

Elaine Liu

**Title: Gefitinib as a Potential Treatment for Aggressive CLL**

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### **Abstract**

Chronic lymphocytic leukemia (CLL) is presently an incurable disease that is expected to increase in prevalence, as a result of the aging population. Currently, the standard treatment used is chlorambucil or fludarabine, combined with cyclophosphamide and rituximab. The disease has a variable course. Recently, it has been shown that the expression of the tyrosine kinase, ZAP-70 in CLL is a strong indicator of poor prognosis, with a greatly reduce time to treatment from diagnosis, compared with ZAP-70 negative patients. Therefore, therapy that specifically targets ZAP-70 positive CLL might improve these patients' outcomes. The tyrosine kinase inhibitor gefitinib has been shown to reduce the phosphorylation levels of several tyrosine kinases including ZAP-70 and SYK. We treated several ZAP-70 positive and ZAP-70 negative patient samples with gefitinib and found that gefitinib had a much lower IC 50 for ZAP-70 positive patients than it did for ZAP-70 negative patients. Also, gefitinib induced more apoptosis in the ZAP-70 positive cell line Jurkat than it did in cells lines not expressing ZAP-70. These results support a role for gefitinib in the treatment of ZAP-70 positive CLL.

## Introduction:

Chronic Lymphocytic Leukemia is the most common type of Leukemia found in North America and Europe. Survival is relatively long with many patients surviving 10 years after diagnosis but the disease is incurable. CLL B cells have the characteristic immunophenotype of CD5+, CD19+, CD20dim, CD23+, and surface immunoglobulin dim. The most heavily contributing risk factor is positive family history of the disease, as 8-10% of patients have a family member with the disease. On the chromosomal level, 2q21.2 is associated with the inheritance of CLL (1). The peak incidence occurs between the ages of 60 to 80, with a male to female ratio of 2:1. The disease is mostly diagnosed at routine check-ups when patients are still in the asymptomatic stage. Some clinical manifestations include symmetrical, non-tender lymphadenopathy, immunosuppression from hypogammaglobulinemia and cellular dysfunction, and hepatosplenomegaly. Early in the disease course, patients tend to experience bacterial infections; in advanced disease, viral and fungal infections dominate. Advanced disease is also characterized by normocytic normochromic anemia, autoimmune hemolytic anemia, immune thrombocytopenia, neutropenia, and red cell aplasia. CLL prognosis can be described using Rai staging, in which Rai stage 0 which describes the mildest disease is associated with an expected survival of 12 years, and Rai stage 4 with less than 4 years of survival. Currently, the standard treatment for CLL is FCR (fludarabine, cyclophosphamide and rituximab). Campth is used on patients that are resistant to FCR. There are also treatments to manage the specific complications of CLL, such as the use of prednisolone for bone marrow failure, corticosteroids for autoimmune hemolytic anemia or thrombocytopenia, radiotherapy to reduce the size of particularly large lymph nodes in late stages of the disease, and immunoglobulin replacement for patients with hypogammaglobulinemia (2). About 5% of CLL patients undergo Richter's transformation, which is disease transformation into diffuse large B cell lymphoma (3).

There are certain characteristics that CLL cells have in common with each other. Research has found that hypermethylation of DNA, an epigenetic abnormality, is present. Also, a fraction of CpG islands in CLL cells are found to be methylated in ways that are different from the methylation pattern found in normal B cells. In addition, most CLL cells also show methylation of DAPK1 (death associated kinase I), which silences the kinase. There are however, differences between CLL cells associated with particularly aggressive disease, and those associated with more indolent disease. In normal B cells, the immunoglobulin heavy chain undergoes hypermutation when the cells come into contact with antigens. In about half of CLL cells, mutated immunoglobulin heavy chain mutations are present. In the other half, they are not (1). The absence of mutations is associated with a more aggressive disease course with a shorter time to treatment from the time of diagnosis. (1,4,5,6). However, an even stronger predictor of negative outcomes in CLL patients and decreased time to treatment is expression of the tyrosine kinase called zeta associated protein (ZAP 70) (4,7). A study has shown that patients with ZAP positive CLL B cells have a significantly shorter time to treatment of 3 years, compared to those with ZAP70 negative cells, regardless of IgH<sub>V</sub> mutational status. Among Zap 70 negative patients, the absence of mutations was associated with a median time to treatment

of approximately 6 years, and the presence of mutations was associated with a median time to treatment of about 10 years. ZAP 70 expression is also associated with genetic mutations of del17p13 and del 11q22 that are also poor prognostic indicators (4).

CLL B-cells that express Zap 70 (zeta associated protein) have enhanced B cell receptor (BCR) signaling. ZAP 70 is a tyrosine kinase, which is an enzyme that moves a phosphate group from ATP onto the tyrosine residue of a different protein. This kind of protein phosphorylation often is an integral part of intracellular signaling pathways, which regulate cell activities including cell division. Tyrosine kinases are involved in mediating the B cell receptor signaling pathway, which is essential for the clonal expansion of B cells after antigen encounter that facilitates the immune response and cell survival. There is significantly higher tyrosine phosphorylation of proteins after BCR ligation in ZAP-70 positive cells compared with ZAP 70 negative cells (5, 8). Zap-70 is a protein tyrosine kinase expressed in normal T cells and is involved in T cell receptor signaling. After a T cell receptor is ligated, SRC family tyrosine kinases phosphorylate tyrosine residues in the accessory molecules of the T cell receptor. Zap 70 is recruited to the motifs and gets activated and in turn activates the Tec family protein tyrosine kinases, the Ca<sup>2+</sup> signaling pathway, and the Ras/mitogen activated protein kinase pathway. Instead of Zap 70, normal B cells use the tyrosine kinase Syk for signal transduction following activation of the B cell receptor(5). B cells normally do not express ZAP 70. When a B cell receptor is stimulated by antigen in normal B cells, Syk is recruited to the B cell receptor and gets phosphorylated by Lyn, an Src family kinase. This activates Syk, which allows it to phosphorylate other molecules. Syk then phosphorylates intermediates including B cell linker protein, Pi3K, and phospholipase Cy2. This is followed by the activation of downstream molecules including Akt, extracellular signal-regulated kinase (ERK), c-JUN-NH2-terminal kinase and nuclear factor (NF)-kB transcription factor (9). Syk is also important in the pathogenesis of CLL, being constitutively phosphorylated at Y352, in the absence of BCR engagement (9-13). The presence of ZAP 70 has been shown to increase Syk phosphorylation although total Syk levels are similar regardless of how much ZAP 70 is present (5,8,14). Since the B cell receptor signaling pathway induces up-regulation of MCL-1, an antiapoptotic protein, continuous activation of the pathway as a result of constitutive activity of Syk leads to decreased cell death in CLL cells. The inhibition of Syk causes apoptosis. ZAP 70 is also associated with increased BCR signaling. Hence, ZAP70 positive cells have increased Mcl-1 and are more resistant to cell death. Therefore, drug therapy that targets ZAP 70 will increase apoptosis in CLL B cells and will have clinical benefit in ZAP70 positive CLL. The tyrosine kinase inhibitor R406 has been found to inhibit Syk as well as increase the effect of fludarabine. R406 inhibition of Syk causes subsequent inhibition of the Akt signaling pathway that is activated by BCR stimulation. Prolonged Akt kinase activation causes increased MCL-1 levels and thus by inhibiting this kinase through Syk even when BCR engagement is present, R406 increases apoptosis (9,15).

A third tyrosine kinase that has relevance to prognosis in CLL is Lyn. Because Lyn phosphorylates Syk in the BCR signaling cascade, Lyn inhibition reduces Syk phosphorylation, which inhibits the BCR signaling cascade and leads to decreased antiapoptotic proteins (9). Lyn is overexpressed in CLL, and has an increased level of phosphorylation in CLL cells. In particular, there is an increased amount of Lyn present in the plasma membrane of leukemia cells. In normal B cells, Lyn is found in lipid rafts, but in CLL cells, is spread evenly over the plasma membrane. (16). The inhibition of lyn also causes apoptosis, and is considered a therapeutic target.

Gefitinib is a tyrosine kinase inhibitor that was initially used because it inhibits epidermal growth factor receptor (EGFR) enzymatic activity. It competes with ATP for EGFR, thus preventing its activation by phosphorylation. Gefitinib lowers vascular endothelial growth factor (VEGF) which is required for the development of neovasculature in tumours. It was originally used to treat non-small cell lung cancer (NSCLC) that was resistant to standard chemotherapy. Some patients benefited more than others. Sequencing studies have found that patients with certain mutations in the EGFR of their NSCLC tumors experienced longer survival with gefitinib than others. Also, tumours that stained positive for phosphorylated Akt (active form of AKT), which is a protein downstream of EGFR, were found to have a greater response to gefitinib than tumours without phosphorylated Akt (17). As mentioned earlier, Akt phosphorylation in CLL cells upregulates MCL-1, and the fact that gefitinib may have a greater effect on cells with this poor prognostic factor is promising for CLL. Most NSCLC demonstrate activated EGFR signaling pathways. When EGFR is activated, phosphorylation of tyrosine residues in the kinase domain of the receptor occurs. The phosphotyrosines then activate partner proteins which activate signaling cascades via the mitogen-activated protein kinase and phosphatidylinositol-3-kinase pathways; these are pathways that protect against apoptosis and allow tumour cells to metastasize and develop neovasculature. Gefitinib is a tyrosine kinase inhibitor was developed with the intention to block the prosurvival effects of the EGFR pathways (18). Fortunately clinical trials have found tumours with EGFR mutations that cause them to be constitutively active, implying poor prognosis, to be especially responsive to gefitinib, as evidenced by these patients longer progression free survival compared to those on carboplatin and paclitaxel. Gefitinib was also found to work especially well in people of Asian descent, non smokers, and women (19-21).

The recommended dose for gefitinib is 250mg orally once daily. The half life is 48 hours, with a bioavailability of 60%. It is metabolized mostly by CYP3A4 and also by CYP3A5 and CYP1A1 on a smaller scale. Radiographic monitoring is required with this drug but should not occur more often than once every 6 weeks. Continuation of use is required even once response

is lost, as tumor progression will accelerate once the drug is taken off. Benefits of gefitinib over other chemotherapeutic agents include that it does not cause myelosuppression, neuropathy, alopecia, or severe nausea. However, its side effects do include rash, diarrhea, and rarely interstitial lung disease. If interstitial lung disease occurs the drug needs to be discontinued. Interestingly, people that develop a rash in general benefit more from gefitinib. Patients with deletions in exon 19, L858R, or a point mutation in exon 21 also tend to have better outcomes (18).

## Rationale

CLL cells are different from lung cancer cells in that they do not express EGFR (22,23). However, although gefitinib was originally used to target EGFR, it also has effects on other tyrosine kinases as well, including ZAP-70, Syk and Lyn (24,25). Since gefitinib reduces the phosphorylation of such kinases, we decided to investigate whether gefitinib would cause a greater level of apoptosis in ZAP70 positive CLL cells compared with ZAP 70 negative cells. This would allow us to make recommendations regarding the use of gefitinib, a drug that is already in use for non-small cell lung cancer, and whose safety profile is known, in CLL patients. Therefore a more rapid translation into the clinic would be possible. Studies have already shown that gefitinib induces cell death in acute myeloid leukemia and acute lymphoblastic leukemia (24,22). The use of gefitinib for CLL is of particular interest since it affects the tyrosine kinase, ZAP 70, associated with aggressive CLL. Specifically, ZAP 70 contributes to poor prognosis as a result of its effect of increasing B cell receptor signaling, which activates the AKT and NFkB downstream signaling pathways that increase MCL-1, an anti-apoptotic protein (5,8,9,15). Therefore, a drug that targets ZAP-70 will inhibit this anti-apoptotic effect and aid in improving the poor prognosis of ZAP-70 +patients. As previous studies have shown that combining Syk and Lyn inhibitors to other chemotherapeutic agents results in synergy, we also evaluated the degree of cell death when gefitinib was combined with the standard chemotherapeutic agent, chlorambucil.

Hypothesis: We hypothesize that gefitinib will induce apoptosis in ZAP-70 positive cells to a greater extent than ZAP 70 negative cells and combination of gefitinib and chlorambucil will increase apoptosis.

## Materials and Methods

### Cytotoxicity Assay

The human T-cell leukemia cell line Jurkat, the human lymphoma cell line BJAB, and chronic lymphocytic leukemia cell line I83 were maintained in suspension cultures in RPMI medium supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin and

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streptomycin in a 5% CO<sub>2</sub> incubator. Cells were seeded at  $5 \times 10^5$  cells per ml in 6 wells plates at 1 ml per well, and treated with varying concentrations of gefitinib dissolved in DMSO (0, 0.1, 1, 5, 10, 20, and 30uM), and in separate plates with varying concentrations of chlorambucil dissolved in acidified ethanol (0, 1, 2.5, .10, 25, 50, and 100uM). The cells were treated for 24 hours, and assessed for the degree of cell death present using acridine orange and ethidium bromide staining, with fluorescent microscopy. Briefly, after 24 hours of incubation, cell suspensions were centrifuged at 2000rpm for 3 minutes, resuspended in phosphate buffered saline, combined with acridine orange/ethidium bromide, and viewed under a fluorescent microscope. Orange cells were counted as dead and green cells were counted as live. Afterwards, all three cell lines were treated with a combination of 5uM gefitinib and 10uM chlorambucil for 24 hours, and assessed for cell death using the same technique as above.

### Cell lysate preparation

Jurkat, BJAB, and I83 cells were pelleted via centrifugation at 1200rpm for 5 minutes. The cells were resuspended in lysis buffer containing protease inhibitor, phosphatase inhibitor, phenylmethanesulfonylfluoride, and sodium vanadate. Lysis of the cells was carried out with alternating cycles of vortexing and incubation of the suspensions on ice.

### Western blot analysis

The protein was western blotted for total Syk, Lyn, and ZAP70. Briefly, 55ug of protein were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis at 100V. The protein was transferred to nitrocellulose (BioRad Laboratories, Hercules, CA, USA) and then probed with antibodies for total ZAP-70, Syk, and Lyn (Cell Signalling Technology Inc., Danvers, MA, USA). The blots were then stripped and re probed with anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a loading control. The pre-B cell line NALM-6, was treated with gefitinib dissolved in DMSO at 0, 30 uM, and with DMSO alone as a control, for varying amounts of time (3, 6, and 18 hours), and western blotted for phospho-lyn. Again, the blot was then stripped and re probed for actin as a loading control.

### MTT Assays

We also examined the effect of gefitinib on patient CLL cells with known ZAP 70 status. Peripheral blood samples drawn from the enrolled patients were centrifuged to isolate the buffy coat from which B cells were then purified using Ficoll separation. The B cells were washed with Hanks buffered saline solution and seeded in 12 tubes at  $3 \times 10^7$  cells per tube. The

seeded cells were either treated with gefitinib at 0, 1, 2.5, 10, or 15uM, or with fludarabine at the same concentrations for 24 hours. DMSO, the solvent used to dissolve the drugs, was used in the 0uM tubes as a control. Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells treated with the different concentrations of gefitinib and fludarabine were seeded in 96 well plates at 200ul per well, at four different cell concentrations ranging from  $1 \times 10^6$  to  $4 \times 10^6$  per ml. The plates were incubated at 37 degrees Celcius, 5% CO<sub>2</sub>, for 3 days. MTT solution was added to each well and the plates were incubated for another 4 hours at 37 degrees Celcius, 5% CO<sub>2</sub>. The cells were pelleted, resuspended in DMSO, and read for absorbance to determine the IC 50 of fludarabine and gefitinib.

### Statistical analysis

Student's paired two-tailed t-test was used to determine if there were statistically significant differences between various aspects of the treatment responses observed. A p value less than 0.05 was considered statistically significant.

### Results

Treatment of Jurkat, BJAB, and I83 cell lines with gefitinib showed that Jurkat cells underwent the greatest amount of cell death at 5uM, 20uM, and 30uM. Although this was not statistically significant, statistical significance was being approached with p values ranging from 0.08 to 0.21 (Figure 1b). There was no difference between the effect of chlorambucil and gefitinib on Jurkat cells with the concentrations used ( $p=0.51$ ). However, the treatment of the cell lines with chlorambucil showed that there was a statistically significant greater amount of cell death in I83 cells compared with both Jurkat ( $p=0.0028$ ) and BJAB ( $p=0.0011$ ) (Table 1a). I-83 cells were also significantly more responsive to chlorambucil than to gefitinib ( $p=0.009$ ). When treating with a combination of 5uM gefitinib and 10uM chlorambucil, there was a 45% cell death in Jurkat, 40% cell death in I-83 and 19% cell death in BJAB. These differences between the combination treatment in the different cell lines were not statistically significant, but more repetitions of the experiment would be required to determine for sure if there is a difference (Jurkat vs I83  $p=0.91$ , Jurkat vs BJAB  $p=0.14$ ). All of the cell lines showed increased cell death with a combination treatment of 10uM chlorambucil and 5uM gefitinib compared with either of the two drugs alone at these same concentrations. The effect of combining the two drugs was greatest in Jurkat cells, in which there was a 45% cell death with the combination treatment compared with 21% with 10uM chlorambucil alone ( $p=0.19$ ) or 25% with 5uM of gefitinib alone ( $p=0.13$ ). Combination treatment in BJAB cells seemed to have the least increased cell death compared with single drug treatment with either drug. The amount of cell death with the combination treatment was almost identical to that seen with gefitinib

treatment alone. Although I-83 cells seemed to respond to the combination with a similar amount of cell death as in Jurkat cells, it is clear from the graph that the combination of chlorambucil with DMSO caused a similar amount of cell death as the combination treatment, which suggests that it is the DMSO that contributed most of the additional cell death on top of what was caused by chlorambucil. Therefore, the combination treatment worked best in Jurkat cells, notably causing more cell death than the combinations of gefitinib and ethanol, or chlorambucil and DMSO. There was no significant difference between gefitinib and gefitinib with ethanol or between chlorambucil and chlorambucil with DMSO in the amount of cell death caused. Overall, BJAB cells showed the least amount of cell death compared with both I-83 ( $p=0.06$ ), and Jurkat ( $p=0.11$ ), but this was not statistically significant (Table 1c).

Overall, the results show that Jurkat cells, are more sensitive to gefitinib both alone and in combination with chlorambucil, but this needs to be further validated with more experiments in order to be shown to be statistically significant

To confirm our results in CLL cells, I used primary CLL cells isolated from a patient in the CLL clinic at CancerCare Manitoba. Using MTT assays, we found that the  $IC_{50}$  of gefitinib for the two ZAP-70 positive CLL patient samples tested were around 4 $\mu$ M. The  $IC_{50}$  of gefitinib for the two ZAP-70 negative patients were over 15 $\mu$ M. This suggests that CLL cells with ZAP-70 positive status are more responsive to gefitinib in vitro than CLL cells with a ZAP-70 negative status. The  $IC_{50}$  of fludarabine determined for one ZAP-70 positive patient was 5 $\mu$ M, and was 8 $\mu$ M and >15 $\mu$ M for the two ZAP-70 negative patients. This suggests that there is no correlation between ZAP-70 status and sensitivity to fludarabine (figure 2).

The expression of tyrosine kinases in the various cell lines tested were evaluated. Western blotting showed Jurkat cells express ZAP70, as shown by the band at 70 kDa but I-83 and BJAB cells failed to expression ZAP-70. (Figure 3 c). However, BJAB and NALM 6 were both positive for Lyn at 56kDa (figure 3a) and Syk at 72kDa (figure 3b). A slight reduction in Lyn phosphorylation was seen in the NALM-6 cells that were treated with 30 $\mu$ M gefitinib dissolved in DMSO, for 3, 6, and 18 hours, compared with control cells that were not treated (figure 4). Therefore, it is likely that gefitinib plays a role in inhibiting ZAP-70 and Lyn phosphorylation.

## Discussion

The experimental results seemed to show that CLL cells expressing ZAP 70 undergo a greater amount of cell death when treated with gefitinib than cells that are ZAP70 negative. This was true both for gefitinib alone and in combination with chlorambucil. Overall, the results obtained with cell lines and with patient cells were agreeable in terms of ZAP 70 status. However, the differences between ZAP 70 positive and ZAP 70 negative outcomes were not shown to be statistically significant for the cell lines. More experiments may be able to establish

with statistical significance that cell sensitivity to gefitinib is dependent on ZAP70 status. In the patient samples, gefitinib had an  $IC_{50}$  of around 4 $\mu$ M in ZAP70 positive patients, which is much greater than the clinically used plasma concentration of 0.3 $\mu$ M. However, because an  $IC_{50}$  of 4 $\mu$ M is still considerably less than  $IC_{50}$  of 15 $\mu$ M in ZAP-70 negative patients, there is still indication that gefitinib will be more effective in ZAP-70 positive patients. Also, because the cells were only incubated for 24 hours with the drug, a greater response may be observed in patients as they would be treated for longer periods of time. An effect of gefitinib on ZAP-70 cells would be plausible due to the fact that the inhibition of ZAP-70 would interfere with B cell receptor signaling (references), which would lead to decreased amounts of the anti-apoptotic protein, MCL-1, causing apoptosis.

Western blotting showed that Syk and Lyn are expressed in BJAB cells, which did not experience as much cell death when treated with gefitinib alone or in combination. This may support the idea that gefitinib response is associated in particular with ZAP70 expression. If this is the case, gefitinib may play a unique role in the clinical management of CLL patients, where it is only given to patients who have ZAP 70 positive status. As such, determination of ZAP 70 status in CLL patients may become an important part of patient evaluation and treatment planning. There have also been other studies in the past evaluating the ability of other tyrosine kinase inhibitors to induce cell death in CLL. One study evaluated the use of sunitinib, an inhibitor of tyrosine kinases associated with VEGF receptors in CLL patients. The rationale behind this study was that VEGF had been known to influence CLL B cell migration and higher levels of VEGF contributed to faster clinical deterioration. However, sunitinib was not found to be useful (26). On the contrary, the tyrosine kinase inhibitors dasatinib and imatinib have been found to be useful. Imatinib targets the tyrosine kinase c-abl, which functions to increase DNA repair. Overexpression of c-abl in CLL increases resistance to alkylating agents such as chlorambucil, and therefore imatinib is able to increase sensitivity to alkylating agents in CLL by inhibiting its DNA repair (27, 28). Dasatinib has also been found to increase CLL sensitivity to chlorambucil and fludarabine, also because it inhibits c-abl (29). A conclusion drawn from these findings is that the degree of therapeutic value of a particular tyrosine kinase inhibitor for CLL is dependent on which tyrosine kinase it targets. Therefore, to further understand the role that gefitinib plays in ZAP-70 positive cells, we could characterize its effects on some of the proteins involved downstream of ZAP-70 in the B cell signaling pathway. This would provide us with more information in order to make better understand how to use gefitinib to treat ZAP-70 positive CLL cells. As it is a drug that is already used in the treatment of non small cell lung cancer and its safety profile is thus known, translation into clinical use for CLL would be more rapid and efficient than other tyrosine kinase that are in clinical development.

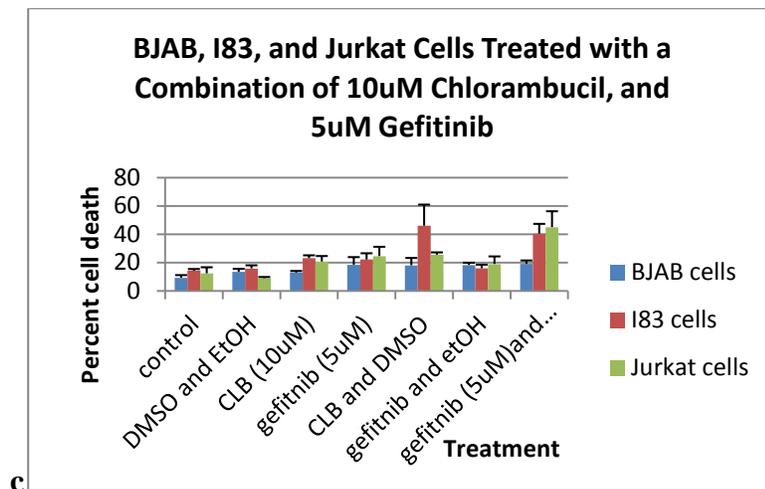
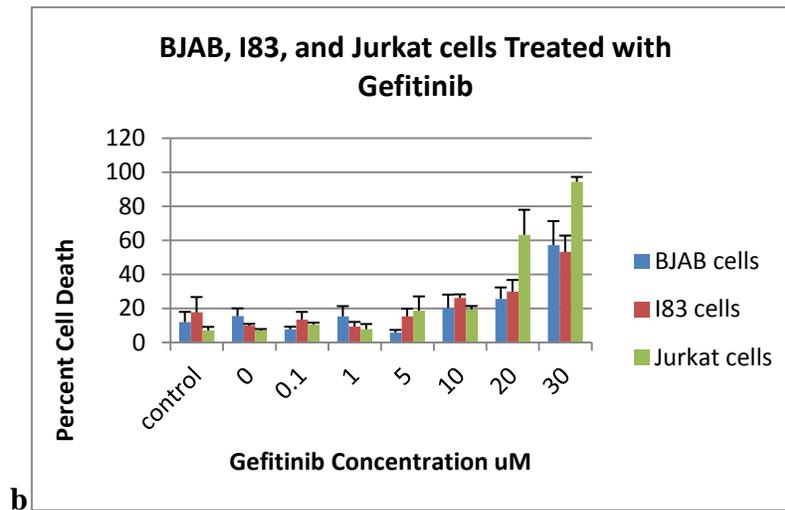
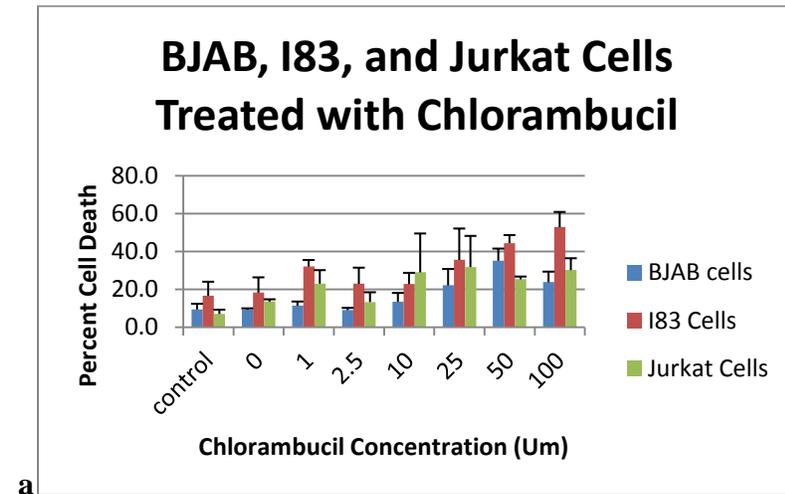
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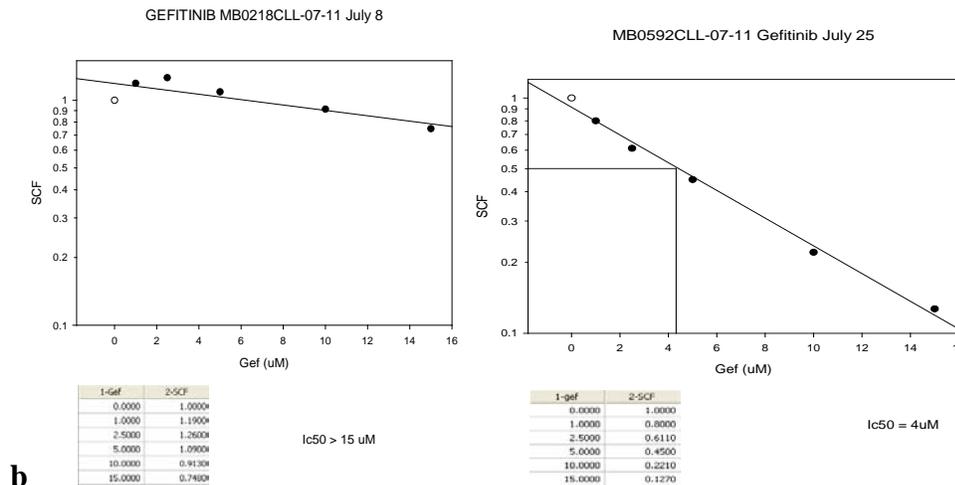
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Tables and Figures

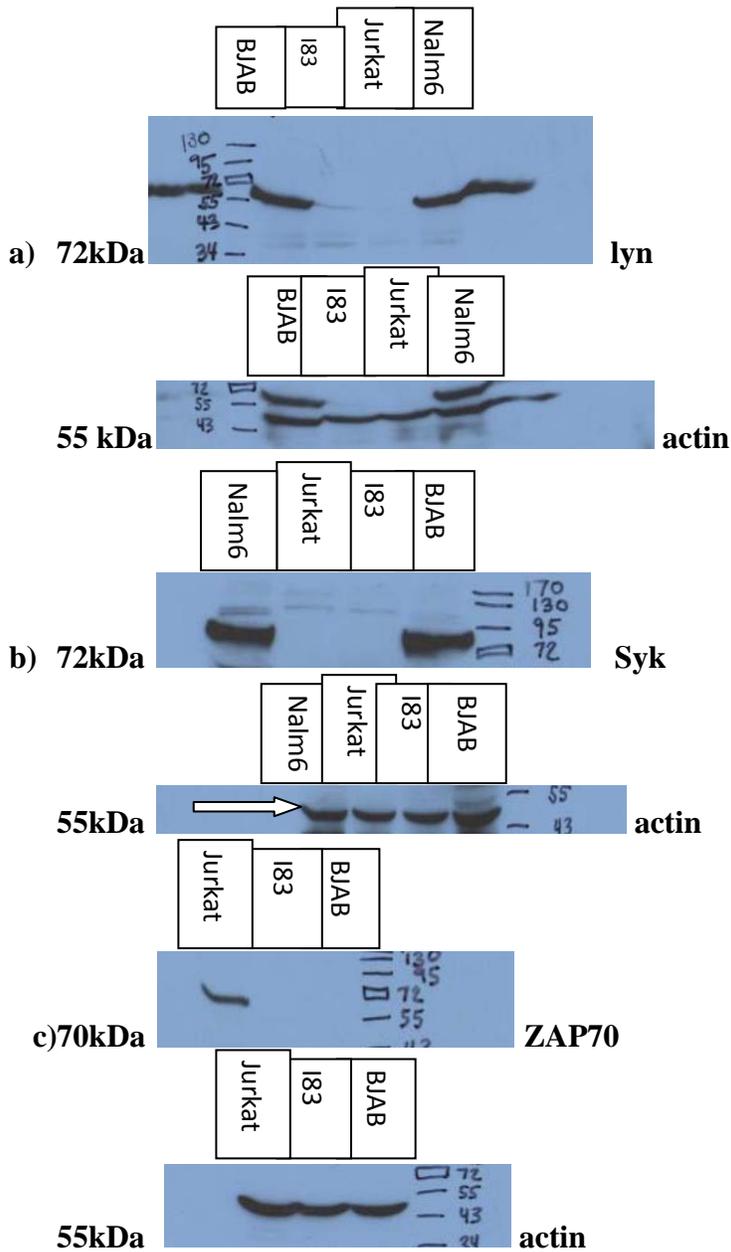


**Figure 1. a) BJAB, I83, and Jurkat cells were incubated for 24 hours with 0, 1, 2.5, 10, 25, 50, or 100uM of chlorambucil dissolved in acidified ethanol. In the control well, an equivalent volume of RPMI was added. An equivalent volume of DMSO was added into the '0' well. b) BJAB, I83, and jurkat cells were treated with 0, 0.1, 1, 5, 10, 20, or 30 uM of gefitinib dissolved in DMSO. In the control well, and equivalent volume of RPMI was added. In the 0 well, an equivalent amount of the solvent DMSO was added. c) BJAB, I83, and Jurkat cells were treated with a combination of 5uM gefitinib and 10uM chlorambucil for 24 hours.**

Patient	ZAP 70 status	IC 50 of fludarabine	IC 50 of gefitinib
1	ZAP 70 positive	N/A	4
2	ZAP 70 positive	5	4
3	ZAP 70 negative	8	>15
4	ZAP 70 negative	>15	>15

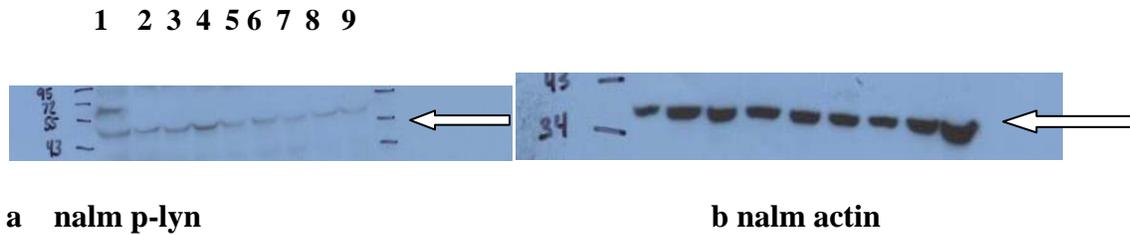


**Figure 2. a) CLL cells from 4 patients whose ZAP-70 status were known were treated with either gefitinib at 0, 1, 2,5, 10, or 15uM, or with fludarabine at the same concentrations for 24 hours. DMSO, the solvent used to dissolve the drugs, was used in the 0uM tubes as a control. The IC 50 of fludarabine and chlorambucil were determined using the MTT assay. B) gefitinib IC 50 graphs of (from left), a ZAP-70 negative patient, and a ZAP70 positive patient (low IC 50).**



**Figure 3. A-b)** Cell lysates made from BJAB, I83, Jurkat, and Nalm 6 cells were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with Syk and Lyn.

The blots were stripped and probed for actin was used as a loading control c)BJAB, I83, and jurkat cells were probed for total ZAP-70, then probed for actin as a loading control.



**Figure 4**

a)The pre-B cell line NALM-6, was treated with gefitinib dissolved in DMSO at 30 uM, for varying amounts of time (3, 6, and 18 hours). Cells were also treated with DMSO alone, or left untreated, as control again for 3, 6, and 18 hours. Lysate preparations were western blotted for phospho-lyn. 1=18 hours control, 2=6 hours control, 3= 3 hours control, 4=3 hours with DMSO, 5=3 hours with 30uM gefitinib, 6=6 hours with 30 uM gefitinib, 7= 18 hours with 30uM gefitinib, 8= 18 hours with DMSO. B) Blot stripped and reprobed for actin as a loading control.