Combination of chloroquine treatment has possibility of overcome T315I mutated Ph⁺ALL cells: establishment of two cell lines of double Ph⁺ALL with or without T315I mutation from one clinical course.

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ABSTRACT
Resistance to imatinib is still problematic in Ph-positive acute lymphoblastic leukemia (Ph⁺ALL). In this study, we established a Ph⁺ALL cell line, DPAL, which had the wild-type version of BCR/ABL, and a T315I mutant Ph⁺ALL cell line, DPAL/T315I, from one clinical course. At diagnosis, this patient had double Philadelphia chromosomes with some additional chromosomal abnormalities, but no point mutation in BCR/ABL. After combination treatment of imatinib with chemotherapy, the T315I mutation appeared, and the DPAL/T315I cell line also had double Philadelphia chromosomes without additional chromosomal abnormalities. This result suggested that some heterogeneous populations of BCR/ABL mutants exist in Ph⁺ALL at diagnosis. Imatinib combined treatment might be only select the dominancy of mutant gene. Our established cell line, DPAL/T315I had severe resistance to imatinib and 2nd tyrosine kinase, dasatinib, though, chloroquine combined treatment with imatinib or the HDAC inhibitor, SAHA induced cell death in DPAL/T315I cells in vitro. The utilize of chloroquine may prove to be a new strategy for eliminating imatinib-resistant Ph⁺ ALL cells, especially T315I mutant positive cells.

INTRODUCTION
Philadelphia chromosome-positive adult acute lymphoblastic leukemia (Ph⁺ALL) had been proved a very low cure rate. Recently, chemotherapy with tyrosine kinase inhibitor against BCR/ABL, imatinib, has improved the remission rate and prognosis for Ph⁺ALL patients. In the GRAAPH-2003 study, imatinib-combined chemotherapy and stem cell transplantation yielded an overall survival rate of 65% and disease-free survival of 51% at 18 months, but the
relapse rate at 18 months was 30% [1], which is quite high. A high relapse rate is a severe problem in adult Ph⁺ALL after the use of imatinib. At the time of disease progression or relapse, various point mutations on BCR/ABL genes, which convey tolerance against imatinib, frequently appear. Second-generation tyrosine kinase inhibitors (2nd TKIs), such as nilotinib, dasatinib, and INNO-406, have an inhibitory effect on imatinib-resistant point mutations of BCR/ABL [2-4]. Nonetheless, a gatekeeper mutation, T315I, is severely resistant against even the second-generation TKIs. Many point mutations appear in the p-loop at the BCR/ABL position, and second-generation TKIs can bind to the ATP-binding site with higher affinity than imatinib [5]. However, the position of the T315I mutation is the binding site of imatinib, and previous studies using crystal structure data revealed that various TKIs could not bind to the product of the mutated gene of T315I [6]. It has remained unknown why these point mutations appear after imatinib treatment, and there are currently no clinical treatment strategies against T315I-positive leukemia.

To date, the effects of several drugs against the mutation of T315I have been studied, such as HDAC inhibitors and Aurora kinase inhibitors [7-9]. These inhibitors affect not only BCR/ABL but also other molecules in cell signaling that are necessary for the growth of Ph⁺ALL cells. As another target in BCR/ABL-positive leukemia, blocking autophagy in the cell death pathway in CML cells has been studied in in vitro assays. In recent reports, various mechanisms of autophagy in cancer cells have been described. One of these is protection of cancer cells from cytotoxic agents. Various in vitro assays have demonstrated that genetic or pharmacological inhibition of autophagy enhances the cytotoxicity of cancer chemotherapeutic agents [10-12]. Bellodi et al. described how imatinib induced autophagy in CML/blast-crisis cell lines, CML/primary cells, and p210 BCR/ABL-expressing myeloid precursor cells. Combining TKIs with autophagy inhibitors resulted in complete elimination of phenotypically and functionally defined CML stem cells [13]. We also previously reported that combination treatment of imatinib with autophagy inhibitor, chloroquine (CQ) induced cell death in both wild type BCR/ABL-transfected BaF3 cells (BaF3/wild) and T315I mutant-type-transfected BaF3 cells (BaF3/T315I) [14].

In this study, we established two Ph⁺ALL cell lines, one having the wild type of BCR/ABL and the other the T315I mutant, from one clinical course of one Ph⁺ALL patient. Using these cell lines, we analyzed the mechanisms of appearance of the T315I mutation from these cell lines and studied the effect of combination treatment with CQ and imatinib or HDAC inhibitor, SAHA.

**MATERIALS AND METHODOLOGY**

**Established cell lines**

We collected bone marrow samples from the Ph⁺ALL patient at the time of diagnosis and we also derived the peripheral blood samples of the same patient at the time of first relapse after treatment with imatinib-combined chemotherapy. After separation of mononuclear cells, we co-cultured the cells with normal human fibroblast in RPMI1640 with 10% fetal bovine serum with penicillin and streptomycin. During establishment of DPAL/T315I cell line, we added 5 μM imatinib to the medium to select the cells with T315I mutation and performed single-cell culture and cloning. Although DPAL cells could obtain the ability of self-proliferation without fibroblast, DPAL/T315I cells were needed co-culture with fibroblasts for stabilization and maintenance. They had been cultured continuously for 36 months at the time of submission of this report.

**Genetic and molecular analysis**

Total RNA of our established cell lines was extracted using TRizol reagent (Invitrogen, CA, USA), and reverse transcription was performed with primerScript RTase (TAKARA, CA, USA). PCR was performed with sense primer, 5’-GAACTCGCAACAGTCTTTCG-3’ (on exon 1 in BCR), and antisense primer, 5’-TGAGGCATCTCAGGCACGTC-3’ (on exon 10 in Abl). DNA sequencing was performed with sense primer, 5’-CCAAAGCGCAACAAGCCACGCAC-3’ (on exon 4 in Abl), and antisense primer, 5’-ACAGCCCCACGGACGCCTTG-3’ (on exon 10 in Abl), with Applied Biosystems 3730 DNA Analyzer. FISH analysis and G-band assay of both cell lines were performed by Nihon Gene Research Laboratories, Inc (Miyagi, Japan). FISH probes were Vysis™ DNA FISH probes, dual fusion dual color.
WST assay

The analyses of imatinib and dasatinib-dose response of DPAL and DPAL/T315I cell lines were performed by WST assay. Additionally, dose response to SAHA with or without CQ to our established cell lines were analyzed by WST assay. The cells were plated into 96-well plates and cultured with various concentrations of these chemicals respectively. In the incubation of DPAL/T315I cells, we co-cultured with fibroblast for cell stability. The time of incubation was 72 hour in imatinib-plate and 48 hour in other chemicals-plate. After incubation, the living cell numbers were assessed using a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan) by combining 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfolphenyl)-2H-tetrazolium (WST-8) and 1-methoxyphenazine methosulfate (1-methoxy-PMS). WST-1 (10% final concentration) was incubated with cells at 37°C for 2 hour and read using microplate reader at 440nm wavelength. The data represent the average of triplicate experiments.

Cell-death assay

Various concentrations of imatinib mesylate (LKT Laboratories, Inc, MN, USA.) or SAHA (Sigma-Aldrich, MO, USA) were added with or without 25 μM chloroquine diphosphate (Sigma-Aldrich, MO, USA) to DPAL and DPAL/T315I cells. In the incubation of DPAL/T315I cells, we co-cultured with fibroblast for cell stability. After 48 hours of incubation, TUNEL assay was performed using an in situ cell-death detection kit (Roche, IN, USA) according to the manufacturer’s protocol. The dUTP-labeled cells were analyzed using FACScan (Becton Dickinson). In FACS analysis, we counted 10,000 cells/assay and performed experiments 3 times or more. The data are the average of triplicate experiments.

Western blotting

After incubation with imatinib with or without CQ, each cell was homogenized in lysis SDS sample buffer. Thirty micrograms of protein was separated in SDS-PAGE. The proteins on the gel were transferred to a PVDF membrane (Bio-Rad, Hercules, CA). The membranes were blocked using casein and were incubated with each antibody. The antibodies used for immunoblotting included anti-LC3 (MBL, Nagoya, Japan), anti-phosphorylated c-abl, anti-phospho-stat5, and anti-phospho-CrkL multiplex western detection cocktail, anti-c-abl, anti-stat5, anti-CrkL (Cell Signaling, MA, USA), anti-phosphorylated AKT, anti-AKT, and anti-beta-actin antibody (Santa Cruz, CA, USA). The proteins on the membrane were detected using ECL-Plus systems (Amersham Pharmacia Biotech, Buckinghamshire, UK). The data were obtained three times or more experiments and the each band intensity was determined by densitometric analysis using ImageJ 1.41o software (National Institute Health) digitizer technology.

Statistical analysis

All experiments were carried out in triplicate and repeated three times or more. Statistical significances of differences were determined by Student’s t test with the level of significance set at p<0.05.

RESULTS AND OBSERVATIONS

Clinical Cases

A 56-year-old man, who had previously undergone resection of esophageal adenocarcinoma, had high fever and fatigue. His laboratory data were WBC 15,400/μL, hemoglobin 9.1 g/dl, and platelet 0.3×10^10/μL, and coagulation data demonstrated DIC. Bone marrow aspiration showed hypercellular bone marrow containing 95% monoblastoid cells. Flow cytometry demonstrated that the immunological cell markers CD10, CD13, CD19, CD22, CD34, HLA-DR, and TdT were positive. Fluorescence in situ hybridization (FISH) analysis showed BCR/ABL fusion signals. G-band analysis demonstrated 47,X,-Y,+5,+8,-9,t(9;22)(q34;q11),+der(22)t(9;22) (Fig. 1A). The patient had trisomy 22 and double fusion chromosomes of t(9;22). The patient was diagnosed with Ph*-positive B-ALL. Chemotherapy combined with imatinib (JALSG202 protocol [15]) was started, and the patient achieved cytogenetic remission after induction treatment. Then, one course of methotrexate (MTX) and cytarabine (Ara-C) regimen as consolidation therapy and a second course of imatinib monotherapy were followed, as indicated by the protocol. Before the third course of consolidation chemotherapy, on day 104, the patient had relapsed. PCR analysis demonstrated that the mRNA of BCR/ABL was 2.6×10^5 copies/μg and bone marrow contained...
15% blast cells.

Fig. 1. The schema of clinical course and establishment of cell line. DPAL cell line was established from the sample at diagnosis as Ph⁺ALL. The sample at diagnosis showed wild type of BCR/ABL by sequence and 47,X,-Y,+t(8;9)(p22;q34),+t(9;22)(q34;q11),+der(22)q23 by G-band analysis. DPAL cells also showed wild type of BCR/ABL in sequence. DPAL/T315I cell line was established from sample at the first relapse after the patient receiving imatinib-combined chemotherapy. The sample at first relapse showed both T315I-mutated BCR/ABL and wild type of that by sequence. DPAL/T315I cells also showed same result of sequence as the sample at the first relapse and showed 47,X,-Y,t(9;22)(q34;q11),+der(22)q23 by G-band analysis.

The patient received Hyper-CVAD (adriamycin, vincristin, cyclophosphamide, and predonine) treatment, and went into a second cytogenetic remission. The population of blast cells was 0.5% in the bone marrow, and the BCR/ABL fusion signal was not detected by FISH analysis. PCR analysis revealed that the mRNA of BCR/ABL was 5.5×10² copies/μg (second relapse). After treatment with a high dose of MTX and Ara-C, cord blood transplantation was performed, nonetheless, the patient died because of disease progression.

Establishment and characterization of Ph⁺ALL cell lines

We established two cell lines of Ph⁺ALL from the clinical samples, at diagnosis and first relapse as described in Materials and Methods. Figure 1 showed the flow chart of our experienced clinical case, sampling, and the characteristics of cell lines. We then named the first cell line DPAL and the second cell line DPAL/T315I. G-band analysis of DPAL/T315I cells demonstrated 47,X,-Y,t(9;22)(q34;q11),+der(22)q23. DPAL/T315I cells had double Philadelphia chromosomes, the same as the sample at diagnosis, whereas additional chromosomal abnormalities, which had been present in the sample at diagnosis (Fig. 2A), had disappeared (Fig. 2B). Sequence analyses were derived from the clinical samples at diagnosis and demonstrated no point mutation on BCR/ABL gene. Similarly, the sequence of DPAL cells only exhibited the wild type of BCR/ABL gene. From the sample of at the first relapse, a double peak of thymine and cytosine at base 315 were detected. That meant that both point mutation
such as a threonine to isoleucine translocation at base 315 (T315I mutation) and wild type of BCR/ABL were positive. Similarly, the sequences of DPAL/T315I cells demonstrated both wild-type and T315I-mutated BCR/ABL gene (Fig. 3A). FISH analysis of DPAL/T315I cells showed three fusion signals of BCR/ABL in one cell. One more Abl signal, which remained from chromosome 22, had been depleted (Fig. 3B). A similar pattern was shown in the FISH analysis of this patient at diagnosis (data not shown). From these results, we considered that one of double Ph- chromosome had wild type of BCR/ABL gene and the other acquired T315I mutation. In addition, it could be also suggested that DPAL/T315I cells were not transformed from DPAL cells but from a subpopulation of diagnosis cells and each cell line was derived from an independent population.

![Fig. 2](image)

**Fig. 2.** G-band analyses of Ph⁺ALL cells at diagnosis and of DPAL/T315I cells and imatinib dose response of established cell lines. (A) The chromosomes of our clinical Ph⁺ALL patient at diagnosis, demonstrating 47,X,-Y,+5,+8,-9,t(9;22)(q34;q11),+der(22)t(9;22). (B) The chromosomes of DPAL/T315I cells, with 47,X,-Y,t(9;22)(q34;q11),+der(22)t(9;22). Additional chromosomal abnormalities that existed at diagnosis disappeared.

Additionally, we analyzed the dose response of imatinib and dasatinib in these cell lines by WST
assay. This assay revealed that imatinib and dasatinib were effective for DPAL cells in a dose-dependent manner, whereas DPAL/T315I cells had severe tolerance to both imatinib and dasatinib (Fig. 3C).

Fig. 3. Genetic analysis and characterization in our established cell lines. (A) The sequences of BCR/ABL in our established cell lines, DPAL and DPAL/T315I. DNA sequences were obtained with sense primer, 5'-CCAAAGCGCAACAAGCCCAC-3' (on exon 4 in Abl), and antisense primer, 5'-ACAGCCCCACGGACGCCTTG-3' (on exon 10 in Abl). The master mixture analyzed with an Applied Biosystems 3730 DNA Analyzer. DPAL cells expressed a normal pattern of sequences of BCR/ABL. DPAL/T315I cells had a double peak of thymine and cytosine on the 315th amino acid. (B) FISH analysis of BCR/ABL in DPAL/T315I cells. (C) WST assay demonstrated the imatinib and dasatinib dose response curves of DAPL and DPAL/T315I cells.
Efficacy of chloroquine in combination treatment in DPAL and DPAL/T315I cell lines

As our WST assay demonstrated, imatinib had no effect on DPAL/T315I cells. For a new strategy for the treatment of DPAL/T315I cells, we analyzed the effect of the blocking of autophagic pathway. CQ is an autophagy inhibitor and inhibits the digestion of autophagosomes after fusion with lysosomes, consequently inhibiting the dissolution of autophagosomes [16]. We treated DPAL and DPAL/T315I cells with imatinib (0.5 and 5.0 μM, respectively) with or without 25 μM CQ for 48 hours and then performed TUNEL assay. We found that CQ treatment increased imatinib-induced cell death in DPAL cells (p=0.038). CQ treatment also increased apoptotic cell death in DPAL/T315I cells (p=0.006) (Fig. 4A). CQ only treatment showed no effect to DPAL cells and it showed a tendency of an increase of apoptosis in DPAL/T315I cells, although there were not significant differences (p=0.266).

To confirm whether CQ indeed inhibited autophagy in combination treatment with imatinib, we performed immunoblotting analysis of LC3. LC3-II is known as a protein that is expressed on the membrane of autophagosomes and used as one of the markers of autophagosomes [17, 18]. Our immunoblotting analyses showed that expression of LC3-II was increased by CQ in both DPAL and DPAL/T315I cells with or without imatinib (Fig. 4B). These
assays demonstrated the possibility of the existence of autophagy during imatinib treatment and inhibition of autophagic digestion by CQ treatment.

To clarify the effect of other autophagy inhibitors, we treated DPAL and DPAL/T315I cells by imatinib with or without bafilomycin A or 3MA. Both bafilomycin A and 3MA are known to inhibit autophagy and those regulate the function of lysosomes. Nonetheless, in our study, neither bafilomycin A nor 3MA accelerated imatinib induced cell death in DPAL and DPAL/T315I cell lines (data not shown).

Fig. 5. The treatment of HDAC inhibitor, SAHA, for DPAL and DPAL/T315I cells. (A) DPAL and DPAL/T315I cells were treated with 0.5 μM SAHA with or without 25 μM CQ for 48 hours. After treatment, we performed TUNEL assay as described in Materials and Methods. We analyzed 50,000 cells for each sample and performed experiments 3 times or more. The data of graphs are shown as the average of each group of experiments and error bars indicate the standard errors (*: p=0.05). (B) SAHA with CQ dose-dependent curve in DPAL and DPAL/T315I cells. After 48 hour incubation, WST assay demonstrated the effect of each dose of SAHA and that of additional CQ to both cells.

Next, we investigated the signaling pathways during the simultaneous treatment of imatinib and CQ in our cell lines. First, we examined the activation of BCR/ABL and its downstream signaling pathways in both cell lines. Imatinib treatment induced decreases of phosphorylation of BCR/ABL and STAT5 in DPAL cells, although, 5 μM imatinib had no effect on these signals in DPAL/T315I cells. The single-agent treatment of CQ did not exert any effect on phosphorylation of BCR/ABL and STAT5 in both cell lines. In addition, combination treatment of CQ with imatinib also did not inhibit the phosphorylation of BCR/ABL and STAT5 in DPAL/T315I cells (Fig. 4B). Otherwise, it is known that a part of the signaling pathways of autophagy is dependent on the PI3K/AKT pathway [19]. Our data showed that phosphorylation of AKT was decreased by treatment of imatinib, and single-agent treatment of CQ did not affect phosphorylation of AKT in DPAL cells. On the other hand, imatinib-only treatment or CQ-only treatment could not significantly affect phosphorylation of AKT in DPAL/T315I cells. Co-treatment of CQ with imatinib induced clear suppression of phosphorylation of AKT in DPAL/T315I cells (Fig. 4B). The densitometric analysis of Western blotting demonstrated
statistically significant differences of phosphorylation of BCR/ABL in DPAL cells between control cells and imatinib-treated cells (p=0.038) and of phosphorylation of AKT in DPAL/T315I cells between control cells and imatinib with CQ-treated cells (p=0.006) (Fig. 4C). These results suggested that imatinib with CQ did not directly inhibit the activation of BCR/ABL pathway, but inhibited the signaling pathway of AKT in DPAL/T315I cells.

Next, to investigate the efficacy of the inhibition of autophagy in cell death of Ph′ALL cells, we studied the effect of HDAC inhibitor SAHA, instead of imatinib, on the induction of both apoptosis and autophagy in BCR/ABL-positive cells [20]. Carew et al. reported that the combination treatment of SAHA and CQ induced cell death in not only wild-type BCR/ABL-positive cells but also in T315I-positive cells. They described how autophagy resisted apoptosis in SAHA-treated BCR/ABL-positive cells and inhibition of autophagy by CQ induced an increase of apoptotic cells [21]. We treated DPAL and DPAL/T315I cells with 0.5 µM SAHA with or without CQ for 48 hours, then we performed TUNEL assay. This study demonstrated that SAHA induced cell death in both DPAL and DPAL/T315I cells. Moreover, CQ accelerated SAHA-induced cell death in DPAL/T315I cell lines (p=0.05), whereas CQ did not show an additional effect on DPAL cells (Fig. 5A). Then, we analyzed the dose response of DPAL and DPAL/T315I cells to SAHA with or without 25 µM CQ by WST assay. In this study, a dose of 0.5 µM or more SAHA was effective for both cell lines and an additional effect of CQ was seen in only DPAL/T315I cells. (Fig. 5B).

DISCUSSION

DPAL cell line had double Philadelphia chromosomes as well as other additional chromosomal abnormalities. Many physicians have described how additional chromosomal abnormalities of Ph′ALL and CML influenced poor prognosis and resistance to imatinib [22, 23]. The appearance of double Philadelphia has also been considered in relation to the amplification of BCR/ABL gene and resistance to imatinib. Our patient was briefly in remission and then immediately relapsed with resistance against imatinib.

Furthermore, the clinical cases of Ph′ALL and the aggressive phase of chronic myelogenous leukemia after treatment with imatinib often acquire a T315I mutation on the BCR/ABL gene [5, 24, 25]. How T315I mutagenesis occurs is unknown. DPAL/T315I, which was derived from the relapse, had the T315I mutation in one of two Philadelphia chromosomes and no extensive additional chromosomal abnormalities. This fact strongly suggested that dominant cells with the wild-type BCR/ABL gene with many additional chromosomal abnormalities did not develop the T315I mutation and that BCR/ABL-positive cells were heterogeneous before imatinib treatment. We speculated how the cells acquired the chromosomal abnormalities in our case. First, normal karyotype chromosomes underwent transformation of (9;22). Then, trisomy 9 and 22 were added and double transformation of (9;22) occurred. Next, the positive clone of one of the wild-type double Philadelphia chromosomes acquired many additional chromosomal abnormalities and the clone grew dominantly. One of the small populations obtained the T315I mutation in one of the double Philadelphia chromosomes. Dominant populations with wild-type BCR/ABL disappeared following imatinib with combination chemotherapy, and subsequently, small populations with imatinib-resistant T315I mutant increased (Fig. 6). Our result suggested that there are cases with heterogeneous populations in BCR/ABL-positive cells and that it is possible that minor populations with T315I mutation in BCR/ABL cells existed in previous treatment. Pfeifer et al. detected kinase domain mutations in the minor subpopulation of leukemic cells in 40% of newly diagnosed and imatinib-naive patients. They reported that remission duration did not differ significantly between patients with or without a detectable early mutation. However, only the small subgroup of patients with a T315I mutation had a shorter median time to progression than the patients with no mutations [26]. The latent existence of a heterogeneous population of BCR/ABL should be a very important point in designing a strategy of clinical treatment of Ph′ALL. The imatinib-combined chemotherapy, even with a second-generation TKI, could not eliminate BCR/ABL-positive leukemia.
Fig. 6. Hypothesis of chromosomal change in our clinical case. First, normal karyotype of chromosomes underwent transformation of (9;22). Next, the cells obtained additional 9 and 22 chromosomes and developed double Philadelphia chromosomes. Then, one of the populations developed the many additional chromosomal abnormalities and other populations got T315I mutations. Dominant populations with wild-type BCR/ABL, which has various additional chromosomal abnormalities, disappeared by treatment of imatinib with combination chemotherapy. After that, gradually, one of the minor populations with T315I showed an increase.

To obtain new strategies to treat BCR/ABL-positive leukemia with tolerance for TKIs, trials of various combinations of kinase inhibitors have been started in vivo and in vitro. The target of autophagy is a new strategy for BCR/ABL-positive leukemia. Cytoprotection with autophagosome formation by imatinib was observed in various leukemia and cancer cell lines as well as normal murine embryonic fibroblasts. Complete inhibition of autophagy by knockdown of atg5 attenuated the cytoprotective effect of imatinib [27].

Kamitsuji et al. described that INNO-406, a second-generation TKI, promoted autophagy and that blocking autophagy with CQ or knockdown of an autophagy-related gene, beclin1, enhanced INNO-406-induced cell death [28]. Recently, we reported that combination of imatinib with CQ was very effective for CML cell line with T315I mutation [14]. In the study, imatinib (0.5 µM) and CQ significantly accelerated apoptosis in K562- and BCR/ABL-mutated BaF3 cells, and also, 5 µM imatinib with CQ induced apoptosis in BaF3/T315I
cells that had mutated BCR/ABL with T315I mutant for BaF3 cells. In addition, in the present study, co-treatment of imatinib with CQ induced an increase of apoptosis in our established Ph-positive ALL cell lines, even DPAL/T315I cells. The combined treatment of imatinib with CQ might be a new strategy to eliminate the Ph-positive ALL cells with T315I mutation.

On the other hand, the HDAC inhibitor, SAHA, could induce cell death in both DPAL cells and DPAL/T315I cells. Furthermore, combination treatment of SAHA with CQ increased cell death in only DPAL/T315I cell lines. We could not clearly understand the mechanisms of the difference of the effect of combined SAHA with CQ treatment between DPAL and DPAL/T315I cells, but it was a very interesting finding. It might be possible that SAHA with CQ induced respective effects in two cell lines. SAHA treatment might induce more autophagy for DPAL/T315I cells than for DPAL cells, so the block of autophagy by CQ could be more effective for DPAL/T315I cells.

Carew et al. described how disruption of the autophagy pathway dramatically augments the antineoplastic effects of SAHA in CML cell lines and primary CML cells expressing wild-type and imatinib-resistant mutant forms of BCR/ABL, including T315I. SAHA with CQ modulates the expression and subcellular localization of cathepsin D, which is an important mediator of anticancer activity [21]. Furthermore, CQ with an HDAC inhibitor, vorinostat, significantly reduced tumor burden and induced apoptosis in a colon cancer xenograft model. Autophagy inhibition with CQ induced ubiquitinated protein accumulation, and vorinostat potentiated CQ-mediated aggregate formation, superoxide generation, and apoptosis [29]. Combination treatment HDAC inhibitor with CQ might be a promising drug to eliminate TKI resistant-Ph ALL. It would be important to study how SAHA and CQ interact to increase cell death in the imatinib resistant cell line.

Although in our assay, other autophagy inhibitor, bafilomycin3A and 3MA could not influence our established cell lines. From there result, we could not determine the effect of CQ is inhibition of autophagy or not. The role of chloroquine might depend on other pathway except for inhibition of autophagy. Western blotting analysis showed CQ treatment could not influence the main signaling pathway of BCR/ABL, and could inhibit phosphorylation of AKT, which is autophagy-related signal. The expressions of autophagy-marker, LC3-II, accelerated under CQ-treatment. CQ monotherapy did not affect our established cell lines, although combination treatment with imatinib or SAHA could induce cell death. So, we could consider the target of chloroquine is not only construction of cells and mechanisms of inside of cells, such as inhibition of autophagy. CQ is an anti-malarial drug, but it has been recognized as having various effects to cancer disease or inflammatory disease such as systemic lupus nephritis. As another effect of CQ on Ph^+ALL cell lines, CQ promotes p53-dependent cell death that does not require caspase-mediated apoptosis [30]. CQ activated the p53 pathway and induced cell death in human glioma cells or colon cancer cells [31, 32]. CQ was also described as an inducer of both p53-dependent and p53-independent cell death and CQ-induced cell death may be mediated by multiple pathways [33].

The concentration of CQ might have been high in our experiments. The actual concentration of CQ in vivo in the microenvironment is unknown. We cannot apply our data to an in vivo context directly, but our study suggests that CQ and inhibition of autophagy play key roles as a novel therapy against imatinib-resistant Ph-positive ALL cells.

CONCLUSION

Our established cell lines were very useful in considering the onset of the BCR/ABL mutations and could be valuable in screening various T315I inhibitors. And the combination of chloroquine treatment with imatinib or SAHA has the potency the new target of the therapy against imatinib-resistant Ph^+ALL cells with T315I mutation.

DISCLOSURE STATEMENT

The authors have no conflict of interest.
REFERENCES


