all-trans retinoic acid. All-trans retinoic acid changes malignant leukemia to treatable cell forms by inducing differentiation of these leukemia cells into drug-sensitive cells. Other drugs that induce differentiation include guanosine triphosphate, herbimycin A, and butyrate for erythroid differentiation of chronic myelogenous leukemia (CML),14-16 and 12-O-tetradecanoylphorbol-13-acetate (TPA), HIV-tat protein, thrombopoietin, and dimethyl sulfoxide (DMSO) for the megakaryocytic differentiation of CML or APL.17-20

In our previous studies, we found that lapatinib induced autophagic cell death in CML and hepatoma.21,22 In this study, we focused on lapatinib-induced cytotoxicity mechanisms in AML cells. We found that lapatinib inhibits the growth of several leukemia cell lines in a dose- and time-dependent manner via either autophagic or apoptotic cell death depending on the different cell types. The induction of autophagic cell death and macrophagic differentiation by lapatinib has been demonstrated in U937 cells. We also found a synergistic effect of lapatinib and other cytotoxic drugs in AML U937 cells. The results of our study suggest that lapatinib may be a potentially useful therapy for leukemia.

Materials and methods

Cell culture

The following human leukemia cell lines were used in our study: AML-derived HL-60 and U937 cells,23 CML-derived MEG-01 and KU812 cells, APL-derived NB4 cells,22 and Jurkat T lymphoma. These cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA) and penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C under 5% CO2. Genetic recombination experiments in our study were approved by the Biosafety Committee of Chang Jung Christian University.

Cell treatments

Lapatinib (GlaxoSmithKline) or erlotinib (Hoffman-La Roche Ltd., Basel, Switzerland) tablets were ground and dissolved in DMSO (Sigma-Aldrich Co., St Louis, MO, USA) to prepare a 1,000-fold stock solution. Autophagy inhibitor 3-methyladenine (3-MA) was dissolved in an RPMI 1640 medium. The pan caspase inhibitor Z-VAD-fmk (R&D Systems, Inc., Minneapolis, MN, USA), etoposide (Sigma-Aldrich Co.), paclitaxel (Sigma-Aldrich Co.), and autophagy inducer thapsigargin (Sigma-Aldrich Co.) at concentrations of 20 nM, 1 µM, 25 µM, and 150 µM, respectively, were dissolved in DMSO to prepare a 1,000-fold stock solution. Treatment with protein kinase C activator TPA (Sigma-Aldrich Co.) was used as the positive control for the differentiation of leukemia cells.24 The 10,000-fold TPA stock solution (1 mM) was prepared in DMSO.

Viability analysis

After treatments, cell viability was analyzed using the trypan blue assay (Thermo Fisher Scientific) or the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Promega Corporation, Fitchburg, WI, USA) according to the manufacturer’s instructions. Cell numbers or optical density (OD) values of the control cells (DMSO vehicle-treated) were calculated as 100% viability. Data from triplicate (or more) experiments were presented as mean ± standard deviation (SD). P-values (P<0.05, P<0.01, P<0.001) were calculated by Student’s t-test between lapatinib-treated and DMSO control cells or as indicated.

Observation of morphology and LC3 aggregation by microscopy

After treatments with drugs for 3 days, the cells were centrifuged on slides using cytospin apparatus (Thermo Fisher Scientific), and were fixed and stained with Liu’s stain (Muto Pure Chemicals, Tokyo, Japan) according to the manufacturer’s instructions. Cell morphology was observed under a microscope. For the observation of LC3 aggregation, the stable expression of GFP-LC3 in U937 leukemia cells was induced by the transduction of a GFP-LC3-containing lentivirus and blasticidin selections as described previously.25

Flow cytometry

For the detection of apoptotic cells with DNA laddering (hypodiploid or sub-G1 phase) by flow cytometry
We then tested the mechanisms involved in lapatinib-induced cytotoxicity. We found a dramatic induction of apoptosis by lapatinib in HL-60 cells, and also induction of apoptosis and autophagic cell death in K562 cells. Approximately, 30%

Transmission electron microscopy
After treatment, U937 cells were spun and then fixed with a 2.5% glutaraldehyde-containing cacodylate buffer (pH 7.4). Then the cells were stained with 1% osmium tetroxide, dehydrated, and embedded in Spurr’s resin according to the manufacturer’s instructions (Electron Microscopy Science, Fort Washington, PA, USA). The cell morphology was observed by transmission electron microscopy (JEM-1200EXII; JEOL, Tokyo, Japan).

Knockdown of autophagy-related genes by shRNA expression system
A lentiviral short hairpin RNA (shRNA) expression system from the RNAi Consortium (TRC) was applied to knockdown the expression of the autophagy-related genes: ATG5, ATG7 (clone #1 or #2, TRCN0000007584 or TRCN0000007587, respectively), Beclin-1 (or ATG6, clone #1 or #2, TRCN0000033549 or TRCN0000033552, respectively), and RFP (as nontargeting shRNA control) as described previously.21 We obtained these pLKO.1-shRNA constructions from the National RNAi Core Facility in Taiwan. The shRNA construct preparation and lentiviral production and infection were performed according to the TRC and the Core Facility protocol as described previously.21,22 After puromycin selection for at least 1 week, cells were treated with lapatinib, and cell viability was validated by trypan blue and MTS assays as described earlier.

Results
Lapatinib-induced apoptotic and nonapoptotic cell death in several kinds of leukemia cells
In this study, we first performed a comparison of the effect of lapatinib on several leukemia cell lines. We used both MTS and trypan blue assays for viability validation. As shown in Figure 1, lapatinib reduced cell viability in a dose- and time-dependent manner in AML-derived U937, ALL Jurkat T, and CML-derived KU812 leukemia cells. Compared to other leukemia cell lines, KU812 is less sensitive to lapatinib-induced mitochondrial collapse in U937 cells (Figure S1). These findings in relation to cell viability reduction and mitochondrial collapse are consistent with the cytotoxic effects of lapatinib in CML-derived K562 and MEG-01 and AML-derived HL-60 and NB4 cells observed in our previous study.22

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Figure 1 Lapatinib inhibits the growth of several leukemia cell lines in a dose- and time-dependent manner.

Notes: Human U937 (A, B), Jurkat (C), and KU812 (D) cells were incubated with either DMSO (vehicle control) or 0.5–10 µM lapatinib for 1–3 days as indicated. The relative percentages of survival rates were analyzed by MTS (A, C, D) or trypan blue (B) assays. Mean OD values or cell numbers of vehicle control were defined as 100%.

*P < 0.05, **P < 0.01, and ***P < 0.001 (Student’s t-test) as compared to the DMSO control.

Abbreviations: DMSO, dimethyl sulfoxide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; OD, optical density.

of the lapatinib-treated K562 cells showed DNA fragmentation.22 As shown in Figure 2A, in lapatinib-treated U937 cells, growth inhibition was mainly caused due to the presence of high percentages of dead cells, in contrast to the low percentages of sub-G1 cells. The low percentages of sub-G1 cells suggest nonapoptotic cell death in lapatinib-treated U937 cells. These data are similar to those obtained for CML-derived MEG-01 (Figure 2) and K562 cells.22 However, lapatinib induced production of massive amounts of sub-G1 cells in NB4 and Jurkat T cells (Figure 2E and F), like in AML-derived HL-60 cells.22 In addition, we confirmed the induction of apoptosis by lapatinib in U937 and Jurkat cells in the presence or absence of the broad-spectrum caspase inhibitor Z-VAD-fmk. As shown in Figure 2C and F, the percentages of sub-G1 cells were effectively reduced by the caspase inhibitor Z-VAD-fmk in Jurkat T cells but not in U937 cells. In agreement, we found that Z-VAD-fmk could not protect most of the U937 cells from lapatinib-induced cell death (Figure 2B), suggesting nonapoptotic cell death in lapatinib-treated U937 cells. Thus, according to our results (Figure 2, and our previous study),22 lapatinib induced apoptosis in AML HL-60, APL NB4, Jurkat T lymphoma, and small portions of CML K562 cells, but induced nonapoptotic cell death in the majority of K562, U937, and CML MEG-01 cells.

Autophagic cell death in lapatinib-treated AML U937 leukemia cells

Since lapatinib effectively induces nonapoptotic cell death in AML U937 cells, we wondered whether lapatinib induced autophagic cell death, the second type of programmed cell death in U937 cells. As shown in Figure 3A, after treatment with lapatinib, massive cells with vacuoles (autophagic morphology) were observed. These morphological data were
similar to those obtained for cells treated with the differentiation inducer TPA. To further confirm lapatinib-induced autophagic cell death in U937 cells, we used the GFP-LC3 overexpression system and fluorescence microscopy. In U937 cells, we detected cells with punctuated GFP-LC3 (LC3 aggregates) after lapatinib or autophagy inducer thapsigargin (TPA) treatment (Figure 3B).

To further confirm the autophagic cell death of U937 cells, we used acridine orange staining. As shown in Figure 3C, high numbers of acridine orange-positive cells were detected in lapatinib-treated cells. We also determined whether lapatinib-induced cytotoxicity was blocked in the presence of the autophagy inhibitor 3-MA. As shown in Figure 3D, 3-MA effectively blocked lapatinib-induced cytotoxicity in U937 cells. In addition, we checked the expression levels of these autophagy-related proteins. As shown in Figure 3E, we found a clear increase in Beclin-1 (ATG6), ATG5, ATG7, LC3, and BNIP in lapatinib-treated leukemia by immunoblotting.

Upon further examination of lapatinib-induced autophagic cell death by transmission electronic microscope (TEM), we found massive cells with vacuoles enclosing a lot of unhealthy mitochondria (Figure 4). Furthermore, we performed autophagy inhibition by using U937 cells with shRNA expression corresponding to autophagy regulatory proteins ATG7, ATG5, and Beclin-1 (Figure S2). U937 leukemia cells were transduced with shRNAs containing plasmids from TRC as described in the “Materials and methods” section and previous reports. As shown in Figure 5, the expression of shRNA against ATG7, ATG5, and Beclin-1 (but not nonspecific shRNA against RFP) inside the cells dramatically decreased lapatinib sensitivity according to examination by the MTS (Figure 5A) and trypan blue assays (Figure 5B). These data, showing the expression of shRNA, further confirmed lapatinib-induced autophagic cell death in AML U937 cells.

**Differentiation induction by lapatinib in AML leukemia cells**

Since we have previously demonstrated the induction of megakaryocytic differentiation marker CD61 by lapatinib in K562 cells, we determined whether lapatinib could also cause differentiation in U937 cells. As shown in Figure 6A and B, we found lapatinib induced the upregulation
of CD14 and CD68 surface markers in U937 cells. This is similar to the effects caused by treatment with the differentiation inducer TPA.\textsuperscript{28–30} TPA induces the differentiation of AML cells into monocyte/macrophage-like cells or polymorphonuclear neutrophil cells.\textsuperscript{31} For further confirmation of differentiation in lapatinib-treated cells, phagocytic activity and ROS production assays were performed.\textsuperscript{24} As shown in Figure 6C and D, phagocytosis ability was dramatically increased with lapatinib treatment, similar to the positive-control TPA treatment. The cellular ROS level was also
Figure 4 The induction of massive vacuoles inside cells by lapatinib in U937 cells.
Notes: U937 cells were treated with (A) DMSO or (B) 10 µM lapatinib for 3 days. Cells were fixed, stained, and observed by TEM. The magnification information of each figure is as indicated.
Abbreviations: DMSO, dimethyl sulfoxide; TEM, transmission electron microscope.

Figure 5 The RNAi-mediated downregulation of autophagy-related genes hinders lapatinib-induced autophagic cell death in U937 cells.
Notes: U937 cells with shRNA expression were prepared. Cells were transduced with shRNA against ATG5, 7, Beclin-1 (BECN), and RFP (nonspecific) mRNA, selected with puromycin, and treated with DMSO (D) or 5–10 µM lapatinib for 2 days. The relative percentages of survival rates were detected using the MTS (A) and trypan blue (B) assays. Two shATG7 or shBECN clones (#1 or #2) were used. *P<0.05, **P<0.01, and ***P<0.001 (Student’s t-test) as compared to the DMSO control.
Abbreviations: RNAi, RNA interference; shRNA, short hairpin RNA; DMSO, dimethyl sulfoxide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; Unif, uninfected cells.
Figure 6 The upregulation of CD14 and CD68 macrophagic differentiation markers and the phagocytosis ability of lapatinib in AML U937 leukemia cells.

Notes: (A, B) U937 cells were treated with DMSO, 5–10 µM lapatinib, or 0.1 µM TPA (differentiation inducer) for 3 days. Cells were harvested, stained with antibodies against CD14 or CD68, and positive cells were analyzed by flow cytometry. (C–E) U937 cells were treated with DMSO, 2.5–10 µM lapatinib, or 0.1 µM TPA (differentiation inducer) as indicated for 3 days. Cells were harvested, incubated with fluorescent latex beads (C, D) or DCFH-DA (E) for the detection of the phagocytosis ability of cells (C, D) or the ROS production of cells (E) by flow cytometry. (D) MFI in (C) is expressed; ***p<0.001 (t-test) as compared to the DMSO control.

Abbreviations: AML, acute myeloid leukemia; DMSO, dimethyl sulfoxide; TPA, 12-O-tetradecanoylphorbol-13-acetate; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; ROS, reactive oxygen species; MFI, mean fluorescence intensity.
increased in lapatinib-treated U937 cells (Figure 6E). The upregulation of CD14 and CD68 surface markers and cellular ROS level suggests the induction of monocyte/macrophage differentiation by lapatinib treatment in AML U937 cells, similar to the effects of TPA treatment.

Synergistic effects of lapatinib and other anticancer drugs in U937 leukemia cells

To reduce the side effects and increase the therapeutic effects, cotreatment is a useful strategy for clinical therapies. We, therefore, wondered whether a combination of lapatinib and other anticancer drugs would show enhanced cytotoxic effects in leukemia. We first choose erlotinib, a targeted therapy drug and EGFR inhibitor that shows off-target effects in AML leukemia cells and patients.4,5,32–38 As shown in Figure 7 and consistent with previous reports, erlotinib alone failed to effectively inhibit U937 cell growth. However, in combination with low doses of lapatinib, both drugs showed dramatic growth inhibition of U937 cells. Again, a combination of low doses of lapatinib and etoposide or paclitaxel effectively increased the cytotoxic effects as compared to a single drug treatment. These results suggest the synergistic effects of lapatinib and other anticancer drugs in U937 leukemia cells. These synergistic effects of lapatinib and other anticancer drugs suggest the possible benefits of the development of combination therapies in leukemia treatment.

Discussion

In this study, we found lapatinib inhibits the growth of several leukemia cell lines in a dose- and time-dependent manner (Figure 1) either via autophagic or apoptotic cell death depending on the type of cell lines (Figure 2).22 This includes apoptosis induction in AML HL-60, APL NB4, Jurkat T lymphoma, and a minority of CML K562 cells, and the induction of nonapoptotic cell death in AML U937, CML MEG-01, and the majority of K562 cells by lapatinib. In U937 cells, autophagic cell death induced by lapatinib was demonstrated by massive vacuole formation observed by TEM (Figure 4) and microscopy (Figure 3A); LC3 aggregations (Figure 3B); acridine orange-positive cells (Figure 3C); cell death inhibition by 3-MA (Figure 3D) (but not Z-VAD-fmk [Figure 2B and C]); upregulation of the autophagy-related proteins ATG5, ATG7, Beclin, BNIP, and LC3 (Figure 3E); and cell death inhibition by knockdown of shRNA against ATG5, ATG7, and Beclin (Figure 5).26 Moreover, we also found induction of differentiation by lapatinib in U937 cells in correlation with the upregulation of monocyte/macrophage markers CD14 and CD68 (Figure 6A and B).23,38 Lapatinib also increased the phagocytosis ability of U937 cells, as shown by massive incorporation of fluorescent beads and higher ROS production (Figure 6C–E).23 We further found that the use of lapatinib and other cytotoxic drugs, including erlotinib, etoposide, and paclitaxel, in U937 cells has a
synergistic effect that increased tumor cytotoxicity (Figure 7). These findings suggest that a combination therapy using lapatinib and other anticancer drugs would further benefit anti-AML applications.

A few studies have focused on lapatinib-induced autophagic cell death. This study is consistent with our previous findings regarding the induction of autophagic cell death in hepatoma cells, induction of autophagic cell death, apoptosis, and differentiation by lapatinib in CML K562 cells. In addition to our findings, some studies have also discussed the induction of autophagy by lapatinib, such as in HCT116 colon cancer cells and in lapatinib and obatoclax cotreated breast cancer cells.

Consistent with the findings of our study, some EGFR inhibitors, for example, sorafenib, gefitinib, erlotinib, and quizzatinib (AC220), have been reported as targeted therapies for AML treatment in preclinical studies. Irwin et al also found the antiproliferation effect of lapatinib in human ALL Z119 and Z181 cell lines with the Philadelphia chromosome and ErbB2 expression. Also, similar to the findings of our study, cytotoxic activity and differentiation induction were found for gefitinib and erlotinib and other targeted therapy drugs against EGFR, in leukemia cells and clinical responses. According to the previous findings and our preliminary results (data not shown), there are no clear expression patterns for EGFR or ErbB2 in AML or in some of the tested cell lines, such as HL-60, despite the demonstration of a EGF-EGFR autocrine loop in U937 cells. Thus, the real targets of lapatinib, as well as gefitinib and erlotinib EGFR inhibitors need to be further investigated.

**Conclusion**

We found that lapatinib induced cytotoxicity in several leukemia cell lines via either autophagic or apoptotic cell death. Lapatinib-induced autophagic cell death was observed in AML U937 cells. We also found differential induction by lapatinib and that treatment with lapatinib and other cytotoxic drugs (in AML U937 cells) had a synergistic effect. Our study suggests that a combination of lapatinib and other anticancer drugs may potentially be used as an AML therapy for clinical application.

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**Disclosure**

The authors report no conflicts of interest in this work.

**References**


Supplementary materials

Figure S1 Lapatinib-induced mitochondria collapse in U937 cells. Notes: U937 cells were treated with DMSO or 2.5 µM lapatinib for 2 days. Cells were stained with DiOC6 (molecular probes) and the MTP inside the cells was analyzed by flow cytometry. Abbreviations: DMSO, dimethyl sulfoxide; MTP, mitochondria transmembrane potential.

Figure S2 Expression of ATG7, ATG5, and Bcln in shRNA knockdown cells. Notes: U937 cells with shRNA expression as indicated were prepared. Cell lysates from shRNA expressing U937 cells were collected and subjected to Western blot analysis for determining the expression of ATG7, ATG5, Bcln, and actin (loading control). Two shATG7 or shBecn clones (#1 or #2) were used. Abbreviations: Bcln, Beclin-1; shRNA, short hairpin RNA.